J. Membrane Biol. 136, 199–214 (1993)

The Journal of
Membrane
Biology

Kinetics of Interaction of Disopyramide with the Cardiac Sodium Channel: Fast Dissociation from Open Channels at Normal Rest Potentials

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Received: 7 April 1993

Abstract. Block of cardiac sodium channels is enhanced by repetitive depolarization. It is not clear whether the changes in drug binding result from a change in affinity that is dependent on voltage or on the actual state of the channel. This question was examined in rabbit ventricular myocytes by analyzing the kinetics of block of single sodium channel currents with normal gating kinetics or channels with inactivation and deactivation slowed by pyrethrin toxins. At -20 and -40 mV, disopyramide 100 μM blocked the unmodified channel. Mean open time decreased *45* and *34%* at -20 and -40 *mV* during exposure to disopyramide. Exposure of cells to the pyrethrin toxins deltamethrin or fenvalrate caused at least a tenfold increase in mean open time, and prominent tail currents could be recorded at the normal resting potential. The association rate constant of disopyramide for the normal and modified channel at -20 mV was similar, $\sim 10 \times 10^6$/M/sec. During exposure to disopyramide, changes in open and closed times and in open channel noise at -80 and -100 mV are consistent with fast block and unblocking events at these potentials. This contrasts with the slow unbinding of drug from resting channels at similar potentials. We conclude that the sodium channel state is a critical determinant of drug binding and unbinding kinetics.

Key words: Sodium channel — Disopyramide — Antiarrhythmic drug — Deltamethrin — Patch clamp — Extracellular pH

Introduction

Despite their potential for inducing serious adverse cardiac effects, the local anesthetic-class drugs remain the most important group of antiarrhythmic agents [9]. A part of their antiarrhythmic action results from frequency-dependent block of the inward sodium current. The mechanism(s) of sodium channel blockade remain unclear. The increased blockade which occurs during repetitive depolarization may result from increased affinity dependent on voltage and or the actual state of the channel [29, 30]. In the state-dependent models of block, the activated and the inactivated states are presumed to have greater affinity for drug compared with the resting state. The activated state includes both the state that precedes opening and the actual open state. Studies with a number of sodium channel blockers in cardiac muscle suggest that the unbinding kinetics from the activated state may play a more critical role in drug blockade than heretofore realized [2, 7, 18, 53]. The actual kinetics of the interaction of drug with the activated state can only be indirectly inferred. Membrane voltage can provide an additional or alternate mechanism of frequency-dependent block. The distinction between the various models has been difficult to make because of the problem of defining the actual state of the sodium channel at specific times following excitation. In both nerve and cardiac muscle, it has been shown that at a time when the macroscopic current is still increasing, a substantial fraction of sodium channels has already inactivated [1, 5]. The current time course cannot be divided into periods in which the channels are exclusively open or inactivated. The problem of defining the actual channel state is even more difficult with upstroke velocity measurements.

In addressing these problems, our first strategy was to use single sodium channel recordings. It is possible to identify, unequivocally, the open state of the sodium channel. However, single sodium channel currents can usually only be recorded over

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a limited range of membrane potential. At potentials positive to the threshold for channel activation, the process of inactivation also develops rapidly. In addition to studies of the normal channel, we modified sodium channel gating pharmacologically such that channel openings could be elicited both at potentials positive to threshold and at the normal resting potential where inactivation is minimal. The pharmacologic modification may modify drug binding kinetics [40]. However, significant literature on the properties and drug blockade of modified sodium channels in native and artificial bilayers is largely corroborated by findings with unmodified channels [10, 41, 45, 48, 54]. The possibility of recording open sodium channels at the normal resting potential permitted an evaluation of the role of channel state, open *vs.* rested, in the binding kinetics of antiarrhythmic drugs with the sodium channel. The much wider range of potential over which open channel currents could be recorded permitted a more complete analysis of the effect of the membrane field on binding kinetics.

The second experimental strategy was to select an antiarrhythmic drug that produces identifiable block of open sodium channels. We selected disopyramide for these studies. Although block of open sodium channels had not directly been demonstrated for disopyramide, both upstroke velocity and whole-cell sodium current measurements suggest that disopyramide is an activated state blocker [35, 37, 52]. A study of the blocking action of nerve sodium channel by disopyramide and some of its derivatives is also consistent with open channel block [59]. Recently, Courtney [14] has suggested that among the tertiary amine class of local anesthetic antiarrhythmic drugs, open channel block may be related to drug size. His molecular modeling studies suggest that tertiary amine sodium channel blockers with an XY dimension of 45 $Å^2$ or greater are open channel blockers. Disopyramide has an XY dimension of 47 $Å^2$.

Open channel block of both normal and pyrethrin-modified sodium channels by disopyramide was demonstrated in the present studies. Fast dissociation of disopyramide from open channels occurs on a millisecond time scale at normal resting potentials, whereas dissociation from closed channels at these potentials has been reported to occur in the time frame of tens of seconds [6]. Low extracellular pH markedly slows the rate of dissociation of antiarrhythmic drugs, including disopyramide, from rested sodium channels [6]. Our results show that the dissociation rate of disopyramide from open sodium channels is little influenced by extracellular pH. The experiments lead to the conclusion that the kinetics of block of sodium channel is more dependent on channel state rather than on voltage. Some of these results have been reported in abstract form [22].

Materials and Methods

The experiments were performed on enzyme-dissociated rabbit ventricular myocytes. The procedure of cell isolation is similar to those previously described from this laboratory [20]. After the initial dispersion, we added a final step of exposure to 0.02 mg/ml deoxyribonuclease 1 (Sigma IV) for 10 min at 37°C. The exposure to deoxyribonuclease increased the success rate of giga-ohm seal formation [58]. Cells were stored in an incubator at 37°C until used.

For cell-attached recordings, the ventricular myocytes were superfused with a high potassium solution of the following composition (mM): KCl 70, K aspartate 80, NaCl 5, $MgCl_2$ 3, EGTA 0.05, HEPES 5, NaH_2PO_4 12 and glucose 10. The pH was adjusted to 7.4 with KOH. The high potassium concentration in the superfusate depolarized the cell to approximately 0 mV. Transmembrane potential in cell-attached patches is quoted in absolute voltages. The micropipettes were filled with a solution of the following composition (mM): NaCl 180, CsCl 1, $MgCl_2$ 5, $CaCl_2$ 0.01 and HEPES 5. The pH was adjusted to 7.4 with NaOH. In those experiments during which single sodium channel current tails were examined at potentials negative to −60 mV, 0.1 mM barium was substituted for calcium and magnesium in the micropipette solution in most of the experiments. All divalent cations produce block of sodium channel at negative potentials [15]. However, this effect is least with barium.

The pH dependence of the block of open sodium channels by disopyramide was studied at pH 6.5–8.0. pKa values ranging from 8.36 to 10.4 have been reported [12, 16, 27]. Substantial modulation of rest recovery from disopyramide block has been reported with pH changes in the range of 7.4 to 8. Further, the range of 6.5–8 used in the present study is relevant to the pH levels that may be attained in vivo. For the pH of 6.5, HEPES was replaced by 2-(N-Morpholino) ethanesulfonic acid (MES); for the pH of 8.0, HEPES was replaced by Tris(hydroxymethyl) methylamino-propanesulfonic acid (TAPS).

Disopyramide was obtained from Sigma. DPI 201-106 was kindly provided by Dr. A. Lindermann (Sandoz, Basel, Switzerland); deltamethrin by Drs. R. H. Brooke (Wellcome Research Laboratories, UK) and P. Joublin, (Roussel UCLAF, Paris, France). A 0.1 M solution of disopyramide was prepared by dissolving the base in 0.1 N HCl and then adjusting the pH to 7 with NaOH. Racemic DPI 201-106 dissolved in HCl was added to the micropipette solution to give a final concentration of 3 μM. A 10 mM stock of deltamethrin was prepared in dimethyl sulfoxide. Aliquots were added to the superfusate to give a final concentration of 10 μM.

