

Open-Channel Block by Internally Applied Amines Inhibits Activation Gate Closure in Batrachotoxin-Activated Sodium Channels

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ABSTRACT We have studied the action of several pore-blocking amines on voltage-dependent activation gating of batrachotoxin(BTX)-activated sodium channels, from bovine heart and rat skeletal muscle, incorporated into planar lipid bilayers. Although structurally simpler, the compounds studied show general structural features and channel-inhibiting actions that resemble those of lidocaine. When applied to the cytoplasmic end of the channel, these compounds cause a rapid, voltage-dependent, open-channel block seen as a reduction in apparent single-channel amplitude (companion paper). Internal application of phenylpropanolamine, phenylethylamine, phenylmethylaniline, and diethylamine, as well as causing open-channel block, reduces the probability of channel closure, producing a shift of the steady-state activation curve toward more hyperpolarizing potentials. These gating effects were observed for both cardiac and skeletal muscle channels and were not evoked by addition of equimolar *N*-Methyl-D-Glucamine, suggesting a specific interaction of the blockers with the channel rather than a surface charge effect. Kinetic analysis of phenylpropanolamine action on skeletal muscle channels indicated that phenylpropanolamine reduced the closed probability via two separate mechanisms. First, mean closed durations were slightly abbreviated in its presence. Second, and more important, the frequency of the gating closures was reduced. This action was correlated with the degree, and the voltage dependence, of open-channel block, suggesting that the activation gate cannot close while the pore is occluded by the blocker. Such a mechanism might underlie the previously reported immobilization of gating charge associated with local anesthetic block of unmodified sodium channels.

INTRODUCTION

Class 1 antiarrhythmics and their relatives inhibit batrachotoxin(BTX)-activated cardiac and skeletal muscle sodium channels (Zamponi et al., 1993a-c; Zamponi and French, 1993, 1994) in at least two ways. One observed mode of action was a rapid, flickery, open-channel block, which was favored by depolarizing potentials, did not distinguish between sodium channel isoforms from heart and skeletal muscle (Zamponi et al., 1993a), and appeared to be caused primarily by binding of the positively charged amino terminal of the drug molecule to the narrow region of the pore (Zamponi and French, 1993). Open-channel block prevented transitions to a long-lived, cardiac-specific population of voltage-independent gating closures with features characteristic of inactivation (Zamponi et al., 1993c). However, we were unable to investigate a potential effect of these agents on voltage-dependent gating, because the flickery nature of the open-channel block interfered with our analysis of the rapid, voltage-dependent gating closures.

In a companion paper, we examine the blocking actions of a series of relatively simple, lidocaine-related compounds on BTX-activated cardiac sodium channels (Zamponi and French, 1994). Three of these compounds, phenylmethylaniline, phenylethylamine, and phenylpropanolamine, blocked the channels as effectively as lidocaine. However, block appeared to occur on a much faster timescale than channel gating and, thus, was seen as a clean reduction in

single-channel amplitude, without any apparent increase in open-channel noise, therefore causing no interference with the recording of brief gating events. Thus, these compounds are especially useful probes to investigate the effects of open-channel block on voltage-dependent gating. Furthermore, because these compounds are such effective open channel blockers (e.g., the apparent dissociation constant for phenylpropanolamine at 0 mV was 3.6 mM; Zamponi and French, 1994), all experiments can be performed at concentrations below 20 mM, so that the small net increase in ionic strength associated with the addition of positively charged blocking ions does not result in significant surface charge-related effects on channel gating (Cukierman et al., 1988).

Here, we report the effects of these three compounds on voltage-dependent gating of BTX-activated rat skeletal muscle and bovine cardiac sodium channels. For both channel subtypes, the probability of channel closure decreased in the presence of open-channel blockers, effectively shifting the activation curve toward more hyperpolarizing potentials. A detailed kinetic analysis carried out for phenylpropanolamine block of the skeletal muscle subtype suggested that there is some closed-state binding that allosterically enhances recovery from channel closure. Nonetheless, the principal action was that binding to the receptor for open-channel block prevented activation gate closure. This latter effect may explain the immobilization of gating charge previously reported for local anesthetic block of unmodified channels (Cahalan and Almers, 1979a).

MATERIALS AND METHODS

Membrane vesicle preparations from bovine heart and rat skeletal muscle, formation of neutral lipid bilayers, and channel incorporation were as described by Zamponi et al. (1993a). In general, only single-channel incorporations were used for experiments. In three cases, a second channel with

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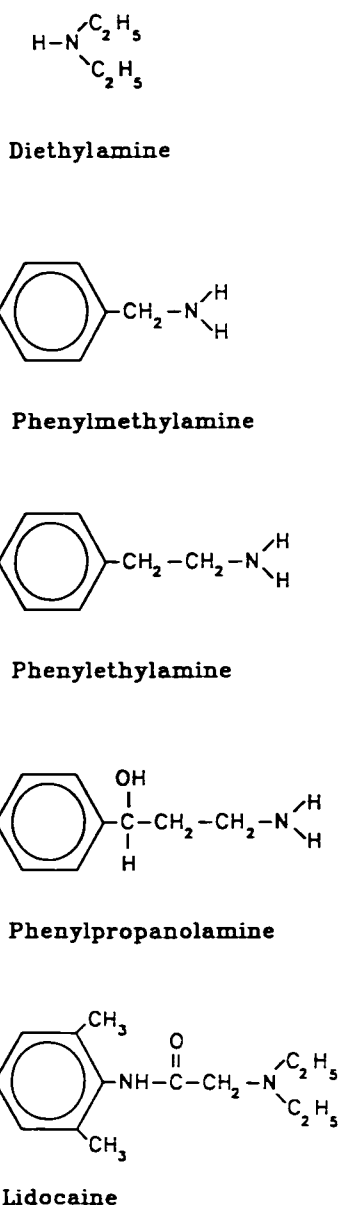


FIGURE 1 Chemical structures of the blockers used in the present study, in comparison with the chemical structure of lidocaine. Phenylpropanolamine, phenylethanamine, and phenylmethanamine resemble the core structure—an aromatic ring, an aryl-amine link, and an amino terminal—of the lidocaine molecule. To investigate a potential role of the aromatic ring, we included diethylamine in our study. All of these compounds have been shown to mimic open-channel block by lidocaine (Zamponi and French, 1994).

opposite orientation was incorporated while an experiment was in progress. These channels were blocked by $14 \mu\text{M}$ tetrodotoxin.

All of the blockers studied were prepared as concentrated stock solutions and simply added to the intracellular side of the channel. The drug stock solutions used were as follows: 100 mM phenylpropanolamine (PhPrOHNH_2), 200 mM NaCl, 20 mM MOPS; 200 mM phenylethanamine (PhEtNH_2), 200 mM NaCl, 20 mM MOPS; 200 mM phenylmethanamine (PhMeNH_2), 200 mM NaCl, 20 mM MOPS; 1 M diethylamine, 3 M NaCl, 50 mM MOPS. All solutions were titrated to a pH of 7.0. The chemical structures of the blockers studied are displayed in Fig. 1. Although we consistently refer to all of the compounds as amines, their relatively high

pKs ensure that the protonated (ammonium) form predominates in our solutions.

Data were acquired in continuous segments of 10 s to 2 min under each condition. Data were filtered at 500 Hz and sampled at 1 kHz during transcription into a personal computer (Compaq 386) and analyzed using pClamp software (Axon Instruments, Foster City, CA). The mean durations of the gating events and the associated open probabilities were determined from events lists created at a bandwidth of 150 Hz. No events were ignored during the creation of events lists; however, filtering at 150 Hz results in the loss of events shorter than 1.2 ms (McManus et al., 1987). We did not correct for missed events. The degree of open-channel block was determined from the reduction in apparent single-channel amplitude, which was measured by cursor from the digitized records. The kinetic analysis of our data was carried out as described in the Appendix. Preparation of figures and all fits were conducted using Sigmaplot v. 5.0 (Jandel Scientific, Corte Madera, CA). Confidence intervals for the regression lines were calculated according to the formalism presented in Larsen and Marx (1981) using MathCAD v. 2.0 (Mathsoft Inc., Cambridge, MA).

