Interactions of Monovalent Cations with Sodium Channels in Squid Axon

II. Modification of Pharmacological Inactivation Gating

JAY Z. YEH and GERRY S. OXFORD

From the Department of Pharmacology, Northwestern University School of Medicine, Chicago, Illinois 60611; the Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27514; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT The time-, frequency-, and voltage-dependent blocking actions of several cationic drug molecules on open Na channels were investigated in voltage-clamped, internally perfused squid giant axons. The relative potencies and time courses of block by the agents (pancuronium [PC], octylguanidinium [C8G], QX-314, and 9-aminoacridine [9-AA]) were compared in different intracellular ionic solutions; specifically, the influences of internal Cs, tetramethylammonium (TMA), and Na ions on block were examined. TMA+ was found to inhibit the steady state block of open Na channels by all of the compounds. The time-dependent, inactivation-like decay of Na currents in pronase-treated axons perfused with either PC, 9-AA, or C8G was retarded by internal TMA+. The apparent dissociation constants (at zero voltage) for interaction between PC and 9-AA with their binding sites were increased when TMA+ was substituted for Cs+ in the internal solution. The steepness of the voltage dependence of 9-AA block found with internal Cs+ solutions was greatly reduced by TMA+, resulting in estimates for the fractional electrical distance of the 9-AA binding site of 0.56 and 0.22 in Cs+ and TMA+, respectively. This change may reflect a shift from predominantly 9-AA block in the presence of Cs+ to predominantly TMA+ block. The depth, but not the rate, of frequency-dependent block by QX-314 and 9-AA is reduced by internal TMA+. In addition, recovery from frequency-dependent block is not altered. Elevation of internal Na produces effects on 9-AA block qualitatively similar to those seen with TMA+. The results are consistent with a scheme in which the open channel blocking drugs, TMA (and Na) ions, and the inactivation gate all compete for a site or for access to a site in the channel from the intracellular surface. In addition, TMA ions decrease the apparent blocking rates of other drugs in a manner analogous to their inhibition of the inactivation process.

Address reprint requests to Dr. Gerry S. Oxford, Dept. of Physiology, University of North Carolina, Medical Science Research Wing 206H, Chapel Hill, NC 27514.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/85/04/0603/18 $1.00

Volume 85 April 1985 603–620
Multiple occupancy of Na channels and mutual exclusion of drug molecules may play a role in the complex gating behaviors seen under these conditions.

**INTRODUCTION**

In squid giant axons, the inactivation process of voltage-dependent Na channels during a step voltage-clamp depolarization proceeds as a single-exponential decay of the conductance (from its peak) to a nonzero steady state level. After removal of this process by intracellular perfusion with pronase or N-bromoacetamide, a similar time-dependent decay can be reinstated by introducing a variety of drug molecules into the axon interior. The list of effective compounds includes many local anesthetics, N-methyl strychnine, alkyl-guanidinium compounds, pancuronium, and 9-aminoacridine (Cahalan and Almers, 1979b; Kirsch et al., 1980; Yeh and Narahashi, 1977; Yeh and Armstrong, 1978; Yeh, 1979).

When the inactivation process remains intact, several of these compounds can interact with the inactivation "gate" and the internal region of the open Na channel to produce "frequency-dependent" cumulative block of the channel population during repetitive stimulation. The importance of the inactivation mechanism to this behavior is emphasized by its disappearance after pronase treatment. For a review of time- and frequency-dependent block of Na channels by drugs, see Yeh (1982).

A common property of the compounds mentioned above is that they are all cationic. The most convincing mimicry of inactivation is seen for those compounds that can also develop hydrophobic interactions with the channel. The similarities between the normal process of Na channel inactivation in squid and the time-dependent block of open Na channels by these compounds has led to the view that the inactivation mechanism is represented by a cationic membrane particle tethered near the inner surface of the channel that responds to activation (opening) of the channel by mechanically or electrostatically blocking ion flow in a manner not unlike that by which a bathtub plug restricts water flow (e.g., Armstrong and Bezanilla, 1977; Rojas and Rudy, 1976). Experimental evidence has even led to the suggestion that a physical realization of the hypothetical plug particle might be the cationic guanidinium group of an arginine residue (Eaton et al., 1978; Oxford, 1980).

While the similar behaviors of normal and drug-induced inactivation support this notion, they could simply be coincidental, and there are quantitative differences between the phenomena in terms of the rate of recovery from inactivation (Yeh, 1982). Correlations can be observed in the modulation of other gating phenomena by both normal and pharmacological inactivation that provide more stringent tests of the validity of the "plug" hypothesis. The "immobilization" of charge movements associated with Na channel activation (gating currents) by both normal inactivation and intracellularly applied drugs represents such a correlation (Cahalan and Almers, 1979a, b; Armstrong and Bezanilla, 1977; Yeh and Armstrong, 1978; Yeh, 1982; Armstrong and Croop, 1982). The converse approach of searching for correlations in the modulation of both normal and pharmacological inactivation by experimental manipulations can also provide
evidence relevant to the validity of an ionic block mechanism. In this paper, we
demonstrate alterations in the time- and frequency-dependent block of Na
channels by several compounds induced by changes in the intracellular mono-
vvalent cation environment. These effects are correlated with the observations on
normal inactivation presented in the preceding paper (Oxford and Yeh, 1985)
and provide further support for the involvement of a "tethered inactivation
particle" in some aspect of Na channel inactivation.

