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9th International Workshop on Plant Membrane Biology

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UNIVERSITY OF CALIFORNIA

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PROGRAM

Sunday, July 19

Event #

1. Registration (3:00-8:00 P.M.). In **De Anza Foyer** next to Main Lobby (Atrium) of the Doubletree Hotel.

2. Wine and Cheese Reception (5:30-8:30 P.M.). In **De Anza Ballroom.**

Monday, July 20

3. Workshop Opening (8:15-8:25 A.M.) LINCOLN TAIZ, University of California, Santa Cruz. (In **Steinbeck Forum Theatre**)

4. Session I (8:25-10:35 A.M.)

   Plasma Membrane H^+-ATPases (Chair: CHRISTER LARSSON, University of Lund, Sweden)

   8:25-8:30 Session Introduction

   8:30-8:55 CAROLYN W. SLAYMAN, Yale University, USA. "Site-Directed Mutagenesis of the Yeast Plasma Membrane H^+-ATPase."

   8:55-9:20 MARC BOUTRY, University of Louvain, Belgium. "Regulation of the Expression of Four Genes Encoding the Plasma Membrane ATPase of Tobacco."

   9:20-9:45 JEFFREY F. HARPER, Scripps Research Institute, USA. "The Plasma Membrane H^+-ATPase Multigene Family in *Arabidopsis thaliana*."

   9:45-10:10 KUNIHIRO KASAMO, National Food Research Institute, Japan. "Regulation of Plasma Membrane H^+-Pumping by Molecular Species of Phosphatidylcholine."

   10:10-10:35 STACEY F. SHARTZER, University of California, Davis, USA. "Regulation of Plasma Membrane Transport Activity is Dependent on a Phosphorylation/Dephosphorylation Cycle."

5. Coffee/Tea Break (10:35-11:05)

6. Session II (11:05 A.M.-12:50 P.M.)

   Plasma Membrane Ca^{2+}-ATPase (Chair: FRANCES DUPONT, USDA, USA)

   11:05-11:10 Session Introduction

11:10-11:35 HUBERT H. FELLE, University of Glessen, Germany. "Ca^{2+} Homeostasis in *Sinapsis* root hair tips: the Role of the Plasma Membrane Ca^{2+}-ATPase."

11:35-12:00 MARIA I. DE MICHAELIS, University of Messina, Italy. "A Biochemical Characterization of the H^+-ATPase and of the Ca^{2+}-ATPase in Plasma Membrane Purified from *Arabidopsis thaliana*."

12:00-12:25 MARIANNE SOMMARIN, University of Lund, Sweden. "Comparison of Ca^{2+}-ATPase Activities in Purified Plasma Membrane and Endoplasmic Reticulum from Wheat."

12:25-12:50 PER ASKERLUND, University of Oxford, UK. "Reconstitution, Characterization and Cellular Localization of a Calmodulin-Stimulated Ca^{2+}-Pumping ATPase Purified from *Brassica oleracea* L.

7. Free Time (12:50-3:00 P.M.)

8. Session III (3:00-5:10 P.M.)

Plant-Environment Interactions (Chair: ENRICO MARTINOIA, Eidgenossische Technische Hochschule, Switzerland)

3:00-3:05 Session Introduction

3:05-3:30 LEON V. KOCHIAN, U.S. Plant Soil and Nutrition Laboratory, Cornell University, USA. "Biophysical and Genetic Studies on the Role of Root Cell Ion Transport Processes in Aluminum Toxicity and Tolerance."

3:30-3:55 BEN-ZION GINZBURG, The Hebrew University of Jerusalem, Israel. "A Study of the Physiology of Halotolerance in *Dunaliela* Using Mutants."

3:55-4:20 PAUL M. HASEGAWA, Purdue University, USA. "NaCl Regulation of Plasma Membrane and Tonoplast ATPase Gene Expression in Glycophytes and a Halophyte during Salt Adaptation."

4:20-4:45 EDUARDO BLUMWALD, University of Toronto, Canada. "Identification of the Vacuolar Na^+/H^+ Antiport."

4:45-5:10 NIGEL CRAWFORD, University of California, San Diego, USA. "Characterization of the CHL1 gene from *Arabidopsis*."

9. BARBECUE IN MEMORY GARDENS (5:30-9:00 P.M.)

Tuesday, July 21

10. Session IV (8:15-10:25 A.M.)

Membrane Receptors and Signal Transduction (Chair: WENDY BOSS, North Carolina State University, USA)

8:15-8:20 Session Introduction

8:20-8:45 HELENE BARBIER-BRYGOO, Institut des Sciences Vegetales, CNRS, France. "Perception of the Auxin Signal at the Plasma Membrane of Tobacco Mesophyll Protoplasts."

8:45-9:10 YOUNGSOOK LEE, Prohang Institute of Science and Technology, Korea. "Abscissic Acid-Induced Inositol Phospholipid Turnover in Guard Cell Protoplasts of *Vicia faba*."

9:10-9:35 ALAN WISE, University of Leeds, UK. "Receptors Coupled to Guanine Nucleotide-Binding Proteins in Higher Plant Cells."

9:35-10:00 ALICE HARMON, University of Florida, USA. "Calcium-Dependent Protein Kinases and Their Roles in Signal Transduction."

10:00-10:25 WINSLOW E. BRIGGS, Carnegie Institution of Washington, Stanford, USA. "The Possible Molecular Basis of a Blue Light Response."

11. Coffee/Tea Break (10:25-10:55)

12. Session V (10:55 A.M. -12: 45 P.M.)

Channels: Physiology and Regulation (A) (Chair: CLIFFORD SLAYMAN, Yale School of Medicine, USA)

10:55-11:00 Session Opening

11:00-11:25 J.. ANDREW SMITH, University of Oxford, UK. "A Malate-Selective Ion Channel at the Vacuolar Membrane of CAM Plants."

11:25-11:55 E. JOHANNES, University of York, UK. "Calcium Channels at the Tonoplast."

11:55-12:20 BARBARA PICKARD, Washington University, St. Louis, USA. "Epidermal Mechanosensory Calcium-Selective Channels Detect Transmembrane Potential, Temperature, Mural pH, and Auxin."

12:20-12:45 MAARTEN J. CHRISPEELS, University of California, San Diego, USA. "Molecular and Functional Analysis of Tonoplast Protein (TIP).

13. Free Time (12:45-2:30 P.M.)

14. Set up all posters in **Serra I.** (2:30-3:00 P.M.)

15. Poster Session I (3:00-6:00 P.M.) Authors of abstracts **63-124** will present their posters.

16. Free Time (6:00-7:30 P.M.)

17. Techniques Workshop I (7:30-9:30 P.M.) In **Steinbeck Forum Theater**.

Fluorescence Imaging (Chairs: SIMON GILROY, University of California, Berkeley, USA and DOUG BUSH, Rutgers University, USA)

7:30-7:50 DOUG BUSH and SIMON GILROY. "Overview of Intracellular Ion Measurements Using Fluorescent Probes."

7:50-8:10 JOE KAO, University of Maryland, USA. "Principles of Dye Design and Use."

8:10-8:30 COLIN BROWNLEE, The Marine Biological Association, UK. "Temporal and Spatial Monitoring of Cytoplasmic Calcium In *Fucus* Zygotes: Developments Using Photometry and Confocal Ratio Imaging."

8:30-8:50 MARK D. FRICKER, University of Oxford, UK. "Confocal Fluorescence Ratio Analysis of Ion Concentrations in Plant Cells."

8:50-9:10 ANTON NOVACKY, University of Missouri, USA. "Alterations of Intracellular pH During Bacterial Hypersensitive Reaction: A Confocal Laser Scanning Microscopy Study."

9:10-9:30 SIMON GILROY. "Manipulation of Intracellular Ion Concentration by Caged Probes."

Wednesday, July 22

18. Session VI (8:00-10:10 A.M.)

Channels: Physiology and Regulation **(B)** (Chair: HIDDE B. A. PRINS, University of Groningen, The Netherlands)

8:00-8:05 Session Introduction

8:05-8:30 NAVA MORAN, Weizmann Institute of Science, Israel. "Proton Block of K^+ Channels in the Plasma Membrane of Guard Cell Protoplasts: A Patch Clamp Study."

8:30-8:55 SARAH M. ASSMANN, Harvard University, USA. "Second Messenger Regulation of Ion Transport in Guard Cells of *Vicia faba*."

8:55-9:20 JULIAN I. SCHROEDER, University of California, San Diego, USA. "Two Types of Voltage-Dependent Anion Currents in Guard Cells."

9:20-9:45 PATRICE THULEAU, University of California, San Diego, USA. "Purified Calcium Channel Blocker-Binding Protein from Carrot Cells Forms Calcium-Permeable Ion Channels After Reconstitution."

9:45-10:10 RAINER HEDRICH, University of Hannover, Germany. "Plant Growth Hormones Control Ion Channels in the Plasma Membrane of Guard cells."

19. Coffee/Tea Break (10:10-10:40 A.M.)

20. Session VII (10:40 A.M.- 12:50 P.M.)

Sugar Carriers (Chair: WIDMAR TANNER, University of Regensburg, Germany)

10:40-10:45 Session Introduction

10:45-11:10 NORBERT SAUER, University of Regensburg, Germany. "Sugar Transporters of the Plant Plasma Membrane."

11:10-11:35 DANIEL R. BUSH, USDA-ARS, University of Illinois, USA. "Proton-Coupled Sucrose and Amino Acid Symports: Substrate Recognition, Photoaffinity Labeling and Molecular Cloning."

11:30-11:50 SERGE DELROT, Université de Poitiers, France. "The Sucrose Carrier of the Plant Plasmalemma: Partial Purification and Reconstitution."

11:50-12:10 GERA D. EYTAN, Technion-Israel Institute of Technology, Israel. "Sugar Transporters Involved in Phloem Unloading."

12:10-12:30 WOLF B. FROMMER, Institut fur Genbiologische Forschung, Germany. "Expression Cloning of Metabolite Transporters from Higher Plants."

12:30-1:00 ULF-INGO FLUGGE, University of Wurzburg, Germany. "Insights into the Structure of the Chloroplast Triose Phosphate-Phosphate Translocater Protein."

21. Free Time (1:00-2:30 P.M.)

22. Poster Session II (2:30-4:00 P.M.) Authors of abstracts **125-184** will be available to present their posters.

23. Poster Discussion in **Steinbeck Forum Theater** (4:00-6:00 P.M.) (Chair: MICHAEL SUSSMAN, University of Wisconsin, USA.)

Six posters will be selected by a panel for brief (10') presentations by the authors. (The authors will be notified ahead of time.) The remainder of the session will be taken up by informal discussions of the posters.

24. Transit to the Monterey Bay Aquarium (6:00-7:00 P.M.)

25. Strolling Dinner through the Monterey Bay Aquarium (7:00-10:00 P.M.)

Thursday, July 23

26. Session VIII (8:15-10:00 A.M.)

Vacuolar H^+-Pumps (A) (Chair: BERNARD MARIN, Unite Fonctionnelle de Biotechnologie, France)

8:15-8:20 Session Introduction

8:20-8:45 HEVEN SZE, University of Maryland, USA. "Vacuolar-type H+-Translocating ATPases in Plant Endomembranes: Subunit Organization and Multigene Family."

8:45-9:10 EMMA JEAN BOWMAN, University of California, Santa Cruz, USA. "Structure and Expression of Genes Encoding the Major Subunits of Vacuolar and Mitochondrial ATPases of *Neurospora crassa* and Strategies for Mutating ATPase Genes."

9:10-9:35 J. PETER GOGARTEN, University of Connecticut, USA. "Evolution and Isoforms of the Plant V-ATPase."

9:35-10:00 MASASHI TAZAWA, Fukui Institute of Technology, Japan. "Inhibition of Vacuolar pH Regulation by Balifomycin A1 in Cells of *Chara corallina*."

27. Coffee/Tea Break (10:00-10:30 A.M.)

28. Session IX (10:30 A.M.-12:40 P.M.)

Channels: Biophysics and Molecular Biology (Chair: ENID A. C. MacROBBIE, University of Cambridge, UK)

10:30-10:35 Session Introduction

10:35-11:00 STEVEN D. TYERMAN, The Flinders University, Australia. "Ion Channels in the Energized Membrane of Wheat Root Protoplasts."

11:00-11:25 F. A. SMITH, University of Adelaide, Australia. "Comparative *In Vivo* Studies of Anion Channels in *Chara*, Fungi and Roots."

11:25-11:50 ADAM BERTL, Yale School of Medicine, USA. "Gating of Ion Channels in Plasmalemma and Tonoplast of Yeast."

11:50-12:15 RICHARD GABER, Northwestern University, USA. "Isolation of plant K^+ channel cDNA by function in yeast."

12:15-12:40 HERVE SENTENAC, ENSA-M/INRA/CNRS, France. "Cloning and Expression in Yeast of a Plant K^+ Transport System."

29. Free Time (12:40-2:00 P.M.)

30. Luncheon Meeting for 1995 Workshop Organizers (12:40-2:00 P.M.)

31. Techniques Workshop II (2:00 - 4:25 P.M.) In **Steinbeck Forum Theater**

Patch-Clamp and Membrane Transport Techniques (Chair: RAINER HEDRICH and JULIAN SCHROEDER)

2:00-2:05 Workshop Introduction

2:05-2:30 THEO ELZENGA, University of Washington, USA. "Characterization of Ion Channels in the Plasma Membrane of Leaf Epidermis Cells of *Pisum sativum*, Argenteum Mutant."

2:30-2:55 C. ZEILINGER, University of Hannover, Germany. "Identification of a Potassium Channel in Mesophyll and Guard cell Plasma Membranes of *Vicia faba* leaves."

2:55-3:20 MICHAEL R. BLATT, University of London, UK "pH Controls K^+ Gating in *Vicia* Guard cells."

3:20-3:45 KLAUS RASCHKE, University of Gottingen, Germany. "Ion Channels in the Plasmalemma of Xylem Parenchyma Cells From Roots of Barley (*Hordeum vulgare* cv. Apex)."

3:45-4:10 KATHRYN BOORER, AFRC-IACR Rothamsted Experimental Station, UK. "Functional Expression of Plant Plasma Membrane Transporter in *Xenopus* Oocytes."

4:10-4:25 MICHAEL DELAY, Axon Instruments, USA. "Instrumentation Used in the Aquisition and Analysis of Plant Electrophysiological Data."

32. Poster Session III (2:00-5:45 P.M.) Authors of abstracts **185-246** will be present from 4:30-5:45 P.M.

33. Take down posters (5:45-6:00 P.M.)

34. Patch-Clamp Demonstration in **Serra I.** (4:30-6:00 P.M.) RAINER HEDRICH, HEINER BUSH AND IRENE MARTEN, University of Hannover, Germany.

35. Free Time (6:00-7:30 P.M.)

36. KEYNOTE ADDRESS (7:30-9:00 P.M.)

BERT SAKMANN, 1991 Nobel Laureate, Max-Planck Institüt für Medizinische, Forschung, Heidelberg, Germany

"Structure-Function Relationships of the ACh Receptor as Probed by Point Mutations"

Friday, July 24

37. Session X (8:15-10:30 A.M.)

Vacuolar H^+-Pumps (B) (Chair: RONALD J. POOLE, McGill University, Canada)

8:15-8:20 Session Opening

8:20-8:45 THOMAS RAUSCH, University of Frankfurt, Germany. "The *Daucus carota* V-type H^+-ATPase: Recent Progress in Understanding the Coordinate Expression of Its Genes."

8:45-9:10 MINOBU KASAI, Okayama University, Japan. "Regulatory Role of Intracellular pH, Ca^{2+}, and Abscisic Acid on the Two Active H^+-Pumps of Tonoplast from Barley Root."

9:10-9:35 DALE SANDERS, University of York, UK. "Potassium Transport into Plant Vacuoles is Directly Energized by the Tonoplast Inorganic Pyrophosphatase."

9:35-10:00 PHILIP A. REA, University of Pennsylvania, USA. "Characterization of Genes Encoding Vacuolar H^+-Pyrophosphatase of *Arabidopsis thaliana* and *Beta vulgaris*."

38. Coffee/Tea Break (10:00-10:30 A.M.)

39. Session XI (10:30-11:50 A.M.)

Cells, Organelles and Signaling (Chair: INGO DAHSE, Friedrich-Schiller University, Germany)

10:30-10:35 Session Introduction

10:35-11:00 JAQUES JOYARD, CNRS, Grenoble-cedex, France. "Pivotal Role of Diacylglycerol in Glycerolipid Biosynthesis by Chloroplast Envelope Membranes."

11:00-11:25 MICHAEL G. HAHN, University of Georgia, USA. "Specific Recognition of Oligosaccharide Elicitors by Binding Protein(s) in Soybean Membranes."

11:25-11:50 SHARON R. LONG, Stanford University, USA. "Response of Root Hair Cells to *Rhizobium* NOD factors."

40. EPILOGUE AND FORECAST (11:50 A.M.-12:00 noon)

WILLIAM J. LUCAS, University of California, Davis, USA.

SITE-DIRECTED MUTAGENESIS OF THE YEAST PLASMA-MEMBRANE [H^+]ATPase

Rao, R., Nakamoto, R.K., Verjovski-Almeida, S., and Slayman, C.W.
Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven CT 06510, USA

The primary transporter in the plasma membrane of *Saccharomyces cerevisiae* is a P-type [H^+]ATPase, encoded by the *PMA1* gene (1). Based on hydropathy analysis, the 100 kDa ATPase polypeptide is thought to be anchored in the membrane by eight hydrophobic segments, four at each end of the molecule. The central hydrophilic portion displays striking sequence conservation throughout the family of P-ATPases; significantly, it contains the binding site for ATP and the site (D378) at which the protein is transiently phosphorylated during the reaction cycle.

To probe the yeast [H^+]-ATPase by site-directed mutagenesis, we have devised a novel expression system (2) in which the final step of plasma membrane biogenesis is blocked by a temperature-sensitive *sec6* mutation, leading to a massive accumulation of secretory vesicles when the cells are shifted to 37°C. At the same time, regulated promoters are used to switch expression from the wild-type chromosomal copy of the *PMA1* gene (required for growth) to the mutant copy (introduced on a plasmid). The mutant ATPase becomes trapped in the secretory vesicles, which are readily isolated and assayed for ATP hydrolysis and H^+ pumping.

Using this strategy, we have explored structure-function relationships within the highly conserved phosphorylation domain of the ATPase:

(376) C S D K T G T L T

Three different substitutions of the phosphorylated residue (D378N, S, and E) led to a surprisingly drastic defect in ATPase structure and biogenesis. In each case, significant amounts of newly synthesized mutant ATPase could be immunoprecipitated from a total membrane fraction, but did not reach the secretory vesicles; instead, it accumulated at an earlier point in the secretory pathway (probably the Golgi). Partial trypsinolysis suggested that the block in biogenesis was due to an abnormal, incompletely folded conformation. Interestingly, all three D378 alleles behaved as dominant lethals, consistent with the idea that the mutant and wild-type polypeptides interact with one another or with limiting amounts of some component of the secretory pathway.

For the remaining residues that were studied, nonconservative substitutions (e.g., K to Q, T to A) led to inactive enzyme that was, at least in part, capable of reaching the secretory vesicles. Even conservative substitutions (K379R, T380S, T382S, T384S) reduced ATP hydrolysis and H^+ pumping to 16-45% of wild-type levels. There were only minor changes in K_m for ATP and pH optimum. By contrast, the mutant enzymes were all significantly less sensitive to vanadate, with 3- to >500-fold elevation of K_i. The simplest interpretation is that the residues immediately adjacent to D378 form part of the phosphate/vanadate binding site; alternatively, replacement of these residues may shift the equilibrium between the E1 and E2 conformations of the enzyme, altering vanadate sensitivity indirectly. Further work will be required to distinguish between these possibilities.

1. Serrano, R., Kielland-Brandt, M.C., and Fink, G.R. (1986) Nature 319, 689-693.
2. Nakamoto, R.K., Rao, R., and Slayman, C.W. (1991) J.Biol.Chem. 266, 7940-7949.

REGULATION OF THE EXPRESSION OF FOUR GENES ENCODING THE PLASMA MEMBRANE ATPase OF TOBACCO

Boutry, M., Bogaerts, P., Michelet, B., Moriau, J. and Perez, Ch.
Unité de Biochimie Physiologique, University of Louvain
Place Croix du Sud, 2-20, B-1348 Louvain-la-Neuve, Belgium

The plasma membrane H^+-ATPase from *Nicotiana plumbaginfolia* is encoded by two gene subfamilies which diverged before the separation of the genera Dilleniidae and rosidae. The first subfamily is composed of three genes (*pma1-3*) whose identity at the protein level is close to 96% (Boutry *et al.*, 1989; Perez *et al.*, 1992). However analysis of their transport level in different organs indicated that they are differentially expressed both in a quantitative and a qualitative fashion (Perez *et al.*, 1992). The second subfamily seems to only contain a single gene (*pma1*) which is 92% identical (at the protein level) to *pma1-3*.

A more precise analysis of the regulation at the tissue or cellular level of the *pma* genes was undertaken by two complementary approaches. *In situ* hybridization was conducted with nucleic acid probes specific to each gene. On the other hand, *pma* transcription promoters were fused to the *gus* reporter gene and introduced in tobacco by genetic transformation. GUS activity was revealed in situ by a histochemical reaction. These approaches revealed that some genes are specifically expressed in particular tissues, some of them being engaged in active transport processes (ovules, pollen, transmission tissues, epidermal cells of the active area of the root system,...).

Transcript mapping revealed signs of a possible regulation at the translation level: the mRNAs have an unusually long leader (transcribed and untranslated 5' region of the mRNA) containing a small upstream open reading frame (uORF) of 5 to 10 codons according to the gene. Chimeric genes were prepared by an in frame fusion of the *gus* reporter gene to the uORF or to the PMA reading frame of *pma1* in order to compare the translation efficiency of both cistrons. Transgenic plants revealed that indeed the ratio of the expression of both constructions varies according to the organ. For instance, in some flower tissues, the uORF-GUS was expressed much more than the PMA-GUS, suggesting a regulation mechanism by which ribosomes preferentially translate either the uORF or PMA.

Finally, to better understand the role of the plasma membrane H^+-ATPase in the plant physiology, we introduced antisense genes controlled by a strong transcription promoter (CaMV 35S). Preliminary observations of transgenic plants showed that plants with antisense RNA specific to *pma3* were modified in their growth rate in the presence of synthetic media containing different salt concentrations.

References

Boutry, M., Michelet, B., and Goffeau, A. (1989) Biochem. Biochim. Res. Comm. **162**, 567-574.

Perez, C., Michelet, B. and Boutry, M. (1992) J. Biol. Chem. **267**, 1204-1211.

The Plasma Membrane H+-ATPase Multigene Family in *Arabidopsis thaliana*.

Harper[1], Jeffrey F. and Sussman[2], Michael R. [1]Dept. of Cell Biology, Scripps Research Institute / MB8, 10666 N. Torrey Pines Rd., La Jolla CA. 92037. [2] Dept. of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, WI. 53706.

In plant cells, the plasma membrane H+-ATPase is thought to drive numerous cotransport systems by generating a proton gradient across the membrane. At least 10 putative plasma membrane H+-ATPase isoforms (AHAs) have been identified in *Arabidopsis* by DNA cloning. Some of these isoforms appear to have unique tissue specific patterns of expression. GUS-reporter gene analysis indicates that isoform AHA3 is expressed primarily in the phloem tissue of both root and shoot organs (1). The expression pattern for isoform AHA2 appears to be very different. Northern blot analysis indicates that isoform AHA2 is expressed primarily in roots (2). GUS-reporter gene analysis further indicates that within the root AHA2 is expressed in the epidermal and cortical cell layers. In shoot structures, AHA2 expression is strongest in leaf hydathodes and various parts of the flower. Analysis of regulatory sequences controlling the expression of AHA2 indicate that sequences associated with the first large intron function to significantly increase expression of AHA2 in non-root tissues. Expression patterns for two AHA2/GUS translational fusions, 2P and 2PI, were compared in multiple transgenic *Arabidopsis* plants; 2PI differs from 2P by inclusion of the first large intron and 14 additional amino acids (*i.e.* up to residue E_{21}). Strong expression was observed in roots of both 2P and 2PI plants. However in leaves, only 2PI plants displayed strong activity -- activity in 2P plants was near background levels.

It is not known how activity of a plant H+-ATPase is regulated, but speculation has focused on a putative autoinhibitor located in the C-terminus. In 9 plant H+-ATPase isoforms examined, two conserved motifs are present in the C-terminus with 80% to 85% identity to AHA2. One of these regions, A_{859} to Q_{886} of AHA2, has been proposed to function as an autoinhibitor, based on *in vitro* inhibition experiments using a peptide corresponding to this domain (3). One new isoform, AHA10, has recently been identified and appears distinct from other isoforms. In AHA10 the two "conserved regions" of the C-terminus are only 30% to 35% identical to other isoforms. This difference may indicate that activity of this isoform is regulated by a mechanism distinct from other plant H+-ATPases.

1. DeWitt, N.D., Harper, J.F., and Sussman, M.R. (1991). *The Plant Journal* **1**:121-128.
2. Harper, J.F., Manney, L., DeWitt, N.D., Yoo, M.H., and Sussman, M.R. (1990). *The Journal of Biological Chemistry* **265**; **23**:13601-13608.
3. Palmgren, M.G., Sommarin, M., Serrano, R., and Larsson, C. (1991). *The Journal of Biological Chemistry* **266**; **30**:20470-20475.

4

REGULATION OF PLASMA MEMBRANE H$^+$-PUMPING BY MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE

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Plasma membrane (PM) H$^+$-ATPase is tightly bound and integral transmembrane protein. We decided the nucleotide sequence of full length cDNA and deduced amino acid sequence of rice PM-ATPase (1). Participation of this enzyme in H$^+$ transport might be confirmed by proteoliposomes. During processes of purification and reconstitution of the PM-ATPase, the enzyme was found to require a molecular species of phosphatidylcholine (PC) for activation (2, 3). In this study, the dependence on phospholipids of H$^+$ pumping was examined by incorporating the enzyme into liposomes of different phospholipid composition (4), with subsequent comparison of the effects of molecular species of PC on the solubilized ATPase activity (3).

The activity of PM-ATPase from rice (Oryza sativa L.) culture cells decreased with increase in the length and the degree of saturation of acyl chain of PC. Remarkable activation was observed when PC posessing 1-palmitoyl (16:0)-2-oleoyl (18:1) fatty acid was added to the reaction mixture. H$^+$-pumping be could detected in proteoliposomes when they were prepared with PC with 1- or 2-unsaturated and 2- or 1-saturated fatty acid, such as 1-palmitoyl-2-oleoyl-PC, but hardly in that with 1-, 2-unsaturated or 1-, 2-saturated fatty acid such as 1-palmitoyl (16:0)-2-stearoyl (18:0)-PC or dilinolenoyl (1-18:3 2-18:3)-PC.

These results indicate H$^+$-pumping to be closely related to ATPase activity and strongly dependent on the nature of lipid polar head group, the length and the degree of saturation of the acyl chains of PC.

1. Wada, M., Takano, M., and Kasamo, K. (submitted)
2. Kasamo, K. and Nouchi, K. (1987) Plant Physiol. 83. 323-328.
3. Kasamo, K. (1990) Plant Physiol. 93, 1049-1052.
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REGULATION OF PLASMA MEMBRANE TRANSPORT ACTIVITY IS DEPENDENT ON A PHOSPHORYLATION / DEPHOSPHORYLATION CYCLE

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The internodal cell surface of *Chara corallina* exhibits a characteristic banding pattern of alternating acid and alkaline regions. This activity has been attributed to the operation of a Class II H^+ transporter acting as a H^+-ATPase in the acid regions and a highly controlled H^+ channel in the alkaline areas (1). These regions of specialized plasma membrane transport activity are sensitive to changes in light intensity. Upon removal of light for brief periods of time the extracellular current pattern is reduced and the membrane potential hyperpolarizes by 30-70 mV. Light activation, the return of the extracellular current pattern to the original intensity, occurs within 3-6 min of reillumination while a transient depolarization of the membrane potential is followed by a return to the original resting potential. A new system has been designed to simultaneously measure the extracellular current density and the membrane potential to elucidate the cellular basis for spatial and temporal control of light activation (2). Our model for the signal transduction pathway of light activated transport function proposes a putative phosphorylation / dephosphorylation cycle that controls the functional state of transporters at the plasma membrane. By the application of a phosphatase inhibitor, or a kinase activator, we can alter the phosphorylation status of cellular proteins and follow the subsequent changes in membrane potential and extracellular current pattern. Incubation of cells in okadaic acid (2.5 μM), a potent inhibitor of serine/threonine phosphatases, resulted in a three-fold reduction in the rate of light activation as compared to control cells. Illuminated cells incubated for 1h with 50 μM 1,2-dihexanoylglycerol (1,2-DG6), a synthetic diacylglycerol that activates protein kinase C, did not exhibit a change in the membrane potential or the extracellular current pattern. However, these same cells, when placed in the dark for 6 min and then reilluminated, had a 100% inhibition of light activation and an uncharacteristically slow depolarization and subsequent unstable establishment of a new hyperpolarized resting potential. As a control, we incubated cells with 1,3-dioctanoylglycerol (1,3-DG8), an isomer of the protein kinase activating diacylglycerol(1,2-DG8) that does not stimulate kinase activity. 1,3-DG8 (74 μM) had no effect on either the membrane potential or the extracellular current pattern during long term illuminated exposure or light activation after a 6 min dark treatment. These results strongly suggest that light dependent recovery of the plasma membrane transport activity is dependent on a dephosphorylation event that is inhibited by okadaic acid or overriden by the enhanced phosphorylation activity in the presence of diacylglycerol analogs.

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A BIOCHEMICAL CHARACTERIZATION OF THE H^+-ATPase AND OF THE Ca-ATPase IN PLASMA MEMBRANE PURIFIED FROM ARABIDOPSIS THALIANA

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Arabidopsis thaliana is the plant material of choice for genetic and molecular biological studies due to its small genome and short life cycle. However its small size poses serious problems to biochemical studies, specially of relatively low abundant and labile membrane proteins.

Here we report on the purification of a plasma membrane (PM) fraction from Arabidopsis seedlings by the phase partitioning technique. This procedure allows the recovery of a highly purified PM fraction with a reasonably high yield (about 3 mg prot per 100 g fr wt of three days old seedlings).

Both the specific activity (about 1 μmol Pi x min^{-1} x mg^{-1} prot) and the biochemical characteristics of the PM H^+-ATPase activity are very similar to those of the enzyme from other plant materials. Also the transport and hydrolytic activity of the PM Ca-ATPase (about 140 nmol Pi x min^{-1} x mg^{-1} prot) have characteristics very similar to those observed in PM isolated from other plant materials.

Comparison between the effects of different fluorescein derivatives on the two ATPase activities shows that also in Arabidopsis the Ca-ATPase is much more sensitive to inhibition by fluorescein derivatives than the H^+-ATPase. Moreover, the effectiveness of the different derivatives tested is very different for the two enzymes: the sequence of inhibitory efficiency is eosin B > erythrosin B > rose bengal for the Ca-ATPase and the opposite for the H^+-ATPase. Thus, using eosin B or erythrosin B it is possible to very selectively inhibit the Ca-ATPase in the PM.

The possibility to obtain from Arabidopsis seedlings a PM fraction suitable for biochemical studies will be of great advantage since it will allow the integration of the biochemical, genetic and molecular biological approaches to the study of the PM H^+-ATPase and Ca-ATPase as well as of other PM proteins.

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COMPARISON OF Ca^{2+} ATPase ACTIVITIES IN PURIFIED PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM FROM WHEAT

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The cytoplasmic free calcium concentration in higher plant cells is below 1 μM, but may be transiently raised as a response to hormonal or environmental signals. The maintenance of the low cytoplasmic Ca^{2+} concentration against the high organellar and extracellular levels (mM range) requires highly efficient transport systems for sequestering Ca^{2+} into organelles or mediating its efflux out of the cell. It is yet not clear under which physiological conditions the respective transport systems operate most efficiently. We have therefore compared ATP-dependent Ca^{2+} transport in highly purified vesicles of plasma membrane and endoplasmic reticulum, respectively, from wheat (*Triticum aestivum* L.).

Sealed inside-out plasma membrane vesicles were purified by counter-current distribution in an aqueous polymer two-phase system, and the ATP-binding site of the Ca^{2+} transport system in plasma membranes was confirmed to be situated on the inner cytoplasmic surface (1). Pure endoplasmic reticulum was obtained by sucrose gradient centrifugation (2). In the presence of ATP a rapid accumulation of $^{45}Ca^{2+}$ was seen in inside-out plasma membrane vesicles, as well as in endoplasmic reticulum vesicles, while no appreciable uptake was observed in the absence of the nucleotide. The Ca^{2+} ionophore A23187 caused a quick release of 90% of the Ca^{2+} accumulated, indicating that the major part of the Ca^{2+} was loaded into the vesicles as free Ca^{2+}. We will present data on the characterization and comparison of these Ca^{2+} transporting systems.

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RECONSTITUTION, CHARACTERIZATION AND CELLULAR LOCALIZATION OF A CALMODULIN-STIMULATED Ca^{2+}-PUMPING ATPase PURIFIED FROM *BRASSICA OLERACEA* L.

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A calmodulin (CaM)-stimulated Ca^{2+}-ATPase was purified about 100-fold from cauliflower (*Brassica oleracea* L.) microsomes using CaM-affinity chromatography. The purified fraction showed a Coomassie-stained polypeptide at 115 kDa together with several polypeptides with lower M_r. Only the 115 kDa polypeptide formed a phosphorylated intermediate. The ATPase was reconstituted into liposomes by CHAPS-dialysis. The proteoliposomes showed ATP-dependent Ca^{2+} uptake and ATPase activity, both of which were stimulated about 4-fold by CaM. The specific ATPase activity was high [ca 5 μmol min^{-1} (mg protein)$^{-1}$] and the Ca^{2+}/ATP ratio was 0.1 when the ATPase was reconstituted with entrapped oxalate. Both ATPase activity and Ca^{2+}-pumping by the reconstituted enzyme were characterized with respect to inhibitor and ionophore sensitivity, stimulation by mono- and divalent cations (including Ca^{2+}) and CaM, nucleotide specificity and pH dependence.

Plasma membranes of high purity obtained from the microsomal fraction by two-phase partitioning showed a 2-fold stimulation of ATP-dependent Ca^{2+} transport by CaM. However, an intracellular membrane fraction depleted in plasma membranes showed an even stronger (ca 4-fold) stimulation by CaM and about 90% of the total CaM-stimulated Ca^{2+} transport was present in this fraction. After separation of the microsomal membranes on a continous sucrose gradient the peak of CaM-stimulated Ca^{2+} transport coincided with the lighter of two peaks of antimycin A-insensitive NADH-Cyt c reductase activity (an ER marker) at a density of 1.12 g ml^{-1}, but a significant part of the CaM-stimulated Ca^{2+} transport was found on the very top of the gradient where no or very little NADH-Cyt c reductase activity could be detected. The protonophore FCCP had no effect on CaM-stimulated Ca^{2+} transport in any of the fractions from the sucrose gradient, indicating that a Ca^{2+}/H^+ antiporter was not involved. In summary our data indicate that CaM-stimulated Ca^{2+}-pumping ATPases are present in at least two types of membranes in *Brassica oleracea*, the plasma membrane and the endoplasmic reticulum.

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BIOPHYSICAL AND GENETIC STUDIES ON THE ROLE OF ROOT-CELL ION TRANSPORT PROCESSES IN ALUMINUM TOXICITY AND TOLERANCE

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Aluminum (Al) toxicity is the primary environmental stress limiting crop and forest productivity on acid soils. Despite the considerable research effort that has been directed towards this problem, the cellular basis for Al toxicity and the mechanisms involved in Al tolerance are still poorly understood. It is often suggested in the literature that Al-induced disruptions in root-cell ion transport processes are a fundamental aspect of Al phytotoxicity. We have been investigating the influence of Al exposure on root ion transport processes in both wheat cultivars that exhibit dramatic genetic differences in Al sensitivity, and near-isogenic wheat lines where tolerance or sensitivity is linked to single-gene differences. In this study, we have used vibrating voltage and ion-selective microelectrode techniques to investigate the influence of Al exposure on various root-cell ion transport processes during the initial stages (< 1 hr) of Al phytotoxicity.

Using both static ion-selective microelectrodes and a sensitive vibrating ion-selective microelectrode system, we demonstrated that under conditions of Al exposure that ultimately resulted in toxicity symptoms, Ca^{2+} influx into cells of the root apex was immediately inhibited by Al exposure. This was the only ion flux influenced by Al during the time period prior to the onset of toxicity symptoms. The rapidity of this inhibition and the rapid reversal of the inhibition upon removal of Al from the bathing solution suggest that Al is acting extracellularly, at the plasmalemma surface. Concentration-dependent kinetic analyses of the Al-Ca interactions were consistent with competitive inhibition of Ca^{2+} influx by aluminum. These results suggest that Al is blocking Ca^{2+} channels in the plasmalemma of root cells. The Al-induced inhibition of Ca^{2+} transport in root apical cells was closely correlated with Al toxicity and differential Al tolerance in both wheat cultivars that differed greatly in their Al-sensitivity, and near isogenic wheat lines where tolerance was associated with a single gene. Based on these results, we have hypothesized that the ability of root cells to resist Al-induced blockage of Ca^{2+} channels is an important aspect of Al tolerance. This hypothesis is currently being investigated further at the single cell and membrane levels.

As a starting point for the identification of Al tolerance genes, we have been screening for Al resistance mutants in *Arabidopsis*. In preliminary screening studies of ~90,000 EMS-mutagenized M2 generation seedlings, we have identified 16 putative mutants in which root growth is resistant to Al. Two of the putative mutants, which we call *alr-3* and *alr-4*, have been rescreened in the M3 generation and are quite resistant to Al, showing considerable root growth in the presence of toxic levels of Al. Following genetic characterization of the mutants, molecular studies will be conducted aimed at identifying and cloning the genes responsible for Al tolerance in these mutants. If these genes can be found, their identification will provide the key for understanding Al tolerance.

A STUDY OF THE PHYSIOLOGY OF HALOTOLERANCE IN *DUNALIELLA* USING MUTANTS

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Research into the extraordinary halotolerance of the unicellular green alga *Dunaliella* has been largely directed towards elucidation of mechanisms of osmoregulation. The question arises whether this is enough to explain the ability of Dunaliella cells to grow at very high salt concentrations (>4M NaCl) and/or tolerate big changes in salt concentration or whether there are specific sensitivities to the ions Na^+ and Cl^- in terms of modified membrane properties, ion transport systems or other cellular systems.

The control of halotolerance may be assumed to be at least in part at the gene level. To test this assumption protein profiles of cells grown at high and low salt concentrations were compared and an extra polypeptide of 140-160 kDa has been found in high salt cells (1,2). A second approach has been to develop mutants with altered degrees of halotolerance. Two mutants isolated in our laboratory grow at roughly the same rate in 0.5M NaCl; one (low-salt mutant) does not grow above 1M NaCl while the other grows faster than the wild type at high concentrations. The low-salt mutant synthesizes glycerol at the same rate as the wild-type when transferred to 1M or 1.5M NaCl but appears to have a high osmotic-water permeability (Lp) and an impaired system of volume control. Na^+ and Cl^- measurements will be described. The correlation of all the physiological processes known to be involved in halotolerance in *Dunaliella* will be discussed in the light of the results.

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NACL REGULATION OF PLASMA MEMBRANE AND TONOPLAST ATPASE GENE EXPRESSION IN GLYCOPHYTES AND A HALOPHYTE DURING SALT ADAPTATION

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The detrimental effects of salinity are the result of both water deficit and the deleterious actions of ions on critical physiological and biochemical processes. Plants cope with salinity by regulating the rate and degree of salt exposure to cells that are actively growing and photosynthesizing and through the intracellular accumulation and compartmentation of inorganic and organic solutes for osmotic adjustment. It is presumed that in saline environments there is increased H^+-electrochemical gradient demand to facilitate transport across the plasma membrane and tonoplast. We have therefore focused on the regulation of plasma membrane and tonoplast H^+-ATPase gene expression in glycophytes and the halophyte *Atriplex nummularia* during and after NaCl adaptation. Increased plasma membrane and tonoplast (70 kDa subunit) H^+-ATPase mRNA levels were detected in cultured cells in response to NaCl treatment. The NaCl responsiveness is apparently developmentally dependent since cells in the rapid expansion phase had the greatest accumulation of ATPase transcripts in response to NaCl treatment. Cells that have been previously adapted to NaCl but have been returned to medium without NaCl (deadapted cells), accumulated higher levels of ATPase mRNAs in response to salt than unadapted cells. The deadapted cells also have a greater capacity for salt adaptation, increased K^+ uptake and reduced Na^+ uptake. When adapted and growing in a saline environment, cells accumulated the same levels of ATPase mRNAs as unadapted cells suggesting that NaCl adapted cells have H^+-electrochemical gradient requirements under steady state ion transport conditions similar to those of unadapted cells. In the glycophyte tobacco, plasma membrane H^+-ATPase mRNA did not accumulate in roots or leaves in response to NaCl. However, in *A. nummularia* plants, the plasma membrane H^+-ATPase mRNA increased during NaCl adaptation in roots but not in leaves or stems. This differential regulation between glycophyte and halophyte roots could reflect a greater ability of halophytes to regulate symplastic Na^+ uptake into roots through an energy dependent plasma membrane efflux mechanism coupled to the H^+ electrochemical gradient. The 70 kDa subunit mRNA was induced by NaCl in old leaves but not in roots or young leaves of a glycophyte indicating that in saline environments promoting vacuolar compartmentation of ions in older leaves involves the tonoplast H^+-ATPase.

IDENTIFICATION OF THE VACUOLAR Na^+/H^+ ANTIPORT

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Vacuolar sodium accumulation is important for the survival of plants in saline environments. The vacuolar Na^+/H^+ antiport has been characterized in sugar beet (Beta vulgaris) (1). The increase in the NaCl concentration in the growth media resulted in an enhanced Na^+/H^+ exchange activity that was correlated with the increased synthesis of a 170 kDa tonoplast polypeptide. A polypeptide with the same molecular mass was also found to incorporate [^{3}H]-MIA, an amiloride analog (2). Pretreatment of beet tonoplast vesicles with antibodies generated against this polypeptide completely inhibited the Na^+/H^+ antiport activity (3). Western analysis of tonoplast proteins revealed that the antibody specifically precipitated the 170 kDa polypeptide in beets and barley, but showed no cross reaction in tomato (a glycophyte that lacks vacuolar antiport activity). Gene expression at the mRNA level was investigated in cells grown in different salt concentrations. In vitro translation products were purified by immunoprecipitation and compared with tonoplast proteins synthesized in vivo in the presence of increasing NaCl concentrations. The use of the 170 kDa polypeptide antibody for immunopurification and reconstitution, and for the molecular cloning of the antiport will be discussed.

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CHARACTERIZATION OF THE CHL1 GENE FROM *ARABIDOPSIS*

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One can isolate mutants that are defective in nitrate assimilation by selecting for resistance to chlorate, the chlorine analog of nitrate. Almost all chlorate resistant plants have been found to be defective in chlorate and nitrate reduction (nitrate reductase deficient). Mutations at the *chl1* locus of *Arabidopsis*, however, result in defects in chlorate and nitrate uptake and have little effect on nitrate reduction (1). It is possible that the *CHL1* gene encodes a transport protein that facilitates nitrate and chlorate uptake.

We have obtained a *chl1* mutant from a population of T-DNA-transformed *Arabidopsis* plants that contain a T-DNA insert at or near the *chl1* locus. DNA sequences flanking the T-DNA insert were cloned from wildtype plants and shown to contain a gene that encodes a 2 kb mRNA. Using RFLP markers, we found that the cloned gene mapped to the top of chromosome 1 where *chl1* is located. In addition, a *chl1* mutant was identified in which the cloned gene was deleted. The Chl1$^-$ phenotype of the deletion mutant could be complemented with a wildtype copy of the gene. Thus, the gene we cloned from the T-DNA insertion mutant is the *CHL1* gene.

Analysis of the sequence and expression of the *CHL1* gene has provided support for the hypothesis that it encodes a transporter. The predicted protein is very hydrophobic and may contain 12 transmembrane segments. *CHL1* mRNA is found primarily in roots and is regulated by nitrate. Experiments are now in progress to study the activity of the *CHL1* gene product in heterologous systems.

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ABSCISIC ACID-INDUCED INOSITOLPHOSPHOLIPID TURNOVER IN GUARD CELL PROTOPLASTS OF VICIA FABA

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Abscisic acid (ABA), a phytohormone stimulating stomatal closure, elevates cytosolic [Ca^{2+}] in a subpopulation of plant guard cells (1). Microinjected inositol 1,4,5-trisphosphate ($InsP_3$), one of the second messengers of phosphoinositide cycle, elevates cytosolic [Ca^{2+}] and stimulates stomatal closure in Commelina communis (2). Also, this $InsP_3$ closes inward K^+-channels in plasma membrane of guard cells of Vicia faba (3). Therefore, stomatal closure could be stimulated by ABA via $InsP_3$, released from hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$).

We have quantified the changes in the level of phospholipids of guard cell protoplasts of Vicia faba during 10, 60, 180, 300, and 600s of ABA treatment. The phospholipids in guard cells were labeled by incubating the cells in ^{32}[P]-orthophosphate-containing medium, separated by TLC, identified by co-separation with unlabeled standards, and the radioactivity in each band determined.

Upon treatment with ABA, inositolphospholipid levels in guard cells changed by up to 20-30%. $PtdInsP_2$ levels initially decreased (at 10 sec) followed by a gradual increase (60 to 180 sec). A second large decrease in $PtdInsP_2$ levels was observed at 300 sec of treatment, perhaps indicating a second pulse of lipid hydrolysis. Changes in the levels of phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate (PtdInsP) were consistent with our hypothesis that ABA initiates a cascade of events leading to pulsatile $PtdInsP_2$ hydrolysis which is rapidly followed by activation of PtdIns 4-kinase and PtdIns(4)P 5-kinase and $PtdInsP_2$ resynthesis. In contrast, phosphatidylcholine and phosphatidylethanolamine levels changed less than 5% compared over the entire course of ABA treatment (figure).

In Vicia faba and Commelina communis, it has been reported that only one-third of guard cells elevate cytosolic [Ca^{2+}] upon ABA treatment (1,4). Thus it is possible that hydrolysis of $PtdInsP_2$ occurs in a subpopulation of guard cells only. Then the extent of changes in $PtdInsP_2$ level in the subpopulation of cells may be much larger than we observed in the analysis of whole population. Our results suggest that inositol phospholipid turnover may play an important role in one intracellular signal transduction pathway mediating ABA and stomatal closure in Vicia faba.

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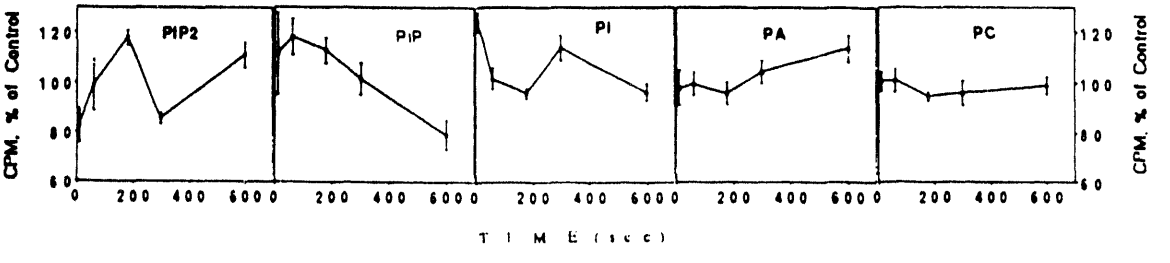

Figure. ABA-induced changes in the phospholipid levels in guard cell protoplasts of Vicia faba. Averages and standard errors from 5 separate experiments are plotted.

RECEPTORS COUPLED TO GUANINE NUCLEOTIDE-BINDING PROTEINS IN HIGHER PLANT CELLS.

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Recently, a gene, *GPA1*, coding for a heterotrimeric G protein α subunit has been cloned and sequenced in *A. thaliana* (1). Affinity chromatography of detergent solubilised microsomal membranes from etiolated *Z. mays* hypocotyls was carried out using a 16-amino acid peptide corresponding in sequence to the C-terminus of the *A. thaliana GPA1* gene product as affinity ligand. Solid-phase microsequencing of a 37 kDa protein, which was present throughout column elution with a 0-2 M NaCl gradient, allowed identification of 12 N-terminal hydrophobic amino acids which did not show homology with any protein sequences currently within the OWL composite protein database. However, due to its hydrophobicity, this sequence could act as part of a membrane-spanning domain. Since there is evidence indicating that receptors interact with the C-terminal region of G protein α subunits (2,3) it may be that this protein represents a G protein-coupled receptor. Secondly, the binding of [^{35}S]GTPγS to *P. sativum* plasmalemma was found to be stimulated by 50% in the presence of the mastoparan-related amphiphilic tetradecapeptide, Mas 7. The latter is known to activate mammalian Gα subunits (4), possibly by mimicking the third cytoplasmic loop of G protein-coupled receptors. This data indicates that G proteins in plants may be regulated in an analogous manner to their mammalian counterparts.

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CALCIUM-DEPENDENT PROTEIN KINASES AND THEIR ROLES IN SIGNAL TRANSDUCTION

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Calcium-dependent protein kinase (CDPK) is present in numerous plant and algal species (5). In flowering plants it is found in roots, stems, cotyledons, leaves, and pollen. Immunological and biochemical evidence shows that CDPK is associated with the plasma membrane (6), F-actin (3), and the symbiosome membrane (7). Molecular genetic evidence indicates that there are several CDPK isoenzymes (1, 2, 5). The existence of a family of calcium-dependent protein kinases having different substrate specificities, different sensitivities to calcium and/or other regulators, and different cellular locations would help explain the involvement of calcium ions in diverse signal transduction pathways.

One role that CDPK may play is to mediate the inhibition of cytoplasmic streaming by Ca^{2+}. The distribution of CDPK in the green alga *Chara* is similar to that of myosin (4), a mechanoenzyme which is thought to be responsible for the movement of the endoplasm and organelles along F-actin cables. In vitro CDPK phosphorylates the regulatory light chain of smooth muscle myosin. In perfused internodal cells of *Chara*, a 16-18 kDa protein tentatively identified as a myosin light chain is phosphorylated in a calcium-dependent manner. Phosphorylation of this protein is blocked by previous perfusion with monoclonal antibodies directed against soybean CDPK. Taken together these data support the hypothesis that the calcium-stimulated phosphorylation of myosin inhibits cytoplasmic streaming.

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THE POSSIBLE MOLECULAR BASIS OF A BLUE LIGHT RESPONSE

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Blue light leads to the phosphorylation of a hydrophobic plasma membrane protein ranging in molecular mass from 114 to 130 kDa in etiolated tissues of all species tested to date. An *Arabidopsis thaliana* mutant showing impaired phototropism shows impaired light-driven phosphorylation. Progress in sequencing and characterizing this protein from pea will be reported. The reaction can be driven in vivo, in vitro in highly purified plasma membrane vesicles, or in plasma membrane vesicles solubilized with non-ionic or zwitterionic detergents. Evidence is presented that the photoreaction is flavoprotein-mediated, and that the action of light is to activate the kinase moiety, rather than to expose substrate sites for phosphorylation by a constitutive kinase. The reaction requires three elements: the phosphorylation substrate, the kinase activity, and the photoreceptor moiety. We are currently addressing the question as to whether one, two, or perhaps three or more polypeptides are involved. Preliminary evidence indicates that the substrate protein contains an ATP-binding site--a prerequisite for kinase activity--and a covalently bound flavin--a prerequisite for photoreceptor activity. We are testing the hypothesis that all three elements reside with a single polypeptide.

A MALATE-SELECTIVE ION CHANNEL AT THE VACUOLAR MEMBRANE OF CAM PLANTS

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Malate is an abundant anion in plant vacuoles, often being the major anionic solute involved in charge balance with inorganic cations such as K^+ and Ca^{2+}. Following its synthesis in the cytosol, malate must be transported across the tonoplast into the vacuole, but the molecular pathway for movement of malate has not been fully clarified. We have been studying plants showing crassulacean acid metabolism (CAM), in which malate accumulates to high concentrations in the vacuole as a result of nocturnal fixation of CO_2. The aim of these experiments has been to establish the transport mechanism for malate influx across the tonoplast.

We have applied the patch-clamp technique to isolated vacuoles of the CAM plant *Kalanchoë daigremontiana* to record the electrical currents associated with malate transport. In symmetrical solutions containing malate as the only permeant ion, large currents indicative of malate influx could be detected in the whole-vacuole configuration at inside-positive potentials (averaging 1.96 nA at +100 mV). Recordings from isolated patches of membrane revealed the presence of small single-channel currents corresponding to a channel with a mean unitary conductance of 3.4 pS in symmetrical 100 mM malate solutions. The channel had a relatively high open probability, a high area density, and accounted quantitatively for the characteristics of the whole-vacuole currents. Further, the anion dependence of these currents showed the same selectivity sequence as found in experiments with tonoplast membrane vesicles.

The properties of this inward-rectifying malate channel are distinct from other channels in the tonoplast that can conduct anions, such as the well-characterized SV and FV channels. We believe it represents a novel type of voltage-gated ion channel responsible for malate influx across the tonoplast in CAM plants.

CALCIUM CHANNELS AT THE TONOPLAST

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Calcium release from the vacuole is thought to play a central role in several types of stimulus-response coupling. We report here the characterization of two Ca^{2+} channels, which both mediate entry of Ca^{2+} to the cytosol from the vacuole.

An inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} pool in plants is associated with the vacuolar membrane (tonoplast) (1). Using tonoplast-enriched microsomes from beet storage root, we demonstrate that the properties of Ca^{2+} release are remarkably similar to those of $InsP_3$-elicited Ca^{2+} release in animal cells, despite the fact that the two receptors are localised at different endomembranes. Thus the plant and animal systems resemble each other with respect to $K_{0.5}$ for $InsP_3$, $InsP_3$ specificity, so-called "quantised release" and inhibition by TMB-8 and by low M_r (4 - 6 kDa) heparin (2). Inhibition by heparin is competitive with $InsP_3$, and the derived K_i is 34 nM. The potency of heparin decreases dramatically as the degree of polymerization in increased.

Characterization of $InsP_3$-specific binding to beet membranes is not possible: formate anion exchange chromatography reveals extensive metabolism of $InsP_3$. The $InsP_3$ receptor from beet storage root was therefore solubilized, and its ligand-binding properties studied by displacement of [^{3}H]-$InsP_3$. $InsP_3$-specific binding accounts for around 40% of total $InsP_3$ binding, is optimal in alkaline conditions and co-purifies with tonoplast. Scatchard plots of $InsP_3$-specific binding yield a single binding site of K_d 56 nM and B_{max} = 8 fmol/mg in microsomes, rising to 30 fmol/mg in partially-purified tonoplast. Low M_r heparin also competes for the $InsP_3$-specific binding site. All these properties - with the exception of membrane location - confirm the similarities of the plant and animal $InsP_3$ receptors.

We have also identified a second pathway for Ca^{2+} release in the tonoplast-enriched microsome preparation (3). This pathway is voltage-sensitive, $InsP_3$- and heparin-insensitive and, unlike $InsP_3$-elicited Ca^{2+} release, is potently inhibited Gd^{3+}. The location of the voltage-sensitive pathway has been confirmed by patch clamp of intact vacuoles, where a voltage-gated, Ca^{2+}-selective channel bearing a similar K_i for Gd^{3+} has been discovered. Possible reasons for the evolution of discrete pathways for Ca^{2+} release from higher plant vacuoles will be discussed.

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EPIDERMAL MECHANOSENSORY CALCIU' 'ELECTIVE CF NNELS DETECT TRANSMEMBRANE POTENTIAL, TEMPERA E, "MURAL" AND AUXIN

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Epidermal cells of onion leaf sheath and bulb scale contain stretch-sensitive Ca^{2+}-selective cation channels. Sensory function is implied by effects of xenobiotics: the lanthanide Gd^{3+} and the cytoskeleton-perturbing herbicide ethyl-N-phenylcarbamate, selective inhibitors for gravitropism, act on the channels at micromolar levels.

The channel system is a complex of linkable equivalent conductance units or "cochannels". As many as 8 or 9 cochannels can open together. Open time for cochannel quadruplets and quintuplets tends to be relatively long with 2 mM Mg^{2+} and no added Ca^{2+} at the cytosolic face. In patches, at least, the channel system "fatigues" or "adapts" when strongly stimulated, and "recovers" when tension is released. Under some circumstances, channel activity is synchronized in rhythmic pulses.

Mechanical tension is the only stimulus known to activate these channels. However, sensitivity to tension is modulated by at least the following four physiological agents (see Posters for details).

1. TRANSMEMBRANE POTENTIAL. In the physiological range, hyperpolarization to more negative V_m reversibly increases stretch-elicited activity. (When membrane potentials are clamped in the positive, nonphysiological, range, however, polarization to more positive V_m enhances sensitivity.)

2. TEMPERATURE. Decrease of temperature in the range ~26°C - ~6°C reversibly increases stretch-elicited activity. When temperature is lowered a degree further, sensitivity drops sharply and irreversibly.

3. AUXIN. Concentrations of ~0.5 μM auxin (indoleacetic acid) appear to enhance sensitivity to tension, while higher levels such as 2 or 3 μM appear to cause decline of sensitivity toward its basal level. Perfusion with 0 μM auxin partially reverses the decline, which may often have a component "fatigue". Repeated reversibility of the effects of each single concentration step must be demonstrated before these interesting results can be interpreted definitively.

4. pH. Within the range 4.5 to 7, lowering pH at the mural membrane face lowers sensitivity. Changes within this range are repeatedly reversible.

While it is mechanistically important that responses to these modulators are self-contained within excised patches, it is physiologically important that suction activates channel currents in protoplasts patched in the whole-cell mode. Further, either touching a current-clamped whole-cell preparation or changing its level of bathing osmoticum can drastically alter V_m and initiate a series of complex changes which, in the latter case, are sensitive to Gd^{3+}. Experiments of this pe may increase understanding of interactions between the several kinds of plasmalemmal channels and of channel roles in vivo. At this moment, the mechanosensory channels seem likely to integrate a variety of signals for a large number of plant processes.

MOLECULAR AND FUNCTIONAL ANALYSIS OF TONOPLAST INTEGRAL PROTEIN (TIP).

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The tonoplasts of many plant cells contain an abundant 27 kD integral membrane protein called tonoplast intrinsic protein or TIP that exists as several isoforms and is encoded by a small gene family. The different members of this family are expressed in an organ specific manner, or in response to certain stimuli. So far four different isoforms have been found: αTIP is an embryo (seed) specific form, γTIP is specific for vegetative and reproductive organs, water stress induced TIP is expressed when plants are dehydrated, and NOD26 is expressed in symbiotic nodules of legumes. Construction of a parsimonious phylogenetic tree of 7 genes shows that sequences from different plants that have similar expression patterns are clustered. The TIP proteins belong to a superfamily that is known as MIP and has representatives in bacteria, yeast, insects and mammals. The prototype protein after which this family is named is the membrane intrinsic protein (MIP) of the gap junctions of bovine lens fibers. The amino acid sequence identity between these different proteins is low (28-35%), but all the proteins have six membrane spanning domains and probably have the same topology with the amino and carboxy termini in the cytosol.

The expression of γTIP in *Arabidopsis thaliana* has been examined in considerable detail using both GUS fusions and in situ hybridisation. The GUS fusions show strong expression in young roots, and elongating stems (especially in the vascular bundles) in stipules and in the receptacle of the flower. There is no expression in meristematic tissues such as root tips or the center of the rosette. In situ hybridisations confirm this pattern and show a very clear correlation in roots and stems with cell elongation. How do we interpret such results? At the time of cell elongation the tonoplast probably acquires new function(s) that are not present in the tonoplasts of the small vacuoles of meristematic cells.

We have taken several approaches to understand the function of TIP: patch clamp experiments with tobacco leaf vacuoles that have transgenic αTIP from bean seeds, and uptake of radiolabeled metabolites by such vacuoles from transgenic tobacco; on Xenopus oocytes injected with α-TIP and γ–TIP mRNA we performed voltage clamp experiments as well as swelling assays. The latter experiments were patterned after similar experiments carried out with CHIP28, a member of the MIP family characterized in erythrocytes (Preston et al, Science 256:385-387, 1992). Xenopus oocytes expressing α–TIP, γ–TIP or CHIP28 when exposed to a hypo-osmotic solution quickly swell and may burst within a few minutes. The swelling of oocytes that express TIP is inhibited by mercuric chloride, a typical inhibitor of water transport in red cells and kidney tubules. We interpret these reults to mean that TIP like CHIP28, is a water channel. The pattern of TIP expression in seeds, elongating cells and as a result of water stress is consistent with the physiological need for rapid water transport into the vacuole.

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OVERVIEW OF INTRACELLULAR ION MEASURMENTS USING FLUORESCENT PROBES

Bush, Douglas, Rutgers University.

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PRINCIPLES OF DYE DESIGN AND USE

Kao, J. , University of Maryland.

TEMPORAL AND SPATIAL MONITORING OF CYTOPLASMIC CALCIUM IN *FUCUS* EGGS AND ZYGOTES: DEVELOPMENTS USING PHOTOMETRY AND CONFOCAL RATIO IMAGING

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A variety of techniques now exist for spatial and temporal monitoring of cytoplasmic calcium. In plant cells, the AM ester and free acid forms of fura-2 and indo-1 have limited applications and interpretation of data obtained with these forms of the dyes needs appropriate caution. Fura-2 and indo-1 both have short cytoplasmic residence times when injected as free acid into *Fucus* eggs and rhizoids. This limits time available for useful measurements of cytoplasmic calcium to 20-30 min following injection.

Dextran-conjugated dyes can largely overcome this problem. Fura-2 dextran (10,000 mr) can be iontophoretically injected into *Fucus* eggs and has been used to monitor cytoplasmic calcium during fertilization. Transient elevations can be observed corresponding to the onset of the fertilization potential. The intracellular behaviour of dextran-linked dyes is critically evaluated.

Longer wavelength calcium indicators, such as fluo-3 and calcium green can potentially be used in most scanning laser confocal microscopes for high resolution imaging. These single wavelength dyes must be used with caution, especially in thick pigmented cells or tissues since attenuation of the exciting laser can give artifactual information. This problem can be largely overcome by ratioing against a calcium-insensitive dye. We have pressure injected constant proportions of calcium green dextran and the pH dye, SNARF dextran into recently fertilized *Fucus* zygotes. SNARF fluorescence emission, monitored at the pH isosbestic point provides a baseline against which to ratio the calcium green signal. This technique has allowed calcium gradient formation to be followed in the same zygote during polarization and germination over 24h. Confocal ratio images show a highly localized Ca^{2+} gradient developing at the growing rhizoid end. Localized elevations can be observed in rhizoid cells of the same age injected with dye either 1 or 24 h previously. Alternative digital deconvolution techniques for high resolution imaging with fura-2 are also discussed.

CONFOCAL FLUORESCENCE RATIO ANALYSIS OF ION CONCENTRATIONS IN PLANT CELLS.

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Confocal scanning laser microscopy (CLSM) permits non-invasive blur-free optical sectioning from intact, living tissues. The volume sampled (voxel) is well defined and quantitative measurements of fluorescence intensity are not corrupted by out of focus information. The realised spatial resolution improves localisation of dye signals in particular sub-cellular compartments. When combined with fluorescence ratio analysis (FRA), CLSM is the optimum solution for quantitative imaging in living cells.

Our BioRad MRC600 CLSM system has been modified to increase the number of excitation wavelengths available in addition to the 488nm & 514nm argon-ion lines. The dual emission UV-dye, Indo-1, was imaged after microinjection into a guard cell using a 325nm He-Cd laser, but biological experiments could not be performed conveniently with the UV-microscope configuration. $[H^+]_i$ and $[Ca^{2+}]_i$ were successfully imaged with alternating dual excitation at 442nm and 488nm and dual channel imaging at 520nm and 600nm using BCECF and Fura-Red. Shuttering between lasers was synchronised to the data collection software (TCSM, ^{TM}BioRad Microsciences Ltd). Sampling rates depended on the size of the image scanned, the number of frames integrated and the disc access time for image storage. Line scans allowed sub-second sampling, whilst 2-D ratio images typically required about 2s. Alternatively, the average ratio from a number of user-defined regions, such as whole cells or areas of the same cell was calculated on-line during the experiment. Graphical presentation of the ratio allowed dynamic changes to be followed during the experiment and synchronised with treatments via annotation of the traces.

Loading of probes into the cytoplasm of plant cells is problematic. Microinjection has proved reliable in a number of tissues and also provides a means to introduce other compounds, such as caged probes. With conventional camera imaging, fluorescence from the dye in the electrode inteferes with measurements from the cytoplasm, unless the electrode can be positioned outside the field of view. Optical sectioning uisng CLSM excludes the signal from dye in the electrode, which can therefore remain in place during fluorescence measurements. This can be exploited for simultaneous electrophysiological recording to allow correlation of ion transients with I/V analysis of membrane transporters.

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ALTERATIONS OF INTRACELLULAR pH DURING BACTERIAL HYPERSENSITIVE REACTION: A CONFOCAL LASER SCANNING MICROSCOPY STUDY.

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During the bacterial hypersensitive reaction (HR) suspension-cultured cells release K^+ and other ions into the surrounding medium and the medium alkalinizes. Atkinson and coworkers (1) hypothesized that the interior of the cell acidifies correspondingly; however, the hypothesis has never been substantiated in cells with intact intracellular compartments. We have demonstrated that the cytosol of suspension cultured cells acidifies during HR using a fluorescent pH probe and Confocal Laser Scanning Microscopy (Bio-Rad MRC-600 confocal system with argon laser.) Cotton suspension cultured cells (*Gossypium hirsutum*, cv Im 216) were loaded with the pH probe SNARF-1 (Molecular Probes, Inc.) prior to treatment with the nonhost bacterial pathogen *Pseudomonas syringae* pv. *tabaci*. A change in fluorescence indicating acidification was observed in some cells as early as 1 h. By 18 h most cells in the bacterially-treated samples exhibited cytosolic acidification in contrast to media-treated controls. These results indicate that fluorescent probes in conjunction with Confocal Laser Scanning Microscopy will facilitate the study of early cytosolic and vacuolar changes during HR.

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MANIPULATION OF INTRACELLULAR ION CONCENTRATION BY CAGED PROBES

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Visualization of the levels of cytosolic ion concentrations using ratio analysis or confocal imaging can reveal the potential role of these ions in the pathways of signal transduction in plant and animal cells. Such studies have already indicated a close association between increases in cytosolic Ca^{2+} and the induction of stomatal closure (1-3). However, these investigations have generally been correlative in nature. For example, although we have visualized localized increases in cytosolic Ca^{2+} associated with the induction of stomatal closure in guard cells of *Commelina communis*, this does not directly indicate the role of these changes in the signal transduction process (2).