RESULTS

Tertiary amines reduce the probability of channel closure as well as causing fast, open-channel block

A variety of aromatic and nonaromatic amines, including phenylpropanolamine, phenylethanamine, and phenylmethanamine, block BTX-activated bovine cardiac sodium channels (Zamponi and French, 1994) by rapidly occluding the pore. This type of block is observed as a reduction in apparent single-channel amplitude without an increase in open-channel noise. A preceding study (Zamponi et al., 1993a) indicated that this "fast block" did not distinguish between the skeletal muscle and the cardiac sodium channel isoforms. As expected from these results, we observed a similar type of block when these three compounds were applied to the intracellular side of BTX-activated rat skeletal muscle sodium channels.

Fig. 2 shows current traces recorded from a skeletal muscle channel in the absence and presence of two fast open-channel blockers. In the absence of blockers, this particular channel was open about 80% of the time, showed a single-channel amplitude of 1.45 pA at a membrane potential of -80 mV , and exhibited rapid, voltage-dependent gating closures. In the presence of 18 mM phenylpropanolamine or 18 mM phenylethanamine, the single-channel current amplitude was reduced by about 50% without an apparent increase in open channel noise. These data resemble the action of these two compounds on the BTX-activated bovine cardiac sodium channel (Zamponi and French, 1994) and suggest a very rapid blocking action beyond the temporal resolution of the recording. In addition to the reduced single-channel amplitude, phenylpropanolamine (20 experiments), phenylethanamine (5 experiments), and phenylmethanamine (9 experiments, data not shown) reduced the frequency of voltage-dependent gating closures (see Fig. 2), hence resulting in an apparent increase in open probability (what we refer to as "open probability" is really the probability of not being in a closed state of the channel). The effect on both single-channel amplitude and gating closures was reversed upon washout. Application of 18 mM *N*-Methyl-Glutamine (NMG) had no noticeable

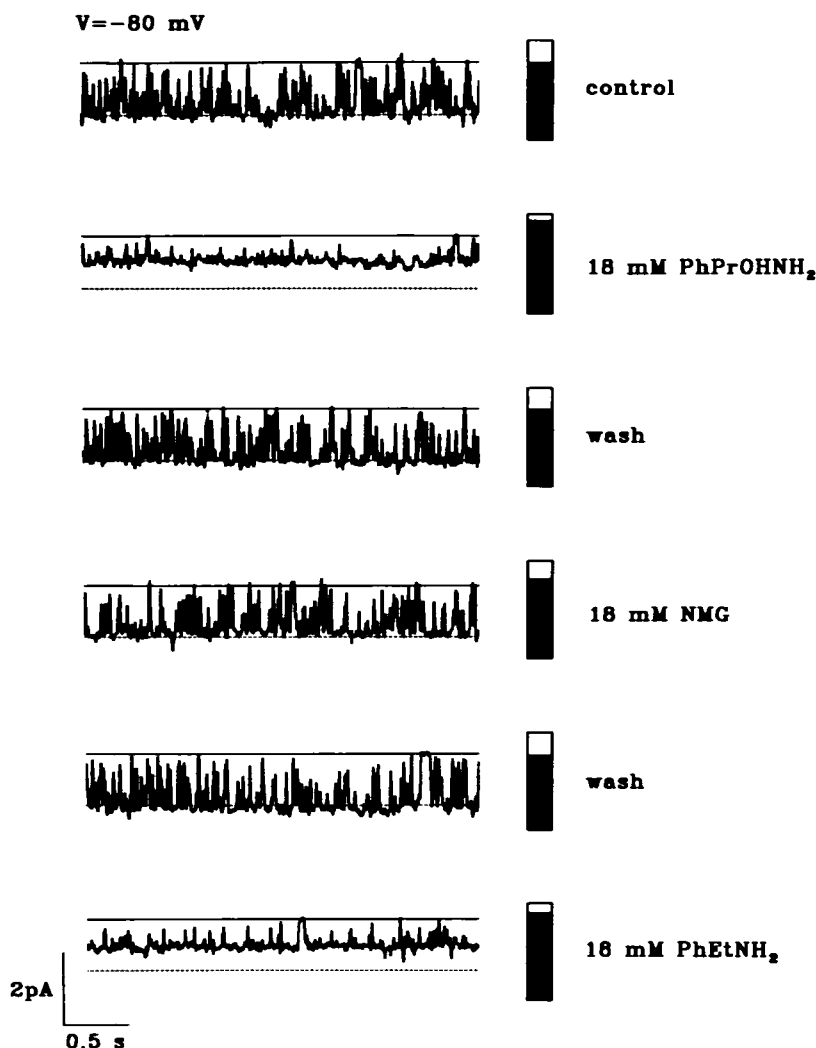


FIGURE 2 Current traces recorded from a single, BTX-activated rat skeletal muscle sodium channel. The records were filtered at 50 Hz; solid lines indicate the closed level, and dashed lines indicate the open level in absence of the blocker. The solid bars represent the apparent open probability on scale from 0 to 1. In absence of blockers, the channel showed rapid voltage-dependent gating closures at a holding potential of -80 mV. In presence of 18 mM phenylpropanolamine or phenylethylamine, the apparent single-channel amplitude was reduced, presumably because of very rapid block that was not resolved as discrete transitions between the open and the blocked states. In addition, the frequency of the gating closures was drastically decreased, resulting in an apparent increase in open probability. The effect was fully reversed upon washout. 18 mM NMG had no noticeable effect on either single-channel amplitude or gating.

effect on either single-channel amplitude or gating, suggesting that the enhanced apparent open probability did not result from a surface charge effect. Paired t-tests indicated probabilities of 0.93 (6 experiments, 18 mM NMG), 0.036 (3 experiments, 18 mM phenylethylamine), and 0.046 (7 experiments, 18 mM phenylmethethylamine) of the observed effects on channel gating occurring due to chance. Thus, both of the blockers appear to cause a significant increase in apparent open probability.

Fig. 3 illustrates the effect of 18 mM phenylpropanolamine on the steady-state activation curve of the channel. In the presence of phenylpropanolamine, the activation curve is shifted toward more hyperpolarizing potentials. A t-test based on three experiments indicates that the shifts of the midpoints (8.7 ± 2.3 mV, mean \pm SD) were statistically significant ($P < 0.03$). We did not resolve, however, a consistent change in the slope of the fits. The observed hyperpolarizing shifts of the steady-state activation curves are consistent with phenylpropanolamine increasing the apparent open probability over the full range of voltage-dependent channel gating.

