

Amine Blockers of the Cytoplasmic Mouth of Sodium Channels: A Small Structural Change Can Abolish Voltage Dependence

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ABSTRACT Many drugs block sodium channels from the cytoplasmic end (Moczydlowski, E., A. Uehara, X. Guo, and J. Heiny. 1986. Isochannels and blocking modes of voltage-dependent sodium channels. *Ann. N.Y. Acad. Sci.* 479:269–292.). Lidocaine, applied to either side of the membrane, induces two blocking modes, a rapid, voltage-dependent open-channel block, and a block of the inactivated channel that occurs on a 1000-fold slower timescale. Here we describe the actions of several lidocaine-related amines on batrachotoxin(BTX)-activated bovine cardiac sodium channels incorporated into planar lipid bilayers. We applied blocking amines from the intracellular side and examined the structural determinants of fast, open-channel block. Neither hydroxyl nor carbonyl groups, present in the aryl-amine link of lidocaine, were necessary, indicating that hydrogen bonding between structures in the aryl-amine link and the channel is not required. Block, however, was significantly enhanced by addition of an aromatic ring, or by the lengthening of aliphatic side chains, suggesting that a hydrophobic domain strengthens binding while the amine group blocks the pore. For most blockers, depolarizing potentials enhanced block, with the charged amine group apparently traversing 45–60% of the transmembrane voltage. By contrast, block by phenylhydrazine was essentially voltage-independent. The relatively rigid planar structure of phenylhydrazine may prevent the charged amino end from entering the electric field when the aromatic ring is bound. The relation between structural features of different blockers and their sensitivity to voltage suggests that the transmembrane voltage drops completely over less than 5 Å. We raise the possibility that the proposed hydrophobic binding domain overlaps the endogenous receptor for the inactivation gate. If so, our data place limits on the distance between this receptor and the intrapore site at which charged amines bind.

INTRODUCTION

We have recently studied lidocaine, QX-314, procainamide, and diethylamine block of batrachotoxin(BTX)-activated sodium channels (Zamponi et al., 1993a–c; Zamponi and French, 1993). All of these compounds caused fast open-channel block that was favored by depolarizing membrane potentials. Although the voltage dependence was similar for all four agents, their blocking affinities varied over about an order of magnitude. Molecular modeling suggested that the difference in potency between procainamide and lidocaine block could be caused by relative orientation of their aromatic rings or differences in the structure of the aryl-amine link (Zamponi et al., 1993c). However, it is not known which of the structural components of these class 1 antiarrhythmic drugs are the most important determinants of the blocking affinity.

Several investigators have addressed this question (e.g., Courtney, 1980, 1983; Bokesch et al., 1986; Ehring et al., 1988; Sheldon et al., 1991). Ehring et al. (1988) suggested a correlation between the potency of a compound and its lipid solubility and molecular weight. The study by Sheldon et al. (1991) revealed correlations between the efficacy of the drugs at displacing [³H]-batrachotoxinin- α -benzoate ([³H]-BTX-B) from its binding site and both the number of carbons in the aryl-amine link and the number of amino-terminal carbons. A more recent study by the same group (R. S. Sheldon, personal communication) indicates that sub-

stituents on the aromatic ring are also important determinants of the strength of binding of class 1 antiarrhythmics to sodium channels. These results show that small structural changes in different parts of the molecule can modify drug binding, but they do not pinpoint the structural features that are essential to make an effective class 1 antiarrhythmic agent.

We have argued that the aromatic ring in the lidocaine molecule is mainly responsible for binding to the inactivated state of the channel, whereas the protonated amine head group causes block of the conducting pathway (Zamponi and French, 1993). In our present study, we attempt to determine the importance of different parts of the lidocaine molecule for the open-channel blocking mode. For this purpose, we have investigated the blocking action of 11 internally applied amine blockers on BTX-activated bovine cardiac sodium channels. In general, compounds that lacked the aromatic ring were more than one order of magnitude less potent than comparable ring compounds. We propose that the aromatic moiety of the drug molecule interacts with a hydrophobic domain within the internal mouth of the channel, whereas the charged amine group binds to a cation-binding site in the pore. Apart from helping to define the minimal structural requirements for open-channel block, the compounds studied are useful molecular probes of the structural features of the inner vestibule of the sodium channel. A novel inference is that rigid phenylhydrazinium ion appears to occlude the conducting pore without penetrating significantly into the transmembrane electric field.

MATERIALS AND METHODS

Membrane vesicles (3.5 mg protein/ml) from bovine cardiac tissue were prepared and preincubated with BTX as described previously (Zamponi et al., 1993a) before attempting to incorporate Na channels into bilayers.

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Planar bilayers were formed from uncharged synthetic lipids (40 mg/ml phosphoethanolamine, 10 mg/ml phosphatidylcholine in decane) on a hole, 150 μm in diameter, in a plastic coverslip partition between two aqueous solutions, and were voltage-clamped as described previously (Zamponi et al., 1993 a, c). Holes were constructed as described by Wonderlin et al. (1990). The bilayers were bathed symmetrically by 1.5 ml of 200 mM NaCl, 20 mM MOPS at pH 7.0 at room temperature ($\sim 22^\circ\text{C}$). The total capacitance ranged from 50 to 80 pF. Between 4 and 20 μl of membrane vesicle suspension were added to the *cis* side while continuously stirring. The voltage was alternated between +70 and -70 mV, and channel incorporation was observed as an increase in conductance. Orientation of channels in the bilayer was determined from the voltage dependence of their gating (Krueger et al., 1983).

All drugs, with the exception of hydrazine and phenylhydrazine, were made up in 200 mM NaCl/20 mM MOPS, and the pH was adjusted to 7.0. Drugs were obtained from the sources indicated below and were used without further purification. Concentrations of the stock solutions were as follows: tetrapropylammonium (TPrA·HCl, Eastman Kodak Co., Rochester, NY) and phenylpropanolamine (PhPrOHNH₂·HCl, Sigma Chemical Co., St. Louis, MO), 100 mM; phenylethylamine (PhEtNH₂·HCl, Sigma), phenylmethylamine (PhMeNH₂·HCl, Sigma), and butanolamine (ButOHNH₂, Aldrich Chemicals, Milwaukee, WI), 200 mM; tetraethylammonium (TEA·Br, Eastman Kodak), propanolamine (PrOHNH₂, Aldrich), phenyltrimethylammonium (PhNMe₃·Br, Sigma), phenylethylhydrazine (PhEtHyd·H₂SO₄, Aldrich), and ethanolamine (EtOHNH₂·HCl, Sigma), 400 mM. Hydrazine (Hyd·2HCl, Sigma) and MOPS were dissolved in water to make a stock

solution of 200 mM hydrazine, 8 mM MOPS at pH 7.0. Phenylhydrazine (PhHyd·HCl, Sigma) was prepared as a stock of 200 mM phenylhydrazine, 200 mM NaCl, and 200 mM MOPS. An elevated MOPS concentration was necessary to dissolve phenylhydrazine at the required final concentration. Hence, the final MOPS concentration in the bath reached up to 72 mM. To test for direct effects of this high concentration of MOPS, we applied 72 mM Na-MOPS to one channel; no effect was observed. To rule out the possibility that phenylhydrazine and MOPS formed a nonblocking complex, we prepared a solution of 50 mM phenylhydrazine/200 mM NaCl/20 mM MOPS (pH 7.0) and used this to perfuse the chamber facing the intracellular side of the channel in two experiments. The observed block was consistent with that seen after the simpler procedure of adding phenylhydrazine from a concentrated stock (see Results). In all other experiments, blockers were added from the concentrated stock solutions described above. The structures of the compounds studied are displayed in Figs. 1 and 2. Lidocaine is included for comparison in Fig. 1.

Tertiary amines and hydrazines are referred to in the text as the tertiary compounds, but at neutral pH would be present predominantly in their protonated forms. It is the protonated forms that are presumed to be active in causing fast open-channel block, e.g., phenylpropanolammonium and phenylhydrazinium (cf. Zamponi et al., 1993a).

