British Journal of Pharmacology (1996) 119, 555-563

A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells

*Tomokazu Watano, [1]Junko Kimura, *Tominori Morita & Hironori Nakanishi

Department of Pharmacology, Fukushima Medical College, 1 Hikarigaoka, Fukushima 960-12, Japan and *Department of Biology, New Drug Research Laboratories, Kanebo Co. Ltd., 1-5-90 Tomobuchi-cho, Miyakojima-ku, Osaka 534, Japan

1 The effects of No. 7943 on the Na^+/Ca^{2+} exchange current and on other membrane currents were investigated in single cardiac ventricular cells of guinea-pig with the whole-cell voltage-clamp technique.

2 No. 7943 at 0.1-10 μM suppressed the outward Na^+/Ca^{2+} exchange current in a concentration-dependent manner. The suppression was reversible and the IC_{50} value was approximately 0.32 μM.

3 No. 7943 at 5-50 μM suppressed also the inward Na^+/Ca^{2+} exchange current in a concentration-dependent manner but with a higher IC_{50} value of approximately 17 μM.

4 In a concentration-response curve, No. 7943 raised the K_mCa^{2+} value, but did not affect the I_{max} value, indicating that No. 7943 is a competitive antagonist with external Ca^{2+} for the outward Na^+/Ca^{2+} exchange current.

5 The voltage-gated Na^+ current, Ca^{2+} current and the inward rectifier K^+ current were also inhibited by No. 7943 with IC_{50}s of approximately 14, 8 and 7 μM, respectively.

6 In contrast to No. 7943, 3',4'-dichlorobenzamil (DCB) at 3-30 μM suppressed the inward Na^+/Ca^{2+} exchange current with IC_{50} of 17 μM, but did not affect the outward exchange current at these concentrations.

7 We conclude that No. 7943 inhibits the outward Na^+/Ca^{2+} exchange current more potently than any other currents as a competitive inhibitor with external Ca^{2+}. This effect is in contrast to DCB which preferentially inhibits the inward rather than the outward Na^+/Ca^{2+} exchange current.

Keywords: Na^+/Ca^{2+} exchange current; No. 7943; 3',4'-dichlorobenzamil (DCB); whole-cell clamp; heart; cardiac myocyte; competitive inhibition; Na^+ current; Ca^{2+} current; K^+ current

Introduction

The Na^+/Ca^{2+} exchange is one of the major mechanisms for regulating the intracellular Ca^{2+} concentration in cardiac myocytes. In normal cardiac cells, the Na^+/Ca^{2+} exchanger extrudes Ca^{2+} from the sarcolemma to maintain the intracellular Ca^{2+} concentration 10^{3-4} times lower than the extracellular concentration. However, in ischaemic cardiac cells where intracellular pH decreases, it has been proposed that the intracellular Na^+ concentration rises through the Na^+/H^+ exchange system, which in turn increases the intracellular Ca^{2+} concentration through the Na^+/Ca^{2+} exchange system (Allen *et al.*, 1993; Scholz *et al.*, 1993; Ver Donck *et al.*, 1993). This Ca^{2+} increase leads to Ca^{2+} overload which induces various pathological conditions including arrhythmia. If an effective inhibitor of Na^+/Ca^{2+} exchanger is available, it may prevent such Ca^{2+} overload during cardiac ischaemia and associated reperfusion injury. However, there are few molecules that have been shown to inhibit the Na^+/Ca^{2+} exchanger (Kaczorowski *et al.*, 1989).

Heavy metals such as, La^{3+}, Cd^{2+}, Mn^{2+} and Ni^{2+} are known to block the Na^+/Ca^{2+} exchanger (Trosper & Philipson, 1983; Kimura *et al.*, 1987). These divalent or trivalent cations, are not specific because they also inhibit Ca^{2+} channels. Synthetic compounds reported as Na^+/Ca^{2+} exchange inhibitors are amiloride derivatives (Kaczorowski *et al.*, 1985; Kleyman & Cragoe, 1988). Among them, DCB (3',4'-dichlorobenzamil) has been demonstrated to inhibit the Na^+/Ca^{2+} exchanger in cardiac membrane vesicles with a relatively low IC_{50} value of 17-30 μM (Siegl *et al.*, 1984; Kaczorowski *et al.*, 1985; Kleyman & Cragoe, 1988; Murata *et al.*, 1995). DCB, however, also inhibits the voltage-gated Na^+ channel (Kleyman & Cragoe, 1988) and T-type and L-type Ca^{2+} channels even more potently than the Na^+/Ca^{2+} exchange current in pituitary cells (Suarez-Kurtz & Kaczorowski, 1988).

Philipson (1984) reported that cationic amphiphiles, such as dodecylamine, dodecyltrimethylamine and laurylcholine, are potent inhibitors of Na^+/Ca^{2+} exchange (~50% at 20 μM) in cardiac sarcolemmal membrane. In addition, lysophosphatidylcholine also inhibits Na^+/Ca^{2+} exchange (Bersohn *et al.*, 1991). A polypeptide, called exchanger inhibitory peptide (XIP), is the most selective inhibitor of the Na^+/Ca^{2+} exchanger so far reported (Li *et al.*, 1991; Chin *et al.*, 1993). This polypeptide is a part of the amino acid sequence (20 amino acids) of the internal loop of the exchange molecule and has a homology to calmodulin binding site which serves as an autoinhibitory domain. XIP, however, must be applied from inside the cell to inhibit the Na^+/Ca^{2+} exchanger.

No. 7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate) (Figure 1) is a Na^+/Ca^{2+} exchanger inhibitor, newly synthesized at New Drug Research Laboratories, Kanebo Co. Ltd. We examined the effect of No. 7943 on the Na^+/Ca^{2+} exchange current and on various other membrane currents in single ventricular cells from the guinea-pig heart with the whole-cell voltage-clamp technique.

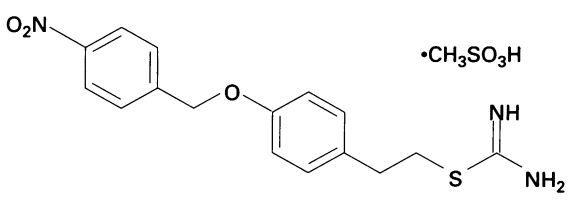

Figure 1 Chemical structure of No. 7943.

[1] Author for correspondence.

Methods

Cell preparation

Guinea-pigs weighing 250–400 g were anaesthetized by intraperitoneal injection of pentobarbitone. The chest was opened under artificial ventilation and the aorta was cannulated *in situ*. The heart was then dissected out and perfused with Tyrode solution (solution 1 in Table 1) on the Langendorff apparatus. After the blood had been washed out, the solution was changed to Ca^{2+}-free Tyrode solution. When the spontaneous heart beat ceased, the perfusate was changed to Ca^{2+}-free Tyrode solution containing collagenase (WAKO, 10 mg per 50 ml) and alkaline protease (Nagase, 1 mg per 50 ml) for about 20 min. The collagenase solution was washed out with high-K^+, low-Cl^- solution (solution 2) and then the ventricles were dissected into the same solution. The ventricular cells were dissociated and stored at 4°C. The temperature of all solutions was maintained at 36±0.5°C with a water jacket.

Electrophysiological experiments

Electrophysiological experiments were carried out using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). The patch-clamp amplifier was Model TM-1000 (Act ME, Tokyo). Patch pipettes were filled with one of the intracellular solutions listed in Table 1 (solution 5 or 6). The cells were dispersed in an experimental chamber which was perfused by the Tyrode solution (solution 1). After a gigaohm seal was formed, the extracellular solution was changed to Ca^{2+}-free Tyrode solution. Then a higher negative pressure was applied inside the pipette to rupture the patch membrane to establish the whole-cell mode. The extracellular solution was subsequently switched to one of the solutions listed in Table 1 (solutions 3 or 4).

