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Inhibition by SEA0400, a Selective Inhibitor of Na^+/Ca^{2+} Exchanger, of Na^+-Dependent Ca^{2+} Uptake and Catecholamine Release in Bovine Adrenal Chromaffin Cells

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Abstract. The effects of SEA0400, a selective inhibitor of the Na^+/Ca^{2+} exchanger (NCX), on Na^+-dependent Ca^{2+} uptake and catecholamine (CA) release were examined in bovine adrenal chromaffin cells that were loaded with Na^+ by treatment with ouabain and veratridine. SEA0400 inhibited Na^+-dependent $^{45}Ca^{2+}$ uptake and CA release, with the IC_{50} values of 40 and 100 nM, respectively. The IC_{50} values of another NCX inhibitor KB-R7943 were 1.8 and 3.7 μM, respectively. These results indicate that SEA0400 is about 40 times more potent than KB-R7943 in inhibiting NCX working in the reverse mode. In intact cells, SEA0400 and KB-R7943 inhibited CA release induced by acetylcholine and DMPP. The IC_{50} values of SEA0400 were 5.1 and 4.5 μM and the values of KB-R7943 were 2.6 and 2.1 μM against the release induced by acetylcholine and DMPP, respectively, indicating that the potency of SEA0400 is about a half of that of KB-R7943 in inhibiting the nicotinic receptor-mediated CA release. The binding of [^{3}H]nicotine with nicotinic receptors was inhibited by SEA0400 ($IC_{50} = 90\ \mu$M) and KB-R7943 ($IC_{50} = 12\ \mu$M). From these results, it is concluded that unlike KB-R7943, SEA0400 has a potent and selective action on NCX in bovine adrenal chromaffin cells.

Keywords: SEA0400, Na^+/Ca^{2+} exchanger, Ca^{2+} uptake, catecholamine release, adrenal chromaffin cell

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is an ion transporter located in the plasma membrane of most cell types. The NCX exchanges Na^+ and Ca^{2+} bidirectionally through the forward mode (Ca^{2+} extrusion) or the reverse mode (Ca^{2+} influx), depending on the ion gradients across the plasma membrane and the membrane potential; and it plays an important role in the regulation of cytosolic Ca^{2+} concentration (1 – 3). In cardiac muscles, for example, the NCX operating in the forward mode is the principal mechanism by which Ca^{2+} is extruded from cells (4). In addition to its role in Ca^{2+} efflux, there are also considerable data indicating that Ca^{2+} influx is induced through the reverse mode under pathophysiological conditions such as cardiac ischemia-reperfusion injury (5, 6).

A major isoform of the NCX1 clone encoding for NCX was reported to be expressed in bovine adrenal chromaffin cells (7). Functional studies using adrenal chromaffin cells demonstrated that inhibiting the Na^+ pump or lowering the extracellular Na^+ concentration increases basal catecholamine (CA) release and/or augments release evoked by secretagogues (8 – 10), and these studies identified Na^+-dependent Ca^{2+} fluxes (11 – 13). These data are consistent with NCX that is located in the plasma membrane of adrenal medullary cells and that mediates the transfer of Ca^{2+} between the extracellular fluid and cytosol.

A number of compounds such as amiloride derivatives and inorganic cations have been reported to inhibit NCX, but at concentrations that also block many other transporters and ion channels. Selective inhibitors of NCX would be useful for clarifying the physiological

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and pathological implications of this transporter. Recently, SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-ethoxyaniline) has been discovered to be a potent and highly selective inhibitor of NCX. SEA0400 potently inhibits Na^+-dependent Ca^{2+} uptake through the reverse mode of the NCX in neuronal cells and cardiomyocytes; and it inhibits at an effective concentration range that does not affect other ion transporters, ion channels, receptors, and enzymes (14 – 18). Furthermore, SEA0400 exhibits protective effects against cerebral (14, 19), myocardial (15, 20), and renal ischemia-reperfusion injury (21); and thus its clinical use for treatment of ischemic events such as heart attack, stroke, and brain edema is expected (18).