For most compounds, data were acquired for periods of 5 s to 1 minute under each set of conditions; for tetrapropylammonium, data were recorded in continuous segments of up to 2 min. Data were filtered at 100 Hz and sampled at 200 Hz during transcription into a personal computer (Compaq 386) and analyzed using pClamp software (Axon Instruments Inc., Foster

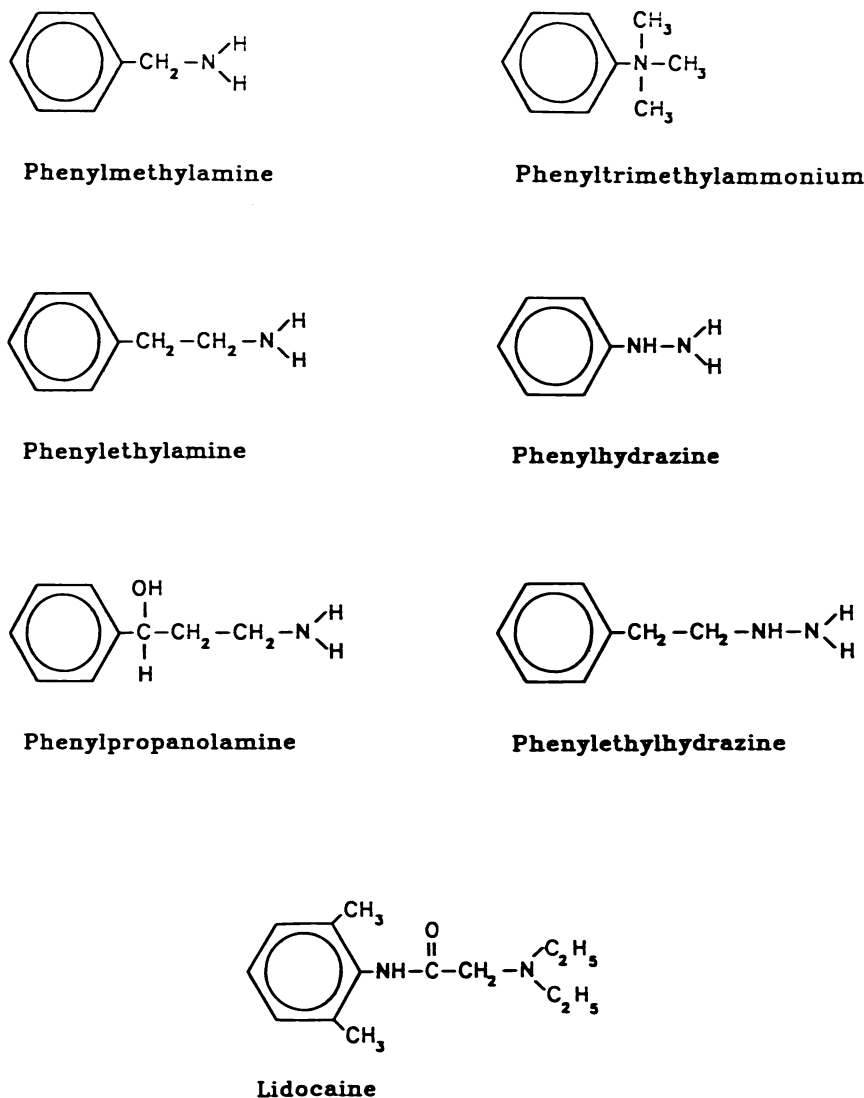
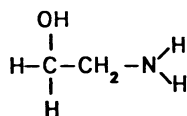
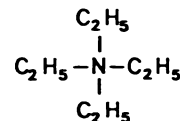


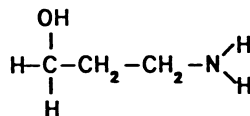
FIGURE 1 Structures of the studied aromatic compounds in comparison with the structure of lidocaine. Note that neither of the studied compounds carries methyl groups on the aromatic ring, a carbonyl group in the aryl-amine link, or ethyl groups on the terminal nitrogen (as in the case of lidocaine).



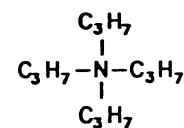
Ethanolamine



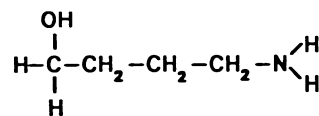
Tetraethylammonium



Propanolamine



Tetrapropylammonium



Butanolamine

FIGURE 2 Chemical structures of the nonaromatic compounds investigated in the present study.

City, CA). For tetrapropylammonium, dwell times and open probabilities were determined from events lists created at a bandwidth of 25 Hz, without correction for missed events. Under these conditions, the dead time of the recording system is 7 ms, compared with a mean blocked time for TPrA of approximately 150 ms at +40 mV. For all other compounds, the amount of block was determined from the reduction in apparent single-channel amplitude. Figures were prepared and fits carried out using Sigmaplot (Jandel Scientific, Corte Madera, CA).

RESULTS

Many ammonium compounds block the open channel

The top trace in Fig. 3 A depicts a typical example of a recording from a BTX-activated cardiac sodium channel. In the absence of blockers, these channels generally show a constant open probability in the range of 0.5–0.9 at potentials more positive than –60 mV. In Fig. 3 A, all of the traces were recorded from the same channel at a membrane potential of +40 mV. Application of 3 mM phenylmethanamine, 1 mM phenylpropanolamine, 3 mM phenylethylamine, or 36 mM ethanolamine resulted in about 50% reduction in apparent single-channel current amplitude without a visible increase in noise. This decrease in apparent single-channel current amplitude is consistent with block occurring on a timescale well beyond the resolution of the system. Although the current decrease can be used to determine the magnitude of the equilibrium dissociation constant, K_d , it does not allow the determination of the individual rate constants for block and unblock. The presence of 36 mM tetraethylammonium also resulted in a reduction in single-channel amplitude; however,

here this effect was accompanied by a distinct increase in noise, which is probably caused by poorly resolved transitions between the open and the blocked states. This suggests that block by tetraethylammonium occurs on a slower time scale than block by the above compounds. Application of 1 mM tetrapropylammonium caused discrete blocked events, with a mean duration of about 150 ms, without a decrease in current amplitude. Because the blocking events were seen as discrete closures, we were able to obtain the individual rate constants for block and unblock (at 0 mV the values were $0.75 \text{ mM}^{-1} \text{ s}^{-1}$ and 7.4 s^{-1} , respectively). The bottom trace was recorded after washout of the final drug to demonstrate the reversibility of the block (similar records were obtained after washout of each compound). Not shown here are traces recorded in the presence of propanolamine, butanolamine, phenyltrimethylammonium, phenylethylhydrazine, and phenylhydrazine (but see Fig. 7). Qualitatively, the effects of these compounds were similar to those of others, such as phenylmethanamine.

Fig. 3 B shows the blocking action of tetrapropylammonium in greater detail. Under control conditions, this channel was open about 95% of the time at both +40 and –40 mV. Application of 12 mM tetrapropylammonium resulted in rapid, but discrete transitions between the open and the blocked states. As can be seen from the record, block was more pronounced at +40 mV, and open times were dramatically reduced in the presence of the blocker.

