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SPECIAL REPORT

Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents

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The effects of 2-[4-[(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline (SEA0400), a newly synthesized Na^+-Ca^{2+} exchanger (NCX) inhibitor, on the NCX current and other membrane currents were examined in isolated guinea-pig ventricular myocytes and compared with those of 2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea (KB-R7943). SEA0400 concentration-dependently inhibited the NCX current with a 10 fold higher potency than that of KB-R7943; 1 μM SEA0400 and 10 μM KB-R7943 inhibited the NCX current by more than 80%. KB-R7943, at 10 μM, inhibited the sodium current, L-type calcium current, delayed rectifier potassium current and inwardly rectifying potassium current by more than 50%, but SEA0400 (1 μM) had no significant effect on these currents. These results indicate that SEA0400 is a potent and highly selective inhibitor of NCX, and would be a powerful tool for further studies on the role of NCX in the heart and the therapeutic potential of its inhibition.
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Keywords: SEA0400; KB-R7943; sodium-calcium exchanger; heart

Abbreviations: KB-R7943, 2-[2-4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea; NCX, Na^+-Ca^{2+} exchanger; SEA0400, 2-[4-[(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline

Introduction The Na^+-Ca^{2+} exchanger (NCX) is involved in myocardial Ca^{2+} regulation; its major role being Ca^{2+} extrusion from the cytoplasm during diastole (reviewed by Bers, 2000). It is also considered to be the major pathway for the influx of Ca^{2+} on reperfusion after myocardial ischemia (Tani, 1990). Some researchers postulate that, during early systole, Ca^{2+} influx occurs through the NCX acting in the reverse mode, which might contribute to triggering of Ca^{2+} release from the sarcoplasmic reticulum (Levi *et al*., 1993). Specific pharmacological agents to modify NCX activity would be a powerful tool for studies on its role in myocardial Ca^{2+} handling. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea), an isothiourea compound, was considered to be the most selective non-peptide inhibitor of NCX and has been used in various studies (reviewed by Shigekawa & Iwamoto, 2001) including those on the myocardium (Watano *et al*., 1996). However, as KB-R7943 is known to affect various proteins at concentrations used to inhibit NCX, an NCX inhibitor with higher specificity is desired. SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-ethoxyaniline; Figure 1) is a newly synthesized compound which was shown to be a potent and selective inhibitor of NCX in cultured neurons, astrocytes and microglia (Matsuda *et al*., 2001). It had negligible affinities towards other transporters, ion channels and receptors. Thus, in the present study, we examined the effects of SEA0400 and KB-R7943 on the myocardial NCX using voltage clamped guinea-pig ventricular myocytes. We found that SEA0400 inhibits myocardial NCX current with 10 fold higher potency than KB-R7943, and that the compound has virtually no effect on Na^+, Ca^{2+} and K^+ channels.

Methods All experiments were performed in accordance to the 'Guiding Principles for the Care and Use of Laboratory Animals' approved by The Japanese Pharmacological Society. Isolated ventricular cardiomyocytes were obtained from male guinea-pig hearts by Langendorff perfusion and collagenase treatment as described previously (Kato *et al*., 1996), and membrane currents were recorded with standard whole cell voltage clamp techniques as described previously (Nishimaru *et al*., 2001). The composition of external and internal solutions is listed in Table 1 and the maximum value of pipette resistance was 2 MΩ for NCX current, 1 MΩ for Na^+ current and 3 MΩ for other currents. For NCX current, the holding potential was set to −30 mV and ramp voltage-clamp pulses with a speed of $\pm$180 mV s^{-1} ranging from −120 mV to +60 mV were applied at 0.1 Hz. NCX current was measured as the current sensitive to 5 mM Ni^{2+} as previously described (Kimura *et al*., 1987; Nishimaru *et al*., 2001). EC_{50} values were calculated by interpolation between the two data points close to 50% inhibition. The free Ca^{2+} concentrations of the internal solution for NCX current measurement was calculated to be 153 nM using Fabiato & Fabiato (1979) equations with the modification by Tsien & Rink (1980). The Na^+ current was measured as the peak inward current on 20 ms depolarization to various membrane potentials ranging from −130 mV to 60 mV from a holding potential of −90 mV. The L-type Ca^{2+} current and inwardly rectifying K^+ current were measured as the peak inward current and steady state current elicited by 300 msec test pulses to various potentials from a holding potential of −40 mV. The delayed rectifier potassium current was

