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Effects of bisaramil, a novel class I antiarrhythmic agent, on heart, skeletal muscle and brain Na^+ channels

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Abstract

The effects of bisaramil, a novel diazabicyclononane antiarrhythmic agent, were compared to those of lidocaine, a clinically used class Ib antiarrhythmic agent, on heart, skeletal muscle and brain Na^+ channels expressed in *Xenopus laevis* oocytes using a two-electrode voltage clamp. Both bisaramil and lidocaine produced a concentration-dependent tonic block of Na^+ current that was most effective on cardiac channels, but bisaramil was more potent than lidocaine. Both drugs produced a concentration-dependent shift in the voltage-dependence of inactivation and delayed recovery from inactivation. Bisaramil produced marked frequency-dependent block of heart channels and mild frequency-dependent block of skeletal muscle and brain channels, whereas lidocaine produced marked frequency-dependent block of all three channel types. Therefore, bisaramil shows tonic and frequency-dependent blockade that is most potent against the heart Na^+ channel, which may account for its potent antiarrhythmic efficacy in vivo, and may result in reduced central nervous system toxicity compared to clinically used agents such as lidocaine. © 1998 Elsevier Science B.V.

Keywords: Bisaramil; Lidocaine; Antiarrhythmic class I; Xenopus oocyte; Na^+ channel

1. Introduction

The function of voltage-gated Na^+ channels is to mediate a rapid membrane depolarization during the early phase of an action potential in excitable cells. The propagating action potential may behave as a wave of electrical excitation across myocardial tissue resulting in contraction of the heart, or proceed down axons of nerves resulting in contraction of striated muscle or synaptic transmission. The diverse functions associated with Na^+ channels within various tissues and the differences in pharmacological properties of Na^+ currents have provided evidence for distinct channel subtypes. Molecular biological studies have demonstrated the existence of at least 8 unique but homologous mammalian Na^+ channel α subunits encoded by distinct genes (reviewed by Goldin, 1995). The α subunit is the primary, pore-forming subunit of the channel, although the functional properties are modified by accessory β subunits (Noda et al., 1986; Isom et al., 1992; Goldin, 1994).

The Na^+ channel can exist in resting (closed), open (active) and inactive (non-conducting) states, between which transitions are both voltage- and time-dependent (Hodgkin and Huxley, 1952). Local anesthetic and antiarrhythmic drugs such as lidocaine have been shown to interact differentially with each of the states of the channel (reviewed by Strichartz et al., 1987). Hille (1977) proposed a Modulated Receptor Hypothesis for the state-dependent interaction of local anesthetics with the neuronal Na^+ channel. Concurrently, Hondeghem and Katzung (1977) proposed a similar model for the interaction of antiarrhythmic drugs with the cardiac Na^+ channel. Both models suggest that the affinity of the drug is greatest for the open and inactive states of the channel. This specificity is of fundamental importance for the drug treatment of cardiac arrhythmias.

Lidocaine is a local anesthetic that is used in the clinical setting to suppress ventricular arrhythmias. It is both a tonic and use-dependent blocker of cardiac Na^+ currents, and is classified as a Ib antiarrhythmic agent according to Vaughan Williams (1984). Lidocaine has been shown to be relatively more selective for cardiac Na^+ currents than for neuronal or skeletal muscle currents (Bean et al., 1983).

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Initial studies suggested that the cardiac selectivity resulted from increased affinity of the drug for the inactive state of the channel (Bean et al., 1983; Sanchez-Chapula et al., 1983; Matsubara et al., 1987), which occurs more frequently in cardiac muscle because of the long duration (~300 ms) of the cardiac action potential. However, recent studies examining drug interactions with Na^+ channel isoforms suggest that the cardiac selectivity may result from intrinsic differences in the affinity of lidocaine for the different α subunits of the channel (Nuss et al., 1995; Wang et al., 1996).

Bisaramil is a novel, heterobicyclic compound similar in structure to the plant alkaloid, sparteine (Hiraoka et al., 1993). Bisaramil has been shown to effectively suppress experimental ischemic (Paroczai et al., 1990; Haruno and Hashimoto, 1995) and reperfusion (Paroczai et al., 1996) arrhythmias. Bisaramil decreased the maximum upstroke velocity (V_{max}) of the action potential in isolated papillary muscle (Sunami et al., 1991), and it produced a direct concentration-dependent block of Na^+ current in isolated rat myocytes (Pugsley and Saint, 1995). Pugsley and Saint (1995) showed that bisaramil produced both tonic and marked use-dependent block of cardiac Na^+ currents in isolated myocytes. Thus, the antiarrhythmic activity of bisaramil may result from its ability to reduce the excitability of cardiac cells by inhibition of Na^+ currents. Sunami and Hiraoka (1996) have recently proposed that bisaramil block of cardiac Na^+ currents may result from its interaction with an active but closed state of the channel.

The potential use of bisaramil for therapeutic purposes has been examined in both phase I and II clinical trials (reviewed by Hiraoka et al., 1993). The phase I clinical study examined the pharmacokinetic and safety profile of bisaramil in healthy volunteers. The drug was administered orally as a single dose in the range of 12.5–150 mg. The results demonstrated that bisaramil, with a half-life of 11 h, produced no significant side effects. When the highest dose (150 mg) of bisaramil was administered, the P-Q, QRS, and Q-T_c intervals of the electrocardiogram were prolonged. The phase II study examined the effectiveness of bisaramil to treat tachyarrhythmias. The results of that study demonstrated a 76% reduction in the incidence of premature ventricular contractions and a 75% improvement rate for paroxysmal supraventricular tachycardias at the highest dose examined (150 mg/day). Thus, bisaramil has the potential to be a safe and effective antiarrhythmic drug for the treatment of supraventricular arrhythmias in humans.

In this study, we characterize the electrophysiological actions of bisaramil on rat cardiac (rH1), skeletal muscle (rSkM1) and brain (rBIIA) Na^+ channels expressed in *Xenopus* oocytes. We show that bisaramil blocks the heart Na^+ current in a tonic- and frequency-dependent manner with greater efficacy than it blocks either the skeletal muscle or neuronal Na^+ channels.

2. Materials and methods

2.1. Transcription of RNA and expression in Xenopus oocytes

The plasmid pVA2580 contains the coding region for the rat brain IIA (rBIIA) Na^+ channel α subunit (Auld et al., 1990), pSkM2 contains the coding region for the rat cardiac (rH1) Na^+ channel α subunit (Kallen et al., 1990), and μ1 contains the coding region for the rat skeletal muscle (rSkM1) α subunit (Trimmer et al., 1989). Plasmid DNA was linearized by digestion with Not I (rBIIA and rSkM1) or Ase I (rH1), and RNA transcripts were synthesized using the message machine T7 (rBIIA and rSkM1) or SP6 (rH1) RNA polymerase transcription kit (Ambion, Austin, TX). Stage V oocytes were obtained from adult female *Xenopus laevis* frogs, defolliculated with collagenase and injected with 50 nl of in vitro transcribed RNA at a concentration to obtain current amplitudes between 1 and 4 μA, as previously described (Goldin and Sumikawa, 1992). The rBIIA and rSkM1 RNA were co-injected with β_1 RNA transcribed in vitro from a plasmid encoding the rat β_1 subunit (Isom et al., 1992). The oocytes were incubated for 48 h at 20°C in ND96 (96 mM NaCl; 2 mM KCl; 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.5) with 0.1 mg/ml gentamicin, 0.5 mM theophylline and 0.55 mg/ml pyruvate.

