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Block of Na^+ channel by bepridil in isolated guinea-pig ventricular myocytes

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Abstract

The effects of bepridil, a potent antiarrhythmic agent, on the Na^+ current (I_{Na}) of single guinea-pig ventricular myocytes were studied using the whole-cell patch-clamp technique. Bepridil inhibited I_{Na} in a dose-dependent manner without causing any change in the *I-V* relationship for I_{Na}. Bepridil suppressed I_{Na} with K_d values of 342 and 40 μM when cells were clamped to holding potentials of -140 and -90 mV, respectively. 10 μM bepridil shifted the steady-state inactivation curve for I_{Na} toward more negative potentials by 7.7 mV ($n = 6$). Bepridil also produced marked use-dependent block with a rapid onset. Recovery of I_{Na} from inactivation was retarded (time constant 290 ms) at a holding potential of -140 mV in the presence of 10 μM bepridil. When the onset of I_{Na} block was studied in experiments using a double-pulse protocol, bepridil blocked I_{Na} by 11.5% after a 4-ms pre-pulse, but significantly blocked it after pre-pulses longer than 16 ms. These results suggest that: (1) bepridil has a higher affinity for the inactivated state than the resting state of Na^+ channel; (2) the drug also produces an open channel block; and (3) the drug shows a lidocaine-like fast kinetic block of Na^+ current.

Keywords: Bepridil; Voltage clamp, whole cell; Na^+ current; Myocyte, single, ventricular

1. Introduction

Bepridil is a relatively new Ca^{2+} channel antagonist which possesses antianiginal action. In addition to a vasodilator action, this agent is also useful for various arrhythmias, such as AV nodal reentrant tachycardia, supraventricular tachycardia using accessory pathway, atrial fibrillation and ventricular arrhythmias (Touboul et al., 1987; Rowland et al., 1985; Perelman et al., 1987), and, hence, it is expected to be developed as a unique antiarrhythmic agent.

According to recent electrophysiological studies, it has been demonstrated that bepridil decreases maximum upstroke velocity of the action potentials and induces an action potential prolongation in atrial and ventricular muscles (Winslow and Kane, 1981; Kato and Singh, 1986), although it has been shown that the drug causes a shortening of action potential duration in Purkinje fibers (Kane and Winslow, 1980; Kato and Singh, 1986). In addition, this agent is known to cause an inhibition of slow action potentials in cultured chick heart cells as well as decrease the spontaneous firing frequency, rate of diastolic depolarization and action potential amplitude in rabbit nodal tissues (Li and Sperelakis, 1983; Kato and Singh, 1986). With respect to the underlying ionic currents, it has been demonstrated that bepridil blocks both Ca^{2+} current and Na^+ current (I_{Na}) (Vogel et al., 1979; Yatani et al., 1986). Additionally, recent findings have shown that this drug also blocks repolarizing K^+ currents and behaves like a class III agent (Berger et al., 1989). Thus, this drug shows potential antiarrhythmic activity through class Ia or Ib, III and IV mechanisms. However, the precise mechanisms underlying bepridil induced block of I_{Na} has not been

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fully investigated. Hence, we conducted whole-cell voltage-clamp experiments to clarify the blocking mechanisms of bepridil on I_{Na} in guinea-pig ventricular myocytes.

2. Materials and methods

Guinea pigs weighing 300–500 g were anesthetized with pentobarbital sodium (30 mg/kg). A dissected heart was mounted on a Langendorff apparatus and perfused with a Ca^{2+}-free Tyrode solution (NaCl 130, KCl 4.8, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 12.5 mM and pH adjusted 7.4 with NaOH). The heart was then perfused with Ca^{2+}-free tyrode solution containing collagenase (Yakult, Japan; 0.1 mg/ml) at 37°C. After 30 min of the collagenase treatment, the heart was perfused with a storage solution of the following composition (in mM): KOH 70, KCl 40, L-glutamic acid 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, Hepes 10, EGTA 0.5 with pH adjusted to 7.4 with KOH. The ventricles were subsequently minced with fine scissors and the cells were filtered through nylon mesh and kept in the storage solution. The cells were then incubated in storage solution containing alkaline protease (Nagase, Japan; 0.04 mg/ml) for 10 min. The cells were washed twice by centrifugation and stored at 4°C in the storage solution.

