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Protective effects of SEA0400, a novel and selective inhibitor of the Na^+/Ca^{2+} exchanger, on myocardial ischemia–reperfusion injuries

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Abstract

The Na^+/Ca^{2+} exchanger (NCX) is involved in myocardial ischemia–reperfusion injuries. We examined the effects of 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), a potent and selective inhibitor of NCX, on myocardial ischemia–reperfusion injury models. In canine cardiac sarcolemmal vesicles and rat cardiomyocytes, SEA0400 potently inhibited the Na^+-dependent $^{45}Ca^{2+}$ uptake with an IC_{50} value of 90 and 92 nM, compared with 2-[2-[4-(4-nitrobenzyloxy)phenyl]isothiourea (KB-R7943, 7.0 and 9.5 μM), respectively. In rat cardiomyocytes, SEA0400 (1 and 3 μM) attenuated the Ca^{2+} paradox-induced cell death. In isolated rat Langendorff hearts, SEA0400 (0.3 and 1 μM) improved the cardiac dysfunction induced by low-pressure perfusion followed by normal perfusion. In anesthetized rats, SEA0400 (0.3 and 1 mg/kg, i.v.) reduced the incidence of ventricular fibrillation and mortality induced by occlusion of the left anterior descending coronary artery followed by reperfusion. These results suggest that SEA0400 is a most potent NCX inhibitor in the heart and that it has protective effects against myocardial ischemia–reperfusion injuries.

Keywords: SEA0400; Na^+/Ca^{2+} exchanger; Myocardial injury; Ischemia; Reperfusion; Inhibitor

1. Introduction

The Na^+/Ca^{2+} exchanger (NCX) is one of the major mechanisms involved in regulating intracellular Ca^{2+} concentrations via the forward mode (Ca^{2+} extrusion) or the reverse mode (Ca^{2+} influx) in excitable cells (Hryshko and Philipson, 1997; Matsuda et al., 1997). It has been suggested that activation of the reverse mode of NCX contributes to myocardial ischemia–reperfusion injury (Tani and Neely, 1989; Cross et al., 1998). Myocardial ischemia is characterized by the ATP depletion and the intracellular acidosis. These changes lead to inactivation of the Na^+/K^+ ATPase and to activation of the Na^+/H^+ exchanger (NHE), respectively, resulting in intracellular Na^+ accumulation (Van Emous et al., 1998; Imanishi et al., 1998). During the early phase of reperfusion, the Na^+ accumulation is further accelerated by the activation of NHE, which follows washout of extracellular H^+ (Lazdunski et al., 1985). The reverse mode of NCX is then activated and intracellular Ca^{2+} overload takes place (Murphy et al., 1999). The pathological increase in intracellular Ca^{2+} concentration leads to various cell injuries (Tani, 1990). Therefore, NCX is most likely to play a crucial role in cell injuries during myocardial ischemia and reperfusion, in close relation to NHE. The involvement of NHE has been well recognized by the evidences showing the protective effects of cariporide, a typical selective NHE inhibitor, in ischemic and reperfused hearts (Scholz et al., 1995; Miura et al., 1997; Aye et al., 1997).

There were so far many reports demonstrating the beneficial effects of NCX inhibitors on myocardial ischemia–reperfusion injuries (Weiss et al., 1990; Kawada et al., 1992). However, as the NCX inhibitors used in those studies have other non-specific actions including NHE inhibition and Ca^{2+} channel blockade (Kaczorowski et al., 1989), the

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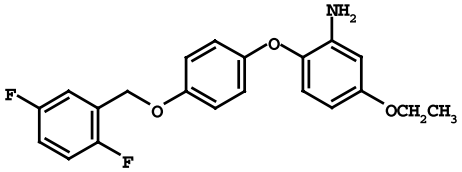

Fig. 1. Chemical structure of SEA0400.

question remains as to whether their benefits were due to inhibition of cardiac NCX. Recently, 2-[2-[4-(4-nitrobenzyloxy)phenyl]isothiourea (KB-R7943) has been introduced as a potent and selective inhibitor of NCX (Iwamoto et al., 1996; Watano et al., 1996) and the pathophysiological roles of NCX were investigated in heart (Nakamura et al., 1998; Ladilov et al., 1999; Mukai et al., 2000), brain (Schröder et al., 1999; Li et al., 2000) and kidney (Kuro et al., 1999). However, it is also unclear if KB-R7943 is indeed a good tool for a selective inhibition of NCX, since there are now increasing reports showing the additional effects of KB-R7943 on various proteins including Ca^{2+} channels, Na^+ channels and K^+ channels at concentrations used to inhibit NCX (Sobolevsky and Khodorov, 1999; Pintado et al., 2000; Arakawa et al., 2000; Matsuda et al., 2001; Tanaka et al., 2002). Therefore, a more selective inhibitor of NCX is required for studies on physiological and pathological roles of NCX.

We found that a newly synthesized compound, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400, Fig. 1), is the most potent and selective inhibitor of NCX reported to date (Matsuda et al., 2001). SEA0400 potently inhibited the Na^+-dependent Ca^{2+} uptake in cultured neurons, astrocytes and microglia with IC_{50} values of 5–33 nM, as compared with KB-R7943 (IC_{50} values, 2–4 μM), but did not significantly affect other 32 proteins examined. In addition, SEA0400 attenuated Ca^{2+} paradox injuries in cultured astrocytes and reduced infarct volumes in rats with transient middle cerebral artery occlusion. These results indicate that NCX plays an important role in cerebral ischemia–reperfusion injuries and that SEA0400 is a valuable tool for reevaluation of the protective effects of NCX inhibitors employed in previous studies on myocardial ischemia–reperfusion injuries.

We now report that SEA0400 is a most potent inhibitor of NCX in the heart. In addition, we show that SEA0400 has protective effects against cell death, cardiac dysfunction and cardiac arrhythmias after myocardial ischemia–reperfusion.

2. Material and methods

All studies reported here have been reviewed by the Taisho Pharmaceutical Animal Care Committee and have met the Japanese Experimental Animal Research Association Standards as defined in the *Guidelines for Animal Experiments* (1987).

