

# Internal Block of Human Heart Sodium Channels by Symmetrical Tetra-alkylammoniums

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**ABSTRACT** The human heart Na channel (hH1) was expressed by transient transfection in tsA201 cells, and we examined the block of Na current by a series of symmetrical tetra-alkylammonium cations: tetramethylammonium (TMA), tetraethylammonium (TEA), tetrapropylammonium (TPrA), tetrabutylammonium (TBA), and tetrapentylammonium (TPeA). Internal TEA and TBA reduce single-channel current amplitudes while having little effect on single channel open times. The reduction in current amplitude is greater at more depolarized membrane potentials. Analysis of the voltage-dependence of single-channel current block indicates that TEA, TPrA and TBA traverse a fraction of 0.39, 0.52, and 0.46 of the membrane electric field to reach their binding sites. Rank potency determined from single-channel experiments indicates that block increases with the lengths of the alkyl side chains (TBA > TPrA > TEA > TMA). Internal TMA, TEA, TPrA, and TBA also reduce whole-cell Na currents in a voltage-dependent fashion with increasing block at more depolarized voltages, consistent with each compound binding to a site at a fractional distance of 0.43 within the membrane electric field. The correspondence between the voltage dependence of the block of single-channel and macroscopic currents indicates that the blockers do not distinguish open from closed channels. In support of this idea TPrA has no effect on deactivation kinetics, and therefore does not interfere with the closing of the activation gates. At concentrations that substantially reduce Na channel currents, TMA, TEA, and TPrA do not alter the rate of macroscopic current inactivation over a wide range of voltages (-50 to +80 mV). Our data suggest that TMA, TEA, and TPrA bind to a common site deep within the pore and block ion transport by a fast-block mechanism without affecting either activation or inactivation. By contrast, internal TBA and TPeA increase the apparent rate of inactivation of macroscopic currents, suggestive of a block with slower kinetics.

## INTRODUCTION

Internal tetraethylammonium (TEA) inhibits many K currents by a rapid open-channel blocking mechanism with relatively high affinity (0.1–20 mM). The block by

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TEA is both voltage and concentration dependent. Analysis of the voltage dependence indicates that TEA traverses ~15% of the membrane electric field to reach its binding site (Yellen, Jurman, Abramson, and MacKinnon, 1991). Point mutations in the so-called H5 region, which is believed to line the pore of K channels, reduce the TEA block (Yellen et. al., 1991; Kirsch, Drewe, Hartmann, Taglialatela, De Biasi, Brown, and Joho, 1992), suggesting that the TEA binding site is located in the permeation pathway. In addition to blocking K channels, internal TEA slows the inactivation of macroscopic currents (Choi, Aldrich, and Yellen, 1991). The fractional block of the amplitude of macroscopic K current is proportional to the reduced rate of macroscopic current inactivation, indicating that TEA binding and inactivation are mutually exclusive. Because the K channels in these studies inactivate by a "ball-and-chain" mechanism (Hoshi, Zagotta, and Aldrich, 1990), the data suggest that internal TEA competes with the inactivation ball for a common or overlapping site at the inner mouth of the K channels. In noninactivating K channels (Armstrong, 1971; French and Shoukimas, 1981), or in K channels in which the inactivation ball is deleted by mutagenesis (Choi, Mossman, Aubé, and Yellen, 1993), long chain derivatives of TEA, applied internally, mimic inactivation, suggesting that normal inactivation occurs by a similar blocking mechanism. Overall, biophysical and mutagenesis studies indicate that internal TEA binds superficially to the inner mouth of K channels. When bound to this site, TEA blocks the pore and interferes with the closing of the inactivation gate.

Although the ball-and-chain mechanism of inactivation was originally proposed for Na channels (Armstrong and Bezanilla, 1977), the evidence for this mechanism is weaker in Na channels than in K channels. Mutagenesis studies of Na channels have identified a putative inactivation gate, a short cytoplasmic loop connecting the third and fourth homologous domains of Na channels (Stühmer, Conti, Suzuki, Wang, Noda, Yahagi, Kubo, and Numa, 1989; West, Patton, Scheuer, Wang, Goldin, and Catterall, 1992). Rather than four inactivation particles, as is the case in K channels, Na channels are postulated to use this single loop, anchored at each end, as the inactivation gate (West et. al., 1992). How this "hinged lid" causes inactivation is not known.

In this paper we have used a series of symmetrical tetra-alkylammonium (TAA) cations as intracellular blockers of the human heart Na channel (hH1). Our goal was to characterize the mechanism of TAA block of hH1 Na channels and explore its effects on macroscopic current inactivation. Preliminary results have appeared in an abstract (O'Leary, Chahine, Chen, Kallen, and Horn, 1993).

## METHODS

### *Functional Expression in tsA201 Cells*

The cDNA for hH1 (Gellens, George, Chen, Chahine, Horn, Barchi, and Kallen, 1992) was obtained from Drs. L.-Q. Chen and R. G. Kallen, and subcloned into the XbaI and HindIII sites of the pRc/CMV plasmid (Invitrogen Corp., San Diego, CA). Transient transfection was used to express hH1 in tsA201 cells, a transformed human kidney (HEK 293) cell line stably expressing an SV40 temperature sensitive T antigen.

The procedure for transfection was as follows. 20 µg DNA (10 µg of plasmid encoding hH1

and 10  $\mu\text{g}$  of salmon sperm DNA [Sigma Chemical Co., St. Louis, MO] were placed into an eppendorf tube containing 0.5 ml of 250 mM  $\text{CaCl}_2$ . This DNA/ $\text{CaCl}_2$  mixture was added slowly to 0.5 ml of a 2x HeBS solution (2x HeBS is [in millimolar]: 274 NaCl, 40 Hepes [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 12 dextrose, 10 KCl, 1.4  $\text{Na}_2\text{HPO}_4$  [pH 7.05]). This mixture was left at room temperature for 20 min and then dripped onto a 100-mm dish of cells,  $\sim 50\%$  confluent, initially containing 10 ml of tissue culture medium (DMEM enriched with 10% fetal bovine serum and 1% penicillin-streptomycin [GIBCO Laboratories, Grand Island, NY]). After  $\sim 12$  h the cells on this dish were split onto 35-mm tissue culture dishes that were used as chambers for patch recording. Typically  $\sim 50$ – $75\%$  of the cells had large ( $> 1$  nA at  $-10$  mV) Na currents 2–3 d after transfection.