A nonaromatic blocker, diethylamine, also antagonizes voltage-dependent gating closures

We have shown that aromatic amines were more effective open-channel blockers than their nonaromatic derivatives (Zamponi and French, 1994). Our data indicated that block was primarily caused by binding of the amino terminal end of the blocker to a hydrophilic site, but that block was enhanced by binding of the aromatic portion of the blocker to a hydrophobic region of the channel. To test whether the antagonistic effect of phenylpropanolamine on the gating closures was caused by the hydrophilic or the hydrophobic ends of the molecule, we investigated the action of diethylamine, a compound that does not carry an aromatic ring. We have previously shown that diethylamine caused fast block of BTX-activated rat skeletal muscle sodium channels; however, the blocking affinity ($K_d(0 \text{ mV}) = 67.5$ mM; Zamponi and French, 1993) was much lower than, for example, that of phenylpropanolamine ($K_d(0 \text{ mV}) = 3.6$ mM; see Fig. 8). Hence, block at negative potentials requires diethylamine concentrations larger than 100 mM. Under our usual experimental conditions,

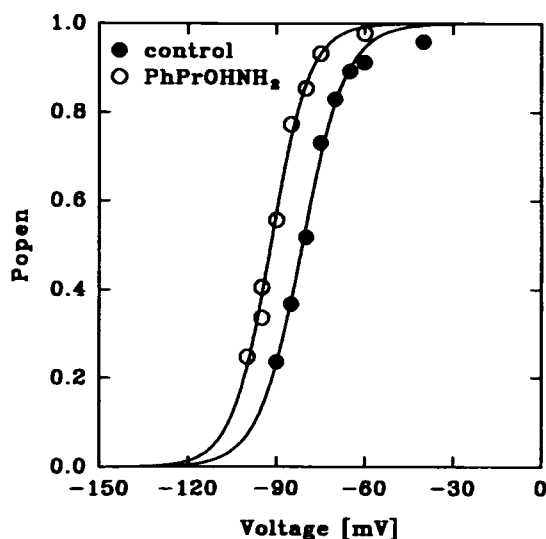
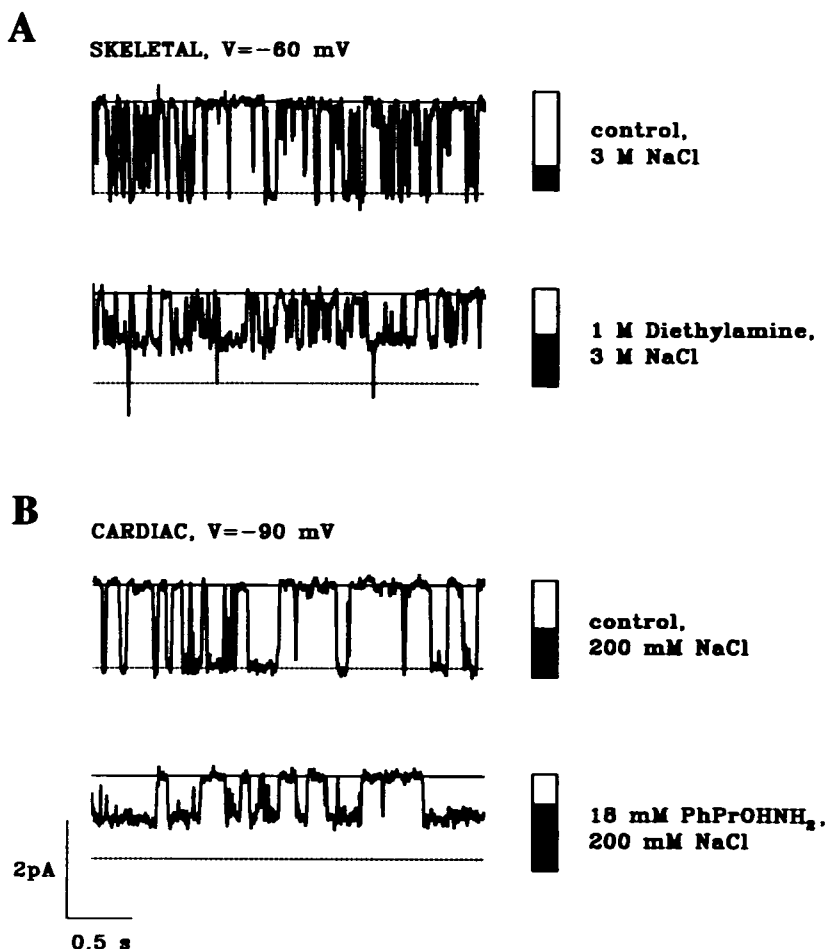


FIGURE 3 Steady-state activation curves recorded from a BTX-activated skeletal muscle channel in absence and presence of 18 mM phenylpropanolamine. The data were approximated with the Boltzman equation $P_{\text{open}} = 1/(1 + \exp(-(V - V_H)/25.4))$, where V is the membrane potential, z is the effective gating charge (and an indication of the steepness of the fit), and V_H is the potential at which $P_{\text{open}} = 0.5$. Phenylpropanolamine (18 mM) shifted the activation curve to more negative potentials, reflecting the reduced frequency of voltage-dependent gating closures. The fitting parameters were as follows: control, $z = 3.5$, $V_H = -81.2$ mV; phenylpropanolamine, $z = 3.9$, $V_H = -91.9$ mV.

the addition of 100 mM of any positively charged ion would result in significant surface charge screening and, thus, would lead to a hyperpolarizing shift of the steady-state activation curve (Cukierman et al., 1988). However, it has been shown that surface charge is effectively screened when recording at sodium concentrations larger than 500 mM (Cukierman, 1991). Hence, in high ionic strength solutions, even addition of large concentrations of diethylamine would not be expected to cause a surface charge-related shift of channel gating. This approach was recently used by Cukierman (1993) to study direct effects of divalent cations on sodium channel gating.

Fig. 4 A shows records obtained in symmetric 3 M NaCl/50 mM MOPS at a pH of 7.0. In the absence of diethylamine, the channel showed pronounced voltage-dependent gating closures at a membrane potential of -60 mV ($P_{\text{open}} = 0.25$). Perfusion of the chamber facing the intracellular side of the channel with 1 M diethylamine/3 M NaCl/50 mM MOPS (pH 7.0) resulted in fast block (again, seen as a reduction in apparent single-channel amplitude), as well as in an apparent increase in open probability ($P_{\text{open}} = 0.54$). When the chamber was perfused with 1 M NMG/3 M NaCl/50 mM MOPS (pH 7.0), the apparent open probability was not significantly affected (data not shown). Based on a paired t-test, the probability of occurrence of a change in

FIGURE 4 (A) Traces recorded from a BTX-activated rat skeletal muscle sodium channel. In this particular experiment, the recording solution was 3 M NaCl/50 mM MOPS at a pH of 7.0. In absence of blockers, this channel was closed most of the time at a membrane potential of -60 mV. Perfusion of the chamber facing the intracellular side of the channel with 1 M diethylamine in 3 M NaCl/50 mM MOPS resulted in pronounced block and a pronounced increase in open probability. The three downward deflections in the diethylamine trace reflect bilayer instability, not opening of a second channel. (B) Current traces recorded from a BTX-activated bovine cardiac sodium channel, bathed in symmetric 200 mM NaCl/20 mM MOPS (pH 7.0), at a membrane potential of -90 mV. As in Fig. 2, application of 18 mM phenylpropanolamine caused fast block and an increase in apparent open probability. For both panels A and B, solid lines superimposed on the records indicate the closed level, and the dashed lines indicate the open level in absence of blockers. All records were filtered at 50 Hz. The solid bars to the right of the traces reflect the apparent open probability on a scale from 0 to 1.



apparent open probability, due to chance alone, was 0.043 for diethylamine (4 experiments), but 0.912 for NMG (3 experiments), indicating that the small, protonated diethylamine molecule was a sufficient structure to inhibit closure of the channel, and that surface charges were effectively screened by sodium ions when recording in 3 M NaCl.

Because phenylpropanolamine, phenylethylamine, phenylmethylamine, and diethylamine appeared to show similar effects on the channel gating, we focused subsequent experiments on the most effective blocker, phenylpropanolamine.

Inhibition of voltage-dependent gating closures by phenylpropanolamine is not tissue-specific

To test whether the effect of phenylpropanolamine on the voltage-dependent gating closures was tissue-specific, we studied the effect of 18 mM internally applied phenylpropanolamine on BTX-activated bovine cardiac sodium channels (3 experiments). Fig. 4 B shows traces recorded from a typical BTX-activated bovine cardiac sodium channel. As with the skeletal muscle channels, the voltage-dependent gating closures of the cardiac isoform were antagonized in the presence of 18 mM phenylpropanolamine.