Micropipettes were fabricated from 1.6 mm OD Pyrex glass tubing (Drummond Scientific, Bromall, PA). The micropipettes were coated up to their tips with Sylgard 184 (Dow Corning, Midland, MI) and fire-polished immediately prior to use. Single channel currents were recorded with a Dagan 3900 patch clamp amplifier (Dagan, Minneapolis, MN) [24]. Each microelectrode was coupled to the input of the amplifier by a silver/silver chloride wire coated with Teflon up to its tip. The bath reference electrode was fashioned from a similar silver/silver chloride electrode embedded in agar- micropipette solutions. Offset voltages were typically 1–3 mV and nulled before initiating recordings. Single channel currents were analogue-filtered prior to digitization. The corner frequency set on the 8-pole Bessel filter (Model 902 LPF, Frequency Devices, Haverhill, MA) depended on the experimental protocol. For sodium channel currents elicited at depolarized potentials, a corner frequency of 2–3.3 kHz was used. Between cells, the holding potential was varied such that some nulls were observed at the test potential. The use of averaged null sweeps

was a much more effective way of reducing the leakage and capacitive currents during analysis. However, for a given experiment, control and drug data were obtained from the same holding potential. For tail currents elicited at potentials more negative than −60 mV, single channel current amplitude was large. Therefore, a corner frequency of 5 KHz was used in some experiments. Filtered currents were digitized at 20–25 kHz and stored on the fixed drive of a microcomputer (Compaq 386/20). Voltage command pulses were provided by an IBM XT Personal Computer equipped with an A/D–D/A interface (TLI interface with Labmaster Boards, Axon Instruments, Burlingame, CA). Actual voltage clamp protocols are described in the Results.

Data Analysis

The techniques of data analysis for experiments done in the absence of a channel modifier were similar to those reported in previous studies from this laboratory [31]. Current during each depolarizing trial was scanned and those without events (nulls) collected. The nulls were averaged and the averaged current subtracted from each trial to remove the residual capacitive and leakage current. Single channel current amplitude was determined from histograms of leakage-subtracted current trials or as the mean of clearly resolved events. An automatic detection scheme with the threshold set at 0.5 times the single channel amplitude was used to identify channel openings. The performance of the algorithm was routinely checked by comparing raw current traces and the idealized records. For experiments with sodium channel modifiers, the depolarizing trials were scanned. Only those trials during which repetitive nonoverlapping channel openings exceed 10% of the total duration of depolarization were selected for analysis. The openings of modified channels usually lasted at least 50% of the total duration of depolarization. Openings of modified channels in which the channel failed to close before the end of the pulse were excluded from analysis. Mean open times of modified channels are therefore underestimated. Histograms of open and closed time distributions were fitted to exponentials using a least-squares procedure. The bin width was adjusted depending on the sampling rate. Validity of the techniques used to select bin width and fitting of exponentials to such fits have been validated by theoretical analysis and simulation done in our laboratory [31, 49]. Closed times were measured during prolonged bursts only when a single channel is operative. The closed-time histograms at hyperpolarized membrane potentials (−80 and −100 mV) were truncated because of the large fraction of empty bins beyond 2.5 msec. Open channel noise during exposure to sodium channel modifiers and disopyramide was compared using spectral analysis. Power spectral density was computed from the Fourier transform of filtered data segments using a fast Fourier transform algorithim [42]. Unless specified otherwise, data are quoted as mean ± SD.

Results

Studies of the Unmodified Sodium Channel

The first goal was to establish the nature of the blocking process of normal sodium channels by disopyramide. Such experiments required the recording of single sodium channel currents under control conditions, exposure to disopyramide, followed by a repetition of the protocols during drug exposure. Because of the known time-dependent changes of sodium channel current kinetics recorded in cell-attached patches in the absence of drug exposure, it was important to document the extent of such changes under the recording conditions used in the present study [20, 34].

In five unselected cells, single sodium channel currents were recorded from cell-attached membrane patches 10, 20 and 30 min after giga-ohm seal formation. Results from one cell and summary data from all cells are presented in Fig. 1. Depolarizing voltage pulses applied from a holding potential of −90 mV to a test potential of −40 mV elicited characteristic single sodium channel currents. Single channel current amplitude was 1.5 pA and mean open time 0.69 msec (*A*). Currents recorded 20 min later (a total of 30 min after giga-ohm seal formation) are shown in *B*. Single channel current amplitude was 1.4 pA and mean open time decreased to 0.56 ms. There was also a decrease in the peak averaged currents. Summary data from all five cells are presented in *C* and *D*. Over a 20 min period, mean open time decreased from 0.75 ± 0.011 msec to 0.67 ± 0.67 msec ($P > 0.5$). Normalized peak current decreased by 50%. While the average change in open time was relatively small, the change in normalized peak current was substantial. The latter was related at least in part to a decrease in the apparent number of functioning channels in the patches. For the remainder of the studies, records were obtained from patches after a 10 min interval without intervention. If rundown was not apparent, we proceeded with drug exposure or other studies. The control experiments suggest that parameters of channel gating that are critically dependent on the number of functioning channels in the patch need to be interpreted with caution.

To determine the effect of disopyramide on the unmodified cardiac sodium channel, control records were obtained 10 min after giga-ohm seal formation. The cell was then exposed to the drug for 10–15 min and the pulse protocol repeated. A full data set was obtained in five cells at a test potential of −20 mV and four cells at −40 mV. In a single experiment, we were also able to obtain data 30 min after washout of disopyramide. Results of that experiment are presented in detail; summary results from all cells are presented in the Table. During control (Fig. 2, left panel), 40-msec depolarizing voltage pulses from a holding potential of −120 mV to a test potential of −20 mV elicited single sodium channel currents with short latency. The number of functioning channels estimated from the maximum number of overlapping openings was two. The distribution of all

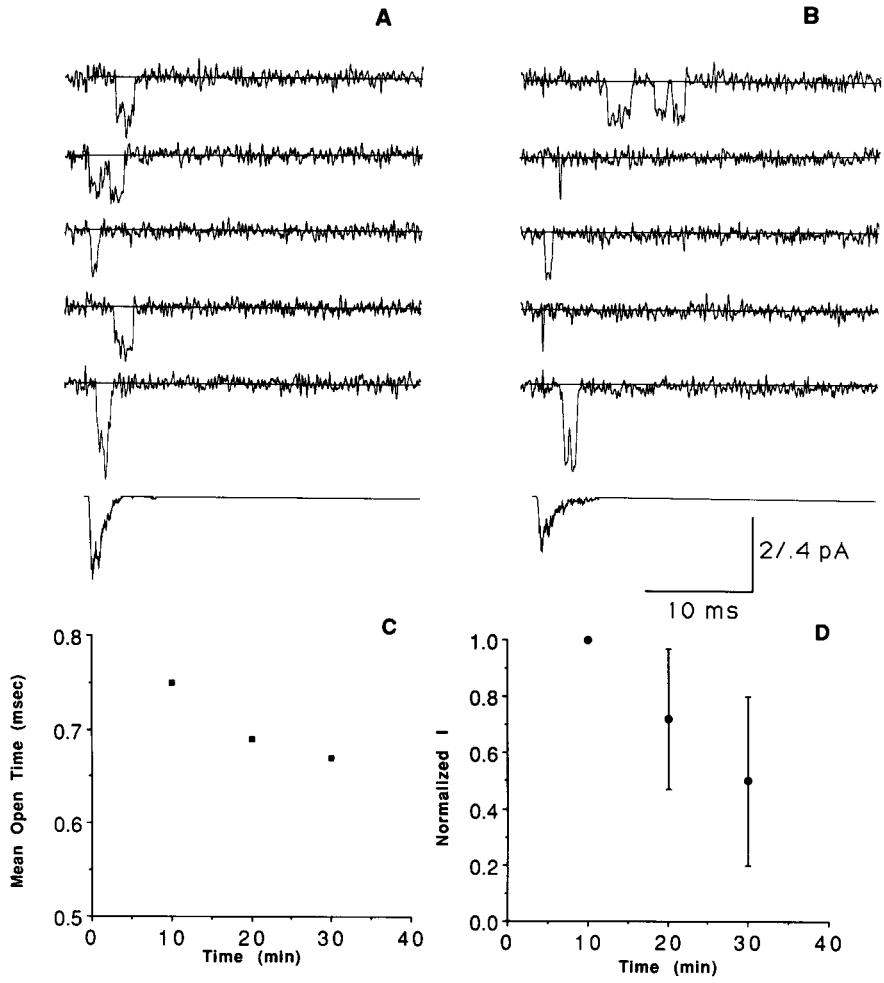

Fig. 1. Time-dependent changes in single channel current. *A* and *B* show single channel currents during five consecutive depolarizing pulses from a holding potential of −90 mV to a test potential of −40 mV. The averaged current from depolarizing trials is shown in the lowest trace. Records in *A* were obtained 10 min after gigaohm seal formation; those in *B* 20 min later. The filter corner frequency was 2.5 kHz. The 2 pA current calibration refers to the single channel current; the 0.4 pA calibration to the averaged current. The mean open time at 10, 20 and 30 min from a total of five patches is plotted in *C*. The averaged current at 20 and 30 min was expressed as a fraction of that at 10 min and plotted against time in *D*.

open times determined from 100 depolarizations was well fitted by a single exponential with a mean closing rate of 1.4/msec. The corresponding mean open time was 0.6 ± 0.59 msec.