METHODS

Experiments were performed on single giant axons isolated from Loligo pealei at the
Marine Biological Laboratory, Woods Hole, MA. Experimental procedures for voltage-
clamping, intracellular perfusion, and data collection were the same as in the preceding
paper (Oxford and Yeh, 1985) and have been also detailed elsewhere (Oxford et al.,
1978; Oxford, 1981).

The experimental solutions and temperatures were also the same as in the preceding
paper. Sources of the drugs used were as follows. 9-Aminoacridine hydrochloride (9-AA)
was purchased from K & K Laboratories, Inc. (Plainview, NJ) and pancuronium bromide
(PC) was obtained from Organon, Inc. (West Orange, NJ). Octylguanidinium (C8G)
nitrate was the kind gift of Dr. Richard A. Hudson (Wayne State University) and C8G
sulfate was kindly provided by Dr. John Dutcher (National Institutes of Health). QX-314
was kindly provided by Dr. Bertil Takman (Astra Pharmaceuticals). All average values of
parameters presented in the text are expressed as means ± SD, unless specifically indicated
otherwise.

RESULTS

Cation Effects on Time-dependent Drug Block of Na Channels

PC (0.1–1 mM) selectively and reversibly blocks Na channels when applied to
the internal surface of the squid axon membrane. In pronase-treated axons, a
time-dependent decay of Na current is seen that reflects the time course of PC
equilibration with its blocking site in the channel (Yeh and Narahashi, 1977). As
illustrated in Fig. 1A, the Na current at +60 mV measured at 8 ms in //250 Cs,
after having been blocked to 4% of the control value by 0.3 mM PC, returns to
83% of the control level upon washing the axon with drug-free solution. A
similar recovery from PC block in //250 TMA was observed, even though the
degree of block by PC in TMA+ (57%) was less than that observed in Cs+ (96%).
This incomplete recovery was invariably observed in all pronase-treated axons
and the degree of recovery from drug block was independent of the degree of
block before washing. Therefore, we have attributed this incomplete recovery
to a rundown of the preparation. To overcome this complication, an experiment
was designed to bracket the drug exposure period by two control periods. The
average value of Na current before and after washing with drug-free solution is
taken as the true control. This control value was used to evaluate the potency
and voltage dependence of PC block.

TMA affects PC block in three ways: (a) it slows the time course of block, (b)
its decreases the apparent potency of PC, and (c) it decreases the voltage depend-
ence of block.
TIME COURSE OF PC BLOCK  PC causes a time-dependent decrease in Na currents in axons in which Na inactivation has been destroyed by pronase or N-bromosuccinimide treatment. This time-dependent decrease in Na currents in the presence of channel blockers in many ways resembles the decay of current caused by normal inactivation mechanisms. As shown in the preceding paper, TMA⁺ slows the time course of Na inactivation and makes it much less complete. Similarly, in the presence of TMA⁺, the PC-induced decay of Na current is slower and less complete than in axons perfused with Cs⁺. In Cs⁺ perfusion

![Figure 1](image)

FIGURE 1. Time-dependent block of pronase-treated Na channels by PC during Cs⁺ vs. TMA⁺ perfusion. (A) Outward Na currents at +60 mV in a pronase-treated, Cs-perfused axon before, during, and after internal exposure to 0.3 mM PC. (B) Outward Na current in the same axon perfused with TMA⁺ before, during, and after PC exposure. Na current (data points) during PC block in Cs⁺ (C) and TMA⁺ (D) and superimposed fits of a two-component exponential decay (solid lines) for each case. Parameters of the fit are given in the text.

medium, 0.3 mM PC caused a rapid decay of the current during a voltage step to +60 mV (Fig. 1C). Detailed kinetic analysis reveals that the decay reflects the sum of two exponential components. The major component in this example decayed with a time constant of 0.5 ms and the slower component had a time constant of 2.53 ms. This slow component contributed <10% of total current. Our previous analysis, based on 35-mm film records, could not resolve this slow component (Yeh and Narahashi, 1977). In TMA⁺-containing solutions, there remain two components of decay in the Na current (Fig. 1D); however, the slower component predominates (time constant, 5.8 ms), while the fast time constant (0.46 ms) becomes a minor component.

CATION EFFECTS ON APPARENT POTENCY OF PC BLOCK  TMA decreased
the potency of PC in blocking Na channels. $K_D$ values at 0 mV were estimated for PC block and compared between axons perfused with $//250$ TMA and those perfused with $//250$ Cs. Estimates assumed that one PC molecule binds to one Na channel, resulting in total loss of conductance in the drug-bound channel. The $K_D$ values found in TMA$^+$ averaged $0.37 \pm 0.10 \text{mM}$ (mean $\pm$ SD, $n = 5$) and were significantly different ($P < 0.005$) from those in Cs$^+$ ($0.14 \pm 0.04 \text{mM}$, $n = 4$).