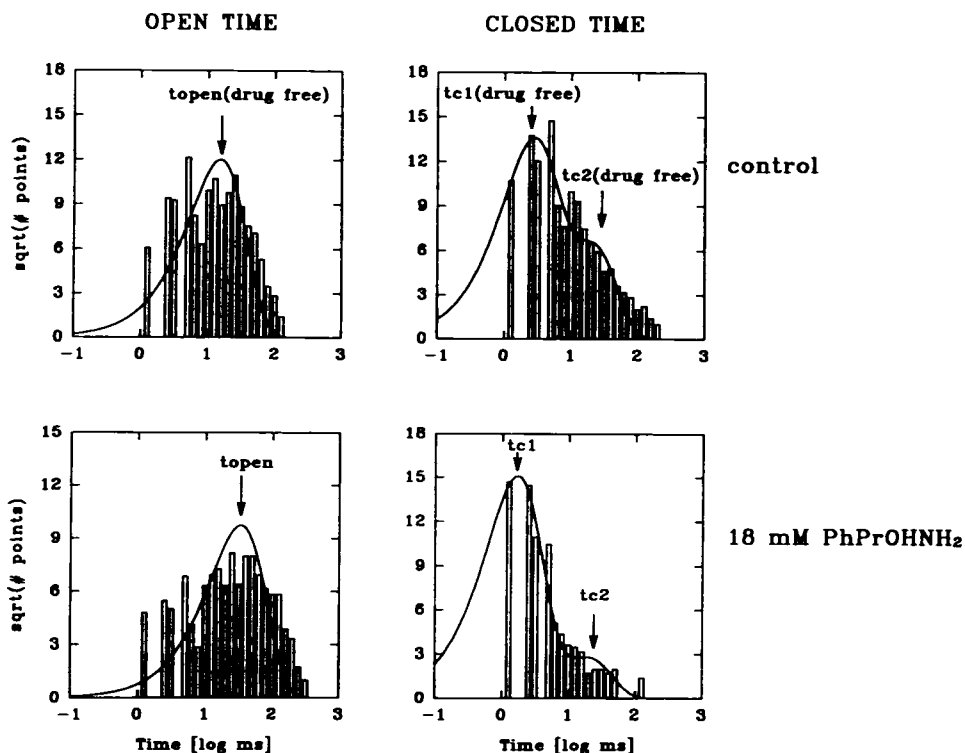
Unlike BTX-activated skeletal muscle sodium channels, the BTX-activated cardiac isoform shows a population of long, voltage-independent gating closures (mean time = 150–600 ms), with features characteristic of inactivation (French et al., 1990; Zamponi et al., 1993b; Zamponi and French, 1993). We have shown that these inactivating events are antagonized by internally applied fast blockers such as

procainamide, QX-314, or diethylamine (Zamponi et al., 1993c; Zamponi and French, 1993). In the present study, a selective effect of phenylpropanolamine on the inactivating events could only account for a fraction of the observed increase in apparent open probability (in absence of blockers, the three channels studied here showed only a low probability of closure at positive potentials), suggesting that we were indeed observing an antagonistic effect on voltage-dependent gating closures. However, a detailed kinetic analysis of the drug-induced effects on voltage-dependent gating closures of the cardiac subtype is complicated by the presence of these inactivating events. Moreover, aromatic compounds cause a slower, cardiac-specific blocking mode by binding to, and stabilizing, the long closed state (Zamponi et al., 1993b; Zamponi and French, 1993), thereby decreasing the open probability. For these reasons, we restricted our detailed kinetic analysis of effects on voltage-dependent gating to the skeletal muscle subtype.

Phenylpropanolamine affects open and closed durations

Fig. 5 shows dwell-time distributions for a typical BTX-activated rat skeletal muscle sodium channel in absence and presence of 18 mM phenylpropanolamine at a membrane potential of -80 mV. In absence of blockers, the open-time distribution was well described with one time constant, t_{open} (drug free) of about 15 ms. The channel showed two populations of voltage-dependent gating closures, a very rapid component, t_{c1} (drug free), with mean durations from 2 to 6 ms, and a slower component, t_{c2} (drug free), with mean du-

FIGURE 5 Dwell-time histograms, in the form devised by Sigworth and Sine (1983) obtained from a BTX-activated skeletal muscle sodium channel in absence and presence of 18 mM phenylpropanolamine at a membrane potential of -75 mV. Apparent open times were fitted with one time constant, t_{open} (drug free), and closed times were fitted with two components, t_{c1} (drug free) and t_{c2} (drug free). Application of 18 mM phenylpropanolamine caused an increase in apparent mean open time. In addition, the briefer closed times were shortened, whereas the longer closures did not appear to be as strongly affected. The time constants obtained from the fits were as follows: t_{open} (drug free) = 15.3 ms; t_{open} = 34.0 ms; t_{c1} (drug free) = 2.46 ms; t_{c1} = 1.7 ms; t_{c2} (drug free) = 19.6 ms; t_{c2} = 18.6 ms.



rations ranging from 15 to 30 ms. As illustrated in Fig. 2, phenylpropanolamine block was not resolved as discrete transitions between the open and the blocked states. Both open and blocked events appeared as part of an apparent "open" state with a reduced single-channel amplitude (see Appendix). Hence, what we refer to as "apparent mean open times" are, strictly speaking, the mean durations between the gating closures and include the unresolved fast blocked events. Addition of 18 mM phenylpropanolamine increased this apparent mean open time to 34 ms and slightly shortened the mean duration of the shorter gating closures. There was little, if any, effect on the longer closures (but see Discussion).

Fig. 6 illustrates the concentration dependences of the dwell-time components for 11 channels at a membrane potential of -80 mV. The apparent mean open times increased linearly with phenylpropanolamine concentration (Fig. 6 A), consistent with the idea that phenylpropanolamine block prolongs the apparent open state. According to the kinetic analysis presented in the Appendix, the inverse of the slope of the fit (7.7 mM) reflects the equilibrium dissociation constant, K_D , for phenylpropanolamine block. The mean durations of the the two types of gating closures seemed to decrease

slightly with phenylpropanolamine concentration (Fig. 6, B and C), indicating that phenylpropanolamine enhanced the recovery from channel closure; however, this effect was not as strongly concentration-dependent as the increase in apparent mean open time. In particular, the mean durations of the slower gating component (Fig. 6 C) showed a large scatter, which probably reflects a limited resolution of this component in the dwell-time distribution. In our experiments with diethylamine, both types of gating closures were shortened in the presence of the blocker, suggesting that the presence of an aromatic ring on the blocker was not required for the faster recovery from channel closure.