All experiments were performed according to guidelines established by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Solutions and drugs

All oocyte experiments were performed at room temperature (20–22°C) in ND96 bath solution. Bisaramil (3-methyl, 7-ethyl, 9-α-(4-chlorobenzoyloxy)-3,7-diazabicyclo[3.3.1]-nonane HCl, a gift from The Chemical Works of Gedeon Richter, Budapest, Hungary) and lidocaine (Sigma Chemical, St. Louis) were solubilized in distilled water as 10 mM stock solutions prior to dilution to the final concentrations in the ND96 bath solution. Bisaramil was used at concentrations ranging from 1 to 1000 μM, and lidocaine was used at concentrations ranging from 1 to 10 000 μM. A low volume (0.75 ml) plexiglass recording bath allowed for efficient exchange (20–40 s) between control and drug solutions from gravity-flow reservoirs. A suction device ensured continuous perfusion at a flow rate of 1–2 ml/min and maintained a constant fluid level.

2.3. Data recording and analysis

Recording electrodes were prepared from borosilicate glass using a two-stage P-87 puller (Sutter Instrument, Novato). Microelectrodes were filled with filtered 3 M KCl/0.5% low melting point agarose and had resistances

between 0.5 and 1.0 MΩ. Currents were recorded using a virtual ground circuit, and the data were filtered at 3 kHz on-line and digitized at a sampling frequency of 12.5 kHz. Currents were recorded and analyzed using pCLAMP 6.0.3 software (Axon Instruments, Foster City, CA). Capacitance transients and leak currents were corrected by P/4 subtraction with the depolarizations for subtraction applied after each protocol. Non-linear curve fitting was performed using SigmaPlot® (version 4.0, Jandel Scientific, San Rafael, CA). Data are shown as the mean ± standard deviation for n experiments. Statistical analyses were performed using SigmaStat® statistical software (Jandel Scientific), with P less than 0.05 being considered statistically significant.

Concentration-response curves for bisaramil and lidocaine were determined by measuring peak inward current for cells depolarized from −100 mV (for rSkM1 and rBIIA) or −120 mV (for rH1) to −10 mV in the absence and presence of either bisaramil (1–1000 μM) or lidocaine (1–10 000 μM). The more negative holding potential (−120 mV) was necessary for recording from the rH1 channel to allow for complete recovery from slow inactivation (Pugsley and Goldin, unpublished data). Currents were allowed to recover from slow inactivation for 10 min before beginning any electrophysiological protocol. Drugs were then perfused for 5 min into the bath before recording current. The resulting fractional block of Na^+ current by each drug at each concentration examined was plotted against the log concentration of drug, and fitted with the Hill equation, $I_{Na} = [1 + ([A]/EC_{50})^n]^{-1}$. In this equation, I_{Na} is the fractional block of Na^+ current, [A] is the concentration of drug (either bisaramil or lidocaine), and 'n_H' is the Hill coefficient describing the stoichiometry of drug binding to the channel.

The voltage-dependence of Na^+ channel conductance (G) was calculated by measuring the peak current at test potentials ranging from −90 mV to +55 mV evoked in 5 mV increments and dividing by $V - V_{rev}$, where V is the test potential and V_{rev} is the reversal potential for Na^+. Reversal potentials were determined by individually fitting current–voltage data as described previously (Kontis and Goldin, 1993). Conductance values were obtained in the absence and presence of either bisaramil (30 or 300 μM) or lidocaine (100 or 1000 μM), which were perfused onto the oocyte for 5 min before determining conductance. Peak conductance values were fit with a two-state Boltzmann equation of the form $G = 1 * [1 + \exp(-0.03937 * z_{app} * (V - V_{1/2})]$, in which V is the potential of the voltage pulse, $V_{1/2}$ is the half-maximal voltage for activation, and z_{app} is the apparent gating charge.

The voltage-dependence of Na^+ channel inactivation was determined using 500 ms conditioning pre-pulses from a holding potential of −100 mV (−120 mV for rH1) to +15 mV in 5 mV increments, followed by a test pulse to −5 mV for 22.5 ms. The peak current amplitude evoked during the test depolarization was normalized to the maximum current amplitude, and plotted as a function of the conditioning pre-pulse potential in the absence and presence of either bisaramil (30, 100 and 300 μM) or lidocaine (100 and 1000 μM). The data were fit with a two-state Boltzmann equation of the form $I = I_{max} * [1 + (\exp(V - V_{1/2})/k)]^{-1}$, in which I_{max} is the maximal current evoked, V is the potential of the voltage pulse, $V_{1/2}$ is the voltage at which 50% of the current is inactive (the midpoint of the inactivation curve), and k is the slope factor.

Recovery from inactivation was measured from a holding potential of −100 mV (−120 for rH1) with a 500 ms depolarizing pre-pulse to −10 mV followed by a variable recovery period from 1500 ms to 10 ms, which in turn was followed by a 22.5 ms test pulse to −10 mV. Peak current amplitudes during the test pulse were normalized to the current amplitude of a 22.5 ms pulse to −10 mV immediately preceding the recovery protocol. The experiments were carried out in the absence and presence of either bisaramil (300 μM) or lidocaine (1000 μM). The peak current was plotted as a function of the duration of the recovery interval. The data were individually fit to either a single ($I = 1 - a * \exp(-t/\tau 1)$) or double ($I = 1 - [(a * \exp(-t/\tau 1) + b * \exp(-t/\tau 2))]$) exponential equation, in which a and b represent the proportion of channels recovering with time constants $\tau 1$ and $\tau 2$, and t is the recovery time interval.

The frequency-dependent effects of bisaramil and lidocaine were examined from a holding potential of −100 mV (−120 mV for rH1) with 25 depolarizations to −10 mV for 24.8 ms each. Trains of pulses were delivered at frequencies of 1, 5 and 30 Hz in the absence and presence of bisaramil (30 and 300 μM) and lidocaine (100 and 1000 μM). Current amplitude during each pulse was normalized to the peak maximal current (pulse number 1) and plotted as a function of pulse number.