The method of recording the Na^+/Ca^{2+} exchange current was similar to those described previously (Kimura *et al.*, 1987; Miura & Kimura, 1989). The current-voltage (*I-V*) relation was obtained by ramp pulses as shown in Figure 2a in all the ramp experiments. The holding potential was usually at −40 mV except in one set of experiments (Figure 7) where it was adjusted to E_{NaCa} (reversal potential of the Na^+/Ca^{2+} exchange current) which varied depending on $[Ca^{2+}]_o$. The shape of the ramp pulse and the corresponding current are shown in Figure 2a. The ramp pulse was initially depolarized to +60 mV, then hyperpolarized to −120 mV and depolarized back to the holding potential at a speed of 720 mVs^{-1}. The total current in response to the ramp pulse (Figure 2a) was plotted as the current-voltage (*I-V*) relation in Figure 2b (left). The current corresponding to the descending limb of the pulse and that to the ascending limb are separated by the capacitative current ((v)–(vi) and (iii)–(iv)). The descending limb current ((iv)–(v)) was plotted after the capacitative current compensation (Figure 2b, (ix)–(viii)). This *I–V* curve was obtained under control conditions for the outward Na^+/Ca^{2+} exchange current, where the Ca^{2+} current, K^+ current and Na^+/K^+ pump current were blocked by D600, Cs^+ and ouabain, respectively.

As shown in Figure 2c (left), under these conditions, the outward Na^+/Ca^{2+} exchange current was induced by changing the external solution from nominally free Ca^{2+} solution to one containing 1 mM Ca^{2+} for about 15 s. The control current and the peak response during the 1 mM Ca^{2+} perfusion were superimposed. The patch pipette was filled with the intracellular solution containing 20 mM Na^+ and pCa 7.02 (solution 5). When the same protocol was repeated in the same cell in the presence of 5 mM Ni^{2+}, a blocker of Na^+/Ca^{2+} exchanger, the outward current failed to develop (Figure 2c right), confirming that this outward current was indeed the Na^+/Ca^{2+} exchange current (Kimura *et al.*, 1987). All the *I–V* curves shown were from the descending limb of the ramp after the capacitative current compensations unless otherwise stated.

Table 1 Composition of solutions (concentrations expressed in mM)

Solution no.	*1*	*2*	*3*	*4*	*5*	*6*
Na^+	140	–	140	–	20	–
Li^+	–	–	–	140	–	–
K^+	5.4	130	–	–	5	5
Cs^+	–	–	–	–	120	130
Ca^{2+}	1.8	–	0–3	1	6	10
Mg^{2+}	1	3	1	1	8	8
Cl^-	151	46	142–148	144	26	36
Phosphate	0.33	20	–	–	–	–
Asparate	–	–	–	–	50	8.9
l-Glutamate	–	50	–	–	–	–
ATP	–	–	–	–	5	5
Creatine phosphate	–	–	–	–	5	5
Taurine	–	20	–	–	–	–
Glucose	5.5	10	–	–	–	–
BAPTA	–	–	–	–	20	20
EGTA	–	0.5	–	–	–	–
HEPES	5	10	5	5	10	20
Ouabain	–	–	0.02	0.02	–	–
D600	–	–	0.004	0.004	–	–
Ryanodine	–	–	0.005	0.005	–	–
pH	7.4	7.2	7.2	7.2	7.2	7.2
pCa (free $[Ca^{2+}]_i$) (nM)					7.02 (96)	6.64 (231)

The data were acquired on-line and analysed later by a computer (NEC, PC-9801RX) with the handmade software called RAM5 (National Institute for Physiological Sciences in Okazaki). The data are expressed as mean±s.e. mean (number of data).

Solutions and drugs

Composition of all the solutions used are listed in Table 1. To see the outward exchange current, solutions 3 (external) and 5 (internal) in Table 1 were used. To see the inward exchange current, solutions 3 and 4 (external) and 6 (internal) were used. The pH of all the solutions were controlled by HEPES buffer. To record the Na^+/Ca^{2+} exchange current, most other membrane currents were blocked by 4 μM methoxy-verapamil (D600) or 3 μM nifedipine for Ca^{2+} channels, external and internal Cs^+ without K^+ to block K^+ channels, 20 μM ouabain for the Na^+-K^+ pump, and by 5 μM ryanodine for Ca^{2+} release channels of the sarcoplasmic reticulum.

No. 7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate, Kanebo, Osaka, Japan, Figure 1) and DCB (3',4'-dichlorobenzamil, Kanebo) were first dissolved in dimethylsulphoxide (DMSO) and added to extracellular solutions so that the final concentration of DMSO was ≤0.1% which did not affect the Na^+/Ca^{2+} exchange current. Neither No. 7943 nor DCB changed the pH of the external solution.

Results

Effect of No. 7943 on the outward Na^+/Ca^{2+} exchange current

Figure 3a shows a set of typical recordings of the effect of No. 7943 on the outward Na^+/Ca^{2+} exchange current. The control external solution contained 140 mM Na^+ (solution 3) without Ca^{2+} and the pipette solution contained 20 mM Na^+ and 96 nM free Ca^{2+} (solution 5). When the external Ca^{2+} concentration was changed from nominally free to 1 mM for about 15 s, the outward Na^+/Ca^{2+} exchange current was induced (Figure 3a(i)). This current was verified as the Na^+/Ca^{2+} exchange current because of its Ni^{2+} sensitivity (see Figure 2c). After the *I–V* curve returned to the original con-