Following exposure to 100 μM disopyramide for 10 min, depolarizations with single channel openings occurred in clusters separated by depolarizations without openings. A segment of consecutive depolarizations with openings was selected for presentation. The estimate of the number of functioning channels remained at two. Single channel mean open time was diminished, mean 0.29 ± 0.29 msec. While depolarizations during control usually contained less than three openings, (maximum number of overlapping events: 2), many depolarizations during disopyramide exposure contained a greater number. Some of the repetitive openings were grouped together as to suggest bursts of openings. If only depolarizations in which one or more channels opened are considered, the averaged number of openings η, per depolarization was 1.75 during control and 2.45 during exposure to disopyramide. Total charge transfer ($\eta \cdot \langle t_o \rangle$ where $\langle t_o \rangle$ is the mean open time of the sodium channel) per depolarization with events was decreased from 1.15 to 0.71 fC. Because of the large increase in the number of null sweeps during drug exposure, a total of 300 depolarizations was obtained during drug exposure to acquire sufficient events for analysis. The distribution of open times was fitted by a single exponential with a mean closing rate of 4.3/msec. In this particular experiment, the holding potential was sufficiently negative to remove resting sodium channel inactivation and there was no change in the apparent number of functioning channels. It appeared reasonable to compare the probability, P_f, that a channel would fail to open during control and drug exposure. P_f was 0.37 during control and 0.5 during exposure to disopyramide. These effects of disopyramide were reversible on washout. Bursts of openings were no longer apparent and the mean closing rate decreased to 1.9/msec. Summary results are presented in the Table. There was no significant difference in the change in mean open time between test potentials of −20 and −40

Table. Effects of disopyramide on single sodium channels

Exp	Exp No.	i	$\langle t \rangle$	No. trials	Fraction of Nulls
			−20 mV		
Control	080189	1.6	0.64 ± 0.54	341	0.15
Drug		1.6	0.35 ± 0.36	630	0.66
Control	032690	1.5	0.61 ± 0.52	197	0.1
Drug		1.4	0.50 ± 0.60	376	0.3
Control	033090	1.6	0.62 ± 0.59	99	0.14
Drug		1.4	0.29 ± 0.29	300	0.25
Control	033000	1.4	0.66 ± 0.54	200	0.03
Drug		1.2	0.31 ± 0.26	397	0.06
Control	070590	1.4	0.7 ± 0.56	100	0.1
Drug		1.3	0.34 ± 0.31	400	0.25
			−40 mV		
Control	032690	1.5	0.62 ± 0.5	97	0.08
Drug		1.5	0.5 ± 0.5	100	0.42
Control		1.8	0.74 ± 0.66	99	0.07
Drug	033090	1.7	0.31 ± 0.26	300	0.1
Control	033000	1.8	0.68 ± 0.63	200	0.03
Drug		1.6	0.3 ± 0.3	100	0.12
Control	070590	1.8	0.76 ± 0.69	100	0.12
Drug		1.6	0.41 ± 0.39	500	0.59

i = Single channel current amplitude (pA).
$\langle t \rangle$ = Mean open time (msec).

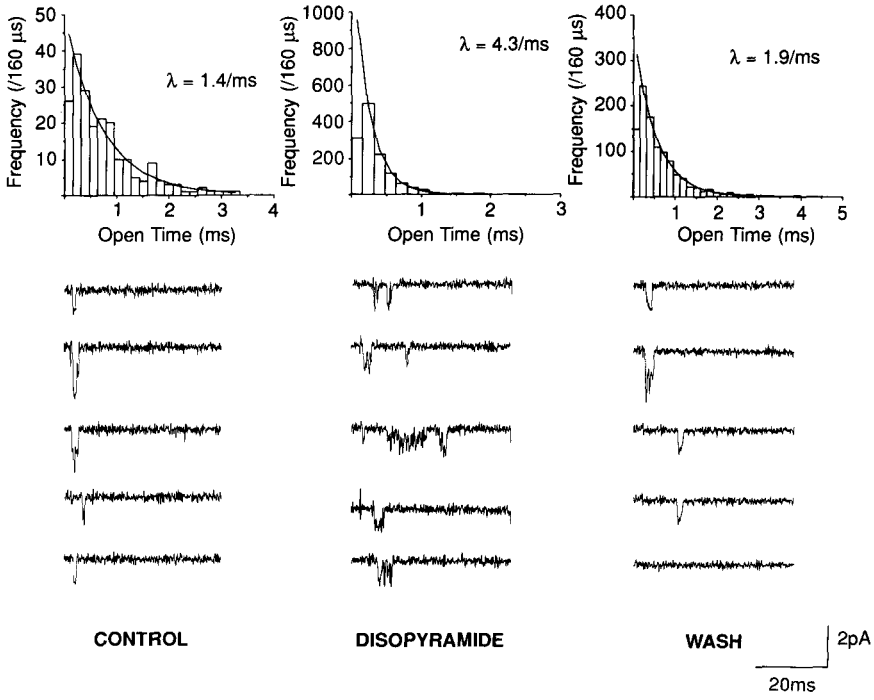

Fig. 2. Block of single sodium channel currents by 100 µM disopyramide. Each column shows the open time histogram and five consecutive single channel current responses to depolarizing voltage pulses from a holding potential of −120 mV to a test potential of −20 mV. The continuous lines are single exponential fits to the distribution of open times. The current records were filtered at a corner frequency of 3.3 kHz and sampled at 25 kHz.

mV. The association rate constant estimated from the reduction in mean open time was 12 and 14 × 10^6/M/sec at −20 and −40 mV respectively. We did not calculate an off rate for the unmodified channel. At the early times during a voltage step when the unmodified channel opens, more than one channel was functioning. Therefore, the closed times could not be interpreted unequivocally.

These results show that disopyramide blocks single sodium channels. Further, block of open channels can be effected without application of disopyramide to the external membrane surface. It was

possible to investigate the blocking action of disopyramide on the unmodified sodium channel over a very limited range of potential. At potentials positive to −20 mV, the reduced single channel amplitude made the signal to noise ratio unfavorable. At a potential of −60 mV or negative to that value, the probability of channel opening was so low as to make acquisition of enough events for analysis very difficult. Therefore, to extend the voltage range over which the analysis could be performed, we were forced to modify sodium channel kinetics pharmacologically.

Some Properties of Deltamethrin-modified Sodium Channels

The choice of an agent to modify the sodium channel and the properties of the modified channel are briefly discussed. In principle, if inactivation and deactivation were markedly slowed, it should be possible to record Na channel currents over a wide range of potentials, including the normal resting potential. A number of agents were tried including the piperazinyl-indole derivatives DPI 201-106 and DBF 9145, and anthropleurin A [17, 36, 55]. As has been reported previously, the piperazinyl derivatives produced open times that were highly voltage dependent, deactivation was little affected, and at some potentials, the frequency of channel closure by deactivation and block was similar. Anthropleurin A slowed deactivation. However, channel modification tended to disappear irreversibly, particularly during drug exposure. The pyrethrin toxins, deltamethrin and fenvalerate, proved to be the most suitable agents [13, 28]. Both deactivation and inactivation were slowed to produce an increase in open time of at least one order of magnitude, and tail currents were prominent.

