Evidence for a Direct Interaction between Internal Tetra-alkylammonium Cations and the Inactivation Gate of Cardiac Sodium Channels

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ABSTRACT The effects of internal tetrabutylammonium (TBA) and tetrapentylammonium (TPeA) were studied on human cardiac sodium channels (hH1) expressed in a mammalian tsA201 cell line. Outward currents were measured at positive voltages using a reversed Na gradient. TBA and TPeA cause a concentration-dependent increase in the apparent rate of macroscopic Na current inactivation in response to step depolarizations. At TPeA concentrations <50 μM the current decay is well fit by a single exponential over a wide voltage range. At higher concentrations a second exponential component is observed, with the fast component being dominant. The blocking and unblocking rate constants of TPeA were estimated from these data, using a three-state kinetic model, and were found to be voltage dependent. The apparent inhibition constant at 0 mV is 9.8 μM, and the blocking site is located 41 ± 3% of the way into the membrane field from the cytoplasmic side of the channel. Raising the external Na concentration from 10 to 100 mM reduces the TPeA-modified inactivation rates, consistent with a mechanism in which external Na ions displace TPeA from its binding site within the pore. TBA (500 μM) and TPeA (20 μM) induce a use-dependent block of Na channels characterized by a progressive, reversible, decrease in current amplitude in response to trains of depolarizing pulses delivered at 1-s intervals. Tetrapropylammonium (TPrA), a related symmetrical tetra-alkylammonium (TAA), blocks Na currents but does not alter inactivation (O'Leary, M. E., and R. Horn. 1994. *Journal of General Physiology.* 104:507-522.) or show use dependence. Internal TPrA antagonizes both the TPeA-induced increase in the apparent inactivation rate and the use dependence, suggesting that all TAA compounds share a common binding site in the pore. A channel blocked by TBA or TPeA inactivates at nearly the normal rate, but recovers slowly from inactivation, suggesting that TBA or TPeA in the blocking site can interact directly with a cytoplasmic inactivation gate.

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INTRODUCTION

Tetra-alkylammonium (TAA) compounds are well-known inhibitors of potassium (K) channels. Internal TAA compounds block K channels by binding to a site near the cytoplasmic mouth of the channel, where they have two apparently opposite effects on the kinetics of K currents. In slowly inactivating K channels, large TAA compounds increase the apparent inactivation rate (French and Shoukimas, 1981; Choi, Mossman, Aubé, and Yellen, 1993). However, in *Shaker* K channels having A-type inactivation, internal tetraethylammonium (TEA) reduces the amplitude of K currents and slows the apparent rate of macroscopic inactivation (Choi, Aldrich, and Yellen, 1991). These data suggest that the larger TAA compounds have slow blocking kinetics that have the appearance of inactivation, whereas smaller compounds, such as TEA, are rapid blockers that can compete with the native inactivation "ball" for its binding site in the channel.

In the accompanying paper we show that a series of symmetrical TAA compounds with alkyl side chains of 1–4 carbons in length block hH1 Na channels (O'Leary and Horn, 1994). Compounds with side chains up to three carbons in length have no effect on inactivation, suggesting that they bind too deep within the pore to be sensed by a cytoplasmic inactivation gate. Unlike these other TAA compounds, tetrabutylammonium (TBA; four carbon alkyl side chains) and tetrapentylammonium (TPeA; five carbon alkyl side chains) increase the apparent rate of macroscopic current inactivation. Here we examine the effects of TBA and TPeA in detail. The data suggest that both compounds bind within the pore and stabilize the Na channel in an inactivated conformation by directly interacting with a cytoplasmic inactivation gate.

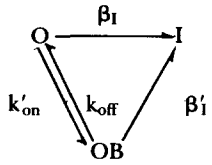
METHODS

Experimental methods are given in the accompanying paper (O'Leary and Horn, 1994). The Na gradient was set so that currents were outward at positive voltages. The external solution consisted of (in millimolar): 140 choline-Cl, 10 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES pH 7.4. The pipette solution contained (in mM) 100 NaF, 30 CsF, 10 CsCl, 5 EGTA and 10 Cs-HEPES pH 7.4. For the experiments of Fig. 5 changes in external [Na] were effected by equimolar substitution for choline. All experiments were done at 12°C in order to measure accurately the decay kinetics at positive voltages.

Model of TPeA Block

We assume that activation (i.e., channel opening) is essentially complete during the decay of outward currents at voltages $> +10$ mV. This assumption implies that, by the time the current reaches its peak value, no further gating transitions will occur between the open state and preceding closed states in the activation pathway. The justification for this assumption derives from estimates of the voltage dependence of gating transitions (e.g., Vandenberg and Horn, 1984; Vandenberg and Bezanilla, 1991; Scanley, Hanck, Chay, and Fozzard, 1990; recently reviewed in Patlak, 1991). We further predict that at these positive voltages, an open channel may inactivate directly or be blocked by TPeA and that a blocked channel may also inactivate.

These assumptions lead to the following kinetic model for decay of outward current.



Channels that can contribute to the decay of current will either be in the open state, *O*, with initial probability P_o, or the blocked state, *OB*, with initial probability $1\text{-}P_o$. If a single molecule of TPeA blocks the channel, k'_{on} is linearly dependent on [TPeA], i.e., $k'_{on} = k_{on}$[TPeA]. In the absence of contradictory evidence, we have assumed that TPeA, like the smaller TAAs, has no effect on activation gates (O'Leary and Horn, 1994). This means that closed channels can be blocked by TPeA, which will have no effect on the rate of activation. Note that the inactivated state is absorbing; this is consistent with the nearly complete decay of Na current in the presence or absence of TPeA. If $k_{off} > 0$, the above model predicts that the decay of current will be a double exponential (e.g., see Goldman, 1976) with rates r_1 and r_2, where

$$r_1, r_2 = (k'_{on} + k_{off} + \beta_I + \beta'_I)/2 \pm \sqrt{[(k'_{on} + k_{off} + \beta_I - \beta'_I)/2]^2 - k_{off}(\beta_I - \beta'_I)}.$$

The fractional weight, w_1, of the fast exponential is

$$w_1 = [(k'_{on} + k_{off} + \beta_I - r_2)P_o - k_{off}]/[P_o(r_1 - r_2)].$$

Two time constants of current decay, the inverses of r_1 and r_2, and their relative weights, were measured from Na currents at [TPeA] > 20 μM, using the program CLAMPFIT (Axon Instruments, Inc., Burlingame, CA). In the absence of TPeA a single time constant was measured and its inverse was used as an estimate of β_I. We estimated k_{on}, k_{off}, and β'_I by minimization of nonlinear least squares, using a variable metric algorithm (Powell, 1978). P_o was determined at each TPeA concentration by the reduction of averaged peak Na current at +50 mV for a population of cells. Adaptively trimmed means (Lehmann, 1983) were 4,683 ± 699 pA, n = 13 cells, control; 3,386 ± 384 pA, n = 10, 50 μM TPeA; 1,086 ± 532 pA, n = 7, 100 μM TPeA. Because of cell-to-cell variability, the estimates of P_o were not as reliable or consistent as the other measured quantities. However, the estimates of rate constants were not very sensitive to the assumed values of P_0.

