Molecular Determinants of Na\(^+\)/Ca\(^{2+}\) Exchange (NCX1) Inhibition by SEA0400*

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SEA0400 is a potent and selective Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) inhibitor. We evaluated the inhibitory effects of SEA0400 on Na\(^+\)-dependent \(^{45}\)Ca\(^{2+}\) uptake and whole-cell Na\(^+\)/Ca\(^{2+}\) exchange currents in NCX-transfected fibroblasts. SEA0400 preferentially inhibited \(^{45}\)Ca\(^{2+}\) uptake by NCX1 compared with inhibitions by NCX2, NCX3, and NCX2X. SEA0400 also selectively blocked outward exchange currents from NCX1 transfectedants. We searched for regions that may form the SEA0400 receptor in the NCX1 molecule by NCX1/NCX3 chimeraic analysis. The results suggest that the first intracellular loop and the fifth transmembrane segment are mostly responsible for the differential drug responses between NCX1 and NCX3. Further site-directed mutagenesis revealed that multiple mutations at Phe-213 markedly reduced sensitivity to SEA0400 without affecting that to KB-R7943. We also found that Gly-833-to-Cys mutation (within the α-2 repeat) greatly reduced the inhibition by SEA0400, but unexpectedly the NCX1 chimera with an α-2 repeat from NCX2 possessed normal drug sensitivity. In addition, exchangers with mutated exchanger inhibitory peptide regions, which display either undetectable or accelerated Na\(^+\)-dependent inactivation, had a markedly reduced sensitivity or hypersensitivity to SEA0400, respectively. To verify the efficacy of the NCX inhibitor, we examined the renoprotective effect of SEA0400 in a hypoxic injury model using porcine renal tubular cells. SEA0400 protected against hypoxia/reoxygenation-induced cell damage in tubular cells expressing wild-type NCX1 but not in cells expressing SEA0400-insensitive mutants. These results suggest that Phe-213, Gly-833, and residues that eliminate Na\(^+\)-dependent inactivation are critical determinants for the inhibition by SEA0400, and their mutants are very useful for checking the pharmacological importance of NCX inhibition by SEA0400.

The Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is a sarcolemmal transporter that is expressed in many mammalian cell types. This exchanger is bidirectional, being controlled by membrane potential and transmembrane gradients of Na\(^+\) and Ca\(^{2+}\) (for reviews, see Refs. 1–3). In cardiac muscle, the exchanger plays the primary role in the extrusion of intracellular Ca\(^{2+}\) (Ca\(^{2+}\)\(_i\)) during each excitation-contraction coupling, although it is capable of introducing Ca\(^{2+}\) into cardiomyocytes under certain conditions (4). In other cell types, however, its importance in Ca\(^{2+}\) handling remains to be well defined. Under pathological conditions such as cardiac ischemia/reperfusion injury (5), the exchanger is thought to cause Ca\(^{2+}\) overload due to elevated intracellular Na\(^+\) (Na\(^+_i\)) concentration, leading to mechanical and electrical dysfunction of cardiomyocytes. Such Ca\(^{2+}\) overloading is also observed in other ischemic organs (1).

The mammalian NCX forms a multigene family of highly homologous proteins (~70% identity to one another) comprising three isoforms, NCX1, NCX2, and NCX3 (6–8). NCX1 is highly expressed in the heart, kidney, and brain and at much lower levels in other tissues, whereas the expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle (9). These three isoforms presumably have similar molecular topologies consisting of nine transmembrane segments and a large central cytoplasmic loop (10, 11). The former part, particularly the α-repeat regions, may participate in ion transport and the interaction with NCX inhibitors and ionic modulators (12–16); the latter part, possessing the exchanger inhibitory peptide (XIP) region, regulatory Ca\(^{2+}\) binding sites, and phosphorylation sites, is primarily involved in various regulatory properties (17–21). NCX1 has been shown to be secondarily regulated by the transport substrates Na\(^+\) and Ca\(^{2+}\) \((22, 23)\). Ca\(^{2+}\)\(_i\) at submicromolar level activates NCX activity by promoting the recovery of the exchanger from the "I\(_2\) inactivation state," whereas high Na\(^+_i\) restrains the exchange by facilitating the entry of the exchanger into the "I\(_1\) inactivation state." The physiological significance of such regulation, however, is poorly understood.

