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Direction-independent block of bi-directional Na^+/Ca^{2+} exchange current by KB-R7943 in guinea-pig cardiac myocytes

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1 We investigated the inhibitory effect of KB-R7943 on 'bi-directional' Na^+/Ca^{2+} exchange current (i_{NCX}) with the reversal potential of i_{NCX} (E_{NCX}) in the middle of the ramp voltage pulse employed.

2 Bi-directional i_{NCX} was recorded with 'full' ramp pulses given every 10 s from the holding potential of −60 mV over the voltage range between 30 and −150 mV under the ionic conditions of 140 mM $[Na]_o$, 20 mM $[Na]_i$, 1 mM $[Ca]_o$ and 433 nM $[Ca]_i$ with calculated E_{NCX} at −50 mV.

3 KB-R7943 (0.1–100 μM) concentration-dependently inhibited the current, which reversed near the calculated E_{NCX}, indicating that the blocked current was i_{NCX}.

4 The inhibition levels were not significantly different between outward and inward i_{NCX} measured at 0 and −120 mV, respectively. IC_{50} of KB-R7943 was approximately 1 μM for both directions of i_{NCX}.

5 Under the bi-directional ionic conditions, only an outward or inward i_{NCX} was induced by positive or negative 'half' ramp pulses, respectively, from the holding potential of −60 mV. KB-R7943 inhibited both direction of i_{NCX} and the concentration-inhibition relations were superimposable to the ones obtained by 'full' ramp pulses.

6 These results indicate that KB-R7943 inhibits i_{NCX} direction-independently under bi-directional conditions. This conclusion is different from that of our previous results obtained from i_{NCX} under uni-directional ionic conditions, where KB-R7943 inhibited i_{NCX} direction-dependently. The difference could be attributed to slow dissociation of the drug from the exchanger.

Keywords: Sodium-calcium exchange; inhibitor; cardiac myocytes; guinea-pig; patch clamp technique; ion exchanger; transporter; heart; KB-R7943

Abbreviations: DCB, 3′,4′-dichlorobenzamil; DMSO, dimethylsulphoxide; E_{NCX}, reversal potential of Na^+/Ca^{2+} exchange current; i_{NCX}, Na^+/Ca^{2+} exchange current; KB-R7943, 2-[2-[4-nitrobenzyloxyl]phenyl]ethyl isothiourea methanesulphonate

Introduction

The Na^+/Ca^{2+} exchanger is a sarcolemmal transporter that plays an important role in regulating intracellular Ca^{2+} concentration in cardiac myocytes (reviews by Hryshko & Philipson, 1997; Reeves, 1998; Yashar *et al*., 1998). Unlike some other transporters that have selective inhibitors such as cardiac glycosides for Na^+/K^+ pump, there has been no specific inhibitor of the Na^+/Ca^{2+} exchanger. Even including non-specific inhibitors, only a small number of drugs have been found to inhibit the Na^+/Ca^{2+} exchanger. An amiloride derivative, DCB (3′,4′-dichlorobenzamil) was one of the earliest synthetic compounds reported to inhibit the exchanger with a K_i value of 17–30 μM (Kleyman & Cragoe, 1988; Lipp & Pott, 1988; Watano *et al*., 1996). KB-R7943 (initially called No. 7943; 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulphonate) is another synthetic drug which inhibits the cardiac Na^+/Ca^{2+} exchange current (i_{NCX}) with higher affinity than DCB. KB-R7943 blocked the outward i_{NCX} more potently ($IC_{50}=0.3$ μM) than the inward i_{NCX} ($IC_{50}=17$ μM) (Watano *et al*., 1996). Iwamoto *et al* (1996) also observed direction-dependent block of the drug with flux studies in cardiomyocytes, smooth muscle cells and the Na^+/Ca^{2+} exchanger-transfected cells. Under physiological or pathological ionic conditions, Na^+/Ca^{2+} exchange is likely to reverse its direction, because the reversal potential of the exchange current can be calculated within the physiological voltage range at a stoichiometry of 3 Na^+: 1 Ca^{2+} (Kimura *et al*., 1986, 1987; Ehara *et al*., 1989). Therefore, it is necessary to investigate the effect of KB-R7943 under the ionic conditions where the exchange current reverses and the current flow can be seen in both the outward and inward directions (bi-directional ionic conditions). As a result, we found that KB-R7943 inhibited both directions of the exchange current with an equal potency under bi-directional ionic conditions.