The aim of this study was to clarify whether SEA0400 is also a selective inhibitor of NCX in bovine adrenal chromaffin cells. For this purpose, we examined the effects of SEA0400 on Na^+-dependent Ca^{2+} uptake and CA release in Na^+-loaded cells, its effects on CA release induced by nicotinic receptor stimulation, and its binding activity with nicotinic receptors in intact cells. These effects of SEA0400 were compared with those of another NCX inhibitor, KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea, ref. 22).

Materials and Methods

Isolation and culture of adrenal chromaffin cells

Adrenal chromaffin cells were isolated from bovine adrenal glands by retrograde perfusion through the adrenal vein with a Ca^{2+}-free physiological salt solution (154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 10 mM glucose, pH 7.0) containing collagenase and deoxyribonuclease-I. The isolated chromaffin cells were purified by density gradient centrifugation on Percoll as described previously (23). The purified chromaffin cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and several antibiotics and then plated in collagen-coated 12- or 24-well tissue culture dishes at variable densities depending on the kind of experiment. Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. The culture medium was replaced every 3 days. Cells were used for experiments after 2 – 9 days in culture.

Measurement of Na^+-dependent $^{45}Ca^{2+}$ uptake

Na^+-dependent $^{45}Ca^{2+}$ uptake experiments were carried out according to the method reported by Pintado et al. (13) with minor modification. Cells cultured at the density of 1×10^6 cells/well in 12-well dishes were used. Before the experiment, cells were preincubated for 45 min with Krebs-HEPES solution (140 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM glucose, 10 mM HEPES, pH 7.4) at 37°C. In order to measure the $^{45}Ca^{2+}$ uptake induced by the gradient of cytosolic Na^+ (NCX in the reverse mode), cells were first loaded with Na^+ in the absence of extracellular Ca^{2+}. Cells were maintained for 40 min with Ca^{2+}-free Krebs-HEPES solution containing ouabain (100 μM) to inhibit Na^+ pumping; and during the last 10 min, veratridine (50 μM), a Na^+ channel activator, was added to the ouabain solution to increase the Na^+ loading of the cells. After this period, veratridine was washed out and the cells were treated with SEA0400 (3 – 1000 nM) or KB-R7943 (0.03 – 10 μM) for 8 min. Simultaneously, the cells were treated with 3 μM nicardipine (an L-type Ca^{2+} channel blocker) and 0.3 μM ω-conotoxin MVIIC (an N-, P-, and Q-type Ca^{2+} channel blocker) to inhibit $^{45}Ca^{2+}$ uptake mediated by voltage-dependent Ca^{2+} channels. To assay the NCX activity, Na^+-dependent $^{45}Ca^{2+}$ uptake was initiated by incubation of the cells for 10 min with Na^+-free Krebs-HEPES solution (choline chloride substitution) containing $^{40}Ca^{2+}$ (1 mM) plus $^{45}Ca^{2+}$ (2.5 μCi/ml) as a tracer in the presence of NCX inhibitors and Ca^{2+} channel blockers. Basal $^{45}Ca^{2+}$ uptake was determined by incubation of the cells with normal Krebs-HEPES solution instead of Na^+-free medium. Then, the medium was rapidly aspirated. The reaction was stopped by addition of cold Ca^{2+}-free Krebs-HEPES solution containing 10 mM $LaCl_3$ and 2 mM EGTA, and cells were washed five times with this solution.

To measure the radioactivity retained, the cells were lysed with a radioimmune precipitation assay protein lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0). Cell lysate from each sample was dissolved in scintillation fluid and counted in a liquid scintillation counter (LSC-5100; Aloka, Tokyo). Na^+-dependent $^{45}Ca^{2+}$ uptake was estimated by subtracting $^{45}Ca^{2+}$ uptake in normal Krebs-HEPES solution from that in Na^+-free medium.

Na^+-dependent CA release

Cells cultured at the density of 2×10^5 cells/well in 24-well dishes were used. The experimental protocol (buffer solutions, incubation periods, and drug treatments) before sampling was exactly the same as that used in the Na^+-dependent $^{45}Ca^{2+}$ uptake study described in the preceding paragraph. After the incubation of Na^+-loaded cells with Na^+-free Krebs-HEPES solution (basal CA release, normal Krebs-HEPES solution), the incubation medium was collected in perchloric acid (0.4 M final concentration) for estimation of the CAs released into the medium. For determination of the CAs remaining in the cells, perchloric acid (0.4 M) was added to the cells adherent to the culture dish, and the cells

were lysed by brief sonication.