A potent and selective NCX inhibitor will be extremely useful in the study of the physiological and pathophysiological roles of NCX and to clarify the reaction mechanism of this transporter. Moreover such an inhibitor may offer therapeutic potential as a new remedy for several ischemic diseases, arrhythmias, heart failure, and essential hypertension. In 1996, KB-R7943 was introduced for the first time as a selective NCX inhibitor (24, 25). This agent has some interesting features. KB-R7943 inhibits the reverse mode (i.e. Ca\(^{2+}\) influx) by NCX much more effectively than the forward mode (24–27), although there is no difference in the selectivity of the agent for the reverse mode under conditions allowing bidirectional currents (28). In addition, KB-R7943 is 3-fold more effective on NCX3 than NCX1 and NCX2 (29). Recently we have investigated the inhibitory mechanism of KB-R7943 using mutational analysis and have

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‡ The abbreviations used are: NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; XIP, exchanger inhibitory peptide; Ca\(^{2+}\)\(_i\), intracellular Ca\(^{2+}\); Na\(^+_i\), intracellular Na\(^+\); Ca\(^{2+}\)\(_o\), extracellular Ca\(^{2+}\); Na\(^+_o\), extracellular Na\(^+\); K\(^+_o\), extracellular K\(^+\); BSS, balanced salt solution; BAPTA, 1,2-bis(2aminophenoxy)ethane-N\(_2\),N\(_2\),N\(_2\),N\(_2\)-tetraacetic acid.

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identified amino acids Val-820, Gin-826, and Gly-833 in the α-2 repeat that are at least partly critical for the interaction of the exchanger with KB-R7943 (16). Since 1996, KB-R7943 has been widely used as a pharmacological tool to study the roles of the exchanger at the cellular and organ levels. For example, KB-R7943 has been shown to efficiently guard against toxicity by cardiac glycosides (26, 30) and ischemia/reperfusion injury of the heart (24, 27, 31–33), kidney (34, 35), and brain (36, 37). More recently, however, nonspecific effects of KB-R7943 have been suggested. KB-R7943 at relatively low doses is reported to block ion channels, neuronal nicotinic acetylcholine receptor, N-methyl-D-aspartate receptor, and norpinephrine transporter (25, 38–40), although this agent actually has a lower potency for the Na+/H+ exchanger, Na+/K+-ATPase, Ca2+-ATPase, and some other receptors (24, 40, 41).

In 2001, Matsuda et al. (40) reported on SEA0400, a newly developed, potent, and selective inhibitor of NCX. SEA0400 potently inhibits Na+-dependent Ca2+ uptake via the reverse mode of the exchanger in three kinds of cultured neuronal cells (40) and in cultured cardiomyocytes (42); their IC50 values were 5–33 nM and 92 nM, respectively. The inhibitory potency of SEA0400 was 80–100 times more powerful than that of KB-R7943. SEA0400 also has an excellent specificity against several ion channels, transporters, and receptors. Recently, SEA0400 has been reported to reduce cerebral infarct volume in a transient middle cerebral artery occlusion model (40) and also markedly attenuate several ischemia/reperfusion injuries in Langendorff-perfused hearts and acute myocardial infarction models (42, 43) and in acute ischemic renal failure models (44). These reports further suggest that the abilities of SEA0400 to guard against these ischemic injuries are more effective than those of KB-R7943. Therefore, the pharmacological research on SEA0400 is expected to become more active in the near future. Currently, however, little is known about the inhibitory mechanism of SEA0400 and the location of molecular determinants of this agent in the exchanger molecule.