Methods

Cell isolation

The method of cell isolation was essentially similar to that described elsewhere (Yazawa *et al*., 1990). Briefly, guinea-pigs of either sex weighing 250–450 g were deeply anaesthetized with pentobarbital. Under artificial ventilation, the heart was extracted and mounted on a Langendorff apparatus. After 12–15 min perfusion of Ca^{2+}-free Tyrode solution containing 10% collagenase (Wako, Tokyo, Japan) and 1% alkaline-protease (Nagase, Japan), single ventricular myocytes were dissociated. The cells were stored in KB (high K^+ low Cl^-) solution at 4°C for the experiments.

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Solutions

Tyrode solution contained (mM); NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1 NaH_2PO_4 0.33, Glucose 5.5, HEPES 5 (pH 7.4). KB solution contained (mM); KOH 70, KCl 40, glutamic acid 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 1, HEPES 10 (pH 7.4). The 'bi-directional' external solution contained (mM); NaCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 5 (pH 7.2), CsCl 5, ouabain 0.02, ryanodine 0.01, nifedipine 0.01. The 'bi-directional' pipette solution contained (mM); NaCl 20, CsOH 120, aspartic acid 60, $MgCl_2$ 3, MgATP 5, BAPTA 20, $CaCl_2$ 13 (free Ca^{2+} 433 nM), HEPES 20 (pH 7.2 with aspartic acid). KB-R7943 was provided by Kanebo Co. Ltd. (Osaka, Japan). KB-R7943 was dissolved in dimethylsulphoxide (DMSO) as 10 or 1 mM stock solution. The bath solution was prewarmed to 35°C by water jacket.

Whole-cell clamp

Whole-cell patch clamp recording of the single ventricular myocytes was performed according to the method developed by Hamil *et al*. (1981). The patch-clamp amplifier was TM-1000 (Act ME, Tokyo, Japan). Ramp pulses were given every 10 s from the holding potential of −60 mV initially depolarizing to 30 mV and then hyperpolarizing to −150 mV and depolarizing back to −60 mV with a constant speed of 0.72 Vs^{-1}. The descending limb of the pulse was used to obtain the data (Watano *et al*., 1996). The capacitance compensation was not performed in the illustrations of I–V curves. The data were stored on-line on a computer (PC-9801 RX, NEC, Tokyo).

Data analysis

The data were expressed as mean ± s.e.mean (number of data). The significance of the data was analysed by Tukey method.

Definition of terms

Uni-directional Na^+/Ca^{2+} exchange current Outward or inward Na^+/Ca^{2+} exchange current induced under uni-directional ionic conditions.

Uni-directional ionic conditions Ionic conditions where either Na^+ or Ca^{2+} is lacking or minimum in the external or pipette (intracellular) solution, so that one-direction of the exchange current can flow, but not or minimally in the other direction. More precisely, an outward uni-directional exchange current was induced by transiently changing $[Ca^{2+}]_o$ from 0 to 1 mM in the presence of 20 mM $[Na^+]_i$, 140 mM $[Na^+]_o$, and 96 nM free $[Ca^{2+}]_i$ (20 mM BAPTA and 6 mM $CaCl_2$). An inward uni-directional exchange current was induced by changing $[Na^+]_o$ from 0 (substituted by 140 mM $[Li^+]$) to 140 mM in the presence of 231 nM $[Ca^{2+}]_i$ (20 mM BAPTA and 10 mM $CaCl_2$) and 1 mM $[Ca^{2+}]_o$ without $[Na^+]_i$. Other components of the pipette solution are essentially the same in the both solutions and that for inducing the bi-directional exchange current.

Bi-directional Na^+/Ca^{2+} exchange current Na^+/Ca^{2+} exchange current induced under bi-directional ionic conditions by either 'full' or 'half' ramp pulses. In the case of 'full' ramp, the exchange current was first outward and then inward for a ramp from +30 to −150 mV with the holding potential of −60 mV (= apparent reversal potential). A 'half' ramp pulse induced either outward or inward Na^+/Ca^{2+} exchange current under the bi-directional ionic conditions. A positive 'half' ramp was to +30 mV and a negative 'half' ramp was to −150 mV from −60 mV.