An examination of the above equations shows that if k_{off} is significantly smaller than the other three rate constants at [TPeA] > 20 μM, and if β_I is of the same order of magnitude as β'_I, then $r_1 \cong k_{on}$[TPeA] + β_I, and $r_2 \cong \beta'_I$. Under these conditions the weight of the fast component will tend to be large, >0.8. Analysis of our data (see Results) are in accord with these simplifying conditions.

Because this model assumes that a channel may inactivate while blocked, it is possible to determine the fraction of channels that inactivate from the blocked state, i.e., that inactivate by the pathway $OB \rightarrow I$. The number of times a channel will visit the open state before inactivating has a geometric distribution (e.g., see Aldrich, Corey, and Stevens, 1983). The probability of an open channel inactivating directly is

$f_o = \beta_I/(\beta_I + k'_{on})$. The probability of a channel inactivating directly from the blocked state is $f_b = \beta'_I/(\beta'_I + k_{off})$. An open channel may be blocked and unblocked repeatedly before inactivating from the open state. The probability it will visit the open state i times before inactivating from the open state is

$$p_i = f_o\,\omega^{i-1},$$

where ω, the probability that an open channel will be blocked and reopen, is $(1 - f_o)(1 - f_b)$. Therefore, the probability that a channel will eventually inactivate while unblocked, given that it starts in the open state, is

$$p_{un} = f_o \sum_{i=1}^{\infty} \omega^{i-1} = f_o[1/(1 - \omega)]. \quad (1)$$

If the channel starts in the blocked state *OB,* the probability that it will eventually inactivate from the open state is $p_{un}(1 - f_b)$. If the initial fraction of unblocked channels is P_o, then the probability of eventually inactivating from the open state is

$$Pr[O \rightarrow I] = p_{un}\,P_o + p_{un}(1 - f_b)(1 - P_o) \quad (2)$$

The fraction of channels which inactivate from *OB* is $1 - Pr[O \rightarrow I]$.

Molecular Dynamics Simulation

The structure and flexibility of TAA compounds was examined with the molecular mechanics program Discover on a Silicon Graphics workstation. The structure of each compound, in the absence of solvent, was iterated 1,000 cycles, corresponding to conformational transitions during ~1 ps at 300°K.

RESULTS

TBA and TPeA Increase the Apparent Inactivation Rate of Na Channels

Na currents were measured at positive voltages (+10 to +70 mV) under ionic conditions that promote outward Na currents. In the absence of a TAA blocker, we assume that the decay of macroscopic Na currents measured at large depolarizations ($\geq$ +20 mV) reflects primarily the Na channel inactivation rate from the open state, with little kinetic contamination by deactivation or reactivation (Vandenberg and Horn, 1984; Yue, Lawrence, and Marban, 1989). Internal TBA and especially TPeA cause concentration-dependent increases in the apparent rate of Na current inactivation at +60 mV (Fig. 1), as well as decreases in the peak current amplitude (data not shown). Higher concentrations of TBA reduced the amplitude of macroscopic Na currents so drastically that we could not obtain accurate measurements of kinetics. Therefore, we focused on TPeA in most of the studies reported below.

Inactivation time constants were measured as a function of both membrane potential and TPeA concentration ([TPeA]). At [TPeA] < 50 μM the current decay is well fit by a single exponential at all voltages. At higher [TPeA] a second exponential component is observed, with the faster component contributing at least 80% to the decay (Fig. 2 *C*). A double exponential time course is observable in Fig. 1 *B* at 100 μM TPeA. The two time constants of the double exponential fit at 100 μM TPeA typically differed by a factor of 5–10 (Fig. 2, *A* and *B*). The slower time constant is not

affected by a twofold change of [TPeA], and is marginally slower than the single time constant measured in the absence of TPeA (Fig. 2 *B*). The faster time constant ([TPeA] > 50 μM) and the single time constant ([TPeA] < 50 μM) both decrease as a function of internal [TPeA] (Fig. 2 *A*).

These data may be explained by a block of open channels by TPeA, assuming that the blocking kinetics for this compound are slow enough to be resolved. This hypothesis could be tested by single channel recording. However, attempts to determine the effects of TPeA at the single channel level were unsuccessful in part because TPeA induces a long lived closed state resulting in brief channel openings interspersed with prolonged quiescent periods (data not shown). These periods probably reflect times when a channel is both blocked and inactivated (see data on use-dependent block, below). Therefore, we restricted our kinetic analysis to whole cell Na currents. If an open channel can either inactivate directly with rate β_I, or be blocked by TPeA with a rate [TPeA]k_{on}, and if inactivation is irreversible at these voltages, as suggested by the decay of Na current nearly to the baseline under all

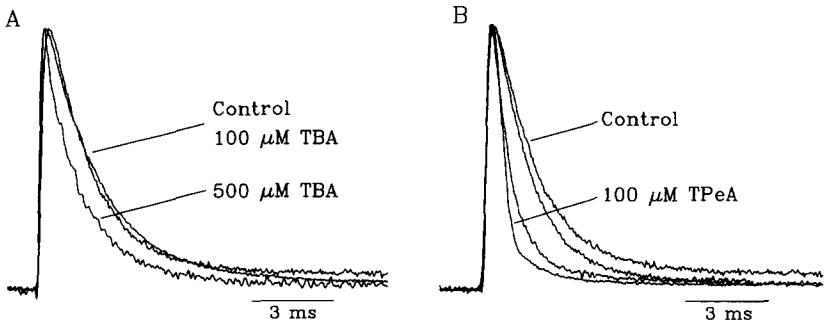


FIGURE 1. Effects of internal [TBA] and [TPeA] on outward Na currents. Whole-cell currents normalized to the same peak value. Cells were held at −120 mV and stepped to +60 mV for 15 ms. (*A*) Currents for 0, 100, and 500 μM TBA. (*B*) Decay was progressively more rapid for [TPeA] of 0, 10, 50, and 100 μM.

conditions, then we can represent the kinetics of decay by the three-state model described in Methods.