3. Results

3.1. Bisaramil blocked cardiac Na^+ channels with greater potency than lidocaine

Because bisaramil can be used to effectively block cardiac arrhythmias (Paroczai et al., 1990), the drug might demonstrate some selectivity for the cardiac Na^+ channel compared to channels that are expressed in skeletal muscle or neurons. To test this possibility, the effects of bisaramil on the electrophysiological properties of rat heart (rH1), skeletal muscle (rSkM1) and brain (rBIIA) Na^+ channels expressed in *Xenopus* oocytes were examined using a two-electrode voltage-clamp. For comparison, the effects of lidocaine were examined on the same 3 Na^+ channel isoforms. Oocytes were held at −100 mV for skeletal

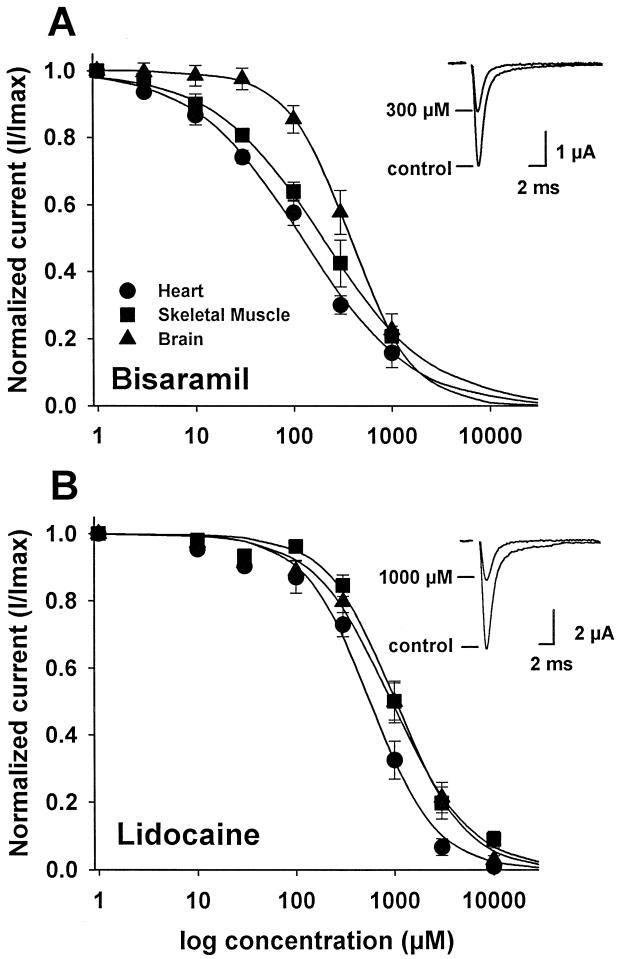

Fig. 1. Concentration-response curves for the effect of (A) bisaramil and (B) lidocaine on heart (●), skeletal muscle (■) and brain (▲) Na^+ channels. Oocytes were injected with Na^+ channel RNA and perfused with ND96. Peak Na^+ currents, evoked every 3 s, were measured at test potentials that elicited maximum inward current (−10 mV). Peak Na^+ currents were measured again after 5 min perfusion of the cell at a flow rate of 1–2 ml/min of ND96 containing increasing concentrations of bisaramil (A) or lidocaine (B). Data points represent the means of at least 6 individual oocytes, and error bars represent standard deviations. The curves described by the solid lines were fit by the Hill equation as described in Section 2. The insets show the current traces during depolarizations to −5 mV in the absence and presence of 300 μM bisaramil (A) and 1000 μM lidocaine (B).

muscle and brain channels and −120 mV for heart channels, and currents were evoked by depolarizations to −10 mV every 3 s. This infrequent pulsing protocol minimizes the effects of frequency dependent block, and therefore provides a reasonable estimation of the the extent to which bisaramil produces tonic block of the Na^+ current.

Fig. 1 shows the concentration-response curves for bisaramil (A) and lidocaine (B) on heart, skeletal muscle and brain Na^+ currents. The smooth lines represent the best fits of the data using the Hill equation, with the parameters of the fits shown in Table 1. Although the stoichiometry of bisaramil and lidocaine binding to the heart Na^+ channel were significantly different from each other (n_H in Table 1), the values are close enough to unity that it is likely that only one drug molecule is necessary to block the channel. Both bisaramil and lidocaine demonstrated some selectivity for heart Na^+ channels compared to skeletal muscle or brain channels. Comparing EC_{50} values, bisaramil blocked heart channels with a potency that is 1.5-fold greater than for skeletal muscle channels and 3.3-fold greater than for brain channels (Table 1). Lidocaine had a potency on cardiac channels that was 1.9-fold greater than for skeletal muscle channels and 1.7-fold greater than for neuronal channels (Table 1). Bisaramil was more potent than lidocaine in blocking cardiac Na^+ channels by a factor of 4.6.

3.2. Bisaramil did not affect the voltage-dependence of Na^+ channel activation

To determine if bisaramil blocked Na^+ channel conductance in a voltage-dependent manner, the effects of 300 μM bisaramil on the voltage-dependence of Na^+ channel activation were compared to the effects of 1000 μM lidocaine. Fig. 2 shows the effects of bisaramil (A) and lidocaine (B) on the heart Na^+ channel isoform. The concentrations of bisaramil and lidocaine were chosen to block 75% of heart channel current (EC_{75}), which would ensure that a sufficient fraction of channels would be blocked to reveal any effects on conductance. The smooth curves represent the best fits to the data using a two-state

Table 1
Inhibition of heart, skeletal muscle and brain sodium channels by bisaramil and lidocaine

Drug	Heart		Skeletal muscle		Brain	
	EC_{50} (μM)	'n_H'	EC_{50} (μM)	'n_H'	EC_{50} (μM)	'n_H'
Bisaramil	122 ± 16	0.9 ± 0.2	189 ± 8	1.0 ± 0.2	400 ± 47	1.3 ± 0.1
Lidocaine	563 ± 22	1.4 ± 0.2	1083 ± 122	1.3 ± 0.1	935 ± 100	1.1 ± 0.1

Concentration-response curves for the effects of bisaramil and lidocaine on the heart (rH1), skeletal muscle (rSkM1) and brain (rBIIA) sodium channels were determined as described in Section 2 in the absence and presence of either bisaramil (1–1000 μM) or lidocaine (1–10 000 μM). The fractional block of sodium current by each drug at each concentration was plotted against the log concentration of drug (Fig. 1) and fit with the Hill equation ($I_{Na} = [1 + ([A]/EC_{50})^n]^{-1}$). In this equation, EC_{50} is the concentration of drug that produced half-maximal block of the sodium current and 'n_H' is the Hill coefficient that describes the stoichiometry of drug binding to the channel. Results are mean ± standard deviation for at least 6 individual cells.