Bi-directional ionic conditions Ionic conditions where Na^+ and Ca^{2+} are present in both the external and pipette (intracellular) solutions, so that the exchange current flows outwardly at voltages positive to the reversal potential and inwardly at voltages negative to the reversal potential of I_{NCX} (E_{NCX}), respectively. Calculated E_{NCX} is at −50 mV and the apparent E_{NCX} at −60 mV (= holding potential) at 140 mM $[Na]_o$, 20 mM $[Na]_i$, 1 mM $[Ca]_o$, 433 nM $[Ca]_i$ (20 mM BAPTA and 13 mM $CaCl_2$).

Results

Effect of KB-R7943 on 'full' ramp exchange current under bi-directional ionic conditions

Figure 1 shows a typical time-course and the magnitude of the blocking effect of 1 μM KB-R7943 on i_{NCX} recorded by 'full' ramp pulses with 10 s intervals. The shape of the ramp pulse is illustrated in Figure 1A. KB-R7943 at 1 μM superfusion

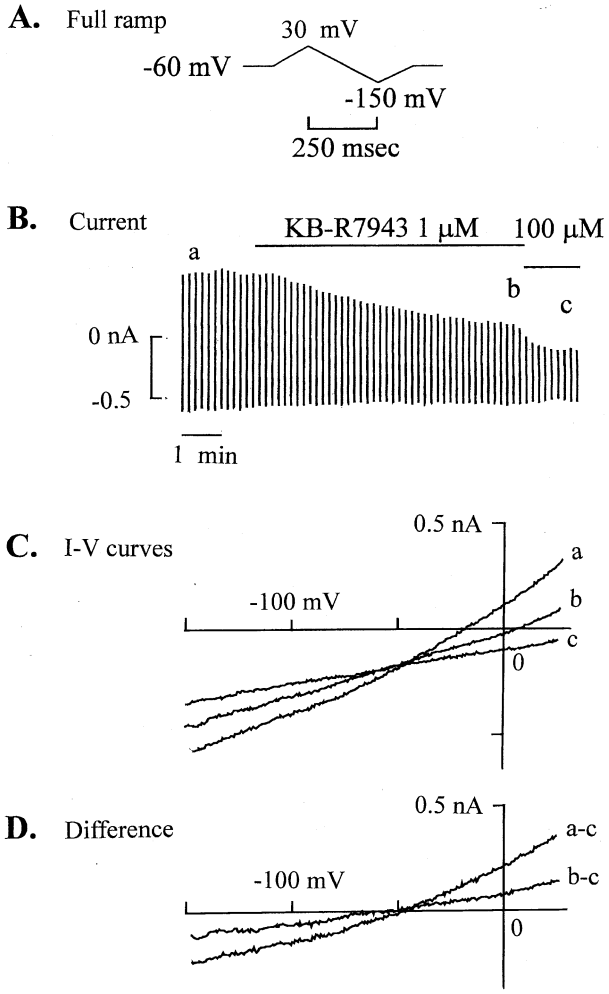

Figure 1 Effect of KB-R7943 1 μM on i_{NCX} induced by 'full' ramp pulses under bi-directional ionic conditions. (A) Shape of a 'full' ramp pulse. The holding potential is −60 mV. (B) Chart recording of the current. Pulse interval was 10 s. Bars above indicate the period that KB-R7943 was superfused. (C) I–V curves obtained from corresponding labels in B; control (a), KB-R7943 1 μM (b) and 100 μM (c). (D) Difference between the I–V curves from C; a–c and b–c.

inhibited both outward and inward components of the current as shown by the envelope of the current trace (Figure 1B). When the inhibition reached the steady state, a high concentration of 100 μM KB-R7943 was applied in the bath to block the exchange current completely. In Figure 1C, the current-voltage relations of the control before and during 1 and 100 μM KB-R7943 applications were superimposed. The difference currents between the control and 100 μM KB-R7943, and between 1 and 100 μM KB-R7943 are superimposed in Figure 1D. Both difference currents crossed with the voltage axis at around -50 mV, indicating that these I–V curves are i_{NCX}. The magnitude of the current was measured at 0 and -120 mV for the outward and inward exchange current components, respectively. KB-R7943 at 3 μM inhibited the exchange current to $52\pm2\%$ ($n=20$) of the control at 0 mV and to $56\pm3\%$ ($n=20$) at -120 mV. The values of percent inhibition were not significantly different between the two voltages.