If the unblocking rate constant k_{off} in our model is very small, i.e., a blocked channel will never reopen, then the decay of the current will be exponential with a rate of β_I+[TPeA]k_{on}, which will depend linearly on [TPeA]. The fact that we observe two exponential components under some conditions requires that blocked channels may reopen. Note also that we allow a blocked channel to inactivate with rate β'_I, which may differ from the inactivation rate constant β_I for an open channel. We fit our decay time courses with one exponential ([TPeA] = 0), or with two exponentials ([TPeA] = 50 or 100 μM). These time constants and their relative weights were used to generate estimates of k_{on}, k_{off}, β_I, and β'_I (see Methods).

Fig. 3 *A*, *B*, and *D*, plot these estimates as a function of membrane potential. The blocking rate constant (k_{on}) increases, and the unblocking rate (k_{off}) decreases, with depolarization. These results are expected if the blocking site for TPeA is located within the membrane electric field. Although neither of these estimated rate

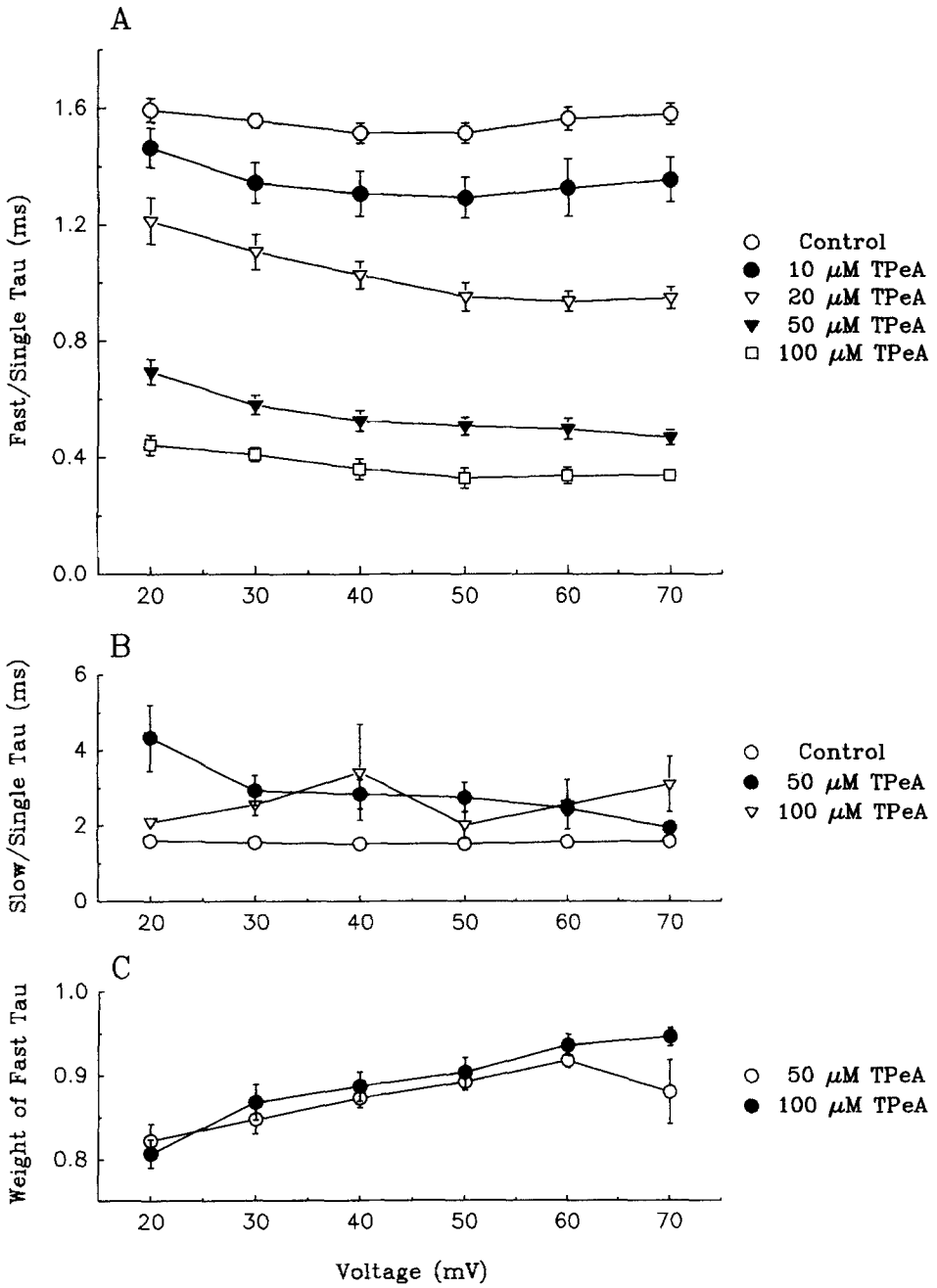


FIGURE 2. Inactivation time constants of TPeA-modified Na currents at positive voltages. The decay of currents were fit to single exponentials for [TPeA] of 0, 10, and 20 μM and two time constants for [TPeA] of 50 and 100 μM. (*A*) Single or faster time constants. (*B*) Single or slow time constant. (*C*) Fractional weight of fast component. Data show mean ± SEM for 3–7 cells.

