MODIFICATION OF SODIUM AND POTASSIUM CHANNEL KINETICS BY DIETHYL ETHER AND STUDIES ON SODIUM CHANNEL INACTIVATION IN THE CRAYFISH GIANT AXON MEMBRANE

by

Bruce Palmer Bean

Submitted in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by:

Dr. David A. Goldstein (Department of Radiation Biology and Biophysics)

Dr. Peter Shrager (Department of Physiology)

Department of Radiation Biology and Biophysics

University of Rochester

School of Medicine and Dentistry

Rochester, New York 14642

1979

DISCLAIMER

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VITAE

The author was born in [redacted]. He attended school in the Niskayuna, New York school system and the Hotchkiss School, Lakeville, Connecticut. He attended Harvard College from 1969 to 1973, graduating with a degree in Biology. In the year 1973-1974 he worked as a technician in the Cardiac Research laboratory at the Children's Hospital Medical Center, Boston. He married Claire Sampson on August 17, 1974. Their daughter, Jessica, was born on October 8, 1978. He began studies in the Department of Radiation Biology and Biophysics in September, 1974. He worked in membrane biophysics, first in the laboratory of Dr. Adil Shamoo and then under the dual advisorship of Dr. David Goldstein of the Department of Radiation Biology and Biophysics and Dr. Peter Shrager of the Department of Physiology, doing his thesis research in Dr. Shrager's laboratory.

ACKNOWLEDGEMENTS

I am more grateful than I can say to Dr. Peter Shrager for giving me the opportunity to work in his laboratory; for many hours of patient personal instruction in electrophysiology and electronics; for facilitating the construction of the apparatus used in this work; for his always excellent suggestions and advice, and especially for his daily example of dedication and care in his work. It has been a great privilege to have worked in his laboratory.

I am very grateful also to Dr. David Goldstein for many discussions of my problems and results and for his stimulating comments and advice. I hope that some of his rigor has rubbed off.

I am grateful to the Department of Radiation Biology and Biophysics for its generous financial support and particularly to Dr. Helen Eberle, who as Education Officer permitted the unorthodox research arrangement, and to Marilyn Campbell, whose handling of all the administrative matters couldn't have been as effortless as it seemed.

The microcomputer and digital electronics system was conceived and designed by Dr. Shrager, John H. Young, and Vasant Saini, and was built primarily by John Young, Vasant Saini, and Larry Crosby. I am especially and eternally grateful to John Young for his constant willingness, during the dark days of debugging the system, to spend yet one more evening helping and teaching me.

Gerry Harris of the Physiology Shop was always willing to help with construction problems and always had a better suggestion.

I cannot imagine a better situation for graduate work than the one I have been in, with easy access to so many good scientists in two departments. I am very grateful to Dr. Paul Horowicz and all the members of the Department of Physiology for treating me as if I were a member of the department. I am, in particular, tremendously grateful to Dr. Ted Begenisich for his interest and encouragement, for his unequalled generosity with his equipment, and especially for the innumerable hours of informal teaching and discussions of membrane physiology, in which he always made things clearer.

DEDICATION

To Claire, who made all the sacrifices

ABSTRACT

The effects of ether and halothane on membrane currents in the voltage clamped crayfish giant axon membrane were investigated. Concentrations of ether up to 300 mM and of halothane up to 32 mM had no effect on resting potential or leakage conductance. Ether and halothane reduced the size of sodium currents without changing the voltage dependence of the peak currents or their reversal potential. Ether and halothane also produced a reversible, dose-dependent speeding of sodium current decay at all membrane potentials. Ether reduced the time constants for inactivation measured with the double pulse technique, and also shifted the midpoint of the steady-state inactivation curve in the hyperpolarizing direction. Large concentrations of ether or halothane resulted in an irreversible shift, in the depolarizing direction, of the voltage-dependence of a process of slow sodium channel inactivation, without changing the voltage-dependence of other sodium channel parameters. Potassium currents were smaller with ether present, with no change in the voltage dependence of steady-state currents. The activation of potassium channels was faster with ether present; the time to half maximum potassium current was decreased at all membrane potentials. There was no apparent change in the capacitance of the crayfish giant axon membrane with ether concentrations of up to 100 mM.

Experiments on sodium channel inactivation kinetics were performed using 4-aminopyridine to block potassium currents. The spatial uniformity of voltage clamp currents was checked by the method of Cole and Moore (J. Gen. Physiol. 44, 123), using a closely spaced pair of electrodes to measure relatively localized current density. Sodium currents decayed with a time course generally fit well by a single exponential. The time constant of decay was a steep function of voltage, especially in the negative resistance region of the peak current vs voltage relation. The time course of inactivation measured with the double-pulse procedure was very similar to that of the decay of the current at the same potential. The measurement of steady-state inactivation curves with different test pulses showed no shifts along the voltage axis of the type described in other axons and predicted by some sodium channel models. Measurements of the voltage-dependence of the integral of sodium conductance were made in order to test models of sodium channel inactivation in which channels must open before inactivating; the results appear inconsistent with some of the simplest cases of such models.

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CHAPTER ONE: MODIFICATION OF SODIUM CHANNEL INACTIVATION BY ETHER AND HALOTHANE

INTRODUCTION

The action potential in nerves is produced by voltage- and time-dependent changes in the ion permeability of the nerve membrane (Hodgkin et al., 1952). These permeability changes are almost certainly due to pores (Armstrong, 1975) composed at least partially of protein (Armstrong et al., 1973; Shrager, 1974, 1975).

The gating mechanisms governing the opening and closing of the ionic channels are not well understood. One question which must be asked is to what extent the lipid milieu surrounding the ionic channels might influence their gating properties. Campbell and Hille (1975) suggested that the differences in the rates of gating seen between sodium channels in frog nerve and those in frog muscle might be due to different membrane material surrounding the channels, which otherwise had nearly identical properties. Chiu et al. (1979) recently noted a sharp break in the temperature dependence of sodium channel inactivation in rabbit nerves and suggested that possibility that this break might reflect a membrane lipid phase transition. More directly, Redmann et al. (1979) found that addition of cholesterol to the squid giant axon changed the rate of sodium channel gating, possibly by altering the lipid environment of the channel. It seems that the hypothesis that the gating process in voltage-dependent channels might be influenced by the state of the surrounding lipids is worth being pursued for its

possible help in understanding the physical mechanisms of ionic channel gating.

Gaseous or volatile liquid general anesthetics are capable of perturbing the lipid structure of bilayer membranes. Most of the changes observed can be lumped together as reflecting an increase in the "fluidity" of the membrane with general anesthetics present: the order estimated by the average tilt of nitroxide spin labels on fatty-acid chains is decreased (Trudell et al., 1973; Boggs et al., 1976; Mastrangelo et al., 1977); the rotational and lateral diffusion rates of various molecules are increased (Vanderkooi, et al., 1977); native and carrier-mediated ionic permeabilities are increased (Johnson and Miller, 1970; Johnson et al., 1973; Pang et al., 1979).

The work described here was conceived as an attempt to use general anesthetics as a tool to perturb the lipid matrix of a nerve membrane. There are two apparent effects of the general anesthetics ether and halothane on the gating kinetics of the sodium and potassium channels in the crayfish giant axon membrane: sodium channel inactivation appears faster and potassium channel activation is speeded. This chapter describes the apparent changes in sodium channel inactivation produced by ether and halothane and discusses some of the difficulties in unambiguously interpreting these effects as true changes in the inactivation process. Chapter Two describes the speeding of potassium channel kinetics and some other effects of ether and halothane on the electrical properties of the crayfish axon.

METHODS

Voltage clamp

Giant axons from the crayfish Procambarus clarkii were dissected, cleaned, and cannulated with a piggy-back voltage clamp electrode as described by Shrager (1974).

The voltage clamp currents were measured in two different ways in different experiments. In earlier experiments, the current signal was obtained from the current flowing to a central 2 mm length of platinized platinum foil flanked by 5 mm guard regions. In later experiments, current density was measured with a differential electrode consisting of two closely spaced platinum wires, a technique which was devised by Cole and Moore (1960) for measuring currents from a smaller length of axon and which is described in greater detail in Chapter 3.

Series resistance compensation (Hodgkin et al., 1952) was included in the voltage clamp circuit. In ealier experiments, feedback for 3-4 ohms-cm^2 could be employed before oscillations were induced in the voltage clamp system. In later experiments, the current signal was filtered at 12 KHz before being fed back into the circuit, a technique which allows full series resistance compensation (Sigworth, 1979). Compensation for 10-13 ohms-cm^2 was used in these later experiments; the series resistance measured in one axon was 11 ohms-cm^2. Many experiments were performed

with low sodium in the bathing solution in order to reduce the size of the sodium currents and further minimize errors from the series resistance.

The fundamental results reported in this Chapter were the same with either current recording method and with various levels of series resistance compensation; there were minor quantitative differences attributable to different amounts of uncompensated series resistance.

Digital recording system

The current signal was amplified and then digitized at various rates with a sample-and-hold amplifier (Teledyne Philbrick 4855) and a 12-bit analog-to-digital converter (Teledyne Philbrick 4133). Each sweep consisted of 255 points, with the first 128 and the next 127 digitized at different intervals of from 5 to 1000 μsec. The digital data were temporarily stored in an IMSAI 8080 microcomputer using direct memory access, then transferred to audio cassette tape. The data were later sent to a PDP 8/E minicomputer for analysis.

In addition to gathering the digitized current data, the microcomputer generated the timing for the voltage clamp command pulses and the triggers for A/D conversions, using INTEL 8253 programmable timers. All timing was synchronized to the microcomputer's internal clock. Optical isolators were used between the digital timing pulses and the analog command pulses.

Temperature control

The nerve chamber was cooled by two Peltier devices and by pre-cooling the bathing solution with ice-water coils. Temperature was measured with a small thermistor (Fenwal GC32 J1) mounted within a few hundred microns of the axon. Ninety-five milliseconds after each current sweep, the temperature signal was digitized and stored as the 256^{th} point of the current sweep, and also converted to degrees and displayed at the computer teletype.

Preparation and delivery of anesthetic solutions

Ether (Baker 2-9239) was redistilled and stored in wood-corked glass flasks. Solutions were made up by pipetting ether through a small hole in the cork of a flask of swirling saline. Solutions were led into the nerve chamber via glass and polyethylene tubing. Solutions both with and without ether flowed through the chamber (about 10 ml capacity) at rates of from 10-40 ml/minute. In control experiments, the constancy of the aqueous ether concentrations in the chamber was checked using ultra-violet absorption spectroscopy to measure the ether concentrations; there is a strong absorption peak at 190 nm. Ether concentrations in the chamber were constant to within 3 percent of the nominal values with flow rates as low as 5 ml/minute.

Halothane (Ayerst) was redistilled to remove the preservative thymol and was stored in light-tight glass containers. Halothane solutions were made up by dilution of a solution of saline

saturated with halothane at room temperature. Halothane concentrations are calculated using a value of 32 mM for this saturated solution (Secher, 1971).

The nerve chamber and associated apparatus were mounted on a vibration-damped platform inside a chemical fume hood in order to protect the experimenter from the deleterious physical (Stevens et al., 1975) and mental (Bruce et al., 1974) effects of the anesthetic vapors.

Solutions

The basic solution employed was van Harreveld's (1936) solution with the substitution of HEPES buffer for bicarbonate buffer and with the addition of 1 mM 4-aminopyridine (4-AP) to block potassium currents (Meves and Pichon, 1975; Yeh et al., 1976). The basic solution was: 205 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2.3 mM HEPES, 1 mM 4-AP, pH 7.6-7.7. Some experiments were performed with solutions in which the NaCl and KCl were partially replaced by iso-osmotic tetramethyl ammonium chloride (TMA). Solutions were 433-438 mOsm before the addition of anesthetic.

Dissection and preparation of the axon were done in normal van Harreveld's solution.

Methods of analysis

In most cases, leak and capacitative currents were subtracted from records of total current by using linearly scaled currents in

response to hyperpolarizations. In some cases linearly scaled currents for small depolarizations (too small to produce any sodium channel activation) were used.

For depolarizations to membrane potentials above 0 mV, there was some relief of the block of potassium currents by 4-AP (Yeh et al., 1976). However, these potassium currents were small and slow enough that the decay of sodium current was nearly complete by the time there was significant potassium current, and the time constant for decay could be determined quite accurately, as was confirmed in experiments employing TTX subtraction to correct exactly for the residual potassium currents. In some experiments, leak, capacitative, and residual potassium currents were obtained by preceding the test pulse by a prepulse long and large enough to totally inactivate sodium current, a procedure which is illustrated in Figure 9 of Chapter 3.

The time constant for the decay of sodium currents was obtained by a least-squares fit (using weighted residuals) to the falling phase of the currents. Usually the fit was made between the times at which the current had decayed to 75 percent and 20 percent of its peak value.

Peak sodium current and the time-to-peak were obtained by fitting a third order polynomial to the points in the region of the peak and calculating the root of the polynomial's derivative corresponding to the peak (Hille, 1971). The end points for the fit were adjusted under visual control to provide an accurate fit.

In the experiments using double pulse inactivation, the pulse patterns were given at 1/3 Hz with automatic incrementing of the prepulse duration. Every fifth pulse was a test pulse with no prepulse; this allowed for correction for a slight use-dependent decrease in peak height (1-5 percent for a sequence of 18 pulses at 1/3 Hz). The double pulse data at a given membrane potential consisted of two series of 16 or 18 pulses with prepulse duration increments differing by a factor of 5 or 10. Provencher's (1975) method for fitting sums of exponentials was used in calculating the time constants and asymptotes for double pulse inactivation data. Dr. Provencher kindly supplied a copy of his DISCRETE computer program.

The parameters in the expression $1/(1 + \exp (V_h - Vh/K)$ were fit to the steady-state inactivation data by plotting the data points on semi-log paper as $(1/h_\infty - 1)$ vs. membrane potential, where $1/h_\infty$ is the steady-state level of inactivation. These plots were usually fairly linear and the line was fit by eye. In some cases these plots showed asymmetry (similar to that shown by Chiu, 1977); in these cases the points corresponding to greater inactivation (larger depolarizations) were weighted the most heavily in making the fit.

RESULTS

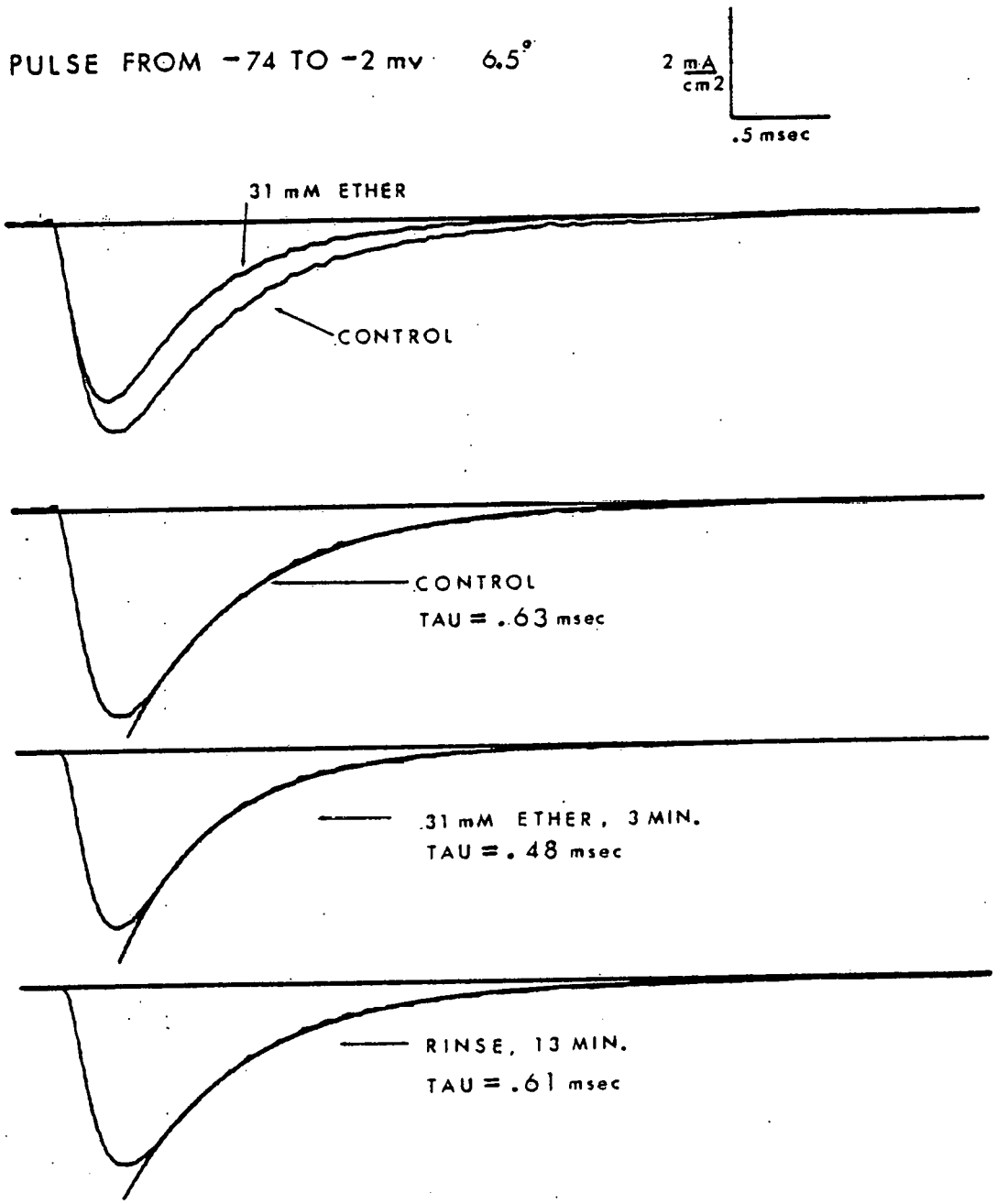
Faster decay of sodium currents

The most obvious change in sodium currents when ether is applied to an axon is that the decay phase of the currents is faster. Figure 1a shows sodium currents at -2 mV before and during

the administration of 30 mM ether. With ether present, the early rising phase is almost identical to that of the control, but the peak occurs earlier and the decay phase is more rapid. Parts b, c, and d of Figure 1 show the currents before, during, and after ether, with the falling phase of the currents fit with an exponential decline to zero; the decay time constant decreases by about 25 percent when ether is applied, and the decrease is reversible when the solution containing ether is rinsed out. However, the peak current following the wash-out of ether is about 15 percent smaller than in the control.

In general, the kinetic changes produced by ether appear nearly as fast as the solutions are exchanged (a few minutes). The reversal is complete or nearly complete after 15-20 minutes of rinsing with ether-free saline. The reversal is essentially total up to ether concentrations of at least 200 mM. However, in addition to the kinetic changes, the application of ether produces changes in the magnitude of the currents; some of these changes appear slowly and are reversed slowly or not at all. The basis of some of these changes in magnitude is discussed in Chapter 2. However, under many conditions "extra" changes in magnitude are lacking and in these cases it appears that the only difference in sodium currents with and without ether is the rate of decay. In parts c and d of Figure 2 are shown, at two potentials, a typical case in which there was no "extra" change in magnitude; the rising phases superimpose and the decline in peak current is seemingly due entirely to the faster decay with ether present.

Figure 1. Effect of ether on sodium current decay. Depolarization from -74 mV to -2 mV. (a) Control current and current in 30 mM ether superimposed. (b,c,d) Control, ether, and wash-out currents superimposed with exponential fits to the falling phases. Fit between 70 percent and 5 percent of the peak current values. Virtual ground current electrode. Full Na, Full K, 1 mM 4-AP. Holding potential -74 mV. Temperature 6.5^{o}


PULSE FROM −74 TO −2 mv 6.5°
2 mA/cm2
.5 msec
31 mM ETHER
CONTROL
CONTROL
TAU = .63 msec
31 mM ETHER, 3 MIN.
TAU = .48 msec
RINSE, 13 MIN.
TAU = .61 msec

The faster decay produced by ether does not depend on the direction of sodium current flow and is of similar magnitude over the entire range of membrane potentials at which sodium currents are activated. Parts a and b of Figure 2 show currents at two membrane potentials, inward currents at -20 mV and outward currents at +65 mV, before, during, and after an application of 100 mM ether; the time constants for decay decrease by similar percentages (42 percent at -20 mV, 48 percent at +65 mV) at the two membrane potentials.

The time constants for decay over the range of membrane potentials from -40 to 95 mV are shown in Figure 3 for the same application of 100 mM ether from which the records in Figure 2 were taken. The percentage decrease in time constant is slightly larger at large depolarizations (53 percent at +75 mV, 59 percent at +95 mV than for small or moderate depolarizations (46 percent at -40 mV, 34 percent at 0 mV). This is typical of most experiments. It is also true in many experiments that for currents in the membrane potential range -40 mV to -30 mV the effect of ether on the decay time constant is not perfectly reversible, as at -35 mV and -30 mv in Figure 3. This may be connected to the fact that the currents in this range often show biphasic decay kinetics, possibly due to spatial non-uniformities of current, as discussed in Chapter 3.

The dependence on ether concentration of the decrease in decay time constant is shown in Figure 4. Each point is a mean value obtained from applications of ether to a number of axons.

Figure 2. Ether-induced kinetic changes for inward and outward currents. (a) Application and wash-out of 100 mM ether at -20 mV. Currents are superimposed with exponential fits to falling phases. Fit between 75 percent and 25 percent of the peak values. (b) Same, for outward currents at +65 mV. (c) Superimposed currents from part a, with ether and following wash-out. (d) Superimposed currents from part b, with ether and following wash-out. Parts a and b are from the same application of ether. 1/4 Na, 1/2 K, 1 mM 4-AP. Differential current electrode. Prepulse subtraction technique (Figure 9, Chapter Three) used for leak and capacitative current correction. Holding potential -80 mV. Temperatures: (a) 3.2^{o}C for control, ether, wash-out. (b) 3.1^{o}C for control, 3.8^{o}C for ether, 3.6^{o}C for wash-out.

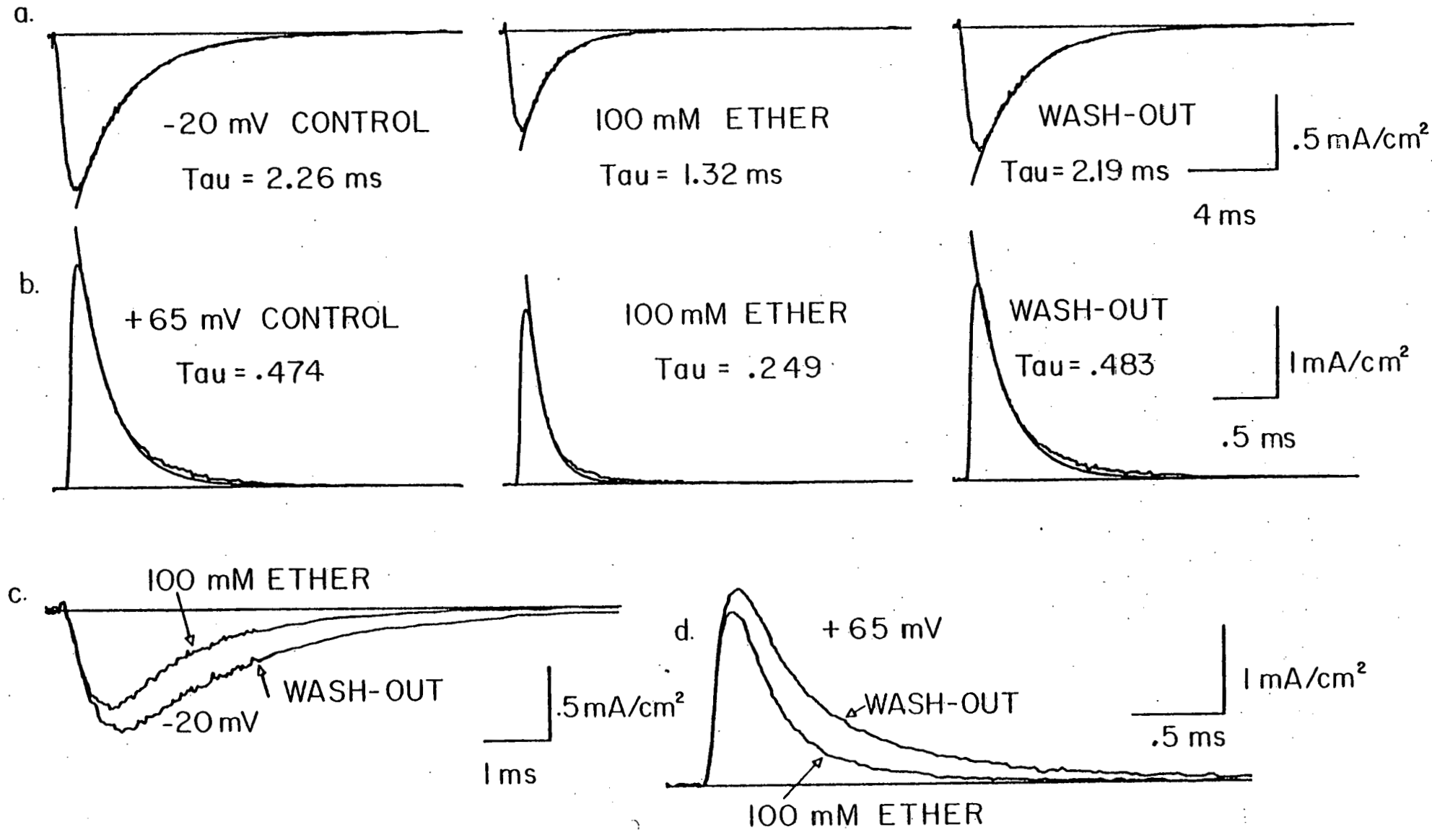
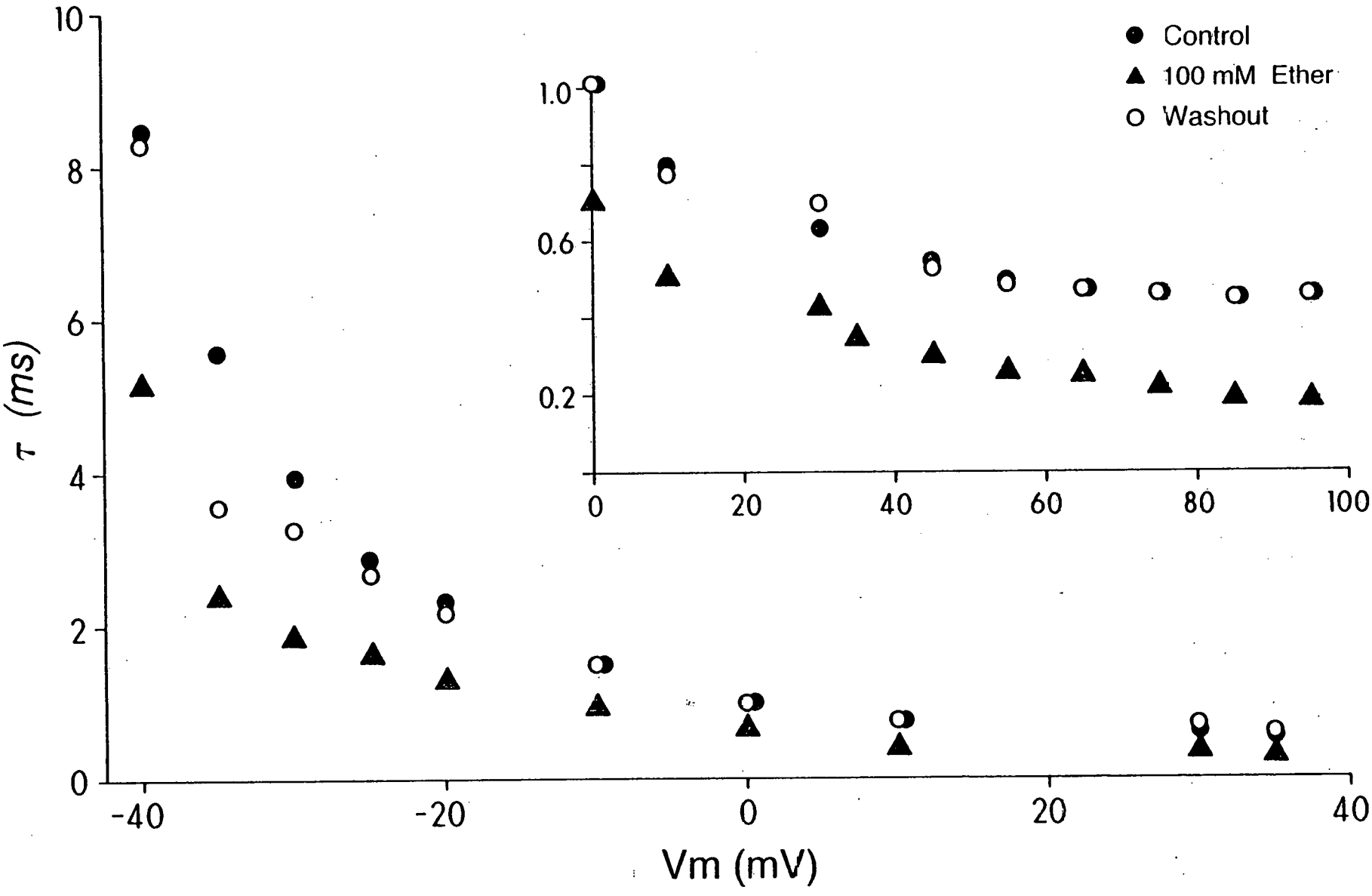

a.
-20 mV CONTROL
Tau = 2.26 ms
100 mM ETHER
Tau = 1.32 ms
WASH-OUT
Tau = 2.19 ms
.5 mA/cm²
4 ms
b.
+65 mV CONTROL
Tau = .474
100 mM ETHER
Tau = .249
WASH-OUT
Tau = .483
1 mA/cm²
.5 ms
c.
100 mM ETHER
WASH-OUT
-20 mV
.5 mA/cm²
1 ms
d.
+65 mV
WASH-OUT
1 mA/cm²
.5 ms
100 mM ETHER

Figure 3. Decay time constant as a function of membrane potential before, during, and after 100 mM ether. Ether was applied for 9 minutes and rinsed for 18 minutes. 1/4 Na, 1/2 K, 1 mM 4-AP. Differential current electrode. Holding potential -80 mV. Temperatures: Control, 3.1-3.5^{o}C; 100 mM ether, 3.0-3.8^{o}C; wash-out, 3.3-3.6^{o}C. At most potentials, control, ether, and wash-out temperatures are matched to within .2-.3^{o}C.