**VOLTAGE DEPENDENCE OF PC BLOCK** TMA$^+$ not only decreases the potency of PC block, it affects the voltage dependence of block as well. Fig. 2 illustrates that this voltage dependence varies with the nature of the cation used to substitute for internal K. The data are plotted according to the following equation (see Coronado and Miller, 1982):

$$\ln[(g_{Na, \text{control}}/g_{Na, \text{PC}}) - 1] = \ln([\text{PC}]/K(0)) - zdFV/RT,$$

where [PC] is the PC concentration, $K(0)$ is the zero-voltage dissociation constant for PC, and $d$ is the apparent electrical distance for the blocking site from the internal channel surface. Values of $[g_{Na, \text{control}}/g_{Na, \text{PC}} - 1]$ are plotted semilogarithmically against membrane potential for axons perfused with $//250$ Cs (open circles) and $//250$ TMA (filled circles). The voltage dependence of PC block was much steeper in the presence of Cs than in TMA, and this is reflected in the estimated values for the electrical distance of the PC binding site ($0.79$ in Cs, $0.33$ in TMA). The average value in $//250$ Cs is $0.76 \pm 0.046$ ($n =$
4). In //250 TMA, two out of five axons demonstrated a clear voltage-dependent block at voltages between −20 and +50 mV, but the effect saturated at potentials above +50 mV. In the three other preparations, no clear voltage-dependent block was observed over a voltage range from −20 to +80 mV.

In our previous report (Yeh and Narahashi, 1977), the voltage dependence of PC block was measured in the presence of internal TEA+, and the effective electrical distance was estimated to be 0.42. This value is smaller than the value of 0.76 seen in Cs+ perfusion solutions, which suggests that TEA+ may also affect the blocking action of Na channels by PC and other blockers.

**C8G Block of Na Channels in TMA-containing Solutions**

The time-dependent block of pronase-treated Na channels by C8G ions closely resembles the normal inactivation process in many respects (Kirsch et al., 1980; Oxford and Yeh, 1982). Channel block by C8G is also modulated by monovalent cations in a fashion resembling inactivation, as seen in Fig. 3. Outward Na currents in a control axon (inactivation intact) perfused consecutively with //250 Cs and //250 TMA (Fig. 3A) can be compared to a C8G-perfused (0.01 mM) pronase-treated axon (Fig. 3B) under the same conditions. The characteristic "cross-over" of current traces reported in the preceding paper upon exchange

![Figure 3](image-url)

**FIGURE 3.** TMA ions inhibit normal inactivation and C8G block similarly. (A) Outward Na current at +100 mV for a control axon consecutively perfused with //250 Cs and //250 TMA as labeled. (B) Na currents at +100 mV for a different pronase-treated axon perfused consecutively with //250 Cs and //250 TMA both containing 10−5 M C8G. Note the "cross-over" of records during both normal inactivation (A) and C8G block (B). Temperature, 8.2°C.

of internal Cs+ by TMA+ is strikingly similar in both cases. Thus, C8G is a more potent blocker in axons perfused with Cs+ than in those perfused with TMA+.

**9-AA Block of Na Channels in TMA-containing Solutions**

The most dramatic decrease in drug potency induced by internal TMA+ perfusion occurred during experiments examining block of pronase-treated Na channels by 9-AA. Fig. 4 depicts Na current families from a pronase-treated axon consecutively perfused with //250 Cs (A) and //250 TMA (B), each containing 0.1 mM 9-AA. The reduction in steady state 9-AA block induced by TMA+ is obvious at all membrane potentials examined, but is most prominent for outward currents during large depolarizations. A direct comparison of current traces at +100 mV from each case (Fig. 4C) reveals the greatly diminished potency of 9-AA in TMA+-perfused axons. The ratio of steady state to peak Na current at
+100 mV during 9-AA block in this example was 0.13 in //250 Cs and 0.54 in //250 TMA.

After the equilibrium binding treatment used above for PC block (Eq. 1), we estimated values for the zero-voltage dissociation constant, $K(0)$, and the effective electrical location of the binding site, $d$, for 9-AA block. $K(0)$ values averaged 58.56 ± 11.61 μM (mean ± SD n = 5) in //250 Cs, but were increased to 177.5 ± 74.55 (n = 4) in //250 TMA. Values for $d$ fell from 0.56 ± 0.07 in //250 Cs to 0.22 ± 0.08 in //250 TMA. Thus, both the potency and voltage dependence of block by 9-AA are diminished by TMA$^+$. The time course of 9-AA block was only slightly changed by TMA$^+$. Time constants for block in pronase-treated axons by 0.1 mM 9-AA were 0.81 ± 0.21 ms in Cs$^+$ (n = 6) and 0.76 ± 0.23 ms in TMA$^+$ (n = 6).

![Figure 4](image)

**Figure 4.** Interference with 9-AA block of Na channels by TMA$^+$. Families of Na currents for a pronase-treated axon perfused with 10-4 M 9-AA in //250 Cs (A) and //250 TMA (B). External solution was 1/3 Na-SW. Voltage steps were applied from a holding potential of -80 mV to membrane potentials of -40, -20, 0, 20, 40, 60, 80, and 100 mV. (C) The records at +100 mV from A and B are superposed. Note the dramatic inhibition of 9-AA block by TMA$^+$.

**Cation Effects on Frequency-dependent Block of Na Channels**

In axons in which the normal inactivation mechanism remains intact (no pronase treatment), the block of open Na channels by many cationic drug molecules (e.g., 9-AA and QX-314) accumulates during repetitive application of depolarizing voltage steps that open Na channels. This results in a continuous decrease in peak current with every applied step, the rate of which depends upon the frequency of pulses. Drug molecules become trapped in the channel between pulses, and recovery is not completed during the interpulse interval. Therefore, more channels become blocked during the next voltage step, which activates them.