2.1. NCX activity in sarcolemmal vesicles

Sarcolemmal vesicles were prepared from dog ventricles, according to Jones (1988). The reverse mode NCX activity was determined by measuring Na^+-dependent $^{45}Ca^{2+}$ uptake into vesicles, as described (Philipson and Nishimoto, 1982). Briefly, 2 μl of Na^+-loaded vesicles (0.5 mg/ml) were diluted into 98 μl of uptake medium containing 20 mM 3-morpholinopropanesulfonic acid (MOPS)/Tris (pH 7.4), 160 mM KCl and 20 μM $^{45}CaCl_2$ at 25 °C. The $^{45}Ca^{2+}$ uptake was terminated at 10 s by adding ice-cold washing medium containing 160 mM KCl and 1 mM $LaCl_3$. Vesicles were rapidly harvested by filtration and washed three times with the same medium. The quantity of $^{45}Ca^{2+}$ remaining in the filtered vesicles was determined by liquid scintillation. Na^+-dependent Ca^{2+} uptake was estimated by subtracting the uptake into K^+-loaded vesicles as a blank from that into Na^+-loaded ones. SEA0400 and KB-R7943 were added to the $^{45}Ca^{2+}$ uptake medium.

2.2. NCX activity in cardiomyocytes

Primary cultured cardiomyocytes were prepared from 17-day-old Wistar rat fetuses according to Sadoshima et al. (1992). Isolated cells were plated in 24-well tissue culture plates at a density of 1×10^5 cell/cm^2, and grown for 2 days with culture medium containing 20 mM HEPES/Tris (pH 7.4), 135 mM NaCl, 5 mM KCl, 5.5 mM glucose, 2.4 mM $CaCl_2$ and 0.7 mM $MgCl_2$. The reverse mode NCX activity was determined by measuring Na^+-dependent $^{45}Ca^{2+}$ uptake into cardiomyocytes, as described (Wakabayashi and Goshima, 1981). Briefly, cardiomyocytes were pre-loaded with Na^+ by incubation in a Ca^{2+}-free medium containing 20 mM HEPES/Tris (pH 7.4), 135 mM NaCl, 5 mM KCl, 5.5 mM glucose and 0.1 mM EDTA at 37 °C. After incubation in the Ca^{2+}-free medium for 10 min, the medium was replaced by a Na^+-free medium containing 20 mM HEPES/Tris (pH 7.4), 135 mM choline chloride, 5 mM KCl, 5.5 mM glucose and 1 mM $^{45}CaCl_2$. The $^{45}Ca^{2+}$ uptake was terminated at 1 min by removal of the Na^+-free medium and the cardiomyocytes were washed three times with ice-cold medium containing 20 mM HEPES/Tris (pH 7.4), 135 mM NaCl, 5.5 mM glucose and 1 mM $CaCl_2$. Cardiomyocytes were solubilized in 0.3 N NaOH and the aliquots were used for determination of radioactivity by liquid scintillation. SEA0400 and KB-R7943 were added to both Ca^{2+}-free and Na^+-free media.

2.3. Ca^{2+} paradox injury

The Ca^{2+} paradox experiments were carried out using the same rat cardiomyocytes described above, according to Thollon et al. (1995). Briefly, Ca^{2+} paradox was induced by a 1-h incubation of rat cardiomyocytes in a Ca^{2+}-free medium containing 20 mM HEPES/Tris (pH 7.4), 140 mM NaCl, 5.4 mM KCl, 5.5 mM glucose and 1 mM EDTA,

followed by a 24-h incubation in a Ca^{2+}-containing Dulbecco's modified Eagle medium (DMEM)/F-12 with 1 mM $CaCl_2$ at 37 °C. After a 1-h exposure to the 1 mM Ca^{2+}-containing medium, the control cells were incubated in the Ca^{2+}-containing medium for 24 h. SEA0400 and KB-R7943 were added to each successive incubation medium then cell survival was determined after 24 h by a colorimetric assay using fluorescein diacetate (FDA) (Kong and Rabkin, 2000).

2.4. Cardiac dysfunction in isolated hearts

Male Wistar rats, weighing 180–250 g, were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). The heart was excised and rapidly mounted on a Langendorff apparatus (IPH-W, Labo Support, Osaka, Japan) via the aorta, and then perfused at a constant pressure of 65 mm Hg with Krebs–Henseleit bicarbonate buffer containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose at 37 °C. Also, the heart was constantly warmed by circulating water jacket at 37 °C. The buffer was gassed with 95% O_2 and 5% CO_2 at pH 7.4. For the measurement of left ventricular pressure, a 21-gauge needle connected to a Statham pressure transducer P23 Db, was inserted into the left ventricular cavity. Heart rate was monitored from the left ventricular pressure. Coronary flow was monitored by a flow meter (LWBF-1, Labo Support). After stabilization for 15 min, the perfusion pressure was reduced to 7 cm H_2O for 60 min then returned to 65 mm Hg for 60 min. SEA0400, KB-R7943 and cariporide were added to the perfusion buffer immediately after the return to normal perfusion (65 mm Hg) for 10 min. When severe and irreversible arrhythmias occurred during hypoperfusion and reperfusion, the hearts were excluded from the study. As a result, in total 11 out of 80 animals evenly from each group were excluded.