Epinephrine and norepinephrine were assayed using high performance liquid chromatography with electrochemical detection (LC-4C; Bioanalytical Systems, West Lafayette, IN, USA). CA release (epinephrine plus norepinephrine) was calculated as a percentage of the total CA content (medium plus cells). Na^+-dependent CA release was estimated by subtracting the basal CA release in normal Krebs-HEPES solution from that in Na^+-free medium.

CA release induced by acetylcholine (ACh), DMPP, and KCl

Cells cultured at the density of 5×10^5 cells/well in 12-well dishes were used. Cells were preincubated for 1 h with Locke solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, pH 7.4) at 37°C. The solution was replaced with fresh Locke solution containing SEA0400 (0.3 – 10 μM) or KB-R7943 (0.3 – 10 μM). After 10-min incubation, the cells were treated with ACh (30 μM), DMPP (10 μM), or KCl (40 mM) for 3 min. The effects of dihydro-β-erythroidine (3 – 300 μM), a selective nicotinic receptor antagonist, on CA release induced by ACh or DMPP were also examined. Furthermore, the effects of SEA0400 and KB-R7943 on basal CA release were also examined in the absence of the secretagogues. Sampling and CA measurement methods were the same as those described in the preceding paragraph.

Nicotinic receptor binding assay

A nicotinic receptor binding assay was carried out according to the methods reported by Wang et al. (24) with minor modification. Cells cultured at the density of 1×10^6 cells/well in 12-well dishes were preincubated for 45 min with Locke solution at 37°C. The solution was replaced with fresh Locke solution containing SEA0400 (1 – 100 μM), KB-R7943 (0.3 – 30 μM), or dihydro-β-erythroidine (a positive control for nicotinic receptor binding, 30 – 1000 μM). After 16 min incubation at room temperature, the cells were treated with 5 nM [^{3}H]nicotine (0.4 μCi/ml, 80 Ci/mmol) for 30 min on ice. After these incubations, cells were washed five times with cold Locke solution on ice and lysed with the radioimmune precipitation assay protein lysis buffer. Extracts were clarified by centrifugation in a microcentrifuge at $15{,}000 \times g$ for 10 min at 4°C. Cell lysate from each sample was dissolved in scintillation fluid and counted in a liquid scintillation counter. Nonspecific binding was estimated by adding 1 mM cold nicotine to the binding mixture, and specific binding was determined by subtracting nonspecific binding from each value.

Analysis of data

The results were expressed as the means ± S.E.M. Analysis of variance was used for statistical analysis of data. When analysis of variance showed a statistical difference, Dunnett's test was used to determine the significance level. P values smaller than 0.05 were considered to be statistically significant.

Drugs

The drugs used were SEA0400 (Taisho Pharmaceutical, Tokyo); KB-R7943 (Nippon Organon, Osaka); acetylcholine chloride (Daiichi Seiyaku, Tokyo); DMPP (Aldrich, Milwaukee, WI, USA); nicardipine, ω-conotoxin MVIIC, (–)-nicotine, dihydro-β-erythroidine (Sigma, St. Louis, MO, USA); $^{45}CaCl_2$ and (–)-[*N*-methyl-^{3}H]nicotine (Amersham Biosciences, Buckinghamshire, UK). SEA0400, KB-R7943, and ω-conotoxin MVIIC were first dissolved in dimethylsulfoxide (DMSO) and then diluted to the required concentration with the buffer solution used in the experiment. The final concentration of DMSO did not exceed 0.3% and the same concentration of DMSO was added to the control. Other drugs were first dissolved in distilled water and diluted to the required concentrations with buffer solution.