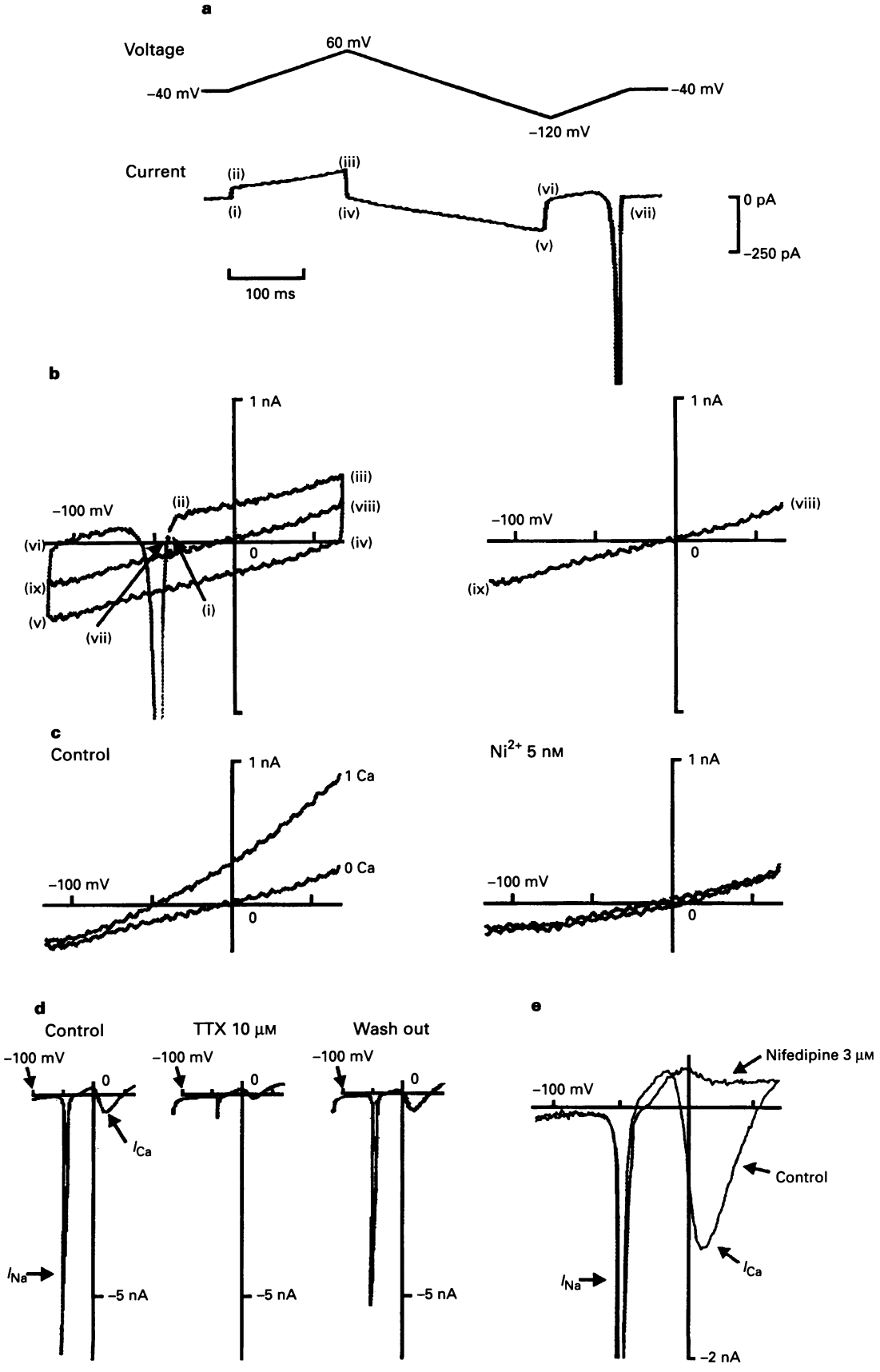

Figure 2 Ramp clamp protocol for recording the Na^+/Ca^{2+} exchange current, Na^+ current and Ca^{2+} current. (a) Voltage ramp pulse (upper trace) and a current response (lower trace) under the control conditions with solutions 4 (external) and 5 (internal). (b) Current-voltage (*I-V*) relation of (a) (left). *I-V* curve ((viii)–(ix)) corresponds to the descending limb current (iv)–(v)) after the capacitative current ((viii)–(iv) or (ix)–(v)) compensation. The *I-V* curve ((viii)–(ix)) alone is plotted on the right. (c) *I-V* curves obtained before (0 Ca) and during (1 Ca) 1 mM external Ca^{2+} perfusion in the absence (left) and the presence (right) of 5 mM Ni^{2+}(d). *I-V* curves of the Na^+ current and the Ca^{2+} current obtained by ascending limbs of the ramp pulse. Na^+ current was reversibly inhibited by 10 μM TTX. (e) Ca^{2+} current in response to the ramp pulse was blocked by 3 μM nifedipine. Control and the current in the presence of nifedipine have been superimposed.

trol level, the external solution was switched to one containing No. 7943 at 0.3 μM. After about 3 min of No. 7943 superfusion, the external Ca^{2+} was raised to 1 mM again. This time the outward Na^+/Ca^{2+} exchange current was suppressed by about 45% compared with the control (Figure 3a(ii)). When the concentration of No. 7943 was raised further to 1 μM in the same cell, application of 1 mM Ca^{2+} did not induce any current, indicating that the exchange current was completely suppressed (Figure 3a(iii)). The current inhibited by No. 7943 recovered after 5 min of washing out the drug (Figure 3a(iv)). Similar experiments were performed in 3 to 6 cells for each concentration of No. 7943 and percentage inhibition of the current was calculated.

A concentration-response relation of the net outward exchange current measured at +50 mV is shown in Figure 3b. The inhibitory effect of No. 7943 on the outward Na^+/Ca^{2+} exchange current was concentration-dependent with IC_{50} of approximately 0.32 μM. To examine whether or not the inhibitory effect of No. 7943 was voltage-dependent, the concentration-response relation was also plotted using the current magnitude measured at 0 mV. The IC_{50} value of No. 7943 was similar to that at +50 mV (data not shown), indicating that the effect of No. 7943 was not voltage-dependent.

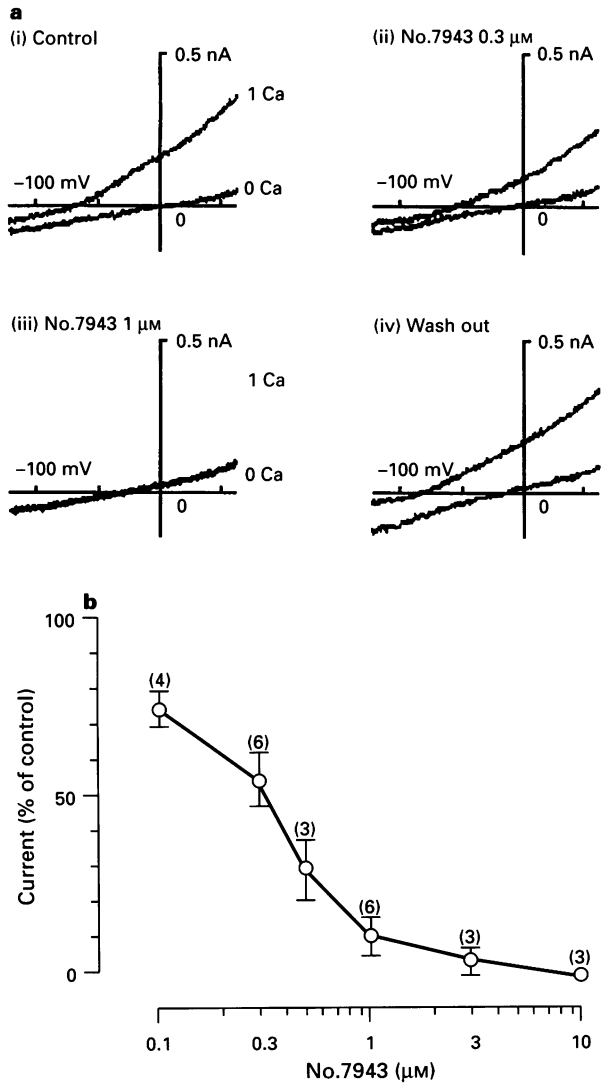

Figure 3 Effect of No. 7943 on the outward Na^+/Ca^{2+} exchange current. (a) *I-V* curves of the outward Na^+/Ca^{2+} exchange current obtained before (0 Ca) and during (1 Ca) 1 mM external Ca^{2+} perfusion in the absence ((i) and (iv)) or the presence (0.3 μM, (ii); 1 μM, (iii)) of No. 7943. (b) Concentration-response curve of No. 7943 and the net outward Na^+/Ca^{2+} exchange current measured at +50 mV. The amplitude of the outward Na^+/Ca^{2+} current in the presence of No. 7943 was expressed as % of the control current. Each point represents the mean±s.e.mean with the number of data indicated in parentheses.

Effect of No. 7943 on the inward Na^+/Ca^{2+} exchange current

Figure 4a depicts typical *I–V* curves showing the effect of No. 7943 on the inward Na^+/Ca^{2+} exchange current. The control external solution contained 140 mM Li^+ and 1 mM Ca^{2+} without Na^+ (solution 4). The pipette solution contained 130 mM Cs^+ without Na^+ and 231 nM free Ca^{2+} (solution 6). The inward Na^+/Ca^{2+} exchange current was induced by changing the external solution from 140 mM Li^+ to 140 mM Na^+ (solution 3) for about 15 s (Figure 4a(i)). After the *I–V* curve returned to the original level, the external solution was changed to one containing No. 7943 at 10 μM. After about 3 min of No. 7943 superfusion, the external solution was changed from 140 mM Li^+ to 140 mM Na^+ again in the presence of No. 7943. The inward Na^+/Ca^{2+} exchange current was suppressed by about 30% of the control (Figure 4a(ii)). When 30 μM No. 7943 was applied, the inward exchange current was completely suppressed in this cell (Figure 4a(iii)). The current inhibited by No. 7943 recovered after 5 min of washing out the drug (data not shown). The inhibition of No. 7943 on the inward Na^+/Ca^{2+} exchange current at −100 mV was concentration-dependent with IC_{50} of approximately 17 μM. The concentration-response relation was also plotted using the data measured at −50 mV, but the IC_{50} value of No. 7943 was similar to that measured at −100 mV (data not shown), indicating again that the inhibitory effect of No. 7943 is not voltage-dependent.