2.5. Ischemia–reperfusion arrhythmias

Male Sprague–Dawley rats, weighing 302–390 g, were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). The animals were intubated and artificially ventilated with room air (volume 15 ml/kg, rate 54 strokes/min). The femoral vein was cannulated for drug administration. The systemic blood pressure was monitored via a catheter inserted into the carotid artery and connected to a Statham pressure transducer P23 Db. The electrocardiogram (ECG) was recorded from standard limb lead II and the heart rate was monitored from the ECG. The chest was opened by a left thoracotomy, and a 6/0 braided silk suture was placed around the left anterior descending coronary artery. After stabilization for 15 min, the coronary artery was occluded by pulling on the suture for 5 min. Successful occlusion was confirmed by an immediate ST segment elevation and by a 20–30% decrease in blood pressure. Reperfusion was performed by releasing the suture for 10 min, and successful reperfusion was confirmed by an increase in blood pressure (Aye et al., 1997). As a result, a total of 18 out of 128 animals evenly from each group were excluded from the study because of inadequacy of ischemia or reperfusion. In preliminary studies, we also confirmed that the ischemic zone sizes were constant, resulting in 25–35% of the total heart volume when measured using blue dye (data not shown). SEA0400, cariporide and lidocaine were administered intravenously 1 min before reperfusion. Arrhythmias were evaluated according to the guidelines of the Lambeth Conventions (Walker et al., 1988). Ventricular tachycardia was defined as a run of four or more consecutive ventricular premature beats. Ventricular fibrillation was defined as a signal for which individual QRS deflections could no longer be distinguished. Mortality was defined as an irreversible ventricular fibrillation with a severe decrease in blood pressure for 3 min or longer.

2.6. Statistical analysis

Data are expressed as the means ± S.E.M. Statistical analysis was carried out using Dunnett's test. Data from ischemia–reperfusion arrhythmias were analyzed using Fisher's exact test. Values of $P < 0.05$ were considered to be significant.

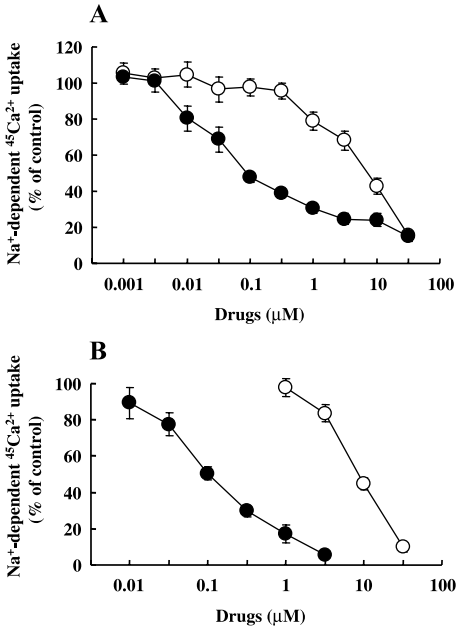

Fig. 2. Effects of SEA0400 and KB-R7943 on Na^+-dependent $^{45}Ca^{2+}$ uptake in dog cardiac sarcolemmal vesicles (A) and cultured rat cardiomyocytes (B). SEA0400 (closed circles) and KB-R7943 (open circles) at the indicated concentrations were added to the $^{45}Ca^{2+}$ uptake medium (A) and to both Ca^{2+}-free medium and $^{45}Ca^{2+}$ uptake medium (B). Results are means ± S.E.M. of four independent determinations (A) and 4–6 wells obtained from six separate preparations (B).

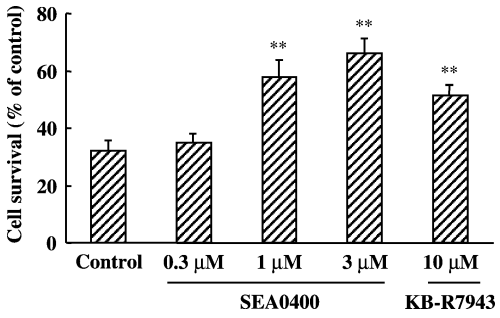

Fig. 3. Effect of SEA0400 and KB-R7943 on Ca^{2+} paradox injury in cultured rat cardiomyocytes. Drugs at the indicated concentrations were added to both Ca^{2+}-free medium and Ca^{2+}-containing medium. Results are means ± S.E.M. of 10–12 wells obtained from six separate preparations. $^{**}P<0.01$ compared with control (Dunnett's test).

2.7. Drugs

Drugs were obtained from the following sources: $^{45}CaCl_2$, Amersham (Tokyo, Japan); FDA, Sigma (St. Louis, MO); DMEM/F-12, Gibco (Grand Island, NY). SEA0400, KB-R7943 and cariporide were synthesized in Taisho Pharmaceutical (Saitama, Japan). Lidocaine was purchased from Fujisawa (Tokyo, Japan). All other chemicals were obtained commercially and were of analytical grade. For in vitro studies, all drugs were dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.1%. In an in vivo experiment, SEA0400 was administrated as a lipid emulsion containing 10% soybean oil, and cariporide and lidocaine were dissolved in saline.

The dosages of SEA0400 (Matsuda et al., 2001), KB-R7943 (Iwamoto et al., 1996; Nakamura et al., 1998), cariporide (Scholz et al., 1995; Aye et al., 1997) and lidocaine (Scholz et al., 1995) were chosen on the basis of previous reports. The dosage of SEA0400 for the anesthetized rat study was also based on the tissue concentration, which is enough to inhibit NCX, in our preliminary study.

Table 1
Effects of SEA0400, KB-R7943 and cariporide on heart rate and coronary flow in isolated rat Langendorff hearts