Results

Effects of SEA0400 and KB-R7943 on Na^+-dependent $^{45}Ca^{2+}$ uptake

Na^+-dependent $^{45}Ca^{2+}$ uptake into Na^+-loaded chromaffin cells was estimated by subtracting basal $^{45}Ca^{2+}$ uptake during the incubation with normal Na^+ solution from $^{45}Ca^{2+}$ uptake during the incubation with Na^+-free solution. The Na^+-dependent $^{45}Ca^{2+}$ uptake in the absence of NCX inhibitors as a control was 1892 ± 289 cpm/1×10^6 cells (n = 14) in both SEA0400 and KB-R7943 experiments. The effects of SEA0400 and KB-R7943 on Na^+-dependent $^{45}Ca^{2+}$ uptake, indicated as % inhibition, are shown in Fig. 1. SEA0400 (3 – 1000 nM) and KB-R7943 (0.03 – 10 μM) inhibited Na^+-dependent $^{45}Ca^{2+}$ uptake in a concentration-dependent manner, and significant inhibition (P<0.01) was observed at concentrations higher than 10 nM and 1 μM, respectively. IC_{50} values calculated from the mean concentration-inhibition curves of SEA0400 and KB-R7943 for Na^+-dependent $^{45}Ca^{2+}$ uptake were 40 nM and 1.8 μM, respectively (Table 1). These results indicate that SEA0400 is 45 times more potent than KB-R7943 in inhibiting Na^+-dependent $^{45}Ca^{2+}$ uptake.

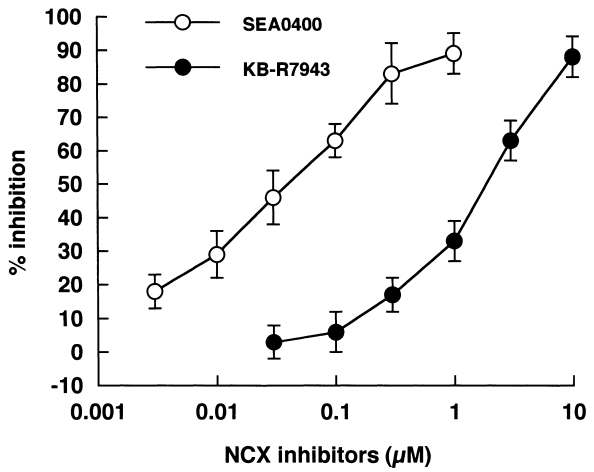

Fig. 1. The effects of SEA0400 and KB-R7943 on Na^+-dependent $^{45}Ca^{2+}$ uptake in bovine adrenal chromaffin cells in which Na^+ was loaded by the treatment of ouabain and veratridine. Na^+-dependent $^{45}Ca^{2+}$ uptake was estimated by subtracting basal $^{45}Ca^{2+}$ uptake during the incubation with normal Na^+ solution from $^{45}Ca^{2+}$ uptake during the incubation with Na^+-free solution. The ordinate shows % inhibition against the control, and the abscissa shows concentrations of NCX inhibitors. Symbols and vertical bars represent means ± S.E.M. obtained from 7 wells.

Effects of SEA0400 and KB-R7943 on Na^+-dependent CA release

Na^+-loaded cells prepared with the same protocol as used in the Na^+-dependent $^{45}Ca^{2+}$ uptake experiments released markedly large amounts of CAs during incubation in the Na^+-free solution. The CA release during the incubation with normal Na^+ solution was defined as the basal CA release from Na^+-loaded cells. Na^+-dependent CA release was estimated by subtracting the basal CA release in the normal Na^+ solution from the CA release in the Na^+-free solution. Na^+-dependent CA release in the absence of NCX inhibitors, as a control, was $13.6 \pm 0.6\%$ of the total CA content (n = 16) in both SEA0400 and KB-R7943 experiments. The effects of SEA0400 and KB-R7943 on Na^+-dependent CA release, indicated as % inhibition, are shown in Fig. 2. SEA0400 (3 – 1000 nM) and KB-R7943 (0.03 – 10 μM) inhibited Na^+-dependent CA release in a concentration-dependent manner, and significant inhibition ($P<0.05$ or $P<0.01$) was observed at concentrations higher than 30 nM and 0.3 μM, respectively. IC_{50} values of SEA0400 and KB-R7943 for Na^+-dependent CA release were 100 nM and 3.7 μM, respectively (Table 1). These results indicate that SEA0400 is 37 times more potent than KB-R7943 in inhibiting Na^+-dependent CA release.

