Interactions of Monovalent Cations with Sodium Channels in Squid Axon

I. Modification of Physiological Inactivation Gating

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ABSTRACT   Inactivation of Na channels has been studied in voltage-clamped, internally perfused squid giant axons during changes in the ionic composition of the intracellular solution. Peak Na currents are reduced when tetramethylammonium ions (TMA^+) are substituted for Cs ions internally. The reduction reflects a rapid, voltage-dependent block of a site in the channel by TMA^+. The estimated fractional electrical distance for the site is 10% of the channel length from the internal surface. Na tail currents are slowed by TMA^+ and exhibit kinetics similar to those seen during certain drug treatments. Steady state I_{Na} is simultaneously increased by TMA^+, resulting in a "cross-over" of current traces with those in Cs^+ and in greatly diminished inactivation at positive membrane potentials. Despite the effect on steady state inactivation, the time constants for entry into and exit from the inactivated state are not significantly different in TMA^+ and Cs^+. Increasing intracellular Na also reduces steady state inactivation in a dose-dependent manner. Ratios of steady state I_{Na} to peak I_{Na} vary from ~0.14 in Cs^+- or K^+-perfused axons to ~0.4 in TMA^+- or Na^+-perfused axons. These results are consistent with a scheme in which TMA^+ or Na^+ can interact with a binding site near the inner channel surface that may also be a binding or coordinating site for a natural inactivation particle. A simple competition between the ions and an inactivation particle is, however, not sufficient to account for the increase in steady state I_{Na}, and changes in the inactivation process itself must accompany the interaction of TMA^+ and Na^+ with the channel.

INTRODUCTION

Na and K channels of excitable membranes open and close in response to changes in the transmembrane potential. The steady state and kinetic properties of such

"gating" behavior are generally thought to depend only upon the membrane electric field and upon temperature. Beyond the much-studied effects of divalent cations upon channel gating, which are thought to be mediated by changes in the electric field (e.g., Hille et al., 1975; however, see Gilly and Armstrong, 1981), little is known about the possible influence of ionic environment on Na and K channel gating under voltage clamp.

Recently, changes in monovalent ion composition have been shown to influence the gating of other channels in both natural and artificial membranes. Increasing the concentration of either Cs^+ or Na^+ in solutions bathing gramicidin-doped bilayers increases the lifetime of the open state of the gramicidin channel (Kolb and Bamberg, 1977). The lifetime of single amphotericin B channels in bilayers is prolonged when nitrate replaces chloride (Ermishkin et al., 1977). The average lifetime of acetylcholine-activated channels at the toad endplate is greater in Li^+- or Na^+-containing solutions than when bathed in Ringers containing Cs^+ or K^+ (Gage and VanHelden, 1979). The reverse was observed for acetylcholine-activated fast excitatory channels of Aplysia neurons, where Cs^+ prolongs the lifetime relative to Na^+ or Li^+ (Marchais and Marty, 1979). In addition, extensive determinations of endplate channel lifetimes in frog during the passage of a variety of organic and inorganic cations reveal dramatic differences among ionic species (Adams et al., 1981).

The involvement of ionic mechanisms in the gating of Na channels has been proposed for the inactivation behavior observed with maintained depolarizations. This view of inactivation gating suggests a relatively mobile cationic charge tethered to the axoplasmic surface of each Na channel near the pathway for ion movement (Rojas and Rudy, 1976; Armstrong and Bezanilla, 1977). Upon depolarization, the hypothetical inactivation charge would, after some delay, physically or electrostatically block further movement of Na ions through the channel, thus decreasing the conductance. Similar ionic blocking schemes have been proposed for the "anomalous rectifier" K channel of muscle and of starfish eggs (for a review, see Hille and Schwarz, 1978). In Na channels in which inactivation has been chemically destroyed (Armstrong et al., 1973; Oxford et al., 1978), certain drugs (Yeh and Narahashi, 1977; Cahalan, 1978), alkyl-ammonium compounds (Rojas and Rudy, 1976), and alkyl-guanidinium compounds (Kirsch et al., 1980) applied to the inner membrane surface can induce a time-dependent decay of Na conductance, which qualitatively resembles normal inactivation. The similarity between drug-induced and natural inactivation tempts one to imagine normal inactivation as occurring via a direct ionic block mechanism; however, such mimicry could be coincidental. Here we examine inactivation under a variety of conditions in order to determine the extent of the phenomenological analogy.

Possible interactions between blocking cations and the Na conductance pathway have serious practical implications for the use of ionic substitutes that may otherwise be presumed to be inert (see, e.g., Begenisich and Cahalan, 1980). In particular, experimental substitution of internal K by certain cations is frequently used to eliminate contributions of ion movement through K channels from ionic current records under voltage clamp. The influences of such substitutions upon

the gating behavior of Na channels have not been previously examined and thus the assumptions of "inertness" remain largely unsupported.

The aim of the present investigation was to examine possible interactions between certain internal monovalent cations routinely substituted for K and the Na channel gating mechanism in terms of kinetic and steady state inactivation behavior. Our data suggest a competitive type of interaction that can lead to altered inactivation behavior. These results and those in the following paper (Yeh and Oxford, 1985) provide evidence in support of ionic blocking models of the normal inactivation process while calling into question previous evidence for the existence of multiple open states of normal Na channels in squid axon. A preliminary account of this work has been published (Oxford and Yeh, 1979). In addition, a related report from another laboratory has appeared (Horn et al., 1981) that complements this presentation, and our experiments and results are similar to some of those previously published for *Myxicola* giant axons (Schauf, 1983).

METHODS

Experiments were performed on single giant axons isolated from *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. Axons were cleaned of most adhering tissue, and the axoplasm was squeezed out with a tiny rubber roller and replaced with an artificial internal solution introduced via a micrometer syringe. Axons were then mounted in a Plexiglas chamber, continuously perfused both internally and externally, and voltage-clamped with conventional axial wire techniques. Details of the electronics and procedures of voltage-clamping have been published (Oxford et al., 1978; Oxford, 1981).