### *Electrophysiology*

Standard whole cell and outside-out patch recordings were made as described previously (Horn and Vandenberg, 1984; Vandenberg and Horn, 1984). Briefly, sylvargard (Dow Corning Corp., Midland, MI), fire-polished pipettes of Corning 8161 glass were used. Currents were recorded and filtered (5 kHz,  $-3\text{dB}$ ) with an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc., Burlingame, CA), and data were acquired with pCLAMP (Axon Instruments, Inc.). In whole cell recordings, the cells were dialyzed at least 15 min before recording data, the series resistance was  $< 4$  M $\Omega$ , 80% of which was compensated. To avoid use-dependent effects of TAA compounds, all stimuli for whole cell experiments were delivered at  $\leq 0.2$  Hz. Temperature was regulated by a TC-10 controller (Dagan, Minneapolis, MN) connected to two peltier devices, and measured by a thermistor  $< 1$  mm from the recording pipette. Single channel recordings were made at room temperature,  $21$ – $23^\circ\text{C}$ . The pipette and bath solutions were varied in different experiments, as indicated in the figure legends. In general the Na concentration was kept low enough, by substitution with intracellular Cs or extracellular choline, to prevent series resistance errors  $> 3$  mV. All TAA compounds were used as chloride or fluoride salts and were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other solution ingredients were obtained from Sigma Chemical Co.

Whole cell data were analyzed by a combination of pCLAMP programs, Sigmaplot (Jandel Scientific, San Raphael, CA), and our own FORTRAN programs. Single-channel records were idealized using TRANSIT (Dr. A. VanDongen, Duke University), and further analyzed with our own programs. Amplitudes of single-channel currents were derived from fits to amplitude histograms. Mean open times were calculated by the method previously described in (Horn, Vandenberg, and Lange, 1984).

## RESULTS

The mechanisms of blockers are easiest to understand when studied at the single-channel level, where the kinetics and voltage dependence of block may be examined without ambiguity caused by interactions between the gating mechanisms of the channel and the presence of a blocker in its site. We will therefore first describe the effects of TAA compounds on single-channel currents, and then show their effects on macroscopic currents. Both single-channel and whole-cell data were obtained from tsA201 cells transiently transfected with a plasmid encoding hH1.

### *Open-Channel Block by TAA Compounds*

TMA is a fast open-channel blocker of Na channels of rat skeletal muscle (Horn, Patlak, and Stevens, 1981). Fast blockers produce brief interruptions in open channel events, caused by short duration residence times in the pore. The inability to

accurately resolve these fast events, due to low-pass filtering, results in an apparent concentration-dependent reduction in the single-channel current amplitudes (Yellen, 1984). Fig. 1 shows the effects of internal TEA (17.5 mM) and TBA (0.25 mM) on single hH1 Na channels in outside-out patches. The reduction in the amplitudes of single channel currents is more obvious at  $-20$  mV than at  $-40$  mV, consistent with

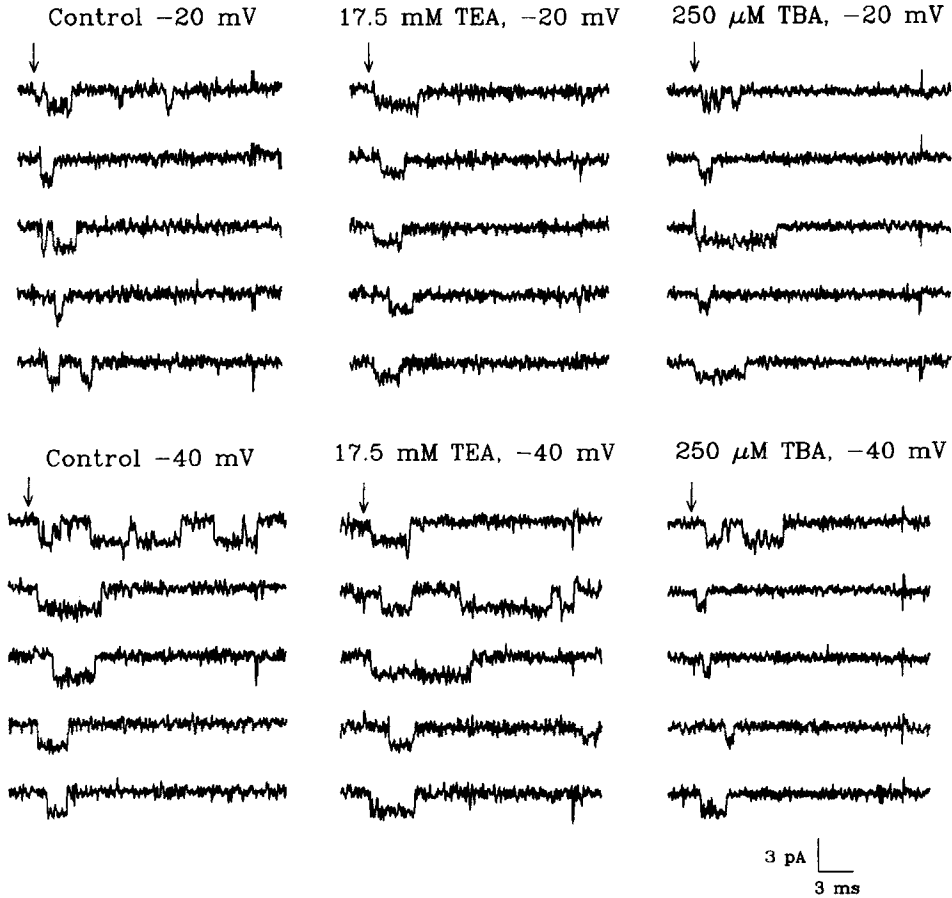


FIGURE 1. Effect of internal TEA (17.5 mM) and TBA (0.25 mM) on single-channel currents from outside-out patches of tsA201 cells transiently expressing hH1. Holding potential,  $-120$  mV; depolarizations (*arrows*) to  $-20$  and  $-40$  mV. Displayed data low-pass filtered at 5 kHz. For analysis these data were further digitally filtered at 3 kHz. Bath solution (in millimolar): 150 NaCl, 2 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 Na-HEPES, pH 7.4. Pipette solution (in millimolar): 130 CsF, 10 CsCl, 5 EGTA, 10 Cs-HEPES, pH 7.4.