Effects of SEA0400 and KB-R7943 on CA release induced by ACh, DMPP, and KCl

ACh (30 μM), DMPP (10 μM), and KCl (40 mM) induced CA release from $1.4 \pm 0.2\%$ (basal release, n = 12) to $11.9 \pm 0.4\%$ (n = 22), $13.5 \pm 0.6\%$ (n = 22), and $8.4 \pm 0.3\%$ of total CA content (n = 12), respectively, in the absence of NCX inhibitors or dihydro-β-erythroidine in intact chromaffin cells. SEA0400 (1 – 10 μM) and KB-R7943 (1 – 10 μM) significantly ($P<0.05$ or $P<0.01$) inhibited the ACh- and DMPP-induced CA release (Fig. 2). For inhibition of ACh- and DMPP-induced CA release, the IC_{50} values of SEA0400 were 5.1 and 4.5 μM and the values of KB-R7943 were 2.6 and 2.1 μM, respectively (Table 1). These results indicate that the ability of SEA0400 to inhibit the nicotinic receptor-mediated CA release is about a half of that of KB-R7943. The KCl-induced CA release was slightly but significantly ($P<0.05$) inhibited 10 μM SEA0400 (21%) and 10 μM KB-R7943 (27%). The basal CA release was not affected by even 10 μM SEA0400 ($1.9 \pm 0.3\%$ of total CA content, n = 6), but was slightly enhanced by 10 μM KB-R7943 ($2.7 \pm 0.3\%$,

Table 1. IC_{50} values of SEA0400 and KB-R7943 for Na^+-dependent $^{45}Ca^{2+}$ uptake, CA release (Na^+-dependent, ACh-, DMPP-, and KCl-induced), and [^{3}H]nicotine binding in bovine adrenal chromaffin cells

Parameter	IC_{50} (μM)		Relative potency[a]
	SEA0400	KB-R7943	
Na^+-dependent $^{45}Ca^{2+}$ uptake	0.04	1.8	45
CA release			
Na^+-dependent	0.1	3.7	37
ACh	5.1	2.6	0.5
DMPP	4.5	2.1	0.5
KCl	>10	>10	nd[b]
[^{3}H]Nicotine binding	90	12	0.1

[a] The relative potency of SEA0400 against KB-R7943 was calculated by dividing the IC_{50} value of KB-R7943 by that of SEA0400. [b] not determined.

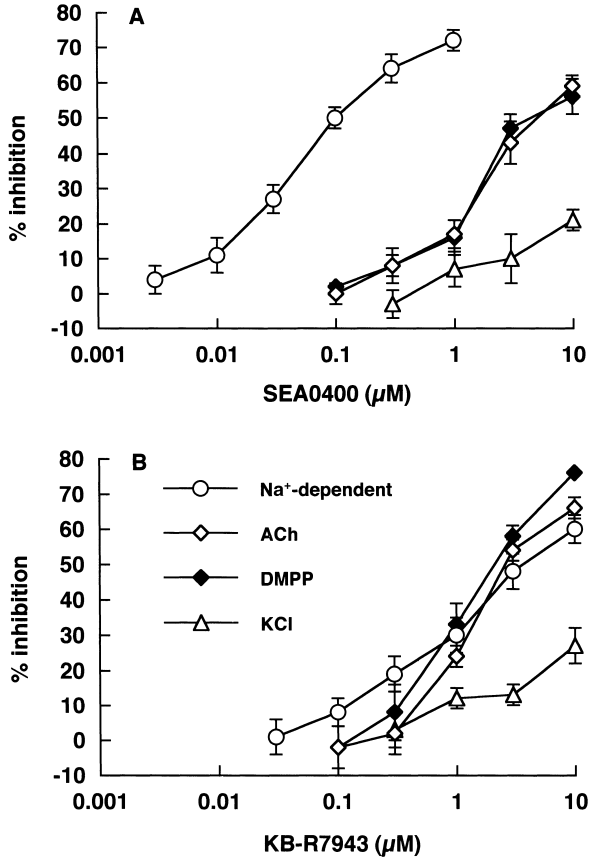

Fig. 2. The effects of SEA0400 (A) and KB-R7943 (B) on Na^+-dependent CA release and ACh-, DMPP-, and KCl-induced CA release in bovine adrenal chromaffin cells. Na^+-dependent CA release was estimated by subtracting basal CA release during the incubation of Na^+ loaded cells with normal Na^+ solution from CA release during the incubation with Na^+-free solution. Na^+ loading of cells was done by the treatment with ouabain and veratridine. In intact cells, CA release was induced by incubation with ACh (30 μM), DMPP (10 μM), or KCl (40 mM). The ordinate shows % inhibition against the control and the abscissa shows concentrations of NCX inhibitors. Symbols and vertical bars represent means ± S.E.M. obtained from 6 – 8 wells.

n = 6).