However, we have been able to follow the stomatal response in guard cells where Ca^{2+} or inositol 1,4,5-trisphosphate ($InsP_3$) has been released to the cytosol from its caged (photoactivatable) form. Cytosolic Ca^{2+} levels were simultaneously monitored using the fluorescent Ca^{2+} indicator Fluo-3. Increasing Ca^{2+} levels from basal (100 nM) to greater than 600 nM through photolysis of caged-Ca^{2+} induced stomatal closure. Increases to below this critical threshold were ineffective at modulating the stomatal response. Similarly, release of $InsP_3$ from its caged form initiated an increase in Ca^{2+} in the cytosol which preceded the induction of stomatal closure. These results suggest a direct role for Ca^{2+} in triggering events leading to the stomatal response and implicate $InsP_3$ in mobilizing Ca^{2+} from intracellular stores as part of the signal transduction pathway in the guard cell. This potential for the use of caged probes to directly investigate the role of changes in the levels of signaling molecules in single, living plant cells will be discussed.

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PROTON BLOCK OF K CHANNELS IN PLASMA MEMBRANE OF GUARD CELL PROTOPLASTS: A PATCH-CLAMP STUDY.

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Due to the intermittent activation of the proton pump during movement of stomata, pH in guard cell apoplast varies between 7.2 and 5.1 in closed and open stomata, respectively . During these movements and pH changes, massive K^+ fluxes cross the cellular plasma membrane accompanying the osmotic changes of turgor and volume of guard cells. Therefore, we examined the effect of extracellular pH on K^+ fluxes via plasmalemmal K channels in *Vicia faba* guard cell protoplasts using patch clamp. This method permits an accurate control of the composition of the solutions facing both the external and the cytoplasmic surfaces of the cell membrane. We calculated membrane conductance from membrane currents, in "whole-cell" configuration, or measured directly single channel conductance in excised patches, in "outside-out" configuration. The "macroscopic", i.e., average, steady-state conductance, g_{ss}, can be described in terms of $=NbP_o\gamma$, where N is the total number of channels, γ is the single channel conductance, b is the probability of a channel not to be blocked by protons, and P_o is the probability of channel gates to be in the open state. We fitted Po to the conductance-voltage relationship, based on a three state model for channel gating, C-C-O (closed-closed-open). Thus, $Nb\gamma$ is voltage independent, and P_o embodies the voltage dependence of channel gating.

Increasing $[H^+]_o$ from 7 nM to 40 μM (pH 8.1 to pH 4.4) shifted the time constants (τ's) of activation and deactivation towards more depolarized potentials, and decreased the steady-state conductance of the Depolarization-dependent K channels (K_D channels) by about 70 %. In contrast to the obvious effect of protons on kinetics (and therefore on gating), P_o did not appear changed by pH (this can be interpreted on the basis of the particulars of the CCO model fitted to the data). Since γ was also not affected significantly by protons, the major effect in steady state had to be attributed to a decrease in Nb. Assuming that this is due to a protonation of a single site, we estimated its dissociation constant as 10 μM (pKa=5.0). This pKa was independent of voltage.

Increasing $[H^+]_o$ from 7 nM to 3 μM (pH 8.1 to pH 5.5) increased the time constants (τ's) of activation and decreased the steady-state conductance of the Hyperpolarization-dependent K channels (K_H channels) by about 20 %. The blocking effect of protons on gating of the K_H channel was reflected also in changes in the parameters of the fitted P_o. The effect of protons on the steady-state conductance could be described in terms of a protonation of a single site , embedded within 1/4 of the distance of the membrane electrical field, with an apparent voltage-dependent pKa, ranging approximately between 5.4 and 4.6, at membrane potentials between -120 and -200 mV, and extrapolated to 6.7 at 0 mV applied potential. These findings contrast sharply with the promoting effect of protons on K_H channels activity at pH range 8.1 - 5.5 observed in intact guard cells (Blatt, M. J.Gen.Physiol.1992, In Press).

Interestingly, further increase of $[H^+]_o$ in protoplasts to 40 μM (pH 4.4) caused an increase of K_H channel "macroscopic" conductance, roughly by 30%, as well as a decrease of τ's of activation.

Examining these channels at the single-channel level will help resolving more specifically which of the components comprising g_{ss} is responsible for each of the pH effects.

SECOND MESSENGER REGULATION OF ION TRANSPORT IN GUARD CELLS OF *VICIA FABA*

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Changes in guard cell turgor resulting from ion uptake or efflux modulate the apertures of stomatal pores, through which photosynthetic CO_2 uptake and transpirational water loss occur. Guard cells are responsive to many of the environmental signals that affect photosynthesis and plant water status, including light, CO_2, drought, and ambient humidity. However, the mechanisms by which these environmental signals are translated into alterations in guard-cell ion content and turgor are only beg ng to be unravelled. In this presentation, current evidence regarding lipids, G-proteins, and cytosolic Ca^{2+} concentrations as second messengers regulating guard-cell ionic fluxes will be discussed (1,2,3). In addition, evidence will be presented for G-protein regulation of outward K^+ channels in mesophyll cells of *Vicia faba*.

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TWO TYPES OF VOLTAGE-DEPENDENT ANION CURRENTS IN GUARD CELLS

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Transpirational loss of water by plants is reduced by closing of stomatal pores in the leaf epidermis. Stomatal closing is mediated by efflux of anions and K^+ ions from guard cells (1,2). Anion channels in the plasma membrane of guard cells may provide a key molecular mechanism for the control of stomatal closing in leaves: guard cell anion channels carry anion efflux (3,4) and simultaneously cause depolarization, which drives K^+ efflux through non-inactivating outward rectifying K^+ channels (5). However, central questions regarding the regulation, diversity and function of anion channels in guard cells have remained unanswered. We have now found that two highly distinct types of depolarization-activated anion currents operate in the plasma membrane of *Vicia faba* guard cells (6). One previously described (7) type of anion channel was activated rapidly within 50 ms by depolarization, inactivated during prolonged stimulation and deactivated rapidly at hyperpolarized potentials ("R-type" anion current). The other depolarization-activated anion current showed extremely slow voltage-dependent activation and deactivation ("S-type" anion current) (6). In contrast to R-type channels, S-type anion channels remained activated for prolonged durations and may therefore account for the long-term anion efflux required for stomatal closure. As anion currents have been shown to be enhanced by depolarization (3,4) and by elevation in the cytosolic Ca^{2+} concentration (3), an abscisic acid-activated Ca^{2+} permeable conductance (8) may contribute to anion channel activation. The distinct voltage- and time-dependencies of S-type and R-type anion channels suggest that they may play different roles during depolarization-associated signal transduction in higher plant cells and that these anion channels may contribute to different processes in the regulation of stomatal movements. In particular, the newly characterized slow and non-inactivating nature of S-type anion channels (6) leads us to hypothesize that Ca^{2+}-dependent (3) S-type anion channels may provide a central molecular mechanism for the control of stomatal closing which is known to be accompanied by long-term anion efflux and depolarization (1,2,9).

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PURIFIED CALCIUM CHANNEL BLOCKER BINDING PROTEIN FROM CARROT CELLS FORMS CALCIUM-PERMEABLE ION CHANNELS AFTER RECONSTITUTION

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A signal-induced increase in the cytosolic Ca^{2+} concentration is considered to be among the most important intracellular mediators for initiation of cellular and developmental events in higher plants. The influx of Ca^{2+} into the cytosol of plant cells is mediated by Ca^{2+}-permeable signal-regulated ion channels which traverse the plasma membrane and vacuolar membrane (for review see 1 and 2).

In spite of the demonstration that Ca^{2+} influx systems are involved in physiological processes in plants, the molecular structure of Ca^{2+} channel components as well as mechanisms of their regulation remain unidentified to date in higher plant cells. Ca^{2+} channel blockers of the phenylalkylamine family and bepridil are known to interfere with a variety of plant functions and to specifically inhibit Ca^{2+} influx into carrot protoplasts (3). Recently, using a phenylakylamine azido-derivative, a 75-kDa plasma membrane binding protein has been identified in carrot cells (4).

We have now purified this Ca^{2+} channel blocker binding protein by lectin-affinity and ion exchange chromatographies and incorporated the protein into phosphatidylcholine vesicles. Subsequently, the small vesicles were transformed into giant liposomes by the dehydration-rehydration technique. Single-channel patch-clamp studies on these proteoliposomes show the presence of Ca^{2+}-selective channel currents (5). These Ca^{2+}-selective channels are not stable. Prolonged recordings give rise to non-selective ion channels with a permeability to calcium and chloride ions. These non-selective Ca^{2+}-permeable ion channels, in contrast, are stable. The addition of bepridil, 10 µM, leads to the inhibition of these non-selective Ca^{2+} channels by reducing the probability of channel opening (5).

Our studies show that patch-clamping of giant liosomes provides a potent approach for determining the function of biochemically characterized membrane transport proteins in plants. Furthermore, these results suggest that the 75-kDa calcium channel blocker binding protein from carrot cells may function as a Ca^{2+} conducting component of higher plant calcium channels.

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PLANT GROWTH HORMONES CONTROL ION CHANNELS IN THE PLASMA MEMBRANE OF GUARD CELLS

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The opening of stomatal pores in the epidermis of plant leaves is caused by an increase in turgor pressure of the guard cells as a result of the accumulation of potassium salts. Although growth hormones have been shown to affect stomatal opening (1), the transduction pathways by which growth regulators exert their effects on stomatal action are largely unknown. Here we report that auxins can elecit stomatal opening. These phytohormones modulate anion channels (2,3) in the plasma membrane in what may be an initial step in regulated volume increase in guard cells.

Auxins shifted the activation potential and alter the amplitude of the anion cannel in a dose-dependent manner. Channel-modulation was hormone-specific as FC, GA, Cytokinin and ABA did not alter anion channel properties. Furthermore the auxin action was channel-specific as K^+ channels which coexist with the anion channels in the plasma membrane were not affected.

Our patch-clamp experiments demonstrate that auxins can directly interact with the extracellular face of the channel. As a result, its activation potential is shifted towards the resting potential of the cell to favour transient channel opening.

In order to prove whether the activation of an auxin-receptor channel is a key step in hormone transduction current analysis are concentrated on auxin-sensitvity mutants, azido-derivatives (4) and high-affinity ligands (5).

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SUGAR TRANSPORTERS OF THE PLANT PLAMSA MEMBRANE

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Sugar transport across the plasma membrane has been found in many different organisms. In bacteria a set of four different types of transporters has been described representing different families of structurally or functionally related proteins, using different types of driving forces like H^+-gradients, Na^+-gradients, phosphoenolpyruvate or ATP. Sugar carriers using a Na^+-gradient across the plasma membrane have also been identified in and cloned from the intestine of mammals, but there was no detectable structural similarity between these carriers and any of the bacterial sugar transport systems. In yeast cells and in other mammalian tissues, however, a great number of transporters has been found catalyzing only facilitated diffusion and not depending on any of the different driving forces mentioned above. These transporters exhibit strong similarity to the H^+/xylose and H^+/arabinose cotransporters from *E. coli*, but not to the H^+-cotransporting lactose permease or any of the other sugar transporters.

Sugar transport across the plant plasmalemma has been shown to be active using an H^+-gradient as driving force. We have cloned an H^+/monosaccharide cotransporter from the lower plant *Chlorella kessleri* and, using this clone as heterologous hybridization probe, a family of transporters from the higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*. Northern blot analyses show interesting organ specific expression differences of some of these putative transporters. All of these plant sugar carriers show similarity to the bacterial H^+/xylose and H^+/arabinose cotransporters and to the carriers catalyzing facilitated diffusion in yeast and mammals. No similarity could be found to the intestinal Na^+/glucose cotransporter, which was suggested by results from other groups according to antibody crossreactivity.

The energy dependence, substrate specificity, and kinetic parameters of these transport proteins were further investigated by heterologous expression in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. These expression systems were also used for testing the influence of *in vitro* mutations on the activity of the *Chlorella kessleri* H^+/sugar cotransporter.

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PROTON-COUPLED SUCROSE AND AMINO ACID SYMPORTS: SUBSTRATE RECOGNITION, PHOTOAFFINITY LABELING, AND MOLECULAR CLONING

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Sucrose and amino acids are actively transported across the plasma membrane of plant cells by proton-coupled symports. In the last three years, several laboratories have provided significant advances in describing the transport properties and bioenergetics of these transport systems using isolated plasma membrane vesicles and imposed proton electrochemical potential differences. After briefly summarizing those data, I will present recent experimental results that provide new insight into the structural determinants involved in substrate recognition and inhibitor binding. Additionally, new data regarding ongoing efforts to identify the symport proteins and clone the genes encoding them will be presented.

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THE SUCROSE CARRIER OF THE PLANT PLASMALEMMA: PARTIAL PURIFICATION AND RECONSTITUTION

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In plasma membranes purified from sugar beet leaves, differential labeling by N-ethylmaleimide in the presence of sucrose has allowed the identification of an intrinsic polypeptide that migrated at 42 kDa after SDS-PAGE, and that was specifically protected by sucrose [1]. Polyclonal antibodies raised against the 42 kDa polypeptide inhibit the uptake of sucrose into protoplasts [2], or into plasma membrane vesicles [3], but they do not inhibit the uptake of hexoses and amino-acids [2,3].

Plasma membranes differentially labeled in the presence of sucrose have been solubilized by 1% CHAPS, and the proteins solubilized were separated by gel filtration under non-denaturating conditions. Under these conditions, a peak of differential label was observed at 120 kDa. When the 120 kDa peak was recovered, denaturated by SDS, and injected on the same column, a peak of differential label was observed at 40 kDa. In parallel experiments, unlabeled membranes were used, and the fractions eluted from the column were monitored by ELISA for their ability to be recognized by anti-42 kDa sera. The results confirmed those obtained by differential labeling, i.e. a major ELISA reactive peak was found at 120 kDa, before denaturation, and at 40 kDa after denaturation by SDS. The 120 kDa peak from unlabeled membranes was used to design a reconstitution procedure with soybean asolectin. Upon energization by an artificial proton motive force the reconstituted 120 kDa fraction exhibited active sucrose transport, but did not transport valine. The 120 kDa fraction was further separated by ion-exchange chromatography on a Mono-Q column eluted by a NaCl gradient. The fractions eluted were monitored by ELISA with the anti-42 kDa serum as above, and reconstituted. A single ELISA-reactive peak was eluted at 0.31 M NaCl. This peak exhibited a high transport activity. SDS-PAGE showed that it was enriched with two very close bands at 42 kDa. It is concluded that a 42 kDa polypeptide is a component of the sucrose transport system. At least under *in vitro* conditions, this polypeptide may trimerize, or agregate with other polypeptides to give a 120 kDa cluster [4].

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SUGAR TRANSPORTERS INVOLVED IN PHLOEM UNLOADING

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Apoplastic phloem unloading involves release of photoassimilates from phloem and/or companion cells and their uptake by the relatively abundant target cells. Despite extensive research in the past, the transporters involved in these processes have not been characterized. The approach we have employed was to isolate plasma membrane vesicles from red beet storage tissue and analyze the sugar transporters in them with particular attention to sucrose and glucose transport.

Analysis of a purified plamalemma fraction revealed two sub-populations of vesicles: Most of the vesicles contained separate proton/glucose and proton/sucrose cotransporters, presumably, originating from the sink cells and involved in active uptake of sugars. A minor fraction of the vesicles, comprising about 10 % of the volume of the vesicles, contained a highly active facilitative sugar transporter. This transporter exhibited the characteristics expected of a mechanism allowing release of sugars from the phloem and/or companion cells to the apoplasm.

The vesicles containing the facilitative sugar transporter were further purified five folds. This fraction was stained by the plasmalemma-specific stain, acidic phosphotungstic acid, bound concanavalin A and was enriched in the plasmalemma markers, Vd^{+}-sensitive ATPase and glucan synthase II.

This transport system exhibited an activation energy of about 11 Kcal/mole and was inhibited by high concentrations (mM range) of sulfhydril-reagents such as $HgCl_2$, pCMBS and NEM.

The facilitative sugar transporter transported both sucrose and glucose. It exhibited a Km for glucose of 180 mM and Vmax of 2.3 μmoles/min per mg protein. Competitive inhibition and trans-stimulation studies have indicated that this system was capable of transporting all monossachrides tested, some disaccharides, such as maltose and sucrose, and some trisaccharides, such as raffinose and maltotriose. It was not affected by amino acids, phospho sugars and some disaccharides, such as lactose.

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EXPRESSION CLONING OF METABOLITE TRANSPORTERS FROM HIGHER PLANTS

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Assimilates are produced in the source organs, e.g. leaves, and transported via the vascular system to the sink organs which serve, e.g. for reproduction. In most plants, the main transfer molecules for carbon and nitrogen are sucrose and amino acids respectively. Long distance transport is necessary to bring the assimilates from the place of synthesis to the the places of metabolization or storage. In tobacco, strong evidence supports the hypothesis that sucrose enters the phloem via the apoplast, requiring specific permeases to allow crossing of the membranes (1). Uptake studies with purified plasma membrane vesicles have demonstrated sucrose and amino acid permease activities (2,3,4). In order to study the regulation and function of the transporters it seems important to isolate the respective genes. Due to the difficulties associated with the identification and purification of membrane transporters the genes for plasma membrane and tonoplast permeases have so far not been cloned. For the Na^+-dependent glucose transporter from rabbit, the problems were circumvented by developing an expression cloning system in oocytes (5). We have used both standard and artificial complementation systems to isolate cDNA clones encoding metabolite transporter genes such as sucrose permease genes from plants. We have used the expression system as a tool to characterize the transport systems.

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INSIGHTS INTO THE STRUCTURE OF THE CHLOROPLAST TRIOSE PHOSPHATE-PHOSPHATE TRANSLOCATOR PROTEIN

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The chloroplast envelope membrane is the site of different metabolite translocators coordinating the metabolism in the cytosol and in the stroma (1). The major envelope membrane polypeptide with an apparent molecular mass of 29 kDa represents the chloroplast phosphate translocator that mediates the export of fixed carbon from the chloroplasts in form of triose phosphates and 3-phosphoglycerate (2). Hydrodynamic and rotational diffusion studies of the isolated translocator-detergent micelle revealed that, in its functional state, the translocator exists as a dimer consisting of two identical subunits (3, 4). Recently, the cDNA sequences of this nuclear-coded protein from both spinach and pea chloroplasts have been determined (5, 6). Based on hydrophobicity distribution analyses of the mature protein and on additional information a tentative model of the arrangement of the phosphate translocator can be proposed. Each monomer of the phosphate translocator contains six transmembrane α-helices. It is proposed that, in the functional dimeric phosphate translocator protein, the 12 transmembrane helices are arranged in such a way that the hydrophilic regions are directed towards the inside of the protein thus forming a hydrophilic pore through which the substrates could be transported across the membrane.

Model structures of the helices were generated by using standard geometrical parameters for α-helices. Subsequently, a three-dimensional structure of the membrane part of the translocator was built by means of computer-graphics and was energy-minimized using the AMBER force field (7). The resulting structure with a C_2-symmetry axis perpendicular to the membrane plane has a total length of about 5 nm and a width of 2.6 nm which values rather fit those obtained by hydrodynamic studies (3, 4). The corresponding values for the hydrophilic translocation channel are 2.2 nm and 0.8 nm, respectively. In combination with site-directed mutagenesis experiments information can be obtained as to what part of the translocation channel is involved in substrate binding. A system for the functional expression of the chloroplast phosphate translocator in yeast cells has recently been developed (Loddenkötter and Flügge, manuscript in preparation). This approach, together with computer-aided molecular-design modelling of the translocation pore, has opened the way to insights into structure-function relationships of the translocator protein.

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VACUOLAR-TYPE H^+-TRANSLOCATING ATPases IN PLANT ENDOMEMBRANES: SUBUNIT ORGANIZATION AND MULTIGENE FAMILY

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Acidification of endomembrane compartments by the vacuolar-type H^+-translocating ATPase (V-ATPase) is vital to the growth and development of plants (1). The V-ATPase purified from oat roots is a large complex of 650 kDa that contains 10 different subunits of 70, 60, 44, 42, 36, 32, 29, 16, 13, 12 kDa (2). This set of ten polypeptides is sufficient to couple ATP hydrolysis to proton pumping after reconstitution of the ATPase into liposomes (3). Unlike some animal V-ATPases, the purified and reconstituted V-ATPase from oat is directly stimulated by Cl^-. The peripheral complex of the ATPase includes the nucleotide-binding subunits of 70 and 60 kDa and polypeptides of 44, 42, 36 and 29 kDa. Six copies of the 16 kDa proteolipid together with three other polypeptides are thought to make up the integral sector that forms the H^+ conducting pathway. Release of the peripheral complex from the native membrane completely inactivates the pump; however the peripheral subunits can be reassembled with the membrane sector to form a functional H^+ pump (4).

Comparison of V-ATPases from several plants indicate considerable variations in subunit composition (1). Hence several forms of the V-ATPase may exist among and probably within plant species. At least four distinct cDNAs encode the 16 kDa proteolipid subunit in oat (5). Multiple genes could encode different subtypes of the H^+ pump that are regulated by the developmental stage and physiological function specific to the cell or tissue.

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STRUCTURE AND EXPRESSSION OF GENES ENCODING THE MAJOR SUBUNITS OF VACUOLAR AND MITOCHONDRIAL ATPases OF *NEUROSPORA CRASSA* AND STRATEGIES FOR MUTATING ATPase GENES

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Subunits A and B of the vacuolar ATPase and subunits α and ß of the mitochondrial ATPase evolved from a common ancestral gene (1). We have isolated and compared the genes encoding these polypeptides in *N. crassa* to see if they share common structural or regulatory features. The four genes have short (55-332 nucleotides), multiple (5-7) introns that are often positioned near the 5' end of the coding region. Coding regions of genes for A and B subunits use 57 and 55 codons, while those of genes for α and ß employ 45 and 44 codons; use of fewer codons is a feature associated with highly expressed genes in fungi. Northern-blot analysis shows that compared to ß-tubulin, messages for A and B subunits are approximately one-third as abundant, while messages for α and ß are about 3-fold more abundant. All four genes appear to be expressed constitutively throughout mycelial growth. Upstream regions of all four genes contain putative GC boxes but no other obviously common elements. However, three genes encoding vacuolar ATPase subunits (A, B, and proteolipid) share two regions of sequence similarity which are being tested for regulatory importance.

We are using two strategies to obtain mutants of vacuolar ATPase genes: (a) screening of strains able to grow at pH 7.5 in the presence of bafilomycin A_1, a specific inhibitor of vacuolar ATPases (2), and (b) inactivation of the ATPase genes by taking advantage of a novel mechanism in *N. crassa* called RIP (repeat-induced point mutations) (3).

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EVOLUTION AND ISOFORMS OF THE PLANT V-ATPase

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V-ATPases that energize the endomembranes of eukaryotes have evolved from the archaebacterial coupling factor ATPases (1,2). The vacuolar type ATPase and the archaebacterial coupling factor ATPase share a high degree of sequence similarity, however, the tertiary and quaternary structure of the archaebacterial ATPases appears to be more similar to the F-ATPases of eubacteria, mitochondria and chloroplasts. The switch in function from ATP synthase/ATPase (F- and archaebacterial type) to exclusively proton pumping ATPase (V-type) is correlated with a change in subunit stoichiometry and an increased proteolipid size. The duplication and fusion event that gave rise to the larger proteolipid type of the V-ATPase occurred early in the evolution of the eukaryotes (2).

Transformation of *Daucus carota* with a gene encoding an antisense mRNA to the A subunit of the vacuolar ATPase resulted in plants with retarded growth, larger central vacuoles and an altered leaf morphology. Vacuolar ATPase activity and the immuno detectable A subunit were decreased in the tonoplast, but remained unchanged in Golgi enriched membrane fractions, suggesting that in carrots at least two organelle specific isoforms exist which respond differently to the antisense mRNA (3).

Two isoforms have been described for the B subunit in humans (4); however, only one gene was found to encode the A subunit in bovine (5). In higher plants two genes encoding the A subunit can be characterized by an intervening sequence of different size. This intron is surprisingly conserved and its unequal size in the two isoform encoding genes allows their easy detection by PCR across the exon intron boundaries (6). This intron is also found in *Coleochaete scutata*, a green alga that is considered to be closely related to the higher plants, whereas other green algae (e.g. *Zygnema*), various fungi and *Euglena* have no intron in the corresponding position. These findings suggest that this intron was already present in the ancestor of the land plants. The duplication that gave rise to the two isoforms, distinguished by the differently sized introns, occurred early in the evolution of the land plants. With one exeption (*Arabidopsis*) the two genes characterized by sequence and size of the intron were found in all ferns, gymno- and angiosperms studied so far (14 species). These two isoforms are not a characteristic of higher eukaryotes in general; their presence appears to be restricted to higher land plants.

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INHIBITION OF VACUOLAR PH REGULATION BY BAFILOMYCIN A1 IN CELLS OF *CHARA CORALLINA*

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The vacuolar pH(pH_v) of *Chara corallina* is maintained at about 5 for a wide range of external pH (1). The capacity of pH_v regulation was clearly demonstrated by the experiment to see the recovery of pH_v from either more acid or more alkaline pH shifted by vacuolar perfusion (1). The pH_v regulation is sensitive to DCCD(1), suggesting involvement of the tonoplast H^+-ATPase.

Bafilomycin A1, which inhibits specifically vacuolar-type H^+-ATPases of *Neurospora crassa* or *Zea mays* (2), was tested for its effect on the pH_v regulation in *Chara* cells. pH of the fluid effused out by vacuolar perfusion was measured as pH_v. When bafilomycin(100 nM) was applied to the cell from the outside, no change in pH_v was observed, but it slightly retarded the recovery of pH_v which had been elevated to 7.3 through replacement of the cell sap with a buffered artificial solution. When an unbuffered artificial cell sap(pH about 5.5) containing bafilomycin was introduced into the vacuole, pH_v increased significantly. The recovery of pH_v from 7.3 was also significantly inhibited, when bafilomycin was involved in the artificial cell sap. Both the H^+-ATPase activity and the H^+-pumping capacity of tonoplast vesicles (3) were also inhibited by bafilomycin. The membrane potential, from which the tonoplast was removed by vacuolar perfusion of a medium containing a Ca^{2+}-chelator EGTA (4), was not affected by direct application of bafilomycin to the cytoplasm.

Following conclusions are drawn. 1) The plasma membrane is less permeable to bafilomycin than the tonoplast in *Chara* cells. 2) It does not affect the electrogenic H^+-ATPase of the plasma membrane. 3) It interferes with the pH_v regulation by specifically inhibiting the tonoplast H^+-ATPase activity.

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ION CHANNELS IN THE ENERGISED PLASMA MEMBRANE OF WHEAT ROOT PROTOPLASTS

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Our aim is to characterise the ion channels and electrogenic pumps in the plasma membrane of root cells. Protoplasts isolated from wheat roots can be easily patch clamped in the cell-attached, whole-cell and outside-out configurations. Electrogenic pumping (ATP dependent, DCCD sensitive) is often observed in cells perfused with low K^+ and Cl^- concentrations. Membrane potentials (after cell perfusion) of between -160 and -220 mV are often observed in 1 mM external KCl. The pump current is rather small (ca 4 mA m^{-2}) and cells which display hyperpolarised PDs have very low conductance and low channel activity such that individual channels can be observed in the whole-cell mode. Inward rectifier channels (anion and cation) appear to limit the extent of the hyperpolarisation.

Various combinations of four types of rectifier currents are observed. At PDs positive of zero, a fast activating current and a slowly activating current are present in various proportions. The slow current more often dominates when the external KCl concentration is 1 mM. The activation PDs for both currents are similar and vary with the equilibrium potential for K^+. The slow current is blocked by verapamil as has been observed with *Amaranthus* protoplasts (see Terry *et al* these proceedings) and can be reconstructed (activation and deactivation) from activation and deactivation sequences of the outward K^+ rectifier channel in outside-out patches. The K^+ channel is shown to be able to carry inward going currents when the reversal potential for the channel is positive or when treated with verapamil (see Terry *et al* these proceedings).

Two types of inward activated currents are observed: a smoothly activating current displaying single exponential kinetics and a highly variable high conductance channel(s) which opens only briefly (spike current). Both currents are activated at PDs less negative than the hyperpolarised PDs observed in energised protoplasts. The reversal PD of tail currents from the smoothly activating inward current and inhibition by TEA of this current indicates that the current is carried by potassium. The current also appears to have a significant anion permeability. Single channels with a conductance of about 5 pS (100 KCl) which open at PDs negative of about -100 mV have similar K/Cl selectivity as the smoothly activating current.

COMPARATIVE *IN VIVO* STUDIES OF ANION CHANNELS IN *CHARA*, FUNGI AND ROOTS

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The plasma membrane of *Chara corallina*, like that of other plant cells, contains channels for Cl^-, the operation of which is regulated by a variety of signals, both chemical and electrical (Tyerman, 1992). For example, efflux of ^{36}Cl from *C. corallina* is greatly increased by cytoplasmic acidification produced by weak acids (Smith and Reid, 1991). We have found that the same treatment also increases the efflux of ^{32}P, presumably as $H_2PO_4^-$. The question as to whether Cl^- and $H_2PO_4^-$ pass through the same channel or through different channels is being addressed by comparing time-courses of efflux of ^{36}Cl and ^{32}P from *C. corallina* in response to cytoplasmic acidification. Other treatments known to increase Cl^- efflux such as action potentials and metabolic inhibition have also been tested with respect to ^{32}P efflux. The effect of these treatments on efflux of other anions from *C.corallina* is also under study.

As well as working with *C. corallina*, we are investigating anion fluxes from mycorrhizal fungi and plant roots, with the aim of resolving the specificity of anion channels *in vivo*.

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GATING OF ION CHANNELS IN PLASMALEMMA AND TONOPLAST OF YEAST

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Observations. Recent patch-electrode experiments on *Saccharomyces cerevisiae* have defined two types of ion channels: a large cation channel in the tonoplast (**YVC1**), and a smaller K^+ channel in the plasmalemma (**YPK1**; see Bertl et al., prev. abstr.). For YVC1, overall open probability (P_o) rises sigmoidally from near zero at positive membrane voltages (V_m; cytoplasm + to vacuole) toward a maximum near -80 mV. Hence the normal V_m of the yeast tonoplast--probably near -40 mV--would facilitate appreciable opening of these channels when other conditions require it. But for YPK1, P_o is near zero at all negative voltages (cytoplasm - to cell exterior) and rises only at *positive* voltages; thus the likely resting V_m (-100 to -200 mV) would strongly inhibit channel opening. That is essential for maintenance of a resting V_m negative to the K^+ equilibrium, and is common to many plant and fungal plasma membranes which are dominated by the electrogenic H^+ pump. Appreciable P_o's for YVC1 require mild *reducing conditions*, which at present could be viewed either **a)** as the basis for dynamic redox regulation of the channel or **b)** as the channels' equivalent of general enzyme protection by antioxidants. Raising cytoplasmic pH, at least within the range 5.5 to 7.5, also increases P_o for YVC1. And finally, for both YVC1 and YPK1, raising cytoplasmic Ca^{++} concentration within a quasi-physiological range (ca. 1 μM and below) enhances P_o; but in the case of YVC1, the calcium effect creates positive feedback, since that channel itself is Ca^{++}-permeant and since stored vacuolar $[Ca^{++}]$ is much higher than free cytoplasmic $[Ca^{++}]$.

Physiological functions. Many possible functions could be listed for regulated K^+ channels in generalized plant and fungal plasma membranes. Under different circumstances these include **I)** to provide a passive, low-affinity entry route for concentrating cytoplasmic K^+ from replete environments, by means of the (H^+-pump driven) V_m; **II)** to buffer V_m--itself vital to other transport functions--against disasters such as moderate membrane damage or temporary energy depletion; and **III)** to assist in turgor modulation by admitting the cation component of salt movement. New experiments are underway to test regulation of YPK1 in relation to these points.

But on another point, **Iv)** mediation of charge-compensation and/or cytoplasmic H^+-buffering during proton-coupled uptake of organic solutes, considerable information is already available. Membrane depolarization and K^+ loss seem to accompany H^+-coupled maltose uptake (Serrano, *EJB* 80:97, 1977); and either K^+ uptake or K^+ loss (Pulver & Verzár, Nature 140: 823, 1940; Van de Mortel et al., *BBA* 936:421, 1988) can accompany H^+-independent glucose uptake, depending upon conditions. At least in the latter case, dye measurements indicate membrane *hyper*polarization to occur with the K^+ flux. Such observations imply that sugar entry causes K^+ channels to open, but the gating messenger should be different for the two sugars. The co-flux of protons with maltose depolarizes yeast plasmalemma, which could open YPK1 via the voltage dependence; net K^+ efflux would then occur provided that V_m went positive to E_{K+}. No such depolarization would accompany glucose influx, however, so another mechanism is needed. Cytoplasmic $[Ca^{++}]$ rises upon glucose entry (Eilam et al., J.Gen. Microbiol. 136:2537, 1990), which should stimulate YPK1 opening. A fast component of that calcium rise probably originates extracellularly, (Eilam & Othman, ibid 136:861, 1990), but a slower component is likely to originate from the vacuole. Release of vacuolar Ca^{++} through YVC1 should be triggered by the reducing surge during initial glucose oxidation, then further enhanced by the positive feedback effect of rising cytoplasmic $[Ca^{++}]$.

ISOLATION OF A PLANT K^+ CHANNEL cDNA BY FUNCTION IN YEAST

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Saccharomyces cerevisiae harbors two genes encoding potassium transporters, *TRK1* and *TRK2* (1,2). Cells deleted for *TRK1* and *TRK2* are viable but require up to 100 mM potassium in the medium to support growth. We have exploited the inability of *trk1Δtrk2Δ* cells to grow on non-potassium-supplemented media to detect the functional expression of genes encoding potassium- transporter proteins from other organisms including plants (3,4). A cDNA clone (*KAT1*) isolated from *Arabidopsis thaliana* that completely suppresses the K^+ transport defect in *trk1Δtrk2Δ* cells was found to encode a 78-kD protein containing hallmarks of the known K^+ channels. These include i) a hydrophobic core of 6 putative transmembrane domains located near the amino terminus, ii) a putative voltage sensing region consisting of Arg/Lys-X-X repeats within the fourth hydrophobic domain and iii) a sequence between the fifth and sixth putative transmembrane domains highly related to the pore regions of other K^+ channels. KAT1 shares the greatest amino acid sequence identity with the cyclic nucleotide-gated ion channels and *ether-a-go-go* ,(eag) a putative K^+ channel from Drosophila. Consistent with this, KAT1 also contains a putative cyclic nucleotide binding domain in the C-terminal half of the protein.

KAT1 function is inhibited *in vivo* by known potassium channel blockers; *KAT1*-dependent growth of *trk1Δtrk2Δ*cells on low-potassium medium is completely blocked by Ba^{2+} and tetraethylammonium ions. Expression of *KAT1* in *Xenopus* oocytes produce a potassium-selective inward current only upon hyperpolarization of the oocyte membrane. Our results indicate that *KAT1* encodes an inward-rectifying potassium channel.

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CLONING AND EXPRESSION IN YEAST OF A PLANT K^+ TRANSPORT SYSTEM

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A mutant (1) of *Saccharomyces cerivisiae*, unable to grow on low-K^+ medium, and belonging to the same complementation group as the *TRK1* (2) transport system, was complemented with a cDNA library (3) made from *Arabidopsis thaliana* seedlings. An *Arabidopsis* clone (AKT1, for *Arabidopsis* K^+ Transport system 1) was able to complement the yeast mutant. In the low (micromolar) K^+ concentration range, the K^+($^{86}Rb^+$) uptake rates were similar in the wild-type and complemented yeast strains; both were much higher than in the mutant strain. When the K^+ concentration was increased to the millimolar range, the uptake rate reached a saturation plateau in the wild-type strain, but in the complemented strain increased further with increasing K^+ concentration. The kinetics of the K^+ transport in the complemented strain was complex, and could not be described as a Michaelian saturation kinetics. The capacity of AKT1 to accumulate K^+ was verified by transferring complemented yeasts into a K^+-free medium. After an initial loss of K^+, which increased the external K^+ concentration to 10 µM, a net influx developed, decreasing the external K^+ concentration to a value of 0.65 µM. Under these conditions, the cytosolic K^+ concentration was estimated to be 0.17 M. Thus, AKT1 maintained a high K^+ accumulation ratio (2.6×10^5), which corresponds to an equilibrium potential difference (E_K) of about -320 mV. No estimate of the actual membrane potential difference is available, and it is not possible to determine whether AKT1 mediates passive or active K^+ transport.

The AKT1 predicted amino acid sequence (838 amino acids) has three domains (5): a channel-forming region, homologous to animal K^+ channels [Shaker family channels (4)], a cyclic nucleotide-binding site, and an ankyrin-like region (6). The original combination of a cyclic binding site and of an ankyrin motif in the *Arabidopsis* K^+ channel will allow us to address new questions concerning the regulation of K^+ transport in plants.

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CHARACTERIZATION OF ION CHANNELS IN THE PLASMA MEMBRANE OF LEAF EPIDERMIS CELLS OF PISUM SATIVUM, ARGENTEUM MUTANT

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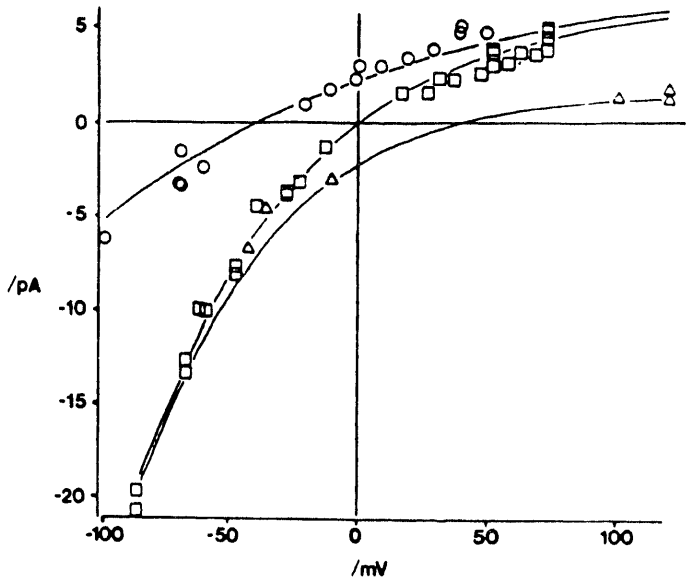
The epidermal cells of young expanding leaves of the argenteum mutant of pea (Pisum sativum) are used to study two light dependent processes: 1) young leaves when exposed to bright light exhibit an increased growth which is due to cell expansion and depends on the availability of K^+ (or Rb^+) in the medium, and 2) light induces a transient initial depolarization. We found indications that part of the depolarization is connected to an efflux of Cl^-. Currently we are in the process of characterizing the different ion channels present in the plasma membrane. Two channels which conduct cations, one with a high selectivity for K^+ and conductance of 20 pS, the other with little discrimination between K^+ and Na^+ and a conductance of 35 pS (in symmetrical 100 mM K^+ solutions) are partly characterized. Also present in the plasma membrane is a channel with a very high conductance. Single channel I-V analysis illustrates that the reversal potential with different bath and pipette media compositions is close to the reversal potential for chloride. Furtermore the I-V relation proved to be very asymmetric with a maximal conductance of about 300 pS between -60 and -100 mV with symmetrical 110 mM Cl^- solutions in pipette and bath, but with a conductance of only 50 pS at positive holding potentials. Fitting the I-V data was possible with a four state, class I model and resulted in kinetic parameters indicating that the Cl^- binding constants on cytoplasmic and apoplastic side of the membrane differ fourfold (see figure). At positive membrane potentials opening and closing times are short, resulting in a flickering behavior, while at negative membrane potentials both opening and closing times are much longer.



I-V relation of single channel currents in inside-out plasma membrane patches of pea leaf epidermal cells. The potential is expressed as the cytoplasmic minus the extracellular potential, current is taken as positive when positive charge is carried out of the cytoplasm. The chloride concentra-tion (cytoplasmic/extracellular) was □: 114/109, ○: 24/109 and △: 114/19 (data of 13 different protoplasts are compiled). The solid curve was fitted through the data points with a cyclic 4-state, class I model (Hansen et al. (1981) J. Membr. Biol. 63: 165-190)

IDENTIFICATION OF A POTASSIUM CHANNEL IN MESOPHYLL AND GUARD CELL PLASMA MEMBRANES OF V. faba LEAVES.

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Ion transport are essential for plant growth, development and movements. Ion channels in the plasma membrane allow a selective entry of ions such as K^+, Cl^- or Ca^{2+} into the cytoplasma of plant cells. In order to identify, characterize and isolate the K^+ channel proteins plasma membranes were prepared from leaves of *Vicia faba L.* by aqueous two-phase partitioning. Transport activity of these vesicles was followed by $^{86}Rb^+$ flux measurements (1). Rb^+ uptake was inhibited by tetraethylammonium. In a following step the plasma membranes were solubilized by preserving transport properties. After reconstitution of transport active protein fractions TEA-sensitive $^{86}Rb^+$ uptake could be restored. The transport activity co-purified with a 67 kDa protein.

Incorporation of the purified protein into planar lipid bilayers elicited K^+ selective channel fluctuations. From the open-channel amplitude a ionic conductance of 40 pS was computed in symmetrical 200 mM KCl solution.

A monoclonal antibody was raised against the purified channel protein (2). $^{86}Rb^+$ uptake of plasma membrane vesicles was inhibited in presence of the antibody. Two polypeptides in the plasma membrane of mesophyll and guard cell were identified by immunoblot analysis. Finally partial aminoacidsequences were obtained from the 67 kDa polypeptide after microsequencing tryptic protein fragments.

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pH CONTROLS K^+ CHANNEL GATING IN *VICIA* GUARD CELLS

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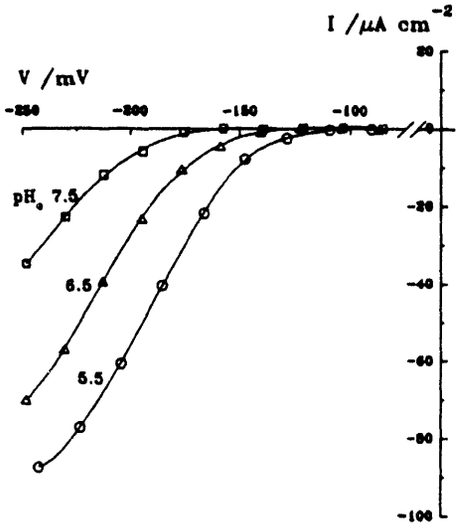
It has long been recognised that $[H^+]$ could be pivotal in controlling transport across the plant plasma membrane[1-3]. The idea continues to hold a special attraction because of the potential for feedback and integration implicit in the link with H^+-coupling economy of the plant membrane -- and this despite a paucity of direct evidence for pH control beyond the well-documented role for H^+ in pump activity and membrane energisation[4].

Data collected from *Vicia* guard cells now indicates that the K^+ channels which mediate K^+ flux for stomatal movements are exceptionally responsive to pH, both intracellular and extracellular[5]. The steady-state, whole-cell currents shown in the Figure (from one cell in 10 mM KCl) are typical of the K^+ inward-rectifier ($I_{K,in}$) response to extracellular pH; currents recorded at any one voltage under voltage clamp were found to rise and the "gating voltage" to shift positive-going under mild acid conditions, a response comparable to the effects of submicromolar $[Ca^{2+}]_i$. This pH_o dependence was not attributable to H^+ permeation and analyses of current kinetics were not consistent with an effect of $[H^+]$ on surface charge screening. Instead, voltage- and pH-dependencies for $I_{K,in}$ followed a simple titration surface function, as if H^+ bound to a single site deep within the membrane and with a pK_a near 7. Additional data to be presented distinguish extracellular and intracellular H^+ action on $I_{K,in}$ and highlight a comparable sensitivity of the K^+ outward-rectifier to *intracellular* pH.



The evidence for $I_{K,in}$ activation by H^+ represents a new departure in understanding the controls on ion channels in plants and sets the K^+ channel apart from inward-rectifiers known in animal cells. It presents a mechanistic basis for channel integration with the plasma membrane H^+-ATPase over a wide range of environmental conditions[6]. Furthermore, the H^+-sensitivities of both inward- and outward-rectifiers intimate roles for pH in controlling K^+ flux during stomatal movements.

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ION CHANNELS IN THE PLASMALEMMA OF XYLEM PARENCHYMA CELLS FROM ROOTS OF BARLEY (*Hordeum vulgare* cv. Apex)

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In order to elucidate mechanisms of salt release from the xylem parenchyma into the xylem vessels in roots, we developed a method to specifically isolate xylem parenchyma cells from decorticated steles of nodal roots of barley, taking advantage of the patterns of cell wall lignification in this particular cultivar, about 0.5 to 1.5 cm from the apex. Cells of early metaxylem were the main source of protoplasts gained by enzymatic digestion of the stele, with no contamination by phloem cells occurring. We investigated the properties of ion channels in the plasmalemma of these cells with the patch-clamp technique in the whole-cell and the outside-out patch-configuration.

In whole-cell experiments, hyperpolarising voltage steps below -110 mV induced a strongly voltage-dependent increase in membrane conductance, due to the activation of inward rectifying channels, in about 80 out of 100 protoplasts. The reversal potential of tail currents was close to the Nernst potential of K^+, indicating a strong selectivity for K^+ versus Cl^-, Mg^{2+} and Ca^{2+}, the other ion species present. The unitary conductance as derived from outside-out patches was 30 pS. In these respects, the K^+ channel closely resembled the inward rectifier found in guard cell protoplasts (SCHROEDER et al. 1987). Adding Ba^{2+} to the bath blocked the channel in a voltage-independent manner. The apparent Ki was about 1 mM. External Ca^{2+} had a weak effect on inward currents, causing a slight inhibition below -180 mV. Lanthanum ions strongly inhibited inward currents in a voltage dependent manner and shifted the threshold potential in a depolarising direction. In contrast to K^+ channels in guard cells (SCHROEDER and HAGIWARA 1989), the cytoplasmic Ca^{2+} concentration (0.15 to 10 µM) did not affect the inward rectifier in xylem parenchyma cells.

An outward rectifying K^+ channel, like the one in guard cells, was also observed, but less frequently than the inward rectifier; TEA^+ (20 mM) in the bath reduced the outward current to 20 %. In several experiments, a Cl^- channel was detected resembling the slow Cl^- channel in guard cells (LINDER and RASCHKE, this workshop), though the unitary conductance appeared to be somewhat higher. Single-channel events could be resolved in the whole-cell configuration.

We suggest that these K^+ and Cl^- channels participate in the ion exchange between symplast and apoplast in the stele.

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FUNCTIONAL EXPRESSION OF A PLANT PLASMA MEMBRANE TRANSPORTER IN *XENOPUS* OOCYTES

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Xenopus oocytes efficiently transcribe and translate injected genetic information and the protein products are correctly assembled, processed and targeted to the proper subcellular compartment. Many animal plasma membrane proteins have been cloned by functional expression in *Xenopus* oocytes including a barley α-amylase (1) and the ethylene-forming enzyme of tomato (4). Many animal plasma membrane proteins have been cloned by functional expression in *Xenopus* oocytes (3), however there have been no reports of the functional expression of higher plant plasma membrane proteins in oocytes.

Recently the STP1 gene of *Arabidopsis* has been cloned and sequenced and shown to encode a H^+/glucose cotransporter (2). Dr. Norbert Sauer kindly provided us with a full-length STP1 cDNA clone which was sub-cloned into the transcription vector pGEM-3Z. The STP1 mRNA, transcribed *in vitro* from the STP1 cDNA, was injected into oocytes and the oocytes were incubated at 18°C for up to 5 days to allow expression of the STP1 mRNA. Uptake at 22°C of ^{14}C-labelled 3-*O*-methyl-D-glucose (3-OMG), was used to assess hexose transport by the oocytes.

Maximum rates of 3-OMG uptake were observed 3 to 4 days after injection with STP1 mRNA. Depending on the batch of oocytes, the pH of the bathing solution during the period of 3-OMG uptake and the time after injection, the oocytes injected with STP1 mRNA took up 3-OMG at a rate between 20 times and several thousand times faster than the water-injected controls. The optimum pH for 3-OMG uptake was 5 and electrophysiological experiments with oocytes injected with STP1 mRNA showed a hexose-elicited depolarization of the oocyte membrane potential, confirming the activity of a H^+/hexose cotransport system.

Following the demonstration that a higher plant membrane transport protein can be functionally expressed in *Xenopus* oocytes we are attempting to use this system to functionally express and to clone the plasma membrane nitrate transporter of barley roots.

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INSTRUMENTATION USED IN THE ACQUISITION AND ANALYSIS OF PLANT ELECTROPHYSIOLOGICAL DATA

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Axon Instruments offers a variety of equipment useful to the plant electrophysiologist. An overview of this equipment, both hardware and software, will be provided along with selected highlights of recent advances in instrumentation technology.

Axon Instruments' voltage- and patch-clamp amplifiers include the Axopatch 200A and the Axopatch-1D. Both are designed for low-noise single-channel and whole-cell recording, and both use resistive feedback current-to-voltage converters in whole-cell mode. For single-channel recordings, however, the Axopatch 200A uses a new proprietary headstage technology based on the capacitative feedback current-to-voltage converter which results in reduced noise, increased dynamic range, wider bandwidth and improved linearity when compared to the resistive method used in the Axopatch-1D. The Axopatch 200A user can employ a single headstage for both cell and bilayer recordings, while the Axopatch-1D offers a few more ease-of-use features.

Data acquisition and analysis software and hardware are available for both IBM-PC and Macintosh computer platforms. For the IBM-PC series of computer, the pCLAMP v5.5 software suite provides extensive functionality with respect to stimulating, recording and analyzing whole-cell and single-channel records and is used with the TL-1 data acquisition interface. AxoBASIC is a programming environment, similar to BASIC-23, which is based on Microsoft QuickBASIC and allows users to quickly develop custom programs for unusual acquisition and analysis situations. For the Macintosh II (or better) computer, Axon Instruments offers the program AxoData for data acquisition in current clamp, voltage clamp and other experiments in which simultaneous stimulation and acquisition are necessary. AxoData allows great flexibility in creating complex protocols using icons and graphical drawing tools. Supported data acquisition hardware include the Instrutech Corp. ITC-16. Integrated analysis and graphics are provided by the program AxoGraph that is oriented towards the types of data records encountered in electrophysiology, features very fast fitting and can read binary data files directly from both AxoData and the pCLAMP suite.

Axon Instruments also offers the Data Acquisition Platform, a computer system based on the Intel 33 MHz 80486 which can be configured with requested Axon Instruments hardware and software and is extensively tested before shipment.

THE DAUCUS CAROTA V-TYPE H^+ATPASE: RECENT PROGRESS IN UNDERSTANDING THE COORDINATE EXPRESSION OF ITS GENES

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A carrot V-typ H^+ATPase A subunit cDNA sequence and its corresponding partial genomic sequence have been reported previously (1,2), and the existence of three independent genes for the A subunit has been demonstrated (3). Recent results suggest the existence of (organell-specific, stress-induced, developmentally controlled) V-type H^+ATPase isoforms in higher plants (3,4,5).
Using PCR technology we have amplified partial cDNA sequences of the carrot B- and c-subunit starting with 1.strand cDNA. The partial B-subunit and c-subunit sequences are highly homologous to their *Arabidopsis thaliana* (6) and *Avena sativa* (5) counterparts, respectively. Using genomic DNA as template PCR amplification suggests the presence of a small gene family for the B-subunit.
The coordinate expression of two subunits of the V_1-part (A and B) and one subunit of the V_o-part (c) are now being analyzed in several experimental systems: a) carrot suspension-cultured cells grown in the absence/presence of 100 mM NaCl, b) evacuolated protoplasts during the process of revacuolation, c) carrot storage roots after hormonal induction of cell division. Interestingly, moderate salt-stress leads to an apparent change in the coupling ratio of H^+-transport/ATP-hydrolysis, and PCR amplification of 1.strand cDNA obtained from salt-stressed cells indicates expression of a B-subunit isoform.
Finally, the previously published partial characterization of the A-subunit promotor (2) has been extended by analysis of a set of promotor deletions and gel retardation experiments with the corresponding promotor fragments.
The results will be discussed with respect to coordinate gene expression and the existence of isoforms of the "housekeeping" plant V-type H^+ATPase(s).

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REGULATORY ROLE OF INTRACELLULAR PH CA2+ AND ABSCISIC ACID ON THE TWO ACTIVE H+ PUMPS OF TONOPLAST FROM BARLEY ROOT

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In higher plant cells, active H^+ pumps in membranes of the cellular organelles play an important role in plant metabolism, growth and development. Here we report the regulatory role of intracellular H^+, Ca^{2+}, and phytohormone abscisic acid (ABA) on vacuolar PPi- and ATP-dependent H^+ pump activities of barley roots.

Barley roots were incubated for 24 hrs in various solutions containing chemicals causing the intracellular acidification, alkalinization, or Ca^{2+} mobilization and membrane vesicles rich in tonoplasts were prepared from the roots. Incubations with acetate, CCCP, vanadate, and Cl^- (for intracellular acidification) enhanced PPi-dependent H^+ pumping activity of the membrane vesicles. In contrast, the activity decreased with K^+, and methylamine(for intracellular alkalinization). Treatment with Ca^{2+} ionophore A23187 increased the activity. As decrease in cytoplasmic pH can lead to increase in the cytoplasmic Ca^{2+}, and vice versa, these results suggest a new signal transduction mechanism where cytoplasmic H^+ or Ca^{2+} may work as a second messenger for the regulation of PPi-dependent H^+ pumping activity.

NO_3^--sensitive ATP-dependent H^+ pumping activity was not significantly changed by all the above treatments. However, ABA was very effective on enhancing the activity. ABA also increased the PPi-dependent H^+ pumping activity. It seems plausible that the increased activity of PPi-dependent H^+-pump depends on the ability of ABA to behave as a Ca^{2+} agonist.

POTASSIUM TRANSPORT INTO PLANT VACUOLES IS DIRECTLY ENERGIZED BY THE TONOPLAST INORGANIC PYROPHOSPHATASE

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Vacuolar K^+ accumulation is opposed by an inside-positive membrane potential of about +20 mV (1), thus necessitating energised K^+ transport across the tonoplast. No H^+-coupled K^+ uptake systems have been characterized. However, the tonoplast H^+-translocating inorganic pyrophosphatase (H^+-PPase), which is activated by K^+, could symport K^+ with H^+ into the vacuole. The following evidence in support of this hypothesis has been obtained by the patch-clamp technique, which has been used to measure the membrane currents generated by the H^+-PPase in isolated vacuoles from Beta vulgaris.

A pyrophosphate-dependent inward current into the vacuoles has been shown to be activated by K^+ at the cytoplasmic side but not at the vacuolar side of the membrane (2). Conversely, an inorganic phosphate-dependent outward current (putative reversal of the H^+-PPase) is activated by K^+ at the vacuolar side.

Moreover, if K^+ were to activate the enzyme without being transported, it should not change the equilibrium of the reaction. In fact, the reversal potential of the (pyrophosphate + phosphate)-dependent currents (E_{rev} : the potential at which the pump is poised at equilibrium) was found to be a function of the K^+ gradient (and not the absolute concentration of K^+) as well as the H^+ gradient across the tonoplast membrane.

Analysis of E_{rev} values suggested that the H^+-PPase is capable of directly translocating K^+ and H^+ into the vacuole, with an overall stoichiometry of 3 positive charges per catalytic cycle.

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CHARACTERIZATION OF GENES ENCODING VACUOLAR H⁺-PYROPHOSPHATASE OF *ARABIDOPSIS THALIANA* AND *BETA VULGARIS*

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Full-length cDNAs encoding the substrate-binding subunit of the vacuolar H⁺-translocating inorganic pyrophosphatase (H⁺-PPase) (1, 2) have been cloned and sequenced from *Arabidopsis thaliana* (3) and *Beta vulgaris* (4). Both classes of cDNA isolate (designated AVP and BVP for ***A****rabidopsis* and ***B****eta* **V**acuolar **P**yrophosphatase, respectively) encode a highly hydrophobic protein with a deduced molecular mass of 81 kDa containing 13-16 amphipathic membrane spans. AVP and BVP show greater than 93% sequence identity at the polypeptide level and both deduced amino acid sequences precisely align with direct sequence data acquired from the MgPPi-protectable, [^{14}C]-N-ethylmaleimide-reactive subunit of the H⁺-PPase isolated from *Beta* (2, 3). While two of the predicted cytosolic hydrophilic domains of the polypeptides encoded by AVP and BVP contain sequence motifs reminiscent of the MgPPi-binding sites of soluble PPases, the clones do not otherwise contain sequence identities with any other class of ion translocase or PPi-dependent enzyme. The results of Southern and Western analyses do, however, indicate that AVP and BVP and the polypeptides they specify are homologous to the reversible H⁺-PPi synthase of *Rhodospirillum rubrum* and *Rhodospirillum capsulatus*. Common evolutionary origins are therefore inferred for the vacuolar H⁺-PPase and the energy-tranducing H⁺-PPi synthase of photosynthetic bacteria.

Northern analyses of poly (A)⁺ RNA extracted from *Arabidopsis* demonstrate pronounced differences between developmental stages with respect to the levels of AVP expression and hybridization screens of an *Arabidopsis* genomic library constructed in λDASH have yielded three classes of genomic clones with distinct restriction patterns. Developmental regulation of expression and the existence of additional genes encoding the vacuolar H⁺-PPase are therefore indicated. The genomic clones are being characterized, and parallel biochemical analyses of transport-competent preparations of the reconstituted enzyme are being conducted, to determine if any of the additional genes encode isoforms of the substrate-binding subunit or additional, as yet unidentified, sequence-related subunits.

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PIVOTAL ROLE OF DIACYLGLYCEROL IN GLYCEROLIPID BIOSYNTHESIS BY CHLOROPLAST ENVELOPE MEMBRANES

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Plastid membranes are characterized by the presence of specific glycerolipids: galactolipids (monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG), sulfolipid (sulfoquinovosyldiacylglycerol, SQDG) and phosphatidylglycerol (PG). Most of these lipids share the same structural feature, i.e. a diacylglycerol backbone containing C18 and C16 fatty acids respectively at the *sn*-1 and *sn*-2 position of the glycerol and synthesized within plastids by a series of enzymes located on the inner envelope membrane (1,2). We have analyzed the functionning of two enzymes of this pathway, the phosphatidate phosphatase, which catalyzes dephosphorylation of phosphatidic acid into diacylglycerol, and the MGDG synthase, which converts diacylglycerol into MGDG.

We first demonstrated that the envelope phosphatidate phosphatase has unique biochemical properties (optimum pH, sensitivity to cations...) and is strikingly different from its extraplastidial and animal counterparts. Among the possible mechanisms involved in the regulation of phosphatidate phosphatase activity, feedback inhibition probably plays a major role (3). Using isolated intact spinach chloroplasts and envelope membranes from thermolysin-treated chloroplasts, we demonstrated that diacylglycerol is a powerful inhibitor of the enzyme (Ki 70 μM). Obviously, inhibition of phosphatidate phosphatase by diacylglycerol would favor channeling of phosphatidic acid towards PG.

We have also performed kinetic studies of MGDG synthase purified from spinach chloroplast envelope membranes (4). The affinity of the enzyme for its substrate is not very high (Km 180 μM), therefore availability of the substrate at the level of the active site of the enzyme could be a limiting factor of the reaction. Assays of the enzyme activity in presence of several diacylglycerol molecular species strongly suggest that structural specificities of MGDG could be due to properties of MGDG synthase. Finally, we have performed two-substrates kinetic analyses and we demonstrated that MGDG synthase is a random bireactant system.

The relevance of these results with the regulation of MGDG, SQDG and PG biosynthesis within chloroplast envelope membranes will be discussed.

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SPECIFIC RECOGNITION OF OLIGOGLUCOSIDE ELICITORS BY BINDING PROTEIN(S) IN SOYBEAN MEMBRANES

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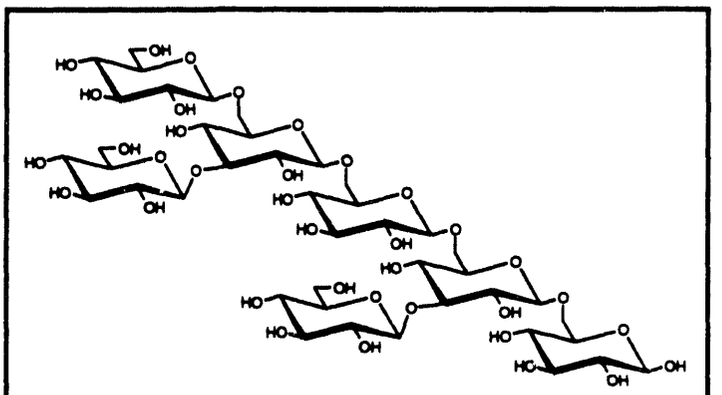

Figure 1: Structure of the hepta-β-glucoside elicitor.

The induction of pterocarpan phytoalexin biosynthesis in soybean in response to oligoglucoside elicitors is being used as a model for the study of cellular signaling mechanisms in plants. Our research has focussed on the first step in this signal pathway, namely the specific recognition of a hepta-β-glucoside elicitor (Fig. 1) by binding protein(s) (presumptive receptors) in soybean cells. The presence, in microsomes derived from the plasma membrane, of a single class of high-affinity (apparent K_d = 0.75 nM), proteinaceous binding sites for the hepta-β-glucoside was demonstrated in binding assays using a radio-iodinated tyraminylated derivative of the elicitor [Cheong & Hahn, Plant Cell 3: 137-147 (1991)]. Competitive inhibition of binding of the radio-labeled hepta-β-glucoside elicitor by structurally related oligoglucosides demonstrated a direct correlation between the binding affinities and the elicitor activities of these oligoglucosides. These results suggest that the structural elements previously identified as being important for the biological activity of the elicitor [Cheong *et al.*, Plant Cell 3: 127-136 (1991)] are also the structural elements recognized by the membrane-localized elicitor binding protein(s).

The hepta-β-glucoside elicitor binding protein(s) have been solubilized using several non-ionic detergents. The solubilized binding protein(s) retained the binding affinity (apparent K_d = 1.42 nM) for the radiolabeled elicitor determined previously for the membrane-localized binding protein(s). The solubilized binding protein(s) also showed the same specificity for elicitor-active oligoglucosides as did the membrane-localized binding protein(s). Current research is directed toward the identification, purification, and cloning of the hepta-β-glucoside binding protein(s). Techniques being utilized include photo-affinity labeling, affinity chromatography, and cloning by functional expression in a heterologous cell type. Purification and characterization of the hepta-β-glucoside binding protein(s) or their corresponding cDNAs is a first step toward elucidating, at the molecular level, how the hepta-β-glucoside elicitor triggers the signal transduction pathway that ultimately leads to the *de novo* synthesis of phytoalexins in soybean. (Supported by NSF grant DCB-8904574 and in part by the USDA/DOE/NSF Plant Science Centers Program through funding by DOE grant DE-FG09-87ER13810).

RESPONSE OF ROOT HAIR CELLS TO *RHIZOBIUM* NOD FACTORS

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The nitrogen fixing bacterium, *Rhizobium meliloti*, causes nodule formation on host plants such as alfalfa. This process involves altered cell morphogenesis in root hairs and cell division in the root cortex. The mechanism of this has recently been shown to involve Nod factors secreted by Rhizobium that act as morphogens on the plant. These factors are modified oligomers of N-acetylglucosamine. The factors in *R. meliloti* are characterized by an N-acyl group and a C-6 acetyl on the non-reducing Glc-NAc and by a C-6 sulfate on the reducing end. Our group and others are working on the relationship of bacterial nod genes to factor structure; our particular interest is in in vitro synthesis of Nod factors. The identification of the Nod factors now makes it possible to study early (possibly rapid) plant responses to a timed, chemically defined stimulus. How does the plant respond to *Rhizobium* Nod factors? What is the receptor? What events happen at the host cell membrane?

We used a spot-inoculation test to investigate the effect of bacterial supernatants and purified nod factors on roots. We found that both supernatants and Nod-factors act as true morphogens, in that the chemical Nod factors elicit development of complete nodules on plants. Given that Nod factors cause events in both epidermal and cortical cells, it is possible that a secondary signal mediates the effect and connects the events in the two plant cell types.

Some secondary signals are accompanied by changes in ion flux. We have found that alfalfa root hair cell membrane potentials can be measured stably by impalement with a single microelectrode, as shown by Brownlee for tomato, and used this technique to assay root hair responsiveness to Nod factors. Alfalfa, but not a non-legume plant (tomato), responds to Nod-RmIV(S) with a depolarization of root hair membrane potential, followed by recovery of the potential and desensitization of the cell to further stimulation. This cell-autonomous assay may be of use in following early events associated with response to Nod-factors, and in assaying which cell types may have the hypothesized Nod-factor receptor.

We have also followed root hairs during response to Nod factors by laser scanning confocal microscopy, using interference contrast and fluorescence optics. We observed a number of cytoplasmic events on the same time scale as the potential change: these include nuclear migration, breakup of a hair vacuole, and aggregation of endoplasmic reticulum-rich cytoplasm at the hair tip. The Nod factor approach makes it possible to associate the time course of this and other responses with the putative secondary signal leading to changes in ion flux.

DOES WATER LEAVE MOTOR CELLS THROUGH H+ PUMPS DURING TURGOR MOVEMENTS?

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When tendrils of the pea plant are mechanically perturbed (MP) on the adaxial surface, a concave curvature develops in the first 20-40 min. This is due to the efflux of water from the adaxial surface, but not from the abaxial surface (1). The resulting difference in turgor causes the tendril to bend around the support against which it rubbed. The bending is preceded by an efflux of H^+. We have used inhibitors of the PM redox chain (which is known to generate an internal accumulation of H^+) and of the PM H^+ pump inhibit both MP-induced coiling and the H^+ efflux associated with it. Since the H+ efflux through the pump, it seemed possible that the water also effluxed through the open pump channel. The same inhibitors that block the pump also block water efflux from MP-treated tendrils. Furthermore, the effective concentration ranges are the same for both processes. Since water is known to efflux animal cells through the action of proton pumps (2), we conclude that the MP-induced turgor change in the adaxial cells occurs, at least in part, by water movement associated with MP-activated proton pumps.

The water efflux across the PM is associated with the action of the proton pump. We ask whether the water molecules actually move thorough the pump with the H^+, or if they move across the lipid bilayer by mass flow in response to the osmotic effect of the accumulation of H^+ in the extracellular cell wall matrix. When the extracellular H+ concentration is increased, it induces coiling and enhances MP-induced coiling. This suggests that the MP-induced water efflux may occur osmotically, by mass flow. To differentiate between the two cases, we plan to use polymeric H^+ buffers (e.g., polyaspartate, polyglutamate) to allow H^+ to be pumped across the membrane, but not to accumulate outside the cell. The results of these experiments will be presented.

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ELECTRICAL CHARACTERISTICS OF CELLS WITH AND WITHOUT ATP: I/V AND G/V PROFILES IN STEADY STATE

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The role of ATP in ion transport across plasmalemma of plant cells is emerging as increasingly complex: not only does it supply energy for the proton pump (1) and the proton pump-driven cotransports, but also it affects K^+ channels (2,3) and Ca^{++} channels (4,5). The current-voltage (I/V) method (6) was applied to distinguish and characterise transport systems while the concentration of ATP in the cell was controlled.

Characean cells were perfused with media containing ATP or hexokinase and glucose (to deplete ATP produced by the cell after perfusion). There are two types of the perfusion method: (a) the cell ends are ligated after perfusion and normal turgor is regained and (b) cells are left open and the outside and inside media are osmotically balanced. For review of the methodology see (1, 4). Changes in turgor pressure are known to affect various transport systems (7), so both (a) and (b) were employed in our experiments.