Effect of KB-R7943 on outward i_{NCX} induced by positive 'half' ramps

Similar inhibition levels between the outward and the inward i_{NCX} were different from our previous results obtained under uni-directional ionic conditions (Watano *et al*., 1996). We examined the drug effects on the 'half' ramp i_{NCX} under the bi-directional ionic conditions, because conditions where the current was always outward (for the positive 'half' ramps) or always inwards (for the negative 'half' ramps) might conceivably lead to different block by KB-R7943, compared to that with the 'full' ramps where the current was first outward and then inward. As shown in Figure 2, positive 'half' ramp pulses from the holding potential of -60 to 30 and back to -60 mV induced an outward exchange current. KB-R7943 at 1 μM blocked the outward current. After the effect had reached the steady state, KB-R7943 100 μM was applied. I–V curves of the control and at 1 and 100 μM KB-R7943 are shown in Figure 2C. The difference I–V curves are superimposed in Figure 2D. The difference I–V curves again crossed each other at the holding potential of -60 mV that is near an expected E_{NCX} value of -50 mV. The current magnitude was measured at 0 mV. The 'half' ramp outward current was inhibited to $54\pm4\%$ ($n=5$) of the control by KB-R7943 1 μM.

Effect of KB-R7943 on inward i_{NCX} induced by negative 'half' ramps

The negative 'half' ramp pulse (Figure 3A) activated the inward exchange current under the bi-directional ionic conditions. A representative chart recording (Figure 3B) shows the inward i_{NCX} inhibited partially by 3 and completely by 100 μM KB-R7943. The current magnitude was measured at -120 mV. The negative 'half' ramp-induced inward i_{NCX} was inhibited to $61\pm4\%$ ($n=14$) and $27.1\pm9.4\%$ ($n=5$) of the control by 1 and 3 μM KB-R7943. These values were not significantly different from those for the outward i_{NCX} induced by positive 'half' ramps represented in Figure 2. Thus the inhibitory effect of the drug was not significantly different between the inward and the outward i_{NCX} under the bi-directional ionic conditions.

Concentration-response curves

Concentration-response curves were obtained by varying the concentrations of KB-R7943 between 0.1 and 10 μM by the

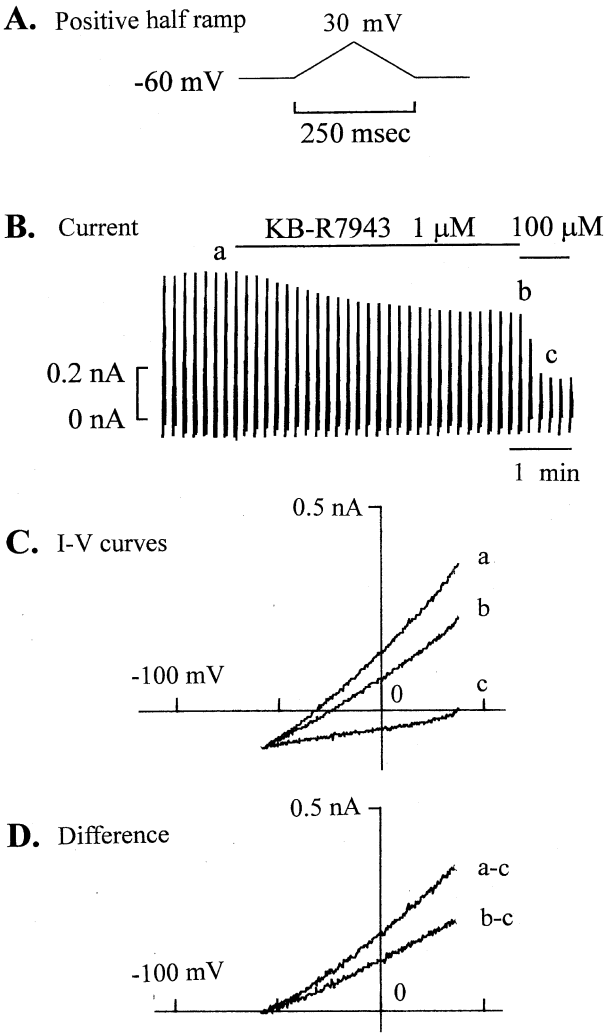

Figure 2 Effect of KB-R7943 1 μM on outward i_{NCX} induced by 'half' ramp pulses under bi-directional conditions. (A) Shape of a positive 'half' ramp pulse. (B) Current trace in response to the 'half' ramp pulses. (C) I–V curves plotted from the corresponding traces in B. (D) Difference I–V curves between a and c (a–c) and b and c (b–c) from C.