In this study, we used three NCX isoforms and NCKX2, a Na+/Ca2+-K+ exchanger type 2, to determine which region(s) and amino acid residues contribute to the different drug responses of these exchangers. We compared the functional properties of these exchangers and their mutants and identified some residues that are critical in conferring the sensitivity to SEA0400 in the NCX1 molecule. Moreover, we applied their mutants, which markedly lose drug sensitivity, to verify the pharmacological consequence of the inhibition of the exchanger by SEA0400 as there is a possibility that the agent actually has unknown nonspecific effects (45). A portion of this work was previously presented in abstract form (46).

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Chinese hamster lung fibroblast LLC-PK1 cells and their NCX transfectants were maintained in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere gassed with 5% CO2, 95% air at 37 °C. Porcine tubular epithelial LLC-PK1 cells and their NCX transfectants were grown in Dulbecco’s modified Eagle’s medium supplemented with 4% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

**Construction and Stable Expression of Wild-type, Chimeric, and Mutant NCX1 Exchangers**—We used major splice variants of cDNAs for NCXs that are prevalent in dog hearts (NCX1.1) and rat brains (NCX2.1 and NCX3.3) (9). As described previously (16, 19, 29), NCX cDNAs were cloned into SacII and HindIII restriction sites in pCRII (Invitrogen). NCX1/NCX3 chimeras shown in Fig. 3A were constructed as detailed in previous references (14). Other chimeras, shown in Fig. 5B, were constructed by transfecting the homologous segments of NCX3 into the corresponding regions of pCRII-NCX1 using the newly introduced restriction enzyme sites or endogenous sites (SacII, NruI, SpeI, BamHI, XmaI, EcoRV, and ClaI). These sites in NCX1 were located at amino acid positions –52, 40, 72, 108, 192, 230, and 445 (numbers based on Ref. 47), respectively. Substitution of amino acid residues in NCX1 was performed by site-directed mutagenesis using a polymerase chain reaction-based strategy as described previously (14). Successful construction of the modified cDNAs was verified by sequencing (ABI PRISM 3100 Genetic Analyzer). These cDNAs were transferred into SacII and HindIII sites in the mammalian expression vector pKCRH.

**Rat NCKX2** cDNA was cloned into EcoRI and KpnI restriction sites in the mammalian expression vector pcDNA3.1 (Invitrogen) as described previously (16). For construction of NCX1/NCKX2 chimeras within the α repeat regions, two pairs of restriction enzyme sites (SacII and BamHI) were newly introduced into pCRII-NCX1 at amino acid positions 106 and 138 for α-1 region and 807 and 837 for α-2 region. Generation of SacII sites resulted in substitution of amino acids (I107A and K137A) in NCX1 with that of NCKX2 (designated NCX1/ NCKX2(α-1) and NCX1/NCKX2(α-2), respectively). To obtain stable expression of wild-type, chimeric, and mutant exchangers, pKCRH or pcDNA3.1 plasmids carrying exchanger cDNAs were transfected in the presence of Lipofectin (Invitrogen) into CCL39 fibroblasts or LLC-PK1 cells (19, 20). Cell clones highly expressing NCX activity or NCKX activity were selected by a Ca2+ killing procedure as described previously (16).

**Assay of 45Ca2+ Uptake**—Na+-dependent Ca2+ uptake into cells expressing the wild-type or mutated NCXs was assayed as described in detail previously (29). Briefly confluent transfecteds in 24-well dishes were washed with Na+-free assay medium (replacing NaCl with equimolar choline chloride) or to normal BSS, both of which contained 0.1 mM CaCl2 (1.5 μL/cm2) and 1 mM ouabain. After a 30-s incubation, 45Ca2+ uptake was terminated by washing cells four times with an ice-cold solution containing 10 mM Hepes/Tris, pH 7.4, 120 mM choline chloride, and 10 mM LaCl3. Cells were then solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity. When present, SEA0400 and KB-R7943 were added before the start of the 30-s incubation. 45Ca2+ uptake into cells expressing NCKX2 was performed under the same conditions as that for NCX.

