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Blockade of cardiac Na^+ channels by a charged class I antiarrhythmic agent, bisaramil: possible interaction of the drug with a pre-open closed state

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Abstract

The mechanism of cardiac Na^+ channel block by a charged class I antiarrhythmic agent, bisaramil, was studied in guinea-pig ventricular myocytes using patch-clamp techniques of whole-cell, cell-attached and inside-out configurations. Bath application of bisaramil caused the use-dependent block of whole-cell Na^+ current (I_{Na}) in a concentration-dependent manner and EC_{30} value was 2.0 μM. At 5 μM bisaramil, the degree of the use-dependent block of I_{Na} with a short (5 ms) pulse protocol (44.9 ± 5.7% of the first pulse I_{Na}) was comparable to that with a long (200 ms) pulse protocol (42.8 ± 5.9%). In cell-attached patches, bisaramil applied to the bath solution (external application) concentration dependently blocked macropatch Na^+ currents (50.3 ± 3.1% inhibition with 10 μM bisaramil). Internal application of bisaramil decreased the inside-out macropatch currents (82.6 ± 1.3% inhibition with 10 μM bisaramil). Blocking effects of bisaramil applied to the bath solution were greater than those seen on the pipette application in all of the whole-cell, cell-attached and inside-out configurations. In cell-attached patches containing a single active channel, bath application of 10 μM bisaramil increased the null sweeps with a significant ($P < 0.001$) nonrandom clustering and decreased the total number of openings, whereas no changes in the number of openings per active sweep, unitary current amplitude, mean open time and mean closed time were observed. While the peak average current was decreased by 51.0 ± 5.6% with 10 μM bisaramil, the number of active sweeps was decreased by 31.4 ± 6.2%. In the presence of 10 μM bisaramil, the mean values of first latencies were significantly ($P < 0.05$) increased and the peak value of the first latency density function was decreased by 15.8 ± 3.6%. From these results, we conclude that a charged tertiary amine, bisaramil interacts with cardiac Na^+ channels preferentially in the activated state. Interactions with pre-open closed states might contribute to the activated channel block by the drug.

Keywords: Cardiac Na^+ channel; Na^+ channel block; Antiarrhythmic agent, class I; Bisaramil

1. Introduction

Local anesthetic types of antiarrhythmic agents have common features to exhibit use-dependent block of cardiac Na^+ channels. According to the modulated receptor hypothesis (Hondeghem and Katzung, 1977, 1984), use-dependent block occurs when the recovery time from block is longer than the diastolic interval and the drugs have higher affinities for the activated and/or the inactivated state of the channel. Hondeghem and Katzung (1977) used the term 'activated state block' for the early component block during depolarization and 'inactivated state block' for the late component. A number of whole-cell studies to demonstrate the blocking actions of local anesthetics on cardiac Na^+ channels were based on this assumption (e.g. review of Hondeghem and Katzung, 1984). However, measurements of the macroscopic currents and analysis of their time courses cannot fix the actual channel states (Aldrich et al., 1983; Berman et al., 1989). For this reason, many studies using single Na^+ channel currents have been reported to characterize the blocking properties of Na^+ channels by class I agents (e.g. Nilius et al., 1987; Grant et al., 1989; Undrovinas et al., 1989), although most of them were carried out on multichannel patches and still provided no information on the closed-channel blockade.

Bisaramil (*syn*-3-ethyl-7-methyl-3,7-diazabicyclo[3.3.1]non-9-yl *p*-chlorobenzoate) mainly depresses the maximum upstroke velocity ($\dot{V}_{max}$) of action potentials

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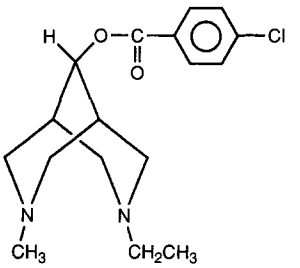

Fig. 1. Chemical structure of bisaramil.

(Sunami et al., 1991b) and it has been proven highly effective in suppressing a variety of experimental arrhythmias (Haruno and Hashimoto, 1993). However, the blocking mechanism of the drug at the level of Na^+ currents remains unclear. On the other hand, bisaramil has a unique physico-chemical property, i.e. despite its form of a tertiary amine with two tertiary amino moieties (Fig. 1), because of a high pK_a of 12.3 at one of the two moieties, it is positively charged (net charge of +1) over a wide range of pH 6–8 (> 99.999%) (Hiraoka et al., 1993). While there have been extensive reports exploring the blocking mechanisms of tertiary ammonium antiarrhythmic agents, the interpretations of the binding site and the sidedness of block are complicated by their existence in an equilibrium mixture of neutral and charged forms at physiological pH (Hille, 1992). From Hille's proposal (Hille, 1977) that charged local anesthetics selectively block open channels through the hydrophilic pathway, bisaramil is expected to be an open channel blocker.

In the present study, we used whole-cell and single channel recordings to determine the actual state of cardiac Na^+ channels required for the blockade by a charged class I agent, bisaramil. We report here that bisaramil reaches the binding site of the Na^+ channels from the outside of the membrane as well as from the inside, and interactions with pre-open closed states might contribute to the activated channel block by the drug. In the text, we used the term 'pre-open closed states' to represent the several closed states in the activation process after depolarization as was done by previous reports (Strichartz and Wang, 1986; Chernoff and Strichartz, 1989; McDonald et al., 1989).

2. Materials and methods

2.1. Myocyte preparation

Single ventricular myocytes from guinea-pig hearts were prepared by an enzymatic dissociation procedure as described previously (Hirano and Hiraoka, 1988).

2.2. Solutions

For whole-cell recordings, the external solution contained (mM): NaCl 10.0, tetramethylammonium chloride 130.0, $CaCl_2$ 1.8, $CoCl_2$ 1.0, CsCl 5.0, $MgCl_2$ 1.2, glucose 11.0, and Hepes 20.0 (pH 7.3, adjusted by tetramethylammonium hydroxide). The pipette solution was composed of (mM): NaF 5.0, CsF 125.0, K_2ATP 5.0, K_2 creatine phosphate 5.0, EGTA 5.0, and Hepes 5.0 (pH 7.2, adjusted with CsOH).

For cell-attached and inside-out patch recordings, the isolated myocytes were superfused with a high-K^+ solution of the following composition (mM): K-aspartate 140.0, NaCl 4.5, $MgCl_2$ 0.5, EGTA 1.0, glucose 5.5, and Hepes 5.0. The pH was adjusted to 7.3 with KOH. This high-K^+ solution was used to depolarize cells to approximately 0 mV. The pipette solution contained (mM): NaCl 140.0, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8, glucose 5.5, and Hepes 5.0; pH was adjusted to 7.3 by adding NaOH.

Bisaramil (bisaramil hydrochloride, Gedeon Richter, Budapest, Hungary) was applied to the bath solution or in the pipette solution as indicated in the text.