	Before ischemia	After reperfusion			
	0 min	70 min	80 min	90 min	120 min
Heart rate (beats/min)					
Control (DMSO)	327 ± 19	349 ± 12	345 ± 9	344 ± 11	349 ± 16
SEA0400, 0.1 μM	314 ± 10	250 ± 23	278 ± 27	287 ± 22	275 ± 24
SEA0400, 0.3 μM	355 ± 7	329 ± 11	323 ± 17	319 ± 19	340 ± 49
SEA0400, 1 μM	351 ± 13	308 ± 25	317 ± 15	312 ± 16	303 ± 15
KB-R7943, 3 μM	323 ± 10	318 ± 30	320 ± 38	280 ± 14	295 ± 16
KB-R7943, 10 μM[a]	306 ± 15	259 ± 18	254 ± 27	262 ± 28	281 ± 41
Cariporide, 1 μM	332 ± 13	374 ± 41	385 ± 46	359 ± 36	348 ± 41
Cariporide, 3 μM	321 ± 15	334 ± 13	319 ± 15	320 ± 18	319 ± 21
Coronary flow (ml/min)					
Control (DMSO)	7.8 ± 0.6	6.0 ± 0.7	5.0 ± 0.6	4.8 ± 0.6	4.1 ± 0.5
SEA0400, 0.1 μM	8.4 ± 0.4	6.6 ± 0.5	6.1 ± 0.4	5.9 ± 0.4	5.0 ± 0.3
SEA0400, 0.3 μM	7.9 ± 0.5	6.4 ± 0.7	6.1 ± 0.6	6.0 ± 0.6	5.2 ± 0.6
SEA0400, 1 μM	9.0 ± 0.3	6.9 ± 0.6	6.7 ± 0.6	6.4 ± 0.6	5.1 ± 0.4
KB-R7943, 3 μM	7.6 ± 0.5	7.4 ± 0.5	6.2 ± 0.4	5.9 ± 0.5	5.3 ± 0.4
KB-R7943, 10 μM[a]	7.7 ± 0.3	7.3 ± 0.5	6.7 ± 0.6	6.7 ± 0.5	5.6 ± 0.3
Cariporide, 1 μM	7.8 ± 0.5	6.4 ± 0.7	5.6 ± 0.4	5.4 ± 0.4	4.1 ± 0.3
Cariporide, 3 μM	6.4 ± 0.7	6.7 ± 0.5	6.2 ± 0.5	6.2 ± 0.4	5.0 ± 0.2

Values are means ± S.E.M. of 8–10 separate hearts after reperfusion.
[a] $P<0.05$ compared with the control group (Dunnett's test).

3. Results

3.1. Effects on NCX activity

In canine cardiac sarcolemmal vesicles, SEA0400 and KB-R7943 inhibited the Na^+-dependent $^{45}Ca^{2+}$ uptake in a concentration-dependent manner with IC_{50} values of 90 nM

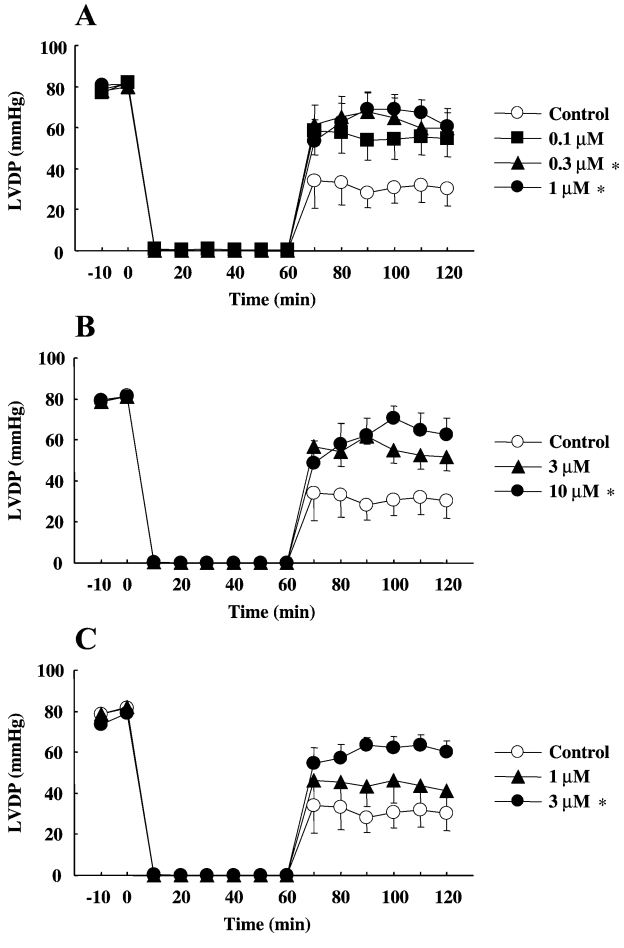

Fig. 4. Effect of SEA0400 (A), KB-R7943 (B) and cariporide (C) on the recovery of left ventricular developed pressure after a 60-min hypoperfusion in isolated rat perfused hearts. Drugs at the indicated concentrations were added to the perfusion buffer immediately after normal perfusion for 10 min. Results are means ± S.E.M. of 8–10 separate hearts. $^{*}P<0.05$ compared with control (Dunnett's test).

and 7.0 μM, respectively (Fig. 2A). On the basis of the IC_{50} value, SEA0400 was about 80 times more potent than KB-R7943. In rat cardiomyocytes, SEA0400 was also about 100 times more potent than KB-R7943 in inhibiting Na^+-dependent $^{45}Ca^{2+}$ uptake (Fig. 2B). The IC_{50} value for SEA0400 was 92 nM and that for KB-R7943 was 9.5 μM. We already reported that SEA0400 has a highly selective profile for NCX, compared with KB-R7943 (Matsuda et al., 2001).

3.2. Effects on Ca^{2+} paradox injury

In cultured rat cardiomyocytes, control cell survival rate after Ca^{2+} paradox injury was 32.1 ± 3.7%, and SEA0400 at 1 and 3 μM increased this rate up to 57.9 ± 6.0% and 66.1 ± 5.1%, respectively (Fig. 3). KB-R7943 at 10 μM also prevented cell death. In this model, SEA0400 was more than 10 times as potent as KB-R7943.