During the early stages of this work, Na current records during voltage clamp were obtained from photographs of oscilloscope traces and analyzed by hand. Later, currents were recorded using a 12-bit analog-to-digital converter (model MP6912, Analogic Corp., Wakefield, MA; or model MAS-1202, Analog Devices, Inc., Norwood, MA) interfaced directly to a PDP 11/03 or PDP 11/23 computer (Digital Equipment Corp., Maynard, MA) for data storage and analysis. The temperature was maintained at $8-12 \pm 0.1\,°C$ by a Peltier device and associated electronic feedback circuitry.

The compositions of external and internal solutions used in these experiments and the abbreviations of those solutions are given in Table I. Sucrose was added to all internal solutions to adjust the osmolarity to ~1,040 mosmol, while the external solutions were adjusted ionically to ~1,010 mosmol. External and internal pH values were adjusted to 8.0 and 7.3, respectively. Junction potentials between internal and external solutions were electronically nulled at the beginning of each experiment. It should be noted that junction potentials of between +6 and +10 mV were consistently observed between internal TMA^+ and Cs^+ solutions ($Cs^+ - TMA^+$) and have been corrected as indicated in the legends of some figures. Average values of parameters in all tables and the text are expressed as means $\pm$ SEM.

RESULTS

Na Currents in the Absence of Internal K^+

Under voltage clamp, a step depolarization of a normal squid axon membrane results in a transient Na current followed by a steady K current. The outward K current can be eliminated by internal perfusion with an impermeant cation

replacement for K. The two most widely used internal K substitutes are Cs and tetramethylammonium (TMA$^+$) ions. Fig. 1 compares the transient Na current at two membrane potentials obtained with perfusion of Cs$^+$ (A) followed by TMA$^+$ (B). At both potentials, the peak Na current is obviously reduced when TMA$^+$ replaces Cs$^+$. In contrast, the steady state ionic current during each pulse is increased upon replacement of Cs$^+$ with TMA$^+$. These effects result in a "cross-over" of the current traces for the corresponding voltages in the two solutions. This effect is better illustrated for another axon in Fig. 1C, where the internal perfusate was changed from //250 Cs (trace I) to //250 TMA (trace II) and is reversed (trace III) upon returning to //250 Cs. The currents shown here could be completely blocked by 300 nM tetrodotoxin, which indicates that contributions from channels other than voltage-dependent Na channels were negligible.

TABLE I

Composition of External and Internal Solutions

External Solutions*	Na$^+$	TMA$^+$	Ca^{++}	K$^+$	Cl$^-$
	mM	mM	mM	mM	mM
ASW//	450	0	50	10	560
K(−)SW//	450	0	50	0	550
1/4 Na-SW//	112	338	50	0	550
1/3 Na-SW//	150	300	50	0	550
TMA-SW//	0	450	50	0	550

Internal Solutions‡	Na$^+$	Cs$^+$	TMA$^+$	K$^+$	Gluta-mate$^-$	F$^-$	Sucrose
	mM	mM	mM	mM	mM	mM	mM
//SIS	50	0	0	350	320	50	310
//300 Cs	0	300	0	0	250	50	345
//300 TMA	0	0	300	0	250	50	345
//300 Na	300	0	0	0	250	50	345
//250 Cs	50	250	0	0	250	50	345
//250 TMA	50	0	250	0	250	50	345

* External solutions were buffered to pH 8.0 with 5 mM HEPES.
‡ pH was adjusted to 7.3 with either 15 mM phosphate buffer (SIS) or 10 mM MOPS.

The markedly incomplete decay of Na current in the presence of internal TMA$^+$ is further revealed in steady state inactivation measurements. In Fig. 2, the relative amount of Na current obtained for a voltage step to 0 mV preceded by a 50-ms conditioning voltage step is plotted as a function of the conditioning voltage for an axon perfused with //300 Cs (closed circles) and then //300 TMA (open circles). The characteristic inactivation of the Na conductance with depolarization is essentially identical up to a membrane potential of ~0 mV. For progressively more positive conditioning voltages, however, the "foot" of the steady state inactivation curve differs greatly in these two cases. An increasing fraction of the channels fails to close in //300 TMA, so that at +100 mV only 30% of the channels are inactivated. In contrast, 85% of the Na$^+$ channels are
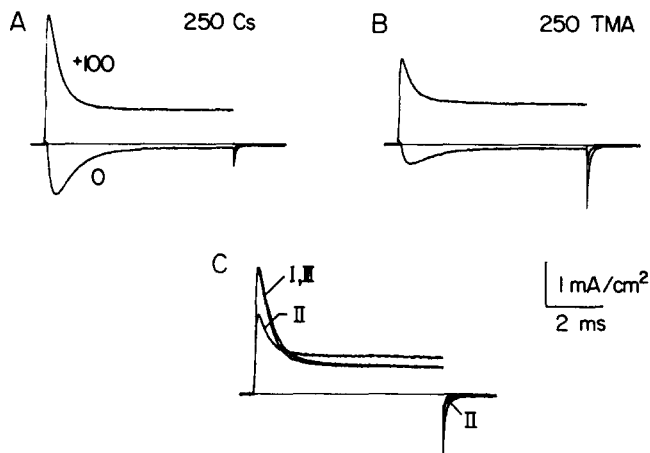
FIGURE 1.   Comparison of Na currents in Cs⁺-perfused and TMA⁺-perfused squid axons. (*A*) Na current records at 0 and +100 mV for an axon in 1/3 Na-SW//250 Cs. (*B*) Na currents from the same axon after internal perfusion with //250 TMA. (*C*) "Cross-over" effect of Na currents in another axon when the internal perfusate is changed from //250 Cs (trace I) to //250 TMA (trace II). Reversal of "cross-over" (trace III) occurs upon return to Cs⁺ solution.
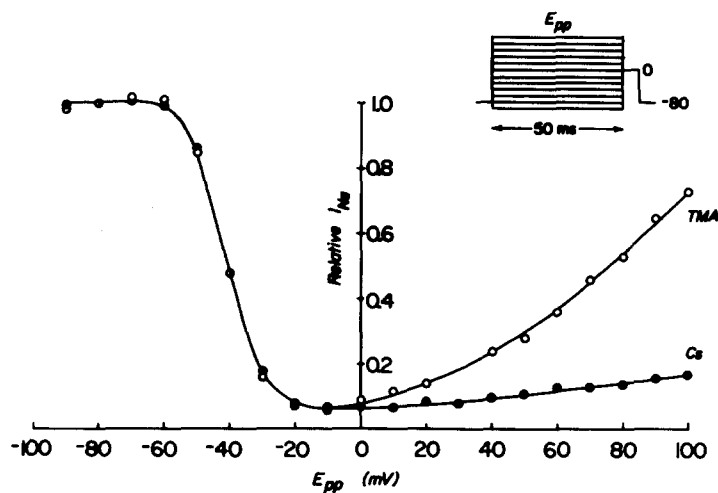