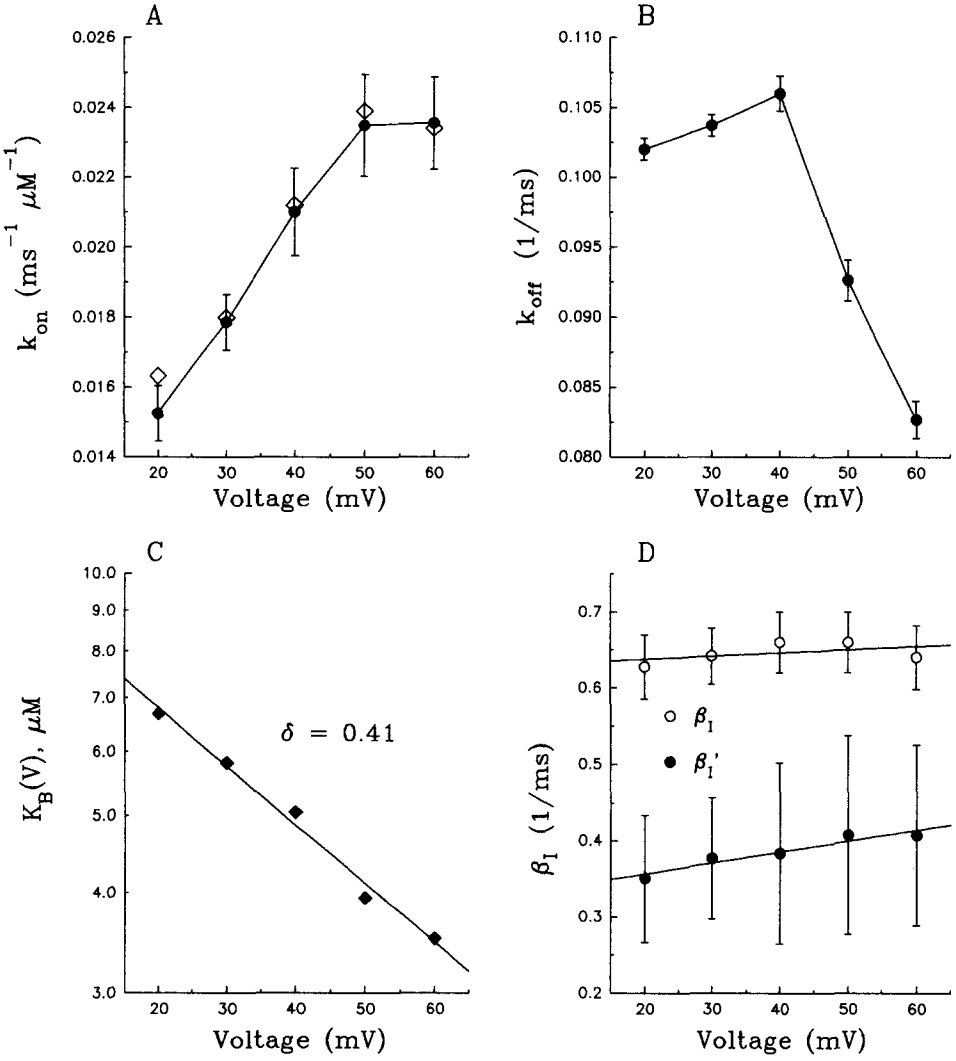


FIGURE 3. Estimates of rate constants for a three-state model. Estimates and standard errors are plotted. (*A*) Association rate k_{on} in units of μM^{-1} ms^{-1}. (*Open diamonds*) Slopes of linear regressions of fast rate vs [TPeA] for 0, 50 and 100 μM. (*B*) Unblocking rate k_{off} in units of ms^{-1}. (*C*) Inhibition constant $K_B(V)$, i.e., k_{off}/k_{on}, in units of micromolar. (*D*) Inactivation rates β_I and β'_I in units of ms^{-1}.

constants shows an exponential voltage dependence, their ratio, an estimate of the inhibition constant, $K_B(V)$, decreases exponentially with depolarization, as if the site were located a fractional distance of $\delta = 0.41 \pm 0.03$ into the membrane field from the inside. This distance is comparable with the estimates we obtained for smaller tetra-alkylammonium (TAA) compounds, which bind ~45% of the way into the electric field (O'Leary and Horn, 1994). Extrapolation of this relationship to 0 mV gives an inhibition constant of 9.8 μM. Fig. 3 *D* plots the estimates of the inactivation

rates for open and blocked channels. Open channels have a slightly greater inactivation rate than drug-blocked channels, i.e., $\beta_I \approx 1.7\beta'_I$. Clearly however, a blocked channel is not prevented from inactivating. Neither of these inactivation rates is strongly voltage dependent.

The fast and slow time constants for the above model are complicated functions of all four rate constants. However, if we assume that k_{off} is small and $\beta_I \approx \beta'_I$, then our model makes two relatively simple predictions (see Methods for details): (*a*) The faster decay rate will approximately equal to k_{on}[TPeA] + β_I, and therefore, will have a linear dependence on [TPeA] with slope k_{on} and Y-intercept β_I, and (*b*) β'_I will approximately equal to the slower decay rate. Fig. 4 shows the fast decay rate, or the single decay rate for [TPeA] < 50 μM, plotted against [TPeA]. The relationship is, as predicted, approximately linear. The plotted lines are the predictions of the faster rate from our three-state model. Note that the decay rates at TPeA < 50 μM fall below the theory lines. This is expected, because at these TPeA concentrations, the

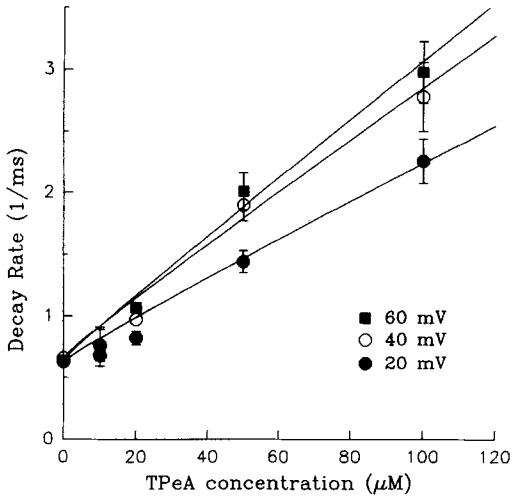


FIGURE 4. Rate of decay of Na current vs [TPeA]. A replot of the data of Fig. 2. The lines are the predictions from the estimates obtained for the 3-state model.

single resolvable time constant that we obtain is actually a composite of fast and slow time constants. The slope (k_{on}) increases with depolarization (Fig. 4), in accord with the voltage dependence of estimates of k_{on} (Fig. 3 *A*). The slopes from linear regression fits of similar plots, excluding the data at 10 and 20 μM TPeA, are plotted in Fig. 3 *A* as open diamonds, and agree with estimates obtained from a least squares fit to the complete three-state model. Furthermore, the estimates of β'_I (Fig. 3 *D*) are closely approximated by the inverse of the slower time constant of decay (Fig. 2 *B*), in accordance with the second prediction. Overall the analysis of our data is consistent with a voltage-dependent block of open channels by internal TPeA. Unlike the case for TAA compounds with side chains shorter than TBA (O'Leary and Horn, 1994), this block has measurable kinetics.