2.3. Recording techniques

Recordings were made in whole-cell, cell-attached and inside-out configurations (Hamill et al., 1981) at room temperature (22–25°C) using a patch-clamp amplifier (AXOPATCH-1D, Axon Instruments, Foster City, CA, USA). Details of the recording techniques have been described in our previous reports (Sunami et al., 1991a,1993). In short, at the start of each experiment, the junction potential was set to zero by adjusting the compensation circuit in the bath solution and was checked again after the end of each experiment. If the junction potential of the 2nd determination exceeded the first one by ±2 mV, the value of the membrane potential was corrected accordingly. In whole-cell recordings, we used electrodes with low resistance ranging from 0.3 to 0.6 MΩ to optimize voltage control. Patch pipettes with resistances ranging between 0.4 and 0.8 MΩ were used to record from cell-attached and inside-out patches containing 9–54 channels (macropatch). To record the channel current from cell-attached patches containing only a single active channel, we used small pipettes with resistances ranging between 7 and 10 MΩ. Current signals were stored on a PCM cassette recorder (PC-108M, Sony Tokyo, Japan) or digitized online by a 12-bit resolution Labmaster A/D converter (TecMar Scientific Solutions, Burlingame, CA, USA) under the control of a personal computer (IBM compatible) and stored on a hard disk. Channel currents recorded from the macropatch and single-channel patches were low-pass filtered at 5 and 2 kHz (−3 dB, 4-pole, Bessel), respectively, and digitized at 10 kHz.

2.4. Experimental protocols

To study use-dependent block of whole-cell Na^+ current (I_{Na}) by bisaramil, trains of 50 depolarizing pulses of 5-, 10- and/or 200-ms duration were applied from a

holding potential of −100 mV to −10 mV with an interpulse interval of 500 ms. Use-dependent block was defined as a diminution in I_{Na} at the 50th pulse relative to the first pulse. Bisaramil was applied to the bath solution or in the pipette solution as indicated in the text. In case of bath application of the drug, after an equilibration period of at least 7 min, pulse trains were repeated in control solutions and then in test solutions, in which each pulse train was separated by a recovery interval of 3 min, which was sufficient to permit full recovery from bisaramil block based on our previous report (Sunami et al., 1991b). The bath solution could be replaced completely within 30 s by switching from one solution to another. Drug effects were tested at least 5 min after bath application of the drug. For the internal application of the drug, bisaramil was dissolved in the pipette solution and applied intracellularly by diffusion through the recording suction pipette. Internal effects of the drug were judged at least 30 min after the rupture of the cell membrane.

For cell-attached and inside-out patch recordings, trains of 300 pulses with 40 ms duration were applied from a holding potential of −120 mV to −50 mV at 2 Hz. We started to record channel currents at least 10 min after cell-attached GΩ seal formation. In case of bath application of bisaramil in cell-attached (external application) and inside-out patch recordings (internal application), the usual approach was to obtain the control, bisaramil and then washout data in the same patch. The drug and washout effects were usually judged 10 min after drug application and after washout of the drug, respectively. As another mode of external application of bisaramil, the outside of the membrane was exposed to the drug in the pipette in cell-attached and inside-out patches, and the drug effect was examined at least 10 min after exposure to the drug. In the drug and washout experiments, a quiescent period of 3 min preceded the start of each pulse train.

2.5. Single channel data analysis

The kinetics and conductance properties of the single channel were analyzed with patches containing only one active channel using a software package, pCLAMP 5.5.1 (Axon Instruments) on a computer (IBM 486 compatible). The capacitive transient was partially compensated by analog circuitry, and the residual transient was removed by subtracting the average current from steps to the same potential without openings. Channel openings were detected using a half-amplitude threshold. Evidence that the patch contained only one channel is based upon the absence of observing any double amplitude openings in more than 2000 sweeps. Open time and closed time histograms were obtained from all of the open events and closed intervals between openings, respectively. They could be fitted to a single exponential after exclusion of the first bin (0.1 ms). First latency was measured as the time from the start of depolarization to the first channel opening and its histogram was constructed with bin size of 0.5 ms. The data of first latency were treated as described below. We also had approaches to quantify the drug effect on the clustering of sweeps with or without openings using a 'runs analysis' (Horn et al., 1984). The curve and model fitting procedures were done using a simplex nonlinear least-squares algorithm on a software program SigmaPlot (Jandel Scientific, Sausalito, CA, USA).

For analysis of first latency, the cumulative frequency histogram of the observed first latency was constructed and fitted to a distribution function derived from a three-state model with one open state and two closed states (C_1-C_2-O) (Patlak and Horn, 1982; Kunze et al., 1985):

$$F(t) = 1 + R2\exp(-R1t)/(R1 - R2) - R1\exp(-R2t)/(R1 - R2) \quad (1)$$

where $R1$ and $R2$ are parameters determined by exit rate constants from two closed states, and t is time. The time derivative of $F(t)$ gives its density function as follows:

$$P(t) = -R1R2(\exp(-R1t) - \exp(-R2t))/(R1 - R2) \quad (2)$$

In order to judge the applicability of the fitting, we used a χ^2 distribution test based on Pearson theorem. Following equation

$$p_i = F(t_i) - F(t_{i-1}), i = 1,2,....,n \quad (3)$$

was also used to predict the observed first latency histograms which were plotted in Fig. 8. p_i is the increment of $F(t)$ over a bin and approaches $P(t)$ as bin width decreases.

2.6. General data analysis

All the values were expressed as means ± S.E. Statistical analysis except that specifically described in the text was done using paired t-test or Dunnett's test for the comparison between two groups of mean values, and $P < 0.05$ was considered significant.

3. Results

3.1. Use-dependent block of whole-cell Na^+ current by bisaramil

We examined the use-dependent block of whole-cell Na^+ current (I_{Na}) by bisaramil. In the control condition, application of a train of 50 depolarizing pulses of 10 or 200 ms duration with a diastolic interval of 500 ms produced little or no change in the peak I_{Na} with successive pulses (Table 1). After bath application of bisaramil, I_{Na} was suppressed by successive pulses in a use-dependent manner and a typical example of block development with 5 μM bisaramil is illustrated in Fig. 2 (the peak I_{Na}

Table 1
Effects of pulse durations on use-dependent block (UDB) of whole-cell Na^+ current (I_{Na}) by bisaramil

Drug	Concentration (μM)	Pulse duration (ms)	n	UDB (%)
Control		10	6	4.5±0.7
Control		200	6	3.9±1.0
Bisaramil	5	5	6	44.9±5.7
Bisaramil	5	10	6	43.8±6.0
Bisaramil	5	200	6	42.8±5.9

Trains of 50 depolarizing pulses of various durations (5, 10, 200 ms) were applied from a holding potential of −100 mV to −10 mV at a diastolic interval of 500 ms. Bisaramil was applied to the bath solution. Values are means±S.E. n indicates the number of experiments

at the 50th depolarizing pulse was decreased to 49.4% of the first pulse). The degree of use-dependent block of I_{Na} increased with increasing concentrations of bisaramil (11.1 ± 1.2, 30.8 ± 2.2, 51.0 ± 4.7 and 85.4 ± 5.7% at the concentration of 1, 2, 5 and 10 μM, respectively, $n = 5–6$) and EC_{30} value (the concentration required to produce the degree of use-dependent block of 30%) was 2.0 μM (95% confidence limit, 1.6–2.3 μM). However, a 'steady state' use-dependent block might not be achieved with 50 pulses because the block development by bisaramil was slow (for example, in case of Fig. 2, the onset rate obtained by a single exponential fitting was 0.036 $pulse^{-1}$) as previously reported (Sunami et al., 1991b).