Single channel currents recorded from a deltamethrin-treated patch are illustrated in Fig. 3. The holding potential was set at −100 mV and 200-msec depolarizing pulses were applied to test potentials of −20 and −40 mV. For the test potentials of −80 and −100 mV, 20-msec depolarizing pulses were applied to −40 or −20 mV to activate the sodium channel followed by 180-msec test pulses to −80 and −100 mV. Membrane current during depolarizing trials showed brief openings of normal channels and or prolonged openings of modified channels. Trials with modified channels are shown in Fig. 3. Analysis was restricted to trials with modified channels without overlapping openings. Mean open times were 19.6, 28, 18 and 40 msec at −20, −40, −80 and −100 mV, respectively. During many depolarizing trials, the channel failed to shut before the termination of the pulse. Those openings have been excluded from analysis. The resulting means may have underestimated the true population means. For a similar reason, no analysis of total burst duration was performed. At potentials positive to the threshold of the normal sodium channels, open times during the bursts were at least ten times greater than that of the unmodified channel. As the blocking rate depends on $1/\langle t_o' \rangle - 1/\langle t_o \rangle$ where $\langle t_o' \rangle$ and $\langle t_o \rangle$ is the mean open time during drug exposure and control, the longer open times of the deltamethrin-modified Na channels compared with other modifiers minimize the impact of changes in mean open time with voltage. In these experiments, $1/t_o \ll 1/t_o'$.

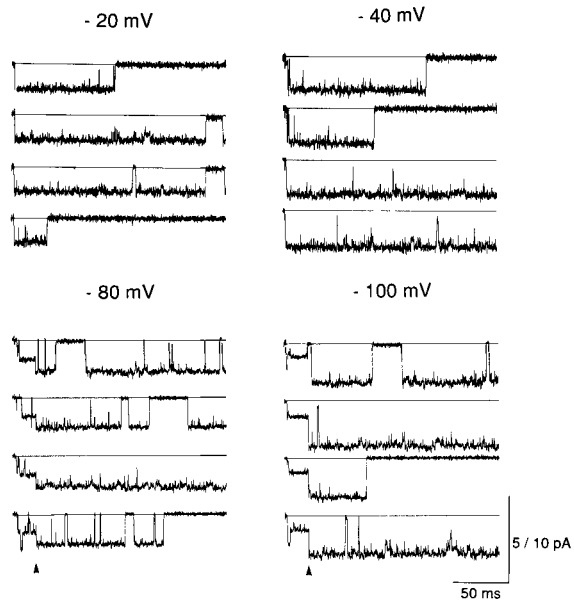

Fig. 3. Single sodium channel currents during exposure to deltamethrin. Selected current recording from a single patch show prolonged nonoverlapping openings at test potentials of −20, −40, −80 and −100 mV. The test pulses to −80 and −100 mV were preceded by 20-msec conditioning depolarizing pulses to −40 and −20 mV, respectively. The arrowheads mark the end of the conditioning pulse. The records show prominent tail current during the test pulses. Current records were filtered at 2.5 kHz. The 5 pA calibration refers to the current recordings at −20 and −40 mV.

In the preliminary experiments with deltamethrin-modified channels, a marked increase in open channel noise and a nonlinear current voltage relationship were observed. This was interpreted as fast block of open sodium channel by calcium and magnesium. For most of the subsequent experiments, Ca^{2+} and Mg^{2+} were replaced by Ba^{2+} as prior studies have suggested that barium is a less potent blocker of open sodium channel than calcium or magnesium [15]. With a barium pipette solution, the current-voltage relationship was linear with a chord conductance of 36 ± 2.5 pS.

Both whole-cell and single channel studies have shown that the kinetics of block of open sodium channels depends on the transmembrane sodium gradient. Recently, it has been suggested that the sodium ions entering the cell during the transient inward current may be confined to a restricted space of about 100 Å, the so-called "fuzzy space." [38, 39]. The movement of sodium ions into such a limited diffusional space was predicted to double $[Na]_i$ and to decrease the driving force for sodium movements into the cell. The prolonged openings throughout the 200-msec depolarizations provided an opportunity to examine this question. If sodium ions are moving into a restricted space, one would predict a decrease in single channel conductance late during the depolarization. The distribution of current amplitudes during the initial and final half of the depolarizations is shown in Fig. 4. The holding potential was −100 mV and the test potential −40 mV. Actual current records are shown in the inset. The mean current amplitude for the initial and final half of the depolarization was 2 pA. Apparently, sufficient ion accumulation is not occurring during the prolonged opening to change single channel current amplitude. This result is significant because the open times during the bursts are so much longer than those of the unmodified channel.

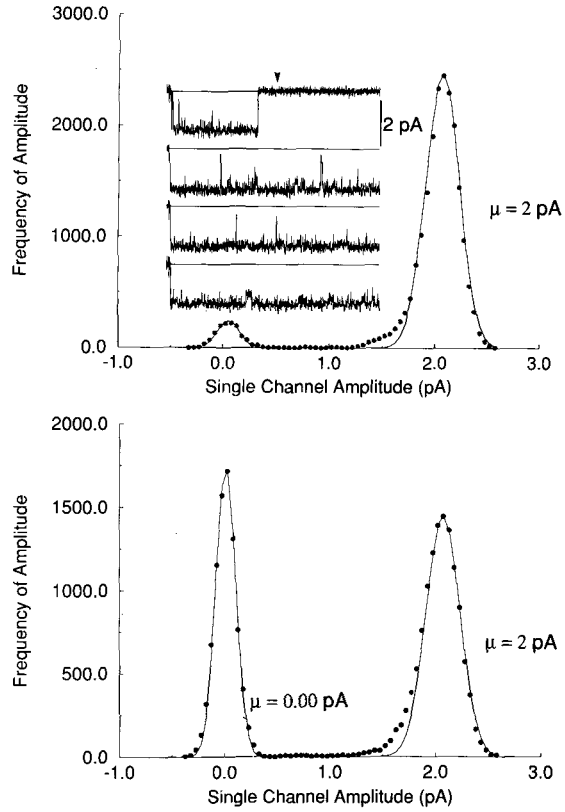

Fig. 4. Single channel current amplitude at early and late times during depolarization with prolonged nonoverlapping openings. The single channel current during prolonged openings has been divided into an initial early and late 100-msec segments. The upper and lower panels show a histogram during the early and late segments, respectively. The frequency of single channel current amplitude at the open channel level has been fitted with normal curves. At both early and late times, the mean was 2 pA. There was a systematic deviation of the initial phase of the distribution from normality. Representative single channel currents filtered at 2 kHz are shown in the inset.

Block of Deltamethrin-modified Sodium Channels by Disopyramide: Influence of Drug Concentration

We assumed the simple bimolecular reaction:

$$C \longleftrightarrow O \underset{k_{off}}{\overset{k_o D}{\rightleftharpoons}} OD$$

where C and O are the drug-free closed and open channel states, OD a drug-associated state of the channel, D, the disopyramide molecule and k_o and k_{off} the association and dissociation constant of the drug with the receptor site. Justification for this model includes the exponential distribution of open times which will be described below. The rate constants are related to the experimentally derived distribution of open and shut times according to the following relationships:

$$k_o = \{1/\langle t_o' \rangle - 1/\langle t_o \rangle\}/D \quad (1)$$

$$k_{off} = 1/\langle t_c \rangle \quad (2)$$

where $\langle t_o \rangle$ and $\langle t_o' \rangle$ are the mean open times in the absence and presence of disopyramide respectively, and $\langle t_c \rangle$ is the mean closed time. Equation (1) also explicitly relates the distribution of open times with drug concentration.

The influence of 20–100 μM disopyramide on the block of deltamethrin-modified sodium channels was studied in a total of 11 experiments. A single drug concentration was examined in each patch. Results of one set of experiments are presented in Fig. 5. The holding potential was set at −90 mV and test pulses were applied to −20 mV. Records during control and at each concentration of disopyramide were obtained from separate seals in the same group of cells. The cells were exposed to each concentration of disopyramide for a minimum of 20 min before each data set was obtained. Analysis was limited to depolarizing trials with prolonged bursts of openings. With increasing drug concentration single channel open time decreased. The association rate