**Measurement of Whole-cell Exchange Currents**—Unidirectional outward and inward exchange currents from NCX transfectants were measured using the whole-cell patch clamp technique as described previously (15, 16). For recording the outward current, the external solution contained 150 mM LiCl (replacing NaCl), 1 mM MgCl2, or 1 mM CaCl2, 20 μM ouabain, 2 μM nicardipine, 5 μM ryanodine, and 5 mM Hepes (pH 7.2), whereas the pipette solution contained 100 mM NaOH, 20 mM CsOH, 1.1 mM MgCl2, 20 mM tetrathylammonium chloride, 2 mM MgATP, 2 mM creatine phosphate, 19.8 mM CaCl2, 50 mM EGTA, and 5 mM Hepes (pH 7.2). The ionized Ca2+ concentration in the pipette solution was calculated to be 0.16 μM. The outward current was activated by the application of 1 mM Ca2+-free BSS. For recording the inward current, the external solution contained 140 mM choline chloride or NaCl, 1 mM MgCl2, 20 μM ouabain, 2 μM nicardipine, 10 μM ryanodine, and 5 mM Hepes (pH 7.2), while the pipette solution contained 30 mM CsCl, 90 mM NaOH, 50 mM aspartic acid, 3 mM MgCl2, 16 mM CaCl2, 5 mM MgATP, 5 mM dipotassium creatine phosphate, 20 mM BaPTA, and 20 mM Hepes (pH 7.2). The ionized Ca2+ concentration in the pipette solution was calculated to be 1.75 μM. The inward current was induced by switching the choline chloride-containing external solution to the NaCl-containing external solution. All experiments were performed at 35°C with the holding and test potentials set at ±40 mV. All data were acquired and analyzed by the pCLAMP software (Axon Instruments).

**Hypoxia and Reoxygenation in LLC-PK1—** LLC-PK1 cells and their NCX transfectants were grown in 96-well microplates at 2 × 104 cells/well. After 2 days, the medium was changed to Hepes-buffered Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were exposed to hypoxic conditions in an Anaero Pack Pouch (Mitsubishi Gas Chemical, Tokyo, Japan) in which the oxygen concentration was less than 1% within 1 h as described previously (35). After 6 h of hypoxia, the cells were put in a humidified incubator gassed with 5% CO2, 95% air for 1 h in Hepes-buffered Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. After the hypoxia/reoxygenation treatment, lactate dehydrogenase activity in the medium was measured using a lactate dehydrogenase cytoytic test kit (Wako Pure Chemicals). SEA0400 (1 μM) was added to the medium at the beginning of reoxygenation. Lactate dehydrogenase activity
release was expressed as a percentage of total cellular lactate dehydrogenase activity.

Statistical Analysis—Data are expressed as means ± S.E. of three or four independent determinations. Differences for multiple comparisons were analyzed by an unpaired t test or one-way analysis of variance followed by Dunnett’s test. Values of p < 0.05 were considered statistically significant.

Materials—CCL39 and LLC-PK1 cells were purchased from American Type Culture Collection (Manassas, VA). SEA0400 (2-[4-[2,5-difluorophenyl]methoxy]phenoxyl]-5-ethoxyaniline) was synthesized by Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). KB-R7943 (2-[4-[4-(nitrobenzyloxyl)phenyl]-ethyl]-isothiourea methanesulfonate) was provided by Nippon Organon (Osaka, Japan). 45CaCl2 was purchased from Amersham Biosciences. All other chemicals were also of the highest grade available.