a voltage-dependent, fast blocking mechanism. To quantitate this voltage dependence, the fractional block was calculated and the effective electrical distance determined. The effective electrical distance  $\delta$  of the TAA binding site within the membrane field can be estimated from the slopes of  $\ln([1-F_u]/F_u)$  vs  $V_m$  plots (Fig. 2A), where  $F_u$  is the fraction of unblocked current, obtained by measuring the

amplitude of single-channel currents in the presence and absence of the blocker, and  $V_m$  is the membrane potential (Horn et al., 1981). Internal TEA, TPrA and TBA block single Na channels by binding to sites with  $\delta$  values of  $0.39 \pm .07$ ,  $0.52 \pm .07$ , and  $0.46 \pm .02$ , respectively, of the distance into the membrane electric field from the cytoplasmic mouth of the channel.

The apparent reduction of single-channel current by TEA is not accompanied by effects on the mean open times of single channels between  $-70$  and  $-10$  mV (Fig. 2 B), showing that (a) the occupancy of the blocking site by TEA does not affect the closing rate of an open channel, and (b) the dwell time of TEA in its binding site is too brief to be resolved in our recordings. TBA, by contrast, slightly reduced the mean open time over a range of voltages (Fig. 2 B). This result is reminiscent of those obtained for intracellular QX-314, which acts as both a fast blocker (reducing

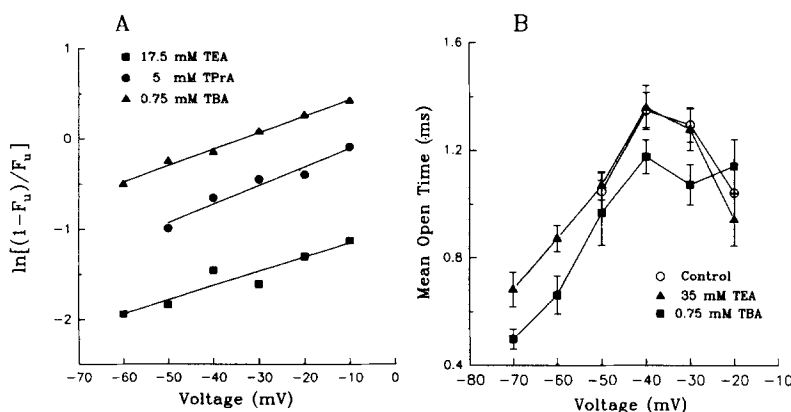


FIGURE 2. Effect of internal TAA compounds on amplitude and mean open time of single-channel currents. (A) Semi-log plot of the fraction of blocked-to-unblocked current amplitude vs voltage. Block was determined from single-channel records in the presence and absence of TAAs. Slopes of regression lines give voltage dependences of  $\delta = 0.39 \pm .07$ ,  $0.52 \pm .07$ , and  $0.46 \pm .02$  for TEA, TPrA, and TBA, respectively. (B) Effect of 35 mM TEA and 0.75 mM TBA on open times.

apparent amplitude of single-channel currents) and a slow blocker (reducing open time) of cardiac Na channels (Gingrich, Beardsley, and Yue, 1993).

#### *Potency of TAA Compounds*

The potency of TAA compounds increases with the length of the alkyl side chain (i.e., TBA > TPrA > TEA > TMA). For example, at  $-10$  mV  $500 \mu\text{M}$  TBA (four carbon side chains) inhibits  $\sim 50\%$  of the amplitude of single channel currents, while TMA (one carbon side chains) at a 200-fold higher concentration blocks at most 15% of Na currents. Assuming a first order binding reaction the blocking dissociation constants ( $K_B(V)$ ) for the reduction of single channel currents were calculated and plotted versus membrane potential (Fig. 3 A). The straight lines are consistent, on this semi-logarithmic plot, with an exponential voltage dependence of TAA binding

( $\delta = 0.45$ ), and provide estimates of the blocking constants of these compounds for their site at zero membrane potential [ $K_B(0)$ ] (Fig. 3 B). The affinities for the TAA compounds at 0 mV increase exponentially (i.e., the binding energy increases linearly) with the alkyl chain length over a range of nearly four orders of magnitude.

At concentrations that affected macroscopic Na currents ( $> 1 \mu\text{M}$ ; see below), we were unsuccessful, in preliminary experiments, in measuring single channel currents in the presence of TPeA. This is due in part to the fact that TPeA causes a use-dependent block of Na channels (O'Leary, Kallen, and Horn, 1994). In subsequent experiments, using hH1 Na channels mutated to remove inactivation (West et al., 1992), and expressed in *Xenopus* oocytes, we have found that internal TBA blocks with similar potency and voltage dependence as we report here (O'Leary et al., 1993),

