Blocking Effect of Bepridil on Na\(^+\)/Ca\(^{2+}\) Exchange Current in Guinea Pig Cardiac Ventricular Myocytes

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ABSTRACT—We examined the effect of bepridil, a class IV antiarrhythmic drug, on Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{\text{NCX}}\)) in single guinea pig cardiac ventricular cells using the whole-cell voltage clamp technique. I\(_{\text{NCX}}\) was recorded by ramp pulses from the holding potential of −60 mV in the presence of 140 mM Na\(^+\) and 1 mM Ca\(^{2+}\) in the external solution and 20 mM Na\(^+\) and 119 mM free Ca\(^{2+}\) (7 mM Ca\(^{2+}\) and 20 mM BAPTA) in the internal solution. Bepridil suppressed I\(_{\text{NCX}}\) in a concentration-dependent manner. The IC\(_{50}\) value was 8.1 µM with a Hill coefficient of 0.8. Intracellular treatment with trypsin via the pipette solution attenuated the blocking effect of bepridil, suggesting that the inhibitory site is on the cytosolic side of the Na\(^+\)/Ca\(^{2+}\) exchanger. In the absence of albumin in the external solution, 10 µM bepridil inhibited I\(_{\text{NCX}}\) by 46 ± 7% (n = 8), while bepridil blocked it by 28 ± 8% (n = 6) in the presence of albumin. Bepridil inhibited I\(_{\text{NCX}}\) in a supra-therapeutic concentration range.

Keywords: Antiarrhythmic drug, Bepridil, Na\(^+\)/Ca\(^{2+}\) exchange current, Whole-cell clamp, Cardiac myocyte

Bepridil (1-N-benzylanilino-2-pyrodinino-3-isobutoxypropane) is an effective drug for the treatment of angina pectoris (1, 2) and supraventricular and ventricular arrhythmias (3). It is a class IV antiarrhythmic drug, but it also has class I and III antiarrhythmic drug properties in the Vaughan-Williams classification. Bepridil also has negative inotropic and chronotropic effects on the heart (4). In electrophysiological studies on single cardiac myocytes, bepridil has been reported to block Na\(^+\) channels (5); L- (5, 6) and T- (7) type Ca\(^{2+}\) channels; three voltage-gated K\(^+\) channels, i.e., the delayed rectifier K\(^+\) current (I\(_{K1}\)) (8, 9); the inward rectifier K\(^+\) current (I\(_{K}\)) (8, 9); the transient outward K\(^+\) current (I\(_{\text{to}}\)) (8); and two ligand-gated K\(^+\) channels, i.e., the muscarinic acetylcholine receptor-operated K\(^+\) current (I\(_{\text{KACCh}}\)) (10), Na\(^+\)-activated K\(^+\) current (I\(_{\text{KNa}}\)) (11) and the hyperpolarization-activated non-selective cation current (I\(_{h}\)) (8).

Garcia et al. (12) using flux studies reported that bepridil inhibits Na\(^+\)/Ca\(^{2+}\) exchange in cardiac sarcoplasmic membrane vesicles. However, electrophysiological studies on the effect of bepridil on Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{\text{NCX}}\)) have not been performed with cardiac myocytes. In the present study, using the whole-cell voltage clamp, we examined the effect of bepridil on I\(_{\text{NCX}}\) in single guinea pig cardiac ventricular cells.

MATERIALS AND METHODS

Isolation of cells

All experiments were performed under the regulations of the Animal Research Committee of Fukushima Medical University. Guinea pigs weighing 250 – 400 g were anesthetized by intraperitoneal injection of pentobarbital. The chest was opened under artificial ventilation, the aorta was cannulated in situ, and the heart was removed. After washing out the blood with Tyrode solution, the heart was mounted in a Langendorff perfusion system. The perfusate was changed to Ca\(^{2+}\)-free Tyrode solution to stop the heartbeat and then to one containing 0.01% w/v collagenase (Wako, Osaka) and 0.002% w/v alkaline protease (Nagase, Tokyo). After about 20 min, the perfusate was changed to a high K\(^+\), low Cl\(^-\) solution (modified KB solution; 13, 14). Incisions were made in cardiac ventricular tissue in the modified KB solution and the tissue was shaken gently to isolate the cells. The cell suspension was stored at 4°C for later use. The Tyrode solution contained: 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 5.5 mM glucose and 5 mM HEPES-NaOH (pH 7.4). The modified KB solution contained: 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM taurine,
20 mM KH₂PO₄, 3 mM MgCl₂, 10 mM glucose, 0.2 mM EGTA and 10 mM HEPES-KOH buffer (pH 7.2).