RESULTS

Inhibitory Properties of SEA0400—We first compared the inhibitory effects of SEA0400 on Na+-dependent 45Ca2+ uptake into CCL39 cells with a stable transfection of NCX isoforms (NCX1, NCX2, and NCX3) or K+-dependent Na+/Ca2+ exchanger (NCKX2). SEA0400 (up to 3 µM) inhibited dose-dependently the initial rate of 45Ca2+ uptake into NCX1 and NCX2 transfectants with I50 values of 0.056 ± 0.007 and 0.98 ± 0.05 µM (n = 3), respectively; but it did not significantly affect uptake into NCX3 and NCKX2 transfectants (Fig. 1), indicating that SEA0400 has a selectivity to NCX1. In NCX1 transfectants, SEA0400 was about 70 times more inhibitory than KB-R7943 (I50 = 4.1 µM, see Fig. 6). To check whether SEA0400 competes with extracellular Ca2+ (Ca2+) on the exchanger, the rate of Na+-dependent 45Ca2+ uptake into NCX1 transfectants was measured under standard conditions as a function of Ca2+ concentration (0.06–2 mM) in the presence or absence of 0.05 µM SEA0400. Their double reciprocal plots of uptake rate versus Ca2+ concentration were linear (data not shown). SEA0400 increased the half-maximal Ca2+ concentration (IC50) value from 0.22 ± 0.03 mM of the control to 0.39 ± 0.05 mM (p < 0.05, n = 3) and decreased the corresponding maximal velocity (Vmax) value from 24 ± 2.6 nmol/mg/30 s of the control to 11 ± 1.4 nmol/mg/30 s (p < 0.05), suggesting a type of mixed (competitive and noncompetitive) inhibition.

Next we examined the effects of SEA0400 on the outward and inward exchange currents induced in NCX1 transfectants by the application of 1 mM Ca2+; the cell was placed in drug-free bath solution and then in bath solution containing 0.3 µM SEA0400 for 6 min followed by washing for 6 min with drug-free solution. B, the inward exchange currents were successively evoked by the application of 140 mM Na+ with or without 3 µM SEA0400 in a manner similar to that in A. C, dose-response curves for the effects of SEA0400 or KB-R7943 on the outward and inward exchange currents. Data are presented as percentage of the peak currents obtained in the absence of the drug. Data are means ± S.E. of three independent experiments.
Chimeric Analysis of the Inhibitory Effect of SEA0400—

Taking advantage of the fact that NCX1 and NCX3 differ distinctly from each other in their sensitivity to SEA0400 despite their high sequence homology, we performed chimeric analysis between these isoforms to identify important region(s) in the NCX1 molecule for the interaction with SEA0400. We constructed a series of chimeras in which one or two segments from NCX3 were transferred into NCX1 in exchange for the homologous segment(s) (Fig. 3). All chimeras constructed exhibited exchange activities similar to that of the wild-type NCX1 (see the legend to Fig. 3).

Fig. 3 shows the effect of 0.3 μM SEA0400 on the rate of Na⁺, dependent ⁴⁵Ca²⁺ uptake into cells expressing wild-type and chimeric exchangers between NCX1 and NCX3. A and B, left, a series of chimeras were constructed by substituting segments of NCX1 with homologous segments of NCX3. These chimeras are named based on the amino acid numbers for NCX1. The restriction enzyme-cut sites are shown by broken lines, and the segments substituted with NCX3 sequences are indicated by hatched boxes. Linear models of the exchangers are indicated at the top and bottom, and the numbered open boxes show positions of transmembrane segments. Right, the initial rates of Na⁺, dependent ⁴⁵Ca²⁺ uptake into cells were measured in the presence or absence of 0.3 μM SEA0400 as described under “Experimental Procedures.” The uptake rates in cells expressing these chimeras were 5–13 nmol/mg/30 s. Data are presented as percentage of the values obtained in the absence of the drug. Data are means ± S.E. of three independent experiments. *, p < 0.05 versus NCX1.