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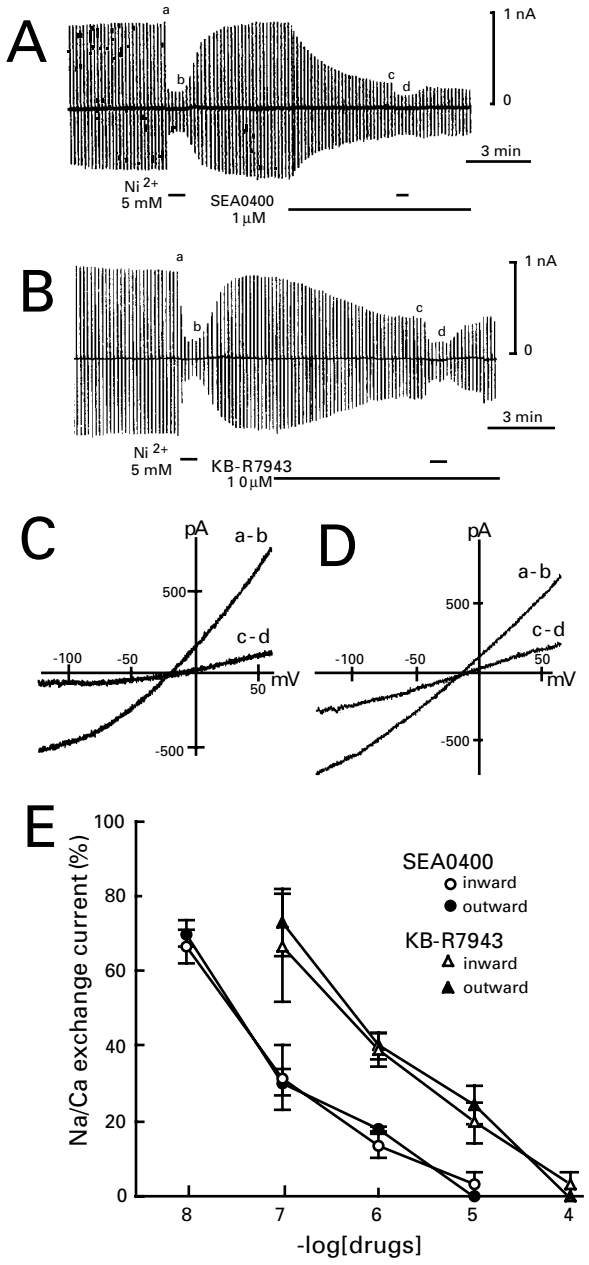

Figure 1 Effect of SEA0400 and KB-R7943 on NCX. NCX current was elicited by ramp voltage clamp pulses at a frequency of 0.1 Hz. A and B: time course of the effects of 1 mM SEA0400 (A) and 10 mM KB-R7943 (B). C and D: superimposed records of the Ni^{2+}-sensitive NCX current in the absence (a-b) and presence (c-d) of SEA0400 (C) and KB-R7943 (D). E: concentration-response relationships for the inhibitory effects of SEA0400 and KB-R7943 on inward and outward NCX current measured at −80 mV and +30 mV respectively. Symbols with bars indicate the mean±s.e.m. from three to four experiments.

measured as the peak tail current on repolarization to −30 mV after 2 s test pulses to various potentials. Measurement of Na^+ current was performed at 22–23°C and that of other currents at 35–36°C. All values are expressed as means±s.e.m. The cells used to obtain a mean value were from different animals. Statistical significance of difference between means was evaluated by the paired *t*-test. SEA0400 and KB-R7943 were provided by Taisho Pharmaceutical Company, Ltd.