ramp protocols shown in Figures 1, 2 and 3 under the bi-directional ionic conditions. Two pairs of concentration-response curves, a total of four curves, are superimposed in Figure 4. One pair is those of the full ramp outward and inward i_{NCX} measured at 0 and -120 mV, respectively. Another pair is those measured also at 0 and -120 mV in response to positive and negative 'half' ramps, respectively, under the bi-directional ionic conditions. Each pair of the concentration-response curves was not significantly different either between the 'half' and 'full' ramps or between the outward (at 0 mV) and inward (at -120 mV) i_{NCX}. These results indicate that there is no significant difference in the levels of inhibition of i_{NCX} by KB-R7943 regardless of the current direction under the bi-directional ionic conditions.

Time course of inhibition by KB-R7943

We measured the time-course of the onset and washing off of the inhibition at 3 μM KB-R7943. Ramp pulses with 3 s intervals recorded the bi-directional i_{NCX}. As shown in Figure 5, the onset time of inhibition was almost single-exponential and took 60 s to reach the maximum inhibition. The washing off or offset time of the inhibition was sigmoid and took about

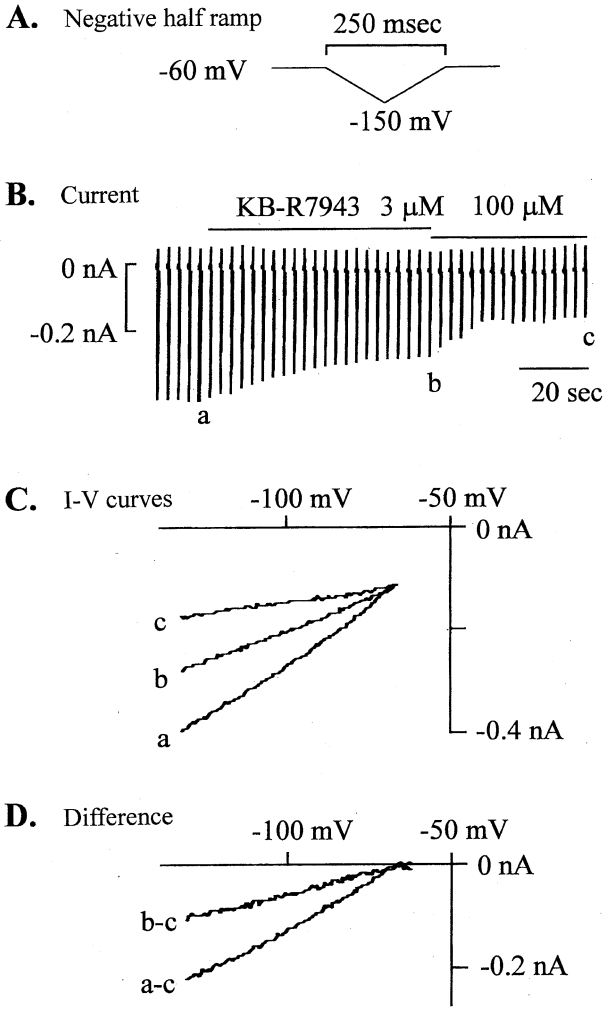

Figure 3 Effect of KB-R7943 at 3 μM on the inward i_{NCX} under the bi-directional ionic conditions. (A) Shape of the negative 'half' ramp pulse. (B). Chart recording of the current in response to regular ramp pulses with 3 s intervals. (C) I–V curves of the corresponding labels in B. (D) Difference I–V curves from C; a–c and b–c.

