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CONF-8910303--1

LA-UR--89-3232

DE90 000687

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TITLE THE GTP BINDING PROTEIN-DEPENDENT ACTIVATION AND DEACTIVATION OF cGMP PHOSPHODIESTERASE IN ROD PHOTORECEPTORS

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SUBMITTED TO Cyclic Nucleotide Phosphodiesterase 2nd Int'l Conf.
Tokushima, Japan
October 4-6, 1989

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The GTP Binding Protein-Dependent Activation and Deactivation of cGMP Phosphodiesterase in Rod Photoreceptors

(Running Title: Rod cGMP Phosphodiesterase)

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Cyclic GMP (cGMP) has a crucial role in visual transduction. Recent electrophysiological studies clearly indicate the existence of cGMP-activated conductance in photoreceptor plasma membranes (29). In darkness, Na^+, Ca^{++}, and Mg^{++} enter rod outer segments (ROS) through cGMP-activated channels while light closes channels by lowering cGMP concentrations through activation of cGMP phosphodiesterase (PDE). Many excellent reviews (1,17,23,25) reference the mechanism of PDE activation in photoreceptors. However, recent progress in understanding the mechanisms regulating cGMP hydrolysis has raised an important question in the PDE-regulation: how does the three-dimensional movement of a subunit of transducin (retinal G protein) (20) relate to the PDE activiation? Associated with that question, the mechanism of PDE regulation appears to vary at different stages of evolution, for example, frog and bovine photoreceptors. This review examines recent progress of the cGMP hydrolysis mechanism by focusing on the subunit interactions between transducin and PDE.

A. GTP hydrolytic cycle in rod photoreceptors

The light-activated PDE cascade is composed of these functional proteins: the photopigment rhodopsin, transducin, and PDE. Signal communication from rhodopsin to PDE is dependent upon the light-mediated binding of GTP to transducin. Transducin is composed of three subunits: $T\alpha$ (Mr 39,000), $T\beta$ (Mr 36,000) and $T\gamma$ (Mr ~7,000). Photon capture by rhodopsin facilitates GTP binding to $T\alpha$, which depends upon the presence of $T\beta,\gamma$. The resulting GTP·$T\alpha$ complex is rapidly released from ROS membranes (20) and somehow activates PDE (10). Following hydrolysis of GTP, the GDP·$T\alpha$ complex returns to ROS membranes (35). A detailed, stepwise mechanism for the events of the GTP hydrolytic cycle (GTP binding, GTP hydrolysis, and GDP release) has been described (32,36).

1. GTP binding. Direct characterization of GTP binding to $T\alpha$ remains unclear, since purification of guanine nucleotide free $T\alpha$ has not been accomplished. All published data describes an exchange reaction between GTP (or hydrolysis resistant GTP analogues) and bound

GDP. When guanosine 5'-0-(3-thiotriphosphate) (GTPγs) was used as a ligand for Tα, the initial rate of GTPγs binding to toad (Bufo marimus) Tα was increased more than 90 fold by increasing illuminated, urea-washed ROS disc membranes in a reconstituted system using specific quantities of purified Tα and Tβ,γ (36). In addition, the initial rate of GTPγs binding to Tα was markedly enhanced by increasing the amount of Tβ,γ with a constant amount of ROS membrane. With very large additions of bleached rhodopsin, the ratio of Tβ,γ to Tα required for a half-maximal rate of binding is very small, reflecting the high probability of Tα interaction with bleached rhodopsin. Conversely, very small additions of bleached rhodopsin the ratio of Tβ,γ to Tα required for the half-maximal rate of GTPγs binding becomes large. Thus, one can experimentally generate a very wide spectrum of apparent catalytic efficiencies for Tβ,γ. Recent data (36) suggest that Tβ is essential for the Tβ,γ dependent stimulation of the rate of GTPγs binding. Moreover, Tγ alone is not sufficient for the Tβ,γ-dependent stimulation of the rate of GTPγs binding (36).