Voltage-clamp experiments were conducted by the single-pipette whole-cell clamp technique. The pipette and tissue bath solutions were essentially identical to those previously described by Follmer et al. (1986). The internal solution was composed of (in mM): NaF 15, CsF 135, Hepes 10 and titrated to a pH of 7.2 with CsOH. I_{Na} was measured during superfusion with low-Na^+ test solution with the following composition (in mM): NaCl 20, CsCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, TMA-Cl 120, $CoCl_2$ 3, glucose 5.6 and Hepes 5 (pH = 7.4 with TMAOH). All experiments were carried out at room temperature (20–22°C). Bepridil hydrochloride (Sigma) was dissolved in deionized water and diluted to the desired concentrations from a 10^{-3} M stock concentration in each experiment.

A voltage-clamp amplifier (ACT ME, 1100T, Japan) designed by M. Yoshii (Narahashi et al., 1987) was used. Suction pipettes were made using a vertical 2-stage pipette puller (Narishige PP-83, Tokyo, Japan). When filled with the aforementioned internal solution, the pipettes had tip resistances of 0.8–1.3 MΩ. The series resistance was compensated by 70 ± 10% after membrane rupture. Digitized current signals were recorded on videotape with a Panasonic NV-F500 video recorder (Osaka, Japan) through a digital cassette recorder (SHOSHIN EM, PCM-DP16, Japan). Each pulse sequence was applied at an interval longer than 30 s to avoid accumulation of the use-dependent effects of the drug (unless otherwise specified). These data were analyzed by a digital oscilloscope (Nicolet 310C) and a computer (NEC PC 9801, Japan or Macintosh IIvx, Fremont, CA, USA). Statistical analysis were made by Student's paired t-test and the data are expressed as mean ± S.D.

3. Results

3.1. Steady-state block of Na^+ current by bepridil

Fig. 1A shows the original current traces of I_{Na} elicited on a test potential to −30 mV from a holding potential of −140 mV before and after superfusion of 300 μM bepridil at 0.1 Hz. Fig. 1B shows the I-V relationship for I_{Na}. Under control conditions, Na^+ current was generated on depolarization to −60 mV, attained at a maximum at −30 mV and reversed polarity at +10 mV. After application of 300 μM bepridil, I_{Na} was reduced by 45 ± 5% without causing any change in the I-V relationship for I_{Na} ($n = 6$). I_{Na} was restored to the control level on wash-out of the drug for 10 min. To verify that the phasic current was carried entirely by ions passing through Na^+ channels, the effect of tetrodotoxin (TTX) on the membrane current was examined. Fig. 1C shows current traces elicited from a holding potential of −90 mV to various potentials ranging from a −60 to 0 mV. At each test potential, 30 μM TTX completely blocked the current transients, indicating that the phasic current was carried by ions passing through Na^+ channels.

The dose-response curves for bepridil block of sodium current are shown in Fig. 2. I_{Na} values relative to the control either from a holding potential of −90 or −140 mV are plotted against the drug concentrations. Bepridil reduced the I_{Na} in a dose-dependent manner and the dose-response curve is shifted in the direction of lower concentrations at a holding potential of −90 mV, suggesting bepridil has a higher affinity in the inactivated state than in the resting state of sodium channel. The apparent dissociation constant (K_d) were 40.3 ± 3.1 and 342.1 ± 4.5 μM at holding potentials of −90 and −140 mV, respectively ($n = 6$). Hill coefficients were 1.12 and 1.15 at holding potentials of −90 and −140 mV, respectively, as calculated using the following equation:

$$\text{Relative } I_{Na} = 1/\left[1 + (C)^n/K_d\right]$$

where C is bepridil concentration, K_d is an apparent dissociation constant and n is the Hill's coefficient. In each experiment, the effect of bepridil was reversible following 10 min of washing.

