Dual Actions of Procainamide on Batrachotoxin-activated Sodium Channels: Open Channel Block and Prevention of Inactivation

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ABSTRACT   We have investigated the action of procainamide on batrachotoxin (BTX)-activated sodium channels from bovine heart and rat skeletal muscle. When applied to the intracellular side, procainamide induced rapid, open-channel block. We estimated rate constants using amplitude distribution analysis (Yellen, G. 1984. *J. Gen. Physiol.* 84:157). Membrane depolarization increased the blocking rate and slowed unblock. The rate constants were similar in both magnitude and voltage dependence for cardiac and skeletal muscle channels. Qualitatively, this block resembled the fast open-channel block by lidocaine (Zamponi, G. W., D. D. Doyle, and R. J. French. 1993. *Biophys. J.* 65:80), but procainamide was about sevenfold less potent. Molecular modeling suggests that the difference in potency between procainamide and lidocaine might arise from the relative orientation of their aromatic rings, or from differences in the structure of the aryl-amine link. For the cardiac channels, procainamide reduced the frequency of transitions to a long-lived closed state which shows features characteristic of inactivation (Zamponi, G. W., D. D. Doyle, and R. J. French. 1993. *Biophys J.* 65:91). Mean durations of kinetically identified closed states were not affected. The degree of fast block and of inhibition of the slow closures were correlated. Internally applied QX-314, a lidocaine derivative and also a fast blocker, produced a similar effect. Thus, drug binding to the fast blocking site appears to inhibit inactivation in BTX-activated cardiac channels.

INTRODUCTION

We have recently described the action of lidocaine on BTX-activated sodium channels from heart and skeletal muscle (Zamponi et al., 1993a, b). Here, we investigate the action of procainamide on the two channel subtypes. The electrophysiological effects of procainamide on the cardiac action potential have been observed since the 1950s (Weidmann, 1955) and extensively studied (e.g., Rosen et al., 1972; 1973), however, some of the results are controversial (e.g., whether procainamide blocks the open or the inactivated state; (Sada et al., 1979; Ehring et al., 1988)). However, there has been no attempt to identify different blocking modes or to describe state dependence of procainamide action.

The purpose this paper is to compare the blocking action of lidocaine with the structurally similar procainamide. We recently presented evidence that BTX-activated cardiac sodium channels show some residual inactivation which appears as a distinct population of long gating closures with mean closed times of 150–600 ms (Zamponi et al., 1993b). Thus, this channel type constitutes an excellent model for the study of state-dependent interactions between the sodium channel and antiarrhythmic agents. For lidocaine, binding of the uncharged form of the drug to this residual inactivated state resulted in pronounced slow block (mean blocked durations generally greater than 1 s (Zamponi et al., 1993b)).

In contrast, the charged form of lidocaine caused fast, open channel block with mean blocked times of less than 1 ms (Zamponi et al., 1993a). A comparison of the potency and state dependence of block by various class 1 antiarrhythmic agents may help to identify structural parts of the drugs which are involved in binding and, hence, provide valuable clues about the nature of the class I antiarrhythmic receptor. However, since the presence of batrachotoxin affects ion selectivity, single channel conductance (e.g., Quandt et al., 1982; Garber and Miller, 1987; Correa et al., 1991) and affinity for local anesthetics (e.g., Khodorov et al., 1975), it is important to remember that the properties of BTX-activated channels are altered when extrapolating our data to unmodified sodium channels.

Procainamide induces rapid, flickery block of both channel subtypes. The rate constants for blocking and unblocking were estimated using the amplitude distribution analysis introduced by Yellen (1984). Procainamide block is qualitatively similar to fast lidocaine block. Block from the intracellular side probably occurs via a hydrophilic route, while from the extracellular side, procainamide may approach the receptor via a direct, hydrophobic pathway. The equilibrium dissociation constant for procainamide block measured at 0 mV, however, was sevenfold larger than that for lidocaine block. Procainamide also appeared to be capable of the type of slow block effected by lidocaine, but only at very high concentrations (>100 mM). This is consistent with the low concentration of the uncharged form of procainamide ($pK_a = 9.1$) present at a pH of 7.0. A more pronounced effect of procainamide on the "inactivation" events was a reduction in their frequency. Kinetic analysis indicated that this reduction was correlated with the binding of procainamide to the fast block receptor. We observed similar effects of fast

block by QX-314 and lidocaine. Our data suggest that binding to the fast block receptor inhibits, either directly or allosterically, inactivation in BTX-activated cardiac sodium channels.

METHODS

Membrane preparations

Membrane vesicles from bovine cardiac and rat skeletal muscle tissue were prepared and incubated with BTX as previously described (Zamponi et al., 1993a). Protein concentrations were 5.4 and 6.2 mg/ml for rat skeletal muscle membranes from two preparations, and 3.5 mg/ml for bovine cardiac membranes from one preparation.

Bilayer methods and single channel recordings

Bilayers, made of a decane solution of uncharged synthetic lipids (40 mg/ml 1-palmitoyl-2-oleoylphosphatidylethanolamine, 10 mg/ml 1-palmitoyl-2-oleoylphosphatidylcholine in decane), were formed on a planar plastic partition (made from a microscope coverslip, cat. no. 12-547, Fisher Scientific Co., Pittsburg, PA) separating two chambers, each containing 1.5 ml of 200 mM NaCl and 20 mM 3-N-morpholinopropanesulfonic acid (MOPS) at pH 7.0 at room temperature ($\approx22°C$). Holes were constructed as described in Wonderlin et al. (1990), all experiments were performed with a hole diameter of 130 μm. The total capacitance was in the range of 50–80 pF. Between 2 and 20 μl of membranes were added to the *cis* side which was continuously stirred. Voltages were alternated between +70 and −70 mV, until channel incorporation was indicated by an increase in membrane conductance. The gating voltage dependence of the channels was used to determine their orientation in the bilayer (Krueger et al., 1983). Cardiac channels generally were oriented with the cytoplasmic end facing the *cis* chamber, skeletal muscle channels usually were in reverse orientation. Recordings were limited to single channel incorporations. Bilayers were voltage clamped as described previously (Zamponi et al., 1993a). The duration of the recordings (10–80 min) was determined by the stability of the bilayers.

Chemicals and solutions

For most experiments, procainamide (purchased from Aldrich Chemical Co., Milwaukee, WI) was dissolved in water to make a stock solution of 200 mM. The pH was adjusted to 7.0 with NaOH. The drug solution was usually added to the chamber facing the intracellular side of the channel. External application of the drug required 20-fold higher concentrations for block. Thus, we prepared a stock solution of 300 mM procainamide and 200 mM NaCl at a pH of 7.0 which allowed us to apply high concentrations (up to 100 mM) of the drug without altering ionic conditions. Both stock solutions produced identical effects when added intracellularly to give 20 mM procainamide. QX-314 was dissolved in water to give a stock solution of 200 mM. Lidocaine and MOPS were dissolved in water to make a stock of 200 mM lidocaine/200 mM MOPS at pH 6.7.