Fig. 3 C shows records obtained in presence of 12 mM aniline. This compound is structurally similar to phenylmethanamine, but lacks an extended aryl-amine link. Although

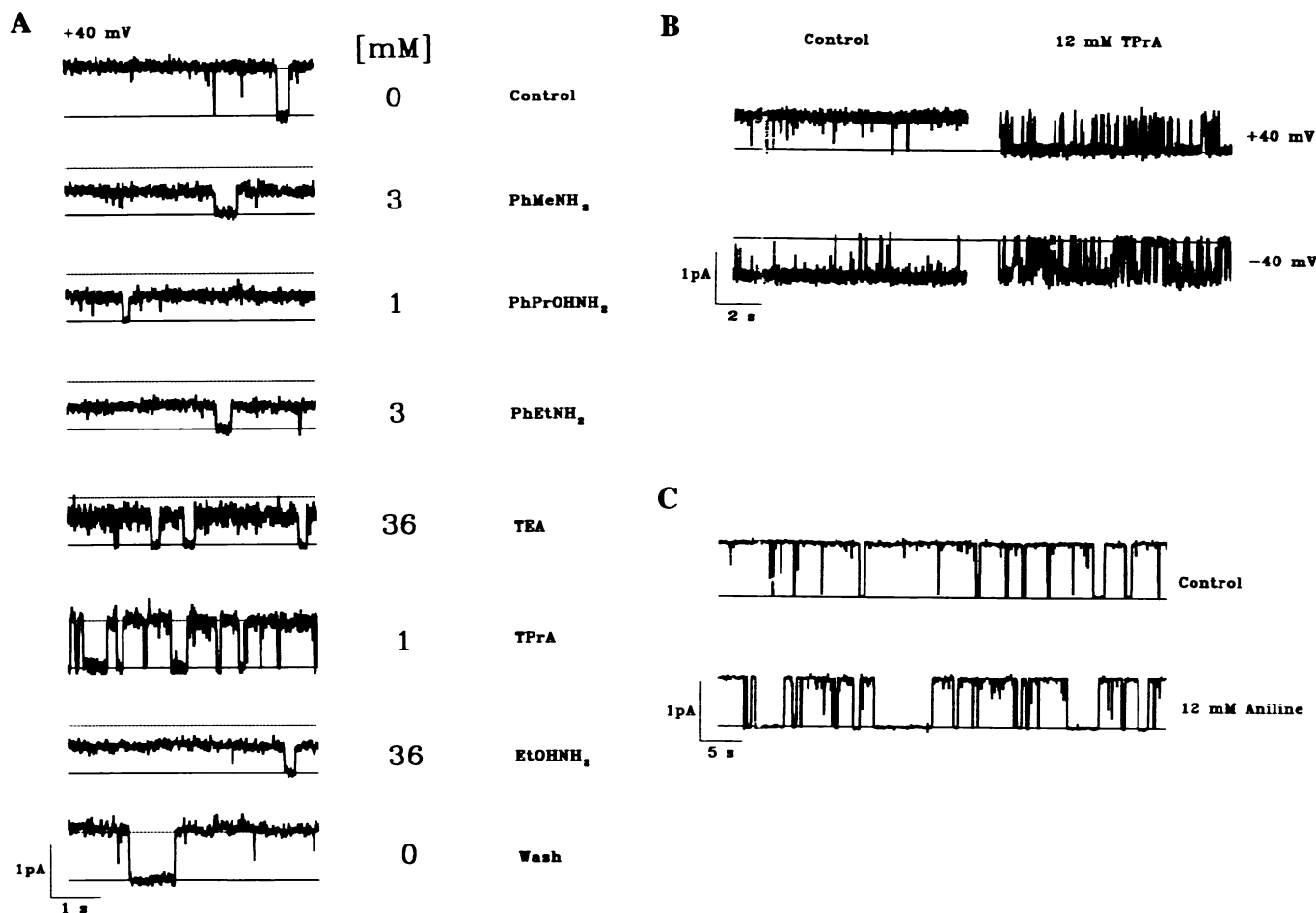


FIGURE 3 (A) Traces recorded from a BTX-activated cardiac sodium channel at a membrane potential of +40 mV. The records were filtered at 50 Hz; solid lines indicate the closed level; dashed lines indicate the control current amplitude of 0.9 pA. In this particular experiment, all shown traces were recorded from the same channel. The order of the displayed records follows the time course of the experiment; however, traces recorded after washout between application of the individual compounds are not shown. In absence of blockers, the channel was open most of the time. In the presence of 3 mM phenylmethylamine (PhMeNH₂), 1 mM phenylpropanolamine (PhPrOHNH₂), 3 mM phenylethylamine (PhEtNH₂), and 36 mM ethanolamine (EtOHNH₂), the apparent single-channel current amplitude was reduced by about 50% without an apparent increase in noise, suggesting very fast blocking action of these compounds. 36 mM tetraethylammonium (TEA) resulted in flickery transitions between the open and the blocked states, which at the used bandwidth, appears as an apparent increase in open-channel noise. 1 mM tetrapropylammonium (TPrA) caused discrete transitions between the open and the blocked states with a mean blocked time of about 150 ms. (B) Records from a BTX-activated cardiac sodium channel in absence and presence of tetrapropylammonium. In absence of the drug, the channel showed an open probability of 0.95 at both +40 and -40 mV. Application of 12 mM tetrapropylammonium resulted in discrete blocking events that were more pronounced at negative potentials ($P_{\text{open}}(+40 \text{ mV}) = 0.25$, $P_{\text{open}}(-40 \text{ mV}) = 0.70$). Solid lines indicate the closed level; the records were filtered at 50 Hz. (C) Effect of 12 mM aniline on a BTX-activated cardiac sodium channel. The traces were filtered at 12.5 Hz, +40 mV. Aniline induces long-lived closures by binding to a nonconducting state of the channel, but does not affect the open state.

aniline did induce the slow blocking mode like that previously described for lidocaine and phenol (block of the "inactivated" state of the channel; Zamponi et al., 1993b; Zamponi and French, 1993), it failed to produce any detectable open-channel block at concentrations as high as 27 mM. This is consistent with aniline ($pK_a = 4.5$) mainly occurring in its uncharged form at a pH of 7.0. In contrast, the permanently charged quaternary aniline derivative, phenyltrimethylammonium, induced potent open-channel block, but no slow block, consistent with our previous observations that only uncharged aromatic compounds induce this type of block.

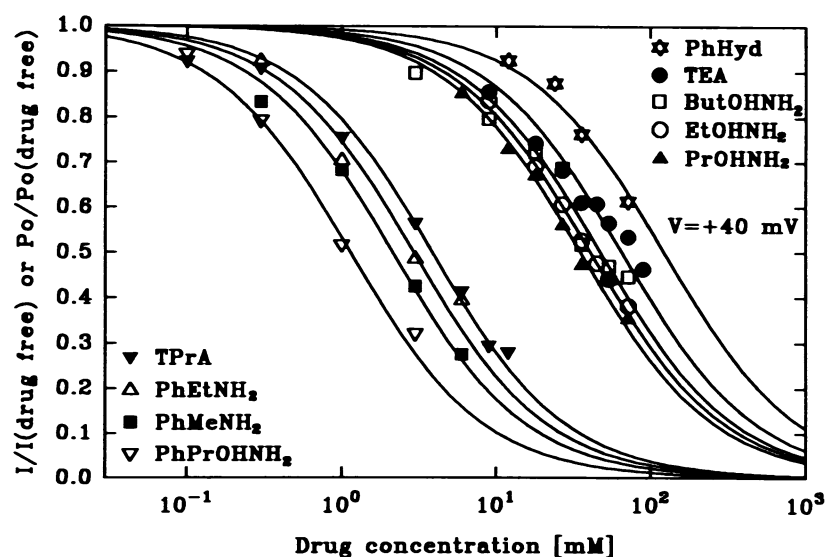
Aromatic rings enhance the blocking affinity

Fig. 4 displays dose-response curves for most of the compounds. The data were generally well fitted with a Hill co-

efficient of 1, suggesting a 1:1 interaction between the channel and the drugs. Note that compounds containing an aromatic ring were more potent blockers, and are thus found in the left portion of the panel. Exceptions were phenylhydrazine, which was the weakest of all of the drugs, and tetrapropylammonium, which approached some of the aromatic compounds in potency.