To study the affinity of bisaramil for the 'activated' and 'inactivated channels', we compared the amount of use-dependent block among pulse trains with three different pulse durations (5, 10 and 200 ms) with a constant diastolic interval of 500 ms. The results are summarized in Table 1. Application of 5 μM bisaramil to the bath solution produced a marked use-dependent block of I_{Na} with three test pulse durations. The degrees of use-dependent block at 5, 10 and 200 ms pulse durations were similar and there were no significant differences among these values (Table 1). Here, we assumed that 5 ms pulses predominantly represented the 'activated state' of the channel and prolongation of pulse durations increased the contribution of 'inactivated states' (Hondeghem and Katzung, 1977, 1984). Since bisaramil blocked I_{Na} use dependently to a similar extent for pulses with short (5 ms) duration and those with long (200 ms) duration, the drug was suggested to have a high affinity for the 'activated state' of the channel.

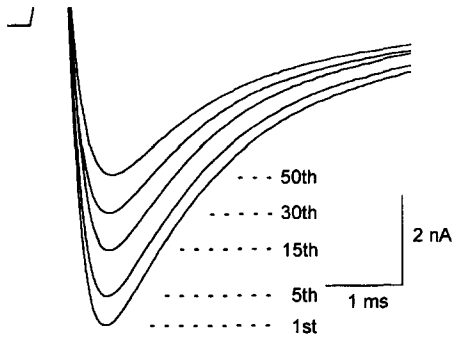

Fig. 2. Superimposed whole-cell Na^+ current (I_{Na}) records at corresponding numbers of the test pulses during a train of 50 pulses in the presence of 5 μM bisaramil in the bath solution. For clarity, only pulses 1, 5, 15, 30, and 50 are shown. A train of 50 depolarizing pulses of 10 ms duration was applied from a holding potential of −100 mV to −10 mV with a diastolic interval of 500 ms.

3.2. Sidedness of block development by bisaramil

Bisaramil molecules are present in their charged form under experimental conditions used (pH 7.3). Therefore, bisaramil is likely to utilize a hydrophilic pathway to approach the binding site of the channel, as proposed by Hille (1977). In nerve tissues, charged local anesthetics are suggested to block I_{Na} by binding to a specific site accessible from the cytoplasmic side of the membrane (Hille, 1977; Schwarz et al., 1977). To examine whether or not this also applies to cardiac cells, we first compared the effects on use-dependent block of I_{Na} with internal or external application of bisaramil using the whole-cell configurations. When 5 μM bisaramil was applied to the pipette solution (internal application), the degree of use-dependent block of I_{Na} was 4.7% of the first pulse I_{Na} and this value was significantly smaller ($P < 0.001$) than that during the exposure to 5 μM bisaramil in the bath solution (external application) (43.8%) (Table 2). With 10 times higher concentration (50 μM) of internally applied bisaramil, the degree of use-dependent block became prominent (38.7%) and there were no significant changes between those by internal 50 μM and external 5 μM bisaramil (Table 2). Here, we cannot simply compare the effectiveness of actions between the external and internal application of bisaramil from the above results, since the intracellular concentration of bisaramil was not known with the pipette application of the drug using whole-cell configurations.

We further examined the sidedness of block development by bisaramil using the cell-attached and inside-out

Table 2
Comparison of use-dependent block (UDB) of whole-cell Na^+ current (I_{Na}) between external and internal application of bisaramil

Drug	Concentration (μM)	Site of application	n	UDB (%)
Control			6	4.5±0.7
Bisaramil	5	External	6	43.8±6.0
Bisaramil	5	Internal	5	4.7±0.9 [a]
Bisaramil	50	Internal	5	38.7±7.1

A train of 50 depolarizing pulses of 10 ms duration was applied from a holding potential of −100 mV to −10 mV at a diastolic interval of 500 ms. Values are means±S.E. n indicates the number of experiments. [a] $P < 0.001$, significantly different from external application of 5 μM bisaramil.

macropatch configurations. A typical example of macropatch Na^+ currents is illustrated in Fig. 3 before and after application of bisaramil to the bath solution. When 10 μM bisaramil was applied to the bath solution in the cell-attached patch (external application), channel activities were decreased and the peak average current reduced to 44.9% of the control (Fig. 3A). After washout of bisaramil, the peak average current was recovered to 69.4% of the control. The blocking effects were also obtained on the inside-out patch when the drug was added at the same concentration to the bath solution (internal application) (Fig. 3B). The peak average current declined to 21.0% of the control. This decline was recovered to 54.8% of the control after washout of bisaramil. The results are summarized in Fig. 4A. In cell-attached recordings, the bath application of bisaramil concentration dependently blocked the macropatch Na^+ currents. On average, 10 μM bisaramil produced 50.3 ± 3.1% ($n = 7$) inhibition of the control in cell-attached macropatch currents (external application). Internal application of 10 μM bisaramil also decreased the inside-out macropatch currents markedly (82.6 ± 1.3% inhibition, $n = 7$) (Fig. 4A). These findings indicate that bisaramil at least enters the cell membrane in spite of the fact that bisaramil molecules are present in the charged form in aqueous solution over a wide range of pH and the drug is effective to block the Na^+ channel current from the inside of the membrane as well as from the outside. Here, we cannot simply compare the blocking degrees of bisaramil applied to the bath solution on cell-attached patches to those on inside-out patches, since gating changes of channels are caused by the replacement of the cytoplasm by the bath solution after excision (Horn and