FIGURE 2.   Conventional steady state Na inactivation as a function of voltage for an axon perfused with //300 Cs (filled circles) followed by //300 TMA (open circles). Na currents were measured isochronally during the test pulse (0 mV) at the time of peak maximum inward current, normalized to this value, and plotted against conditioning prepulse voltage. Similar results were obtained with "gaps" between prepulse- and test pulses and for maximum current measurements during the test pulse regardless of time (see Chandler and Meves, 1970*b*).
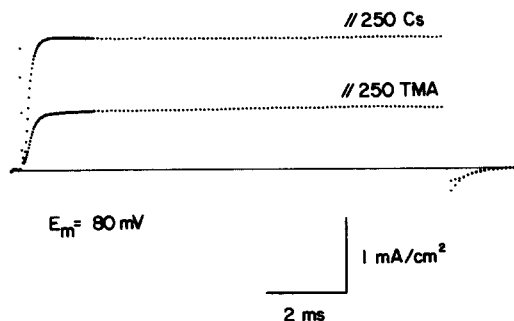
FIGURE 3. Block of open Na channels by internal TMA ions. Outward Na currents for a pronase-treated axon bathed in 1/4 Na-SW and consecutively perfused with //250 Cs and //250 TMA. Membrane potential, +80 mV. Temperature, 10.2°C.

inactivated after a conditioning step to +100 mV in //300 Cs. Qualitatively similar results were obtained with voltage protocols that included returns to the holding potential (gaps) for various periods (0.7–3 ms) between conditioning and test pulses. The apparent loss of inactivation at high depolarization has been previously reported in squid by Bezanilla and Armstrong (1977, Fig. 7) using internal TMA^+ and by Chandler and Meves (1970b, Fig. 5) using high internal Na.

Pronase Treatment Reveals Block by TMA^+

The more complete inactivation of Na conductance with internal Cs^+ might suggest that Cs ions block Na channels in a time-dependent manner, resulting in an artifactually small steady state current. The large steady state currents seen in TMA^+-perfused axons, on the other hand, could perhaps reflect an additional conductance state normally present at high positive potentials (Chandler and Meves, 1970b; Bezanilla and Armstrong, 1977). Several observations argue against this view. In axons in which inactivation is removed with either internal pronase or N-bromoacetamide treatment, Na currents during TMA^+ perfusion are simply scaled down from those during Cs^+ perfusion (Fig. 3). This reduction was completely reversible. Thus, TMA ions may enter and block open Na channels. Table II documents the changes in peak and steady state (8-ms) Na currents at +80 mV in TMA^+-perfused axons as percentages of values during Cs^+ perfusion both in axons with intact inactivation and after pronase treatment.

In pronase-treated axons, the block of Na channels by TMA^+ is apparently

TABLE II
Effect of TMA^+ on Peak and Steady State Na Currents

Condition	$I_{Na}(TMA)/I_{Na}(Cs)$	
	Peak I_{Na}	I_{Na} (8 ms)
Inactivation intact ($n = 9$)	0.66±0.02*	1.27±0.06
Pronase treated ($n = 4$)	0.64±0.02	0.63±0.03

* SEM.

voltage dependent. A comparison of instantaneous Na current-voltage relations in a pronase-treated axon perfused consecutively with //250 Cs and //250 TMA reveals a marked decrease in Na conductance at large depolarizations in the presence of TMA$^+$ (Fig. 4A). Assuming that no block of the channel by Cs$^+$ occurs, an electrical distance for the TMA$^+$ blocking site from the inside surface
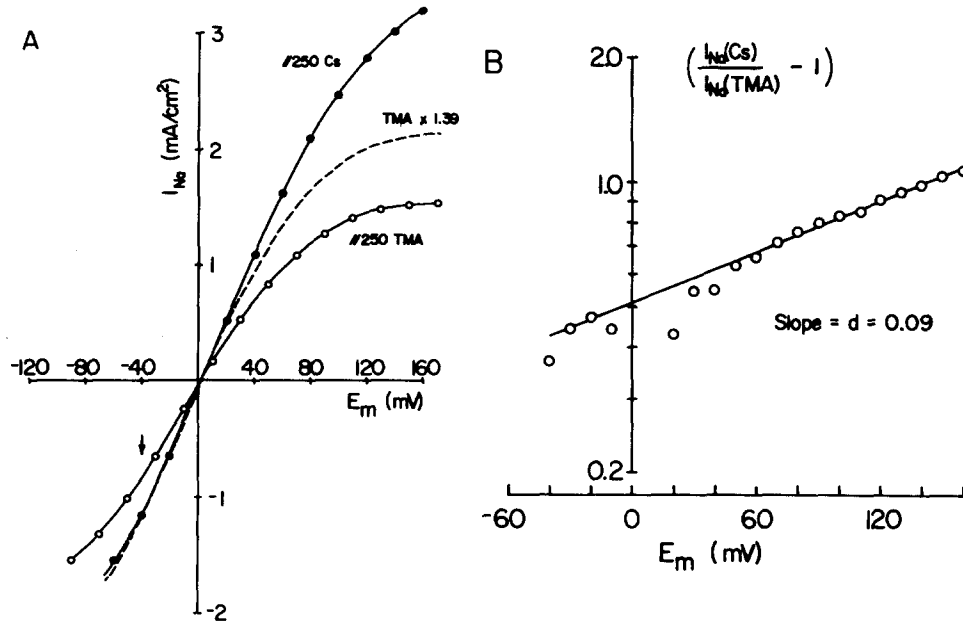