3.3. Effects on cardiac dysfunction

In the ischemia–reperfusion model of isolated rat Langendorff hearts, the mean baseline values of left ventricular developed pressure, heart rate and coronary flow were 81 ± 1 mm Hg, 328 ± 5 beats/min and 7.7 ± 0.2 ml/min, respectively (n = 69). There was no difference in these parameters between experimental groups (Table 1). During hypoperfusion, left ventricular developed pressure decreased rapidly, almost reaching 0 mm Hg, and was sustained until normal perfusion was restored (Fig. 4). After reperfusion with normal pressure, left ventricular developed pressure recovered from a zero level to about one-third of the pre-ischemic level in the control group (Fig. 4). In this model, ischemia–reperfusion was confirmed by a change of coronary flow from pre-ischemic value (7.7 ± 0.2 ml/min) to 0.1 ± 0.1 ml/min at 10 min after hypoperfusion and 6.7 ± 0.2 ml/min at 10 min after reperfusion. Neither SEA0400 nor cariporide had any significant effect on heart rate and coronary flow, as compared with the control DMSO-treated group when estimated with the area under curve (AUC), but KB-R7943 at 10 μM did produce a significant decrease in heart rate and an increase in coronary flow (Table 1). SEA0400 at 0.3 and 1 μM improved the recovery of left ventricular developed pressure after a 60-min reperfusion period from 32.1 ± 8.6% (control), when estimated with the AUC, to 66.7 ± 10.3% and 66.8 ± 7.5%, respectively (Fig. 4A). Both KB-R7943 at 10 μM (Fig. 4B) and cariporide at 3 μM (Fig. 4C) were also effective in preventing cardiac dysfunction. In this model, SEA0400 was at least more than 10 times as potent as KB-R7943 and cariporide.

3.4. Effects on ischemia–reperfusion arrhythmias

In the ischemia–reperfusion arrhythmia model of anesthetized rats, the mean baseline values of mean blood pressure and heart rate were 85 ± 1 mm Hg and 443 ± 5 beats/min, respectively (n = 110). Four minutes after ischemia, the mean values of mean blood pressure and heart rate were reduced to 61 ± 1 mm Hg and 429 ± 5 beats/min, respectively. There was no difference in these parameters between experimental groups (Table 2). Neither SEA0400 nor cariporide had any significant effect on mean blood pressure and heart rate, as compared with each vehicle, but lidocaine did produce a significant reduction in mean blood pressure and heart rate (Table 2). After a 5-min ischemia–reperfusion in the control vehicle-treated group, the incidence of ventricular tachycardia, the incidence of ventricular fibrillation and mortality rates were 100%, 80% and 70%, respectively (Fig. 5A). SEA0400 at any dose examined did not affect the incidence of ventricular tachycardia, but at 1 mg/kg it did reduce the incidence of ventricular fibrillation to 30% and the mortality rate to 20% (Fig. 5A). The mortality rate was also reduced to 20% by 0.3 mg/kg of

Table 2
Effects of SEA0400, cariporide and lidocaine on mean blood pressure and heart rate in anesthetized rats

	Mean blood pressure (mm Hg)			Heart rate (beats/min)		
	Before ischemia	After ischemia		Before ischemia	After ischemia	
		Before treatment	After treatment		Before treatment	After treatment
Vehicle	88 ± 7	61 ± 6	73 ± 7	435 ± 20	417 ± 24	416 ± 23
SEA0400, 0.03 mg/kg	84 ± 5	59 ± 4	71 ± 5	457 ± 11	442 ± 15	444 ± 14
SEA0400, 0.1 mg/kg	82 ± 4	57 ± 4	63 ± 4	463 ± 9	445 ± 14	445 ± 13
SEA0400, 0.3 mg/kg	89 ± 4	65 ± 5	79 ± 4	449 ± 14	440 ± 19	438 ± 18
SEA0400, 1 mg/kg	86 ± 4	61 ± 4	81 ± 5	455 ± 13	440 ± 9	450 ± 8
Saline	93 ± 5	63 ± 6	75 ± 6	428 ± 18	403 ± 17	412 ± 17
Cariporide, 0.3 mg/kg	78 ± 1	61 ± 5	70 ± 7	419 ± 11	413 ± 14	415 ± 13
Cariporide, 1 mg/kg	84 ± 3	61 ± 4	80 ± 4	441 ± 13	428 ± 15	444 ± 13
Cariporide, 3 mg/kg	81 ± 2	59 ± 3	71 ± 6	441 ± 22	437 ± 20	434 ± 18
Lidocaine, 1 mg/kg	88 ± 4	58 ± 6	53 ± 3[a]	445 ± 17	434 ± 21	384 ± 18[a]
Lidocaine, 3 mg/kg	86 ± 4	66 ± 4	51 ± 3[a]	446 ± 12	439 ± 17	347 ± 13[a]

Values are means ± S.E.M. of 10 separate animals.
[a] P < 0.01 compared with the control saline-treated group (Dunnett's test).

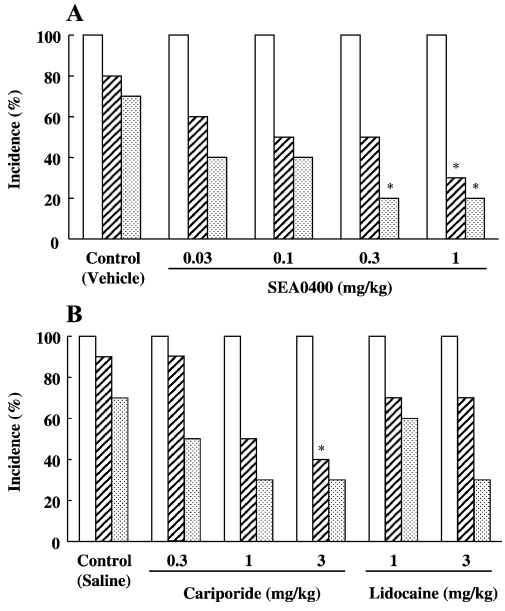

Fig. 5. Effect of SEA0400 (A), cariporide and lidocaine (B) on the incidence of ventricular tachycardia (open bar), ventricular fibrillation (hatched bar) and mortality (shaded bar) induced by reperfusion after a 5-min ischemia in anesthetized rats. Drugs at the indicated concentrations were administered intravenously 1 min before reperfusion. Each group consisted of 10 individual animals. *$P<0.05$ compared with each control (Fisher's exact test).

SEA0400 (Fig. 5A). In another control saline-treated group, the incidence of ventricular tachycardia, the incidence of ventricular fibrillation and mortality rates were 100%, 90% and 70%, respectively (Fig. 5B). Cariporide at 3 mg/kg reduced the incidence of ventricular fibrillation to 40%, but the effect on mortality rate was not statistically significant (Fig. 5B). Lidocaine at 1 and 3 mg/kg did not show any significant protection against ischemia–reperfusion arrhythmias (Fig. 5B). In this model, SEA0400 was at least more than three times as potent as cariporide.