The rate of GTPγs binding to Tα shows a strong dependence upon the concentration of Mg^{++} when small amounts of illuminated, urea-washed ROS disc membranes were present in a reaction mixture. Mg^{++} markedly reduces the quantity of illuminated ROS membranes required for comparable binding of GTPγs Tα (32). Mg^{++} also serves to enhance sensitivity to the photic stimulation, especially over the lower end of the dynamic range of rod photoreceptors (32). Thus, Mg^{++} would profoundly influence the amplification step in which hundreds of GTP·Tα complexes can be generated by a single bleached rhodopsin (11).

2. GTP hydrolysis. Tα is rapidly released from illuminated ROS membranes following the binding of GTP or its analogues (20). In the presence of GTP or its analogues, Tα is readily extracted from illuminated ROS membranes even in the presence of 5 mM Mg^{++} and/or high concentration of salt (an isotonic buffer). In contrast, the dissociation of Tβ,γ from illuminated ROS membranes is reduced in the presence of 5 mM Mg^{++} even if the membranes are washed with hypotonic buffer after extraction of Tα with GTP (36).

Following the rapid release of the GTP·Tα complex from ROS membranes, GTP (bound to Tα) is hydrolyzed (in the soluble phase) in the absence of Tβ,γ, which remains primarily associated with ROS membranes (36). This conclusion is supported by the kinetic analysis of the rate of GTP hydrolysis (36). In the presence of increasing concentration of Tβ,γ, the GTP hydrolysis rate shows clear evidence of saturation throughout the Tβ,γ concentration range. This behavior is predicted by a model in which Tα is solely responsible for the hydrolysis of GTP. Moreover, the conclusion is also supported by the topological perspective that the binding of GTP to Tα rapidly terminates the interaction with Tβ,γ (20). Under the conditions that abolish the effect of Mg^{++} on GTP binding to Tα (in the presence of large quantities of illuminated, urea-washed ROS membranes), Mg^{++} is not required for the hydrolysis of bound GTP (32). Moreover, these experiments also show that Mg^{++} increases the rate of GTP hydrolysis by only 50% (32).

3. GDP release. Following hydrolysis of GTP to GDP, GDP·Tα returns to ROS membranes (35). Return of GDP·Tα to the ROS membrane surface is stimulated by the presence of membrane-bound Tβ,γ (9,36). GDP is subsequently released even in the absence of other guanine nucleotides. In a reconstituted system using purified components, the rate of GDP release is very slow in the absence of guanine nucleotides, since GDP also binds to Tα with a high affinity (32,36). Illuminated ROS membranes and Tβ,γ are both required for the release of GDP from Tα in the absence of guanine nucleotides (32,36). The slow GDP release is detected in the presence or absence of Mg^{++}, and the rate of GDP release is not stimulated by the presence of Mg^{++}.

In contrast, when crude ROS membrane preparations were incubated with [^{3}H] GTP, rapid GDP displacement is readily detected once the free GTP pool is exhausted (36). This GDP release is dependent upon the presence of Mg^{++} (32). [^{3}H]GDP bound to Tα in crude membrane preparations appears quite stable in the absence of Mg^{++}.

Summary. As shown in Figure 1, all of the known functions of Tβ,γ (including the requirement of Tβ,γ for GTP binding to Tα, for the return of GDP·Tα to the ROS membranes, and for the release of GDP from the GDP·Tα complex) are expressed essentially at the surface of the ROS disc membranes. In contrast, the hydrolysis of GTP by Tα occurs in the soluble fraction and does not depend upon the presence of ROS membranes or Tβ,γ. Mg^{++} plays a fundamental role in all reactions that occurr at the surface of the ROS disc membrane. However, Mg^{++} is not crucial for the GTP hydrolysis detected in the soluble fraction.

B. GTP·Tα-dependent PDE activation.

PDE in rod photoreceptors is composed of three subunits: Pα (Mr 88,000), Pβ (Mr 84,000), and Pγ (Mr 11-13,000) (3). cGMP hydrolysis has been suggested to be catalyzed by Pα,β while Pγ is an inhibitory subunit (18). The GTP·Tα complex somehow stimulates PDE activity (10), presumably resulting in a decline in cytoplasmic cGMP levels. How is each step of the GTP hydrolytic cycle of transducin related to the mechanism of PDE activation?