Identification of Residues Involved in Drug Sensitivity—Fig. 4A shows amino acid sequences of NCX1 and NCX3 in SpeI-BamHI (amino acids 73–108 in NCX1) and XmaI-EcoRV segments (i.e. the fifth transmembrane region) from NCX3, which contained eight residues unique to each isoform. To identify the critical residues involved in drug sensitivity, these unique residues in NCX1 were exchanged with the corresponding residues in NCX3, one or two residues at a time. Five mutants derived from the SpeI-BamHI region did not produce any significant change in sensitivity to inhibition by 0.3 μM SEA0400 (Fig. 4B). On the other hand, among seven mutants derived from the XmaI-EcoRV region, only N1-P213L mutant (Phe-213 mutated to Leu) exhibited a significantly decreased SEA0400 sensitivity to a level almost comparable with that observed in the wild-type NCX3 (Fig. 4B). We further performed multiple amino acid substitution at Phe-213 to analyze its importance for drug sensitivity. Fig. 4C shows the dose-response profiles of these mutants for inhibition of Na⁺, dependent ⁴⁵Ca²⁺ uptake

Fig. 3. Effects of SEA0400 on Na⁺, dependent ⁴⁵Ca²⁺ uptake into cells expressing the wild-type and chimeric exchangers between NCX1 and NCX3. A and B, left, a series of chimeras were constructed by substituting segments of NCX1 with homologous segments of NCX3. These chimeras are named based on the amino acid numbers for NCX1. The restriction enzyme-cut sites are shown by broken lines, and the segments substituted with NCX3 sequences are indicated by hatched boxes. Linear models of the exchangers are indicated at the top and bottom, and the numbered open boxes show positions of transmembrane segments. Right, the initial rates of Na⁺, dependent ⁴⁵Ca²⁺ uptake into cells were measured in the presence or absence of 0.3 μM SEA0400 as described under “Experimental Procedures.” The uptake rates in cells expressing these chimeras were 5–13 nmol/mg/30 s. Data are presented as percentage of the values obtained in the absence of the drug. Data are means ± S.E. of three independent experiments. *, p < 0.05 versus NCX1.
NCX1 Inhibition by SEA0400

**Fig. 4. Amino acid comparison between NCX1 and NCX3 and effects of SEA0400 on Na\(^+\)-dependent \(^{45}\text{Ca}^2\) uptake into cells expressing NCX1 mutants substituted at their unique residues.** A, amino acid alignment of regions responsible for drug sensitivity (positions 73–108 and 193–230 of NCX1) in NCX1 and NCX3. Conserved amino acids between the two exchangers are indicated with dots. The box shows the position of Phe-213 whose mutation alters the SEA0400 sensitivity. B, we constructed NCX1 mutants in which the unique residues in regions indicated in A were exchanged with the corresponding residue of NCX3 (B) or Phe-213 was substituted for multiple amino acids (C). In B, the initial rates of \(^{45}\text{Ca}^2\) uptake into cells expressing these NCX1 mutants were measured in the presence or absence of 0.3 \(\mu\text{M}\) SEA0400; in C, the concentration of the drug was varied to determine the dose-response curves for NCX1 mutants. The uptake rates in cells expressing NCX1 mutants were 6–11 nmmol/mg/30 s. Data are presented as percentages of the values obtained in the absence of the drug. Data are presented as means ± S.E. of three independent experiments. * \(p < 0.05\) versus NCX1.

by SEA0400. N1-F213R and N1-F213C, as well as N1-F213L, were least sensitive to SEA0400; the agent at the highest dose (3 \(\mu\text{M}\)) reduced \(^{45}\text{Ca}^2\) uptakes of these two mutants by only 20%. N1-F213A showed intermediate drug sensitivity between wild-type NCX1 and N1-F213L. The IC\(_{50}\) value for N1-F213A was 0.91 ± 0.1 \(\mu\text{M}\) (n = 3), which was about 15 times as large as that for the wild-type NCX1. N1-F213Q and N1-F213E mutants were not analyzed because they were non-functional exchangers.