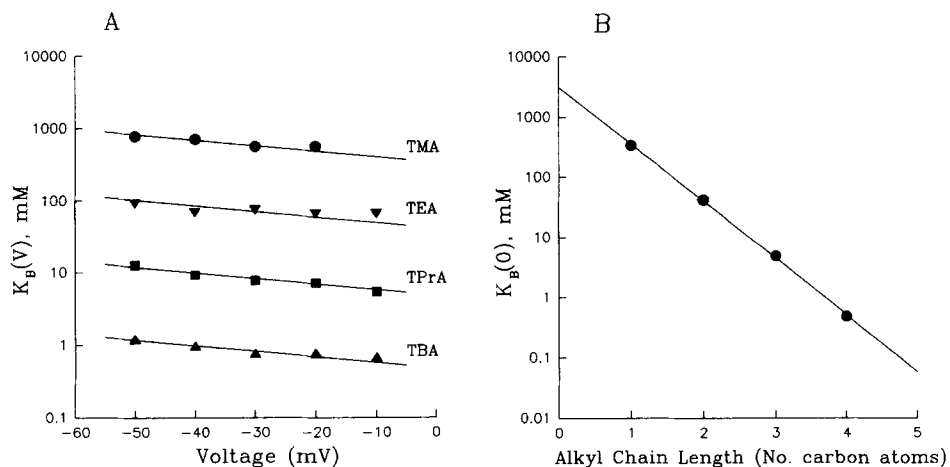


FIGURE 3. Voltage and alkyl chain-length dependence of TAA block. (A) Semi-log plot of inhibition constant  $K_B(V)$  for TAA block, calculated from fractional reduction in amplitudes of single-channel currents, vs voltage. Data and fit with a slope of  $\delta = 0.45$  are drawn to a one-site blocking model. The estimated blocking constants at 0 mV [ $K_B(0)$ ] are:  $335 \pm 15$  mM, TMA;  $41.4 \pm 3.3$  mM, TEA;  $4.84 \pm 0.16$  mM, TPrA;  $0.48 \pm 0.02$  mM, TBA. (B) Semi-log plot of extrapolated  $K_B(0)$  vs alkyl chain length. The regression line shows that  $K_B(0)$  is an exponential function of chain length, decreasing by a factor of 9.46 for each added methylene.

but that TPeA reduces the mean open time while having only minor effects on the amplitude of the single-channel currents (O'Leary and Horn, manuscript in preparation). These data suggest that TPeA has slower unblocking kinetics than the other TAA compounds we have examined. These ideas, with supporting data, are elaborated in the accompanying paper (O'Leary et al., 1994).

#### TAA Inhibition of Whole-Cell Na Currents

Fig. 4 shows the effects of internal TEA, TBA, and TPeA on the whole-cell hH1 Na currents. At concentrations that reduce the amplitudes of single-channel currents by  $\sim 50\%$ , TEA and TBA inhibit outward more than inward Na currents, consistent with a voltage dependent block. Qualitatively similar effects are observed with 115 mM

TMA and 5 mM TPrA (data not shown). To better illustrate the voltage dependence of TAA block the peak Na currents were normalized to the expected inhibition of single-channel currents at  $-40$  mV, using the blocking parameters from Fig. 3. Fractional peak current versus voltage plots (Fig. 5 *A*) show that the block produced by internal TAA compounds increases with depolarization; i.e., these compounds change the shape of the  $I$ - $V$  relationship, reducing peak current more strongly at

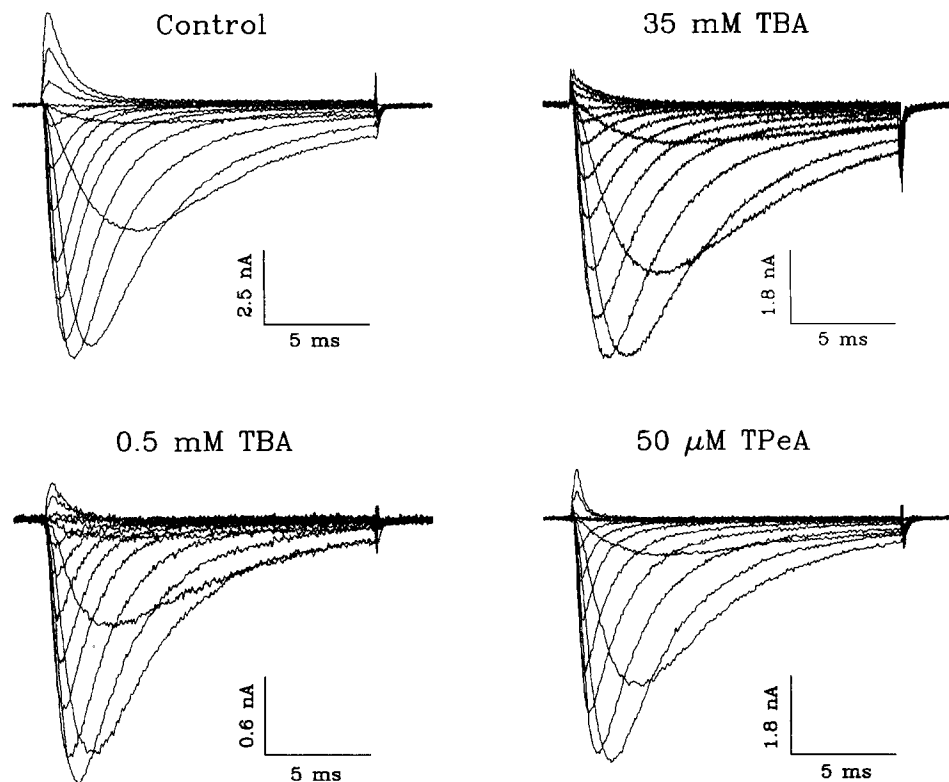


FIGURE 4. Effect of intracellular TEA (35 mM), TBA (500  $\mu$ M), and TPeA (50  $\mu$ M) on whole-cell recordings of hH1 Na currents in tsA201 cells. Each panel shows a family of currents obtained by voltage steps in 10-mV increments, between  $-80$  and  $+70$  mV, from a holding potential of  $-120$  mV. External solution (in millimolar): 75 NaCl, 75 choline Cl, 2 KCl, 1.5  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , 10 glucose, 10 Na-HEPES (pH 7.4). Internal solution (in mM): 95 CsF, 35 NaCl, 10 CsCl, 5 EGTA, 10 Cs-HEPES (pH 7.3). Temperature  $15^\circ\text{C}$ .

more positive voltages. Despite inhibiting whole-cell Na currents, however, internal TMA, TEA, TPrA, and TBA do not appear to alter the kinetics of macroscopic current inactivation. This is shown more clearly for outward currents, below.