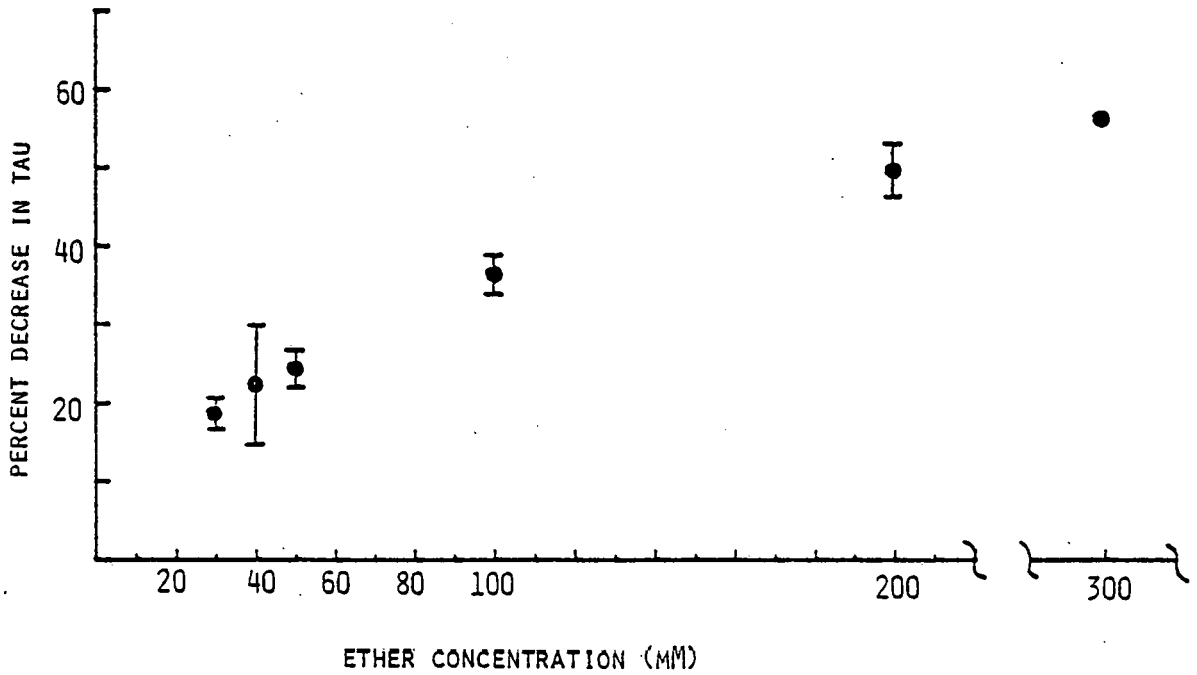
Inset: Data for 0 to +100 mV on expanded scales.


Control
100 mM Ether
Washout
τ (ms)
Vm (mV)
10
8
6
4
2
0
-40
-20
0
20
40
1.0
0.6
0.2
0
20
40
60
80
100

Experiments were performed with a variety of external sodium and potassium concentrations and with holding potentials from -72 mV to -105 mV; there were no obvious differences in effects with changes in any of these parameters. Most experiments were done in the temperature range -1^{o} to 8^{o}C; a few experiments at 25^{o}C showed changes in kinetics of about the same magnitude, but this point was not investigated in detail.

The experiments summarized in Figure 4 were all done with 1 mM 4-AP to block potassium currents, but it was clear that the presence of 4-AP was not required for the effect of ether on sodium channel kinetics. A number of experiments were done with no 4-AP, with potassium currents present, and the time-to-peak for the early transient current peak was decreased to a similar extent in these experiments. Also, in one experiment sodium currents were obtained by a TTX-subtraction procedure with no 4-AP present: 50 mM ether was applied and rinsed, 100 nM TTX was applied to block sodium currents, and 50 mM ether was applied again. The reversibility of the effect of ether on the potassium currents in this axon allowed accurate determinations of sodium current time course by subtracting the TTX-insensitive currents both with and without ether. The time constant for sodium current decay at -15 mV was decreased by 25 percent with ether present, in good quantitative agreement with the results summarized in Figure 4 for 50 mM ether with 4-AP.

Figure 4. Concentration dependence of decrease in decay time constant. Each point is the mean ± SEM for applications of ether to a number of different axons (5 for 30 mM, 4 for 40 mM, 4 for 50 mM, 7 for 100 mM, 3 for 200 mM, 1 for 300 mM). The value for each axon was determined as the mean for a number of membrane potentials between -20 mV and +90 mV.

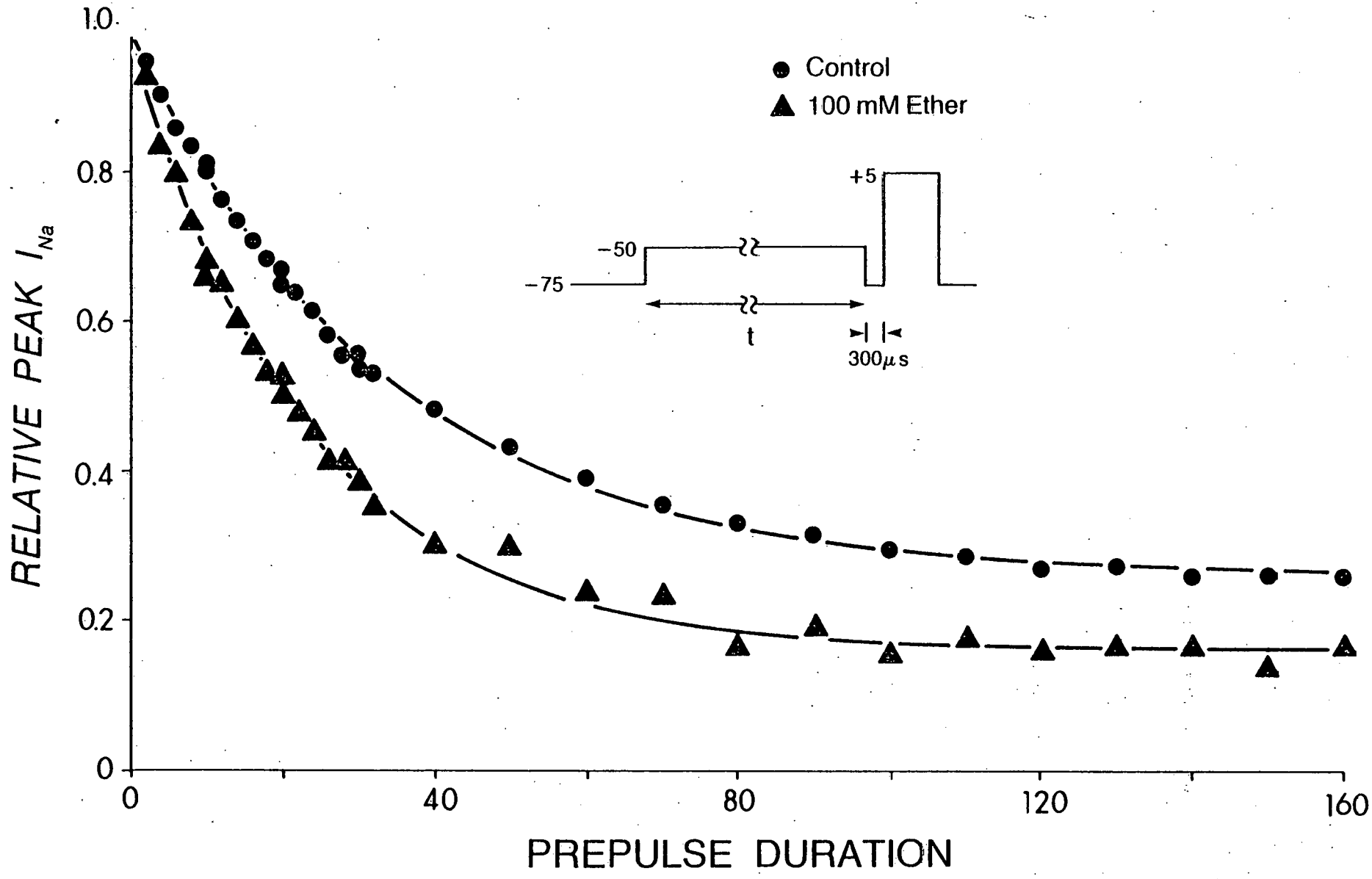

PERCENT DECREASE IN TAU
60
40
20
20
40
60
80
100
200
300
ETHER CONCENTRATION (MM)

Double-pulse experiments

The faster decay of sodium currents with ether present is reminiscent of the faster decay seen with internal pancuronium ion (Yeh and Narahashi, 1977) and quaternary strychnine derivatives (Shapiro, 1977; Cahalan and Almers, 1979). In the case of these compounds, the increased rate of decay appears to be due to time-dependent block of open sodium channels rather than to a speeding of the normal inactivation mechanism. One experiment leading to this conclusion in the case of pancuronium is that when the rate of inactivation is determined by the double pulse method for small depolarizations, where there is very little opening of sodium channels, the time constant for inactivation is not affected by pancuronium (Yeh and Narashashi, 1977). It seemed important to check this point for ether.

Double pulse experiments with ether show that the time constant for inactivation is decreased by ether at small depolarizations and to a degree similar to the change in decay time constant for the single pulse currents at larger potentials. The results of one such experiment are shown in Figure 5. The membrane was depolarized from its resting potential of -75 mV to -50 mV for a variable length of time and, following a brief return to rest, the level of inactivation reached during the prepulse was assayed by the peak sodium current flowing during the test pulse to +5 mV. For the example shown in Figure 5, the time course of inactivation measured in this way is quite well described, both with and without

Figure 5. Effect of ether on the time course of inactivation determined by the double pulse method. Pulse pattern is illustrated in the inset and described in the text. Peak current during the test pulse is plotted as a function of the prepulse duration. Points are fit with an exponential decay to an asymptote. Control: $y = .731 \exp(-t/33.2\ ms) + .259$; Ether: $y = .818 \exp(-t/22.9\ ms) + .162$. Full Na, Full K, 1 mM 4-AP. Holding potential -75 mV. Temperatures: Control, 1.9-2.0^{o}C; Ether, 1.8-2.1^{o}C.


1.0
0.8
0.6
0.4
0.2
0
RELATIVE PEAK I_{Na}
Control
100 mM Ether
+5
−50
−75
t
300μs
0
40
80
120
160
PREPULSE DURATION

ether, by a single exponential decay to a non-zero asymptote. With 100 mM ether present, the time constant for the decay is reduced about 31 percent and, in addition, the asymptotic value is smaller; in the same axon, 100 mM ether decreased the decay time constant for currents near 0 mV by 25-30 percent. Time constants and steady-state values for double pulse inactivation were smaller with ether in all axons examined. Results of double pulse experiments are given in Table I.

Steady-state fast inactivation

It is evident from the data shown in Figure 5 and Table I that inactivation at small depolarizations is more complete with ether present. This effect of ether was examined in greater detail by measuring "steady-state" fast inactivation over an appropriate range of membrane potentials. A long (50-500 ms) prepulse to a variable membrane potential is followed by a test pulse of fixed height (Hodgkin and Huxley, 1952a); the peak current during the test pulse is taken as an index of the level of inactivation achieved during the prepulse. In principle, the prepulse is long enough that an asymptotic value of inactivation is reached. In Figure 6 are shown typical steady-state inactivation curves determined in this manner with and without 100 mM ether. There are two changes produced by ether: first, the midpoint of the curve is shifted to the left along the membrane potential axis, in the hyperpolarizing direction; second, the curve is significantly

TABLE 1

EFFECT OF ETHER ON DOUBLE PULSE INACTIVATION

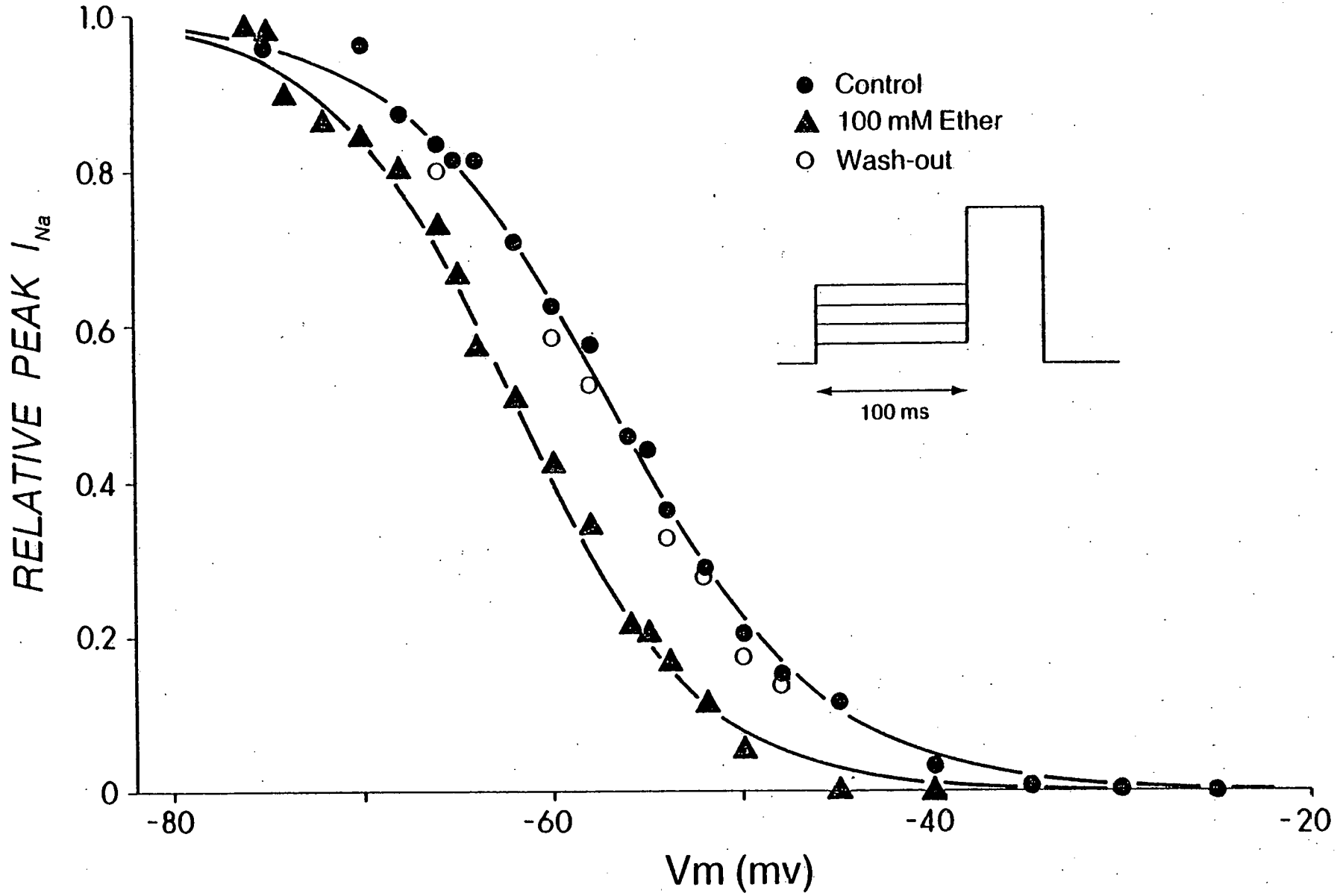
A. ONSET

		CONTROL			100 mM ETHER		
Axon	Membrane potential	Tau (ms)	Asymp.	Temp.	Tau (ms)	Asymp.	Temp.
1	-50 mV	33	.26	1.9-2.0°C	23	.16	1.2-2.0°C
2	-55 mV	46	.56	3.0°C	23	.13	2.3-2.6°C
	-45 mV	28	.10	3.2-3.3°C	9.1	.04	2.9-3.0°C
	-35 mV	8.6	.06	3.5-3.6°C	3.4	.03	3.8-3.9°C

B. WASH-OUT

		100 mM ETHER			WASH-OUT		
Axon	Membrane Potential	Tau (ms)	Asymp	Temp.	Tau (ms)	Asymp.	Temp.
3	-31 mV	2.1	0	1.4-2.0°C	2.7	0	2.7-3.3°C

Figure 6. "Steady-state" fast inactivation vs. membrane potential; with and without ether. Peak sodium current during the test pulse is plotted as a function of the membrane potential during the 100 ms prepulse. Holding potential -80 mV, test potential +50 mV. Reference potential for normalization is -80 mV. 1/10 Na, 2/5 K, 1 mM 4-AP. Differential current electrode. Temperatures: Control, 1.7-2.1^{o}C; Ether, 0.5-1.9^{o}C; Wash-out, 1.9-2.4^{o}C.


1.0
0.8
0.6
0.4
0.2
0
RELATIVE PEAK I_{Na}
-80
-60
-40
-20
Vm (mv)
Control
100 mM Ether
Wash-out
100 ms

steeper with ether present. It is convenient to describe these changes by fitting an analytical expression to the data points; the smooth curves in Figure 6 are drawn according to the expression (Hodgkin and Huxley, 1952a) $1/(1 + \exp (V - Vh)/K)$, where Vh is the value, in mV, of the membrane potential at which inactivation is one-half complete, and K is a parameter determining the steepness of the curve. In the example shown in Figure 6 (Axon II-3 in Table II), the control Vh value of -57 mV was shifted to -62 mV by ether, and K declined from 5.6 to 4.8. These changes were mostly reversible by washing out the ether. Table II gives Vh and K values for ether applications to two other axons under different conditions; the changes in Vh and K were always seen. The reversibility of the effect of ether on steady-state fast inactivation is emphasized by the results from Axon II-2 in Table II: the application of 100 mM ether in this axon was made after 200 mM ether had been applied and washed out; the changes in Vh and K are as large as for the other axons, in which 100 mM ether was applied to the fresh axon.

There are difficulties in defining and measuring steady-state fast inactivation. One problem is the existence of a process of slow inactivation with a potential-dependence which overlaps that of fast inactivation (Shrager, 1977; Starkus and Shrager, 1978). Fast inactivation has time constants of 20-60 ms in the range from -50 to -70 mV (3°), so that prepulse durations of at least 100 ms are required for a reasonable approximation to steady-state

TABLE II.

EFFECT OF ETHER ON STEADY-STATE FAST INACTIVATION

Axon	Prepulse Duration	Reference Potential	Control Vh(mV)	Control k	100 mM Ether Vh	100 mM Ether k	Rinse Vh	Rinse k
II-I	50 ms	-93 mV	-50	7.3	-63	5.5		
	50 ms	-73 mV	-58	6.4	-60	4.8		
	500 ms	-73 mV	-58	6.6	-63	3.8		
II-2	150 ms	-97 mV	-61	5.7	-67	4.9	-64	5.6
II-3	100 ms	-80 mV	-57	5.6	-62	4.8	-58	5.1
	100 ms	-100 mV	-60	6.9	-64	5.8	-60	6.5

values. Slow inactivation has a time constant of about 150 ms at -115 mV (8°C) (Shrager, 1977) so that in an axon with substantial slow inactivation present at the holding potential, it is impossible to establish a saturating value for the test pulse current, since for increasingly large hyperpolarizations, relief from slow inactivation produces increasingly large test pulse currents. One strategy for checking that the apparent effect of ether on fast inactivation was not an artifact due to an effect on slow inactivation was to confine the measurements to membrane potentials greater than the holding potential; that is, to use the holding potential as the reference potential for normalizing the test pulse height. Although the resulting inactivation curve may be distorted from the true one, due to some fast inactivation present at the holding potential, any effect of ether is almost certainly on fast inactivation since slow inactivation has time constants of more than a second in the range -75 to -55 mV (8°C) (Shrager, 1977). A second strategy was to use low-potassium solutions to hyperpolarize the axons and, presumably, remove most slow inactivation at the resting potential.

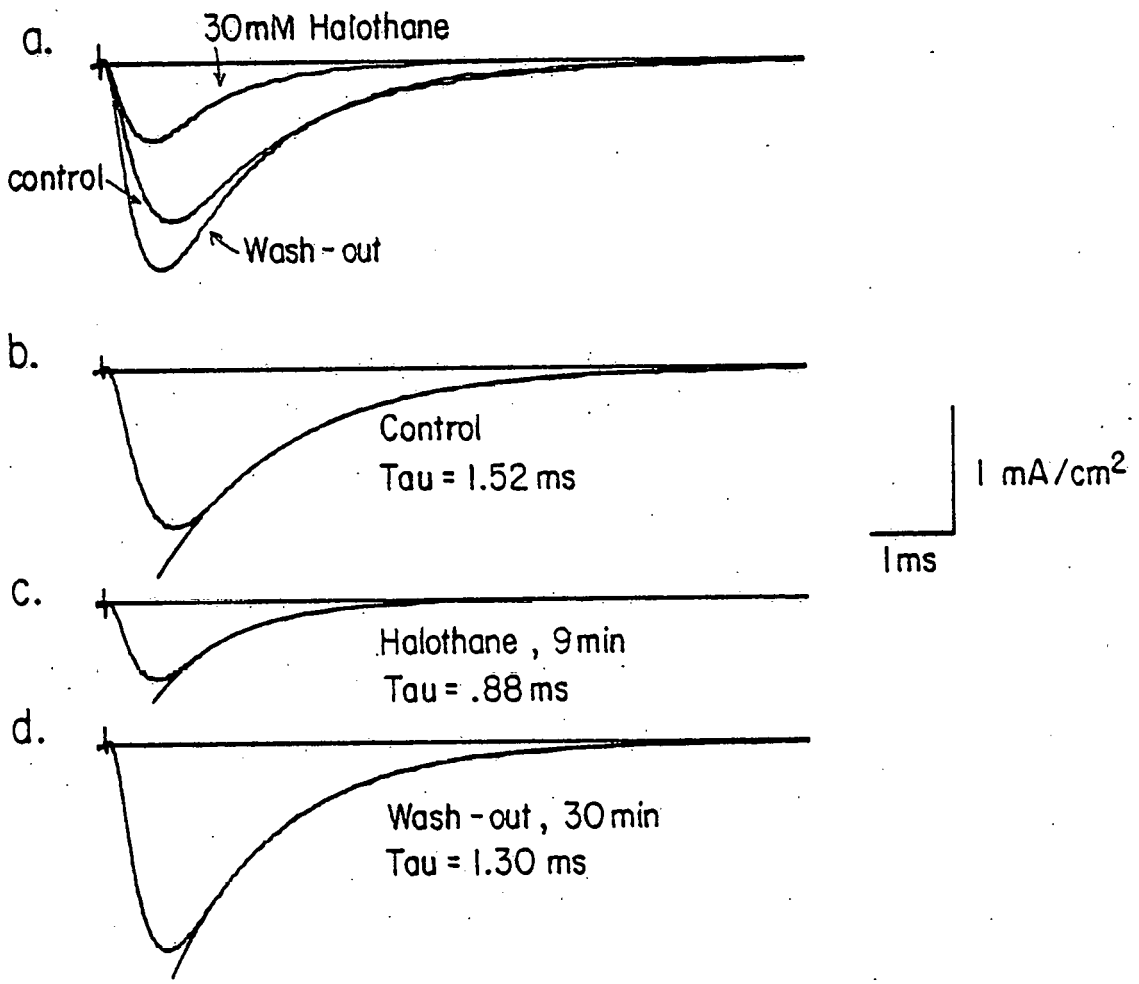
The experiments summarized in Table II were performed with a variety of holding potentials, prepulse durations, and reference potentials. The shift of Vh and decrease in K were always seen. It seems reasonable to conclude that these changes represent a true alteration in steady-state fast inactivation.

Effect of halothane on inactivation kinetics

Halothane is an inhalation anesthetic with basic pharmacological effects similar to those of ether. Halothane's effects on sodium current kinetics in the cray fish axon appear to be essentially identical to those of ether. Figure 7 shows sodium currents at -2 mV before, during and after exposure to 32 mM halothane. The exponential fits to the decay phases of the currents are shown in Parts b, c, and d of the Figure; as for ether, the decay time constant is considerably decreased by halothane. As with ether, time constants are reduced at all membrane potentials, both for inward and outward currents.

Halothane solutions change the kinetics of sodium currents with a time course of many minutes, in contrast to the rapid action of ether. The reversal on wash-out is also slow. After 9 minutes exposure in the application of halothane shown in Figure 7, the decay time constant was still decreasing, and after 30 minutes of rinsing with halothane-free solution, recovery was still not complete. It seems likely that the slowness of halothane's action and reversal relative to ether's is due to the difference in water:membrane partition coefficient between the two compounds. The oil:water partition coefficient for halothane is 285 (olive oil, 25^{o}C) and for ether it is 4 (Regan and Eger, 1967). (The values for membrane:water partitioning are probably less (Mastrangelo et al., 1978) but the relative values for halothane and ether are probably still similar). It is possible that the membranes of the

Figure 7. Effect of halothane on sodium current kinetics. Depolarization from -72 mV to -12 mV. (a) Control, 30 mM halothane, wash-out currents superimposed. (b,c,d) Currents superimposed with exponential fits to falling phases. Currents fit between 70 percent and 10 percent of peak values. Full Na, Full K, 1 mM 4-AP. Virtual ground current electrode. Temperatures: Control, 2.5^{o}C; Ether, 2.5^{o}C; Wash-out, 2.6^{o}C.


a.
30mM Halothane
control
Wash - out
b.
Control
Tau = 1.52 ms
1 mA/cm2
1ms
c.
Halothane , 9min
Tau = .88 ms
d.
Wash - out , 30 min
Tau = 1.30 ms

Schwann cell layer surrounding the axon act to buffer the halothane concentration near the axon membrane.

The slowness of halothane's effects, coupled with the necessity for high flow rates to prevent loss of concentration due to evaporation, made it impractical to deliver halothane long enough to reach a steady-state; this made a detailed study of the concentration-dependence of halothane's effects impossible. In two cases in which 6 mM halothane was applied, there was very little change in sodium current kinetics; the decay time constant decreased by less than 10 percent for a four-minute application. However, this relative lack of effect may simply have been due to failure of the halothane to reach the axon membrane at a significant concentration.

DISCUSSION

Block of open sodium channels or modification of inactivation rate processes?

The central question in understanding the mechanism by which ether produces faster decay of sodium currents is whether the existing inactivation process is in some way accelerated by ether or whether the faster decay is due to block of open sodium channels, as appears to be the case with pancuronium (Yeh and Narahashi, 1977) and N-methylstrychnine (Shapiro, 1977; Cahalan, 1978, Cahalan and Almers, 1979). Distinguishing between these alternatives, or even deciding if they are distinguishable, is not easy.

It is clear that the mechanism of action of ether and halothane is very unlikely to be similar in detail to the mechanisms of pancuronium, N-methylstrychnine, or other relatively large cations. Ether and halothane are small, neutral molecules with high lipid solubility. If they do act by preferential blocking of open sodium channels, it is not because of a lack of access to closed sodium channels, as may be the case with the hydrophilic cations. It also seems unlikely that ether and halothane would block channels by physical or electrostatic occlusion of the pore. A "blocking" of open sodium channels by ether and halothane would most likely be due to activated channels possessing a conformation with a receptor site for anesthetic molecules which did not exist in the closed state. It should also be noted that such a

hypothetical receptor would have to be fairly nonspecific; ether (H_5C_2-O-C_2H5) and halothane (HBrClC-CF_3), a halogenated ethane derivative, have significantly different structures.

The only real similarity between effects on sodium currents of internal pancuronium or N-methylstrychnine and those of ether or halothane is the faster decay of the currents. Pancuronium, N-methylstrychnine and other internal large cations produce characteristic hooked tail currents when the membrane is repolarized after a depolarization, and also produce tail currents which persist long after the sodium current has decayed completely (Cahalan and Shapiro, 1976; Yeh and Narahashi, 1977; Calahan, 1978; Cahalan and Almers, 1979). Tail currents in ether and halothane appear normal.

A second difference, mentioned in RESULTS, is that pancuronium has no effect on the rate of inactivation or on steady-state inactivation for depolarizations to membrane potentials more negative than -50 mV, where a small fraction of the sodium channels are activated (Yeh and Narahashi, 1977); application of ether, on the other hand, results in both more complete steady-state inactivation and in faster kinetics. It is tempting to use the observation that ether speeds inactivation at -55 mV, where a small fraction of sodium channels is open, to a similar (relative) extent as at large positive potentials, where a large fraction is open, to argue that ether does not act by blocking open sodium channels.

But, attempts to develop this argument quantitatively make it seem unconvincing. It is necessary to realize that although the fraction of sodium channels open at small depolarizations is so small as to make the currents un-measurable with single pulses, there may still be enough open so that block of open sodium channels can proceed at a sufficiently rapid rate to significantly speed the time course of inactivation measured with the double-pulse procedure. This point was made in a similar context by Bezanilla and Armstrong (1977). A simplified calculation illustrates this point: for large depolarizations, where activation is maximal, the decay time constant is decreased from about .4 msec to about .2 msec by 100 mM ether (Figure 3). If ether were blocking open sodium channels, the effective rate constant for blocking would be about 2.5 ms^{-1}. (This assumes that the opening of the channels is fast compared to inactivation, which is a good approximation for large membrane potentials.) Could this same blocking rate constant significantly affect the rate of inactivation at -55 mV, where few channels are activated at any given time? The effective rate constant for "inactivation" through blocking by ether will be approximately given by the fraction of open channels multiplied by the blocking rate constant of 2.5 ms^{-1}. The fraction of open channels at -55 is too small to measure directly but experiments employing signal-averaging of sodium currents at small depolarizations (unpublished) yielded the result that the peak conductance at -50 mV is about .01 of the peak

conductance at large depolarizations and that the peak conductance changes e-fold with a change in membrane potential of 3.7 mV in the range -50 mV to -40 mV; extrapolating this result, the fraction of open channels at -55 mV would be .0026. Thus, the effective rate constant for "inactivation" through blocking would be (.0025) x (2.5 ms^{-1}) = .0065 ms^{-1}. In axon I-2 in Table I, the inactivation time constant was 46 ms and the steady-state non-inactivated fraction of channels was .56; this yields, for a first-order reversible reaction, rate constants of .0096 and .0122 ms^{-1} in the forward and backward directions. Thus the effective rate constant for "inactivation through blocking" is comparable to the rate constants for normal inactivation and the net rate of "inactivation" determined by the double-pulse method would be substantially faster with ether present.