FIGURE 4. Voltage dependence of TMA$^+$ block. (A) Instantaneous Na current-voltage relation for a pronase-treated axon perfused with either //250 Cs (filled circles or //250 TMA (open circles). The external solution was 1/4 Na-SW//. A conditioning depolarization of 4 ms duration to 0 mV was followed by steps to various potentials between −100 and +160 mV. The sampling interval was 20 μs and the data points in the graph were obtained 100 μs after the voltage transition. Solid lines were fit by eye. The data points for //250 TMA were shifted by 8.5 mV to the right along the voltage axis to correct for the measured junction potential. The dashed curve was calculated by scaling the TMA$^+$ data by the ratio of Na currents in Cs$^+$ to that in TMA$^+$ at −40 mV (arrow). Note the increasing discrepancy between the dashed curve and the Cs$^+$ data with increasing depolarization. (B) Semilogarithmic plot of [I_{Na}(Cs)/I_{Na}(TMA)] − 1 vs. membrane potential. Data points obtained from instantaneous I-V shown in A. The slope corresponds to the fractional electrical distance of a hypothetical TMA$^+$ binding site from the internal membrane surface, assuming a one-ion pore.

of the channel can be derived from the difference between currents in Cs$^+$ and TMA$^+$ at several potentials (see Woodhull, 1973; Horn et al., 1981). Fig. 4B is a plot of [I_{Na}(Cs)/I_{Na}(TMA)] − 1 on a logarithmic scale vs. membrane potential, where I_{Na}(Cs) and I_{Na}(TMA) represent the instantaneous current measurements in Cs$^+$ and TMA$^+$, respectively, from Fig. 4A. The slope of this relationship provides an estimate for the electrical distance of a hypothetical TMA$^+$ binding

site from the internal membrane surface. This estimate assumes the Na channel to be a one-ion pore (Hille, 1975) under these experimental conditions (however, see Begenisich and Cahalan, 1980). The average fractional electrical distance determined in this manner was 0.10 ± 0.02 (three axons). This value is considerably different from the value of 0.89 obtained from single channel measurements in rat myotubes (Horn et al., 1981) and will be discussed later.
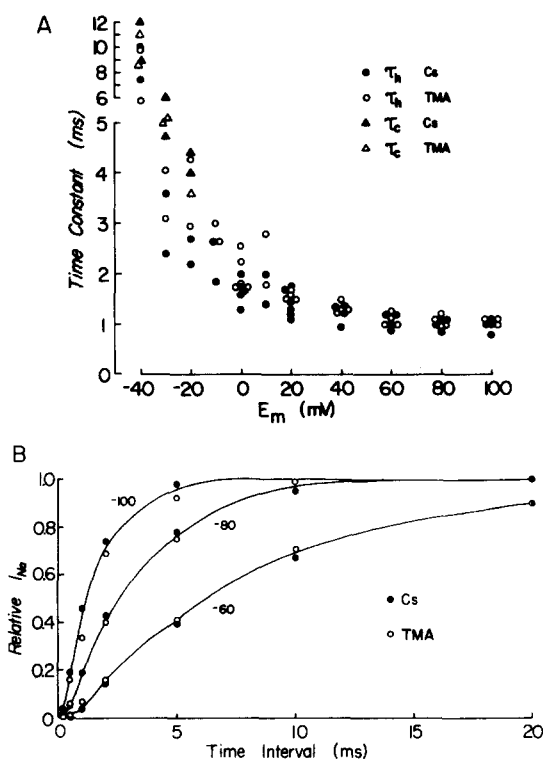

FIGURE 5.   Na inactivation kinetics in the presence of internal Cs^+ or TMA^+. (A) Time constants for the decay of Na current during a maintained depolarization (τ_h, circles) and for the reduction in Na conductance during a conditioning pulse (τ_c, triangles) as a function of membrane potential. Filled data points were obtained in //250 Cs; the open data points are for //250 TMA. (B) Recovery from inactivation at −100, −80, and −60 mV in //250 Cs (filled circles) and //250 TMA (open circles). Data points represent $(I_{Na} - I_{Na}^0)/(I_{Na}^* - I_{Na}^0)$ where the superscripts represent peak current (+20 mV) at the beginning ($t = 0$) and end ($t = 50$ ms) of the recovery interval. The conditioning prepulse was to 0 mV for 20 ms.

Inactivation Kinetics Do Not Differ Markedly in Cs^+ vs. TMA^+

Comparisons of the rate of decay of Na currents ($1/\tau_h$), the rate of inactivation during conditioning depolarization ($1/\tau_c$), and the rate of reactivation during intervals between depolarizations ($1/\tau_r$) were made in axons perfused sequentially with Cs^+ and TMA^+. Small differences in the inactivation time constants, τ_h and τ_c, were found between Cs^+- and TMA^+-perfused axons (Fig. 5 A). TMA^+ slowed

τ_h by ~20% at all voltages examined. Na channel reactivation rates were also determined and were found to be essentially unchanged by the ion substitution (Fig. 5B).

Na Inactivation in Internal Cs^+ Resembles Nonperfused Axons

The appearance of a residual steady state Na conductance during depolarizations of perfused axons under voltage clamp has been regarded by some as an artifact of the perfusion technique, as these residual currents were not described in the original voltage-clamp studies of Hodgkin and Huxley (1952). Recently, digital techniques for data acquisition and improved methods of temperature control have allowed more accurate determinations of Na currents in intact axons (or K^+-perfused axons) by subtraction of current records before and after tetrodotoxin (TTX) treatment. Shoukimas and French (1980) report a steady state Na^+ current very similar to that seen in Cs^+-perfused axons. We have also observed such currents with TTX subtraction procedures. Table III documents ratios of steady state to peak I_{Na} (I_{ss}/I_p) for intact axons as well as for K^+-, Cs^+-, and TMA^+-perfused axons. The remarkable similarity among the values for non-perfused,

TABLE III

Ratios of Steady State to Peak Na Current

Condition	I_{ss}/I_p at +80 mV*
Nonperfused axons[‡] ($n = 2$)	0.12±0.02
SIS-perfused axons[‡] ($n = 4$)	0.13±0.06
Cs-perfused axons ($n = 12$)	0.16±0.04
TMA-perfused axons ($n = 14$)	0.44±0.04
Na-perfused axons ($n = 9$)	0.39±0.03

* All measurements were made at a temperature of 10–12°C.

‡ Determined from difference currents before and after 300 nM TTX.