Raising External Na Concentration Relieves TPeA Effects on Decay Rate

Our previous study suggests that TAA compounds inhibit Na currents by binding deep within pore (O'Leary and Horn, 1994). This conclusion derives primarily from the effective electrical inhibition distances for TAA compounds with alkyl side chains of one to four carbons. To further analyze TAA binding, we examined the interaction between internal TPeA and external Na ions. Competition between external Na and internal TPeA for a common binding site is most likely to occur within the confines of the channel pore. As shown above, internal TPeA increases the apparent rate of Na channel inactivation. If Na ions can displace TPeA from its internal binding site, then these time constants should increase at higher external Na concentration ([Na]). In the presence of 20 μM TPeA, raising the external [Na] from 10 to 100 mM increases these time constants (Fig. 5), consistent with a relief of block by TPeA. In contrast, raising external [Na] under control conditions slightly decreases the rate of Na channel inactivation. External Na is least effective at antagonizing TPeA effects at

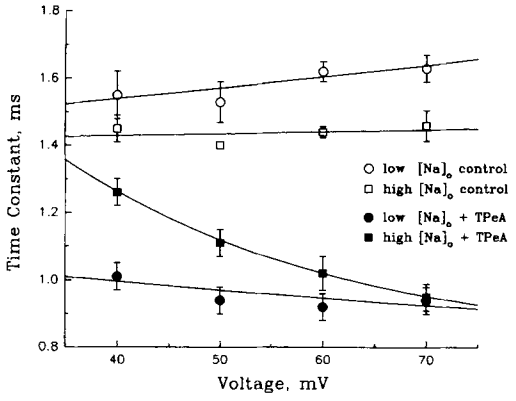


FIGURE 5. Effects of external [Na] on decay time constants. The inactivation time constants in the presence and absence of TPeA (20 μM) were determined at low (10 mM) and high (100 mM) external Na concentrations. Plotted lines represent fits to these data assuming exponential voltage dependence to the time constants. The voltages dependences were e-fold/248 mV (low [Na], control), /1.1 V (high [Na], control), /111 mV (low [Na] + TPeA), and /26 mV (high [Na] + TPeA).

more depolarized voltages, which tend to drive Na out of the channel. The data are consistent with a model in which increased Na occupation of a site within the channel reduces TPeA binding, therefore, partially relieving its effects on the decay of macroscopic Na current.

Use Dependence

In addition to accelerating inactivation, TPeA also produces a use-dependent block of Na channels. A train of 18-ms pulses to +50 mV at a rate of 1 Hz has little effect on the amplitudes of control Na currents (Fig. 6 *A*). In the presence of internal TPeA (20 μM) the identical stimulation results in a progressive decrease in Na current amplitude (Fig. 6 *B*). This use dependence is reminiscent of that described for local anesthetics (Hondeghem and Katzung, 1977), and shows that TPeA slows the recovery of inactivated channels, in spite of the very negative holding potential of −120 mV. After a train of depolarizations, complete recovery is attained by interpulse intervals of 60–90 s (data not shown).

The slow recovery from inactivation in the presence of TPeA has one of two likely origins. The first possibility is that the slow recovery reflects the slow unblocking rate, k_{off}, of TPeA-blocked channels. However, the estimate of k_{off} at +20 mV is ~ 0.1 ms^{-1} (Fig. 3 *B*), corresponding to a blocked dwell time of ~ 10 ms. Although we cannot measure k_{off} at −120 mV, its voltage dependence suggests that it will be larger at −120 than at +20 mV. Therefore the expected dwell time of TPeA in its blocking site is much too brief to be able to explain the use dependence. A more likely explanation is that when a drug-blocked channel inactivates, it traps the TPeA in its site, and this blocked-inactivated channel is slower to recover from inactivation than an unblocked channel.

Fig. 7 *A* plots the data of Fig. 6 as a relative reduction of peak current. After 1 s at −120 mV, 79% of the current recovers from inactivation with [TPeA] = 20 μM. According to our kinetic model this means that at least 21% of Na channels inactivate while blocked by TPeA. To understand the significance of this value, we used the calculated rate constants for our three-state model to estimate the fraction of channels that inactivate directly from the blocked state, *OB*, using Eq. 2 in Methods.

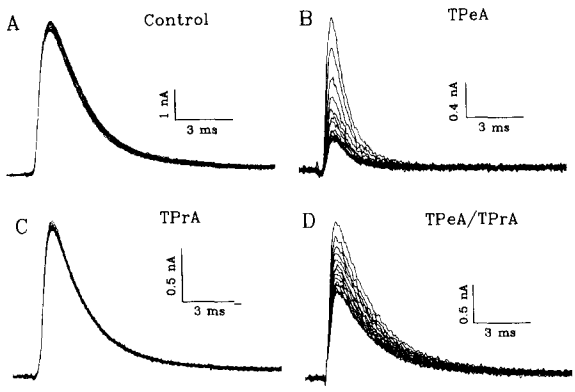


FIGURE 6. TPeA-induced use dependence and antagonism by internal TPrA. Each panel shows the response to a train of 16 depolarizing pulses to +50 mV for 18 ms from a holding potential of −120 mV and delivered at 1 Hz. (*A*) Control. (*B*) 20 μM internal TPeA. (*C*) 5 mM TPrA. (*D*) 20 μM TPeA and 5 mM TPrA.

At +50 mV and [TPeA] = 20 μM, we estimate that 44% of Na channels inactivate while blocked. This calculation shows that approximately half of the inactivated-blocked channels recover during the 1-s interval at −120 mV. These estimates imply a recovery time constant for inactivated-blocked channels of ~ 1.4 s, compared to a control value of < 60 ms in the absence of TPeA (data not shown). Fig. 7 *A* shows that 500 μM TBA exhibits use dependence similar in rate and extent as that seen for 20 μM TPeA. Both of these compounds, therefore, inhibit the recovery from inactivation.