Another illustration of the difficulty of unambiguously eliminating a "blocking of open sodium channels" mechanism for the general anesthesia is to note that if normal inactivation only occurred after the channels have opened, as for example in the model proposed by Bezanilla and Armstrong (1977), then a speeding of normal inactivation rate processes would be kinetically indistinguishable from blocking of open sodium channels.

Sodium channel inactivation models

If it is assumed that ether and halothane act by altering rate processes in the existing inactivation mechanism, are the results of any use in distinguishing between various kinetic models for

sodium channel inactivation? In principle, the results might be useful, but in practice, the necessary distinctions seem too fine to make with any confidence. Both strictly coupled models, where channels must open before inactivating, and the Hodgkin-Huxley model, with independent activation and inactivation processes, can give the general result of the currents with and without ether matching well in the early part of the rising phase of the current while having different decay rates. (These simulations simply assumed that the effect of ether was to increase the forward rate constant for inactivation in each model.) There are differences in the predictions of the two models: with the Hodgkin-Huxley model, the rising phases do not match as well for as long as in coupled models; since inactivation starts at time zero in the Hodgkin-Huxley model, faster inactivation will produce smaller currents immediately. With coupled models inactivation proceeds with a lag which, since it depends on activation, is about the same for different inactivation constants. A related difference is that for a given amount of speeding of the decay phase, the Hodgkin-Huxley model predicts a greater decline in the peak current size than coupled models do. Another expression of the difference is that if the exponential fit to the decay phase is extrapolated back to time zero, the intercept will not be changed with a change in the Hodgkin-Huxley model; in coupled models, the intercept of the extrapolation is larger with faster inactivation. With the ether data, in cases in which it is estimated that there is no

"extra" change in current magnitude, the intercept of the extrapolated fit is smaller with ether for moderate depolarizations and larger with ether for large depolarizations. However, such calculations depend critically on the assumption of no "extra" change in current magnitude, which is impossible to independently check; also, the data for large depolarizations may be enough distorted by the finite time for charging of the membrane capacitance to seriously affect the results. In any case, the quantitative differences between the various models are relatively small and probably well within the bounds of errors from various sources in the experimental data.

One difficulty in fitting the data by simple changes in the Hodgkin-Huxley model should be noted; this concerns the changes in the steady-state inactivation curve produced by ether. The changes in the decay time constant with ether can be fit fairly well with a simple scaling of βh, the forward rate constant for inactivation, over the entire membrane potential range. However, simply scaling βh shifts the steady-state inactivation curve with no change in its steepness, while with ether both the midpoint and the steepness are changed (Table II). There appear to be no simple changes in the Hodgkin-Huxley expressions for the voltage-dependence of αh and βh which will produce the characteristic change in the steady-state inactivation curve seen with ether.

Comparison with previous results

There are two previous reports of voltage clamp work using general anesthetics. Shrivastav et al. (1976) studied the effects of trichloroethylene on currents in the squid axon; they found that the steady-state inactivation curve was shifted in the hyperpolarizing direction, as with the results with ether on the crayfish axon. Kendig et al. (1979) reported a similar shift produced by ether in the frog node. Neither group reported on the detailed kinetics of sodium currents.

A result reported by Heinbecker and Bartley (1940) may be closely connected to the speeding of inactivation reported here. They reported that 24 mM ether produced a dramatic increase in the rate of accommodation to a subthreshold depolarization in frog nerve. The rate of accommodation depends on the rate of sodium channel inactivation and the rate of potassium channel activation; in the frog nerve, where the potassium conductance is relatively small, the rate of accommodation at small depolarizations may primarily reflect the rate of inactivation.

General anesthetics have been found to speed the decay of end-plate currents (Gage and Hamill, 1976a, 1976b) and to decrease the mean channel lifetime estimated from current noise measurements (Gage et al., 1979); as with general anesthetics in the axon, it is not clear whether this represents a "blocking" mechanism or a speeding of the gating process. However, it is clear that the observations with the end-plate channels and the present ones with

sodium channels may depend on the same basic mechanism. The fact that potassium channel activation kinetics are faster with ether (Chapter Two), an effect which can not be attributed to a "blocking" mechanism, perhaps gives some extra weight to the possibility that in all three channels the effects are due to a speeding of gating processes. It would seem reasonable to propose that the ability of the general anesthetics to increase the "fluidity" of lipid bilayer membranes (Trudell et al., 1973; Boggs et al., 1976; Mastrangelo et al., 1977; Vanderkooi, et al., 1977) might underly all three effects, as one possibility proposed by Gage and Hamill (1976a) for their results.

Observations probably related to results reported here were made by Oxford and Swenson (1979), studying the effect of octanol on sodium currents in the squid axon. They found both that steady-state inactivation was shifted in the hyperpolarizing direction and that the time constant for sodium current decay was reduced. Furthermore, they found no sign of time-dependent blocking by octanol in axons treated with internal pronase to remove inactivation, and suggested that their results probably represented effects of octanol on the normal inactivation mechanism. Octanol has effects similar to those of general anesthetics on end-plate channels (Gage et al., 1978); just how far its effects on the structure of lipid bilayers resemble those of general anesthetics is not clear. It seems possible that the mechanisms of effects on inactivation by octanol and by general anesthetics are similar.

The results reported here may be more useful when the results of more experiments like those of Redmann et al. (1979) and Chiu et al. (1979), which use other means to alter the lipid environment of the sodium channel, are available. It will be particularly interesting to see if the selective action on inactivation and not activation kinetics is true for other methods of lipid alteration.

Pharmacological significance

It seems almost certain that the production of unconsciousness by general anesthetics is due to block of synaptic transmission in the central nervous system (Richards, 1973; Richards et al., 1975; Richards and white, 1975). Larger concentrations of anesthetics are required for block of nerve conduction than for block of synaptic transmission or for clinical general anesthesia (Larrabee and Posternak, 1952; Richards, 1973; Richards and White, 1975). However, the results reported here suggest that the generation of action potentials may be affected in more subtle ways if changes in sodium channel kinetics were produced by the levels of anesthetic present during general anesthesia.

How do the ether concentrations used in the present research compare with clinical concentrations? The blood concentrations of ether during anesthesia of dogs at 37^{o} are 15-24 mM (Ronzoni, 1923; Haggard, 1924; Robbins, 1945) these correspond to the same concentrations in saline since the blood:saline partition coefficient is about unity (Steward et al., 1973). The smallest concentration of ether which had a consistent, significant effect

on sodium channel inactivation was 30 mM, which decreased the decay time constant by an average of about 20 percent. However, there is a problem in relating these studies, done at low temperature in a peripheral axon of an invertebrate poikliotherm, with possible effects at 37°C in central neurons of vertebrates. Cherkin and Catchpool (1964) found that goldfish adapted to 37°C required 25 mM ether for narcosis while at 10°C, they required 34 mM. Such a temperature dependence would be consistent with action in a membrane; water:membrane partition coefficients show variation in the proper direction. If general anesthetics do act by dissolving into membranes, the concentrations in the membrane would probably vary significantly with the lipid composition of the membrane, which might vary considerably between mammals and poikliotherms. With these uncertainties, it would not seem surprising if vertebrate sodium channels were affected by clinical levels of anesthetics, but, at face value, the present work suggests that such effects would be small.

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CHAPTER TWO: MODIFICATION OF SODIUM CHANNEL SLOW INACTIVATION AND POTASSIUM CHANNEL KINETICS BY ETHER AND HALOTHANE

INTRODUCTION

The reasons for being interested in studying the effects of general anesthetics on axons were outlined in the introduction to Chapter One; what seemed the most interesting effect, the apparent speeding of sodium channel inactivation, was treated in Chapter One. In this chapter, other effects of ether and halothane on the electrical properties of the crayfish axon are discussed. Most of these effects have not been investigated in very much detail, and many of the results and conclusions presented here must be regarded as preliminary.

Briefly, ether and halothane had no significant effects on resting potential or leakage conductance even at large concentrations. The magnitudes of both sodium and potassium currents were depressed, partially reversibly. The reversal potential of the sodium channel was unchanged. The application and wash-out of large concentrations of anesthetic were found to cause an irreversible shift in the voltage-dependence of a slow inactivation process in the sodium channel. The kinetics of potassium channel activation were faster when ether was present, although the dependence of this effect on ether concentration was variable from axon to axon. Finally, a measurement of the membrane capacity of an axon showed no change with ether present.

METHODS

The basic experimental precedures, data collection system, and the methods for analyzing sodium currents were described in Chapter One. Most of the experiments described here were performed using the guarded plate electrode for current collection; one of the experiments showing the shift in slow inactivation was done using the differential electrode for recording currents, in order to be sure that neither the basic properties of slow inactivation nor its shift by ether were artifacts due to spatial variation in membrane potential. They were not.

The level of steady-state slow inactivation was measured by changing the holding potential while giving test pulses to a constant potential. The time constant for changes in the amplitude of the test pulse current was about 6 seconds at -78 mV at 9^{o}; the process was a bit faster at more negative potentials and slower at less negative potentials. Usually a holding potential was established for 15 to 30 seconds for membrane potentials of -80 to -100 mV, or 30 seconds to 3 minutes for less negative potentials; these durations seemed sufficient for the test pulse currents to nearly reach a steady-state value. There is probably an even slower process of inactivation: following the establishment of a series of more negative potentials from a resting potential of -80 mV or -75 mV, after re-establishment of the resting potential the currents were often slightly larger than they had been in the control, and they relaxed back to their original size with a time

course of several minutes. However, the magnitude of this "hysteresis" was not more than about 10 percent and probably did not contribute much error to the measurements.

Potassium currents were obtained using normal van Harreveld's solution with 100 nM tetrodotoxin added to block sodium currents. Leak and capacity currents were subtracted by adding scaled currents from hyperpolarizing pulses.

The method for measuring capacitance is described in the text.

RESULTS

Resting potential and leakage current

Up to 300 mM ether had no effect on the resting potential of fresh, healthy axons, and in a single application of 400 mM ether, the resting potential of -74 mV declined only to -72 mV. All applications of large amounts of ether were performed at 6° or below; up to 20 mM ether had no effect on resting potential at 25°. Similarly, up to 32 mM halothane had no effect on resting potential at low temperatures. At 25°, 1-3 mM halothane had no consistent effect on resting potential; there were occasionally small hyperpolarizations or depolarizations, but these were usually transient.

In axons which had been used for more than a few hours or which appeared otherwise decrepit, application of large amounts of ether or halothane sometimes caused a decline in resting potential, usually irreversibly.

Up to 400 mM ether had no effect on the leakage current measured in response to hyperpolarizing pulses.

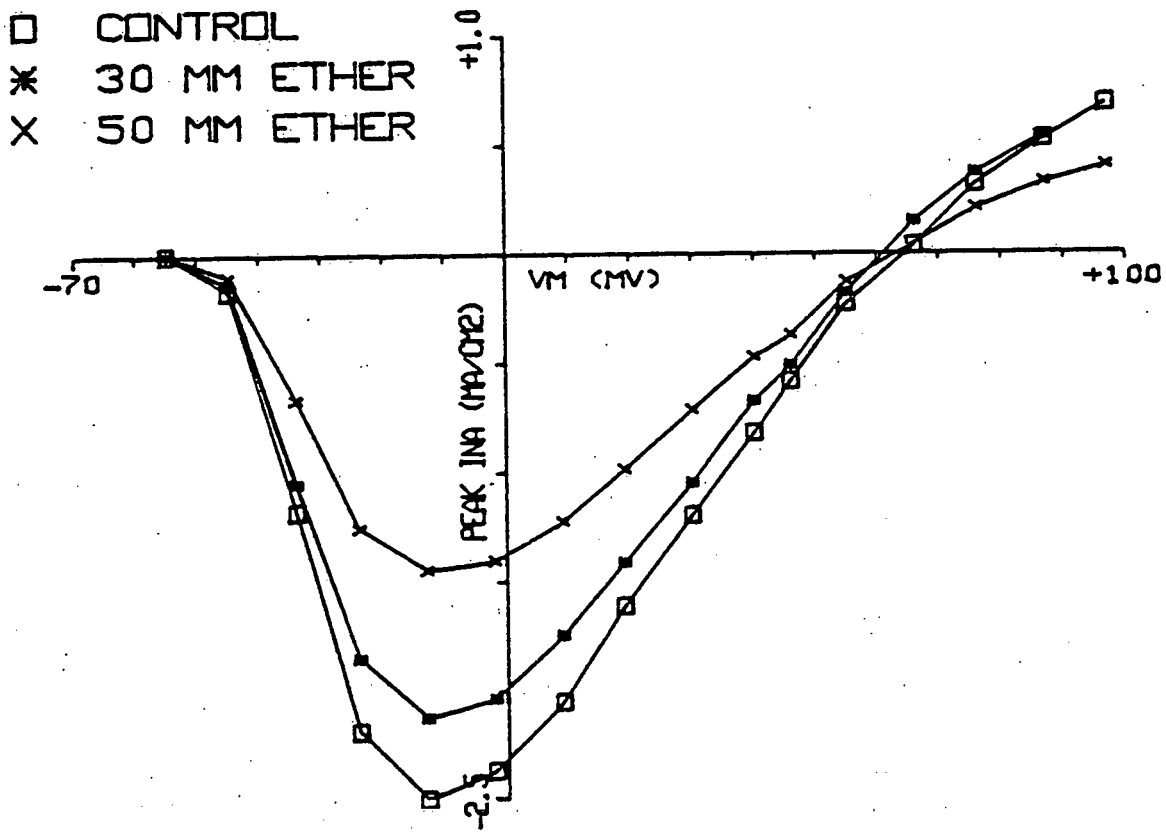
Partial block of sodium currents

Applications of concentrations of ether of 20 mM and above decreased the size of sodium currents, with no significant effect on the voltage-dependence of the sodium conductance or on the reversal potential of the sodium channel. The peak sodium current

as a function of membrane potential is shown for a typical application of moderate amounts of ether in Figure 1. In this experiment, 30 mM ether was applied for 10 minutes, 40 mM ether was applied for 13 minutes, 50 mM ether was applied for 12 minutes, and finally the axon was rinsed with ether-free solution. The peak current vs. membrane potential curves shown are for the control currents, the currents after 6 minutes of 30 mM ether, and the currents after 7 minutes of 50 mM ether. Peak current heights were reduced in 30 mM ether to about 85 percent of the control and in 50 mM ether to 55 percent of the control; after 25 minutes of rinsing with ether-free solution, the currents recovered partially, to 80 percent of the control. The lack of complete reversibility with 30-50 mM ether is usual.

There is no experimentally significant change in the reversal potential with ether present. There is also essentially no change in the voltage-dependence of the relative peak current heights. (There are very slight differences in most cases in that the peak currents at large depolarizations are usually decreased less relative to the control than are currents for moderate depolarizations. This is because the decrease in peak current heights produced by ether is due partially to the faster inactivation with ether present. The relative rates of activation and inactivation determine to what extent the faster inactivation will affect the peak current height and are such that peak current is decreased more for moderate depolarizations than for very large depolarizations (or very small depolarizations). This may be seen

Figure 1. Decrease in peak sodium currents by 30 and 50 mM ether. Times of application of ether are noted in the text. Note the lack of change in the general shape of the I-V curve and in the reversal potential. Full Na, Full K, 1 mM 4-AP. Temperature: $8.5^{o}C$.


CONTROL
30 MM ETHER
50 MM ETHER
+1.0
-70
VM (MV)
+100
PEAK INA (MA/CM2)
-2.5

in the currents in Figure 2, Parts C and D, in Chapter One: at -20 mV, 100 mM ether decreased the peak currents by 20 percent relative to wash-out, and at +65 mV, by only about 12 percent. This effect may be partly the cause of the 30 mM ether curve in Figure 1 deviating, near the reversal potential, from being a scaled version of the control curve. However, this deviation is probably mainly due to errors in reading the small currents near the reversal potential.)

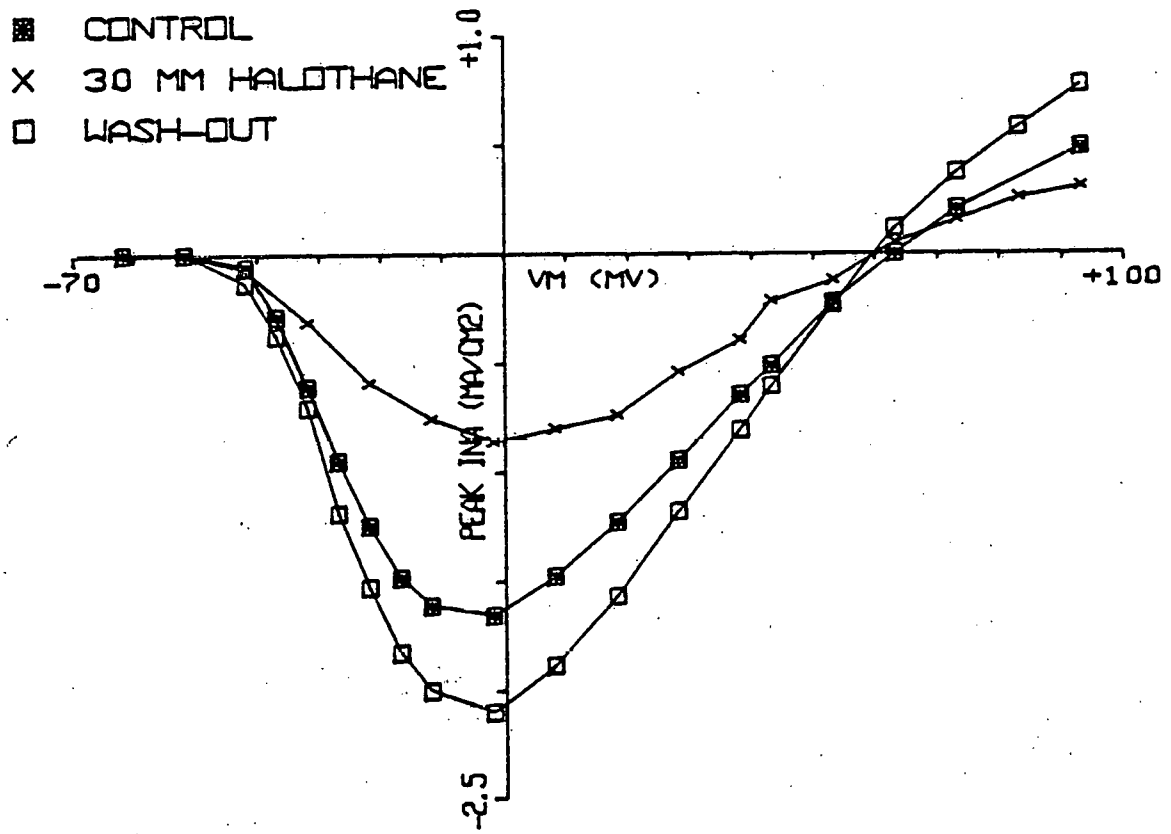
Halothane had a similar effect in depressing sodium currents without significantly changing the reversal potential or the voltage-dependence of the currents (Figure 2).

Shift in sodium channel slow inactivation

A curious and consistent result observed with ether was that when concentrations greater than 60 mM were applied to an axon with a holding potential near -75 mV (the usual resting potential near $4^{o}C$ with normal potassium in the bathing solution), the sodium currents after rinsing out the ether with ether-free solution were invariably larger than the control currents had been. This was also seen with large concentrations of halothane, as in Figure 2.

Further experiments have shown that this effect seems to be due to an irreversible shift in the voltage-dependence of a slow inactivation process which governs the maximal sodium conductance available when the axon is held at various membrane potentials. Slow inactivation has been previously described in the crayfish axon by Shrager (1977), and Starkus and Shrager (1978) described an

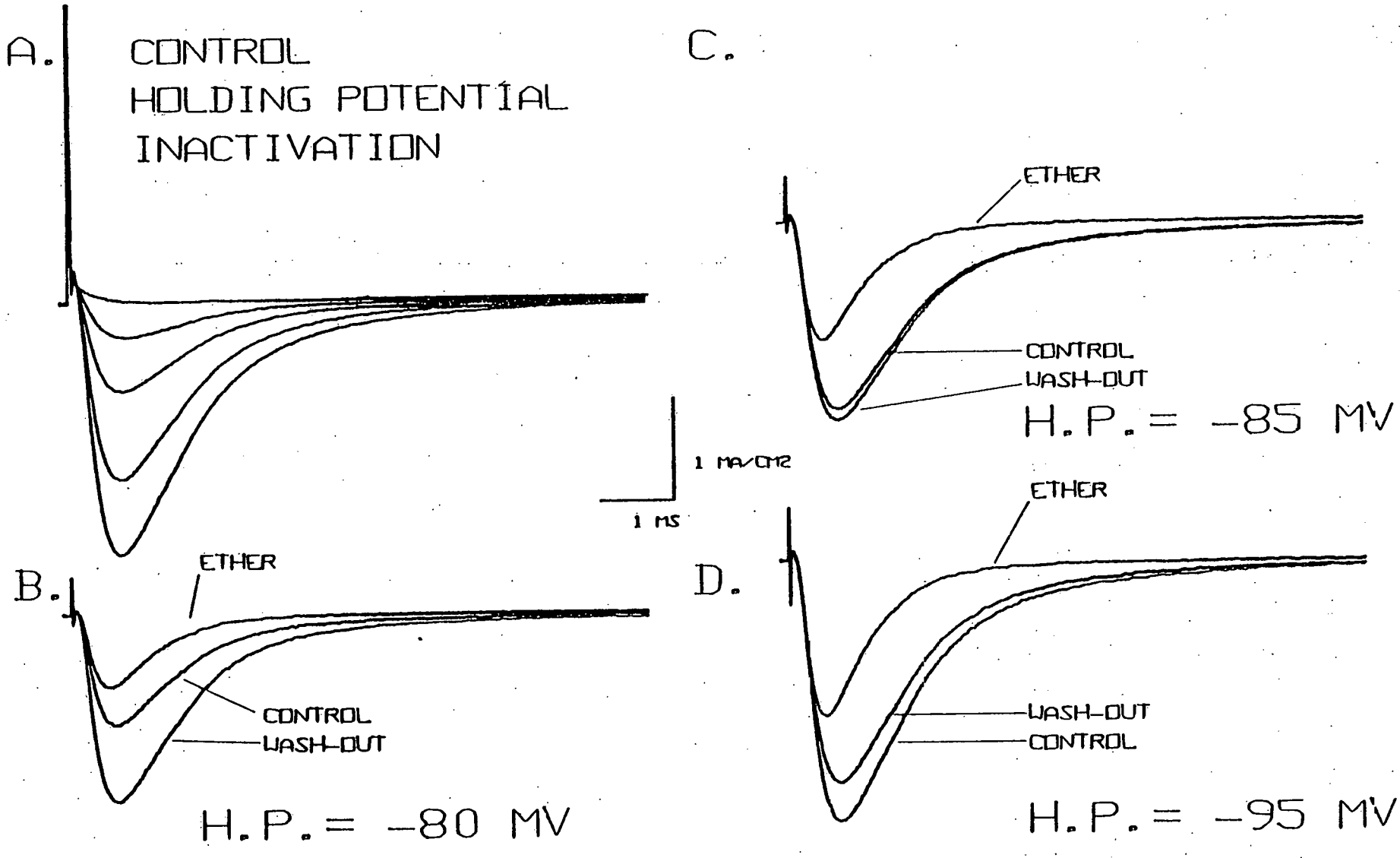
Figure 2. Decrease in peak sodium currents by halothane. 30 mM halothane applied for 9 minutes and rinsed for 30 minutes. Full Na, Full K, 1 mM 4-AP. Holding potential -72 mV. Temperatures: Control, 2.5^{o}C; Ether, 2.5^{o}C; Wash-out, 2.6^{o}C.


CONTROL
30 MM HALOTHANE
WASH-OUT
+1.0
-2.5
-70
VM (MV)
+100
PEAK INA (MA/CM2)

irreversible shift in the voltage-dependence of the process, in the depolarizing direction, when the axon was treated internally with trypsin. The application and removal of large concentrations of ether or halothane appears to produce a similar shift, but of smaller magnitude.

The basic phenomenon of slow inactivation observed through changes in the holding potential is shown in Part A of Figure 3. The holding potential is varied and the sodium conductance available is assayed by measuring the peak sodium current flowing during a test pulse to a constant potential. Each holding potential is established for 30 seconds or more to allow the test pulse sodium current to reach nearly a steady-state value. In Figure 3a, the currents for a test pulse to +5 mV are shown for holding potentials of -65, -75, -80, -85, and -95 mV; the sodium current was very small with a holding potential of -65 mV and reached a nearly saturating maximum size at -95 mV. The current kinetics do not change significantly with holding potential in such experiments; usually the decay time constants change by less than 10 percent and the changes are consistent with reasonable values of residual uncompensated series resistance. (This lack of significant change in kinetics with large changes in current size is reassuring evidence for the lack of significant current-dependent errors in the establishment of voltage control by the voltage clamp.)

Figure 3. Inactivation with changes in holding potential, and effects of 200 mM ether. (a) Control currents in response to depolarizations to +5 mV from holding potentials of -65, -75, -80, -85, and -95 mV. Currents are larger for more negative holding potentials (b) Control, ether, and wash-out currents for a depolarization to 5 mV from a holding potential of -80 mV. (c) Same, but for -85 mV holding potential (d) Same, but for -95 mV holding potential. Full Na, Full K, 1 mM 4-AP. Resting potential -75 mV. Temperatures: (a) 1.6-1.7^{o}C; (b) Control, 2.5^{o}C; ether; 2.3^{o}C; wash-out, 3.8^{o}C (c) Control, 1.7^{o}C, ether, 2.5^{o}C, wash-out, 2.6^{o}C (d) Control, 1.7^{o}C, ether, 3^{o}C, wash-out 2.9^{o}C.

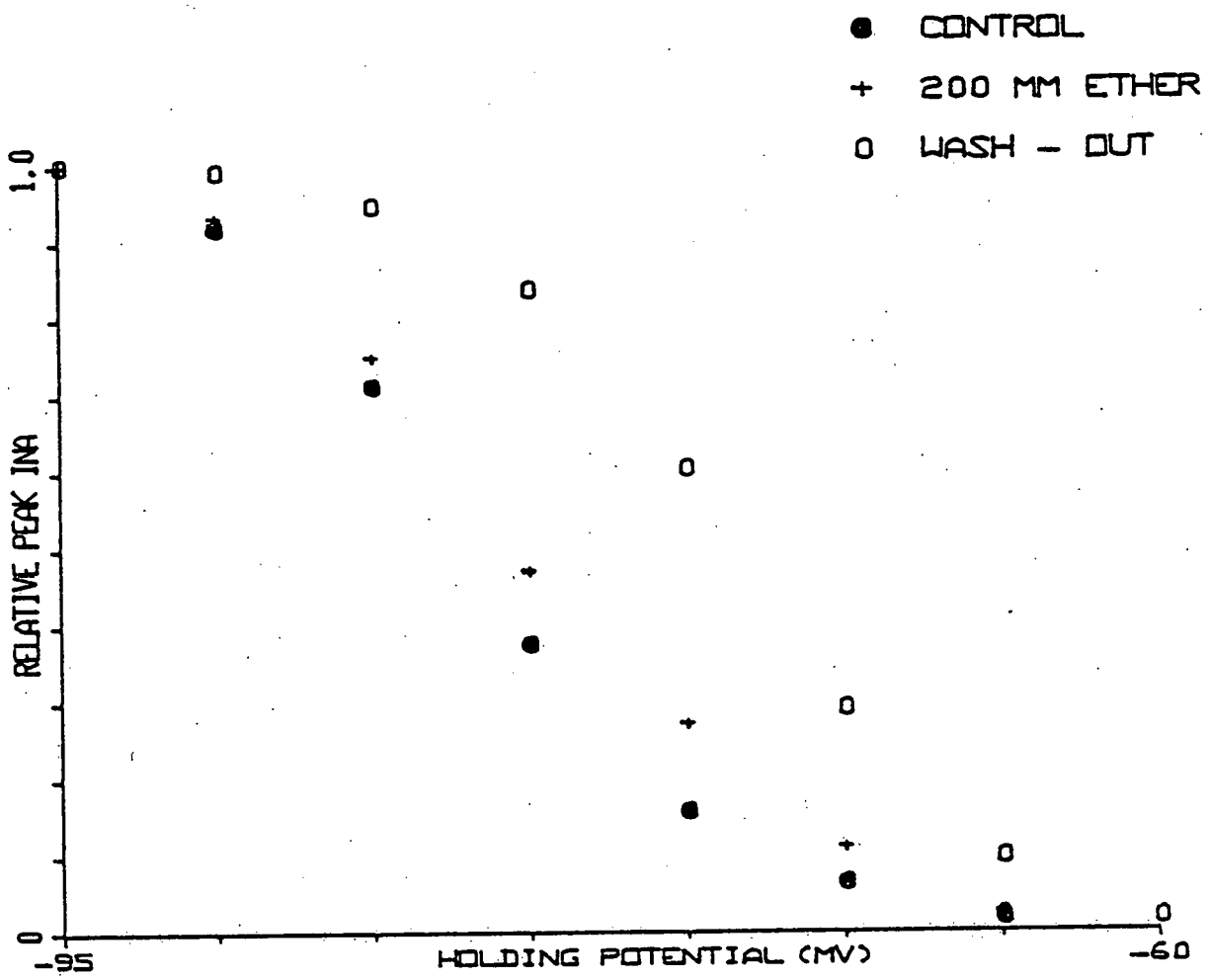

A.
CONTROL
HOLDING POTENTIAL
INACTIVATION
1 MA/CM2
1 MS
B.
ETHER
CONTROL
WASH-OUT
H.P.= -80 MV
C.
ETHER
CONTROL
WASH-OUT
H.P.= -85 MV
D.
ETHER
WASH-OUT
CONTROL
H.P.= -95 MV

In Figure 4, the peak currents during the test pulse to +5 mV are plotted as a function of holding potential before, during, and after the wash-out of 200 mM ether. After 20 minutes of 200 mM ether, the mid-point of the curve is shifted from its control value of -82 mV to -81 mV; following 20 minutes of rinsing with ether-free solution after a total of 25 minutes of 200 mM ether, the curve is shifted to the right, with a midpoint of -74 mV. In another application of 200 mM ether to a different axon, the control mid-point was -83 mV, and the mid-point following wash-out was -78 mV.