In frog rod photoreceptors, the activation of PDE by transducin is accompanied by the physical release of PDE inhibitor from PDE-bound ROS membranes suspended in an isotonic, GTP-containing buffer (35). Washing illuminated disc membranes with an isotonic buffer released 86% of the peripheral proteins without any release of inhibitor. Subsequent washing with the same buffer containing GTP released 80% of the inhibitor. PDE activity was stimulated about 10 times by washing membranes with an isotonic, GTP-containing buffer even if the enzyme activity was measured in the absence of GTP or hydrolysis-resistant GTP analogues. Densitometric scanning demonstrated that the amount of a protein (Mr 13,000) was reduced to only 15% of the quantity present in the membranes washed with the GTP free buffer. The GTP-washed membranes contained 85% of the Pα,β found in membranes which were washed with the GTP free buffer (33). Thus, about 60 70% of 13,000 protein was extracted by the isotonic buffer containing GTP, in close agreement with published data (35).

When PDE inhibitor was eluted from the frog ROS membranes with guanosine 5'-(β,γ-imino)triphosphate, Gpp(NH)p, it appeared in fractions with an apparent molecular weight of 60,000 upon chomatography in a Sephadex G-100 column (35). Our recent studies (33) confirmed these data using GTPγs; following purification of the inhibitor using a Blue Sepharose CL-6B column and a TSK 250 size exclusion column in a FPLC system (Pharmacia), these inhibitory fractions contained Tα and Pγ. The molecular ratio of Pγ to Tα in the peak fraction was 0.7:1. These data clearly show that in frog photoreceptor systems the PDE activation by transducin resulted from the release of the inhibitory subunit Pγ (complexed with GTP·Tα) from the membrane-bound PDE.

In contrast, several studies have not detected the release of an inhibitory subunit from the membrane-bound PDE of bovine photoreceptors in isotonic buffers. Sitaramayya et al (24) suggested that Pγ was not released from the PDE catalytic complex during bovine PDE activation. They showed that kinetic properties (Km for cGMP and Kd for inhibitor) of the light-activated and trypsin-activated PDE were quite different and suggested that light-activated PDE exists as a complex with transducin (Pα,β·Tα·Pγ or Pα,β·Tα). Wensel and Stryer (27) found that following the washing of bovine ROS membranes with an isotonic, GTP-containing buffer, Pγ was conspicuously absent from the supernatant. Navon and Fung (22) reached the same conclusion using coimmunoprecipitation of GTPγs·Tα with holoenzyme of PDE. Hingorani et al (16) suggested, using bifunctional cross-linking reagents, that the bovine PDE activation involves a direct interaction between Tα·Gpp(NH)p and Tβ,γ with Pα,β (without Pγ release). We have confirmed that our published procedure (35) did not cause the release of Pγ from bovine PDE (33). Neither Tα-bound nor Tα-free Pγ was detected in the supernatant after washing ROS membranes with isotonic, GTP-containing buffers. It is possible that, in bovine ROS membranes (suspended in an isotonic, GTP-containing buffer) during PDE activation, either Pγ is released from Pα,β, and the Pγ·Tα complex is not released from the membrane, or Pγ remains complexed with Pα,β while interacting with Tα. Wensel and Stryer (27) showed

that the binding of Pγ to trypsin-treated Pα,β in bovine ROS is very tight and that its dissociation constant is less than 10 pM. The tight binding of Pγ to Pα,β may prevent the release of Pγ with GTP·Tα from bovine PDE. Our recent data (33) indicated that 24 nM Pγ was required for 50% inactivation, of Pγ-free PDE in ROS membranes (0.39 μg) washed with an isotonic, GTP-containing buffer. These data indicate that in bovine photoreceptors PDE activation does not relate to the three-dimensional movement of Tα.

If PDE activation by trypsin (18) is functionally equivalent to PDE activation by GTP, and trypsin activates 100% of PDE, then only 40% of PDE was activated by washing the frog ROS membranes with an isotonic, GTP-containing buffer. However, SDS polyacrylamide gel electrophoresis revealed that 60-70% of Pγ was extracted from the membrane-bound PDE by washing with GTP-containing buffer. If frog PDE contains one Pγ, and the removal or destruction of Pγ is the only PDE activation mechanism, there is no simple explanation for the discrepancy in PDE activation by Tα and trypsin. However, if frog PDE contains two Pγs per PDE as suggested in bovine rod photoreceptors (8), these data suggest that binding of the first Pγ to Pγ-free PDE inhibits the majority of PDE activity. The second Pγ may have other role(s) in the PDE cascade, for example, the stimulation of cGMP binding to noncatalytic sites on PDE (31).