4. Discussion

We obtained evidence that SEA0400 is an extremely potent inhibitor of NCX in dog cardiac sarcolemmal vesicles and cultured rat cardiomyocytes, the IC_{50} values being 90 and 92 nM, respectively. The potency of SEA0400 in inhibiting the NCX activity was about 100 times that of KB-R7943. These findings are consistent with our recent observations (Matsuda et al., 2001) that SEA0400 strongly inhibited NCX activity in cultured rat neurons, astrocytes and microglia with IC_{50} values of 5–33 nM, compared with KB-R7943 (2.0–3.8 μM). We also reported that SEA0400 has a highly selective profile for NCX in comparison with KB-R7943 (Matsuda et al., 2001). SEA0400 did not significantly affect ion transporters (NHE, Na^+/K^+ ATPase, Ca^{2+} ATPase), ion channels (Ca^{2+} channels, Na^+ channels and K^+ channels), 15 receptors including adrenergic and adenosine receptors, and five enzymes including phospholipase A_2 and phospholipase C examined at concentrations up to 3 μM, although KB-R7943 at 3 μM did affect L-type Ca^{2+} channels, site 2 of the Na^+ channel which is one of the binding sites of neurotoxins acting on the inactivation of voltage-gated Na^+ channels, muscarinic acetylcholine, leukotriene B_4, platelet activating factor and norepinephrine transporter bindings. Tanaka et al. (2002) also confirmed that SEA0400 at 1 μM which inhibited NCX currents by more than 80% had no significant effects on the Na^+, L-type Ca^{2+}, delayed rectifier K^+ and inwardly rectifying K^+ currents, whereas an equipotent concentration of KB-R7943 (10 μM) inhibited those currents by more than 50% in isolated guinea-pig ventricular myocytes. In addition, KB-R7943 has been reported to inhibit the nicotinic acetylcholine receptor (Pintado et al., 2000), *N*-methyl-D-aspartate channels (Sobolevsky and Khodorov, 1999) and store-operated Ca^{2+} entry (Arakawa et al., 2000). Taken together, these results strongly indicate that SEA0400 is a most potent and selective inhibitor of NCX. Therefore, SEA0400 is apparently a more favorable compound than KB-R7943 to use as a new tool for reinvestigating the pathophysiological roles of NCX. We reported that SEA0400 attenuates the Ca^{2+} paradox-induced cell death in cultured rat astrocytes and reduced the infarct volumes after a transient middle cerebral artery occlusion in rats (Matsuda et al., 2001).

In the present study, we asked if the selective NCX inhibitor SEA0400 would attenuate myocardial ischemia–reperfusion injuries, in vitro and in vivo. We first used the Ca^{2+} paradox model which can mimic ischemia–reperfusion injury, since previous studies demonstrated the participation of NCX in this model (Chapman and Tunstall, 1987; Matsuda et al., 1996). In cultured rat cardiomyocytes, SEA0400 dose-dependently inhibited Ca^{2+} paradox-induced cell death, but a nearly complete inhibition (>80%) of NCX (Fig. 2B) was definitely needed to show beneficial effects (Fig. 3) with SEA0400 (1 μM). Such a difference in potency between NCX inhibition and protection from Ca^{2+} paradox injury was also observed in cultured rat astrocytes (Matsuda et al., 2001). We cannot fully explain the reason, but it seems that the injury is entirely dependent on Ca^{2+} overload, which is triggered mainly by the reverse mode of NCX and any residual Ca^{2+} accumulation may lead to cell injuries. In addition, the involvement of other mechanism(s) such as Ca^{2+} channel(s) and non-selective cation channel(s) cannot be ruled out. In contrast, KB-R7943 (10 μM) showed a lesser but significant protection compared with SEA0400 (Fig. 3) with a relatively weak inhibition (about 55%) of NCX (Fig. 2B). This may be related to additional inhibitory effects of KB-R7943 on the Ca^{2+} channel (Watano et al., 1996; Matsuda et al., 2001; Tanaka et al., 2002), which is at least in part involved in Ca^{2+} paradox injury (Chapman and Tunstall, 1987). It should be noted that SEA0400 up to

3 μM did not affect the Ca^{2+} channel (Matsuda et al., 2001).

In another in vitro ischemia–reperfusion study using the Langendorff perfused rat heart, the compounds were administered for 10 min just after reperfusion. In this protocol, SEA0400 improved the cardiac dysfunction after reperfusion, as did KB-R7943 and cariporide. No clear relation existed between the ability of SEA0400 to increase the left ventricular developed pressure and the effects on heart rate or coronary flow. Therefore, it is more likely that the improved recovery of left ventricular developed pressure by SEA0400 was related to the inhibition of NCX. Cariporide attenuates myocardial injuries during ischemia and reperfusion via NHE inhibition, in in vitro and in vivo models (Scholz et al., 1995; Miura et al., 1997; Aye et al., 1997). These results indicate that NCX as well as NHE plays an important role in myocardial ischemia–reperfusion injuries. In the case of KB-R7943, it seems that the mechanism of improvement in left ventricular developed pressure recovery is a little complicated since KB-R7943 is less selective for NCX as described above and it decreased the heart rate and increased the coronary flow (Table 1), and may therefore have contributed to the improvement. Nevertheless, the protective effect of KB-R7943 in this model was likely to due to the NCX inhibition. Nakamura et al. (1998) reported that post-ischemic treatment of KB-R7943 improved the cardiac dysfunction in a similar model in which neither the Ca^{2+} channel blocker diltiazem nor the Na^{+} channel blocker lidocaine did so. In addition, a similar degree (60–70%) of NCX inhibition (Fig. 2) was required for both KB-R7943 and a selective inhibitor SEA0400 to show protective effects (Fig. 4). Therefore, Ca^{2+} and Na^{+} channels that affect heart rate and coronary flow are not involved in the protective effects of SEA0400 and KB-R7943, at least in this model of rat Langendorff hearts.