Competition between TAA Compounds

In the accompanying paper, we show that unlike TPeA and TBA, short chain TAA compounds (i.e., 1–3 carbon side chains) block Na currents but have no effect on inactivation (O'Leary and Horn, 1994). To further characterize TPeA binding, we examined the interaction between TPeA and tetrapropylammonium (TPrA), a symmetrical three-carbon side chain TAA that binds $\sim 50\%$ of the way through the

electrical field. We were specifically interested to test if internal TPrA would compete with TPeA for its binding site. Examination of TPeA-modified Na currents with and without internal TPrA provides an indirect method of assaying TPeA binding. If both compounds compete for the same or overlapping sites, then addition of TPrA should displace TPeA, resulting in a decrease of the use-dependent block and reduced rates of current decay.

TPeA-induced use dependence was measured in the presence and absence of TPrA. TPrA (5 mM) should inhibit ~70% of Na currents at +50 mV (O'Leary and Horn, 1994), but is not use dependent (Figs. 6 *C* and 7 *A*). Simultaneous application of TPeA and TPrA reduces the extent of the use dependence seen with TPeA alone (Fig. 6 *D* and 7 *A*), consistent with a displacement of TPeA from its binding site.

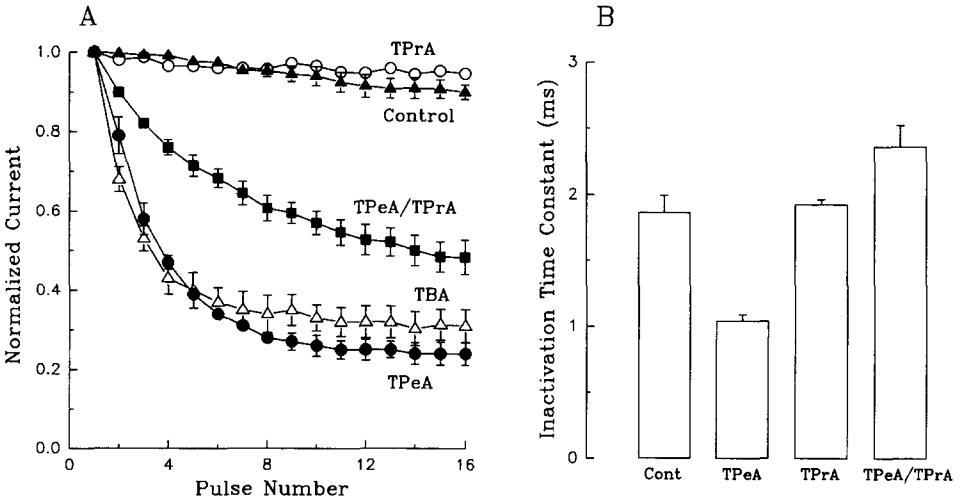


FIGURE 7. Antagonism of 20 μM TPeA effects by internal 5 mM TPrA, and use dependence of 500 μM TBA. Data obtained from currents similar to those in Fig. 6. (*A*) Effect of 1 Hz stimulation on normalized peak current at +50 mV from $n = 3$ cells. Peak currents for each pulse of the train are expressed as a fraction of that from the first pulse. (*B*) TPrA effects on decay time constants obtained at +50 mV from a holding potential of −120 mV.

These data also show that a sharp transition occurs between TPrA and TBA, because only TAA compounds larger than TPrA are strongly use dependent.

In addition to antagonizing use dependence, TPrA also inhibits the TPeA-induced increase in apparent inactivation rate (Figs. 6 *D* and 7 *B*). Internal TPeA (20 μM) reduces the decay time constant to 56% of the control value ($P < 0.02$, *t* test), while TPrA alone has no discernible effect ($P > 0.5$). Simultaneous addition of both compounds increases the apparent inactivation time constants slightly above control values ($P < 0.05$). These results suggest that TPeA and TPrA compete for a common binding site located within the pore. TPeA binding to this site both blocks Na channels, as evidenced by increases the apparent rate of inactivation, and stabilizes the channels in an inactivated state.

DISCUSSION

TPeA Is a Pore Blocker

We have shown previously that symmetrical TAA compounds with 1–4 carbon alkyl side chains act like fast, open channel blockers by reducing the amplitudes of single channel currents (O'Leary and Horn, 1994). The data presented here indicate that TPeA is also a reversible, voltage-dependent open channel blocker. This conclusion is supported by the following observations:

(*a*) The faster of two apparent inactivation (i.e., decay) rates at [TPeA] $\geq$ 50 μM is linearly dependent on [TPeA] with a Y-intercept approximately equal to the measured inactivation rate in the absence of TPeA (Fig. 4). This result is quantitatively consistent with our three-state model (see Methods) if the unblocking rate constant k_{off} is small, and if the inactivation rate from the open state is comparable in magnitude to the inactivation rate from a TPeA-blocked state. The estimates of rate constants (Fig. 3) are in accord with these limiting conditions.

(*b*) Two time constants of Na current decay are observed when [TPeA] $\geq$ 50 μM. The biexponential decay indicates that blocked channels can reopen, i.e., that the block is reversible. At lower [TPeA] the blocked state is not sufficiently occupied to contribute significantly to a slow rate of decay.

(*c*) Both blocking and unblocking rates are voltage dependent and contribute to the voltage-dependent occupancy of the TPeA binding site (Fig. 3). The voltage dependence, equivalent to the movement of a single positive charge a fractional distance of 0.41 $\pm$ 0.03 into the membrane field from the inside, is similar to that observed for smaller TAA compounds ($\delta \approx 0.45$; O'Leary and Horn, 1994), suggesting that all TAA compounds block at the same site. Further support for this idea is that TPrA, at a concentration that blocks ~70% of Na current, interferes with two of the effects of TPeA, namely the increased decay rate and the use dependence (Fig. 6).