C. Roles of transducin in PDE turnoff mechanism.

GTP hydrolysis is a prelude to effector turnoff in GTP-dependent signal transduction mechanisms. Cassel and Selinger (4) first proposed that hydrolysis of GTP is a mechanism for turnoff of activated adenylate cyclase in turkey erythrocyte membranes. In rod photoreceptors, GTP hydrolysis also has been shown to be a turnoff mechanism of GTP-activated PDE (28). In ROS membrane preparations, GTP bound to Tα is hydrolyzed resulting in bound GDP and the concomitant inhibition of membrane-bound PDE (35). Using a Sephadex G-100 column, all inhibitory activity in the GTP-washed supernatant was eluted in the same fraction as a complex

of Pγ·GTPγs·Tα (Mr 57,000) (33). In the column chromatography, no inhibitor activity was found in fractions around molecular weight 13,000. In these studies the Pγ·GDP·Tα complex was purified using a Blue Sepharose CL-6B column and a TSK 250 column. Densitometric scanning of the peak fraction in a TSK 250 column chromatography revealed that purified complex was composed of Pγ and Tα, and that the ratio of Pγ to Tα was 1:1. These data indicate that Pγ remains in a complex with Tα after GTP is hydrolyzed to GDP and that the binding of Pγ to GDP·Tα is not readily reversed.

How does the Pγ·GDP·Tα complex inhibit Pγ-free PDE? There are two possibilities: (1) the complex interacts first with Pγ-free PDE leading to the release of GDP·Tα which returns to Tβ,γ, or (2) the complex interacts with membrane-bound Tβ,γ, releasing Pγ which subsequently binds to Pγ-free PDE. Recent data (33) suggest the second possibility, that the Pγ·GDP·Tα complex interacts first with Tβ,γ. When Pγ·GDP·Tα was incubated with Tβ,γ in the presence of urea-treated disc membranes (which appeared to contain none of the known components of PDE cascade except rhodopsin) Pγ was detected in the supernatant following the association of GDP·Tα with ROS membranes. In contrast, Pγ was found associated with membrane-bound PDE when membranes contained active PDE. These data and our published data (30) indicate that, at least in frog rod photoreceptors, GTP hydrolysis is necessary but not sufficient for the PDE inactivation. Tβ,γ appears necessary for the release of Pγ from the GDP·Tα complex in order to inactivate Pγ-free PDE. This conclusion is also supported by the data, suggesting that the reassociation of transducin with ROS membranes was not effected by the presence of Pγ or PDE, singly or in combination (35). In contrast, the reassociation of Pγ with disc membranes was affected by the presence of (active) PDE.

So far no data has been published about the mechanism of PDE turnoff following GTP hydrolysis to GDP in bovine systems. However, Kroll et al (19) found that the addition of large amounts of GDP·Tα to trypsin-treated PDE resulted in 90-100% inhibition of the enzyme activity. The GDP·Tα complex also inhibited the stimulation of cGMP hydrolysis by GTPγs·Tα.

This inhibition could be reversed by excess GTPγS·Tα, as well as by Tβ,γ, indicating that the binding site for the activated Tα species is in close proximity and/or overlaps the binding site for the GDP·Tα complex on the enzyme. These data suggest that PDE turnoff results from a direct interaction between GDP·Tα and the catalytic moiety of PDE (Pα,β), although the role of Pγ in the turnoff mechanism remains unclear.