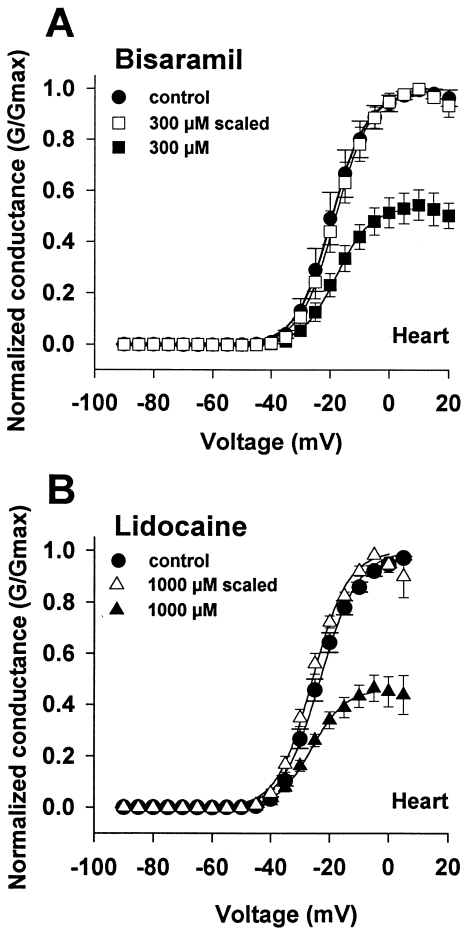

Fig. 2. Effects of bisaramil (A) and lidocaine (B) on heart (rH1) Na^+ channel conductance (G). Oocytes injected with rH1 Na^+ channel RNA were held at -120 mV and Na^+ currents elicited with a 12 ms depolarizing pulse from -90 mV to $+55$ mV in 5 mV increments. The voltage-dependence of peak conductance is compared in the absence (●) and presence (■) of 300 μM bisaramil (A). The curves described by the smooth solid lines represent a two-state Boltzmann function as described in Section 2. When the conductance curve in the presence of the 300 μM concentration of bisaramil is scaled to the maximum (□), neither a shift in the half-maximal activation voltage ($V_{1/2}$) nor a change in apparent gating charge (z_{app}) is observed. The parameters of the fits for the control are $V_{1/2} = -20 \pm 3$ mV, $z_{app} = 4.7 \pm 0.3$ e_0, and for 300 μM bisaramil are $V_{1/2} = -19 \pm 2$ mV, $z_{app} = 4.6 \pm 0.3$ e_0. (B) The voltage-dependence of peak conductance in the absence (●) and presence (▲) of 1000 μM lidocaine. When the conductance curve in the presence of 1000 μM lidocaine is scaled to the maximum (△), neither a shift is apparent in the half-maximal activation voltage ($V_{1/2}$) nor a change in apparent gating charge (z_{app}) is observed. The parameters of the fits for the control are $V_{1/2} = -24 \pm 1$ mV, $z_{app} = 4.8 \pm 0.1$ e_0, and for 1000 μM lidocaine are $V_{1/2} = -22 \pm 1$ mV, $z_{app} = 5.0 \pm 0.3$ e_0.

Boltzmann function, with the parameters of the fits given in the legend. When the curves for conductance in the presence of drug are scaled to the control maximum (open symbols), it becomes obvious that there is no significant change in either $V_{1/2}$ or the slope factor (z_{app}) for either bisaramil or lidocaine. Similar results were observed for the effects of bisaramil and lidocaine on the voltage-dependence of conductance of the skeletal muscle and brain Na^+ channels (data not shown). Therefore, neither bisaramil nor lidocaine significantly altered the voltage-dependence of activation for any of the Na^+ channel isoforms.

3.3. Bisaramil caused hyperpolarizing shifts in the voltage-dependence of inactivation

Many antiarrhythmic drugs, such as lidocaine, are known to bind preferentially to the inactive state of the Na^+ channel. This preferential binding results in a hyperpolarizing shift in the inactivation curve of Na^+ channels in the presence of drug. To determine if this were the case for bisaramil, the effects of bisaramil on the voltage-dependence of inactivation were examined and compared to the effects of lidocaine. Inactivation was examined using a two-pulse protocol with a 500 ms inactivation pre-pulse to ensure that all of the channels were inactive. Drug concentrations were utilized to obtain minimal current block (30 μM bisaramil and 100 μM lidocaine) and marked current block (300 μM bisaramil and 1000 μM lido-caine). The effects of 300 μM bisaramil (A–C) and 1000 μM lidocaine (D–F) on the heart (A and D), skeletal muscle (B and E) and brain (C and F) channels are shown in Fig. 3. The smooth curves represent the best fits to the data with a two state Boltzmann function, with the parameters of the fits shown in Table 2.

In contrast to the lack of effect on the voltage-dependence of activation, both bisaramil and lidocaine altered the voltage-dependence of inactivation, with the degree of alteration dependent upon both drug concentration and Na^+ channel isoform. Bisaramil shifted the $V_{1/2}$ of inactivation in the hyperpolarizing direction for all three of the isoforms in a concentration-dependent fashion, with a higher concentration causing a larger shift. For example, 300 μM bisaramil shifted the curve for the heart channel by -6 mV, for the skeletal muscle channel by -4 mV and for the brain channel by -9 mV (Table 2). A similar trend was observed for lidocaine, but with a different rank order for the isoforms. Lidocaine (1000 μM) shifted the $V_{1/2}$ for the skeletal muscle channel by -15 mV, for the brain channel by -14 mV, and for the heart channel by -11 mV. Bisaramil did not significantly change the slope factor for any of the channel isoforms, but 1000 μM lidocaine significantly increased the slope factors (decreased the steepness) for all 3 channel isoforms. Therefore, bisaramil does shift the voltage-dependence of Na^+ channel inactivation in the hyperpolarizing direction.

3.4. Bisaramil significantly affected recovery from inactivation

The differential binding of an antiarrhythmic drug to the various states of the Na^+ channel (closed, open or inactive) is likely to affect the kinetics of recovery from inactivation. To characterize the effects of bisaramil on