Post-ischemic administrations of SEA0400 and cariporide at 1 min before reperfusion also reduced the incidence of ventricular fibrillation and subsequent cardiac death in our in vivo ischemia–reperfusion model using anesthetized rats. However, the effect of cariporide on mortality was not statistically significant. In this respect, Aye et al. (1997) pointed out that post-ischemic administrations of cariporide especially at the onset of reperfusion reveal a lesser protection against the ischemia–reperfusion arrhythmias compared with the pre-ischemic administrations. They assumed that cariporide administered at the onset of reperfusion did not reach the binding site of NHE quickly enough to suppress the arrhythmias. In general, cardiac arrhythmias in this model occur mostly within 1 min after reperfusion. The pharmacokinetic properties of cariporide have yet to be published, but the tissue concentrations of SEA0400 at 0.3 and 1 mg/kg in the rat heart are 0.75 and 2.1 μg/g tissue, respectively, at 1 min after intravenous administration, hence sufficient concentrations to inhibit NCX. In contrast, lidocaine produced no significant effect even at doses that produced significant decreases in the mean blood pressure and the heart rate. Although the analysis of ancillary ECG parameters which provides an in-depth understanding of the molecular action for the antiarrhythmic effect was not performed in the present study, other evidence suggests that the concentrations used for SEA0400 are unlikely to be very non-selective. These results suggest that activation of NCX in close relation to NHE is mainly involved in cardiac arrhythmias after reperfusion.

Very recently, Reuter et al. (2002) reported that a low concentration of SEA0400 (0.1 μM) reduced the Ca^{2+} transients in embryonic heart tubes obtained from $NCX^{-/-}$ mice. These results suggest that SEA0400 has an NCX-independent effect that is responsible for the depression of the Ca^{2+} transients. However, there seems to be some adaptations in $NCX^{-/-}$ heart tubes and the mechanisms of Ca^{2+} handling in this $NCX^{-/-}$ preparation as well as the target molecule of SEA0400 other than NCX are not known. In addition, the data were obtained using embryonic tissue and cannot be generalized to adult myocardium since the Ca^{2+} handling in embryonic versus adult is different. Furthermore, there is no evidence that the NCX-independent mechanism exists in adult myocardium. It is clear that SEA0400, unlike KB-R7943, does not affect the known ion transport systems such as channels and pumps (Matsuda et al., 2001; Tanaka et al., 2002). Therefore, although the possibility that an NCX-independent effect of SEA0400 is related to its protective effects on ischemia–reperfusion injuries cannot be ruled out, the lack of selectivity might be observed only under the limited conditions.

In conclusion, we found SEA0400 to be a most potent and selective inhibitor of NCX in the heart and its beneficial effects in in vitro and in vivo models of myocardial ischemia–reperfusion injuries. Therefore, our results support the previous studies showing the NCX contribution to myocardial ischemia–reperfusion injuries.

References

Arakawa, N., Sakaue, M., Yokoyama, I., Hashimoto, H., Koyama, Y., Baba, A., Matsuda, T., 2000. KB-R7943 inhibits store-operated Ca^{2+} entry in cultured neurons and astrocytes. Biochem. Biophys. Res. Commun. 279, 354–357.

Aye, N.N., Xue, Y.X., Hashimoto, K., 1997. Antiarrhythmic effects of cariporide, a novel Na^{+}–H^{+} exchange inhibitor, on reperfusion ventricular arrhythmias in rat hearts. Eur. J. Pharmacol. 339, 121–127.

Chapman, R.A., Tunstall, J., 1987. The calcium paradox of the heart. Prog. Biophys. Mol. Biol. 50, 67–96.

Cross, H.R., Lu, L., Steenbergen, C., Philipson, K.D., Murphy, E., 1998. Overexpression of the cardiac Na^{+}/Ca^{2+} exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. Circ. Res. 83, 1215–1223.

Hryshko, L.V., Philipson, K.D., 1997. Sodium–calcium exchange: recent advances. Basic Res. Cardiol. 92 (Suppl. 1), 45–51.

Imanishi, K., Kusuoka, H., Hashimoto, K., Yoshioka, J., Yamaguchi, H., Nishimura, T., 1998. Intracellular sodium accumulation during ischemia as the substrate for reperfusion injury. Circ. Res. 84, 1401–1406.

Iwamoto, T., Watano, T., Shigekawa, M., 1996. A novel isothiourea deriv-

ative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J. Biol. Chem. 271, 22391–22397.

Jones, L.R., 1988. Rapid preparation of canine cardiac sarcolemmal vesicles by sucrose flotation. Methods Enzymol. 157, 85–91.

Kaczorowski, G.J., Slaughter, R.S., King, R., Garcia, M.L., 1989. Inhibitors of sodium–calcium exchange: identification and development of probes of transport activity. Biochim. Biophys. Acta 988, 287–302.

Kawada, T., Yoshida, Y., Sakurai, H., Imai, S., 1992. Myocardial Na^+ during ischemia and accumulation of Ca^{2+} after reperfusion: a study with monensin and dichlorobenzamil. Jpn. J. Pharmacol. 59, 191–200.

Kong, J.Y., Rabkin, S.W., 2000. Palmitate-induced apoptosis in cardiomyocytes is mediated through alterations in mitochondria: prevention by cyclosporin A. Biochim. Biophys. Acta 1485, 45–55.

Kuro, T., Kobayashi, Y., Takaoka, M., Matsumura, Y., 1999. Protective effect of KB-R7943, a novel Na^+/Ca^{2+} exchange inhibitor, on ischemic acute renal failure in rats. Jpn. J. Pharmacol. 81, 247–251.

Ladilov, Y., Haffner, S., Balser-Schafer, C., Maxeiner, H., Piper, H.M., 1999. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na^+/Ca^{2+} exchanger. Am. J. Physiol. 276, H1868–H1876.

Lazdunski, M., Frelin, C., Vigne, P., 1985. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. J. Mol. Cell. Cardiol. 17, 1029–1042.