Table 1 Composition of solutions for whole cell recordings (concentrations expressed in mM)

	I_{NCX}	I_{Na}	$I_{Ca\ and\ I_{K1}}$	I_K
External solutions				
NaCl	140	60	143	
choline-Cl		80		145
KCl			5	
$CaCl_2$	2	0.1	1.8	1
$MgCl_2$	2	2.5	0.5	1
CsCl	2	5		
$BaCl_2$	1			
$CoCl_2$		3		
HEPES	10	10	5	5
NaH_2PO_4	0.33	0.33	0.3	
KH_2PO_4				0.33
glucose	5.5	5.5	5.5	5.5
nisoldipine	0.002			0.002
ouabain	0.02			
atropine				0.002
Internal solution				
KOH			110	100
KCl			40	40
CsOH	160	100		
CsCl	20			
NaOH	20			
NaCl		20		
$CaCl_2$	29			
$MgCl_2$	2	1	1	1
TEA-Cl	20	20		
aspartic acid	42	80	70	70
HEPES	10	10	5	5
EGTA	42	10	10	10
CP-Na_2			5	
ATP-Mg	10	5	5	
ATP-K_2				5

The pH of external solutions were adjusted to 7.4 with NaOH. The pH of internal solution for the delayed rectifier K^+ current was adjusted to 7.2 with KOH and other internal solutions by CsOH. CP; creatine phosphate. I_{NCX}: Na^+-Ca^{2+} exchanger current, I_{Na}: Na^+ current, I_{Ca}: L-type Ca^{2+} current, I_{K1}: inwardly rectifying K^+ current, I_K: delayed rectifyer K^+ current.

Results *Effect of SEA0400 and KB-R7943 on NCX current* The NCX current was measured as the Ni^{2+}-sensitive current elicited by ramp voltage clamp pulses (Figure 1). The effect of 5 mM Ni^{2+} reached steady state in about 30 s and was completely reversed on washout. Both SEA0400 and KB-R7943 inhibited the NCX current in both directions; the inward and outward NCX currents measured at −80 mV and +30 mV, respectively, were reduced to 17.5±0.6% and 13.4±3.2%, respectively, by 1 μM SEA0400. The effects of both compounds were concentration-dependent, SEA0400 being more potent than KB-R7943; the EC_{50} value of SEA0400 for the inward and outward NCX current was 40 nM and 32 nM, respectively, and that of KB-R7943 263 nM and 457 nM, respectively.

Effect of SEA0400 and KB-R7943 on ion channel currents Effects of 1 μM SEA0400 and 10 μM KB-R7943,

which inhibited the NCX current by about 80%, on ion channel currents were examined. The Na^+ current was elicited by depolarizing pulses in the presence of nisoldipine and Cs^+ (Figure 2). SEA0400 had no effect while KB-R7943 significantly decreased the current; the peak inward Na^+ current on depolarization to -20 mV in the presence of SEA0400 and KB-R7943 was $96.0 \pm 1.1\%$ and $45.9 \pm 14.5\%$ of that in their absence, respectively. The L-type Ca^{2+} and inwardly rectifying K^+ currents were elicited by test pulses to various potentials from a holding potential of -40 mV (Figure 3). SEA0400 had no effect on the currents while KB-R7943 significantly decreased both currents. The peak inward L-type Ca^{2+} current at 10 mV in the presence of SEA0400 and KB-R7943 was $91.0 \pm 2.2\%$ and $44.8 \pm 7.5\%$ of that in their absence, respectively. The inward rectifying K^+ current at -60 mV in the presence of SEA0400 and KB-R7943 was $96.0 \pm 2.5\%$ and $6.3 \pm 4.4\%$ of that in their absence, respectively. The delayed rectifier K^+ current was not affected by SEA0400 but was significantly reduced by KB-R7943 (Figure 4). The tail current on repolarization to -30 mV from 50 mV in the presence of SEA0400 and KB-R7943 was $98.0 \pm 10.0\%$ and $26.8 \pm 1.2\%$ of that in their absence, respectively. The effects of SEA0400 (1 μM) and KB-R7943 (10 μM) were summarized in Figure 5. At 10 μM, SEA0400 inhibited the L-type Ca^{2+} current at $+10$ mV and inwardly rectifying K^+ current at -60 mV to $77.3 \pm 5.0\%$ ($n=5$) and $87.7 \pm 3.5\%$ ($n=4$), respectively. The delayed rectifier K^+ current at $+50$ mV was not affected by 10 μM SEA0400; the tail current amplitude in the presence of the drug was $102.8 \pm 4.0\%$ ($n=5$) of that in its absence.