3.2. Voltage-dependent block of I_{Na} by bepridil

To study the high affinity of bepridil to inactivated Na^+ channels, the effects of bepridil on the steady-state inactivation curve was examined using a standard double-pulse protocol (Fig. 3, inset). After a 3-s conditioning pulse to potentials ranging from −140 to −60 mV in 10-mV steps, a 20-ms depolarizing test pulse to −30 mV was applied and resultant currents were recorded before and

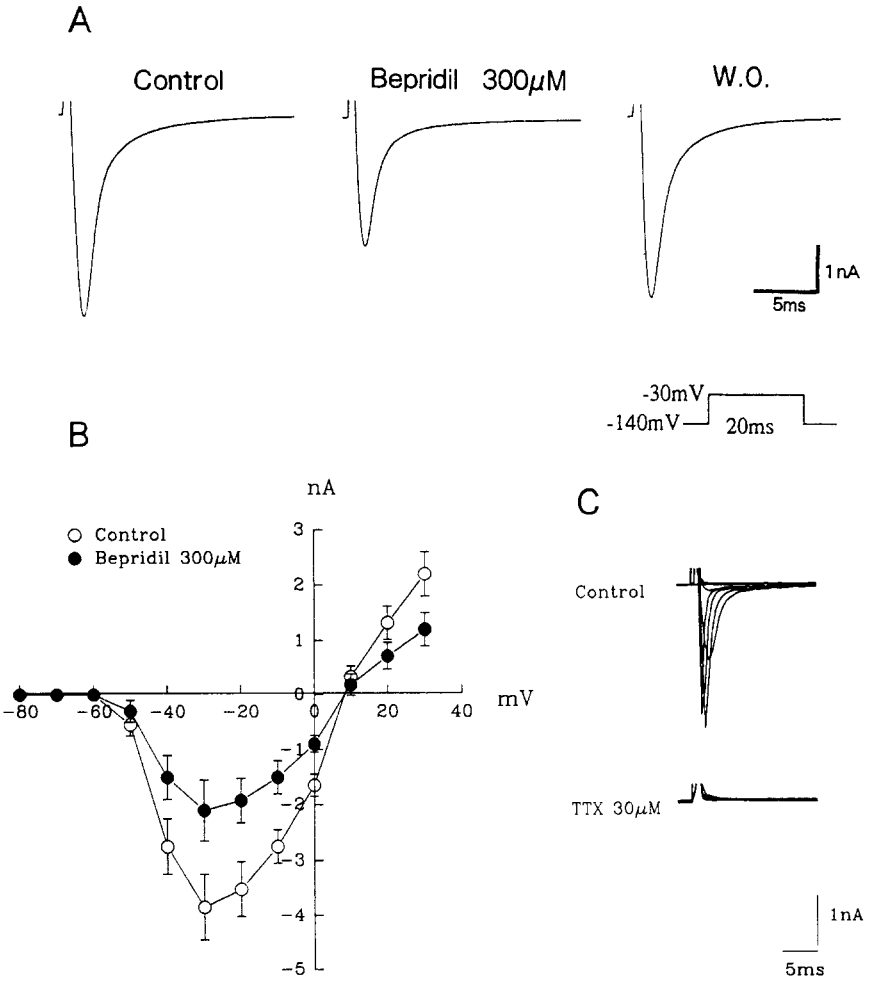

Fig. 1. Effect of bepridil on the *I-V* relationship for I_{Na}. Panel A shows representative current traces of I_{Na} elicited by a test potential to −40 mV from a holding potential of −140 mV before (left), during (center) superfusion with bepridil 300 μM and following washout (right). Currents were recorded at a frequency of 0.1 Hz. Panel B shows *I-V* relationship of I_{Na} under control conditions and in the presence of 300 μM bepridil. I_{Na} was blocked by bepridil without causing any change in *I-V* relationship. Each symbol and vertical bar represents the mean ± S.D. ($n = 6$). Panel C shows effects of 30 μM TTX on the membrane current. Na^+ current was elicited from a holding potential of −90 mV to various potentials ranging from −60 to 0 mV.