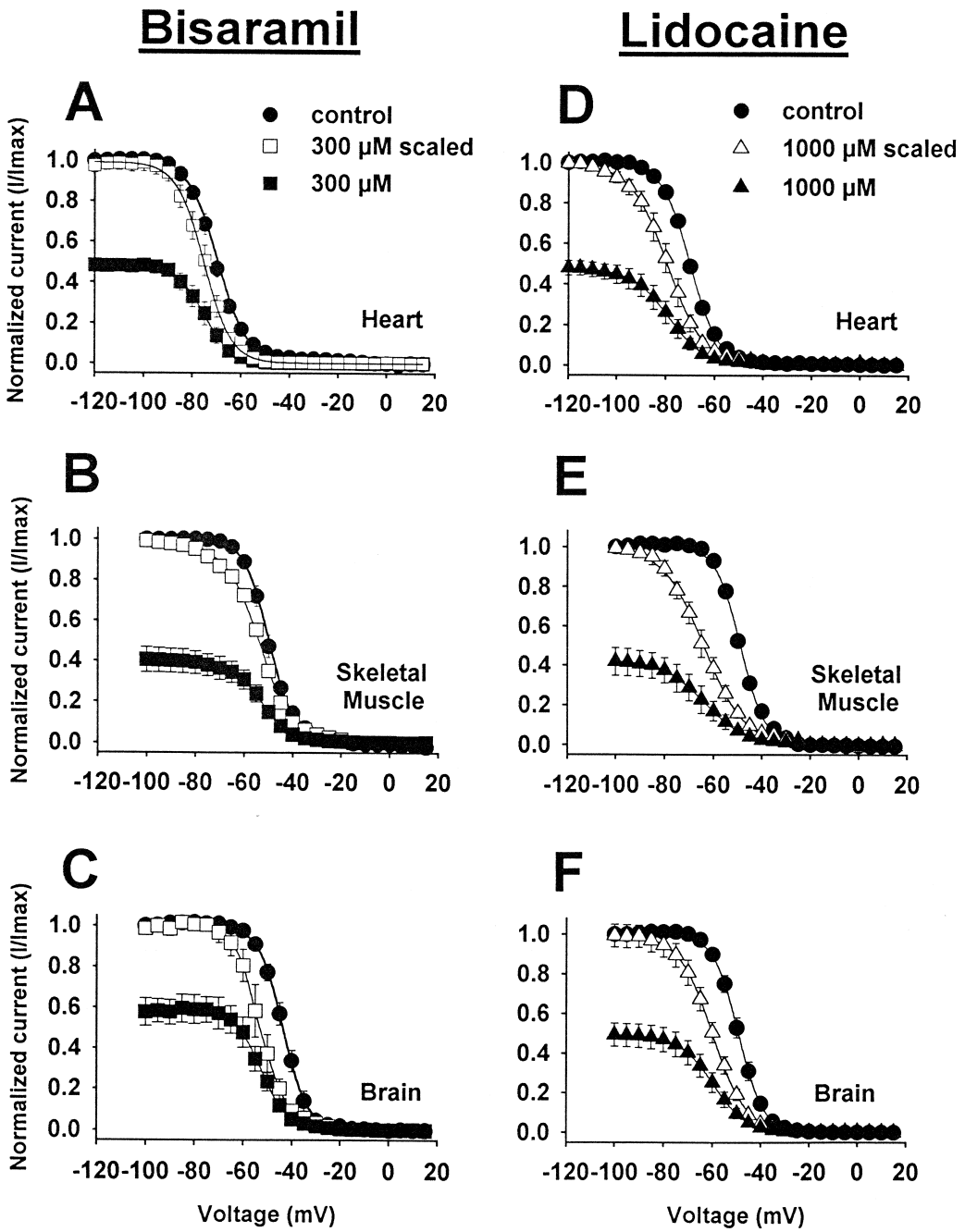

Fig. 3. Effects of 300 μM bisaramil on the voltage-dependence of inactivation of heart (A), skeletal muscle (B) and brain (C) Na^+ channels. The effects of 1000 μM lidocaine on heart (D), skeletal muscle (E) and brain (F) channels are also shown. Inactivation was examined using a two-pulse protocol in which oocytes were held at -100 mV (-120 mV for the heart channel) and depolarized from -90 to $+15$ mV for 500 ms, followed by a test-pulse to -5 mV for 22.5 ms to determine channel availability. The data points in all panels were determined from at least 4 oocytes and error bars represent the standard deviations. All curves were fit with a two-state Boltzmann function as outlined in Section 2. Inactivation curves are shown in the absence (●) and presence of 300 μM bisaramil (■) (A–C) or 1000 μM lidocaine (▲) (D–F). The data for 300 μM bisaramil (□) (A–C) or 1000 μM lidocaine (△) (D–F) are scaled to the maximum to demonstrate the shifts in the voltage-dependence of inactivation.

recovery from inactivation, we used a double-pulse protocol as outlined in Section 2. As in the experiments to examine the voltage-dependence of inactivation, drug concentrations were utilized to obtain minimal current block (30 μM bisaramil and 100 μM lidocaine) and marked current block (300 μM bisaramil and 1000 μM lidocaine). The results for recovery from inactivation for the three channel isoforms are plotted in Fig. 4 for 300 μM bisaramil (A–C) and 1000 μM lidocaine (D–F). The smooth curves represent exponential fits to the data, using 1 or 2

Table 2
Effects of bisaramil and lidocaine on the voltage-dependence of inactivation

	Heart		Skeletal muscle		Brain	
	$V_{1/2}$ (mV)	k (mV)	$V_{1/2}$ (mV)	k (mV)	$V_{1/2}$ (mV)	k (mV)
Bisaramil						
Control	-70 ± 1.3	6.1 ± 0.2	-50 ± 1	5.1 ± 0.4	-44 ± 1	5.0 ± 0.4
30 μM	-69 ± 0.4	6.7 ± 0.2	-50 ± 1	5.1 ± 0.6	-47 ± 2	5.4 ± 1.1
100 μM	-71 ± 0.3	7.1 ± 0.4*	-51 ± 1	5.5 ± 0.3	-49 ± 1*	5.3 ± 0.5
300 μM	-76 ± 2.0*	5.7 ± 0.4	-54 ± 1*	6.7 ± 1.2	-53 ± 2*	5.4 ± 0.7
Lidocaine						
Control	-70 ± 1.0	6.0 ± 0.1	-49 ± 1	5.0 ± 0.1	-49 ± 1	5.1 ± 0.2
100 μM	-71 ± 1.0	6.5 ± 0.3	-57 ± 1*	6.7 ± 0.5*	-55 ± 2*	4.9 ± 0.3
1000 μM	-81 ± 3.0*	7.9 ± 0.5*	-64 ± 2*	8.3 ± 0.2*	-63 ± 2*	6.3 ± 0.4*

Data were fit to a two-state Boltzmann function as outlined in Section 2. $V_{1/2}$ is the voltage at which half-maximal current inactivation occurs and k is the slope factor.
* Indicates a statistically significant difference from control at $P < 0.05$.

time constants depending on the isoform. The parameters of the fits are shown in Table 3.

With no drug present, recovery from inactivation was a fast mono-exponential process for the skeletal muscle and brain isoforms. However, a second slow component (τ_2) was observed for the heart isoform. In the presence of either bisaramil or lidocaine, recovery from inactivation of all three isoforms was slowed. Both of the time constants for recovery of the heart channel were increased, and the percentage of current recovering with the slow time constant increased significantly. Similar effects were observed for the skeletal muscle and brain channels, with an increase in the fast time constant and the appearance of a second, slow component of recovery.

3.5. Bisaramil caused use-dependent block of only the cardiac Na^+ channel

Differential block of Na^+ channels in closed, open or inactive states by antiarrhythmic drugs can result in use-dependent block, which is an important parameter to assess efficacy against arrhythmias characterized by rapidly firing action potentials. We therefore examined use-dependent block of the three Na^+ channel isoforms by bisaramil and lidocaine. Steady-state tonic block of Na^+ current was achieved using single depolarizing current pulses delivered every 3 s in the absence and presence of bisaramil or lidocaine. As in the previous experiments, two drug concentrations were utilized to obtain minimal current block and marked current block. Following steady-state drug block (tonic block), trains of depolarizing pulses were delivered to assess the extent of phasic block at different frequencies. The results are shown in Fig. 5 for bisaramil (A–C) and lidocaine (D–F) examined at a pulse frequency of 30 Hz. The percent of current block at the 25th pulse is shown at frequencies of 1, 5 and 30 Hz in Table 4.