D. Modulation of GTPase activity with Pγ.

Recent data (33,35) indicate that in frog rod photoreceptors, each step of the GTP-hydrolytic cycle of transducin is closely related to the regulation of PDE activity. Following GTP binding to Tα, release of the PγGTP·Tα complex from the ROS disc membranes parallels the increase in PDE activity. Following hydrolysis of GTP, the PγGDP·Tα complex reassociates with the membrane with concurrent reduction in PDE activity. The termination of PDE activation is regulated by subunits of transducin. Thus, it was of interest to investigate the effect of Pγ on the GTPase activity of transducin. As shown in Figure 2, GTPase activity of transducin was inhibited by the addition of Pγ fractions in Blue Sepharose CL-6B column chromatography. In this experiment, Pγ was extracted with an isotonic, GTP-containing buffer following extensive washing of illuminated ROS disc membranes. When the effect of Pγ on GTPase activity was measured in the reconstituted system using purified components, 92 nM Pγ was required for 50% deactivation. The Pγ-sensitive site(s) in the GTP-hydrolytic cycle remain unclear; however, the data suggest a close interaction between Pγ and transducin subunits. The Pγ-dependent inhibition of GTPase activity has also been reported recently in bovine rod photoreceptors (21). However, Pγ-sensitive steps in the GTP hydrolytic cycle are uncertain since the GTP hydrolytic cycle responsible for PDE turnoff has not been analyzed. In bovine systems, the GTP hydrolytic cycle related to PDE turnoff may not be the same as the GTP-hydrolytic cycles of Tα which is free of Pγ (27).

E. Noncatalytic cGMP binding sites on PDE.

The change in cGMP concentration in rod photoreceptors is critical in visual transduction. Total acid-extractable cGMP is estimated to be 60 μM in frog rod outer segments prepared in a 1 mM Ca^{++} buffer (5); however, the concentration of free cGMP has been estimated to be only 1-6 μM (2). These data suggest that most of cGMP in rod photoreceptors is bound and may be inaccessible for hydrolysis by light-activated PDE. In frog rod photoreceptors, PDE has been shown to be one of the cGMP-binding components (34) and the concentration of PDE is about 30 μM (29), which could bind most of the measured cGMP. Thus, it is possible that a large drop in free cGMP concentration might produce a relatively small decline in total cGMP concentration during transduction. The large declines to a lower steady-state cGMP level observed after longer light exposure may indicate another type of light-induced biochemical response, perhaps related to light adaptation (6).

In frog rod photoreceptors two classes of high affinity, cGMP-specific binding sites have been found in PDE (34). Scatchard analysis revealed the presence of two classes of cGMP binding sites with apparent Kd values of 0.16 and 0.83 μM. These high affinity cGMP-specific binding sites are distinct from the PDE catalytic site. The cGMP-binding sites have been shown to be extremely sensitive to tryptic proteolysis. The most interesting point is that cGMP binding to the noncatalytic cGMP binding sites are enhanced by Pγ (31). As described, Pγ is an inhibitory subunit of PDE and Pγ is released with GTP·Tα in frog rod photoreceptors. Thus, the physiological activators of PDE, light, and GTP, have been shown to reverse the Pγ effects on both the enzyme activity of PDE and the binding of cGMP to its noncatalytic sites on PDE. This data was recently confirmed in toad rod photoreceptors (7).

In bovine rod photoreceptors, PDE has been shown to have cGMP binding sites (13). Preparations of purified bovine rod PDE contained 1.8 ± 0.3 mole of tightly bound cGMP per mole of PDE (13). Scatchard analysis of cGMP binding indicated the presence of two classes of binding sites on PDE with extraordinarily slow dissociation rates. These data suggest that Pγ is

not released from PDE with GTP·Tα during PDE activation, since cGMP release from the noncatalytic steps has been detected frog rod photoreceptors when Pγ is released from PDE with GTP·Tα. In contrast to rod PDE, bovine cone PDE binds at least 10-fold more cGMP/mole of PDE than rod PDE (12). cGMP binds to this noncatalytic site with high affinity (Kd = 11 nM). These data suggest that the nature of frog rod PDE is more comparable to bovine cone PDE than to bovine rod PDE.