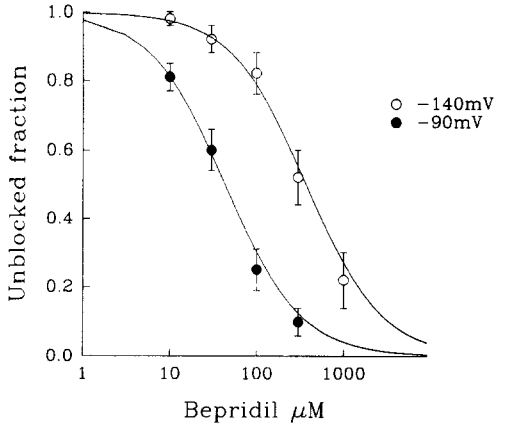

Fig. 2. Dose-response curve for the bepridil-induced block of Na^+ current. Dose-response curves obtained at holding potentials of −140 and −90 mV are shown. Relative values of I_{Na} normalized by taking the controls as unity are plotted against the drug concentrations. Each measurement represents the mean ± S.D. based on 6 experiments. Currents were recorded at a frequency of 0.1 Hz.

during superfusion with 10 μM bepridil. The steady-state inactivation ($h\infty$) curve was obtained by normalizing the current values with its peak I_{Na} at −140 mV as unity. The line drawn through the data points represents the following equation:

$$h\infty = 1/\{1 + \exp(V_m - V_h)/k\},$$

where V_m = conditioning pulse potential, V_h = conditioning pulse potential where I_{Na} is one-half maximal and k is the slope factor (mV). Under control conditions, $h\infty$ was nearly 0 at −60 mV and was maximal at about −120 mV. This $h\infty$ curve was shifted toward more negative potentials by 7.7 mV although the slope factor was unchanged by 10 μM bepridil. On average from 6 experiments, V_h was −77.4 ± 3.8 mV in the control and −85.1 ± 3.5 mV with 10 μM bepridil. The slope factor was measured as 4.89 ± 0.6 mV during the control and 4.86 ± 0.7 mV during superfusion with 10 μM bepridil. The result that bepridil blocks I_{Na} in a voltage-dependent manner suggests that

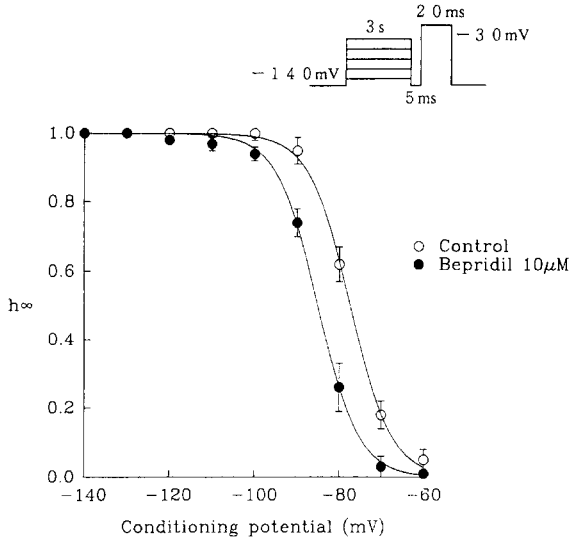

Fig. 3. Steady-state inactivation curves for I_{Na} in the presence or absence of 10 μM bepridil. Na^+ currents were measured using a standard two-pulse protocol (see inset). The steady-state inactivation curve obtained by normalizing the current values with its peak I_{Na} at −140 mV as unity is shown. Bepridil shifted the steady-state inactivation curve toward more negative potentials by 7.7 mV although its slope factor remained unaltered ($k = 4.9$). Each symbol and vertical bar represents the mean ± S.D. ($n = 6$). Each two-pulse sequence was applied at 30-s intervals.

this drug has a higher affinity for the inactivated state of the channel rather than the resting state. Washout of bepridil resulted in a nearly complete recovery of V_h, indicating that the shift in $h\infty$ during superfusion with beridil was not caused by time-dependent changes in $h\infty$ (Table 1).