Fig. 5 shows the voltage dependence of the equilibrium dissociation constants, K_d , of all the compounds. As in Fig. 4, the drugs containing an aromatic ring, with the exception of phenylhydrazine, show the lowest K_d values and, thus, most potent block. With the exception of phenylhydrazine, which does not appear to show a significant voltage dependence, all of the other drugs appear to show a similar voltage dependence ($\alpha\delta$ generally between 0.4 and 0.65), consistent with the idea that these drugs bind to one common receptor. The

FIGURE 4 Dose-response curves recorded from BTX-activated cardiac sodium channel at a membrane potential of +40 mV. The curves were fitted with simple hyperbolas $I/I(\text{drug free}) = 1/(1 + [C]/K_d)$, where I and $I(\text{drug free})$ are the apparent single-channel current amplitudes in presence and absence of the drugs, respectively, $[C]$ is the drug concentration, and K_d is the equilibrium dissociation constant for the drug. For tetrapropylammonium, we plotted the normalized open probability as a function of concentration, fitting of the data was conducted using the same formalism as for the other compounds. The obtained K_d values were as follows: phenylpropanolamine, 1.4 mM; phenylmethylaniline, 2.1 mM; phenylethylamine, 2.9 mM; tetrapropylammonium, 3.9 mM; propanolamine, 35.5 mM; ethanolamine, 41.3 mM; butanolamine, 47.9 mM; tetraethylammonium, 65.7 mM; phenylhydrazine, 123.4 mM. All symbols represent means from two to five experiments, except for phenylhydrazine, where data from only one experiment are displayed.



fitting parameters for Fig. 5 are summarized in Table 1. The low value for $\alpha\delta$ for phenylhydrazine reflects the lack of significant voltage dependence. This also becomes clear from inspection of Fig. 5 C, where the apparent electrical distances and their SDs are displayed for each drug. To test whether the hydrazine portion, per se, on the phenylhydrazine molecule was responsible for the loss of voltage dependence, we studied the blocking action of phenylethylhydrazine. As can be seen from Fig. 5 and Table 1, phenylethylhydrazine block was voltage-dependent, and was 20-fold more potent at 0 mV, than phenylhydrazine. For three channels, at which phenylhydrazine block was determined at both +50 and -50 mV, a paired t-test suggested identical equilibrium dissociation constants at the two voltages ($P = 0.38$). In contrast, a similar test showed a clear, and significant, voltage dependence of the block by the closely related compound, phenylethylhydrazine ($P = 0.014$).

Our data suggest that phenylhydrazine is only able to interact with the channel in a conformation in which the charged amino terminal is prevented from a significant entry into the transmembrane electrical field.

Despite its negligible penetration into the electric field, phenylhydrazine blocks within the pore

Because phenylhydrazine shows blocking properties that are quite distinct from those of other compounds, it seemed possible that the phenylhydrazine molecule could bind to a distinct site located outside the ion-conducting pathway. To investigate this possibility, we tested the permeation of hydrazine and the dependence of block by internal phenylhydrazine on external sodium ions.

Fig. 6 depicts current-voltage relations recorded from the same channel under three different experimental conditions. In all cases, the external solution was 200 mM NaCl/20 mM MOPS at pH 7.0. Current-voltage relations were recorded in internal 200 mM NaCl/20 mM MOPS, internal 200 mM hydrazine-HCl/8 mM MOPS, and finally in internal 200 mM *N*-methyl-D-glucamine-HCl (NMG)/20 mM MOPS. With

symmetric sodium, the current-voltage relation was essentially linear with a reversal potential of 0 mV. When internal sodium was replaced by hydrazine, we also obtained a linear relation; however, here the reversal potential was -11.5 mV, suggesting that hydrazine is more permeant than sodium. In presence of the channel-impermeant ion NMG, the relation was linear at negative membrane potentials, but asymptotically approached zero at positive membrane potentials. Data from three experiments indicated a permeability ratio $P_{\text{Hyd}}/P_{\text{Na}}$ of 1.7 ± 0.09 as determined by the Goldman-Hodgkin-Katz equation and corrected for incomplete ionization of hydrazine ($pK_a = 8$; Hille, 1971) at a pH of 7.0. Because hydrazinium is able to pass easily through the channel, the hydrazine group on the phenylhydrazine molecule should not prevent this molecule from entering the channel deeply.

Fig. 7 shows current traces recorded from one channel at +40 mV in presence and absence of phenylhydrazine, with symmetric 200 mM NaCl/20 mM MOPS, or with the extracellular solution replaced with 200 mM NMG. In presence of external sodium ions, 60 mM phenylhydrazine reduced the apparent single-channel amplitude to 87% of the control value. In presence of NMG on the extracellular side, the current was reduced to 72%. In three experiments, 60 mM phenylhydrazine caused a reduction in apparent single-channel amplitude to $81 \pm 5.5\%$ in the presence of external sodium and $68 \pm 5.5\%$ in its absence. According to a paired t-test, these values are significantly different ($P = 0.03$). In addition, the replacement of sodium ions by NMG did not alter the minimal voltage dependence of phenylhydrazine block (not shown). These data are similar to previous observations on QX-314 and diethylamine block (Zamponi et al., 1993a; Zamponi and French, 1993) and suggest that phenylhydrazine blocks the channel within the conducting pathway of the channel.

DISCUSSION

The initial goal of this study was to determine the minimal structural requirements for a drug to mimic open-channel

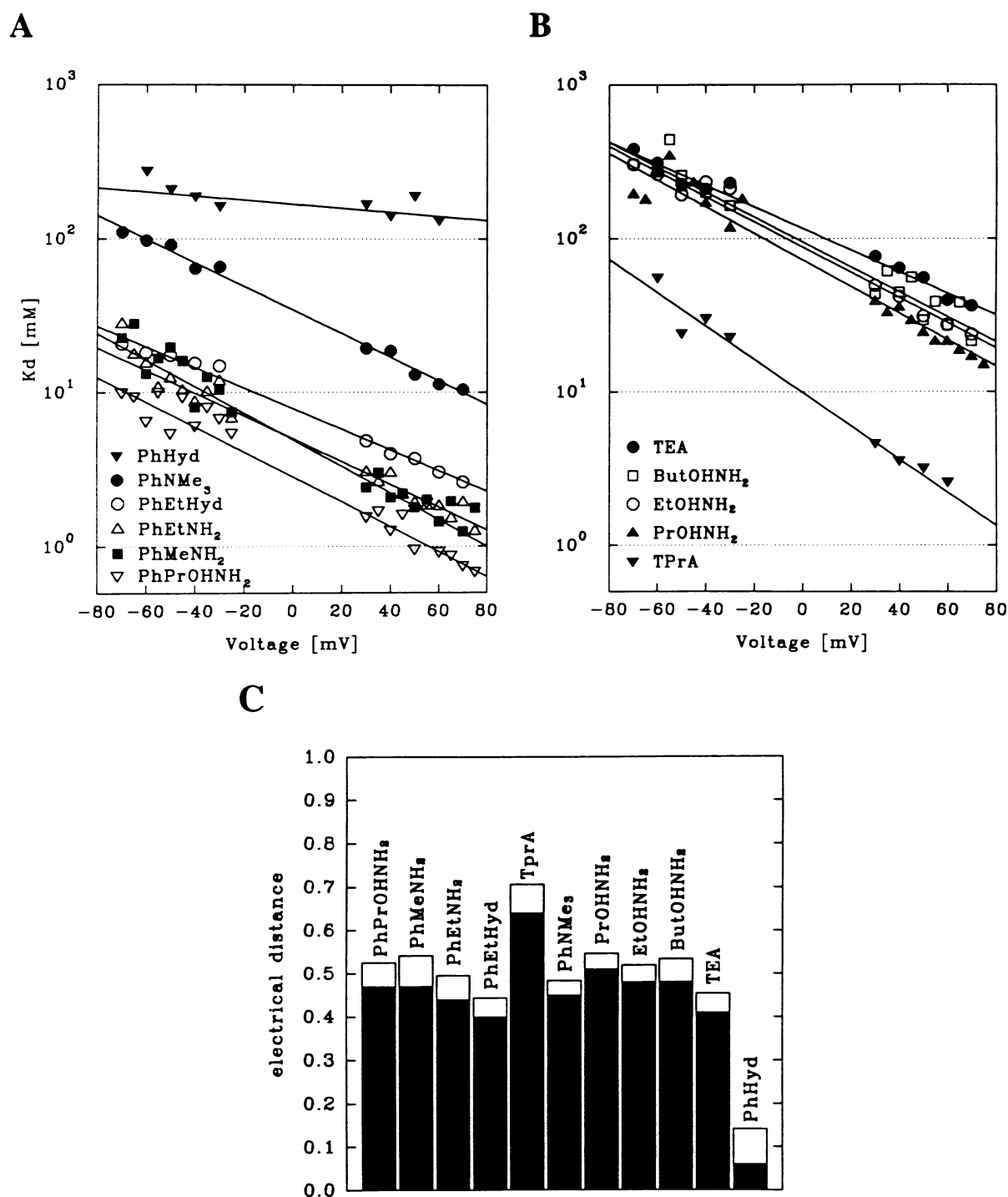


FIGURE 5 Voltage dependences of the equilibrium dissociation constants, K_d , of the aromatic (A) and nonaromatic (B) compounds. All data points represent means from three to eight experiments; solid lines are least-square fits to the data, based on the relation $K_d(V) = K_d(0)\exp(-z\delta V/25.6)$, where V is the membrane potential, $z\delta$ is the effective electrical distance, and $K_d(0)$ is the equilibrium dissociation constant. The slopes of the regression lines reflect the penetration of the transmembrane voltage by the individual compounds (Woodhull, 1973). The obtained values for $z\delta$, the values of the equilibrium dissociation constants $K_d(0)$, and the regression coefficients, r , are summarized in Table 1. (C) Comparison of the electrical distances for all of the compounds. The filled areas indicate the slopes, $z\delta$, obtained from the fits in panels A and B. The open bars indicate the magnitudes of the 95% confidence intervals as calculated according to the formalism in Larsen and Marx (1981).