Dihydro-β-erythroidine (10 – 300 μM) significantly (P<0.05 or P<0.01, n = 8) inhibited the ACh- and DMPP-induced CA release, with IC_{50} values of 100 and 86 μM, respectively.

Nicotinic receptor binding assay

Specific binding with nicotinic receptors was estimated by subtracting 5 nM [^{3}H]nicotine binding in the presence of 1 mM cold nicotine (non-specific binding) from the binding in the presence and absence (control) of NCX inhibitors or dihydro-β-erythroidine. The specific binding of the control was 8248 ± 1143 cpm/1 × 10^6 cells (n = 24) in all experiments. The concentration-displacement curves of SEA0400 and

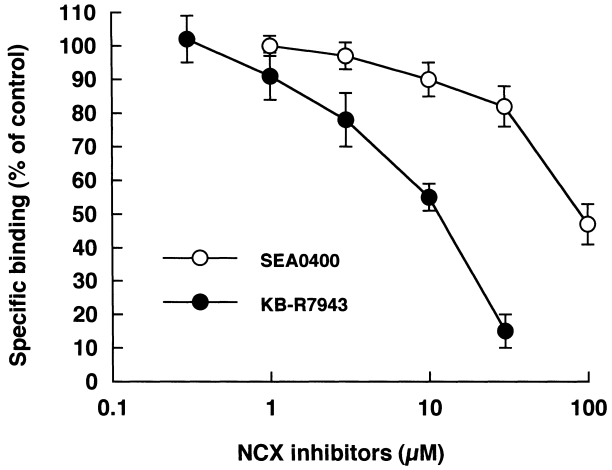

Fig. 3. Concentration-displacement curves of SEA0400 and KB-R7943 for [^{3}H]nicotine binding in bovine adrenal chromaffin cells. Specific binding with nicotinic receptors was estimated by subtracting 5 nM [^{3}H]nicotine binding in the presence of 1 mM cold nicotine (non-specific binding) from the binding in the presence and absence (control) of NCX inhibitors. The ordinate shows % of the control, and the abscissa shows concentrations of NCX inhibitors. Symbols and vertical bars represent means ± S.E.M. obtained from 8 wells.

KB-R7943 for [^{3}H]nicotine binding are shown in Fig. 3. SEA0400 (30 and 100 μM) and KB-R7943 (3 – 30 μM) significantly (P<0.05 or P<0.01) inhibited [^{3}H]nicotine binding. The IC_{50} values of SEA0400 and KB-R7943 calculated from mean concentration-displacement curves were 90 and 12 μM, respectively (Table 1). These results indicate that the ability of SEA0400 to inhibit the [^{3}H]nicotine binding is about 1/10 of that of KB-R7943.

Dihydro-β-erythroidine (100 – 1000 μM) significantly (P<0.05 or P<0.01, n = 8) inhibited [^{3}H]nicotine binding, with an IC_{50} value of 360 μM.

Discussion

Na^+-dependent Ca^{2+} uptake was induced by the incubation of Na^+-loaded bovine adrenal chromaffin cells with a Na^+-free medium containing the L-type Ca^{2+} channel blocker nicardipine and the N-, P-, and Q-type Ca^{2+} channel blocker ω-conotoxin MVIIC. The cytosol was loaded with Na^+ by treatment with ouabain and veratridine to inhibit the Na^+ pump and to activate Na^+ channels, respectively. Under these conditions, it is likely that Na^+-dependent Ca^{2+} uptake is mostly due to activation of the NCX working in the reverse mode (13). As expected, SEA0400 at 10 – 1000 nM inhibited Na^+-dependent $^{45}Ca^{2+}$ uptake, and the IC_{50} value was 40 nM. The effective concentration range of SEA0400 in the present study is consistent with that inhibiting Na^+-dependent $^{45}Ca^{2+}$ uptake in three kinds of cultured rat