Data analysis

For experiments involving internal or external fast block, data were acquired for up to 1 min at each voltage. Because we were unable to resolve the fast blocking events as discrete steps, we used the amplitude distribution analysis introduced by Yellen (1984) to analyze fast block. The analysis was implemented as described for fast lidocaine block (Zamponi et al., 1993a).

To study the effect of procainamide, QX-314, and lidocaine, on the long gating closures for the cardiac subtype, slow gating events were recorded in continuous records of up to 20 min. Data were filtered at 200 Hz and sampled at 500 Hz during transcription, and digitally filtered at 25 Hz to create events lists in pCLAMP version 5.5 (Axon Instruments Inc., Foster City, CA). For studies of open times, closed events shorter

than 70 ms were ignored to eliminate contributions from fast gating and blocking events. For generation of closed time histograms we did not ignore events, however, filtering at 25 Hz eliminates events shorter than 7 ms (McManus et al., 1987). We did not correct for missed events.

Dwell time distributions and amplitude histrograms were generated and fitted in pCLAMP, preparation of figures and all other fits were conducted in Sigmaplot (Jandel Scientific, Corte Madera, CA).

Molecular modeling

The MACROMODEL software package (Mohamadi et al., 1990), based on the AMBER (Weiner et al., 1984) molecular mechanics force field and extensions to it, was used to search for low energy conformations of lidocaine and procainamide. Calculations were simplified by considering only the key inner fragments of each molecule. Specifically, methyl rather than ethyl groups were attached to the tertiary nitrogen atoms, and for procainamide, the para -NH$_2$ moiety was omitted. Usage-directed Monte Carlo searching of conformational space was used to examine 3000 conformers with tabulation of those conformers within 50 kJ·mol^{-1} of the lowest energy structure.

RESULTS

Procainamide causes fast block

In the absence of blockers, BTX-activated sodium channels generally show a constant open probability ($P_{open} > 0.5$) at potentials more positive than about −60 mV. Figs. 1, *A* and *B*, show examples of records in absence and presence of procainamide for both bovine cardiac and rat skeletal muscle sodium channel subtypes. BTX-activated cardiac sodium channels generally show a distinct population of long, trypsin-sensitive gating closures (Zamponi et al., 1993b) with mean durations ranging from 150 to 600 ms. A typical example is the closed event at +40 mV. These types of gating events are only rarely seen for the skeletal muscle subtype. For both subtypes, addition of 12 mM procainamide resulted in a reduction in apparent single channel amplitude and an increase in open channel noise. As can be clearly seen from the records, the effect was more pronounced at depolarizing potentials. The apparent increase in noise is probably caused by blocked events that, at a bandwidth of 50 Hz, are not resolved as discrete transitions from the open to the closed state. Block appears as a flickery open state, with a reduced apparent single channel amplitude and a broadened amplitude distribution. This can clearly be seen in Fig. 1 *C*. In absence of blockers, the normalized amplitude distribution is comprised of two Gaussians representing the open (defined as 1) channel and the baseline (defined as 0) amplitudes. In presence of 12 mM procainamide (*middle panel*), the mean current amplitude is decreased and the apparent open channel noise is increased, as shown by a leftward shift and broadening of the peak representing the open channel. This becomes more obvious when the peak representing the baseline is subtracted (*bottom panel*). Assuming a two state, open-blocked model, the shape of the remaining histogram may be described theoretically by the filter output density function of Yellen (1984):

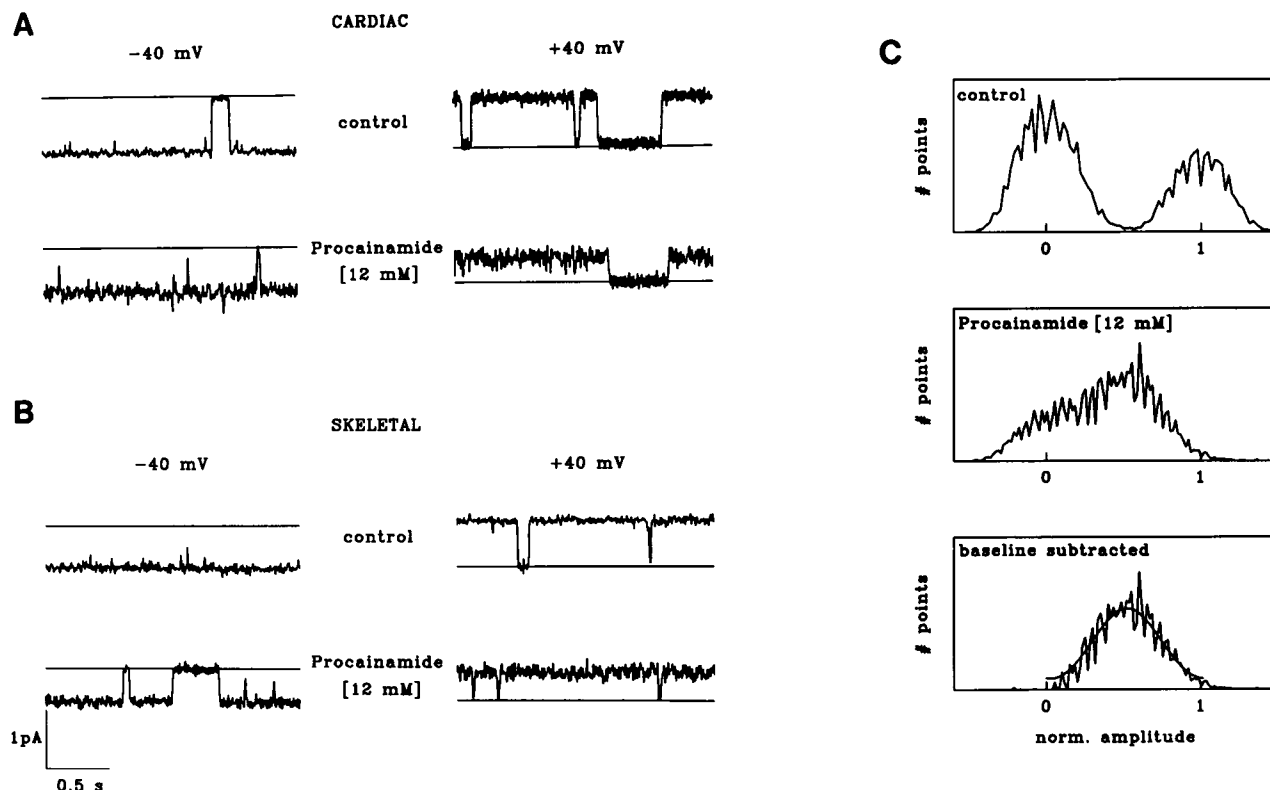$$f(y) = \frac{y^{a-1}(1-y)^{b-1}}{\int y^{a-1}(1-y)^{b-1} \, dy}.$$