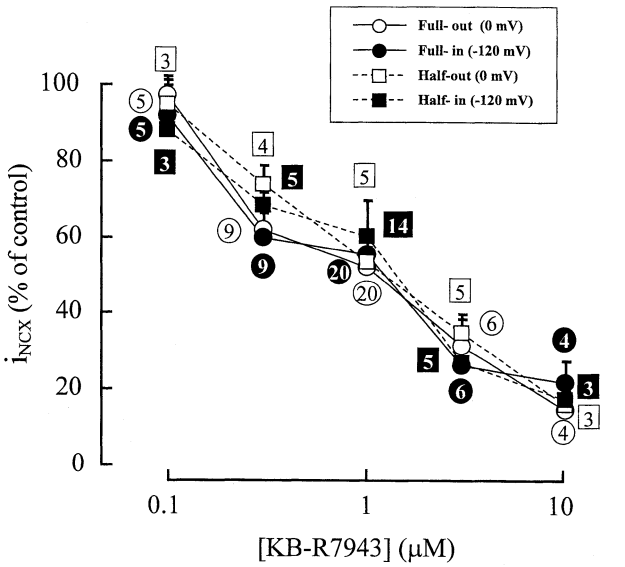

Figure 4 Superimposed concentration-inhibition curves of KB-R7943 under bi-directional ionic conditions. Current magnitudes were measured at 0 mV for outward i_{NCX} and at -120 mV for inward i_{NCX} in response to both 'full' and 'half' ramp pulses. All the points at a given concentration of the drug are not significantly different. Numbers of cells are indicated in the corresponding symbols near each point.

90 s for 100% recovery. The average half time of the onset was 21 ± 4 s ($n=7$) and that of washing-off was 35 ± 4 s ($n=6$).

Discussion

We reported previously that KB-R7943 blocked the outward i_{NCX} more potently ($IC_{50}=0.3$ μM) than the inward i_{NCX} ($IC_{50}=17$ μM) (Watano *et al.*, 1996, see Figure 6). This result was obtained under 'uni-directional ionic conditions' where the outward i_{NCX} was induced by elevating external Ca^{2+} from 0 to 1 mM in the presence of 20 mM $[Na^+]_i$, 140 mM $[Na]_o$ and 96 nM free $[Ca^{2+}]_i$ in the pipette solution. The inward i_{NCX} was induced by changing the external solution from 140 mM $[Li^+]_o$ to $[Na^+]_o$ in the presence of 231 nM Ca^{2+} without Na^+ in the pipette solution.

The present results indicate that KB-R7943 equally inhibited the outward and inward i_{NCX} measured at 0 and -120 mV, respectively; the membrane potentials 60 mV away from the holding potential of -60 mV which almost coincided with the recorded reversal potential of i_{NCX}. The IC_{50} values of KB-R7943 were approximately 1 μM for the 'full' ramp under the bi-directional ionic conditions. Similar IC_{50} values were obtained for the positive and negative 'half' ramps under the bi-directional ionic conditions (Figure 4). Thus KB-R7943 inhibits i_{NCX} direction-independently under the bi-directional ionic conditions.

In Figure 6, the concentration-inhibition curves include a pair obtained under the uni-directional ionic conditions (curves fitted in the Figure 3 and 4 of Watano *et al.* 1996) and another curve of the 'full' ramp i_{NCX} measured at 0 and -120 mV (fitted to Figure 4 of the present paper). It can be clearly seen that the concentration-inhibition curve of the bi-directional i_{NCX} locates between those of the uni-directional inward and outward i_{NCX}.

Why is the inhibitory effect of the drug direction-dependent under the uni-directional ionic conditions, while it is direction-independent under the bi-directional ionic conditions? One possible explanation is schematically presented in Figure 7. We found previously that KB-R7943 was a competitive inhibitor with respect to external Ca^{2+} on the exchanger (Watano *et al.*, 1996; Watano & Kimuta, 1998), although it was noncompetitive in the data of Iwamoto *et al.* (1996). Assuming that the drug is competitive to external Ca^{2+}, and that there are at least two different conformations in the exchanger molecule (for the general concept see Colquhoun, 1998); E_1 as a Na^+-binding formation and E_2 as a Ca^{2+}-binding formation, the most likely conformation of the exchanger for binding KB-R7943 is E_2. Under uni-directional ionic conditions, outward i_{NCX} was induced by elevating extracellular Ca^{2+} concentration from 0 to 1 mM. When the extracellular Ca^{2+} is at 0 mM under this condition, most of the exchanger molecule may exist in the E_2 form of Figure 7A. Therefore the IC_{50} value of 0.3 μM KB-R7943 may reflect the high affinity of the drug to E_2 form. In contrast, changing external Li^+ into Na^+ induced uni-directional inward i_{NCX}. In Li^+ external solution, most of the exchanger may exist in the E_1 form (Figure 7B). The IC_{50} value of 17 μM KB-R7943 under these conditions may reflect its low affinity to the E_1 form. In this scheme, the E_2 form also exists. However, if the period of the exchanger in the E_2 form is very brief, there may be no time for the drug to bind with the E_2