#### *Voltage Dependence of TAA Inhibition of Macroscopic Currents*

We analyzed the voltage dependence of block by internal TEA, TBA, and TPrA from  $-55$  to  $+20$  mV, using the previously described method (Fig. 2 *A*), and the data are

plotted in Fig. 5 *B*. The fractional block of  $0.43 \pm .04$ , obtained from the slope of this plot, is consistent with that measured for block of single channels. We plotted the expected  $I$ - $V$  relationship for block by TEA, TBA, and TPrA in Fig. 5 *A* as theory lines. The altered shapes of the  $I$ - $V$  curves are largely explained, over a wide voltage range, by the voltage-dependent reduction of single-channel currents. This result is surprising, because TEA derivatives typically show a greater voltage dependence of block for macroscopic K currents than observed at the single-channel level (Choi et al., 1993). This is primarily attributed to the fact that TEA can only block K channels when they are open. Open probability and block by internal TEA, both of which increase with depolarization, combine to contribute to the reduction of macroscopic

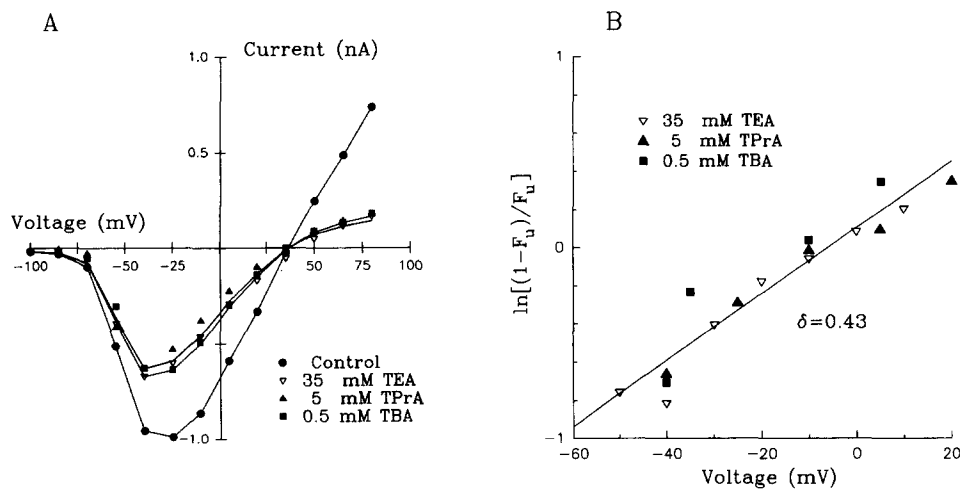


FIGURE 5. Effect of TAA compounds on peak current-voltage curves. (A)  $I$ - $V$  plots. Data obtained as in Fig. 4. The peak currents in presence of TAA compounds were normalized for the amount of block expected for single-channel block at  $-40$  mV. The currents were more reduced by TAAs at more positive voltages. The lines for  $I$ - $V$  curves in the presence of TAAs were obtained by multiplying the  $I$ - $V$  plot for the control by the expected fraction of unblocked current in the presence of the TAAs, using the slope of the relationship plotted in B. (B) Voltage dependence of reduction of macroscopic current. Semi-log plot of the fraction of blocked-to-unblocked current amplitude vs voltage. Data obtained from the normalized  $I$ - $V$  curves shown in Fig. 5 A. The effective electrical distance was obtained as in Fig. 2 A.

K current. In our experiments (Fig. 2 A and Fig. 5 B), the similar voltage dependence of single-channel and macroscopic block over a range where the peak open probability of Na channels increases steeply indicates that TAA compounds, to a first approximation, do not distinguish channels with closed or open activation gates, blocking both with equivalent efficacy.

To test this idea in more detail, we examined the effects of TPrA on the kinetics and voltage dependence of deactivation. Specifically we tested whether an open channel can close while TPrA occupies its blocking site. If not, we predict that deactivation kinetics will be slowed by the blocker, exactly as expected for the slowing of inactivation kinetics by TEA in K channels (Choi et al., 1991). This assumes that



channel closing (at voltages of  $-120$  to  $-60$  mV) is well-represented by a single transition that can only occur if the blocking site is empty. The deactivation rate constant will then be scaled by the fraction of unblocked channels,  $F_u$ , as predicted by the voltage-dependent block of single-channel currents (Fig. 3). Fig. 6, *A* and *B*, show