It was not practical to follow the time course of the development of the shift with complete holding potential inactivation curves, but the time course could be followed by observing the test pulse current heights for a single holding potential near -75 mV. In such cases, there was an initial reduction in peak current as soon as the ether solution was applied; after 10 minutes or so, the peak current size would go through a minimum and then slowly begin to rise: it seems likely that this rise represents the beginning of the shift in slow inactivation. As ether concentrations could conveniently only be applied for 20 minutes or so, it is unclear whether the shift would become complete with ether solution present, or whether washing out the ether solution is necessary to give the complete shift.

Parts b, c, and d of Figure 3 compare, at each of three holding potentials, the currents before, during, and after 200 mM ether. Notice that at -80 mV, the current after wash-out is substantially

Figure 4. Shift of slow inactivation by ether. Peak current during a test pulse to +5 mV is plotted versus holding potential before, during, and after 200 mM ether. Peak current heights are normalized with respect to that with -95 mV holding potential. Actual peak current heights with -95 mV holding potentials were: control, 2.5 mA/a cm^2; Ether, 1.5 mA/cm^2; Wash-out, 2.1 mA/cm^2. Full Na, Full K, 1 mM 4-AP. Temperatures: Control, 1.6-1.7^{o}C; Ether, 2.5 - 3.3^{o}C; Wash-out, 1.6 - 2.3^{o}C.

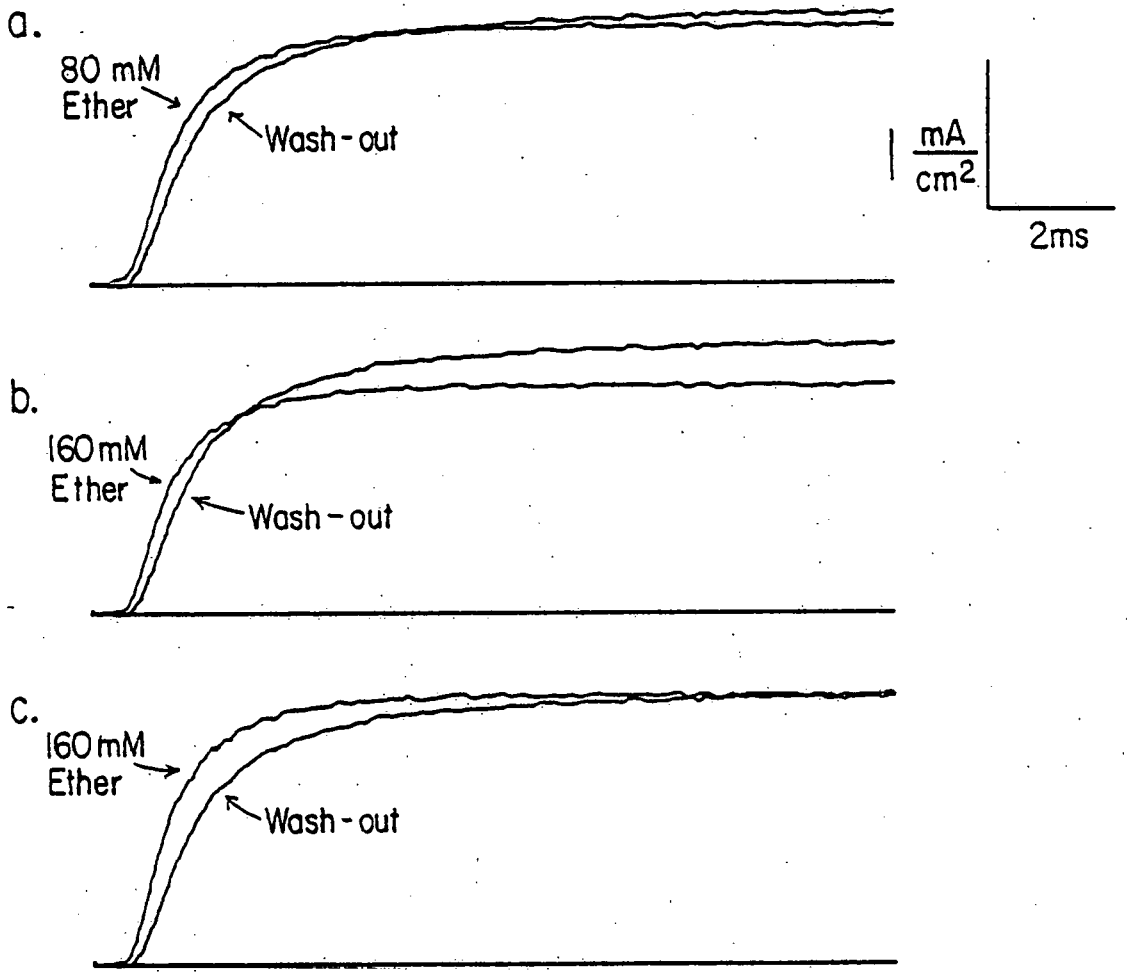

CONTROL
200 MM ETHER
WASH - OUT
1.0
0
RELATIVE PEAK INA
-95
HOLDING POTENTIAL (MV)
-60

the control; while at -95 mV, where presumably recovery from slow inactivation is nearly complete in all cases, there is a loss of about 15 percent of the current in the wash-out compared to the control. At -85 mV, in this particular axon, the effects of the shift in slow inactivation and the irreversible loss of 15 percent of the current nearly balanced one another, and the currents before and after the wash-out of ether are nearly identical. Part C is a nice illustration that the shift in slow inactivation is not the result of a generalized shift in the voltage-dependent gating properties of the sodium channel: if either the activation or the (fast) inactivation processes had been shifted by even a few mV, the kinetics in these two currents would not match so well. Part d of this Figure also makes the point that nearly all of the decrease in peak current height due to 200 mM ether may be due to the change in fast inactivation kinetics; at -95 mV, where recovery from slow inactivation is probably saturated both with and without ether, the initial rising phases of the currents are nearly identical with and without ether.

Effects of ether on potassium current kinetics

In most axons tested, application of ether resulted in faster turn-on of the potassium currents; this effect was reversible. An example of currents with and without ether is shown in Figure 5. In this axon, 40 mM ether was applied for 12 minutes, 80 mM ether was applied for 15 minutes, 160 mM ether was applied for 15

Figure 5. Changes in potassium current kinetics with ether. Depolarization from -75 mV to +90 mV. Sequence of application of ether concentrations is described in the text. (a) Current in 80 mM ether superimposed with the current after washing-out 160 mM ether. (b) Current in 160 mM ether superimposed with the current after rinsing. (c) The currents from (b) scaled so that their steady-state values match. Full Na, Full K, 100 nM TTX. Temperatures: (a) Ether, 4.4^{o}C; wash-out, 4.8^{o}C (b,c) Ether 4.8^{o}C, Wash-out 4.8^{o}C.


a.
80 mM
Ether
Wash - out
mA
cm2
2ms
b.
160 mM
Ether
Wash - out
c.
160 mM
Ether
Wash - out

minutes, and the axon was rinsed with ether-free solution. Since there was an irreversible decrease in current sizes from the beginning to the end of the sequence, the currents with 80 and 160 mM ether are shown, in Parts a and b, superimposed with the currents after recovery from ether, in order to emphasize the differences in kinetics with and without ether. Although the currents with ether have smaller steady-state values, they are actually larger than the current with no ether for the first 2 milliseconds or so. The change in kinetics is further emphasized in Part C, where the currents with and without 160 mM ether have been scaled so that their steady-state values match.

The production of faster potassium channel kinetics by ether was a fairly but not perfectly consistent finding. Nine axons were used for experiments with ether on potassium currents. Seven of the axons showed clear changes in kinetics, with "cross-over" between control and ether currents like that seen in Figure 5, with ether concentrations from 40 to 480 mM; the reverse effect was seen upon washing out the ether, although usually the currents following wash-out were not exactly the same as in the control. In two axons, applications of ether did not have any significant effect on potassium current kinetics. These results are summarized in Table. I.

TABLE I

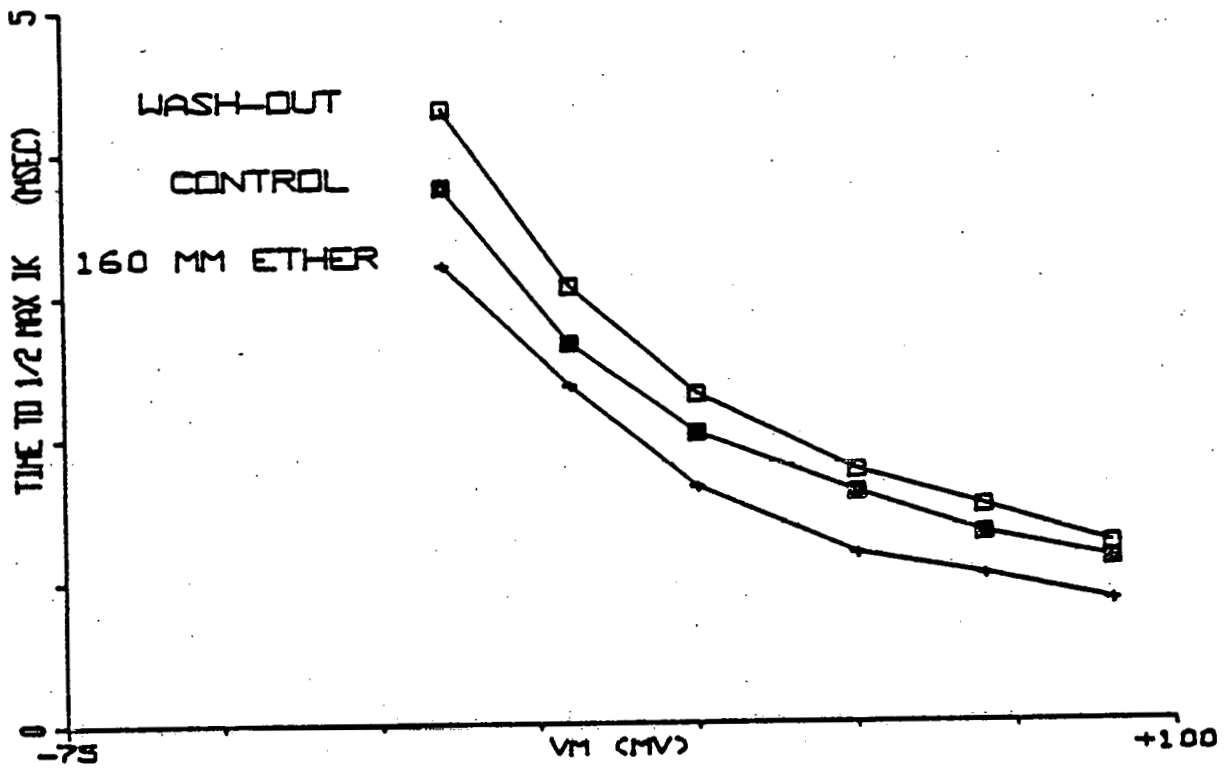
EFFECT OF ETHER ON POTASSIUM CURRENT KINETICS

			Percent decrease in time to half-maximum	
Axon	Ether Concentration (mM)	Crossover	Compared to control	Compared to washout
1	50	YES	20	30
	50	YES	25	
2	60	YES		23
3	50	NO		
4	40	YES	25	
5	50	NO		
6	40	NO	2	
	80	YES	17	
	160	YES	24	32
7	100	YES	17	22
	300	YES	20	25
8	80	YES	13	
9	160	YES	28	34
	480	YES	40	

The speeding of activation kinetics was quantitated by measuring the time taken to reach half of the maximum potassium current. Figure 6 shows data from the same experiment from which Figure 5 is taken (Axon 6 in table I); the time to half maximum current is plotted as a function of membrane potential for the control, in 160 mM ether (following applications of 40 and 80 mM ether), and following the wash-out of ether. The kinetics are changed at all membrane potentials to a roughly similar degree; usually, as in Figure 6, the percent decrease in the time to half maximum current is somewhat greater for the larger depolarizatons. Figure 6 is typical of most of the axons in that the currents following the rinsing out of an application of ether are slightly slower than the control currents before ether was applied.

There seems to be variablility from axon to axon in the concentrations of ether necessary to produce a given change in potassium channel kinetics. In Axon 4 in Table I, 40 mM ether produced a large effect while in Axon 6 the same concentration barely had any effect. The experimental conditions were similar in the two axons; however, it is interesting to note that the control kinetics were substantially faster in Axon 6: the time to half maximum at +90 mV was 1.3 msec ($4.5^{o}C$) while in Axon 4 the time to half maximum at +91 mV was 2.7 msec ($2.6^{o}C$). It seems likely that the speeding of potassium kinetics by ether would have been seen in all axons if large enough concentrations had been used.

Figure 6. Time to half maximum potassium current with and without 160 mM ether. Same axon as in Figure 5. Full Na, Full K, 100 nM TTX. Temperatures: Control, 4.6°C; Ether, 4.6-5.0°C; wash-out, 4.7-4.8°C.


WASH-OUT
CONTROL
160 MM ETHER
TIME TO 1/2 MAX IK (MSEC)
5
0
-75
VM (MV)
+100

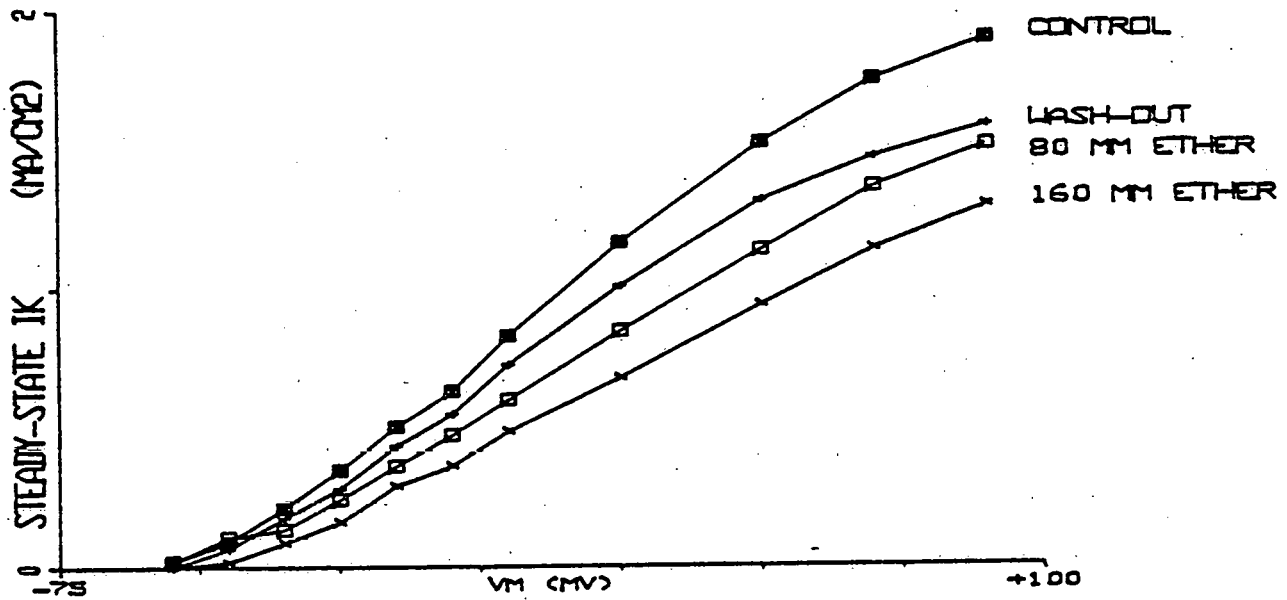
Block of potassium current

In addition to speeding activation kinetics, 40 to 160 mM ether decreased steady-state current levels by 10-30 percent, usually only partly reversibly. Figure 7 (which is again from Axon 6 of Table I) shows the steady-state potassium currents as a function of membrane potential for the control, in 80 mM ether, in 160 mM ether, and after rinsing. The voltage-dependence of the potassium currents was not significantly different in ether. Comparison of Figure 7 with Figure 6 shows that the change in current kinetics with ether is not the result of a generalized shift of the voltage-dependence of the channel.

Membrane capacitance

General anesthetics perturb the structure of lipid bilayers (Trudell et al., 1973; Vanderkooi et al., 1977; Johnson and Miller, 1970); this perturbation might underly the pharmacology of general anesthetics. Recently, two groups suggested a possible mode of coupling between a change in membrane lipid structure and changes in nerve membrane function. Haydon et al. (1977a, 1977b) observed that the capacitance of artificial bilayers saturated with n-alkanes varied according to the number of carbons in the alkane; presumably the differences in capacitance reflect differences in bilayer thickness due to adsorption of alkane. They noticed that the changes in capacitance were correlated with the ability of the

Figure 7. Steady-state potassium current versus membrane potential, with and without 80 and 160 mM ether. Same axon as in Figures 5 and 6. Holding potential -75 mV. Full Na, Full K, 100 nM TTX. Temperatures: Control, 4.6°C; 80 mM ether, 4.3-4.5°C; 160 mM ether, 4.5 - 5.0°C, wash-out, 4.6 - 4.8°C.

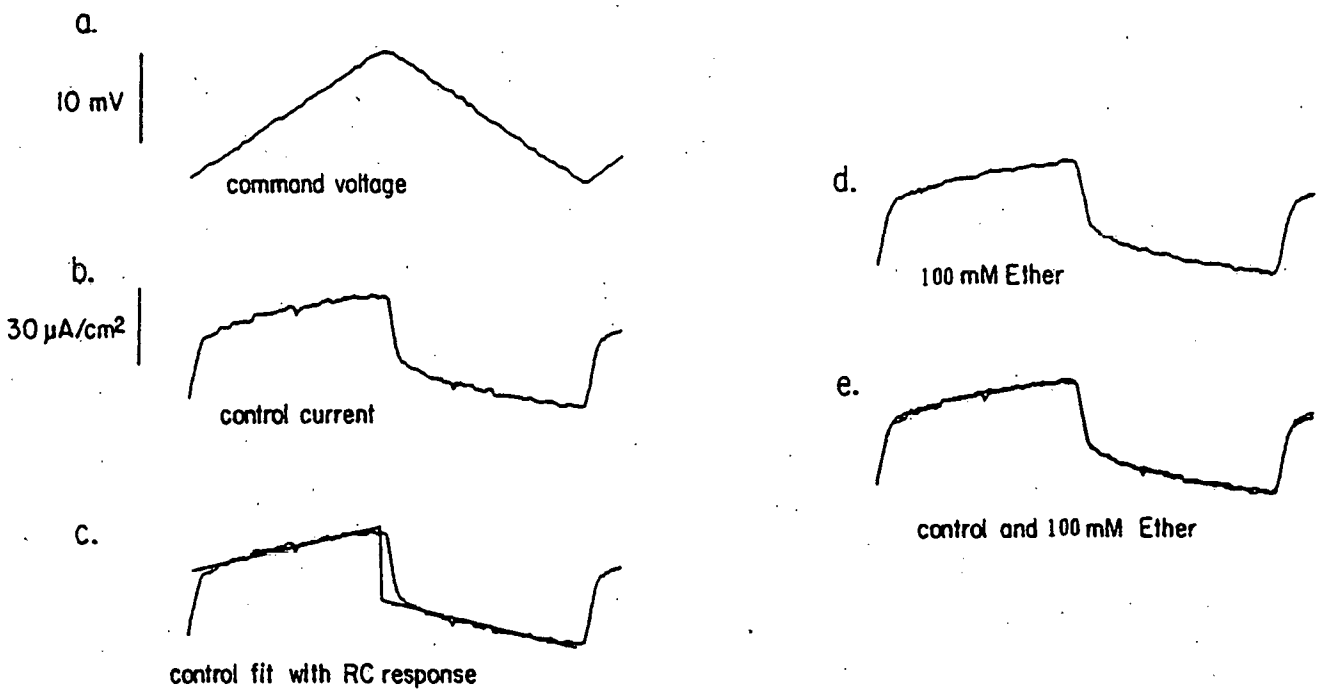

STEADY-STATE IK (MA/CM2)
2
0
-75
VM (MV)
+100
CONTROL
WASH-OUT
80 MM ETHER
160 MM ETHER

n-alkanes to block nerve conduction in the squid axon and frog sciatic nerve, and suggested a molecular mechanism by which the alkane might inhibit the nerve impluse: adsorption of alkane into the membrane would thicken the lipid bilayer of the membrane and would reduce the stability of open sodium channels by requiring them to span a longer distance. Ashcroft et al. (1977a, 1977b) observed decreases in the capacitance of artificial lipid bilayers when benzyl alcohol was present, and made an essentially identical proposal for how benzyl alcohol might block nerve impulses.

A giant axon provides a convenient preparation for studying possible drug-induced changes in capacitance. In the crayfish giant axon, ether concentrations up to 100 mM appeared to have no detectable effect on membrane capacitance, at least with the relatively crude measurements I have made. Up to 50 mM ether had no effect on the the capacitative transient produced by a voltage step under voltage clamp; when the analog signal of the capacitative transient was displayed on a storage oscilloscope with a fast time base, the transients with and without ether superimposed within the width of the trace. In order to make measurements of capacitance which could conveniently be digitized for analysis, I used a triangular wave-form under voltage clamp. The method and its results are illustrated in Figure 8. Part a shows the command voltage form, a 14.3 mV peak-to-peak 1.2 Hz triangular wave applied at a holding potential of -81 mV. The current signal in response to this wave-form is shown in Part b; it

is close to the response expected from a resistor and capacitor in parallel. The values of membrane resistance (380 ohms-cm^2) and capacitance (.89 $\mu f/cm^2$) are estimated by fitting the data with the theoretical response of an RC combination. In Part d, the current in 100 mM ether was recorded; it is essentially identical to the control current, as is emphasized by Part e, where the currents with and without ether are superimposed. Probably a change in capacitance greater than about 5 percent would have been detected in this experiment.

Figure 8. Membrane capacitance with and without 100 mM ether. A triangular wave-form (14.3 mV peak-to-peak, 1.2 KHz) is applied under voltage-clamp. (a) Voltage wave-form. (b) Membrane current (c) Fit of the expected response of a parallel RC combination. Fit was made by estimating RC values and adjusting the values by eye for best fit; fit shown is with R = 380 ohm-cm^2, C = .89 $\mu f/cm^2$. (d) Same as part (b), but with 100 mM ether. (e) Currents with and without ether superposed. 1/4 Na, 1/2 K, 1 mM 4-AP, 100 nM TTX. Holding potential - 81 mV. Temperature 3.7^{o}C.


a.
10 mV
command voltage
b.
30 μA/cm²
control current
c.
control fit with RC response
d.
100 mM Ether
e.
control and 100 mM Ether

DISCUSSION

Comparison with previous voltage-clamp results

There are two previous reports of voltage clamp studies on the effects of general anesthetics on nerve membranes. Kendig et al. (1979) found that ether in the frog node depressed sodium currents without changing the sodium channel reversal potential, in agreement with the results reported here. Shrivastav et al. (1976) studied the action of trichloroethylene on the squid axon and found, in contrast to the results with ether and halothane on the crayfish axon, that the resting potential was decreased, the leakage conductance was decreased, the steady-state conductance curve was shifted along the membrane potential axis and that the reversal potential for the peak transient current was shifted. Whether the differences lie with the anesthetics or the preparations is not clear.

Change in potassium channel kinetics

Probably the most useful of the observations reported here is the speeding of potassium channel activation by ether. Two other ionic channels show altered gating kinetics with general anesthetics; both end-plate currents (Gage and Hamill, 1975; Gage et al., 1979) and sodium currents show faster decay kinetics. In both these cases it is ambiguous whether the faster decay is caused by some sort of time-dependent block of open channels or whether the native gating processes are being affected. In the case of the

faster potassium kinetics, the change is clearly not the result of channel blocking or inactivation by anesthetic: its seems that the normal gating process is made faster when ether is present. This gives credence to the possiblity that native gating processes are also being affected in the cases of the end-plate channel and the sodium channel. The fact that all three of the ionic channels so far examined show changes in gating kinetics may also add some weight to the idea that the changes might be mediated by changes in membrane lipid structure, seen with general anesthetics in model systems (Trudell et al., 1973; Vanderkooi et al., 1977).

It is interesting that sodium channel activation kinetics are not affected by ether while potassium channel activation is speeded, since one might expect the basic gating mechanisms to be similar. It is possible that the two processes have different types of rate limiting processes, though, especially considering the difference in their time scales.

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CHAPTER THREE: STUDIES ON SODIUM CHANNEL INACTIVATION

INTRODUCTION

When a nerve membrane is sufficently depolarized from its resting potential, the permeability to sodium ions rises and then becomes small again (Hodgkin and Huxley, 1952a). A first step in understanding the kinetic mechanism of the sodium channel is to understand the relation of the inactivating phase of the sodium conductance to the initial activating phase. Hodgkin and Huxley's postulate of independent activation and inactivation gates, both of which must be open for the sodium channel to conduct ions (Hodgkin and Huxley, 1952b) appears inconsistent, at least in its original form, with some recent observations on sodium channel kinetics.

One observation which is apparently common to all experimental preparations is that inactivation develops with a lag which roughly parallels the time course of the opening of the channels (Armstrong, 1970; Goldman and Schauf, 1972; Peganov, 1973; Schauf and Davis, 1975; Bezanilla and Armstrong, 1977) rather than with the exponential time course expected from an independent, first-order process. Lags in the recovery from inactivation when the axon is repolarized after a long depolarization have also been reported (Schauf, 1976; Chiu, 1977).

Another experiment with results useful for distinguishing between inactivation models has different results reported in

different nerves. Goldman and Schauf (1973) reported that, in the Myxicola giant axon, the rate of inactivation measured with the prepulse inactivation method of Hodgkin and Huxley (1952a) was substantially slower than the rate of decay of sodium currents at the same potential. The same result was reported for lobster giant axons. (Oxford and Pooler, 1975). However, in the squid giant axon (Hodgkin and Huxley, 1952b; Chandler et al., 1965; Bezanilla and Armstrong, 1977) and in the frog node (Chiu, 1977) the rates determined by the two methods are nearly identical. The difference between the two results is that in lobster and Myxicola axons the rate of decay of sodium currents is only mildly voltage-dependent and is quite rapid for small depolarizations, while in squid and frog nerve the rate is sharply voltage-dependent and is slow for small depolarizations.

Other types of experiments on inactivation have been performed in Myxicola axons but have not been reported in comparable detail for other preparations. One experiment is the measurement of steady-state fast inactivation by the method of Hodgkin and Huxley (1952a) in which a long prepulse to a varying potential produces inactivation, the extent of which is assayed by the peak sodium current during a test pulse of fixed height following the prepulse; in the Myxicola axon the steady-state inactivation curve constructed with this method is different for different test pulse potentials (Goldman and Schauf, 1972; Schauf et al., 1976). This experiment was suggested by Hoyt (1968) for distinguishing between

inactivation models; Goldman (1976) has discussed experimental details and precautions. Another result from Myxicola is that over a fairly wide range of membrane potentials, sodium currents decay almost to zero while inactivation measured with the prepulse method is far from complete (Goldman and Schauf, 1973). This is an important result, as Goldman (1976) has pointed out, since it can rule out many models in which activation and inactivation are independent processes.

Because of the importance of inactivation experiments for narrowing the field of possible kinetic models for the sodium channel, and because of the seemingly different results from different preparations, it seemed worthwhile to perform the same and similar experiments in the voltage clamped crayfish axon preparation developed by Shrager (1974). In doing so I have tried to assess and reduce some errors associated with the voltage clamp method used.

There are two major possible sources of error in voltage clamping giant axons with the axial wire technique. One is the problem of unavoidable resistance in series with the membrane, which complicates establishing a constant membrane potential (Hodgkin et al., 1952). The second is the possibility of non-uniformities of potential and current in the region of membrane from which current is recorded. Despite the extensive theoretical and experimental consideration given to this problem by Cole and Moore (1960), Taylor, Moore and Cole (1960), and Chandler, FitzHugh

and Cole (1962), the extent to which it influences the results of specific experiments is not usually known or estimated.

In the work reported here I have used a technical advance by Sigworth (1979) for more complete series resistance compensation, and a technical advance by Cole and Moore (1960) for estimating and reducing errors due to spatial non-uniformity. The remaining errors are estimated to be small enough to allow reasonable confidence to be attached to the kinetic data obtained. The experimental results are similar to those in squid and frog nerve but differ from them in a number of details. The results seem inconsistent with both the simplest models with independent activation and inactivation processes and with the simplest models in which only open channels can inactivate. Many classes of intermediate cases cannot be excluded.