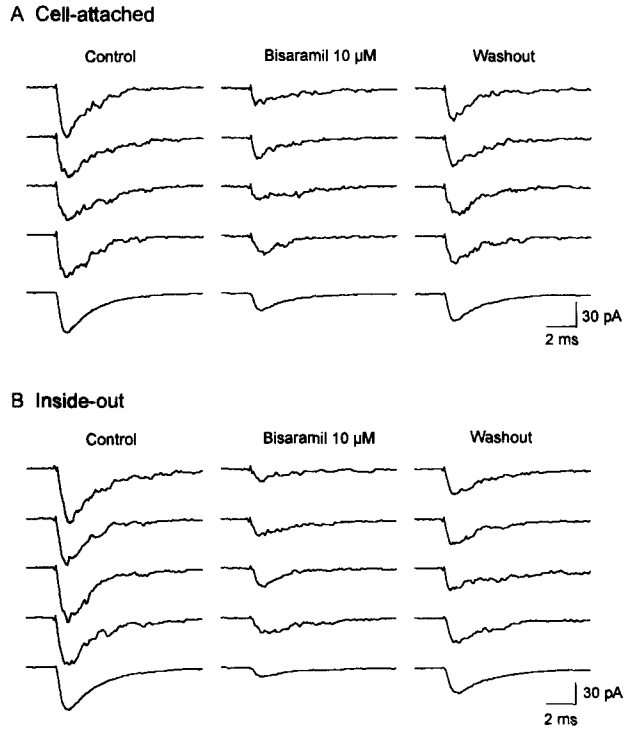

Fig. 3. Effects of bisaramil applied to the bath solution on macropatch Na^+ currents recorded from cell-attached and inside-out patches. Panel A: macropatch currents and ensemble average current records from a cell-attached patch. Currents were elicited by a train of 300 pulses with 40 ms duration. Pulses were applied from a holding potential of −120 mV to −50 mV at 2 Hz. The left, middle and right column show 4 consecutive sweeps (from the top) and an ensemble average current (the bottom) obtained from 300 sweeps before and after application of 10 μM bisaramil to the bath solution, and after washout of bisaramil, respectively. The patch contained 32 channels. Panel B: macropatch currents and ensemble average current records from an inside-out patch. Currents were elicited by the same protocol shown in panel A. The left column shows 4 consecutive sweeps (from the top) and an ensemble average current (the bottom) obtained from 300 sweeps under control condition, the middle those in the presence of 10 μM bisaramil in the bath solution and the right those after washout of bisaramil. The patch was different from that in panel A and contained 54 channels.

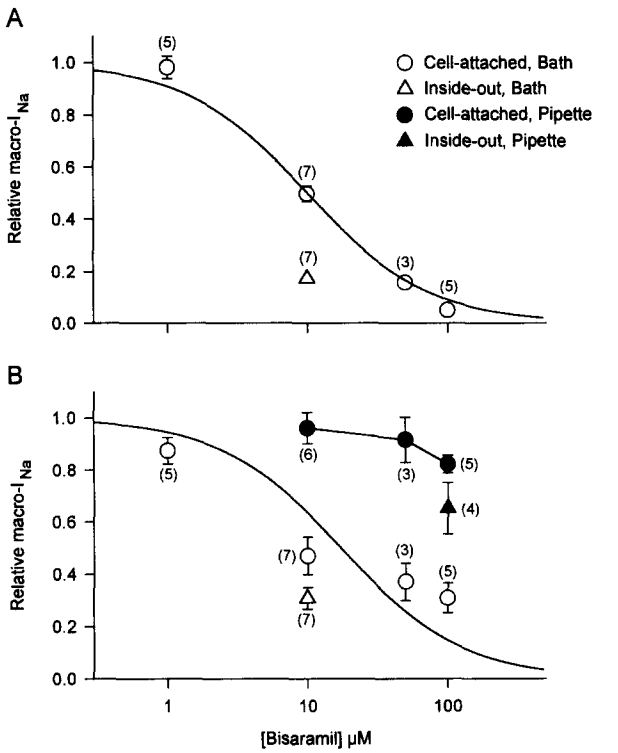

Fig. 4. Concentration-response relations for the block of macropatch Na^+ currents by bisaramil applied to the bath or pipette solution. In panel A, the peak amplitudes of average currents obtained from 300 sweeps in the presence of indicated concentrations of bisaramil were normalized to that in the absence of bisaramil and they were plotted as relative macropatch Na^+ currents (relative macro $-I_{Na}$). In panel B, the peak average currents for the last 10 pulses in a train of 300 pulses were normalized to that for the first 10 pulses in the presence of indicated concentrations of bisaramil and they were plotted as relative macropatch Na^+ currents (relative macro $-I_{Na}$). Continuous lines are fits of a first-order Hill saturation function (relative macro $-I_{Na} = 1/\{1 + ([\text{bisaramil}]/K_d)\}$, where K_d is the dissociation constant and [bisaramil] is the concentration of bisaramil) to the data points (for the bath application of bisaramil in cell-attached configurations, (○) with K_d values of 10.1 (panel A) and 17.3 μM (panel B). Currents were elicited by the same protocol shown in Fig. 3 and bisaramil was applied to the bath (○, △) or to the pipette solution (●, ▲) in cell-attached and inside-out configurations. Numbers in parentheses indicate number of experiments.

Vandenberg, 1986; Kirsch and Brown, 1989). In Fig. 4B, blocking effects of bisaramil applied to the pipette solution are compared to those seen on the bath application for the cell-attached and inside-out patches. Since there were no control data in case of the pipette application of the drug and a quiescent period of 3 min preceded the start of each pulse train, the blocking effectiveness of bath and pipette applications of the drug was judged from 'apparent' use-dependent block, which was determined by a diminution in the peak average current for the last 10 pulses compared to that for the first 10 pulses in a train of 300 pulses. Consequently, the block was greater when the drug was applied to the bath solution in both cell-attached and inside-out experiments.

3.3. Modulation of single channel kinetics by bisaramil

Bisaramil applied to the bath solution concentration dependently blocked macropatch Na^+ currents in cell-attached patches, as described above. Therefore, we tried to clarify the precise mechanism of the Na^+ channel block by bisaramil in cell-attached patches containing only one active channel. Fig. 5 illustrates a typical example of the elementary Na^+ channel currents, before and during the application of 10 μM bisaramil to the bath solution, and after washout of the drug. Although 10 μM bisaramil decreased the peak average current by 51.0 ± 5.6% ($n = 5$) in single active channel patches, the unitary current amplitude was not changed (control vs. 10 μM bisaramil; 1.55 ± 0.01 vs. 1.55 ± 0.01 pA, $n = 5$). Therefore, we first examined the effect of bisaramil on open times. A typical example of the distribution of the channel open time at −50 mV is given in Fig. 5. The open time histograms during the control, exposure to 10 μM bisaramil and the washout were best expressed by a single exponential distribution with time constants of 0.66, 0.64 and 0.65 ms, respectively. In five patches, the mean open time was 0.66 ± 0.09 ms in the control and 0.65 ± 0.08 ms in the presence of 10 μM bisaramil, and there were no significant changes between the two values.