We further examined the cross-reaction of SEA0400 on KB-R7943 on each critical mutant, N1-F213L or N1-G833C, that has been shown to exhibit a very low sensitivity to the latter (16). As shown in Fig. 5A, SEA0400 (0.01–3 \(\mu\text{M}\)) had almost no effect on \(\text{Ca}^{2+}\) uptake by N1-G833C. In contrast, KB-R7943 (0.3–100 \(\mu\text{M}\)) normally inhibited uptake by N1-F213L, being equal to that by the wild-type NCX1, indicating that Phe-213 is a specific determinant for inhibition by SEA0400. Because Gly-833 is located in the \(\alpha-2\) repeat region (Fig. 5A), we further analyzed the importance of \(\alpha-2\) repeat for drug sensitivity using the newly developed chimera NCX1/NCKX2(\(\alpha-2\)) in which the \(\alpha-2\) region in NCX1 was exchanged with that of NCKX2. Intriguingly NCX1/NCKX2(\(\alpha-2\)) possessed exchange activity and affinity for \(\text{Ca}^{2+}\) \(K_{\text{m}} = 0.15 \mu\text{M}\) similar to those of the wild-type NCX1. SEA0400 and KB-R7943 inhibited \(\text{Ca}^{2+}\) uptake by NCX1/NCKX2(\(\alpha-2\)) to levels comparable to those seen in the wild-type NCX1, although both inhibitors had no effect on the activity of the wild-type NCKX2 (see Fig. 1). We also constructed NCX1/NCKX2(\(\alpha-1\)) in which \(\alpha-1\) region was similarly exchanged, but it was non-functional.

**Effects of SEA0400 on NCX Mutants with Altered Regulatory Properties**—We also examined the effects of NCX inhibitors on several mutants that display the altered kinetics of Na\(^+\)-dependent inactivation (i.e., I\(_1\) inactivation). In CCL39 cells expressing the wild-type NCX1, the whole-cell outward current evoked by 1 mM \(\text{Ca}^{2+}\), was typically recorded as an initial peak and a subsequently decayed pattern (Fig. 6A). This current decay was dependent on the Na\(^+\) concentration and displayed a good fit with two exponential models as reported previously (48, 49), suggesting it is due to the I\(_1\) inactivation. The XIP region (amino acids 219–238) has been reported to be involved in the I\(_1\) inactivation (17). As shown in Fig. 6A, XIP region mutants XIP-4YW, which was produced by the mutations of Y224W/Y226W/Y228W/Y231W (49), and N1-F223E exhibited completely eliminated and accelerated I\(_1\) inactivation, respectively. These phenotypes were consistent with previous reports (17, 49). Interestingly XIP-4YW and N1-F223E mutants exhibited a markedly reduced sensitivity and hypersensitivity, respectively, to inhibition by SEA0400 or KB-R7943 (Fig. 6B). The difference between IC\(_{50}\) values of SEA0400 for XIP-4YW and NCX1 was similar to that of KB-R7943, whereas the difference in SEA0400 between N1-F223E and NCX1 tended to be smaller than that of KB-R7943 (see the legend to Fig. 6). Additionally, we examined the effects of SEA0400 on \(\Delta246–672\), in which a large cytoplasmic loop (amino acids 246–672)
containing a Ca\(^{2+}\) binding site was deleted to display a deregulated phenotype for both I\(_{1}\) inactivation and Ca\(^{2+}\)/H11001 regulation (49, 50), and D447V/D498I, in which the Ca\(^{2+}\)/H11001 binding site was mutated to display a phenotype for a low Ca\(^{2+}\)/H11001 affinity (Ca\(^{2+}\)/H11001 concentration at half-maximal activation (\(K_{h}\)) 1246–672, like XIP-4YW, showed diminished sensitivity to inhibition by SEA0400 (IC\(_{50}\) 2.8 \(\mu\)M), whereas D447V/D498I possessed a normal sensitivity to inhibition by SEA0400 (IC\(_{50}\) 0.057 \(\mu\)M).