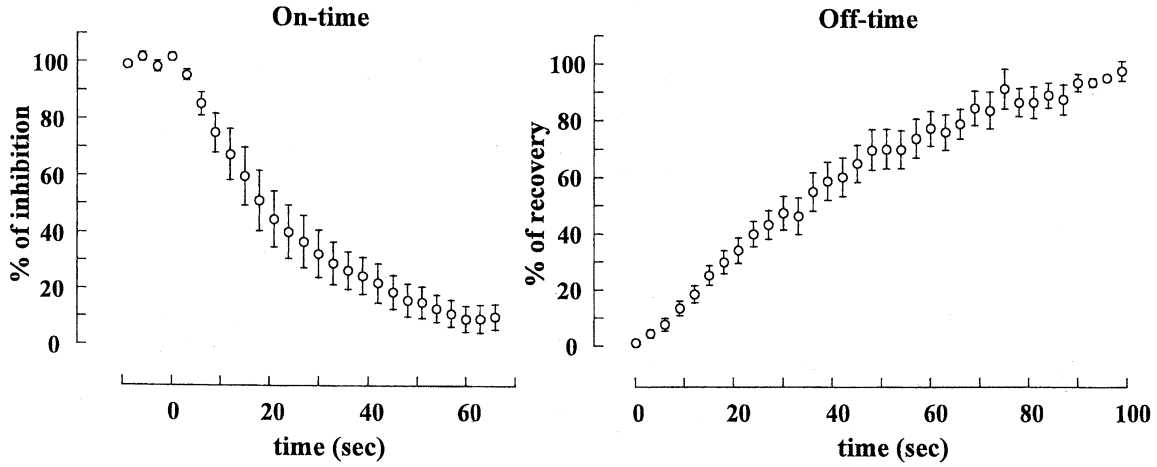

Figure 5 Time course of inhibition of the onset upon application (left panel) and recovery upon washing off (right panel) 3 μM KB-R7943. The pulse was applied with 3 s intervals.

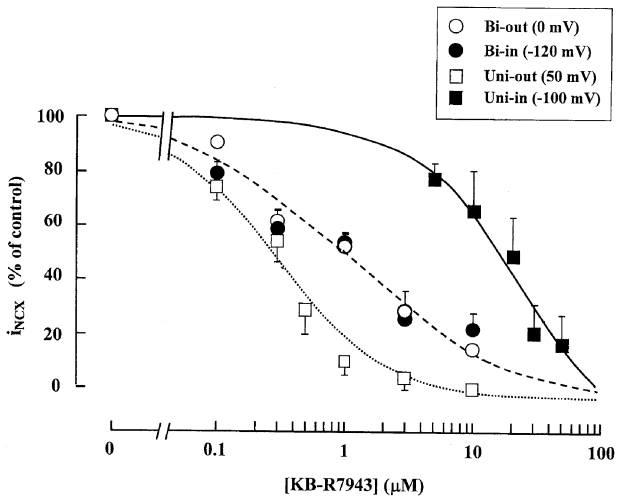

Figure 6 Comparison of concentration-response curves of KB-R7943 and i_{NCX} under the uni-directional (uni) and bi-directional (bi) ionic conditions. The curves under uni-directional ionic conditions were fitted to the data in Watano *et al.* (1996; Figure 3 and 4). The bi-directional data are the same as those for the 'full' ramp shown in Figure 4 and the curve was fitted on the outward i_{NCX} (out) at 0 mV. 'in' indicates inward i_{NCX}, 'out' indicates outward i_{NCX}.

form (Figure 7B). Likewise, it may be plausible that the E_1 form exists very briefly in the uni-directional outward exchange current mode in Figure 7A.