The increase in apparent mean open time is correlated with phenylpropanolamine block

Fig. 7 shows dose-response curves obtained from the degree of phenylpropanolamine block and from the increase in apparent mean open time. The degree of phenylpropanolamine block is reflected in a decrease of the apparent single-channel amplitude (e.g., Zamponi and French, 1993). The concentration dependence of the amplitude decrease was well fitted with a simple hyperbola, suggesting a 1:1 interaction be-

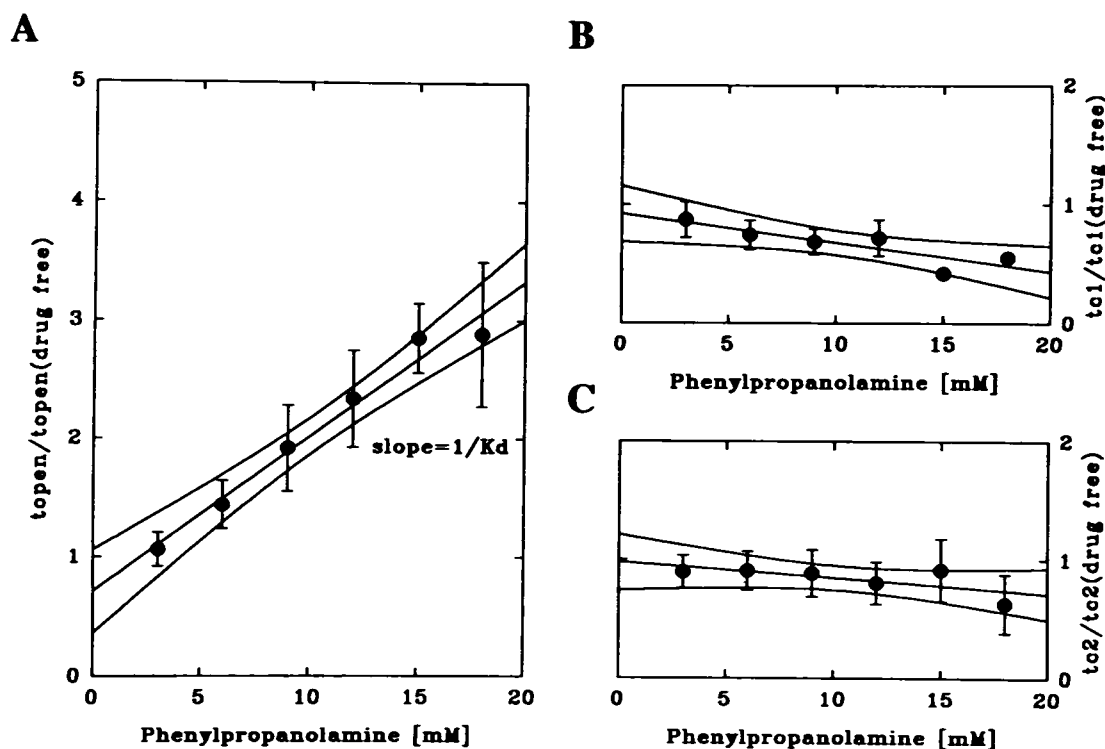


FIGURE 6 Dependence of the dwell times on the phenylpropanolamine concentration at a membrane potential of -80 mV for the skeletal muscle isoform. The symbols represent means from one to seven data points, error bars indicate standard errors, solid straight lines are linear regressions, and the curved lines are 95% confidence intervals. Data from 11 experiments were included in the figure. (A) Apparent mean open times increase with increasing phenylpropanolamine concentration. According to the kinetic analysis described in the Appendix, the inverse of the slope of the fit ($r = 0.99$, slope = 0.131 ± 0.063 at 95% confidence) reflects the equilibrium dissociation constant, K_D , for phenylpropanolamine block (7.7 mM). (B) The mean durations of the faster gating component, t_{cl} , decrease with increasing phenylpropanolamine concentration ($r = 0.86$, slope = -0.024 ± 0.018 at 95% confidence). (C) The mean durations of the longer gating closures, t_{cs} , also appear to decrease with phenylpropanolamine concentration ($r = 0.67$, slope = -0.014 ± 0.036 at 95% confidence). A statistical analysis of the correlation coefficients (Larsen and Marx, 1981) indicated that the slopes differ significantly from zero ($P > 0.99$). Note that the intercepts of the fits were allowed to vary from 1. In panel A, when a regression was formed constraining the intercept to 1, the obtained slope was 0.106, indicating a K_D for phenylpropanolamine block of 9.5 mM.

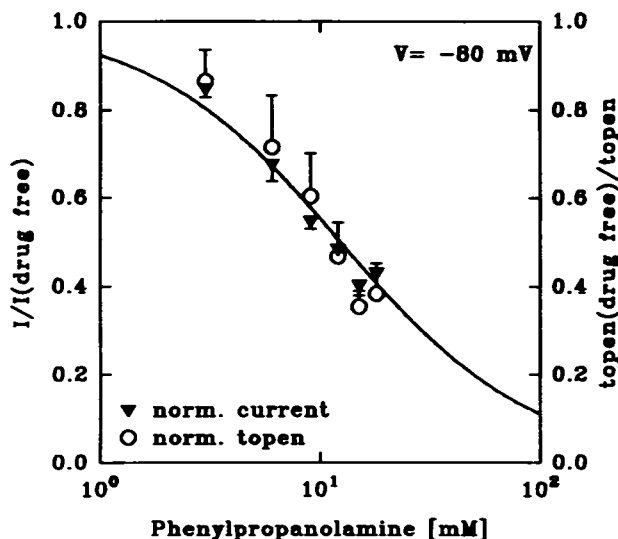


FIGURE 7 Dose dependence of the effects of phenylpropanolamine on the apparent single-channel amplitude (∇ , downward error bars) and on the apparent mean open times (\circ , upward error bars) for the skeletal muscle isoform at a membrane potential of -80 mV. Error bars indicate SEs; the figure includes data from 11 experiments. Each data set was fitted with a simple hyperbola (—), $1/(drug\ free) = 1/1 + [P]/K_d$ or $t_{open}(drug\ free)/t_{open} = 1/1 + [P]/K_d$, where $[P]$ is the phenylpropanolamine concentration and K_d is the concentration at half block. The K_d values obtained from the amplitude decrease, and the increase in apparent mean open time were 12.4 and 12.3 mM, respectively, and hence, the fits overlap.

tween the blocker and the channel. The concentration at the midpoint of the fit reflects the equilibrium dissociation constant, K_d , for phenylpropanolamine block.

In the Appendix, we present a linear four-state closed-closed-open-blocked model in which, because of the rapid nature of the blocking events, the open and the blocked states are observed as a single, apparent open state with a reduced single-channel amplitude and increased lifetime. In this model, increasing the concentration of phenylpropanolamine shifts the equilibrium toward the apparent open state, thereby increasing the mean lifetime, t_{open} , of this state. A similar analysis was used to describe the antagonistic effect of another fast blocker, procainamide, on voltage-independent gating closures in BTX-activated bovine cardiac channels (Zamponi et al., 1993c) and to account for the competition between tetraethylammonium and the inactivation gate of voltage-dependent potassium channels (Choi et al., 1991). In the present case, plotting the ratio $t_{open}(drug\ free)/t_{open}$ as a function of phenylpropanolamine concentration yields a simple hyperbola with a half-maximal concentration equal to the K_d for phenylpropanolamine block (see Appendix). As can be seen from Fig. 7, the fits of both the fractional block and the apparent open times virtually overlap, suggesting that, at least at a membrane potential of -80 mV, the occupancy of the open channel blocking site and failure of channel closure are closely correlated.

The K_d value obtained from the fit of the gating data in Fig. 7 (12.3 mM) is somewhat larger than the value obtained

from the slope in Fig. 6 A (7.7 mM). However, because both Figs. are based on the same data set (but apparent open times are inverted in Fig. 7), we attribute this discrepancy, in part, to a difference in the fitting procedures. The inversion of the data in Fig. 7 results in a different weighing of the data points. Furthermore, when the intercept in Fig. 6 A was held at the theoretical value of 1, the obtained K_d value was 9.5 mM, closer to that obtained in Fig. 7.

Fast open-channel block is favored by depolarization. If phenylpropanolamine block prevents channel closure, one would expect to detect a similar voltage dependence for the increase in apparent mean open time. Fig. 8 A shows the voltage dependences of the equilibrium dissociation constant, when calculated either directly from the fractional block or indirectly from the increase in apparent mean open time (see Eq. 7, Appendix). As can be seen from the figure, the voltage dependence of the increase in mean open time ($z\delta = 0.56$) closely approximates that of the fractional block ($z\delta = 0.44$). In addition, the K_d values obtained by either method are similar in magnitude. Fig. 8 B illustrates the voltage dependence of phenylpropanolamine block determined over a larger voltage range. The voltage dependence ($z\delta = 0.44$) is consistent with that obtained in Fig. 8 A, indicating that the voltage range of 45 mV used in Fig. 8 A was sufficient to provide an indication for the voltage dependence of phenylpropanolamine block.