Mode of inhibition of the outward Na^+/Ca^{2+} exchange current by No. 7943

We next investigated the mode of inhibition of the outward Na^+/Ca^{2+} exchange current by No. 7943. The inhibition pattern, i.e. competitive, noncompetitive or uncompetitive, was assessed as a function of external Ca^{2+} concentration by determining the values of the apparent maximum current density (I_{max}) and the apparent K_m value of $[Ca^{2+}]_o$ (K_mCa^{2+}). As shown in Figure 5, the concentration-response relations between $[Ca^{2+}]_o$ and the outward exchange current were obtained in the absence and presence of No. 7943 at 0.3 or 1 μM under the following conditions. $[Ca^{2+}]_i$, $[Na^+]_o$ and $[Na^+]_i$ were set at constant concentrations of 96 nM, 140 and 20 mM, respectively, using solutions 3 (external) and 5 (internal), while $[Ca^{2+}]_o$ was changed to six different concentrations between 0.15 and 3 mM. To maintain $[Ca^{2+}]_i$ constant, the membrane potential was held at a calculated equilibrium potential of the Na^+/Ca^{2+} exchange current (E_{NaCa}), where there would be no net flow of the exchange current at any $[Ca^{2+}]_o$. Thus the holding potential was set at E_{NaCa} of −40, −72, −91, −107, −114 and −122 mV at 0.15, 0.5, 1, 1.5, 2 and 3 mM $[Ca^{2+}]_o$, respectively. This time $[Ca^{2+}]_o$ was raised stepwise and each $[Ca^{2+}]_o$ was perfused for about 1 min. The ramp pulse was given every 10 s to record the steady state exchange current, and whenever $[Ca^{2+}]_o$ was changed, the holding potential was adjusted to the corresponding E_{NaCa} at each $[Ca^{2+}]_o$.

Figure 5a superimposes the *I–V* curves obtained at the six different $[Ca^{2+}]_o$ in the absence (i) of No. 7943, at 0.3 μM (ii) and at 1 μM (iii). The concentration-dependent suppression of the exchange current by No. 7943 can be seen clearly. The current magnitude was measured at the potentials 50 mV more positive to E_{NaCa} at each $[Ca^{2+}]_o$, and the magnitude of the net exchange current was obtained by subtracting the current at 0.15 mM $[Ca^{2+}]_o$ from the currents at each higher $[Ca^{2+}]_o$. Each value was converted to the current density by dividing with the capacitance of the cell. Similar experiments were repeated at least in 3 cells for each $[Ca^{2+}]_o$ and the mean values are plotted in Figure 5b. The I_{max} and K_mCa^{2+} values were obtained by directly fitting the data by computer using the

Marquardt method. The I_{max} values were 5.42 ± 0.66 ($n=5$), 5.56 ± 1.14 ($n=3$) and 5.31 ± 1.00 ($n=3$) at the control, 0.3 and at 1 μM No. 7943, respectively. The I_{max} values were not significantly altered by No. 7943 statistically (one-way ANOVA). The $K_m Ca^{2+}$ values were 1.30 ± 0.36 ($n=5$), 1.83 ± 0.75 ($n=3$) and 8.49 ± 1.86 mM ($n=3$) at the control, 0.3 and at 1 μM

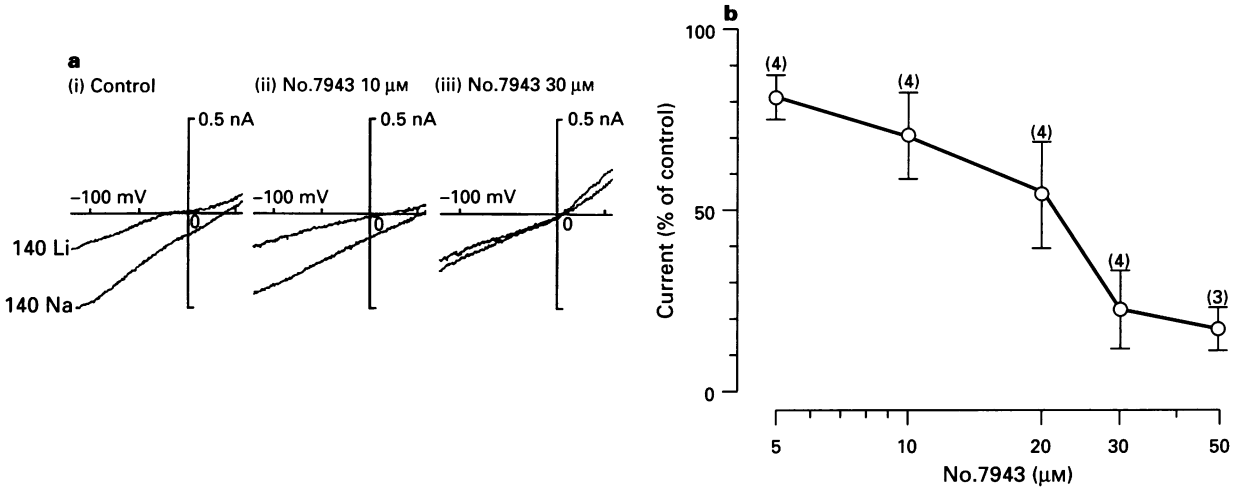

Figure 4 Effect of No. 7943 on the inward Na^+/Ca^{2+} exchange current. (a) *I-V* curves of the control in 140 mM Li^+ external solution (140 Li) and the inward Na^+/Ca^{2+} exchange current obtained in 140 mM Na^+ (140 Na) in the absence (i) and presence of No. 7943 (10 μM, (ii); 30 μM, (iii)). (b) Concentration-response curve of No. 7943 (5, 10, 20, 30 and 50 μM) and the net inward Na^+/Ca^{2+} exchage current measured at −100 mV. The amplitude of the inward Na^+/Ca^{2+} exchange current in the presence of No. 7943 was expressed as % of the control current. Points represent the mean ± s.e.mean with the number of data shown in parentheses.