FIGURE 1   Current traces recorded from single BTX-activated sodium channels from (*A*) bovine heart and (*B*) rat skeletal muscle in absence and presence of procainamide. In absence of the drug both channel types are open most of the time at potentials more positive than −60 mV. Procainamide, applied to the intracellular side, acts similarly on both channel subtypes by inducing rapid blocking events. At the illustrated bandwidth of 50 Hz the fast blocking events were not resolved as discrete, rectangular steps, but rather appear as an increase in open channel noise with a concomittant decrease in the apparent single channel current amplitude. The solid lines indicate the closed level. Note that the traces for the cardiac subtype at +40 mV and −40 mV were recorded from two different bilayers with different baseline noise. (*C*) Amplitude distributions for the same cardiac channel traces at +40 mV as in *A*. In absence of procainamide, the normalized amplitude distribution shows two Gaussian-distributed peaks (*upper panel*). Here, the open level has been defined as 1, and the closed level as 0. In presence of 12 mM procainamide (*middle panel*), the apparent single channel amplitude is decreased and the open channel noise is increased. Hence, in the amplitude distribution this appears as a broadening and shift of the the open level peak toward smaller amplitudes. After subtraction of the baseline (*lower panel*), the remaining peak is fitted with the filter output density function outlined under Results. The fit shown in this panel yielded blocking and unblocking rates of 6487/s and 7018/s, respectively.

This, when convolved with a Gaussian function describing the baseline noise and then fitted to the amplitude distribution as shown in the bottom panel, provides estimates for the association and dissociation rate constants of the blocker ($k_{on} = a/\tau$, $k_{off} = b/\tau$ with $\tau = 0.228/f$, where *f* is the frequency at 3-dB attenuation for an eight-pole Bessel filter, and 0.228 is an empirically determined constant (Yellen, 1984)). We have recently used this type of analysis to determine blocking and unblocking rate constants for lidocaine (Zamponi et al., 1993a).

Procainamide block is voltage-dependent and similar for both channel subtypes

Fig. 2 shows the voltage dependences of the individual rate constants and the equilibrium dissociation constants of internally applied procainamide for both channel subtypes. The blocking rate was favored by depolarizing potentials (Fig. 2 *A*) which is consistent with a positively charged blocker entering the transmembrane voltage from the cytoplasmic side of the channel. Unblock was enhanced at negative voltages

(Fig. 2*B*) as expected for a positively charged blocker exiting the internal mouth of the channel down the electric potential gradient. Since the equilibrium dissociation constant (K_d) is determined by the ratio of unblocking to blocking rate, it decreased with more positive potentials (Fig. 2 *C*). The voltage dependence of the K_d suggests that the positively charged amine group located at the procainamide tail penetrates the transmembrane voltage 43% (for the cardiac subtype) and 31% (for the skeletal muscle subtype) from the cytoplasmic end of the channel (Woodhull, 1973). Both channel subtypes appeared to show similar rate constants for procainamide block.

The similar effect of internal procainamide on the channels from both tissues is also illustrated in Fig. 3. Fig. 3 *A* shows dose response curves at +40 mV. The open probability (the probability that a channel is not blocked while in an open gated state) was calculated from the rate constants. Note that this definition contrasts with the definition of open probability used in the analysis of slow gating gating events later in this paper. The fast-block data were fitted with a first order hyperbola suggesting a 1:1 inter-

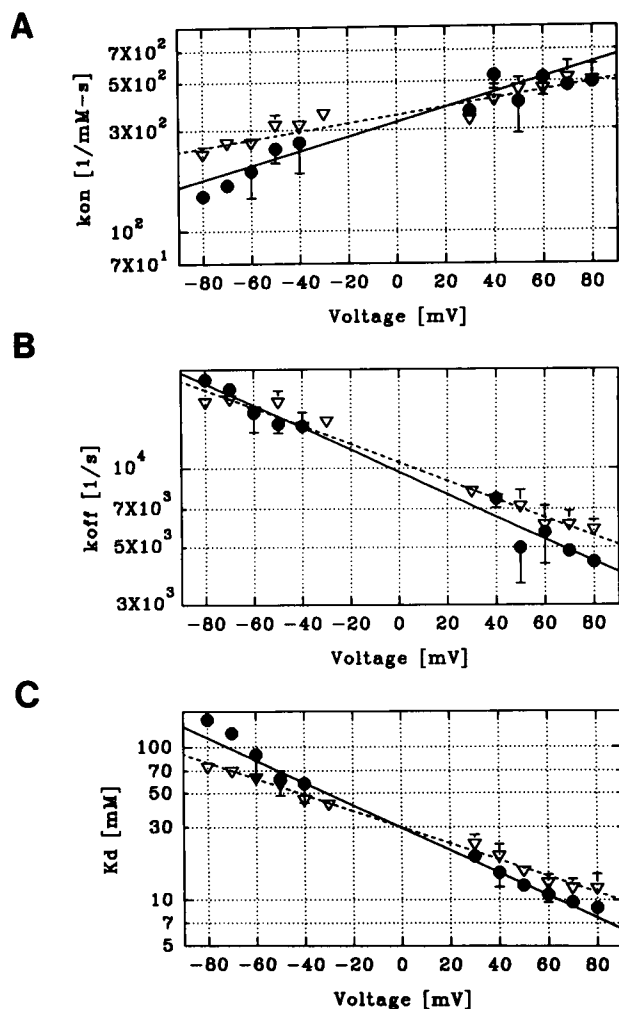**A**

B

C

FIGURE 2   Voltage dependence of the rate constants and equilibrium dissociation constant for internal procainamide block of bovine cardiac (*circles, solid lines, downward error bars*, six experiments) and rat skeletal muscle (*triangles, dashed lines, upward error bars*, six experiments) sodium channels. Rate constants were obtained using the amplitude distribution analysis introduced by Yellen (1984). Lines are least square fits, error bars indicate standard deviations. (A) The block rates increase with depolarization, consistent with the idea of a positively charged blocker entering the transmembrane electric field from the cytoplasmic end of the channel (cardiac: $(z\delta)_{on} = 0.18$, skeletal: $(z\delta)_{on} = 0.11$). (B) The unblock rates increase with hyperpolarization (cardiac: $(z\delta)_{off} = 0.25$, skeletal: $(z\delta)_{off} = 0.21$). (C) The equilibrium dissociation constant, K_d, is calculated from the ratio of unblock to block rate ($K_d = k_{off}/k_{on}$). The voltage dependences of the K_d values (cardiac, $z\delta = 0.43$; skeletal, $z\delta = 0.31$) suggest binding sites located 43 and 31% of the way across the transmembrane voltage from the cytoplasmic end of the channel, for the cardiac and the skeletal muscle subtypes, respectively.

action between the blocker and its binding site. The concentrations required to block the channel half of the time (14.9 mM for the cardiac subtype, 19 mM for the skeletal muscle subtype) are consistent with the K_d values obtained from Fig. 2 C.