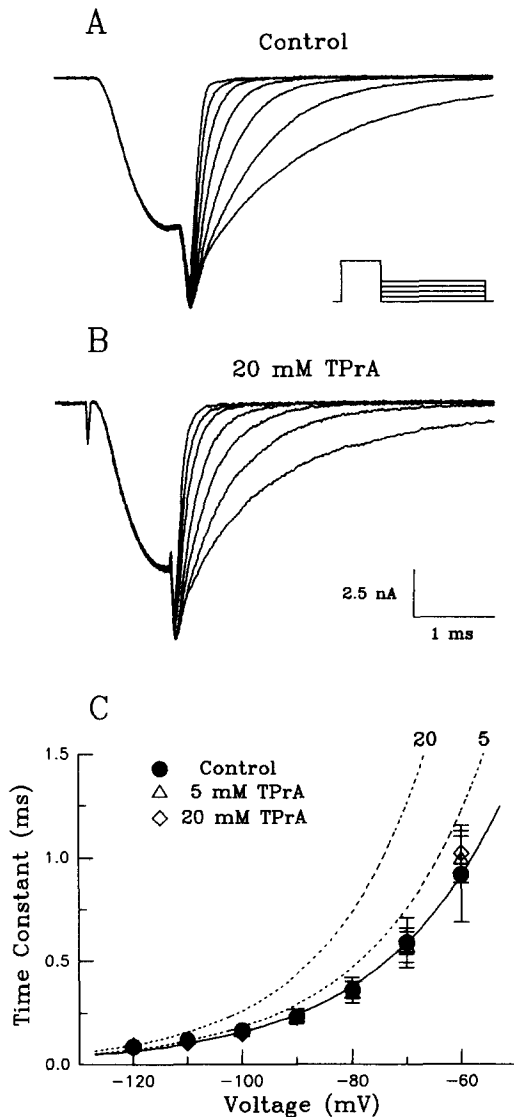


FIGURE 6. Lack of effect of internal TPrA on deactivation kinetics. (*A*) Control; and (*B*) 20 mM TPrA currents. Currents activated at  $-20$  mV, and tail currents were measured between  $-120$  and  $-60$  mV in 10-mV increments. (*C*) Deactivation time constants plotted for 0, 5, and 20 mM TPrA. The time constants of deactivation show an exponential dependence on voltage (*solid line*), changing e-fold for 22.7 mV. The predicted effects of TPrA (*dashed lines*) were calculated assuming a blocking constant  $K_B(0)$  of 5 mM and a site  $\delta = 0.45$  into the membrane field (see Fig. 3). External solution (in millimolar): 150 NaCl, 2 KCl, 1.5  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , 10 Na-HEPES (pH 7.4). Internal solution (in millimolar): 130 CsF, 10 CsCl, 5 EGTA, 10 Cs-HEPES (pH 7.3). 5 mM TPrA was simply added to the internal solution. 20 mM TPrA was substituted for Cs. The average series resistance for eight experiments was  $2 \text{ M}\Omega$  (before 80% compensation). Average cell capacitance, 20 pF. Temperature  $15^\circ\text{C}$ .

tail currents, measured at  $15^\circ\text{C}$ , in a control cell and in a cell dialyzed with 20 mM TPrA. This concentration is predicted to reduce the amplitude of the single-channel current at  $-90$  mV, for example, to a factor of 0.54 of its original value, and simultaneously cause a 1.85-fold  $[(0.54)^{-1}]$  increase in the deactivation time constant,

according to the above model. However, no change in the time course of tail currents is observed, either at 5 mM or 20 mM TPrA, over a range of  $-120$  to  $-60$  mV. These currents are well fit by a single exponential decay; the time constants are plotted in Fig. 6 C along with the predicted effects of TPrA (*dashed lines*). These results show that we should be able to resolve an effect on deactivation kinetics. The lack of effect supports our previous findings showing that activation gates are insensitive to the presence of these small TAAs in their blocking sites.

In contrast to TMA, TEA, and TPrA, TPeA causes an apparent increase in the rate of macroscopic inactivation, especially at depolarized voltages (Fig. 4). We will show similar properties for TBA at high concentration and depolarized voltages.

#### *Effect of TAA Compounds on Macroscopic Inactivation*

In *Shaker* K channels, internal TEA slows the rate of macroscopic current inactivation, suggesting that TEA competes with the inactivation ball for its binding site on the

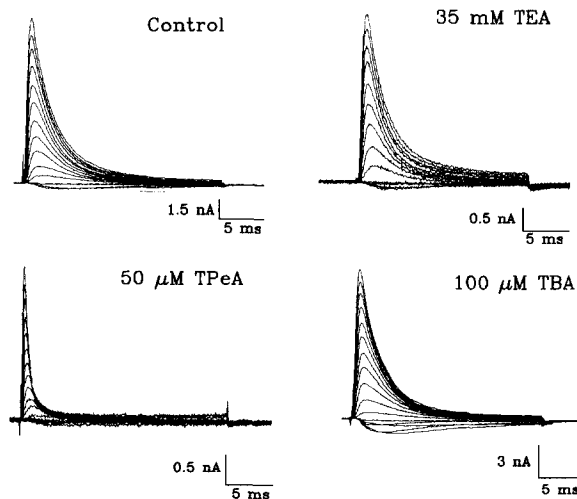


FIGURE 7. Effect of TAA compounds on outward Na currents, using a reversed Na gradient. Cells were held at  $-120$  mV and pulsed for 20 ms in 10-mV increments between  $-70$  and  $+80$  mV. External solution (in millimolar): 75 NaCl, 75 choline Cl, 2 KCl, 1.5  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , 10 glucose, 10 Na-HEPES (pH 7.4). Internal solution (in millimolar): 95 CsF, 35 NaCl, 10 CsCl, 5 EGTA, 10 Cs-HEPES (pH 7.3). Temperature  $12^\circ\text{C}$ .

inside of the channels (Choi et al., 1991). Our data contrast with these observations in K channels, in that TAAs either have no effect or increase the apparent rate of inactivation in Na channels. To better quantify the effects of TAA compounds on inactivation we examined the block of outward Na currents at large depolarizing potentials, where the rate of inactivation from the open state should be much greater than the rate of deactivation (Vandenberg and Horn, 1984; Yue, Lawrence, and Marban, 1989). Measuring the relaxation of Na currents at large depolarizations therefore yields an estimate of the Na channel inactivation rate that is minimally contaminated by deactivating or opening Na channels. To enhance the resolution of outward Na currents, we used a reversed Na gradient (5 mM Na outside//75 mM Na inside) and cooled the cells to  $12^\circ\text{C}$  to slow the kinetics. Measuring the TAA effects on inactivation under these conditions has the added advantage that the potency of these compounds is greater at depolarized potentials. Fig. 7 shows the effects of TEA (35 mM), TBA (100  $\mu\text{M}$ ), and TPeA (50  $\mu\text{M}$ ) on outward Na currents. As expected

from our previous whole-cell experiments, the kinetics of outward Na currents are not significantly altered by TEA or TBA. This is in stark contrast to internal TPcA which dramatically increases the rate of outward current inactivation.