3.3. Onset of I_{Na} block by bepridil

To clarify the time course of the development of I_{Na} block, the current amplitude was measured as a function of various durations of conditioning pulse shown in Fig. 4. The inset denotes pulse protocol. Pre-pulses of various durations were applied to −30 mV from a holding potential of −140 mV. After a 250-ms recovery period at −140 mV, which allowed drug-free channels to recover fully, a 20-ms test pulse was applied again to −30 mV. Panel A shows traces demonstrating the effects of increasing conditioning pulse duration on the test pulse in the presence of 10 μM bepridil. In panel B, the relative values of I_{Na} are plotted as a function of the conditioning pulse duration. In the presence of 10 μM bepridil, the I_{Na} was reduced by 11.5 ± 1.5% after a pre-pulse of 4 ms in duration ($n = 6$). Since a 4-ms pre-pulse was considered long enough to activate I_{Na} channel but too short to inactivate it, I_{Na} block observed here could be attributed to I_{Na} block in the activated state. On the other hand, when the pre-pulse duration was increased to 16 ms or longer, a much greater reduction in the test I_{Na} was observed, indicating that bepridil preferentially bound to the channel in the inactivated state. The effect of bepridil was reversible following 10 min of washing.

3.4. Use-dependent block of I_{Na} by bepridil

Fig. 5A shows actual current traces elicited by 10 consecutive depolarizing pulses to −30 mV from a holding potential of −140 mV in the presence of 10 μM bepridil. The current amplitude for each pulse was normalized relative to that of the first pulse in the train and was plotted as a function of the pulse number in Fig. 5B. Bepridil produced use-dependent block with a rapid onset. The effect of bepridil was reversible in drug-free solution.

3.5. Effect of bepridil on recovery from inactivation

Recovery of I_{Na} from inactivation was assessed using a standard two-pulse protocol shown in the inset in Fig. 6. A conditioning pulse to −30 mV for 3 s was followed by various recovery periods and then by a test pulse to −30

Table 1
Summary of the data

K_d	40.3 ± 3.1 μM (holding potential = −90 mV), 342.1 ± 4.5 μM (holding potential = −140 mV)	$n = 6$
V_h	−77.4 ± 3.8 mV (control), −85.1 ± 3.5 mV (bepridil 10 μM), −80.6 ± 3.4 mV (washout)	$n = 6$
Slope factor	4.89 ± 0.6 mV (control), 4.86 ± 0.7 mV (bepridil 10 μM), 4.73 ± 0.8 mV (washout)	$n = 6$
Onset of I_{Na} block (% block)	0.2 ± 0.1% (control, PI 1000 ms), 1.5 ± 1.2% (control, PI 500 ms), 2.1 ± 1.3% (control, PI 200 ms)	$n = 6$
	10.3 ± 3.5% (bepridil 10 μM, PI 1000 ms), 21.4 ± 4.2% (bepridil 10 μM, PI 500 ms), 32.8 ± 5.5% (bepridil 10 μM, PI 200 ms)	$n = 6$
Recovery time constant	15 ± 5 ms (control), τ fast; 30 ± 4 ms, τ slow; 290 ± 34 ms (bepridil 10 μM)	$n = 5$ $n = 5$

K_d, apparent dissociation constant; V_h, potential showing $h\infty = 0.5$; PI, pulse interval; τ, time constant.