Overall, our data suggest that the activation gate of the channel cannot close when a phenylpropanolamine molecule is blocking the open channel.

A decrease in closed times contributes to the increase in apparent open probability

As we have shown in Fig. 6 B, the mean closed durations appeared to be slightly shortened by the presence of phenylpropanolamine. To illustrate the contribution of this effect to the overall apparent open probability, the data are mathematically scaled so that they, in theory, show Michaelis-Menten dependence on the phenylpropanolamine concentration (see Appendix, Eq. 10) and plotted. This method has the particular advantage that it does not require the fitting of dwell-time histograms and, thus, is not dependent on resolution of individual dwell-time components. Fig. 9 compares the concentration dependence of the transformed data with that of the fractional block (and thus, the effect on the apparent mean open time, see Fig. 7). When the data reflecting the apparent open probability were fitted with a simple hyperbola, we obtained an ED_{50} value of 5.6 mM, which is less than half of the concentration required for a 50% amplitude reduction. Because the effect on the apparent mean open time was nearly perfectly correlated with the degree of block (Figs. 7 and 8 A), the lack of correlation between the apparent open probability and open-channel block must be caused by an enhanced rate of channel opening. Hence, we propose that phenylpropanolamine can bind to a closed state of the channel and thereby enhance recovery from closure.

For an empirical description of the effect of phenylpropanolamine on the apparent open probability, we expand the previously introduced linear four-state closed (C2)- closed (C1)- open (O)- blocked (B) model, such that binding to a putative, saturable site allosterically increases the transition rate from C1 to O by a concentration-dependent factor (see Appendix). The newly introduced factor does not affect or invalidate the kinetic analysis of the apparent mean open times described in the preceding section. However, the apparent open probability of the channel is now increased by phenylpropanolamine via two separate processes: first, by prolonging open durations by preventing activation gate closure while bound to the open-channel blocking site; and second, by enhancing the recovery from channel closure by binding to a separate site accessible when the channel is in a closed state. Thus, the data reflecting the apparent open probability in Fig. 9 are described via three fitting param-

eters, one that reflects the equilibrium dissociation constant, K_d , for open-channel block, and two additional parameters that describe the binding affinity and maximal effect of the putative allosteric site. When the data were fitted according to this expanded model (fit in Fig. 9), the obtained K_d value for open-channel block was 13.1 mM, which is very close to the value obtained from Fig. 7. The additional fitting parameters describing the concentration dependence of the introduced allosteric factor, a , indicate that the rate of recovery from the closed state would be increased up to 26-fold (the maximum value is reached when the site is saturated at a concentration of about 3 M), and that a half-maximal effect is obtained at a phenylpropanolamine concentration of 377 mM. At the largest concentration used in our experiments (18 mM), the opening rate is increased only about twofold (see also Fig. 6 B).

Our data suggest that the apparent open probability of the channel is enhanced by the presence via two separate mechanisms: most important, phenylpropanolamine binding to the open channel prevents activation gate closure; and second, binding to the closed channel enhances channel opening.

DISCUSSION

Lidocaine-related amines cause fast-open channel block of skeletal muscle Na channels

In cardiac sodium channels, activated by BTX, several lidocaine-related amines cause rapid open-channel block (Zamponi and French, 1994). We note here, in passing, that at least three of these compounds block BTX-activated rat skeletal muscle sodium channels in a similar manner and with similar affinities. This is not surprising, because we have previously shown that rapid open-channel block by lidocaine, QX-314 (Zamponi et al., 1993a), procainamide

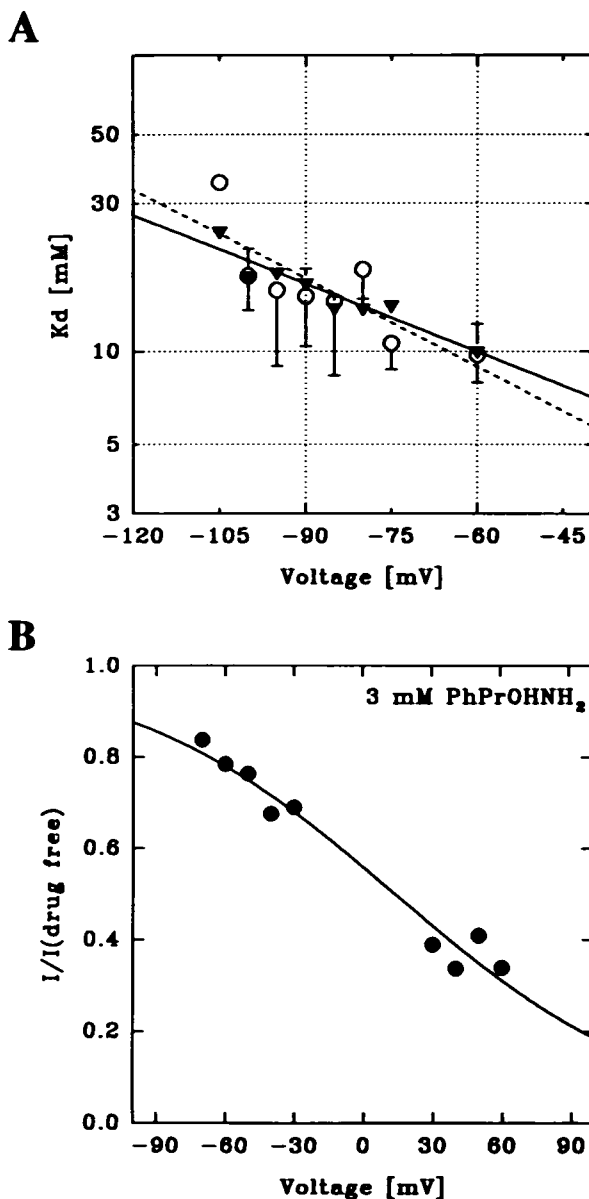


FIGURE 8 (A) Voltage dependence of the equilibrium dissociation constant for phenylpropanolamine block of the skeletal muscle isoform, determined either directly from the reduction in apparent single-channel amplitude (∇ , upward error bars, —), or from the concentration-dependent increase in t_{open} (\circ , downward error bars, ---). Each single point was calculated from the expressions presented in Fig. 7 (see also Appendix). Data from 16 experiments are included in the figure; the symbols are means from 1 to 28 data points. The regression lines are least-square fits; the error bars indicate SEs. The voltage dependence of the increase in apparent mean open time parallels that of fast block. The slopes of the regression lines reflect the fractional distance, $z\delta$, of the blocking site across the transmembrane voltage from the cytoplasmic end of the channel (Woodhull, 1973). We obtained $z\delta$ values of 0.56 and 0.44 when the K_d was calculated from gating and from the reduction in single-channel amplitude, respectively. (B) Voltage dependence of fast block by 3 mM phenylpropanolamine determined over a larger voltage range than in panel A. The data were obtained from a single experiment; the solid line is a fit based on the Boltzmann equation $1/(1 + \exp(z\delta(V_H - V)/25.6))$, where $z\delta$ reflects the steepness of the fit and is the apparent electrical distance from the cytoplasmic solution to the binding site, V is the membrane potential, and V_H is the potential at half-block. The apparent electrical distance of 0.44 is consistent with the result in panel A, suggesting that the phenylpropanolamine molecule penetrates the transmembrane voltage 44% of the way from the cytoplasmic end of the channel. The parameter V_H was 13.8 mV.