To quantify the effects of TAA compounds on macroscopic inactivation we measured the time constants ( $\tau_h$ ) of Na current decay between  $-20$  and  $+80$  mV. These decays were usually well fit by a single exponential. Fig. 8 shows  $\tau_h$  of outward Na currents in the presence and absence of internal TAA compounds. Concentrations of TMA, TEA, and TPrA that significantly block Na currents do not alter the time course of macroscopic current inactivation. These data are consistent with the proposal that internal TMA, TEA, and TPrA act as voltage dependent open-channel blockers without interfering with the closing of the inactivation gate of Na channels. In contrast to these smaller TAA compounds,  $500 \mu\text{M}$  TBA (fivefold higher than the concentration used in the experiment of Fig. 7) and  $50 \mu\text{M}$  TPcA both increase the apparent rate of inactivation (Fig. 8), suggesting that TBA and TPcA enhance rather than hinder Na channel inactivation, a result that is opposite from that previously

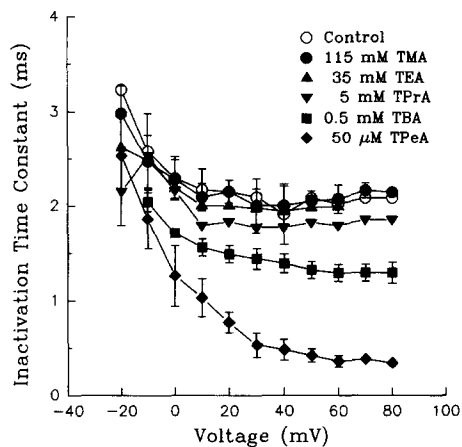


FIGURE 8. Effect of TAA compounds on inactivation time constants. Data from outward currents using reversed Na gradient. Time constants were obtained from single exponential fits for current decay from data similar to those shown in Fig. 7.

described for the effects of internal TEA on *Shaker* K channels. A more detailed examination of the effects of TBA and TPcA on Na current inactivation is found in the accompanying paper (O'Leary et al., 1994).

#### DISCUSSION

We have investigated the block of human heart Na channels by a series of tetra-alkylammonium compounds. TMA, TEA and TPrA inhibited whole-cell Na currents when applied to the inside of the channels without significantly altering the kinetics of macroscopic current inactivation. In excised outside-out patches, internal TMA, TEA, TPrA, and TBA reduced the single-channel current amplitudes, consistent with a fast open-channel blocking mechanism. TAA inhibition of both whole-cell and single-channel currents is more pronounced at depolarizing membrane potentials, showing that the block is voltage dependent. Our data therefore describe a

process in which internal TAA compounds transiently occlude hH1 Na channels by rapidly binding and unbinding from a site deep within the pore.

#### *Voltage Dependence of TAA Binding*

The voltage dependence of intra-pore blockers can be estimated from single-channel inhibition data (Fig. 2A). The parameter  $\delta$  represents the equivalent electrical distance across the membrane electric field traversed by a blocker to reach its binding site (Woodhull, 1973). Analysis of the voltage-dependence of TAA block indicates that these compounds bind to a site  $\sim 45\%$  of the distance across the electric field. Although suggestive of an intra-pore blocking mechanism, other interpretations are possible, such as a voltage-dependent change in affinity of the TAA binding site. More definitive evidence that TAA compounds inhibit Na currents by binding within the pore could be obtained by examining the interaction between external permeant cations and the internal blockers. We provide evidence in the accompanying paper that both external Na and internal TPrA antagonize the effects of TPeA, suggesting that it blocks Na channels by binding within the pore (O'Leary et al., 1994).

Several studies have examined the effects of TAA compounds on Na channels. TMA causes a voltage-dependent block of single Na channels from rat muscle (Horn et al., 1981;  $\delta = 0.89$ ) and the macroscopic Na currents of squid (Oxford and Yeh, 1985;  $\delta = 0.10$ ). In another study of squid Na currents, the apparent  $\delta$  decreased from 0.9 to 0.1 with depolarization (Keynes, Meves, and Hof, 1992). TMA inhibits the single-channel currents of batrachotoxin-activated Na channels with a blocking constant at zero mV [ $K_B(0)$ ] of 370 mM and effective electrical distance of 0.48 (Moczydlowski, Uehara, and Hall, 1984). These latter results are in close agreement with our single-channel data from hH1 Na channels [i.e.,  $K_B(0) = 335$  mM,  $\delta = 0.45$ ]. These results also show that the inactivation process, removed by batrachotoxin, has little effect on the properties of open channel block by TMA. The variabilities in voltage dependence may reflect differences in experimental conditions, or in the preparation (e.g., squid versus mammalian Na channels). Caution is required, however, in interpretation of macroscopic currents, because of the possibility that the block may depend on the state of the activation or inactivation gates. When the inactivation of squid axonal Na channels is destroyed by proteolytic enzymes, TEA induces a time-dependent block of Na currents (Rojas and Rudy, 1976) reminiscent of the block of K channels by long-chain derivatives of TEA (Armstrong, 1971). In general, our data are in fair agreement with most of the previous studies on internal TAA block of Na channels, showing a voltage-dependent block about half-way through the membrane field.

#### *Effects of TAA Binding on Kinetics of Inactivation and Activation*

At concentrations that block 50% or more of the Na currents internal TMA, TEA, and TPrA do not alter the rate of inactivation of whole-cell Na currents. This finding is in agreement with our single channel data showing that TEA has no effect on mean open times. TBA, by contrast, causes a slight reduction in open time and an apparent increase in the rate of inactivation. The larger compound TPeA also increases the apparent inactivation rate. We find no evidence, therefore, that internal TAAs interfere with the closing of the inactivation gate, in contrast with *Shaker* K channels where internal TEA simultaneously blocks currents and slows the rate of macroscopic

current inactivation (Choi et al., 1991). The *Shaker* data suggest that TEA directly competes with the "inactivation ball" for its binding site on the mouth of the channel (Choi et al., 1993). The block of K channels by internal TEA is weakly voltage-dependent ( $\delta = 0.15$ ), suggesting that TEA binds near the mouth of the channels (Yellen et al., 1991). It is therefore likely that TEA binding within the pore is deep for Na channels but relatively shallow for K channels. Differences in TAA binding must stem from intrinsic differences in the structure of the internal mouths of these ion channels.