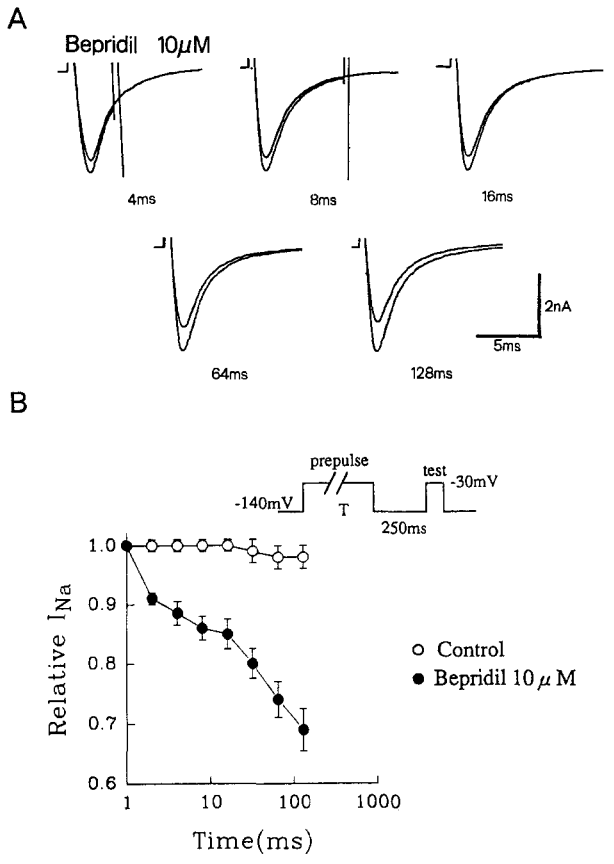

Fig. 4. Time course of bepridil binding to Na^+ channels. Panel A shows traces elicited on conditioning potentials and on test potentials during superfusion with 10 μM bepridil for varying pre-pulse durations. In panel B, the relative values of I_{Na} are plotted as a function of the conditioning pulse duration. With a short pre-pulse of 4 ms in duration, bepridil produced a small reduction in relative I_{Na} whereas pre-pulses longer than 16 ms resulted in a greater reduction of relative I_{Na}. Each symbol and vertical bar represents the mean ± S.D. ($n = 6$). Each two-pulse sequence was applied at 30-s intervals.

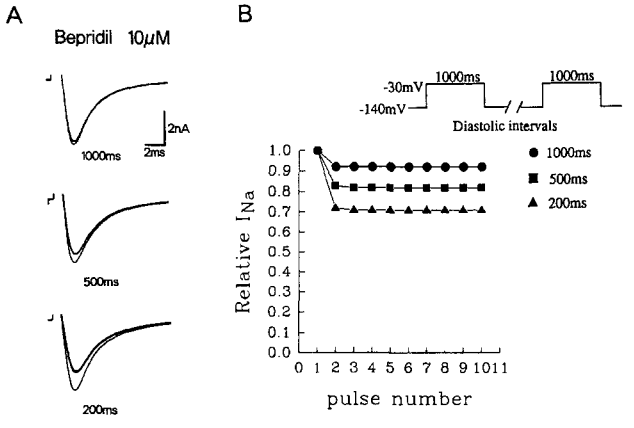

Fig. 5. Use-dependent block of I_{Na} by bepridil. 10 consecutive pulses to −30 mV (1000-ms duration) were delivered at various pulse intervals ranging from 200 to 1000 ms from a holding potential of −140 mV. The peak I_{Na} for each pulse was normalized to that for the first pulse. Note a use-dependent block of I_{Na} with rapid onset. Each 10-consecutive pulse sequence was repeated at 30-s intervals to avoid accumulation of the use-dependent block.