Li, S., Jiang, Q., Stys, P.K., 2000. Important role of $Na^+–Ca^{2+}$ exchange in spinal cord white matter injury at physiological temperature. J. Neurophysiol. 84, 1116–1119.

Matsuda, T., Takuma, K., Nishiguchi, E., Hashimoto, H., Azuma, J., Baba, A., 1996. Involvement of $Na^+–Ca^{2+}$ exchanger in reperfusion-induced delayed cell death of cultured rat astrocytes. Eur. J. Neurosci. 8, 951–958.

Matsuda, T., Takuma, K., Baba, A., 1997. $Na^+–Ca^{2+}$ exchanger: physiology and pharmacology. Jpn. J. Pharmacol. 74, 1–20.

Matsuda, T., Arakawa, N., Takuma, K., Kishida, Y., Kawasaki, Y., Sakaue, M., Takahashi, K., Takahashi, T., Suzuki, T., Ota, T., Hamano-Takahashi, A., Onishi, M., Tanaka, Y., Kameo, K., Baba, A., 2001. SEA0400, a novel and selective inhibitor of $Na^+–Ca^{2+}$ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J. Pharmacol. Exp. Ther. 298, 249–256.

Miura, T., Ogawa, T., Suzuki, K., Goto, M., Shimamoto, K., 1997. Infarct size limitation by a new $Na^+–H^+$ exchange inhibitor, Hoe 642: difference from preconditioning in the role of protein kinase C. J. Am. Coll. Cardiol. 29, 693–701.

Mukai, M., Terada, H., Sugiyama, S., Satoh, S., Hayashi, H., 2000. Effects of a selective inhibitor of Na^+/Ca^{2+} exchange, KB-R7943, on reperfusion-induced injuries in guinea pig papillary muscles. J. Cardiovasc. Pharmacol. 35, 121–128.

Murphy, E., Cross, H., Steenbergen, C., 1999. Sodium regulation during ischemia versus reperfusion and its role in injury. Circ. Res. 84, 1469–1470.

Nakamura, A., Harada, K., Sugimoto, H., Nakajima, F., Nishimura, N., 1998. Effects of KB-R7943, a novel Na^+/Ca^{2+} exchange inhibitor, on myocardial ischemia–reperfusion injury. Folia Pharmacol. Jpn. 111, 105–115.

Philipson, K.D., Nishimoto, A.Y., 1982. $Na^+–Ca^{2+}$ exchange in inside-out cardiac sarcolemmal vesicles. J. Biol. Chem. 257, 5111–5117.

Pintado, A.J., Herrero, C.J., Garcia, A.G., Montiel, C., 2000. The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. Br. J. Pharmacol. 130, 1893–1902.

Reuter, H., Henderson, S.A., Han, T., Matsuda, T., Baba, A., Ross, R.S., Goldhaber, J.I., Philipson, K.D., 2002. Knockout mice for pharmacological screening. Testing the specificity of $Na^+–Ca^{2+}$ exchange inhibitors. Circ. Res. 91, 90–92.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J., Izumo, S., 1992. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. J. Biol. Chem. 267, 10551–10560.

Scholz, W., Albus, U., Counillon, L., Gögelein, H., Lang, H.J., Linz, W., Weichert, A., Schölkens, B.A., 1995. Protective effects of HOE642, a selective sodium–hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc. Res. 29, 260–268.

Schröder, U.H., Breder, J., Sabelhaus, C.F., Reymann, K.G., 1999. The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 protects CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury. Neuropharmacology 38, 319–321.

Sobolevsky, A.I., Khodorov, B.I., 1999. Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the Na^+/Ca^{2+} exchange inhibitor KB-R7943. Neuropharmacology 38, 1235–1242.

Tanaka, H., Nishimaru, K., Aikawa, T., Hirayama, W., Tanaka, Y., Shigenobu, K., 2002. Effects of SEA0400, a novel inhibitor of sodium–calcium exchanger, on myocardial ionic currents. Br. J. Pharmacol. 135, 1096–1100.

Tani, M., 1990. Mechanisms of Ca^{++} overload in reperfused ischemic myocardium. Annu. Rev. Physiol. 52, 543–559.

Tani, M., Neely, J., 1989. Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts: possible involvement of $H^+–Na^+$ and $Na^+–Ca^{2+}$ exchange. Circ. Res. 65, 1045–1056.

Thollon, C., Iliou, J.P., Cambarrat, C., Robin, F., Vilaine, J.P., 1995. Nature of the cardiomyocyte injury induced by lipid hydroperoxides. Cardiovasc. Res. 30, 648–655.

Van Emous, J.G., Schreur, J.H.M., Ruigrok, T.J.C., Van Echteld, C.J.A., 1998. Both $Na^+–K^+$ ATPase and $Na^+–H^+$ exchanger are immediately active upon post-ischemic reperfusion in isolated rat hearts. J. Mol. Cell. Cardiol. 30, 337–348.

Wakabayashi, S., Goshima, K., 1981. Kinetic studies on sodium-dependent calcium uptake by myocardial cells and neuroblastoma cells in culture. Biochim. Biophys. Acta 642, 158–172.

Walker, M.J.A., Curtis, M.J., Hearse, D.J., Campbell, R.W.F., Janse, M.J., Yellon, D.M., Cobbe, S.M., Coker, S.J., Harnrss, J.B., Harron, D.W.G., Higgins, A.J., Julian, D.G., Lab, M.J., Manning, A.S., Northover, B.J., Parratt, J.R., Riemersma, R.A., Riva, E., Russell, D.C., Sheridan, D.C., Winslow, E., Woodward, B., 1988. The Lambeth conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. Cardiovasc. Res. 22, 447–455.

Watano, T., Kimura, J., Morita, T., Nakanishi, H., 1996. A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells. Br. J. Pharmacol. 119, 555–563.

Weiss, R.G., Lakatta, E.G., Gerstenblith, G., 1990. Effects of amiloride on metabolism and contractility during reoxygenation in perfused rat hearts. Circ. Res. 66, 1012–1022.