Despite the fact that the cytoplasmic "inactivation ball" of K channels carries a net positive charge, the rate of microscopic inactivation is relatively voltage-independent (Zagotta and Aldrich, 1990), implying that "the ball" does not enter the membrane electric field to reach its binding site. Like K channels, Na channel inactivation is weakly voltage-dependent (Aldrich, Corey, and Stevens, 1983; Greeff and Forster, 1991). By analogy, the cytoplasmic inactivation gate (Stühmer et al., 1989; West et al., 1992) is likely to bind on the internal mouth of Na channels. This is consistent with our finding that concentrations of TMA, TEA, and TPrA that significantly block Na currents have no effect on the rate of Na channel inactivation. Evidently when bound within the pore, these TAA compounds are sufficiently distant from the cytoplasmic mouth of the channel that they do not hinder the closing of the inactivation gate. The exceptions to this finding are the two largest TAA compounds we examined, TBA and TPcA, both of which cause an increase in the apparent rate of macroscopic current inactivation. We provide evidence in the accompanying paper that TBA and TPcA both block the pore and interact directly with the inactivation gate (O'Leary et al., 1994).

Block of K channels by TEA and its long-chain analogs requires that the channels be open (Armstrong, 1971; French and Shoukimas, 1981; Choi et al., 1993). Furthermore, K channels cannot close until the TEA leaves the channel. These data show that the activation gates of K channels are exquisitely sensitive to the presence of a TAA blocker in its binding site. Our Na channel data contrast with those for K channels. Three separate experiments suggest that activation gates are insensitive to the presence of the smaller (TMA, TEA, and TPrA) TAA compounds inside the Na channel.

First, single-channel open times are not increased by 35 mM TEA (Fig. 2 B); i.e., the closing rate is not decreased by the presence of TEA in its blocking site. The closing rate is a sum of deactivation and inactivation rate constants (Horn et al., 1984). However at more negative voltages ( $V < -30$  mV) the deactivation rate constant, representing the closing of the activation gate, dominates. If the activation gate cannot close while TEA is in its site, then at  $-40$  mV 35 mM TEA is expected, based on its reduction of the amplitude of single-channel current, to decrease the deactivation rate constant by 30%. We saw no effect of TEA on open times.

Second, TAA block of macroscopic currents has the same voltage dependence as that of single channels (Figs. 2 A and 5), implying that the affinity of the channels for these compounds is the same, whether channels are open or closed. In other words, the smaller TAAs can enter and block a closed channel.

Finally, TAA block does not prevent channel closing, as determined by kinetics of tail currents (Fig. 6). These data all suggest that the opening and closing of the activation gates occur at a location closer to the external surface of the channel than

the TAA binding site. Therefore, a TAA molecule in its putative site in the middle of the pore appears to lie between the activation and inactivation gates, neither of which sense its presence. This interpretation suggests a remarkable isolation of the middle of the permeation path from the gates of the channel.

#### *Characteristics of the TAA Binding Site*

The cationic charge on quaternary ammoniums such as the TAA series used here is broadly distributed throughout the methyl and methylene groups that surround the nitrogens, resulting in a lower than predicted charge density (Pullman, Courriere, and Coubeils, 1971; Pullman and Courriere, 1972), a situation that is exacerbated by increasing the length of the alkyl side chains. The linear relationship between free energy of binding and the number of methylene groups (Fig. 3 B) is consistent with hydrophobic interactions playing a large role in TAA binding (Tanford, 1980). Our data further imply that a hydrophobic binding site for TAA molecules is located within the pore. This surprising conclusion is consistent with previous findings suggesting that both Na and K channels have hydrophobic patches within their ion channels (Armstrong, 1975; French and Shoukimas, 1981; Miller, 1982).

It has been suggested that binding sites for quaternary ammonium compounds can be formed by aromatic amino acids (Dougherty and Stauffer, 1990; Gao, Chou, and Auerbach, 1993). Electrostatic interactions between the cationic charge and the  $\pi$  electrons of the aromatic rings may stabilize TAA binding. Recent data support this hypothesis for TAA binding to ion channels. Introducing aromatic amino acids into the external mouth of K channels increases the affinity and weakens the voltage dependence of external TEA block (Heginbotham and MacKinnon, 1992). Both effects are consistent with the creation of a high-affinity TEA binding site composed of four tyrosine residues, one from each of the K channel subunits. The temperature dependence of external TEA binding (Heginbotham and MacKinnon, 1992) is also consistent with a cation- $\pi$  bond mechanism. A ring of four aromatic residues could in principle accommodate both the cationic charge and the hydrophobic side chains of TAA molecules. Aromatic residues may also be capable of forming a K-selective binding site (Kumpf and Dougherty, 1993), leading to the possibility that such residues are responsible for both selectivity and TAA blocking sites in the pore of K channels (Miller, 1993). It is not clear whether aromatic residues also play a role in the selectivity of Na channels or in the block by TAAs. However there are other examples of quaternary ammonium binding sites that are composed primarily of aromatic amino acids, e.g., the binding gorge of acetylcholinesterase (Sussman, Harel, Frolow, Oefner, Goldman, Toker, and Silman, 1991), and the ligand binding site of the nicotinic acetylcholine receptor (Tomaselli, McLaughlin, Jurman, Hawrot, and Yellen, 1991; O'Leary and White, 1992). It is likely that TAA compounds will prove to be useful probes of the interior mouth of the Na channel pore.

We thank Vincent Santarelli for transfections and care of the cells, Drs. L.-Q. Chen and Roland G. Kallen for providing us the cDNA of hH1, Dr. David T. Yue for sharing a copy of his manuscript before publication, and Drs. R. G. Kallen and Robert French for comments on the manuscript.

This work was supported by NIH grant AR41691.

*Original version received 8 November 1993 and accepted version received 8 April 1994.*

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