K^+-perfused, and Cs^+-perfused axons suggests that Cs ions do not appreciably alter normal inactivation behavior. Alternatively, K ions could exert an effect similar to that of Cs^+ and play a role in inactivation gating in situ.

Na^+ Tail Currents Are Slower in TMA^+-perfused Axons

The characteristic exponential decline of Na conductance upon repolarization of the membrane to negative potential levels (deactivation) was compared in axons perfused with Cs^+ and TMA^+. In Fig. 6A, Na tail currents at −120 mV are shown after prepulses of either 1 or 8 ms to +20 mV. After a 1-ms prepulse, it is evident that the tail currents in the presence of internal TMA^+ are significantly slower than in Cs^+, and the conductance, as indicated by the amplitude of the current, is suppressed. In addition, a slight "hook" in the tail current in TMA^+ is present that is very much faster than, but reminiscent of, those seen in the presence of certain blocking drugs such as pancuronium (Yeh and Narahashi, 1977). After an 8-ms prepulse, the relative amplitudes of the tail currents are reversed, however, and the slower time course in the presence of TMA^+ is still apparent. Time constants are given in the figure legend. The switch in relative amplitudes of tail currents in the two solutions between 1 and 8 ms is expected

from the relationship of peak and steady state Na conductance during the prepulse (see Fig. 1).

The effect of TMA$^+$ on the kinetics of deactivation is independent of the inactivation gating mechanism. After internal perfusion with pronase, the slower tail current kinetics can still be observed in the presence of TMA$^+$ (Fig. 6B). Such effects are also observed in pronase-treated axons perfused with certain blocking agents (e.g., Cahalan, 1978).

Reduction of Inactivation by High Internal Na

The increase in the noninactivating fraction of Na channels in the presence of TMA$^+$ (Fig. 2) is reminiscent of a previous report in squid axons perfused with
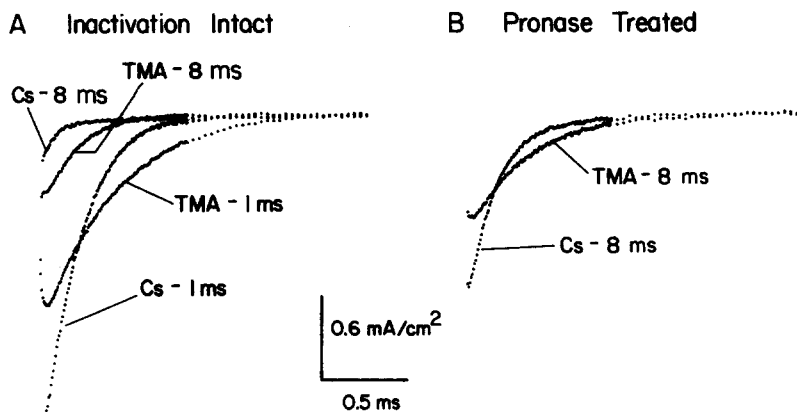

FIGURE 6. Na current tails in internal Cs$^+$ or TMA$^+$. (A) Tail current records recorded at −120 mV after either a 1- or 8-ms voltage step to +20 mV in //250 Cs or //250 TMA. The external solution was 1/3 Na-SW//. Note the rapid "hook" in the current tail in TMA$^+$ and the somewhat slower decay. The measured time constants for exponentials fit to the late portions of each tail current are 90 (Cs, 8 ms), 170 (TMA, 8 ms), 183 (Cs, 1 ms), and 378 μs (TMA, 1 ms). (B) Na current tails measured following an 8-ms pulse in the same axon after pronase treatment. Time constants were 192 and 363 μs for Cs and TMA traces, respectively. Temperature, 6.1 °C.

high internal NaF (Chandler and Meves, 1970a). We re-examined Na channel inactivation in the presence of high internal Na and compared the data with our observations with internal Cs$^+$ and TMA$^+$. In Fig. 7, comparisons of inward Na$^+$ currents in K(−)SW//300 Cs (B) and outward Na$^+$ currents in TMA-SW//300 Na (A) are illustrated for several membrane potentials. Direct comparisons between inward and outward currents are shown for +40 (C) and +80 mV (D) at two time bases. It appears from these records that both the degree and rate of Na channel inactivation are lower for outward Na$^+$ movement than for inward Na$^+$ movement at a constant membrane potential. The ratio of steady state current to peak current is much greater for outward currents, while the time constant of inactivation is somewhat slower than for inward currents. The

expanded time scale reveals, on the other hand, that Na channel activation occurs at nearly the same rate for inward and outward currents.

It is unlikely that these effects arise from either uncompensated series resistance or from Na^+ accumulation/depletion phenomena in the periaxonal space, since they are observed when the current magnitudes are reduced with 5 nM TTX. In addition, 100% of the measured series resistance was compensated during this experiment (see Oxford, 1981).

The question arises as to whether the reduction in inactivation seen with outward Na current is related to the direction of ion movement through Na channels, to the magnitude of Na current, or to the elevation of internal Na.
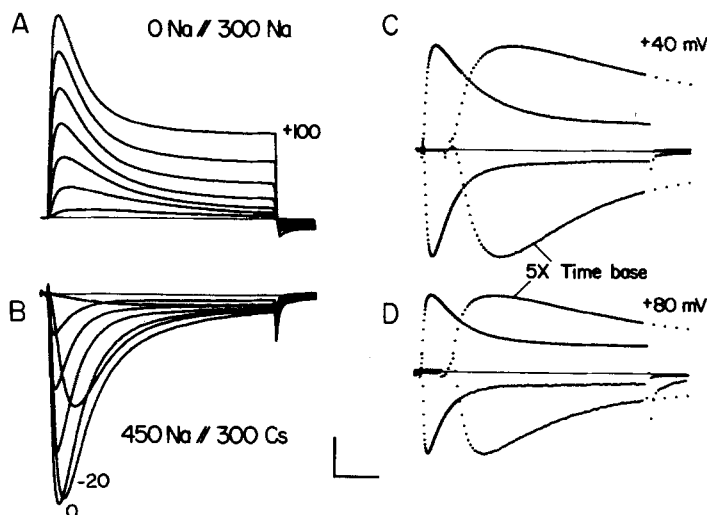
FIGURE 7. Comparison of inward and outward Na currents at the same membrane potentials. (*A*) A family of outward Na currents in TMA-SW//300 Na for a series of voltage steps between −20 and +100 mV. (*B*) Inward currents from the same axon in Na-SW//300 Cs for voltage steps from −60 to +80 mV. (*C* and *D*) Direct comparison of inward and outward currents at +40 and +80 mV, respectively, depicted on two time bases. Peak currents have been scaled arbitrarily to the same amplitude to allow comparisons of kinetics and steady state currents. Calibration bars represent 1 $mA \cdot cm^{-2}$ and 2 ms except for the expanded time scales in *C* and *D* (0.4 ms) and arbitrary amplitude scales in *C* and *D*.