The degree of frequency-dependent block of Na channels by both 9-AA and QX-314 is greatly reduced in TMA$^+$-perfused axons as compared with Cs$^+$-perfused axons. Fig. 5 illustrates the frequency-dependent reduction (1 Hz) of $I_{Na}$ at +80 mV by 0.1 mM 9-AA in //250 Cs (A) and then in //250 TMA (B).
The equilibrium reduction of peak current after the application of five depolarizing steps is clearly smaller in TMA⁺ than in Cs⁺. The time course of cumulative block is shown in Fig. 5C for this axon. Here it can be seen that the time constant for block in Cs⁺ (1.15 s) does not differ substantially from that in TMA⁺ (1.55 s), whereas the equilibrium block by 9-AA is 68% in Cs⁺ but only 26% in TMA⁺.

![Figure 5. TMA⁺ reduces frequency-dependent block of Na channels by 9-AA. (A) Outward Na current records elicited by six repetitive (1-Hz) depolarizations to +80 mV in an axon perfused with //250 Cs containing 10⁻⁴ M 9-AA. Note the progressive reduction of peak current with consecutive pulses. (B) An identical experiment performed on the same axon perfused with //250 TMA and 10⁻⁴ M 9-AA. Note the diminished reduction in peak current for successive pulses. (C) Time course of the peak current reduction seen in A and B and for a separate experiment on the same axon perfused with //SIS and 20 mM TEA⁺ (raw currents not shown). Data points are peak Iₚ relative to that obtained with the first pulse in the sequence. Solid lines represent single-exponentials fit to each set of data points.](image)

The equilibrium block is also shown for the same axon during perfusion with //SIS plus 20 mM TEA⁺ and is not very different from that seen in //250 Cs.

TMA⁺ exerts the same inhibitory effect on frequency-dependent block of Na channels by QX-314, as shown in Fig. 6. In Fig. 6A, the currents from five consecutive voltage steps (1 Hz) to +80 mV in an axon perfused with //250 Cs
containing 0.4 mM QX-314 demonstrate a greater cumulative block than when the axon is perfused with 250 TMA containing QX-314 (B). The average onset and depth of block for four experiments is illustrated in Fig. 6C and could be approximated by a single-exponential time course. The average time constants were 1.0 ± 0.12 and 1.2 ± 0.14 s, respectively, for 250 Cs and 250 TMA. The steady state block, expressed in terms of percent $I_{Na}$ relative to the resting block level was 0.40 ± 0.04 in Cs$^+$ and 0.59 ± 0.08 in TMA$^+$; these values are significantly different ($P < 0.025$).

**Figure 6.** TMA$^+$ reduces the frequency-dependent block of Na channels by internal QX-314. Outward current records elicited from an axon by five consecutive (1-Hz) voltage steps to +80 mV during perfusion with either 250 Cs (A) or 250 TMA (B) in the presence of 0.4 mM QX-314. (C) Average time course of frequency-dependent QX-314 block for $I_{Na}$ for four axons perfused with Cs$^+$ (open circles) or TMA$^+$ (filled circles). Solid lines represent single-exponentials fit to each set of data points. Data are expressed as means ± SD ($n = 4$).

**Voltage Dependence of Phasic Block** TMA$^+$ decreases the voltage dependence of frequency-dependent block by 9-AA and QX-314 as compared with that observed in the presence of internal Cs$^+$. The voltage dependence of this phenomenon was determined by measuring the remaining $I_{Na}$ associated with a test pulse after a train of conditioning potential steps ranging from −80 to +80 mV. The normalized Na currents were plotted as a function of the conditioning potential (Fig. 7). The depth of frequency-dependent block was enhanced for increasingly more positive conditioning potentials in the presence of either internal TMA$^+$ or Cs$^+$. It is evident, however, that TMA$^+$ dramatically suppressed the voltage dependence of block at potentials above −20 mV.
RECOVERY FROM FREQUENCY-DEPENDENT BLOCK The mechanism by which TMA⁺ decreases the frequency-dependent block of Na channels could result from an accelerated dissipation of block during interpulse intervals induced by TMA⁺. That is, the recovery from frequency-dependent block could be

![Graph](image)

**Figure 7.** TMA⁺ reduces the voltage dependence of phasic block of Na channels. An initial voltage step to 0 mV was applied to an axon to obtain a control inward $I_{\text{Na}}$. 20 conditioning voltage steps (1 Hz) at various potentials were then applied to produce phasic drug block and a final test step again to 0 mV was used to assess the magnitude of remaining $I_{\text{Na}}$ (see the inset in A). The current during the last step relative to that during the initial step is plotted as a function of the conditioning voltage for block by 0.1 mM 9-AA in //250 Cs and //250 TMA (A) and for another axon perfused with 0.8 mM QX-314 in //250 Cs and //250 TMA (B).
speeded by TMA⁺. To test this possibility, the time course of recovery from block was measured and compared in //250 TMA and //250 Cs. The time constants of recovery from QX-314 block were 17.0 ± 5.0 (n = 3) and 22.0 ± 6.0 s (n = 3) in TMA⁺ and Cs⁺, respectively. The time constants of recovery from 9-AA block of Na channels were 13.8 ± 2.55 (n = 6) and 11.2 ± 4.25 s (n = 6) in Cs⁺ and TMA⁺, respectively. In both cases, the small differences in the time constants between TMA⁺ and Cs⁺ cannot account for the profound differences in frequency-dependent block caused by either 9-AA or QX-314. These results are consistent with the observation reported in the preceding paper that TMA⁺ does not interfere with the Na inactivation recovery process.