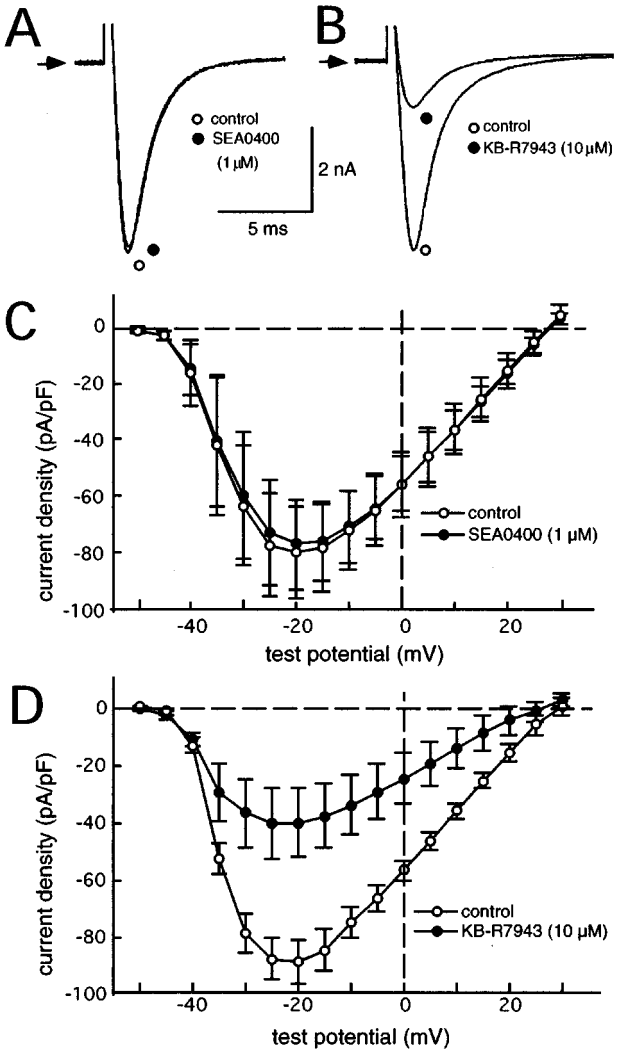

Figure 2 Effect of SEA0400 and KB-R7943 on Na^+ current. A and B: superimposed current records on depolarization to -20 mV from a holding potential of -90 mV in the absence and presence of 1 mM SEA0400 (A) and 10 mM KB-R7943 (B). Arrows indicate zero current level. C and D: current-voltage relationships in the absence and presence of SEA0400 (C) and KB-R7943 (D). Symbols with bars indicate the mean $\pm$ s.e.m. from four experiments.