To test the voltage dependence of procainamide independently of the assumptions of the amplitude distribution analysis, we calculated the voltage-dependent reduction in mean current in the presence of 12 mM procainamide. The results
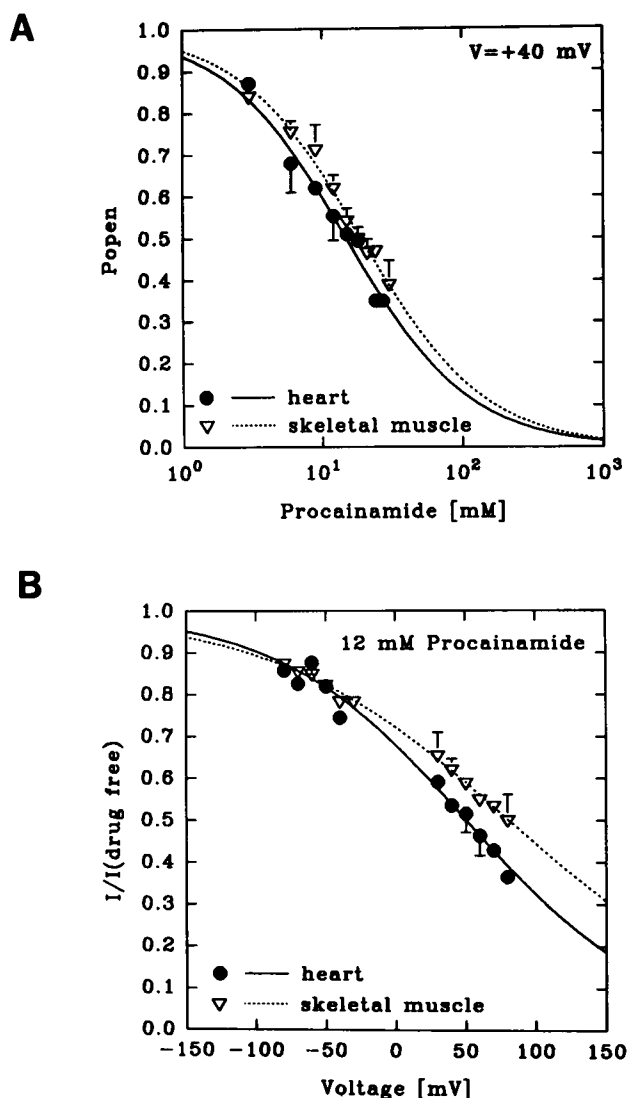
A

B

FIGURE 3   (A) Dose response curves for block of bovine cardiac and rat skeletal muscle sodium channels by internally applied procainamide at +40 mV. The open probability, P_{open}, was calculated from the rate constants as follows: $P_{open} = k_{off}/(k_{on} + k_{off})$. Data points from skeletal muscle (*upward error bars*, six experiments) and heart (*downward error bars*, six experiments) were fitted with a first order hyperbola $P_{open} = 1/(1 + [P]/K_d)$, where [P] is the drug concentration and K_d is the equilibrium dissociation constant. The K_d values obtained from the fit are 19.0 mM for the skeletal muscle and 14.9 mM for the cardiac subtype. Error bars indicate standard deviations. (B) The voltage dependence of procainamide block was also directly determined from the reduction in apparent single channel amplitude. The data were fitted with a Boltzmann relation $I/I(drug\ free) = 1/(1 + exp\{z\delta (V - V_H)/25.4\})$, where V is the membrane potential, V_H is the potential at half block and $z\delta$ is proportional to the slope of the fit (c.f. Woodhull, 1973). The $z\delta$ values obtained from the fits suggest that the procainamide binding sites are located 38 and 30% of the way through the transmembrane voltage for the cardiac and the skeletal muscle subtype respectively. These data are consistent with the results obtained from amplitude distribution analysis and illustrated in Fig. 2 C. Symbols, error bars and number of experiments are as described in A.

are presented in Fig. 3 B. The data points were fitted with a Boltzmann relation, the steepness of the slope indicates electrical distances of 0.38 and 0.30 for the cardiac and the

skeletal muscle subtypes, respectively. These values are close to those obtained from Fig. 2 C.

Block from the extracellular side is weak

In a clinical setting, antiarrhythmic drugs approach the target cell from the extracellular medium. They can then act directly from the extracellular side (Alpert et al., 1989; Baumgarten et al., 1991) or pass through the cell membrane to act via a membrane-delimited pathway (Zamponi et al., 1993a; Hille, 1977a, b) or from the intracellular side (Hille 1977a, b; Strichartz, 1976). We therefore applied the drug to the chamber facing the extracellular side of the membrane. We did not observe any block at concentrations around the K_d for internal fast block. At concentrations of 100 mM, the apparent single channel amplitude was reduced by 25% at +80 mV and by about 10% at -70 mV for the cardiac subtype. We did not attempt to expose the channel to higher drug concentrations. At +40 mV, data from 2 experiments suggest an apparent K_d for external block of approximately 400 mM. In addition, block appeared to be less voltage-dependent than for internal application ($z\delta = 0.17$ for equilibrium block, from one cardiac channel). In general, the increase in ionic strength on the extracellular side due to the large drug concentrations resulted in a shift of the steady state activation curve toward more depolarizing potentials for both channel types. For the channels in these experiments, the midpoint of the steady-state activation curve for the muscle subtype occurred at more positive potentials than that of the cardiac channels. Hence, the increase in ionic strength on the extracellular side resulted in rapid gating transitions for the skeletal muscle subtype at potentials more negative than -50 mV, making a quantitative analysis of fast block impossible. Because of more negative midpoints of the steady state activation curves, some analysis was possible for the cardiac channels, as described above.

Procainamide antagonizes long gating closures of the cardiac subtype

Cardiac sodium channels, unlike the skeletal muscle subtype, show a distinct population of long closures with time constants between 150 and 600 ms (French et al., 1990). This is illustrated in Fig. 4 A. The single channel record and the corresponding dwell time histogram showed at least two distinct types of closures, a short-lived closed state on the order of 20 ms and a longer closed state with a mean time of about 330 ms. In the presence of procainamide (nine experiments), the frequency of the long closures was reduced, as can be seen from the record in Fig. 4 B and the smaller peak (t_{c2}) in the corresponding dwell time histogram. The duration of the long gating events was not affected by the drug.

Fig. 5 A demonstrates that the time between the long gating events increased linearly with procainamide concentration. For this analysis, events shorter than 70 ms were ignored, thus, the open and the fast blocked states as well as the the short-lived gating closures between successive long closures are combined as a single "open" state (see also Zamponi et al., 1993b). Only the long gating events appeared as closures. According to our kinetic model presented in the Discussion, the slope of the regression line reflects the K_d for fast internal procainamide block. The value obtained from this fit (17.9 mM) is close to the 14.9 mM obtained from the dose response curve for fast block in Fig. 3 A. Procainamide did not affect the mean duration of the long gating closures (Fig. 5 B). The closed times were obtained from fits to dwell time histograms as in Fig. 4, A and B, and here, no events were ignored during the analysis. Our data suggest that a cardiac channel is unable to enter the long closed state when blocked by procainamide and that the drug neither binds to, nor enhances recovery from, this state.