EFFECT OF INTERNAL Na ON 9-AA BLOCK OF Na CHANNELS High concentrations of internal Na ions interfere with Na inactivation in a manner similar to that of internal TMA⁺. Na inactivation is less complete under both conditions (Oxford and Yeh, 1985). Since the inactivation process has been shown to play an important role in the phenomenon of frequency-dependent drug block of Na channels, one might expect that elevated internal Na⁺ concentrations would suppress frequency-dependent block, as does TMA⁺. Such is the case for block by 9-AA.

Frequency-dependent block by 0.1 mM 9-AA was compared in axons perfused with either //250 Cs (50 mM internal Na⁺) or //300 Na. The time course of frequency-dependent block was not significantly different in the two cases, but the depth of block in //300 Na was significantly less than that seen with only 50 mM Na⁺ inside. The time constants for the onset of frequency-dependent block were 1.23 ± 0.32 (n = 6) and 1.43 ± 0.16 s (n = 6) for 50 and 300 Na⁺, respectively. In contrast, the fraction of unblocked channels, expressed in terms of $I_{Na}$ after prepulse conditioning relative to $I_{Na}$ without conditioning, was 0.32 ± 0.09 (n = 5) in 50 mM Na⁺ and 0.56 ± 0.04 (n = 6) in 300 mM Na⁺.

The recovery from frequency-dependent 9-AA block was not very different in the two solutions. The time constants of recovery were 13.8 ± 2.55 s (n = 6) in 50 mM Na⁺ and 10.9 ± 2.0 s (n = 4) in 300 mM Na⁺. This again suggests that differences in frequency-dependent block induced by high internal Na⁺ are not likely to arise from accelerated drug dissociation at rest. Rather, they must arise from interactions between Na channels and Na ions during the depolarizing phase.

DISCUSSION

TMA⁺ and other organic cations have often been used as ion substitutes for intracellular K in studies of the gating or pore properties of Na channels. Although TMA⁺ is impermeable through the Na channel (Hille, 1971), it is far from inert in terms of both the physiology and pharmacology of the Na channel. In the preceding paper (Oxford and Yeh, 1985), we addressed the physiological effects of TMA ions on Na channels: they block Na channels and retard the inactivation process. A similar manifestation of this ion-channel interaction is TMA⁺ interference with the actions of open channel blocking drugs. Among them, PC, C8G, QX-314, and 9-AA have been studied in detail in axons with intact Na inactivation mechanisms and in axons where the Na inactivation has.
been removed by pronase treatment. TMA+ affects open channel block in three ways, as illustrated using PC as an example: (a) it slows the time course of PC block, (b) it decreases the potency of PC block, and (c) it decreases the voltage dependence of PC block.

In pronase-treated or N-bromoacetamide-treated axons, many open channel blocking compounds induce a time-dependent decay of Na current. The time course of decay of Na currents in the presence of these blockers usually follows a single-exponential time course. In some cases (e.g., PC), the decay is better approximated as the sum of two exponentials. This time-dependent reduction in currents resembles normal Na channel inactivation in many respects (see review of Yeh, 1982). The single-exponential decay of Na current can be simulated by a kinetic model in which the inactivation (or drug blockade) is coupled to the activation of channels, as illustrated below:

```
R (drug blocked)
↓
A ← B ← C ← D*
↓
P (TMA blocked)
```

State R represents the drug-blocked state of the channel, D* is the conducting state, and A–C are nonconducting intermediate states in the activation gating sequence. In this scheme, TMA ions compete with other open channel blockers for the open channel state and add an additional nonconducting, gate-open, TMA-blocked state to the scheme (P). This interaction between open channel blockers and TMA ions, which themselves are open channel blockers, is analogous to the model described in the previous paper to account for the effect of TMA ions on Na inactivation. In that kinetic scheme, TMA+ interacted with a binding site or access to a binding site for a natural inactivation particle.

The two blocked states, one for TMA+ and the other for other open channel blockers (PC, C8G, or 9-AA), are assumed to be exclusive. A more complex possibility would incorporate multiple occupancy of the Na channel by two compounds (e.g., TMA and 9-AA). This is conceivable because of the very different d values estimated for the binding sites for each molecule. Simulation of this possibility is complicated and we cannot rule out the involvement of such a mechanism at this time.

In TMA+ containing solutions, the activation of Na channels is modeled as in normal solutions. The rate constants for TMA block and unblock are chosen to be very rapid; thus, in the absence of another drug, no time dependence of TMA block is discernible, which is in agreement with experimental results (Oxford and Yeh, 1985).

A direct consequence of the interaction pictured in Scheme I is that both the potency and time course of channel block caused by other open channel blockers are affected in the presence of TMA ions. This is actually seen experimentally. The block of control (pronase-treated) Na current by TMA+ is simulated in Fig. 8A, where the blocking and unblocking rate constants for TMA+ are 20 and 40
ms\(^{-1}\), respectively. Simulation of the currents in Cs\(^+\)-perfused axons during block with 0.1 mM 9-AA was achieved (Fig. 8B) by providing 9-AA blocking and unblocking rate constants, \(k_{dr}\) and \(k_{ud}\), respectively, derived from experimental data on the steady state level and the time constant of block (see Yeh and Narahashi, 1977). The simple introduction of the TMA\(^+\)-associated rate constants, \(k_{dp}\) and \(k_{pd}\), into the simulation does not, however, reproduce the experimental records of 9-AA block in TMA\(^+\)-perfused axons. It thus appears that a competitive interaction between TMA\(^+\) and 9-AA molecules alone is insufficient to explain our observations. To account for the degree of “cross-over” seen experimentally, it was necessary to increase the 9-AA unblocking rate constant twofold and to decrease the blocking rate constant threefold. This alteration is