neuronal cells (IC_{50} = 5 – 33 nM, ref. 14) and in cultured rat cardiomyocytes (IC_{50} = 92 nM, ref. 15). On the other hand, inhibition by KB-R7943 was observed at 1 μM or more, and the IC_{50} value was 1.8 μM, which is in accord with the result obtained in a similar experiment using bovine adrenal chromaffin cells (IC_{50} = 5.5 μM, ref. 13). Therefore, SEA0400 is 45 times more potent than KB-R7943 in inhibiting Na^+-dependent (NCX-mediated) Ca^{2+} uptake in bovine adrenal chromaffin cells.

Three genes for mammalian NCX have been cloned. NCX1 is highly expressed in the heart, brain, and kidney and expressed at much lower levels in other tissues, whereas the expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle (17). It was reported that bovine adrenal chromaffin cells express NCX1 (7). SEA0400 predominantly blocked NCX1, only mildly blocked NCX2, and exerted almost no influence on NCX3 when its blocking action on Na^+-dependent $^{45}Ca^{2+}$ uptake was assessed in NCX-transfected fibroblasts (25). These investigators also reported that KB-R7943 was more effective on NCX3 than on NCX2 and NCX1 in the same preparation (26). These studies indicate that SEA0400 (IC_{50} = 56 nM) is more potent than KB-R7943 (IC_{50} = 4.3 μM) in inhibiting NCX1. The effective concentration ranges and the IC_{50} values of the NCX inhibitors for Na^+-dependent $^{45}Ca^{2+}$ uptake in the present study are in accord with those for Na^+-dependent $^{45}Ca^{2+}$ uptake mediated by NCX1 in NCX-transfected fibroblasts. Therefore, it is conceivable that NCX1 is involved in Na^+-dependent $^{45}Ca^{2+}$ uptake in bovine adrenal chromaffin cells.

CAs are released from adrenal chromaffin cells by exocytosis triggered by an increase in the cytosolic concentration of Ca^{2+}. Several investigators have shown that inhibiting the Na^+ pump or lowering extracellular Na^+ concentration increases basal CA release and/or augments CA release evoked by secretagogues in adrenal medullary cells (8 – 10). These results suggest that the CA release is triggered by Na^+-dependent Ca^{2+} uptake under these conditions. In the present study, Na^+-dependent CA release experiments were carried out according to an experimental protocol similar to that in the Na^+-dependent $^{45}Ca^{2+}$ uptake experiments. Markedly large amounts of CAs were released from Na^+-loaded cells during incubation with Na^+-free medium, suggesting that the CA release is due to Ca^{2+} influx through the reverse mode exchange of the NCX. The Na^+-dependent CA release was inhibited by 30 – 1000 nM SEA0400 (IC_{50} = 100 nM) and 0.3 – 10 μM KB-R7943 (IC_{50} = 3.7 μM). These results are consistent with those obtained in the Na^+-dependent $^{45}Ca^{2+}$ uptake experiment in this study and indicate that SEA0400 is 37 times more potent than KB-R7943 in inhibiting Na^+-dependent CA release.

Physiological release of CAs from the adrenal gland is initiated by activation of nicotinic receptors by ACh released from the splanchnic nerve. Activation of nicotinic receptors promotes Na^+ and Ca^{2+} influx through receptor-linked ion channels, the resulting depolarization produces Ca^{2+} influx through voltage-operated Ca^{2+} channels, and the subsequent elevation of cytosolic concentration of Ca^{2+} triggers adrenal CA release (27, 28). In the present study, the effects of SEA0400 and KB-R7943 on CA release in response to nicotinic receptor stimulation were examined in intact chromaffin cells. SEA0400 at 1 – 10 μM inhibited CA release induced by ACh and the nicotinic receptor stimulant DMPP, with IC_{50} values of 5.1 and 4.5 μM, respectively. These results indicate that the concentrations required to inhibit the nicotinic receptor-mediated CA release are about 50 times larger than those to inhibit Na^+-dependent CA release (IC_{50} = 100 nM). On the other hand, the concentrations of KB-R7943 required to inhibit CA release induced by ACh (IC_{50} = 2.6 μM) or DMPP (IC_{50} = 2.1 μM) are almost the same as those necessary to inhibit Na^+-dependent CA release (IC_{50} = 3.7 μM). These results indicate that SEA0400 but not KB-R7943 has a potent and selective action on NCX in bovine adrenal chromaffin cells.