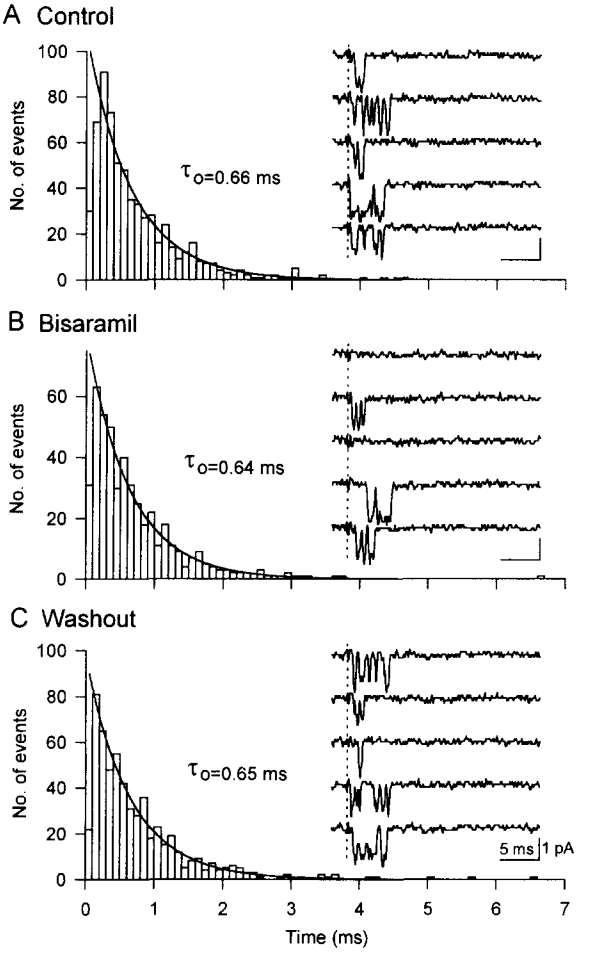

Fig. 5. Effects of bisaramil on the open time of the Na^+ channel currents recorded from a cell-attached patch with a single active channel. Panel A shows the open time histogram and 5 consecutive sweeps of single channel currents (a dotted line: the start of depolarizing pulse) in the control condition, panel B those in the presence of 10 μM bisaramil applied to the bath solution and panel C those after washout of bisaramil. The time constants (τ_o) of the open time were obtained from single exponential fits to the data for all the cases. Currents were elicited by a train of 300 pulses with 40 ms duration. Pulses were applied from a holding potential of −120 mV to −50 mV at 2 Hz.

The effect of bisaramil on the closed time is shown in Fig. 6. Closed times were measured as closed intervals between openings. The closed time distributions were fitted to a single exponential with a time constant of 0.47 ms in the control, 0.44 ms with 10 μM bisaramil and 0.44 ms after washout of the drug. On average, the closed time constants in the absence and presence of bisaramil were 0.43 ± 0.04 and 0.37 ± 0.03 ms ($n = 5$), respectively and these two values were not significantly different.

In the presence of bisaramil, sweeps without channel openings (null sweeps) were frequently seen. Therefore, we examined whether bisaramil block was associated with increased numbers of nulls. Fig. 7 shows a typical example of the number of openings against the sequence of the sweeps. After application of 10 μM bisaramil, the number of nulls increased from 62 to 135 sweeps in 300 depolarizing steps and the total number of openings decreased from 585 to 387. These changes were confirmed in four other patches. On average, the number of nulls significantly increased from 84 ± 9 (control, $n = 5$) to 152 ± 15 sweeps (10 μM bisaramil) ($P < 0.01$) in 300 sweeps and that of the current-containing sweeps (active sweeps) decreased by 31.4 ± 6.2%. Consequently, the total number of openings significantly decreased from 507 ± 60 (control, $n = 5$) to 342 ± 49 (10 μM bisaramil) ($P < 0.01$). On the other hand, there were no differences in the number of openings per active sweep (control vs. 10 μM bisaramil; 2.3 ± 0.2 vs. 2.3 ± 0.2 openings, $n = 5$).

Since null sweeps seemed to appear in clusters in Fig. 7B, the degree of grouping of active sweeps and null

sweeps was examined using a 'runs analysis' (Horn et al., 1984). In the control of Fig. 7, a 'runs analysis' revealed no clustering, and null and active sweeps tended to appear alternately. On the other hand, 10 μM bisaramil induced significant ($P < 0.001$) nonrandom clustering of null sweeps (Fig. 7B). In all five patches we tried, after application of 10 μM bisaramil, null sweeps appeared in a significant ($P < 0.001$) nonrandom manner, whereas significant clustering of null sweeps was not observed in the control, which was similar to the previous reports using patches with mostly more than one functioning Na^+ channel (Kohlhardt and Fichtner, 1988; Undrovinas et al., 1989). In this way, bisaramil block was associated with a decrease in the number of open events based on the induction of nonrandom clustering of null sweeps although it was not a sole cause for a decrease in the peak open probability by the drug as described below. Since clustering of null sweeps with bisaramil obtained from single active channel patches seems to reflect the slow cycling of channels between drug-free and drug-bound states, the cluster times may be a useful parameter for estimation of the rate of drug binding. Such an evaluation was not obtained because of the limited number of sweeps (300 sweeps).

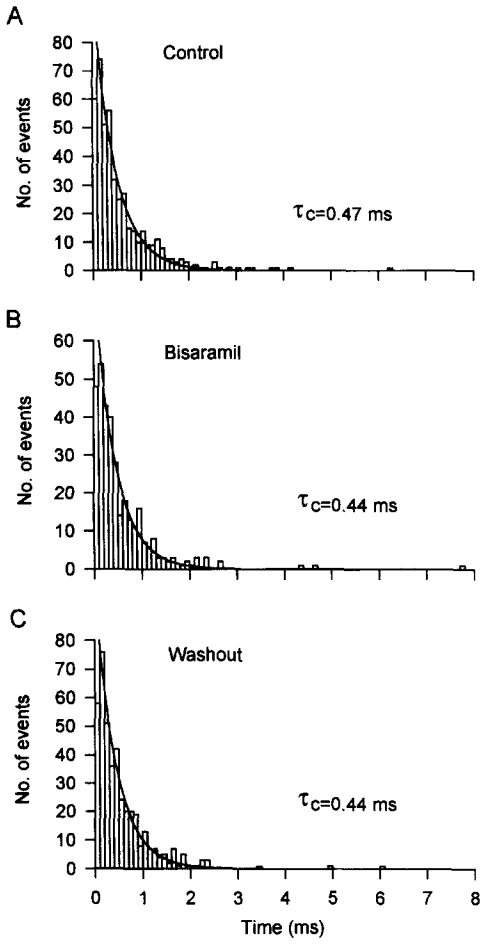

Fig. 6. Effects of bisaramil on the closed time of the Na^+ channel currents recorded from a cell-attached patch with a single active channel. Closed times were measured as intervals between openings. Panel A shows the closed time histogram in the control condition, panel B that in the presence of 10 μM bisaramil applied to the bath solution and panel C that after washout of bisaramil. The time constants (τ_c) of the closed time were obtained from single exponential fits to the data for all the cases. The data were obtained from the same experiment shown in Fig. 5.