Under the bi-directional ionic conditions in Figure 7C, the holding potential is almost equal to the equilibrium potential of i_{NCX}. Therefore, the conformation of E_1, E_2 and all the other formations may be equally distributed at the holding potential at the steady state. Furthermore, KB-R7943, once bound, dissociates from the exchanger with the dissociation rate (35 s, see Figure 5) significantly slower than a ramp pulse duration (500 ms). Thus although the direction of the exchange current reverses during a ramp pulse, the bound drug blocks both direction equally. The IC_{50} value of 1 μM under the bi-directional ionic conditions regardless of 'full' or 'half' ramp may reflect the mixed affinity for both E_1 and E_2 conforma-

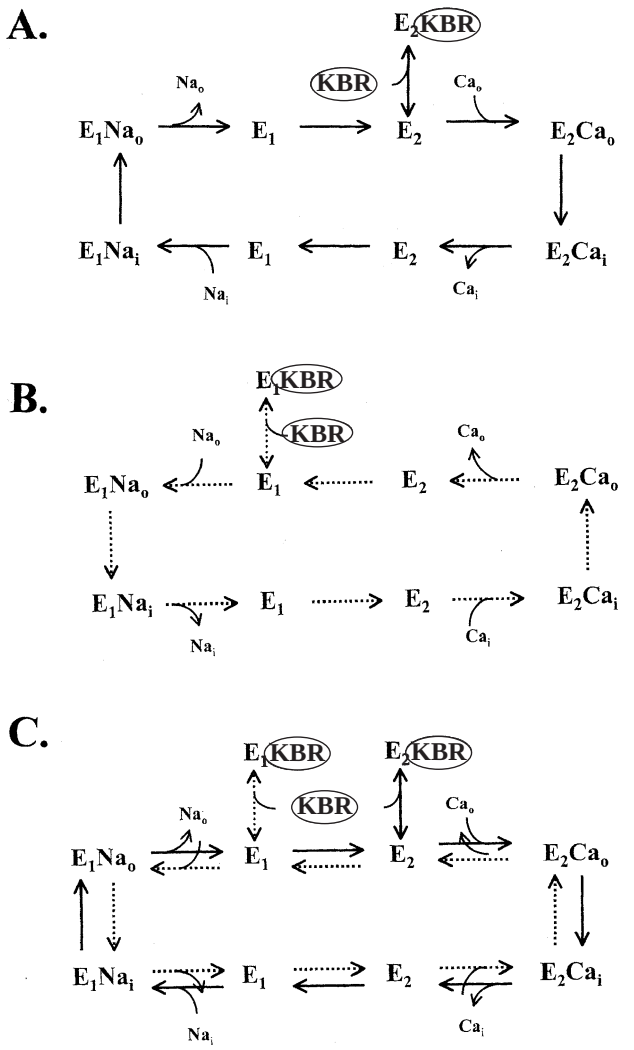

Figure 7 Schematic models of Na^+/Ca^{2+} exchange. (A) Outward uni-directional exchange current model. (B) Inward uni-directional exchange current model. (C) Bi-directional exchange current model. E_1 and E_2 represent the different conformations of the exchanger. E_1; Na^+-binding conformation. E_2: Ca^{2+} binding conformation. The lower case letters of o and i indicate extracellular and intracellular, respectively.

tions, since the value is between the IC_{50} values of 0.3 and 17 μM for the outward and inward uni-directional i_{NCX}, respectively.

Mg^{2+} also inhibits i_{NCX} in a direction-dependent manner under uni-directional ionic conditions (Kimura, 1996). This tendency was also observed in the inhibition by Ni^{+} and DCB (unpublished observations). It will be interesting to investigate how KB-R7943 inhibits i_{NCX} under various physiological or pathological conditions of the cardiac myocytes.

Finally, it is worth noting that the results we have obtained in bi-directional conditions conform to what is required from thermodynamic considerations. In a completely reversible system, an intervention that could affect transport in one direction more than another would risk changing the equilibrium potential, which is strictly impossible without a continuous supply of external energy (such as ATP splitting). Very close to the equilibrium potential therefore our results could be viewed as a thermodynamic necessity. What is significant is that the results apply even as far as 60 mV on either side of the reversal potential, which strongly implies that the dissociation of the inhibitor from the exchanger protein must be much slower than the speed of the ramp clamps.

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