The *Chara* cells in group (a) perfused with 1 mM ATP displayed hyperpolarized resting potentials at -220 ±10 mV (7 cells) and sigmoid I/V (current-voltage) characteristics normally associated with the pump state (8). Near the resting potential the clamp currents stabilised within ~1 sec. If the cells were depolarized to an excitable level, the resting potential stayed at -60±13 mV (7 cells) and the I/V characteristics changed drastically, displaying a conductance increase near the resting level and a negative conductance region between -100 and -200 mV. The cells perfused with hexokinase and glucose showed depolarized resting potentials at -80±10 mV (5 cells) and upwardly concave I/V curve. The clamp currents exhibited large capacitive spikes. Excitation was abolished.

The cells in group (b) tended to lower resting potentials both with and without ATP, particularly if $Pb(NO_3)_2$ was used to stabilise them (1). However, the shapes of the +ATP and -ATP I/V curves were similar to those found in cells from group (a).

The results are discussed in terms of the proton pump I/V characteristics and the background channel conductances.

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SPATIAL VARIATIONS OF THE EXTRA- AND INTRA-CELLULAR pH OF CHARACEAN INTERNODE

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Using fluorescein-di-acetate (FDA) the relative cytoplasmic pH (pHc) was optically estimated as a function of position in Characean internode in relation to the acidic/alkaline regions. FDA-acetoxymethylester (FDA-AM) can penetrate the membrane and accumulates in the cytoplasm where FDA-AM is splitted into FDA and AM by cytoplasmic esterases. FDA can no longer get through the membrane out of the cytoplasm and acts as a pH-probe. The intensityies of fluorescence ratio at 540nm excited by 480nm/420nm is pH-dependent. In the case of light-on transition, it was found that the pHc at the latent alkaline region increased higher than that at the neighbouring acidic regions.

Recently, the time dependencies of the apparent macroscopic electrical parameters, including the membrane potential and conductance along the cell have been measured by scanning the water-film-electrode as a function of position. At the light-on transition, it has been shown that the membrane potential at the latent alkaline region depolarizes with a less increase in apparent conductance than the neighbouring latent acidic regions. After the depolarization, however, the membrane conductance at this particular region increases significantly up to four times or more larger than the neighbouring acidic regions. The resting potential is usualy found to be more negative than -150mv, due to the activity of H-pump; up to -300mv and -200mv at the acidic and the alkaline regions respectively. The light-off transition induces a remarkable decrease in conductance and a significant perturbation in potential. Then, getting uniform in both potential and conductance, the cell reaches a steady state within 2hrs in the routine experimental condition.

These observations enable us to give a possible interpretation on the alkaline region formation along the surface of Characean internode: At the light-on transition, the activity of cytoplasmic OH-ion in the latent alkaline regionis getting higher than the neighbouring regions. In the latent alkaline region, the H-pump activated by light is partially inhibited by OH-ions and the membrane is depolarized with a decrease in membrane conductance. Then, the increased OH-ions open the putative OH-channel. The alkalinity fixes the diffusible dissolved inorganic carbon (DIC) into $CaCO_3$ as a CO_2 reservoir.

RELATIONSHIPS BETWEEN H^+ PUMP ACTIVITY AND INTRACELLULAR pH

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Previous work, mainly carried out with the water plant *Elodea densa*, has shown that: i) the activation of H^+ extrusion ($-\triangle H^+$) by K^+ and by fusicoccin plus K^+ (FC+K^+) is associated with an alkalinization of both vacuole and cytosol (1); ii) the inhibition of $-\triangle H^+$ by erythrosin B (EB) or vanadate is accompanied by a significant acidification of both compartments (2); iii) the intracellular acidification induced by high (mM) concentrations of weak acids is associated with a large increase in $-\triangle H^+$ (3). This suggested that the H^+ pump activity can modulate the pH_{in} and is, in turn, influenced by its values.

To bear out this hypothesis and test whether it is generalizable, we examined some more experimental conditions in *Elodea densa*, and extended the investigation also to *Arabidopsis thaliana*, a plant material particularly suitable for the selection of mutants. Some results are here reported on the changes in $-\triangle H^+$ and in pH_{in} induced either by treatments with weak bases, or by changes of the pH_{out}, or by treatments with activators and inhibitors of the H^+ pump. For some conditions we also measured the changes in malate level and in buffer capacity of the cell sap associated with the changes in pH_{in}.

We found that: a) 5 mM nicotine increased vacuolar and cytosolic pH by 0.37 and 0.21 pH units, and strongly reduced $-\triangle H^+$ in *Elodea* leaves; b) the increase in $-\triangle H^+$ induced by the increase in pH_{out} (from 5.5 to 6.5) was associated with an increase in pH_{in}, more evident in *Arabidopsis*; c) the activation of the H^+ pump by K^+ or FC+K^+ (or the inhibition by EB) strongly increased (or decreased) both the pH_{in} and the buffer capacity of the cell sap in *Arabidopsis* ; d) the changes in buffer capacity were substantially due to the changes in malate level.

These results confirm the view of a modulating effect of the H^+ pump on the pH_{in} and, conversely, of the pH_{in} on the H^+ pump activity.

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MEMBRANE POTENTIAL IN RELATION TO CYTOPLASMIC PH MODIFIED BY LIGHT, ACID- AND ALKALINE-LOADING IN *EGERIA* AND *CHARA* CELLS

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In order to study the relationship between cytoplasmic pH(pH_c) and membrane potential (E_m), internodal cells of *Chara corallina* and leaves of *Egeria densa* were either illuminated, acid- or alkaline- loaded, and their pH_c and E_m were simultaneously measured with double-barelled pH micro-electrodes fabricated according to Reid and Smith (1988) (1) with a small modification. The basic external solution was an artificial pond water of pH_o 7.5.

When internodal cells of *Chara* were illuminated, pH_c increased and E_m often hyperpolarized. On the other hand, in leaf cells of *Egeria*, pH_c transiently increased and E_m hyperpolarized (Fig. 1). Fusicoccin (10 μM) did not affect both pH_c and E_m in *Chara* but induced in *Egeria* membrane hyperpolarization without causing a significant change in pH_c. When *Chara* and *Egeria* cells were treated with acetate (5 mM) or butyrate (5 mM) at pH_o 5, pH_c instantly decreased and the membrane potential hyperpolarized (Fig. 2). On the other hand, when cells were subjected to NH_4Cl (10 mM in *Chara*, 1 mM in *Egeria*) at pH_o 9, pH_c increased and the membrane potential depolarized. Thus, acidification of the cytoplasm always induces the membrane hyperpolarization which reflects an activation of the H^+ pump of the plasma membrane. But there are also cases where the membrane hyperpolarization can occur without coupling with the cytoplasmic acidification.

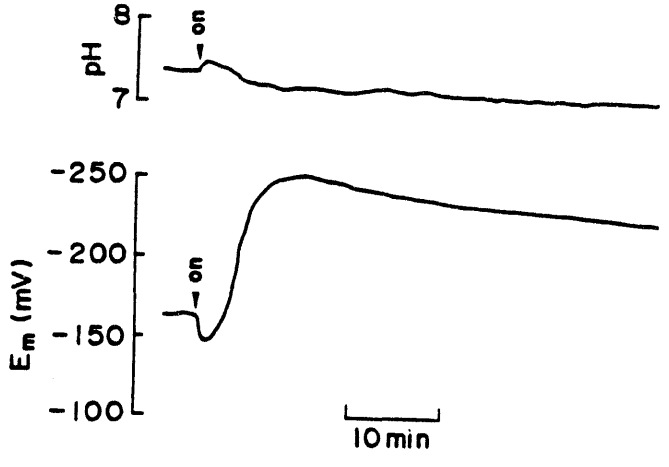

Fig. 1 : Changes in pH_c and E_m of *Egeria* cell on illumination.

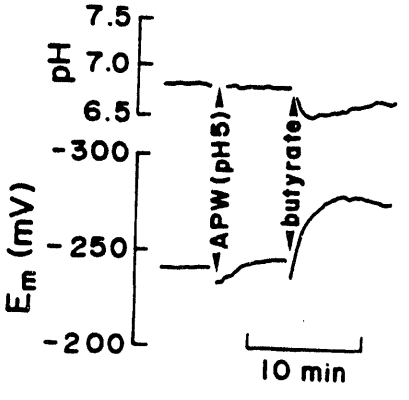

Fig. 2 : Changes in pH_c and E_m of *Egeria* cell after treatment of 5 mM butyrate at pH 5.

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Ultrastructure of *Chara Corallina* using Impedance Spectroscopy

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Introduction

For the last twenty years our department has specialised in the development of impedance spectroscopy. We have used such techniques to characterise conduction, dielectric and geometrical properties and electro-chemical processes of the sub-microscopic layers in synthetic membranes and at solution electrode interfaces. However, the underlying motivation for this development is the *in vivo* characterisation of cell ultrastructure.

Measurements and interpretations of impedance spectra are heavily dependent on complicated mathematics. It has been difficult to find a descriptive presentation which by-passes the mathematics and reveals the physics. We have recently developed a presentation, based on Network (or Transfer) Functions used in Control and Systems Analysis, which we feel provides such a physical insight. Here we present impedance spectra from *Chara Corallina*, using this presentation.

Materials and Methods

Four Terminal measurements of impedance[1] were made on *Chara* with our version of the Ogata 'water film electrode' apparatus[2,3]. The effect of cable properties in the long cylindrical cells were accounted for[4].

Results

A typical Network Function for *Chara* (a transformation of the impedance spectra) is shown in Fig A and depicts several peaks. The location of the peaks define frequency constants (reciprocal time constants) which arise from electrical geometrical properties of *Chara* ultrastructure. Our analysis suggests that the first two peaks arise from the plasmalemma and tonoplast, the third from plasmalemmasomes[5], the fourth from the Plasma Membrane Coat[6] and the fifth, with a frequency constant at very high frequencies (not shown), from the static cytoplasm[3].

Fig B shows the Cauche integral of the Network Function. The step sizes at the peaks are the reciprocal of the capacitances of the ultrastructures. In some instances, when the dielectric constants of ultrastructures are known, these capacitances can be used to calculate the thicknesses of structures and/or other geometrical features such as plasmalemmasomes[7]. However, such interpretations do not apply to the first two peaks which characterise the plasmalemma and tonoplast. These peaks and the corresponding 'reciprocal capacitance' steps arise from phenomenological effects such as those described by Coster's Double Fixed Charge model[8] for these membranes.

Discussion

We assume that the tonoplast impedance is at most 1/3 that of the plasmalemma[9], that the dielectric geometric capacitance of an unfolded plasmalemma[10] is 10mF/m^2 and that our algorithm accurately corrects for cable properties[4]. Given these assumptions, our model returns physiologically reasonable values for the electrical geometrical properties of *Chara* ultrastructure quoted in the literature[3-15].

Acknowledgments

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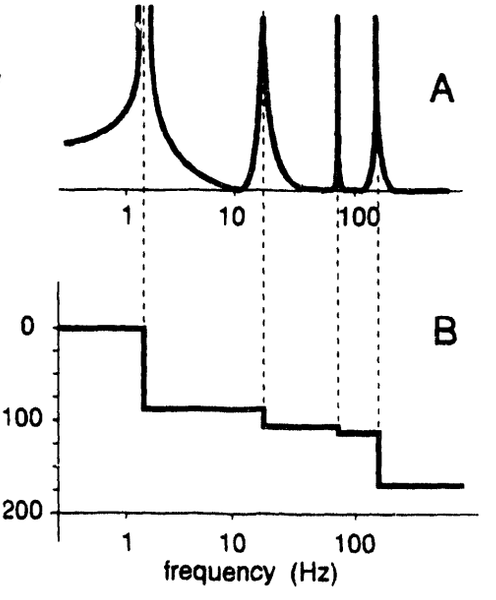

IDENTIFCATION AND BIOCHEMICAL CHARACTERIZATION OF THE PLASMAMEMBRANE H^+-ATPase IN GUARD CELLS OF *Vicia faba*.

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The plasma membrane H^+-pump in guard cells generates the driving force for rapid ion fluxes required for stomatal opening (1). In order to investigate the cell specific biochemical properties of this proton-translocating ATPase plasmamembrane vesicles were from isolated from guard and mesophyll cells of *V. faba*. The capability of the H^+-ATPase to create a proton gradient is maintained in vesicles derived by high pressure homogenization and polymer-separation (2). Furthermore two polypeptides in the range of 100 and 92 kDa were identified by Western blot analysis.

Comparison of either H^+-pumping or ATP-hydrolysis in isolated plasmamembrane vesicles revealed a 2-fold higher specific activity in guard cells than in mesophyll cells, which is consistent with recent patch clamp studies (3). Results from immuno-blot analysis indicate that these differences in enzyme activities were traceable to a higher abundance of the electroenzyme per unit membrane protein in guard-cell plasmamembrane.

We suggest that the high H^+-pump capacity is necessary to enable guard cells to react to sudden changes in the environment by a change in stomatal aperture.

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EPITOPE-TAGGING OF A PHLOEM-SPECIFIC PLASMA MEMBRANE H +-ATPase ISOFORM OF *ARABIDOPSIS*

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The plasma membrane H^+-ATPases of plants are encoded by a multi-gene family, with at least ten members identified in *Arabidopsis*. The various H^+-ATPases have different tissue-specific patterns of expression, possibly providing cell-types such as phloem cells, guard cells, and root hairs with specific isoforms to meet their diverse physiological requirements. The extensive amino acid similarity between the H^+-ATPase isoforms prevents cursory prediction of differences in enzyme function, and impedes production of isoform-specific antibody probes. Isoforms may be immunologically and biochemically indistinguishable, however nuances in sub-cellular localization, activity, regulation, and associations with cellular components could have important consequences for the cells in which they exist. Presently we are employing the method of epitope-tagging to permit surveillance of individual *Arabidopsis* H^+-ATPase isoforms. This method entails cloning the antigenic determinant for an available monoclonal antibody into the coding region of an H^+-ATPase gene, and regenerating transgenic plants which produce "tagged" H^+-ATPase proteins.

We have regenerated transgenic tobacco and *Arabidopsis* plants that produce epitope-tagged *Arabidopsis* H^+-ATPase isoform 3 (AHA3). Previous studies have indicated that AHA3 is a 100 kd plasma membrane protein that is expressed specifically in phloem cells, pollen, and regions of the ovule. Antigenic determinants for c-myc, influenza hemagluttinin, and the entire coding sequence for β-glucuronidase (GUS), were individually cloned into the cytoplasmic carboxy-terminus of AHA3. Transgenic plants expressing the "tagged" genomic sequences were regenerated using standard transformation techniques. The c-myc and influenza hemagluttinin monoclonal antibodies immunoreact with a 100 kd membrane protein in transgenic, but not untransformed control plants. Furthermore, preliminary data suggest that GUS-tagged AHA3 is targeted and expressed in the plasma membrane of phloem cells.

EEDQ INHIBITION OF MAIZE ROOT P-TYPE ATPASE AND ITS RELATIONSHIP TO ENZYME STRUCTURE AND FUNCTION

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The biochemical events that convert the chemical energy from the hydrolysis of ATP to the generation of an electrochemical gradient by transport proteins are poorly understood. The inhibition of the plasma membrane ATPase from corn roots by N-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) was compared to that of ATPase solublized with N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (3-14) to provide insight into the minimal functional unit. The chromatographic behavior of the 3-14 solublized ATPase activity during size exclusion chromatography and glycerol gradient centrifugation indicated that the solublized enzyme was in a monomeric form. Both plasma membrane-bound and solublized ATPase were inhibited by EEDQ in a time- and concentration-dependent consistent with a first-order reaction. When the log of the reciprocal of the half time for inhibition was plotted as a function of the log of the EEDQ concentration, straight lines were obtained with a slope of approximately 0.5 and 1.0 for membrane-bound and 3-14 solublized ATPase, respectively. The results are discussed in terms of a change in the number of polypeptides per functional ATPase complex from a dimer in plasma membranes to a monomer after solublization with 3-14, and support the conclusions attained previously by irradiation inactivation (Briskin, DP, Reynolds-Niesman, I. 1989. Plant Physiology 90:394-397).

Localization of H^+-ATPases in the plasma membrane and in the cytoplasm of lily pollen: an electronmicroscopic study.

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H^+-ATPases in the plasma membrane of plant cells play an essential role in plasmalemma transport, cellular metabolism, and plant growth, particularly tip growth. Most tip growing plant cells drive an ionic current through themselves, and it has been suggested that a heterogeneous distribution of H^+-ATPases in the plasma membrane of the cell is involved in the observed current patterns [1]. For H^+-ATPases labelling, sections of pollen grains and tubes from *Lilium longiflorum* were treated with monoclonal antibodies from mice (clone 44B8A1) and gold-conjugated anti-mouse antibodies [2].

The results show that immunochemical cross-reaction with the monoclonal antibody occurs in the plasma membrane of pollen grains (5.7 μm^{-1}) and in the plasma membrane of the tube tip, but with half the density (3 μm^{-1}) of that found in the pollen grain. No binding of the antibody could be detected in membranes of vesicles that will fuse with the plasma membrane of the tube tip. Binding sides for antibodies are absent in mitochondrial, liposomal, and amyloplastic membranes, and also almost absent in the plasma membrane of the generative cell. Sometimes antibodies binding to ER and dictyomsome membranes could be observed.

The reported results support the proposal that a heterogeneous distribution of H^+-ATPases could be found in the plasma membrane of tip growing lily pollen. Epitopes to which the antibody binds have also been observed in cytoplasmic membranes belonging to the secretory pathway (ER and golgi). Therefore, with this technique it would also be possible to investigate a change in the turnover of H^+-ATPases which is supposed to occur during plant growth hormone action [3].

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MOLECULAR CLONING OF VANADATE-SENSITIVE ATPASE FROM MARINE ALGA AND RICE WITH PCR METHOD

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Animal Na^+,K^+-ATPase, H^+,K^+-ATPase and Ca^{2+}-ATPase and H^+-ATPase from higher plants and fungi are grouped in E-type ATPase which activity is inhibited by orthovanadate and localized on plasma membrane or sarcoplasmic reticulum. The vanadate sensitive ATPases form phosphorylated intermediate during reaction cycle. Two parts of those enzymes are highly conserved, the phosphorylation site and ATP binding site are also common with 7 to 12 amino acids sequence among them (1).

Synthesized oligonucleotides (both 20 bps) from the two parts were used for PCR primers to amplify this part between phosphorylation and ATP binding sites of vanadate-sensitive ATPases from cDNA of rice and marine alga *Heterosigma akashiwo* (2). Approximate 800 bp DNAs were amplified from both cDNA libraries, respectively. The amplified DNAs were used for probe to screen for ATPase cDNAs. Resultant clone of rice, OSA1, showed high homology to H^+-ATPases from other higher plants. The deduced amino acid sequence homologies of OSA1 compared with that from tomato (*L. esculentum*), tabacco (*N. plumbaginifolia*), *A. thaliana* and *S. cereviceae* were 87.8, 87.4, 79.2 and 35.5 %, respectively. Another clone (HAA13) from marine alga *H. akashiwo* (Raphidophyceae) showed the highest homology (52.0 %) with H^+-ATPase from protista *Leishmania donovani*, but the low homology (25.0 %) with animal Na^+,K^+-ATPase.

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THOSE PROTON PUMPS MAY BE TOXIC! EXPRESSION OF THE *NEUROSPORA* PLASMA MEMBRANE ATPase IN CULTURED MAMMALIAN CELLS

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Objectives. Heterologous expression of a fungal plasma-membrane H^+-ATPase gene in mammalian cells has been undertaken with three principal objectives: I) to develop a preparation which is readily amenable to whole-cell patch recording, for electrophysiological study of mutated ion pumps; II) to examine the physiologic regulation of this transporter in a biological membrane normally lacking such a system; and III) conversely, to study the H^+-pump's effect on metabolism of cells which normally regulate cytoplasmic pH by other mechanisms.

Methods. cDNA coding for the plasma-membrane H^+-ATPase from *Neurospora crassa* (Hager et al., *PNAS* 83:7693, 1986), was introduced into the pSV2 vector and cotransfected into NIH3T3 cells along with pSV2neo (neomycin resistance gene) for selection. Out of 18 cell lines which showed both antibiotic resistance and hybridization (southern blots) with a cDNA probe for the H^+-ATPase gene, eight proved stable and were carried into further experiments.

Results. For all eight lines, integration of the *Neurospora* gene into the mammalian genome was low: 1-2 copies each. Application of three different restriction-digestion strategies yielded different patterns for each of the lines, implying integration of pSV2 (SV40 promoter) and the H^+-ATPase gene at a different point in the genome for each. All eight were immunopositive for the ATPase protein, probed with the Hager polyclonal antibody (ibid), by western blots and immunofluorescent cytochemistry. The level of expression of immunoreactive material was sensitive to age and growth temperature, as well as to the presence or absence of the (SV40) transcription-inducing agent, sodium butyrate. Bona fide *Neurospora* enzyme bands on SDS gels at 100 kD, and six of the lines showed this band, though it was low in two (<0.02 ng/μg membrane protein) and high in one (>0.8 ng/μg) compared with the average (0.2-0.3 ng/μg), under optimal conditions. An unexpected immunoreactive band at 120 kD was observed at 37°C, but disappeared on incubation at 26°C.

Immunofluorescent cytochemistry indicates the appearance of *Neurospora* gene product predominately in endoplasmic reticulum for three cell lines thus far examined, and in plasma membrane for two. The latter two lines, in particular, have been tested for proton-pumping and cytoplasmic buffer capacities, and both display enhanced cytoplasmic buffering in response to pulsed acid loading of the cells, when compared either with the normal 3T3 cells, with a vector-transfected control, or with vanadate-treated transfected cells. [The proton-pumping experiments have necessarily been carried out during inhibitor suppression of the native Na^+/H^+ exchanger.] Electrophysiological experiments to measure possible ionic currents from the expressed proton pump are under way.

Growth of all eight transfected lines is slow compared with that of normal 3T3 cells, and suggests a potential toxic effect of the *Neurospora* protein in mammalian cells. These results clearly differ from those reported earlier upon expression of the corresponding *Saccharomyces* gene in mammalian fibroblasts (Perona & Serrano, *Nature* 334:438-440, 1988; Gillies et al., *PNAS* 87:7414, 1990).

The authors are indebted to Drs. Carolyn Slayman and Toni Claudio for continued encouragement, advice and support, and to Dr. Robert Nakamoto for assistance with many technical problems.

EXPRESSION OF FUNCTIONAL PLANT PLASMA MEMBRANE H^+-ATPase IN YEAST INTERNAL MEMBRANES

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We have developed a system for heterologous expression of the plant plasma membrane H^+-ATPase in yeast (J. Biol. Chem. (1992) in press). One of the isoforms (AHA1) of the plasma membrane H^+-ATPase from Arabidopsis thaliana was produced in a yeast expression system comprising a multi-copy plasmid and the strong promoter of the yeast PMA1 gene. Western blotting with a specific monoclonal antibody showed that the plant ATPase is one of the major membrane proteins made by the t[illegible]med cells. The plant ATPase synthesized in yeast i[illegible]y active. It hydrolyzes ATP, pumps protons, and the reaction cycle involves a phosphorylated intermediate. Unlike the situation in plants, however, most of the plant ATPase is not expressed in the plasma membrane. Rather, the enzyme appears to remain trapped into the endoplasmic reticulum. In this location the plant ATPase can be purified with high yield (70 mg from 1 kg yeast) from membranes devoid of endogenous yeast plasma membrane H^+-ATPase.

Using this convenient expression system two other isoforms of the plant ATPase (AHA2 and AHA3) have been produced and characterized. In addition, the yeast system has allowed us to characterize some mutants of the plant ATPase produced by in vitro mutagenization.

IMMUNOLOGICAL APPROACHES TO THE STUDY OF PLANT CALMODULIN-STIMULATED CALCIUM PUMPS

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Calmodulin-stimulated calcium pumping ATPase (CaM-ATPase) activity has been purified and characterised from plants ([1] and Askerlund and Evans, this volume). This poster describes work resulting in the production and characterisation of a small family of monoclonal antibodies to purified CaM-ATPase and preliminary studies to use them to determine the subcellular localisation of this activity.

Female balb/c mice were immunised at 4-week intervals with affinity purified CaM-ATPase. Of 469 hybridoma cell lines screened by ELISA, 20 recognised epitopes present in the column eluent. All MABs were class IgM and none were affected by periodate oxidation, indicating that they did not recognise carbohydrate epitopes of glycoproteins. Secondary screening was by Western blotting against CaM affinity column eluent, microsomes and purified plasma membranes. Six cell lines were found to recognise a c140,000 M_r polypeptide in the column eluent, likely to be the intact CaM ATPase.

Light microscope immunofluorescence studies of onion and maize root cells using the antibodies indicated predominantly non-plasma membrane staining. Fluorescence staining appeared to be co-localised with endoplasmic reticulum. Localisation of the CaM-ATPase at both the endoplasmic reticulum and the plasma membrane was suggested by Western blotting aqueous two-phase partition- and sucrose density gradient-purified membranes. Here, the MABs recognised a polypeptide of around 150,000 M_r which may be the CaM-ATPase running at an anomolously high M_r. Biochemical evidence supporting the proposition that the CaM-ATPase has a dual subcellular location is presented by Askerlund and Evans (this volume).

When a range of MABs to mammalian SR/ER (A4H3, Y1f4, Y2E9, Y3G6, 12H7;[2]) and PM calcium pumps (gift of J.T. Penniston) were Western blotted against purified plant CaM-ATPase no recognition of high M_r polypeptides was indicated, although the anti-mammalian PM CaM-ATPase MAB gave a similar immunofluorescence pattern to the anti-plant MABs. The immunoblotting results were in contrast to earlier work using an anti-erythrocyte CaM-ATPase polyclonal antibody[1].

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CHANGES IN ATP-DEPENDENT Ca^{2+} TRANSPORT IN SEALED PLASMA MEMBRANE VESICLES ISOLATED FROM SENESCING PARSLEY LEAVES

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It has been suggested that the capability to maintain cytoplasmic Ca^{2+} at homeostatic levels is impaired during plant senescence. To test whether this could be due to changes in the Ca^{2+} pumping activity, we have characterized the ATP-dependent Ca^{2+} transport activity in plasma membrane vesicles isolated from leaves of parsley (Petroselinum crispum) at various senescence stages. Based on three senescence-associated activities (chlorophyll loss, proteolysis and lipid oxidation), six consecutive senescence stages were defined in detached parsley leaves undergoing dark senescence (stages A-F). Sealed plasma membrane vesicles were obtained in high purity from microsomal fractions of these senescing leaves by aqueous two-phase partitioning. Based on the analysis of a range of markers, the plasma membrane preparations (U_2 phase), which exhibited a vanadate-sensitive, Mg^{2+}-dependent H^+-ATPase activity, were shown to be devoided of mitochondria membranes or acid-phosphatase activity, and showed only trace amounts of tonoplast contaminations. All the vesicles were oriented inside-out and exhibited ATP-driven ^{45}Ca transport at pH 7.2. During senescence of detached parsley leaves, the specific activities of the H^+-ATP hydrolysis, decreased by 50% during the initial 24 h, and then leveled off. On the other hand, the ATP-dependent uptake of Ca^{2+}, which declined too by 50% during the initial 24 h, decreased to zero within the subsequent 48 h. These senescence-induced changes in the activities of the membraneous enzymes preceded the onset of chlorophyll loss and the rapid accumulation of aldehydes and amino acids. The results suggest that loss of the ability of extruding Ca^{2+} out of the cell is an early event in senescence of parsley leaves. Such an event is likely to result in elevated levels of Ca^{2+} in the cytoplasm, which may be the signal that triggers turnover of membrane phospholipids, leading irrevocably to cell death.

INVESTIGATION OF CALCIUM-TRANSPORTING ATPases IN RED BEET

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For calcium to function effectively as a secondary messenger its concentration within the cytoplasm must be carefully regulated and maintained at a low resting level. The plasma membrane, tonoplast and endoplasmic reticulum (ER) are thought to play important roles in calcium homeostasis through the action of direct Ca^{2+}-pumping ATPases (ER and PM) and a Ca^{2+}/H^{+} antiport (tonoplast) (Evans et al. 1991).

The plasma membrane and ER calcium pumps show similar properties with respect to their calcium transporting activities. Therefore this study aims to differentiate between these two systems by studying the phosphorylated intermediates formed when incubating ER-enriched and plasma membrane-enriched fractions with [γ-^{32}P]ATP as substrate.

Purified plasma membrane was obtained by aqueous two phase partitioning of a red beet microsomal fraction. The absence of NADH-Cyt c reductase activity in this fraction indicated the lack of ER contamination. In contrast the lower phase obtained following aqueous-two phase partitioning of an ER-containing-sucrose gradient fraction was highly enriched in NADH-Cyt c reductase activity. Western blotting, using a polyclonal antibody to the plasma membrane proton pump, revealed that this ER-enriched fraction was only slightly contaminated by plasma membrane. The phosphorylated intermediates formed in both of these fractions were characterized. Calcium-dependent phosphoenzymes were observed in both fractions which exhibited rapid turnover, hydroxylamine sensitivity and vanadate sensitivity. The effects of erythrosin B, thapsigargin, cyclopiazonic acid, and a number of K^{+} salts were studied to investigate possible differences in the properties of the ER and PM calcium transporters.

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A PROTEIN WITH HOMOLOGY TO CALCIUM-TRANSPORTING ATPASES IS FOUND IN THE CHLOROPLAST ENVELOPE

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A partial *Arabidopsis thaliana* cDNA clone has been selected from an expression library using an antiserum directed against proteins from spinach (*Spinacea oleracea*) chloroplast envelope. Sequence analysis reveals that this clone contains an open reading frame encoding a 96 kDa polypeptide. The deduced amino acid sequence contains all of the features common to P-type ATPases as well as regions of homology unique to calcium-transporting ATPases from animal, plant and fungal sources. The highest homology was found to mammalian plasma membrane Ca^{2+}-ATPases (31% amino acid identity with rat plasma membrane Ca^{2+}-ATPase).

Localization of the polypeptide to the chloroplast envelope was confirmed immunochemically. A fusion protein was expressed in *E. coli* from the cDNA and an antiserum was raised against the fusion protein. This antiserum was used to probe preparations of pea chloroplast envelope, thylakoid membranes and microsomes on immunoblots. Strong specific cross-reactivity was observed to a 90-95 kDa polypeptide in the chloroplast envelope fraction. Slight cross-reactivity was observed to a larger polypeptide in the microsomes and little or no cross-reactivity was observed in the thylakoid preparation.

Experiments are in progress to identify ion-transporting ATPases in chloroplast envelope preparations. Experiments are planned to analyze targeting of the polypeptide to the chloroplast envelope and to elucidate the physiological function of this enzyme.

CHARACTERISATION AND LOCALISATION OF A Ca^{2+}-DEPENDENT ATP HYDROLYSIS IN THE MICROSOMAL FRACTION OF WHEAT ROOT.

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A Ca^{2+}-dependent ATP hydrolysis present in the microsomal fraction of wheat roots has been previously reported[1,2]. In this poster we present data which more fully characterises this activity.

The pH profile of Ca^{2+}-dependant ATP hydrolysis in the microsomal fraction revealed two distinct activity peaks at pHs of 5.5 and 7.5. Density gradient centrifugation on an 8-45% (w/w) continuous sucrose gradient indicated that at both pHs the Ca^{2+}-dependent ATP hydrolysis was maximal in fraction 14 (F_{14}). This fraction had a sucrose density of 1.045 g ml^{-1} and was tonoplast enriched, as demonstrated by maximal nitrate sensitive Mg^{2+},Cl^-ATPase activity. In F_{14} the Ca^{2+}dependant ATP hydrolysis was 38-51% higher than the Mg^{2+}-dependent hydrolysis. The nucleotide specificity of both the Ca^{2+} and Mg^{2+}-dependent hydrolysis in F_{14} was high for nucleotide triphosphates but low for di- and mononucleotides and *p*-nitrophenylphosphate. The Ca^{2+}-dependent hydrolysis was more ATP specific at pH 5.5 than 7.5.

ATP hydrolysis in F_{14} had a K_m of 8.12 $\pm$0.63 μM for $[Ca^{2+}]_{free}$. The Ca^{2+}-dependent ATP hydrolysis at both pH 5.5 and 7.5 was insensitive to vanadate, azide and baflinomycin, however, the activity at pH 7.5 was sensitive to KCl.

These results indicate the presence of a Ca^{2+}-dependent ATPase on the tonoplast of wheat roots. The activity of this enzyme will be low at cellular levels of free Ca^{2+} (low nanomolar range) but may become activated during Ca^{2+} "spikes" associated with various signal transduction processes[3]. This enzyme may therefore have a role in purging the cytoplasm of Ca^{2+}.

Work is currently proceeding to determine the relative H^+-pumping activities of the Mg^{2+} and Ca^{2+}-ATPases in tonoplast vesicles.

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CALCIUM AND VANADATE ENHANCE PROTEIN PHOSPHORYLATION AND INHIBIT H^+-ATPASE ACTIVITY IN THE PLASMA MEMBRANE OF TOBACCO, CUCUMBER AND ARABIDOPSIS THALIANA

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Plasma membranes were isolated from leaves of tobacco, cucumber and *Arabidopsis thaliana* by aqueous two phase partitioning. Protein kinases and phosphoproteins were highly enriched in the plasma membrane. Specific activity of protein kinases in the plasma membrane was > 100 times higher than in soluble protein fraction and > 7 times higher than in endomembranes of all three plant species. *In vitro* phosphorylation of plasma membrane proteins was Ca^{2+}, but not calmodulin-dependent. A few proteins showed strong phosphorylation in the absence of free Ca^{2+}. The phosphorylation of pp60, a phosphoprotein present in all three plants, was inhibited by free Ca^{2+}. Vanadate, a potent H^+-ATPase inhibitor, strongly and specifically enhanced the phosphorylation of plasma membrane proteins at concentrations from 100 to 500 μM. Vanadate had no effect on the phosphorylation of soluble and endomembrane proteins. Vanadate had no effect on the thiophosphorylation of plasma membrane proteins, whereas Ca^{2+} greatly enhanced thiophosphorylation. Results from chase experiments after pulse labeling in the presence and absence of vanadate suggested that vanadate increased the rate of accumulation of phosphorylated proteins in the plasma membrane by inhibiting dephosphorylation. The enhancement in protein phosphorylation by Ca^{2+} and vanadate was additive. Plasma membrane protein kinases were highly sensitive to serine/threonine kinase inhibitors, but not to tyrosine kinase inhibitors. Okadaic acid had no effect on the plasma membrane protein phosphorylation. Plasma membrane phosphoproteins were highly phosphorylated at the serine residue. Tyrosine phosphorylation was not detected. The ratio of phosphoserine to phosphothreonine was about 7. Ca^{2+} and vanadate strongly inhibited H^+-ATPase activity, whereas EGTA and EDTA strongly promoted H^+-ATPase activity and reversed the inhibition by Ca^{2+}, but not by vanadate. The results suggest that H^+-ATPase in the plasma membrane may regulated by protein phosphorylation.

ACTIVATION OF THE GUARD CELL PLASMA MEMBRANE H^+-ATPASE OF *VICIA FABA* BY PLANT GROWTH REGULATORS

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Stomatal movement is controlled by external and internal signals such as light, phytohormones or cytoplasmic calcium.

We have studied the dose-dependent effect of auxins on the modulation of stomatal opening as mediated through the activity of the plasmamembrane H^+-ATPase. The auxin-dependent opening was characterized by bell-shaped dose-response-curves.

The patch-clamp technique was used to elucidate the electrical properties of the H^+-ATPase as effected by growth regulators and seasonal changes. H^+ currents through the ATPase were characterized by a voltage-dependent increase in amplitude positive to the resting potential, reaching a plateau at more depolarized values. Auxin-stimulation of this electroenzyme resulted in a rise in the outward-directed H^+ current and membrane hyperpolarization, indicating that modulation of the ATPase by the hormone may precede salt accumulation as well as volume and turgor increase.

Annuale cycles in pump activity were expressed by a minimum in pump current during january and february. Resting potentials and plasma membrane surface area, on the other hand, did not exhibit seasonal changes.

The pump activity per surface area was approximately 2-3 fold higher in guard cells then in mesophyll cells and thus correlates with their physiological demands.

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RE-EXAMINATION OF THE ACID GROWTH THEORY OF AUXIN ACTION: AUXIN-INDUCED PROTON EXCRETION AND ACID-INDUCED CHANGES IN THE MECHANICAL PROPERTIES OF THE CELL WALL.

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We re-examined the acid growth theory of auxin action concerning the following two points.

1)Auxin-induced proton-excretion.

IAA was applied to Vigna hypocotyl segment by xylem perfusion. IAA stimulates the electrogenic proton pump at the plasmalemma that excretes protons to the cell wall apoplast. However, a large amount of protons is trapped in the cell wall as a pH-buffer (1). We attempted to release protons from the cell wall by application of cations that can exchange with protons on the cell wall polysaccharides (2).

2)The changes in the mechanical properties of the cell wall during acid growth: comparison with that under auxin-induced growth.

An aerosol of HCl solution was applied to the abraded Vigna hypocotyl segment during xylem perfusion. The cell wall extensibility and the effective turgor were increased under the acid-induced growth as similar to auxin-induced growth (3). However, turgor pressure, which scarcely changed under auxin-induced growth (3), largely decreased under acid-induced growth. We consider this discrepancy in the behaviour of turgor pressure as follows. Auxin stimulates both the surface and the xylem proton-pumps simultaneously (4), then enhanced the active water uptake as simultaneously as it loosens the cell wall (5). On the other hand, acid-aerosol does not stimulate the proton pumps although it loosens the surface cell wall.

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CALCIUM FLUX IN EXCHANGE FOR PROTONS IN OAT 'ACID-GROWTH'

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The acid-growth theory of IAA action to cause elongation growth of plant cells (1) states that IAA causes cells to extrude protons which acidify the cell walls causing them to loosen. Questions over the theory include (i) whether IAA reliably reduces wall pH (6); (ii) whether extruded protons exchange with load-bearing calcium cross links to loosen the walls; (iii) whether the major bonds are not calcium cross links but other unknown bonds that break enzymatically at low pH (7).

We have measured Ca^{2+} and H^+ net fluxes following IAA or fusicoccin treatment of split oat coleoptiles which were pretreated for between 1 and 4.5 hours in aerated, unbuffered 0.1 mM KCl + 0.1 mM $CaCl_2$, at pH 6. Net fluxes were measured using the non-invasive Microelectrode Ion Flux Estimation (MIFE) technique to measure electrochemical gradients (3). Using the weak acid Donnan-Manning (WADM) model of the wall (4,5), and avoiding buffers or chelators, we have inferred conditions in the wall from the measured net fluxes and external quantities. Fusicoccin (1 μM) causes immediate and massive H^+ extrusion (to a peak of 350 nmol m^{-2} s^{-1}) from oat coleoptile cells, with about two thirds of the extruded H^+ exchanging for Ca^{2+} in the cell walls in a process that we have quantitatively modelled. IAA causes smaller net effluxes (~15 nmol m^{-2} s^{-1}, each ion) but with a 10 minute delay to the start of H^+ efflux. Our results appear to validate the acid-growth theory for IAA action in oats, with acid breakage of Ca^{2+} cross links being a possible mechanism. Interestingly, the IAA-induced calcium efflux appears to start with no delay, as if the IAA initiates an immediate release of calcium. Preceding the well-known membrane hyperpolarisation caused by IAA, we observe transient H^+ influx to the tissue and membrane depolarisation.

Our hypothesis is: IAA releases Ca^{2+} from some location (not load-bearing) in the wall. Some of this Ca^{2+} enters the cell (perhaps via Ca^{2+} channels that are themselves opened by IAA binding to the plasmalemma). The rest of the Ca^{2+} released leaves the wall, H^+ moving into the wall to maintain charge balance during those first few minutes. The 'second messenger' Ca^{2+} entering the cytoplasm causes a rise in pCa, observed by Felle (2), and initiates H^+ extrusion with a total delay of 10 minutes. This H^+ causes 'acid-growth' by exchange-release of Ca^{2+} from load-bearing bonds between polymer molecules. The Ca^{2+} leaves the wall, together with the residue of H^+. What other ion fluxes maintain charge balance is unknown.

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CELL GROWTH AND MEMBRANE ACTIVITIES IN PLANTS

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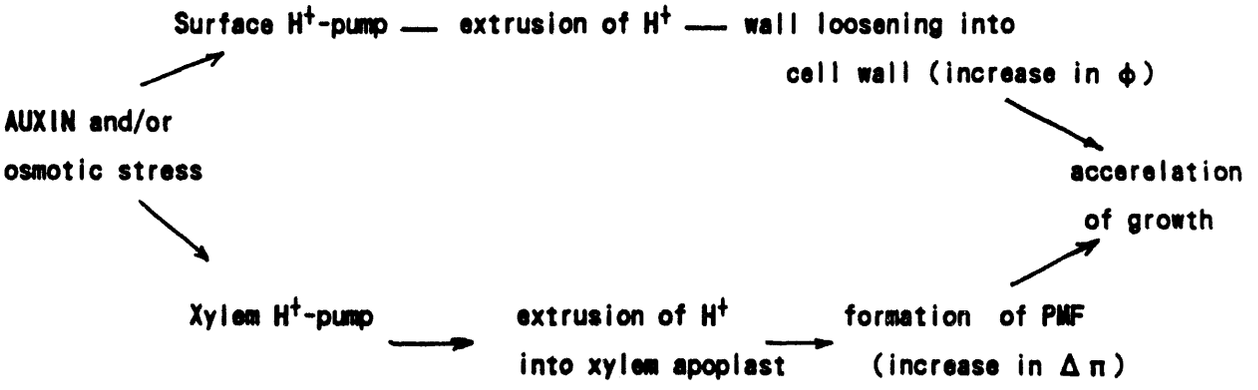
We had developed a model of physiological structure in plant axial organs (1). Later, we investigated the physiological role of the main elements in this model, and reached a conclusion expressed schematically as follows (2):



Then we have examined whether both proton pumps really increases wall extensibility (ϕ) or osmotic potential difference, by means of pressure jump method(4) together with an improved pressure probe(6), based on the KATOU-FURUMOTO's diagram (3). We discovered that IAA and /or osmotic stress increased not only ϕ and $\Delta \pi$, but also the effective turgor P-Y (5), where P is the turgor and Y is the yield threshold of the cell wall. Since P is not changed by IAA, Y must be decreased(5,6). In the absence of GA, IAA did not increase ϕ , but only decreased Y (7).

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RELATIONSHIPS BETWEEN THE CHANGES OF MALATE AND GLUCOSE-6-P LEVELS AND OF INTRACELLULAR pH ASSOCIATED WITH K^+- AND NH_4^+-DEPENDENT ELECTROGENIC H^+ EXTRUSION

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Previous work has shown that: a) the activation of H^+ extrusion ($\triangle H^+$) by IAA or by fusicoccin (FC) in the presence of K^+ is associated with an increase in malate via activation of malate synthesis by PEP carboxylase; b) the increase in malate is associated with an increase in glucose-6-P and an inhibition of PPP activity; c) K^+- and (K^+ plus FC)-induced $-\triangle H^+$ stimulation is associated with - and appears the cause of - an alkalinization of the cytosol. This suggested the hypothesis of the cause-effect chain of events: $-\triangle H^+$ → increase of pH_{cyt} → increase of malate → malic enzyme-mediated increase of NADPH → inhibition of glucose-6-PDH → increase of glucose-6-P (1).

To test this hypothesis we started from the finding that $NH_4^+{}_{out}$ and (NH_4^+ plus FC) induce in Elodea densa leaves stimulations of $-\triangle H^+$ very similar to those induced by $K^+{}_{out}$ and by (K^+ plus FC), while cytosolic pH is markedly increased by $K^+{}_{out}$ (by about 0.2, without, and by 0.4, with FC (1,2)) and very little or not at all by $NH_4^+{}_{out}$ (± FC). In leaves thus treated we also measured the changes of malate, glucose-6-P, $NADP^+$ and NADPH. We found that: i) both malate and glucose-6-P significantly increased in the (K^+ plus or minus FC), but not in the (NH_4^+ plus or minus FC)-treated leaves; ii) NADPH and $NADP^+$ levels (6.8 and 8.1 nmol/g FW respectively) were not significantly changed by any of the treatments.

These results confirm the cause-effect relationship between $-\triangle H^+$-associated changes of pH_{cyt} and of malate level, as well as the parallelism between the responses of malate and of glucose-6-P to treatments influencing $-\triangle H^+$ and pH_{cyt}. The failure to detect simultaneous changes of NADPH and $NADP^+$ is in disagreement with our former hypothesis according to which glucose-6-P increase would be a consequence of the high pH-induced increase in malate, and thus in NADPH/$NADP^+$ ratio. The possibility remains open of a modulating effect of cytosolic pH on the activity of NADPH in inhibiting glucose-6-PDH (as demonstrated for the chloroplast enzyme).

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INCUBATION OF INTACT *AVENA*-ROOTS WITH FUSICOCCIN INCREASES THE ATPASE-ACTIVITY IN PLASMA MEMBRANE VESICLES.

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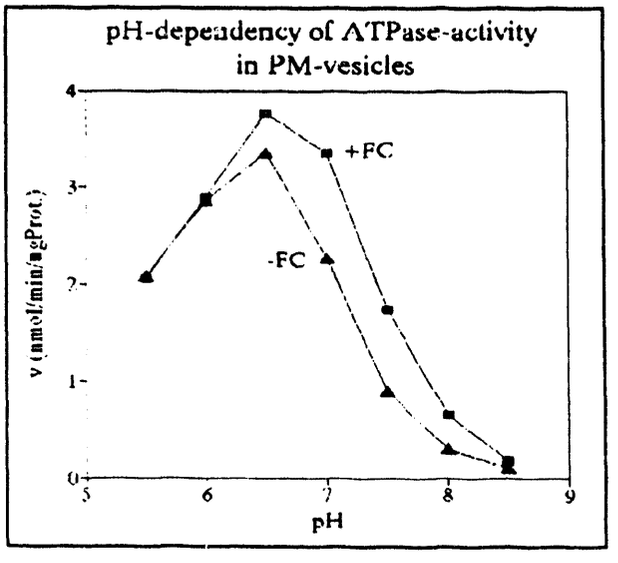
The phytotoxin Fusicoccin (FC) is known for its induction of auxin-like responses in plant tissues (Marré 1979). It promotes growth and it affects ion-fluxes across the plasma membrane (PM). In roots from *Plantago media* and *Avena sativa* the following responses to FC were observed: hyperpolarization of the membrane potential and stimulation of both proton efflux and potassium influx.

It is generally accepted that FC stimulates the PM-H^+-ATPase (Marré 1979). Other effects of FC on transport processes would have their origin in that stimulation. Recently, an alternative explanation has been presented. A FC-induced decrease in the membrane conductances of passive proton- and potassium- fluxes would also result in the observed hyperpolarization and changes in proton- and potassium-fluxes, even without an increased PM-H^+-ATPase-activity (Blatt and Clint 1989).

We investigated the effects of FC on ion-fluxes in protoplasts with the patch-clamp technique and in PM-vesicles with biochemical techniques. In this presentation we discuss the results obtained with the PM-vesicles. The results obtained by patch-clamping protoplasts are discussed in an other presentation (see Vogelzang, Lanfermeijer and Prins).

Incubation of intact *Avena*-roots with FC prior to PM-vesicle isolation resulted in a stimulation of PM-ATPase-activity in the vesicles. This stimulation was most pronounced at pH-values higher than the pH-optimum of the ATPase-activity in vesicles from untreated roots. FC-treatment shifted the pH-optimum of the PM-ATPase-activity to more alkaline values with about 0.3 pH-values. A more detailed study of the stimulation by FC revealed that the stimulation primarily resulted from an increase of the V_{max} of the PM-ATPase. Apparent affinities (K_m) were not affected by FC.



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THE PLASMALEMMA H+-ATPase IS MODULATED BY A CALCIUM CALMODULIN DEPENDENT PHOSPHORYLATION

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The plasmalemma H+-ATPase is a key enzyme which is involved in the regulation of many cellular and physiological processes. Its main function is the production and maintenance of an electrochemical proton gradient across membranes using the metabolic energy from ATP hydrolysis. Such an electrochemical gradient may be utilized to energize the secondary transport of nutrients, osmoregulation, cell elongation, control of intracellular and extracellular pH (1). The relevant importance of this enzyme makes it a possible target for modulation. It has been recognized that the response to extra- and intracellular stimuli that signals a modification of a physiological status may increase or decrease the activity of this enzyme. Evidence obtained in the recent years suggests the possibility that the modulation of the H+-ATPase might be linked to a phsphorylation/dephosphorylation system and that the Ca-Cam complex may be involved (2,3).

Aim of our work has been the determination of a possible involvement of the Ca-Cam complex on the regulation of the H+-ATPase activity through a covalent modification. A fraction from maize primary roots enriched in plasma membranes was prepared by using the partitioning aqueous/polymer two-phase technique. The H+-ATPase activity in the plasma membranes was decreased when Ca^{2+} and Cam were present together either when measured as ATP hydrolysis or as H^+ pumping activity. In both cases the decrease was around 50%. To understand whether the effect of Ca-Cam was direct on the H+-ATPase, plasma membranes were incubated in a condition suitable for protein phosphorylation and in the presence of Ca^{2+} and Cam and successively reisolated to eliminate Ca^{2+} and Cam. Also in this case a decrease by 35/45% in the H+-ATPase activity was determined. Phosphorylation of membrane protein show an increase in the incorporation of ^{32}P when Ca^{2+} and Cam were present together. Autoradiography of the SDS-PAGE of phosphorylated plasma membranes shows an increase in the pattern of phosphorylated proteins. In particular the phosphorylation of a band around 100kD which can be the H+-ATPase was increased by more than 50%. The data presented seem to confirm that the negative effect of Ca^{2+}-Cam on the H+-ATPase activity is sustained by a phosphorylation mechanism.

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MUTANTS OF ARABIDOPSIS THALIANA ALTERED IN FUSICOCCIN SENSITIVITY

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Fusicoccin (FC) is a toxin strongly activating the plasmalemma H^+-ATPase. In Arabidopsis, as in other species, the toxin induces at the cellular level hyperpolarization, acidification of the external medium and increased uptake of cations and, at the plant level, stomatal opening and wilting.

We took advantage of the capacity of FC to promote solute uptake for developing a procedure which kills Arabidopsis thaliana wildtype seedlings exposed to FC and to a toxic cation (Paraquat). M_2 progenies from 2020 M_1 plants mutagenized with EMS were screened and and three FC-Paraquat resistant seedlings came out. The trait was transmitted to advanced generation and, starting from M_5, homogenous progenies were assayed for resistance to Paraquat alone and for sensitivity to FC with three additional tests (resistance to FC plus hygromycin, resistance to wilting and stomatal opening). The three selected lines appeared all sensitive to Paraquat at a concentration five times higher thant that used in the selective system, and insensitve to FC in the three additional tests used. In one line (5-2), the trait is transmitted according to a monogenic dominant (or codominant, depending on the test used) type of inheritance. A preliminary characterization of the physiological properties of the mutant will be reported.

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CONTROLLED PROTEOLYSIS AFFECTS THE REGULATORY PROPERTIES OF THE PLASMA MEMBRANE H^+-ATPase AND Ca-ATPase

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Controlled trypsin treatment increases the activity of the plasma membrane (PM) H^+-ATPase of fungal and plant cells, by removing a C-terminal inhibitory domain. Also the Ca-ATPase of erythrocytes, an enzyme closely related to the plant cell (PM) Ca-ATPase has a C-terminal inhibitory domain, the removal of which by controlled trypsin treatment increases the enzyme affinity for Ca^{2+} and abolishes its activation by calmodulin (CaM).

We have investigated the effect of controlled trypsin treatments of PM isolated from radish or Arabidopsis seedlings, on the activities of the H^+-ATPase and of the Ca-ATPase, with special regard to their sensitivity to activation respectively by fusicoccin (FC) and CaM.

The activation of the PM H^+-ATPase induced by controlled proteolytic treatment is strongly dependent on the pH of the assay medium and markedly increases with the increase of pH from 6.6 to 7.5, similarly to what observed for the activation by FC. The proteolytic treatment abolishes FC-induced stimulation of the PM H^+-ATPase, while only scarcely affecting FC binding to its receptor.

Controlled treatment with trypsin in the presence of the nucleoside triphosphate substrate ITP stimulates the PM Ca-ATPase activity, increasing its affinity for Ca^{2+} and abolishing its stimulation by exogenous CaM.

These results taken as a whole indicate that both the H^+-ATPase and the Ca-ATPase of the PM of plant cells are endowed with a regulatory domain which controls their activities under physiological conditions. Effectors such as FC or CaM may modulate enzyme activity by interacting with the regulatory domains or by modifying their interaction with the enzyme.

RE-EXAMINATION OF THE ACID GROWTH THEORY OF AUXIN ACTION: AUXIN-INDUCED PROTON EXCRETION AND ACID-INDUCED CHANGES IN THE MECHANICAL PROPERTIES OF THE CELL WALL.

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We re-examined the acid growth theory of auxin action concerning the following two points.

1)Auxin-induced proton-excretion.
IAA was applied to Vigna hypocotyl segment by xylem perfusion. IAA stimulates the electrogenic proton pump at the plasmalemma that excretes protons to the cell wall apoplast. However, a large amount of protons is trapped in the cell wall as a pH-buffer (1). We attempted to release protons from the cell wall by application of cations that can exchange with protons on the cell wall polysaccharides (2).

2)The changes in the mechanical properties of the cell wall during acid growth: comparison with that under auxin-induced growth.
An aerosol of HCl solution was applied to the abraded Vigna hypocotyl segment during xylem perfusion. The cell wall extensibility and the effective turgor were increased under the acid-induced growth as similar to auxin-induced growth (3). However, turgor pressure, which scarcely changed under auxin-induced growth (3), largely decreased under acid-induced growth. We consider this discrepancy in the behaviour of turgor pressure as follows. Auxin stimulates both the surface and the xylem proton-pumps simultaneously (4), then enhanced the active water uptake as simultaneously as it loosens the cell wall (5). On the other hand, acid-aerosol does not stimulate the proton pumps although it loosens the surface cell wall.

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THE EFFECT OF LIGHT-INDUCED CHANGES IN CYTOSOLIC pH AND pCa ON PLASMALEMMA TRANSPORT

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Stimulation of photosynthetic activity by light induces transient increases in pH and pCa in the cytosol which influence the transport activity of the proton pump and of the potassium channel in the plasmalemma. Alkalinization could originate from two mechanisms: uptake of protons across the envelope as a consequence of the depletion of protons in the stroma due to the generation of the transthylakoid pH-gradient or by uptake of CO_2 as caused by increased activity of the Calvin cycle. As increased CO_2-fixation is promoted by an increased supply of ATP and thus also by the transthylakoid pH-gradient, the two candidates for the cytosolic increase in pH could not be distinguished by means of previous kinetic investigations (1) as the time-constant τ_4 of the changes in transthylakoid pH-gradient would dominate the kinetics of the light-induced changes in the activity of the proton pump in either case.

Leaves of *Aegopodium podagraria* were used in order to compare the effects of the modulation of light intensity, CO_2- and O_2-concentrations on chlorophyll-fluorescence (2) and on plasmalemma potential. All three input signals resulted in kinetically equivalent responses of the activity of the plasmalemma proton-pump, i.e. in all three cases the time- constant of the induced depolarisation was identical to τ_4 (10 to 40s) of the induced changes in chlorophyll-fluorescence which is an indicator of changes in the transthylakoid pH-gradient. A direct action of CO_2 on cytosolic pH would have induced much faster responses.

In addition, a direct CO_2-effect on plasmalemma potential via a direct action of CO_2 on cytosolic pH could be excluded by studying the dependence on light intensity of the time-constant of CO_2- and O_2-action on plasmalemma potential. It was found to be strongly dependent on light intensity as τ_4 related to the transthylakoid pH-gradient is. This would not be expected for a direct action of CO_2 on cytosolic pH.

The light-induced decrease in transport activity of the K^+-channel is supposed to be caused by an uptake of calcium-ions into the chloroplasts (1). This is caused by an unknown mechanism, who becomes obvious in the kinetics of chlorophyll-fluorescence as an individual component related to the time-constant τ_{5a}. The uptake of calcium results in a light-induced increase in the velocity of cytoplasmic streaming as measured by laser velocimetry (3).

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ROLE OF CYTOPLASMIC INORGANIC PHOSPHATE IN LIGHT-INDUCED ACTIVATION OF H^+ PUMPS IN THE PLASMA MEMBRANE AND TONOPLAST OF *CHARA CORALLINA*

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The plasma membrane of characean cells hyperpolarizes upon illumination (Fig. 1) with simultaneous acidification of the vacuole (Fig. 2). These phenomena are known to result from the activation of H^+ pumps in the plasma membrane and the tonoplast through photosynthesis. We investigated the mechanism of the light-induced H^+ pump activation in characean cells using perfusion technique (1) and reported that changes in adenine nucleotide levels in the cytosol may control the activity of the H^+ pump in the plasma membrane (1).

It is known that the cytoplasmic Pi level decreases in the light (2). Biochemical studies have already shown that Pi inhibits ATPase activities noncompetitively (3). In the present study, we found that in *Chara* cells, both the electrogenic activity of the plasma membrane H^+ pump and the ATP- and PPi- dependent H^+ transport across the tonoplast were noncompetitively inhibited by Pi. On the other hand, in an unique variant strain of *Chara* which contained a very low level of Pi in the vacuole, the cytoplasmic Pi level decreased upon illumination in concert with the light-induced hyperpolarization of the plasma membrane and the acidification of the vacuole (Fig. 3). These results suggest that the cytoplasmic Pi level is one of the regulators of photosynthesis-mediated activation of H^+ pumps in the plasma membrane and the tonoplast (4).

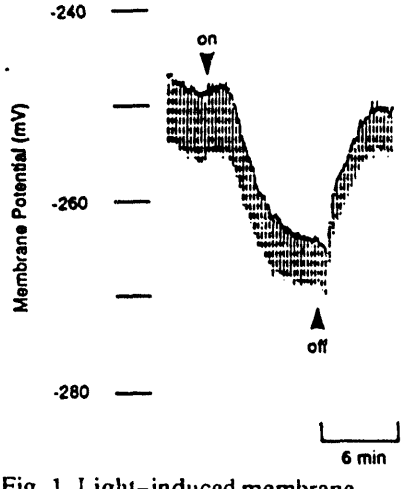

Fig. 1. Light-induced membrane hyperpolarization.

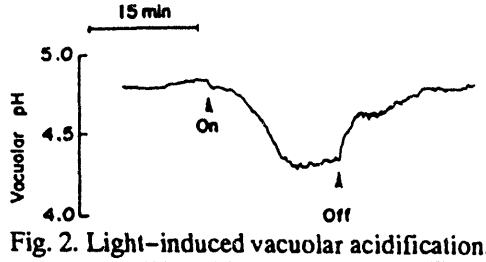

Fig. 2. Light-induced vacuolar acidification.

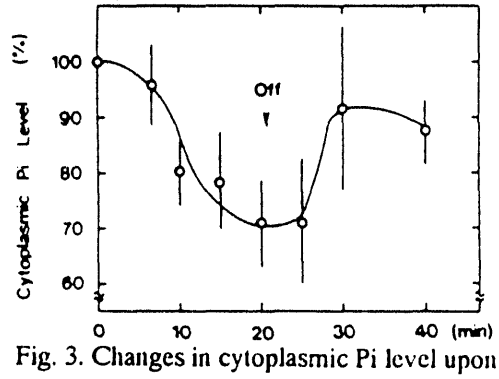

Fig. 3. Changes in cytoplasmic Pi level upon illumination.

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ORIENTATION OF THE BINDING SITE FOR THE AUXIN TRANSPORT INHIBITOR NAPHTHYLPHTHALAMIC ACID IN ZUCCHINI PLASMA MEMBRANE VESICLES

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The phytohormone auxin (indole-3-acetic acid) plays an essential role in the growth and development of plants. Auxin is synthesized at the growing tip of a plant shoot and transported in a polar fashion to the base of the shoot, where it exerts effects on growth (1). Several synthetic inhibitors of auxin transport, or phytotropins, have been described (2). One such inhibitor is *N*-1-naphthylphthalamic acid (NPA). NPA inhibits polar auxin transport both in plant seedling sections (3) and in isolated plasma membrane vesicles (4). Binding of NPA is thought to inhibit efflux of auxin through a basally-located efflux carrier. In order to gain a clearer understanding of the mechanics of auxin transport regulation , we have determined the orientation of the NPA binding site within the plant plasma membrane.

Previous studies of NPA binding have been performed using crude microsomal preparations. In recent years, an aqueous two-phase partitioning procedure has been developed which allows one to obtain relatively pure preparations of plasma membranes (5). The plasma membranes which are purified by this procedure are primarily in the right-side-out orientation. However, a freeze/thawing procedure may be used to prepare inside-out vesicles for comparison with the right-side-out ones.(6).

Plasma membrane vesicles of both orientations were prepared from zucchini. Binding activity of [^{3}H]NPA was found to be higher in inside-out vesicles, although the affinity of the NPA binding protein for NPA was unaltered. A similar result was obtained upon osmotic shock of right-side-out vesicles. Osmotic shock revealed latent NPA binding activity. In addition, NPA binding protein was much more sensitive to trypsin in inside-out vesicles than in right-side-out ones. These results all suggest an inward-facing orientation for the NPA binding protein in plant cells. The difference in NPA binding between right-side-out and inside-out vesicles was not as great as the difference observed for a similarly-oriented marker activity, ATP-dependent proton pumping. This was found to be due to a rapid diffusion of NPA across the plasma membrane.

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BIOCHEMICAL CHARACTERIZATION OF NAPHTHYLPHTHALAMIC ACID BINDING IN THE ZUCCHINI PLASMA MEMBRANE

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Indole-3-acetic acid (IAA) is transported from the apex of stems toward the base in a polar fashion. This polar transport can be inhibited by phytotropins such as naphthylphthalamic acid (NPA). Phytotropins are believed to interact with the IAA efflux carrier, where they in some way block the exit of the IAA anion from plant cells. Radiolabeled NPA binding assays can be used to identify proteins in the plasma membrane which specifically bind this molecule. We have developed a new assay which allows quantitative analysis of [^{3}H]NPA binding and the inhibition of this binding by other phytotropins, with high reproducibility and low background. This assay utilizes the Millipore Multiscreen® filtration apparatus to separate the unbound [^{3}H]NPA from protein-[^{3}H]NPA complexes by vacuum filtration through cellulose membranes. This assay procedure was used to analyze [^{3}H]NPA binding in saturation curves, Lineweaver-Burke plots, Hill plots, and Scatchard plots. The Scatchard plots were generated both through direct plots and through computer curve fitting with the program Ligand (1). These binding analyses indicate that there is a single binding site for NPA in zucchini plasma membranes which binds [^{3}H]NPA with high affinity (K_d~10 nM).

Using this plasma membrane assay system, it is also possible to study the interactions between IAA and NPA binding. The mechanism by which NPA blocks auxin transport is currently unknown. It is possible that NPA acts by altering the affinity of IAA for the efflux carrier, either through inhibition of binding or inhibition of release of IAA. This first possibility was examined by analysis of the interactions between IAA and NPA binding in plasma membranes. The effect of IAA on [^{3}H]NPA binding was tested and IAA was found to be a noncompetitive inhibitor of NPA binding. This result suggests that these two compounds have distinct, but interacting binding sites. The effect of IAA on NPA binding was also assessed using the photoaffinity label 5-azido-[7-^{3}H]IAA ([^{3}H]-N_3IAA) (2). This label has previously been used to identify several auxin binding polypeptides (3,4) and has been shown to be relatively specific under certain conditions. Plasma membrane proteins were labeled with [^{3}H]N_3 IAA using the procedure of Hicks *et al.* (3). The covalent attachment of [^{3}H]-N_3IAA decreases subsequent [^{3}H]NPA binding, just as the noncovalent binding of IAA decreased [^{3}H]NPA binding. The effect of NPA on [^{3}H]-N_3IAA binding was also assessed using this system. These initial experiments suggest that there are interactions between IAA and NPA binding in zucchini plasma membranes. In a recent report by Zettl *et al.*, a parallel approach has been used to demonstrate that the binding of [^{3}H]-N_3NPA to a corn plasma membrane protein is also blocked by IAA (5). They also found that NPA blocked [^{3}H]-N_3IAA labeling to the same protein (6). Their results offer further support that this approach will facilitate understanding of the nature of the action of auxin transport inhibitors.

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SOME PROPERTIES OF BLUE LIGHT-DEPENDENT RESPONSE OF STOMATA

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Signal transduction process involved in the blue light-dependent response of stomata was investigated in the Vicia guard-cell protoplasts and Commelina epidermis. Blue light-dependent proton pumping was inhibited by ML-9, an inhibitor of Ca^{2+}/CaM-dependent myosin light chain kinase (MLCK), and by W-7, trifluoperazine and HT-74, the CaM antagonists, suggesting the involvement of MLCK in the blue light response of stomata. In accord with the effect of drugs on the proton pumping, light-dependent stomatal opening in Commelina epidermis was suppressed by W-7 and ML-9 (1).

Blue light-dependent proton pumping was inhibited completely by DCCD, an inhibitor of H^+-ATPase at 10 μM, and by phenylmercuric acetate at 5μM, which reduces the ATP level to 10% (2). The results suggest the pump activity was mediated by plasma membrane H^+-ATPase. The pump activity was inhibited by verapamil(1mM), but it was restored by the further addition of fusicoccin (FC) which activates the proton pump. However, the pump activity could not be restored by FC in the presence of DCCD. The results indicate that the process by which the perception of blue light is transduced into the activation of proton pump is suppressed by verapamil. Verapamil seems to inhibit the signal transduction process but not the proton pump itself. Effect of verapamil on the light-dependent stomatal opening in the Commelina epidermis will be reported.

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THE PLASMA MEMBRANE H^+-ATPASE : PURIFICATION BY FPLC, RECONSTITUTION, AND REGULATION

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The plant plasma membrane H^+-ATPase plays a central role in plant physiology. The enzyme pumps protons from the cytoplasm to the cell exterior using ATP as the energy source. The eletrochemical gradient produced across the plasma membrane is believed to be the driving force for nutrient uptake. Acidification of the cell wall seems to be a prerequiste for cell wall extension, and alkalinization of the cytoplasm may be the factor triggering cell division. *In vivo*, H^+ pumping across the plasma membrane is regulated by plant hormones and light.

In vitro, the H^+-ATPase is stimulated by certain lipids such as lysophospholipids (1). It is also activated by proteolytic removal of the C-terminal region. It has been suggested that activation by proteases and lysophospholipids occurs by a similar mechanism, namely the removal (irreversibly or reversibly) of an autoinhibitory domain in the C-terminal region, and that this autoinhibitory domain may be the ultimate target for *in vivo* regulation (2).

We have solubilized the H^+-ATPase from isolated plasma membranes and purified the enzyme to near homogeneity by FPLC. Regulation by proteolytic removal of the C-terminal region, and by lysophosphatidylcholine has been studied.

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ACTIVATION AND INTRACELLULAR SIGNALLING IN THE *FUCUS* EGG.

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The mechanism of egg activation has been extensively studied in various animal systems. Upon fertilization a wave of free calcium is observed propagating (via calcium induced calcium release (CICR)) through the whole of the egg (1). This increase in cytoplasmic calcium is shown to be a release from internal stores of the egg (2, 3) and is thought to be responsible for it's activation.