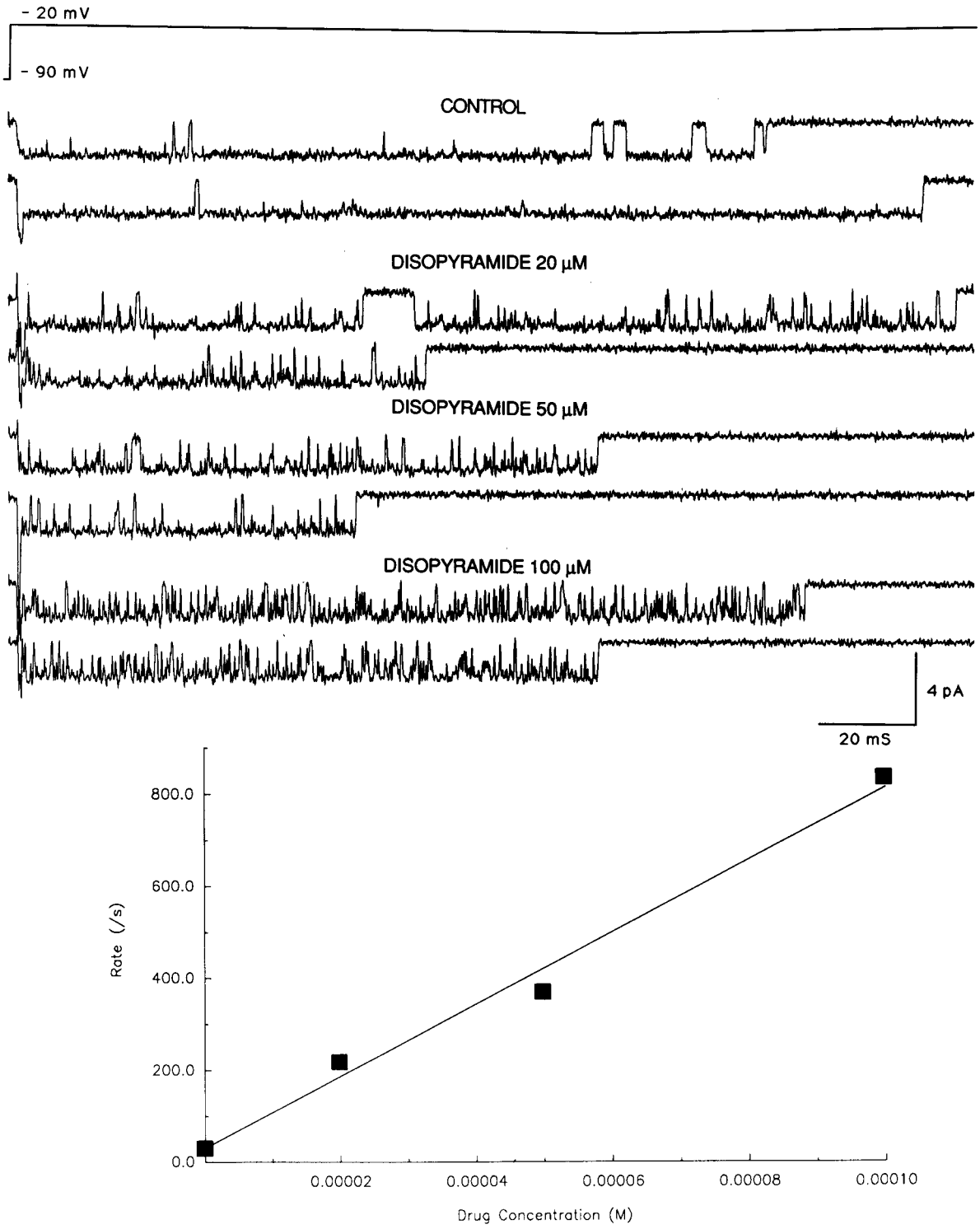

Fig. 5. Relationship between drug disopyramide concentrations and the association rate with single sodium channel currents. Single sodium channel currents were elicited with 200-msec pulses applied from −90 to −20 mV. Representative recordings obtained during control and exposure to 20, 50 and 100 μM disopyramide in separate patches are shown. The derived association rates are plotted against drug concentration. The relationship was linear. From the slope, an association rate constant of 7.7×10^6/M/sec was obtained.

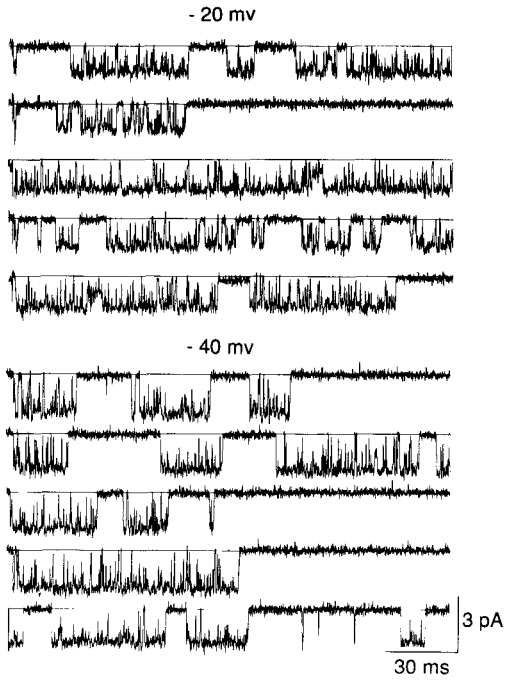

Fig. 6. Block of deltamethrin-modified channels by 100 μM disopyramide. The upper and lower panel show single channel currents recorded during depolarizing voltage pulses from a holding potential of −100 to test potentials of −20 and −40 msec. Depolarizing trials with nonoverlapping bursts of opening were selected for analysis and presented in the figure. The records were filtered at 2.5 kHz.

($k_o[D]$) is plotted against drug concentration in the lower panel of the figure. The relationship was linear (consistent with Eq. 1). From the slope of the least-squares fit to the line, an association rate constant of 7.7×10^6/M/sec was obtained. In two other sets of experiments, association rate constants of 7.9 and 11.5×10^6/M/sec were obtained. In all three sets of experiments, the mean closed time did not depend on drug concentration at the test potential.

Influence of Voltage

The possibility of recording single sodium-channel currents, both at potentials positive to threshold and current tails on return to the normal rest potential, permitted an analysis of the voltage dependence of block over a wide range of potentials. Figure 6 shows bursts of openings recorded from a cell at test potentials of −20 and −40 mV during exposure to 100 μM disopyramide. The prolonged openings evident in the disopyramide-free recordings now have multiple brief interruptions, indicative of blocking events. At −20 mV, prolonged bursts were evident in 23% of depolarizing trails. There was a total of 1,528 openings with a mean open time of 1.4 ± 1.4 msec. At −40 mV, prolonged bursts were evident in 8% of depolarizations. There was a total of 339 events with a mean of 2 ± 1.8 msec. At both test potentials, the distribution of open time was well fitted by single exponentials. It is evident from Fig. 6 that the distribution of closed times is more complex. There are prolonged shut times dividing the open time into bursts and brief closures within bursts. At least two exponentials with a fast and a slow time constant would be required to describe such a distribution. However, despite the fact that closed times greater than 2.5 msec account for most of the shut periods, they were too few to define the slow time constant. If the distribution is constrained to events of duration less than 2.5 msec, mean closed times of 0.48 and 1.1 msec are obtained at −20 and −40 mV, respectively.

A qualitatively similar blocking process was evident in the range of normal rest potentials such as that recorded at −100 mV in Fig. 7. Because of the much larger single channel currents at −100 mV (for example, the single channel current amplitude was 5.2 and 5.6 pA in the upper and lower panels of Fig. 7), the records were obtained at a filter corner frequency of 5 kHz. The pulse paradigm consisted of a conditioning pulse to −20 mV to activate the sodium channel, followed by a return to −100 mV. The depolarizations shown are examples of depolarizing trials where channel openings at the end of the conditioning pulse produced tail currents on return to −100 mV. During control, the mean open time was 33 ± 27 msec and the average number of openings was 4.5 per depolarization with prolonged openings. During exposure to 100 μM disopyramide, the mean open time decreased to 6.4 ± 6.6 msec, and there was an average of 17.2 openings per depolarization. The mean closed times were 0.09 ± 0.07 and 0.25 ± 0.47 msec during control and exposure to disopyramide. The increase in the number of openings per depolarization and the presence of both prolonged and brief shut periods during drug exposure indicate that the drug can dissociate rapidly from open channels at normal rest potentials.

The equilibrium dissociation constant K_D is given by k_{off}/k_o. k_{on}, k_{off} and K_D are plotted as a function of membrane potential in Fig. 8. The relationships are log-linear over the range of membrane potential −20 to −100 mV. The apparent voltage dependence of K_D results primarily from the voltage dependence of k_o. Woodhull [56] has proposed a simple blocking model for a charged species blocking on ion channel within the membrane field.