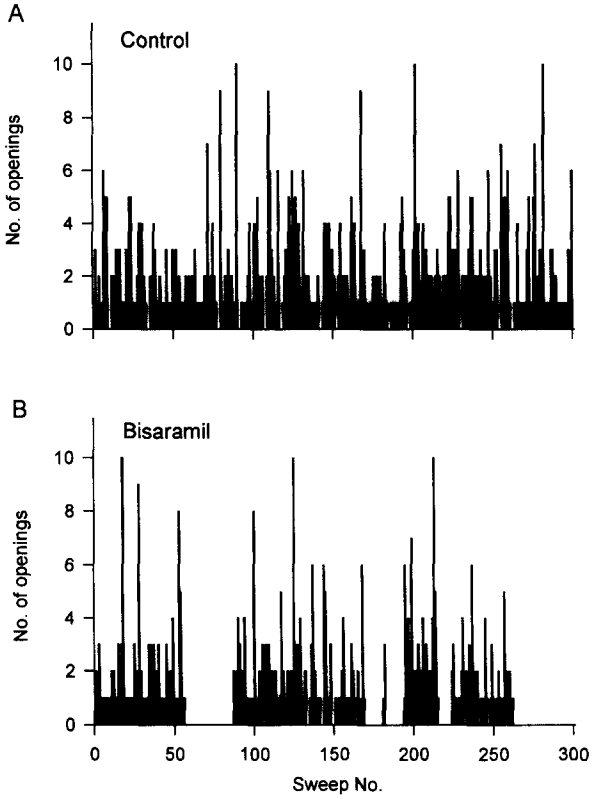

Fig. 7. Effects of bisaramil on null sweeps of the channel currents. The numbers of openings are plotted against the sequence of the sweeps before (panel A) and after application of 10 μM bisaramil to the bath solution (panel B). The data were obtained from a cell-attached single active channel patch different from that shown in Figs. 5 and 6. Openings were elicited by the same protocol shown in Fig. 5.

Here, it should be noted that if a decrease in the number of open events alone based on increasing null sweeps causes bisaramil block, the degrees of decreasing the peak average currents and the number of active sweeps are expected to be similar because no change in the number of openings per active sweep was observed before and after application of the drug. However, the observation that 10 μM bisaramil decreased the peak average current by 51% and the number of active sweeps by 31% was inconsistent with the above expectation. Therefore, the effects of bisaramil on the fast gating process were further analyzed with first latencies. In the presence of 10 μM bisaramil, the mean values of first latencies were significantly increased from 2.68 ± 1.41 to 2.98 ± 1.49 ms ($n = 5$, $P < 0.05$). Reversibility of this effect on washout was confirmed in three experiments we completed. We further quantified the effects of bisaramil on the first latencies by

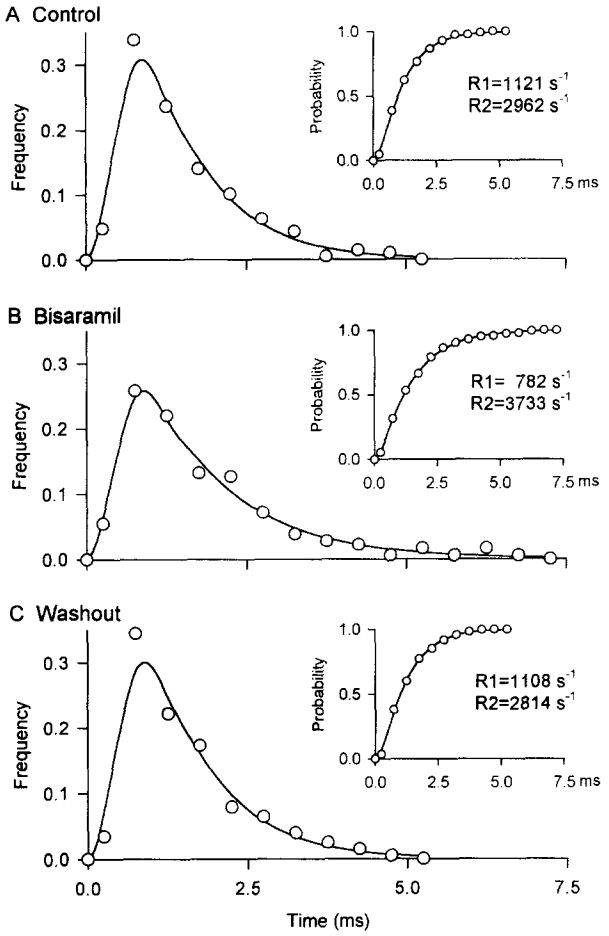

Fig. 8. Effects of bisaramil on the first latency of the Na^+ channel currents recorded from a cell-attached single active channel patch. The histograms of the first latency and the cumulative first latency (top corner) are shown in the absence (panel A) and presence of 10 μM bisaramil in the bath solution (panel B), and after washout of bisaramil (panel C). The smooth curve in each first latency histogram is p_i curve (Eq. 3) derived from the distribution function, $F(t)$ (Eq. 1). The cumulative frequency of the observed first latency is fitted to the distribution function, $F(t)$ using a three-state model with one open state and two closed states (C_1-C_2-O) (Patlak and Horn, 1982) and two parameters ($R1$, $R2$) determined by exit rate constants from two closed states are indicated. The data were obtained from the same protocol as that shown in Fig. 5.

applying a three-state model with one open state and two closed states (Patlak and Horn, 1982), which is a model of Na^+ channel activation. In Fig. 8, control, bisaramil and washout data of cumulative first latency were fitted to the distribution function, $F(t)$ (Eq. 1) (Kunze et al., 1985). In the control, two parameters ($R1$ and $R2$, parameters determined by exit rate constants from two closed states) were 1121 and 2962 s^{-1}. After application of 10 μM bisaramil to the bath solution, the value of $R1$ decreased to 782 s^{-1} and $R2$ increased to 3733 s^{-1}. In four patches well fitted with a three-state model, $R1$ decreased from 1456 ± 229 to 983 ± 105 s^{-1} (not significant) and $R2$ increased from 3895 ± 608 to 4947 ± 630 s^{-1} ($P < 0.05$) by 10 μM bisaramil. After washout, $R1$ and $R2$ values were restored close to the control values (1108 and 2814 s^{-1}, respectively) (Fig. 8C). Such changes by bisaramil implied the significant effects of bisaramil on closed states and the following changes were caused in the first latency distributions. (i) 15.8 ± 3.6% ($n = 4$) decrease in the peak value of $P(t)$ (Eq. 2), which was calculated using the values of time to peak (T_{peak}) obtained from Eq. (4); (ii) 4.7 ± 1.2% increase in probability of observing first openings after T_{peak}, which was presented by $1 - F(T_{peak})$ (see Eq. 1), or the area under the $P(t)$ curve after T_{peak}; (iii) no change (0.1 ± 0.3%) in T_{peak}, which was calculated by

$$T_{peak} = (\ln R1 - \ln R2)/(R1 - R2) \quad (4)$$

From these analyses of first latency distribution, it seems likely that bisaramil interacts with the closed states of the channel in an activation process (pre-open closed states), which in part contributes to the reduction of macroscopic Na^+ current, but has no influence on the time to peak of Na^+ current. In this way, bisaramil blocked the cardiac Na^+ channels with an increase in the first latency as well as with a decrease in the number of open events based on increasing null sweeps.

4. Discussion

The present study demonstrated that a charged class I antiarrhythmic agent, bisaramil, interacted with cardiac Na^+ channels in the 'activated state' having access from the outside of the membrane as well as from the inside. The pre-open closed states might be required for the 'activated channel block' by the drug.