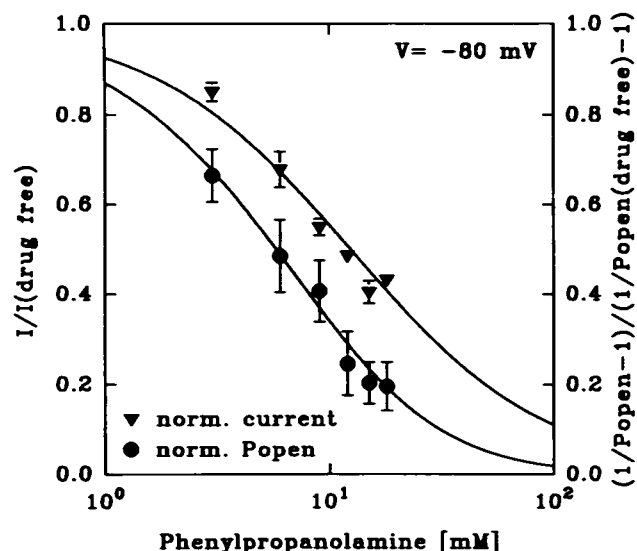


FIGURE 9 Phenylpropanolamine dose dependence of the apparent single channel amplitude (▼) and a normalized transform of the apparent open probability (●) for the skeletal muscle isoform at a membrane potential of -80 mV. Error bars indicate SEs, and the figure includes data from 11 experiments. The amplitude data, $1/I(\text{drug free})$, are identical to those in Fig. 7 and are included in this figure to facilitate comparison. The data reflecting the apparent open probability were fitted, according to the kinetic analysis presented in the Appendix, with the equation $(1/P_{\text{open}} - 1)/(1/P_{\text{open}}(\text{drug free}) - 1) = 1/((1 + [P]/K_d)^a)$, with $a = 1 + \max/(1 + K_s/[P])$, $[P]$ is the phenylpropanolamine concentration, K_d is the equilibrium dissociation constant for phenylpropanolamine block, K_s is the equilibrium dissociation constant for binding to the putative allosteric site, and \max reflects the saturability of the allosteric binding site. The fitting parameters were as follows: $K_d = 13.1$ mM, $K_s = 377$ mM, $\max = 25.9$. The obtained K_d value is close to the value obtained in Fig. 7.

(Zamponi et al., 1993c), and diethylamine (Zamponi and French, 1993) did not distinguish between channels from these two tissues. Our new data lend further support to the idea that the cytoplasmic end of the sodium channel pore is similar for both channel subtypes.

Amine pore blockers reduce the probability of channel closure

Intracellular application of phenylpropanolamine resulted in a shift of the steady-state activation curve toward more hyperpolarizing potentials. This shift reflects a decrease in the closed probability of the channel in the presence of the blocker. It has been shown previously that intracellular application of divalent cations results in a leftward shift of the steady state-activation curve, whereas externally divalent applied cations cause a shift in the opposite direction (e.g., Cukierman et al., 1988). It has been suggested that the effect does not require the screening of membrane surface charges, because it also occurs with channels incorporated into neutral lipid bilayers (e.g., Cukierman et al., 1987) and because the same concentration of divalent cations applied to the extracellular side results in a larger shift than internal application (e.g., Cukierman and Krueger, 1990, 1991). More recent work by Cukierman (1993) suggests that both internally and

externally applied barium ions shift the steady-state activation curve of BTX-activated rat brain channels through a specific interaction with the gating machinery in addition to possible screening of surface charges on the channel protein.

Our experiments suggest a similar conclusion for amine pore blockers. We recorded from sodium channels incorporated into uncharged phospholipid bilayers, ruling out membrane surface charge as a factor. Moreover, application of 18 mM NMG had no appreciable effect on channel gating, suggesting that the simple addition of 18 mM inert salt was not sufficient to screen negative charges on the channel surface. This is supported by our experiments carried out in 3 M NaCl. In solutions with such high ionic strength, essentially all of the available surface charges should be screened by sodium ions (Cukierman, 1991, 1993), and yet, diethylamine enhanced the apparent open probability of the channel, whereas an equimolar concentration of NMG had no effect. These observations suggest that lidocaine's open-channel blocking relatives decrease the closed probability of the channel by binding to a specific site at the intracellular mouth of the channel.

Recovery from channel closure is enhanced by amine pore blockers

In absence of blockers, we resolved two populations of voltage-dependent gating closures, brief ones (2–6 ms) and somewhat longer ones (15–30 ms). The mean durations of the shorter closures appeared to decrease with increasing phenylpropanolamine concentrations. The mean durations of the longer gating events showed a weaker concentration dependence (Fig. 6 C). According to the linear four-state model, C2-C1-O-B, the longer gating component, t_{c2} , is comprised of an electrically silent burst between the two closed states, C1 and C2. If phenylpropanolamine selectively enhances the transition rate from C1 to O, the burst duration and, hence, the closed time component t_{c2} , would decrease with increasing phenylpropanolamine concentrations. This decrease would be expected to be about 2 times as steep than the one observed in Fig. 6 C, but would still lie within the 95% confidence intervals. The data points in Fig. 6 C are quite scattered, because the longer events did not always occur sufficiently frequently for us to resolve properly this gating component in the dwell-time distributions (see Fig. 5). In six selected experiments in which the longer closures were more clearly resolved, the mean duration of these closed events decreased more strongly with phenylpropanolamine concentration and, hence, we feel justified in using the linear four-state kinetic model.

There is little evidence about the nature of the putative allosteric binding site. Because diethylamine also shortened closed durations, the site is probably distinct from the aromatic-binding domain thought to potentiate open-channel block (e.g., Wang, 1990; Zamponi and French, 1994). This points to an interaction between the charged amino terminal of the blocker with a hydrophilic region of the channel, similar to Cukierman's (1993) suggestion to explain the inter-

action between internal barium ions and sodium channel gating. In our case, the allosteric action was introduced to improve the fit obtained with the simple linear-four-state model (Scheme 2, Appendix).

Open-channel block prevents activation gate closure

A more clear-cut effect of phenylpropanolamine was the prevention of activation gate closure. The analysis of this effect is easier, because the open time distributions have only one time constant. Moreover, in contrast with the closed durations, the apparent mean open times were prolonged by phenylpropanolamine, hence facilitating the resolution of mean open times.

The increase in the mean durations between gating closures was correlated with both the degree and the voltage dependence of open-channel block, consistent with a model in which the activation gate is unable to close whenever a blocker is bound to the receptor for open-channel block. Our data contrast with the conclusions of Yeh and Tanguy (1985), who argued that the activation gate might be able to trap local anesthetics inside the channel. The difference between the study by Yeh and Tanguy and our results could arise from the batrachotoxin modification of the our channels. However, the arguments by Yeh and Tanguy were based on the voltage dependence of recovery from use-dependent block, rather than on direct measurement of dwell times. A previous study by the same group on 9-aminoacridine block of single *N*-bromoacetamide-modified sodium channels (Yamamoto and Yeh, 1984) indicated that channels could not close while blocked by 9-aminoacridine, consistent with our present findings and in contrast with the interpretation by Yeh and Tanguy. Our observations also parallel data by Cahalan and Almers (1979b), who demonstrated that *N*-methylstrychnine, an open-channel blocker, prevented the closure of both the activation and the inactivation gates of sodium channels from squid giant axons. Furthermore, for pronase-treated squid giant axons, Cahalan (1978) suggested that *N*-methylstrychnine, 9-aminoacridine, procaine, or QX-572 also prevented activation gate closure.