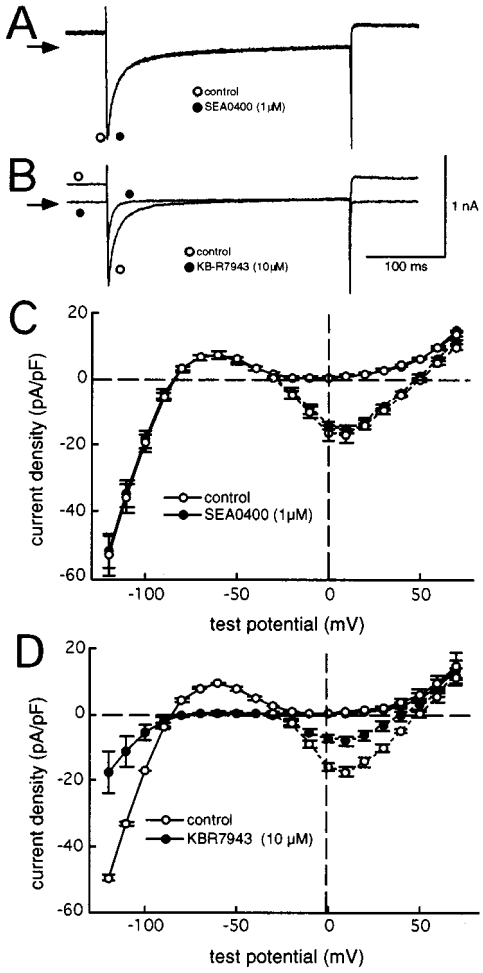

Figure 3 Effect of SEA0400 and KB-R7943 on Ca^{2+} and K^+ currents. A and B: superimposed current records on depolarization to 10 mV from a holding potential of -40 mV in the absence and presence of 1 mM SEA0400 (A) and 10 mM KB-R7943 (B). Arrows indicate zero current level. C and D: current-voltage relationships in the absence and presence of SEA0400 (C) and KB-R7943 (D). Symbols with bars indicate the mean $\pm$ s.e.m. from four experiments.

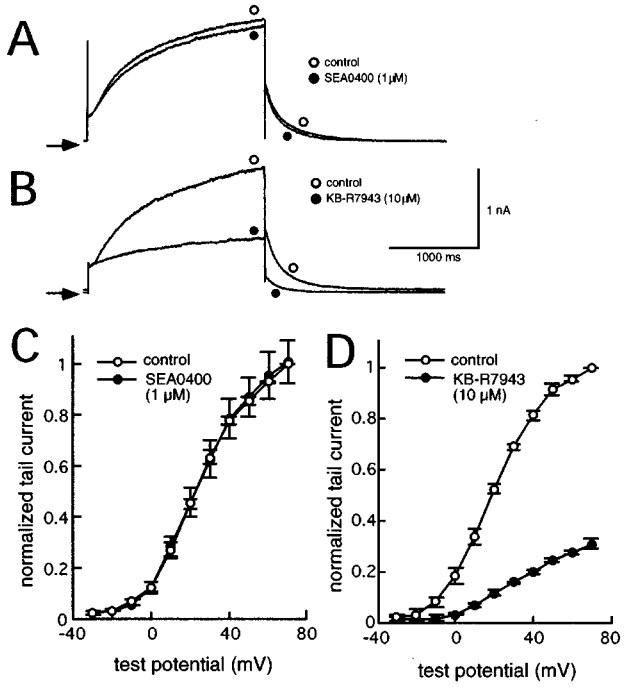

Figure 4 Effect of SEA0400 and KB-R7943 on delayed rectifier K^+ current. A and B: superimposed current records on depolarization to 50 mV and return to the holding potential of −30 mV in the absence and presence of 1 mM SEA0400 (A) and 10 mM KB-R7943 (B). Arrows indicate zero current level. C and D: current-voltage relationships for the peak tail current in the absence and presence of SEA0400 (C) and KB-R7943 (D). Symbols with bars indicate the mean ± s.e.m. from four experiments.