(*d*) The effect of TPeA on current decay rate is antagonized by raising external [Na] (Fig. 5). The simplest explanation for this effect is that Na ions can occupy a site in the pore and inhibit TPeA binding. Similar *trans*-side effects have been observed for other cytoplasmic blockers of Na channels, such as the quaternary lidocaine derivative QX-314 (Cahalan and Almers, 1979*b*; Gingrich, Beardsley, and Yue, 1993; Zamponi, Doyle, and French, 1993), and strychnine (Shapiro, 1977). The mechanism of the antagonism of TPeA binding by external Na could be due either to a competitive occupancy of the same site, or to a destabilization (i.e., knock off) of the TPeA when a Na ion occupies an adjacent site within the pore. In principle, these mechanisms are distinguishable, since only the latter will have an effect on the unblocking rate k_{off}. Our data show that the antagonism by external Na is voltage dependent (Fig. 5), as might be expected for the voltage-dependent occupancy of a site by Na within the pore. Depolarization tends to drive a Na ion out of the channel into the external solution, thereby reducing its effect on TPeA binding. This voltage dependence, e-fold for 26 mV, could be explained by a binding site for Na nearly 100% across the membrane field from the outside. This voltage dependence is larger than might be expected for a simple competition between Na and TPeA for the same site, and therefore, favors a knock-off mechanism (see discussion in Hille, 1975).

Inactivation Traps TBA and TPeA inside the Channel

The use dependence seen with TBA and TPeA block is reminiscent of that observed in the presence of other large cationic blockers of Na channels, such as QX-314 (Cahalan, 1978; Yeh, 1978), 9-aminoacridine (Yeh, 1979), octylguanidinium (Kirsch, Yeh, Farley, and Narahashi, 1980), pancuronium (Yeh and Narahashi, 1977), and *N*-methyl strychnine (Cahalan and Almers, 1979*a*). The tertiary amine lidocaine also causes a use-dependent block of Na channels (Hondeghem and Katzung, 1977). Except for octylguanidinium, the use dependence of these blockers is reduced or abolished if the inactivation is removed enzymatically, for example by cytoplasmic pronase treatment of squid axon. This result has led several authors to propose that a drug-blocked channel can inactivate, and that a blocked-inactivated channel recovers very slowly at negative holding potentials, leading to a cumulative reduction of current for repetitive pulses. Our data are in accord with this idea. Our estimates of the unblocking rate k_{off} for TPeA are too large to be able to account for the slow recovery from inactivation. Therefore, a channel must be both inactivated and blocked to account for the use dependence we observe. Because both TBA and TPeA bind deeply within the pore, and the inactivation gate is believed to be a cytoplasmic "lid" (West, Patton, Scheuer, Wang, Goldin, and Catterall, 1992), it is reasonable to suppose that the lid traps these blockers within the site, and that the trapped blockers inhibit the lid from opening at negative holding potentials.

A Comparison of TAA Compounds

The TAA compounds we have studied, ranging in size from TMA (1-carbon chain length) to TPeA (5-carbon chain length), all block Na channels in a voltage-dependent manner, presumably by binding at the same site in the pore. However there are some notable differences between smaller and larger TAAs. For example, none of the smaller TAAs, from TMA to TPrA, influence the apparent rate of inactivation. Also, use dependence is observed only for compounds as large as TBA and TPeA. Finally, neither the activation nor inactivation gates can sense the presence of the smaller TAAs in their binding site (O'Leary and Horn, 1994). Our data suggest, however, that the inactivation gate is inhibited from opening by both TBA and TPeA. We cannot exclude the possibility that the activation gate is also involved in trapping the larger blockers, i.e., TBA and TPeA, in the channel, as observed in K channels (Armstrong, 1971).

Some of the differences between small and large TAAs may be explained simply by a higher affinity of the larger compounds for a binding site. Extrapolation of the exponential relationship between inhibition constant and alkyl chain length, determined from the TAA-induced reduction of single channel current amplitude (Fig. 3 *B* in O'Leary and Horn, 1994), predicts that the inhibition constant for TPeA at 0 mV will be 58 μM. This value is reasonably close to that obtained (i.e., 9.8 μM) by a very different method, namely the kinetic analysis used in this paper. The decrease of the inhibition constant as chain length increases could be accounted for by a monotonic decrease in the unblocking rate constant, k_{off}. As the TAA compounds increase in size, this change will lead to measurable blocking kinetics, which are unmeasurably fast in compounds smaller than TBA. A similar gradual change from a fast to a slow

blocker with increase in chain length has been observed for these same compounds in K channels (French and Shoukimas, 1981). Although this kinetic effect can explain the actions of TBA, and especially TPeA, on the apparent inactivation rate, it cannot explain the sudden appearance of use dependence for these two compounds because, as noted above, the estimated unblocking rate is much faster than the recovery from inactivation of blocked channels.

To understand the origin of use dependence, we used a molecular dynamics analysis to explore the dimensions and conformational flexibility of the TAA compounds. Fig. 8 shows scaled models of each of the TAAs, simulated in the absence of solvent. The elimination of solvent is permissible because our interest is on conformation and minimization of adverse steric interactions, which we did not want biased by solvent or binding site interactions. A conservative estimate is that differences ≥ 0.1 nM (1 Å) are significant. Our simulations show that the atoms of

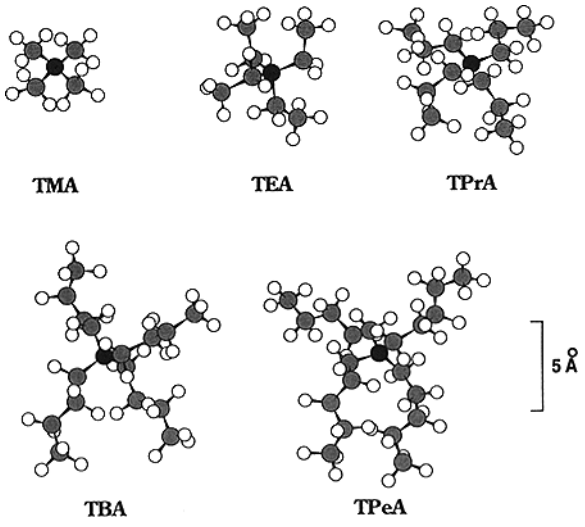


FIGURE 8. Structure of tetraalkylammonium cations based on molecular dynamic simulations. Conformations shown after 1,000 iterations.

TBA and TPeA do not explore any different geometry than those of the other TAAs, except that the alkyl chains are all *trans* from TMA to TPrA, whereas larger TAAs can adopt gauche conformations, in which the arms tend to draw together. Table I shows minimum and maximum distances from the nitrogen (*N*) to the end of the alkyl chain, and the distances from end-to-end, for all compounds. The variations in *N*-to-end distances are much less than those of end-to-end distances, showing that the four arms are relatively rigid, but can move closer and further apart, like the flippers of an applauding seal. In fact the shortest end-to-end distance for TPeA, 8.5 Å, is shorter than that for TPrA. This minimum distance for TPeA is just a bit larger than the closest approach permitted by the van der Waals radii. The maximum end-to-end distances increase monotonically with chain length. We find no evidence that the larger TAAs can collapse into a ball.