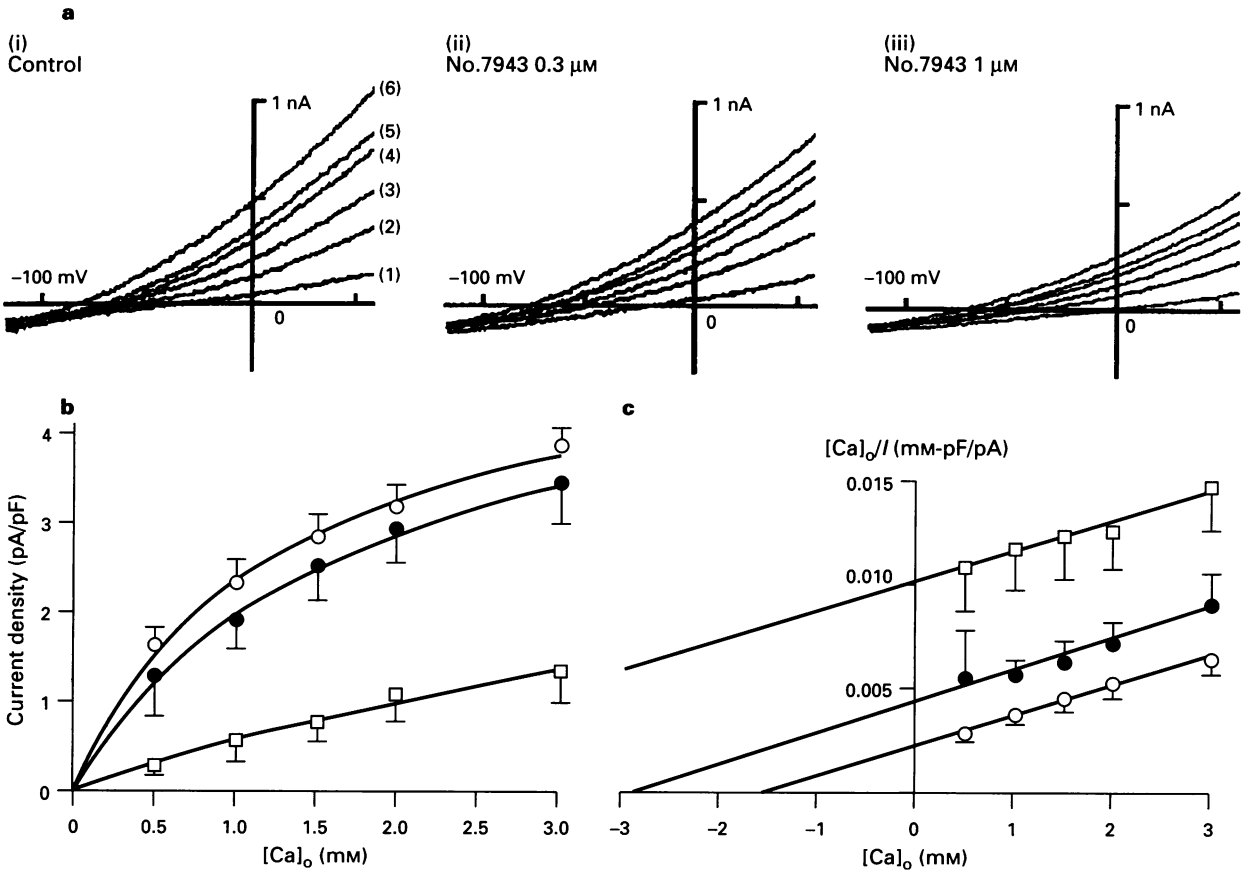

Figure 5 Effect of varying external Ca^{2+} on outward Na^+/Ca^{2+} exchange current in the absence and presence of No. 7943 (a) *I-V* curves of the outward Na^+/Ca^{2+} exchange current induced by (1) 0.15, (2) 0.5, (3) 1, (4) 1.5, (5) 2 and (6) 3 mM $[Ca^{2+}]_o$ in the absence (i) and the presence (0.3 μM (ii) and 1 μM (iii)) of No. 7943. The holding potential was adjusted to E_{rev} at each $[Ca^{2+}]_o$ as described in the text. The external solution 3 and the internal solution 5 were used. (b) Concentration-response curves between $[Ca^{2+}]_o$ and the net outward exchange current measured at 50 mV more positive to each E_{NaCa} in the absence (○, $n=5$ at each $[Ca^{2+}]_o$) and in the presence (0.3 μM, ●, $n=3$ each; 1 μM, □, $n=3$ each) of No. 7943. The curves were fitted by a computer programme 'Kotaro' (Sankaido, Japan) with Marquardt methods. Correlation coefficients (R) > 0.84 for all curves. (c) Hanes-Woolf plots of the data in (b). The lines were drawn by eye.

No. 7943, respectively. The K_mCa^{2+} values were shifted statistically significantly toward higher values by the drug (one-way ANOVA). These results suggest that No. 7943 is a competitive antagonist with respect to $[Ca^{2+}]_o$ for the outward Na^+/Ca^{2+} exchange current. To illustrate the mode of inhibition more clearly, a Hanes-Wolf plot ($[Ca^{2+}]_o/i$ versus $[Ca^{2+}]_o$) of the data was employed as shown in Figure 5c. The three fitted lines appear to be parallel, suggesting that the mode of inhibition of No. 7943 is competitive with respect to $[Ca^{2+}]_o$.

Effect of DCB on the Na^+/Ca^{2+} exchange current

DCB has been reported to inhibit the Na^+/Ca^{2+} exchanger (Siegl *et al.*, 1984; Kaczorowski *et al.*, 1985; Kleyman & Cragoe, 1988; Murata *et al.*, 1995); we therefore compared the effects of DCB and No. 7943. Figure 6a shows a set of representative data of the effect of DCB on the inward Na^+/Ca^{2+} exchange current. The experimental conditions were the same as those used for the effect of No. 7943 on the inward Na^+/Ca^{2+} exchange current. When the external solution was changed to one containing DCB at 10 μM for about 3 min, the inward Na^+/Ca^{2+} exchange current was suppressed by approximately 30% (Figure 6a(ii)). When 30 μM DCB was applied, suppression of the exchange current was almost complete (Figure 6a(iii)). The current inhibited by DCB recovered after 5 min of washing out the drug (data not shown). A concentration-response relation between the net inward exchange current measured at -100 mV and the concentration of DCB is shown in Figure 6c (open circles). The inhibition of DCB on the inward Na^+/Ca^{2+} exchange current was concentration dependent with IC_{50} of approximately 17 μM.

Figure 6b shows the effect of DCB on the outward Na^+/Ca^{2+} exchange current. The experimental conditions were the same as those used for the effect of No. 7943 on the outward Na^+/Ca^{2+} exchange current. After the external solution was changed to one containing DCB at 10 or 30 μM for about 3 min, the outward Na^+/Ca^{2+} exchange current was not significantly changed (Figure 6b(ii)). The concentration-response relation between the net outward exchange current measured at $+50$ mV and DCB was also plotted in Figure 6c (filled circles). DCB inhibited the inward exchange current but not the outward Na^+/Ca^{2+} exchange current in the concentration range between 10 and 30 μM DCB.

Effect of No. 7943 on various other currents

We also tested the effect of No. 7943 on the membrane currents other than the Na^+/Ca^{2+} exchange current, such as the Na^+ current, Ca^{2+} current and the inward rectifier K^+ current. Figure 2D and E illustrates the basic experiments to verify the Na^+ and Ca^{2+} currents in response to ramp pulses. The external solution was Tyrode solution (solution 1) containing 3 mM Cs^+ to block the inward rectifier K^+ current. The pipette solution contained 120 mM Cs^+ (solution 5). The ramp protocol was the same as described in Figure 2A and the depolarizing ramp currents were plotted. The Na^+ current was induced by the depolarizing ramp pulse over the potential range negative to -40 mV and the Ca^{2+} current at the potentials positive to -40 mV. The Na^+ current was blocked by 10 μM TTX reversibly (Figure 2D), and the Ca^{2+} current by 3 μM nifedipine (Figure 2E).