Bisaramil blocked the heart channel in a use-dependent manner, so that 60% of the current was blocked by 300 μM bisaramil at 30 Hz. This effect was less pronounced than that observed with 1000 μM lidocaine, which blocked 85% of the current at 30 Hz. Bisaramil minimally blocked ($\sim 10\%$) the skeletal muscle and brain channels in a use-dependent manner. In contrast, lidocaine blocked the skeletal muscle and brain Na^+ channels with a degree of use-dependence comparable to that observed for the heart channel, with 1000 μM lidocaine inhibiting 71% and 70%

Table 3
Effect of bisaramil and lidocaine on recovery from inactivation

	Heart				Skeletal muscle				Brain			
	τ_1 (ms)	%	τ_2 (ms)	%	τ_1 (ms)	%	τ_2 (ms)	%	τ_1 (ms)	%	τ_2 (ms)	%
Control	9 ± 1	76 ± 4	381 ± 74	24 ± 3	130 ± 22	100	ND[a]	ND[a]	46 ± 4	100	ND[a]	ND[a]
Bisaramil 300 μM	31 ± 2	38 ± 2	$\sim 15{,}000$[b]	62 ± 2	52 ± 3	25 ± 5	$\sim 5{,}000$[b]	75 ± 5	95 ± 17	31 ± 3	$\sim 16{,}000$[b]	69 ± 3
Lidocaine 1000 μM[c]	96 ± 9	19 ± 2	$\sim 23{,}000$[b]	81 ± 2	78 ± 11	24 ± 3	$\sim 13{,}000$[b]	76 ± 3	74 ± 13	35 ± 3	$\sim 10{,}000$[b]	65 ± 3

Data represent the mean $\pm$ standard deviation for 4 oocytes.
[a] ND indicates 'not determined,' because recovery of these channels was best fit with a single exponential equation.
[b] Precise values for τ_2 could not be determined, because the longest recovery interval that was used was 1500 ms.
[c] Lidocaine control data is not shown in the table, but is not statistically different from bisaramil control data. The τ_1 and τ_2 values were 9 ± 0.4 and 295 ± 19 ms for the heart channel, while the τ_1 value was 110 ± 7 for the skeletal muscle channel and 55 ± 12 for the brain channel.

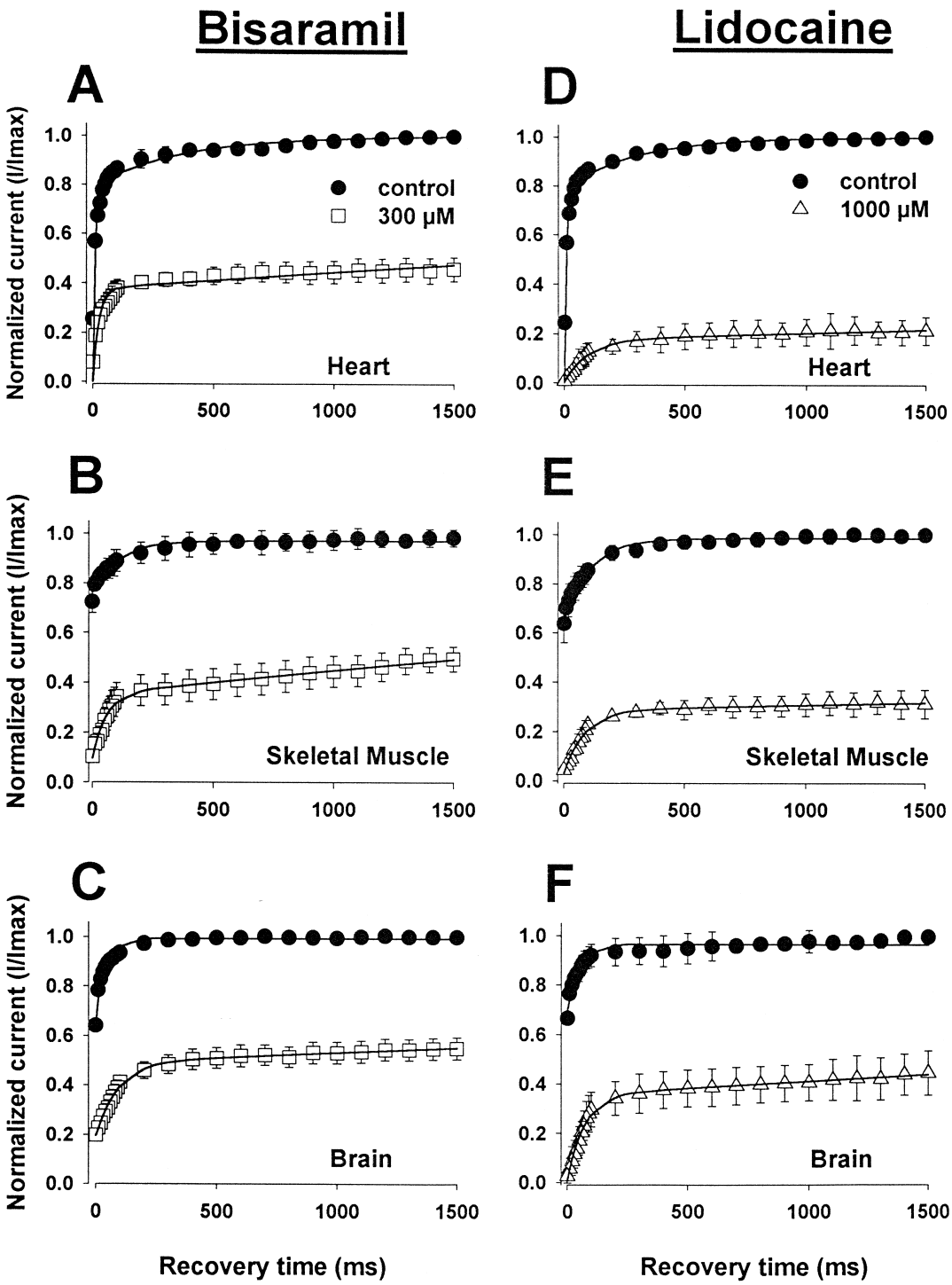

Fig. 4. Effect of 300 μM bisaramil on recovery from inactivation of heart (A), skeletal muscle (B) and brain (C) Na^+ channels. The effects of 1000 μM lidocaine on heart (D), skeletal muscle (E) and brain (F) channels are also shown. Oocytes were held at -100 mV (-120 mV for the heart channel) and depolarized with an inactivating pulse to -10 mV for 500 ms. This was followed by a variable recovery interval of 10 ms duration up to a change in recovery time of 100 ms, followed by 100 ms changes up to a recovery time of 1500 ms, at either -100 mV or -120 mV for the heart channel. The final depolarization was a 20 ms test pulse to -10 mV. The peak current amplitude elicited during the test pulse was normalized to a 22 ms pulse elicited from a holding potential of -100 mV or -120 mV for the heart channel immediately prior to the recovery protocol. The mean $\pm$ standard deviation value for the normalized current is shown plotted against the recovery time between pulses for 5 oocytes either in the absence of drug (●) or in the presence of 300 μM bisaramil (□) or 1000 μM lidocaine (△). The smooth curves represent fits of the data to either a single (skeletal muscle and brain without drug) or double exponential equation as outlined in Section 2.