**Patch clamp recording**

Membrane currents were recorded by the whole-cell patch-clamp method (15). Single ventricular cells were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Nikon, Tokyo) and superfused at 5 ml/min with the Tyrode solution. The temperature of the bath was kept constant at 36 ± 0.5°C. Patch pipettes were forged from glass capillaries with an external diameter of 1.5 mm (MC-15; Nippon Rikagaku Kikai, Tokyo). The pipette resistance was 2–3 MΩ when filled with the pipette solution. The pipette solution contained: 20 mM NaCl, 20 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), 7 mM CaCl₂ (free Ca²⁺ concentration of 119 nM), 120 mM CsOH, 50 mM aspartate, 3 mM MgCl₂, 5 mM MgATP and 10 mM HEPES (pH 7.2 with CsOH). The extracellular solution contained: 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.02 mM ouabain, 0.01 mM nifedipine, 0.005 mM ryanodine and 5 mM HEPES-NaOH (pH 7.2). The Ca²⁺ current (IₙCa), K⁺ currents, Na⁺/K⁺ pump current and Ca²⁺ release channels of the sarcoplasmic reticulum were blocked by nifedipine, Cs⁺, ouabain and ryanodine, respectively.

The electrode was connected to a patch-clamp amplifier (TM-1000; Act ME, Tokyo). Recording signals were filtered at 2.5 kHz bandwidth, and the series resistance was compensated. Current signals were stored on-line and analyzed with a computer (PC-9801RX; NEC, Tokyo) using software called RAM5.

The current-voltage (I-V) relationship was obtained by ramp pulses as described previously (16, 17). The holding potential was set at −60 mV. The ramp pulse was initially depolarized to −60 mV, then hyperpolarized to −110 mV and depolarised back to the holding potential at a speed of 640 mV·s⁻¹. The ramp pulses of 500-ms duration were given with 10-s intervals. The descending limb current was plotted for I-V curves without capacitance compensation.

IₙCa was induced by 1 mM Ca²⁺ and 140 mM Na⁺ in the external solution and 20 mM Na⁺ and 119 nM free Ca²⁺ in the pipette solution. Under these ionic conditions, the reversal potential of IₙCa was calculated to be −84.5 mV at a stoichiometry of 3Na⁺:1Ca²⁺.

**Drugs**

Bepridil, ouabain, nifedipine and ryanodine were purchased from Sigma Chemical Co., St. Louis, MO, USA. KB-R7943 (2-[2-[4-(4-nitrobenzoyl)phenoxy]ethyl]isothio-urea methanesulfonate) was a kind gift from Kanebo Co., Ltd. (Osaka). Bepridil, nifedipine and KB-R7943 were dissolved in DMSO and added to the extracellular solutions with DMSO at a final concentration of ≤0.1%, which did not affect IₙCa. Trypsin (2.5 μg/ml) (Difco laboratories, Detroit, MI, USA) was dissolved directly in the pipette solution. All chemicals were of the highest grade available.

**Data analyses**

All the values are presented as the mean ± S.E.M. (number of experiments). Student’s t-test and analysis of variance were used for statistical analyses. P values of less than 0.05 were considered significant.

**RESULTS**

**Effects of bepridil on IₙCa**

Figure 1A shows a typical chart recording of the current under the whole-cell clamp. When the external solution was switched to one containing 30 μM bepridil, the current was gradually suppressed. After the effect of bepridil reached a steady state, a high concentration of KB-R7943 (100 μM), a potent inhibitor of the exchanger, was applied to completely block IₙCa. Figure 1B illustrates the I-V relationships of the control (a), at the maximum effect of 30 μM bepridil (b) and in the presence of KB-R7943 (c). The I-V curves of the bepridil-sensitive and the KB-R7943 sensitive components were obtained by subtraction (Fig. 1C). Both I-V curves crossed the voltage axis at the negative voltages expected for the reversal potential of IₙCa, indicating that these currents are IₙCa. Inhibition of bepridil was reversible (figure not shown).