![Figure 8](image_url)

**Figure 8.** Simulation of time-independent TMA block and time-dependent 9-AA block of Na current in a pronase-treated axon. The kinetic model of Scheme II was used to simulate Na currents at +80 mV. The control Na current (Cs-containing perfusate) was simulated with the following rate constants: \(k_b = k_r = 25 \text{ ms}^{-1}\); \(k_{ud} = 20 \text{ ms}^{-1}\); \(k_{dr} = k_{dp} = k_{pd} = 0\). The TMA block was reproduced with \(k_{dp} = 20 \text{ ms}^{-1}\) and \(k_{pd} = 40 \text{ ms}^{-1}\) (A). The 9-AA block in Cs\(^+\) (B) was reproduced with \(k_{dr} = 0.9 \text{ ms}^{-1}\) and \(k_{ud} = 0.2 \text{ ms}^{-1}\). When the internal solution contained both 9-AA and TMA\(^+\), Scheme II was used to produce the current with the same rate constants for TMA block, but with the 9-AA blocking rate constant, \(k_{dr}\), being slowed down to 0.3 ms\(^{-1}\), and the unblocking rate constant, \(k_{ud}\), being speeded up to 0.4 ms\(^{-1}\). The cross-over of the time-dependent decay of Na currents is satisfactorily reproduced. Na current is calculated as \(I_{Na} = g_{Na}D^*(E - E_{Na})\), where \(g_{Na} = 150 \text{ mS/cm}^2\) and \(E_{Na} = 50 \text{ mV}\).

This phenomenon might arise in the following manner.
If the binding sites for TMA\(^+\) and 9-AA are located in the channel and are exclusive, they are likely to be sequentially accessed from the intracellular side. The larger values of \(d\) found for 9-AA block suggest that this site may reside deeper in the channel than that for TMA\(^+\) and that 9-AA molecules would have to pass the TMA site as they block. After the dissociation of a TMA ion from its site, the channel could retain a "memory" of its interaction with TMA\(^+\), which might be manifest as a change in the rate constants governing both entry and exit of 9-AA from its binding site as it passes the TMA site. As TMA\(^+\) binding itself is voltage dependent, so too would be the change in the 9-AA rate constants. Alternatively, occupancy of the shallower site by a TMA ion could exclude a 9-AA molecule and thereby impart an apparent decrease in voltage dependence to the observed 9-AA block. That is, as TMA is introduced, the net block shifts from a deep site to a shallow site and the voltage dependence actually reflects a larger contribution of TMA block.

In axons whose Na inactivation processes are intact, 9-AA and QX-314 produce frequency- and voltage-dependent block of Na channels (Yeh, 1978; Yeh, 1979; Cahalan, 1978). Factors required to produce a frequency-dependent block of Na channels have been discussed in detail by Yeh (1982). According to the paradigm of voltage-clamp pulses applied, the drug-channel interaction can be divided into two phases: a depolarizing phase and a hyperpolarizing phase. During the depolarizing phase, the drug-channel interaction leads to a net blocking action, and during the hyperpolarizing phase, the drug-channel interaction favors an unblocking action. Since many pulses are required for the drug-channel interaction to reach a balance between the blocking and unblocking action, phenomenologically the process manifests as a frequency-dependent block.

The degree of frequency- and voltage-dependent block caused by these two drugs is more intense in Cs-containing solutions than in TMA-containing solutions. TMA ions, in principle, could suppress drug blocking action during a depolarizing phase and/or accelerate the unblocking action during a hyperpolarizing phase. The contribution from the latter effect must be minimal since the time course of recovery from the frequency-dependent block, indicative of the unblocking action, is not appreciably affected by TMA ions. During the depolarizing phase, two factors might contribute to TMA\(^+\) effects on frequency- and voltage-dependent block: a direct open channel blocking action as discussed above, and an indirect effect on Na inactivation. Inasmuch as Na inactivation has played an essential role in producing frequency-dependent block of Na channels by most local anesthetics (Yeh, 1982), and because TMA\(^+\) interferes with the Na inactivation process, an effect of TMA\(^+\) on frequency-dependent block would be expected.

Using 9-AA as a model drug for producing the frequency-dependent block of Na channels, we have simulated TMA\(^+\) effects on frequency-dependent block by a kinetic model, illustrated in Scheme II. Scheme II represents an expansion of Scheme I in which the original drug blocked state (\(R\)) is now represented by a series of states (\(A'\), \(B'\), \(C'\), \(R\), and \(E'\)), each of which is considered to be drug bound.
These states represent an activation-inactivation gating sequence for channels that have an associated 9-AA molecule and the rate constants governing transitions between these states are the same as those for transitions between the normal, drug-free channel states. For example, states $E$ and $E'$ represent the inactivated states of the free Na channel and of the drug-bound channel, respectively. The schemes can thus be viewed as parallel populations of channels that activate and inactivate similarly, but one population is drug-blocked, while the other is normal. Transitions between the two populations occur exclusively between the free open state ($D^*$) and the blocked open state ($R$).