The experiment shown in Fig. 8 reveals that the large noninactivating component of Na current is not dependent upon current direction. In this experiment, the mole fraction of internal Na^+ and Cs^+ was varied while the total concentration of both cations was maintained at 300 mM. The external solution was TMA-SW//. As internal Na^+ was replaced by Cs^+, the outward currents were reduced and the degree of inactivation increased. Scaling the records in Fig. 8*A* to normalize the peak Na current (*B*) reveals a progressive reduction of inactivation in higher internal Na. Either the rise in internal Na^+ concentration or the resulting increase in outward current magnitude appears to be responsible for

the diminished inactivation. Experimental discrimination between these possibil-
ities is not feasible since pharmacological reduction of macroscopic Na currents
is generally all-or-nothing at the level of each channel. Thus, attempts at manip-
ulating current magnitude without changing ion concentrations are ambiguous.

Since both Na^+ and TMA^+ appear to inhibit inactivation, it might be expected
that substitution of internal Na^+ by TMA^+ would not change the fraction of
inactivating channels significantly. This expectation is verified and illustrated in
Fig. 8, C and D, where internal Na^+ was progressively replaced by TMA^+. The
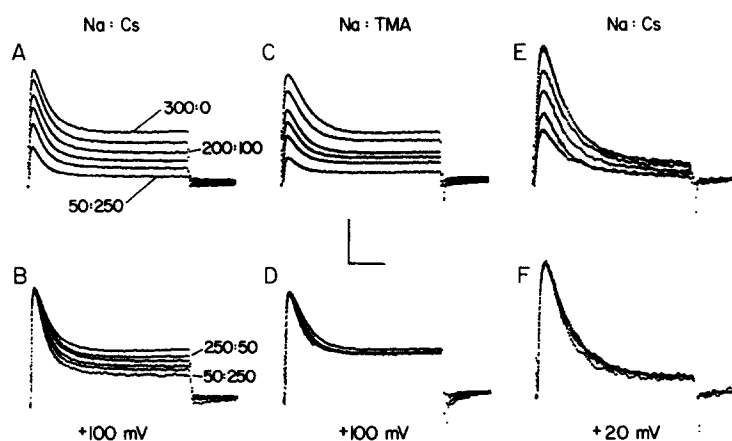records that are normalized at peak current show no significant differences in

FIGURE 8.   Internal Na^+ and TMA^+ inhibit Na channel inactivation. (A) Outward
Na currents at +100 mV as the internal Na^+ was replaced by Cs^+ in 50-mM
increments from a Na/Cs ratio of 300:0 (top trace) to 50:250 (lower trace). (B)
Same records as in A scaled to match the peak values of current. Again, the top and
bottom traces represent Na/Cs ratios of 300:0 and 50:250, respectively. (C) Outward
Na currents in the same axon in which TMA^+ replaces Na^+ in the same format as
A and B. (D) Same records as in C scaled to match peak currents. (E) Na currents
at +20 mV during a Na/Cs exchange as in A. (F) Same records as in E scaled to
match peak current. The voltages in the figure have not been corrected for junction
potentials.

the ratio of peak to steady state current. As with TMA^+, the apparent inhibition
of inactivation by internal Na^+ is also voltage dependent. Scaling records in
different Cs^+–Na^+ mixtures at a more negative potential (+20 mV) demonstrate
a nearly constant level of inactivation (Fig. 8, E and F).

DISCUSSION

The results presented above reveal a significant interaction between certain
monovalent cations exposed to the internal membrane surface of the squid giant
axon and the characteristic inactivation mechanism of voltage-dependent Na
channels. In the presence of relatively high internal concentrations of either
TMA or Na ions, inactivation is retarded and is much less complete at large

positive membrane potentials in comparison with axons perfused with Cs^+ or K^+. Paradoxically, the resultant increase in steady state Na^+ current in TMA^+-perfused axons is accompanied by a reduction in peak current that simultaneously reflects an increase in the noninactivating channel population and a direct block of a fraction of the total channels, respectively. The reduction in Na current by TMA^+ in pronase-treated axons and the similarities between drug-treated and TMA^+-treated Na tail currents provide evidence in support of a direct and rapid block of Na channels at the internal surface. This block is slightly voltage dependent and, interpreted within the context of a single energy barrier, one-ion pore scheme, suggests an electrical distance for the TMA^+ interaction site that is ~10% of the way across the channel from the inner membrane surface.

In evaluating the effects of internal TMA^+ and Na^+ on the inactivation process of Na channels, our assumption is that Cs^+ is inert for purposes of providing a standard for comparison. While it has indeed been our experience that internal Cs^+ (as a K^+ substitute) rarely and then only slightly reduces Na currents and is clearly not the blocker of Na channels that TMA^+ is, we cannot unequivocally reject the proposal that Cs^+ (and perhaps even K^+) may have effects upon normal inactivation gating. In this regard, it is noteworthy that Na channels appear to inactivate completely in frog muscle (Campbell and Hille, 1976), mammalian pituitary and neuroblastoma cells (Dubinsky and Oxford, 1984; Matteson and Armstrong, 1984; Aldrich et al., 1983), crayfish axons (Bean, 1981), and lobster axons (Oxford and Pooler, 1975). Perhaps all of the residual steady state Na conductance observed in squid axons reflects ion-gating interactions. In any event, the observed differences between Cs^+ on the one hand and TMA^+ or Na^+ on the other are clear, and Cs^+ is taken as a convenient, appropriate reference cation.