METHODS

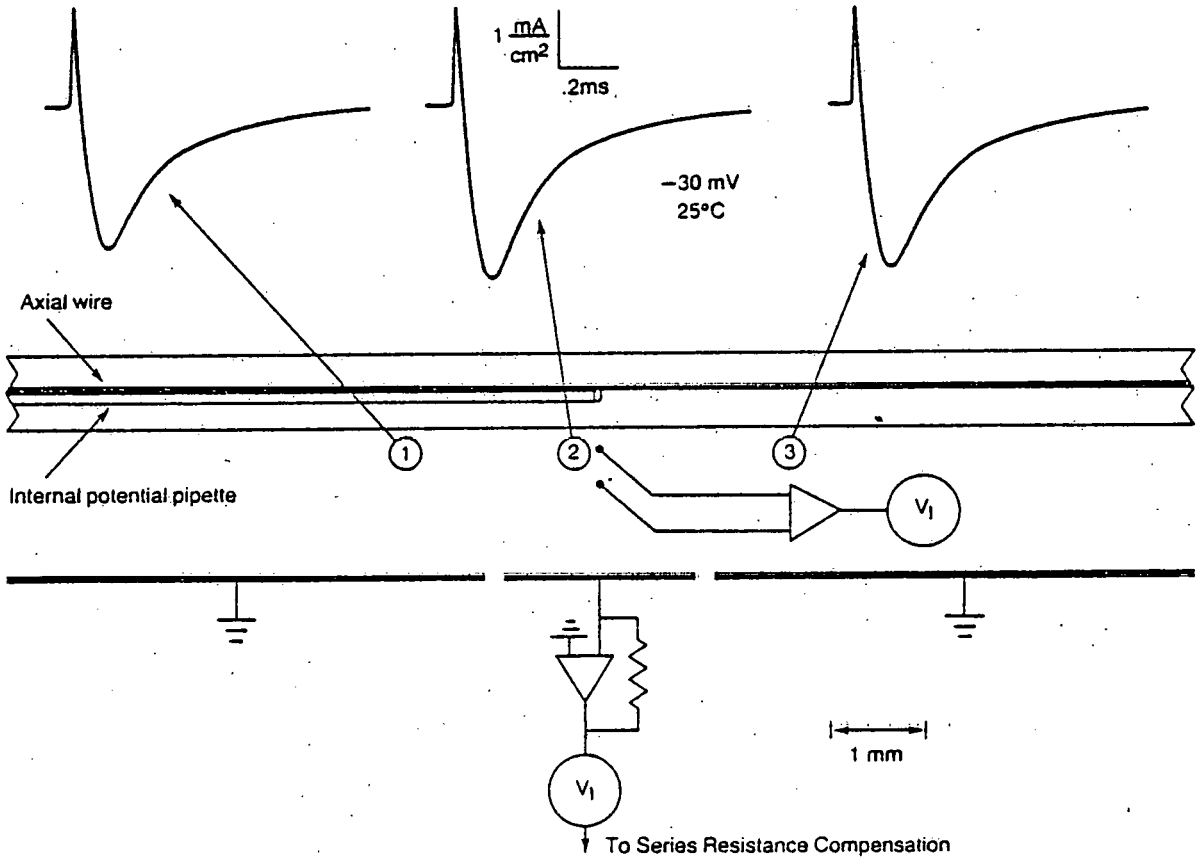
Medial giant axons from Procambarus clarkii were dissected, cleaned, and voltage clamped by the axial wire technique described by Shrager (1974). Crayfish were from Louisiana and California; there were no apparent systematic differences in the properties of their axons.

The microcomputer system for data collection, the control and measurement of temperature, and the methods of analysis have been previously described (Chapter 1).

Current measurement

To clamp the membrane potential, current was passed with the internal axial wire to an external electrode consisting of three platinized platinum plates of lengths 5 mm, 2 mm, and 5 mm, as shown in Figure 1. Membrane current density was measured by recording current density in the external solution as the potential drop between a pair of platinized platinum wire electrodes separated by 180 μ and oriented perpendicular to the axon (Figure 1), a technique devised by Cole and Moore (1960; see also Cole, 1968) to permit measurement of relatively localized membrane current density. The wires had diameters of 25 μ and were insulated except for the final 170 μ. The nearer wire was usually within 80 μ of the axon when the electrode pair was in its final position at the "control point" opposite the tip of the internal potential pipette. The signal from the differential electrode was

Figure 1. Method of current measurement. Not drawn to scale in the vertical direction; a typical axon diameter is 200 μ, and the external platinum plate ground electrodes were usually about 3 mm from the axon. The currents shown were recorded with the differential electrode opposite the control point, 2 mm to the left, and 2 mm to the right; the nearer wire of the electrode was about 700 μ from the axon. Current density was calculated by comparing current signals recorded with the differential electrode with those from the central plate; in this axon, the calibrating factor was 1 mA/cm^2 per .73 mV between the wires in the differential electrode. Full Na, Full K, 1 mM 4-AP. Temperatures: $2.5^{o}C$.


1 mA/cm²
2ms
−30 mV
25°C
Axial wire
Internal potential pipette
1
2
3
V1
V1
1 mm
To Series Resistance Compensation

amplified and high-pass filtered at .06 Hz and low-pass filtered at 14 KHz before being digitized.

The spatial resolution of the electrode pair was calibrated as shown in Figure 2. A pipette "point" current source in the nerve chamber simulated a small piece of axon membrane, and the electrode pair was moved parallel to the axis of the pipette. The size of the signal recorded at various positions relative to that recorded opposite the mouth of the pipette ($x = 0$) is shown in Figure 2. The solid line in Figure 2 gives the values expected if the field due to the source falls off as $1/r^{3/2}$ in the plane parallel to the floor of the chamber. This approximation is reasonably good, judged both by the fit to the data in Figure 2 and by direct measurement of potentials in the chamber. Knowing how the current from a small source will spread in the chamber, together with the geometry of the axon and electrode pair, permits an estimation of the magnitude of the contributions of various parts of the axon membrane at various distances to the total recorded current signal.

Series resistance compensation

In order to increase the amount of series resistance compensation which could be employed without inducing oscillations in the voltage clamp system, the current signal was low-pass filtered at 12 KHz before being fed back into the voltage clamp amplifier (Sigworth, 1979). The current signal from the central plate of the external current electrode was used for series

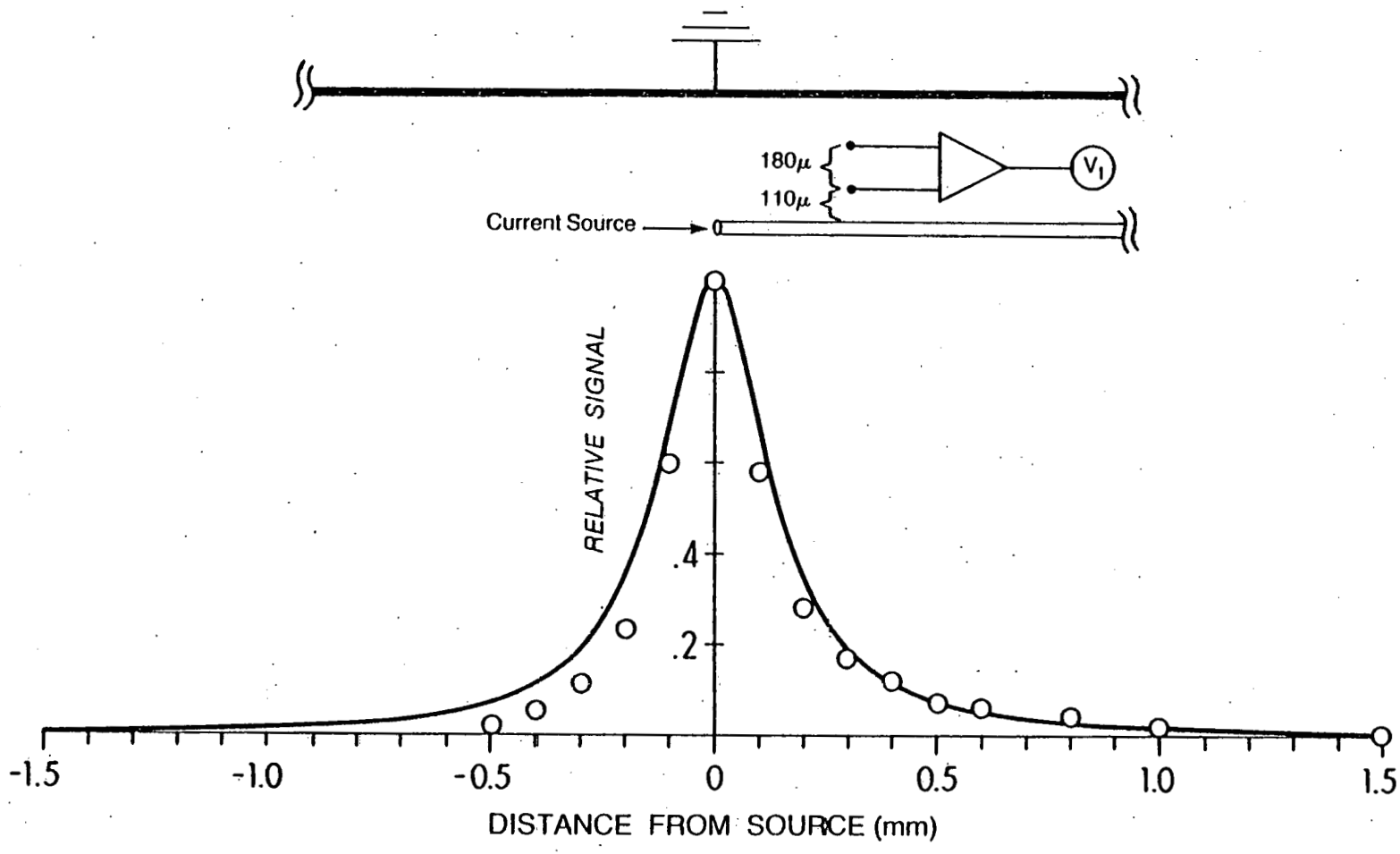

180μ
110μ
V_1
Current Source
RELATIVE SIGNAL
.4
.2
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
DISTANCE FROM SOURCE (mm)

Figure 2. Spatial resolution of the differential electrode. The pipette current source was in the chamber in the position normally occupied by the axon, about 3 mm from the ground electrode and about 100 μ up from the floor of the chamber. Points give the recorded voltage signals normalized with respect to that recorded opposite the mouth of the pipette. The solid line is drawn according to

$$y = 1/(x^2 + a^2)^{1/4} - 1(x^2 + (a + b)^2)^{1/4}$$

with a = distance of nearer wire from source = 110 μ

b = separation of wires = 180 μ

resistance compensation since the signal from the differential electrode pair had an unstable DC component. Compensation for 10-13 ohm-cm^2 was used. The series resistance measured from the time constant of the capacitative transient, in an axon in which the membrane capacitance was independently measured, was 11 ohm-cm^2.

Except for the currents shown in Figures 3 and 5, all the experiments reported in this paper were performed with external sodium concentrations of 52 mM or below, in order to further reduce errors due to series resistance.

Solutions

Solutions were modifications of van Harreveld's (1936) solution: 205 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2.3 mM $NaHCO_3$, pH 7.5-7.6. All solutions containing 4-aminopyridine (4-AP) had the bicarbonate replaced by 2.3 mM HEPES buffer, and were adjusted to pH 7.6-7.7. "1/4 Na; 1/2 K; 1 mM 4-AP" has 52 mM NaCl, 2.7 mM KCl, normal $CaCl_2$ and $MgCl_2$, 1 mM 4-AP, with tetramethylammonium chloride (TMA) to yield 433-438 m Osm. The solutions named with other fractional sodium or potassium concentrations were the same except for greater or less substitution by TMA.

RESULTS

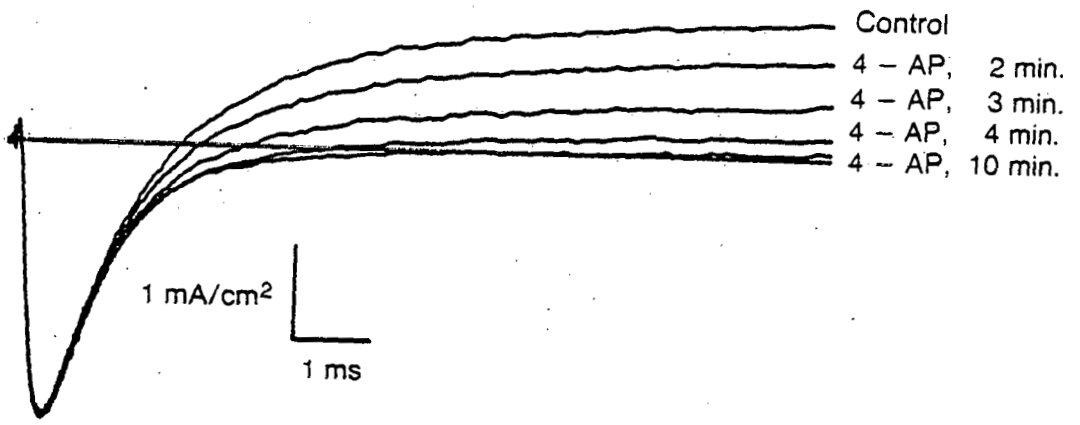
Use of 4-amino-pyridine to block potassium current

In most of the experiments reported in this thesis, bathing solutions contained 1 mM 4-amino-pyridine (4-AP) to block potassium currents (Meves and Pichon, 1975; Yeh et al., 1976). I therefore checked to see if there were any evident effects of 4-AP on sodium currents. Figure 3 shows currents at various times during the onset of block of potassium current by 4-AP. Although potassium current is blocked nearly completely after ten minutes, there is no change in the rise and early decay of sodium current. There was never a decrease in the size of peak early currents; in some axons, peak early currents increased slightly at all potentials, with no apparent change in kinetics. Also, in a number of experiments sodium currents were obtained from axons in the absence of 4-AP by using tetrodotoxin (TTX) to block sodium current and then subtracting the TTX - insensitive current from the total current. The currents obtained in this way were quantitatively similar in every way examined to those obtained in axons with 4-AP present. It thus seems reasonable to conclude tentatively that 4-AP has no effect on sodium channel kinetics.

Methods of obtaining sodium current from total current

Figure 4a shows membrane currents obtained in a bathing solution containing low (52 mM) sodium and 1 mM 4-AP, the usual

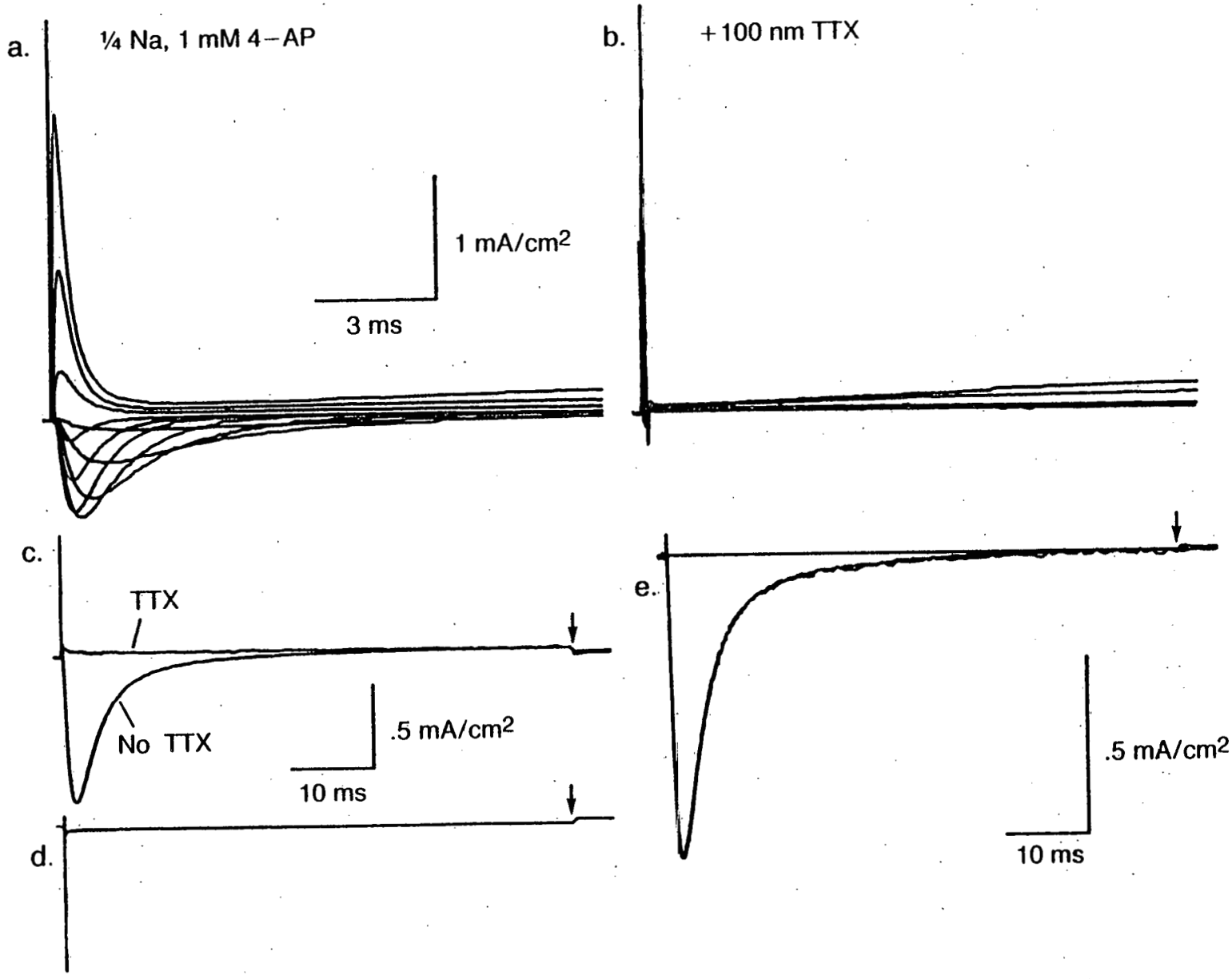
Figure 3. Block of potassium currents by 1 mM 4-AP. Depolarization from -75 mV to +15 mV. Time for complete replacement of the solution in the chamber is several minutes. Full Na, Full K. Temperature: 1.5^{o}C.


Control
4 – AP, 2 min.
4 – AP, 3 min.
4 – AP, 4 min.
4 – AP, 10 min.
1 mA/cm²
1 ms

solution employed in these experiments. Except for the early capacitative current, a small leakage current, and, at the higher depolarizations, some residual potassium current, the currents are sodium currents as defined by their TTX-sensitivity (Figure 4b). In the crayfish axon, potassium currents are blocked essentially completely by 4-AP for depolarizations to potentials less than 0 mV. For these potentials, corrections for leak and capacitative currents could be made by addition of appropriately scaled currents for hyperpolarizing pulses. Currents obtained in this way appeared to be purely sodium currents except for an initial, small outward current which is almost certainly gating current (Armstrong and Bezanilla, 1974). Parts c, d, and e of Figure 4 compare two methods of correcting for leak and capacitative current. Part c shows two currents superimposed, both in response to a pulse from -86 mV to -26 mV, one with and one without TTX. Part d shows the capacitative and leakage currents in response to a pulse from -86 mV to -166 mV. In Part e, two current sweeps have been superimposed; one is the sodium current obtained by subtracting the two sweeps in Part c, the second is the current obtained by scaling the sweep in Part d by 8/6 and adding it to the non- TTX sweep in Part c. Except for the initial outward blip of presumed gating current which is present in the second but not in the first, the two currents match nearly perfectly.

For potentials greater than 0 mV, block by 4-AP was usually not quite complete. Small potassium currents, with slow kinetics (Yeh,

Figure 4. Ionic currents in low sodium and 4-AP. (a) Currents for depolarizations from -82 mV to -42, -32, -22, -12, -2, +8, +18, +33, +53, +93 mV. Reversal potential is about +22 mV. 1/4 Na, 1/2 K, 1 mM 4-AP. Temperature 3.8°C. (b) Same, but with 100 nM TTX added to bathing solution. (c) Current (in a different axon) for depolarization from -86 mV to -26 mV, with and without TTX. 1/5 Na, 1/5 K, 1 mM 4-AP. Temperature 3.3°C. (d) Same for hyperpolarization from -86 mV to -166 mV. (e) Superimposition of sodium currents obtained by two methods described in text. Initial outward current is only present in the case in which capacitance and leak are corrected by current from hyperpolarization. Otherwise the two currents match perfectly.


a.
¼ Na, 1 mM 4–AP
1 mA/cm2
3 ms
b.
+100 nm TTX
c.
TTX
No TTX
.5 mA/cm2
10 ms
d.
e.
.5 mA/cm2
10 ms

et al., 1976), can be seen for the two largest depolarizations in Figure 4b. In addition, leak conductance was non-ohmic and was usually significantly greater for larger depolarizations than for hyperpolarizations and small depolarizations. For larger depolarizations, sodium current was isolated from total current in two ways. One was simply to add TTX and subtract the TTX-insensitive current. An equally satisfactory but much more convenient method was to record two current sweeps; in one, the test pulse was preceded by a conditioning prepulse sufficiently long and strong to totally inactivate the sodium current. The prepulse was separated from the test pulse by a short return to rest so that the capacitative current would be the same in the test pulse with the prepulse as in that without the prepulse. This procedure is illustrated in Figure 9. The prepulse inactivates the sodium current but has no apparent effect on leak or residual potassium current; subtracting the two current sweeps yields sodium current. This procedure appeared entirely equivalent to TTX subtraction in axons in which both were used. (In principle there is also gating current present with the pre-pulse subtraction procedure, but for outward sodium currents it is obscured. When the prepulse subtraction technique is used for inward currents, especially near the sodium reversal potential, gating current is evident.)

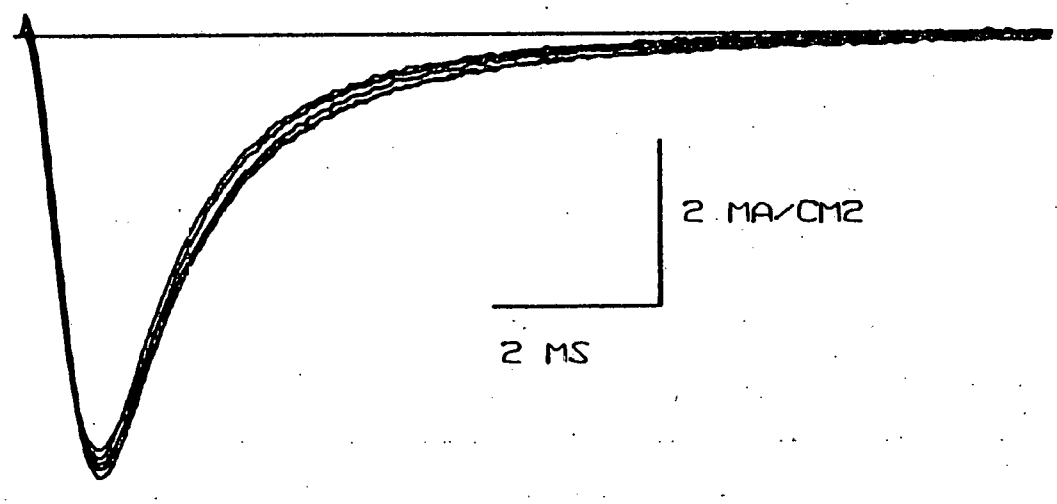
Spatial uniformity of membrane currents

The uniformity of sodium currents along the length of the axon was examined by moving the differential electrode pair described in Methods to various positions. The general result was that currents were reasonably uniform in magnitude and time course over lengths of axon extending a few millimeters in either direction from the control point opposite the tip of the internal potential pipette. Figure 5 shows currents recorded at various points spanning a 4 mm length of axon centered at the control point. The currents shown are in response to a step in potential from -75 mV to -25 mV, in the heart of the negative-resistance region of the membrane, where both current amplitudes and kinetics are sensitive to small changes in membrane potential and where the membrane potential is inherently unstable. Despite this, there are only relatively minor differences among the currents.

It is useful to express the differences in currents recorded at various locations as differences in the "effective" membrane potential at the different locations. Differences in the effective membrane potential can be estimated by comparing, at the different locations, the curves of peak sodium current vs. potential or τ_h, the time constant for the decay of sodium current, vs. potential. Figure 6 shows peak sodium current as a function of membrane potential recorded at the control point and at 1 mm in either direction from the control point. The magnitude and the potential dependence of the currents at the control point and at 1

Figure 5. Currents in response to a depolarization from -75 mV to -25 mV were recorded at three positions: opposite the control point, 1 mm right, 2 mm right, 1 mm left and 2 mm left. The nearer wire of the electrode was 300-400 μ from the axon. Leak and capacitative currents were corrected for with current from a hyperpolarizing pulse. Full Na, Full K, 1 mM 4-AP. Temperature 1.8^{o}C.

UNIFORMITY OF SODIUM CURRENTS OVER 4 MM



mm right were very similar; the currents at 1 mm left (toward the direction from which the piggy-back electrode had been inserted) were effectively shifted along the potential axis in the hyperpolarizing direction by about 2-3 mV, both in the position of the negative-resistance region and in the value of the reversal potential. Also, the currents at 1 mm left are about 20 percent smaller in the positive limb of the current-voltage relation relative to those at the control point. Figure 7 shows plots of τ_h vs. membrane potential taken over a 2 mm span (in a different axon). Above -20 mV the time courses of the currents were essentially identical at all three positions; below -20 mV, where the τ_h vs. potential curve is steep, differences are evident. The points from currents recorded 1 mm left are shifted by about 3 mV relative to those at the control point; this is seen most clearly in the -35 to -20 mV range. The points from currents recorded at 1 mm right are very similar to those from the control point; the effective shift at any potential is less than 1 mV. Considering the whole span, then, it seems reasonable to conclude that for any given voltage pulse, the currents over the 2 mm length originate from areas of membrane differing in potential by 4 mV or less.

The uniformity of effective membrane potential determined in 5 axons over spans of 2 mm and 4 mm, using the shift in τ_h vs. membrane potential, is shown in Table I. Usually the shift was determined in the region around -25 mV; here the curve was

Figure 6. Peak sodium current vs. membrane potential at three positions along the axon. The nearer wire was 100-200 μ from the axon. 1/4 Na, 1/2 K, 1 mM 4-AP. Holding potential -83 mV. Temperature 3.7°C.

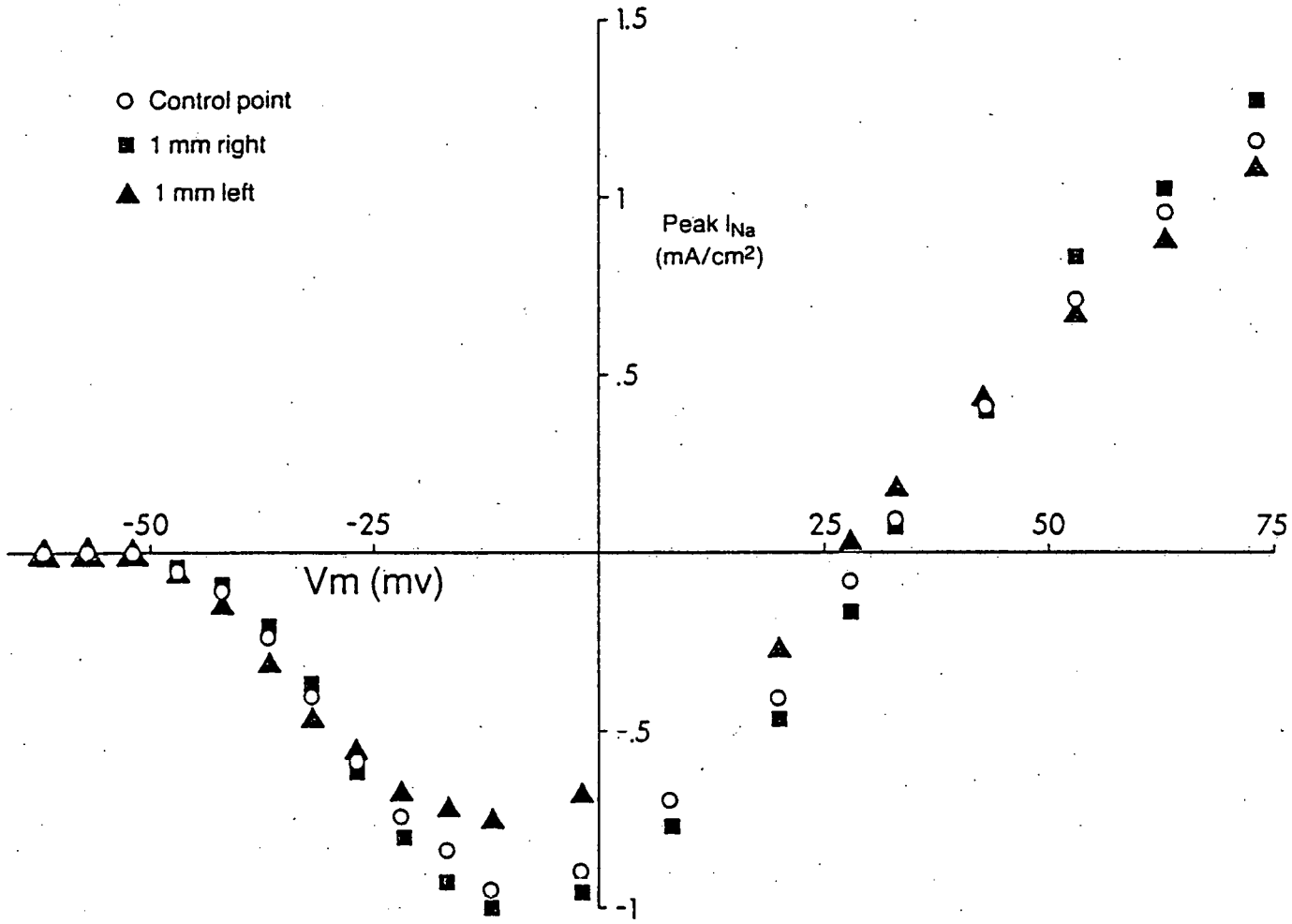
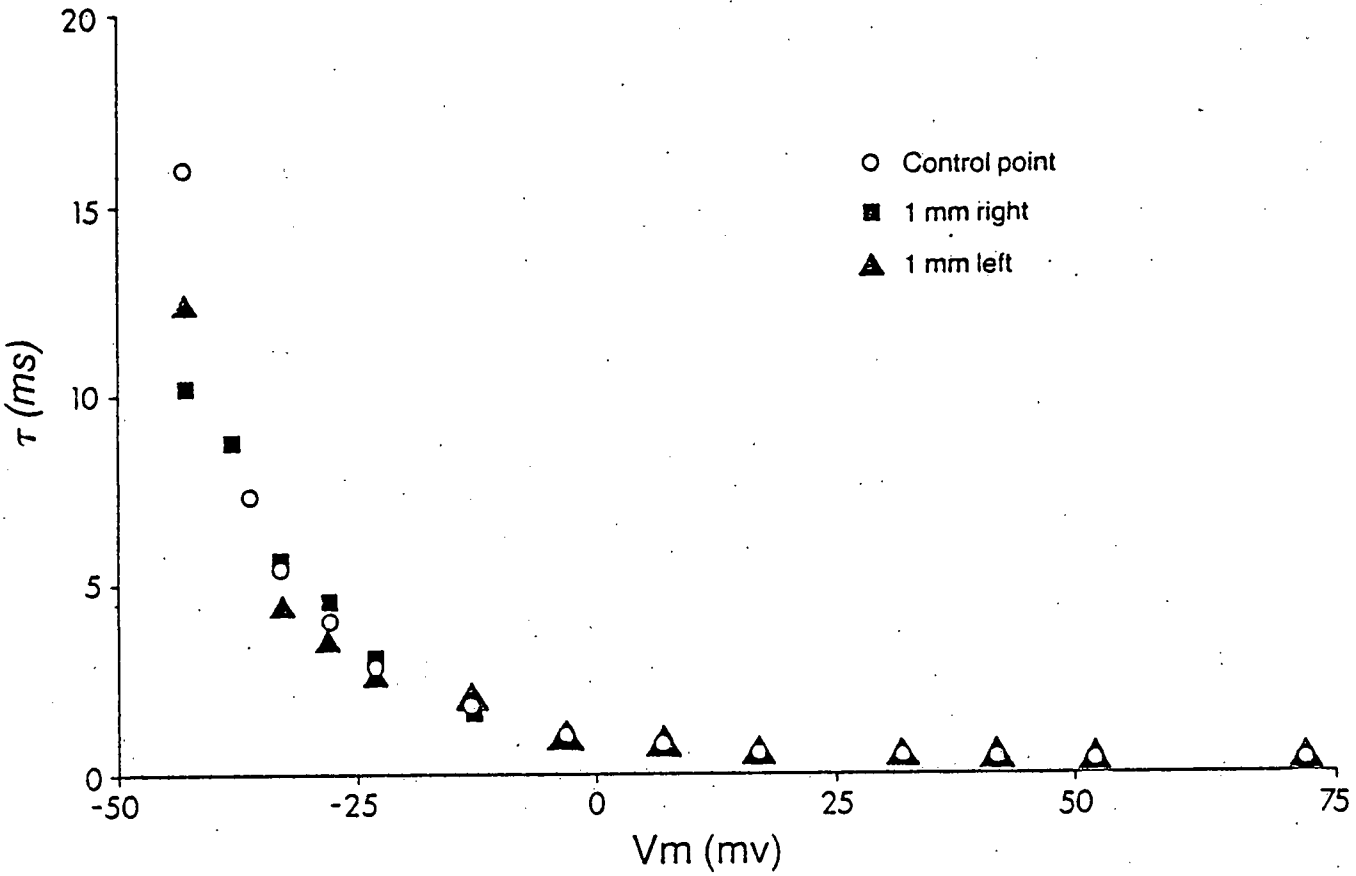

Control point
1 mm right
1 mm left
1.5
1
.5
-.5
-1
Peak I_{Na}
(mA/cm²)
-50
-25
25
50
75
Vm (mv)

Figure 7. Decay time constant vs. membrane potential at three positions along the axon. The nearer wire was 100-200 μ from the axon. 1/10 Na, 2/5 K, 1 mM 4-AP. Holding potential -83 mV. Temperature 4.2-4.4^{o}C.