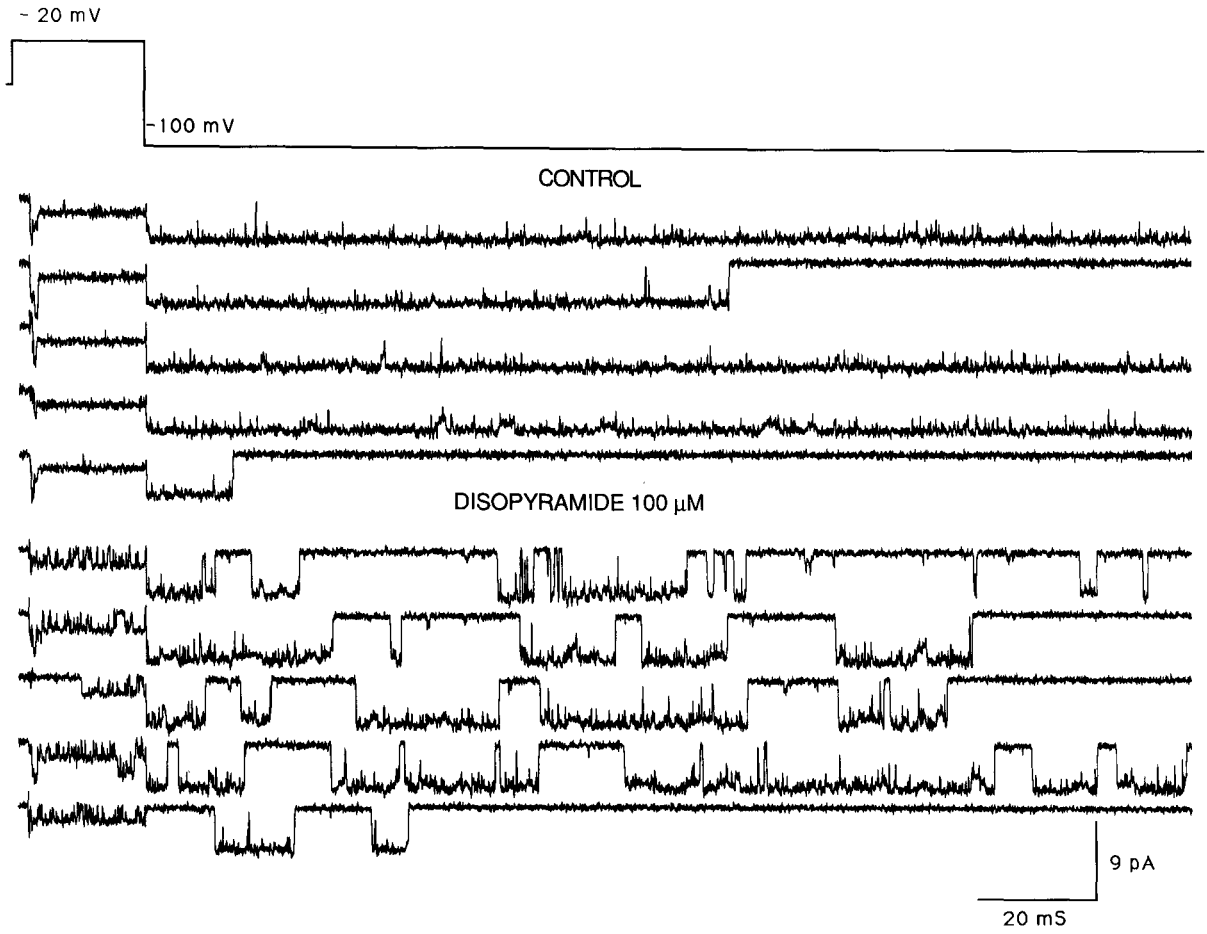

Fig. 7. Block of single deltamethrin-modified sodium channel currents at hyperpolarized potentials by disopyramide. Single sodium channel currents were elicited by depolarizing conditioning pulses to −20 mV followed by test pulses to −100 mV. The first five current recordings were obtained from a drug-free membrane patch. The lower five recordings were obtained from another patch exposed to 100 μM disopyramide for 30 min. The recordings were filtered at 5 kHz. The difference in the blocking kinetics at −20 and −100 mV can be compared in the early and late portions of the current recordings.

The voltage dependence of K_D is given by the following relationship:

$$K_D(V) = K_D(0) \exp(-\delta\, Ve/kT) \quad (3)$$

where $K_D(0)$ is the equilibrium dissociation constant at zero mV, δ is the fraction of the membrane field sensed at the blocking site, V the membrane potential, and e, k and T have their usual meanings. The Woodhull model is potentially useful in that it might provide insight as to the location of the drug binding site. When the data in Fig. 8 were fitted to the relationship, a value of 1.1 was obtained for δ. As δ is a fractional distance, then $0 \leq \delta \leq 1$. The value outside this range raises two possibilities: (i) the blocking species is multivalent and/or (ii) the equilibrium dissociation rate constants at hyperpolarized potentials (e.g., −80 and −100 mV) have been overestimated. The first possibility is not likely as disopyramide has a single pKa of 8.36 or 10.4.

The series of experiments from which the K_D was obtained at −100 and −80 mV were filtered at a corner frequency of 2 kHz such that they would be directly comparable with those at −20, −40 and −60 mV. In four other experiments, where a corner frequency of 5 kHz was used for recordings obtained at −100 mV, the mean k_o was 1.6×10^6/M/sec compared with 0.26×10^6/M/sec obtained from records filtered at 2 kHz. This suggests that there is significant bandwidth limitation to the resolution of blocking events at −100 mV. A comparison of the noise level when the channel is open and shut in Fig. 7 strongly supports such a conclusion.

If channel noise induced by blocking and unblocking events exceeds the bandwidth of the amplification system, then one will expect an increase in

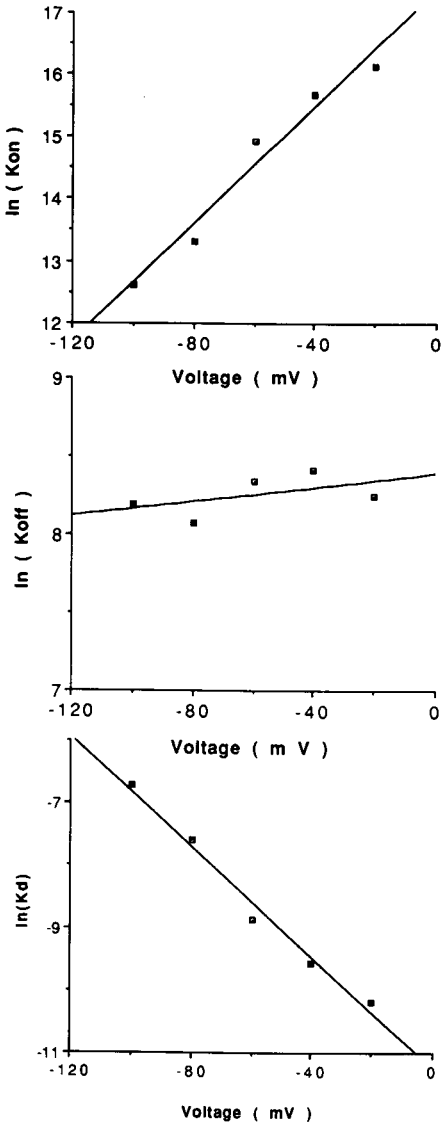

Fig. 8. Voltage dependence of blocking descriptors. The natural logarithm of the association and dissociation rates and the dissociation rate constants, k_{on}, k_{off} and K_D are plotted in the upper, middle and lower panels against membrane voltage; lines are least-square fits. The apparent voltage dependence of K_D results primarily from the voltage dependence of the association rate.