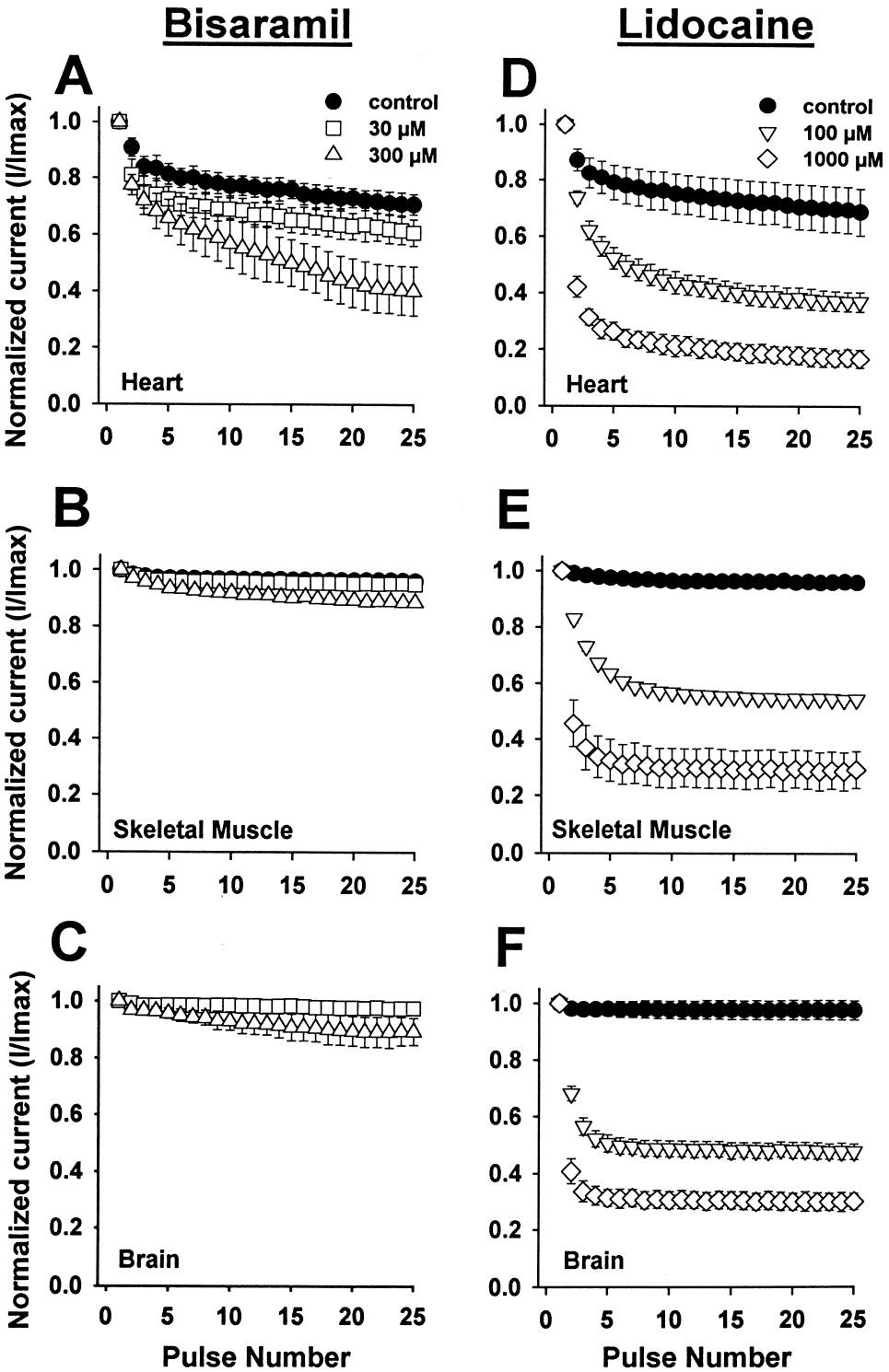

Fig. 5. Effects of bisaramil and lidocaine on the use-dependent block of heart (A, D), skeletal muscle (B, E) and brain (C, F) Na^+ channels in the absence (●) or presence of bisaramil [30 μM (□) or 300 μM (△)] or lidocaine [100 μM (▽) or 1000 μM (◇)]. A series of depolarizing pulses of 20 ms duration to −10 mV were applied from a holding potential of −100 mV at 30 Hz. The peak currents were normalized to the current during the first pulse, and plotted as a function of pulse number. The peak currents measured in the presence of drug were normalized to the current during the first pulse in the presence of drug, even though those currents measured ~90% (for low drug concentrations) and ~40% (for high drug concentrations) compared to drug-free current amplitudes. This normalization emphasizes the amount of use-dependent block that developed independently of the tonic block by each drug.

Table 4
Frequency-dependent blocking actions of bisaramil and lidocaine on sodium currents evoked at 1, 5 and 30 Hz

Drug	1 Hz			5 Hz			30 Hz		
	heart	skeletal muscle	brain	heart	skeletal muscle	brain	heart	skeletal muscle	brain
Bisaramil									
Control	4.5 ± 1.1	0	0	4.1 ± 2.4	1.4 ± 0.2	1.2 ± 0.1	29 ± 4	4.0 ± 0.9	1.4 ± 0.4
30 μM	11 ± 7*	2.1 ± 1.4	2.3 ± 1.4	3.6 ± 1.3	1.8 ± 0.4	2.3 ± 1.2*	39 ± 5*	7.8 ± 4.1	1.5 ± 1.4
300 μM	28 ± 6*	7.2 ± 2.4*	4.8 ± 1.5*	11 ± 3*	14 ± 0.8*	4.1 ± 1.2*	60 ± 8*	12 ± 1*	11 ± 4*
Lidocaine									
Control	3.7 ± 1.0	0	0	4.2 ± 2.1	7.3 ± 2.4	0	33 ± 8	4.1 ± 0.5	2.2 ± 1.3
100 μM	7.4 ± 0.9*	2.1 ± 1.4	2.3 ± 1.3	11 ± 1*	23 ± 2*	12 ± 1*	65 ± 3*	46 ± 1*	53 ± 2*
1000 μM	11 ± 1*	8.9 ± 2.3*	3.7 ± 1.2*	19 ± 1*	35 ± 2*	32 ± 5*	85 ± 3*	71 ± 7*	70 ± 3*

Percent reduction of the peak sodium current measured at pulse number 25 of a series of depolarizing trains delivered to the oocyte at 1, 5 and 30 Hz. Data are expressed as mean ± S.D. for 5 individual oocytes.
* Indicates a statistically significant difference from control at $P < 0.05$.

of the current at 30 Hz. Therefore, bisaramil selectively blocked the heart Na^+ channel in a use-dependent manner.

4. Discussion

The pharmacological actions of bisaramil on the rat heart, skeletal muscle and brain Na^+ channels have been compared to those of lidocaine, a clinically used class Ib antiarrhythmic agent. Bisaramil produced a concentration-dependent tonic and frequency-dependent blockade of Na^+ currents, with some selectivity for the heart channel. These results confirm and extend previous in vivo and in vitro studies with bisaramil (Sunami et al., 1991; Pugsley and Saint, 1995; Haruno and Hashimoto, 1995; Sunami and Hiraoka, 1996).

The tonic component of bisaramil block of Na^+ channels was compared to lidocaine using an infrequent pulsing protocol, which demonstrated that bisaramil is more potent than lidocaine in blocking all 3 Na^+ channel isoforms. Tonic block may result from drug interaction with the resting state of the channel, as has been shown to be the case for other Na^+ channel blocking drugs (Hondeghem and Katzung, 1984). We have observed that the resting state component of tonic block by 300 μM bisaramil resulted in only 8% block of heart, 5% block of skeletal muscle and 3% block of brain Na^+ currents when the channels were maintained in the resting state by continuously holding the cells at −100 mV (−120 mV for heart channel) (Pugsley and Goldin, unpublished observations). These levels of block are significantly less than the 40–60% block observed for 300 μM bisaramil in this study when the channel was depolarized every 3 s (Fig. 1). These results show that bisaramil blocks the Na^+ channel primarily by interaction with either the active or inactive state of the channel. The data concerning bisaramil's effect on Na^+ channel inactivation help to distinguish between these two possibilities.