20
15
10
5
0
τ (ms)
-50
-25
0
25
50
75
Vm (mv)
Control point
1 mm right
1 mm left

sufficiently steep to be sensitive, while the currents were big enough for accurate determinations of τ_h. These were typical axons which were "good" in the sense that they had stable resting potentials in the range -72 to -76 mV in van Harreveld's solution at 3-4°C. A few axons had low or unstable resting potentials (and were not used for experiments); most had been seen to be damaged during entry of the piggy-back electrode. These "bad" axons often had spectacularly non-uniform currents, lending some confidence to the resolving power of the differential electrode.

Knowing approximately the magnitude of variations in potential along the length of the axon, and knowing the resolving power of the electrode, as shown in Figure 2, permits a rough estimation of how much variability in membrane potential there will be in the areas of membrane which contribute significant amounts of current density to the differential electrode when it is in its final position opposite the control point. Assuming that the electric field due to a current source spreads as $1/r^{(3/2)}$, as in the theoretical line in Figure 2, then for the differential electrode with spacing 180μ, with the nearer wire 70μ from the axon, and assuming an axon diameter of 200μ, simple numerical approximations lead to the result that 75 percent of the signal would originate from about a .7 mm length of axon centered at the control point. 90 percent of the signal would originate from about a 1.5 mm length. (This assumes equal membrane current densities along the axon's length.) Therefore, the estimations given in Table I of

TABLE I

SPATIAL UNIFORMITY OF EFFECTIVE MEMBRANE POTENTIALS

Axon	External sodium concentration	Potential variation In 2 mm length (mV)	Potential variation in 4 mm length (mV)
1	Full	3	5
2	Full	4	5
3	1/4	5	7
4	1/4	4	6
5	1/4	3	5
6	1/4	6	
7	1/10	4	

membrane potential uniformity over a 2 mm span translate fairly directly into estimations of the potential uniformity represented by the signal recorded at the control point by the differential electrode. Of course, the largest contributions come from the nearest portions of membrane, which are closest in potential to the ideal command potential pipette.

The most common pattern of deviation from strict uniformity was that seen in Figures 6 and 7: the effective membrane potentials 1 mm right were very similar to those at the control point, but the membrane 1 mm left was effectively more depolarized by 2-4 mV by the same pulse. This pattern of deviation is easily understood; the axial wire of the piggy-back electrode was fastened to the potential pipette by a dab of laquer about 400μ long, located about 1 mm behind the tip of the pipette. The current density delivered by the axial wire was, therefore, not at all homogeneous in this region, and some non-uniformity in membrane potential must be expected. In contrast, direct measurements of current density when current was passed with the axial wire in salt solution showed that the delivered current density was quite uniform to the right of the tip of the pipette, in agreement with the near uniformity of membrane currents in the part of the axon where current was supplied by this part of the wire.

A condition which was not optimal for recording currents from the smallest possible area of membrane was the use of ground plates on only one side of the axon. In several experiments, two plates

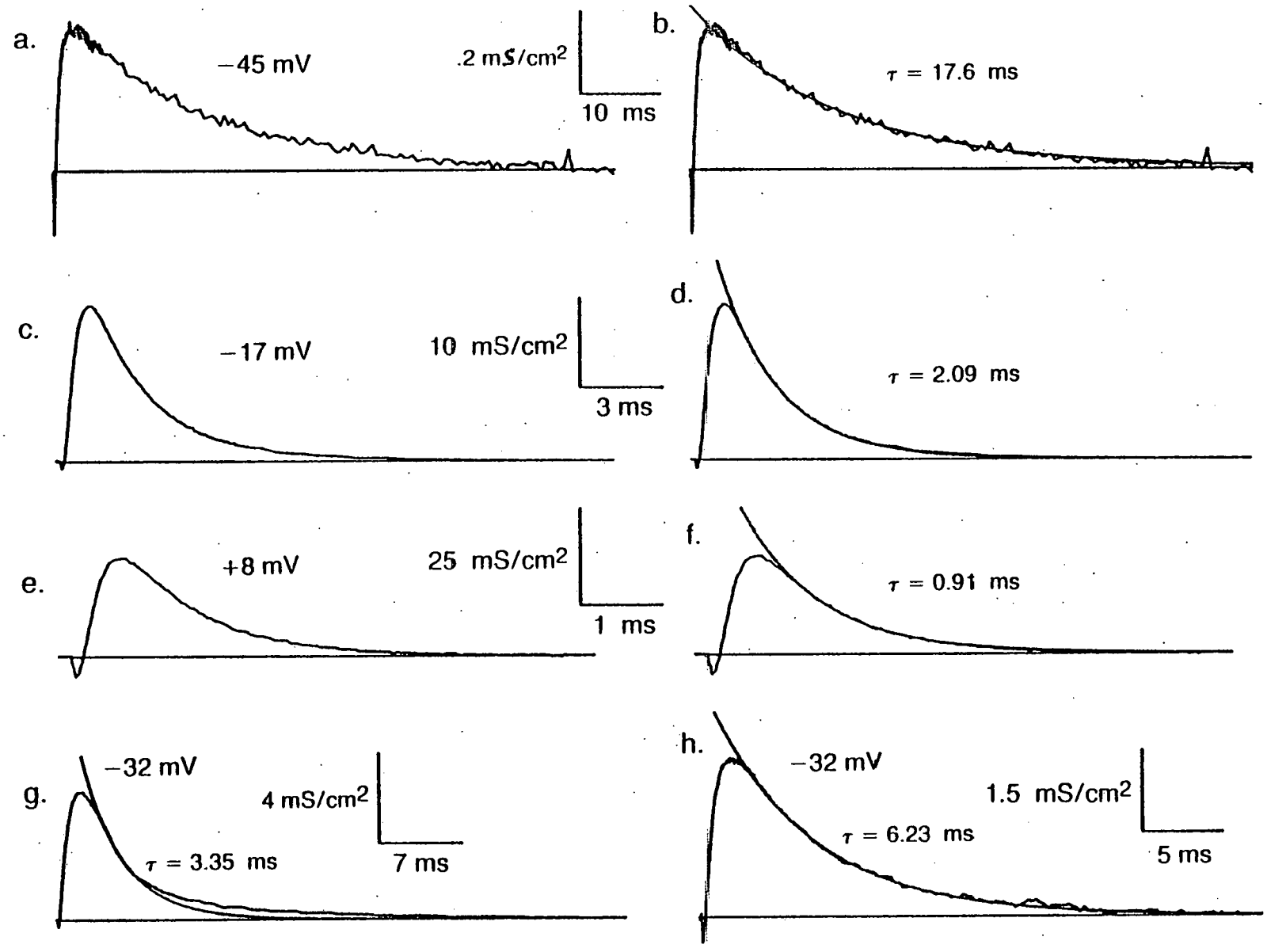
were used, one on either side of the axon. The spatial uniformity, judged both by measurement of currents along the length of the axon and by the biphasic decay of currents in the -30 mV to -35 mV range (see Results), was not significantly better with two plates, and the small increase in the effective resolving power of the electrode with this arrangement, due to less relative contribution by the areas of membrane on the far side of the axon, did not compensate for the smaller total signal and consequent decrease of the signal-to-noise ratio.

Decay of sodium conductance

At most membrane potentials, sodium conductance decays exponentially to zero. In Parts a,c, and e of Figure 8 are shown sodium conductances at three potentials in a single axon. The initial downward deflection is due to gating current; leak and capacitative current were corrected for by adding currents from hyperpolarizations. In Parts b, d and f are shown the same currents with the falling phase fit with an exponential declining to zero. The fit is very good at all three potentials. The exponential has been fit between the times at which conductance had declined to 75 percent and 30 percent of its peak value, in order to emphasize any deviation from a single exponential at longer times.

There were three classes of exceptions to the usual single exponential decay to zero. In most axons, currents between -35 mV

Figure 8. Exponential decay of sodium conductance. Currents have been divided by the driving force (membrane potential - reversal potential) to give the conductances. (a,c,e) Conductances at -45, -17, +8 mV in the same axon. (b,d,f) Same conductances superimposed with exponential fits to falling phases. Fits were made between 70 percent and 30 percent of the peak value. (g) Deviation from single exponential decay at -32 mV; same axon as (a-f). (h) Single exponential decay at -32 mV in a different axon. In all parts, leak and capacitative currents were corrected for by adding currents from hyperpolarizations. (a) and (b) are signal averaged sums of 16 depolarizing and 16 hyperpolarizing sweeps. (c-g) are sums of single sweeps, one depolarizing and one hyperpolarizing. (h) is the signal averaged sum of 4 depolarizing and 4 hyperpolarizing sweeps. (a-g): 1/4 Na, 1/2 K, 1 mM 4-AP; 3.5^{o}C (h): 1/10 Na, 2/5 K, 1 mM 4-AP; 3.3^{o}C


a.
−45 mV
.2 mS/cm2
10 ms
b.
τ = 17.6 ms
c.
−17 mV
10 mS/cm2
3 ms
d.
τ = 2.09 ms
e.
+8 mV
25 mS/cm2
1 ms
f.
τ = 0.91 ms
g.
−32 mV
4 mS/cm2
τ = 3.35 ms
7 ms
h.
−32 mV
1.5 mS/cm2
τ = 6.23 ms
5 ms

and -30 mV exhibited biphasic decay. Figure 8g shows a relatively severe example. However, the degree of deviation from a single exponential varied from axon to axon and in axons with the best spatial uniformity characteristics the deviation was usually quite small. An example is shown in Figure 8h. It seems likely, therefore, that the deviation usually seen is due to spatial variations in membrane currents and is not an intrinsic property of the sodium channels.

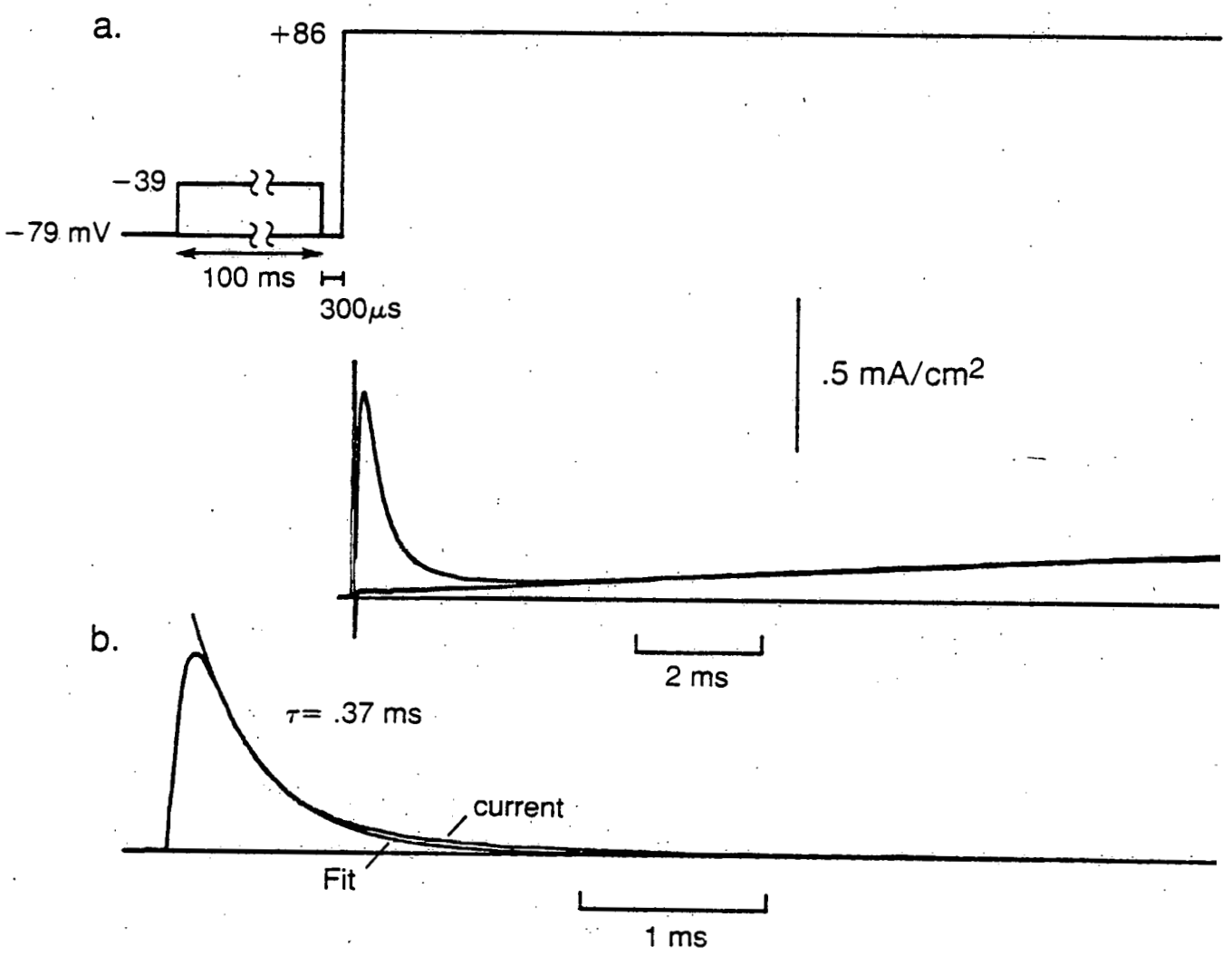
A second region of membrane potential where the fit to a single exponential was generally not excellent is for larger depolarizations, to potentials greater than +40 mV or so. There was usually a small, slower component to the decay of currents at these potentials. A typical example is shown in Figure 9. In Figure 9b, an exponential has been fitted to the current between the times at which it has decayed to 75 percent and 25 percent of its peak value; there is a clear foot in the current record relative to the fit. This foot is not an artifact of the prepulse subtraction technique since it also appeared in currents obtained by TTX subtraction.

The third region where exceptions were sometimes seen is at very small depolarizations, in the region -45 mV to -40 mV. In some axons, the decay appeared to proceed to a non-zero asymptote. The asymptote was always less than 10 percent of the peak value; the decay to the asymptote appeared exponential within the accuracy of the determination.

Figure 9. Prepulse subtraction technique for leak and capacitative current correction.

(a) Pulse protocol and currents. The test pulse to +86 mV was preceded by a 100 msec prepulse to -39 mV in order to inactivate sodium current; the resultant current was subtracted from the test pulse current with no prepulse to yield sodium current.

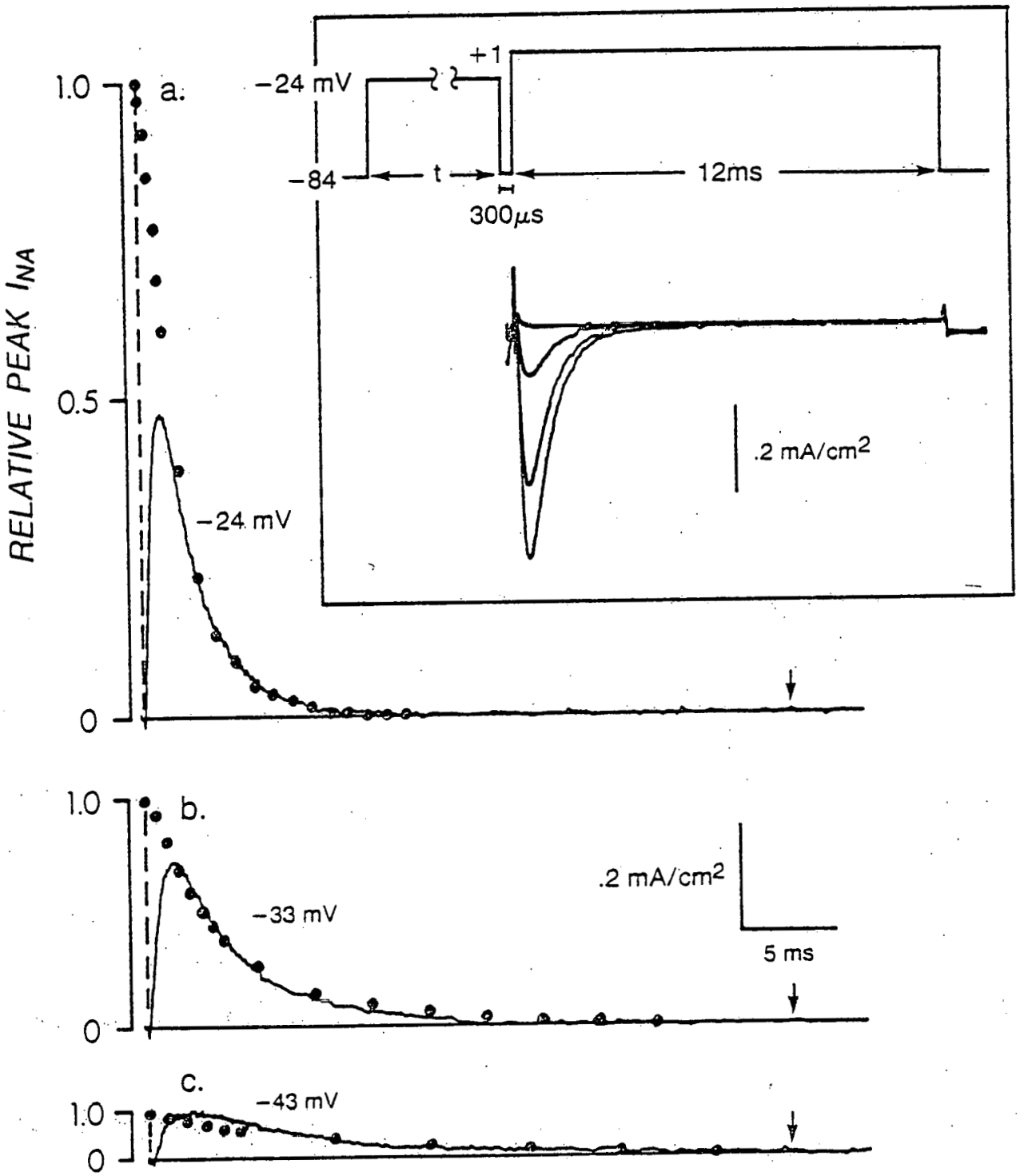
(b) Current obtained in this way superimposed with least-squares exponential fit; fit was between 75 percent and 25 percent of the peak current. Note second component 1/4 Na, 1/2 K, 1 mM 4-AP. 3.9^{o}C


a.
+86
−39
−79 mV
100 ms
300μs
.5 mA/cm2
2 ms
b.
τ= .37 ms
current
Fit
1 ms

Double pulse measurement of inactivation

The double pulse method of Chandler, Hodgkin, and Meves (1965) was also used to follow the development of inactivation at various membrane potentials. The procedure is illustrated in the inset of Figure 10. A prepulse of variable duration is applied to the membrane and the level of inactivation reached during the prepulse is assayed by the size of the current during a test pulse following the prepulse and separated from it by a return to the holding potential for 300 microseconds. The purpose of the return to rest is to allow the non-inactivated fraction of sodium channels to begin, as nearly as possible, from the same initial condition with the prepulse as without it. The duration of 300 microseconds was selected to be short enough to allow almost no recovery from inactivation (there was recovery of only a few tenths of a percent at -82 mV) while long enough that the kinetics of currents after prepulses were only very slightly different from those in the absence of prepulses. In the inset to Figure 10 are shown the test pulse currents with various prepulse durations; with sufficiently long prepulses, in this case a 12 millisecond pulse to -24 mV, there is virtually no sodium current remaining during the test pulse. This was confirmed in experiments employing TTX subtraction. Consequently, correction for leak, capacitative, and any residual potassium current could be made in a simple way by subtracting the test pulse currents following a prepulse sufficient for complete inactivation.

Figure 10 Comparison of double pulse inactivation with sodium current decay. Inset: the pulse pattern used in Part a. Details of the performance and analysis of double pulse experiments are given in METHODS of Chapter One. The currents shown in the inset are for prepulse durations of 0, 1, 3, and 12 msec. The cpacitative transients have been truncated to save space. (a,b,c) Comparison of double pulse inactivation time course with single current decay. The normalized double pulse points have been scaled to match the currents when the decay of the currents is about 50 percent complete. The currents are all inward currents and have been plotted with inward current positive for convenient comparison with the double pulse points. Dashed lines indicate start of the depolarization; arrows indicate termination. In the currents shown in a-c, leak and capacitative currents were corrected for by adding currents for hyperpolarizations. 1/4 Na, 1/2 K, 1 mM 4-AP. Temperatures: 3.2^{o}C for the single pulse currents; 3.5 - 3.6^{o}C for double pulse experiments in (a) and (b); 3.3 - 3.4^{o}C for the double pulse experiment in (c).


1.0
a.
−24 mV
+1
−84
t
12ms
300μs
0.5
RELATIVE PEAK I_{NA}
.2 mA/cm²
−24 mV
0
1.0
b.
.2 mA/cm²
−33 mV
5 ms
0
c.
−43 mV
1.0
0

In Parts a, b, and c of Figure 10 the time course of inactivation determined by the double pulse method is compared to the time course of decay of single pulse current at the prepulse potential. The double pulse points have been superimposed on the current records and scaled so that the two curves match when the single pulse current has decayed to about half of its peak value. At all potentials, the kinetics of inactivation followed by the two methods appear similar over the time at which they may be compared. Of particular interest for kinetic models of sodium channels are the kinetics of inactivation for small depolarizations; it is in this region of membrane potential that Goldman and Schauf (1973) found, in the Myxicola axon, that single pulse currents decayed much faster than the time course of inactivation determined by the prepulse method. Figure 11 shows data like that in Figure 10 but from an axon in which signal averaging was employed to obtain a clearer record of the single pulse current at -42 mV, where peak conductance is small (1/7 of the peak conductance at $^{+}$36 mV) and decays slowly, with a time constant of about 10 milliseconds. The time courses are closely similar.

Pooled data from three axons are given in Figure 12. Both measurements show that the rate of inactivation is sharply dependent on membrane potential in the region from -50 to 15 mV, which is also the region in which peak sodium conductance varies steeply with membrane potential. The small differences in time

Figure 11. Time courses of double-pulse inactivation and sodium current decay at -42 mV. Current is the signal averaged sum of 16 depolarizing and 16 hyperpolarizing pulses. Double pulse points are scaled to match when the current has decayed about halfway. Dashed line indicates start of pulse; arrow indicates end. 1/4 Na, 1/2 K, 1 mM 4-AP. Temperature: 3.5 - 3.6^{o}C.

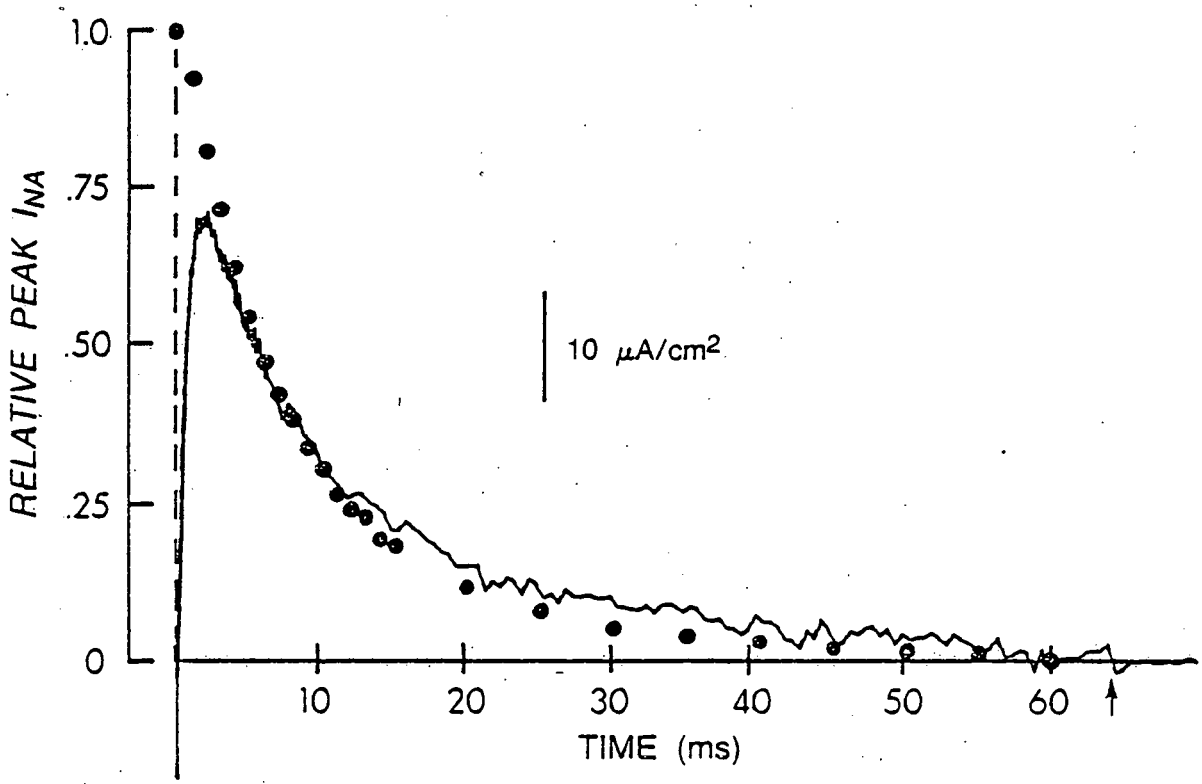
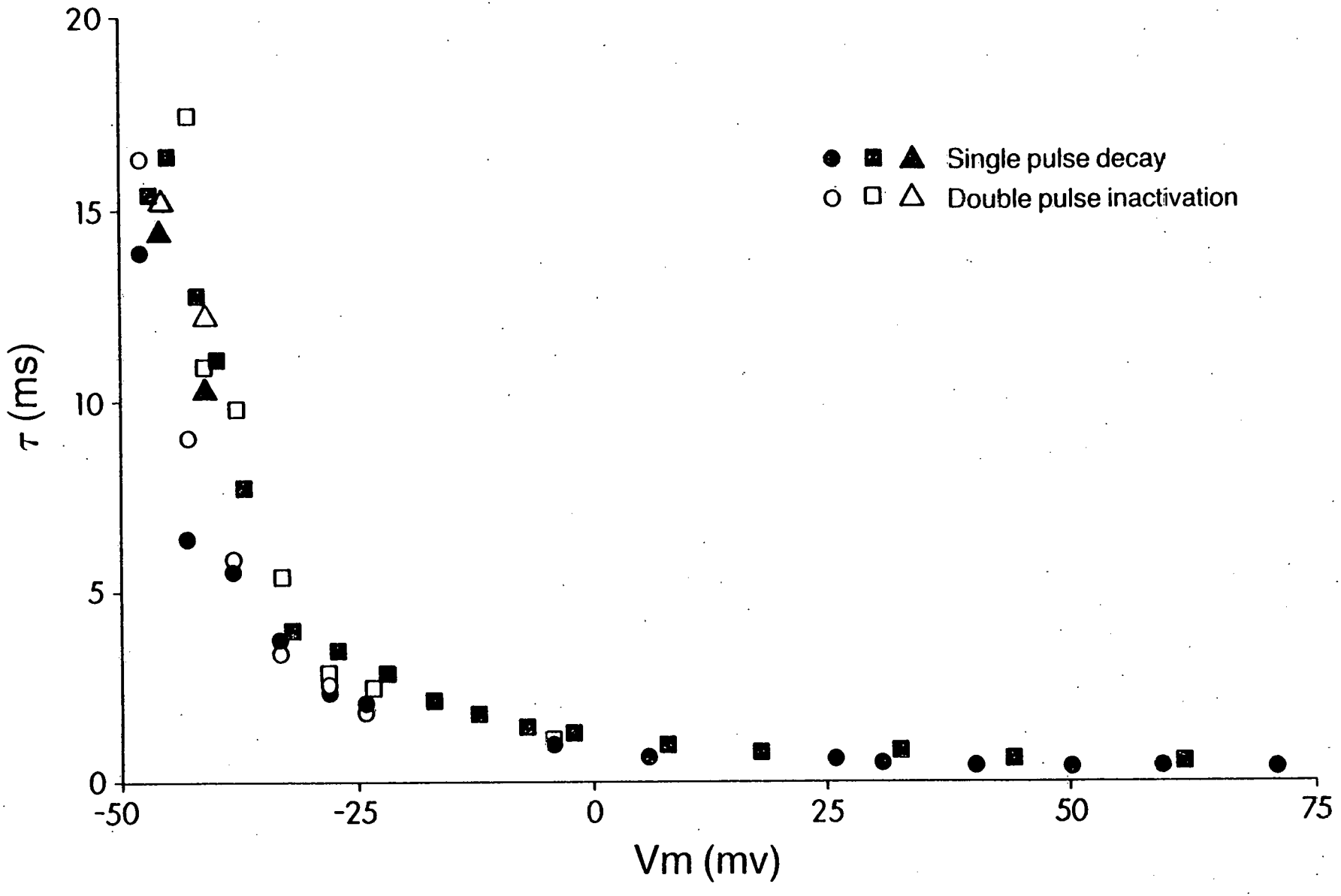

1.0
.75
.50
.25
0
RELATIVE PEAK I_{NA}
10 μA/cm²
10
20
30
40
50
60
TIME (ms)

Figure 12. Collected double pulse inactivation and current decay data for three axons. Time constants were obtained from exponential fits to the decay phases of the currents and to the double pulse inactivation curves after the initial lag. 1/4 Na, 1/2 K, 1 mM 4-AP for all axons. Experiments performed at 3.2 - 3.6^{o}C.