F. Conclusions.

Photoreceptors are highly specialized neurons. Although many proteins detected in rod photoreceptors have not been identified (14), the primary proteins have been purified and their roles have been identified in the light-activated PDE cascade. Thus, photoreceptors have been a useful model for the study of signal transduction mechanisms. This review shows that the frog rod PDE cascade may be especially unique. The three-dimensional movements of the $P\gamma T\alpha$ complex in an isotonic buffer give the frog PDE cascade unique utility for the study of GTP-dependent signal transduction systems, especially in the studies of interaction between transducin and PDE subunits. These differences between frog and bovine systems may stem from differences in one or more transducin subunit(s) (15) and/or PDE (26). The clarification of these differences in the mechanisms of PDE activation will enhance the study of signal transduction, although they do not appear to be fundamental differences. Moreover, the studies of the signal transduction mechanism would be enhanced further through integration of the data concerning G-protein-effector interaction from invertebrate visual systems and hormonal regulation of adenylate cyclase systems.

Acknowledgements

I thank Drs. John S. George and John C. Fowler for critical reading of the manuscript, and Juanita Lujan and Kathryn Umbarger for proficient editorial assistance. This review was supported by grant EY07545 from the National Eye Institute (NIH).

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Figure Legends

Figure 1. Scheme of GTP hydrolytic cycle. α,β,γ: transducin subunits Tα, Tβ, and Tγ, respectively. Reaction 1: GTP binding to Tα; Reaction 2: release of GTP·Tα; Reaction 3: hydrolysis of bound GTP; Reaction 4: return of GDP·Tα; and Reaction 5: GDP release.

Figure 2. Inhibitory effect of Pγ on the GTPase activity of transducin. Illuminated ROS membranes from 50 frogs were washed with 5 ml of a buffer (10 mM Tris·HCl (pH 7.5), 5 mM DTT, 5 mM Mg SO_4, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.005 mM pepstatin and 0.005 mM leupeptin) (x7) and with 5 ml of a buffer (100 mM Tris·HCl (pH 7.5), 5 mM DTT, 5 mM $MgSO_4$, 0.1 mM PMSF, 0.005 mM pepstatin and 0.005 mM leupeptin) (x7) and Pγ was extracted from the washed membranes with 5 ml of the same buffer containing 400 μM GTP. The supernatant containing Pγ was applied to a Blue Sepharose CL-6B column (9 x 300 mm). The proteins were eluted with KCl gradient (0-1.5 M) in a buffer (10 mM Tris·HCl (pH 7.5), 1 mM DTT, 5 mM $MgSO_4$, and 0.1 mM PMSF). Following lyophilization of each fraction (40 μl) Pγ effects on Pγ-free PDE activity (●) and GTPase activity (○) were examined (33,36). 100% of PDE activity represents 12 μ mole cGMP hydrolyzed/mg/min. 100% of GTPase represent 4.2 μ moles GTP hydrolyzed/mg/min. The peak fraction (66) of protein (▲) contain 0.19 μg/100 μl.