block by lidocaine in kinetics, potency, and voltage dependence (for BTX-activated cardiac channels: $K_d(0 \text{ mV}) = 4.1 \text{ mM}$, $z\delta = 0.38$; Zamponi et al., 1993a). The structural requirements of class 1 antiarrhythmic agents and local anes-

thetics have been the focus of several previous studies. Data by Sheldon et al. (1991) suggest that optimal displacement of [^3H]-BTX-B occurred for drugs with four or more amino terminal carbons and with two carbons in the aryl-amine link.

TABLE 1 Equilibrium parameters measured at 0 mV

Drug	K_d (0 mV) [mM]	$z\delta$	r	# Experiments
Phenylpropanolamine (PhPrOHNH ₂)	2.8	0.47	0.95	5
Phenylmethylamine (PhMeNH ₂)	4.9	0.47	0.92	3
Phenylethylamine (PhEtNH ₂)	4.9	0.44	0.89	8
Phenylethylhydrazine (PhEtHyd)	7.6	0.40	0.96	3
Tetrapropylammonium (TPrA)	9.9	0.64	0.96	4
Phenyltrimethylammonium (PhNMe ₃)	33.1	0.45	0.96	3
Propanolamine (PrOHNH ₂)	72.4	0.51	0.98	4
Ethanolamine (EtOHNH ₂)	87.7	0.48	0.98	4
Butanolamine (BuOHNH ₂)	94.8	0.48	0.97	3
Tetraethylammonium (TEA)	116.1	0.41	0.90	5
Phenylhydrazine (PhHyd)	166.0	0.06	0.35	4

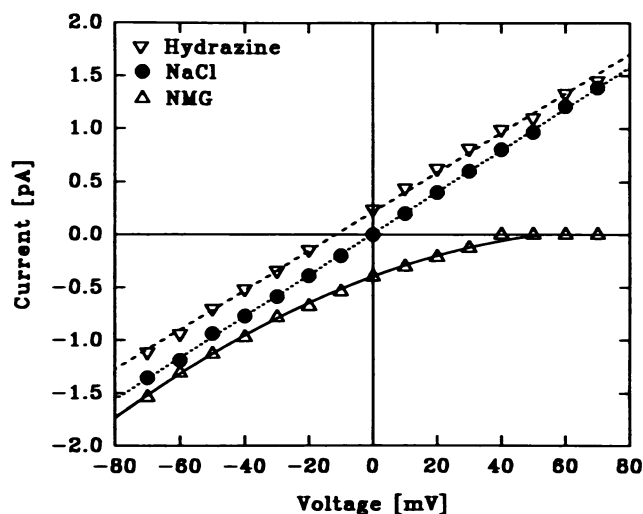


FIGURE 6 Current-voltage relations recorded from the same channel under three different conditions on the intracellular side. The solution on the extracellular side was 200 mM NaCl/20 mM MOPS (pH 7.0). First, a current-voltage relation was recorded under symmetric 200 mM NaCl/20 mM MOPS. Then the internal solution was exchanged for 200 mM hydrazine/8 mM MOPS (pH 7.0), and a second current-voltage relation was recorded. The chamber facing the intracellular side of the channel was subsequently perfused with 200 mM NMG/20 mM MOPS (pH 7.0) and another I/V relation was obtained. Finally, the original symmetric sodium conditions were restored to rule out potential offsets. The reversal potential in symmetric sodium was 0 mV at both the beginning and the end of the experiment, indicating that no drift had occurred. In presence of hydrazine, the reversal potential shifted to -11.5 mV. The straight lines (NaCl, hydrazine) are least-square fits to the data; the curved line (NMG) is merely a smooth approximation to the data points.

Courtney (1980, 1983, 1988) suggested that activity was correlated with lipid solubility and molecular weight of the drugs. On frog sciatic neurons, Bokesch et al. (1986) reported a correlation of tonic block with hydrophobicity and with the number of terminal carbons, but not with molecular weight or pK_a of the drugs. However, in these studies the drugs were applied from the extracellular side and, hence, block requires the diffusion of the drugs through a hydrophobic pathway (see also Zamponi et al., 1993a). In addition, the drugs may interact with more than one kinetic state of the channel, making it difficult to define the structural requirements for in-

teraction with one particular state or binding site. To circumvent these potential problems, we applied the drugs from the intracellular side. Because all of the drugs studied occur predominantly in their charged form at a pH of 7.0, they presumably approach the receptor directly via a hydrophilic pathway. Hence, pK_a and lipid solubility are not an issue here. Moreover, in single-channel experiments one can more clearly separate drug effects on the different kinetic states of the channel, which is not always possible in whole cell approaches or binding studies. Here we restricted our analysis to a single mode of action: fast, open-channel block.

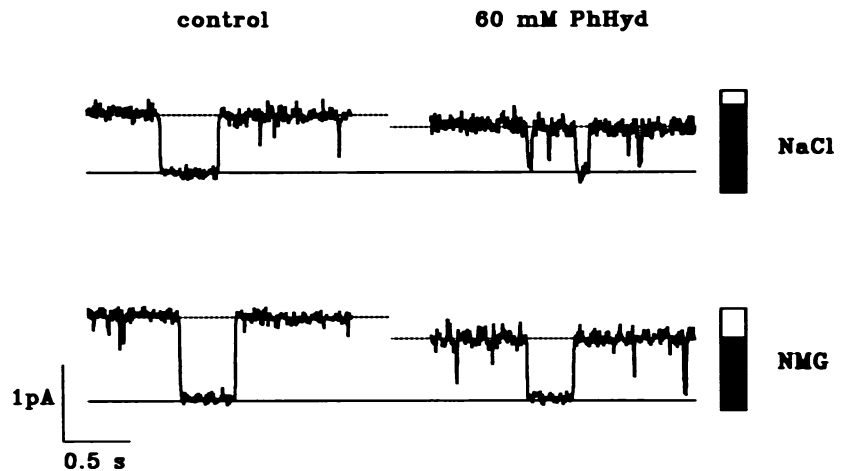
All of the compounds, with the exception of phenylhydrazine, showed a voltage dependence comparable with that of lidocaine. Block by the relatively small molecules diethylamine (Zamponi and French, 1993) and tetramethylammonium (Uehara and Moczydlowski, 1986) is also similarly voltage-dependent. These data suggest that all of these blockers, except phenylhydrazine, bind to a receptor located about roughly one-third to one-half of the way across the transmembrane voltage.

Roles of the aromatic ring and the aryl-amine link

The magnitudes of the equilibrium dissociation constants presented in Table 1 indicate that an aromatic ring generally increases the blocking affinity. With exception of tetrapropylammonium, all of the nonaromatic compounds produced block that was more than an order of magnitude weaker. The importance of the aromatic ring is directly suggested by a comparison of the K_d values for ethanolamine (87.7 mM) and phenylethylamine (4.9 mM), and the K_d values of propanolamine (72.4 mM) and phenylpropanolamine (2.8 mM). We did not study tetramethylammonium action here, but we have previously shown that open-channel block is almost identical for cardiac and skeletal muscle channels (Zamponi et al., 1993a; Zamponi and French, 1993). The K_d for tetramethylammonium block of BTX-activated skeletal muscle channels was 350 mM (Uehara and Moczydlowski, 1986), whereas phenyltrimethylammonium, in our experiments, blocked with a 10-fold higher affinity. Hence, the addition of an aromatic ring generally appears to increase blocking affinity by at least an order of magnitude.