We have investigated the role of free calcium in the activation of the eggs of the brown marine alga, *Fucus serratus*. Eggs were microinjected with the calcium sensitive dye fura-2 dextran (10,000mw) and average egg cytoplasmic calcium monitored using a dual wavelength excitation photometric system. Membrane potentials were simultaneously monitored. Resting egg free calcium is measured between 100-300nM. Transient elevations of cytoplasmic calcium upto 1-3μM were observed with the onset of the fertilization potential. $^{45}Ca^{2+}$ uptake increased transiently in the first minutes following fertilization suggesting a role for Ca^{2+} uptake across the plasma membrane. Localized Ca^{2+} microinjection activated eggs only locally, suggesting CICR may not be involved.

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ASSOCIATION O' \CTIN WITH PLASMA MEMBRANE VESICLES ISOLATED FROM CAULIFLOWER

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The dynamic, plasma membrane-associated cytoskeleton is important for cell morphogenesis and organization in plants as well as in other organisms. In isolated animal plasma membranes, cytoskeletal components, e.g. actin, have been detected. We wanted to see whether similar components were present in isolated plant plasma membranes, as a first step towards an understanding of the dynamics of the microfilament system.

Highly purified plasma membranes from cauliflower inflorescences were obtained by aqueous polymer two-phase partitioning. A monoclonal antibody against chicken gizzard actin (Amersham N 350), recognized a polypeptide having the MW of actin (42-43 kDa) in Western blots of such preparations. The actin was recovered in a detergent-insoluble fraction of the plasma membrane (1% Triton X-100, 100,000g pellet) ruling out the possibility of vesicle-enclosed monomeric actin. It was furthermore enriched in the plasma membrane fraction to the same extent as glucan synthase II, a specific marker for this membrane, and depleted in fractions depleted in plasma membrane. The effect of different preparation conditions on the recovery of plasma membrane-associated actin will be described.

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IS CYTOPLASMIC PH AN INDUCING FACTOR OF ROTATIONAL CYTOPLASMIC STREAMING IN EGERIA DENSA ?

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When leaves of Egeria densa are incubated in the dark for more than one night, chloroplasts are localized on the periclinal face of cells on the adaxial side. On illumination cytoplasmic streaming is activated, chloroplasts are displaced to the anticlinal face, and a vigorous rotational streaming of cytoplasm containing chloroplasts is induced along the anticlinal face. Treatment of L-histidine (L-His) of 0.1-1.0 mM can also induce this movement of chloroplasts. Both light and L-His caused a slight alkalization of the cytoplasm, which was measured by in vivo ^{31}P-NMR method (1).

We then hypothesized that the alkalization of cytoplasm may be involved in the activation of rotational cytoplasmic streaming. As for the light-induced cytoplasmic alkalization, it is supposed that light activates the electrogenic H^+-pump in the plasma membrane to alkalize the cytoplasm. As for the alkalization with L-His, we assumed the following mechanism. L-His enters cells via symport with H^+ and the resultant acidification of cytoplasm activates the H^+ pump so much that the cytoplasmic pH (pH_c) is increased. To see whether the membrane potential is depolarized by the symport with H^+, we measured E_m on application of L-His. E_m was not affected by L-His which had been freshly prepared. But it was depolarized with L-His which had been placed for several days at room temperature. It was shown that NH_4^+ having a strong depolarizing effect was found in the old solution of L-His.

Next we measured pH_c simultaneously with E_m using double-barelled pH microelectrode containing a liquid ion exchanger. On illumination pH_c increased at first and then decreased, while E_m greatly hyperpolarized. On application of L-His both pH_c and E_m showed no significant change. To alkalize the cytoplasm, we applied NH_4Cl (1 mM) at pH 9 and methylamine (0.2-200 mM) at pH 7.5. The rotational streaming of chloroplasts was induced in a small percent of cells. To acidify the cytoplasm, isobutyric acid (5 mM) was applied at pH 5. It reversibly inhibited the L-His-induced rotational streaming of chloroplasts.

Thus the present results are not necessarily confirmative to support the pH_c-hypothesis on the induction of rotational cytoplasmic streaming.

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HORMONAL REGULATION OF Ca^{2+} TRANSPORTERS LEADS TO RAPID CHANGES IN STEADY-STATE LEVELS OF CYTOSOLIC Ca^{2+} IN THE CEREAL ALEURONE

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The cereal aleurone responds to gibberellic acid (GA) by expressing genes whose products promote germination of the grain. Although the early events in hormone action in this tissue are not known, we have recently shown that steady-state changes in cytosolic Ca^{2+}, ($[Ca^{2+}]i$), occur within minutes of treatment with GA (1). We have hypothesized that these changes in $[Ca^{2+}]i$ are induced by hormonal regulation of Ca^{2+}-transporters at the plasma membrane. We have tested this hypothesis by measuring the effect of inhibitors of Ca^{2+} transport on $[Ca^{2+}]i$ in GA-stimulated cells. Measurements of $[Ca^{2+}]i$ in individual aleurone cells were performed using the Ca^{2+}-sensitive fluorescent probe, fluo-3 (1). Fluo-3 was loaded into cells by incubating isolated aleurone layers in 5 μM fluo-3 for 20 min. Fluorescence from fluo-3 measured in individual cells using a highly-sensitive photometry system.

Blocking extracellular influx either by removal of extracellular Ca^{2+} or by the addition of nifedipine, a Ca^{2+}-channel blocker, prevented the steady-state rise in $[Ca^{2+}]i$ that is induced by GA. Nifedipine had no effect on $[Ca^{2+}]i$ in the absence of GA while removal of extracellular Ca in the absence of GA leads to a slight decline in $[Ca^{2+}]i$. These results indicate that the rise in $[Ca^{2+}]i$ that is induced by GA requires influx of Ca^{2+} across the PM and are consistent with the hypothesis that GA activates a nipedipine-sensitive Ca^{2+} channel at the plasma membrane. In order to determine whether the efflux Ca^{2+}-ATPase is also regulated by GA, we measured the ability of cells to restore $[Ca^{2+}]i$ after a pulse of intracelluar Ca^{2+}. GA caused a reduction in rate of recovery compared to cells treated without GA or with abscisic acid (ABA). Measurement of changes in $[Ca^{2+}]i$ induced by the Ca^{2+}-ATPase inhibitors, vanadate and erythrosin B, indicate that PM Ca^{2+}-ATPase is the dominant pump for efflux of Ca^{2+} from the cytosol. In summary, our data indicate that GA induces a steady-state rise in $[Ca^{2+}]i$ through the coordinate regulation of a Ca^{2+} influx channel and a Ca^{2+} efflux ATPase at the plasma membrane.

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BARLEY ALEURONE PROTOPLASTS: A MODEL SYSTEM FOR SECRETION AND INTRACELLULAR PROTEIN TRANSPORT

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The aleurone layer of barley has been widely used as a model system to study the intracellular transport and secretion of hydrolytic enzymes [1]. This tissue is ideally suited for such investigations since it consists of only one cell type that neither grows nor divides, but whose capacity to synthesize enzymes such as α-amylase can be regulated by the plant hormones gibberellic acid (GA) and abscisic acid (ABA) [2]. The presence of a thick cell wall surrounding the aleurone protoplast poses a serious limitation to studies of exo- and endocytosis, however. The cell wall occupies as much as 30-40% of the volume of the aleurone layer and it acts as a buffer, retaining secreted proteins in the wall and impeding access of externally added probes. Protoplasts isolated from the aleurone layer circumvent many of these problems. Although populations of barley aleurone protoplasts respond to GA and ABA, [2,3], little is known about the response of individual cells to these hormones. Do all protoplasts in a population respond to GA and ABA and do they do so synchronously? We have developed a method for monitoring the secretion of α–amylase from single aleurone protoplasts. Protoplasts are immobilized in a matrix of low-melting-point agarose containing solubilized starch, and α-amylase secretion is monitored by digestion of the starch from the area immediately adjacent to the protoplast. The starch-free area around individual cells is visualized using light microscopy as a clear halo following staining with iodine. Using this method we have shown that only about 50% of protoplasts isolated from an aleurone layer respond to GA. This provides the necessary information for interpretation of inhibitor studies, and experiments using extensive physical and/or chemical manipulation after incubation in Ca^{2+} and GA and prior to embedding in the gel matrix. For example we have shown that Ca^{2+} and monensin rapidly alter the rate of α-amylase secretion from single cells. Because protoplasts can also be perfused in the gel with solutions of differing composition, this thin-layer method is suitable for studies of the effects of various modulators of exocytosis after embedding in the matrix. The utility of this technique for single-cell studies will be described.

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DEFAULT SECRETION IN BARLEY ALEURONE PROTOPLASTS

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Over the years, cereal aleurone has proved to be a useful model system for the study of enzyme secretion in plants. In response to gibberellic acid (GA) the aleurone layer of cereal grains synthesizes and secretes a large number of hydrolases which play an important role in the mobilization of food reserves stored in the endosperm. This hormone-controlled activation of the aleurone layer is accompanied by dramatic changes in the ultrastructure of the cells.

We investigated whether aleurone protoplasts can be used as a model system to study default secretion by barley aleurone. For this purpose, we studied transient synthesis of heterologous proteins in GA-induced aleurone protoplasts.
Aleurone protoplasts were transfected with a construct encoding the prokaryotic cytoplasmic protein phosphinotricin acetyltransferase (PAT), preceded by a signal peptide. The enzyme was found to be secreted by the aleurone protoplasts. The same approach has successfully been used by Denecke et al (1) to demonstrate default protein-secretion in tobacco leaf protoplasts.
Leakage of the aleurone protoplasts was excluded by cotransfection of the cells with a construct encoding the prokaryotic cytoplasmic protein chloramphenicol acetyltransferase (CAT). All CAT activity remained inside the protoplasts.
Furthermore, we established that the PAT secreted by the aleurone protoplasts passed through the endomembrane system of the cells, by studying the routing of the gene product of a construct encoding the signal peptide-PAT, followed by a C-terminal KDEL sequence. It has been demonstrated that in tobacco protoplasts, like in mammalian cells, a KDEL sequence functions as a signal for retention of soluble proteins in the ER (2). In the presence of the KDEL sequence we found that most PAT activity was retained by the cells.

Now that a system is operational for monitoring the default secretory pathway in barley aleurone cells, it will be interesting to compare the default PAT-secretion to secretion of barley hydrolases. Some data on the effect of GA and its antagonist abscisic acid on PAT and hydrolase secretion will be discussed.

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THE CONTRIBUTION OF THE PLASMA MEMBRANE TO GRAVISENSING IN CHARA INTERNODAL CELLS.

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When an internodal cell of Chara is oriented vertically, the cytoplasm streams down 10% faster than it streams up (1). However, when the cells are treated with various impermeant proteases that act at the plasma membrane-extracellular matrix junction, gravisensing is inhibited even though streaming continues. The gravireceptor is sensitive to Proteinase K, thermolysin and collagenase, but not to trypsin, α-chymotrypsin or carboxypeptidase B. Moreover, the tetrapeptide RGDS inhibits gravisensing in a concentration-dependent manner, indicating that the gravireceptor may be an integrin-like protein. Through localized treatments, we have determined that the gravireceptor protein is localized at the ends of the cell (2).

Further evidence that the gravireceptor is localized at the plasma membrane-extracellular matrix junctions at the ends of the cell comes from the observation that a unidirectionally-applied hydrostatic pressure (490 Pa) mimics the effect of gravity in inducing a polarity of cytoplasmic streaming. The pressure-induced polarity has identical requirements and sensitivities to inhibitors as the gravity-induced polarity (3).

Lastly, the magnitude and the sign of the graviresponse is modulated by the external [Ca^{2+}]. The Ca^{2+} required for gravisensing appears to enter the cell through two classes of channels. Each one is pharmacologically distinct from the Ca^{2+} channel involved in E-C coupling. We have not yet determined the relationship between the gravireceptor protein and the channels.

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THE EFFECT OF LIGHT-INDUCED CHANGES IN CYTOSOLIC pH AND pCa ON PLASMALEMMA TRANSPORT

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Stimulation of photosynthetic activity by light induces transient increases in pH and pCa in the cytosol which influence the transport activity of the proton pump and of the potassium channel in the plasmalemma. Alkalinization could originate from two mechanisms: uptake of protons across the envelope as a consequence of the depletion of protons in the stroma due to the generation of the transthylakoid pH-gradient or by uptake of CO_2 as caused by increased activity of the Calvin cycle. As increased CO_2-fixation is promoted by an increased supply of ATP and thus also by the transthylakoid pH-gradient, the two candidates for the cytosolic increase in pH could not be distinguished by means of previous kinetic investigations (1) as the time-constant τ_4 of the changes in transthylakoid pH-gradient would dominate the kinetics of the light-induced changes in the activity of the proton pump in either case.

Leaves of *Aegopodium podagraria* were used in order to compare the effects of the modulation of light intensity, CO_2- and O_2-concentrations on chlorophyll-fluorescence (2) and on plasmalemma potential. All three input signals resulted in kinetically equivalent responses of the activity of the plasmalemma proton-pump, i.e. in all three cases the time- constant of the induced depolarisation was identical to τ_4 (10 to 40s) of the induced changes in chlorophyll-fluorescence which is an indicator of changes in the transthylakoid pH-gradient. A direct action of CO_2 on cytosolic pH would have induced much faster responses.

In addition, a direct CO_2-effect on plasmalemma potential via a direct action of CO_2 on cytosolic pH could be excluded by studying the dependence on light intensity of the time-constant of CO_2- and O_2-action on plasmalemma potential. It was found to be strongly dependent on light intensity as τ_4 related to the transthylakoid pH-gradient is. This would not be expected for a direct action of CO_2 on cytosolic pH.

The light-induced decrease in transport activity of the K^+-channel is supposed to be caused by an uptake of calcium-ions into the chloroplasts (1). This is caused by an unknown mechanism, who becomes obvious in the kinetics of chlorophyll-fluorescence as an individual component related to the time-constant τ_{5a}. The uptake of calcium results in a light-induced increase in the velocity of cytoplasmic streaming as measured by laser velocimetry (3).

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SPATIAL DISTRIBUTION OF ROOT CALMODULIN-BINDING PROTEINS

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In order to further understand Ca^{2+}-mediated processes in roots, extracts of Vicia faba roots were analyzed for the presence of calmodulin (CaM)-binding proteins. Using ^{125}I-CaM overlay assays of blots containing Vicia extracts fractionated by SDS-PAGE, many Ca^{2+}-dependent CaM-binding proteins were detected, of which two were predominantly localized in the root. In 1.5 week old seedlings, CaM-binding proteins with M_r = 115 kDa and 62 kDa were detected over the length of the primary root (16 cm.). The 115 kDa band appeared in extracts made from the root tip up to the transition zone. The 62 kDa band appeared in extracts made from regions starting from 1 centimeter above the root tip up to and slightly past the transition zone. Roots were dissected and extracts made from tissues comprising the primary root. Both the 115 kDa and 62 kDa proteins were detected in extracts of both stele and cortex, however the 62 kDa band was found to a lesser extent in axillary roots. Following ultracentrifugation of extracts, the majority of root CaM-binding proteins were found in the microsomal pellet, including the 115 kDa protein. The 62 kDa protein, however, was found predominantly in the supernatant and represented the major CaM-binding protein in that fraction. Further purification and characterization of both the 115 kDa and 62 kDa proteins will be performed with the goal of identifying their roles in Ca^{2+}-mediated signal transduction in root.

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CALCIUM AND CALMODULIN COORDINATE THE ACTIVATION OF THE SECRETORY APPARATUS IN BARLEY ALEURONE CELLS IN RESPONSE TO GIBBERELLIC ACID AND ABSCISIC ACID.

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Secretion of α-amylase from barley aleurone cells is stimulated by gibberellic acid (GA_3) and Ca^{2+}, and this increase in secretion is reversed by abscisic acid (ABA). Gibberellin increases the level of cytosolic Ca^{2+} from approximately 100 nM to 300 nM after 6 to 8 h, and this new level is stably maintained. The increase in cytosolic Ca^{2+} is localized to the peripheral cytoplasm of the cell and may result from accelerated Ca^{2+} influx across the plasma membrane. Abscisic acid reverses the effect of GA_3 on cytosolic Ca^{2+} levels within 2 to 3 h, preceding the first measureable effects on the synthesis and secretion of α-amylase. These changes in cytosolic Ca^{2+} level may play a role in coordinating the activity of the secretory apparatus in response to GA_3 and ABA.

Gibberellic acid and abscisic acid also regulate the activity of Ca^{2+} transport into the endoplasmic reticulum (ER). α-Amylase is synthesized in the lumen of the ER. It is a Ca^{2+} containing metalloenzyme that requires at least one bound Ca^{2+} per enzyme molecule to maintain its activity. Thus, micromolar levels of Ca^{2+} must be maintained in the lumen of the ER to support the GA_3-induced formation of new α-amylase molecules. Gibberellic acid stimulates the activity of the Ca^{2+}-ATPase responsible for Ca^{2+} uptake by ER in the aleurone cell and increases the level of Ca^{2+} in the lumen of the ER. Abscisic acid reverses these effects. The activity of this ER Ca^{2+}-pump is stimulated approximately two fold by calmodulin (CaM) in membranes from non-GA_3-treated cells. Gibberellin increases both the level of CaM in the cell and the amount of CaM associated with ER membranes. The increase in CaM levels occurs within 4 to 6 h of GA_3 treatment, concurrent with the activation of the Ca^{2+} pump but preceding the activation of amylase secretion. Thus, changes in CaM level and distribution may provide a mechanism for the activation of the ER Ca^{2+} pump by GA_3. We are currently investigating the hypothesis that Ca^{2+} and CaM are part of the mechanism that coordinates and integrates the activities of the secretory apparatus of the aleurone cell in response to GA_3 and ABA.

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EFFECT OF CALCIUM AVAILABILITY ON CALMODULIN LEVEL AND ON METABOLIC REACTIVATION IN EARLY GERMINATION PHASES OF RADISH SEEDS.

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Calcium and Calmodulin (CaM) control many physiological processes in plants. The level of Ca^{2+}–CaM complex can be modulated by the level of intracellular Ca^{2+} and also by the level of CaM. CaM level appears to play an important role in the metabolic reactivation which characterizes seed germination (1, 2, 3). In radish seeds Ca^{2+}–CaM active complex is also modulated by the presence of a proteinaceous inhibitor (1). Seed germination is also characterized by the reactivation of membrane functions suggesting a precocious recovery of the control of ionic homeostasis. The fact that Ca^{2+} in early germination was released into the medium (1) suggests a high level of cytosolic Ca^{2+}.

The reduction of Ca^{2+} availability during radish seed germination was obtained by incubating the seeds in the presence of the Ca^{2+} chelator EGTA. The presence of EGTA inhibited germination (measured as increase in fr. wt.) at concentrations higher than 1 mM. When Ca^{2+} was administered together with EGTA, the inhibition was removed suggesting that the EGTA effect is due to the reduction of Ca^{2+} availability. EGTA treatment decreased the level of free Ca^{2+} in the medium, but the cation bound to EGTA increased; parallelly Ca^{2+} in the embryo axes decreased. At 1 mM EGTA, the reduction of Ca^{2+} availability only slightly affected metabolic reactivation (measured as RNA and DNA increases) and had no effect on membrane reactivation.

The level and specific activity of CaM in the soluble fraction of radish embryos strongly increased in early germination phases (ca. +450%). 1 mM EGTA greatly reduced (ca.-50%) the increases in level and specific activity of CaM. When the soluble fraction was analyzed on Sephadex G75 column, CaM was eluted in several peaks at MW higher than 30 kD and in particular, at 36 kD, CaM was co-eluted with the proteinaceus CaM inhibitor present in ungerminated seeds. CaM eluted in this zone decreased in the seeds germinated in the presence of EGTA.

The decrease in level and specific activity of CaM was not accompanied by a decrease in germination and metabolic reactivation when the seed germinate at low Ca^{2+} suggesting that the level of the cation might control CaM increase and level, that the level of CaM could contribute to control the level of cytosolic Ca^{2+} and that the Ca^{2+}–CaM dependent activities could also be also controlled by the presence of the CaM inhibitor.

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THE MEASUREMENT OF CALCIUM INFLUX INTO PLANT CELLS

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Despite the perceived importance of Ca^{2+} in almost all aspects of plant growth and development, relatively little is known about the mechanism of its uptake by plant cells. Measurement of Ca^{2+} influx using radioisotopic tracers is complicated by the high capacity of cell walls to bind divalent cations. In complex tissues such as roots there is considerable uncertainty in distinguishing between extracellular bound tracer and actual influx across the plasma membrane. The normal procedure in such experiments is to "desorb" extracellular tracer from the cell wall by rinsing with unlabelled Ca^{2+} or other cations, usually at higher concentrations to accelerate the process. However, it is difficult to test both the efficiency of desorption and the degree of loss of intracellular tracer during the desorption process.

The problems of cell wall binding of Ca^{2+} are considerably reduced when measuring Ca^{2+} fluxes into charophytes, since it is possible with the large cylindrical internodal cells to not only physically separate the cell contents from the cell wall at the end of the influx period, but also to separate tracer activities in the cytoplasm and vacuole. We will report the results of our studies of ^{45}Ca influx into *Chara* cells, describing the kinetics of Ca^{2+} uptake, the relationship of Ca^{2+} uptake to growth and the effects on Ca^{2+} uptake of pH, K^+, salinity, turgor, PD and action potentials.

The characteristics of ^{45}Ca exchange in the cell walls of intact cells and in isolated cell walls of *Chara* will be discussed in relation to the validity of desorption methods used in the measurement of divalent cation fluxes in other tissues.

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MEASUREMENT OF FREE CALCIUM AT HIGH IONIC STRENGTH: ACTIVITIES AND HOMEOSTASIS OF CYTOPLASMIC FREE CALCIUM IN *ACETABULARIA*

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Neutral carrier based Ca^{2+}-selective microelectrodes have been examined according to Ammann [1] for application in concentrated multi-ion solutions. Calculations with data from references in [1] and our calibration series with Ca^{2+}-EGTA buffers (Fig. 1) provide the physicochemical conditions for determination of submicromolar concentrations of free Ca^{2+} in the cytoplasm (with about 400 mM K^+ and 70 mM Na^+) of the marine alga *Acetabularia acetabulum*. Corresponding measurements in seawater-like media with high [Na^+] and low [K^+] were not possible because of the selectivity of the sensor (ETH 129) for Na^+ over K^+. The experimental results for the cytoplasmic amounts of free Ca^{2+} in *Acetabularia* (Fig. 2), show a concentration of 560 nM free Ca^{2+} corresponding to 140 nM activity. Amounts and timecourse of changes in cytoplasmic Ca^{2+} upon removal and readdition of external (10 mM) Ca^{2+} show steady state changes by about 50 nM (following the direction of external Ca^{2+}) which are preceeded by transient overshoots. Fig. 3 shows an example. The similar amounts of the slopes at the rising and at the falling

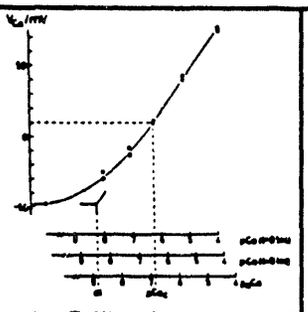

Fig. 1. Calibration curve of electrode for experiment in Fig. 2. *p*Ca: $-\log_{10}$(concentration) of free Ca^{2+} at 0.5 or 0.1 ionic strength, I; $_pI$Ca: $-\log_{10}$(activity) of free Ca^{2+} at I=0.1 M; 0: precalibration, ●: postcalibration.

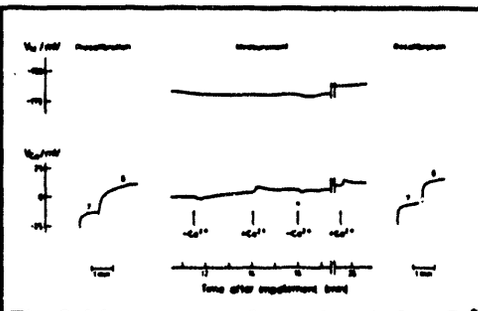

Fig. 2. Measurement of cytoplasmic free Ca^{2+} by Ca^{2+} selective microelectrode, embedded by sections of pre- and postcalibration of the electrode used; upper trace recording of intracellular reference voltage; lower trace: pCa^{2+} trace;-Ca^{2+}: replacement of 10 mM Ca^{2+} by Mg^{2+} + 10 mM EGTA; +Ca^{2+}: replacement of Mg^{2+} and EGTA by 10 mM Ca^{2+}.

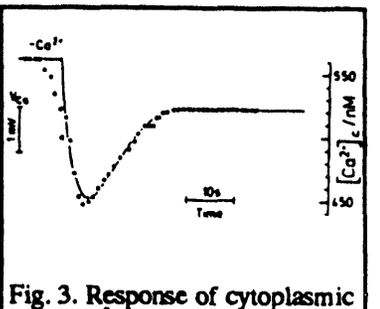

Fig. 3. Response of cytoplasmic free Ca^{2+} upon removal of extracellular Ca^{2+}; points: measured (* in Fig. 2); curve: fitted by the expression $\Delta Ca^{2+} = \exp(-t/\tau_1)\{A_1 sn(x)+A_2 cs(x)\}-A_2$ with $x=2\pi t/\tau_2$, A_1 = 305 nM Ca^{2+}, A_2 = 49 nM Ca^{2+}, τ_1 = 5.8 s, and τ_2 = 47.4 s.

edge of this peak, render a description by damped oscillations of a feedback control system (equation and parameters in Fig. legend 3) more suitable than by two superimposed exponentials with physically reasonable amplitudes. Using the maximum rate of decrease of cytoplasmic Ca^{2+} upon removal of external Ca^{2+}, an unidirectional Ca^{2+} efflux of $\geq$0.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is determined which is considered to mark the steady state turnover of Ca^{2+} at the plasmalemma. This high rate and the high electrochemical gradient for Ca^{2+} (*Camf* about -0.58 V) across the plasmalemma at a resting voltage of about -170 mV, point to a powerful Ca^{2+} export system which cannot sufficiently be fueled by ATP-hydrolysis alone ($\Delta G/F$ for ATP in *Acetabularia*: about -0.45 V [2]) but requires additional energy. -Details in [3]; supported by the DFG.

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The mode of action of gibberellins in the delay of leaf senescence of alstroemeria cut inflorescences.

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Cut inflorescences of alstroemeria (*Alstroemeria pelegrina*c.v. Westland) exhibit a loss of chlorophyll of the vegetative leaves in the dark. A mixture of gibberellins strongly delays chlorophyll loss of cut inflorescences of alstroemeria (1). In this study we have investigated the effects of various chemically pure gibberellins (GA_1, GA_3, GA_4, GA_8, GA_9, GA_{13} and GA_3 methylester, obtained from L.N. Mander, Cambera, Australia) on the retention of chlorophyll both in leaves of cut inflorescences of alstroemeria and in detached leaf tips incubated in solution. GA_1, GA_3, GA_4, GA_9 and GA_{13} caused a delay in chlorophyll loss both in leaves of cut inflorescences of alstroemeria and in detached leaf tips incubated in solution whereas GA_8 and GA_3 methylester had little or no influence on the rate of chlorophyll loss.

To get a first insight in the signal transduction pathway of gibberellin we have searched for gibberellin-binding proteins using a [^{3}H] GA_1 of high specific activity (obtained from P. Davies, Ithaca, USA). Firstly, we have focussed on potential gibberellin-binding proteins present in the plasmamembrane. Therefore, we have isolated plasmamembranes from alstroemeria leaves using an aqueous two-phase partitioning method (2). Purity of the plasmamembrane preparations was determined by marker enzyme analysis, SDS-PAGE and TLC analysis of the extracted lipids. Subsequently, the binding of [^{3}H] GA_1 to the isolated plasmamembranes was determined.

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GTP-BINDING PROTEINS IN LEAF AND ROOT MEMBRANES: CHARACTERISTICS AND MODULATION BY ENVIRONMENTAL CONDITIONS.

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Antisera raised against a highly conserved amino acid sequence ($G_{\alpha\text{-common}}$ peptide) of mammalian GTP-binding proteins cross-reacted with microsomal membranes isolated from roots (melon, *Cucumis melo* L.) and leaves (clerodendrum, *C. speciosum*). The labeled sets of proteins were of M_r=130, 47 and 37 kDa (melon) and M_r=42 and 25 kDa (clerodendrum). Microsomal membranes isolated from roots and leaves bound $GTP_{\gamma}S$ with a high affinity, following the sequence $GTP_{\gamma}S$~GTP>GDP>>ATP. The K_d of $GTP_{\gamma}S$ binding to root and leaf membranes was ~10 and 15 nM, respectively. The number of high affinity binding sites was approximately 5 and 75 pmol/mg total membrane protein, respectively. Purified plasmalemma was relatively rich in $GTP_{\gamma}S$ binding sites.

Root membranes isolated from melon seedlings grown in excess NaCl exhibited a two fold increase in the number of GTP binding sites as well as in the amount of protein labeled with anti-$G_{\alpha\text{-common}}$ antibody; K_d slightly increased. Following chill treatment, membranes isolated from clerodendrum leaves exhibited a reduction in the number of GTP binding sites and the amount of protein labeled with anti-$G_{\alpha\text{-common}}$ antibody was less than in the control.

The findings indicate that plant-membranes contain GTP-binding proteins similar to mammalian GTP-binding proteins. The expression of these proteins seems to be modulated by environmental conditions, suggesting that GTP-binding proteins play a role in the physiological response of plants to the environment.

POLYPHOSPHOINOSITIDE PHOSPHOLIPASE C IN PLANT PLASMA MEMBRANES. PURIFICATION AND CHARACTERIZATION.

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Phospholipase C is a key enzyme in the transduction of agonist-dependent signals across the plasma membrane in animal cells, catalyzing the formation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 is formed by the two-step phosphorylation of phosphatidylinositol (PI) by PI kinase and phosphatidylinositol 4-phosphate (PIP) kinase, enzymes that are present in plant plasma membranes as well (1). As phospholipase C, preferentially active on PIP and PIP_2, also has been identified in plasma membranes from plants (2), the enzymic basis for signal transduction through hydrolysis of polyphosphoinositides is present.

We have localized the active site of the polyphosphoinositide-specific phospholipase C to the cytoplasmic surface of the plant plasma membrane using highly purified inside-out (cytoplasmic side out) and right-side out (apoplastic side out) wheat root plasma membrane vesicles obtained by aqueous polymer two-phase partitioning. The enzyme was dependent on micromolar concentrations of Ca^{2+} for activity, and millimolar Mg^{2+} further increased the activity. The enzyme was solubilized from plasma membranes using octylglucoside and purified 25-fold by hydroxylapatite and ion-exchange chromatography (3). The purified enzyme catalyzed the hydrolysis of PIP and PIP_2 with specific activities of 5 and 10 μmol/ min per mg protein, respectively. PI was not a substrate.

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CHARACTERIZATION OF A PHOSPHATIDYLINOSITOL KINASE ACTIVATOR: AN ALTERNATIVE ROLE FOR INOSITOL PHOSPHOLIPIDS IN SIGNAL TRANSDUCTION

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Phosphatidylinositol kinase (PI kinase) catalyzes the phosphorylation of phosphatidylinositol (PI) to form phosphatidylinositol monophosphate (PIP). The product of PI kinase, PIP, has been shown to increase the activity of the plasma membrane vanadate-sensitive ATPase (1,2) and to affect actin polymerization (3). We have found that the plasma membrane PI kinase can be released from the membrane by phospholipase A_2 and that the released PI kinase can be activated by an endogenous heat-stable factor in the 40,000xg supernatant of homogenate of carrot culture cells (4). There are at least three fractions separated on DEAE-Sepharose CL-6B that activate the solubilized PI kinase. One of the fractions has been purified further and was found to contain a 48 kDa protein. Activation of the PI kinase by the 48 kDa protein is saturated at a concentration of $3x10^{-7}$ M. The sequences of two peptide fragments of the 48 kDa protein are homologous to an actin-binding protein and the elongation factor-1 (EF-1) alpha subunit. The purified activator binds actin. Based on these data and the fact that PIP can affect actin polymerization by binding gelsolin, we propose the following working model as one signal transduction pathway: In response to an external stimulus which increases phospholipase A_2 activity, PI kinase is released from the plasma membrane. The released PI kinase binds to the activator and actin oligomers, and the activated PI kinase reassociates with the membrane increasing the production of PIP. PIP binds to gelsolin which results in an increase in actin polymerization, an alteration in cytoskeletal structure which would affect cell physiology and growth.

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IMMUNOCHEMICAL AND FUNCTIONAL ANALYSIS OF CALCIUM-DEPENDENT PROTEIN KINASE

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Soybean calcium-dependent protein kinase (CDPK) contains three functional domains: a catalytic domain that is most closely related to those of the calmodulin-dependent protein kinases; a regulatory domain that is 40% identical to spinach calmodulin; and a putative autoinhibitory domain (1). The native enzyme binds calcium with high affinity and specificity (2). Upon binding calcium ions, the protein-phosphorylating activity of CDPK is increased 50-100-fold (2,3). Thus the ability both to detect and to transduce the calcium signal reside in a single molecule. These properties make CDPK the quintessential calcium target protein.

To understand the function of the putative autoinhibitory domain, the effect on activity *in vitro* of synthetic peptide corresponding to residues 302-332 in this domain was determined. Inhibition of CDPK by peptide 302-332 was noncompetitive with histone IIIS, syntide-2, and ATP, and the the Ki ranged from 2 to 50 μM, depending on the substrate tested. These results support the identification of residues 302-332 as a functional autoinhibitory domain.

The epitope recognized by an antibody previously used to show that CDPK is associated with F-actin in plant and algal cells (4,5) and with the plasma membrane (6) was determined. Fragments of the cDNA encoding CDPK were produced by BAL 31 digestion or site directed deletion, ligated into vector pET3a, and expressed in *Escherechia coli*. Immunoblotting of the truncated proteins showed that the epitope maps to a 14 residue sequence in the amino-terminal portion of catalytic domain.

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IDENTIFICATION AND CHARACTERIZATION OF TWO PHOSPHOLIPASE A_2 ACTIVITIES IN LEAVES OF Vicia faba

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Recent experimental results suggest that two products of phospholipase A_2 (PLA_2), lysophospholipids and free fatty acids, may serve as second messengers in some plant systems (1,2). Despite the potential importance, biochemical characteristics of the enzyme in plants remain largely unknown. We have performed detailed characterizations of PLA_2 activities in a higher plant, Vicia faba.

Two kinds of PLA_2 activities were identified and characterized from homogenates of leaves of Vicia faba using 1-palmitoyl-2-[1-^{14}C] palmitoyl-glycerophosphocholine and 1,2-dipalmitoyl-glycerophospho-[N-methyl-^{14}C] choline as substrates. Based on TSK G 3000 SW gel filtration, two enzyme activities migrated as molecular weight of 70 kDa and 14 kDa. The first (70 kDa peak) was similar to a lysosomal PLA_2 in animal systems; it was optimally active at pH 4.5 and was not dependent on Ca^{2+} for its activity. In the presence of 5 mM Ca^{2+}, "PLA_1" as well as "PLA_2" activities were shown in 70 kDa peak. The second (14 kDa peak) had similarity to mammalian non-pancreatic secretory type II PLA_2; 1) it was optimally active in pH range of 9-10, 2) it required mM Ca^{2+} for optimal activity, and 3) incorporation of substrates into sodium deoxycholate micelles inhibited the enzyme activity.

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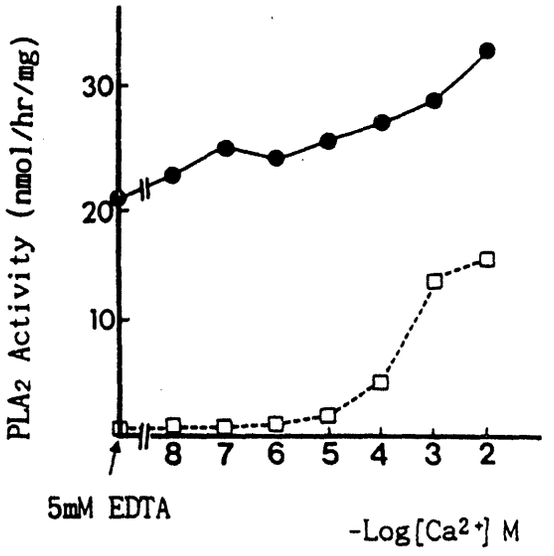

Fig. 1

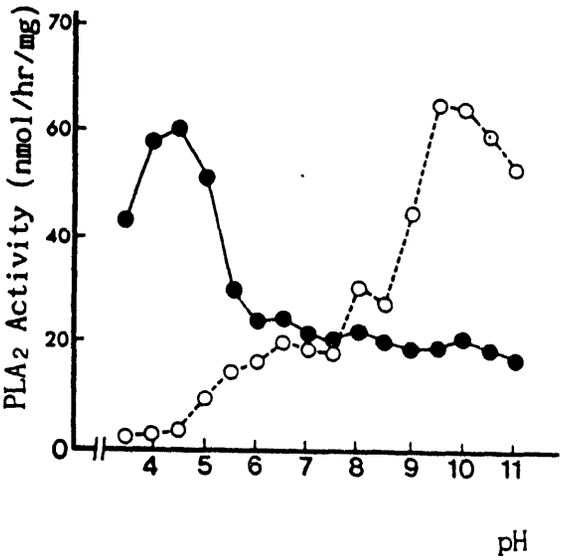

Fig. 2

Figure 1. Ca^{2+} requirements of 70 KDa (●) and 14 KDa (□) PLA_2 activities isolated from Vicia faba leaves.

Figure 2. Effects of pH on 70 KDa (●) and 14 KDa (○) PLA_2 activities isolated from Vicia faba leaves.

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IDENTIFICATION AND PURIFICATION OF GTP-BINDING PROTEINS IN THE PLASMA MEMBRANE OF ZUCCHINI (*Cucurbita pepo* L.)

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We have investigated the possibility that G-protein-like entities may be present in the plasma membrane (PM) of zucchini (*Cucurbita pepo* L.) hypocotyls by examining a number of criteria common to animal and yeast G-proteins. The GTP binding and hydrolysis characteristics of purified zucchini PM are similar to the characteristics of a number of known G-proteins. Our results demonstrate GTP binding to a single PM site having a K_d value between 16-31 nM. This binding has a high specificity for guanine nucleotides, and is stimulated by Mg^{2+}, detergents, and fluoride or aluminum ions. The GTPase activity (K_m=0.49μM) of zucchini PM shows a sensitivity to NaF similar to that seen for other G-proteins. ADP and ATP have a stimulatory effect on GTP binding and hydrolysis at concentrations around 1 μM. (1)

Localization of GTPγ^{35}S binding to nitrocellulose blots of PM proteins separated by SDS-PAGE indicates binding to 31 and 35 kDa proteins. These predominant GTP-binding species can be isolated by affinity chromatography and purified by SDS-PAGE. Sequence analysis of affinity-purified GTP-binding proteins from zucchini hypocotyls will allow us to determine to which class of GTP-binding proteins these belong, and permit us to discuss the possible role(s) of these proteins in plant signal transduction.

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Soluble and membrane-bound inositol-1,4,5-trisphosphate 5-phosphatase activity in plant cells

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In plant and animal cells, cytosolic free calcium is thought to play a central role in signal transduction . The primary site of calcium release in plants appears to be the large vacuole, since several studies have shown that the addition of inositol 1,4,5-trisphosphate causes a specific and saturable transient release of Ca^{2+} from tonoplast vesicles or intact vacuoles (Schumaker and Sze, 1987). Intracellular signals are expected to be transient, and therefore the signal must be metabolized or removed. Indeed, in animal cells, an Mg^{2+}-dependent inositol 1,4,5-trisphosphate 5-phosphatase has been described for different cell types (Berridge and Irvine,1989).

Analysis of the products formed by the incubation of inositol 1,4,5-trisphosphate with vacuolar membranes showed two breakdown products, inositol 4,5-diphosphate and inositol 1,4-diphosphate. In the absence of Mg^{2+}, inositol 4,5-diphosphate was the predominant peak. Addition of Mg^{2+} reduced the appearance of inositol 4,5-diphosphate, and a higher amount of inositol 1,4,-diphosphate could be observed. Hydrolysis of inositol 1,4,5-trisphosphate as a function of the incubation pH showed that inositol 4,5-diphosphate is released mainly under acidic conditions. The pH profile for the activity is similar to that of the vacuolar phosphatase. In contrast, inositol 1,4,-diphosphate, the product formed by the inositol 1,4,5 trisphosphate 5-phosphatase, can only be detected at higher pH values. The pH profile is similar to that of many cytosolic enzymes. Indeed, an inositol 1,4,5-trisphosphate 5-phosphatase would be expected to be localized at the outer surface of the membrane, since the signal acts at the cytosolic face of the vacuole. The Km value calculated is about 30 µM and, therefore, in the same range as most values reported for animal cells. Cytosolic fractions obtained from evacuolated protoplasts also showed a specific inositiol 1,4,5 trisphosphate hydrolysis. About 60 % of the activity can be attributed to the cytosol,and 40 % to the vacuolar membranes.

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ACTIVATION OF PHOSPHOLIPASE A_2 (PLA2) BY AUXIN AND MASTOPARAN GENERATES LYSOPHOSPHOLIPIDS TO ACTIVATE PROTEIN KINASE AND H^+-ATPase

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Activation of PLA2 by 2,4-D is rapid, in cultured soybean cells within 1 min and in zucchini hypocotyls within 15 min. ABA, GA_3, acetic acid had no effect, PAA and 2,3-D were weakly active in soybean cells (all at $5x10^{-4}$ M) which excludes stress as a reason for activation. In microsomes isolated from both organisms 10^{-7} M auxins activate PLA2 and 2,3-D is almost inactive. Mastoparan, a peptide activator of animal G proteins and of PLA2, activates PLA2 in soybean cells and hypocotyls at 2-7 μM. Growth of abraded hypocotyls is stimulated by 70% by 2 μM mastoparan. At 2-7 μM mastoparan PLA2 is activated in microsomes. Hence mastoparan partially mimicks auxin action. Since PLA2 activation is mediated by the known auxin-binding protein (1) the results support the conclusion that PLA2 activation is part of the primary action of auxin.

The two reaction products of PLA2, fatty acids and lysophospholipids, are both potentially biologically active. One particular fatty acid, α-linolenic acid, received much attention recently as the precursor to jasmonic acid, a potent signalling substance (2). Lysophosphatidylcholine (LPC), the second reaction product, was investigated by us as an activator of membrane-associated protein kinase and of H^+-ATase (3). It activates H^+ transport and ATP hydrolysis of both H^+-ATPases in isolated vesicles. Besides LPC and its substitute 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor, PAF), acidic lysophospholipids also activate protein kinase, with LPI, LPG, and LPA in decreasing order. Preliminary tests show that some lysophospholipids stimulate growth. Hence, two new substance classes, the G protein activator-peptide mastoparan and lysophospholipids, can partially mimick auxin action. This is taken as evidence for an auxin-triggered signal transduction chain leading from a receptor-linked and G-nucleotide-sensitive PLA2 to a lysophospholipid-activated protein kinase. Comparison of autoradiography and Western blot shows that in highly purified plasma membranes the H^+-ATPase is a prominent substrate of the kinase.

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CHANGES IN LIPID KINASE AND PHOSPHOLIPASE A ACTIVITY IN PLASMA MEMBRANES OF PETUNIA PETALS DURING SENESCENCE

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The senescence of flower petals is accompanied by modifications in compositional, structural, physical and functional properties of cellular membranes. These processes begin with an early decrease in membrane protein and phospholipids, followed by a decrease in membrane fluidity, membrane enzyme activity, solute uptake ability and in the capacity of the cells to retain water. Changes in the synthesis or catabolism of membrane lipids would affect the distribution of the lipids within the bilayer and thereby could alter the activity of several plasma membrane enzymes. Indeed, modifications in the activity of enzymes involved in PC synthesis were found in a microsomal membrane fraction during petal senescence. In this work we are reporting on changes in the activities of DAG kinase, the inositol phospholipid kinases and phospholipase A (PLA) in relation to petunia petal senescence.

When endogenous lipid kinases were assayed by adding [^{32}P]-ATP to the isolated plasma membranes, the major products formed were PA and PIP. The formation of [^{32}P]-PIP showed no significant changes during the first 4 days, but declined by about 20% in the fifth day following harvest. The amount of [^{32}P]-PA formed in the in vitro assay increased 1.7 and 2.7 fold on day 3 and 4, respectively, and then decreased to one third the initial value on day 5. The experiments were repeated using exogenous substrates for short periods in order to compare the specific activities of the enzymes. In the presence of exogenous PI, there was a significant decrease in PI kinase specific activity after day 2. The activity on day 4 was about 50% of the day 1 value. DAG kinase activity increased on day 3 and 4 and decreased to below day 1 levels on day 5. Thus the changes in the specific activity of DAG kinase were consistent with the observations of PA formation in the absence of exogenous substrate. PI kinase specific activity in the presence of exogenous substrate, however, did not mimic the profile of product formation using endogenous substrate. In addition to lipid kinase activity, we monitored the PLA activity in the plasma membrane-rich fraction, with NBD-PC as a substrate. The PLA specific activity increased continuously over the entire period of the study.

The senescence-related modifications in the activities of these enzymes would affect the distribution of the DAG, PA, PI, PIP, PIP_2, lysolipids and free fatty acids, which are all putative second messengers or their precursors, in the membranes. Therefore, it is plausible that they are involved in modulating flower metabolism during senescence.

OSMOTIC STRESS DECREASES PHOSPHATIDYLINOSITOL (PI) KINASE ACTIVITY IN CARROT CELL PLASMA MEMBRANES: OKADAIC ACID REVERSES THIS EFFECT

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Phosphatidylinositol monophosphate (PIP), produced by phosphatidylinositol (PI) kinase, is a negatively charged phospholipid which is present in plasma membranes of plant cells. In addition to acting as a source of second messengers, PIP has been shown to increase the activity of the plasma membrane vanadate-sensitive ATPase (1,2)

When carrot cells were treated for 5 min hyperosmotically with 0.4 osmolal sorbitol in conditioned medium, the amount of [^{32}P]labeled phosphatidylinositol monophosphate (PIP) produced by isolated plasma membranes during *in vitro* phosphorylation with [γ-^{32}P]ATP decreased to approximately half of the control value. The decrease in PI kinase activity was observed both in the absence and presence of exogenous substrate (PI). In addition, as a result of hyperosmotic stress, plasma membrane ATPase activity decreased. The maximum reduction in ATPase was found after 5 min of treatment. If cells were first treated for 1 h with 100 nM okadaic acid, a potent protein phosphatase 1 and 2A inhibitor, the decreased [^{32}P]PIP production resulting from hyperosmotic stress was not observed. There was no effect of okadaic acid treatment on [^{32}P]PIP production in plasma membranes isolated from control cells. Okadaic acid (1 nM-1 μM) in phosphorylation reaction had no significant effect on [^{32}P]PIP production. While there is no evidence for phosphorylation or dephosphorylation of PI kinase *per se*, these data suggest that the decrease in PIP biosynthesis observed as a result of hyperosmotic stress is mediated by a okadaic acid-sensitive phosphatase.

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PROTEIN KINASE ACTIVITES IN POTATO MITOCHONDRIA

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Protein phosphorylation represents a major regulatory process in animal cells. Protein kinases act together with phosphoprotein phosphatases by reversible phosphorylation/dephosphorylation of specific proteins in the cell.

Protein kinases are present in several subcellular compartments of plant cells, but in general relatively little is known about their function. Potato tuber mitochondria contain several protein kinases localized both outside and inside the inner membrane, phosphorylating approximately 30 polypeptides (1,2). One of these protein kinases is the PDH kinase which is tightly associated with the PDH complex and regulate its activity in the matrix of mitochondria.

Apart from the PDH kinase, potato mitochondria contain at least two other protein kinases. We are currently studying these activities; one being soluble and the other membrane-bound. These two activities are not located in the matrix and might represent two forms of the same enzyme. The membrane-bound activity was solubilized and partially purified by DEAE-Sephacel chromatography. Histone H1 was used as exogenous substrate. The protein kinase is totally dependent on calcium for activity with maximum being activity at 1 μM calcium.

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LIGHT-DEPENDENT H^+ AND Cl^- FLUXES IN THE GREEN ALGA *EREMOSPHAERA VIRIDIS*

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Using ion-sensitive microelectrodes, the Cl^- and H^+ activities in the cytoplasm of the unicellular green alga *Eremosphaera viridis* were measured. In the light, cytoplasmic Cl^- activity was 2.2 mM or lower and cytoplasmic H^+ activity was about $5.4 \cdot 10^{-8}$ M (pH 7.3). Darkening resulted in a transient acidification, which was compensated within 3 to 5 min. While the cytoplasmic pH decreased on darkening the Cl^- activity increased to 3.2 mM. This higher Cl^- level was maintained in the dark. Switching light on again decreased the Cl^- activity to the level previously observed in the light. Simultaneously a transient alkalization of the cytoplasm was observed. The cytoplasmic Cl^- was kept constant within the limits described above. It was independent from (lower) external concentrations. The transient character of the light-dependent pH changes was probably caused by pH-stat mechanisms.

Studies with different photosynthesis inhibitors (DCMU, piretanide, venturicidin) indicated a *direct* relation between the light-driven H^+-flow across the thylakoid membrane and the observed light-dependent Cl^- and H^+ activity changes in the cytoplasm. It is suggested that light-driven H^+-flux across the thylakoid membrane was in part electrically compensated by a parallel Cl^--flux through a voltage-dependent Cl^- channel in the thylakoid membrane (1). This gave rise to changes of the H^+ and Cl^- activity in the chloroplast stroma, and these activity changes were compensated by Cl^- and H^+-fluxes across the chloroplast envelope giving rise to the observed Cl^- and H^+ activity changes in the cytoplasm. There is a light-dependent H^+ flow from the cytoplasm via the chloroplast stroma into the thylakoid lumen (and a reverse flow upon darkening) and this H^+ flow is in part electrically compensated by Cl^-.

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TURGOR REGULATION IN *CHARA BUCKELLII* IN LOW IONIC STRENGTH MEDIUM

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Chara buckellii is a salt tolerant alga collected from a high $MgSO_4$ lake. Unlike freshwater Charophytes, it regulates its turgor in response to osmotic stress (1). We showed previously (2) that regulatory turgor decrease in response to a 150 mOsmol/kg hypotonic stress occurred in about 40 min, and was accompanied by membrane depolarization, an increase in conductance, and a decrease in the rate of cytoplasmic streaming. Decreasing Ca^{2+} concentration in the medium or adding Ca^{2+} channel blockers such as nifedipine inhibited turgor regulation, the increase in conductance and the decrease in streaming, but not the depolarization. These data are consistent with an influx of Ca^{2+} being involved in the signal transduction pathway for turgor regulation.

Electrophysiological characteristics of the membrane are different in saline and low ionic strength medium (3), with membrane potential less negative and conductance higher than in low ionic strength medium. We therefore repeated the turgor regulation experiments in low ionic strength medium with osmolality controled with sorbitol. The time course of turgor regulation in response to the same osmotic stress was similar to that seen in saline medium, as was the increase in conductance and the decrease in streaming rate. The depolarization was less, however, and was preceded by a transient hyperpolarization. Lowering external Ca^{2+} to 10 nM, buffered by EGTA, did not inhibit turgor regulation. 0.1% ethanol, the vehicle for the inhibitors, inhibited turgor regulation in the low ionic strength medium, although it had no effect in the saline medium. Effects of the inhibitors were therefore hard to assess, but nifedipine appeared to have a small effect. The significance of these data for our model of turgor regulation are discussed.

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MEMBRANE POTENTIAL AND ACTION POTENTIAL OF MARINE PLANKTONIC DIATOMS

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Techniques have been developed (both enzymatic and mechanical) to obtain viable protoplasts of several genera of marine planktonic diatoms, raised in culture. Attempts to obtain gigaohm seals required for patch and whole cell voltage clamping have been generally unsuccessful, possible because of a residual coating of the plasmalemma of these protoplasts, as indicated by scanning electron microscopy. However, microelectrodes can be used to measure membrane potentials and to monitor changes in these potentials in response to variations in nutrient conditions and age of the culture.

EFFECTS OF IONOPHORES AND CHANNEL BLOCKERS ON ION CONTENT AND BUOYANCY OF THE MARINE DIATOM Ditylum brightwellii

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The effect of ionophores and channel blockers on the buoyancy of the marine diatom Ditylum brightwellii is being investigated following the observation that the density of a planktonic cell and hence its buoyancy is in part controlled by the concentration of certain cellular ions (see Anderson and Sweeney, 1978). Compounds being used include the K^+ ionophore valinomycin, the Na^+ ionophore monensin, and the K^+ channel blockers tetraethylammonium and lanthanum chloride. Under control conditions (no ionophore), our experiments show that the sinking rate of a population of D. brightwellii is about 1 cm/hour. We are currently manipulating the ionic conditions of the cells and measuring concomitant changes in sinking rates in order to investigate in more detail the ionic mechanism of buoyancy control. The buoyancy of natural populations of diatoms will determine their depth in the ocean, which will in turn affect the overall level of primary productivity of the population.

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ION CHANNELS CHARACTERIZED BY PATCH CLAMP AND VOLTAGE CLAMP IN PROTOPLASTS FROM THE GREEN ALGAE *EREMOSPHAERA VIRIDIS*

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Introduction

A well described signal transduction process takes place in the unicellular green algae *Eremosphaera* as published recently (1, 2). Specific potassium channels in the plasmalemma open during the course of a transient potential (TP) presumably triggered by calcium. Calcium activity might increase by releasing calcium from internal stores or by a calcium influx across specific channels in the plasmalemma. The specific potassium channels causing the TP were characterized in voltage- and current-clamp experiments with regard to their regulation.

Protoplastisolation

For patch clamp measurements protoplasts of E. were isolated similar to the protocol of Rosen et al. (3). The incubation medium contains 5 enzymes (Cellulase, glucuronidase, driselase, pectinase, and hemicellulase) with an osmolarity of nearly 600 mosmol and a pH of 5.7. Even at high enzyme concentration and long lasting incubation time only few protoplasts were obtained at room temperature.

Patch clamp measurements

In many experiments only few giga-seals were obtained. This indicates that the plasma membrane is still covered by some cell wall fragments. Nevertheless in preliminary experiments we found channels with a conductance of approximately 50 pS.

Whole cell measurements

Experiments were carried out with the two electrode voltage clamp set-up. I/V curves were measured during the different phases of the TP. The treatment with a-naphtyl phosphate (NP), A 23187 and trifluoperazine (TFP) created very long lasting TPs (up to 10 min). In the presence of Ba^{2+} (10 μM) and Sr^{2+} (1 mM) so called repetitive TPs were released.

Tetraethyl ammonium (TEA 10 mM) and Ba^{2+} (1 mM) inhibited the TP. This effect was reversible after removing these inhibitors. Experiment with sodium showed an inhibiting effect of this cation on the potassium channels.

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ION CHANNELS IN THE PLASMA MEMBRANE OF THE COCCAL GREEN ALGA *EREMOSPHAERA VIRIDIS*: TWO POTASSIUM CHANNELS AND ONE ANION CHANNEL

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Ion-channels in the plasma membrane of the unicellular green microalga *Eremosphaera viridis* are responsible for an action-potential like excitablitity of the alga, causing a transient hyperpolarization of the membrane potential by potassium efflux. In the maximum of this transient potential change the I/V-curves recorded with the two electrode voltage clamp technique exhibited N-shaped characteristics.

We could demonstrate that these characteristics were caused by three different ion channels in the plasma membrane: the previously described potassium channel (1), an inward rectifying potassium channel and a voltage dependent anion efflux channel.

The inward rectifying potassium channel conducted different cations in a non-ohmic manner with rising conductances at more hyperpolarized membrane potentials. It could be activated at rest, if the anion-channel was inhibited by NPPB ((5-nitro-2-3-phenylpropylamino) benzoic acid, 40 μM) , Zn^{2+} (50 μM) or A9C (Anthracen-9-Carboxic-acid, 0.5 mM). The cation currents did not respond to the potassium channel inhibitors TEA (Tetraethylamoniumsulfate, 1mM), Ba^{2+} (1mM) and Cs^{+} (1mM), but were strongly reduced by 100 μM $AlCl_3$.

The voltage-dependent currents responsible for the negative slope in the I/V-curve were appriciably reduced by the anion-channel inhibitors NPPB (40 μM), $ZnCl_2$ (50 μM) and A9C (0.5 mM). They were observed only at the top of the transient potential change. This suggests the existance of an additional activation step.

These data demonstrate the existance of two different cation channels and one anion channel in the plasmalemma of the coccal green alga *Eremosphaera viridis*. The channels described here showed striking similarities to channels in the plasma membrane of different higher plant cells, especially *Vicia faba* guard cells (2).

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REGULATION OF A PLASMA MEMBRANE CHLORIDE CHANNEL IN *CHARA CORALLINA* BY INTRACELLULAR pH AND PHOSPHORYLATION

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The large depolarizing current during the Charophyte action potential is well known to be carried by Cl^- (efflux) in response to an increase in cytosolic free Ca^{2+} (1,2). However, a novel role for plasma membrane Cl^- channels has been proposed more recently by Smith & Reid (3), who suggested that a dramatic increase in Cl^- efflux during cytoplasmic acidosis might serve the function of offsetting membrane hyperpolarization resulting from enhanced electrogenic H^+ pump activity in these conditions. This raises the possibility that Cl^- efflux is regulated not only by cytosolic free Ca^{2+}, but also by cytosolic pH.

We report here that efflux of $^{36}Cl^-$ from internally perfused, tonoplast-free cells is enhanced as a first-order function of intracellular $[H^+]$ as pH is lowered over the physiological range 7.8 to 6.8 in Ca^{2+}-free conditions. In the presence of nM levels of Ca^{2+}, a second effect is also apparent: the apparent K_d for Ca^{2+} of Cl^- efflux is markedly lowered as intracellular pH decreases. The net result is that cytoplasmic acidosis triggers Cl^- efflux both directly and indirectly *via* enhanced sensitivity of the flux to cytosolic Ca^{2+}. These effects cannot be explained on the basis of voltage-sensitivity of Cl^- efflux, since the perfusion solutions deployed had an insignificant effect on membrane potential.

Furthermore, phosphorylation/dephosphorylation of the putative channel exerts a profound regulatory role on its activity, and this can override the reponse to both $[H^+]$ and $[Ca^{2+}]$. Thus, intracellular perfusion with the catalytic subunit of protein kinase A and ATP results in complete inhibition of Cl^- efflux. Non-hydrolysable ATP analogs are inactive with respect to inhibition. Conversely, perfusion with a phosphatase in conditions normally associated with almost complete inhibition of Cl^- efflux (pH 7.8, free Ca^{2+} = 0) elevates Cl^- efflux to 60% of the maximal level observed with optimal $[H^+]$ and $[Ca^{2+}]$. The Cl^- channel therefore appears to reside in a semi-phosphorylated state *in vivo*. We will discuss the physiological functions of the Cl^- channel in relation to its regulatory characteristics.

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Effects of calmodulin on Ca^{2+}-dependent Cl^--sensitive anion channel Chara plasmalemma: A patch clamp study

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The inside-out patch-clamp technique was applied to plasmolyzed plasmalemma of Chara corallina internode without enzymatic treatment. We found a Cl^--sensitive anion channel. The channel activity was Ca^{2+}- and V_m-dependent and we named this channel Ca^{2+}-dependent Cl^--sensitive anion channel. This channel opened most frequently at about -100 mV when the Ca^{2+} concentration in the cytoplasmic side was 1.0 uM. At 10 uM Ca^{2+} the channel opened less frequently, and at 0.1 uM very scarecely. The channel activity was blocked by several antagonists of calmodulin. These properties of this channel, Ca^{2+}- and V_m-dependence and sensitivity to antagonists of calmodulin, resembled those of the excitable Ca^{2+}-activated Cl^- channel in the Chara internode (1, 2, 3).

The current flowing through the channels at 1.0 uM Ca^{2+} decreased with time showing fluctuations. The decrease in the current was apparently due to a decrease in number of open channels. Application of calmodulin (from spinach) to the cytoplasmic side restored the channel activity transiently or markedly. Besides, the application of calmodulin shifted the V_m, at which the largest current flowed, from -100 to -140 mV. Application of EGTA abolished the CaM effect. These suggest that calmodulin not only activates the Ca^{2+}-dependent Cl^--sensitive anion channel, but also modulates the V_m dependence of the channel activity. Our results support that calmodulin activates the excitable Ca^{2+}-activated Cl^- channel in the Chara internode

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LOCALIZED PATCH CLAMPING OF PLASMA MEMBRANE OF THE POLARIZED *FUCUS* ZYGOTE.

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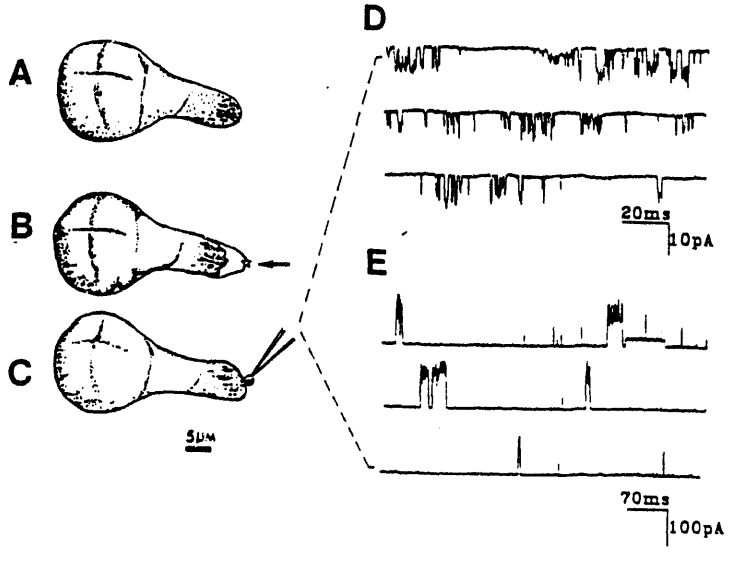
A fundamental problem in plant cell biology concerns the role of plasma membrane ion channels in development and maintenance of polarity. Direct patch clamp recordings from localized regions of polarizing or polarized cells are still lacking. Removal of the cell wall to expose plasma membrane suitable for patch recording is achieved in all but a few cases by enzymatic treatment to produce a spherical protoplast. Such protoplasts are unsuitable in studying the distribution and regulation of ion channels in a polarized cell due to the loss of structural order once the cell wall is removed. In this presentation we describe a novel technique for localized cell wall removal of the *Fucus* zygote rhizoid cell using U.V. laser to access the plasma membrane enabling high resistance patch clamp recordings.

A U.V. laser was introduced via the U.V. port of an inverted microscope and focussed to the diffraction limit. Plasmolysed zygotes were aligned such that the tip of the cell wall was ruptured by pulsing the laser (Fig. 1B). Careful control of cell turgor by microperfusion enabled extrusion of plasmalemma bound cytoplasm through the ruptured wall (Fig 1C). High resistance seals (> 5GΩ) were achieved (Fig 1C) and records of single channel activity on the plasma membrane of the tip of the rhizoid were obtained (Fig 1D,E). Downward diflections represent outward currents in the cell attached configuration. Several channel types have been observed which are currently under investigation. The outward channels in figure 1D are likely to be K^+ channels at the transmembrane potential of -80mV (pipette 0mV). In addition very large inward channels have been observed (Fig. 1E) at the transmembrane potential of -80mV. Both Cl^- and Ca^{2+} ions could carry inward currents at this potential. We are now further characterizing the plasma membrane ion channels, their distribution and regulation during the development and maintenance of polarity.

FIGURE 1.



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SELECTIVITY OF ION CHANNELS: ENZYME KINETICS VERSUS PERMEABILITY

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Selectivity of ion channels is frequently described by relative permeability coefficients as calculated by the Goldman equation which originates from the theory of *independent* electrodiffusion of ions across membranes. We consider this approach inadequate because different substrates will probably not permeate independently through a specific channels but in a competitive mode. Since ion channels are, in fact, enzymes (which catalyze the transition of ions across lipid membranes), an enzyme kinetic treatment of this subject (1) seems to be adequate. An example of this approach has been worked out during patch-clamp studies on a K^+ channel in *Chara*. Experimental conditions and corresponding data are given in Fig. 1, together with the theoretical current-voltage curves as fitted to the data by the enzymatic reaction scheme with the parameters given by Fig. 2.

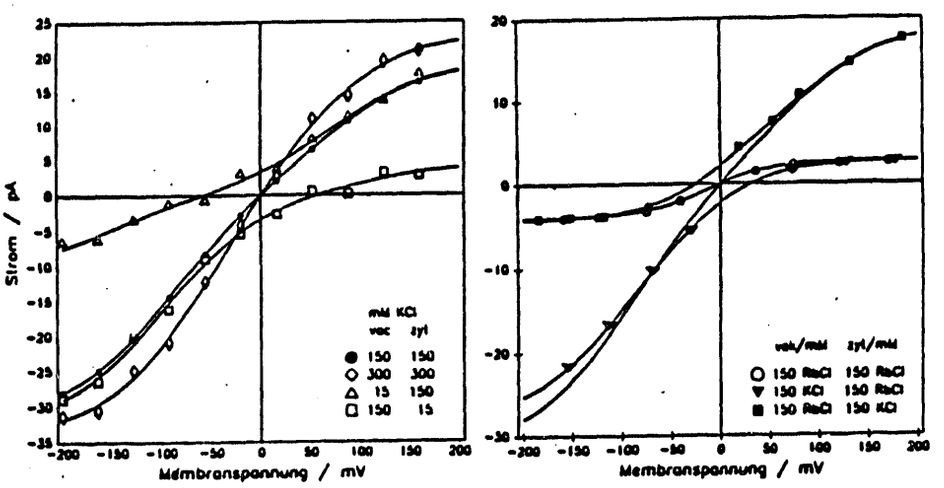
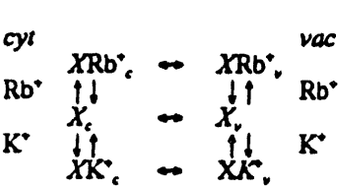


Fig. 1. Open-channel current-voltage relationsships of the prime K^+ channel in the tonoplast of *Chara*, measured on isolated patches from cytoplasmic drops at various ionic conditions at the two sides of the membrane as marked; all solutions with 1 mM Tris/EGTA *p*H 7; symbols measured, curves fitted; reference curve (symmetric 150 mM KCl) in right plot without symbols.

Fig. 2. Reaction scheme (left) of the channel X for K^+/Rb^+ selectivity, parameters (right) fitted to data in Fig 1.