Figure 7a shows the effects of No. 7943 on the Na^+ and Ca^{2+} currents activated by ramp pulses. After recording the control *I-V* curve (Figure 7a(i)), the external solution was changed to one containing No. 7943 at 1 μM. Immediately after application of No. 7943, the Na^+ and Ca^{2+} currents started to diminish and reached a steady state within about 3 min. (Figure 7a(ii)). Subsequent application of 10 and 50 μM No. 7943 further suppressed the currents (Figure 7a(iii) and (iv)). Both the currents recovered after 5 min of washing out the drug (Figure 7a(v),). The concentration-response relation of the Na^+ current and the Ca^{2+} current obtained by ramp

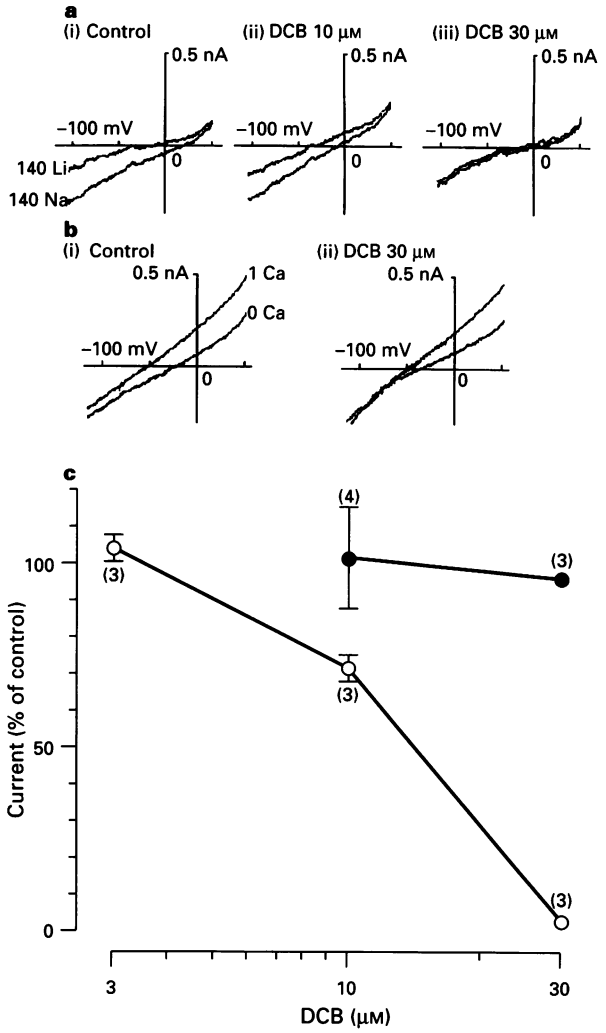

Figure 6 Effect of DCB on the inward and the outward Na^+/Ca^{2+} exchange current. (a) *I-V* curves of the control in 140 mM Li^+ external solution (140 Li) and the inward Na^+/Ca^{2+} exchange current obtained in 140 mM Na^+ (140 Na) in the absence (i) and the presence of DCB. Concentrations of DCB are indicated above. (b) *I-V* curves of the outward exchange current obtained before (0 Ca) and during 1 mM Ca^{2+} superfusion in the absence (i) and in the presence (ii) of 30 μM DCB. (c) Concentration-response curves of DCB (3, 10 and 30 μM) and the net inward exchange current measured at -100 mV (○) and the net outward exchange current measured at $+50$ mV (●). The amplitude of the currents in the presence of DCB were expressed as % of the control currents. Each symbol represents the mean ± s.e. mean.

pulses were plotted in Figure 7d. The inhibition of the Na^+ current (△) and Ca^{2+} current (□) by No. 7943 was concentration-dependent. The IC_{50} values of No. 7943 for the Na^+ and Ca^{2+} currents were approximately 14 and 8 μM, respectively. The inhibition of the Ca^{2+} current was also checked by square pulses. The Ca^{2+} current was activated by depolarizing step pulses of 200 ms duration from the holding potential of -40 mV to various potentials. Figure 7b shows the representative traces of the Ca^{2+} current at $+10$ mV in the absence and presence of No. 7943 at 10 μM. The peak and the sustained components were suppressed by the drug. On average, the peak of the Ca^{2+} current was reduced to 79% ($n=3$) by 10 μM No. 7943.

Figure 7c shows the effect of No. 7943 on the inward rectifier K^+ current. The holding potential was -40 mV. The control external solution was Tyrode solution (solution 1) containing 3 μM nifedipine. The pipette solution was the same (solution 5) as above. The inward rectifier K^+ current was recorded from the descending limb of the ramp pulse (Figure 7c). After recording the control, the external solution was

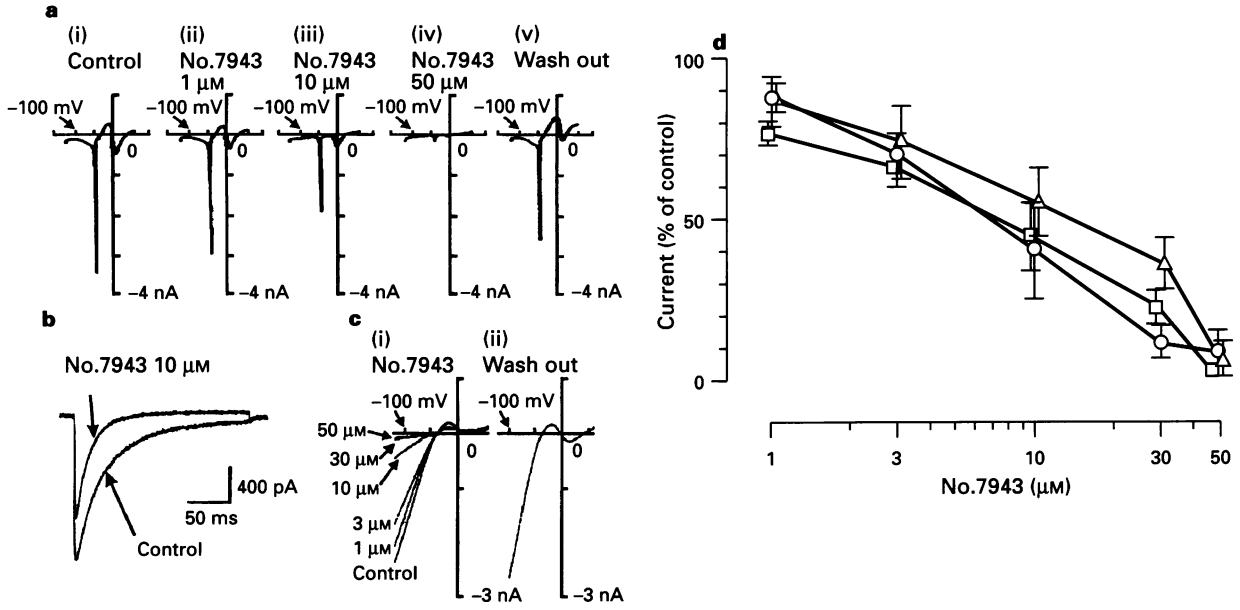

Figure 7 (a) Effect of No. 7943 on the voltage-gated Na^+ and Ca^{2+} currents obtained by ramp pulses. The concentrations of No. 7943 are indicated above each panel. (b) Effect of No. 7943 on the Ca^{2+} current obtained by depolarizing square pulses from −40 mV to +10 mV for 200 ms in the absence (control) and in the presence of No. 7943 10 μM. (c) Effect of No. 7943 on the inward rectifier K^+ current. *I-V* curves before the drug application (control) and in the presence of No. 7943 are superimposed (i); (ii) is after washing out the drug. (d) Concentration-response curves between No. 7943 and the Na^+ current peak values (△, $n=5$ at each concentration), the Ca^{2+} current peaks (□, $n=4$ each) and the inward rectifier K^+ current measured at −100 mV (○, $n=5$ each). Each symbol represents the mean ± s.e.mean.

changed to one containing No. 7943 at 1 μM. After about 3 min of No. 7943 superfusion, the inward rectifier K^+ current was suppressed (Figure 7c(i)). When 3, 10, 30 and 50 μM No. 7943 were applied cumulatively, suppression of the current became progressively more potent (Figure 7c(i)). The K^+ current recovered after 5 min of washing out the drug (Figure 7c(ii)). The magnitude of the inward rectifier K^+ current was measured at −100 mV and the concentration-response curve was superimposed in Figure 7d (○). The IC_{50} value of No. 7943 for the K^+ current was approximately 7 μM.