The fact that local anesthetic block causes an immobilization of gating charges (Cahalan and Almers, 1979a) is consistent with our observations that activation gate cannot close when the channel is blocked from the internal side. Local anesthetic-induced immobilization is similar to that associated with inactivation of the channel (e.g., Armstrong and Bezanilla, 1977). Goldmann (1986, 1988) reported that internal cations (such as cesium) antagonized inactivation of unmodified sodium channels and suggested that these ions prevented the entry of a positively charged side chain of the inactivation gate into the narrow region of the channel. We have previously shown that fast open-channel block prevented inactivation-like closures in BTX-activated cardiac sodium channels, perhaps because of competition between the blocker and a part of the inactivation gate (Zamponi et al., 1993c). These observations, and the results presented in the

current study, suggest that the immobilization of gating charges by inactivation and by local anesthetic block could arise from a common mechanism: either amine pore blockers or a structure on the inactivation gate might bind to the open channel, and occupation of this site might either directly, or allosterically, prevent activation gate closure.

We cannot, however, distinguish rigorously between a direct or an allosteric action. An allosteric mechanism could account for the effects on both open and closed durations. Open-state binding to the site with high affinity could allosterically prevent activation gate closure. When the channel is closed, drug binding to the site (which might shift to lower affinity when the channel is in the closed state) could allosterically enhance channel opening. In case of a direct action, the activation gate would have to be located close to the blocking site (and thus, the narrow region of the pore; Zamponi et al., 1993a; Zamponi and French, 1993, 1994), because the small diethylamine molecule was sufficient to prevent activation gate closure. In case of a single allosteric interaction, because the site appears to be accessible when the channel is closed, the activation gate would have to be located on the extracellular side of the open-channel blocking site. We have observed that rat skeletal muscle sodium channels continue to gate during partial block by one particular μ -conotoxin mutant, R13Q (Becker et al., 1992). Because these toxins seem to occlude the pore at its extracellular mouth, this would indicate that the activation gate is confined to a short stretch in the narrow region of the pore. If so, activation gate closure may involve a constriction of this region, which could be inhibited by impermeant blockers.

Overall, we favor the simple view that physical occlusion of the sodium channel pore by lidocaine-related amines prevents activation gate closure in BTX-activated sodium channels from heart and skeletal muscle. This antagonism may underlie the gating charge immobilization seen with local anesthetic block of unmodified channels.

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APPENDIX

Mathematical treatment of the kinetic models

In absence of blockers, at the bandwidth used, the voltage-dependent gating of BTX-activated rat skeletal muscle channels can be described in form of the linear three-state model:



In Scheme 1, the open probability, P_o (drug free), can be expressed as a function of the transition rate constants between the different kinetic states

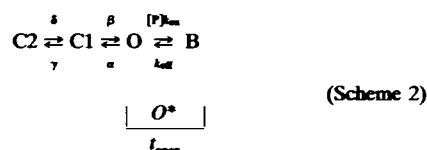
(Zamponi et al., 1993b) and is given by the equation

$$P_o(\text{drug free}) = \frac{1}{1 + \alpha/\beta + (\alpha/\beta)(\gamma/\delta)} \quad (1)$$

The mean time spent in the open state, $t_{\text{open}}(\text{drug free})$, depends only on the transition rate from the open state, O, to the first closed state, C1, and follows the relation

$$t_{\text{open}}(\text{drug free}) = 1/\alpha. \quad (2)$$

Application of an open channel blocker results in the addition of a blocked state, B.



Here, the true open time, t_o , and the true blocked time, t_b , are given by the relations

$$t_o = 1/(\alpha + k_{\text{on}}[P]) \quad (3)$$

and

$$t_b = 1/k_{\text{off}}, \quad (4)$$

where $[P]$ is the phenylpropanolamine concentration. However, because block by phenylpropanolamine is very rapid and appears as a reduction in single-channel amplitude, rather than discrete transitions between the open and the blocked states, we treat the blocked state as part of an apparent open state, O^* , with an apparent mean duration, t_{open} . The apparent open time is comprised of a burst between the open and the blocked states, and can be expressed as

$$t_{\text{open}} = (n+1)t_o + (n)t_b, \quad (5)$$

where n is the number of cycles within the burst (Colquhoun and Ogden, 1988). Because

$$n = k_{\text{on}}[P]/\alpha, \quad (6)$$

combining Eqs. 2–6 yields

$$\frac{t_{\text{open}}}{t_{\text{open}}(\text{drug free})} = 1 + \frac{k_{\text{on}}[P]}{k_{\text{off}}}. \quad (7)$$

Equation 7 is the basis for the plot in Fig. 6 A. Because the ratio $k_{\text{on}}/k_{\text{off}}$ is equivalent to the equilibrium dissociation constant for phenylpropanolamine block, K_d , plotting the relation in Eq. 7 as a function of phenylpropanolamine concentration yields a straight line with a slope equivalent to the inverse of the K_d . Equation 7 is used to calculate one of the sets of K_d values plotted in Fig. 8 A. The inverse of Eq. 7,

$$\frac{t_{\text{open}}(\text{drug free})}{t_{\text{open}}} = \frac{1}{1 + k_{\text{on}}[P]/k_{\text{off}}}, \quad (8)$$

when plotted as a function of phenylpropanolamine concentration, yields a simple hyperbola with a fitting parameter equivalent to the K_d . This Michaelis-Menten form of Eq. 7 is the basis for the plot of the apparent open times in Fig. 7.

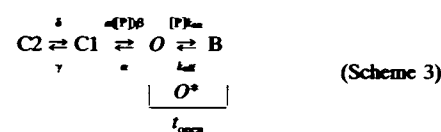
The probability of being in the apparent open state, P_{open} , is given by the sum of the probabilities of being in the true open state and the blocked state, and is given by the expression

$$P_{\text{open}} = \frac{1 + k_{\text{on}}[P]/k_{\text{off}}}{1 + \alpha/\beta + (\alpha/\beta)(\gamma/\delta) + k_{\text{on}}[P]/k_{\text{off}}}. \quad (9)$$

Combining and rearranging Eqs. 1 and 9 yields

$$\frac{1/P_{\text{open}} - 1}{1/P_o(\text{drug free}) - 1} = \frac{1}{1 + k_{\text{on}}[P]/k_{\text{off}}}, \quad (10)$$

which, like Eq. 8, yields a hyperbola when plotted as a function of phenylpropanolamine concentration. However, when we plotted our data in this form in Fig. 9, the K_d values obtained from the fits differed by 50% of those determined directly from the fractional block. As shown in Fig. 6 B, mean closed durations appeared to be shortened in the presence of phenylpropanolamine. Hence, we propose a model in which drug binding allosterically enhances recovery from the closed state, C1. In Scheme 3, we introduce an allosteric factor, a , which reflects the increased rate of channel opening.



In this kinetic model, the expressions for the apparent mean open times are identical to those of Scheme 2, as expected for a Markovian process. However, the newly introduced factor alters the expressions for the apparent open probability given in Eqs. 9 and 10, such that

$$\frac{1/P_{\text{open}} - 1}{1/P_o(\text{drug free}) - 1} = \frac{1}{(1 + k_{\text{on}}[P]/k_{\text{off}})(a[P])}. \quad (11)$$

The factor a is assumed to be concentration-dependent, according to the relation

$$a[P] = 1 + \frac{(\beta_{\text{max}}/\beta) - 1}{1 + K_a/[P]}, \quad (12)$$

where β_{max} is the maximum rate of opening for a channel in presence of phenylpropanolamine at a given membrane potential, and K_a is the equilibrium dissociation constant for drug binding to the putative allosteric site. In our experiments, the rate β is not known, hence we substitute max for $(\beta_{\text{max}}/\beta) - 1$. Hence, fitting the data in Fig. 9 with the combined Eqs. 11 and 12 yields three fitting parameters, K_d , K_a , and max . According to Scheme 3, the K_d value obtained from the fit is equivalent to the equilibrium dissociation constant for phenylpropanolamine block.

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