The current magnitudes were measured at +50 mV at

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**Fig. 1. Inhibition of IₙCa by bepridil. A: Chart recording of the membrane current. The horizontal bars above the current indicate where 30 μM bepridil and 100 μM KB-R7943 were applied externally. B: I-V curves obtained at the corresponding labels in panel A. Trace a is the control; b, in the presence of bepridil; and c, after addition of KB-R7943. C: Difference I-V curves between a and c (a-c) and between b and c (b-c) in panel B.
different concentrations of bepridil and the percent inhibition was calculated. The bepridil concentration-response curve is shown in Fig. 2. Bepridil inhibited $I_{\text{NCX}}$ in a dose-dependent manner. A sigmoidal fitting of the curve yielded an IC$_{50}$ value for bepridil of 8.1 $\mu$M (n = 29). The Hill coefficient is 0.8, indicating that one molecule of bepridil is sufficient for the inhibition.

**Effect of trypsin on bepridil inhibition of $I_{\text{NCX}}$**

We examined whether bepridil inhibited $I_{\text{NCX}}$ from the cytosolic side of the membrane by perfusing trypsin via the pipette solution. Figure 3 shows that when the pipette solution contained trypsin (2.5 $\mu$g/ml), 30 $\mu$M bepridil did not inhibit $I_{\text{NCX}}$. KB-R7943 at 100 $\mu$M still significantly blocked $I_{\text{NCX}}$ after the trypsin treatment. As illustrated in Fig. 4 (see also Fig. 2), 30 $\mu$M bepridil blocked $I_{\text{NCX}}$ by 75 ± 4% (n = 5) in the control without trypsin, while it blocked $I_{\text{NCX}}$ only by 28 ± 3% (n = 4) after trypsin treatment. These data suggest that bepridil acts at the cytosolic side of the Na$^+$/Ca$^{2+}$ exchanger.

**Effect of albumin on bepridil inhibition of $I_{\text{NCX}}$**

It has been reported that bepridil binds to albumin, so the concentration of free bepridil in plasma is low (18). Therefore, we examined the effect of bepridil on $I_{\text{NCX}}$ in external solution containing 1% bovine albumin. As summarized in Fig. 5, 10 $\mu$M bepridil blocked $I_{\text{NCX}}$ by 46 ± 7% (n = 8) in the absence of albumin, while it blocked $I_{\text{NCX}}$ by 28 ± 8% (n = 6) in the presence of albumin. These data suggest that the albumin-free form of bepridil is more effective in blocking $I_{\text{NCX}}$.

**DISCUSSION**

In the present study, we demonstrated that bepridil inhibits $I_{\text{NCX}}$ in guinea pig cardiac ventricular cells with an IC$_{50}$ value of about 8.1 $\mu$M and a Hill coefficient of 0.8. The site of action of bepridil may be on the cytoplasmic side of the exchanger because intracellular treatment with trypsin via the pipette solution attenuated the effect of the drug. We recently reported that trypsin treatment attenuates...
the inhibitory effects of amiodarone (19) and BDM (20) on $I_{\text{NCX}}$ but not that of KB-R7943. The Na+/Ca$^{2+}$ exchanger molecule has 9 transmembrane segments (21, 22) with a large intracellular loop, which contains various regulatory sites (23, 24). ‘De-regulation’ of the exchanger by cytosolic trypsin or $\alpha$-chymotrypsin treatment has been demonstrated with respect to activation by intracellular Ca$^{2+}$ and ATP, Na$^{+}$-dependent inactivation (25, 26), inhibition by H$^+$ (27) and by the calmodulin inhibitor W-7 (28).

Garcia et al. (12) reported that the inhibitory effect of bepridil on the Na$^+$/Ca$^{2+}$ exchanger is direction-dependent, since Na$^+$-dependent Ca$^{2+}$ efflux is more strongly inhibited than Na$^+$-dependent Ca$^{2+}$ uptake in dog cardiac membrane vesicles. This is similar to the effect of dichlorobenzamil (17) but opposite to the effect of KB-R7943 which inhibits the Ca$^{2+}$ entry mode of Na$^+$/Ca$^{2+}$ exchange more potently than the Ca$^{2+}$ efflux mode (17, 29). However, this direction-dependent inhibition of KB-R7943 was seen only when uni-directional $I_{\text{NCX}}$ was induced under distinct ionic conditions. When both directions of $I_{\text{NCX}}$ are induced simultaneously (bi-directional $I_{\text{NCX}}$), KB-R7943 blocks both of them equally (30). Amiodarone (19) and BDM (2,3-butanediol monoxime) (20) also inhibited bi-directional $I_{\text{NCX}}$ direction-independently. In the present study, we could not raise the free Ca$^{2+}$ concentration in the pipette solution sufficiently to measure the inward component of bi-directional $I_{\text{NCX}}$ because of the Ca$^{2+}$-sensitizing effects of bepridil (31, 32), which would facilitate cell contraction during the experiment. However, it is thermodynamically unlikely that any drug inhibits one direction of $I_{\text{NCX}}$ more potently than another under bi-directional ionic conditions (30).