The rate constants for 9-AA molecules to bind and unbind to the open Na channel were estimated from the time-dependent block of Na currents in pronase-treated axons in Cs-containing solution. These rate constants were also used in TMA-perfused axons. The unblocking reaction at the holding potential was assumed to be the same for both schemes since the time courses of recovery from the frequency-dependent block were similar in both solutions. In the TMA-containing solution, the inactivation kinetics of Na channels are affected as explained in the previous article (Oxford and Yeh, 1985). Namely, $k_{ad}$ was speeded by TMA ions (but $k_{da}$, was not). The presence of 9-AA ions is assumed not to alter the activation and inactivation rate constants of Na channels.

Fig. 9A depicts a simulation of the frequency-dependent block of Na currents in the presence of 0.1 mM 9-AA in Cs-containing solution. The hallmark reduction in peak Na currents with repetitive pulses is satisfactorily reproduced in this case. Fig. 9B depicts an attempt to simulate the frequency-dependent Na channel block by 9-AA in the presence of TMA ions. In this case, we assumed that the influence of TMA ions was mediated indirectly via their action on inactivation gating. Thus, the only modifications to Scheme II involved increasing the value of $k_{ad}$ twofold and providing large values for $k_{dp}$ and $k_{pd}$, the TMA$^+$ blocking and unblocking rate constants, respectively (see figure legend). In this simulation, the apparent frequency dependence is only minimally altered from that in Fig. 9A. Thus, our initial suggestion that TMA$^+$ effects on this behavior are mediated solely by altering inactivation gating would seem to be incorrect. By analogy with the modification of Scheme I required to account for time-dependent block, we found it necessary to additionally slow the forward rate constant for 9-AA block ($k_{ad}$) in the presence of TMA$^+$ (Fig. 9C) in order to satisfactorily reproduce the experimental reduction in frequency-dependent block. It appears, therefore, that a common feature of the TMA$^+$ interaction with Na channel inactivation and drug block involves a reduction in the equilibrium association between the inactivation "gate" or a drug molecule and the channel. This change might be mechanistically realized in several ways. TMA ions might, upon dissociation from the channel, leave an open channel with a
FIGURE 9. Simulation of frequency-dependent block of Na channels by 9-AA in Cs- and TMA-perfused conditions. Part A was simulated by Scheme II assuming that the channels were at state A at -80 mV. Upon depolarization to +80 mV, the rate constants for channel activation are: $k_{a} = k_{c \rightarrow a} = k_{a \rightarrow c} = 25 \text{ ms}^{-1}$; $k_{b} = k_{b \rightarrow c} = 20 \text{ ms}^{-1}$. The rate constants for channel inactivation are: $k_{d} = k_{d \rightarrow c} = 1.5 \text{ ms}^{-1}$ and $k_{d} = k_{d \rightarrow c} = 0.15 \text{ ms}^{-1}$. The forward blocking rate constant, $k_{d}$, equals 0.9 ms$^{-1}$ and the backward unblocking rate constant, $k_{d}$, equals 0.2 ms$^{-1}$; $k_{d} = k_{d} = 0$. During the 8-ms depolarizing phase, the Euler method of numerical integration was used. During the interpulse interval, an analytical solution was used to replace the time consuming integration over the 1-s interval. At the end of the depolarizing pulse, all intermediate states in the drug-free population were summed ($F = A + B + C + D + E$). Similarly, all intermediate states in the drug-bound population were summed ($M = A' + B' + C' + R + E'$). The analytical solution has the form

$$Q = M \exp(-t/\tau),$$

where $Q$ is the population of drug-bound channels after the interpulse interval, $t$, and $\tau$ is the time constant of recovery from the frequency-dependent block, 12 s in this case. At the end of an interpulse interval (1 s), therefore, the population of drug-bound channels, $Q$, equals $M \exp(-1/12)$, and the newly available drug-free channels then equal $(M - Q)$. Thus, the total population of drug-free channels becomes $F + M - Q$. For the second depolarizing pulse, the initial conditions assumed were that $A = F + M - Q$, $A' = Q$, and the remaining intermediate states were unpopulated. This operation was repeated five times to produce the traces in A. A similar procedure was used to produce B with the following exceptions: (a) TMA block was incorporated with the forward blocking rate constant, $k_{dp} = 20 \text{ ms}^{-1}$ and the unblocking rate constant, $k_{dp} = 40 \text{ ms}^{-1}$, (b) the inactivation rate constant, $k_{d} = 0.3 \text{ ms}^{-1}$, and (c) the drug-free channels at the end of the 8-ms depolarizing pulse were $F = A + B + C + D + E + P$. To simulate C, Scheme II was used as for B with an additional modification for the rate constants for 9-AA block: $k_{d} = 0.2 \text{ ms}^{-1}$ and $k_{d} = 0.4 \text{ ms}^{-1}$. $I_{Na}$ was calculated as in Fig. 8 with $g_{Na} = 200 \text{ mS/cm}^2$. 
“memory” of the previous binding because of a slowly relaxing image force or a corresponding conformational change that would present a greater energy barrier to subsequent 9-AA binding. Alternatively, TMA ions might be more effective at reducing the local surface potential in the vicinity of the channel inner mouth than Cs ions because of binding to specific internal negative surface charges. This could lower the effective 9-AA concentration in the proximity of the channel and thus reduce the forward blocking rate constant, $k_d$, which is the product of the concentration and the pseudo-first-order rate constant. While such charges have been suggested to exist only near the voltage-sensing mechanisms of channels (Chandler et al., 1965), there is no evidence to rule out their existence near the intracellular surface of the ion pathway (however, see Begenisich, 1975). The evidence does not allow discrimination between these possibilities at the present time.