	K^+	Rb^+
vac binding	10566 $\cdot 10^6\ s^{-1}\cdot M^{-1}$	403 $\cdot 10^6\ s^{-1}\cdot M^{-1}$
vac side debinding	105660 $\cdot 10^6\ s^{-1}$	5070 $\cdot 10^6\ s^{-1}$
cyt side binding	5283 $\cdot 10^6\ s^{-1}\cdot M^{-1}$	300 $\cdot 10^6\ s^{-1}\cdot M^{-1}$
cyt side debinding	15849 $\cdot 10^6\ s^{-1}$	240 $\cdot 10^6\ s^{-1}$
translocation $c \rightarrow v$	1409 $\cdot 10^6\ s^{-1}\cdot f_V$	498 $\cdot 10^6\ s^{-1}\cdot f_V$
translocation $v \rightarrow c$	3369 $\cdot 10^6\ s^{-1}\cdot f_V^{-1}$	4510 $\cdot 10^6\ s^{-1}\cdot f_V^{-1}$

reorientation: $c \rightarrow v$ $270\cdot 10^6\ s^{-1}$, $c \leftarrow v$ $194\cdot 10^6\ s^{-1}$; $f_V = \exp(V_m/(51\ mV))$

If the Rb^+ kinetics were compared with those of K^+ simply on the basis of the different saturation currents for K^+ and for Rb^+ or by the asymmetric reversal voltages, the resulting "relative permeabilities" would be inconsistent. However, the entire set of data is well described by the enzyme kinetic model in Fig. 1 with the unique set of parameters given. It is pointed out, that the relatively slow reorientation of the empty binding site is an essential property of the model: it enables the description of the missing trans-inhibition effects in the data. Another crucial feature is that only the charge-translocations (not the binding equilibria) are voltage-dependent steps. Without this property, the relatively strong curvatures of the curves cannot be described as simply. - Supported by the Deutsche Forschungsgemeinschaft.

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ION CHANNELS IN THE PLASMALEMMA OF HYDRODICTYON AFRICANUM.

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A major component of the conductance of the plasmalemma of the large cells of the freshwater alga, Hydrodictyon africanum, is an outward voltage and time-dependent K^+ conductance, examined in some detail by Findlay and Coleman (1) with standard voltage clamp techniques applied to intact whole cells. Until now there have been no studies of single ion channels in this membrane by patch clamping because of the difficulty in obtaining the necessary access to the membrane without the cell wall. We have developed a simple method, in which a cell is plasmolysed, cut open, and the cytoplasmic contents broken up to some extent, to yield fragments of cytoplasm. The success of this method apparently arises from the nature of the cytoplasm, which is stiff, and whose volume is small compared with that of the chloroplasts. The fragments maintain the curvature they possessed in the intact cell, and it appears that part of the plasmalemma continues to form the convex surface of the fragment.

It is possible to patch clamp the membrane, presumed to be the plasmalemma, on the convex surface of these fragments, and to observe channel activity. Preliminary results for detached patches, with both sides of the patch bathed in a solution containing (in mM) 50 KCl, 5 $CaCl_2$, at pH 5.5, show channels, with substates, and with maximum conductance of about 50 pS, accompanied by a background of considerably smaller channels. Surprisingly, in view of the evidence from intact cells, the behaviour of the this large channel, when ion gradients are applied across the patch, shows that it is not a K^+ channel.

This method of producing membrane fragments, whilst promising, almost certainly needs refinement. It may also be possible to extend it to other large-celled algae, particularly some of the marine algae.

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VOLTAGE-CLAMP STUDIES OF EXCITATION: COMPARISON OF DATA FROM INTACT AND PERFUSED CELLS OF *CHARA* AND *NITELLOPSIS*

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There is now a general agreement that excitation in the *Characeae* involves an inflow of Ca^{++} which in turn activates the Cl^- channels (1-4). However, a detailed dynamics of the currents through the different types of channels have not been worked out. The comparison of two *Characeae* species and the data from perfused tonoplastless cells provides some insights into the excitation phenomenon.

The experiments were performed at the Himeji Institute of Technology (Japan) at the time of MJB visit funded by the Australian Department of Industry, Technology and Commerce. The excitation currents obtained from *Chara corallina* displayed the main features seen previously (5): two negative peaks near the threshold p.d. (potential difference), a large single negative peak between -100 and -60 mV and a prompt positive spike near 0 p.d. level. The excitation currents from *Nitellopsis* also exhibited two negative peaks near threshold, but at p.d.'s more positive than -90 mV the amplitude of negative transients declined and several peaks could be observed. Large positive currents were seen near 0 mV but the prominent positive spike was absent.

The cells were perfused as decribed by Mimura *et al.* 1983 (6). Both species then showed very similar excitation currents. Between the threshold and ~-60 mV total inactivation of the negative currents was absent, but one or two negative peaks could still be distinguished. At more positive p.d.'s the negative currents became transient, similarly to those in intact cells. The negative excitation currents were inhibited by 5 mM TEA (tetraethylammonium). This inhibition was total in *Nitellopsis* and partial in *Chara*.

The results are discussed in terms of Cl^- channels, different types of Ca^{++} channels and K^+ channels.

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SINGLE-CHANNEL CURRENTS IN THE PLASMA MEMBRANE OF *Chara australis.*

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Patch-clamping the plasma membrane of plant cells is made difficult by the presence of a cell wall. In *Chara australis* the cell wall is particularly difficult to remove as it does not respond to any of the enzymatic digestion protocols that have proved successful on higher plant cells. Nevertheless, the fact that many membrane transport processes in the plasma membrane of *Chara australis* have been well characterised using electrophysiological techniques makes this a meaningful system in which to look for single ion channels.

A surgical method for removing the cell wall of *Chara* has been developed [Laver, 1991] which enables access to the plasma membrane through a small opening cut in the wall of a plasmolysed cell. The advantage of this method is that it does not require digestion of the cell wall by enzymes.

Despite gaining access to the membrane surface, high resistance seal formation is still relatively infrequent. To eliminate the possibility that cell wall regeneration may interfere with seal formation, inhibitors of cellulose synthesis have been applied and the effectiveness of these treatments will be discussed.

In cases where high resistance seals have been obtained, single-channel current records reveal a number of different channels in the plasma membrane. Channels with conductances as low as 15 pS have been observed. It is likely that these are the same channels observed previously [Coleman, 1986; Laver, 1991]

Our present study has also identified a channel with a high conductance of up to 100 pS. So far results would suggest that this is a relatively non-selective channel. At present the physiological role of such a channel in the plasma membrane of *Chara* is unclear. Determining whether or not a particular ionic species dominates the conductance will reveal more about the possible function of this channel.

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VOLTAGE DEPENDENT Cl^- CHANNEL ACTIVITY-TRANSIENT AT *CHARA* PLASMA MEMBRANE

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Chara internodal cells were plasmolysed in experimental solution (in mM: 10 KCl, 1 $CaCl_2$, 5 Hepes, pH 7, 320 sorbit) and after cutting a small window through the cell wall the plasma membrane was accessable for the formation of giga-seals (1). With electrodes containing 100 mM $BaCl_2$ it was possible to record (cell attatched) single channel activity with a strongly voltage-dependent gating: The current-voltage relation of the open channel reversed at -50 mV [$(-)V_{pip}$]. This is in the range of voltages expected for the Cl^- equilibrium potential assuming a $[Cl^-]_{cyt}$ of approximately 10 mM (2) and taking into account -45$\pm$ 3 (12) mV free running membrane voltage (measured in current-clamp mode at I=0 after breaking through the membrane). This identifies the current as a Cl^- current. Channels rarely opened at voltages < 0 mV. Clamping to more positive voltages evoked the repeated activation of transient channel activity (1 to 5 sec long) with up to 6 single channell active at one time (Fig.). Activation always began and ended with the lowest conductance level (lg). During the transient period, however, single channel events with much larger amplitudes were regularly observed (hg) and these amplitudes accounted for the sum of 2 or 3 smaller channels respectively. Neither in the opening nor in the closing transition of these large channel events was it possible to resolve switching of smaller components. The large amplitudes may represent entirely different channels with a gating confined to the period of transient activity of multiple Cl^- channels. Alternatively it may result from close cooperative gating of 2 or 3 low conductance Cl^- channels. The activation of transient channel activity was restricted to the cell attatched mode.

The results will be discussed in respect to the mechanism of membrane excitation in Characean algae.

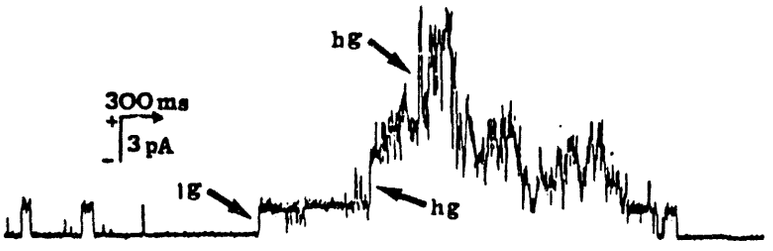

Fig. Recording of voltage evoked activation of multible conductance fluctuations at +65 mV test potential (:-V_{pip}). Low conductance (lg) and high conductance transitions (hg) are indicated by arrows.

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K^+-CHANNELS IN THE PLASMA MEMBRANE OF *CHARA CORALLINA*: MULTIPLE LEVELS OF CONDUCTANCE

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Two methods were used to make the plasma membrane of *Chara* cells accessible for patch clamp experiments in the cell-attached configuration. In one method cells were plasmolysed, one end of the cell wall was cut off and the plasma membrane became exposed as a result of deplasmolysis [1]. In the other method the cells remained plasmolysed during the experiments and a small window was cut through the cell wall to give access to the membrane [2]. The experimental results did not depend on the method used. Under the given ionic conditions (inside about 100 mM K^+, 10 mM Cl^-, <1µM Ca^{2+}; outside 180 mM K^+, 182 mM Cl^-, 1 mM Ca^{2+}) the reversal voltage of about +12mV indicates that the open channel currents are carried by K^+. The I-V relationship was linear over the observed voltage range from -70 mV to +60 mV. At least five different levels of conductance were recorded, which appear to be multiples of about 30 pS. Transitions between zero current and each current level took place without visible stops at the intermediate levels (inset Fig.1). Although it cannot be excluded that the different conductance levels reflect different types of K^+-channels, it is suggested that these levels are due to clusters of 30 pS K^+-channels which open and close in a strongly cooperative manner. Single conductances could be observed in any combination. Fig.1 shows a rare observation were five conductance levels could be identified in one patch.

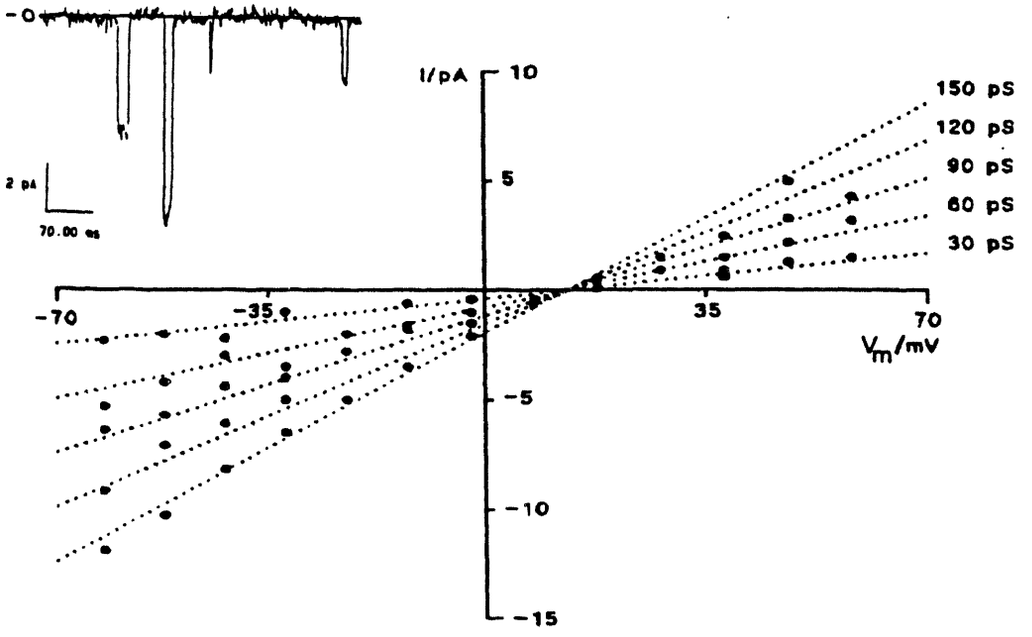

Fig.1: I-V relationship of the open channels; all conductance levels observed in one cell-attached patch; membrane voltages (V_m) are given as the sum of pipette voltage and average resting voltage (+8 mV, measured in the current-clamp mode); bath and pipette solution contained 180 mM KCl, 1 mM $CaCl_2$ and 5 mM Tris/MES, pH 7.2; inset: continuous recording of inward current steps at -42 mV, 300 Hz low-pass filtered

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POTASSIUM UPTAKE IN ROOT CELLS OF *ARABIDOPSIS THALIANA*.

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Potassium is a major crop nutrient, but many controversies still remain about the exact mechanism of K^+ absorption in higher plant roots. At moderate external K^+ concentrations (approx. 0.2 mM and higher) and with membrane potentials (E_m) more negative than the K^+ nernst potential (E_{K+}), potassium ions could be taken up passively (i.e. thermodynamically down hill). K^+ conducting ion channels could very well serve this purpose. However, higher plants can grow in conditions with ext. K^+ concentrations as low as 1 μM. In that case E_{K+} is usually more negative than E_m and K^+ has to be taken up 'actively' (1).

In *Neurospora crassa* (2) active K^+ uptake is driven by a K^+/H^+ symport and in *Chara* and other charophytea the driving force is the Na^+ gradient (3, 4).In higher plants no definitive answer has so far been given to this problem.

We have investigated the plasma membrane transport properties of *Arabidopsis thaliana* root cells in order to elucidate the ways K^+ is taken up by these cells. Membrane potentials of the wild type and a K^+ uptake mutant were measured for K^+ grown and K^+ starved plants. These experiments showed that a) in all conditions used E_{K+} becomes more negative than E_m if the ext. K^+ drops below approx. 30 to 100 μM, b) Na^+ ions do not provide the driving force for K^+ uptake.

Patch clamping in the whole cell and patch mode of these cells has revealed at least 1 inward rectifying K^+ conducting channel, 2 outward rectifying channels of which at least one conducts K^+, and an outward rectifying stretch activated channel also capable of K^+ conduction. Difference curves of whole cell currents (with and without K^+ in the experimental solutions) for the wild type and the mutant are being used to investigate the reversal potential of K^+ transport at the plasma membrane. From shifts in these reversal potentials after defined K^+ changes, together with data about internal K^+ concentrations and net K^+ uptake, we are in the process of distinguishing K^+ transport through ion channels from that through other transporters.

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PATCH-CLAMP STUDIES ON ELODEA LEAF PROTOPLASTS. II. DIFFERENT I/V CHARACTERISTICS OF UPPER AND LOWER CELLS.

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Elodea leaves consist of two cell layers; an upper layer with large cells and a lower layer with small cells. These two cell types respond remarkably different to light: the lower cells extrude protons and take up K^+-ions whereas the upper cells do the opposite. Our interest is in the mechanism of this cell differentiation process and the light regulation of the transport processes involved. To this end protoplasts from the upper and lower surface were isolated mechanically, thereby avoiding the introduction of artifacts due to enzyme incubation.

Solutions (in mM); (supplemented with mannitol to give a final osmolarity of 380 mOsm.

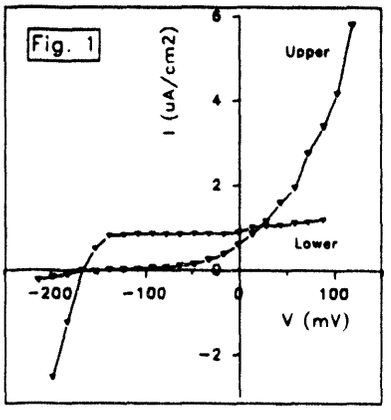
Bath: 10 KCl, 2 $MgCl_2$, 5 $CaCl_2$, 2 $KHCO_3$ (pH 8).

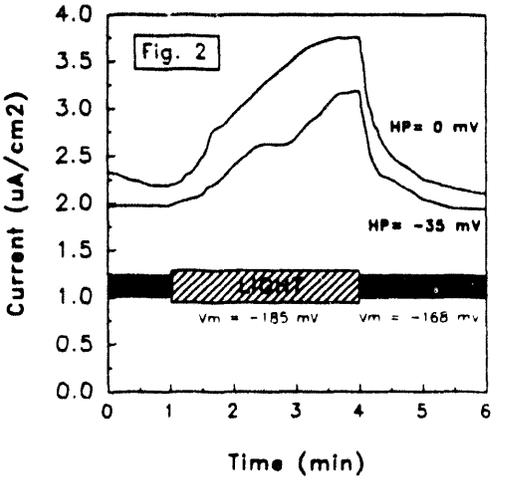
Pipet: 50 KCl, 2 $MgCl_2$, 0.33 $CaCl_2$ (pCa=7), 1.37 EGTA, 2.5 MgATP, 10 Hepes/Tris (pH 7.2).

Results

The differences in the I/V characteristics of a lower and upper cell are striking (Fig. 1). In conformity with the physiology, the lower cells show an inward rectifying current, activated at very negative membrane potential (V < -150 mV) which, in the dark, is mainly carried by K^+. Under identical conditions this current is virtually absent in the upper cells. In contrast, the upper cells exhibit outward rectifying currents, activated at V > -70 to -50 mV; these channels are not activated in the lower cells.

The third difference is the activity of the H^+-pump. The positive current (Fig. 1) in the lower cell over a wide voltage range is likely to be pump activity. This is more clear from Fig. 2 were light induced a rapid increase in positive current. The large cells from the upper surface showed very little pump activity.





Conclusion

Light induced pH polarity in some aquatic angiosperms involves tight regulation of bulk flow of ions over a short time range, like in e.g. stomata. But, unlike stomata, transport in Elodea leaves involves two adjacent and electrically tightly coupled cells, which are permanently or temporarily differentiated. Elodea may serve as a model system to study the mechanism of generation of leaf polarity and cell-cell communication.

DIRECT EFFECTS OF Ca^{2+}-CHANNEL BLOCKERS UPON THE ACTIVITY OF K^+-SELECTIVE CHANNELS IN THE PLASMA MEMBRANE

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Ca^{2+}-channel blockers are known to affect a wide range of processes in plant cells. Here we show that organic and inorganic blockers, used at concentrations greater than 1 μM, affect directly the activity of K^+-selective channels in the plasma membrane of *Amaranthus tricolor* protoplasts. These effects are not mediated by the blockade of Ca^{2+} channels.

The blockers tested included 1,4-dihydropyridines (nifedipine, nicardipine), verapamil, bepridil and the inorganic ions Gd^{3+} and La^{3+}. They were applied to whole-cell and detached outside-out patches of plasma membrane at concentrations from 50 nM to 100 μM. The concentration of Ca^{2+} on the cytoplasmic side of the plasma membrane ($[Ca^{2+}]_{cyt}$) was clamped at either 50 nM or 500 nM by use of pipette solutions buffered with Ca-EGTA (10 mM, in some instances 40 mM).

The principal currents to be observed in whole-cells are due to the activity of cation outward rectifier channels (1). Each of the blockers caused an immediate reduction of the time-dependent outward currents at doses as low as 1 μM. Concentrations for 50% reduction of current ranged from 1.5 μM (verapamil), 5 μM (bepridil & La^{3+}) to 70 μM (nifedipine). Each produced a different, but reversible, kinetic block of the outward current which was independent of the level of $[Ca^{2+}]_{cyt}$. In addition, verapamil activated a sustained inward cation current at negative membrane potentials. The same effects were found with individual channels in detached outside-out patches, and again were independent of the concentration of free Ca^{2+} used in the pipette solutions. Conductance and selectivity of the cation outward rectifiers are unchanged by the drugs.

The results outlined above show 1) that the cation outward rectifiers in *Amaranthus tricolor* are not typical Ca^{2+}-dependent channels, and 2) that all of the Ca^{2+}-channel blockers tested above 1 μM had direct effects upon the activity of K^+-selective channels in the plasma membrane.

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MODULATION OF MECHANOSENSORY Ca^{2+} CHANNELS BY TEMPERATURE

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Gating of associations of mechanosensitive Ca^{2+} cochannels in the plasmalemma of onion epidermal cells (1) has a strong and unusual temperature dependence (2). Tension-dependent activity rises steeply as temperature is lowered from 25°C to about 6°C, but drops to a low level at about 5°C (Fig. A,B). Under the conditions tested (2 mM Mg^{2+} at the cytosolic face of outside-out membrane patches, 1 mM Ca^{2+} at the mural face, and 100 mM KCl at both faces), promotion results both from more bursting at all linkage levels and from longer duration of bursts of cochannels linked as "multiplets", primarily quadruplets and quintuplets. Conductance decreases linearly, and only modestly, with temperature (Fig. C,D). We propose that these and closely related mechanosensitive channels participate in a variety of responses to temperature, including thermonasty, thermotropism, hydrotropism, and both cold damage and cold acclimation.

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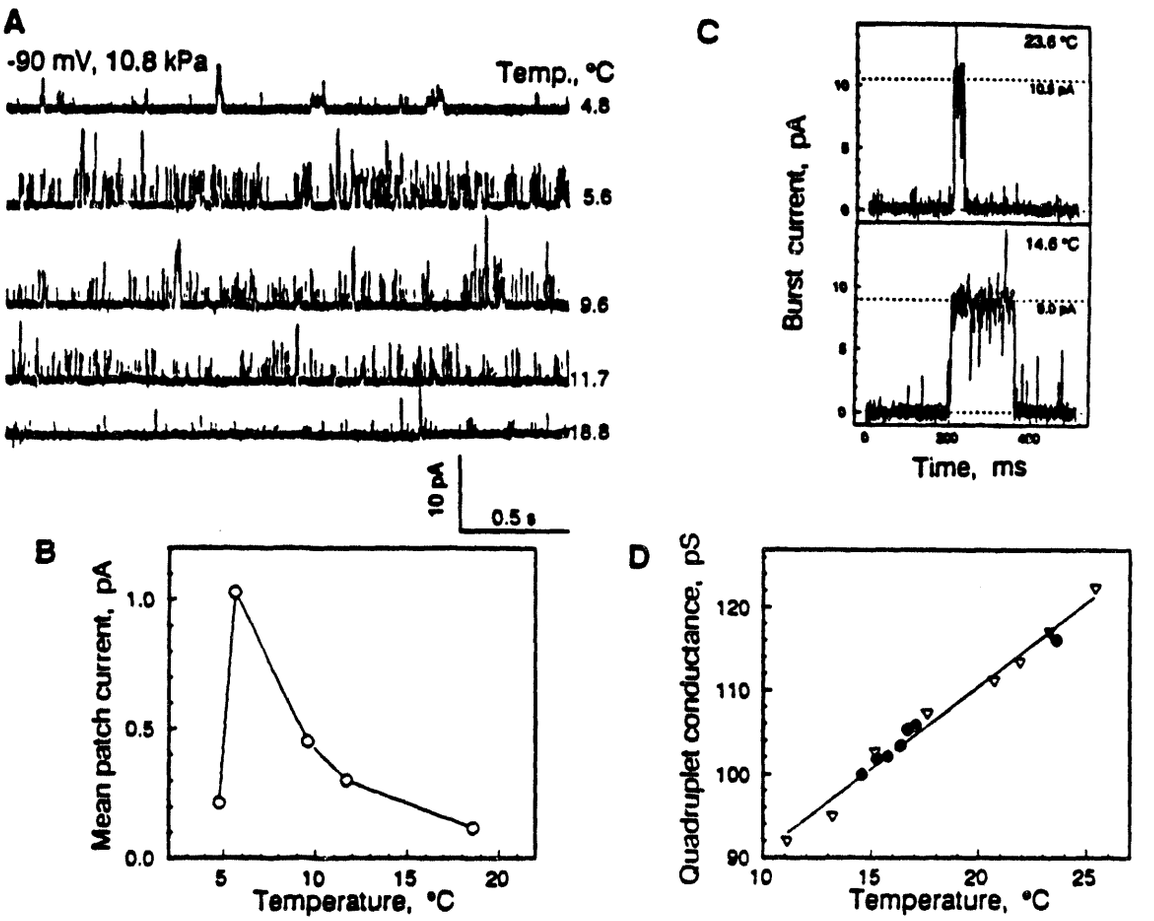

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AUXIN AND pH MODULATE EPIDERMAL MECHANOSENSORY Ca^{2+}-SELECTIVE CHANNELS IN ONION; AUXIN ADDITIONALLY ACTIVATES K^+ CHANNELS

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AUXIN EFFECTS. In isolated patches of cell membrane from the epidermis of onion *(Allium cepa)* bulb scales, the natural auxin indoleacetic acid (IAA) influences two cation channels.

The first is an abundant mechanosensitive Ca^{2+}-selective cation channel which may be viewed as a complex made up of linked or linkable cochannels (1). Sensitivity to membrane tension is modulated by V_m. The channel selects Ca^{2+} over K^+ when both ions are presented together so that, given the activities of the two ions believed to prevail in the wall and given the normal strongly negative V_m of the cells in vivo, this channel will serve importantly in gating Ca^{2+} entry.

Due to the inferred Ca^{2+} recognition process, the channel passes relatively small and spikey currents when presented predominantly with Ca^{2+}. However, the channel is better permeated by K^+ when this ion is presented separately: current bursts are larger and of longer duration. Taking advantage of the more readily assessed K^+ currents, we have shown that IAA modulates the sensitivity of the channels to mechanical stimulation. Application of 1 μM IAA yields up to ten-fold promotion of mean current through the collective channels of the patch.

The second channel influenced by IAA is a K^+ channel. Its gating is not influenced by V_m or membrane stretch, and the channel is inactive in the presence of high Ca^{2+} at the cytosolic but not mural face of the membrane patch.

Application of 0.5-1 μM IAA increases K^+ channel activity; no observations have been made above this concentration. IAA also modestly increases apparent single-channel conductance.

The influences of IAA on the two channels appear to constitute two means by which the hormone exerts physiological actions; in particular, the influences appear to parallel those reported by Loros and Taiz (2) on plasmolysis and deplasmolysis of the epidermal tissue.

pH EFFECTS. Extracellular pH modulates stretch sensitivity of the Ca^{2+} selective channel but does not sensibly influence K^+ channel activity. Within a physiologically realistic range, sensitivity of the mechanosensory channel decreases with increasing acidity. Insofar as IAA can promote acidification of wall space in growing cells, it may be speculated that pH and IAA modulations of the mechanosensory channel might participate in a feedback cycle regulating mechanosensory Ca^{2+} signalling.

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CHANGES OF K^+ UPTAKE THROUGH PLASMA MEMBRANE INDUCED BY SPERMIDINE IN ROOTS OF MAIZE PLANTS

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3d old maize seedlings were grown for 18h in CaSO4 solution in the presence or absence of Spermidine (Spd) up to 1 mM. Plants treated with Spd have a biochemical and hystological behaviour similar to stressed plants (1). In fact, a higher phenolic presence, increased oxidase activities and reduced growth of roots were found in Spd-treated seedlings compared to untreated ones.

A comprehensive analysis of K^+ uptake in roots of intact plants, in freshly cut (To) and washed root segments (washing is known to restore K^+ uptake level to that of intact roots before cutting) (Table 1), both from control and Spd-treated tissues, suggests that the increased K^+ uptake shown in Spd-treated tissues at T_0 is due to an increase of K^+ uptake in roots of intact plants. It is still unknown why K^+ uptake of washed segments from treated roots does not resemble that of corresponding segments in intact plants, as occurs in control seedlings.

Data reported in this work are concerned with some experiments carried out to know the time course of Spd effect on K^+ uptake in intact plants at different Spd concentrations. In addition, we report that the activated K^+ uptake activity caused by Spd is balanced by a parallel increase of K^+ efflux from roots.

Table 1

Treatments	($^{86}Rb^+$) K^+ uptake(μmol g-1FW h-1)		
	Freshly cut segments	Washed segments	Intact roots
Control	0 67	1.43	1.37
Spd 1 mM	2 17	2.03	3.57

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EXPRESSION OF AN OUTWARD RECTIFYING POTASSIUM CHANNEL FROM MAIZE mRNA AND cRNA IN *XENOPUS* OOCYTES

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Two major types of potassium channels have been described in plant cells. One type is responsible for potassium influx upon membrane hyperpolarization and the other type is responsible for potassium efflux upon membrane depolarization (1, 2). We have applied the heterologous expression system of *Xenopus* oocytes (3) with an electrophysiological assay for analysis of plant potassium channel gene expression. Injection of poly $(A)^+$ mRNA from maize roots and shoots and injection of cRNA from a maize expression library induced the formation of an outward rectifying channel in *Xenopus* oocytes (figure and reference 4). This outward rectifying channel was selective for K^+, voltage- and time-dependent, activated at potentials more positive than -40mV and did not inactivate during 900 ms stimulation. These properties are similar to those of outwardly rectifying K^+ currents recorded from higher plant cells using the whole-cell configuration of the patch-clamp technique (1, 2).

Our studies show that *Xenopus* oocytes can be used as an expression system for the functional identification and isolation of plant potassium channel genes as well as for future studies on the relation between the structure and function of plant ion channels.

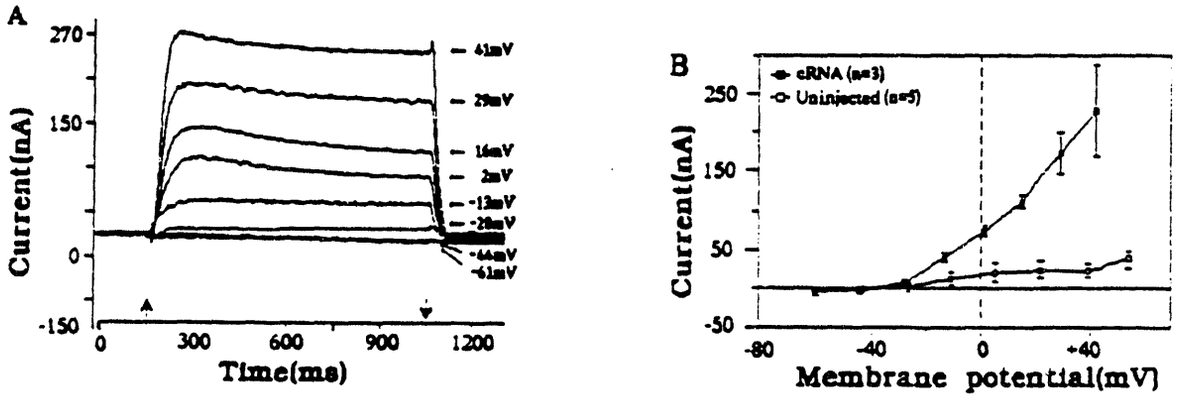

Figure.: (A) Expression of outward currents in *Xenopus* oocytes injected with maize shoot cRNA, upon depolarization from a holding potential of -60mV. (B) Mean of outward currents of cRNA injected oocytes (filled squares) or uninjected oocytes (open squares) as a function of membrane potential.

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ISOLATION AND PATCH-CLAMPING OF ROOT H[illegible]R PROTOPLASTS SHOWS THE PRESENCE OF INWARD-RECTIFYING K^+ CHANNEL CURRENTS

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Despite the central role of K^+ as a macronutrient for higher plant growth, the molecular mechanisms of transmembrane uptake of K^+ remain largely unknown. Studies on *Vicia faba* guard-cell protoplasts have suggested that inward-rectifying K^+ channels can provide a mechanism for low-affinity K^+ uptake (1, 2). To further test the general validity of this hypothesis, we have developed a method to specifically isolate protoplasts from root hairs of wheat (*Triticum aestivum*), as root hairs represent a central uptake site for macronutrients under naturally occurring conditions (3, 4). By modifying a recently published method (5) we obtained root hair protoplasts amenable to patch-clamp studies. Preliminary results from protoplasts in the whole-cell configuration show voltage-dependent activation of an inward current at hyperpolarizing potentials with activation half-times of 50 to 100 ms (Figure). Tail current analyses show that this current is carried by K^+. Further experiments are being pursued to characterize the inward current and the role it plays in low-affinity K^+ uptake by root hair cells.

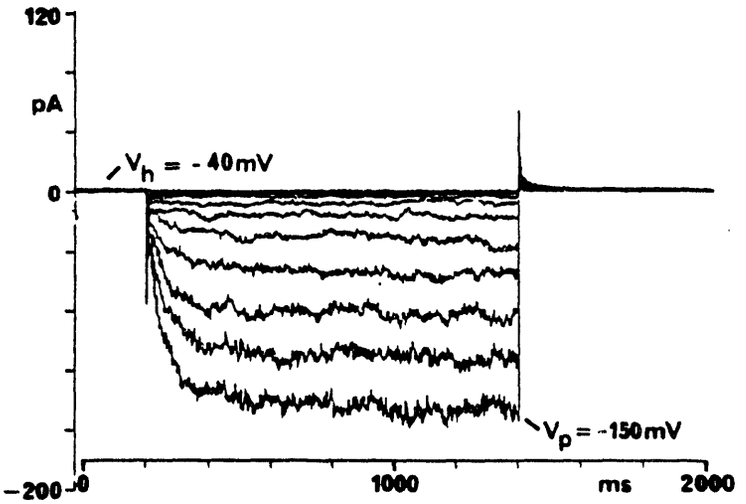

FIGURE: Whole-cell inward currents in the plasma membrane of a root hair protoplast. The voltage was held at -40 mV and stepped to more negative potentials in 10 mV increments. The ionic contents of the bath solution were (in mM): 10 KCl, 2 $MgCl_2$, 1 $CaCl_2$, adjusted to pH 4.5 with 1 mM HCl. The pipette solution contained (in mM): 100 KGlu, 4 MgATP, 2 $MgCl_2$, 2 EGTA, 10 Hepes, and 2.6 KOH at pH 7.2.

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ION CHANNELS IN THE PLASMA MEMBRANE OF *ARABIDOPSIS*: ROLE IN LIGHT-INDUCED VOLTAGE CHANGES

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A characterization of the plasma-membrane ion channels in *Arabidopsis* mesophyll cells was viewed as a prerequisite for studies of their physiological functions as well as useful background for anticipated molecular-genetic studies. By recording single-channel currents from inside-out patches, the selectivities and open-channel current-voltage (Io-Vm) relationships of four distinct channel-types were determined. A summary of their Io-Vm relationships and recordings of each channel-type are shown in Fig. 1. One of the channel-types transports K^+ and Na^+ but not Cl^- (A in Fig. 1), two are selective for K^+ over Na^+ and Cl^- (B and C), and a fourth is a Cl^- channel (selectivity presently unknown) that is stretch-activated (D). Details concerning all but the Cl^- channel are in press (*1*).

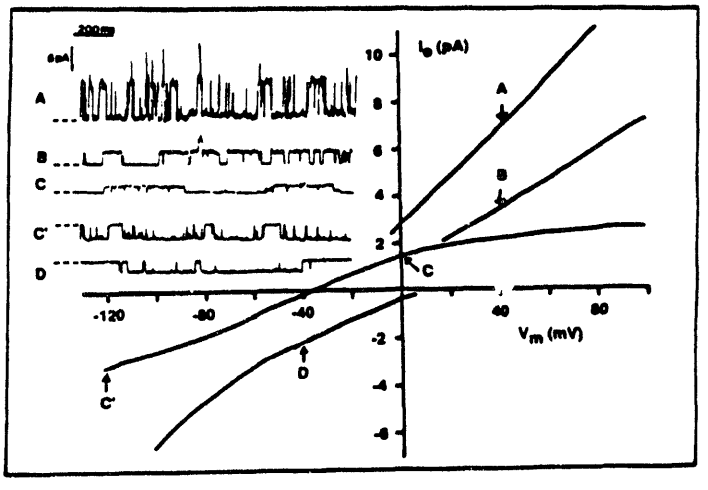

Fig. 1 Open channel current (Io) plotted versus membrane voltage (Vm) for four channel-types in the plasma membrane of *Arabidopsis*. Single-channel records in upper left are labeled with the same letter as the Io-Vm curve for that channel-type. The position of the letter on the curve corresponds to the voltage at which the displayed channel traces were recorded. Data are from inside-out patches with 220 mM KCl in the bath and 50 mM KCl in the pipette. E_K = -38 mV. (- - -) to the left of the channel traces denotes closed-channel current.

Membrane voltages of -150 to -200 mV were measured in intact leaves. White light (25 W · m^{-2}) induces a transient 30-70 mV depolarization. The same light treatment activates at least two channel-types (B and C) in cell-attached, but not excised, patches. Activation of these channels could depolarize the membrane as far as the equilibrium voltage for K^+. DCMU, an inhibitor of photosynthetic electron transport, inhibits the light-induced changes in Vm. We will present evidence that a photosynthetically-produced metabolite activates the K^+ channels which participate in the light-induced voltage transient. Thus, the activity of ion channels in the plasma membrane of *Arabidopsis* mesophyll cells can be influenced by cellular metabolism.

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ION CHANNELS IN THE PLASMA MEMBRANE AND TONOPLAST OF *Arabidopsis thaliana* CULTURED CELLS

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Ion channels were studied, by means of the patch clamp technique in the "whole-cell" configuration, in protoplasts and vacuoles isolated from cultured cells of *A. thaliana*, material of choice due to the ease in obtaining mutants that, if altered in transport mechanisms, could be a useful tool for studying transport processes and their regulation.

In the protoplasts two types of time- and voltage-dependent currents are observed: hyperpolarization-activated inward rectifying currents (1) and depolarization-activated outward rectifying currents. The inward currents are carried by K^+ and other monovalent cations (permeability sequence: $K^+ = NH_4^+ > Na^+ > Li^+$) and inhibited by 10 mM TEA^+, a K^+ channel blocker. The outward currents are carried by K^+ or Cl^- depending on the concentration ratio of the two ions in the external medium; in the cation conducting state the permeability sequence is $K^+ = NH_4^+ > Na^+ = Li^+ > Cs^+$, in the anion conducting state Cl^- and nitrate have the same permeability.

In the vacuoles time-dependent hyperpolarization-activated inward rectifying currents, carried by K^+ and other monovalent cations (K^+ = ammonium = Na^+ = $Li^+ > Cs^+ > Cl^-$ = nitrate) are observed.

Besides, time-dependent currents, activated by depolarizations in protoplasts and by hyperpolarizations in vacuoles and similar from this point of view to those described above, are carried by polyamines (putrescine, spermine, spermidine) (2).

Finally, work is in progress on the effect of changes in temperature on the activity of the plasma membrane and tonoplast channels.

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DIRECT EFFECTS OF Ca^{2+}-CHANNEL BLOCKERS UPON THE ACTIVITY OF K^+-SELECTIVE CHANNELS IN THE PLASMA MEMBRANE

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Ca^{2+}-channel blockers are known to affect a wide range of processes in plant cells. Here we show that organic and inorganic blockers, used at concentrations greater than 1 μM,affect directly the activity of K^+-selective channels in the plasma membrane of *Amaranthus tricolor* protoplasts. These effects are not mediated by the blockade of Ca^{2+} channels.

The blockers tested included 1,4-dihydropyridines (nifedipine, nicardipine), verapamil, bepridil and the inorganic ions Gd^{3+} and La^{3+}. They were applied to whole-cell and detached outside-out patches of plasma membrane at concentrations from 50 nM to 100 μM. The concentration of Ca^{2+} on the cytoplasmic side of the plasma membrane ($[Ca^{2+}]_{cyt}$) was clamped at either 50 nM or 500 nM by use of pipette solutions buffered with Ca-EGTA (10 mM, in some instances 40 mM).

The principal currents to be observed in whole-cells are due to the activity of cation outward rectifier channels (1). Each of the blockers caused an immediate reduction of the time-dependent outward currents at doses as low as 1 μM. Concentrations for 50% reduction of current ranged from 1.5 μM (verapamil), 5 μM(bepridil & La^{3+}) to 70 μM(nifedipine). Each produced a different, but reversible, kinetic block of the outward current which was independent of the level of $[Ca^{2+}]_{cyt}$. In addition, verapamil activated a sustained inward cation current at negative membrane potentials. The same effects were found with individual channels in detached outside-out patches, and again were independent of the concentration of free Ca^{2+} used in the pipette solutions. Conductance and selectivity of the cation outward rectifiers are unchanged by the drugs.

The results outlined above show 1) that the cation outward rectifiers in *Amaranthus tricolor* are not typical Ca^{2+}-dependent channels, and 2) that all of the Ca^{2+}-channel blockers tested above 1 μMhad direct effects upon the activity of K^+-selective channels in the plasma membrane.

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CLONING OF A POTASSIUM CHANNEL GENE IN *ARABIDOPSIS THALIANA* WITH A DEGENERATE OLIGONUCLEOTIDE PROBE

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Voltage-dependent potassium channels from various eukaryotic cells have similar electrophysiological and pharmacological characteristics and share common structural motifs. In the family of K^+ channels that contain six potential membrane spanning segments, two regions are highly conserved: the voltage-sensor, S4, and the pore region which links the fifth and sixth transmembrane segments (S5 and S6). S4 is typified by a series of positively charged amino acids that are situated at every third position along the transmembrane sequence. The channel pore is characterized by a central peptide, TMTTVGYGD, which is present in Shaker-like K^+ channels and is partially conserved in the more distantly related Slo and Eag proteins (1).

To search for homologous genes in *Arabidopsis thaliana* we designed a degenerate oligonucleotide corresponding to the TMTTVGYGD peptide sequence. A genomic library was screened at low stringency and positive plaques were purified by subsequent rounds of hybridization. The isolates were separated into two groups based upon the stringency at which the filters were washed. A 2.7 Kb EcoRI fragment from the high-stringency clone PCAT14 (Potassium Channel *Arabidopsis thaliana*) was subcloned into Bluescript and partially sequenced.

PCAT14 contains a continuous open reading frame that encodes the sequence

SYYLIADRYPHQGKTWTDAIPNFTETSLSIRYIAAIYWSITTMTTVGYGDLHASNTIEMVFITV

S5 S6

This segment includes the conserved pore motif TMTTVGYGD, bounded by hydrophobic regions that are likely to constitute the fifth and sixth membrane spanning segments. Work is in progress to isolate a full-length cDNA clone corresponding to this genomic sequence.

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Outward and inward K^+ currents in protoplasts from cultured tobacco cells

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Ion channels in the plasma membrane of protoplasts isolated from cultured cells of *Nicotiana tabacum* were studied in the whole-cell configuration of the patch-clamp technique.

Besides an important role in osmoregulation and salt uptake, ion channels likely also are essential components of signal transduction pathways. E.g., the plant growth hormone auxin induces fluxes of different ions (e.g., H^+, K^+, Cl^- and Ca^{2+}). Therefore, the study of ion channels will contribtue to the understanding of signal transduction pathways in plant cells. To investigate the role of ion channels in auxin signal tranduction we performed experiments on protoplasts from suspensions of a *Nicotiana tabacum* L. cv Bright Yellow cell line grown in LS-medium containing 8.8×10^{-7} M 2,4D at 25°C in the dark. Protoplasts were prepared by overnight incubation in a solution (about 10 g wet weight cells/150 ml) containing 0.24 M $CaCl_2$, 0.05 g/150 ml cellulase and 0.005 g/150 ml macerozyme. The extracellular solution consisted of (mM): 10 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 1 KOH, 10 Mes (pH 5.5) with osmolarity set with mannitol at 1010 or 570 mOsm. The pipette (= intracellular) solution consisted of (mM): 100 K-gluconate, 2 $MgCl_2$, 0.45 $CaCl_2$, 1.1 EGTA, 4 MgATP, 3 KOH, 10 HEPES (pH 7.0), osmolarity set at 708 mOsm with mannitol.

At least two different ion condutances could be detected in whole-cell voltage-clamp experiments. An outward rectifying conductance and an inward rectifying conductance. From ion substitution experiments we found that both conductances were mainly selective for K^+ ions. Activation of the conductances was voltage and time dependent. The outward as well as the inward current did not show inactivation. The outward current activated for membrane potentials more positive than 0 mV with a sigmoidal time course upon step-wise changes in the holding potential. The inward current activated at potentials more negative than about -70 mV and showed an exponential like activation time course. The outward current was found in about 100% of the protoplasts, while the inward current was only present in about 30% of the protoplasts tested (36 protoplasts tested). The outward current could be inhibited (partly) by potassium ion channel inhibitors such as TEA and quinidine. The inward current was inhibited by 1 mM $BaCl_2$.

In future experiments the effects of auxin (applied both extra-and intracellularly) on these conductances will be tested.

NON-ENZYMATIC PLASMA MEMBRANE ACCESS OF MEDICAGO ROOT HAIRS THROUGH LASER MICROSURGERY.

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The study of the membrane properties of individual plant cells in situ requires the complete removal of the cell wall. The standard approach is the use of enzyme cocktails for the preparation of protoplasts (1). However, there is increasing evidence indicating that enzymes have deleterious effects on membrane properties (2-6) and physiology of protoplasts (5,7). Here, we used the UV laser microbeam technique (8) to perforate the cell wall of root hairs from Medicago sativa under plasmolyzing conditions. This approach gives direct access to the plasma membrane. Since plasmolysis creates an osmotic stress on plant cells by decreasing membrane fluidity (2,9) and modifying the membrane potential (10), different conditions for obtaining a gentle plasmolysis without disturbing the physiology of the root hairs have been tested. The results of the experiments indicated that when a slow and progressive plasmolysis is achieved, cytoplasmic streaming is maintained during the whole process, root hairs continue to develop and the plants to grow after being deplasmolyzed and cultured on an appropriate medium.The opening of the cell wall with the laser microbeam induces an immediate movement of the protoplasma and the partial or complete extrusion of the cell content. The movement of protoplasma depends on the calcium concentration of the extracellular solution. In these conditions, the laser microperforation technique gives direct access to a small area of a few μm^2 of plasma membrane in situ. The formation of protoplasts or subprotoplasts can also be monitored. Metabolic activity and membrane integrity of the protoplasts were tested by fluorescein diacetate staining. Microscopic examination revealed organelle movement and the presence of a nucleus. The plasma membrane was free of cell wall as shown by Tinopal staining. This laser technique should be suitable to prepare native plant material for patch-clamp studies in conditions where (i) the interactions between the cell wall and the plasma membrane are not totally ruptured, and (ii) the cellular polarity and the cell to cell interactions through plasmodesmata are maintained .

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THE HIGHER ORDER HINKLEY-DETECTOR FOR THE ANALYSIS OF PATCH-CLAMP DATA

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Improving temporal resolution in patch-clamp studies results in a strong increase of noise. This requires more powerful methods for the detection of transitions between different levels of current. A better performance as compared with threshold detectors was achieved by the Higher Order Hinkley-Detector (H.O.H.D.), which utilizes nonlinear filter algorithms. The analysis of simulated noisy data reveals a relationship between signal-to-noise-ratio, temporal resolution and the occurrence of false alarms. Under the condition that no false alarms occur, the H.O.H.D. provides a shorter temporal resolution. It leads to a significantly more exact estimation of the durations of the detected events (1). Another benefit of the H.O.H.D. is the sharp cutoff between missed and detected events which makes the theoretical treatment of missed events easier.

The H.O.H.D. was applied to patch-clamp studies of the caesium block of the potassium channel from tonoplast vesicles of *Chara corallina* at a sampling rate of 100 kHz with the anti-aliasing filter set to $f_{3dB} = 25$ kHz. The new detector enabled the detection of very short opening and closing phenomena with time constants of 20 to 100 μs (2).

A difficult and time-consuming task during the analysis of patch-clamp data is the determination of the closed- and open-levels of pipette current. Errors in this stage of the analysis distort the results of the H.O.H.D. or other detection algorithms which are based on the prior knowledge of the levels.

In order to obtain an on-line correction of the levels, the distributions of the amplitudes assigned to a certain level by the H.O.H.D. are calculated. A forgetting factor is introduced in order to track drifting levels. Statistical testing of mean and variance of these distributions opens the possibility of readjusting the levels if they turn out to be not quite correct.

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PATCH-CLAMP STUDIES ON *ELODEA* LEAF PROTOPLASTS. I. EFFECT OF HIGH PH.

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The plasmalemma of the polar leaves of *Elodea* (1) exhibits a pH-dependent proton permeability (P_{H^+}), at high pH, P_{H^+} increases (2,3). Using the patch-clamp we tried to detect possible pH-induced H^+-currents across the plasma membrane of the *Elodea* protoplast.

Solutions (in mM)

Pipette: 50 NaCl, 0.33 $CaCl_2$, 2.04 $MgCl_2$, 1.37 EGTA ($pCa^{2+}=7$) and 10 Hepes/BTP pH 7.5.

Low-pH bath : 50 NaCl, 2 $CaCl_2$, 0.4 $MgCl_2$ and 10 Hepes/BTP pH 7.5.

High-pH bath: 45 NaCl, 2 $CaCl_2$, 3.1 $MgCl_2$ and 10 CAPS adjusted with 5 NaOH to pH 10.5.

All solutions were supplemented with 175 mM Mannitol to a final osmolarity of 275 mM.

Results

Fig. 1 shows the IV-curves obtained at low pH. Currents were measured 4 s after the start of the pulse, departing from a holding potential (V_H) of either 0 (a) or -175 mV (b). Repeating the experiment at high pH resulted in the IV-curves of Fig. 2. Now, changing V_H from 0 mV (a) to -175 mV (b) resulted in a parallel shift of the IV-curve in upward direction; the reversal potential changed from -30 to-90 mV.

Fig. 1.

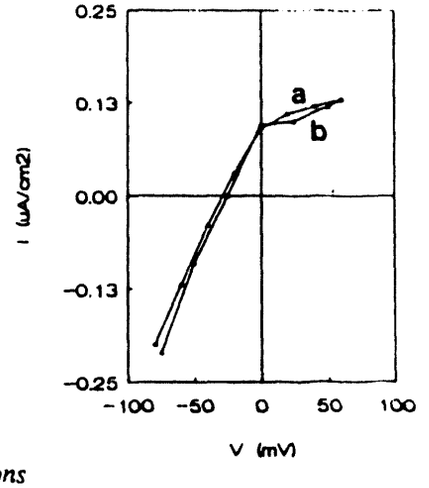
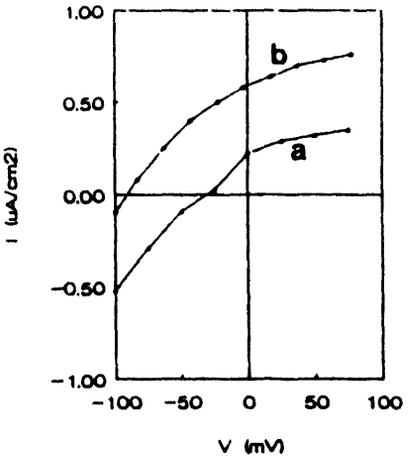


Fig. 2.



Conclusions

The results were interpreted in terms of experimental evidence for the existence of H^+ conducting channels in the *Elodea* plasmalemma. At high external pH, the activation was increased at negative potentials, while the deactivation at more positive potentials was relatively slow and resulted in the observed shift of the IV-curve after changing V_H. At symmetric pH (7.5), changing V_H did not change the IV-curve.

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TRANSPORT PROCESSES IN THE PLASMALEMMA OF *PLANTAGO* ROOT PROTOPLASTS: A PATCH CLAMP STUDY

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Plantago media and *P.maritima* were used as a model for the study of transport mechanisms present in the plasmalemma of root cells. The two plants differ in their strategy towards sodium stress. Cytoplasmic sodium is transported either to the vacuole or to extracellular spaces. The patch clamp technique allows for measurements of electrogenic processes mediated by ion channels and proton pumps. The selectivity, conductivity and kinetics of ion channels have mainly been investigated in the plasmalemma of root protoplasts of *Plantago media*. Hyperpolarisation (hypac) and depolarisation activated (depac) ion channels were measured in cell attached patch (CAP) and outside out patch (OOP) configurations. A 20-30 pS depac potassium conductance was dominantly present in most patches. This ion channel activates upon depolarising potential steps. Hyperpolarisating potentials deactivate the channel and generate an inward current which displays a typical flickering behaviour thus causing a significant decrease in mean open and mean closed time. The values of the P_K/P_{Cl} and P_K/P_{Na} permeability ratios are respectively 60 and 20 or higher. This indicates a high specificity of the channel for potassium. It is therefore unlikely that sodium ions are transported via this channel.

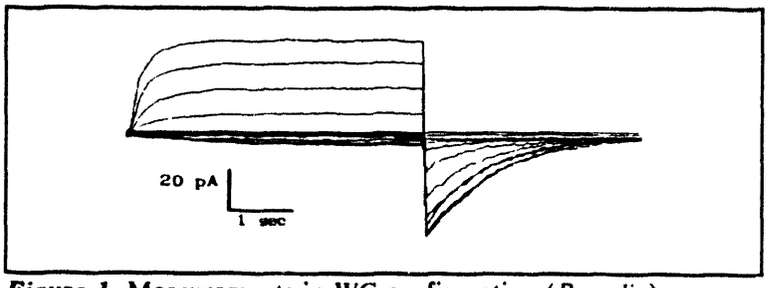

Figure 1 Measurements in WC-configuration (*P.media*)

Whole cell (WC) measurements of *P.media* (Fig.1) were in agreement with the single channel measurements as they also were dominated by a large inward outward potassium conductance which only deactivates at potentials more negative then the potassium Nernst potential (E_k). This conductance was present in both species, however *P.maritima* displayed another large conductance which is not fully characterised yet. The gating characteristics of the potassium conductance is potassium dependent. The WC conductance is half maximal at E_k. This is fully in agreement with (in vivo) microelectrode measurements, in which the membrane potential (V_m) is highly E_k dependent.

A strong transport process can be evoked by addition of fusiccocin (FC). In vivo experiments exhibit a typical response for FC: A hyperpolarisation of the V_m (1), a large proton efflux (2) and a potassium influx (3). The hyperpolarisation of V_m indicates that a change has occurred in the typical potassium dependence of V_m. This would require either a significant increase in pump activity or a reduction of the potassium conductance. Detailed information can be obtained with patch clamp techniques. Preliminary measurements indicate that FC induces a decrease in potassium conductance at low cytoplasmic ATP levels.

EXPRESSION OF AN *ARABIDOPSIS* INWARD-RECTIFYING POTASSIUM SELECTIVE CHANNEL IN *XENOPUS* OOCYTES

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This characterization of the *KAT1* cDNA in *Xenopus laevis* oocytes was initiated to determine the specific function of the protein encoded by *KAT1*. Two *Arabidopsis thaliana* cDNA clones *KAT1* and *AKT1* (1) were recently isolated based on their ability to functionally substitute a K^+ transporter in *Saccharomyces cerevisiae* (2). Large inward currents were activated by hyperpolarization of the membrane potential to values more negative than -102 ± 13 mV in oocytes injected with messenger RNA synthesized from *KAT1* cDNA (Fig. 1A). Currents were not activated by depolarizing pulses in *KAT1*-injected oocytes (Fig. 1B). The *KAT1*-mediated currents were reduced by approximately 70% when 115 mM KCl in the bath solution was replaced with 115 mM RbCl or NH_4Cl. The hyperpolarization-induced currents were reduced by approximately 90% when *KAT1*-injected oocytes were bathed in NaCl, LiCl or CsCl. Tetraethylammonium (TEA^+) and barium reversibly blocked the inward current. The single channel conductance of the KAT1 inward-rectifying K^+ channel was 34 pS with 115 mM K^+ on the extracellular membrane side. The voltage- and time-dependent activation, ionic selectivity, blockage by TEA^+ and Ba^{2+} and single channel conductance of the *KAT1*-mediated current establish that this cDNA encodes an inward-rectifying K^+ channel similar to those found in higher plants (3). KAT1 is the first inward-rectifying K^+ channel to be identified in plants and animals.

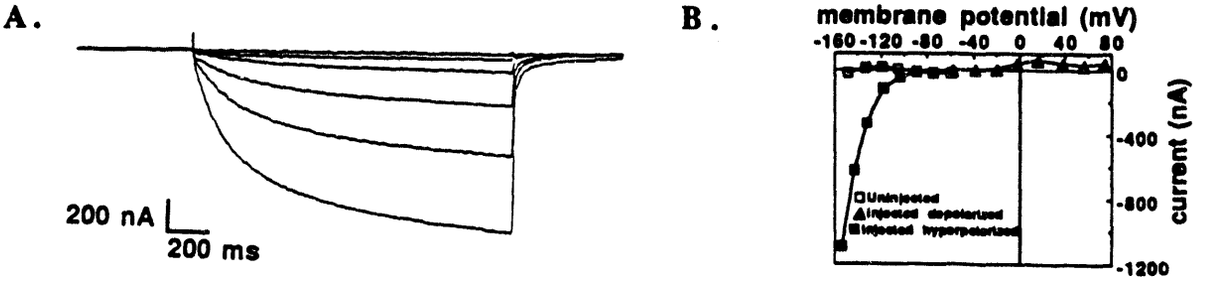

Figure 1 (A) Currents were elicited in response to hyperpolarizing pulses from a holding potential of -60 mV in oocytes injected with *KAT1* mRNA with 115 mM K^+ in the bathing medium. (B) Currents at the end of 1.5 second pulses are plotted as a function of applied voltage pulse potentials.

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ASYMMETRIC DISTRIBUTION OF IONS BETWEEN EPIDERMAL AND MESOPHYLL CELLS IN BARLEY LEAVES

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X-ray microanalysis was used to determine the distributions of several nutrient elements between vacuoles of epidermal and mesophyll cells in barley leaves and the distributions were then related to the concentrations of these nutrients in the shoots. Under the growth conditions used, phosphorus was found only in mesophyll vacuoles, never in the epidermis. In contrast, chloride and calcium were located almost exclusively in the epidermis while potassium and sodium were more evenly distributed between the two cell types. The compartmentation of calcium and chloride in the epidermis was maintained over a wide range of tissue concentrations of these ions. In particular, chloride was excluded from the mesophyll of salt-grown barley until the tissue chloride concentration reached about 170 mM and then it appeared in the vacuoles of these cells but only at low concentrations. In contrast, sodium was not excluded from the mesophyll of salt-grown or potassium-deficient barley and there was evidence that this ion was preferentially accumulated in the mesophyll. The results confirm published results indicating substantial differences in the ionic composition of different leaf cells (1, 2) and further indicate that the distributions are sensitive to nutrient supply and salinity.

The accumulation of only certain nutrients in the vacuole of mesophyll cells indicates that these cells exert control over the nutrients they absorb from the transpiration stream. Presumably mechanisms exist for excluding chloride and calcium from the mesophyll and thus they are absorbed by epidermal cells. This would suggest that mesophyll cells have a very efficient calcium extrusion system since the negative electrical potential across the plasma membrane provides a very strong driving force for uptake of this ion. Similarly they may lack an active chloride transport system which would be necessary for the accumulation to chloride above the low concentrations which passive transport against this membrane potential would permit. In contrast, epidermal cells presumably lack the active calcium extrusion system but have an efficient calcium transport system at the tonoplast in order to effectively compartment this ion in the vacuole. In addition they presumably also possess an active chloride uptake system.

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Abstract

Ion channels he inner chloroplast envelope membrane

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During illumination of chloroplast the stromal pH rises by 0.5 to 0.1 units. This stromal alkalization acts as a regulatory mechanism to facilitate optimal light adaptation of photosynthesis. Due to the pH dependence of the carbon cycle enzymes the regulation of the pH gradient across the chloroplast envelope is important for optimal carbon fixation [1,2]. The mechanism by which the proton gradient across the chloroplast envelope in the light is maintained remains elusive. It has been postulated that a electrogenic ATPase and a monovalent cation channel may be involved in the regulation of K^+ and H^+ fluxes across the chloroplast envelope [2,3]. In reconstituted chloroplast envelope membranes an ion channel with preferential cation permeability and a conductance of 200 pS has been reported previously [4].

Our goal was to identify components of the system in the inner chloroplast envelope membranes which performs regulation of ion fluxes across the inner envelope membrane. For this we conducted electrophysiological studies with the inner chloroplast envelope membrane reconstituted into liposomes in order to characterize possible ion channels in the envelope membrane. Patch clamp measurements on giant liposomes containing reconstituted spinach envelopes and measurements in planar bilayers containing the same inner envelope membrane from spinach chloroplasts were performed. Both techniques revealed the same results, they show that the inner chloroplast envelope membrane contains at least two different types of ion channels, one class of channels mainly permeable to anions and another one mainly permeable to cations. The cation channels revealed a high conductance state of 120-180 pS and a low conductance state of ≅ 10-30 pS in 30 mM - 100 mM KCl at pH 7, while the anion channel revealed open channel conductance of ≅ 60-80 pS. Both, anion and cation channels showed a complex gating behavior, i.e. subconductance levels and flickering. The cation channels were completely blocked by the local anesthetic lidocaine (2Diethylamino-N-[2,6-dimethylphenyl]acetamide) and their gating was strongly effected by Mg^{2+} and Ca^{2+}.

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THE EFFECT OF SALT-INDUCED MEMBRANE VOLTAGES ON THE ENERGETICS AND KINETICS OF THE ULTRA-FAST ELECTRON TRANSFER REACTIONS OF PHOTOSYSTEM II

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Across the thylakoid membrane of spinach chloroplasts a diffusion potential of defined magnitude can be created by rapid changes in the KCl concentration (salt-jumps). The analysis of salt-induced electrochromic absorbance changes indicates that the magnitude of this diffusion potential is given by the Goldman equation. The resulting electric field leads to an increase in the yield of photosystem II (PS II) fluorescence for positive membrane voltages - positive in the inner thylakoid space - and a decrease for negative membrane voltages (Dau and Sauer, 1991, BBA 1098, 49-60).

Now we have studied this phenomenon by measurement of picosecond fluorescence decays. Based on the kinetic PS II model of Schatz et al. (1988, Biophys. J. 54, 397-405), the fluorescence decays were interpreted in terms of the rate constants of primary charge separation (formation of reaction center cation radical and pheophytin anion radical), primary charge recombination (recombination of primary biradical to chlorophyll singlet state) and secondary charge separation (reduction of primary quinone acceptor). For increasingly positive thylakoid voltages, the results are indicative of a relatively small but significant decrease in the rate constant of primary charge separation (by about 8 % per +100 mV thylakoid voltage) and a much larger increase (by about 50% per +100 mV) of the rate constant of primary charge recombination. The free energy difference of the primary radical pair was found to change by 17 meV per 100 mV thylakoid voltage. The relation between the rate constant of primary charge separation and the free energy difference appears to be linear within the accessible range of free energy changes (-15 meV to +22 meV); the rate constant of primary charge separation increases with increasingly negative free energy differences by 6% per 10 meV. This free energy dependence is compared with calculations for different semiclassical and quantum mechanical models of electron transfer reactions.

MULTI-BARRELLED ION-SELECTIVE MICROELECTRODES FOR INTRA- AND EXTRACELLULAR MEASUREMENT OF NH_4^+, NO_3^-, K^+, H^+, AND ELECTRICAL POTENTIAL.

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Multi-barrelled ion-selective microelectrodes were fabricated in which two barrels contained liquid ion exchange resins (LIX) for specific ions, and an additional barrel served as a local potential-sensing electrode. In the past, electrical cross-talk between adjacent high impedance ion-selective barrels has made the use of multi-barreled ion-selective microelectrodes problematic. This difficulty was eliminated by filling alternate barrels in a seven-barrel arrangement (Fig. 1). Initially, the tip of a seven-barrelled micropipet was dipped into low viscosity cyano-acrylic glue, while the barrels which would become the three sensing barrels were pressurized. This step served to fill the alternate barrels and interstial spaces with a non-conducting solid, thereby increasing the impedance betwen the ion-selective barrels. Subsequently, each LIX was front-filled into the appropriate barrel; contamination of the previously filled barrel was prevented by maintaining a positive pressure in the finished barrel while applying suction to the barrel being filled. A four-channel, high input impedance (10^{15} Ω) differential electrometer was constructed and connected to a data acquisition system. The system could be directed to sample each channel sequentially and repeatedly over a specified time period.

With this system it was possible to determine simultaneously net fluxes of a variety of ions, by measuring their activity gradients in the unstirred layer of solution immediately external to the root surface. Alternatively, intracellular ion concentrations could be determined and the specific subcellular compartment (cytoplasm or vacuole) identified by the pH value recorded from a H^+-selective barrel. Interfence by K^+ in the NH_4^+ microelectrode could be corrected by determining the $[K^+]$ using a K^+-selective microelectrode and recalculating $[NH_4^+]$ using the potentiometric selectivity coefficient (K_{ij}^{Pot}) for NH_4^+ over K^+.

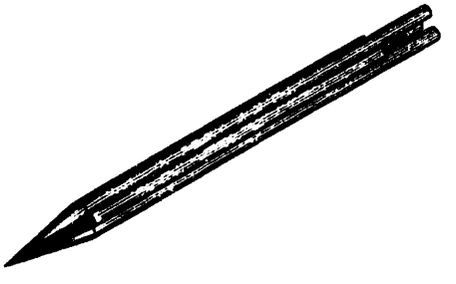

Fig. 1. A schematic of a seven-barrel microelectrode. The tips of the four shorter barrels (*i.e.*, three outer barrels and the central core) are filled with cyano-acrylic glue as depicted by the dark shading. One of the longer barrels contains NH_4^+-selective liquid ion exchanger, another contains NO_3^--selective liquid ion exchanger, and the last contains a salt solution and serves as a local reference electrode.

COMPARATIVE UTILIZATION OF THE VIBRATING PROBE AND ION-SELECTIVE MICROELECTRODE TECHNIQUES TO INVESTIGATE THE ELECTROPHYSIOLOGY OF POLARITY ESTABLISHMENT IN HIGHER PLANTS

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New cell polarity is established in cells adjacent to a wedge-shaped wound in pea roots (1) and in cells at the proximal end of the *Graptopetalum* leaf base following leaf detachment (2). These changes in cell polarity, the timing of which differs between the two systems, precede and initiate wound repair and organogenesis, in pea roots and *Graptopetalum* leaves, respectively. We have used a comparative, quantitative approach using two non-injurious techniques, to investigate both extracellular net ion currents and specific ion fluxes during the initial stages of polarity establishment in these two higher plant systems. In both cases, the vibrating probe technique revealed that very large extracellular ion currents are localized specifically at the sites of future cytomorphological changes in cell polarity (3, 4). Using the ion-selective microelectrode technique, we established that a large Ca^{2+} influx is a component of the net ion currents measured, and that in both plant systems it is a dynamic flux which is inhibitable by antagonists of membrane transport of Ca^{2+} (4, 5). K^+ and H^+ fluxes measured using this same technique, were found to be smaller and relatively steady after leaf detachment in *Graptopetalum* (4). In contrast, dynamic fluxes of K^+ (large efflux) and H^+ (small influx) were recorded after wounding in pea roots (5). Finally, a quantitative comparison of the summed ion fluxes with the net ion currents revealed that Ca^{2+}, H^+ and K^+ transport appears to account for the major electrogenic fluxes during the initial 12 hours after *Graptopetalum* leaf detachment (4), but in wounded pea roots, ion fluxes other than those measured in this study, must occur after wounding (5).

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DETERMINATION OF INRACELLULAR K^+ IN PLANT PROTOPLASTS USING THE FLUORESCENCE PROBE PBFI.

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A tetra(acetoxy-methyl)ester of the potassium-binding benzofuran isophthalat, PBFI-AM, was used to determine the concentration of free K^+ in cells and protoplasts isolated from roots and shoots of young barley plants (*Hordeum vulgare* L. cv. Kara). After loading cells with an acetoxymethyl ester of the dye, the esterase activity in the cytoplasm of the cells is supposed to split the ester to an ion-binding acid form and thereby change the exitation spectra (1). Using ratio imaging microscopy (2,3) the fluorescence intensity was measured at the exitation ratio 340/380 nm. Using protoplasts from shoots, or roots, the fluorescence was increased and stable after 2 hours' incubation with the dye at 4°C in Tris' buffer at pH 5.5. A 5 hours' incubation time was needed at pH 7.0. With the exception of sieve tubes, intact cells of barley took up only small amounts of PBFI-AM. The dye distributed throughout the cytoplasm of the protoplasts but concentrated in the nucleus similarily to smooth muscle cells (4).