Simple compounds like phenylmethylamine and phenylethylamine blocked the channels as strongly as lidocaine. This shows that open-channel block does not require methyl groups on the aromatic ring or the presence of a carbonyl group in the aryl-amine link, which are both features of lidocaine's structure. In lidocaine, the carbonyl group could potentially form hydrogen bonds with the channel. However, neither phenylmethylamine nor phenylethylamine include potential hydrogen bond-forming groups in their aryl-amine links, suggesting that hydrogen bonding by structures in the aryl-amine link with parts of the channel is unlikely to be a major contributor to the fast blocking action of lidocaine. It is possible, however, that addition of methyl groups on the aromatic ring or attachment of ethyl groups on the terminal amino nitrogen would further enhance block. This is strongly

FIGURE 7 Effect of NMG on the phenylhydrazine block at a membrane potential of +40 mV. All traces were recorded from the same channel and filtered at 50 Hz. Solid lines indicate the closed level; dashed lines indicate the mean, baseline-subtracted, open-channel current. The solid bars indicate the normalized apparent single-channel amplitude on a scale from 0 to 1 for the channel in presence of phenylhydrazine. Under symmetric 200 mM NaCl/20 mM MOPS, the channel showed a single-channel amplitude of 0.77 pA. Application of 60 mM phenylhydrazine decreased the amplitude to 0.67 and, hence, to 87% of the control value. Under asymmetric conditions with 200 mM NMG/20 mM MOPS on the extracellular side, the single-channel amplitude in absence of phenylhydrazine was 1.07 pA, reflecting the increase in driving force caused by the removal of external sodium ions. Application of 60 mM phenylhydrazine reduced the apparent single-channel amplitude to 0.78 pA, which constitutes a relative reduction to 72% of the control value.



implied by the data of Sheldon et al. (1991, and personal communication), who showed in binding assays that [³H]-BTX-B displacement was enhanced with an increasing number of amino terminal carbons or with an increasing number of carbons attached to the aromatic ring. The same conclusion is suggested by the potent blocking action of clofilium on BTX-activated rabbit skeletal muscle sodium channels (Nettleton et al., 1990). Clofilium, a quaternary drug with four carbons in the aryl-amine link and 11 amino terminal carbons, blocks these channels in the low micromolar range. Other evidence comes from observations by Uehara and Moczydlowski (1986) on the actions of QX-314 and QX-222. These two molecules are structurally identical with the exception of the terminal nitrogen, which carries three methyl groups in QX-222, but three ethyl groups in QX-314. Consistent with the findings of Sheldon et al. (1991), QX-314 is twice as potent as QX-222.

We also applied aniline, in which an amino group is directly attached to the benzene ring, to the intracellular side of the channel. This compound did not cause any detectable open-channel block. However, aniline ($pK_a = 4.6$) is mostly uncharged at a pH of 7.0, and the lack of open-channel block is thus consistent with the absence of the charged form (cf. Zamponi et al., 1993a). In contrast, phenyltrimethylammonium, a permanently charged aniline derivative with three methyl groups attached to the nitrogen atom, caused pronounced fast block, suggesting that at a sufficiently low pH, aniline might also bind, if the fast blocking site were available under these conditions.

Nonaromatic compounds like tetrapropylammonium or tetrapentylammonium (our unpublished observations) also effectively blocked open cardiac sodium channels. It has been shown that the length of the alkyl chain in *n*-alkyl-quaternary ammonium compounds (Wang et al., 1991) is correlated with the affinity of the drug for the blocking site. Wang et al. (1991) suggested the presence of a hydrophobic pocket within the internal mouth of the channel that can accommodate up to 18 methyl groups. A molecule like tetrapropylammonium, which has long alkyl chains, could in-

teract with this proposed hydrophobic domain, thus accounting for the high affinity of this drug for the channel. It is possible that aromatic rings interact with the same hydrophobic domain to facilitate binding. Wang (1990) suggested, based on the binding affinity of stereoisomers of several local anesthetics, that there are, in fact, two separate hydrophobic domains, one that binds aromatic rings, and a second one that interacts with amino terminal alkyl chains. This is consistent with the observation that both alkyl chains attached to the amino terminal and aromatic rings appear to enhance block (Sheldon et al., 1991). It is difficult, however, to say whether the long chains of *n*-alkyl-quaternary ammonium compounds interact with the "aromatic" site, or with the domain that binds amino terminal chains, or with both.

Individual rate constants for block and unblock are not accessible

We cannot compare the blocking kinetics of the compounds studied in this paper with those of lidocaine. Lidocaine block is characterized by an apparent increase in open-channel noise caused by incompletely resolved blocking events (Zamponi et al., 1993a). We estimated the individual rate constants for lidocaine block using a statistical analysis of the single-channel amplitude distribution (Yellen, 1984) and found a blocking rate that was favored by depolarizing potentials, and an unblocking rate that was enhanced at negative voltages (Zamponi et al., 1993a). This qualitative behavior was also found with QX-314, procainamide (Zamponi et al., 1993a, c), and tetrapropylammonium. However, the blocking kinetics of all the other compounds tested were apparently even faster than those of lidocaine, making them inaccessible to the amplitude distribution analysis.

The presence of an aromatic ring did not result in an increase in a detectable open-channel noise (compare traces for ethanolamine and phenylethylamine in Fig. 3). This might indicate that the enhancement of block by the aromatic ring occurs via an increase in blocking rate, rather than a decrease in the unblocking rate. Although we cannot rule out the pos-

sibility that the unblocking kinetics of the drugs are slowed by the presence of an aromatic ring, but still lie beyond our resolution of our experiments, a selective increase in association rate would be consistent with the two-step binding model outlined below.

Lidocaine-like open-channel block: minimal structural requirements and mechanism

Overall, the available data suggest that effective cytoplasmic, fast, open-channel block generally requires the following: a) a charged amine group, b) an aromatic ring, and c) a somewhat flexible aryl-amine link. A charged amine group appears to be an absolute requirement for block, but because block by small, nonaromatic compounds like diethylamine does occur at higher concentrations, hydrophobic interactions appear to be facilitatory rather than an absolute requirement.

We suggest that block by lidocaine-like compounds may occur in two steps. First, the aromatic portion of the drug molecule could bind to the hydrophobic site. Then the charged amine group would traverse part of the transmembrane electric field to bind to the its receptor, thus occluding the pore in a voltage-dependent manner. In this model, the "aromatic" site would serve as a flexible anchoring point that facilitates the approach of the charged amine group to the receptor for open-channel block. An aromatic blocker would thus be like a detachable inactivation gate in the "ball-and-chain" or "hinged-lid" models of inactivation. Because both steps must be fast, but only one is dependent on blocker concentration, the dose-response relation for fast block reflects 1:1 stoichiometry.

Additional carbons attached to the aromatic ring would enhance the overall hydrophobicity and, thus, binding affinity of the drug for the "aromatic" site, whereas additional amino-terminal carbons could prolong the life time of the blocked state via an interaction between amino terminal alkyl chains and the proposed second hydrophobic site on the channel (Sheldon et al., 1991, and personal communication; Bokesch et al., 1986). We believe that binding of the aromatic ring enhances the open-channel block directly, rather than allosterically, because co-application of phenol with diethylamine did not noticeably affect diethylamine block.