We have previously shown that lidocaine caused pronounced slow blocking events at millimolar concentrations

FIGURE 4 Long gating closures of the bovine cardiac sodium channel are inhibited by procainamide. Histograms are in the form devised by Sigworth and Sine (1987). All data were recorded at +40 mV, traces were filtered at 25 Hz, solid lines indicate the closed level. (A) An example of a typical single channel record from a BTX-activated cardiac sodium channel (closures are downward deflections) and the corresponding closed time histogram. In absence of the drug, the closed time distribution shows at least two distinct populations of closures (time constants $t_{c1} = 20$ ms, t_{c2}(drug free) = 332 ms). The longer closures are characteristic for the cardiac subtype (Zamponi et al., 1993b). (B) Procainamide (12 mM), applied to the intracellular side, causes a reduction in the frequency of the long closures, but does not affect their mean duration. This can be clearly observed in the dwell time distribution as an almost twofold reduction in the number of long events, while the time constants of the fits are essentially the same as in A ($t_{c1} = 17$ ms, $t_{c2} = 335$ ms). The traces used for the generation of the histograms were 385 s in A and 405 s in B.
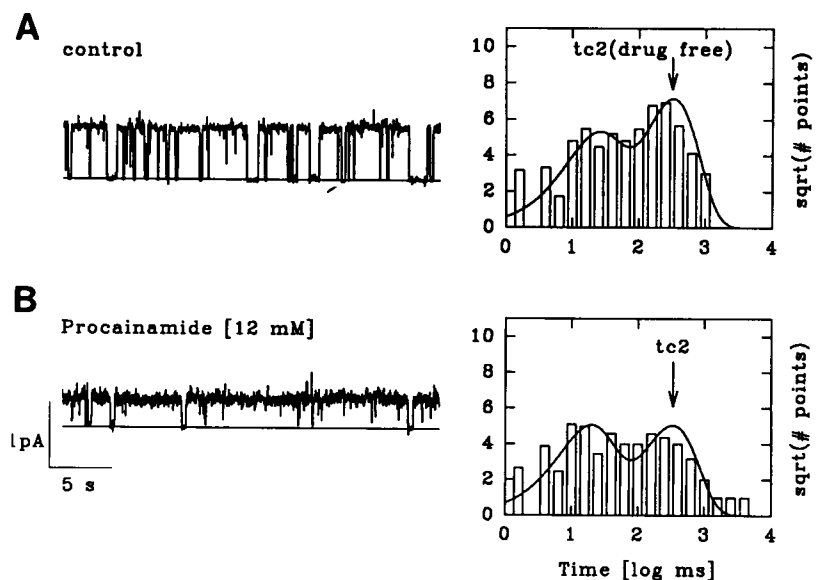
FIGURE 5   (*A*) The times between long gating closures increase linearly with the procainamide concentration. According to the kinetic model presented in the text, the slope of the regression line (r = 0.85, slope = 0.056 ± 0.045 at 95% confidence, probability of nonzero slope > 0.99 (Larsen and Marx, 1981)) represents the inverse of the equilibrium dissociation constant for fast procainamide block. The K_d value obtained from this fit (17.9 mM) is consistent with the results from Fig. 3 *A*. (*B*) The mean duration of the long gating closures is not affected by the procainamide concentration (r = 0.27, slope = 0.004 ± 0.017 at 95% confidence). Closed times were determined from fits to dwell time histograms as in Fig. 4, *A* and *B*. Solid lines in *A* and *B* are least square fits, error bars indicate standard deviations, dashed lines are 95% confidence intervals. Open and closed times were determined as described under Methods. For both *A* and *B*, data from five experiments (*filled circles*, 13 data points with a mean of 65 events measured per data point) are included in the fit. The open circle indicates a value obtained from one experiment where a stock solution of 300 mM procainamide and 200 mM NaCl (pH 7.0) was used. This point was not included in the fit. (*C*) The reduction in frequency of long gating closures follows the same voltage dependence as fast block. Each type of symbol (*circles, upward triangles, downward triangles*) represents data from one channel. All points are single data points except for the upward triangles which are averages from two different concentrations. The K_d for fast block (*filled symbols*) is calculated from the reduction in apparent single channel amplitude (K_d = [*P*]/{(*I*(drug free)/*I*) − 1}), where [*P*] is the procainamide concentration and *I* and *I*(drug free) are the mean baseline subtracted currents in presence and absence of procainamide). K_d values (*hollow symbols*) were also calculated from the reduction in long gating events (K_d = [*P*]/{(t_{open}/t_{open}(drug free)) − 1}; see Discussion). The voltage dependences of the K_d values indicate a $z\delta$ of 0.38 (r = 0.93, slope = 0.0065 ± 0.002 at 95% confidence, probability of non-zero slope >0.99) and 0.41 (r = 0.77, slope = 0.0071 ± 0.0045 at 95% confidence, probability of non-zero slope >0.99), respectively, when calculated directly from the current reduction or from the mean time between long gating closures. The regression line and confidence intervals shown were calculated from the gating data. Note that the data points for fast block are virtually identical to the data presented in Fig. 2 *C*, but were determined here from a different set of experiments.

A

B

C

(Zamponi et al., 1993a). Unlike fast block, this mode of action was correlated with the concentration of the uncharged form of lidocaine. This type of block occurred as a silent burst between the long-lived closed state and the blocked state, hence, mean times in the nonconducting state increased with increasing lidocaine concentrations. At 20 mM procainamide, we did not observe this type of block. However, in one experiment we raised the internal procainamide concentration to 40 mM and observed an almost twofold increase in mean closed time. In another experiment, application of 100 mM procainamide to the extracellular side resulted in the appearance of several distinct long closures which resembled slow lidocaine blocking events. These observations suggest that procainamide does have the ability to produce slow block, but because of its higher pK_a and its lower lipid solubility than that of lidocaine, this type of block does not occur frequently. This is consistent with the observed low potency of fast block by external procainamide (100-fold lower than fast external lidocaine block), which probably also requires diffusion of the uncharged form of the drug through a hydrophobic pathway.
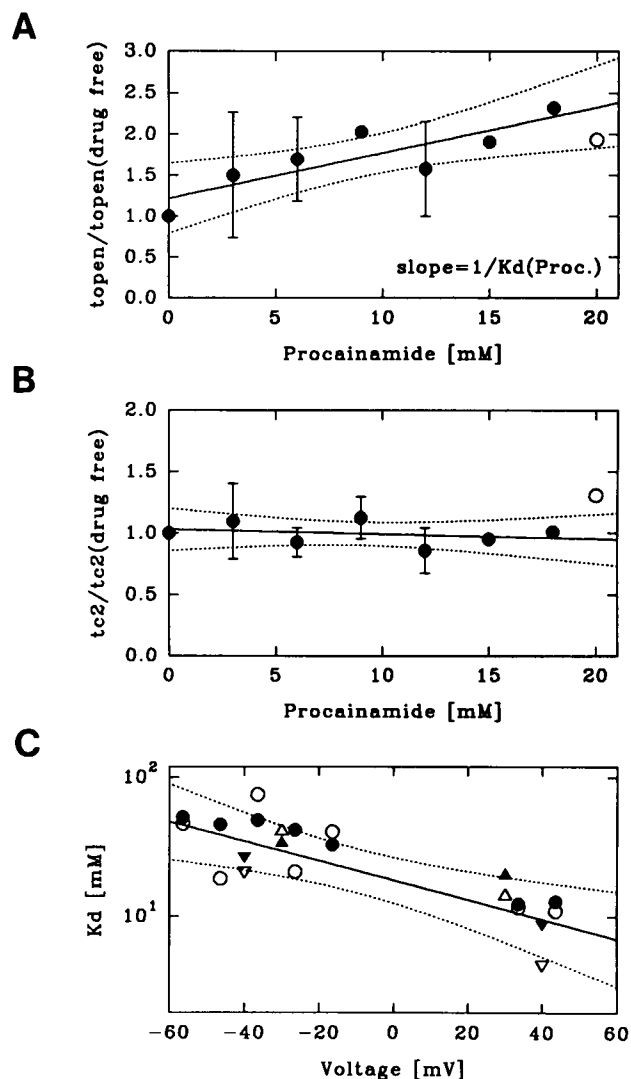
Inhibition of long gating closures is voltage-dependent