At increase of the external K^+ the fluorescence ratio was increased probably due to net transport of K^+. The K^+ transport was faster into root protoplasts than into shoot protoplasts and was still increasing at 20 mM K^+ when the transport mechanism of shoot protoplasts was saturated. The free K^+-concentration of root protoplasts from plants cultivated at 10°C was lower than that of protoplasts from plants grown at 20°C. The opposite result was obtained with sieve tubes from roots, which contained more free K^+ after the plants had been grown at 10°C than at 20°C.

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MEASUREMENT OF INTRACELLULAR FREE MAGNESIUM IN PLANT CELLS USING MAGNESIUM-SELECTIVE MICROELECTRODES.

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It is increasingly apparent that a central role is played by magnesium in the intermediary metabolism of all cells. Several key compounds, including ATP and inorganic pyrophosphate, form complexes with the magnesium ion and these are subsequently utilised as substrates in numerous enzymatic reactions. Moreover some enzymes are also activated by the binding of Mg^{2+} to a specific regulatory site or to the active site itself. Hence cytosolic free magnesium is important in determining the thermodynamic poise and kinetics of the various reactions. This involvement of Mg^{2+} in cell physiology implies that the free ion concentration is homeostatically controlled and thus regulation of the Mg^{2+} transport at the plasma membrane and/or the tonoplast must occur.

Direct measurement of the intracellular concentration of free magnesium is important for an understanding of transport regulation and enzyme cofactor activity. The application of improved ionophores in the manufacture of magnesium-selective microelectrodes presenting a potentially accurate method for such measurements. Neutral carrier sensors ETH 5214 and ETH 4030 incorporated into a PVC-based membrane matrix have been utilised in fabricating double-barrelled microelectrodes. the electrodes have tip diameters of approximately 1μm with a resistance of 10MΩ when filled with 100 mol.m^{-3} KCl solution. Potassium is the most important interfering ion at physiological concentrations. The electrodes made with ETH 5214 and ETH 4030 have detection limits of about 0.2 and 0.07 mol.m^{-3} for Mg^{2+} respectively, in the presence of 120 mol.m^{-3} K^{+}.

LIPID-SOLUBLE ORGANIC CATIONS ENTER FUNGAL CELLS PRIMARILY IN ELECTRONEUTRAL FORM

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Background. We reported previously (pp. 79-80 in "Plant MembraneTransport", ed. J. Dainty et al., 1989) that lipid-soluble organic cations such as tetraphenylphosphonium (TPP^+) and tetraphenylarsonium (TPA^+) do not distribute in equilibrium with the plasma-membrane voltage (V_m) in intact fungal cells. They cannot, therefore, be generally used as voltage probes. These ions do distribute passively into liposomes, but only to a limit of -150 mV (vesicle interior negative; i/o conc. ratio of ~300). With intact cells of *Saccharomyces* or *Neurospora*, however, the ratios rarely reach above 20 (-75 mV), despite directly measured V_m's beyond -200 mV in *Neurospora*. Spurious binding of the ions makes accurate kinetic description of their uptake mechanism(s) difficult at low concentrations (< 100 μM), but becomes less significant at high concentrations (>1 mM). We have therefore examined the mechanism of TPP^+ and TPA^+ entry into *Neurospora* using extracellular concentrations of 1-30 mM.

Methods. ^{3}H-TPP^+ and ^{3}H-TPA^+ were counted in dried, solubilized pellets from suspension cultures of wild type (RL21a) *Neurospora*. Net fluxes of Na^+ and K^+ were determined by flame analysis of acid-extracted pellets; and net fluxes of H^+ were measured with a glass electrode in lightly buffered cell suspensions (2 mM dimethylglutaric acid, pH 5.8; 3 mM HEPES, pH 8.2). V_m's were measured with (1 M) KCl-filled penetrating electrodes, and resistances were computed from simultaneous current- and voltage-pulse data on two (or 3) independent electrodes in single hyphal "cells".

Results. Fluxes. Under most conditions (different pH_o and $[Ca^{++}]_o$; rapid mixing), initial influxes of TPA^+ or TPP^+ were directly proportional to extracellular concentration, with maximal rates well above 100 mM/min (mM = mmoles/kg cell water) for 20 mM $[TPP^+]_o$, high pH_o (8.2), and zero added Ca^{++}. Uptake was logarithmic in time, with an apparent rate constant of 0.5-0.6/min, but leaving (after ca. 5 min) a residual steady influx near 10% of the initial rate. Ca. 70% of uptake was balanced by efflux of K^+ or Na^+ from the cells, but there was no induced efflux of H^+ ions, either transiently or in steady-state.

Voltage and resistance. *Neurospora* depolarized in TPP^+ or TPA^+ at all concentrations above 1 mM, but with highly variable time course and extent, *except* for first trials with the highest concentrations (20-30 mM). During rapid admission of 20 mM TPP^+ (flow rate of 20 chamber-vols/min), depolarization was roughly logarithmic in time and coincided with decay of the cytoplasmic ATP concentration. During slower flow, however, multiple components were visible, especially a rapid initial drop of 50-70 mV accompanied by a 30% increase of membrane conductance (G_m). The implied leakage currents were 6-8 $\mu A/cm^2$, corresponding to fluxes per unit volume of ~12 mM/min. Subsequent phases of depolarization were all accompanied by *decreasing* G_m.

Discussion. There is no single simple interpretation of these results, but the small initial depolarization and current, the large effluxes of Na^+ and K^+, and the absence of measurable net H^+ efflux (membrane current provided by the proton pump) all indicate that only a small fraction of TPP^+ or TPA^+ influx can occur electrophoretically. It follows that the larger fraction of influx must occur electroneutrally, either in exchange for cytoplasmic cations (Na^+ or K^+ ?) or by co-influx with unidentified anions.

SIGNAL TRANSDUCTION IN STOMATAL GUARD CELLS: BIOCHEMICAL, OPTICAL AND ELECTROPHYSIOLOGICAL APPROACHES.

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Cytosolic calcium ($[Ca^{2+}]_i$, protons ($[H^+]_i$) and membrane potential (E_m) interact to give integrated control of a number of ion transporters on the plasma membrane and tonoplast of stomatal guard cells during opening and closing movements. Unambiguous interpretation of the sequence and significance of events during responses to external stimuli demands simultaneous measurement of these parameters *in vivo* , and identification of the transduction pathways that link them to key effectors on the plasma membrane and tonoplast *in vitro*.

Fluorescent probes allow measurement of dynamic changes in ion concentrations in guard cells. Dyes whose spectra alter on binding to the ion permit fluorescence ratio analysis (FRA) which automatically compensates for variation in dye concentration, dye leakage and photobleaching. Combination of FRA with confocal scanning laser microscopy (CLSM) provides the optimum for quantitative fluorescence measurements. Our most recent advance has been development of dual-excitation dual-emission confocal ratio-imaging to allow simultaneous measurement of $[Ca^{2+}]_i$ and $[H^+]_i$. Confocal optical sectioning also excludes signal from dye in the electrode used for injection, which can remain in place during fluorescence measurements. Simultaneous electrophysiological measurements are also possible therefore. Iontophoretic microinjection and voltage-clamping ideally requires triple-barrelled electrodes for controlled current injection. We have initiated combined electrophysiological and optical measurements in guard cells of *Commelina* and *Vicia* .

Changes in $[Ca^{2+}]$ may act directly or link into additional signal transduction systems through Ca^{2+}-binding proteins such as calmodulin and protein kinase cascades. Calmodulin has been identified immunologically in guard cells, whilst a several bands are phosphorylated *in vitro* by endogenous kinases. A number of potential candidates for regulation by phosphorylation have been identified by immunological detection on Western blots after SDS-PAGE, including the plasma membrane and tonoplast H^+-ATPases and PEP-carboxylase. Co-localisation of phosphorylation and antibody detection on 2-D gels is underway but has been hampered by the low number of GCP that can be routinely isolated. Detection of phosphorylated intermediates on acid gels has indicated the presence of two P-type ATPases with characteristics similar to Ca^{2+}-ATPases in other systems. Their identity and sub-cellular localisation awaits further clarification.

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SIMULATION OF THE DIFFUSION OF ION[illegible]OM A PERFUSED P[illegible]CH PIPETTE INTO A GUARD-CELL PR[illegible]OPLAST AND EXPERIMENTAL VERIFICATION OF DIFFUSION TIMES

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We wished to determine equilibration times for ion distribution in a guard-cell protoplast after the composition of the solution in a perfused pipette had been changed (NEHER and ECKERT 1988). A model was constructed in which a conical pipette was attached to a spherical protoplast (r=8 µm) containing a vacuole (r=7.5 µm). Changes in concentration were computed for this system in response to a change within the pipette in an area 100 µm from the the tip. A time course was computed with K^+ as the diffusing ion. The concentration at the pole of the protoplast opposite to the tip of the pipette changed with an effective time constant of 3.14 s. The distribution of K^+ in the cytoplasm was fairly uniform. For Ca^{2+}, EGTA, and gluconate, the time constant was estimated to be 32 s. In a verification experiment, replacement of Cl^- by gluconate occurred with a time constant of 76 s .
Experiments with perfused pipettes allowed to demonstrate a shift in activity of K^+ inward channels by Ca^{2+} (SCHROEDER and HAGIWARA 1989) in one and the same cell, including the reversibility of the Ca^{2+} effect.

The simulation showed in the whole-cell configuration
(1) that the concentration equilibration of ions of the size of Ca^{2+} requires 2 to 3 min,
(2) that the pipette tip is the major resistance (confirming PUSCH and NEHER 1988), and
(3) that the distribution of ions in the cytoplasm is fairly uniform.

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ANION CHANNELS IN THE PLASMALEMMA OF GUARD CELLS: A SLOW ONE EXISTS BESIDES THE FAST ONE AND IT CAN BE ACTIVATED AT LOW MEMBRANE POTENTIALS

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Stomatal closure requires salt efflux from the guard cells and this, in turn, requires a depolarization of the plasmalemma in order to activate the voltage controlled channels for the outward-bound flow of K^+ and anions. Although the anion channels of guard cells described so far (1,2) will ultimately contribute to the depolarization, they cannot initiate it because they themselves require a depolarization of the plasmalemma to values above about -80 mV in order to become activated. This threshold will in most cases be above the equilibrium potential for K^+.

Guard-cell protoplasts were prepared from epidermal fragments obtained by mincing leaves of Xanthium strumarium and Vicia faba in a blender (3). The patch-clamp technique was used to measure whole-cell currents and to record single-channel activities.

We discovered in solutions containing Cs^+ (in order to suppress currents of K^+), with micromolar Ca^{2+} in the cell, Cl^- channels that were characterized by activity at membrane potentials as low as -180 mV and by slow activation kinetics.

Whereas the fast activating channels of V. faba described before had a rise time of about 10 and 20 ms at -40 and -60 mV, respectively (4), the newly found Cl^- current required about 20 s at a potential of -180 mV to reach a steady state; at +60 mV a steady state was not reached even after 90 s (after a holding potential of -200 mV).

In single-channel recordings the fast anion channel had a mean open time of about 2-4 ms, the slow one a mean open time of about 1 s.

We suggest that the slowly activating anion channel has an important function in stomatal closure.

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IS EXTRACELLULAR CALCIUM REQUIRED FOR THE ACTION OF ABSCISIC ACID ON STOMATAL GUARD CELLS ?

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Recent studies have shown that ABA induced-rise in cytoplasmic calcium (1,2) was concomitant with an inward directed current (2). This current was linked to an entry of calcium from the extracellular medium via non-selective permeable channels (2,3). The time course of this event occurs in few seconds. The authors (2) proposed that the entry of extracellular calcium is the initial mechanism of ABA-induced stomatal closure but they do not exclude the possibility of inositol 1,4,5-trisphosphate stimulated-calcium release from internal stores (4,5,6).

This study investigated the effect of ABA on the inward-rectifying potassium current in the absence and presence of extracellular calcium. The experiments were conducted on guard cell protoplasts from *Vicia faba* using the whole-cell patch clamp technique (7).

Regardless of whether extracellular calcium was present or not, ABA was still able to reduce the inward-rectifying potassium current. However, this decrease was slower in the absence of extracellular calcium. These results suggest that ABA induced-stomatal closure does not necessarily require extracellular calcium. Furthermore, the data supports the hypothesis of internal calcium release by a secondary messenger mechanism (4,5,6).

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ROLE OF CALCIUM IN ABA-INDUCED STOMATAL CLOSURE

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There is growing evidence for the involvement of changes in cytoplasmic Ca^{2+} in ABA-induced stomatal closure, but the relative contributions of Ca^{2+} influx at the plasmalemma and release of Ca^{2+} from internal stores remain to be established.

Early events in the signal transduction chain leading to ABA-induced stomatal closure have been established by a number of methods. These include electrophysiological methods in whole cells, by voltage-clamping guard cells of *Vicia faba* and analysing their current-voltage relations. Thus Thiel, MacRobbie and Blatt (1) found that opening of a leak channel, which may be a non-selective channel allowing Ca^{2+} influx, was a very early event in the sequence of changes following ABA treatment, together with inhibition of the inward K^+ channel. These changes preceded the activation of the outward K^+ channel. Very early changes in $^{86}Rb^+$ efflux from *Commelina* guard cells have been observed by MacRobbie (2); the effects of La^{3+} in the external solution on the ABA-induced changes in $^{86}Rb^+$ efflux , presented here, suggest that Ca^{2+} influx at the plasmalemma can play a role in the signalling chain.

However other results presented suggest that internal release of Ca^{2+} is involved. Parmar (3) investigated the patterns of labelling of inositol phospholipids and inositol phosphates, before and immediately after treatment with ABA; this was done by labelling 'isolated' epidermal strips, in which only guard cells are alive, with myo-[^{3}H]inositol. The results presented suggest that there is turnover in the phosphoinositide signalling pathway within 30 s of ABA treatment. Guard cells can respond to release of inositol 1,4,5-trisphosphate within the cytoplasm , by increasing cytoplasmic Ca^{2+}, by showing the electrical changes associated with increased cytoplasmic Ca^{2+}, and by stomatal closure (4,5). Taken together these results suggest that IP_3-triggered release of Ca^{2+} from internal stores forms a part of the response to ABA.

It is probable that both influx of Ca^{2+} at the plasmalemma and internal release are involved.

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EFFECTS OF G-PROTEIN REGULATORS AND Ca^{2+} ON OUTWARD K^+ CHANNEL CURRENT IN MESOPHYLL CELLS OF *VICIA FABA*

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We have used whole-cell patch-clamp techniques to identify an outward, voltage-dependent K^+ current in mesophyll cell protoplasts from *Vicia faba*. With internal solution containing 104 mM K^+, 2 nM free Ca^{2+}, pH 7.2 and external solution containing 13 mM K^+, 1 mM Ca^{2+}, pH 7.2, depolarization of the membrane potential (V_m) to voltages > -20 mV induces channel opening, with average outward current of approximately 500 pA at V_m = +85 mV (n = 6). Currents are not observed in K^+-free solutions, and are eliminated by the K^+ channel blocker, Ba^{2+} (1 mM), implicating current flow through K^+ channels. Introduction of either 500 μM GTPγS (a GTP analog) or 500 ng ml^{-1} cholera toxin (a G-protein activator) to the cytosol results in a decline in outward K^+ current. Conversely, 500 μM GDPβS (a GDP analog) causes a rise in the current. Cytosolic free Ca^{2+} concentrations ≥ 200 nM cause a decrease in outward K^+ current, with complete inhibition at 3 mM free Ca^{2+}. G-proteins (1) and Ca^{2+} may be elements of signal transduction pathways modulating the outward K^+ channel of mesophyll cells in *Vicia faba*.

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IDENTIFICATION AND MODULATION OF A VOLTAGE-DEPENDENT ANION CHANNEL IN THE PLASMA MEMBRANE OF GUARD CELLS BY HIGH-AFFINITY LIGANDS

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Anion channels in the plasma membrane of Vicia faba guard cells catalyze the release of anions during regulated volume decrease (1,2). Furthermore these channels seem to be involved in the targeting of the plant growth hormones auxines (3).

We have analyzed the effect of different anion channel blockers on these voltage-dependent anion channels. Ethacrynic acid, a structural correlate to an auxin, caused a shift in activation potential and simultaneously a transient increase in the peak current amplitude, whereas the other blockers shift and block the voltage-dependent activity of the channel. The capability to inhibit anion currents increase with the sequence: probenecid < A-9-C < EA < niflumic acid < IAA-94 < NPPB. All inhibitors reversibly block the anion channel from the extracellular side. Channel block on the level of single anion-channels is characterized by a reduction of long open-transitions into flickering bursts and a decrease in channel amplitude, indicating an interaction with the open mouth of the channel. Comparison of dose-response curves for shift and block imposed by the inhibitor, indicate two different sites within the channel which interact with the ligand.

IAA-23, a structural analog of IAA-94, was used to enrich ligand-binding polypeptides from the plasma membrane of guard cells. From this protein fraction a 60 kD polypeptide cross-reacted specifically with polyclonal antibodies raised against anion channels isolated from kidney membranes (4).

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KINETICS AND Ca^{2+} PERMEABILITY OF K^+ CHANNELS IN Vicia AND Zea GUARD CELL PROTOPLASTS.

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We have devised a method for the isolati[illegible] cell protoplasts from Zea mays (1), and have used whole-cell patch clamp to compare plasma membrane K^+ current in guard cell protoplasts of this species and Vicia faba. Upon hyperpolarization of the membrane potential (V_m) to activate inwardly rectifying K^+ channels (2), typical steady-state (leak-subtracted) inward K^+ currents are -22 $\mu A.cm^{-2}$ in (Zea), -40 $\mu A.cm^{-2}$ (Vicia) at V_m=-188 mV in 12 mM external K^+.

In both species, we find that K^+ current activation follows a single exponential timecourse, where the rate of channel activation is independent of V_m over the range -128 - -188 mV. Channel activation is faster in Zea than in Vicia: tau at -188 mV = 41 ± 5 ms (Zea) cf. 141 ± 4 ms (Vicia). These results indicate that [illegible]channels conducting inward K^+ current may be considered to open from a [illegible]losed state.

We will present evidence that Ca^{2+} can permeate these channels (3). After a hyperpolarizing voltage step has been used to open channels, depolarization of the membrane results in inward tail current at voltages where there is no electrochemical force to drive K^+ inward. Tail current magnitude and reversal potential are sensitive to shifts in the electrochemical potential of Ca^{2+} but not of other ions. These channels may therefore comprise part of a regulatory mechanism for Ca_i in Vicia and Zea guard cells. Changes in Ca_o and (associated) changes in Ca_i regulate a variety of intracellular processes and ion fluxes including the K^+ fluxes associated with stomatal aperture change (4,5).

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PHOSPHATASE ANTAGONIST OKADAIC ACID INHIBITS K^+ CURRENTS IN INTACT VICIA FABA GUARD CELLS

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We tested the possibility that protein phosphorylation/ dephosphorylation plays a prominent role in the control of the K^+ currents which drive stomatal movement. Intact Vicia faba guard cells were impaled with double-barrelled electrodes for voltage-clamping (1) and treated with okadaic acid** (OA), a membrane permeable inhibitor of phosphatase 1 and phosphatase 2A activity (2). Bathing cells in experimental solution containing OA (200 nM or 1 µM) typically resulted in a progressing and non-reversible decay of K^+ currents through the inward and outward rectifier. In 4 out of 10 cells the treatment completely abolished K^+ currents leaving the potential of these cells comparatively insensitive to changes in $[K^+]o$ (Fig.). The inhibition of both K^+-currents followed the same single exponential kinetics. The decay was preceeded by a lag periode which was variable from cell to cell. In an alternative approach cells were loaded from the voltage recording pipette with the synthetic phosphatase inhibitor Naphthylphosphate (1mM) (3). These experiments gave comparable results in that these cells revealed current-voltage (I-V) relations with very small inward and outward K^+ currents.
The results indicate that both K^+ currents are modulated by a common mechanism which is linked, possibly indirectly, to protein phosphorylation/ dephosphorylation.

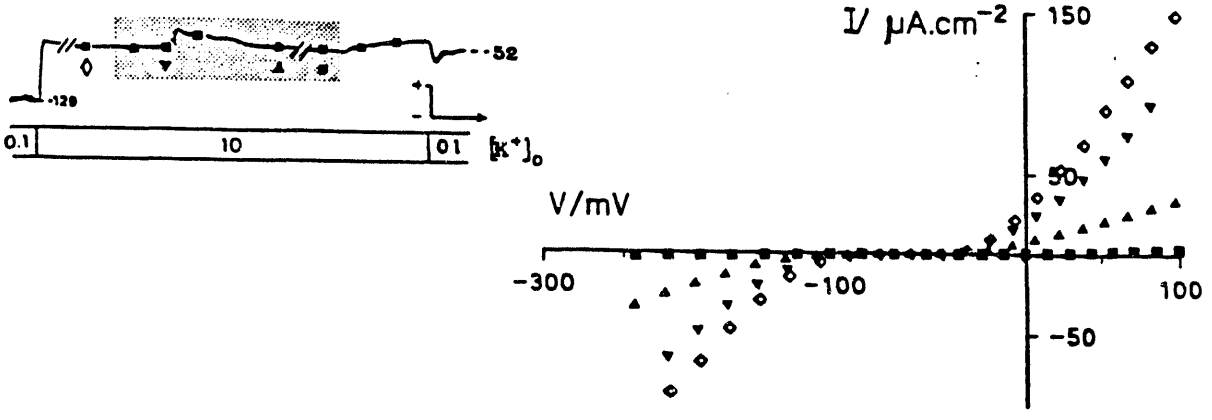

Fig. Effect of 200 nM okadaic acid on steady-state I-V relation of a Vicia faba guard cell (in 10 mM KCl, 5mM Ca-Mes, pH 6.1) before (◇) and after (in min ▼:3; ▲:9; ■:15) adding OA to the incubation chamber. Inset: Membrane voltage and response to changes in $[K^+]o$ ±OA treatment. Presence of OA indicated by shaded area. Scaling: horizontal 3 min, vertical 40 mV. Solid bars indicate times of I-V scans with symbols cross referenced to I-V data.
**OA was a generous gift by Prof. P. Cohen, Dundee University, UK

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CALCIUM CONDUCTANCES IN THE PLASMA MEMBRANE OF *VICIA FABA* GUARD CELL PROTOPLASTS

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In order to resolve small calcium currents across the plasma membrane of guard cells with high resolution, we used the patch clamp technique on protoplasts of *Vicia faba* guard cells in the whole-cell mode, using solutions in which calcium was the only permeant ion (30 mM calcium glucuronate in the bath and 150 mM N-methyl glucamine glucuronate in the pipette). Under these conditions, the plasma membrane initially exhibited a rather flat current-voltage relationship, with an outward current of 3-5 pA, similar to the proton pump current identified previously [1]. Without nucleotides in the pipette, this outward pump current decayed with a halftime of about 30 s, and as it randown, a new, inward-directed conductance arose, reaching a high, stable conductance 3-5 min after patch breakthrough. This current was voltage dependent, with a maximum current of -6 to -20 pA at -200 mV and dropping to zero current as the membrane potential rose to -60 to -40 mV. The inward current was reversibly inhibited by replacement of external calcium with NMG, indicating it was a calcium current. The current was principally comprised of channel currents of small amplitude and long duration (0.1 pA and 100 to 500 ms at -120 mV). Superimposed on this basal current was an additional channel current of higher amplitude, shorter duration, and low open probability (1 pA and 1-5 ms mean open time at -120 mV; see ref [2]). When channel blockers were applied to the external bath, only flunarizine (100 μM) was fully effective at blocking the low-amplitude calcium current. Verapamil and nifedipine (up to 1 mM) were ineffective, as was TFP (10 μM) applied to the cytoplasmic side of the membrane. Lanthanum (1 mM) exerted only a samll (35%) inhibition on the small-amplitude current, whereas it fully blocked the high amplitude current, as did 1 mM gadolinium.

These results, in concert with earlier work, argue for multiple, calcium-permeable conductances in the plasma membrane of guard cells with varying biophysical and pharmacological properties. The coordinate decay of the H^+ pump current with the appearance of the small-amplitude calcium conductance suggests that these two membrane transporters may be regulated in opposite directions by common cytoplasmic factors.

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MALATE TRANSPORT AT THE TONOPLAST STUDIED USING PROTEIN-MODIFYING REAGENTS AND MONOCLONAL ANTIBODIES

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Malate is one of the most widespread solutes in plant vacuoles, playing an important role in charge balance and turgor maintenance in many species. Malate transport into the vacuole seems to occur by an electrophoretic mechanism, driven by the inside-positive membrane potential generated by the tonoplast H^+ pumps (the H^+-ATPase and H^+-PP_iase). In plants showing crassulacean acid metabolism (CAM), the transport pathway for malate across the tonoplast is provided by an inward-rectifying, malate-selective ion channel (J.A.C. Smith et al., these proceedings).

To study the biochemical properties of this transport system, malate transport by tonoplast vesicles has been screened for its sensitivity to a wide range of potential inhibitors using a membrane filtration assay. Covalent modifiers of positively charged amino groups, such as diethyl pyrocarbonate (a histidyl reagent), phenylglyoxal (Arg) and pyridoxal phosphate (Lys), strongly inhibited malate uptake. Substrate-protection experiments indicated that one or more lysine residues may form part of the malate-recognition site of the channel.

A library of monoclonal antibodies has also been raised in mice against the tonoplast membrane from mesophyll cells of the CAM plant *Kalanchoë daigremontiana*. From 97 cell-line supernatants that crossreacted with tonoplast membrane in enzyme-linked immunosorbent assays (ELISA), several monoclonal antibodies have been identified that significantly inhibit malate transport. These antibodies are now being used to characterize the malate channel further at the molecular level.

VACUOLAR CALCIUM CHANNELS

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Free calcium in the cytoplasm of plant cells is important for the regulation of many cellular processess and the transduction of stimuli. Control of cytoplasmic calcium involves the activity of pumps, carriers, and possibly ion channels. The patch-clamp technique was used to study Ca^{2+} channels in the vacuole of sugar beet cells (1). Vacuolar currents showed inward rectification at negative potentials, with a single channel conductance of 40 pS, and an open probability dependent on membrane potential. These channels could participate in the regulation of cytoplasmic calcium by sequestering Ca^{2+} inside the vacuole. Another type of calcium channels were recorded at positive membrane potentials. These outward rectifying currents were increased in the presence of Bay K 8644, a Ca^{2+}-agonist (2). These outward currents would allow the release of Ca^{2+} from the vacuolar storage pool to the cytoplasm, providing a means for the elevation of cytoplasmic Ca^{2+} concentrations.

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CHARACTERIZATION OF VACUOLAR MALATE AND K^+ CHANNELS UNDER PHYSIOLOGICAL CONDITIONS

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Patch-clamp techniques were employed to study the electrical properties of vacuoles from sugar beet cell suspensions at physiological concentrations of cytoplasmic calcium. Vacuoles exposed to potassium malate revealed the activation of instantaneous and time-dependent outward currents by positive membrane potentials. Negative potentials induced only instantaneous inward currents. The time-dependent outward currents were ten times more selective for mal^{2-} than for K^+ and were completely blocked by zinc. Vacuoles exposed to KCl only developed instantaneous currents when polarized to positive or negative membrane potentials.

Our results clearly show that at physiological Ca^{2+}_{cyt} (10^{-7} M), the time-dependent outward channels are active beyond E_{mal2-} thus providing a pathway for the movement of mal^{2-} into the vacuole. A similar role has been confered on the inward rectifying cation channels (1,2), although the activity of these channels has only been observed at unphysiological levels of Ca^{2+}_{cyt} (10^{-3} M) and at more negative potentials than E_{mal2-} (1). In addition to the physiological conditions in which the time-dependent outward channels are active, their higher selectivity for mal^{2-} than for K^+, suggests that these channels are a possible pathway for the movement of mal^{2-} into the vacuole than the inward rectifying channels. Malate inside the vacuole could act as a counterion for K^+ and concomitantly, also be involved in mechanisms of cellular osmoregulation.

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PH AND CA^{2+} MODULATE THE ACTIVITY OF ION CHANNELS IN THE VACUOLAR MEMBRANE OF GUARD CEL. - POSSIBLE INTERACTION WITH CYTOPLASMIC CALMODULIN

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The predominant ion channel in the vacuolar membrane of guard cells from *Vicia faba* has been investigated with respect to its modulation by $[H^+]$, $[Ca^{2+}]$ and CaM using the patch-clamp technique. SV-type currents were recorded at hyperpolarising membrane potentials (negative inside vacuole) in the whole vacuole configuration. Single SV-type channels (1) were resolved in isolated membrane patches. Single-channel analysis revealed a slope conductance of 274±30 pS (in 200 mM KCl). This ion channel is permeable for both, cations and anions, indicated by the relative permeability ratio $P_K : P_{Cl} = 2\text{-}4 : 1$. 100 μM Zn^{2+} on the cytoplasmic side of the membrane was found to completely block the SV-channel within a few seconds in a reversible fashion. Thereby the amplitude of the single-channel current remained unaffected. Application of DIDS known to block the SV-type channel in sugar beet vacuoles did not block the guard cell channel.

In further experiments the modulation of the SV-type channel in guard cells of *Vicia faba* was compared to that of sugar beet tap roots (2). Although the origine of vacuoles strongly differ, the effect of voltage and ligands was comparable. Increasing the cytosolic proton level decreased the channel activity, whereas it was increased by the elevation of the cytoplasmic calcium concentration. The rise in the cytoplasmic calcium-concentration shifted the activation potential towards positive membrane potentials. Additionally, vacuoles were incubated with CaM in order to test whether Ca^{2+} sensitivity of the channel is increased in the presence of cytoplasmic CaM. In agreement with the stimulating effect of CaM W-7 (3) imposed a flickering block on SV-type channels (K_i = 4 μM). W-7 block was reversible and did not change the single-channel conductance. On account of their high sensitivity to cytoplasmic factors the SV-type channels lend themselves to identification of the control of ion release in the vacuole of higher plants.

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PATCH CLAMP STUDY OF A NOVEL PATHWAY FOR INTRACELLULAR Ca^{2+} MOBILIZATION

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Elevation of cytosolic free Ca^{2+} plays a key role in stimulus-response coupling in plant cells and digital imaging of Ca^{2+} dyes in intact cells suggests that the vacuole is a major site of Ca^{2+} release into the cytosol [1]. In addition to inositol 1,4,5 trisphosphate ($InsP_3$)-elicited Ca^{2+} release we have recently discovered an alternative pathway for Ca^{2+} mobilization from the vacuole via a voltage-gated channel [2]. The single channel properties have been investigated in isolated vacuoles from sugar beet taproots with the patch clamp technique.

In biionic conditions (Ca^{2+} on the vacuolar side, K^+ on the cytoplasmic side) single channel current voltage relationships reveal a large conductance for K^+ influx (200 pS with 50 mM K^+) and a small conductance for Ca^{2+} efflux (12 pS with 5 - 20 mM Ca^{2+}). The zero current potentials, however, indicate a 15 - 20 times higher selectivity for Ca^{2+} over K^+. When K^+ is used as a charge carrier, addition of increasing Ca^{2+} concentrations to the vacuolar side causes a progressive decrease in the unitrary K^+ efflux current ($K_{1/2}$ = 0.29 mM at V = 0 mV) indicating ionic competition for an intrapore binding site. The voltage dependence of this process reveals that the binding site is located approximately 9 % of the electrical field into the pore from the vacuolar side.

Channel openings are largely promoted by positive-going shifts in the vacuolar membrane potential. Elevation of vacuolar Ca^{2+} shifts the threshold for voltage activation to less positive potentials and causes a further increase in the open-state probability over the physiological range of vacuolar membrane potentials. Changes in Ca^{2+} on the cytosolic side do not significantly affect Ca^{2+} efflux current but there are several indications that channel gating is also controlled by cytoplasmic regulators. We conclude that this voltage-sensitive Ca^{2+} release pathway could potentially provide a way to achieve a sustained elevation of cytosolic free Ca^{2+} needed to trigger long term responses involved in stimulus-response coupling.

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ISOLATION OF INTACT VACUOLES FROM THE ALEURONE LAYER OF BARLEY

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Barley aleurone cells synthesize and secrete large amounts of hydrolytic enzymes upon stimulation by gibberellic acid. Most of the raw material for these proteins originates within the cell's vacuoles, the so-called protein bodies. These storage organelles have been shown to contain large amounts of protein, phytin, and ions such as phosphate and potassium. We are interested in how these reserves are transported across the tonoplast and how this transport is integrated with cellular function.

The study of vacuoles from the barley aleurone layer has been difficult because an aqueous procedure for isolating intact vacuoles has not been available. We have developed such a procedure. The plasma membrane of isolated aleurone protoplasts is disrupted by mechanical shearing in isotonic buffer to release cellular contents. The resulting lysate is quickly filtered through nylon mesh and layered onto a three-step density gradient, which is centrifuged at 5-10xg. Vacuoles band at the interface of the bottom two layers. Western-blotting analysis with an antibody against barley α-amylase indicates that there is little contamination of the vacuolar fraction by cytoplasmic proteins, and probing with an antibody against the tonoplast intrinsic protein TiP-25 indicates that tonoplast yields are high.

We have begun to examine channel activity in the vacuolar membrane using the patch-clamp technique. Preliminary data based on single channel events suggests that several different channels are present in the tonoplast. Characterization of these is underway and progress will be reported.

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FAST SWITCHING AND COUPLED EXCITATION OF THE Cs^+-BLOCKED K^+-CHANNELS IN TONOPLAST VESICLES OF *CHARA CORALLINA* AS DIRECTLY RESOLVED IN THE TIME SERIES OF PIPETTE CURRENT

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Patch-clamp experiments open the facility of distinguishing between effects on single channel conductivity and on transitions into inactive states. This approach fails if time resolution is not fast enough. The existence of fast undetected switching is indicated by measured beta-distributions (1) or by theoretical considerations (2, 3) especially in the case of the anomalous mole fraction effect (4). A new fast patch-clamp set-up with a powerful detector of current levels in noisy signals (higher order Hinkley-detector, 5, 6) enabled the observation of time-constants up to 10 μs.

The gating kinetics induced by $Cs^+$is a good example for fast opening and closing phenomena which until now were believed to be an influence on single channel current. Dwell-time histograms were created by means of the higher order Hinkley detector from patch-clamp studies on the Cs^+-block of the K^+-channel in the tonoplast of *Chara corallina*. In contrast to previous recordings, two time-constants ranging from 20 μs to 100 μs and from 1 ms to 3 ms, respectively, were required for fitting the data. In order to illustrate that the previously measured effect of Cs^+on the single channel conductivity of K^+-channels is pretended by undetected gating effects, a lower resolution was simulated by averaging blocks of 20 samples (this corresponds to a temporal resolution of 200 μs). Then, the dwell-time histograms could be fitted without the fast time-constant. I-V curves of apparent single channel currents constructed from the averaged and the non-averaged data coincided at positive membrane potentials. At negative potentials the averaged currents were smaller and showed a negative slope. The non-averaged currents were compared with those of the unblocked K^+-channels.

A peculiar effect was found especially at high negative potentials and with Cs^+in the pipette and bath solution, namely, the amplitude histograms could not be fitted sufficiently well by a binomial distribution. The small open propability found under these conditions excludes that the frequently observed simultaneous openings of more than one channel is just fortuitous. Therefore we suggest a mutual influence and excitation of the K^+-channels. This influence is modelled by the correlated bond-percolation which is often used in the theory of Ising-models (7).

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HYDROSTATIC AND OSMOTIC PRESSURE ACTIVATED CHANNEL IN PLANT VACUOLE

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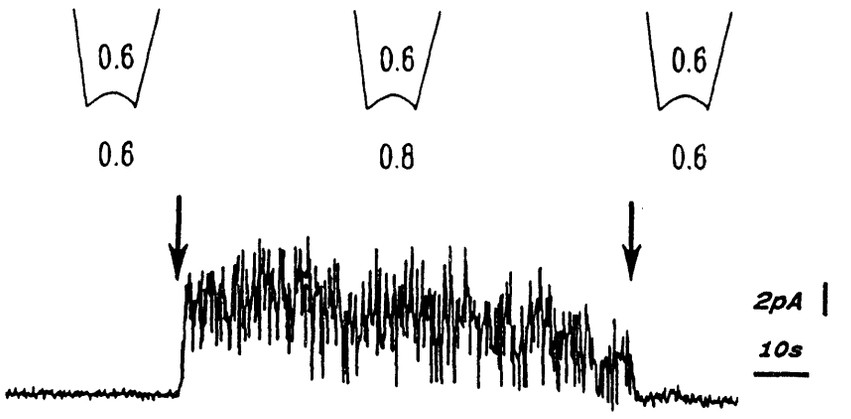
Plants respond to a great range of signals in their environment. For some of these signals, transduction at the cellular level occurs through the gating of various types of ionic channels in the plasma membrane. Channels sensitive to membrane potential, chemical stimulus or mechanical deformation of cell membrane, have already been found in plant cells. We present here patch clamp experiments performed on isolated red beet vacuoles (*Alexandre J, Lassalles JP 1991, Biophysical J, 60, 1326-1336, 1991*), indicating that the tonoplast contains a channel which is activated by stretching the vacuolar membrane. The channel is more permeable for K^+ than for Cl^- ions (P_K / P_{Cl} # 3). The opening probability, p_o, and the conductance of the channel (20 pS in 200 mM KCl symmetrical solutions) are not modified by a change in cytosolic Ca^{2+} or vacuolar potential. There is a strong increase in p_o, when pressure or suction are applied to a patch membrane (a 1.5 kN m^{-2} suction induces an e-fold change in the mean patch current). The channel is blocked when 10 μM Gd^{3+} are applied to the cytoplasmic side of the tonoplast. For the patch currents recorded under isoosmotic conditions, the channel conductance and p_o are not affected when the sorbitol osmolality increases from 0.6 to 1 osmol/ kg. When only a 0.2 osmol/ kg sorbitol gradient is applied to the patch, a strong reversible increase in current is observed even in the lack of externally applied stretch (see figure). Measurements in the whole vacuole configuration (diameter of the vacuole: 45μm) indicate that the osmotic gradient results in a 100-200 pA / current per osmol.



Effect of a 0.2 osmol / kg sorbitol gradient on an isolated patch current. The patch was performed either with 0.8 or with 0.6 osmol / kg sorbitol solutions

The channel described in this report is gated by a change in osmolality, without the need of a change in turgor pressure. This channel represents a possible example of an osmoreceptor in plant cell membranes.

ACTIVATION KINETICS OF CATION CHANNELS IN RADISH VACUOLES

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Voltage dependent gating of ion channels has been studied using the patch-clamp technique in a variety of channels in animal and plant cells. The molecular basis of the opening mechanism has been associated with the movement of charged gating particles sensing the membrane electric field. However, little or nothing is known about the molecular processes associated with the opening of single ion channels. It has been particularly difficult to monitor channel gating in animal cells as they are characterized by fast time constants $tau_{act} \approx 1ms$) and often masked by simultaneous channel inactivation. We have circumvented these problems studying the opening of calcium activated cation - selective channels from radish root vacuoles, which display activation time constants of several hundreds of milliseconds and do not inactivate.

We demonstrate that these channels have two kinetically distinct activation modes which, at a transmembrane voltage of -100 mV, are characterized by fast and slow activation times such that the ratio between the two time constant is in the order of 1:5. Therefore activation times in the order of seconds are observed in the slow mode. Different membrane potential protocols allowed to switch between the two modes in a controlled and reversible manner. This process may represent a modulator of voltage dependent ion channels in other plant and animal systems.

CHARACTERIZATION OF THE TONOPLAST Ca^{2+}/H^+ ANTIPORT SYSTEM FROM MAIZE ROOTS

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Calcium (Ca^{2+}) plays a key role as an activator and regulator of many biological processes in higher plant cells (1). The concentration of free cytosolic Ca^{2+} is maintained at less than 200 nM by the action of high affinity membrane-bound receptors (2). Recently, the vacuole of plant cells was shown to play a major role in the sequestration and storage of Ca^{2+}, and as a reservoir for Ca^{2+} release during signal transduction (1,2,3). Ca^{2+}-transport at the tonoplast is achieved by a Ca^{2+}/H^+ antiport system, driven by the electrochemical potential difference created by the two tonoplast proton pumps : the V-H^+-ATPase and the H^+-PPase (1,3,4). The Ca^{2+}/H^+ antiport system has been studied in isolated membrane vesicles using the tonoplast proton pumps (1,3,4) or by imposing a pH gradient across the tonoplast membrane ("pH jump") (3).

In this study, the "pH jump" technique was used to characterize the Ca^{2+}/H^+ antiport system of maize roots in order to avoid any interference of the tested chemicals with the V-H^+-ATPase or the H^+-PPase. Low density membrane vesicles were prepared as previously described (4). The vesicles were sedimented and resuspended in a buffer (BTP-Mes 25 mM, sucrose 10%) at pH 6. After a 30 min incubation, the vesicles were sedimented, resuspended in a small volume of buffer pH 6 and frozen at -196°C. For the calcium transport experiments, 20 µl of vesicles (40-80 µg protein) were added to 470 µl of ice cold buffer (BTP-Mes 25 mM, sucrose 10%) at pH 8. The vesicles were equilibrated for 10 min to allow a H^+ gradient to be formed before the Ca^{2+} uptake was initiated by the addition of 10 µl of $^{45}Ca^{2+}$ (50'000 cpm). After 2 min, 400 µl of vesicles were filtered on a 0.45 µm filter (Gelman GN-6) which had been pre-wetted with 2 ml of buffer (pH 8). The filter was washed three times with 1 ml of ice cold buffer (pH 8) and radioactivity determined by liquid scintillation spectrometry.

The Ca^{2+}/H^+ antiport activity was completely inhibited by ruthenium red (100 µM), whereas erythrosin B (100 µM) was almost without effect. Surprisingly, DCCD (1 mM) did not inhibit the antiport activity, as opposed to the results of Schumaker and Sze (3) on oat roots. Divalent ions were strongly inhibitory ($La^{2+}>Mn^{2+}>Co^{2+}$), as previously reported (3). The sulfhydryl group reagent *N*-ethylmaleimide (NEM) was only slighdy inhibitory at 1 mM, whereas 5,5'-dithio bis-(2-nitrobenzoate) (DTNB) was witout effect at 100 µM. These results do not support the involvement of SH groups in the transport of Ca^{2+} by the antiport. Chemical modification of different amino acid residues of the antiport will be used to elucidate the amino acid residues participating in the Ca^{2+} transport activity.

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NET K^+ EFFLUX AGAINST THE ELECTROCHEMICAL GRADIENT IN Cs^+- AND FC-TREATED *ELODEA DENSA* LEAVES

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In fusicoccin-treated *Elodea* leaves the addition of Cs^+ completely reversed K^+-induced Em depolarization and inverted the direction of net H^+ and K^+ fluxes, a significanat alkalinization of the medium and a release of K^+ becoming evident in spite of an Em value very close to that observed with fusicoccin alone. The K^+ net efflux was thus occurring against a steep electrochemical gradient ($[K^+]_{in}/[K^+]_{out}$ = 200 and Em = -260 mV). This finding suggested the operation of some non electrogenic K^+ transport mechanism.

The same conclusion is also suggested by the behaviour of *Elodea densa* leaves, where photosynthesis simultaneously induces a strong increase of K^+ uptake and H^+ extrusion at one side of the leaf blade and an increase of H^+ apparent uptake an of K^+ release at the other side (1), while no significant differences in E_m values between the two layer of cells are detectable.

A disagreement between the measured values of K^+ net efflux and those predicted by the electrochemical gradient seems also apparent in the case of ferricyanide-induced K^+ efflux in conditions of inhibited H^+ pump operation, when the depolarization of E_m does not seem large enough to account for the observed K^+ release.

These lines of evidence suggest the exdistance at the plant plasma membrane of some non electrogenic system transporting K^+ and possibly also H^+. Apparently, the requirements for a similar system would be fulfilled by an H^+/K^+ antiport, as that proposed by Felle (2) and by Cooper et al. (3) on the basis of different experimental approaches.

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SODIUM-DRIVEN SYMPORT IN CHAROPHYTES: THE ELECTROGENIC UREA TRANSPORT SYSTEM

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Urea is taken up by *Chara australis* by three parallel processes including a low-Km electrogenic transport [Wilson & Walker, 1988; Wilson, O'Donoghue & Walker, 1988]. The Km for urea influx by this process and for membrane current evoked by urea is 0.35 μM in experiments with 1.0 mM external sodium.

We find that the current evoked by micromolar concentrations of urea requires external sodium, with a Km of about 30 μM. The current has a Vmax of the order of 10 nmol/(sqm.s). Radiotracer studies show that low concentrations of sodium stimulate urea influx from low conccentrations of urea by about 10 nmol/(sqm.s), while low concentrations of urea stimulate sodium influx from low concentrations of sodium by about the same amount. The similar sizes of these stimulations under comparable conditions suggest the transport stoichiometry is 1 urea: 1 sodium. The kinetics of electrogenic urea transport suggest a random-binding symport model [Sanders, 1986] in which sodium binds first at low urea concentrations, while the transport rate is reduced at high urea concentrations by urea binding first. This appears to be the first instance of the cis-inhibition predicted by Sanders.

We have found that membrane current is also evoked, in the presence of external sodium, by urea analogues including N-methyl-urea and acetamide, which inhibit the low-Km component of urea influx [Wilson *et al.*, 1988]. These analogues share one amino group and the carbonyl group with urea.

The low values of Vmax for the electrogenic process suggest that the sodium-urea symport is capable of playing a maintenance role at low urea concentrations but not of providing reduced nitrogen for rapid growth. At concentrations of tens of micromolar the passive route carries a much bigger flux than the electrogenic route; if cis-inhibition also shuts down the latter this would be adaptive.

Membrane current has been found to be evoked, in the presence of sodium, by micromolar urea in *Nitella translucens* [Walker & Sanders, 1991]. Thus sodium-driven symport appears in both extant tribes (*Chareae* and *Nitelleae*) of the *Characeae*. It appears also, by the same token, in mildly acidophile as well as mildly alkalophile species.

A suggested rationale for sodium-driven symport in plants will be discussed.

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CYTOPLASMIC PH REGURATION DURING Pi UPTAKE BY CULTURED PLANT CELLS

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Transport of inorganic phosphate (Pi) across the plasma membrane in plant cells has been extensively investigated, and a proton cotransport mechanism has been proposed on the basis of electrical measurements of Em (1, 2). Recently we found that the addition of small amount of Pi to a cell suspension of *Catharanthus roseus* induced a rapid alkalinization of the medium (3). After exhaustion of external Pi, the pH of medium immediately started to recover the original value. This, found in a system without buffer, clearly demonstrated that Pi is cotransported with protons into the cell, and the $H^+/H_2PO_4^-$ stoichiometric ratio of the cotransport was calculated to be 4 (3), which, in turn, strongly suggested cytoplasmic acidification during Pi uptake.

This was studied by means of a fluorescent pH indicator, BCECF, and ^{31}P-NMR spectroscopy. Cytoplasmic acidification as measured by BCECF started immediately after Pi application. Within a minute or so, a stable state was attained and no further acidification occurred, while Pi absorption was still proceeding. As soon as Pi in the medium was exhausted, cytoplasmic pH started to recover. Coincidentally, the medium pH started to recover toward the original acidic pH. The Pi-induced changes in the cytoplasmic pH were confirmed by ^{31}P-NMR study. Maximum acidification of the cytoplasm induced by 1.7 mM Pi was 0.2 pH units. Vacuolar pH was also affected by Pi. In some experiments but not all, pH decreased reversibly by 0.2-0.3 pH units during Pi absorption. Results suggest that the cytoplasmic pH is regulated by proton pumps in the plasma membrane and in the tonoplast. In addition, other mechanisms that could consume extra protons in the cytoplasm are suggested.

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THE STOICHIOMETRY OF PHOSPHATE/PROTON COTRANSPORT AND THE RESPONSE OF PROTON EFFLUX TO PHOSPHATE IS ALTERED BY PHOSPHORUS DEFICIENCY

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We have previously obtained evidence that in phosphorus adequate (+P) *Trifolium repens* plants, phosphate absorption proceeds via a 1:1 cotransport with protons (1). Growing plants in phosphate free solution for 14 days prior to measurements (-P plants) resulted in a net phosphate absorption rate by roots of intact plants that was double the rate for +P plants.

Measurements of ΔE_{vo} on roots of -P plants showed a rapid transient depolarisation (mean = 28.3 ± 1.8 mV) on addition of 125 μM H_3PO_4 to the buffered, phosphate free nutrient solution bathing the roots. The depolarisation was followed by a gradual persistent repolarisation which resulted in values for ΔE_{vo} more negative than the initial value. For +P plants, there was no significant, comparable effect. This indicates a difference between mechanisms of phosphate transport by +P and-P plants, ie. that the stoichiometry of proton/phosphate transport is 1 in +P plants and >1 in -P plants.

Phosphate transport by both +P and -P plants was inhibited by 300 μM N-ethylmaleimide (NEM) but the inhibition was more severe for +P plants. Vanadate (50 μM) inhibited phosphate transport by a moderate and similar extent in both +P and -P plants.

There were no significant differences in net proton efflux into phosphate free nutrient solution between +P and -P plants. However on addition of phosphate, proton efflux decreased in +P plants but increased in -P plants. In +P plants, NEM added after phosphate severely inhibited proton efflux but there was no significant inhibition in the case of -P plants.

These results point to qualitative differences between +P and -P *T. repens* plants in the transport of phosphate and its interaction with proton movements.

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NITRATE IS ACTIVELY TRANSPORTED AT THE TONOPLAST OF BARLEY ROOT CELLS.

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Nitrate-selective microelectrodes have been used to measure nitrate activity in the cytoplasm and vacuole of barley root cells grown in nutrient solution containing 10 $mol.m^{-3}$ nitrate (1). For epidermal cells, the mean cytoplasmic and vacuolar nitrate activities were 5.0 $mol.m^{-3}$ and 38.9 $mol.m^{-3}$, respectively, while for cortical cells the corresponding values were 2.6 $mol.m^{-3}$ and 67.6 $mol.m^{-3}$. The nitrate electrodes also measure the membrane potential across the plasma membrane and tonoplast. In epidermal cells the mean (± SE) tonoplast membrane potential was +10.6 (± 4.6) mV and in cortical cells it was +12.3 (± 11.2) mV. These values are too small to allow the observed vacuolar nitrate concentrations to be achieved by passive transport so nitrate transport into the vacuole must be an active process. Given the measured electrochemical gradients, a passive mechanism could only achieve vacuolar activities of 8 and 5 $mol.m^{-3}$ for the epidermal and cortical cells, respectively.

Proton-selective microelectrodes were used to measure the pH gradient across the tonoplast to assess whether this nitrate transport could be mediated by H^+/NO_3^- antiport. For epidermal cells, the mean (± SE) cytoplasmic and vacuolar pH values were 7.12 ± 0.06 (n=10) and 4.93 ± 0.11 (n=22), respectively, while for cortical cells the corresponding values were 7.24 ± 0.07 (n=3) and 5.09 ± 0.17 (n=7). Calculations of the energetics for the antiport mechanism indicate that the observed gradient of nitrate across the tonoplast of both epidermal and cortical cells could be achieved by a H^+/NO_3^- antiport with a 1:1 stoichiometry (2).

Recently, nitrate-selective microelectrode measurements of intracellular nitrate have been made in barley root cells grown in 0.1 $mol.m^{-3}$ nitrate nutrient solution for 24 h. These epidermal cell measurements show that the mean cytoplasmic nitrate activity is 4.2 $mol.m^{-3}$ and the vacuole is 20.5 $mol.m^{-3}$, indicating that cytoplasmic nitrate is regulated independently of the external concentration. Furthermore, the intracellular distribution of nitrate at this lower external concentration also requires active transport at the tonoplast.

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Arginine Transport into Mitochondria of *Neurospora crassa*

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Biosynthesis of arginine in *Neurospora crassa* takes place in two different compartments. Synthesis of ornithine from glutamate and conversion of ornithine to citrulline occurs in the mitochondria and synthesis of arginine from citrulline occurs in the cytosol. Arginine biosynthesis is regulated by feed-back inhibition. The first two enzyme of ornithine synthesis, acetylglutamate synthase and acetylglutamate kinase, are feed-back inhibited by high levels of arginine which is produced in the cytosol (1). In order to perform feed-back inhibition as well as to support biosynthesis of mitochondrial proteins, arginine must cross the mitochondrial membranes.

In this study, arginine transport into the mitochondria of *N. crassa* has been examined using a filter retention method. Arginine transport was found to be saturable (K_m = 6.5 mM) and to have a pH optimum of pH 7.5. Uncouplers and substrates of oxidative phosphorylation did not affect the transport rate. The arginine concentration within the mitochondrial matrix after transport was similar to that of the reaction medium. These results indicate that mitochondrial arginine transport is facilitated transport rather than active transport. The mechanism of arginine transport, whether symport requiring an anion or antiport requiring a cation, is not clear at this point. The specificity of the arginine transport system was investigated with various amino acids and arginine analogs. The basic amino acids ornithine, lysine and D-arginine inhibited arginine transport. Arginine analogs which have been modified at the N- or C- terminus inhibit arginine transport; however, an analog which was modified on the guanidine side chain did not inhibit transport. These results suggest that the positive side chain of arginine is important for recognition by the transport system. Arginine transport could be irreversibly blocked by treating mitochondria with a reactive arginine derivative, *N*-nitrobenzyloxycarbonyl arginyl diazomethane.

When arginine is supplied in the medium, the cytosolic arginine concentration of *N. crassa* increases from 0.2 mM to about 15 mM which is higher than the observed K_m value of arginine transport (2). The observed characteristics of mitochondrial arginine transport can explain feed-back regulation of arginine biosynthesis in *N. crassa.*

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GLYPHOSATE TRANSPORT INTO PLASMA MEMBRANE VESICLES ISOLATED FROM COMMON LAMBSQUARTERS LEAVES

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Glyphosate, an inhibitor of EPSP synthase in the shikimic acid pathway, is a widely used postemergence herbicide that is generally sprayed on the surface of the leaf. Since glyphosate is a hydrophilic molecule, it is applied in the presence of surfactants that disrupt the cuticle and allow penetration. Recent studies suggested that those surfactants that enhance glyphosate phytotoxicity may also have a significant effect on the plasma membrane. The objectives of this study were to identify the mechanism of glyphosate transport across the plasma membrane and to determine whether surfactants play a significant role in increasing glyphosate flux. Plasma membrane vesicles (PMVs) were isolated from mature leaves of lambsquarters using the aqueous-phase partitioning method. Vesicles were isolated and suspended in an osmotically balanced medium at pH=7.8. For transport experiments, membrane vesicles were diluted into an acidic uptake solution (pH=6.0) which included radiolabeled substrates in the presence or absence of the protonophore, CCCP. This experimental treatment imposed a transmembrane proton electrochemical potential difference that was capable of driving several proton-amino acid symports, thus indicating that these membrane vesicles were transport competent and functional. Glyphosate, atrazine, and bentazon transport were tested in this experimental system. Atrazine accumulated inside the vesicles, but flux was not influenced by the transmembrane ΔpH. Bentazon also accumulated inside the vesicles and accumulation was driven by the imposed proton concentration difference. Glyphosate flux was very low and unresponsive to the imposed pmf, suggesting the plasma membrane is a barrier to glyphosate uptake. Surfactant additions to the transport solution (0.1 - 0.0001%) were evaluated for their effects on ΔpH (via acetate accumulation) and glyphosate transport. At surfactant concentrations exceeding 0.01%, acetate accumulation was greatly diminished, implicating a significant impact on membrane integrity. At 0.01%, the imposed pH-gradient was still present and, interestingly, glyphosate transport was stimulated 4-fold. Our initial hypothesis suggested agriculturally effective surfactants, *e.g.* cationic *vs* nonionic, might form a molecular complex with glyphosate that contributes to transmembrane flux. However, both classes of surfactants increased glyphosate influx *in vitro*. Thus, no correlation was observed between whole plant efficacy and glyphosate accumulation. Current data suggests that surfactant efficacy may be the result of charged surfactants ability to diffuse away from the cuticle into the subtending apoplastic space w re they act on the plasma membrane to increase glyphosate uptake.

GABA PRODUCTION AND EFFLUX IN RESPONSE TO CELLULAR ACIDIFICATION

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GABA (4-aminobutyrate) is synthesized through the decarboxylation of L-Glu (L-Glu + H^+ ---> GABA + CO_2); and compared to other free amino acids is present in high concentrations in plant cells. GABA levels rise rapidly and dramatically in response to a variety of stress conditions including anaerobiosis (1). Recent papers suggest that GABA production and associated H^+ consumption is part of a metabolic pH-stat which ameliorates the intracellular pH decline associated with anaerobiosis (2,3). Similarly GABA production may be necessary for pH regulation during H^+/L-Glu symport into Asparagus mesophyll cells (4). To test this hypothesis GABA production and efflux have been measured in isolated Asparagus cells in response to three treatments designed to cause intracellular acidification. Acid loads were imposed using 60 min of (i) H^+/L-Glu symport, (ii) anaerobiosis, and (iii) treatment with a permeant weak acid butyrate. Both intra- and extracellular GABA levels increased more than 100% after anaerobiosis; almost 1000% after H^+/L-Glu- symport (light or dark) and almost 500% after addition of 3mM butyrate at pH 5.0. These three experiments support the hypothesis that GABA production is involved in resisting intracellular acidification. Glutamate decarboxylase isolated from these cells has a pH optimum of 6; suggesting that intracellular acidification stimulates the enzyme responsible for GABA synthesis. Fluorescent pH probes are being used in attempts to monitor changes in intracellular pH. The biological significance of GABA efflux is not yet understood.

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TRANSPORT OF NEUTRAL, ACIDIC, AND BASIC AMINO ACIDS IN PLASMA MEMBRANE VESICLES FROM TOBACCO LEAVES

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We have studied amino acid transport in plasma membrane vesicles from tobacco leaves. The vesicles were obtained by partitioning of a microsomal fraction in an aqueous polymer two-phase system (1). Transport was measured after imposition of ΔpH (pHi=7.0 and pHo=5.0) and/or $\Delta\psi$ (inside negative) across the vesicle membrane. In parallel experiments pHi was monitored with vesicle-entrapped pyranine, and changes in $\Delta\psi$ were recorded by using the cyanine dye diS-C3-(5).

In the presence of both gradients, at least 100-fold accumulations of the neutral amino acid L-valine in the vesicles have been observed. At pH 5.0 the imposition of $\Delta\psi$ stimulated the influx of valine by about 30%. Accumulation of the acidic amino acid L-glutamate could also be effected by ΔpH, but the influx was not stimulated by $\Delta\psi$. Conversely, accumulation of the basic amino acid L-lysine could be driven by $\Delta\psi$, but ΔpH had no effect on the influx. These results demonstrate that: (a) transport of L-valine is a H^+-symport; probably this holds for neutral amino acids in general (2); (b) transport of L-glutamate is electrically silent; it is transported as zwitterion without a proton, or as anion plus a proton; (c) transport of L-lysine is a uniport.

Transport of all three amino acids was specific for the L-isomer. In vesicles from the ValR-2 mutant (2,3,4) the influx of valine was lower than in vesicles from wild-type.

Remarkable results were obtained in experiments on the pH-dependency of the valine influx. pH-profiles for the influx at 1 μM and 1 mM were very similar: at both substrate concentrations the influx became fully depressed when the pH was raised from 4.5 to 7.0. Substrate saturation curves at pH 4.5, 5.5 and 6.5 were all clearly biphasic; there was no indication of the two kinetic components merging into a single one when the proton concentration was increased (5).

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STRUCTURAL DETERMINANTS IN SUBSTRATE RECOGNITION BY THE NEUTRAL, PROTON-AMINO ACID SYMPORTS

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Amino acids are actively transported across the plasma membrane of plant cells by proton-coupled symports. Recently, we identified four amino acid symports, including two porters for the neutral amino acids, using isolated plasma membrane vesicles and imposed proton electrochemical potential differences (1,2). In the results reported here we investigated the effect of amino acid analogues on neutral amino acid transport to identify structural features that are important in molecular recognition by Neutral system I (isoleucine) and Neutral system II (alanine and leucine). D-Isomers of alanine and isoleucine were not effective transport antagonists of the L-isomers. These data are characteristic of stereospecificity and suggest that the positional relationship between the α-amino and carboxyl groups is an important parameter in substrate recognition. This conclusion was supported by the observation that β-alanine and analogues with methylation at the α-carbon, at the carboxyl group, or at the α-amino group were not effective transport inhibitors. Specific binding reactions were also implicated in these experiments because substitution of the α-amino group with a space filling methyl or hydroxyl group eliminated transport inhibition. In contrast, analogues with various substitutions at the distal end of the amino acid were potent antagonists. Moreover, the relative activity of several analogues was influenced by the location of sidechain branches and Neutral system I and II were resolved based on differential sensitivity to branching at the ß-carbon. The kinetics of azaserine and *p*-nitrophenylalanine inhibition of leucine transport were competitive. We conclude that the binding site for the carboxyl end of the amino acid is a well defined space that is characterized by compact, asymmetric positional relationships and specific ligand interactions. Although the molecular interactions associated with the distal portion of the amino acid were less restrictive, this component of the enzyme-substrate complex is also important in substrate recognition because the neutral amino acid symports are able to discriminate between specific neutral amino acids and exclude the acidic and basic amino acids.

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EXPRESSION CLONING OF METABOLITE TRANSPORTERS FROM HIGHER PLANTS

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Assimilates are produced in the source organs, e.g. leaves, and transported via the vascular system to the sink organs which serve, e.g. for reproduction. In most plants, the main transfer molecules for carbon and nitrogen are sucrose and amino acids respectively. Long distance transport is necessary to bring the assimilates from the place of synthesis to the the places of metabolization or storage. In tobacco, strong evidence supports the hypothesis that sucrose enters the phloem via the apoplast, requiring specific permeases to allow crossing of the membranes (1). Uptake studies with purified plasma membrane vesicles have demonstrated sucrose and amino acid permease activities (2,3,4). In order to study the regulation and function of the transporters it seems important to isolate the respective genes. Due to the difficulties associated with the identification and purification of membrane transporters the genes for plasma membrane and tonoplast permeases have so far not been cloned. For the Na^+-dependent glucose transporter from rabbit, the problems were circumvented by developing an expression cloning system in oocytes (5). We have used both standard and artificial complementation systems to isolate cDNA clones encoding metabolite transporter genes such as sucrose permease genes from plants. We have used the expression system as a tool to characterize the transport systems.

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A GENE FAMILY CODING FOR SUGAR TRANSPORTERS FROM *ARABIDOPSIS THALIANA*

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Carbohydrates, the main products of photosynthesis, must be transported from the tissues of their synthesis ("source") to the different carbohydrate consuming tissues ("sink").

In plants the transport of substrates can occur either on the symplastic way through plasmodesmata or on the apoplastic way. The apoplastic concept requires the existence of transport proteins. In the literature a monosaccharid transporter and a sucrose transporter have been postulated for the plasma membrane of higher plants. An energy dependant uptake of glucose has been shown to occur across the plasma membrane of pea mesophyll cells. A sucrose uptake has been described for mesophyll protoplasts of *Vicia faba* and for cotyledones of *Ricinus communis*. But all this data are based on measurements of model-systems. An alternative way to study sugar carriers of plants is cloning their genes.

We have cloned and sequenced a gene family coding for putative sugar carriers from *Arabidopsis thaliana*. A cDNA clone of the H^+/hexose cotransporter from the green alga *Chlorella kessleri* was used as hybridization probe for screening an *Arabidopsis* genomic and cDNA library. Until now we have isolated four different clones (STP1 - STP4), which encode different proteins with high homology to other sugar transporters from man, rat, bacteria and fungi. For two of the carriers we have shown the substrate specificity by heterologous expression in the fission yeast *Schizosaccharomyces pombe*. The STP1 (Sugar Transport Protein) protein is a monosaccharid transporter. The expression of STP1 mRNA is low in heterotrophic tissues like roots and flowers. High levels of expression are found in leaves and stems. The STP4 protein is a monosaccharid transporter as well, but its mRNA is most strongly expressed in sink tissues like flowers and roots. With PCR we found, that in *Arabidopsis thaliana* at least eight more transporter genes exist.

REGULATION OF SUGAR TRANSPORT AND SUCROSE STORAGE IN SUGARCANE (SACCHARUM SPEC.)

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Sugarcane cells were grown as suspension culture either in batch or in continuous mode. Sugar uptake proceeds by two high affinity hexose uptake systems (Km 1mM) and a diffusion - like uptake phase, which works for hexoses and sucrose. Growth at various sugar concentrations revealed that the active high affinity uptake systems are sufficient to support maximal growth rates, the diffusion - like phase has no bearing on growth.
In contrast high intracellular sucrose concentrations (above 150 mM) were only achieved at high extracellular sugar concentration, where the diffusion - like uptake system comes into play.
The taken up sugars (hexoses and sucrose) undergo both a rapid synthesis to sucrose (by SPS and SS) and a rapid sucrose hydrolysis (by invertase and SS). The storage of sucrose is the small difference between rapid synthesis and rapid degradation and is regulated by the activity of the involved enzymes.
Uptake of sugar (hexoses and sucrose) into the vacuole is rapid, but passive and hardly saturable. Neither ATP nor uncoupler affect the uptake, however PCMBS does. The passive nature of vacuolar uptake is corroborated by compartmental analysis, which revealed that sucrose in sugarcane cells is equally distributed at the same concentration between vacuole and cytosol.
Experiments with tissue slices of internodes of different developmental stage from sugarcane plants showed that sugar uptake into the cells and the rate of sucrose cycling (synthesis and degradation) are strongly developmentelly regulated in the sense that the enzyme activities become very slow in internodes of high sucrose content. The degree of vacuolisation precedes sucrose loading.

C-DNA CLONES HOMOLOGOUS TO THE SUGAR TRANSPORTER FAMILY, ISOLATED FROM RICINUS COMMUNIS.

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The cotyledon of Ricinus communis shows one of the highest sugar uptake rates reported for higher plants. Hexoses are taken up by a passive transport system (according lack of proton symport and membrane depolarisation) whereas sucrose is taken up by proton symport. Oligonucleotides of a length of 20 - 30 bases were contructed for two regions, which belong to the most conserved ones for all so far known genes, which code for sugar transporters. One is located at the end part of the 10th membrane spanning helix, the other is in the loop immediately after the 12th membrane span. The codon usage of Ricinus was deduced from several known gene sequences of that plant.

A m-RNA preparation from cotyledons was primed in the PCR with the constructed oligonucleotides and a series of c-DNA fragments was obtained. Sequencing them revealed at least 6 different clones, which all exhibited the conserved amino acid regions of sugar transporters, including some of the conserved amino acids which stand on isolated places in the gene. The homology to the published Arabidopsis hexose transporter is 40 - 70 % on amino acid level. Preparations of mRNA from roots did not yield detectable fragments in the PCR.

The full length clones related to the PCR fragments are isolated from a c-DNA library. Determination of organ specificity, developmental expression and substrate specificity will be attempted.