Because the effective electrical distance for TAA binding is approximately the same in all compounds, $\delta \approx 0.45$, it is reasonable to assume that the central *N* has a similar position in the binding site for all compounds, and that this site is deep within the pore. We imagine that one or more alkyl arms extend in the cytoplasmic direction. This physical picture allows us to speculate about the difference in use dependence between smaller and larger TAAs. The maximum *N*-to-end length of TPrA is 6.1 Å, whereas that of TBA is 7.4 Å. This additional 1.3 Å may be sufficient for an alkyl arm of pore-bound TBA to reach the cytoplasmic inactivation gate when it closes. This interaction stabilizes the inactivation gate in its closed conformation, and slows recovery after a depolarization, leading to a use-dependent reduction of current. This interpretation can explain the fact that blocking properties of TAA compounds change gradually with chain length, but that use dependence appears suddenly for molecules larger than TPrA. Because the inactivation rate constant has little voltage dependence (Fig. 3 *D;* see also Greeff and Forster, 1991), and the binding site is about half way through the membrane field, our data imply that 45% of the electric field encompasses a distance of between 6 and 7 Å. Blocking experiments in K channels show that 65% of the electric field drops over 6–7 Å (Miller, 1982), suggesting that hH1 Na channels have a longer pore region.

TABLE I

Minimum and Maxumum Distances between the Nitrogen (N) of TAA Compounds and the End of the Alkyl Chain

No. of C atoms	N-to-end	End-to-end
1	3.5–3.6	6.4–6.6
2	4.5–4.8	7.1–8.8
3	5.9–6.1	8.7–11.1
4	6.4–7.4	10.0–13.8
5	7.4–8.8	8.5–15.2

All distances, given in Angstroms, were obtained from molecular simulations adding 2 Angstroms to the *N*-to-end distances and 4 Angstroms to the end-to-end distances to take into account the van der Waals radii.

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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.* 306:436–441.

Armstrong, C. M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *Journal of General Physiology.* 58:413–437.

Cahalan, M. D. 1978. Local anesthetic block of sodium channels in normal and pronase-treated squid giant axons. *Biophysical Journal.* 23:285–311.

Cahalan, M. D., and W. Almers. 1979*a*. Block of sodium conductance and gating current in squid giant axons poisoned with quaternary strychnine. *Biophysical Journal.* 27:57–74.

Cahalan, M. D., and W. Almers. 1979*b*. Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin. *Biophysical Journal.* 27:39–55.

Choi, K. L., R. W. Aldrich, and G. Yellen. 1991. Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels. *Proceedings of the National Academy of Sciences, USA.* 88:5092–5095.

Choi, K. L., C. Mossman, J. Aubé, and G. Yellen. 1993. The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron.* 10:533–541.

French, R. J., and J. J. Shoukimas. 1981. Blockage of squid axon potassium conductance by internal tetra-*N*-alkylammonium ions of various sizes. *Biophysical Journal.* 34:271–291.

Gingrich, K. J., D. Beardsley, and D. T. Yue. 1993. Ultra-deep blockade of Na^+ channels by a quaternary ammonium ion: catalysis by a transition-intermediate state? *Journal of Physiology.* 471:319–341.

Goldman, L. 1976. Kinetics of channel gating in excitable membranes. *Quarterly Review of Biophysics.* 9:491–526.

Greeff, N. G., and I. C. Forster. 1991. The quantal gating charge of sodium channel inactivation. *European Biophysics Journal.* 20:165–176.

Hille, B. 1975. Ionic selectivity of Na and K channels of nerve membranes. *In* Membranes: A Series of Advances. Vol. 3. Lipid Bilayers and Biological Membranes: Dynamic Properties. G. Eisenman, editor. Marcel Dekker, Inc. NY. 255–323.

Hondeghem, L. M., and B. G. Katzung. 1977. Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochimica Et Biophysica Acta.* 472:373–398.

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

Lehmann, E. L. 1983. Theory of Point Estimation. John Wiley and Sons, Inc., NY. 360–368.

Miller, C. 1982. Bis-quaternary ammonium blockers as structural probes of the sarcoplasmic reticulum K^+ channel. *Journal of General Physiology.* 79:869–891.

O'Leary, M. E., and R. Horn. 1994. Internal block of human heart sodium channels by symmetrical tetra-alkylammoniums. *Journal of General Physiology.* 104:507–522.

Patlak, J. 1991. Molecular kinetics of voltage-dependent Na^+ channels. *Physiological Reviews.* 71:1047–1080.

Powell, M. J. D. 1978. A fast algorithm for nonlinearity constrained optimization calculations. *In* Numerical Analysis. Lecture Notes in Mathematics No. 630. G. A. Watson, editor. Springer-Verlag, Berlin. 144–157.

Scanley, B. E., D. A. Hanck, T. Chay, and H. A. Fozzard. 1990. Kinetic analysis of single sodium channels from canine cardiac Purkinje cells. *Journal of General Physiology.* 95:411–437.

Shapiro, B. I. 1977. Effects of strychnine on the sodium conductance of the frog node of Ranvier. *Journal of General Physiology.* 69:915–926.

Vandenberg, C. A., and F. Bezanilla. 1991. A sodium channel gating model based on single channel, macroscopic ionic, and gating currents in the squid giant axon. *Biophysical Journal.* 60:1511–1533.

Vandenberg, C. A., and R. Horn. 1984. Inactivation viewed through single sodium channels. *Journal of General Physiology.* 84:535–564.

West, J. W., D. E. Patton, T. Scheuer, Y. Wang, A. L. Goldin, and W. A. Catterall. 1992. A cluster of hydrophobic amino acid residues required for fast Na^+-channel inactivation. *Proceedings of the National Academy of Sciences, USA.* 89:10910–10914.

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

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

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

Yue, D. T., J. H. Lawrence, and E. Marban. 1989. Two molecular transitions influence cardiac sodium channel gating. *Science.* 244:349–352.

Zamponi, G. W., D. D. Doyle, and R. J. French. 1993. Fast lidocaine block of cardiac and skeletal muscle sodium channels: one site with two routes of access. *Biophysical Journal.* 65:80–90.