[^{3}H]Nicotine binding with nicotinic receptors was displaced by SEA0400 and KB-R7943, with the IC_{50} values of 90 and 12 μM, respectively. Generally, the IC_{50} value obtained from displacement binding assay is thought to be variable depending on the labeled ligand concentration and number of receptors, and therefore, it does not match the IC_{50} value obtained from the functional response mediated by the receptors. To evaluate their inhibitory effects, we examined the effects of the selective nicotinic antagonist dihydro-β-erythroidine, as a positive control, on CA release induced by ACh and DMPP and on [^{3}H]nicotine binding for nicotinic receptors. The IC_{50} values of dihydro-β-erythroidine were 100 and 86 μM for CA release induced by ACh and DMPP, respectively, and 360 μM for [^{3}H]nicotine binding. These results indicate that the concentration required to inhibit [^{3}H]nicotine binding is about 4 times higher than the concentrations necessary to inhibit the nicotinic receptor-mediated CA release. The ratio well corresponds to the ratio obtained with KB-R7943 (about 5-fold) but not to the ratio obtained with SEA0400 (about 20-fold). Accordingly, the inhibitory effect of KB-R7943 on nicotinic receptor-mediated CA release seems to be due to the inhibition of nicotinic receptors as reported previously (13), whereas the inhibitory effect of SEA0400 cannot be explained by the same mechanism. Multiple neuronal nicotinic receptor subtypes composed of 9 different

α subunits and 3 different β subunits are identified and have been found to display different functional characteristics and different affinities to nicotinic agonists and antagonists (29, 30). Under the same conditions in this study, mecamylamine and hexamethonium, nicotinic antagonists, inhibited the ACh-induced CA release, with the IC_{50} values of 0.2 and 8 μM, respectively, whereas they did not inhibit [^{3}H]nicotine binding even at 100 times higher concentrations or more (data not shown). Therefore, the possibility remains that SEA0400 inhibits the nicotinic receptor-mediated CA release by acting on some nicotinic receptor subtype. Another possible explanation is the contribution of the SEA0400-induced inhibition of NCX to the inhibition of the nicotinic receptor-mediated CA release, since the inhibitory potencies of NCX inhibitors are dramatically altered by intracellular Na^+ concentration and the Na^+-dependent inactivation state (17, 31). However, the exact mechanism underlying the inhibitory effect of SEA0400 on the nicotinic receptor-mediated CA release is not evidenced in this study. Consequently, it is also uncertain whether NCX is responsible for the physiological release of CAs, although it has been suggested that NCX is involved in CA release under the condition in which the Na^+ pump is inhibited or extracellular Na^+ is reduced (8 – 10).

CA release induced by high K^+ was slightly reduced by 10 μM SEA0400 (21%) or 10 μM KB-R7943 (27%). The depolarization-induced CA release is thought to be associated with Ca^{2+} influx due to activation of voltage-operated Ca^{2+} channels. Therefore, it seems likely that the inhibition by SEA0400 and KB-R7943 is related to their blocking of voltage-operated Ca^{2+} channels, as observed in the rat cerebral cortex (14). However, the inhibitory action of SEA0400 on voltage-operated Ca^{2+} channels seems to be negligible when compared with its ability to inhibit NCX.

In conclusion, the present study demonstrated that SEA0400 at nM order concentrations inhibited Na^+-dependent Ca^{2+} uptake and CA release in Na^+-loaded chromaffin cells and at higher concentrations, nicotinic receptor-mediated CA release in intact cells. SEA0400 is about 40 times more potent than KB-R7943 in inhibiting the NCX-mediated responses, whereas the potency of SEA0400 is about a half of that of KB-R7943 in inhibiting the nicotinic receptor-mediated CA release. These results indicate that SEA0400 has a potent and selective action on NCX in bovine adrenal chromaffin cells.

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