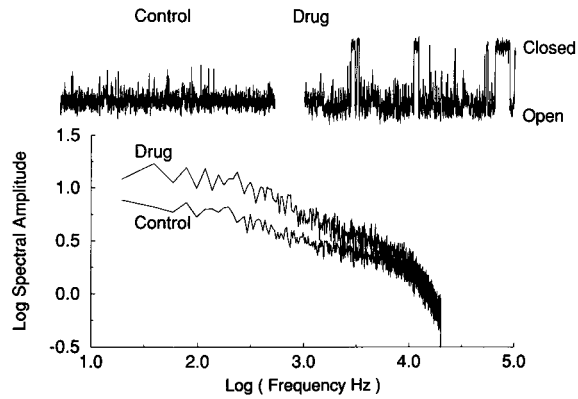

Fig. 9. Single channel recordings and average power spectrum. Shown in upper left is a typical example of open channel noise in the absence of drug. The upper right panel illustrates a comparable recording, including several closed events. Records were obtained at −100 mV. Note the similarity between open channel noise under drug-free conditions and closed channel noise in the presence of drug. Comparison of open channel noise shows several significant differences. The number of short duration closing events is much greater in the presence of drug. Also, the average open channel current appears to exhibit low frequency shifts, indicative of bursts of high frequency closings that exceed the bandwidth of the amplifier system. The lower panel shows the average power spectrum derived from the two conditions.

the low frequency portion of the power spectrum. To test this hypothesis, we compared the average power spectrum of open channel noise in the presence and absence of drug. As shown in Fig. 9, there are obvious differences between the amplitudes of open channel noise. Not only is the amplitude of the high frequency component of the noise increased with drug, but also the amplitude of the low frequency component increases (Fig. 9, upper right and left panels). This observation was verified by computation of the Fourier Transform of the open channel power spectrum. Figure 9, lower panel, shows a plot of the average (n depolarizations) power spectrum under control (n = 19) and drug (n = 16) conditions. Note that the power during drug exposure always exceeds that during control, both low and high frequencies.

A simple noise model without channel blockade, predicts a single Lorentzian process that is flat from zero frequency to some cut-off frequency, fc. We found that the power was not flat in the low frequency range, but rather decreased monotonically at rates that increased with increasing frequency. This departure from a simple Lorentzian process appears to reflect high frequency blocking and unblocking events both in the absence and presence of drug. Candidates for channel blockade in the absence of drug include external divalent cations. The incompletely resolved block results in an analogue aliasing process where high frequency noise appears as low frequency noise. This is a direct result of the asymmetry of noise due to blocking and unblocking events such that the average noise is nonzero.

The Influence of pH

A number of studies in nerve, skeletal and cardiac muscle have shown that the kinetics of interaction of local anesthetic-class drugs with the sodium chan-

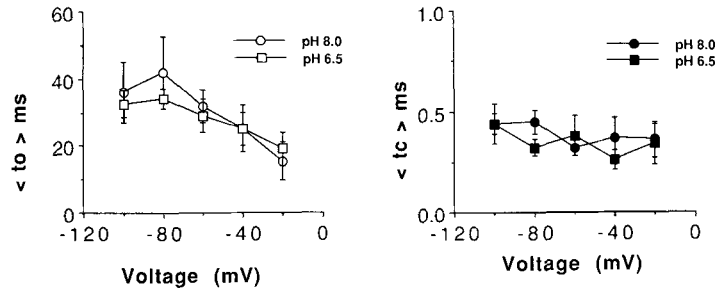

Fig. 10. Influence of external pH on single channel mean open and closed times in the absence of drugs. Single sodium channel currents were recorded in the absence of disopyramide at pH 8.0 and 6.5. Mean values of open and closed time in five experiments at each pH is plotted against test potential. The vertical bars show the standard deviation of the determinations. Mean open and closed times were not significantly influenced by external pH.

nel is modulated by external pH [21, 25, 32, 47]. In particular, lowering of the external pH decreases the rate of dissociation of drug from the resting and/or inactivated state of the channel. The data presented in this manuscript show that drug dissociation from the open sodium channel at normal rest potential may be rapid. In contrast, drug dissociation from the rested channel is very slow. It was of interest to determine the nature of the pH modulation of open channel blockade.

Single channel amplitude and duration at −20 mV to −100 mV were examined at pH 6.5 and 8.0 in the absence of drug. The pH change did not affect single channel amplitude significantly. Mean conductance was 29 ± 0.5 pS at pH 6.5 and 29 ± 0.8 pS at pH 8.0. Single channel mean open and closed times at the two pH studies are plotted against test potential in Fig. 10. Mean open and closed times were not significantly influenced by the pH change.

The kinetics of block of open sodium channels during exposure to disopyramide was determined at pH 8.0 and 6.5. Experiments at each pH were determined in separate patches. Only the pH of the micropipette solution was varied. The pH of the perfusate was fixed at pH 7.4. Therefore, the pH changes should not have affected the distribution of drug across the cell membrane as a whole. Disopyramide 100 μM was included in the micropipette.

The mean open and closed times at −70 to −30 mV have been plotted in Fig. 11. At each pH, mean open time increased at hyperpolarized potentials. However, open times were shorter at pH 8.0 compared with pH 6.5. This is consistent with a faster rate of association of disopyramide with the sodium channel at high pH_o. In contrast, the mean closed times show little dependence on membrane voltage and external pH. This lack of dependence of drug dissociation (as evidenced by constant closed times) contrasts with prior studies showing slow dissociation of the drugs from rested channels and indicates that the influence of pH on the drug dissociation rate depends on the state of the channel.

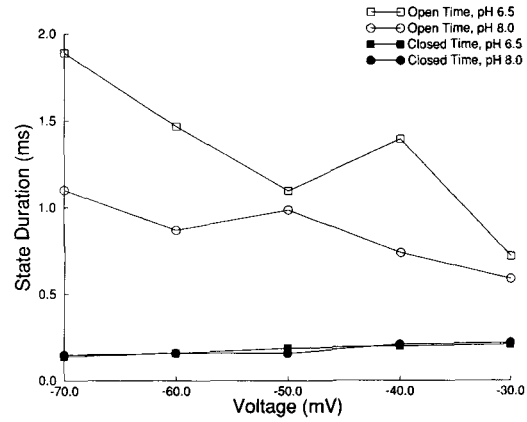

Fig. 11. The influence of external pH on the blocking kinetics of disopyramide. Single sodium channel current was elicited from deltamethrin-treated patches with 200-msec pulses to −70 and −30 mV. Open and closed time durations are plotted in the ordinate; test voltage on the abscissa. The closed time was not influenced by the pH change from 6.5 to 8. However, the open time was abbreviated by high pH.

Discussion

This study has examined the mechanisms of blockade of open sodium channels by disopyramide and the much broader question of the relationship between channel state and the kinetics of block. For the unmodified sodium channel, both control and drug data were obtained during continuous recording from the same patch. This required stable recording from the patch of at least 30 min. Even after a 10-min wait following giga-ohm seal formation, time-dependent changes were still evident. There was a significant decrease in average single channel current and a small decrease in open time. A prior study from this laboratory reported a time-dependent increase in open time [19]. It appears that the important change is a shift in the current-voltage relationship on the voltage axis such that an increase or decrease may be observed, depending on the test voltage. The yield of complete experiments was less than 10% of total seals obtained.

The fact that an effect of disopyramide could be demonstrated without its direct addition to the micropipette solution points to the approach of the drug to its binding site. It is able to access its binding site when excluded from the external membrane surface. This suggests the availability of lateral access to the binding site or through the ion conducting pore. This contrasts with results with the disopyramide derivative, penticainide, which had to be applied to the external membrane surface to block open sodium channels [8]. This may be related to the much lower lipid solubility of penticainide. Penticainide blocking action may be restricted to an external receptor only.

Block of the open unmodified sodium channel could be clearly demonstrated. Single channel open time was abbreviated. The decrease in open time was much larger than the average decrease that we observed during prolonged recordings in the absence of drug. Reversibility of this effect on washout was also demonstrated. In the most complete experiment, it was shown that in depolarizations with one or more openings, the average number of openings increased during disopyramide exposure. Even in the unmodified channel, the drug can associate and dissociate repeatedly with the open channel to produce "flickering." This result differs from that of Benz and Kohlhardt [4] who reported that the observation of repetitive blocking and unblocking events requires the removal of inactivation. In the simple sequential blocking model used in the analysis of the results, the total charge transferred during channel opening should, on average, be unchanged during the presence of drug [43, 44]. Single channel openings are converted to bursts in which total open time during the burst is the same as the open time in the absence of drugs. The presence of multiple channels in all patches analyzed and the very brief duration of the open unmodified channel precluded such burst analysis. However, the substantial reduction in the average charge transferred in the complete experiment suggests that drug-associated open sodium channels can undergo terminal closure. Presumably, the drug-associated open channel undergoes inactivation before the drug dissociates from the channel.