It should perhaps be noted here that our observations pertain only to internal cation effects. It is our experience that TMA^+ is a superior inert substitute for external monovalent cations (see, for example, Oxford, 1981; Wu et al., 1980) and when prepared fresh is better than $Tris^+$ as a replacement for external Na^+. No effects of external TMA^+ on Na channel gating have been reported to our knowledge.

Voltage Dependence of TMA⁺ Block

The general idea of an interaction between TMA ions and a binding site for the hypothetical tethered inactivation particle is supported by experimental analogies seen with time-dependent drug block of pronase-treated Na channels (see the following article). In addition, the relatively weak voltage dependence of TMA^+ binding to open channels and the correspondingly shallow estimate for the electrical distance of the site from the cytoplasmic interface are consistent with the proposal that the normal inactivation gate is principally outside the membrane electric field and derives most of its apparent steep voltage dependence from coupling to the activation process (Armstrong and Bezanilla, 1977).
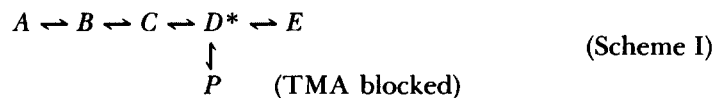
The discrepancy between our results on the voltage dependence of TMA^+ block and those of Horn et al. (1981) using single channel measurements is both interesting and difficult to explain. The single channel measurements were

performed over a different and narrower voltage range than those presented here. If activation-inactivation coupling contributes significantly to inactivation voltage dependence, the block by TMA$^+$, by analogy, might be expected to demonstrate a steeper voltage dependence in the range of activation gating used by Horn and co-workers. However, single channel current amplitudes were the source of the data and should not be expected to reflect gating properties unless the bandwidth of the recording was insufficient to detect rapid gating processes in single channel events (i.e., flicker), which is unlikely in their experiments. In addition, the data points used to estimate the effective electrical distance in Fig. 4B were much more depolarized than the range of activation gating in squid axon. The smaller ratios of instantaneous Na$^+$ currents in Cs$^+$ to those in TMA$^+$, which we observed at potentials more negative than 0 mV, were scattered and may reflect a steeper voltage dependence (e.g., Fig. 4B). However, since the activating voltage prepulse for these measurements was more positive than the activation gating range and since the gating rate constants are apparently slower than the blocking rates for TMA$^+$ (see Fig. 3), the instantaneous I-V will not reflect channel gating at more negative voltages. Our measurements were principally of outward currents in the presence of 50 mM internal Na$^+$, while the single channel measurements were of inward currents with no internal Na$^+$. Since internal Na$^+$ also appears to interact with channel gating, perhaps the relative internal Na$^+$ concentration contributes to the discrepancy. Finally, species differences in Na channel function may be involved, as the inactivation process in mammalian Na channels appears to be much more rapid in relation to activation gating than that observed in squid axon (Aldrich et al., 1983).

Models for Ion Modulation of Channel Gating

Kinetic schemes and some associated molecular interpretations for the inactivation phenomena of Na channels abound in contemporary literature (e.g., Armstrong and Bezanilla, 1977; Nonner, 1980; Aldrich et al., 1983). We therefore approached the question of a theoretical framework in which to explain our findings with caution, recognizing that the numer of possible schemes that would satisfy the data is too large to yield a unique interpretation of our results. The observations reported in the following article do, however, complement mechanisms that incorporate an interaction between internal modulating cations (TMA$^+$ or Na$^+$) and a positively charged natural inactivation particle at a site near the internal surface of the Na channel.

A simplified linear kinetic scheme formalizes the activation-inactivation sequence in a coupled fashion from a closed resting state (A), through several closed intermediates (B and C), to an open conducting state (D^*), and then to an inactivated state (E) that presumably reflects the occupancy of a blocking site by the natural inactivating particle. TMA ions interact with open channels and "compete" with the inactivating particle for its blocking site (or a nearby excluding site) and thus add an additional nonconducting, gate-open, TMA$^+$-blocked state (P) to the scheme:

$$A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D^* \rightleftharpoons E$$
$$\updownarrow \qquad\qquad\qquad\qquad \text{(Scheme I)}$$
$$P \quad \text{(TMA blocked)}$$

Similar open-but-blocked states have been proposed for cationic drug block of Na channels (Yeh and Narahashi, 1977; Cahalan, 1978). The ON and OFF rate constants for TMA$^+$ block are rapid relative to those for the inactivation particle. Thus, the blocked state serves as both a "sink" and a "source" for the conducting state of the channel during the early stages of a depolarizing voltage step. This modification alone is sufficient to generate the "cross-over" observed when
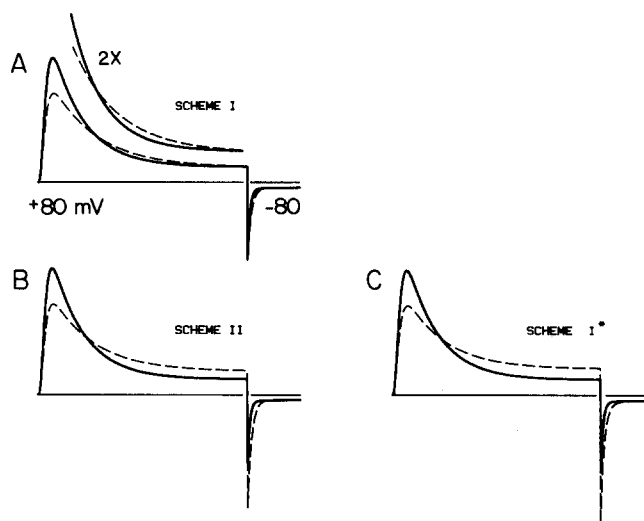

FIGURE 9. Simulations of the TMA$^+$ effect on Na inactivation. All curves are simulated Na current (E_{Na} = +55 mV) for voltage steps to +80 mV from a holding potential of −80 mV. Numerical integration used a modified Euler method with a step size of 5 μs. Rate constants are given in Table IV for all curves. The solid curves in each case represent the same simulation using Scheme I and the rate constants listed as "control" in Table IV. Note the persistent inward I_{Na} upon repolarization, which represents the reflux of channels through the open state during recovery from inactivation. This was not observed experimentally and can be eliminated from the simulations in a number of ways that do not alter the behavior focused upon in these examples. (*A*) The "cross-over" of current traces during TMA$^+$ perfusion simulated with a simple competition model (dashed line, Scheme I). Middle sections of each curve are scaled by 2X to demonstrate the phenomenon clearly. (*B*) Simulation with Scheme II (dashed line), which incorporates at TMA$^+$-induced second open state. (*C*) A modification of the simulation with Scheme I shown in *A* to increase the steady state I_{Na} by increasing the reverse inactivation rate constant, k_{ed}.