In the state-dependent models of block, the whole-cell measurement of the 'activated channel block' could be judged by imposing short depolarization pulses with sufficient block development without further increase in the longer pulses (Hondeghem and Katzung, 1984). We used this criterion and found that bisaramil was judged as an 'activated channel' blocker. However, the time course of the macroscopic current does not represent the period of the actual channel states (Aldrich et al., 1983; Berman et al., 1989). While the 'activated channel block' includes the blockade of pre-open closed and open states with an assumption of minimal contamination of inactivation block, analysis of macroscopic currents alone cannot separate both kinds of blockage. Here, we used the term 'pre-open closed states' to represent the several closed states in the activation process after depolarization as was done by previous reports (Strichartz and Wang, 1986; Chernoff and Strichartz, 1989; McDonald et al., 1989). Therefore, the pre-open closed state is not necessarily consistent with the closed state during the holding potential, the so-called rested (closed) state in macroscopic current experiments. If the drug has a higher affinity for the closed state prior to the open state after depolarization different from that

during the holding potential, we will see the use-dependent block of whole-cell Na^+ current, which will be regarded as 'activated channel block' from the macroscopic current experiments.

To examine whether the block occurs during the states preceding the channel opening (pre-open closed states) or during the open state, we further analyzed single channel data obtained from patches containing only one active channel. Consequently, bisaramil increased null sweeps with clustering and decreased the total number of open events, whereas the number of openings per active sweep was not affected, which was similar to a previous report (Kohlhardt and Fichtner, 1988). In addition, first latencies were reversibly increased with bisaramil and this effect as well as an increasing effect of null sweeps contributed to the reduction of macroscopic Na^+ current. Further, analysis of first latency using C_1-C_2-O model (Patlak and Horn, 1982), which is a model of Na^+ channel activation demonstrated that bisaramil certainly affected exit rate constants from either one or two pre-open closed states. However, for an increase of first latencies we could not completely exclude the possibility of the drug interaction with the closed state during the holding potential and the drug-bound conducting channels with different gating kinetics from drug-free channels (McDonald et al., 1989; Baumgarten et al., 1991). On the other hand, open times were not changed by the drug and we had no evidence for the drug interaction with the open state.

From the observation that bisaramil applied to the bath solution concentration dependently and reversibly blocked macropatch Na^+ currents in cell-attached patches, it was clear that bisaramil at least entered the cell membrane despite its being a charged form in aqueous solution (> 99.999% at pH 7.3). Consistent with this macropatch experiment, the bath application of the drug reversibly caused the kinetic change of the channel in cell-attached patches containing only one active channel. Alpert et al. (1989) reported that a permanently charged lidocaine analogue, QX-314 (2-triethylamino-*N*-[2,6-dimethylphenyl]-acetamide) blocked cardiac whole-cell Na^+ current not only when applied from the inside but also from the outside. This result implied either that an external binding site for QX-314 existed in the cardiac Na^+ channel, or that QX-314 permeated the membrane and reached the binding site accessible from the cytoplasmic side of the membrane. Similarly, Maestrone et al. (1994) showed that a quaternary ammonium compound, pancuronium blocked the whole-cell Na^+ current when externally applied in the dorsal root ganglion sensory neurones and suggested that pancuronium might reach its binding site on the closed channel through the hydrophobic pathway. In general, it is believed that charged molecules cannot cross the lipid membrane easily. Therefore, bisaramil might first interact with negatively charged groups on the membrane compositions such as membrane proteins and positive charge on the drug might be neutralized, which will facilitate the drug permeation in the membrane. On the other hand, Rhodes et al. (1985) suggested that charged forms of dihydropyridines with high partition coefficients might follow a membrane pathway of cardiac sarcolemmal membrane to gain access to the Ca^{2+} channel binding site. According to this view, if bisaramil has a high lipophilicity, the drug might permeate the membrane. Since a lipophilicity as well as a pK_a of the drug is an important determinant of which pathway the drug utilizes to approach the channel receptor, a hydrophilic or a hydrophobic pathway, the partition coefficient of the drug remains to be determined.

It has been known that external Na^+ concentration ($[Na^+]_o$) affects the blocking actions of local anesthetics (Cahalan and Almers, 1979; Barber et al., 1992; Gingrich et al., 1993; Ono et al., 1996). Since we used the low concentration of Na^+ ($[Na^+]_o = 10$ mM) to achieve voltage control in whole-cell experiments, we could not simply compare the blocking degrees of the drug in whole-cell recording to those in cell-attached and inside-out recordings, in which $[Na^+]_o$ of 140 mM was used. In addition to this $[Na^+]_o$ effect, since channel gating changes with the replacement of the cytoplasm by the bath solution after excision (Horn and Vandenberg, 1986; Kirsch and Brown, 1989), we did not compare the blocking degrees for different configurations, but the blocking effectiveness of bath and pipette application of the drug was compared with each other for the same configuration. Consequently, the pipette application of the drug always caused a smaller amount of block than the bath application regardless of the kind of configurations, that is, whole-cell, cell-attached and inside-out configurations. This might be consistently explained by the finding that bisaramil enters the cell membrane. If the drug permeates the membrane, the drug concentration might be lower on both sides of the cell or patch membrane when applied in the pipette because the cell and the bath act as a large sink for the drug in the pipette (Hille, 1977; Alpert et al., 1989) and the gradual decrease of drug concentration may occur on both sides of the membrane.

As discussed above, bisaramil seems to interact with the pre-open closed states of the channel despite its molecule being a charged form, which is contradictory to Hille's proposal in nerve that charged local anesthetics selectively block open channels (Hille, 1977). Courtney (1988) has suggested in his molecular modeling studies that tertiary amine Na^+ channel blockers with an end-on cross-sectional area (XY dimension) of larger than 45 $Å^2$ are open channel blockers. Actually, disopyramide having an XY dimension of 47 $Å^2$ produced flickering block and decreased the mean open time of unmodified channels (Grant et al., 1993). Consistent with this observation, bisaramil having a smaller XY dimension of 40 $Å^2$ (our observation using an atom model) did not reduce the open times. Bisaramil with a maximum X dimension of 6.2 Å, however, was expected to be an open channel blocker from

another criteria of Courtney (1988) suggesting that drugs with a maximum X dimension exceeding 5 Å are open channel blockers. Considering no evidence for the drug interactions with open states, not only the drug size but also other physico-chemical properties of the drug such as a pK_a and a partition coefficient might need to be taken into account. In addition to the size and the lipid solubility of the drug, we need to consider stereoselective interactions between the receptor and the drug, although no studies have yet been done.

In conclusion, a charged tertiary amine, bisaramil, interacts with cardiac Na^+ channels in the 'activated state' and increases first latencies as well as null sweeps. The pre-open closed states of the channels might be required for the 'activated channel blockade' by the drug.

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References

Aldrich, R.W., D.P. Corey and C.F. Stevens, 1983, A reinterpretation of mammalian sodium channel gating based on single channel recording, Nature 306, 436.

Alpert, L.A., H.A. Fozzard, D.A. Hanck and J.C. Makielski, 1989, Is there a second external lidocaine binding site on mammalian cardiac cells?, Am. J. Physiol. 257, H79.