The normal gating kinetics of the sodium channel severely restricts the range of voltage over which the blocking process can be examined. In particular, the unmodified sodium channel opens rarely, if at all, at normal rest potentials. We modified the gating of the sodium channel pharmacologically to extend the range of potentials over which the properties of open channels could be examined. There was a special interest in studying potentials at which the channel is normally in the resting state. A wide variety of enzymes, toxins and drugs that slow sodium inactivation have been described. However, with a majority of these interventions, deactivation persists. This produces rapid channel closure at normal rest potentials. Further, sodium channel open time remains highly voltage dependent when deactivation is intact [36]. This poses a substantial limitation to the analysis of the voltage dependence of blockade. The fraction of channel closures that result from block and from deactivation changes with test potential.

We used the pyrethrin derivatives, deltamethrin and fenvalerate, to slow both inactivation and deactivation. These drugs produced a 10-fold or greater increase in mean open time of the sodium channel, so estimates of the blocking kinetics are not very dependent on the initial value of the channel open time. The marked slowing of deactivation produces prominent tail currents at the normal resting potential. The meaning of "normal resting potential" under our recording conditions of reduced temperature and low divalent cation concentration may be in question. We use the term to mean potentials at which channel availability is approximately one and spontaneous channel openings are rare in the absence of prior activation. In the initial experiments examining tail currents at -80 and -100 mV, a substantial increase in open channel noise and a reduction of the chord conductance was noted. This was interpreted as block of open sodium channels by the divalent cations, calcium and magnesium, present in the normal pipette solution. For the remainder of the experiments, calcium and magnesium were replaced by barium, as calcium and magnesium produce 46 and 42% block of open sodium channels compared with 16% by barium [15].

Generally, is there any utility in performing studies on modified sodium channels? The potential advantages of studying sodium channel currents of long duration over a wide range of membrane potential are clear. However, the modifier could alter channel ion conduction and/or drug-blocking mechanisms. The use of channel modifiers has provided a wealth of information about sodium channel ion conduction in both native membranes and artificial bilayers [e.g., 41, 45, 48, 54].

The results of studies on the modified channel parallel those of the normal channel in native membrane. Of the modifiers that have been used, batrachotoxin appears to produce the most change [33]. Channel cation selectivity is changed and inactivation and activation gating variables are shifted to more negative potentials. Yet the agent proved useful in comparing drug binding local anesthetic-class drugs to the cardiac sodium channel [48]. Rank order of potency and stereoselectivity as assessed from BTX-binding studies parallel the clinically ef-

fective concentrations. Although there are studies in nerve showing a reversal of stereoselectivity for block in nerve [40], this was not the case in cardiac muscle. However, the generalized shift in gating produced by batrachotoxin made it less useful for the present studies.

A generalized shift in gating of the sodium channels is not observed with the pyrethrin toxins. Spontaneous openings at the normal rest potentials remain rare. A normal threshold potential is still observed in the presence of the pyrethrins, and depolarizing pulses must first be applied to potentials positive to this level to activate sodium channels before current tails can be observed at the normal resting potentials. Backx et al. [3] combined sodium channel modification with fenvalerate and point mutation of the skeletal muscle μl sodium channel to define the divalent cation blocking site in the sodium channel. The blocking site identified in the fenvalerate-modified channel was the same as that previously identified in the unmodified channel [46]. Our results include data on both the normal and the unmodified channel. The blocking rates for the normal and modified channel in our study were quite similar, 12×10^6/M/sec and $7.7–11.5 \times 10^6$/M/sec, respectively at a test potential of -20 mV. Taken together, these data suggest that useful insight about channel blockade can be obtained from studies of the pyrethrin-modified sodium channel.

The occurrence of prolonged opening of modified sodium channels provided an opportunity to examine the effects of sodium entry on single channel current amplitude. A number of studies (e.g., reference 38) have suggested the amount of sodium ions entering the cell during the brief (<1 msec) opening of the sodium channel is sufficient to alter the driving force for sodium influx. Such a proposition would be likely only if the sodium ions entering the cell during the brief channel opening are confined to a restricted space. A comparison of the current amplitude of the single channel current at early and late times provided a simple test for the hypothesis. There was no change of single channel amplitude at early and late times. The prolonged open times of the modified sodium channels should have exaggerated any ion accumulation during channel opening. The result suggests that sodium ions entering the cell do so into a space sufficiently large to prevent significant ion accumulation.

The kinetics of block of open sodium channels was studied over a potential range from -20 to -100 mV. The blocking mechanism at potentials positive to the threshold potential is similar to that reported for other open channel blockers [8, 36, 43, 44]. Open time was exponentially distributed. The closing rate increased with increasing drug concentration. The association rate constant is similar to that reported for open channel blockers of other voltage- or receptor-gated ion channels [43, 44]. The similarity of the association rate constant in these diverse systems suggests that some fundamental process such as diffusion may be rate-limiting.

These are the first studies of which we are aware that have examined the blockade of open cardiac sodium channels at potentials in the range of normal resting potential. Blocking and unblocking events similar to those occurring above threshold were observed at -80 and -100 mV. The distribution of closed times appears to be complex, as brief events less than 2.5 msec in duration and longer events of duration tens of milliseconds were evident. Even at a bandwidth of 5 kHz, all the blocking events at -80 and -100 mV were not well resolved. The power spectrum of the open channel shows an excess of noise above the drug-free current at all frequencies. We interpret the excess noise as evidence of blocking and unblocking events. The bandwidth limitation of the recordings compromised the estimate of the fractional electrical distance sensed at the binding site. We can conclude from the voltage dependence of block that a substantial portion of the membrane field is sensed at the binding site. Estimates of the fraction of the field sensed at the blocking site in other systems have been ~0.6–0.7 [41, 57].

The very rapid dissociation observed in the records at -80 and -100 mV contrasts with the much slower dissociation rate observed from resting channels at similar potentials. Campbell [6] has reported a recovery time constant of 12 sec in guinea pig papillary muscle exposed to disopyramide at 37°C. Similarly, Gruber and Carmeliet [23] reported recovery time constants of 71 ± 8 sec at -95 mV in rabbit Purkinje fibers exposed to disopyramide. Whole-cell sodium current measurements done in our laboratory at a reduced temperature of 17°C showed little recovery after rest periods of several minutes. It is only by repetitive stimulation that significant recovery was observed.

A difference between drug interaction with open and rested channels was also evident from the studies that involved changes in external pH. Mean open times were less at pH 8 than at pH 6.5. The faster association rate at pH 8 suggests that the effective concentration of disopyramide at its blocking site is greater. At pH 8, the fraction of disopyramide in its uncharged membrane-permeable form is increased compared with lower pH values. If the passage of external drug to its binding site includes the lipid bilayer, the concentration of disopyramide at its binding site would be increased. There was little difference in the shut times at pH 8.0 and 6.5. The

effects of pH on the block of open sodium channels by disopyramide are similar to those reported for cocaine in skeletal muscle sodium channels expressed in lipid bilayers [54]. Over a pH range of 6.5–8.5 the dissociation rate of cocaine was unchanged. The lack of effect of external pH on the dissociation rate from open sodium channels in these studies contrasts with the studies of the pH dependence of drug dissociation from rested channels [11, 21, 23, 45]. In rabbit Purkinje fibers, the time constant of recovery from resting block doubled as external pH was reduced from pH 8 to 7.4.

When the data in this study and those published by others are reviewed, one is driven to the conclusion that at least for the open channel blocker, disopyramide, the state of the channel is the major determinant of the kinetics of interaction of drug with the sodium channel. The kinetics and pH dependence of drug dissociation from open and rested channels are clearly different.

We had proposed a simplified model of drug interaction with the sodium channel in which binding kinetics is fixed, but access to the receptor site is controlled by the channel gates [51]. The analytical advantage of such a model has been discussed. With fewer binding states, there are relatively simple protocols to determine binding parameters [50]. The results of this study showing the dependence of blocking kinetics on channel state are at variance with those simple assumptions and require the use of blocking models such as the modulated receptor model [26, 33] that include explicit binding parameters with each state of the channel or more complex guard and trap functions in the guarded receptor model may be more appropriate.

The important role of the state of the channel must be incorporated in the analysis of the mechanism by which external factors such as pH, potassium activity and beta-adrenergic stimulation affect block. For example, reduced external pH changes the voltage dependence of the gating kinetics of the sodium channel and/or the distribution of channels in the resting, open and inactivated state at a given potential. Such a redistribution of channels in its various kinetic states may have important consequences for the mechanism of block.

This research was supported in part by grants HL 32708, HL32994 and HL 17676 from the National Institutes of Health, and an American Heart Association (NC Chapter) Fellowship Award.

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