20
15
10
5
0
τ (ms)
-50
-25
0
25
50
75
Vm (mv)
Single pulse decay
Double pulse inactivation

constants for inactivation determined by the two procedures may be accounted for by spatial nonuniformity of membrane potential amounting to a few mV. In general the time course of inactivation determined by the double pulse method was less well described by a single exponential than were the decay phases of the single pulse currents. This may also be understood as the result of spatial nonuniformity.

Lag in double pulse inactivation

The time course of inactivation measured with the double pulse method always showed a "lag", or a delay of .2-2 msec before the rate of development of inactivation reached its maximum. Similar lags in double pulse inactivation are seen in other nerves (Armstrong, 1970; Bezanilla and Armstrong, 1977; Goldman and Schauf, 1972; Peganov, 1973); in these nerves, the time course of the lag appears to be roughly similar to the time for the sodium current during the prepulse to reach its peak. The lag in double pulse inactivation and its relation to the current during the prepulse are shown in Figure 13 for a prepulse to -3 mV. Both the prepulse sodium current and the double pulse inactivation points (crosses in Figure 12a) decay exponentially after about 1.5 msec. The double pulse inactivation points have been scaled to match the sodium current for later times. Also plotted is an exponential decay curve which fits both the double pulse inactivation points and the sodium current for times beyond 1.5 msec; the deviation of

the double pulse points from the exponential curve at earlier times is obvious.

How accurately does the development of double pulse inactivation correlate with the sodium conductance during the prepulse? For the simplest strictly coupled models, in which channels must open before inactivating, the rate of development of inactivation would be proportional to the number of channels able to undergo inactivation, that is, to the number of open channels, and to sodium conductance. Chandler, Hodgkin, and Meves (1965) performed a double pulse experiment designed to test such a mechanism; their data showed no lag and was better fit by an exponential decay than by a curve drawn on the assumption that the rate of inactivation was proportional to sodium current during the prepulse. The result of this same experiment performed in the cray fish axon are shown in Figure 13a; the curve labeled f(t) was calculated assuming that the rate of inactivation is proportional to the sodium current. Although both f(t) and the time course of double pulse inactivation show an initial lag and then exponential decay, they are not identical. The main difference is that the double pulse points show less of a lag at early times; this is shown more clearly in the inset to Figure 13a, where the two curves have been scaled to match at time zero.

Are the differences between f(t) and double pulse inactivation in Figure 13a experimentally significant? There is difficulty in obtaining sodium currents which are precise enough in the first

hundred microseconds or so, where gating current, imperfect capacitative current correction, or imperfect TTX subtraction often introduces some error. Also, since the calculation of f(t) curves depends on taking the integral of the sodium current, small errors in the baseline due to imperfect leak correction can have significant consequences. A method which gives the same information but minimizes the effect such errors have on the comparison is to calculate the rate of inactivation from the double pulse inactivation curve and compare it directly with the time course of sodium current. This is shown in Figure 13b. A smooth curve, the sum of two exponentials and a constant, was fit to match the double pulse inactivation points. Then the derivative of this curve, which gives the rate of development of inactivation, is compared to the sodium current. In this example, and in other cases in different axons, the rate of inactivation is not proportional to sodium current at early times; it rises faster and reaches a peak earlier. It is difficult to know to what degree experimental errors affect such comparisons. One problem is the use of the peak test pulse current as an index of the fraction of non-inactivated channels; it is not clear, without knowing the kinetic mechanism of the channel, exactly how good an index it is under various pulse protocols. It seems quite clear that the general time course of the lag in inactivation is not an artifact due to different relative rates of activation and inactivation during the test pulse with and without prepulses of various

Figure 13. Lag in double pulse inactivation (a) The time course of double pulse inactivation is compared to current decay at the prepulse potential of -2 mV as in Figures 10 and 11. Crosses give the double pulse points. The curve labeled f(t) was calculated as

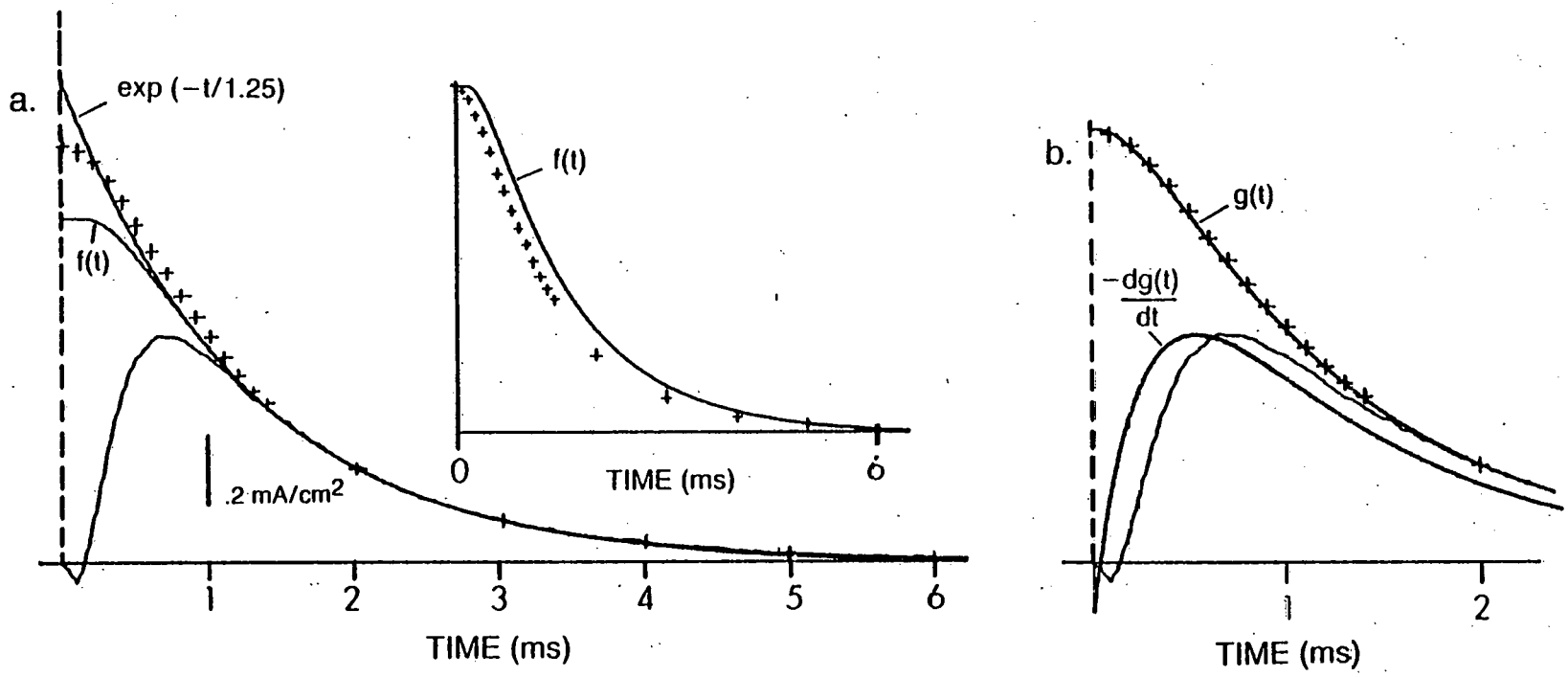
$$f(t) \quad \int_{0}^{t} I_{Na} \cdot dt' \qquad \int_{0}^{t_o} I_{Na} \cdot dt'$$

with t_o = 64 msec.

The curve labeled exp (-t/1.25) is the exponential fit to the falling phase of the current.

Inset: f(t) and double pulse inactivation points scaled to match at time zero.

(b) Same data; g(t) is an empirical fit to the double pulse inactivation points; the negative of its time derivative is plotted scaled to match the peak current height. 1/4 Na, 1/2 K, 1 mM 4-AP. Single pulse current 3.5°C; double pulse points, 3.1 - 3.4°C.


a.
exp (−t/1.25)
f(l)
.2 mA/cm²
1
2
3
4
5
6
TIME (ms)
f(l)
0
TIME (ms)
6
b.
g(t)
−dg(t)/dt
1
2
TIME (ms)

durations; with a 300 µsec return to rest between the prepulse and the test pulse, the time to peak in the test pulse current decreases only very slightly even with large prepulses. Also, the lag is just as evident when a 3 msec return to rest is used instead of 300 µsec, and in this case the time to peak in the test pulse currents does not change at all. A more troublesome problem is the possibility that small non-uniformities in membrane potential might contribute in unknown ways to distort comparisons like those in Figure 13. In axons with poor spatial uniformity the time course of double pulse inactivation often does not correspond very well with the time course of single pulse currents, and it would not be surprising if in axons with good but not perfect uniformity there were large enough errors to affect results like those in Figure 13.

Testing simple coupled models

The simplest model for strictly coupled inactivation kinetics, in which channels must open before inactivating, is

$$\text{Closed} \rightarrow\rightarrow\rightarrow \text{Open} \xrightarrow{K} \text{Inactivated} \quad \text{(Scheme I)}$$

for potentials at which inactivation is complete; for potentials at which inactivation is incomplete, there would have to be a significant rate constant for the Inactivated → Open transition as well. Bezanilla and Armstrong (1977) have shown that models of this type with the rate constant K being voltage independent can account for the main features of inactivation.

Scheme 1 makes predictions about the form of sodium currents which can be tested fairly simply. In this model, the rate of inactivation is given by the fraction of the channels in the open state multiplied by the rate constant K. The results of the previous section suggest that the rate of inactivation is not strictly proportional to the number of open channels and would be inconsistent with Scheme 1. There is another, similar, calculation which appears to rule out the simplest case of scheme 1, with K being voltage independent. This approach is based on the observation that this model predicts that at all membrane potentials where inactivation is complete, the integral of the sodium conductance during a depolarizing pulse will be a constant. The rate of inactivation is proportional to the fraction of open channels:

$$d\,[\text{Inactivated}] / dt = [\text{Open}] \times K$$

Integrating from 0 to ∞, since all the channels become inactivated,

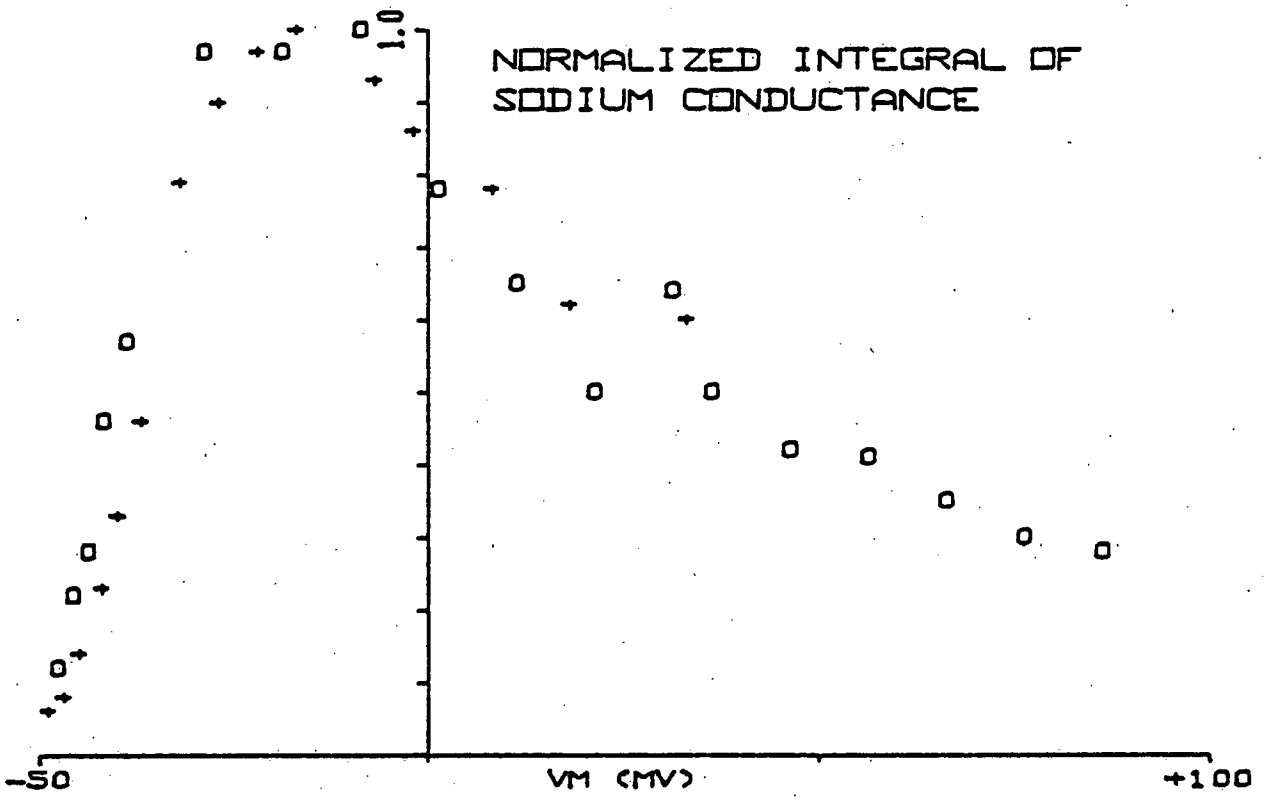
$$1 = \int_0^\infty d[\text{Inactivated}] = \int_0^\infty K \times [\text{Open}]\, dt$$

$$= K \times \int_0^\infty [\text{Open}]\, dt \qquad \text{(Expression 1)}$$

and if K is not voltage dependent, the integral ought to be the same at each membrane potential.

The experimental results are shown in Figure 14. It is clear that the integral of sodium conductance is not independent of membrane potential; the integral is at a maximum in the region -10 to -20 mV, but quickly becomes small at more negative membrane potentials and also becomes small, but not as steeply, at more

Figure 14. Dependence of integrals of sodium conductance on membrane potential. Details of the calculation are given in the text. Data from two axons; the integrals in each axon were normalized with respect to the largest value. Crosses: 1/4 Na, 1/2 K, 1 mM 4-AP, 3.5^{o}C. Leak and capacitative current corrected for by hyperpolarizing pulse currents. Squares: 1/4 Na, 1/2 K, 1 mM-4AP, 3.6^{o}C. Leak and capacitative current corrected for by prepulse inactivation method.


NORMALIZED INTEGRAL OF
SODIUM CONDUCTANCE
1.0
-50
VM (MV)
+100

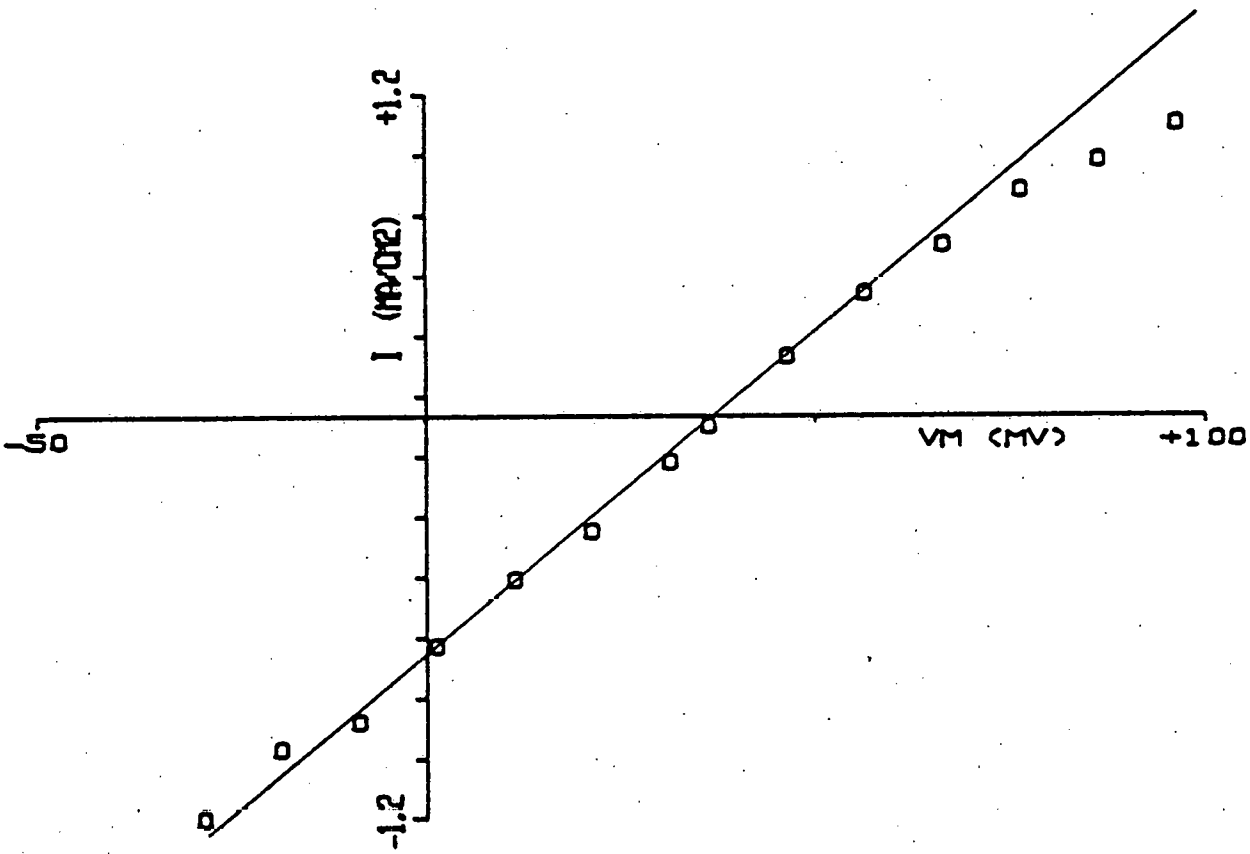
positive potentials. The smaller values at more negative potentials are not simply due to the time constants for decay being so long that the integral cannot be calculated for long enough times; the time constants for decay in the axons shown in Figure 14 were about 10 msec near -40 mV, with the 64 msec pulse duration used in one of the axons the current would have decayed to a fraction of a percent. The fact that inactivation is not quite complete for the points in the -50 mV to -40 mV range (See Figure 16) probably has only a small effect and would be in the direction of making the integrals larger.

The integrals of conductance plotted in Figure 14 were calculated on the basis of an ohmic conductance,

$$G_{Na} = I_{Na} / (V_m - V_{Rev})$$

where V_{Rev} is the reversal potential. Some of the curvature in Figure 14 could be caused by the current-voltage curve for open sodium channels being non-linear. The instantaneous current-voltage curve for the sodium currents in one of the axons in Figure 14 is shown in Figure 15. It is essentially linear from -30 mV to +60. Below -30 mV, it was impossible to accurately measure tail currents for the instantaneous current-voltage curve because of the presence of an extremely fast (10 - 20 μsec) initial decay phase in the tails which makes extrapolating back to time zero impossible; this has also been noted in the Myxicola axon by Goldman and Hahin (1978). For membrane potentials above +60 mV, there is some deviation from linearity in the current-voltage

Figure 15. Instantaneous current-voltage relation for sodium channels. Channels were opened with a .7 msec pulse from -79 mV to +11 mV, followed by a step to a variable membrane potential. The instantaneous current at the new potential was obtained by extrapolating an exponential fit to the first 50-100 µsec back to the transition time. Same axon as represented by squares in Figure 14. 1/4 Na, 1/2 K, 1 mM 4-AP, 2.6^{o}C.


+1.2
I (mA/cm2)
-1.2
-50
VM (MV)
+100

relation but it is not large enough to account alone for the decrease in the integral at these potentials.

The decrease in conductance integrals for both large and small depolarizations relative to -10 to -20 mV means that Figure 14 is inconsistent with any version of Scheme 1 with an inactivation rate constant showing a monotonic dependence on membrane potential.

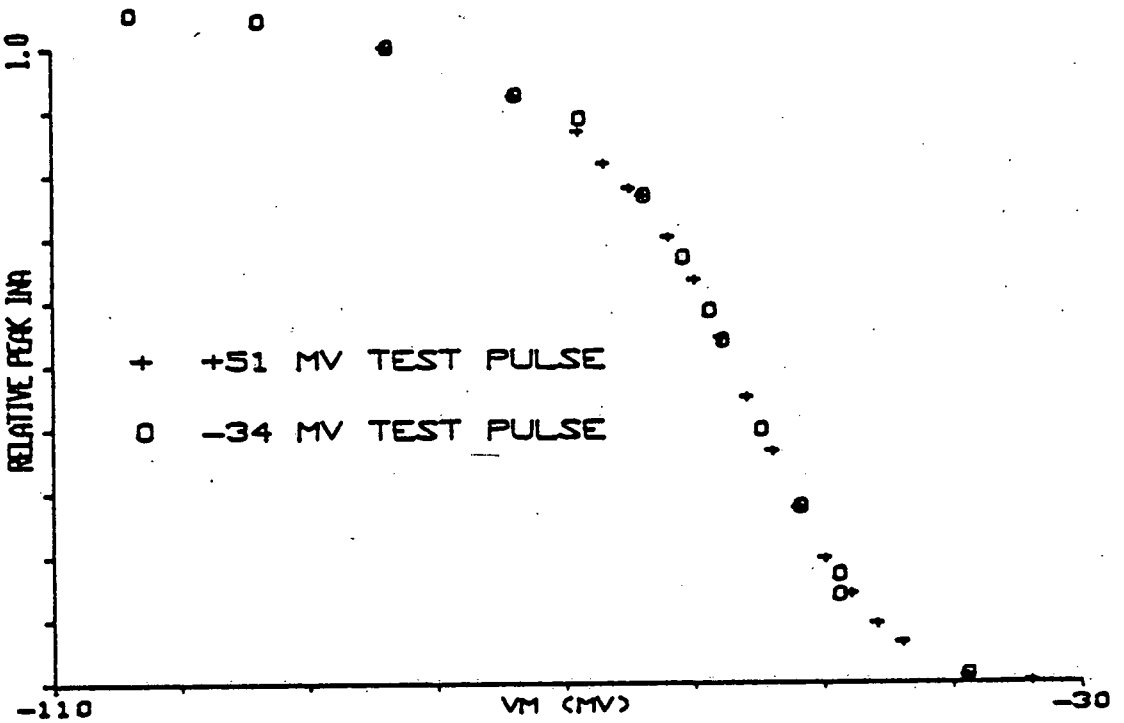
My attempts at finding kinetic models for the sodium channel consistent with the data described in this Chapter will not be detailed here; I found no model which would simultaneously give, in detail, the correct dependences on membrane potential of the peak sodium current activation curve, the decay time constant, and the conductance integral. My efforts concentrated on linear multi-state models in which the probability of inactivation is much greater when the channel is open or is in an intermediate state between the closed and open states; the extremely close correlation in membrane potential between the steep parts of the activation curve and the decay time constant curve make it hard to believe that inactivation is not coupled in some way to activation.

Steady-state inactivation determined with different test pulses

In the Myxicola axon, it is found that the steady-state inactivation curve shifts along the membrane potential axis when test pulses producing different peak levels of conductance are used to assay the inactivation achieved during the prepulse; the significance of such shifts for kinetic models has been discussed by Hoyt (1968) and Goldman (1976).

In Figure 16 are shown the results of an experiment designed to test for this effect in the crayfish axon. Prepulses of 100 msec were followed by test pulses to -34 or +51 mV and the resulting steady-state inactivation curves obtained with the two test pulse potentials are compared. (In the experiment in Figure 16, the prepulse and test pulse were separated by a 300 μsec return to the holding potential so that the capacitative currents would be the same in all test pulses, to allow for accurate subtraction of them). The curves are identical within the accuracy of the data, at all membrane potentials. In this axon, the peak conductance at -34 mV was about 1/5 of that at +51 mV. In the Myxicola axon, the steady-state inactivation curve shifts 5.8 mV for a two-fold increase in peak sodium conductance, so that a shift of a smilar magnitude would have been detected.

Figure 16. Absence of shift in steady-state activation curve with test pulse height. Steady-state inactivation curves were obtained by giving a 100 msec prepulse to a variable potential, returning to rest for 300 µsec, and stepping to the test pulse potential. The peak currents during the test pulses are plotted as a function of the prepulse potential, normalized to the value for a step from -84 mV, the holding potential. (The data points for -94 mV and -104 mV for the -34 mV test pulse show that there was virtually no inactivation at -84 mV; peak current was only 5 percent increased by the 100 msec prepulse to -104 mV and much of this may have been due to removal of slow inactivation. See METHODS, Chapter One.) The curves are identical for test pulses to -34 mV and +51 mV. Peak conductance at -34 mV was .19 of that at +51 mV. 1/4 Na, 1/2 K, 1 mM 4-AP, 3.3 - 3.5^{o}C.


1.0
RELATIVE PEAK INA
+ +51 MV TEST PULSE
O −34 MV TEST PULSE
−110
VM (MV)
−30

DISCUSSION

The main purpose of the work described in this Chapter was to peform in the crayfish axon some of the experiments on sodium channel inactivation necessary to begin to formulate kinetic models for the sodium channel consistent with the macroscopic currents. The results of the experiments are similar in general but different in detail to the results in other nerves; a complete comparison and enumeration of the various similarities and differences nerve by nerve is beyond the scope of this thesis. The basic kinetic properties of inactivations; the sharply voltage-dependent decay time constant and the similarity in time course between current decay and double pulse inactivation, are more like those of the squid axon and frog nerve than the Myxicola axon. The simple exponential decay to zero of sodium current in the crayfish axon makes it an attractive preparation for quantitative experiments on sodium channel kinetics.

The inability of Scheme 1 to fit the conductance integral data may put limitations on how closely the block of channels by internal cations (Armstrong, 1970; Yeh and Armstrong, 1978; Cahalan and Almers, 1979) resembles normal inactivation.

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APPENDIX: PHARMACOLOGY OF GENERAL ANESTHETICS

INTRODUCTION

General anesthetics are gases or volatile liquids which produce unconsciousness when inhaled. Any gas, even an inert gas (Fenn, 1969) can produce general anesthesia if present at sufficient pressure. This fact has led to the idea of a mechanism of general anesthesia which does not depend on the specific chemistry of each gas.

For the purposes of this thesis, barbiturates, opiates, ketamine, and other "intravenous anesthetics" will not be considered to be general anesthetics, although this term is sometimes used to include them. Their actions on the central nervous system appear to differ from those of gaseous and volatile agents (van Harreveld et al., 1951; Price and Dripps, 1970; Brazier, 1972) although the resulting clinical states are similar.

NEUROPHYSIOLOGY OF GENERAL ANESTHESIA

It is not known how the general anesthetics produce unconsciousness. As will become clear, electrical recordings from almost every area of the nervous system show some change during general anesthesia. If there is a specific effect on a particular structure which underlies the loss of consciousness, we do not yet know what it is.

The reticular theory

Until fairly recently, it was widely believed that the loss of consciousness was due to block of the ascending reticular system, with the consequent removal of a tonic excitatory action normally exerted on the cerebral cortex by the reticular system. This view, due mainly to the work of Magoun and his collaborators, was based partly on analogies to states of coma produced by sleep or reticular transection, and partly on the observations that responses in the reticular formation due to peripheral sensory stimulation were greatly reduced during ether or pentobarbital anesthesia (French et al., 1953; Magoun, 1963) and that the "arousal response" seen in the cortex upon stimulation of the reticular formation was blocked during pentobarbital anesthesia (French and King, 1955).

Recent evidence has accumulated which is inconsistent with the reticular theory. Darbinjan, Golovchinsky and Plehotkin (1971), using deep-brain recordings in cats, found that the arousal response during stimulation of the reticular formation was not abolished during ether anesthesia and concluded that the decreases in the reticular formation responses to sensory stimulation were due to depression of the synaptic input to the reticular formation, rather than to depression of the reticular formation itself. Mori and his colleagues found increased activity of reticular neurons during cyclopropane anesthesia (Mori et al., 1972) as did Shimoji and Bickford (1971) during halothane anesthesia. So, although

recordings during barbiturate anesthesia are consistent with the reticular theory (French et al., 1953; French and King, 1955; Darbinjan et al., 1971), those during inhalant general anesthesia are not.

Changes in activity of specific regions

There is a voluminous literature on changes in electroencephalographic recordings during general anesthesia (reviewed by Stockard and Bickford, 1975). Such data is of limited value in determining effects on specific regions of the brain because of the problem of deciding whether changes in the activity of a particular region are "intrinsic" or the result of changes in input from other regions.