Barber, M.J., D.J. Wendt, C.F. Starmer and A.O. Grant, 1992, Blockade of cardiac sodium channels: competition between the permeant ion and antiarrhythmic drugs, J. Clin. Invest. 90, 368.

Baumgarten, C.M., J.C. Makielski and H.A. Fozzard, 1991, External site for local anesthetic block of cardiac Na^+ channels, J. Mol. Cell. Cardiol. 23 (Suppl. I), 85.

Berman, M.F., J.S. Camardo, R.B. Robinson and S.A. Siegelbaum, 1989, Single sodium channels from canine ventricular myocytes: voltage dependence and relative rates of activation and inactivation, J. Physiol. (London) 415, 503.

Cahalan, M.D. and W. Almers, 1979, Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin, Biophys. J. 27, 39.

Chernoff, D.M. and G.R. Strichartz, 1989, Binding kinetics of local anesthetics to closed and open sodium channels during phasic inhibition: relevance to antiarrhythmic actions, in: Molecular and Cellular Mechanisms of Antiarrhythmic Agents, ed. L. Hondeghem (Futura Publishing Company, New York) p. 307.

Courtney, K.R., 1988, Why do some drugs preferentially block open sodium channels?, J. Mol. Cell. Cardiol. 20, 461.

Gingrich, K.J., D. Beardsley and D.T. Yue, 1993, Ultra-deep blockade of Na^+ channels by a quaternary ammonium ion: catalysis by a transition-intermediate state?, J. Physiol. (London) 471, 319.

Grant, A.O., M.A. Dietz, F.R. III Gilliam and C.F. Starmer, 1989, Blockade of cardiac sodium channels by lidocaine: single-channel analysis, Circ. Res. 65, 1247.

Grant, A.O., D.J. Wendt, Y. Zilberter and C.F. Starmer, 1993, Kinetics of interaction of disopyramide with the cardiac sodium channel: fast dissociation from open channels at normal rest potentials, J. Membr. Biol. 136, 199.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann and F.J. Sigworth, 1981, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, Pflüger's Arch. 391, 85.

Haruno, A. and K. Hashimoto, 1993, Antiarrhythmic effects of bisaramil in canine models of ventricular arrhythmia, Eur. J. Pharmacol. 233, 1.

Hille, B., 1977, Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction, J. Gen. Physiol. 69, 497.

Hille, B., 1992, Mechanisms of block, in: Ionic Channels of Excitable Membranes (Sinauer, Sunderland, MA) p. 390.

Hirano, Y. and M. Hiraoka, 1988, Barium-induced automatic activity in isolated ventricular myocytes from guinea-pig hearts, J. Physiol. (London) 395, 455.

Hiraoka, M., A. Sunami and K. Tajima, 1993, Bisaramil, a new class I antiarrhythmic agent, Cardiovasc. Drug Rev. 11, 516.

Hondeghem, L.M. and B.G. Katzung, 1977, Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels, Biochim. Biophys. Acta 472, 373.

Hondeghem, L.M. and B.G. Katzung, 1984, Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs, Annu. Rev. Pharmacol. Toxicol. 24, 387.

Horn, R. and C.A. Vandenberg, 1986, Inactivation of single sodium channels, in: Ion Channels in Neural Membranes, ed. J.M. Ritchie, R.D. Keynes and L. Bolis (Alan R. Liss., New York) p. 71.

Horn, R., C.A. Vandenberg and K. Lange, 1984, Statistical analysis of single sodium channels: effects of *N*-bromoacetamide, Biophys. J. 45, 323.

Kirsch, G.E. and A.M. Brown, 1989, Kinetic properties of single sodium channels in rat heart and rat brain, J. Gen. Physiol. 93, 85.

Kohlhardt, M. and H. Fichtner, 1988, Block of single cardiac Na^+ channels by antiarrhythmic drugs: the effect of amiodarone, propafenone and diprafenone, J. Membr. Biol. 102, 105.

Kunze, D.L., A.E. Lacerda, D.L. Wilson and A.M. Brown, 1985, Cardiac Na currents and the inactivating, reopening, and waiting properties of single cardiac Na channels, J. Gen. Physiol. 86, 691.

Maestrone, E., V. Magnelli, M. Nobile and C. Usai, 1994, Extracellular pancuronium affects sodium current in chick embryo sensory neurones, Br. J. Pharmacol. 111, 283.

McDonald, T.V., K.R. Courtney and W.T. Clusin, 1989, Use-dependent block of single sodium channels by lidocaine in guinea pig ventricular myocytes, Biophys. J. 55, 1261.

Nilius, B., K. Benndorf and F. Markwardt, 1987, Effects of lidocaine on single cardiac sodium channels, J. Mol. Cell. Cardiol. 19, 865.

Ono, M., A. Sunami and M. Hiraoka, 1996, Interaction between external Na^+ and mexiletine on Na^+ channel in guinea-pig ventricular myocytes, Pflüger's Arch. 431, 101.

Patlak, J. and R. Horn, 1982, Effects of *N*-bromoacetamide on single sodium channel currents in excised membrane patches, J. Gen. Physiol. 79, 333.

Rhodes, D.G., J.G. Sarmiento and L.G. Herbette, 1985, Kinetics of binding of membrane-active drugs to receptor sites: diffusion-limited rates for a membrane bilayer approach of 1,4-dihydropyridine calcium channel antagonists to their active site, Mol. Pharmacol. 27, 612.

Schwarz, W., P.T. Palade and B. Hille, 1977, Local anesthetics: effect of pH on use-dependent block of sodium channels in frog muscle, Biophys. J. 20, 343.

Strichartz, G.R. and G.K. Wang, 1986, The kinetic basis for phasic local anesthetic blockade of neuronal sodium channels, in: Molecular and Cellular Mechanisms of Anesthetics, eds. S.H. Roth and K.W. Miller (Plenum Medical Book Company, New York) p. 217.

Sunami, A., Z. Fan, J. Nitta and M. Hiraoka, 1991a, Two components of use-dependent block of Na^+ current by disopyramide and lidocaine in guinea pig ventricular myocytes, Circ. Res. 68, 653.

Sunami, A., T. Sawanobori, H. Adaniya and M. Hiraoka, 1991b, Electrophysiological properties of a new antiarrhythmic agent, bisaramil on guinea-pig, rabbit and canine cardiac preparations, Naunyn-Schmiedeberg's Arch. Pharmacol. 344, 323.

Sunami, A., Z. Fan, T. Sawanobori and M. Hiraoka, 1993, Use-dependent block of Na^+ currents by mexiletine at the single channel level in guinea-pig ventricular myocytes, Br. J. Pharmacol. 110, 183.

Undrovinas, A.I., N.A. Burnashev, V.V. Nesterenko, J.C. Makielski, I.A. Fleidervish, H.A. Fozzard and L.V. Rosenshtraukh, 1989, Single channel sodium current in rat cardiomyocytes: use-dependent block by ethacizin, J. Pharmacol. Exp. Ther. 248, 1138.