If fast block and inhibition of long closures are correlated, one would expect to detect a voltage dependence in the reduction of the number of long gating events which follows the voltage dependence of fast block. Fig. 5 *C* compares K_d values for fast block from three experiments at procainamide concentrations between 6 and 40 mM. In each of these experiments, we recorded at positive and negative potentials from the same channel. The K_d values were calculated via two different ways: 1) directly from the reduction in open channel current amplitude (*filled symbols*) and 2) indirectly from the reduction in frequency of long gating events according to the kinetic model presented in the Discussion (*open symbols*). Both the magnitudes of the K_d values and their voltage dependences were similar when determined by either method. Note that at each of the experiments (*same shaped symbol*) the effect was less pronounced at negative potentials (higher K_d values). For the most complete experiment in which the bilayer was unusually stable (*circles*), we

exposed the channel to 40 mM internal procainamide (from the 300 mM procainamide/200 mM NaCl stock). To ensure that the increase in intracellular ionic strength associated with the high procainamide concentration would not affect the channel gating, we recorded a steady state activation curve as a control. At potentials more positive than −55 mV, the open probability reached a plateau of 90%. Hence, at these potentials (where there is only little voltage-dependent gating), the increase in ionic strength on the intracellular side did not interfere with our measurements.

Other fast blockers also prevent long gating closures

We also observed a reduction in frequency of long gating closures for QX-314 (three experiments). As for procainamide, the effect was concentration- and voltage-dependent and was half-maximal near the K_d for fast block. At +40 mV the K_d for QX-314 block is 3 mM (Zamponi et al., 1993a); here we estimated 2 mM from the reduction in gating closures. In previous experiments on slow lidocaine block (Zamponi et al., 1993b) we did not observe a consistent reduction in the number of long gating events. Most of these experiments were performed at negative potentials with extracellular application of the drug at concentrations up to 6 mM. Because fast block under these conditions is weak, it would be difficult to resolve a consistent change in mean open times. We therefore applied 3 mM external lidocaine at +40 mV and 21 mM external lidocaine at −40 mV (two experiments) and observed twofold and 1.6-fold increases in mean open time, respectively (the expected values under these conditions would be 1.5- and 2.4-fold, respectively). Since open times generally appear to be rather scattered (see Fig. 5 B, and companion paper (Zamponi and French, 1993)), we consider these data to be consistent with the view that fast lidocaine block also inhibits transitions to the long-lived closed state. Overall, the data from all three drugs support the idea that drug binding to the fast block receptor prevents long gating closures.

DISCUSSION

Fast blocking actions of procainamide and lidocaine are similar

We recently performed a detailed study on the blocking action of lidocaine on BTX-activated cardiac and skeletal muscle sodium channels (Zamponi et al., 1993a, b). Internal application of lidocaine caused rapid, open-channel block with similar properties in both cardiac and skeletal muscle channels. The blocking rate was increased by depolarization and the unblocking rate was increased by hyperpolarization. For external application, the fast blocking rate was voltage-independent, an observation which suggested that lidocaine may find a direct hydrophobic path to the blocking site from the extracellular side.

Procainamide appears to block via a similar mechanism. Block is seen as rapid, flickery reduction in open-channel

current, and both the blocking and the unblocking rates show a voltage dependence similar to those for internal lidocaine block. Both the rat skeletal muscle and the bovine cardiac subtype are similarly affected. For lidocaine, we obtained $z\delta$ values of 0.33 (skeletal muscle) and 0.38 (heart) for the voltage-dependence of the K_d; here, for procainamide, the values were 0.31 and 0.43, respectively. This supports the idea that lidocaine and procainamide bind to one common receptor (Sheldon et al., 1987).

When procainamide was applied from the extracellular side of the channel, the block appeared to show a decreased voltage sensitivity (data not shown). A loss in voltage dependence of the blocking rate would be sufficient to account for this effect which would be consistent with the idea that external procainamide acts in the neutral form via a mechanism like that proposed for external lidocaine.

Structural considerations. Why is procainamide less potent than lidocaine?

Internal procainamide exhibits a roughly sevenfold lower blocking affinity than lidocaine. Fast block from the internal side appears to be caused by the charged forms of the drugs, because it can be mimicked by permanently charged analogs, and because raising the internal pH decreases the degree of open channel block (Zamponi et al., 1993a; Strichartz, 1976). However, both drugs exist predominantly in the charged form at neutral pH. Hence, the different pK_a values of lidocaine and procainamide cannot account for the difference in apparent K_d values for internal block.

The structures of lidocaine and procainamide differ in four major points: 1) the aromatic ring in the lidocaine molecule carries two methyl groups, 2) an amino group is attached to the aromatic ring in procainamide, 3) the intermediate chain in procainamide contains an additional methylene group, and 4) the amine-carbonyl (peptide) sequence linking the aromatic ring to the intermediate chain in lidocaine is reversed in procainamide (Fig. 6 A).