TMA$^+$ is substituted for Cs$^+$ (Fig. 9*A*), but will not sustain a larger steady state current, as observed experimentally (Fig. 1).

To account for the increased steady state current (decreased inactivation), a number of mechanisms can be suggested. First, internal perfusion with TMA$^+$ might conceivably induce a second open state of the Na channel. One scheme for such a phenomenon is depicted below:

$$A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D^* \rightleftharpoons E$$

(Scheme II)

$$Q^* \rightleftharpoons P$$
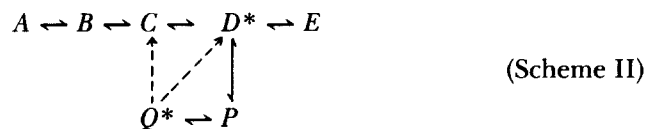
where both D^* and Q^* are conducting states and the Q^* state during a depolarization is loaded exclusively from the TMA^+-blocked state (P). With appropriate rate constants (Fig. 9B), steady state Na^+ current can be produced in this fashion. Upon repolarization, deactivation of the Q^* state could occur either by a transition back to the normal conducting state D^* (Fig. 9B) or a bypass of this state to one of the closed intermediate states (e.g., C). Satisfactory rate constants can be assigned for either possibility.

Alternatively, increasing the rate constant that governs recovery from inactivation in Scheme I (k_{ed}) for more positive test potentials will result in a larger

TABLE IV

Rate Constants for Simulations of Fig. 9

Rate constant	Control	+80 mV			Control	−80 mV		
		A	B	C		A	B	C
ms^{-1}								
k_{ab}	25	25	25	25	0	0	0	0
k_{ba}	0	0	0	0	20	20	20	20
k_{bc}	25	25	25	25	0	0	0	0
k_{cb}	0	0	0	0	20	20	20	20
k_{cd}	20	20	20	20	0	0	0	0
k_{dc}	0	0	0	0	16	16	16	16
k_{de}	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
k_{ed}	0.15	0.15	0.15	0.3	0.15	0.15	0.15	0.3
k_{dp}	—	100	100	100	—	100	100	100
k_{pd}	—	200	200	200	—	200	200	200
k_{pq}	—	—	0.2	—	—	—	0.2	—
k_{qp}	—	—	0.1	—	—	—	0.1	—
k_{dq}	—	—	0	—	—	—	0	—
k_{qd}	—	—	0.1	—	—	—	20	—

steady state I_{Na} (Fig. 9C). This last mechanism implies a "memory" of TMA^+ binding by the channel such that a channel which has previously been bound by TMA^+ exhibits a lower affinity for the natural inactivating particle. Such mechanisms are obviously speculative and require more experimental and theoretical information in order to test their validity.

Previous Observations Related to Steady State I_{Na}

The existence and magnitude of steady state Na conductance during a step depolarization has been examined by others previously, most notably Chandler and Meves (1970a–c). They found that the ratio of steady state to peak I_{Na} was about twice as large in axons perfused with NaF as in those perfused with either CsF or KF (Chandler and Meves, 1970a). In addition, the values for the rate constant for entry into the inactivated state (β_h) in KF-perfused axons were twice

those in NaF-perfused axons, which is consistent with a slowing of the inactivation with high internal Na (Chandler and Meves, 1970c).

A large steady state Na conductance has been reported by Bezanilla and Armstrong (1977) for squid axons internally perfused with 200 mM TMA^+. They determined the fraction of noninactivating channels to be ~0.4 at +80 mV (Fig. 7 of Bezanilla and Armstrong, 1977), which agrees with our determinations in TMA^+-perfused axons (Fig. 2). In dialyzed *Myxicola* giant axons, elevation of internal Na concentrations produces a noninactivating component of Na current that is apparently absent in Cs^+-dialyzed axons (Schauf and Bullock, 1979).

Implications of Results for Future Work

It is clear from these results and those of others (Horn et al., 1981; Schauf and Bullock, 1978, 1979; Schauf, 1983) that the gating properties of Na channels are influenced by the nature of the intracellular cation environment and that indiscriminate use of ion substitutes with the aim of obtaining impermeant, inert contributors to internal ionic strength can lead to an ambiguous interpretation of Na channel kinetic data. With recent technical advances in access to and control of the intracellular composition of very small cells during voltage-clamp studies (Lee et al., 1980; Hamill et al., 1981), manipulation of the internal monovalent cations is being extended beyond the territory of continuously perfused axons. In such cases, caution should be exercised in assuming the inertness of cation substitutes during initial descriptive experiments as well as detailed studies.

Descriptions of Na current data in terms of kinetic or other mathematical models are aimed in part at defining phenomenological states of the Na conductance that might be successfully related to conformational states of protein molecules as more molecular information about the channels becomes available. The presence of kinetic phenomena in ionic or gating current records that are largely or solely a result of ion-channel interactions could result in a misinterpretation of channel behavior in terms of a molecular "state," which is thought to be an inherent feature of the channel protein conformation.

It has previously been demonstrated that the ionic selectivity property of Na channels is a dynamic feature that can be altered by the concentration and type of internal monovalent cations present (Cahalan and Begenisich, 1976; Ebert and Goldman, 1976; Begenisich and Cahalan, 1980). The observation that Na channel gating properties are also labile to monovalent cations suggests that gating and selectivity may not reside in exclusive molecular domains of the Na channel but may in fact possess some common mechanisms of modulation. Studies on the relation between relative ion permeability and gating kinetics for several permeant species in Na channels as have begun for endplate channels (Adams et al., 1981) would be worthwhile.

The results of the following paper reveal that the time-dependent block of Na channels by internally applied drug molecules can also be modulated in a similar manner by monovalent cations. The resulting parallels between drug-induced channel block and normal channel inactivation provide further evidence for some common mechanisms associated with the two phenomena.

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