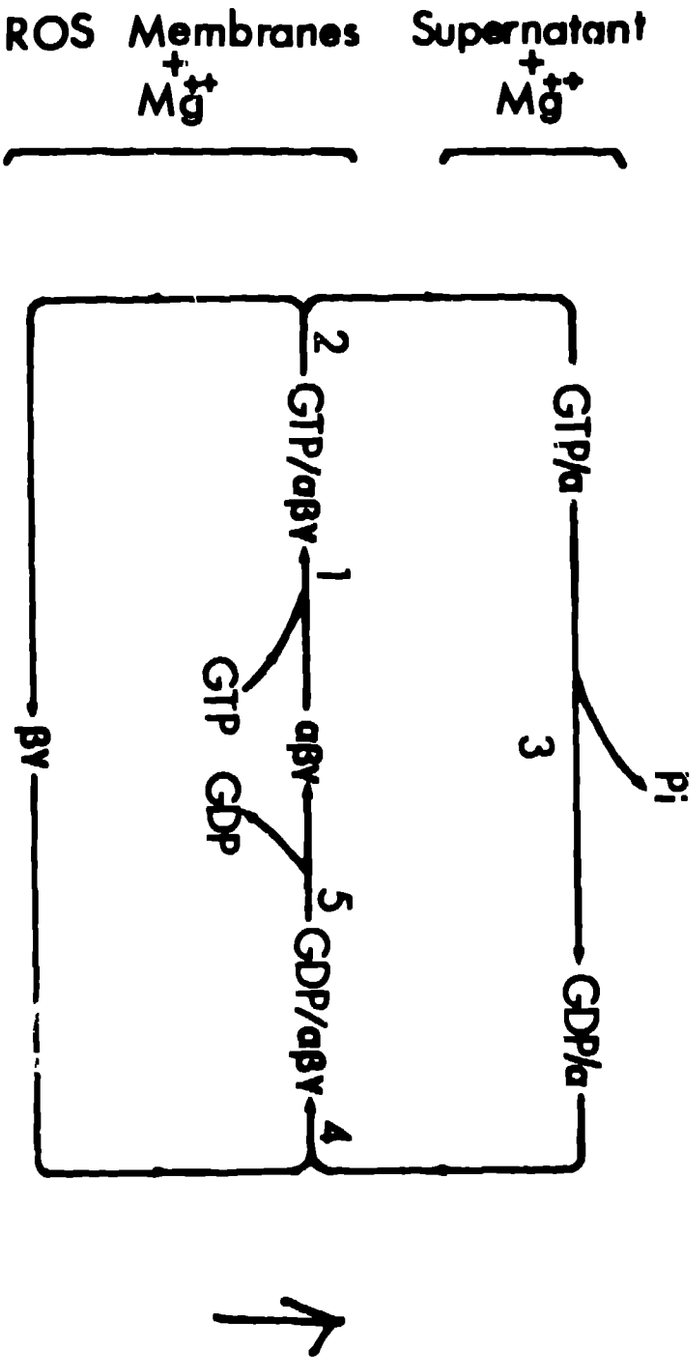
ROS Membranes
Mg^{++}
Supernatant
Mg^{++}
GTP/aβγ
2
GTP
1
GTP/a
aβγ
3
Pi
βγ
GDP
5
GDP/aβγ
4
GDP/a

Fig 1

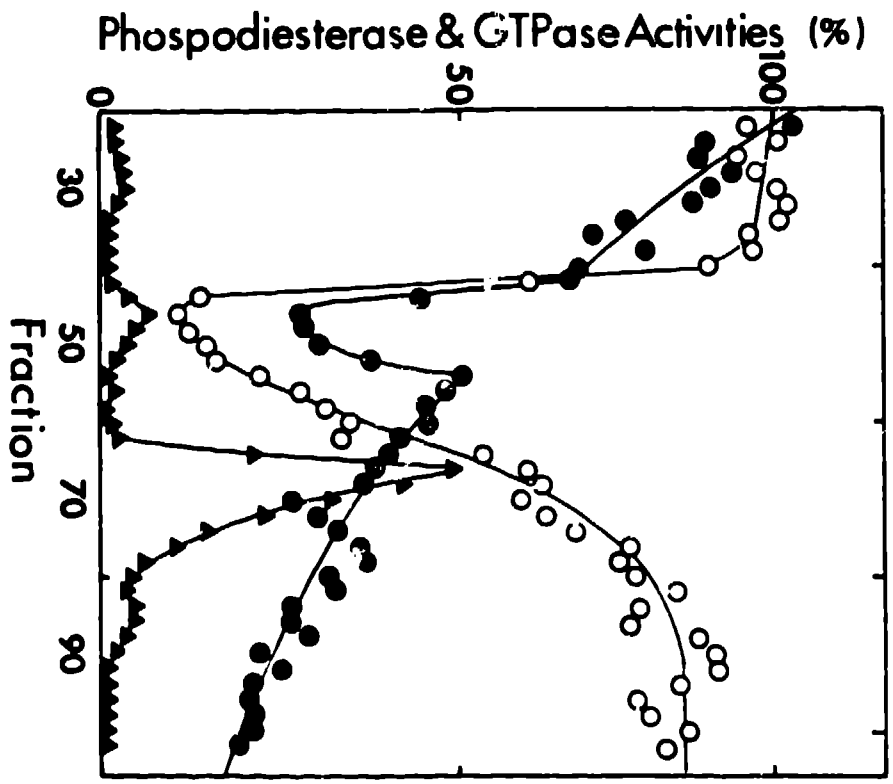
Phospodiesterase & GTPase Activities (%)
0
50
100
30
50
70
90
Fraction

Fig 2