Discussion

We found that No. 7943 reversibly suppressed the outward Na^+/Ca^{2+} exchange current in a concentration-dependent manner with an IC_{50} of 0.32 μM. Suppression of the inward Na^+/Ca^{2+} exchange current by this drug, however, was significantly less potent compared to that of the outward exchange current and the IC_{50} was 17 μM. No. 7943 also inhibited the voltage-gated Na^+ and Ca^{2+} currents and the inward rectifier K^+ current. The IC_{50} values of these effects were similar to that for the inward Na^+/Ca^{2+} exchange current i.e., 14, 8 and 7 μM, respectively and were 20 to 50 fold larger than that for the outward Na^+/Ca^{2+} exchange current. Thus, No. 7943 inhibits the outward exchange current (or the reverse mode) more potently than any other currents investigated.

Effects of the drug on the Na^+ current and the Ca^{2+} current were investigated with ramp pulses in the present study. Since both the Na^+ and Ca^{2+} currents have time-dependent activation and inactivation kinetics, ramp pulses may perturb the channel activity by steadily changing the driving force and the number of conducting channels simultaneously. Thus the concentration-response curve obtained by ramp pulses should be considered as a rough estimation, which though in the present study is sufficient to show that there is a concentration-range of No. 7943 which affects the exchange current exclusively. Since the ramp pulse result may not produce the same result as that obtained by the step pulses, we checked this point. The mean decrease of the Ca^{2+} current measured by ramp pulses was to 43% ($n=3$) of the control at 10 μM No. 7943 (Figure 3), while that measured by the square pulses was to 79% ($n=3$) (Figure 7b). This discrepancy may have derived at least partly from the methods, because when the reduction of the Ca^{2+} current was measured at 20 ms later than the peak during the step pulse, the value became 50% in average ($n=3$). Thus there is a possibility that the IC_{50} for the Ca^{2+} current obtained by the ramp pulse protocol was underestimated. If so, No. 7943 becomes an even more selective blocker of the outward exchange current compared to the Ca^{2+} current.

When the mode of inhibition of the drug was examined, No. 7943 shifted the K_mCa^{2+} value from the control of 1.3 mM to 1.8 mM (1.4 fold) at 0.3 μM, or to 8.5 mM (6.5 fold) at 1 μM. However, the drug did not significantly change the I_{max} values. These results suggest that No. 7943 inhibits the outward Na^+/Ca^{2+} exchanger competitively with respect to external Ca^{2+}.

In the present study, we confirmed that DCB inhibits the inward Na^+/Ca^{2+} exchange current with an IC_{50} of approximately 17 μM. However, to our surprise, DCB did not affect the outward Na^+/Ca^{2+} current even at 30 μM, a concentration that inhibited the inward exchange current completely. In frog atrial cells, DCB inhibited both directions of the exchange current with a lower IC_{50} of 0.4 μM (Bielefeld *et al.*, 1986). In mammalian cardiac membrane vesicles, Na^+-dependent Ca^{2+} efflux was preferentially blocked by DCB and thus DCB was reported to be a competitive inhibitor with Na^+ (Siegl *et al.*, 1984; Slaughter *et al.*, 1988). That the inhibitory effects of DCB are more potent on the inward rather than the outward Na^+/Ca^{2+} exchange current is opposite to the results with No. 7943.

The Na^+/Ca^{2+} exchanger molecule has been cloned (Nicoll *et al.*, 1990), but the binding sites for external Na^+ and Ca^{2+} have not been identified. It has been proposed that the exchanger contains two classes of cation binding sites, a divalent site which can bind either one Ca^{2+} or two Na^+ and a monovalent site which binds only one Na^+ but not Ca^{2+} (Blaustein & Russell, 1975; Reeves & Sutko, 1983). The exchanger is most likely to operate with the ping-pong (or consecutive) kinetics rather than with the simultaneous kinetics

(Khananshvili 1991; Niggli & Lederer, 1991; Li & Kimura, 1991). Thus the conformation of the external site may change from one configuration which has a higher affinity for Ca^{2+} to another with a higher affinity for Na^+. This hypothesis can be supported by the evidence that No. 7943 and DCB inhibit the different modes of exchange.

Different degrees of inhibition with regard to the Ca^{2+} site and the Na^+ site have also been reported for some other drugs. Bepridil caused partial inhibition of Na^+_i-dependent Ca^{2+} uptake but complete block of Na^+_0-dependent Ca^{2+} efflux (Garcia *et al.*, 1988). Bepridil is non-competitive with Ca^{2+} but competitive with Na^+ and thus it is considered to interact at a site which binds Na^+ but not Ca^{2+}. External Mg^{2+} inhibits the outward exchange current more effectively than the inward exchange current, indicating that Mg^{2+} interacts preferentially with the Ca^{2+} binding (Kimura, 1996). On the other hand, inhibition of quinaqurine is similar to amiloride derivatives (De La Peña & Reeves, 1987). This evidence lends further support to the idea that two distinct binding sites or configurations exist in the Na^+/Ca^{2+} exchanger molecule.

We also attempted to apply No. 7943 intracellularly by the pipette solution. No. 7943 at 50 μM in the pipette solution did not prevent the development of the inward or the outward exchange current. The exchange current was not apparently different from the control without No. 7943 in the pipette solution. We cannot exclude the possibility that No. 7943 did not sufficiently diffuse into the cell interior, because of its relatively large molecular weight of 427.5. However considering its K_i value of 0.32 μM for the external Ca site and that we waited for 20 min after establishing the whole cell mode, sufficient current appeared in both outward and inward mode at 50 μM in the pipette led us to conclude that No. 7943 does not affect, or is much less potent from the intracellular side.

It has been hypothesized that cardiac ischaemia causes intracellular acidosis due to anaerobic metabolism (Steenbergen *et al.*, 1977; Allen & Orchard, 1987) and ATP hydrolysis (Dennis *et al.*, 1991). As a consequence, the Na^+/H^+ exchange system is activated and causes Na^+ influx and H^+ efflux (Frelin *et al.*, 1984; Poole-Wilson, 1989). A rise in intracellular Na^+ concentration reduces the Ca^{2+} efflux via the forward Na^+/Ca^{2+} exchange and/or induces Ca^{2+} influx through the reverse mode of the Na^+/Ca^{2+} exchange depending on the Na^+ gradient and the membrane voltage. The Ca^{2+} overload caused by these mechanisms would induce cardiac arrhythmia (Coetzee & Opie, 1987), stunning (Kusuoka *et al.*, 1993) and necrosis during cardiac ischaemia and reperfusion. Cell swelling, which occurs under these conditions, also has an effect on Na^+/Ca^{2+} exchanger current (Wright *et al.*, 1995). If these are the major mechanisms, and if a more specific Na^+/Ca^{2+} exchange inhibitor could be developed from No. 7943, it may be possible to prevent cardiac ischaemia and reperfusion injury by inhibiting the Ca^{2+} influx via the Na^+/Ca^{2+} exchanger.

In conclusion, No. 7943 is a potent antagonist of the outward Na^+/Ca^{2+} exchange current and its blocking mechanism may involve competition with external Ca^{2+}. This is an opposite characteristic to DCB which suppresses the inward Na^+/Ca^{2+} exchange current preferentially. No. 7943 may be a useful tool to investigate the Na^+/Ca^{2+} exchanger.

The authors wish to thank Dr K. Iuchi, and Dr T. Sukamoto of the New Drug Research Laboratories of Kanebo Co., Ltd., for providing T. W. with an opportunity to study at Fukushima Medical College. Ms Sanae Sato's technical assistance is highly appreciated. This work was supported by a grant-in-aid for scientific research (07307025) from the ministry of Education, Science and Culture, Japan.

References

ALLEN, D.G., CAIRNS, S.P., TURVEY, S.E. & LEE, J.A. (1993). Intracellular calcium and myocardial function during ischemia. *Adv. Exp. Med. Biol.*, **346**, 19–29.

ALLEN, D.G., & ORCHARD, C.H. (1987). Myocardial contractile function during ischemia and hypoxia. *Circ. Res.*, **60**, 153–168.