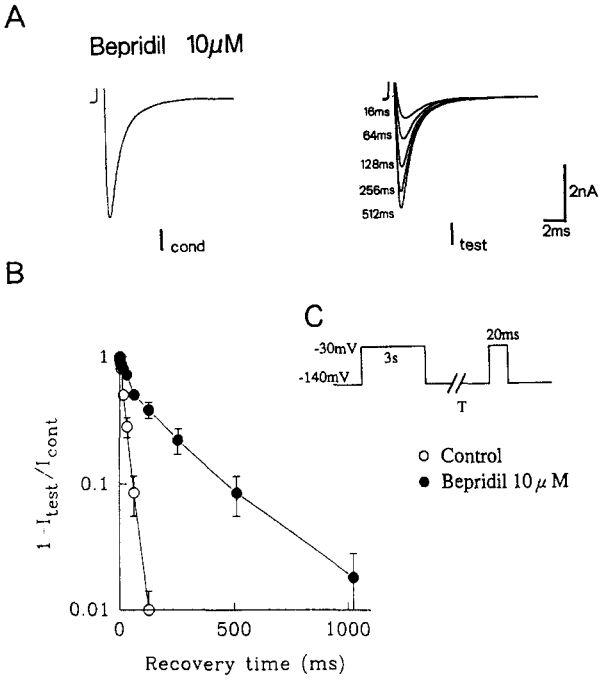

Fig. 6. Effect of bepridil on the recovery of I_{Na} from inactivation. Recovery of I_{Na} from inactivation was assessed using a two-pulse protocol as shown in the inset of B. A conditioning pulse to −30 mV for 3 s was followed by various recovery periods and then by a test pulse to −30 mV. In panel A, records of I_{Na} elicited by the conditioning pulse (I_{cond}) and the test pulse (I_{test}) in the presence of 10 μM bepridil were superimposed. Each two-pulse sequence was applied at 30-s intervals. In panel B, the unrecovered fraction of I_{Na} ($1 - I_{test}/I_{cont}$) was semilogarithmically plotted as a function of the recovery intervals. Under control conditions, the unrecovered fraction was expressed by a single exponential function with a time constant of 15 ± 5 ms, whereas in the presence of 10 μM bepridil the fraction was expressed by a double-exponential function with time constants of 30 ± 4 and 290 ± 34 ms. Each symbol and vertical bar represents the mean ± S.D. ($n = 5$).

mV. Panel A shows representative traces of I_{Na}, in the presence of 10 μM bepridil, during conditioning and test pulses with interpulse durations as indicated.

In panel B, the unrecovered fraction of I_{Na} ($1 - I_{test}/I_{cont}$) was semilogarithmically plotted as a function of the recovery times.

Under control conditions, the unrecovered fraction was expressed by a single-exponential function with a time constant of 15 ± 5 ms, whereas in the presence of 10 μM bepridil, the fraction was expressed by a double-exponential function with time constants of 30 ± 4 ms and 290 ± 34 ms ($n = 5$). These findings suggest that bepridil molecules dissociated with a relatively fast time constant of 290 ms from the inactivated Na^+ channels. Recovery was nearly complete after 10-min recovery period in drug-free solution. The summary of these data is shown in Table 1.

4. Discussion

The present study demonstrated that bepridil blocks Na^+ current in a dose-dependent manner. The K_d of this

drug was 342 μM at a holding potential of -140 and 40 μM at -90 mV, suggesting bepridil has a higher affinity for the inactivated state than for the resting state of the Na^+ channel. The fact that the inactivation curve for the Na^+ channel was shifted in the negative direction also suggested higher affinity for inactivated Na^+ channels of this drug. This could be related to the inhibitory action of this drug on conduction in ventricular muscles and Purkinje fibers which have long action potential duration, and may lead to one of the antiarrhythmic mechanisms against ventricular arrhythmias.

In the present study, all experiments were performed at 20–22°C to obtain adequate space-clamp control. Lowering the temperature would cause a reduction in Na^+ channel conductance and slowing the gating kinetics. Although we could not examine the effect of temperature on the I_{Na} block of bepridil, it is possible that at more physiological temperatures, there would be a decreased I_{Na} block by this drug since decreases in temperature have been shown to enhance the blocking effect of local anesthetic agents (Bradley and Richards, 1984; Sanchez et al., 1987).

It has been reported that therapeutical doses of bepridil plasma concentrations are in the range of 0.5–5 μM (Benet, 1985). Since 97% of the drug is said to be bound to plasma protein, the free serum drug concentrations would be estimated to range from 0.001–0.01 μM (Benet, 1985). Based on this value, and the results of our experiments, the resting block is considered to be a minor role in the effects of bepridil on I_{Na}. On the other hand, the dissociation constant for binding to the inactivated state, as calculated by the following equation (Bean et al., 1983), is 1.5 μM.

$$\Delta V_h = k \ln[1 + [D]/K_{d\,rest}/(1 + [D]/K_{d\,i})]$$

where ΔV_h is the midpoint shift of the inactivation curve, k is the slope factor, $[D]$ is the concentration of bepridil, and $K_{d\,rest}$ and $K_{d\,i}$ are the dissociation constant for binding to rested state and inactivated state, respectively. Using this value, we estimated that the inactivated Na^+ channel block amounts to 9% at a free bepridil concentration of 0.01 μM. According to these numbers, it remains unknown if Na^+ current block actually occurs in the clinical setting. However, i.v. bepridil increased the ventricular and atrial effective refractory periods and prolonged both the QRS duration and the HV interval (Singh et al., 1985; Somberg et al., 1985). These effects are consistent with bepridil's propensity, at high i.v. doses, to block the fast sodium channels in human heart.