Ehring et al. (1988) studied the effects of 12 out of 16 possible compounds, which were based on combinations of these differences, on action potentials in guinea pig papillary muscle. These investigators could not directly attribute changes in substituents to a particular blocking behavior. Block seemed rather to be correlated with molecular weight or with lipid solubility determined from partition of water into octanol (see also Courtney (1981 and 1983)). These factors, however, cannot account for our observed sevenfold difference in K_d values for internal application, because the molecular weights of both drugs are almost identical, and block from the intracellular side occurs through the internal mouth of the channel when the drugs are in the charged, hydrophilic form. This is consistent with the conclusions of Sheldon et al. (1991), who studied the displacement of [³H]-batrachotoxinin A 20α-benzoate by lidocaine derivatives. They suggested that differences in IC_{50} values could not solely be attributed to partition coefficients or pK_a values, but were critically dependent on specific structural features in-

A

Lidocaine   Procainamide

B
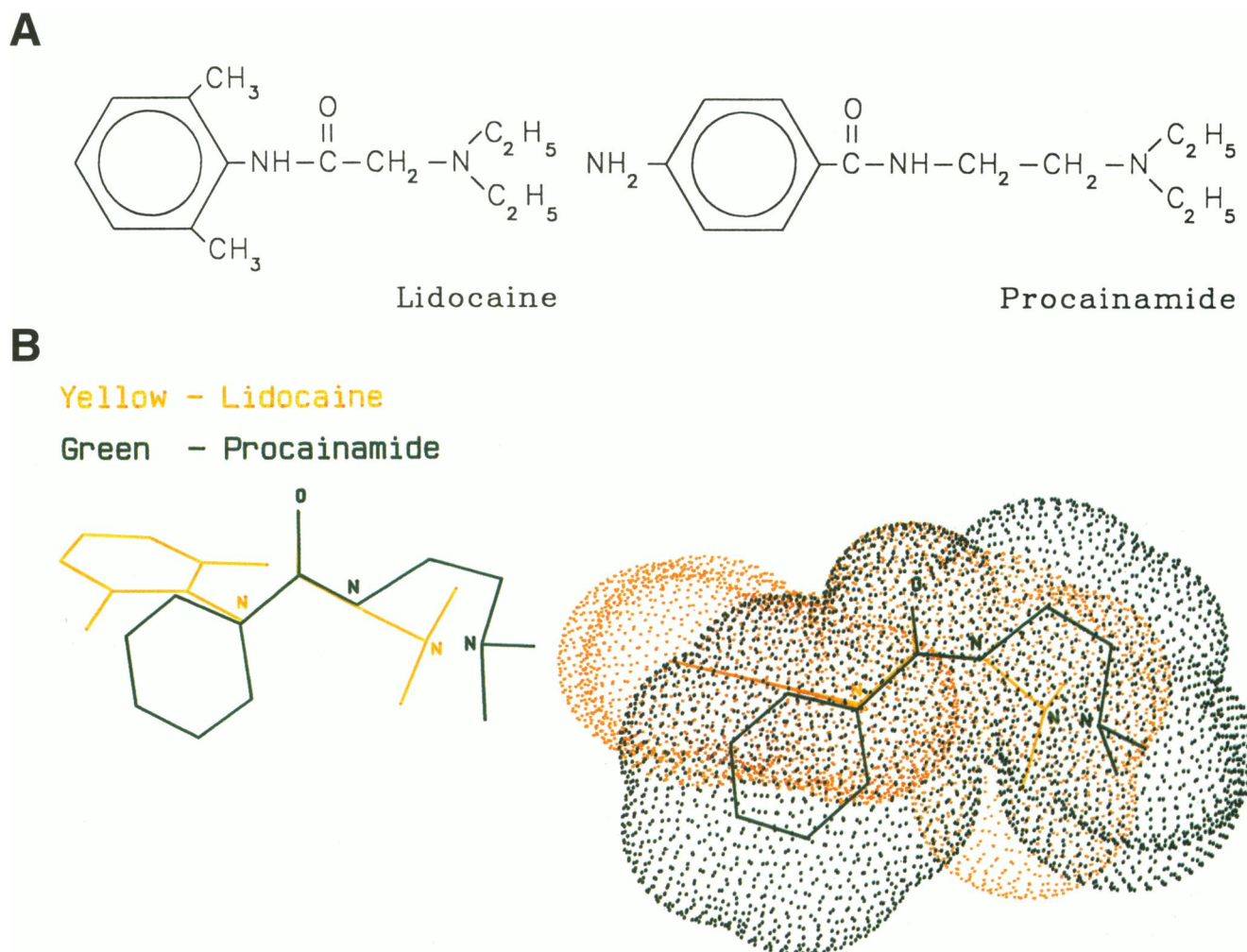
Yellow – Lidocaine
Green – Procainamide

FIGURE 6   (*A*) Chemical structures of lidocaine and procainamide. The voltage dependence of block is thought to arise from passage of the charged terminal tertiary nitrogen through 30–40% of the transmembrane voltage. (*B*) Superposed backbone structures and space-filling models of the key inner fragments of procainamide and lidocaine. The carbonyl *O* atoms in the aryl-amide link have been superposed for reference. The lowest energy conformer for procainamide is shown. The alkyl chain attaching to the lidocaine tertiary amine head group has been folded to reduce to 1.53 Å at the distance between the terminal N atoms in the superposed structures. This lidocaine structure is 0.3 kcal·mol^{-1} higher in energy than the extended, lowest energy conformer (not shown). Note that, the aromatic ring for lidocaine is rotated about 90° with respect to the peptide plane, whereas for procainamide the aromatic ring and the peptide group are coplanar. The orientations of the backbone and space-filling representations are not identical. In the backbone structures, the viewpoint is chosen so that both aromatic rings are easily identifiable; in the space-filling models, the aromatic ring of lidocaine is depicted exactly perpendicular to the plane of the paper.

cluding the number of amino-terminal carbons and the length of the intermediate chain (e.g., insertion of an additional methylene group in the chain increased the IC$_{50}$ by almost twofold). In addition, sidegroups on the aromatic ring appear to be critically involved in BTX displacement (R. Sheldon, personal communication). Uehara and Moczydlowski (1986) showed that procaine, which lacks the amine between the aromatic ring and the carbonyl group, produces fast block which is similar to procainamide block in voltage dependence and potency, suggesting that the amide group in the link between the aromatic ring and the tertiary amine head group is not important for fast block. Overall, it seems likely that the different binding affinities and blocking modes of lidocaine and procainamide are directly related to specific structural differences.

In order to explore other possible structural determinants of drug activity, we performed molecular mechanics calculations, as described under Methods, to examine the likely conformations of key inner fragments of the lidocaine and procainamide structures. The low energy conformers of the two compounds differed in two distinct ways. First, the relative orientation of the aromatic ring to the amide plane was different by 90°, with the ring in the lidocaine structure approximately perpendicular to the amide plane. The rotation in lidocaine arises from the presence of the *o*-methyl groups and resembles the structural motif common to several anticonvulsants recently analyzed by Duke and Codding (1992). Second, comparing the lowest energy conformation of each compound with the carbonyl oxygen atoms superposed, the smallest possible separation of the amine nitrogens is 3.5 Å,

a difference determined primarily by an extended conformation in lidocaine and a folded conformation in procainamide. However, a low energy folded conformer of lidocaine—only 0.3 $kcal\text{-}mol^{-1}$ higher in energy than the extended form—has a carbonyl O to amine N separation similar to that found in the low energy conformer of procainamide. Fig. 6 *B* shows a superposition of the two similar structures. In the figure the carbonyl oxygen atoms have been superimposed and the molecules have been oriented to achieve the closest possible approach of the amine N atoms, which lie within 1.53 Å of one another. The space-filling representation of the two molecules indicates the significant difference in space occupied by the two aromatic rings even though the chain portions of the molecules have fair overlap. This adjusted overlap shows that the chain portions of these molecules could occupy the same recognition site, but, even in that case, the aromatic rings would not both fit snugly into the same hydrophobic pocket. This is consistent with binding studies on stereoisomers of RAC 109 (Postma and Catterall, 1984; Hill et al., 1988) and may account for the lower potency (sevenfold higher K_d, equivalent to a 1.15 $kcal\text{-}mole^{-1}$ difference in binding energies) of procainamide in causing fast block.