BERSOHN, M.M., PHILIPSON, K.D. & WEIS, R.S. (1991). Lysophosphatidylcholine and sodium-calcium exchange in cardiac sarcolemma: comparison with ischemia. *Am. J. Physiol.*, **260**, C433–C438.

BIELEFELD, D.R., HADLEY, R.W., VASSILEV, P.M. & HUME, J.R. (1986). Membrane electrical properties of vesicular Na-Ca exchange Inhibitors in single atrial myocytes. *Circ. Res.*, **59**, 381–389.

BLAUSTEIN, M.P. & RUSSELL, J.M. (1975). Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *J. Membr. Biol.*, **22**, 285–312.

CHIN, T.K., SPITZER, K.W., PHILIPSON, K.D. & BRIDGE, J.H.B. (1993). The effect of exchanger inhibitory peptide (XIP) on sodium-calcium exchange current in guinea pig ventricular cells. *Circ. Res.*, **72**, 497–503.

COETZEE, W.A. & OPIE, L.H. (1987). Effects of components of ischemia and metabolic inhibition on delayed after depolarizations in guinea pig papillary muscle. *Circ. Res.*, **61**, 157–165.

DE LA PEÑA, P. & REEVES, J.P. (1987). Inhibition and activation of Na^+-Ca^{2+} exchange activity by quinacrine. *Am. J. Physiol.*, **252**, C24–C29.

DENNIS, S.C., GEVERS, W. & OPIE, L.H. (1991). Protons in ischemia: where do they come from; where do they go to? *J. Mol. Cell. Cardiol.*, **23**, 1077–1086.

FRELIN, C., VIGNE, P. & LAZDUNSKI, M. (1984). The role of the Na^+/H^+ exchange system in cardiac cells in relation to the control of the internal Na^+ concentration. *J. Biol. Chem.*, **259**, 8880–8885.

GARCIA, M.L., SLAUGHTER, R.S., KING, V.F. & KACZOROWSKI, G.J. (1988). Inhibition of sodium-calcium exchange in cardiac sarcolemmal membrane vesicles. 2. Mechanisms of inhibition by bepridil. *Biochemistry*, **27**, 2410–2415.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

KACZOROWSKI, G.J., BARROS, F., DETHMERS, J.K., TRUMBLE, M.J. & CRAGOE, E.J. Jr. (1985). Inhibition of Na^+/Ca^{2+} exchange in pituitary plasma membrane vesicles by analogues of amiloride. *Biochemistry*, **24**, 1394–1403.

KACZOROWSKI, G.J., GARCIA, M.L., KING, R. & SLAUGHTER, R.S. (1989). Development and use of inhibitors to study sodium-calcium exchange. In *Sodium-Calcium Exchange*. ed. Allen, F.J., Noble, D., & Reuter, H., pp. 66–101. Oxford: University Press.

KHANANSHVILI, D. (1991). Voltage-dependent modulation of ion binding and translocation in the cardiac Na^+-Ca^{2+} exchange system. *J. Biol. Chem.*, **266**, 13764–13769.

KIMURA, J. (1996). Effect of external Mg^{2+} on the Na^+-Ca^{2+} exchange current in guinea-pig cardiac myocytes. *Ann. N. Y. Acad. Sci.*, **119**, 515–520.

KIMURA, J., MIYAMAE, S. & NOMA, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J. Physiol.*, **384**, 199–222.

KLEYMAN, T.R. & CRAGOE, E.J. Jr. (1988). Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.*, **105**, 1–21.

KUSUOKA, H., CAMILION DE HURATADA, M.C. & MARBAN, E. (1993). Role of Sodium/Calcium exchange in the mechanisms of myocardial stunning: Protective effect of reperfusion with high sodium solution. *J. Am. Coll. Cardiol.*, **21**, 240–248.

NIGGLI, E., & LEDERER, W.J. (1991). Molecular operations of the sodium-calcium exchanger revealed by conformation currents. *Nature*, **349**, 621–624.

LI, J.M. & KIMURA, J. (1991). Translocation mechanism of cardiac Na-Ca exchange. *Ann. N. Y. Acad. Sci.*, **639**, 48–60.

LI, Z., NICOLL, D.A., COLLINS, A., HILGEMANN, D.W., FILOTEO, A.G., PENNISTON, J.T., WEISS, J.N., TOMICH, J.M. & PHILIPSON, K.D. (1991). Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J. Biol. Chem.*, **266,** 1014–1020.

MIURA, Y. & KIMURA, J. (1989). Sodium-Calcium exchange current: Dependence on internal Ca^{2+} and competitive binding of external Na^+ and Ca^{2+}. *J. Gen. Physiol.*, **93,** 1129–1145.

MURATA, Y., HARADA, K., NAKAJIMA, F., MARUO, J. & MORITA, T. (1995). Non-selective effects of amiloride and its analogues on ion transport systems and their cytotoxicities in cardiac myocytes. *Jpn. J. Pharmacol.*, **68,** 279–285.

NICOLL, D.A., LONGONI, S. & PHILIPSON, K.D. (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science*, **250,** 562–565.

NIGGLI, E. & LEDERER, W.J. (1991). Molecular operations of the sodium-calcium exchanger revealed by conformation currents. *Nature*, **349,** 621–624.

PHILIPSON, K.D. (1984). Interaction of charged amphiphiles with Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.*, **259,** 13999–14002.

POOLE-WILSON, P.A. (1989). Regulation of intracellular pH in the myocardium; relevance to pathology. *Mol. Cell Biochem.*, **89,** 151–155.

REEVES, J.P. & SUTKO, J.L. (1983). Competitive interactions of sodium and calcium with the sodium-calcium exchange system of cardiac sarcolemmal vesicles. *J. Biol. Chem.*, **258,** 3178–3182.

SCHOLZ, W., ALBUS, U., LANG, H.J., LINZ, W., MARTORANA, P.A., ENGLERT, H.C. & SCHOLKENS, B.A. (1993). Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. *Br. J. Pharmacol.*, **109,** 562–568.

SIEGL, P.K., CRAGOE, E.J. Jr., TRUMBLE, M.J. & KACZOROWSKI, G.J. (1984). Inhibition of Na^+/Ca^{2+} exchange in membrane vesicle and papillary muscle preparations from guinea pig heart by analogs of amiloride. *Proc. Natl. Acad. Sci. U.S.A.*, **81,** 3238–3242.

SLAUGHTER, R.S., GARCIA, M.L., CRAGOE, E.J.Jr., REEVES, J.P. & KACZOROWSKI, G.J. (1988). Inhibition of sodium-calcium exchange in cardiac sarcolemmal membrane vesicles. 1. Mechanism of inhibition by amiloride analogues. *Biochemistry*, **27,** 2403–2409.

STEENBERGEN, C., DELEEUW, G., RICH, T. & WILLIAMSON, J.R. (1977). Effects of acidosis and ischemia on contractility and intracellular pH of rat heart. *Circ. Res.*, **41,** 849–858.

SUAREZ-KURTZ, G. & KACZOROWSKI, G.J. (1988). Effects of dichlorobenzamil on Ca currents in clonal GH_3 pituitary cells. *J. Pharmacol. Exp. Ther.*, **247,** 248–253.

TROSPER, T.L. & PHILIPSON, K. (1983). Effects of divalent and trivalent cations on Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles. *Biochem. Biophys. Acta.*, **731,** 63–68.

VER DONCK, L., MARCEL, B. & VERDONCK, F. (1993). Inhibition of sodium and calcium overload pathology in the myocardium: a new cytoprotective principle. *Cardiovasc. Res.*, **27,** 349–357.

WRIGHT, A.R., REES, S.A., VANDENVERG, J.I., TEIST, V.W. & POWELL, T. (1995) Extracellular osmotic pressure modulated sodium-calcium exchange in isolated guinea-pig ventricular myocytes. *J. Physiol.*, **488,** 293–301.

(Received January 3, 1996
Revised June 10, 1996
Accepted June 19, 1996)