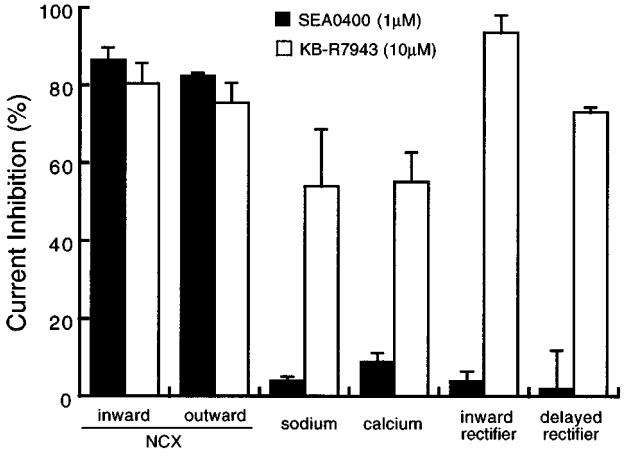

Figure 5 Summarized effects of SEA0400 and KB-R7943 on myocardial ionic currents. The inward and outward NCX currents at −80 mV and +30 mV, respectively, sodium current at −20 mV, L-type Ca^{2+} current at +10 mV, inward rectifying potassium current at +60 mV and delayed rectifier potassium current were measured as described in the text and inhibition of current amplitudes in the presence of SEA0400 (1 μM) and KB-R7943 (10 μM) were expressed as percentages of the value in the absence of compounds.

Discussion The present results demonstrated that SEA0400 is a potent inhibitor of cardiac NCX current with an EC_{50} value of 30–40 nM for both inward and outward directions (Figure 1). The potency of SEA0400 was about 10 fold higher than that of KB-R7943, which also shows direction-independent inhibition of NCX under the present experimental condition (Figure 1; Kimura *et al*., 1999). SEA0400 had no effect on the Na^+ current, L-type Ca^{2+} current, inwardly rectifying K^+ current and delayed rectifier K^+ current at 1 μM (Figures 2–5), a concentration at which NCX was inhibited by more than 80%. Although complete inhibition of NCX may be achieved with 10 μM SEA0400, caution is necessary for its use because the L-type Ca^{2+} current is also partially inhibited at this concentration. Matsuda *et al.* (2001) have reported that SEA0400 inhibits NCX in cultured neurons, astrocytes and microglia with EC_{50} values of 5–33 nM (Matsuda *et al*., 2001). They have also extensively examined binding of SEA0400 to various receptors, ion channels and transporters and found that the compound is highly selective for NCX. Thus, although its precise mechanism of action remains to be investigated, SEA0400 appears to be a powerful pharmacological tool for studies on the physiological role of NCX in the myocardium. The compound enables re-evaluation of earlier conclusions drawn from experimental results with KB-R7943, which markedly inhibits Na^+, L-type Ca^{2+} and K^+ currents, at concentrations to inhibit NCX. For example, whether transsarcolemmal Ca^{2+} influx through the NCX triggers Ca^{2+} release from the sarcoplasmic reticulum on normal myocardial contraction (Levi *et al*., 1993) could be examined in various preparations using this compound. We previously reported that α-adrenergic stimulation of adult mouse ventricular myocardium results in a negative inotropic response (Nishimaru *et al*., 2001). The proposed involvement of enhanced NCX activity in this phenomenon could also be examined with SEA0400. SEA0400 was shown to reduce infarct volumes after transient middle cerebral artery occlusion in rat cerebral cortex and striatum and attenuated Ca^{2+}-induced cell damage in cultured astrocytes (Matsuda *et al*., 2001). In the heart, Ca^{2+}-influx through the reverse-mode NCX on reperfusion after ischemia is considered to be one of the major causes of myocardial damage (Tani, 1990). Thus, it is highly possible that SEA0400 have cardioprotective effects against myocardial ischemia-reperfusion injury. In conclusion, the present study showed that SEA0400 is a potent and highly selective inhibitor of NCX, and would be a powerful tool for further studies on the role of NCX in the heart and the therapeutic potential of its inhibition.

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