Data in the companion paper (Zamponi and French, 1993) suggest that interactions of the aromatic moiety, peptide, and the connecting chain would be strong enough to stabilize similar conformations of lidocaine and procainamide. Fast block by diethylamide, which represents the isolated tertiary amine head group, is about 1/16 as potent as for lidocaine, indicating a net stabilization of only about 1.6 $kcal\text{-}mol^{-1}$ by the combined effects of aromatic ring, peptide group, and alkyl chain. Thus, the critical component for voltage-dependent fast block appears to be the charged amine group. Ancillary interactions do strengthen the binding to some extent, and they are somewhat more effective for lidocaine than for procainamide, probably because of structural differences illustrated in Fig. 6 *B*.

Reduced frequency of long gating closures of cardiac channels is correlated with the degree of fast block

With increasing procainamide concentration, the long gating closures observed for BTX-activated cardiac sodium channels become less frequent, but their duration remains unaffected. In the presence of procainamide, there was about a 50% reduction in the frequency of long gating closures at the K_d for fast block. This is consistent with the idea that procainamide, when bound to the receptor responsible for fast block, prevents long gating closures.

To test this hypothesis, we isolated the long closed events from fast block and fast gating closures by heavily filtering the records (25 Hz) and ignoring closed events shorter than 70 ms. Thus, in absence of the blocker, true openings and short closures are lumped together into one open state, O (mean duration, t_{open}(drug free)), and only long gating events are seen as closures (mean duration, t_{c2}). Procainamide adds

an additional kinetic state (Bf). However, in our analysis of the slow closures, Bf becomes part of the "open" state (mean duration, t_{open}, see Eq. 1). Our analysis assumes that the rates α and β are not affected by the presence of procainamide and does not consider contributions from fast gating events, because at $+40$ mV they are short, and are infrequent compared with the blocking transitions. We have used similar assumptions to analyze slow lidocaine block (Zamponi et al., 1993b). Hence, we consider the following linear three state model:

$$Bf \underset{\delta}{\overset{\gamma[P]}{\leftrightarrows}} O \underset{\beta}{\overset{\alpha}{\rightleftarrows}} C2 \qquad (1)$$
$$\underbrace{}_{t_{open}}$$
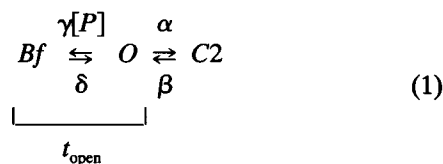
Transitions to the closed state C2 are only permitted from the true open state (O). Since the transition from the open to the fast blocked state (Bf) is concentration-dependent, procainamide shifts the equilibrium away from the closed state C2 and toward the blocked state Bf, resulting in fewer closures and an increase in t_{open}. This concentration-dependent increase is described by the relation:

$$t_{open} = (n + 1)t_{open}(\text{drug free}) + (n)t_{bf},$$

where n is the number of transitions between the true open state (O) and the fast blocked state (Bf). According to Colqhoun and Ogden (1988),

$$n = \gamma[P]/\alpha,$$

where $[P]$ is the procainamide concentration. Since

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

and

$$\delta = \frac{1}{t_{bf}}, \qquad \frac{t_{open}}{t_{open}(\text{drug free})} = 1 + \left(\frac{\gamma}{\delta}\right)[P].$$

Plotting this relation against procainamide concentration yields a straight line with an intercept at 1 and a slope equivalent to $1/K_d$ for fast procainamide block. This plot is the basis for Fig. 5 *A*. Consistent with our kinetic scheme, the K_d obtained from the regression line (17.9 mM) is close to the K_d for fast block (14.9 mM).

If our model is correct, one would also expect the reduction in gating closures to follow the same voltage dependence as fast block. We observed a qualitatively weaker inhibition of long closures at negative potentials in four experiments. For three of these experiments a quantitative analysis was warranted. As can be seen from Fig. 5 *C*, the voltage dependence of the reduction in the frequency of long gating closures was similar to that of fast block. We obtained electrical distances of 0.38 and 0.41 for the voltage dependences of fast block and reduction in the number of long gating events, respectively. These data are consistent with the value obtained from Fig. 2 *C* and provide further evidence for our proposed kinetic model.

We did not devote a detailed study to the effects of QX-314 on the frequency of the long gating closures. However, observations that the effect was voltage-dependent, increased with QX-314 concentration, and was half-maximal near the K_d for QX-314 block ($K_d = 6.4$ mM at 0 mV (Zamponi et al., 1993a)) suggest a similar correlation between the fast blocking action of QX-314 and the inhibition of long gating closures. Together with our observations on lidocaine and diethylamide (see companion paper), our data suggest that drug binding to the fast blocking site causes the inhibition of long closures, i.e., of the residual inactivation seen in BTX-modified cardiac channels.

So far, most studies on procainamide have dealt with the effects of the drug on whole cell action potentials (e.g., Rosen et al., 1972; 1973; Arnsdorf and Bigger, 1975). These authors reported a shortening of the action potential after application of lidocaine or procainamide, as well as a reduction in $(dV/dt)_{max}$. Carmeliet and Saikawa (1981) suggested that this effect was due to block of sodium, and not potassium currents. However, we could not find any data on procainamide action at the single channel level. It is, thus, difficult to view all of our results in context of the effects observed on action potentials. The procainamide-induced open channel block reported here would be consistent with a reduction in the inward sodium current during the initial depolarizing phase of the cardiac action potential (e.g., Rosen et al., 1972). However, we are unable to state whether the antagonizing effect of procainamide on inactivation observed in our experiments is significant under physiological conditions, because few data are available on the effects on procainamide on isolated sodium currents. Investigating the action of procainamide on unmodified, single sodium channels or whole cell sodium currents should help to clarify the interactions of procainamide with the inactivated state.

CONCLUSIONS

We have investigated the interactions between procainamide and BTX-activated sodium channels from heart and skeletal muscle. When internally applied, procainamide causes voltage-dependent open channel block which is similar for both subtypes. Block from the outside is weak, because it possibly requires direct hydrophobic access to the binding site, and procainamide is present predominantly in the charged form. In cardiac channels, fast block by procainamide appears to prevent long duration closures thought to be a residual inactivation of the BTX-modified channels.

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