Our experiments examining the onset of Na^+ channel block demonstrated that the blocked fraction of Na^+ channel was 11.5% with a conditioning pulse duration of 4 ms, which was long enough to allow sodium channels to be fully activated. In contrast, bepridil significantly blocked I_{Na} when pre-pulses were longer than 16 ms. In addition, bepridil had no significant effects on I_{Na} decay kinetics. These results suggest bepridil has a higher affinity for the inactivated state of Na^+ channel although it does have an effect on activated sodium channel to some degree. Although we have not conducted experiments using atrial muscles, it is suggested that open Na^+ channel block of this drug may play some role in the blocking of supraventricular arrhythmias and atrial fibrillation in addition to its effects on Ca^{2+} and K^+ channels. (Yatani et al., 1986; Berger et al., 1989).

Bepridil caused marked use-dependent block with rapid onset. Moreover, recovery of I_{Na} from inactivation was relatively rapid with a time constant of 290 ms in the presence of 10 μM bepridil. These findings suggest that bepridil should be classified as a fast type of class I antiarrhythmic agent because of rapid drug dissociation. Anno et al. (1984a,b) have shown that the recovery of V_{max} from the use-dependent block was rapid with a time constant of 602 ms in guinea-pig ventricular muscles (Anno et al., 1984a,b). On the other hand, Yatani et al. have reported that the time constant for slower recovery phase of I_{Na} was 290 ms in single neonatal rat cells (Yatani et al., 1986). Our current findings, in agreement with these previous studies, also put bepridil in the class of fast kinetic drugs. Recently, proarrhythmic activity of class IC antiarrhythmia agents has attracted increasing attention among elecrophysiologists following the negative results reported in the Cardiac Arrhythmia Suppression Trial (The Cardiac Arrhythmia Suppression Trial Investigators, 1989). In this regard, bepridil might be relatively safe because of its fast kinetics. On the other hand, it is known that bepridil causes QT prolongation and sometimes constitutes increased risk for ventricular proarrhythmic events, such as Torsade de Pointes (Singh, 1992). Although these proarrhythmic effects may be mainly explained by the block of repolarizing K^+ current, there is also a possibility that high dose of bepridil produces a new area of unidirectional block by blocking sodium channels, leading to a proarrhythmic event. Therefore, as is the case with many other drugs with antiarrhythmic activity bepridil should be also used with caution in clinical setting.

Finally, comparing the effects of bepridil on blocking Ca^{2+} and Na^+ channels, it is reported that the half-blocking concentration for Na^+ channels is 100 times as large as that for Ca^{2+} channels (30 vs. 0.5 μM, respectively) (Yatani et al., 1986). On the other hand, I_K was decreased by 1.8 μM bepridil to 70% of its pre-drug amplitude in sheep Purkinje fibers (Berger et al., 1989). Thus, the blocking effect of Na^+ channels is reported to be weaker compared with that of K^+ channel or Ca^{2+} channel. However, as described before, clinical electrophysiological studies have shown that bepridil prolongs infranodal conduction (HV interval), indicating that the depression of Na^+ channel is induced by therapeutical bepridil doses (Singh et al., 1985). Hence, in addition to blocking effect on Ca^{2+} channel and K^+ channel by bepridil, the Na^+ channel block which includes inactivated channel block

and minimal open channel block, might be effective for various arrhythmias in clinical setting.

In summary, it is suggested that: (1) bepridil has a higher affinity for the inactivated state than the resting state of Na^+ channels; (2) the drug also produces an open channel block as well as inactivated channel block; and (3) the drug shows a lidocaine-like fast kinetic block of Na^+ current.

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