Bisaramil shifted the voltage-dependence of inactivation for Na^+ channels in the hyperpolarizing direction (Fig. 3). These results are similar to those observed for lidocaine, which is known to interact predominantly with the inactive state of the channel (Bean et al., 1983; Sanchez-Chapula et al., 1983; Bennett et al., 1995). According to the Modulated Receptor Hypothesis, this shift can result from a higher affinity of the drug for the inactive state of the channel. A prediction of the Modulated Receptor Hypothesis is that recovery of the drug-bound channel from inactivation should be slowed and biphasic, with a fast component reflecting recovery of unbound channels and a slow component reflecting recovery of channels bound to the drug (Hille, 1977; Hondeghem and Katzung, 1984). Consistent with this prediction, the time courses of Na^+ channel recovery from inactivation in the presence of bisaramil for all isoforms were best fit with double exponential equations, as has been observed for many other class I antiarrhythmic Na^+ channel blockers (Nitta et al., 1992; Ragsdale et al., 1994). In addition, bisaramil significantly delayed recovery compared to control conditions, similar to the effects of lidocaine (Fig. 4 and Table 3). These results are consistent with the hypothesis that bisaramil does interact with the inactive state of the Na^+ channel.

However, previous studies with bisaramil suggest that Na^+ channel blockade is not due to preferential block of the inactive state. Pugsley and Saint (1995) showed that the degree of block produced by bisaramil did not increase with a prolongation of the pulse duration. Prolonged depolarization maintains channels in the inactive state for a longer period of time, which would increase block if bisaramil had a high affinity for the inactive state. In addition, single channel studies by Sunami and Hiraoka (1996) suggest that bisaramil blocks an active but closed state of the Na^+ channel. Although bisaramil may bind to the active state of the channel, it did not affect the voltage-dependence of activation of any of the Na^+ channel isoforms (Fig. 2). These results are consistent with previous results (Pugsley and Saint, 1995), and are similar to those observed with other antiarrhythmic and local anesthetic drugs (Clarkson et al., 1988a). Based on all of

the data, bisaramil may interact with both an active and inactive state of the Na^+ channel.

Block of either active or inactive channels would be of benefit in pathological conditions associated with myocardial ischemia. In this setting, a number of significant changes occur in the extracellular and intracellular milieu. For example, accumulation of potassium in the extracellular space depolarizes cardiac myocytes (Cascio et al., 1995), resulting in inactivation of some percentage of Na^+ channels. Class I antiarrhythmic drugs such as lidocaine become highly effective under these conditions by reducing excitability and abolishing myocardial conduction within ischemically-depressed tissue (Janse, 1992). A similar mechanism of action could explain the antiarrhythmic efficacy of bisaramil.

The frequency-dependent block of Na^+ currents by bisaramil can be interpreted in terms of the Modulated Receptor Hypothesis as resulting from selective binding to the active and inactivate states (Hille, 1977; Hondeghem and Katzung, 1977). According to the Modulated Receptor Hypothesis, the interaction between blocking drugs and the Na^+ channel is modulated by higher binding affinities to the active or inactive state compared to the resting state of the channel. The decrease in Na^+ current at high rates of stimulation results from an accumulation of drug-associated channels, since Na^+ channels spend more time in the active and inactive states as the inter-pulse (or diastolic) interval shortens. In the case of bisaramil, the drug may bind to both active and inactive states of the channel.

Bisaramil produced an enhanced inhibition of Na^+ currents at high frequencies of stimulation for the heart channel, but not for the skeletal muscle and brain channels (Fig. 5). These findings are consistent with previous in vivo (Sunami et al., 1991) and in vitro results (Pugsley and Saint, 1995; Sunami and Hiraoka, 1996). In contrast, lidocaine produced marked frequency-dependent block of all three Na^+ channel isoforms. There are at least three possibilities to explain the isoform-specific frequency-dependent block by bisaramil. First, bisaramil may unbind more rapidly from skeletal muscle and brain Na^+ channels, so that those channels completely recover from block before the next depolarization. We consider this possibility unlikely, because the rate would have to be fast enough for complete unbinding within 33 ms, as no use-dependence was observed at 30 Hz. Second, the difference in the affinities of bisaramil for the active or inactive state compared to the resting state may be greater for the heart channel than for either the skeletal muscle or brain channel. It is known that the amino acid sequence of the Na^+ channel α subunit can affect drug binding, because the cardiac Na^+ channel isoform is more sensitive to lidocaine than either the skeletal muscle or brain isoforms (Nuss et al., 1995; Makielski, 1996; Wang et al., 1996). Finally, there may be differential access of bisaramil to the functional binding site in the three channels.

Assuming that the binding site for antiarrhythmic drugs is on the intracellular side, then the charge and lipophilicity of a drug can provide an indication of the mechanism by which it enters the cell to exert its effects. Bisaramil can be considered a permanently charged molecule, because it has a nitrogen moiety with a pK_a of 12.3, which is protonated under physiological conditions (Hiraoka et al., 1993). The positive charge should limit the ability of bisaramil to cross the cell membrane. One mechanism by which the drug could enter the cell is through a hydrophilic pathway that develops as channels open, as was proposed for local anesthetics by Hille (1977). This mechanism would explain why bisaramil predominantly blocks channels that have activated, although it would imply that the channels must also open. Other charged compounds, such as QX-314, tetrodotoxin and certain class Ic agents like flecainide, do produce frequency-dependent block of the Na^+ channel when applied to the outside of the cardiac cell (Clarkson et al., 1988b; Alpert et al., 1989; Nitta et al., 1992). On the other hand, a small amount of bisaramil may be able to cross the cell membrane and bind to the resting state of the channel via a hydrophobic access route in a manner similar to other quaternary charged drugs (Strichartz, 1976; Sunami and Hiraoka, 1996), as has been suggested previously (Hille, 1977).

There is evidence for a specific binding site on the Na^+ channel for antiarrhythmic drugs. Ragsdale et al. (1994, 1996) identified a putative local anesthetic, antiarrhythmic and anticonvulsant drug binding site in the S6 transmembrane spanning region of domain IV of the channel. In addition, a different residue in the S6 region determines external accessibility of the charged local anesthetic QX-314 to the cardiac Na^+ channel (Qu et al., 1995). It is unknown if bisaramil binds to the same region of the channel or enters the cell through a comparable pathway. Isoform-specific differences in these regions may be responsible for the cardiac selectivity of bisaramil that we observed.

In summary, bisaramil is an effective antiarrhythmic drug that blocked cardiac, skeletal muscle and brain Na^+ channels expressed in *Xenopus* oocytes. The drug interacts with the inactive state of the channel, but based on previous results it also probably interacts with an active, but not necessarily open, state of the channel. Bisaramil demonstrated greater selectivity for the cardiac channel than did lidocaine, and in particular it blocked only the cardiac channel in a use-dependent manner. The high efficacy of cardiac Na^+ channel block by bisaramil suggests that this drug may have increased antiarrhythmic efficacy and less central nervous system toxicity compared to other Na^+ channel blockers.

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