THE PROTON-SUCROSE SYMPORT: INHIBITION AND PHOTO-AFFINITY LABELING

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The proton-sucrose symport is an important component of the assimilate partitioning pathway in many plants. The interaction of several compounds which inhibit this porter through covalent modification, including PCMBS, NEM, DEPC, and Hg^{2+}, was examined in isolated plasma membrane vesicles (PMV). NEM was not an effective inhibitor of the symport under both energized (pH 6.0) and unenergized (pH 7.7) conditions. In contrast, PCMBS, DEPC, and Hg^{2+} blocked symport activity. However, in control experiments, it was discovered that Hg^{2+}, but not PCMBS or DEPC, dissipated the proton motive force that drives sucrose accumulation. It was further demonstrated that Hg^{2+} dissipated an imposed pmf in protein free liposomes, thus obscuring its effect on the sucrose symport. In time- and concentration-dependent inactivation experiments, it was shown that DEPC binding is substrate protectable, thereby implicating binding at or near the active site. In contrast, PCMBS activity was not linked to substrate binding. DEPC activity was reversible with hydroxylamine, suggesting interaction with histidine residues, and preloading purified vesicles with free histidine did not slow DEPC-dependent inactivation kinetics. Since the membrane vesicles are predominantly right-side out, the last observation is consistent with a DEPC-sensitive site which is accessible from the outside face of the vesicle. The PCMBS sensitive-site is also on the outside face since this compound cannot cross the membrane.

Two compounds that are known inhibitors of glucose porters, cytochalasin B and forskolin, inhibit proton-sucrose symport activity in PMV isolated from sugar beet leaves. For glucose porters, cytochalasin B binding is reversible and appears to be directed at the substrate binding site. Forskolin also appears to act at the active site, based on cyto B's ability to block forskolin binding. Since these compounds inhibit sucrose transport, we obtained an iodinated phenylazide derivative of forskolin to use as a photoaffinity label for the sucrose symport. It should be noted, that a glucose porter is also present in these PMV. Under conditions that result in sucrose transport inhibition, a single polypeptide of 55 kDa is labeled by the forskolin derivative in intact PMV after activation with uv light. Labeling was not blocked by glucose, sucrose, or cyto B. This wasn't too surprising since the affinity of the analogue for the glucose porter is reported to be much higher than native forskolin and substrate protection is poor. Significantly, however, cytochalasin B is able to protect glucose porters from photolabeling with this forskolin analogue. The absence of cyto B protection here suggests the protein labeled could be the sucrose symport. We are currently obtaining sequence information about this protein for comparison to predicted protein sequences derived from three sugar porters we've cloned from the same source tissue.

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CHARACTERISTICS OF TONOPLAST SUCROSE TRANSPORT INTO PROTEOLIPOSOMES

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Sucrose is the major organic constituent of sugarcane stalk tissue. In mature tissue the concentration of sucrose can approach 0.8M (60% of the dry weight). Most of the sucrose is compartmentalized in vacuoles of the storage parenchyma cells. To reach this compartment, the sugar must cross the tonoplast. We have previously described a system that demonstrates sucrose transport across the tonoplast of sugarcane cells (1, 2). Although sucrose uptake was stimulated by MgATP (1), the mechanism of this stimulation is not clear, since a sucrose-H^+ antiport could not be unequivocally demonstrated. Williams et al. (2), using tonoplast preparation isolated on a sucrose gradient, and Preisser and Komor (3), using isolated vacuoles, could not detect MgATP stimulation of sucrose uptake even though a proton gradient was generated by the addition of MgATP in both cases. Nevertheless, sucrose uptake was relatively high, even in the absence of MgATP. This uptake showed saturation kinetics and pH dependence and could be inhibited by sulfhydryl reagents as well as by an arginyl residue modifying reagent (1). These results indicated that sucrose transport is catalyzed by a protein. We recently prepared monoclonal antibodies against total tonoplast proteins, and several of these antibodies inhibited sucrose uptake into tonoplast vesicles. Using one of these monoclonal antibodies to monitor the protein during solubilization and purification, we partially purified a protein that takes up sucrose when reconstituted into liposomes.

We studied sucrose transport characteristics by using crude as well as partially purified tonoplast proteins reconstituted into liposomes. The effects of MgATP and ΔpH on energization of sucrose transport will be discussed The effects of inhibitors against specific amino acid residues and against specific transmembrane domains of known sugar transporters on sucrose uptake into proteoliposomes are being investigated. The results should lead to a better understanding of both the energization of sucrose transport into vacuoles and the functional requirements of the tonoplast sucrose carrier. (Funded in part by USDA, ARS Cooperative Agreement 58-914-143)

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STACHYOSE, AMINO ACID, GLUCOSE AND SUCROSE TRANSPORT IN PLASMA MEMBRANE VESICLES ISOLATED FROM ZUCCHINI

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Stachyose, a sugar of the raffinose series, is the predominant form of translocated sugar in the Cucurbits. Although recent evidence suggests stachyose is concentrated inside the phloem, the mechanism involved in stachyose loading is not known. We tested the hypothesis that a stachyose transporter on the plasma membrane, similar to the recently described proton-sucrose symport, is responsible for stachyose accumulation in the vascular tissue. Plasma membrane vesicles (PMV) were purified from mature leaves of zucchini using the aqueous phase partitioning method. These vesicles maintained an imposed proton electrochemical potential difference and they exhibited proton-coupled amino acid transport. Moreover, a facilitated glucose transport system was present in the zucchini plasma membrane. In contrast, no evidence of carrier-mediated stachyose or sucrose transport was observed. Since the amino acid symports were present in these PMVs, the absence of active stachyose and sucrose uptake suggests similar carriers were not present, although the possibility of differential inactivation cannot be eliminated. We also investigated transport activity in isolated tonoplast membrane vesicles. Although proton-coupled calcium transport was demonstrated, no active stachyose or sucrose transport was present. Taken together, these data suggest typical proton-coupled carrier proteins may not be responsible for stachyose accumulation in the phloem. Additionally, these observations lend support to the notion that stachyose synthesis in the phloem may account for its accumulation. If this is the loading mechanism, however, the source of free energy driving the synthesis reactions against a large concentration gradient has yet to be described.

MOLECULAR CLONING OF THREE SUGAR TRANSPORTERS FROM MATURE LEAF TISSUE

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A "super-family" of sugar transport proteins has recently been identified (1,2). This is a particularly interesting group of transport systems (≈20 members have been cloned thus far) for several reasons, including: *i)* members transport a variety of monosaccharides, *ii)* members include active (ion-coupled) and passive porters, *iii)* members are found in both prokaryotes and eukaryotes, and *iv)* a disaccharide porter is part of the family. These observations suggest the possibility that the plant's proton-sucrose symport is part of this "super-family" of porters. Therefore, we identified regions of sequence conservation in this family and used them as guides for the design of degenerate primers for RNA-polymerase chain reaction using mRNA extracted from sugar beet leaf tissue. This system was chosen because of our recent work describing the transport characteristics and bioenergetics of the proton-sucrose symport in plasma membrane vesicles isolated from this tissue (3,4). Three DNA fragments, of the predicted size, were amplified and subsequently cloned. Hydropathy plots of the derived amino acid sequences from all three clones are similar to those of other members of this family. Moreover, the amino acid sequence of the clones exhibited 20-30% identity to other members of the family and 25-30% identity among each other. Northern blot analysis of two clones demonstrated differential expression during leaf development. The third clone was not present in Northern blots of leaf derived message. Although this clone may have been derived from contaminating material, it is not homologous to previously cloned porters and, thus, this fragment may have been amplified from message of very low abundance. We believe these clones represent excellent candidates as the genes encoding both the glucose and sucrose symports of the leaf plasma membrane. We are currently attempting to determine, directly, the function of the encoded proteins by expressing these clones in yeast sugar transport mutants.

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SOLUBILIZATION AND RECONSTITUTION OF GLUTAMINE AND SUCROSE CARRIERS FROM TISSUES OF RICINUS COMMUNIS

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Sucrose and amino acids are actively transported into plant cells by proton-coupled carriers (Reinhold and Kaplan 1984). Plasma membrane vesicles, purified by aqueous two phase partitioning, were used previously to carry out an *in vitro* characterization of these proteins found in *Ricinus communis* cotyledons (Williams et al. 1990). Plasma membrane vesicles isolated from *Ricinus communis* roots have also been found to possess a proton-coupled glutamine carrier that has similar properties to that in the cotyledons (Williams et al. 1992).

This report further characterizes the properties of the amino acid carrier, both in root native plasma membrane vesicles and following solubilization and reconstitution into liposomes. Glutamine transport in plasma membrane vesicles was enhanced in the presence of a ΔpH and further stimulated when an internal negative $\Delta\psi$ was imposed across the vesicles. Uptake was inhibited in the presence of the protonophore CCCP. Transport in the reconstituted system showed similar properties.
Data is also presented characterizing sucrose and glutamine transport following solubilization of these carriers from cotyledon plasma membrane and reconstitution into liposomes.

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FUNCTIONAL RECONSTITUTION OF A PURIFIED VACUOLAR H^+-ATPase FROM OAT ROOTS.

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A vacuolar H^+-ATPase (V-type ATPase) of plant cells generates a proton motive force across the tonoplast (acidic and positive inside). This primary energization of the membrane controls the secondary accumulation of other solutes, via H^+-coupled transport and through channels, which is required for the various functions of the vacuole. Purification of the vacuolar H^+-ATPase from oat roots (*Avena sativa* var Lang) indicated that ten polypeptides were associated with ATP hydrolysis activity (1). To determine if this purified enzyme retained H^+ transport capability and to study the regulation of H^+-transport activity in an isolated system, the purified H^+-ATPase was incorporated into phospholipid vesicles.

Formation of sealed proteoliposomes was accomplished by removing Triton X-100 from the purified H^+-ATPase in the presence of sonicated *E. coli* phospholipids. The quenching of acridine orange fluorescence was used to probe the acidification of K^+-loaded proteoliposomes. The K^+ ionophore valinomycin stimulated MgATP-dependent acidification, indicating that H^+ transport was electrogenic. Proton transport was inhibited by Bafilomycin A_1, DCCD, and NO_3^- (inhibitors of V-type ATPases), and was insensitive to azide and vanadate (inhibitors of mitochondrial and plasma membrane H^+ pumps, respectively). Chloride stimulated H^+ transport in the presence of K^+ and valinomycin (2). This result indicated that the plant V-type ATPase, in contrast to some animal V-type ATPases, was stimulated directly by Cl^-.

The purified H^+-ATPase from oats contained ten polypeptides of 70, 60, 44, 42, 36, 32, 29, 16, 13, and 12 kDa. We have now shown that this set of polypeptides was sufficient for coupled ATP hydrolysis and H^+ transport. Significantly, this preparation lacked a 100-115 kDa polypeptide suggested to be required for the H^+-transport activity of some animal V-type ATPases.

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CHANGES IN GENE EXPRESSION ASSOCIATED WITH VACUOLAR TRANSPORT AND INDUCTION OF CRASSULACEAN ACID METABOLISM

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Leaf mesophyll vacuoles play an important role in plants demonstrating crassulacean acid metabolism (CAM), being involved both in cytoplasmic homeostasis and malic-acid storage during the day-night cycle. Pivotal to this role are the transport activities of particular proteins in the vacuolar membrane. In CAM plants, an H^+-ATPase, an H^+-PP_iase, a malate channel, and possibly other transport proteins may be involved in the regulation of vacuolar malic-acid accumulation. Since the transition from C_3 photosynthesis to CAM is under developmental and environmental control, such plants provide a useful system for studying the molecular basis of ion transport at the vacuolar membrane and its relationship to the physiological activities of the cell.

Identification of the genes encoding these and possibly other transport proteins involved in CAM has been pursued by preparing total RNA and mRNA fractions from uninduced (C_3) and induced (CAM) leaf tissue from *Kalanchoë* species. These fractions are being probed with PCR primers designed to conserved regions of three known gene superfamilies for transport proteins: (1) the ATP-binding cassette (ABC) superfamily (which includes the multidrug resistance P-glycoprotein and cystic fibrosis gene product), (2) the *Nod 26* and tonoplast intrinsic protein (TIP) superfamily, and (3) the sugar transporter superfamily. Additional screening of genomic DNA is being performed with cDNA clones from genes for selected transport proteins. For mRNA expression studies, the C_3 to CAM transition has been followed during the course of leaf development in *K. daigremontiana* and during CAM induction by short-day photoperiods in *K. blossfeldiana*. Results of the comparison of steady-state mRNA levels for putative transport proteins in uninduced and induced CAM leaf tissue will be discussed in relation to the control of transport activity at the vacuolar membrane in CAM plants.

CHARACTERIZATION OF cDNAs ENCODING THE 16 kDa PROTEOLIPID OF THE VACUOLAR H^+-ATPase FROM *ARABIDOPSIS*.

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The proton motive force generated by the vacuolar H^+-translocating ATPase provides the primary driving force for transport of numerous ions and metabolites across the plant vacuolar membrane. This multimeric enzyme consists of a nucleotide-binding peripheral sector and an integral sector that forms the H^+ channel (1). The N,N'-dicyclohexylcarbodiimide-binding 16 kDa proteolipid is the major component of the integral sector, essential for both H^+-ATPase activity and assembly of the enzyme complex (2).

As a step towards understanding the physiological and developmental regulation of the vacuolar H^+-ATPase, cDNAs encoding the 16 kDa proteolipid from *Arabidopsis* have been obtained. An *Arabidopsis* leaf cDNA library was screened using the oat cDNA encoding the 16 kDa proteolipid subunit of the vacuolar ATPase as a probe (3). Two similar but distinct cDNAs have been isolated and partially characterized. The cDNAs shared approximately 84% nucleotide sequence similarity. The derived amino acid sequences were nearly identical to the oat 16 kDa polypeptide. The *Arabidopsis* cDNAs differed from each other and the oat cDNA predominantly by third position nucleotide changes. Genomic Southern blots probed with the oat cDNA revealed at least three distinct hybridizing restriction fragments indicating the presence of a small multigene family encoding the proteolipid subunit in *Arabidopsis*.

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Regeneration of the vacuole after evacuolation

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When subjected to density gradient centrifugation (Griesbach and Sink 1983), mesophyll protoplasts can be evacuolated. This results in the formation of miniprotoplasts which contain about 50 to 70% of the activities of the cytosolic enzymes and nearly the full activities of organelle-bound enzymes (Hampp et al. 1985). Fusion of evacuolated protoplasts with evacuolated protoplasts has the advantage that, during the fusion, only one vacuole is present and therefore the risk of the release of hydrolytic activities into metabolically active compartments is reduced. In spite of the potential of this system for the study of the synthesis and processing of vacuolar and tonoplast proteins, and for obtaining additional information about the ontogeny of vacuoles, no study has been performed thus for. Tobacco mesophyll protoplasts were evacuolated by centrifugation in a density gradient. Evacuolation resulted in the quantitative loss of vacuolar hydrolytic activities. The evacuolated miniprotoplasts were cultivated under different conditions. The regeneration of the central vacuole was investigated by light and electron microscopy using the freeze substitution method and by determining the activities of vacuolar marker enzymes. Vacuoles and hydrolytic activities, as well as cell wall material, reappeared faster when the cells were cultivated at low osmotic strength. Replacement of mannitol by NaCl as an osmoticum greatly enhanced the growth rate of the newly formed vacuole. However, synthesis of hydrolytic activities was not enhanced. A newly synthesized tonoplast polypeptide could be detected by using a polyspecific serum raised against barley tonoplast proteins. Expression of the mRNA specific for the 60 kD subunit of the tonoplast ATPase was dependent on the growth rate of the newly synthesized vacuole. Tonoplast-bound pyrophosphatase activity could also be detected. We are now looking for the expression of this proton pump and hope to gain some insight into the role of the two proton pumps in the early development of vacuoles.

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TWO CATALYTIC VACUOLAR H^+-ATPase SUBUNITS ARE DIFFERENTIALLY REGULATED IN RAPIDLY ELONGATING SEED TRICHOMES

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Developing cotton seed trichomes provide a unique opportunity to study vacuolar biogenesis in plants. Cotton seed trichomes are single epidermal cells exceeding one-inch in length that differentiate from the outer integument of the ovule. The onset of trichome elongation at anthesis is accompanied by dramatic morphological changes in the vacuolar compartment. Rapid enlargement of a central vacuole is associated with the accumulation of significant ATPase activity localized in the tonoplast and interestingly, the endoplasmic reticulum (ER). We are therefore interested in investigating the regulation, assembly and transport of vacuolar H^+-ATPases (VATPase) in rapidly elongating cotton trichomes as a focal point to studying vacuolar biogenesis.

The 69 kD catalytic VATPase subunit is encoded by two highly conserved genes in cotton. Gene-specific cDNA probes corresponding to the 5'-untranslated regions were constructed to study the spatial and temporal regulation of the 69 kD VATPase subunits in developing cotton trichomes. Northern and RNA slot blot analysis demonstrated that the VATPase catalytic subunit is developmentally regulated in elongating cotton trichomes. The catalytic subunit mRNAs increase significantly to peak levels at two stages of development corresponding to the onset of trichome elongation at anthesis and again at 10 days-postanthesis (dpa). The increase in 69 kD VATPase mRNAs at 10 dpa coincides with maximum rates of elongation. Moreover, Northern blot analysis coupled with *in situ* RNA hybridization revealed that the two VATPase mRNAs are differentially regulated in developing cotton ovules. Two non-allelic genetic trichome mutants defective in elongation (Li_1 and Li_2) also showed a deviation from normal VATPase catalytic subunit expression patterns in developing trichomes. Future endeavors will be directed towards tracing the route of VATPase transport through the endomembrane system of elongating trichomes in wild-type and genetic trichome mutants.

STRUCTURE OF THE VACUOLAR ATPASE FROM *NEUROSPORA CRASSA* AS DETERMINED BY NEGATIVE STAIN ELECTRON MICROSCOPY

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The vacuolar class of ATP-driven proton pumps is responsible for acidifying regions within the endomembrane network of eucaryotic cells. Biochemical and molecular data suggest the vacuolar-type ATPase to be comprised of at least nine different subunits with a total molecular weight of approximately 700 kDa. Sequence analysis of the major subunits of the vacuolar-type ATPase shows that the enzyme is homologous to the F-type ATPases found in chloroplast, mitochondrial, and eubacterial membranes.

Both the F-type and vacuolar-type ATPases are partitioned into two domains: a peripheral sector which hydrolyzes ATP and a membrane-spanning channel through which protons are passed. We have examined the structure of the vacuolar ATPase of vacuoles from the filamentous fungus *Neurospora crassa*. Whether using intact or detergent-solubilized membranes, the enzyme complex was strikingly similar to, yet distinctly different from, the F-type ATPase structure found on mitochondrial membranes. The structure resembles a "ball-and-stalk" atop a membraneous vesicle. The head domain measured approximately 12.5 nm in diameter and displayed a distinct cleft down the middle of the complex. This domain was perched on a prominent stalk roughly 3 by 8 nm long. Most intriguing was the presence of basal components which appeared to project from the membrane at the base of the stalk.

The peripheral sector can be released from the membrane and examined as a soluble complex. It appeared as a roughly spherical structure, 12.5 nm in diameter. The structural details seen in the membrane-bound ATPase were retained after release from the membrane.

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DIFFERENTIAL EFFECTS OF SULFITE ON ATPASE AND PROTON PUMPING ACTIVITY OF THE YEAST VACUOLAR ATPASE

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Gradient-purified vacuolar H^+-ATPase of S. cerevisieae exhibits nonlinear hydrolysis kinetics characterized by a rapid initial phase (v1) lasting for about 10 minuts, followed by a slower steady state rate (v2). Sulfite strongly stimulates the V-ATPase activity during the v2 phase of the reaction. Since the v2 phase is believed to represent an inhibited sdtate of the enzyme caused by tightly bound ADP at the catalytic site, sulfite may stimulate activity by promoting release of the tightly-bound nucleotide. Whereas ADP inhibition in the presence of chloride is strictly competitive, mixed inhibition is oberved in the presence of sulfite, suggesting the participation of a regulatory site as well. Sulfite also protects the enzyme against cold-inactivation, as well as inhibition by NBD-Cl, NEM and DCCD. These reuslts induicate that sulfite causes a conformational change in the enzyme which is trtansmitted down to the channel.

In contrast to its effects on hydrolytic activity, sulfite inhibits proton pumping by the V-ATPase in sealed native tonoplast vesicles. When the hydrolysis assays are carried out under the same conditions used for proton pumping, i.e. in the presence of glycerol, sulfite inhibits hydrolysis as well. Thus it appears that the effects of sulfite are strongly influenced by $\Delta\mu H+$.

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MOLECULAR ANALYSIS OF THE A SUBUNIT OF THE YEAST VACUOLAR ATPASE: THE ROLE OF CYSTEINES AND THE NONHOMOLOGOUS REGION

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The A subunit (~70 kD) of the eukaryotic V-ATPase contains several conserved cysteine residues within or near the catalytic site which may account for the sensitivity of V-ATPases to the sulfyhdryl inhibitor, NEM. We changed two of these, Cys-254 and Cys-261, to serines and tested for nitrate-sensitive ATPase activity and sensitive to NEM and NBD-Cl. Cys-261 is more highly conserved than Cys-254 and is located adjacent to the putative phosphoanhydride-binding motif: GKTV. We also tested a double mutant as well. All three mutants exhibited nitrate-sensitive ATPase activity, indicating that the cysteines are not required for catalytic activity. Cys261->Ser and the double mutant were both insensitive to NEM and NBD-Cl, whereas the Cys254->Ser mutant was still sensitive to these inhibitors. These results suggest that Cys-261 is the binding site of NEM and NBD-Cl.

We have also conducted experiments to determine the function of the "nonhomologous region." This domain is absent from the F-ATPase β subunits, but is present in archaebacterial ATPases and is believed to represent an evolutionary insert. A stretch of seventy amino acids from Arg-160 to Thr-230 was deleted from the nonhomologous region of the A subunit. The mutant cells containing the shortened A subunit could grow slowly at pH 7.5, suggesting that the V-ATPase is catalytically active. Like the wild-type enzyme, the deletion mutant is stimnulated by sulfite and inhibited by zinc. However, the mutant enzyme appears to be stimulated rather than inhibited by nitrate. The results suggest that the characteristic nitrate-sensitivity of the V-ATPases may be a function of the nonhomologous region.

EFFECT OF NITRATE ON THE PROTON GRADIENT AND MEMBRANE POTENTIAL OF THE TONOPLAST MEMBRANE OF LETTUCE.

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For many plant species nitrate is one of several solutesthat accumulate in the vacuoles to maintain cell turgor. Accumulation of nitrate in the vacuoles may rise to high concentrations (up to 100 mM) and is inversily related to the concentration of organic acids.

Vacuoles contain already a large proton gradient afterisolation, but they do not have a membrane potential. In this condition they do not take up nitrate. Influx of nitrate in the vacuoles is only possible when a membrane gradient is generated at the expense of ATP. Indeed, nitrate uptake is stimulated by ATP. This indicates a coupling between nitrate and ATPase.

Nitrate also inhibits ATPase activity, as shown abundantly in the literature. However, nitrate can also stimulate proton pumping by ATPase, provided that a membrane potential is present. This depends on the order of addition: nitrate inhibits ATPase when the anion is added to the assay before addition of ATP. Nitrate initiates proton pumping when ATP is present at the start of the assay.

To study a situation similar to the one in the vacuoles, vesicles were used with an existing proton gradient, without or with an imposed membrane potential. The change of the proton gradient or membrane potential were monitored with quinacrine or oxonol fluorescence quenching, respectively.

Addition of nitrate to the vesicles results in a transient increase of the proton gradient followed by a dissipation. Addition of PP_i recovers the proton gradient completely. The appearance of the transient increase depends on the nitrate salt used. The slope of the dissipation depends on the the nitrate concentration and obeys Michaelis Menten kinetics. The dissipation likely due to proton leakage, resulting from ATPase inhibition by nitrate.

Addition of nitrate to vesicles with an imposed membrane potential causes a dissipation of the proton gradient. Addition of PP_i does not recover this gradient.

The results will be discussed in the poster.

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THE VOLTAGE-DEPENDENT H^+ ATPASE OF THE SUGAR BEET VACUOLES IS REVERSIBLE

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The vacuolar H^+ ATPase is essential for the creation and maintainance of solute gradients. Knowledge of the reversal potential expressed by the voltage and pH dependence of the pump determines the activity range of the enzyme.

We studied the voltage-dependence of the pump using the whole-vacuolar configuration of the patch-clamp technique (1). In the presence and absence of the pH gradients across the vacuolar membrane voltage-steps to various depolarizing and hyperpolarizing potentials were applied. When the cytoplasmic surface of the vacuolar membrane was perfused with Mg-ATP solutions, inward-directed H^+ currents depolarized the membrane potential. In the presence of Mg-ATP the current-voltage relationship of the pump was characterized by an almost linear increase in inward H^+ in the range of -20 mV to +100 mV. Currents generated by the H^+ ATPase tend to saturate at more positive potentials.

In the presence of a pH-gradient (inside-acid) the application of ADP+P resulted in the release of H^+ from the vacuole and hyperpolarization of the membrane potential. Our results indicate that under conditions which thermodynamically allow ATP-synthesis the physiological direction of the H^+ fluxes through voltage-dependent ATP-hydrolyse can be reversed.

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IN VITRO ASSEMBLY OF THE VACUOLAR-TYPE ATPASE

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The V-type, ATP-dependent proton pump is a complex enzyme composed of integral and peripheral membrane associated sectors. This pump acidifies various endomembrane systems in eukarotic cells, and in higher plants provides a major driving force for transport of substances into the large central vacuole. In some systems, however, this enzyme appears to reside on a membrane that is distinct from the vacuole membrane. We have found, for example, that the membrane distribution of the V-type ATPase is different in vascular vs cortical tissues derived from Apium aveolens. In vascular tissue, the ATPase (as measured by western blot analysis or activity) is found in a rather disperse distribution with virtually no activity in the low density regions as is found in cortical tissues. Interestingly, H^+-PPiase activity is not differentially distributed in this manner, as this enzyme is uniformly found on low density membranes.

Our efforts are focused on determining the molecular basis underlying biosynthesis, assembly, and targeting of the V-ATPase. Initial results indicate that one of the peripheral subunits (the 70 kDa subunit containing the catalytic ATP binding site) is assembled with the integral membrane sector on the E.R. This is substantiated by the observation that mRNA encoding this subunit (but not another peripheral subunit, the 60 kDa regulatory subunit) is associated only with membrane-bound polysomes. Further, in vitro assembly assays indicate the 70 kDa subunit can associate with microsomal membranes, however, only in the presence of other mRNA encoded factors. It is therefor likely that the 16 kDa subunit (the integral membrane proton pore) or other subunits may serve as assembly site. Despite the indication that the catalytic subunit is synthesized and assembled on the E.R. membrane, it appears to be neither translocated through nor inserted into the membrane and it has no apparent cleaved targeting sequence. Interestingly the 60 kDa subunit is not assembled on the E.R. in vitro. These data suggest that the V-ATPase is partially assembled on the E.R., and is subsequently sorted to the vacuolar membrane. At some point during its transit to the vacuole it becomes activated, perhaps through the addition of the 60 kDa regulatory subunit and other ATPase subunits. This is likely to occur sometime before transit through trans Golgi as acidification and ATPase activity have been measured on this organelle. Efforts are presently focused on determining requirements for the assembly of the 70 kDa subunit on the E.R. membrane and those requirements for subsequent sorting to the vacuolar membrane.

KINETICS OF THE VACUOLAR H^+-PYROPHOSPHATASE: THE ROLES OF MAGNESIUM, PYROPHOSPHATE, AND THEIR COMPLEXES AS SUBSTRATES, ACTIVATORS AND INHIBITORS

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The responses of the vacuolar membrane (tonoplast) proton-pumping inorganic pyrophosphatase (H^+-PPase) from oat (*Avena sativa* L.) roots to changes in Mg^{2+} and PPi concentrations have been characterised. The kinetics are complex and reaction kinetic models have been used to determine which of the various complexes of Mg and PPi are responsible for the observed responses. The results indicate that the substrate for the oat root vacuolar H^+-PPase is Mg_2PPi and that this complex is also a non-competitive inhibitor. In addition, the enzyme is activated by free Mg^{2+}, and competitively inhibited by free PPi. This conclusion differs from that reached in previous studies (1, 2, 3) which proposed that MgPPi is the substrate for plant vacuolar H^+-PPases. However, models incorporating MgPPi as a substrate were unable to describe the kinetics of the oat H^+-PPase. Models incorporating Mg_2PPi as the substrate can describe some of the published kinetics of the *Kalanchoë daigremontiana* vacuolar H^+-PPase (3). Calculations of the likely concentrations of Mg_2PPi in plant cytoplasm suggest that the substrate binding site of the oat vacuolar H^+-PPase would be about 70% saturated *in vivo*.

In other studies using the residue-specific reagents, N-ethylmaleimide (NEM) and phenylglyoxal (POG) we have found evidence for the presence of essential cysteine and arginine residues in the H^+-PPase (see also 4, 5). Preliminary results indicate that the histidine-specific reagent, diethylpyrocarbonate, is also strong inhibitor. To investigate further the role of the various Mg and PPi complexes in the activity of the PPase we determined their ability to protect the enzyme from inhibition by these reagents. Experiments with NEM and POG have shown that both Mg_2PPi and free Mg^{2+}, but not free PPi, protect the PPase and that they interact in their protective effects. These findings support the conclusion that Mg_2PPi binds to the enzyme and that it may be the substrate.

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MOLECULAR PROPERTIES OF VACUOLAR H^+-PPase AND INTEGRAL MEMBRANE PROTEIN

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Transport of ions and metabolites at the vacuolar membrane is supported by two proton pumps, H^+-pyrophosphatase (PPase) and H^+-ATPase. We are conducting a series of experiments to characterize the proton pumps and the other elements of vacuolar membrane at molecular level. The H^+-PPase (1) and H^+-ATPase (2) were purified from mung bean hypocotyls previously. In contrast to the ATPase (nine subunits), the PPase is a compact energy-transducing enzyme consisting of a single polypeptide of 73 kD (1). This will be confirmed by reconstitution of the enzyme into liposomes. The functional form of PPase may be a homodimer (3, 4). The antibody against mung bean PPase reacted with the enzymes from marine alga (*Acetabularia acetabulum*, 5) and photosynthetic bacteria (*Rhodospirilum rubrum*, 6), but not with yeast cytoplasmic PPase or rat liver mitochondrial PPase (7). The PPase requires Mg^{2+} as a stabilizer and activator. Calcium ion strongly inhibits the enzyme through formation of CaPPi (7). These enzymatic properties are similar to the soluble PPase. The PPase is a key enzyme for understanding the molecular evolution of H^+-translocating enzymes and the mechanism of biogenesis of vacuolar membrane in elongating cells (8).

Recently, a major integral protein (23 kD, VM23) of vacuolar membrane was purified from radish (9). VM23 was extracted from the membranes by chloroform: methanol, and the hydrophobic carboxyl modifier DCCD bound to it. This hydrophobic protein is one of the abundant, conserved proteins among higher plants. The function of VM23 remains to be investigated.

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RECONSTITUTION OF TRANSPORT FUNCTION OF VACUOLAR H^+-TRANSLOCATING PYROPHOSPHATASE

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With the long-term objectives of defining the precise transport capabilities and structure-function partitioning of the vacuolar H^+-translocating inorganic pyrophosphatase (H^+-PPase) a method is described for reconstitution of the transport function of the enzyme prepared from etiolated hypocotyls of *Vigna radiata*. The method entails sequential extraction of isolated tonoplast vesicles with deoxycholate and CHAPS, admixture of CHAPS-solubilized protein with cholate-dispersed cholesterol:phospholipid mixtures, dialysis and glycerol gradient centrifugation. The final density gradient-fractionated proteoliposome preparation is 9-fold enriched for PPase activity and active in PPi-dependent, electrogenic H^+-translocation. Since PPi hydrolysis and PPi-dependent H^+-translocation by the proteoliposomes are indistinguishable qualitatively from the corresponding activities of native tonoplast vesicles, the functional integrity of the H^+-PPase appears to be conserved throughout the solubilization and reconstitution protocols. These characteristics, in conjunction with the amenability of the preparation to both radiometric membrane filtration and fluorimetric assays, demonstrate its applicability to a broad range of transport assays.

SDS-PAGE analysis of the reconstituted enzyme reveals selective enrichment of the previously identified M_r 66,000, substrate-binding subunit of the H^+-PPase (1, 2, 3) and two additional polypeptides of M_r 20,000 and 21,000. The sufficiency of the M_r 66,000 subunit, alone, for PPi-dependent H^+-translocation and the involvement of the M_r 20,000 and 21,000 polypeptides in PPase function will be discussed with regard to the results of studies directed at determining the transport-competence of reconstituted preparations depleted or enriched for the latter two polypeptides. In the light of recent electrophysiological studies implicating the H^+-PPase in energized K^+ transport across the tonoplast (4), data concerning the capacity of the reconstituted enzyme for $\Delta\mu_{H^+}$-independent, PPi-energized H^+-K^+ symport will also be presented.

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CALCIUM EFFECTS ON THE PYROPHOSPHATASE ACTIVITY OF THE TONOPLAST FROM *ACER PSEUDOPLATANUS* CELLS.

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Two protons pumps, H^+-ATPase (adenosine triphosphatase) and H^+-PPase (pyrophosphatase) are associated with the tonoplast of *Acer pseudoplatanus* cells (1). The proton gradient provide an energy source which can be used for secondary transports. The H^+-PPase has been characterized and purified (1,2). The present paper reports the effects of calcium on both tonoplast bound- and purified PPase in order to investigate its possible regulation by phosphorylation/dephosphorylation reactions catalysed by Ca^{2+}/CaM dependent protein kinases.

Tonoplast vesicles were obtained from isolated vacuoles (3). PPase is purified by gel filtration (Sephacryl HR 200) and anion exchange chromatography (FPLC, Mono Q column). The PPase activity (MgPPi hydrolysis) was measured in presence of free Ca^{2+}, with or without calmodulin (CaM) and in the presence or in absence of CaM antagonists (trifluoperazine or W7).

The tonoplast bound- and purified PPase activities were inhibited by Ca^{2+}. Calcium decreased the tonoplast PPase activity to 50 % at 10 µM Ca^{2+} compared to 20 % for purified PPase. Futhermore, inhibition by Ca^{2+} of tonoplast bound-PPase and purified PPase activities were reversed after addition of EGTA. The addition of CaM did not modify Ca^{2+} effects but CaM antagonists inhibited the activity of tonoplast bound-PPase (I_{50}=100 µM trifluoperazine and I_{50}=300 µM W7) and were uneffective on the purified PPase.

CONCLUSION : Ca^{2+} inhibits both tonoplast bound- and purified PPase activities ; inhibition was immediate and reversible as reported for the tonoplast PPase of mung bean (4). The sensitivity of the purified PPase to Ca^{2+} did not exclude an inhibition of the PPase by Ca^{2+}/CaM dependent phosphorylation reactions. Indeed CaM antagonists inhibited tonoplast bound-PPase activity and not purified enzyme. Taken together, our results suggest that the vacuolar PPase is sensitive to Ca^{2+} and could be regulated by Ca^{2+}/CaM dependent protein kinases.

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ION TRANSPORT SYSTEMS IN ACETABULARIA

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A. Cl^--translocating ATPase in Acetabularia acetabulum

(1) Biochemical studies: A Cl^--translocating ATPase was isolated from A. acetabulum (1). The enzyme consisted of two subunits (a, 54 kDa; b, 50 kDa), and amino acid sequences of peptides isolated from the subunits showed 40 to 100% similarity to the α and β subunits of chloroplast ATPase in higher plants, respectively. Reconstitution studies (2,3) revealed that the binding of 2 Cl^- per enzyme molecule was required for the Cl^-- transport and Br^- and F^- diminished the ATP-driven Cl^- transport activity.

(2) Molecular cloning: A DNA fragment (ca. 160 bp) encoding the a subunit of the Cl^--ATPase was isolated after amplification of a cDNA library.

B. Cation-translocating ATPases in A. acetabulum

The $\alpha\beta\gamma$ complex of the chloroplast ATPase was purified and characterized. A vacuolar ATPase and inorganic pyrophosphatase were identified by their H^+-transport activities and immunoreactivities with the antibodies against the large and small subunits of mung bean vacuolar ATPase, and pyrophosphatase (4). The PCR products encoding the α (270 bp) and β (270 bp) subunits of CF_1-ATPase, and the large (280 bp) and small (690 bp) subunits of vacuolar ATPase were isolated and cloned. The deduced amino acid sequences showed high similarity (over 90%) to the analogous subunit from the higher plant enzymes.

C. Sulfate permease in A. acetabulum

A 359 bp fragment was isolated after amplification by PCR. The deduced amino acid sequence showed 55% identity to Anacystis nidulans CysA protein involved in sulfate permease complex.

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EVIDENCE FOR CA^{++}-GATED PROTON FLUXES IN CHLOROPLAST THYLAKOID MEMBRANES: CA^{++} CONTROLS A LOCALIZED TO DELOCALIZED PROTON GRADIENT SWITCH

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Chloroplast thylakoid ATP formation can be driven by either localized or delocalized proton gradients, and the coupling response can be reversibly switched by several treatments, all of which act through perturbing membrane-bound Ca^{++} (1,2). Direct measurement of lumenal pH using pH-sensitive fluorescent dyes loaded into the lumen showed that the lumen pH did not reach the energetic threshold pH under active phosphorylation ($\Delta pH \leq$ 1.7 units when ≥2.3 units are required) in the localized-mode thylakoids (low-salt stored), but with the delocalized mode thylakoids the ΔpH was ≥2.5 (high-salt stored). The low-salt stored thylakoids gave a $\Delta pH \geq 2.5$ under basal conditions, the same ΔpH range as for high-salt stored membranes in either basal or coupled conditions.

Covalently linked probes (photoaffinity labeling with [^{3}H]chlorpromazine) for tight Ca^{++}:protein adducts showed that the 8 kDa CF_0 part of the H^+ channel either provides, or is close to, the Ca^{++} binding site involved with the H^+ gradient gating events (2). The purified 9 subunit CF_0-CF_1 reconstituted into liposomes also showed a strong photoaffinity labeling of the 8 kDa CF_0 proteolipid.

Evidence for gating of H^+ fluxes at the CF_0 H^+ channel structure suggests that the gating site, and hence the putative Ca^{++} binding site, is at the inner boundary of CF_0 H^+ channel; i.e., at the lumenal side of the CF_0. The evidence for this is that chlorpromazine binding to the 8 kDa proteolipid blocks H^+ flux from both directions. With chlorpromazine bound to the CF_0, electron transport-driven H^+ uptake can occur into sequestered domains, sufficient to drive active, localized $\Delta\tilde{\mu}_{H^+}$-driven ATP formation, but the H^+ ions do not readily equilibrate into the lumen, as judged by the failure of lumenally located amines to give extra H^+ uptake. Chlorpromazine bound to the CF_0 can also block the succinate-driven dark acid-base-driven ATP formation, by blocking lumenal H^+ ions – originating from the succinic acid – from effluxing out through the CF_0 channel. In both cases, the chlorpromazine blocking action is not a complete block, rather it appears that the drug increases the Ca^{++} binding affinity at the gating site; i.e., by decreasing the pK_a of the putative Ca^{++} binding carboxyl groups. By increasing the acidity either by a lower (lumen) pH in the acid part of the acid-base jump or by a greater redox-driven H^+ uptake into the sequestered domains, the chlorpromazine blocking action can be overcome. We interpret this as resulting from the eventual protonation of the Ca^{++} binding carboxyl groups with displacement of the Ca^{++} and gate opening.

The localized ↔ delocalized H^+ gradient gating appears to be coupled to other physiologically important events in chloroplasts including a high light stress protective response involving the violaxanthin - zeaxanthin cycle The V→Z conversion requires pH <5.9 for maximum activity. We found that significant differences occurred in the V→Z conversion when the localized $\Delta\tilde{\mu}_{H^+}$ coupling was compared to delocalized $\Delta\tilde{\mu}_{H^+}$. This could indicate an interesting stress response signal associated with shifting from localized to delocalized energy coupling modes.

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MEMBRANE TRANSPORT MODIFIED BY MICROBIAL PHYTOTOXINS

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The attack of phytopathogens to plant cells often starts with or is accompanied by the release of toxins. On their way to the target(s) the plasmalemma (PL) is the first site of interaction of toxins. Irrespective whether they remain within the PL or pass it, they can modify the membrane properties and functions.

For transport processes, the energization of the PL is an important parameter. The PL proton pump ensures energization by building up and maintaining the electrical potential difference (PD) and the proton gradient across the PL provided ion leakage does not shunt the membrane. This well-regulated process might be disturbed by the interaction with phytotoxins.

On the basis of the GOLDMAN-HODGKIN-KATZ, a modified HODGKIN-HOROWICZ and the GOUY-CHAPMAN-STERN equations, we developed a strategy to investigate such interactions. It includes measurements on planar bilayer membranes (BLM), liposomes, membrane vesicles, protoplasts, vacuoles, membrane patches and the whole cell. The well-known phytotoxins **fusicoccin** (FC) and **tentoxin** as well as the less characterized **coronatine**, **phaseolotoxin** and **syringomycin** were examined with respect to their effects on the model membranes (BLM resistance, surface potential of liposomes) and on biomembranes (PD across the PL, I-U curves and medium acidification by tissues using leaves, leaf cells and their protoplasts of Egeria densa the energization of PL vesicles from Chenopodium rubrum and of vacuoles from Riccia fluitans) as well as on stomatal opening in epidermal strips of Commelina communis.

The results approved the strategy. Full consistency was obtained with FC, syringomycin, and tentoxin. Less conclusive results were obtained for the remaining phytotoxins suggesting that their target(s) are not membrane-bound. The efficacy of variation of this strategy is discussed.

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The results approved the strategy. Full consistency was obtained with FC, syringomycin, and tentoxin. Less conclusive results were obtained for the remaining phytotoxins suggesting that their target(s) are not membrane-bound. The efficacy of variation of this strategy is discussed.

EFFECTS OF BETICOLIN, THE YELLOW *CERCOSPORA BETICOLA* TOXIN ON THE PLASMALEMMA ATPase ACTIVITY AND ON THE TONOPLAST ATPase AND PYROPHOSPHATASE ACTIVITIES

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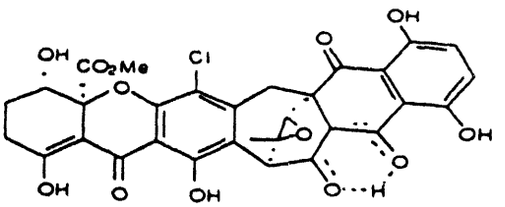
Beticolin was extracted from the mycelium of *Cercospora beticola*. The structure has been elucidated by X-Ray analysis (1). The formula of the compound is $C_{31}H_{23}ClO_{13}$ (MW 638).



The effects of the toxin have been investigated on the maize root plasma membrane ATPase after solubilization and reactivation using lysophosphatidylcholine. Beticolin inhibited the plasma membrane ATPase activity (I_{50}=4.8µM). This inhibition depended on incubation time, pH and lipid/protein ratio. Lineweaver-Burk plot analysis revealed competitive inhibition kinetics (Ki=1.7µM). These effects are similar than those previously reported for CBT (2). Beticolin was assayed on ATPase and PPase activities of tonoplast obtained from isolated vacuoles of sycamore cells. It inhibited both activities but only with a weak incubation time dependency. The ATPase was more sensitive than the PPase ; a total inhibition of the ATPase activity was obtained whereas PPase was very slightly inhibited (15%). As mentioned above for the plasma membrane ATPase, the inhibition of the tonoplast ATPase by beticolin was competitive with respect for ATP (Ki=76nM) and thus the tonoplast ATPase was much more sensitive than the PM ATPase.

Conclusion : We identified a yellow phytotoxin produced by *Cercospora beticola* and studied its inhibitory effects on plasmalemma and tonoplast ATPase activities. Recently, Hossain *et al.* reported the structure of a compound they named "cebetin" which is an isomer of the beticolin studied in the present work (3) All these data suggest that the yellow toxin of *Cercospora beticola* previously called CBT includes different isomers. This hypothesis is supported by experiments in progress in our laboratory which show that *C. beticola* mycelium contains other yellow compounds with the same chemical skeleton. Furthermore, the production of all these toxins varied with the strain cultivated and the growth medium used.

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PURIFICATION AND CHARACTERIZATION OF THE CALLOSE SYNTHASE OF THE PLANT PLASMA MEMBRANE

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The 1,3-β-glucan synthase (callose synthase) was solubilized from cauliflower (*Brassica oleracea* L.) plasma membranes with digitonin and partially purified by ion exchange and gel filtration FPLC using CHAPS in the elution buffers (1,2,3). These initial steps were necessary to obtain specific precipitation of the enzyme during product entrapment, the final purification step. Five polypeptides of 32, 35, 57, 65 and 66 kDa were highly enriched in the final preparation and are likely components of the callose synthase complex. The purified enzyme was activated by Ca^{2+}, spermine and cellobiose in the same way as the enzyme in situ, indicating that no essential subunits were missing. The polyglucan produced by the purified enzyme contained mainly 1,3-linked glucose. However, 8% of the glucose residues were 4-linked which may indicate that the enzyme has the ability to make both 1,3- and 1,4-polyglucans. The protein complex may be identical to the rosettes of the plasma membrane putatively involved in cellulose synthesis (4). Since three of the five polypeptides were N-terminally blocked we have further improved the recovery of functionally active multi protein complex in order to generate enough material for proteolytic cleavage and determination of internal amino acid sequences. By using PCR with oligonucleotide primers based on partial amino acid sequences we intend to clone the genes coding for the polypeptides, and by using antisera raised against synthetic peptides we propose to discern whether the callose and the cellulose synthesizing activities are located in the same multi protein complex.

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TISSUE SPECIFIC REGULATION BY NACL OF TONOPLAST ATPASE GENE EXPRESSION

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Osmotic adjustment and the avoidance of toxic cytosolic concentrations of Na^+ and Cl^- are two of the principal requirements for plants to survive and grow in a saline environment. The generation and maintenance of adequate H^+ electrochemical gradients is critical to facilitated solute transport and consequently is an integral component of physiological adaptation to saline environments. While some of the biochemical and physiological processes inherent to salt tolerance are likely to be similar in all cells of the plant, the organismal aspects of solute uptake and partitioning suggest that there will be variations between different organs and tissues for many processes associated with solute transport.

Examination of the NaCl induced accumulation of mRNA for the 70 kDa subunit of the tonoplast H^+-translocating ATPase reveals an increased accumulation of this message in older expanded leaves of tomato plants treated with NaCl. No increase in accumulation of this message was observed in either the young unexpanded leaves, or in the roots of these plants. This data is in accord with the reported patterns of tissue specific ion accumulation, where Na^+ was found to be preferentially localized in older leaves. The levels of mRNA for the 70 kDa subunit in the roots and young leaves of control plants (no salt) were approximately equivalent to the amount of this message in the expanded leaves of plants treated with 400 mM NaCl. These results not only support a role for altered expression of the tonoplast ATPase genes in salt tolerance, but suggests that the demands for H^+ electrochemical potential vary relative to the demands for osmotic adjustment and ion compartmentation in different tissues.

MEMBRANE POTENTIAL DEPENDANT SODIUM UPTAKE INTO RIGHT-SIDE-OUT PLASMAMEMBRANE VESICLES ISOLATED FROM WHEAT ROOTS.

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We are studying Na^+ transport processes in wheat root plasmamembrane vesicles, prepared by aqueous polymer two phase partitioning[1], in relation to an enhanced K^+/Na^+ discrimination trait located on the 4D chromosome[2]. Enzyme latency studies demonstrated that the vesicles were 80-90% right-side-out, therefore any Na^+ uptake is equivalent to influx *in vivo*. A filtration assay was used to measure $^{22}Na^+$ uptake in response to membrane potentials imposed by either K^+-valinomycin clamps or by inside-acid pH jumps. The existence and magnitude of membrane potentials was demonstrated by the uptake of the lipophilic cation tetraphenylphosphonium which accumulated in proportion to the imposed membrane potential. Na^+ uptake characteristics were determined, and the effects of various ion channel inhibitors investigated to determine whether transport was via specific channels.

Sodium influx was stimulated by inside negative membrane potentials with a log-linear flux vs voltage relationship. The Km for Na^+ at -100 mV was 34.8 $\pm$6 and the pH optima for uptake between 5.5 & 6.5. The ion channel inhibitors tetrodotoxin (TTX), saxitoxin (STX), Ba^{2+}, Cs^{2+}, quinine, verapamil and Ca^{2+} inhibited Na^+ uptake at -100 mV, whereas tetraethylammonium (TEA), ruthenium red, La^{3+}, and Gd^{3+} had no effect. The I_{50} for TTX and STX was 3-4 μM, 100 fold higher than for animal Na^+-channels. Inhibition of Na^+ influx by TTX, STX, Ba^{2+}, and quinine is membrane potential dependant. The range of channel blockers which are inhibitory suggests uptake is via a non-specific cation channel.

Na^+ uptake at -100 mV into vesicles isolated from hexaploid (+4D) and tetraploid (-4D) wheat was not significantly different indicating that transport via this channel does not account for the observed characteristics attributed to the K^+/Na^+ discrimination trait.

Work is now proceeding to determine whether Na^+/H^+ antiport systems are present in the vesicles and whether these are the point of control of the K^+/Na^+ discrimination trait.

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THE EFFECTS OF Na^+ ON ATP HYDROLYSIS AND H^+ TRANSLOCATION IN ROOT PLASMA MEMBRANE VESICLES

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Whole plant studies using *Spergularia marina* have demonstrated that successful Na^+ management in this species is controlled in the roots where unidirectional Na^+ fluxes and net H^+ efflux rates across the plasma membrane are extremely high and where Na^+ does not accumulate to its electrochemical potential (1,2). These data suggest that a substantial $\Delta\mu_{Na+}$ is present as well as a $\Delta\mu_{H+}$. We are characterizing the generation of these forces using root plasma membrane vesicles isolated from plants grown at low and high salinity and determining the degree to which the forces are independent or integrated. We report here the Na^+-dependent modifications of the activities of the P-type H^+-ATPase which generates the $\Delta\mu_{H+}$. ATP hydrolysis activity was determined by the release of Pi. H^+ translocation activity was monitored with absorbance changes in the weak base acridine orange during time courses of vesicle acidification. Time course data were fit with a monoexponential asymptotic decay function to quantify kinetic parameters.

Na^+-dependent reductions in ATP hydrolysis and H^+ translocation activities were pH-dependent (being most pronounced at high pH) and independent of the salinity of the growth media. The addition of NaCl to K^+ media at pH 7.7 reduced the specific ATPase activity by 35% but it produced no change at pH 6.5 or 7. NaCl (but not Na^+-gluconate) reduced vesicle acidification at pH 6.5 and 7 by 30% to 40%; it inhibited it entirely at pH 8, conditions under which a large ΔpH was generated using K^+. The data suggest that Na^+ disrupts K^+-stimulated activities at high pH; the Na^+ effect on vesicle acidification may be Cl^--dependent.

Na^+ protected against vanadate inhibition of both ATP hydrolysis and H^+ translocation activities when it was partially or totally substituted for K^+; in low salinity plants, vanadate inhibition was reduced by 40% to 50%, and in plants grown at high salinity, vanadate inhibition was nearly completely overcome. These data suggest that Na^+ protects against K^+-dependent vanadate inhibition of the plant plasma membrane P-type H^+-ATPAse in a manner similar to that observed for the animal cell plasma membrane P-type (Na^+,K^+)-ATPase (3).

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PLASMA MEMBRANE AND TONOPLAST ATPase AND K^+-STIMULATED PYROPHOSPHATASE ACTIVITIES IN NaCl-ADAPTED ACER PSEUDOPLATANUS CELLS

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The adaptation to Na^+ salts of the glycophites involves an accumulation into the vacuole of Na^+ which lowers cellular osmotic potential and avoids toxic effect in the cytoplasm (1,2). An induction of Na^+/H^+ antiport by NaCl stress has been observed in some materials (3,4). This transport of Na^+ in to the vacuole is energized by the H^+ electrochemical gradient across the tonoplast sustained by the activities of an H^+ ATPase and a K^+-pyrophosphatase.

In this work we compared the activities of the plasma membrane and tonoplast ATPases and of the K^+-PPase in normal and adapted cells (at exponential phase of growth). Cell suspension cultures of Acer pseudoplatanus have been adapted to grow in NaCl concentrations up to 80 mM by a stepwise treatment with increasing salt concentrations. The results show that the activities of vanadate-sensitive and nitrate-sensitive ATPases (measured in 8.000-108.000 g microsomes and in plasma membrane or tonoplast enriched fractions) were not significantly different in the two types of cells. On the contrary, the K^+ stimulated PPase activity was much higher in adapted than in normal cells (from +60 to +200%). pH dependence and ionic requirements for this enzyme were the same in normal and adapted cells. The half maximal rate of K^+-stimulated PPase activity was obtained at about 24 uM PPi in microsomes prepared from both types of cells. The PPase activity measured in microsomes was activated by detergents such as Brij 58R and Triton X-100 more in adapted than in normal cells.

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NA^+ FLUXES IN *CHARA*: A COMPARISON BETWEEN SALT SENSITIVE *CHARA CORALLINA* AND SALT TOLERANT *CHARA BUCKELLII*.

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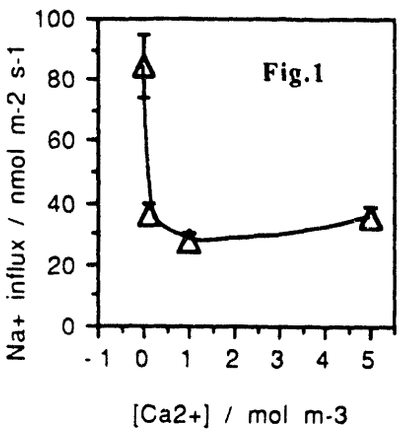
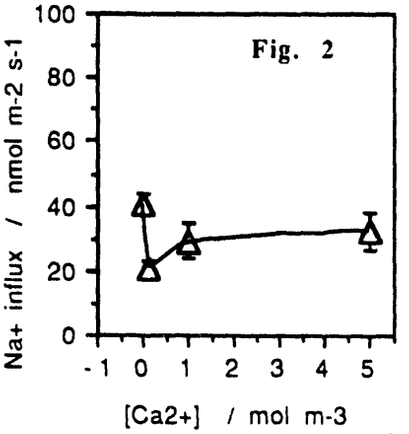
Both the obligate freshwater alga, *Chara corallina* and the euryhaline alga, *Chara buckellii* can survive in solutions containing 70 mol m^{-3} NaCl as long as the Ca^{2+}:Na^+ ratio is maintained at about 1:10 (1,2). *C. buckellii* can survive approximately 5 days in 70 mol m^{-3} NaCl with low (0.1 mol m^{-3}) external Ca^{2+} whereas under the same conditions *C. corallina* usually dies within 24 hours (1,2). Under these conditions cell death has been associated with a cytotoxic influx of Na^+. Previous studies indicate that increased Na^+ influx at elevated salinity is due to the increase in $\Delta\mu_{Na}$, not to a change in P_{Na} (3). The addition of Ca^{2+} acts to decrease P_{Na} and so reduce Na+ influx. Fig. 1 shows the depedance of Na^+ influx on Ca^{2+} concentration for *C. corallina* after 90 minutes of exposure to 20 mol m^{-3} Na^+ and Fig 2. shows the same for *C. buckellii* . After 48 hours exposure to this solution Na^+ influx in *C. buckellii* shows the same dependance on Ca^{2+} concentartion as does *C. corallina* in Fig. 1. The onset of the sensitivity of Na^+ influx to external Ca^{2+} is much slower in *C. buckellii* than in *C. corallina*.





Na^+ influx into *C. corallina* (Fig. 1) and *C. buckellii* (Fig. 2) after 90 minutes in 20 mol m^{-3} NaCl, 0.05 mol m^{-3} K_2SO_4, 2.5 mol m^{-3} HEPES, pH 7. Data points represent the mean and SE of 10 cells.

It has been proposed that Ca^{2+} inhibitable Na^+ influx (for *C. corallina* at least) is due to the passage of Na^+ through K^+ channels, the permeability of which can be increased by high monovalent cation concentrations and can be reduced by a high concentration of divalent cation (4). The mechanisms for Na+ uptake into *C. corallina* and *C. buckellii* will be discussed in relation to the effect of various K^+ channel blockers (eg. La^{3+}, TEA^+, Ba^{2+}) on P_{Na}.

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THE ROLE OF THE V-TYPE ATPASE IN SALT TOLERANCE: EXPLORING THE EFFECTS OF NACL ON TRANSPORT, SUBUNIT COMPOSITION AND GENE EXPRESSION.

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The V-type ATPase may play an important role in salt tolerance, since it is thought to provide the primary driving force for uptake of Na^{2+} and Cl^- into the vacuole. In order to test the hypothesis that the activity of the V-type ATPase is regulated in response to salt (1), the ATPase from control and salt-grown barley (*Hordeum vulgare* cv CM72) roots was extensively characterized in terms of ATP hydrolysis, proton transport, subunit composition and gene expression. When barley seedlings were grown in 100 mM NaCl, there was an increase in rate of proton transport as estimated by rate of quench of acridine orange fluorescence in isolated vesicles. However, there was no change in rate of ATP hydrolysis or amount of the ATPase protein as detected by antibodies against the 68 and 53 kDa subunits. The holoenzyme was purified by Sephacryl column chromatography followed by FPLC, and 10 putative subunits were identified (115, 68, 53, 45, 42, 32, 17, 13, and 12 kDa) (2). Subunit composition on 1D SDS gels was identical for the ATPase from control and salt-grown roots. The B subunit (53 kDa) frequently was resolved as 3 bands on SDS gels; these might be isoforms or products of proteolysis. In order to determine if there is more than one gene for the B subunit, a cDNA library was screened with a heterologous cDNA probe for the B subunit from *Arabidopsis* (3). Two full length cDNAs that code for the B subunit were identified and sequenced. The coding regions were similar but the 5' and 3' untranslated regions of the two cDNAs were different. The results demonstrate that at least two genes for the B subunit are expressed in barley roots. Preliminary results indicate that there was no effect of salt on mRNA levels for the two B subunit genes. Also, there was no effect of salt on mRNA levels for the A subunit (68 kDa) as detected with a heterologous cDNA probe from carrot (4). We have not ruled out the possibility that changes in ATPase activity are related to changes in gene expression for one or more subunits. However, mechanisms for post-translational regulation of the ATPase must also be considered.

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GENETIC AND DROUGHT-INDUCED DICLOFOP RESISTANCE ASSOCIATED WITH RECOVERY OF ELECTROGENIC MEMBRANE POTENTIAL OF THE PLASMALEMMA

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The mechanism of resistance to the herbicide diclofop-methyl, an inhibitor of acetyl-coA carboxylase (ACCase), has been examined in resistant (R) and susceptible (S) biotypes of *Avena fatua* and in susceptible *A. sativa* plants subjected to drought stress, which induces resistance. There were no differences in herbicide uptake, long-distance transport, or metabolism to non-phytotoxic products between R and S *A. fatua* plants. Crude ACCase preparations from R and S *A. fatua* were equally sensitive to diclofop; similar results were obtained with ACCase from drought-stressed and non-stressed *A. sativa*. Control tissues from both species acidified an unbuffered bathing medium; diclofop treatment reversed this, causing rapid alkalinization. Tissue from the R but not the S *A. fatua* biotype re-acidified the medium when the diclofop solution was replaced with a herbicide-free solution. Similar re-acidification was obtained with drought-stressed *A. sativa* or *A. sativa* pretreated with abscisic acid. Diclofop (50 μM) rapidly depolarized the electrogenic membrane potential (E_M) in peeled coleoptiles in both species. However, when diclofop was removed from the bathing solution, E_M was re-established in the R *A. fatua* and the drought-stressed *A. sativa*, but not in their S or non-stressed counterparts. Diclofop (100 μM) had no effect on H^+-ATPase activity in purified plasmamembrane vesicles prepared from the R and S *A. fatua* biotypes. These results suggest that in both instances (endogenous and induced resistance), the ability of the plasma membrane to regenerate E_M following depolarization is associated with herbicide resistance. Previous results (1) indicating that membrane depolarization by diclofop can be blocked by PCMBS suggest the involvement of a membrane protein component in the depolarization. We are currently investigating the connection between membrane depolarization, repolarization, and the resistance of these plants to diclofop and other ACCase inhibitors.

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EFFECT OF WASHING ON THE PLASMA MEMBRANE AND ON STRESS REACTIONS OF CULTURED ROSE CELLS

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Cultured rose cells have been used as a model for studies of responses of plant cells to various stresses, including UV radiation, protein-synthesis inhibitors, and Phytophthora cell-wall elicitors (1-3). Many of the responses involve reactions at the plasma membrane: efflux of K^+, changes in the acid balance between cytoplasm and external medium, synthesis of H_2O_2, inhibition of ferricyanide reduction. In these studies, the cells have typically been washed three times with a solution of 1 mM $CaCl_2$, 0.1 mM KCl, with incubations of 30 min between washings and 120 min after the last wash. We now show that this washing procedure results in an apparent loss of 5 proteins and an increase in one major protein in PEG-dextran phase partition-purified plasma membrane, as determined by isoelectric focusing-SDS-PAGE 2-dimensional electrophoresis and Coomassie Brilliant Blue staining. The specific activity of the vanadate-sensitive ATPase is increased from 29.9 ± 6.7 to 49.1 ± 11.5 umol Pi/mg protein-h (n=5). Washing also alters the responses of the cells to UV-C radiation and Phytophthora elicitor. Compared with the unwashed cells, the washed cells show less net K^+ efflux after UV-C and elicitor treatments; more synthesis of H_2O_2 after UV-C and a pattern of accumulation of H_2O_2 after elicitor treatment that shows a delayed but higher peak; and more inhibition of ferricyanide reduction after UV-C, but not after elicitor treatment. The results suggest that washing has differential effects on the UV and elicitor receptors or on the mechanisms that couple these receptors to the responses.

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CHANGES IN PLASMA MEMBRANE COMPOSITION AND ATPASE ACTIVITY IN NEEDLES OF WHITE SPRUCE (PICEA GLAUCA) SEEDLINGS TREATED WITH TRIADIMEFON AND OSMOTIC STRESS

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White spruce [Picea glauca (Moench) Voss.] seedlings were grown in solution culture and treated with sterol metabolism inhibitor [20 mg l^{-1} triadimefon; 1-(chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)-2-butanol] for 4 weeks and then subjected to osmotic stress with polyethylene glycol 3350 (Zwiazek and Blake 1990). Water potentials and electrolyte leakage were measured in seedlings before and after the seedlings were subjected to osmotic stress with polyethylene glycol and the plasma membranes were isolated from needles and purified using a two-phase partitioning method (Larsson et al. 1988). Plasma membranes were used to study lipid composition and the activity of plasma membrane bound ATPase.

Water potentials drastically declined and electrolyte leakage increased in triadimefon treated seedlings before the seedlings were subjected to osmotic stress with polyethylene glycol. When the seedlings were exposed to osmotic stress, triadimefon treated plants maintained higher water potentials and leaked less electrolytes compared with control plants. Plasma membrane bound ATPase activity was drastically inhibited by both triadimefon and osmotic stress treatments and the observed decrease in ATPase activity could be correlated with the increase in electrolyte leakiness. Numerous changes in the composition of the plasma membranes were observed including a decrease in phospholipid:protein ratios and sterol:protein ratios and an increase in sterol:phospholipid ratios. Both triadimefon and osmotic stress treatments altered the composition of free sterols in the plasma membranes. The results indicate that triadimefon induces mild water stress in plants and many of the observed changes in membrane composition anf function in triadimefon treated plants could develop as the result of water stress.

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TURGOR AND OSMOTIC RELATIONS IN ROOTS OF *ASTER TRIPOLIUM*: WATER AND SOLUTE TRANSPORT THROUGH THE MEMBRANE

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Pressure probe-, nanoliter osmometry- and NMR-technique were used to study water transport phenomena on the cellular level in *Aster tripolium*. The intact plants showed a strong increase in the radial turgor and osmotic pressure gradients (1) from the outside to the inside of the root undependent from the salt stress (i.e. from 0.1 MPa to about 0.6 MPa). Strong differences were found between the penetration of salts and non-electrolytes to the apoplasmic space. In contrast to NaCl, the uptake of sucrose or PEG into the apoplasmic space seemed to be freely possible or - more likely - prevented by the pit-containing boundary. Only in the case of non-electrolytes turgor pressure gradients decreased and osmotic pressure increased, respectively, as was expected for the ideal osmometer behaviour. The much more interesting point of our study was the collapse of the turgor pressure gradients after excision of the root within about 15 to 30 min and its equilibration at the intermediate level of about 0.3 MPa. This value agreed well with the osmotic pressure deduced from plasmolysis tests on excised roots. High resolution NMR-images (2,3) yielded at very short spin-echo times radial cell strands separated by radial regions of air-filled spaces. These and other data allow conclusions about the driving forces for water and solute transport in the root of intact plants and about the function of air-filled radial spaces under control and saline conditions. They also showed simultaneously that excised roots may be artifactual systems.

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KINETIC CHARACTERIZATION OF Fe(III)-REDUCTION BY PLASMA-MEMBRANE VESICLES AND INTACT ROOTS

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Electron transport activities by root cells were determined *in vivo* and with plasma membrane (PM) vesicles from species differing in their ecological amplitude. Plasma membranes were isolated by two-phase partitioning yielding high amounts of sealed, right-side-out orientated vesicles as both H^+-ATPase and redox systems showed high latencies upon the addition of Triton-X 100. PM-bound redox systems were dependent on NADH as an electron donor and reduced both FeEDTA and ferricyanide (FeCN). Intact plants reduced ferric EDTA with a pH optimum of 5.5 whereas the reaction *in vitro* exhibited maximal rates at pH 7. This difference might be due to electrostatic interactions between the substrate and the fixed charges of cell walls which are not present in the *in vitro* assay.

The reduction rates determined with intact, iron-stressed plants were correlated with the specific activities of the NADH-FeEDTA reductase *in vitro* and with the expected amount of soluble iron on the natural stands of the species. Kinetic analysis revealed strict similarities for the reduction of FeEDTA by both systems. As the *in vitro* data were obtained with iron-sufficient tissue, the results are not consistent with the *de novo* synthesis of a low midpoint electrochemical potential ("turbo") reductase under conditions of Fe-deficiency stress. However, differences in the kinetic behaviour and in the specificity of the electron donor between NADH-FeEDTA and NADH-FeCN reductase provide evidence for multiple electron transport activities in the PM. Ferric chelate reduction activity was also found in PM-vesicles isolated from roots of *Hordeum vulgare*, in which no increased reduction activity was observed *in vivo*.

The PM-bound redox activity was increased by the addition of cations without changes in the substrate affinity for NADH, suggesting structural changes in the redox system or electrostatic alterations of the charged lipids surrounding the enzyme by the assay conditions. In the absence of cations, PM vesicles isolated from roots of two *Geum* species revealed biphasic saturation curves for the reduction of FeEDTA leading to two distinct apparent K_m values. This finding was interpreted in terms of negative cooperativity towards FeEDTA as addition of cations (5 mM $MgCl_2$) led to a hyperbolic curve with Michaelis-Menten characteristics. It is concluded, that the activity of the FeEDTA reductase is at least in part regulated by the intracellular concentration of cations.