In the case of the cerebral cortex, there are several studies showing dramatic decreases in the intrinsic activity of neurons. Darbinjan et al. (1971) showed that callosal potentials, evoked in one part of the cortex in response to stimulation of the contralateral hemisphere, and thought to involve only transmission by cortical neurons, were markedly depressed during ether anesthesia. Richards and his collaborators observed depression in the function of specific neuronal tracts in in vitro brain slices from the olfactory cortex (Richards, 1973 a,b; Richards et al., 1975) and from the dentate gyrus of the hippocampus (Richards and White, 1975) at sub-clinical levels of general anesthetics.

CELLULAR ELECTROPHYSIOLOGY OF GENERAL ANESTHESIA

A large number of effects of general anesthetics at the cellular level have been observed in *in vitro* studies: depression of cilial beating (Nunn, 1974), of mitosis (Nunn, 1974), of actomyosin ATPase activity (Merin, 1975), of bacterial luminescence (White *et al.*, 1975), of cell aggregation (Ungar, 1975), of phospholipid metabolism (J. Miller, 1975), of mitochondrial respiration (Rosenberg and Naugaard, 1973). These actions were all seen at clinical concentrations of anesthetics. The following review will deal only with actions of anesthetics on nerve conduction and synaptic transmission, both because it is clearest how these effects may alter the function of the nervous system and because of the subject of this thesis, but the diversity of cellular actions of general anesthetics should be borne in mind.

Anesthetic concentrations and in vitro experiments

It is important to compare the concentration of anesthetics producing *in vitro* effects to the concentrations present during general anesthesia of an animal. Anesthetic concentrations during surgical anesthesia can be measured as blood concentrations or as alveolar concentrations. Eger and his associates (Eger, 1964; Eger *et al.*, 1965; Regan and Eger, 1967) developed the concept of the "minimum alveolar concentration": the alveolar concentration at which 50 percent of the subjects do not move in response to a skin incision. (de Jong and Eger (1975) have pointed out that,

practically speaking, this is below a minimum for a surgeon, since at this concentration 50 percent of his patients would be leaping up from the operating table). Concentrations present in surgical anesthesia range up to two to three times the minimum alveolar concentration, depending on the anesthetic and the level of anesthesia. I will refer to this range as "surgical" or "clinical" concentrations of anesthetic. Respiratory arrest occurs at from two to six times the minimum alveolar concentration, again depending on the anesthetic (Regan and Eger, 1967). I will refer to these as "apneic" concentrations.

Most research (including the experiments of this thesis) has been peformed with ether and with halothane, the most venerable and the most popular (respectively) surgical general anesthetics.

The minimum alveolar concentration for ether anesthesia of dogs is 3.29 percent (V/V) and the apenic alveolar concentration is 9.3 percent (Regan and Eger, 1967) which correspond (assuming equilibrium) to concentrations in saline solution of about 15 mM and 44 mM, respectively, using an air:saline partition coefficient of 12 (Steward et al., 1973; Renzi and Waud, 1977). Blood concentrations of ether during anesthesia of dogs range from about 15 to 24 mM (Ronzoni, 1923; Robbins, 1945; Haggard, 1924), corresponding to saline concentrations of about the same, since the blood:water partition coefficient is about 1 (Steward et al., 1973). Blood concentrations of ether at respiratory arrest are 21 to 26 mM (Ronzoni, 1923). Aqueous concentrations of ether needed

for narcosis of poikliotherms at 37° are similar to those needed for anesthesia of mammals at the same temperature: 25 mM for newts (Miller et al., 1973) and goldfish (Cherkin and Catchpool, 1964). Concentrations needed for narcosis of goldfish which have been adapted to lower temperatures are higher: 34 mM at 10° and 35 mM at 5° (Cherkin and Catchpool, 1964). The requirement for higher concentrations at lower temperatures is consistent with a membrane site of action since the oil:water partition coefficient decreases with temperature.

The minimum alveolar concentration for halothane anesthesia of dogs is .87 percent (v/v) and the apneic concentration is 2.5 percent (Regan and Eger, 1967); assuming equilibrium, these values correspond to .28 mM and .79 mM in saline solution at 37°, using a partition coefficient of .76 for the air:saline partition (Steward et al., 1973)

Synapses

It is widely believed that the production of general anesthesia is due, at the cellular level, to the block of synaptic transmission. As will be seen in the following review, there is ample evidence that synaptic transmission in the spinal cord, in synaptic ganglia, and in the brain itself can be significantly depressed by clinical levels of general anesthetics. However, in most of these cases, there is little understanding of the mechanism by which synaptic transmission is depressed.

Sympathetic ganglia

One of the themes running through cellular electrophysiological studies of general anesthetics is the observation that at clinical levels of anesthetics, synaptic transmission can be depressed with little effect on the ability of nerve fibers to conduct action potentials. The classical work demonstrating this was that of Larrabee and Posternak (1952), who investigated the effect of several general anesthetics both on direct nerve conduction and on conduction through perfused sympathetic ganglia from cats, rats, and rabbits. They found that for a given concentration of anesthetic, the reduction in the height of the (externally recorded) action potential was greater in post-synaptic fibers than in direct-conduction fibers. Clinical concentrations of ether and chloroform decreased the height of post-synaptic action potentials by about 20 percent; significant depression of direct action-potential conduction required anesthetic concentrations two or three times larger than clinical levels. Larrabee and Posternak emphasized that their results did not necessarily imply a selective effect on synaptic processes (now known to be pre-synaptic release of transmitter and post-synaptic membrane conductance changes in response to transmitter); since the safety factor is much higher in axonal conduction than in synaptic transmission, selective block of trans-synaptic excitation could be explained "without assuming an action on synaptic structures which differs in any way from the action on other portions of the neuron".

Sympathetic ganglia synaptic transmission and pressure reversal of general anesthesia

Johnson and Flagler (1950) showed that ethanol narcosis of tadpoles could be reversed by high pressures (100-200 atmospheres). This reversal has since been found also for a variety of gaseous and volatile anesthetics (Halsey and Wardley-Smith, 1975). The observation of pressure reversal has led to the expectation that if there is a single "fundamental" action responsible for general anesthesia, it ought to be reversible by pressure.

Kendig and her collaborators (1975) examined the pressure sensitivity of the block of synaptic transmission in rat sympathetic ganglia by halothane and methoxyflurane. At atmospheric pressure, reduction of the post-synaptic action potential was greater than the reduction of the pre-synaptic (direct conduction) action potential; a surgical concentration of halothane (.25 mM) depressed the post-synaptic action potential by about 20 percent. (This is in good agreement with Larrabee and Posternak's (1953) work with the cat ganglia where a surgical concentration of ether (20 mM) depressed the post-synaptic action potential by 20 percent). Kendig and her collaborators found that depression of transmission in both "slow" (muscarinic) and "fast" (nicotinic) acetylcholine-mediated synapses was not antagonized by pressure: pressure itself depressed transmission and augmented the depression produced by halothane or methoxyflurane. In contrast,

pressure did (slightly) antagonize the depression of pre-synaptic action potentials by higher concentrations of anesthetic (Kendig et al., 1975; Kendig and Cohen, 1977).

Spinal cord

Austin and Pask (1952) recorded extracellular potentials in the spinal cords of cats exposed to various concentrations of ether and found that monosynaptic ventral root potentials in response to stimulation of the sciatic peripheral nerve were markedly depressed by concentrations of ether (measured in the blood) corresponding to surgical anesthetic levels. The dorsal root potential, on the other hand, reflecting only the conduction of action potentials in "type A" fibers, was unaffected up to concentrations of ether which produce respiratory arrest.

Somjen and Gill (1963) and Somjen (1967) used the techniques developed by Eccles and his collaborators to record intracellularly from spinal cord motoneurons in animals which inhaled ether. Excitatory post-synaptic potentials elicited by stimulation of sensory nerves were decreased in amplitude during ether inhalation.

Somjen and Gill also tested the excitability of motoneurons by injecting current with an intracellular electrode, to see if, in addition to the depression of synaptic potentials, the generation of action potentials in response to a given excitation might be affected by ether. They found that although the threshold potential for firing was not regularly affected, the ability to respond to a sustained depolarization with repetitive firing was

depressed, and the degree of accommodation to a gradually increasing current was enhanced. This was true in some but not all neurons; when present, these effects were reversible and reproducible. In these experiments no estimate was made of the ether concentrations.

Richens (1969 a,b) used both extracellular and intracellular recording to investigate the depression by volatile anesthetics of synaptic transmission in the isolated frog spinal cord. Synaptic transmission was completely blocked by concentrations of anesthetics which did not impair conduction of action potentials in pre-synaptic fibers. In attempting to localize the site of action of the anesthetics in blocking synaptic transmission, Richens found that post-synaptic potentials were smaller, that the threshold for firing of the motoneurons was higher, and that intracellularly-recorded motoneuron action potentials were smaller in the pressure of chloroform.

Synapses in the brain

Recently, Richards and his collaborators have demonstrated that general anesthetics can depress excitatory synaptic transmission in the brain itself (Richards, 1973 a,b,c; Richards et al., 1975; Richards and White, 1975). They recorded potentials from the surface of thin in vitro guinea pig brain slices while stimulating tracts containing a large number of parallel axons which synapse

onto a second population of neurons. The evoked field potentials recorded in this way have various components which reflect the action potentials in the stimulated tract, the synaptic potentials in the post-synaptic cells, and also the action potentials arising in the post-synaptic neurons (Richards and Sercombe, 1970; Richards and White, 1975). Because the neuron populations in these tracts are very homogeneous, the signals are quite sharp and stable.

Richards (1973a) and Richards, Russell and Smaje (1975), using brain slices from the olfactory cortex, found that halothane, ether, and methoxyflurane, at concentrations below the minimum alveolar concentrations, depressed the amplitude of the post-synaptic potential. The pre-synaptic action potentials were depressed only at higher concentrations (but these were still within the range of surgical concentrations). Richards and White (1975) made similar observations on slices from the dentate gyrus in the hippocampus.

Richards and his collaborators have attempted to identify the point or points at which synaptic transmission is depressed. It is very difficult to eliminate any possibility. For example, it is clear that at the lowest concentrations which affect transmission, the action potentials in the bulk of the pre-synaptic nerve tract are not failing, but it is impossible to eliminate the possibility that conduction is failing in the very fine pre-synaptic arborizations of these nerves; the small currents from these probably make almost no contribution to the recorded field potentials.

The threshold for firing of the post-synaptic cells could be estimated (in a necessarily crude way) from the size of the synaptic potential necessary to excite a given amplitude of the post-synaptic action potential signal. The threshold did not seem to change with the general anesthetics present, in agreement with the findings of Somjen and Gill (1963) on motoneurons. However, as with the motoneuron experiments, there were suggestions that the generation of action potentials was altered in more subtle ways. Trichloroethylene produced a tendency for repetitive firing not normally seen (Richards, 1973c). Ether simply depressed the firing of some cells but induced latent and repeitive firing in others (Richards et al., 1975). Halothane and methoxyflurane depressed the activity of all cells observed (Richards, 1973a; Richards et al., 1975).

Some of the action of some of the anesthetics could be shown to be post-synaptic. Glutamate applied iontophoretically to the post-synaptic cells produced action potential activity in these cells. Ether, methoxyflurane, and trichloroethylene diminished this activity, suggesting a post-synaptic effect of these anesthetics (Richards, 1973c; Richards et al., 1975; Richards and Smaje, 1976). However, halothane had no effect even at levels which profoundly reduce stimulation-evoked activity (Richards, 1973b; Richards and Smaje, 1976). Smaje (1976) tested the ability of general anesthetics to alter action potential activity produced by iontophoresis of acetylcholine onto these cells (the action of

acetylcholine is of the "slow" type in these neurons). All the gaseous and volatile general anesthetics tried, ether, methoxyflurane, trichloroethylene, and halothane, caused a dose-dependent increase in the firing rate produced by acetylcholine.

Nicoll (1972) also studied synaptic transmission in the mammalian cortex, in this case in the in vivo olfactory bulb of rabbits. Mitral axons (in the lateral olfactory tract) were stimulated; the mitral somas were thus excited antidromically, and the excitatory synapses of the mitral dendrites (which have reciprocal synapses with the dendrites of granule cells (Eccles, 1973)) were thus activated. These events were followed by recording extracellular field potentials. In contrast to the findings of Richards and his collaborators with olfactory cortex synapses, Nicoll found that excitatory synaptic potentials were depressed in these olfactory bulb synapses only at high (approximately apneic) concentrations. Furthermore, depression of the nerve conduction and of synaptic transmission took place at about the some concentrations.

The granule cells onto which the mitral cells synapse in turn have synapses back onto the mitral cell dendrites. These are inhibitory synapses (Eccles, 1973). By using paired stimuli, Nicoll found that the inhibition of mitral cells by the granule cells was markedly prolonged by moderate anesthetic concentrations. There was no way from these results to distinguish between various possible mechanisms: blockage of re-uptake of the transmitter, facilitation of release of the transmitter, and so on).

The most striking facet of the observations made on the actions of general anesthetics on synapses is the complexity and diversity of the effects seen: not only do effects on inhibitory synapses differ from those on excitatory synapses (Nicoll, 1972) but, even within the same neurons, excitation produced by one transmitter is depressed while that produced by another is not (Richards and Smaje, 1976; Smaje, 1976) and even when considering the actions on the excitation produced by a single transmitter on a single type of neuron, halothane is strikingly different in its effects from the other anesthetics (Richards and Smaje, 1976). It is clear that a simple statement of the sort "general anesthesia is produced by block of excitatory synaptic transmission" will not suffice to explain the action of general anesthetics: which synapses in which neurons in which part of the brain must be determined.

Neuromuscular junctions

Action of general anesthetics at the neuromuscular junction has been of interest for two reasons. First, ever since Auer and Meltzer (1914) suggested a "curare-like" action of ether, depression of neuromuscular transmission has been postulated to contribute to the loss of muscle tone seen in deep ether anesthesia. Second, the neuromuscular synapse may provide a model for actions of anesthetics at central synapses. From the point of view of this thesis, the muscle end-plate provides a case where the

actions of general anesthetics in modifying the operation of a voltage-sensitive ionic channel have been described.

Auer and Meltzer (1914) found that in deeply etherized dogs indirect stimulation of muscles via nerves was depressed while directly stimulated muscle contraction was normal. Secher (1951) tried to determine whether this depression was due to nerve failure by recording external nerve action potentials in the rat phrenic nerve-diaphragm muscle preparations; he found that nerve conduction failed at about the same time as muscle contraction. Ether concentrations were not reported.

Karis, Gissen and Nastuk (1966, 1967) found that clinical concentrations of ether completely blocked tension development in the frog sciatic nerve-sartorius muscle system. (Axonal conduction was found to be unaffected by these concentrations "in general agreement with the earlier work of Secher", but Secher found parallel depression except in experiments where an unspecified amount of curare was also present). In these experiments clinical concentrations of ether and halothane diminished the amplitude of end-plate potentials and of miniature end-plate potentials (m.e.p.p.s). The threshold for firing of the muscle membrane was found to be increased.

In attempting to define the site of general anesthetic action on the neuromuscular junction, Galindo (1971) found that clinical concentrations of halothane decreased both the amplitude and the frequency of m.e.p.p.s, suggesting actions both pre- and

post-synaptically. Kennedy and Galindo (1975) found that clinical concentrations of enflurane decreased the amplitude of m.e.p.p.s but not their frequency; the threshold of the muscle membrane was increased, as for ether and halothane.

Waud and Waud (1975 a,b) found that clinical levels of a number of general anesthetics reduced the depolarization produced in the frog end-plate by the acetylcholine analog carbamylcholine. Minimum alveolar concentrations reduced depolarization in response to a given dose of carbamylcholine by about 25 percent.

Recently, Gage and Hamill (1976 a,b) examined miniature end-plate currents (m.e.p.c.s) and the resulting m.e.p.p.s in rat diaphragm muscle in the presence of ether, halothane, chloroform, and enflurane and Gage, Hamill, and Van Helden (1979) examined the effect of ether on m.e.p.c.s and on the current noise induced by acetylcholine in toad sartorius muscles. Subclinical levels of anesthetics increased the rate of decay of m.e.p.c.s with no (rat muscle) or only slight (toad muscle) decreases in m.e.p.c. amplitudes; this results in diminished m.e.p.p. amplitudes. Concentration corresponding to minimum alveolar concentrations caused about 30 percent decreases in the time constant for decay of m.e.p.c.s. Large concentrations of anesthetics (for example, more than 40 mM ether) produced the opposite effect: larger time constants than the control. Gage et al. (1979) used the theory developed by Anderson and Stevens (1973) to calculate the mean channel lifetime from the current noise produced by applied

acetylcholine; the mean channel lifetime decreased progressively with concentrations of ether from 5 to 70 mM. Gage et al. (1979) interpreted their results to suggest that ether in some way reduces the stability of open end-plate channels (the increase in the m.e.p.c. decay time constant with high concentrations of ether appeared to be due to inhibition of acetylcholinesterase and consequent delayed removal of acetylcholine from the synaptic cleft), while pointing out that the alternative hypothesis that ether blocks open end-plate channels could not be ruled out.

Nerve conduction

Many experiments have shown that nerve conduction can be reversibly blocked by general anesthetics. However, since the concentration of anesthetic required for significant block in all nerves so far examined are higher than those needed for induction of general anesthesia, most workers discount nerve conduction block as the basis of general anesthesia.

Following are summaries of work done with the most studied of the general anesthetics, ether and halothane. Unless otherwise noted, action potentials were recorded extracellularly from multi-axon nerves. Where gas concentrations have been converted into saline concentrations, I have used partition coefficients given by Steward et al. (1973), Regan and Eger (1967), and Mastrangelo et al. (1975), with the temperature dependence of the partition coefficients taken into account.

Ether	Minimum alveolar concentration	15 mM
	Apneic concentration	44 mM

Lorente de No (1947) found reversible block of myelinated frog fibers by ether. Ether concentrations were not known.

Larrabee and Posternak (1952) found that 48 mM ether reduced the height of the action potential of type A (myelinated) fibers in the cat cervical ganglion by 40 percent. Type C (unmyelinated) fibers were blocked by only 10 percent at this concentration. At 77 mM, type A fibers were blocked by 90 percent and type C fibers by 40 percent.

Roth (1975) found that 5.6 percent (v/v) ether in the gas phase (54 mM in saline at 25°) blocked frog sciatic nerve action potentials by 30 percent.

Schwartz (1968) found that 90 mM ether was required to block conduction in a single squid giant axon.

Richards, Russell, and Smaje (1975) found that 4.5 percent (v/v) ether (21 mM in saline solution) blocked the action potential from lateral olfactory tract fibers from guinea pigs by 10 percent. Smaller concentrations had no significant effect.

Richards and White (1975) found that 4.5 percent ether blocked conduction in perforant tract neurons by more than 40 percent (equilibrium was not reached).

Halothane	Minimum alveolar concentration	.28 mM
	Apneic concentration	.79 mM

Ellis (1969) found that 4 percent (v/v) halothane (1.3 mM in saline) blocked rat sciatic nerve fibers by 15 percent. 2.5 mM produced 40 percent block. Conduction velocity was slowed 10 percent by 2.5 mM.

Roth (1975) found that 3.6 percent (c/c) halothane (2.0 mM in saline solution at 25^{o}) blocked frog sciatic nerve action potentials by 50 percent; 3 mM blocked by 70 percent.

Diamond, Havdala, and Sabelli (1975) reported that 18 mM halothane blocked the action potential in frog sciatic nerve by 25 percent. (The discrepancy between the result and Roth's is probably due the fact that Diamond et al. did not use desheathed nerves).

Kendig and Cohen (1975) found that .5 mM halothane blocked impulses in the rat cervical ganglian by 15 percent. 1 mM blocked by 25 percent.

Richards (1973a) found a 10 percent decrease in the signal from the lateral olfactory tract fibers of the guinea pigs with 3 percent (v/v) halothane (corresponding to .97 mM in saline solution).

While it is true that relatively large concentrations of anesthetics are required to block conduction significantly in most nerves so far examined, it is clear that there is considerable

variation in the sensitivity of various types of nerves. The data are too scanty to make definite statements about variation of sensitivity with, say, fiber diameter, but it is interesting that the most sensitive neurons are the small (.5 - 1 micron) central neurons. The perforant tract neurons are at least 40 percent blocked by a high clinical level of ether.

A number of people (Wall, 1967; Seeman, 1972; Richards, 1973a; Richards and White, 1975) have suggested that block of conduction in the very fine pre-synaptic arborizations, as small as .1 micron in diameter (Peters et al., 1970), could be important in general anesthesia. (Not all of those who have suggested this think it is likely). This thinking is based on numerous observations of greater sensitivity of smaller fibers to a variety of blocking agents (Gasser and Erlanger, 1929; Uehara, 1960; Staiman and Seeman, 1974). However, these studies have been done with myelinated fibers, while the central arborizations are unmyelinated; also, such studies seem not to have been done with volatile or gaseous anesthetics.

A second point from this nerve block data is that there is a significant level of conduction block of a number of nerve types at apneic concentrations of anesthetic. Thus, while it is probably not important in the production of general anesthesia, nerve block could well be involved in respiratory arrest.

Mechanism of nerve block

There are only a few studies with information about how nerve conduction is blocked by general anesthetics. Most of the available work suggests that the primary cause of block is a depolarization of the nerve fiber. Alcock (1906) found that 50 percent ether (v/v) caused depolarization of frog sciatic nerves, as measured by injury currents through a cut end. Wright (1946), working with cat peroneal and rat tibial fibers, found that dilute ether vapors both reversibly depolarized the nerves and blocked their action potentials, with similar time courses. Lorente de No (1947) made the same observation with frog sciatic nerves, and in addition noted that hyperpolarization restored the excitability of an ether-treated nerve. Ether concentrations were not known.

Depolarization probably causes loss of excitability by inactivation of sodium channels; the fact that the inactivation vs. membrane potential curve is shifted in the hyperpolarizing direction by ether, as reported here in Chapter 1 for the cray fish axon and by Kendig et al. (1979) for the frog node, means that depolarizing the nerve will inactivate an even greater fraction of the sodium channels than in the control case. The loss of available sodium channels through inactivation is consistent with Lorente de No's observation of restoration of action potentials by hyperpolarization; this observation shows clearly that sodium channels can be made available with a sufficiently negative membrane potential. This is also in agreement with the observation

in Chapter 2 that 200 mM ether decreases peak sodium currents by only about 50 percent with holding potentials of -80 to 100 mV. However, in the crayfish axon even large concentrations of ether and halothane did not depolarize the resting membrane, and it is likely that at low temperatures the axon would still be excitable with as much as 200 mM ether. Most of the studies showing depolarization in the other nerves were done at room temperature or above with large concentrations of ether; I did not do experiments with these conditions.

Changes in accommodation and repetitive activity

It is interesting to note that in a number of systems, subtle changes in nerve firing are seen with concentrations of anesthetics too low to block excitation. It is interesting to speculate that at least some of these may be related to the changes in sodium and potassium current kinetics described in Chapters 2 and 3.

Perhaps the most striking observation is one made by Heinbecker and Bartley (1940). They found, in frog sciatic nerves, that 42 mM ether, which depressed the size of single action potentials but did not eliminate them, produced a dramatic increase in the rate of accommodation to a subthreshold depolarization. The rate of accommodation depends on the rate of sodium channel inactivation and the rate of potassium channel activation (Hodgkin and Huxley, 1952); both these rates are increased by ether in the crayfish axon and it is very tempting to relate those effects to the one observed by Heinbecker and Bartley.

Somjen and Gill (1963) also observed cases in which accommodation was enhanced by ether, in cat spinal motoneurons. They found also that the ability to respond to a long depolarization with sustained firing was depressed by ether. Both effects could be explainable by changes in sodium channel inactivation kinetics, and potassium channel activation kinetics, like those seen in the crayfish axon. However, the motoneuron has a much more complex set of voltage-dependent channels and the factors governing accommodation and repetitive firing are likely to be very complicated. The same caution applies to any attempt to interpret the changes in firing patterns seen in the guinea pig olfactory cortex (Richards, 1973c; Richards et al., 1975). Another finding of this type is the depression of post-tetanic repetitive firing seen by Van Poznak (1963) in cat soleus motor axons with clinical concentrations of ether. In any case, it is clear that anesthetics may have significant effects on action potential generation even at concentrations too small to block excitability; even if such effects are not of fundamental importance in the physiology of general anesthesia, it is possible they play a role in more subtle ways, such as determining differences in the detailed effects of the different anesthetics.

Molecular theories of general anesthesia

A cornerstone of most molecular theories of anesthesia is the idea that the anesthetic properties of gases somehow result from their dissolving into cell membranes, a hypothesis originally made

by Overton (1901) as a consequence of noting the excellent correlation between the anesthetic potency of a compound and its olive oil:water partition coefficient.

Before discussing the effects of general anesthetics on membrane structure, it should be emphasized that it is by no means certain that these effects underly general anesthesia. Correlations between anesthetic potency and physical properties other than lipid solubility have been made, including, for example, van der Waals constants (Koski et al., 1975) and ionization potentials (Sandorfy, 1976). Pauling (1961) and S. Miller (1961) independently suggested that anesthetic potency was correlated with the ability of the gas to form micro-crystalline hydrates ("clathrates") of structured water molecules; Pauling proposed that clathrates would form near protein molecules and would affect their activity. However, recently molecules have been found, SF6 for example, for which the correlation is not very good (K. Miller, 1969). A number of workers have pointed out that the correlation of anesthetic potency with lipid solubility would be consistent with anesthetic molecules interacting with hydrophobic regions of probes and causing conformational changes leading to loss of activity (Eyring, 1966; Eyring et al., 1973; Hsia and Boggs, 1975; Ueda et al., 1975; Woodbury et al., 1975; Franks and Lieb, 1978). A few aqueous enzymes have been found whose activity is inhibited by general anesthetics, but clinical concentrations did not produce significant inhibition (Brammall et al., 1973; Ueda and Kamaya, 1973).

Critical volume hypothesis

Mullins (1954, 1973, 1975) suggested that anesthesia occurs when a critical volume of anesthetic has entered a cell membrane, regardless of the structure of the anesthetic molecule. In various forms, this has been a popular idea. K.W. Miller and his collaborators (S. Johnson and K. Miller, 1970; K. Miller et al., 1973; K. Miller, 1974; K. Miller, 1975) have suggested that anesthetic molecules dissolve in some membrane in the cell and cause that membrane to expand by disordering the lipids. This hypothesis was designed in part to account for the pressure reversal of narcosis shown in narcotized tadpoles, newts, and mice (F. Johnson and Flagler, 1950; K. Miller et al., 1973; Lever et al. 1971; Halsey and Wardley-Smith, 1975). Pressure is believed by Miller and his collaborators to reverse the effects of anesthetics by compressing the membrane back to its original volume. From the amount of pressure needed to reverse effects from various doses of anesthetic, they calculated that during general anesthesia the membrane would be expanded by about .4 percent.

Seeman and Roth (1972) have shown that erythrocyte membranes are expanded by about .4 percent with clinical levels of anesthetics. Seeman (1972, 1974, 1975) calculated that the actual occupying volume of anesthetic at these concentrations would be only about .02 percent in the membrane; he suggested that extensive conformational changes in membrane proteins must be involved in the membrane expansion. However, Ueda, Shieh and Eyring (1974) have

found that a purely phospholipid monolayer was expanded by .5 percent in the presence of clinical concentrations of a number of general anesthetics, so that it is not clear that proteins must contribute to the erythrocyte expansion.

It is not clear how expansion of bilayers would produce general anesthesia. Mullins (1975) suggested that the volume increase would cause a local increase in pressure within the bilayer and that this pressure would affect membrane proteins by compressing them. Seeman (1975) suggested specifically that the sodium channel in the nerve membrane is compressed or distorted so that it is blocked. It is difficult to see how these models could account for the pressure reversal of anesthesia, since they postulate that proteins are made inactive by local increases in pressure to begin with.

Changes in membrane fluidity

Trudell, Hubbell, and Cohen (1973a) found that halothane and methoxyflurane decreased the order of fatty-acid chains in phospholipid bilayer vesicles. The order is estimated by the average tilt of nitroxide spin labels on fatty-acid chains, detected by electron spin resonance (Hubbell and McConnell, 1971); "fluidity" is often used to mean the inverse of "order" in this sense. Trudell, Hubbell, and Cohen (1973b) also showed that pressure reversed the decrease in order caused by the anesthetics. They suggested that biological membranes would be similarly affected

and that this is the basis of general anesthesia. Later work has confirmed the increase in fluidity for other anesthetics; while there are some differences in estimates of the concentrations of anesthetics needed for significant effects, it is clear that large concentrations (for example, above 50 mM ether) have clear effects, and clinical levels probably have small, but real, effects (Boggs et al., 1976; Mastrangelo et al, 1977).

Other measurements have also shown perturbation of bilayer structure by general anesthetics. The rotational diffusion rate of the fluorescent probe 1- phenyl-6-phenylhexatriene was shown by Vanderkooi et al. (1977) to be increased in the presence of a variety of general anesthetics; they also showed that the lateral diffusion rate of the fluorescent molecule pyrene was faster with general anesthetics present (for example, increased by a factor of 2.7 by 200 mM ether).

S. Johnson and K. Miller (1970) and Johnson, Miller, and Bangham (1973) showed that general anesthetics at clinical levels increased the cation permeability of liposomes, both native sodium permeability and valinomycin-mediated potassium permeability. They further demonstrated that this effect could be reversed by high pressures.

It is not clear that the effects seen on bilayer systems have any bearing on the physiological phenomenon of general anesthesia. It is possible that the functioning of some of the membrane-bound ionic channels and enzymes in nerves might be influenced by the

state of lipids surrounding them; Gage and Hamill (1976 a,b) and Gage, Hamill and Van Helden (1979) suggested that the reduction of the stability of end-plate channels they observed could possibly be due to an effect of the anesthetics on the lipids surrounding the end-plate channel. However, it is clear that much more must be understood about the functioning of membrane proteins, and about the action of general anesthetics on lipids and on proteins, before the molecular basis for the pharmacological actions of general anesthetics becomes clear.

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