Therefore, it is most plausible that bepridil inhibits both directions of $I_{\text{NCX}}$ equally under bi-directional ionic conditions.

The effects of bepridil on other ionic currents have been investigated. L-type Ca$^{2+}$ current was blocked by bepridil in a voltage- and use-dependent manner with an IC$_{50}$ of 0.5 $\mu$M at a holding potential of $-50$ mV (5). Bepridil blocked T-type Ca$^{2+}$ channels more potently than L-type Ca$^{2+}$ channels (7). Bepridil blocks Na$^+$ current (I$_{\text{Na}}$) with an IC$_{50}$ of 30 $\mu$M in cultured neonatal rat ventricular cells at a holding potential of $-100$ mV (5) and with $K_v$ values of 342 and 40 $\mu$M at holding potentials of $-140$ and $-90$ mV, respectively, in adult guinea pig ventricular myocytes (33, 34).

Most types of voltage-gated K$^+$ channels in adult ventricular cells are affected by bepridil. With the two-micro-electrode voltage-clamp technique in sheep cardiac Purkinje fibres, 1.8 $\mu$M bepridil inhibited I$_{\text{K}}$ and I$_{\text{Kr}}$ by 70% and 30 $\mu$M bepridil inhibited I$_{\text{K}}$ completely (8). Bepridil had no effect on I$_{\text{K1}}$ and I$_{\text{Kr}}$ in neonatal rat ventricular cells (5). The delayed-rectifier K$^+$ currents I$_{\text{K1}}$ and I$_{\text{Ks}}$ were suppressed with IC$_{50}$ values of 13.2 and 6.2 $\mu$M, respectively (9). The ligand-gated K$^+$ current, carbachol-, adenosine- and GTPyS-induced I$_{\text{ACH}}$ of guinea pig atrial cells was also susceptible to bepridil (IC$_{50}$ of approximately 2 $\mu$M) (10). Among the K$^+$ currents, the lowest IC$_{50}$ value, 0.51 $\mu$M, was obtained for I$_{\text{Na}}$ with inside-out membrane patches of guinea pig ventricular cells (11).

Kodama et al. (35) reported that 1 $\mu$M bepridil prolonged the action potential duration (APD) without affecting $V_{\text{max}}$ and slightly increased contraction. Bepridil induced an increase in the affinity of the contractile system, Ca$^{2+}$ sensor protein troponin C, and increased Ca$^{2+}$ sensitivity (31, 36, 37). Thus bepridil promoted the Ca$^{2+}$ sensitivity of contractile proteins, which might cancel the depression of contractile tension owing to the inhibitory effect of I$_{\text{Na}}$ (36, 37).

Bepridil can bind to plasma protein such as albumin and $\alpha$-l-acid glycoprotein (18, 38, 39). In the present study, 10 $\mu$M bepridil inhibited I$_{\text{NCX}}$ by $46 \pm 7\%$ (n = 8) in the absence of albumin, but by $28 \pm 8\%$ (n = 6) in the presence of albumin (Fig. 5). From the concentration-response relationship in Fig. 2, it can be estimated that the concentration of free bepridil was 3 $\mu$M in the presence of 1% bovine albumin. Therefore, 70% of bepridil was bound in 1% bovine albumin. In human plasma, which has a much higher concentration of albumin, fractions of free bepridil of 0.23% (38) or 2% (39) have been reported. The therapeutic concentration range of bepridil is between 0.2 to 2 $\mu$g/ml, which corresponds to 0.5 – 5 $\mu$M (18). If 2% is free, the concentration of free bepridil in plasma would range from 0.001 to 0.01 $\mu$M. This concentration range can suppress I$_{\text{Ca}}$, I$_{\text{Na}}$, I$_{\text{K1}}$, I$_{\text{Ks}}$, I$_{\text{ACH}}$ but not I$_{\text{NCX}}$. Thus, the drug may not inhibit I$_{\text{NCX}}$ at the therapeutic concentration range.
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