Relevance for antiarrhythmic action: some reservations

We note that the simple ability of a drug to block effectively an open cardiac sodium channel does not make it a useful antiarrhythmic agent. There are several critical issues that we do not address here. First, a clinically active drug must be able to penetrate the cell membrane to reach the binding site, which is not easily achieved by permanently charged hydrophilic drugs like tetrapropylammonium, but is readily seen with phenylpropanolamine, phenylethylamine and phenylmethylamine (our unpublished observations). Second, we do not address possible interactions between the compounds and the inactivated state of the channel, which is character-

istic of several class 1 antiarrhythmic agents. Third, drugs with very simple structures are less likely to show channel-subtype specificity. In addition, the presence of BTX alters several properties of unmodified channels like single-channel amplitude, permeation (e.g., Garber and Miller, 1987; Correa et al., 1991) or stereoselectivity (Wang and Wang, 1992b), and affinity for local anesthetics (e.g., Khodorov et al., 1975) and, thus, our results might not hold for unmodified channels. Our data contribute some biophysical understanding of how class 1 antiarrhythmics interact with a site responsible for open-channel block, but they do not necessarily offer direct aid in the design of clinically more effective drugs. Nonetheless, it appears clear that specific structural features determine the potency of open-channel block, and that hydrophobic interactions between the drug and the channel strongly modulate the blocking affinity.

Why is phenylhydrazine block weak and essentially voltage-independent?

The most striking result of our study is the low potency of phenylhydrazine block and its apparent lack of voltage dependence. The simple presence of an additional amine group in the aryl-amine link cannot account for this effect, because both lidocaine and procainamide carry an amide group in this region. The effect cannot be solely caused by the hydrazine portion of the molecule, for two reasons: a) phenylethylhydrazine was an effective, and voltage-dependent, blocker, and b) free hydrazinium is freely permeant (see below). Neither can the uncharged species of the drug be responsible for this effect. Phenylhydrazine ($pK_a = 8.8$, Merck Index, 10th Edition) occurs predominantly in its charged form at a pH of 7.0, with only about 1% of the total phenylhydrazine in its uncharged form. Furthermore, raising the internal pH to from 7.0 to 7.8 (giving a fivefold increase in the concentration of the uncharged drug species) did not increase the degree of phenylhydrazine block (not shown).

The absence of voltage dependence could potentially be explained by phenylhydrazine binding to a different site located outside the ion-conducting pathway. Because phenylhydrazine routinely caused the bilayers to become unstable, a direct competition experiment between a slower acting drug like tetrapropylammonium and phenylhydrazine was not possible. However, removal of external sodium ions increased the affinity of phenylhydrazine for the blocking site, suggesting that the site is located within the conducting pore. These results parallel observations on the effect of external sodium ions on QX-314 block and diethylamine block (Zamponi et al., 1993a; Zamponi and French, 1993), suggesting that phenylhydrazine and the other compounds bind in the conducting pathway. Thus, the aromatic rings of all compounds may interact with the same site.

Protonated hydrazine readily permeates the pore, as described by Hille (1971). He found a permeability ratio P_{Hyd}/P_{Na} of 0.59. Our own experiments (Fig. 6) gave a value of 1.7. The difference might reflect intrinsic differences between cardiac and neuronal channels, or an effect of

batrachotoxin to modify ion selectivity (e.g., Khodorov and Revenko, 1979; Garber and Miller, 1987). The ability of hydrazinium to permeate the pore suggests that the hydrazine portion on the phenylhydrazine molecule should not prevent phenylhydrazine from significantly entering the pore. Despite this, the lack of voltage dependence of its blocking action indicates that the charged amino group does not penetrate the transchannel electric field.

A possible basis for the lack of voltage dependence of phenylhydrazine block is explored in Fig. 8. This naive cartoon assumes that the aromatic ring on the phenylhydrazine molecule interacts with the same hydrophobic binding site proposed for the other ammonium compounds. An aromatic interaction of some kind is supported by the observation that hydrazine permeated the pore readily; hence, it must be the aromatic ring that prevents phenylhydrazine from doing so. Phenylhydrazine is a planar molecule (Koo, 1965; Srinivasan and Swaminathan, 1968); thus, the three-dimensional orientation of the chain portion of the molecule relative to the channel would be spatially defined if the aromatic ring were partially immobilized by binding to the hydrophobic site. Because of the rigidity of the phenylhydrazine structure, it is possible that the phenylhydrazine molecule interacts with the channel in an orientation in which the terminal amine group does not significantly traverse the transmembrane voltage (Fig. 8).

In contrast, the position of the amino terminal in phenyltrimethylammonium would overlap with the position of the uncharged nitrogen atom of phenylhydrazine (Krug and Mueller, 1989) and, thus, as illustrated in Fig. 8, might be able to penetrate the electric field significantly. In the cartoon, for the charged terminal nitrogen of phenylhydrazine to enter the channel while the aromatic ring is anchored, it would have to rotate through a hydrophobic domain on the opposite side of the pore mouth, an energetically unfavorable path for the protonated nitrogen. In molecules like phenylmethylamine, the bond linking the amino terminal and the methyl carbon is tilted by about 60° relative to the aromatic plane (Djinovic et al., 1990) so that the charged amino terminal could enter the transmembrane voltage more deeply. The aryl-amine links of the longer molecules like procainamide, lidocaine, or phenylethylhydrazine, possess a higher degree of flexibility, allowing for possible conformations in which the amino terminal can penetrate the electric field, hence accounting for the "full" voltage dependence of block by these compounds. The energy required to obtain a "blocking" conformation is expected to vary among the different blockers and, thus, to be an important factor for defining the relative blocking affinities of these compounds. We have proposed, based on molecular modeling, such an argument to account for the sevenfold difference between the blocking affinities of lidocaine and procainamide (Zamponi et al., 1993c).

How steep is the voltage gradient in the sodium channel pore?

Block by phenyltrimethylammonium shows the "normal" voltage dependence ($z\delta = 0.45 \pm 0.03$). On the other hand,

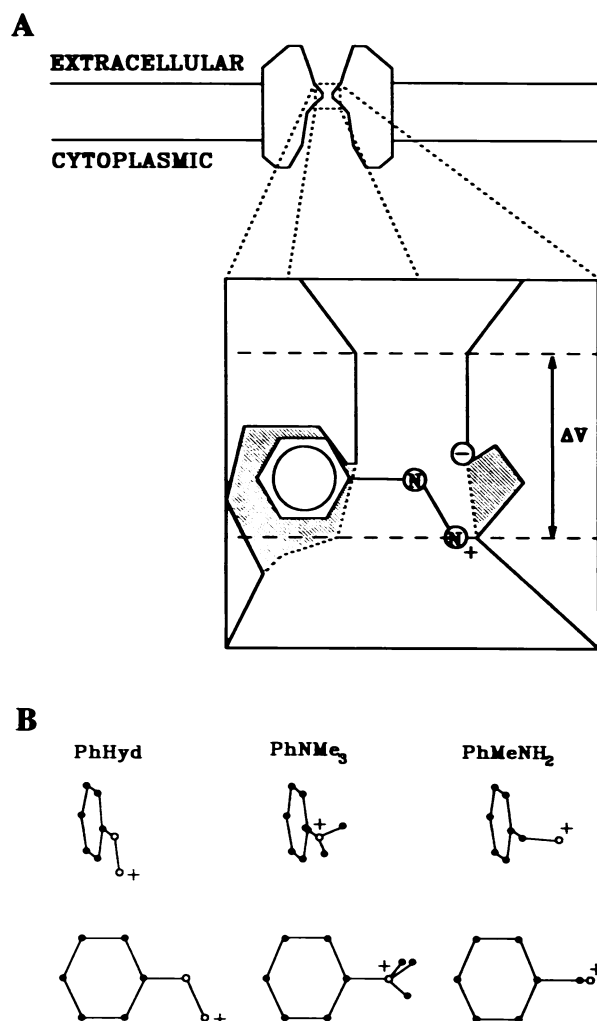


FIGURE 8 (A) Sketch of the immediate vicinity of the narrow region of the pore of the sodium channel, with a bound phenylhydrazine molecule. The arrow indicates the drop of the transmembrane voltage. In the enlarged panel, the dotted lines indicate the borders of potential hydrophobic binding domains of the channel. The negative charge indicates the binding site for ammonium compounds. Binding of the aromatic ring to the hydrophobic domain defines the orientation of the chain portion of the phenylhydrazine molecule, so that the charged terminal nitrogen cannot significantly penetrate the transmembrane voltage. For phenyltrimethylammonium, the position of the terminal amine group would overlap with the position of the uncharged nitrogen atom in phenylhydrazine (see panel B). Hence, the terminal amine group of phenyltrimethylammonium would be located about 45% of the way across the transmembrane voltage during block. The second hydrophobic domain indicated may bind potential alkyl chains attached to the terminal amino nitrogen. (B) Sketch of the orientations of the chains in phenylhydrazine, phenyltrimethylammonium, and phenylmethylamine relative to the aromatic rings in these molecules. The upper and the lower halves of the panel show the molecules at an angle of 75° and -15° , respectively. In addition, the structures in the upper half are tilted forward by 10° to facilitate comparison of the molecules. As can be seen, phenylhydrazine is a planar molecule, whereas in phenylmethylamine the bond between the methyl carbon and the amino terminal is tilted by about 60° relative to the aromatic plane. A comparison between panel A and the lower half of panel B demonstrates that the amino terminal of the phenylmethylamine molecule could penetrate the transmembrane electric field by 45%, whereas the amino terminal of phenylhydrazine might remain outside the field gradient when the aromatic rings of these molecules are bound to the hydrophobic site.