In summary, the effects of TMA and Na ions on the magnitude, kinetics, and voltage dependence of natural inactivation and drug-induced “inactivation” of Na channels are consistent. These data support the involvement of a particle blocking mechanism in at least some aspect of the normal Na channel inactivation process in squid axons. We emphasize that the kinetic schemes for Na channel gating used to explain our observations probably represent simplifications of the inactivation process that only describe that pathway coupled to channel activation and do not address other possible inactivation processes supported by other evidence (Oxford and Pooler, 1975; Aldrich et al., 1983). The view of the channel that emerges incorporates intraluminal binding sites for small monovalent cations and for bulkier, more hydrophobic cationic blocking drugs. These agents may interact via mutual exclusion to alter the apparent kinetics of the drug-receptor interaction and can alter the behavior of the inactivation gate. It is thus apparent that the intracellular experimental ion environment plays an important role in gating behavior and can obscure and mislead investigations aimed at describing inactivation gating in terms of the conformational states of channel protein molecules.

The authors wish to express their appreciation to Drs. Richard Hudson, John Dutcher, and Bertil Takman for generous gifts of some compounds used in this research. This work was supported by National Science Foundation grants BNS79-21505 and BNS82-11580 to G.S.O. and by U. S. Public Health Service grant GM24866 to J.Z.Y.

Original version received 15 March 1984 and accepted version received 13 December 1984.

REFERENCES

Aldrich, R. W., D. P. Corey, and C. F. Stevens. 1983. A reinterpretation of mammalian sodium channel gating based on single channel recording. Nature (Lond.). 306:436–441.

Armstrong, C. M., and F. Bezanilla. 1977. Inactivation of the sodium channel. II. Gating current experiments. J. Gen. Physiol. 70:567–590.

Armstrong, C. M., and R. S. Croop. 1982. Simulation of Na channel inactivation by thiazin dyes. J. Gen. Physiol. 80:641–662.

Begenisich, T. 1975. Magnitude and location of surface charges on Myxicola giant axons. J. Gen. Physiol. 66:47–65.
Cahalan, M. D. 1978. Local anesthetic block of sodium channels in normal and pronase-treated squid giant axons. *Biophys. J.* 23:285–311.

Cahalan, M. D., and W. Almers. 1979a. Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin. *Biophys. J.* 27:39–56.

Cahalan, M. D., and W. Almers. 1979b. Block of sodium conductance and gating current in squid giant axons poisoned with quaternary strychnine. *Biophys. J.* 27:57–73.

Chandler, W. K., A. L. Hodgkin, and H. Meves. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol. (Lond.)* 180:821–836.

Coronado, R., and C. Miller. 1982. Conduction and block by organic cations in a K⁺-selective channel from sarcoplasmic reticulum incorporated into planar phospholipid bilayers. *J. Gen. Physiol.* 79:529–547.

Eaton, D. C., M. Brodwick, G. S. Oxford, and B. Rudy. 1978. Arginine-specific reagents remove sodium channel inactivation. *Nature (Lond.)* 271:473–476.

Hille, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* 58:599–619.

Kirsch, G., J. Z. Yeh, J. M. Farley, and T. Narahashi. 1980. Interaction of n-alkylguanidines with the sodium channels of squid axon membrane. *J. Gen. Physiol.* 76:315–335.

Oxford, G. S. 1980. Biochemical correlates of sodium channel gating in nerve membrane. *Fed. Proc.* 39:ix. (Abstr.)

Oxford, G. S. 1981. Some kinetic and steady-state properties of sodium channels after removal of inactivation. *J. Gen. Physiol.* 77:1–22.

Oxford, G. S., and J. P. Pooler. 1975. Selective modification of sodium channel gating in lobster axons by 2,4,6-trinitrophenol. Evidence for two inactivation mechanisms. *J. Gen. Physiol.* 66:765–779.

Oxford, G. S., C. H. Wu, and T. Narahashi. 1978. Removal of sodium channel inactivation in squid giant axons by N-bromoacetamide. *J. Gen. Physiol.* 71:227–247.

Oxford, G. S., and J. Z. Yeh. 1982. Molecular mechanisms of sodium channel inactivation: discriminations using temperature, octanol, and ion competition. *Biophys. J.* 37:104a. (Abstr.)

Oxford, G. S., and J. Z. Yeh. 1985. Interactions of monovalent cations with sodium channels in squid axon. I. Modification of physiological inactivation gating. *J. Gen. Physiol.* 85:583–602.

Rojas, E., and B. Rudy. 1976. Destruction of the sodium conductance inactivation by a specific protease in perfused nerve fibers from *Loligo*. *J. Physiol. (Lond.)* 262:501–531.

Yeh, J. Z. 1978. Sodium inactivation mechanism modulates QX-314 block of sodium channels in squid axons. *Biophys. J.* 24:569–574.

Yeh, J. Z. 1979. Dynamics of 9-aminoacridine block of sodium channels in squid axons. *J. Gen. Physiol.* 73:1–21.

Yeh, J. Z. 1982. A pharmacological approach to the structure of the Na channel in squid axon. In Proteins in the Nervous System: Structure and Function. Alan R. Liss, New York.

Yeh, J. Z., and C. M. Armstrong. 1978. Immobilization of gating charge by a substance that simulates inactivation. *Nature (Lond.)* 273:387–389.

Yeh, J. Z., and T. Narahashi. 1977. Kinetic analysis of pancuronium interaction with sodium channels in squid axon membranes. *J. Gen. Physiol.* 69:293–323.