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PLASMA MEMBRANE REDOX ACTIVITIES AND QUINONE SITES IN TRANSMEMBRANE SIGNAL TRANSDUCTION

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Plant plasma membranes transport electrons from cytoplasmic electron donors (e.g. NADH) to external acceptors (ferricyanide, ascorbate radical, oxygen). The putative natural electron acceptor is oxygen and the rate limiting transfer of electrons from NADH to oxygen is quinone-dependent and is catalyzed by an intrinsic plasma membrane enzyme designated as an NADH (quinone) oxidase. The oxidase is responsive to plant growth hormones of the auxin type. Growth and oxidase activity are stimulated in parallel in a concentration dependent manner with excised segments and plasma membranes isolated from excised segments, respectively, of etiolated hypocotyls of soybean. When analyzed by SDS-PAGE, the NADH oxidase activity copurifies with protein bands of approximate molecular weights of 72 and 36 kDa. The partially purified enzyme activity remains responsive to auxins. Lysophospholipids and free fatty acids, both products of A-type phospolipases, stimulate the NADH oxidase activity in a time-dependent manner suggestive of stabilization of an activated form of the enzyme.

Inhibitors that block auxin-induced growth of segments of soybean hypocotyl also inhibit the auxin-induced oxidase activity. These include the anticancer drug adriamycin and several sulfonylurea herbicides, e.g. chlorsulfuron, known to interact with quinone sites in target proteins. Mastoparan, a peptide toxin from wasp venom that mimics G protein-coupled receptors, stimulates both the oxidase of isolated soybean plasma membrane vesicles and elongation growth to approximately the same degree as do active auxins. The results implicate redox activities of the plasma membrane involving quinone sites as a potentially important component of the signal transduction pathway of auxin control of cell elongation in plants.

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ARE CYTOCHROME B_5 REDUCTASE AND THE IRON CHELATE REDUCTASE AT THE ENDOPLASMIC RETICULUM OF *PHASEOLUS VULGARIS* RELATED?

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Endoplasmic reticulum vesicles isolated from corn and kidney bean roots reduce iron chelates, *in vitro* (1), including ferrirhodotorulic acid, ferric citrate, ferric EDTA, and ferricyanide. The reductase is NADH specific. This reduction may potentially function to free iron for uptake into organelles, incorporation into enzymes, and transport, possibly as ferrous nicotianamine (2). The only intracellular enzyme previously shown to reduce iron chelates with low reduction potentials is nitrate reductase (3). The subunit of nitrate reductase responsible for iron reduction shares 122 out of 250 amino acids with cytochrome b_5 reductase (4), an endoplasmic reticulum localized NADH-specific enzyme. Cytochrome b_5 reductase is commonly assayed using the iron chelator, ferricyanide. The enzyme contains a flavin adenine dinucleotide prosthetic group. In mammalian cells, cytochrome b_5 reductase faces the cytosolic side of the endoplasmic reticulum, and is attached to the membrane at its N-terminus by myristic acid, a covalently bound N-terminal fatty acid (5).

The similarities between cytochrome b_5 reduction and iron reduction thus suggest that the two activities may be related. To test this, active ferric citrate reducing bands solubilized from the endoplasmic reticulum vesicles of *Phaseolus vulgaris* were identified in native isoelectric focussing gels using the protocol of Holden et al (6). Three active bands were found between pI 6.75 and 7.25. This is more basic than the plasma membrane ferric citrate reductase isolated from tomato roots by Holden et al (6). The endoplasmic reticulum iron reducing bands were separated by SDS-polyacrylamide gel electrophoresis, or were concentrated on centricon filters for spectral analyses, to compare cytochrome b_5 reductase and iron chelate reductase.

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TRANSPLASMA MEMBRANE ELECTRON TRANSPORT: THE POSSIBLE INVOLVEMENT OF A *b*-TYPE CYTOCHROME

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The plasma membrane of higher plant species contains a specific *b*-type cytochrome. An extensive characterization, using highly purified membrane preparations, has revealed midpoint oxidation-reduction potentials (E'_0) between +110 mV and +160 mV and an α-band absorption maximum at about 561 nm of the reduced component (1, 2). The heme protein was readily reduced by Na-ascorbate and constitutes the major cytochrome component (70-80%) of these membranes. So far, no physiological function could be assigned to the electron carrier.

In recent work we were interested to test the possible involvement of the cytochrome in trans-membrane electron transport. Plasma membrane vesicles were prepared that contain high concentrations of ascorbate. These vesicles are capable of transferring electrons to externally added impermeable electron acceptors such as ferricyanide and cytochrome *c* (3).

As expected, the absorption spectrum of these plasma membrane vesicles showed the characteristic α-band maximum of the reduced *b*-type cytochrome. Addition of ascorbate oxidase did not re-oxidize the cytochrome, supporting the idea that ascorbate was effective from the inside of tightly sealed vesicles. Addition of low concentrations of ferricyanide to these preparations caused a transient decrease of the absorption maximum which reversed within a few minutes. These absorbance changes at 561 nm indicate a rapid oxidation of the cytochrome followed by a re-reduction. Ferricyanide becomes fully reduced in this reaction. In accordance with similar observations that were made using ascorbate loaded chromaffin granules that contain a high potential *b*-type cytochrome (4), we hypothesize that the above reaction indicates a transient electron transfer from an internal electron donor (probably ascorbate) to external ferricyanide with the cytochrome operating as an electron carrier. Also the cytochrome redox potential (about +150 mV) makes it a likely candidate in the electron transfer from ascorbate (about +52 mV) to ferricyanide (+400 mV). Other electron acceptors (Fe(III)-citrate, Fe(III)-EDTA) that are commonly used in NADH-mediated plasma membrane electron transport measurements were ineffective in reoxidizing the cytochrome in ascorbate loaded vesicles. This is possibly explained by the low redox potentials of these components and further substantiates our current hypothesis.

If electrons were indeed transferred from the vesicle interior to external ferricyanide, the generation of a membrane potential (positive inside) was to be expected. Absorbance changes obtained with the potential probe oxonol VI confirm this prediction and the ionophore FCCP effectively reversed the charge gradient.

These experiments provide strong evidence that the high potential plasma membrane *b*-type cytochrome is involved in transmembrane electron transfer in higher plants.

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PURIFICATION OF Fe^{3+}-CHELATE REDUCTASE BY NATIVE PAGE AND FPLC

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Plant root plasma membranes (PM) contain a NADH-dependent Fe^{3+}-chelate reductase (1). Since this enzyme plays an important role in iron acquisitation and iron-deficiency responses of dicotyledonuous plants (2), we tried to isolate and purify Fe^{3+}-chelate reductase from tomato root PM.

Material and Methods:

1. PM from tomato roots were obtained by two-phase partitioning as described (3).
2. PM proteins were solubilized with Triton X 100 at a protein:detergent ratio of 1:2.8 or 1:15 (w/w), respectively. In all subsequent steps detergent was included.
3. The Triton-soluble fraction was subjected to native PAGE in a 7.5 % gel. After identification of the enzyme containing region by enzyme-staining of a small vertical section, that part of the gel was electro-eluted in 50 mM $(NH_4)HCO_3$. The eluate was either subjected to FPLC or 2D-PAGE.
4. FPLC: The native PAGE eluate was separated by a salt gradient (20 mM Tris/HCl pH 8.8, 0-1 M NaCl) on a Mono Q column.
5. 2D-PAGE: 1. Dimension: native IEF pH 5-7, enzyme-staining, 2. Dimension 10 % SDS-PAGE.

Table 1: Purification table of Fe^{3+}-chelate reductase and common polypeptide pattern of the fractions.

Fraction	Specific Activity*	Purification (x fold)	Polypeptides (MW) ± 90 kDa	± 50 kDa	± 30 kDa	
1. PM	0.76	--				
2. Triton-soluble	1.29	1.7	98 ± 6	51 ± 1	29 ± 1	(n=3, 2D-PAGE)
3. nat. PAGE eluate	4.61	6.0	96 ± 2	49 ± 1	29 ± 1	(n=3, 2D-PAGE)
4. FPLC fraction	108.56	142	86 ± 2	50 ± 0	28 ± 1	(n=2, 10% SDS-PAGE)

* µmol Fe [min mg Prot.]$^{-1}$

Results:

Enzyme purification based on enrichment of specific activity is shown in Table 1. Fe^{3+}-chelate reductase was purified >140-fold in the FPLC fraction. All active fractions contained multiple polypeptide bands, when analyzed by SDS-PAGE or 2D-PAGE. Polypeptide bands in common, independently of the purification method, at the position of the highest enzymatic activity were only found at ± 30, ± 50, and ± 90 kDa.

Conclusions:

Fe^{3+}-chelate reductase activity from tomato roots can be purified by a combination of native PAGE and HPLC on an anion exchange column. We suggest that the activity (pI = 5.5) contains subunits of 29, 50 and 93 kDa.

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CHARACTERISTICS OF THE PLASMAMEMBRANE BOUND NITRATE REDUCTASE OF CHLORELLA SACCHAROPHILA

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In barley and in the unicellular greenalga Chlorella the existence of a plasmamembrane bound nitrate reductase (PM-NR) is demonstrated. This enzyme is clearly distinct from the well known soluble nitrate reductase (NR) which is located in the soluble cellfraction. The characteristics of the PM-NR are different from the soluble NR in various aspects including the native molecular weight, the hydrophobic pattern, the iso-electric point and the heterodimeric structure. The PM-NR is located on the outside of right-side-out vesicles and can not be removed from the surface of inside-out vesicles. The treatment of right-side-out vesicles using PIPLC resulted in a release of PM-NR from the surface and simultanously changed its hydrophobic pattern. Experiments with the aim to incorporate ^{3}H-ethanolamine in the anchor structure are in progress.

Treatment of barley roots and Chlorella proto-plasts with anti-NR-IgG inhibited nitrate uptake but not that of nitrite. Removal of PM-NR from protoplasts also reduced nitrate uptake significantly. The function of PM-NR in nitrate uptake is discussed with respect to the biochemical characteristics and localisation.

CHARACTERIZATION OF IRON CHELATE REDUCTION BY LEAF MESOPHYLL AND LEAF PLASMA MEMBRANES OF VIGNA UNGUICULATA

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It is now generally accepted that iron uptake by roots of dicotyledonuous (and some monocotyledonuous) plants occurrs after an obligatory reduction of Fe (III) to Fe(II) by a plasma membrane (PM)-bound ferric chelate reductase (FCR) (e.g. 1). However, in the xylem iron is transported as Fe(III) citrate to the shoot (2). In the present study, the question is adressed, whether a reduction step may also be involved in iron uptake by leaf mesophyll cells.

After removal of the abaxial epidermis, vacuum infiltrated leaves of Vigna unguiculata revealed an Fe(III) citrate reducing activity, which was stimulated severalfold by application of saturating red light under CO_2 saturating conditions for photosynthesis. The K_m values observed were similar in the dark and in the light (ca. 100 µM, fig.1), while v_{max} ranged from 20 (dark) to 100 nmol m^{-2} s^{-1} (light). Plasma membranes from leaves were obtained by the aqueous two-phase partitioning method and also revealed Fe(III) citrate reductase activity with a similar K_m as in vivo. The pH optimum of the reductase was 6.5-7, and all data were very similar to biochemical characteristics of PM-FCR from plant roots (1,3). Under iron deficiency, Vigna leaves become strongly chlorotic. Data from these plants will be compared to the controls to evaluate the possible occurence of an iron-stress-induced increase in FCR activity, as it has been found in root PM (1).

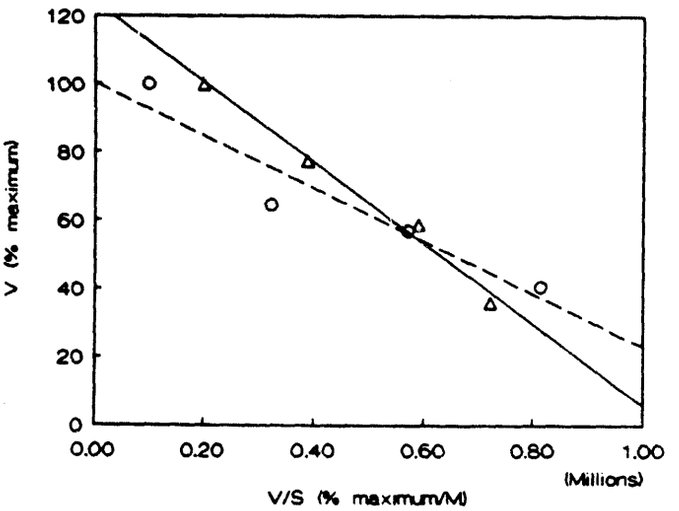

Fig.1: Eadie-Hofstee plots of the kinetics of Fe (III) citrate reduction by leaf mesophyll in the light (o) and by leaf PM (Δ).

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THE EFFECT OF Al^{3+}, La^{3+} AND TEC^{3+} ON NET Ca^{2+} INFLUX MEASURED AT THE APEX OF ALUMINUM-SENSITIVE AND ALUMINUM-TOLERANT WHEAT ROOTS.

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The effects of aluminum (Al), lanthanum (La) and tris(ethylenediamine)cobolt(III) (TEC) on the net flux of calcium (Ca) at the apex of wheat roots was investigated using the vibrating Ca-selective microelectrode technique. It has been previously shown that Al treatments known to inhibit root growth in the Al-sensitive cultivar, "Scout" (10 μM $AlCl_3$, 0.1 $CaCl_2$, pH 4.5), also cause an immediate inhibition of net Ca influx at the root apex. Similar treatments do not inhibit root growth or net Ca influx in the Al-tolerant cultivar, "Atlas" (1). Under similar conditions La inhibited root growth and Ca fluxes in both "Scout" and "Atlas", showing that the relative tolerance to Al in "Atlas" is not a general phenomena with all trivalent cations. It is uncertain whether the differential effect of Al on Ca fluxes in these two cultivars is important to the Al-toxicity response or attributable to the inherent differences between "Scout" and "Atlas". We repeated these experiments with pairs of closely-related lines of wheat which exhibit differential sensitivity to Al. Tolerance to Al, in these lines, is linked to a single, dominant gene difference. Results from the first pair of lines are consistent with those found in "Scout" and "Atlas": root growth and Ca influx were inhibited significantly more by Al in the sensitive line, than in the tolerant line. This suggests that tolerance to Al in wheat roots may be related to the ability to maintain Ca influx during Al-stress. We will report on the results obtained with other closely-related lines, and compare the effects of the trivalent cations Al, La and TEC. Inhibition of Ca uptake by these cations is considered in terms of the blockage of Ca channels and changes to the membrane surface potential. These interactions will be discussed with reference to the potential mechanisms of Al-phytotoxicity.

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CADMIUM TRANSPORT ACROSS THE TONOPLAST OF OAT ROOTS: DIRECT DEMONSTRATION OF A Cd^{2+}/H^+ ANTIPORT ACTIVITY

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Cadmium is a non-essential, potentially toxic, pollutant metal which accumulates in the kidney with a biological half-life exceeding 10 years. Its main pathogenic effects, in humans, are renal tubular damage, pulmonary emphysema and osteomalacia. Cadmium principally occurs in the human diet as a result of its uptake and concentration from soil by crop plants. Little is known concerning mechanisms of Cd^{2+} uptake by plants or animals at the tissue or cell level. Cadmium is known to accumulate in the vacuole of tobacco leaf mesophyll cells[1, 2], however the mechanism is unknown. We have studied the transport of Cd^{2+} across the tonoplast of oat roots (the major tissue of Cd^{2+} accumulation in oat seedlings exposed to 10-20μM Cd^{2+}), using isolated tonoplast vesicles, and have demonstrated the transport of Cd^{2+} into the vesicles via a Cd^{2+}/H^+ antiport activity. Accumulation of Cd^{2+} (monitored directly using ^{109}Cd) was driven by a ΔpH generated by either the V-type ATPase (Fig. 1) or artificially using nigericin to exchange K^+ and H^+ in K^+-loaded vesicles (not shown). Cadmium accumulation was accompanied by efflux of H^+, which was Cd^{2+} concentration dependent (Fig. 2), and ΔpH-dependent Cd^{2+} accumulation showed saturation kinetics with a $Km_{(app)}$ of 7μM. Cytoplasmic Cd^{2+} concentration can be estimated to be between 0.2-2μM which makes this Cd^{2+}/H^+ antiport activity a candidate for transport of this pollutant metal from the cytoplasm to the vacuolar sap under conditions of low as well as high level Cd^{2+} exposure. Exposure of seedlings to 20μM Cd^{2+} for 4 days (from imbibition) caused a 100% increase in tonoplast H^+ pump activity[3]. Effects of growth with Cd^{2+} on Cd^{2+}/H^+ antiport activity and tonoplast proteins are currently being studied.

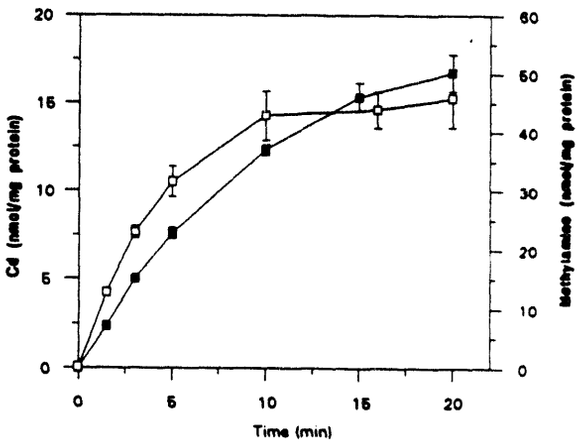

Fig. 1. Cd^{2+} uptake (■), methylamine uptake (□).

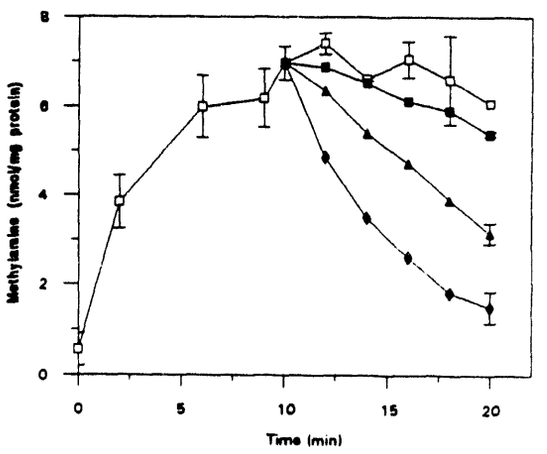

Fig. 2. Effect of Cd^{2+} on an existing ΔpH, Cd^{2+} added after 10 min to a final concentration of 10μM (■), 100μM (▲), and 1000μM (◆).

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A VACUOLAR MEMBRANE ABC-TYPE TRANSPORT PROTEIN IMPLICATED IN PHYTOCHELATIN-MEDIATED HEAVY METAL TOLERANCE

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Exposure of plants and some fungi, such as the fission yeast *Schizosaccharomyces pombe*, to heavy metals induces the synthesis of cysteine rich peptides called phytochelatins (PCs). Enzymatically synthesized from glutathione (γ glu-cys-gly), PCs are small peptides with the general structure $(\gamma \text{ glu-cys})_{n=2-11}$gly that chelate heavy metals by formation of thiolate bonds. Two different forms of PC-metal complexes are found in fission yeast and plant cells exposed to Cd. One species, composed mostly of PCs and Cd, elutes from gel filtration columns with an apparent molecular weight of 3-4 kDa (LMW PC-Cd complex). A second, more highly charged form has an apparent molecular weight of 6-9 kDa (HMW PC-Cd-S^{-2} complex), and contains acid labile sulfide in addition to PCs and Cd. A Cd sensitive fission yeast mutant defective in production of the HMW PC-Cd-S^{-2} complex has been identified. We have isolated a gene, designated *hmt1* (heavy metal tolerance), that complements this mutant. cDNA sequence analysis suggests that the *hmt1* gene product is an integral membrane protein sharing amino acid sequence identity with ABC-type membrane transport proteins. The better characterized members of this family include the bacterial periplasmic permeases and the mammalian *mdr* and *cftr* gene products. Representatives of this group have also been reported in insects, fungi and protozoa.

Plant cells accumulate Cd and PCs in the vacuole, suggesting a link between vacuolar sequestration of PC-Cd complexes and heavy metal tolerance. A procedure for fractionating fission yeast cell components was developed, and intact vacuoles were purified. Immunoblot analysis of this fraction indicates that a chimeric protein produced from an *hmt1-lacZ* translational fusion construct co-purifies with the vacuolar fraction, suggesting that the *hmt1* gene product is a vacuolar membrane transport protein. Analysis of cell components purified from fission yeast strains exposed to Cd indicates that PCs are indeed associated with the vacuolar fraction, and that most of the HMW PC-Cd-S^{-2} complex present in the cell co-fractionates with this organelle. Overexpression of the *hmt1* gene results in markedly increased production of the HMW PC-Cd-S^{-2} complex associated with the vacuole. We propose that the *hmt1* gene product is responsible for transport into the vacuole of PCs, Cd, PC-Cd complexes, or factors required for synthesis of the HMW PC-Cd-S^{-2} complex.

THE REGULATION OF TONOPLAST PROPERTIES BY STRESS

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The tonoplast plays an important role in the metabolic compartmentation of plant cells. Active primary proton transport is associated with the identification of tonoplast-bound ATPase and pyrophosphatase. The tonoplast trans-membrane protonmotive force enables active transport of ions, amino-acids and organic acids. However, combined translocation of certain molecules and protons has been clearly identified and some tonoplast ionic channels has been demonstrated, showing that the membrane is active in movements of ions and metabolites. The paper reports unpublished laboratory work and compares the results with those in the literature.

Severe changes in the environment of plant cells (cultivated or not) cause aggressive stress and cause metabolic changes at various levels. Decompartmentation - in which tonoplast plays a special role - is observed in all cases. This is described in detail in two systems : tonoplast isolated from *Catharanthus roseus* cells (cultured in liquid medium) and lutoids from *Hevea brasiliensis* (rubber tree) latex. Responses to the different types of stress tested included disturbances in basic cell characteristics such intracompartmental pH (and associated proton gradients), redox potentials, ion concentrations, and trans-tonoplast electrical potential gradients. These form a set of signals which modifies cell metabolism. Further data is provided concerning trans-tonoplast transport functions. Bidirectional solute fluxes between the intravacuolar compartment and the cytoplasm modulate the main biosynthesis pathways in plant cells.

The tonoplast is one of the main targets of freezing stress in plant cells. According to the type of the exposure to low temperature, the membrane is unable to achieve active transport of most ions and organic molecules. Membrane damage is correlated with the absence of any trans-tonoplast proton gradient mediated-transfer of sugars, amino-acids, organic acids and hormones. Freeze-drying of tonoplast without cryoprotectants can be seen as an efficient denaturating process.

Substantial metal stress affects tonoplast and considerably modifies barrier effectiveness. Metals modify the structure and/or surface charge characteristics. Cd, Zn, Mn and Al severely affect proton pump capacities. In the ATPase, metals compete with Mg for ATP. They also directly modify the conformation of the ATPase active site and its affinity for the Mg-ATP complex. The kinetic patterns of ATP hydrolysis and proton pumping are completely changed and the transport properties of tonoplast are significantly modified.

The other aggressive sresses tested had similar effects on tonoplast.

Any damage to endocellular membranes, and hence any process leading to the decompartmentation of plant cells, generates metabolic response patterns which are identical under a wide variety of different stress types. The role of tonoplast should be re-investigated and its importance of this aspect taken into account.

KINETICS OF K^+ EFFLUX FROM ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE CULTIVARS OF WHEAT

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Soil acidity is a major stress factor and solubilized aluminum (Al) ions in acid soil have a toxic effect of acid soil on plant growth. Cell membranes may be a primary injury site in the Al toxicity, thereby an ability of normal ion fluxes is strongly related to the mechanism of Al tolerance (1).

The efflux of K^+ of Al tolerant (Atlas 66) and-sensitive (Scout) wheat (kind gift from Dr. C.D. Foy, USAD) was investigated. Wheat seedlings cultured with 100 μM $CaCl_2$ were loaded with 25 mM K_2SO_4 and 200 μM $CaCl_2$ for 2 h, and followed by washing with 200 μM $CaCl_2$ until extrusion of K^+ becomes basal level. The pH values during the treatments was kept at approximately 5.5. Then the samples were transferred into the solution (pH 4.2) with or without various chemicals and efflux of K^+ was monitored for 40 min. The rate of K^+ efflux of Scout was twice as much as that of Atlas 66 in the distilled water adjusted to pH 4.2 with H_2SO_4. Vanadate increased the rate of K^+ efflux markedly to the similar level in both Scout and Atlas 66, and the amounts of extruded K^+ exceeded those of without vanadate. The presence of Al and Ca^{2+} either alone or together repressed the K^+ efflux markedly. The small amount of Al as low as 5 μM repressed the K^+ efflux markedly and the effect was more pronounced in Scout. The repressive effect of Ca^{2+} on K^+ efflux was completely avolished when Ca^{2+} was supplied together with vanadate but Al repressed the K^+ efflux even in the presence of vanadate. Repressive effect of Ca^{2+} and Al on K^+ efflux is completely different. Al in its short time effect may act as a K^+ channel blocker. CCCP, EGTA and verapamil increased the K^+ efflux significantly. Results suggest that K^+ efflux was caused by depolarization due to the transported H^+ into cytoplasm through pH gradient. The permeability of H^+ or H^+-transport pump activity may play an important role on the K^+ efflux at acidic pH and Ca^{2+} have a regulative role on those activities. Thus characteristic difference in terms of K^+ efflux between Atlas 66 and Scout may be explained by the different potential of plasma membrane to regulate the H^+ in the cytoplasm.

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EFFECT OF BORON ON PROTON TRANSPORT AND MEMBRANE PROPERTIES IN SUNFLOWER CELL MICROSOMES

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The requirement for boron by plants is well known, nevertheless its mode of action has not yet been clearly established. Recent works on the effect of boron on membrane potential and transport suggest that boron plays an important role in the maintenance of membrane structure and functionality. The effects of boron deficiency on H^+ pumping and on passive conductance, as well as on fluorescence anisotropy in KI-washed microsomes isolated from sunflower cell suspensions have been investigated. Boron deficiency reduced the total and vanadate-sensitive ATPase activities as well as the vanadate-sensitive ATP-dependent H^+ pumping. Kinetic studies also revealed that boron deficiency reduced the apparent V_{max} of vanadate-sensitive ATPase activity, with no important change in the apparent K_m for Mg^{2+}-ATP. Proton leakeage was increased in microsomal vesicles isolated from boron-deficient cells and incubated in the reaction mixture without added boron, this effect being reversed by the addition of boron to the reaction medium. Fluorescence anisotropy data revealed that DPH and TMA-DPH probes were more immobilized in microsomes grown with 0.1 mM boric acid, indicating a decrease of membrane fluidity by boron deficiency. Taken together these data suggest that inhibition of the proton gradient formation in microsomes from sunflower cells grown in the absence of boron, could be due to the combined effects of reduced H^+-ATPase activity as well as to increased passive conductance across the membrane, possibly derived from an increase in membrane rigidification.

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SURFACE CHARGE DENSITY AND AFFINITY VALUES OF CALCIUM, MAGNESIUM AND SODIUM IONS TO MELON ROOTS PLASMA MEMBRANE: COMPARISON OF SORPTION DATA WITH A COMPETITIVE SORPTION MODEL.

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The presence of calcium in the external solution is of prime importance for proper root elongation under saline conditions. This study measured the competition of Na and Mg with Ca on the sorption sites of plasma membrane (PM) from melon root cells.

Sorption of ^{45}Ca on right-side out PM vesicles of melon roots was studied at various levels of Ca, Na and Mg concentrations . The PM vesicles were prepared by aqueous two-phase partitioning. The amounts of ^{45}Ca sorbed decreased with increasing Na concentration at a constant Ca concentration, and increased with increasing Ca concentration at a constant Na concentration. Magnesium ions compete more strongly than Na with Ca for sorption to the PM. Hundred mM of Na as compared to 5 mM of Mg reduced the amount of Ca sorbed on the PM from 1.093 to 0.44 mmol/g protein in the presence of 1 mM Ca. The calcium ionophore A23187 did not change the amount of Ca sorbed to the vesicles suggesting that Ca did not accumulate inside the vesicles but on both sides of the PM.

The amounts of Ca sorbed on the PM vesicles fit very well a competitive sorption model, which takes into account specific binding to surface sites and the amount of cations in the electrical double layer. It also calculates the effect of surface sites concentration on the degree of sorption and takes into account the depletion of cations from the solution as a result of sorption. The best fit of the model to the experimental data, was obtained for an average surface area of 370 $Å^2$ per charge, and when the binding coefficients used for Na , Mg , and Ca were 0.8, 9 and 50 M^{-1} respectively. The binding constants used in this study are in the range of previously reported values for phospholipid vesicles obtained by different methods indicating that the behavior of biomembranes resembles that of phospholipid bilayers. However, our results indicate that non phospholipid components in the PM contribute significantly to Ca binding. This study might explain the specific role of Ca in relieving salt stress in plant roots.

K^+ IONS AS A TRIGGER FOR INTERDEPENDENT COUPLING OF MOLECULAR H^+-GENERATORS AND K^+-CHANNELS

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The bioenergetical components of Mitchell's equation are strongly dependent on K^+ ion activity both in apoplast and in cytoplasm. Then the problem arises of *how potassium might participate in a transition from E_m to E_{H^+} state expressing the most essential feature of a plant cell.*

Measuring of K^+ fluxes, medium acidification, ionic gradients and electrical parameters of plasma membrane show that the inflow level of K^+ ions promotes a generation of E_{H^+} up to 120 mV (K_m=0.01-0.02 mM K^+) with a concomitant decrease of E_m to circa -120 mV thus maintaining constant thermodynamic equilibrium E_m-E_{H^+}≃-250 mV. This phenomenon stresses the new role of potassium as a crucial element triggering generation of E_{H^+} proton gradient for the secondary anion transport in mineral plant nutrition. It is a characteristic of this $E_m<E_{K^+}$ *intermediate state* that K^+-channels are still closed under electrical control of H^+-current generators, but K^+ influx (K_m=0.01-0.02 mM K^+) is high, suggesting a K^+-stimulated transport ATPase and redox pump. Saturation of E_{H^+} at 0.05-0.1 mM K^+ is in accord with K^+ activity in natural soil solutions and freshwater reservoirs .

Another type of pump-channel coupling is to appear at the critical point of further E_m transition if concentration of K^+ ions increases higher than 1.0-2.0 mM (species specific). This triggers an opening time of K^+-channels which clamp E_m to the corresponding K^+-equilibrium E_{K^+} due to increasing of K^+ permeability and K_{in}/K_{out} exchange . In the transitional range of E_m=E_{K^+} K^+ ions become potential-determining. Chemical K^+ activation of H^+-ATPase at the interface is now dominated by electrical K^+-control utilizing diffusion E_{K^+} variation at the plasma membrane. Thus, interdependent pump-channel coupling is based on K^+ ion triggering effects in electrical control by either K^+-induced H^+-current generators (Epstein's Mechanism I) or K^+-diffusion potential (Epstein's Mechanisms II).

BIOENERGETICAL EXPRESS-TRIAL OF PLANT DROUGHT RESISTANCE

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The aim of the present study is to extent Sullivan's osmotic PEG-test of plant drought resistance towards the state of energy transduction at the plasma membrane. It is presumed that an imposed turgor pressure might induce a membrane deformation associated with the activity of immobilized H^+-current generators.

K^+-activated generation of H^+-potential gradient was measured in intact roots of 5-days-old Spring Wheat seedlings (4 varieties cultivated in the drought region). Concentration-dependent effect of turgor pressure was found to be most pronounced at 8% PEG. Acidifying activity of Wheat roots decreased after 1 day PEG-treatment up to 4 times, after 2 days even up to 8 times. It was in accord with the natural drought resistance of varieties: more tolerable cultivars were more stable under imitation of the environmental stress (well-known Saratovskaya-29 was the best). Variation of H^+-potential gradient under drought stress increased from 2 to 3 times suggesting a selection of the effective donor-plants for further tests. Bioenergetical drought trail was shown to be more sensitive to turgor pressure than the widely used growth reactions of roots and leaflets. It seems to become as a primary tool differentiating varietal drought resistance of plants.

CORRELATION BETWEEN THE VALUE OF MEMBRANE POTENTIAL OF INTACT PLANT CELL AND THE TEMPERATURE SENSITIVITY OF ELECTROGENIC H^+ PUMP OF PLASMA MEMBRANE

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The temperature dependence of membrane potential (E_m) of stem parenchyma cells of a pumpkin (Cucurbita pepo L.) on condition of gradual cooling (1.2-1.5°C×min^{-1}) from 23 to 10°C has been studied. It has been shown that the Arrhenius plots of the E_m have a discontinuity the temperature (T_c) of which correlates with the initial value of the cell E_m. The correlation was examined in the interval of the E_m values from -150 to -195 mV, T_c being changed from 16.0 to 18.5°C. During the analysis of the above correlation the relation between the point of discontinuity on the Arrhenius plot of the E_m and an abrupt temperature-dependent transition in the operation regime of the plasma-membrane electrogenic pump (H^+-ATPase) has been established. From the experimental data for isolated membrane vesicles with using hydrophobic fluorescent probes, i.e. 3-methoxybenzanthrone and pyrene, a thermal phase-structural rearrangement in the membrane lipids is a trigger of the abrupt change in the pump activity. It was suggested that the correlation between E_m and T_c shows a controlling influence of the E_m of intact plant cell on the temperature sensitivity of the electrogenic H^+ pump of the plasma membrane, which is realized by means of potential-dependent modulation of phase state of the membrane lipids.

MECHANISMS INVOLVED IN LIPID REGULATION OF THE PLASMA MEMBRANE H^+- ATPase

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Lipid dependency of the H^+- ATPase suggests a possibility of its regulation via the lipid environment. Effects of lipid modification on the H^+- ATPase activity in plasma membrane preparations from corn seedling roots has been investigated.

Exogenous phospholipase A_2 treament decreased boundary lipid microviscosity around the H^+- ATPase molecule. This process was accompanied by a transition from the H^+- ATPase activation to its inhibition. It was established that the phospholipase A_2 influenced the H^+-ATPase by means of phospholipid hydrolytical products. Lysophosphatidylcholine specifically stimulated the H^+- ATPase without any effect on bilayer dynamic properties. Free fatty acids didn't activate the H^+- ATPase but increased boundary lipid fluidity. Existence of two mechanisms of the H^+- ATPase activity regulation by the phospholipase A_2 was suggested: i) direct action on the H^+- ATPase protein and ii) inderect action via boundary lipid layer. It was demonstrated that effects of plant growth regulators on the H^+- ATPase activity were connected with activation of endogenous phospholipases A_2 and D in plasma membranes.

Lipid modification during the H^+- ATPase solubilization procedure has been studied. Solubilization caused a loss about of 95% phospholipid content with increasing of sterol:phospholipid ratio. The H^+- ATPase preparations solubilized by lysophosphatidylcholine and Zwittergent 3-14 were distinguished by their sterol and phospholipid composition. The role of lipid - protein interaction in the H^+- ATRase regulation is discussed.

SELENIUM-DEPENDENT CHANGES FOR CORTEX AND STELE CELL MEMBRANES OF MAIZE ROOTS

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A root representing an interaction system for tissues with specific functions is a convenient object to study a selenium effect of different concentrations on a condition of cell membranes. A permeability as a functional state whereas a fatty acid content as a structural state for cortex and stele cell membranes of maize root is the objective of the study.

Growth inhibiting portions of selenium have not revealed significant changes in the cell membrane permeability with the electrolyte output rate as a criterion. However, the content of palmitic and stearic acids has increased.

Growth inhibiting portions of selenium have caused a marked washing of electrolytes out of cortex tissues giving an evidence of disturbances in the cell membrane permeability. Revealed changes for the content of fatty acids confirm destructive changes of a lipid component of membranes whereupon the index of unsoluble fatty acids has changed insignificantly.

The membrane permeability has reduced in stele cells, at the same time the index of unsoluble fatty acids has increased. Apparently, large portions of selenium deteriorate functional systems providing biosynthesis of lipids.

It is assumed that counter-directionally revealed selenium-dependent changes for cell membrane permeabilities ofcortex and stele is a consequence of their specific functions.

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MEMBRANE-BOUND AND SOLUBLE FORMS OF ECTO Ca-ATPase (ECTO-NUCLEOTIDASE) IN BARLEY ROOT CELLS

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The enzyme was found in soluble fraction (supernatant 150 000 g) to be similar with the ecto Ca-ATPase of microsomal fraction enriched with plasmalemma fragments (1). The enzyme with optimum activity at pH 6 hydrolyzed all the nucleoside triphosphates and ADP, but not AMP, β-glycerophosphate or p-NPP. The ATPase possesed a high affinity to calcium (7 μm) and magnesium (17 μM) ions.

The distribution of nucleotidase activity between soluble and microsomal fractions was depended on the composition of the homogenizing medium. At alkaline pH or addition of $CaCl_2$ total activity of soluble fraction was increased.

These results indicate the presence of soluble and membrane-bound ecto-nucleotidase in barley root cells. The localization of soluble enzyme form in apoplast is discussed.

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EFFECT OF PHENOLIC COMPOUNDS ON THE ECTO Ca-ATPase OF PLASMALEMMA BARLEY ROOT CELLS

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Ecto Ca-ATPase detected in the microsomal fraction and on the surface of isolated root cells was sensitive to phenolic acids which were released from barley roots subjected to osmotic shock with sucrose. Phenolic compounds such as vanilinic, gallic, p-cumaric, chlorogenic, protocatechic, caffeic and ferulic were determined by thin-layer chromatography. It was shown that after osmotic shock of barley roots its Ca-ATPase activity was increased (1).

Addition of the fractions containing phenolics only to the incubation medium decreased the enzyme activity of the microsomal fraction isolated from the same root material. Among the phenolics only vanilinic (127 μmoles P_i/mg protein min) and gallic (96 μmoles P_i/mg protein min) at the concentration of 10^{-4} M decreased the Ca-ATPase activity (182 μmoles P_i/mg protein min) while the other phenolic acids did not effect on the enzyme activity. However, only vanilinic acid at the concentration at 10^{-5} M (90 μmoles P_i/mg protein min) inhibited Ca-ATPase activity.

These results indicate specific effect of vanilinic acid and its role as a modulator of ecto Ca-ATPase activity.

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ECTO Ca-ATPase (ECTO-NUCLEOTIDASE) OF PLASMALEMMA BARLEY ROOT CELLS: PURIFICATION AND SOME PROPERTIES

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Ecto Ca-ATPase of microsomal fraction was solubilized with 0.25 M KCI. Properties of membrane-bound and solubilized enzyme were found to be similar (hydrolysis ATP, CTP, GTP, UTP and ADP, but not AMP, β-glycerophosphate, pNPP or pyrophosphate; K_m for ATP 36 μM; optimum at pH 6 and 40°C; K_m for Ca_{free} 7 μM, K_m for Mg_{free} 17 μM; inhibition by NaF) (1).

Then the enzyme was purified by $(NH_4)_2SO_4$ precipitation, dialysis and Toyopearl-650 M fractionation. The specific Ca-ATPase activity eluted by bufer peak was increased 150-200 fold, but 0.1 n KCI - 25 fold. SDS gel pattern of bufer peak revealed three major molecular weight protein bands corresponding to 25, 46 and 50 kD while 0.1 n KCI peak - 25, 36 and 38 kD ones. The enzyme preparate hydrolized ATP and ADP, but not β-glycerophosphate (2).

The participation of polypeptides in the regulation of ecto Ca-ATPase activity is discussed.

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CORRELATION BETWEEN THE VALUE OF MEMBRANE POTENTIAL OF INTACT PLANT CELL AND THE TEMPERATURE SENSITIVITY OF ELECTROGENIC H^+ PUMP OF PLASMA MEMBRANE

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The temperature dependence of membrane potential (E_m) of stem parenchyma cells of a pumpkin (Cucurbita pepo L.) on condition of gradual cooling (1.2-1.5°C·min^{-1}) from 23 to 10°C has been studied. It has been shown that the Arrhenius plots of the E_m have a discontinuity the temperature (T_c) of which correlates with the initial value of the cell E_m. The correlation was examined in the interval of the E_m value from -150 to -195 mV, T_c being changed from 16.0 to 18.5°C. During the analysis of the above correlation the relation between the point of discontinuity on the Arrhenius plot of the E_m and an abrupt temperature-dependent transition in the operation regime of the plasma-membrane electrogenic pump (H^+-ATPase) has been established. From the experimental data for isolated membrane vesicles with using hydrophobic fluorescent probes, i.e. 3-methoxybenzanthrone and pyrene, a thermal phase-structural rearrangement in the membrane lipids is a trigger of the abrupt change in the pump activity. It was suggested that the correlation between E_m and T_c shows a controlling influence of the E_m of intact plant cell on the temperature sensitivity of the electrogenic H^+ pump of the plasma membrane, which is realized by means of potential-dependent modulation of phase state of the membrane lipids.

THE ASYMMETRY OF ION TRANSPORT IN ELODEA: THE DISCREPANCY BETWEEN LIGHT MEMBRANE POTENTIAL KINETICS AND POTASSIUM TRANSPORT

NOVAK V.A., MICLASHEVICH A.I.

By using of miniature electrode couples (pH, pK, pBr, pNO_3, platinum-redox and others) we have developed contact method for the investigation of the asymmetry of ion transport and the redox activity in the plasmalemma. The integral and differential kinetics of the light-induced transmembrane potential (MP), ion transport and $Fe(CN)_6$-reduction on the upper and the lower surfaces of single leaf are registered simultaneously. The light induced the acidification near the surface on the lower side leaf and the alkalinization near the surface on the upper side leaf. At a time MP hyperpolarization, activation of NO_3^-, Br^-, K^+ transport and $Fe(CN)_6$-reduction are to a marked degree more on the lower leaf side than upper side. The light induced transitions of MP are characterized by two, at least, clearly expressed phases: (1) primary depolarization (2-4 min) and (2) following progressive increasing of hyperpolarization to a steady state situation. There is a strong correlation between phasic transitions of MP and anion transport (pNO_3, pBr). The kinetics of MP and pK is in counterphasic changes. The alternation of two counterphase pK - K^+ efflux and following increasing of the uptake its to a steady state situation correspond to the second phase of MP hyperpolarization. It is assumed that the K^+ influx (electrogenic H^+/K^+ exchange) and K^+ efflux (electroneutral K^+/H^+ exchange) define K^+ transport across the plasmalemma. Both systems of K^+/H^+ exchange are regulated by cytoplasmic pH in counterphasic change manner. Alkaline pH reaction of the upper leaf side may be accounted by the functioning of electroneutral OH^-/anion exchange in the plant cell plasmalemma. The nature of asymmetry and its role in metabolic and transport regulation in the plant cells will be discussed.

THE TARGET OF FUSICOCCIN ACTION: H^+-ATPase OR PLASMALEMMA REDOX SYSTEM?

MICLASHEVICH A.I., NOVAK V.A., MOROKOVA E.A.

The hypotesis that fusicoccin (FC) affects on the electrogenesis and ion transport by means of the modification of the plasmalemma redox system in the plant cells is examined. We have investigated the stimulating effect of FC on the transmembrane potential (MP), H^+ and K^+ transport and $Fe(CN)_6$-reduction by Elodea leaves. The value of FC effects is determined by energetic state of the cells and FC concentration during FC treatment of the leaves. The light increased the stimulating FC effects up to maximum value, which is in excess of the value of the stimulating FC effect in the dark on the average up to 100%. The stimulating effect of FC on $Fe(CN)_6$-reduction in the dark is increased ca 30-50% by energetic substrates (glucose, ethanol, ascorbate). The decrease of media's pH to pH 4,0 - 4,5 modeled the light stimulating effect of FC on MP and $Fe(CN)_6$-reduction in the dark. The electrons accepting by ferricyanide (0,6 mM) on the outer side of the plasmalemma during FC treatment of the leaves resulted in increasing of stimulating FC effect on $Fe(CN)_6$-reduction ca 80%. Metabolic inhibitor NaN_3 (0,01 mM pH 5) and the uncouper 2,4-DNP (0,02 mM pH 5), which are included in the media of the FC treatment did not influence on the stimulating FC effect on the light as well as in the dark. Our results allow to conclude that the interaction of FC with the plasmalemma is cooperative, independent from metabolic energy process, wich takes place at the protonated state of the reseptor. FC did not change energetic state of the cells: ATP and NADH levels and respiration not alter without transport and redox loading on the plasmalemma. It is assumed that FC affects on plasmalemma redox function by the modification of redox component activity, but not changing of energetic metabolism of the cells. Convincing evidence for the participation of the plasmalemma redox system in FC effects on the electrogenesis and ion transport is obtained during the investigation of the asymmetry of ion transport and plasmalemma redox activity on Elodea leaves. H^+ and K^+ transport MP and $Fe(CN)_6$-reduction are to a marked degree more on thr lower side of the leaf than the upper side. FC stimulated hyperpolarization of MP positively correlated with the value of $Fe(CN)_6$-reduction. The effect of FC on MP absence when $Fe(CN)_6$-reduction on the upper side of leaf is close to 0.

BIOGENESIS OF THE PERIBACTEROID MEMBRANE AND TARGETING OF NODULINS IN SOYBEAN ROOT NODULES

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Endocytotic release of *Rhizobium* inside the legume root cells forms a new subcellular compartment, the peribacteroid membrane (PBM) housing the bacteria. Derived from the plasma membrane, this membrane undergoes many changes (1) as it incorporates nodule-specific proteins (nodulins). Integrity of this membrane is vital for symbiosis. The PBM has properties common to both plasma membrane and vacuolar membrane. We are interested in biogenesis, targeting, and function of PBM nodulins. Nodulin-26 and nodulin-24 are major proteins of the PBM. Nodulin-26 is an intrinsic PBM protein, while nodulin-24 is a surface protein facing the bacteroids. These proteins are targeted to PBM through different mechanisms.

Nodulin-26 represents a family of proteins including MIP-26. The members of this family form channels and are conserved from bacteria to mammals. We have determined the topology of nodulin-26 in PBM. Nodulin-26 consists of 271 amino acids with six potential transmembrane domains and lacks an amino terminal signal sequence. A full length nodulin-26 cDNA and its various deletion derivatives were transcribed *in vitro* after linking them to T3 promoter. *In vitro* translation of these transcripts in a rabbit reticulocyte lysate, in the presence or absence of microsomal membranes, suggested that nodulin-26 is co-translationally inserted into the microsomes without a cleavable signal peptide. The first two transmembrane domains (103 amino acids) of the protein are sufficient for microsomal membrane insertion. Membrane-translocated nodulin-26 binds to Concanavalin A (Con-A) and is sensitive to endoglycosidase-H treatment, suggesting it to be glycosylated. Topological studies on nodulin-26 suggested that both the N and C termini of this protein are on the cytoplasmic surface of the peribacteroid membrane, while the glycosidic residue is on the membrane surface facing the bacteroids. *In vitro* phosphorylation experiments showed that nodulin-26 is phosphorylated by a Ca^{2+}-dependent, calmodulin-independent protein kinase located in the peribacteroid membrane. Based on its homology with several eucaryotic and procaryotic channel-type membrane proteins, nodulin-26 may form a channel translocating specific molecules to the bacteroids during endosymbiosis in legume plants. In order to understand biogenesis of PBM, we are attempting to complement various yeast mutants deficient in endocytosis, membrane biosynthesis, and the intracellular transport of proteins with expression libraries of soybean and *Vigna* cDNAs. Isolation of genes involved in controlling membrane biosynthetic pathways in plants will be of great importance in understanding the signal transduction mechanism that triggers membrane proliferation and formation of subcellular compartments.

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TEMPERATURE-DEPENDENT DYNAMICS FOR GROWTH SUBSTANCES AND PERMEABILITY OF WINTER WHEAT CELL MEMBRANES

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An increase of the auxin activity has been observed in the process of seed swelling within 2-4 hours at +10, 27 and 35°C, while a deviation from 27°C has reduced it. Under these conditions a high inhibiting effect of abscisic acid with R_f 0.92 has been developing with a distinct rhytmicality.

The effect of variable temperatures (35/27, 10/27°C) has induced a substance with R_f 0.85 showing inhibiting or stimulating effects. The identification has given an evidence of the adaptation as a result of induced phenolcarboxylic acid.

It is assumed that the seed adaptation to unfavourable conditions accomplishes both by reserving auxin substances in a bound form and accumulating germination inhibitors.

The auxin and inhibitor status on the organ level in seedlings has been changed due to duration of different temperatures. Uneven effects are the evidence of a temperature influence on the transport of growth stimulants and re-distribution of stimulants among organs.

Of discussing are the results of temperature-dependent changes cell membrane permeabilities of a coleoptile and root and their relationship to variations of the growth substance dynamics.

CAROTENOIDS AND SENSORY TRANSDUCTION OF THE BLUE LIGHT RESPONSE IN GUARD CELLS. Eduardo Zeiger, Department of Biology, University of CAlifornia at Los Angeles.

Guard cells have well characterized responses to blue light, that include stimulation of proton pumping, starch hydrolysis, malate biosynthesis, and stomatal opening. In intact leaves, stimulation of stomatal opening by pulses of blue light given under high fluence rates of background red light irradiation have an induction 1/2 time of ca. 9 sec and a decay 1/2 time of ca. 9 min.

The chemical identity and the localization of the blue light photoreceptor(s) in guard cells remain to be characterized, although it has been widely assumed that the responses belong to the "cryptochrome" type, presumed to be mediated by flavins. On the other hand, we have recently isolated carotenoids from guard cells and their chloroplasts and found that their absorption spectrum was very similar to an action spectrum of the blue light-stimulated stomatal opening. This finding prompted us to investigate whether guard cell chloroplasts have specific blue light responses which could implicate carotenoids as blue light photoreceptors in guard cells.

Obtained results indicate that isolated guard cell chloroplasts have responses to blue light matching those seen with intact guard cells. These responses include starch hydrolysis and the modulation of red light-induced, chlorophyll a fluorescence transients by low fluence rates of blue light. We also found that adaxial (upper) stomata from cotton leaves open more in response to blue than to red light, and show a concomitant, more pronounced blue light-dependent enhancement of red light-stimulated fluorescence quenching than that seen with abaxial (lower) stomata. A wavelength dependence of the blue light-enhanced fluorescence quenching is very similar to the absorption spectrum of carotenoids. In addition, adaxial guard cells have about twice the carotenoid content than their abaxial counterparts.

These data indicate that carotenoids could function as photoreceptors for the sensory transduction of blue light in guard cells. The well-characterized xanthophyll cycle indicates that carotenoids are optimally suited to funnel energy away from the thylakoid membrane, and the polyene-based photoreceptor systems in bacteria and animals indicate that carotenoids are very effective light signal transducers. Carotenoids in the guard cell chloroplasts could act as molecular switches. Depending on the energy charge of the thylakoid membrane, carotenoids could funnel light energy into the antenna pigments and thus stimulate photosynthetic reactions. Alternatively, carotenoids could move away from the chlorophyll molecules and transduce the absorbed energy into stroma and/or cytosolic reactions, expressed as blue light responses. Several properties of the guard cell responses to blue light can be explained by this hypothesis. Supported by NSF, DOE and USDA.

Regulation and Pharmacology of the SV-Channel in the Tonoplast Membrane of *Chenopodium rubrum* L.

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The calcium-dependent slow-vacuolar (SV) cation-selective channel in the tonoplast of Chenopodium suspension cells is reversibly blocked by the calmodulin-antagonists W-7 and W-5; inhibition is cancelled by addition of native calmodulin isolated from Chenopodium, spinach or maize, but not by bovine brain calmodulin [1].

▸ The SV-channel is highly sensitive against the scorpion toxin charybdotoxin which is considered as a rather specific inhibitor of a particular class of calcium-dependent potassium channels in animals. The toxin binds (half-max. at 20 nM) to the closed channel [2,4].

▸ The channel is blocked in a voltage-dependent fashion by the acetylcholine receptor antagonist (+)-tubocurarine. This plant toxin binds to the open channel, from the cytosolic side only, with 7 μM half-maximum inhibition [3,4].

▸ The channel's mean open time is 30 ms, the closed time ranges from 60 to 900 ms. Calcium and voltage control the channel's open probability predominantly through the closed time [5].

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THE MEMBRANE-BOUND AUXIN RECEPTOR IN MAIZE: ANTIBODY PROBES OF STRUCTURE, FUNCTION AND ACTIVE SITE

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A range of antibodies has been developed to probe the structure and function of the maize auxin receptor. The epitopes recognised by a number of polyclonal sera and by five monoclonal antibodies have been mapped with the aid of an epitope mapping kit. The polyclonal sera recognise three principal epitopes, two of which are conserved in other species. Two monoclonals recognise the endoplasmic reticulum retention sequence (-KDEL) at the C-terminus, while another two recognise a region close to the N-terminus. Therefore we have markers for specific parts of the auxin receptor and we are using these to identify functionally active parts of the protein. Additionally, we have a number of sera against a synthetic peptide. This peptide was identified from the published receptor sequence as being likely to contribute to the auxin binding site. The antipeptide sera recognise the receptor on immunoblots and have potent auxin agonist activity on tobacco leaf mesophyll protoplasts, eliciting characteristic hyperpolarisation of the plasma membrane. By contrast, antisera to the native receptor act as auxin antagonists. This auxin agonist activity means that we have identified at least part of the auxin binding site and leaves little doubt that the maize protein is a genuine auxin receptor. We aim to design 'anti-sense peptides' against this region of the protein and to evaluate these for auxin activity. While the electrophysiology data indicate that the receptor is active on the outside of the plasma membrane, the bulk of the receptor protein resides in the endoplasmic reticulum in accordance with its KDEL targeting sequence. This and other questions will be discussed.

CHAOTIC DYNAMICS IN PLANT GROWTH: INDIRECT EVIDENCE FOR A STRAIN RATE SENSOR ON THE PLASMA MEMBRANE OF GROWING CELLS

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Many plants exhibit spontaneous, sometimes oscillatory, fluctuations in stem and root elongation rate over periods of minutes to hours, even under constant environmental conditions. The cause of such fluctuations is unknown, although random fluctuation in internal processes is usually assumed. This poster will present evidence for an alternative view, namely that these fluctuations arise from a nonlinear feedback system which may act to coordinate membrane recruitment with wall relaxation and surface area expansion.

When cucumber seedlings were challenged with step changes in externally-applied pressure (pressure-block device), their growth rates exhibited fast and complex kinetics of recovery. Similarly, the growth response to pressure sinusoids displayed time lags, resonances, frequency-dependent harmonic responses, and period doublings. Analysis of growth-rate time series by nonlinear forecasting indicates an underlying dynamical system that gives rise to a temporal pattern of "deterministic chaos". These features are characteristic signatures of systems with nonlinear feedback .

The results imply rapid feedback (lag of 2-4 min) from a sensor which measures growth rate over an interval of about 1 min. This conclusion, in turn, means that if wall expansion is caused by wall loosening enzymes, their activity must be closely coupled to cellular processes dependent on the growth rate sensor. This complex growth mechanism may serve to coordinate wall expansion with other required processes in growing cells (e.g. membrane recruitment and recycling) and under some conditions may give rise to nutation and related growth oscillations. Likely candidates for this postulated growth rate sensor are stretch-activated ion channels which have the necessary sensitivity to serve as sensitive gauges of membrane strain.

STRETCH-ACTIVATED ION CHANNELS IN PLASMA MEMBRANES: POSSIBLE STRAIN GAUGES FOR REGULATION OF CELL VOLUME AND GROWTH RATE

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Rapid cell expansion presents a hazard to the plasma membrane of walled cells because the mechanical forces generated by turgor can easily rupture the membrane if its elastic limit (2-3% strain) is exceeded. In rapidly growing cells and in guard cells during stomatal opening, this elastic limit would be reached in a few minutes, if membrane recruitment was not coordinated with cell expansion. Stretch-activated ion channels may serve as strain gauges to coordinate the separate processes of cell expansion and membrane recruitment.

Stomata often exhibit oscillatory responses to step changes in water supply or transpiration demand, which suggests a feedback system regulating cell volume changes. Patch-clamp analysis [1] of cell-attached membranes and excised membranes demonstrated that *Vicia faba* guard cell protoplasts have at least three types of stretch-activated channels (chloride-, potassium- and calcium-permeable) which are distinguishable from their spontaneous (not stretch activated) counterparts on the basis of their kinetics, amplitude and selectivity, as well as their sensitivity to membrane stretch.

Cucumber hypocotyls exhibit complex growth dynamics characteristic of a system with nonlinear feedback [2]. These growth dynamics lead me to propose that stretch-activated channels in the plasma membrane of growing cells act as growth rate sensors to supply a signal (calcium?) which quickly modulates wall relaxation processes. Evidence from cell-attached membrane patches shows that cortical cells from the growing region of cucumber hypocotyls have stretch-activated channels in their plasma membranes, as predicted. Strategies for elucidating their possible role in growth rate regulation will be discussed.

[1] Cosgrove, Daniel J. and Hedrich, R. 1991. Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membranes of guards cell s in Vicia faba L. Planta 186: 143-153

[2] Cosgrove, Daniel J. 1992. Chaotic dynamics in plant growth: indirect evidence for a strain rate sensor on the plasma membrane of growth cells. Abstract/Poster at this meeting.

Using Luminous Plants to Measure Intracellular Calcium

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Aequorin is a protein of coelenterate origin which specifically binds calcium ions. It consists of a single polypeptide chain, apoaequorin, and a hydrophobic luminophore, coelenterazine. Upon binding calcium ions a conformational change brings about the release of energy as blue-light. For this reason microinjected aequorin has been much used, especially in animal cells, to detect changes in cytosolic calcium by measuring consequent changes in luminescence. This approach had until recently been limited to single cells of a relatively large size.

By genetically transforming *Nicotiana plumbaginifolia* to express apoaequorin, and by treating these transgenic plants with coelenterazine produces luminous plants whose light-emission directly reports cytosolic calcium. This method allows the measurement of increases in cytosolic calcium in response to a number of stimuli eg. touch, cold-shock and the addition of fungal elicitors. As this approach allows the measurement of cytosolic calcium changes in whole plants for the first time, measurements of the the effects of stimuli relevent to whole plants, *eg.* wind, can be made.

Using a highly sensitive photon-counting camera it is possible to image the aequorin luminescence resulting from various stimuli. In this way some sort of picture of which cells are involved in different calcium-responses can be constructed.

Most recently work has been initiated which involves the use of different coelenterazine analogues which endow aequorin with altered properties, *eg.* changes in calcium-sensitivity, and the preparation of gene-constructs to target aequorin to specific organelles. Hopefully this approach will allow the measurement of organelle free calcium *in vivo*.

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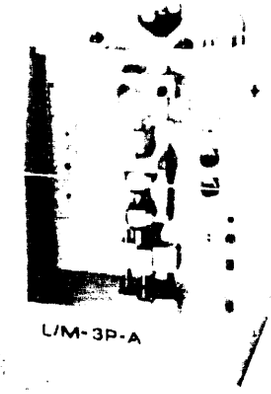

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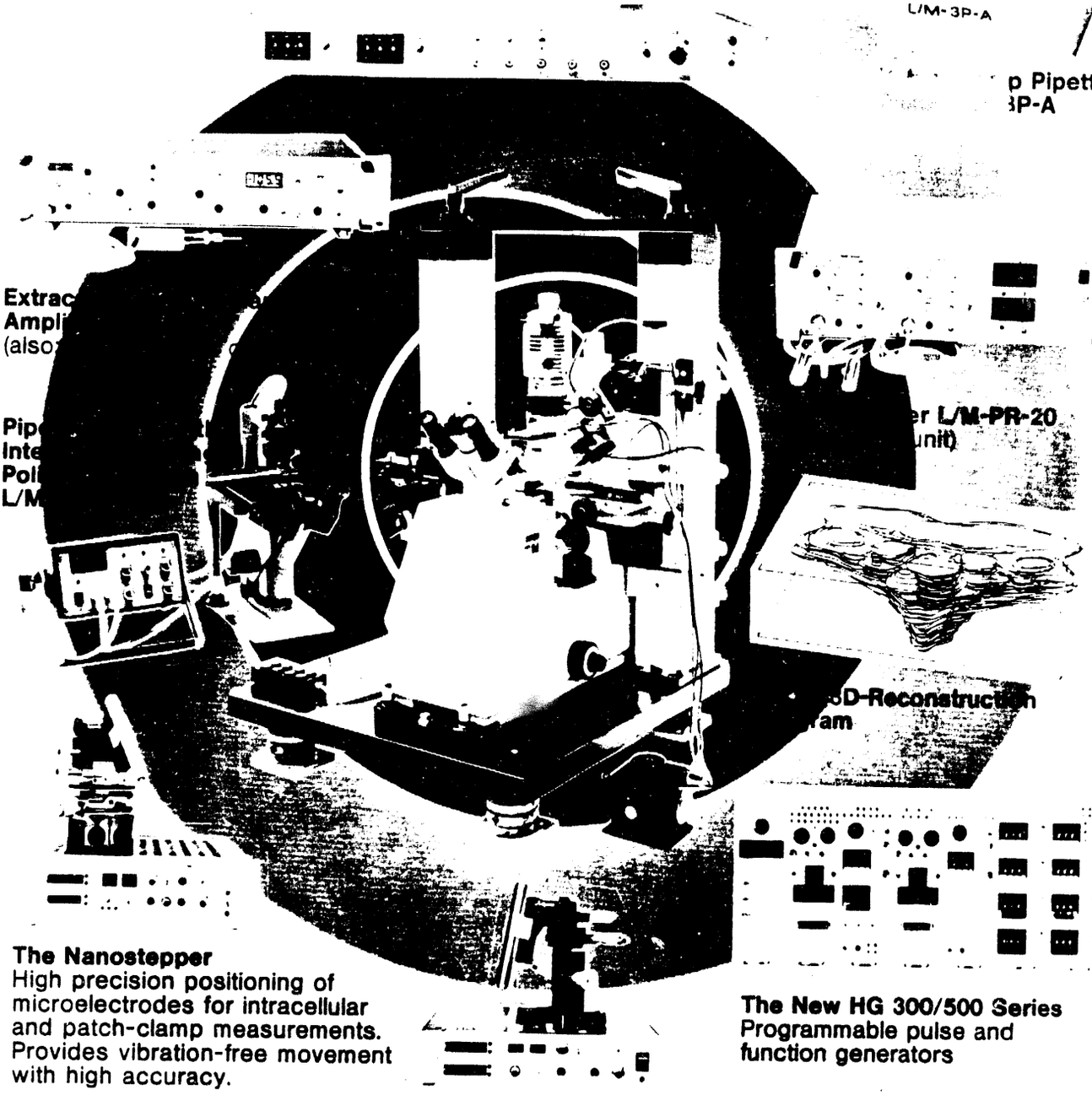

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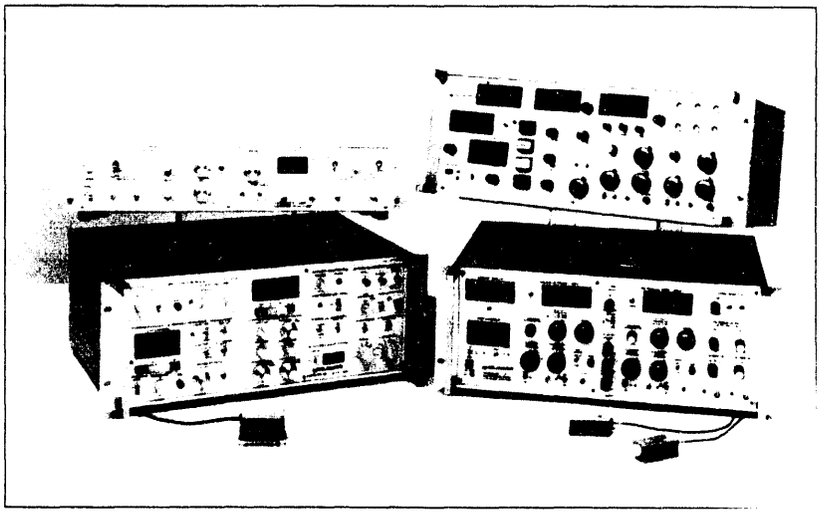

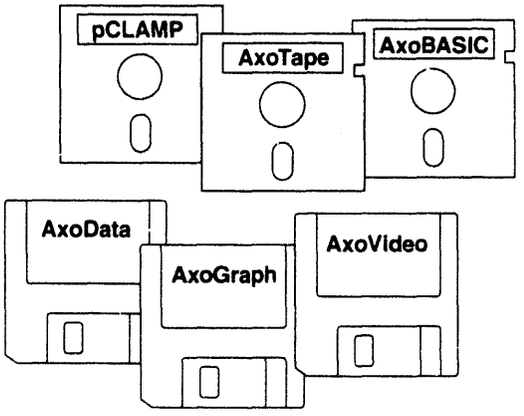

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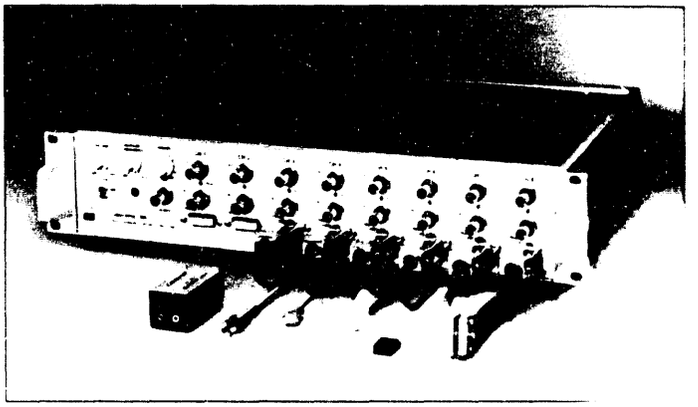

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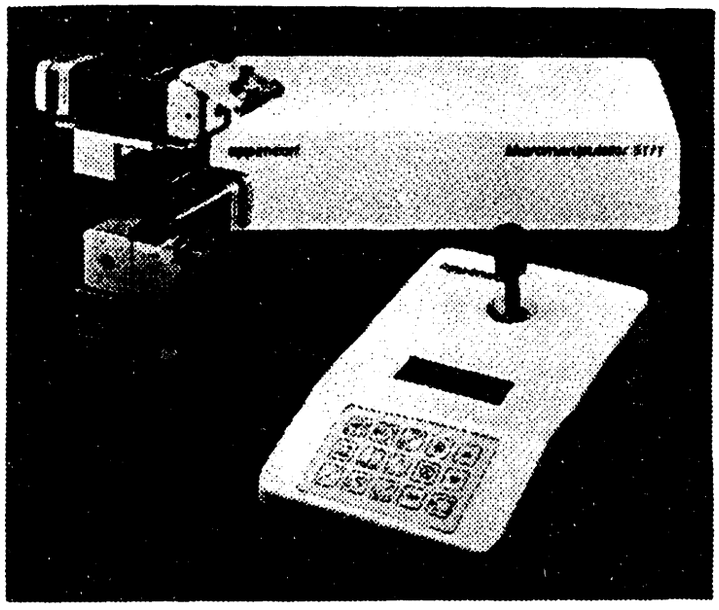

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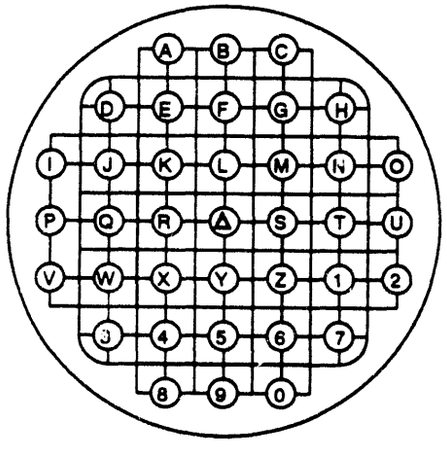

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