the phenylhydrazine molecule blocked at an apparent electrical distance, $z\delta = 0.06 \pm 0.08$. The distances between the terminal nitrogen atoms and the aromatic rings in these two molecules differ by only one bond length (see Figs. 1 and 8). In the most extreme case, in which the bond linking the two nitrogen atoms of phenylhydrazine is oriented parallel to the electric field, the voltage would drop about $40 \pm 10\%$ over one bond length (1.46 Å, (Srinivasan and Swaminathan, 1968)). If the voltage profile is not grossly nonlinear, this implies that the total transmembrane voltage drops over a distance of no more than about 5 Å. All energy barriers and wells for ions traversing the transmembrane voltage would therefore be confined to a very short stretch of the channel. This, not surprisingly, is considerably shorter than for K channels (e.g., Villarroel et al., 1988) that probably accommodate a relatively large number of permeant ions in single file.

Such a steep gradient could be consistent with the somewhat variable electrical distances, estimated for drugs like lidocaine, procainamide, diethylamine, or the compounds studied here which, we suggest, bind to the same site (Zamponi et al., 1993 a, c; Zamponi and French, 1993). Small variations in the chemical structures could result in measurable changes in the electrical distances because of differences in the degree of penetration of the center of charge of the drug into the electric field. Extreme steepness of the potential gradient has other important implications, which we explore in the following sections.

Implications for voltage-dependent block by guanidinium toxins

A relatively steep voltage gradient might also be consistent with the similar voltage dependence of block by differently charged saxitoxin derivatives (Moczydlowski et al., 1984) in BTX-activated skeletal muscle channels. STX derivatives with charges ranging from +2 to -1 all showed the same voltage dependence, perhaps implying that only the positively charged C8 guanidinium group enters the electric field from the extracellular side, whereas the other charges do not sense the applied voltage. Moczydlowski et al. (1984) discounted this possibility based on the steep voltage-dependence of TTX block. These authors reasoned that, for TTX to show such a steep voltage dependence, the lone charge on the TTX molecule would have to penetrate the electric field by a distance greater than the distance between the C8 guanidinium group and the other charges on the STX molecule and, hence, during STX block additional charge would have to enter the electric field. This would be inconsistent with their observations that STX derivatives with different net charges blocked with the same voltage dependence. However, this argument assumed a less steep potential gradient (<60% of the voltage dropping over 4 Å). A voltage gradient as steep as we propose here could account for lack of correlation between the voltage dependence of STX block with the number of charges on the STX molecule: the putative pore-blocking guanidinium groups of STX or TTX

might traverse 60% of the transmembrane voltage over a distance smaller than that between the C8 guanidinium group and the additional charge on the STX molecule.

Interactions among batrachotoxin, drug binding, and inactivation

Batrachotoxin reduces the affinity for the local anesthetic procaine by two orders of magnitude (e.g., Khodorov et al., 1975). However, BTX seems only weakly to affect block of sodium channels by the nonaromatic, tetramethylammonium (compare Uehara and Moczydlowski, 1986; Horn et al., 1981; Oxford and Yeh, 1985). This suggests that batrachotoxin reduces the affinity of local anesthetics for the proposed aromatic-binding part of the fast block receptor. This is distinct from the slow block receptor, which binds uncharged aromatic local anesthetics such as phenol (Zamponi et al., 1993b), and perhaps benzocaine, whose action appears unaffected by BTX (Schneider and Dubois, 1986). Batrachotoxin also reduces the affinity of the inactivation gate for its receptor (Zubov et al., 1983). West et al. (1992) demonstrated the importance, for fast inactivation of the sodium channel, of a cluster of hydrophobic residues, including one phenylalanine residue, located in the cytoplasmic loop connecting repeats III and IV. Phenylalanine, like some of the compounds studied here, possesses an aromatic ring that may enhance binding to the inactivation gate receptor.

We speculate that the proposed hydrophobic site that facilitates fast block might be part of the endogenous receptor for the inactivation gate. Batrachotoxin thus might reduce the affinity for local anesthetics/class 1 antiarrhythmics and the inactivation gate for the hydrophobic site via the same mechanism. Phenyltrimethylammonium appears to be able to bind simultaneously to the hydrophobic site and the amine receptor (substitution of an aromatic ring for one methyl group of tetramethylammonium increases the blocking affinity more than 10-fold). Considering the short distance between the charged aminoterminal and the aromatic ring of phenyltrimethylammonium, this suggests that the receptor for the inactivation gate and the charged-amine binding site are separated by less than 5 Å.

This is consistent with our previous results suggesting an interaction between the inactivation gate and open-channel block. Binding of open-channel blockers reduced the frequency of a population of long gating closures with features characteristic of inactivation (Zamponi et al., 1993c). The compounds studied here acted similarly (data not shown). If aromatic rings on the drug molecules, and on the inactivation gate, were able to bind to the same receptor, one would expect such an antagonistic interaction. In addition, however, nonaromatic compounds such as diethylamine (Zamponi and French, 1993) appear to inhibit the residual inactivation in BTX-activated channels, and analogous behavior is seen in unmodified sodium channels (e.g., Schauf, 1983; Oxford and Yeh, 1985; Goldman, 1988).

Tanguy and Yeh (1988) proposed that batrachotoxin may bind to the receptor for the inactivation gate. Nonetheless,

BTX-modified channels appear to inactivate to some degree (e.g., Wang and Wang, 1992a; Zamponi et al., 1993b). Perhaps BTX occupies only part of the global receptor for the inactivation gate, preventing the closing transition, but not completely masking that portion that normally interacts with the critical phenylalanine residue on the "hinged lid" to stabilize the inactivated state. Thus, the BTX receptor and the proposed aromatic binding domain for local anesthetics would be located in close proximity, as also suggested by observations in the following paper (Zamponi and French, 1994), and consistent with the antagonistic effect of BTX on both inactivation and local anesthetic block.

As mentioned earlier, data of Wang et al. (1991) indicated the presence of a hydrophobic pocket capable of binding long alkyl chains (10–18 carbons), suggesting that the hydrophobic binding pocket is relatively big. A large binding domain would be expected for the inactivation gate, given that a cluster of three adjacent hydrophobic amino acid residues appeared to be important for inactivation (West et al., 1992). This suggests that charged groups on the inactivation gate, in addition to the phenylalanine, might be able to enter the pore mouth. Such a mechanism was previously suggested by Goldman (1986, 1988) and might account for the intrinsic voltage dependence of inactivation in unmodified cardiac sodium channels (Yue et al., 1989). Nonetheless, the different view, that internal tetra-alkylammonium blockers do not inhibit inactivation but can stabilize the inactivated state, is presented by O'Leary and Horn (1994) and O'Leary et al. (1994), based on studies of cardiac sodium channels expressed in mammalian cells. Further work will be needed before these issues are fully resolved.

In summary, although direct evidence is lacking, the idea that batrachotoxin reduces both the affinity for local anesthetics and the inactivation gate, together with evidence that hydrophobic interactions are critical for both inactivation and block by ammonium compounds, and that block by these compounds prevents inactivation, are consistent with overlapping domains of interaction between batrachotoxin, lidocaine-like drugs, and the inactivation gate.

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