### DISCUSSION

SEA0400 is the most potent and selective NCX inhibitor among the existing inhibitors. The inhibitory potency of SEA0400 is over 30 times more powerful than that of KB-R7943, the first developed inhibitor. SEA0400 is expected to be widely applied as a pharmacological tool or as a new remedy for ischemic disease, arrhythmia, hypertension, and so on. In this study, we examined the inhibitory properties of SEA0400 by measuring Na\(^+/\)H11001-dependent 45Ca\(^{2+}\)/H11001 uptake and whole-cell exchange currents and identified critical molecular determinants for its inhibition from chimeric analysis and subsequent site-directed mutagenesis. Finally we established a new approach using the expression system of SEA0400-insensitive mutants to verify the pharmacological consequence of NCX inhibition by SEA0400.
revealed that SEA0400 predominantly blocks NCX1 (IC$_{50}$ = 56 nM), only mildly blocks uptake by NCX2, and exerts almost no influence upon uptake by NCX3 or NCKX2 (Fig. 1). Consequently SEA0400 has an advantage in research targeting the NCX1 isoform. Previously we had found that KB-R7943 is 3-fold more inhibitory to NCX3 than to NCX1 and NCX2, and its derivative KB-R7898 has the same inhibitory potency to all three NCX isoforms (29). Although these NCX inhibitors have a common benzyloxyphenyl substructure (29, 40), they exhibit different isoform selectivities, indicating that the substituents of the benzyloxyphenyl moiety prescribe the isoform selectivity. We have also found that the divalent cation Ni$^{2+}$ displays an inhibition that is 10-fold more potent in NCX1 and NCX2 than in NCX3 and that the monovalent cation Li$^+$ stimulates the...
exchange activity of NCX2 or NCX3 greater than that of NCX1 (14). Taken together, the pharmacological properties are clearly different between NCX1 and NCX3, and the properties of NCX2 are intermediate among them. On the other hand, the three isoforms have been shown to possess similar affinities for transport ions, Ca\(^{2+}\), or Na\(^{+}\) (29, 51). We found evidence that SEA0400, like KB-R7943 (14, 27), did not simply compete with Ca\(^{2+}\). Therefore, we postulate that SEA0400 does not act directly on the Ca\(^{2+}\) binding site but probably interacts with the region close to the transport pathway in which the structure in NCX1 may be functionally different from that in NCX3.

We next evaluated the transport mode selectivity of SEA0400 by measuring whole-cell outward and inward exchange currents from NCX1 transfectants. SEA0400 selectively blocked the outward currents (i.e. reverse mode) induced by the application of 1 mM Ca\(^{2+}\), whereas even at a high dose (10 \(\mu\)M) it had a negligible effect on the inward currents induced by the application of 140 mM Na\(^{+}\) (2). Similarly KB-R7943 showed selectivity for the reverse mode of the exchanger consistent with some studies (24–27). In contrast, other data, obtained by measuring bidirectional exchange currents under constant ionic conditions, have shown that both SEA0400 and KB-R7943 equally block outward and inward exchange currents (28, 52). In the present study, however, we used different ionic conditions (see “Experimental Procedures”) to measure unidirectional outward and inward exchange currents. Therefore, the property of their mode selectivity may depend on the difference in ionic conditions. Although competitive interactions between NCX inhibitors and Ca\(^{2+}\) could offer a plausible explanation for their reverse mode selectivity, we did not obtain evidence of this as described above. Currently NCX is considered to function in a consecutive cycle whereby ion translocation takes place in two steps (53), so at present it seems very difficult to explain the mode selectivity of NCX inhibitors.

Despite the puzzle surrounding the mechanism, some pharmacological data support the reverse mode selectivity of SEA0400 or KB-R7943. Both inhibitors have been demonstrated to clearly protect against cardiac ischemia/reperfusion injury (cardiac dysfunction, arrhythmias, and cell death) by blocking Ca\(^{2+}\) overload via the reverse mode of the exchanger (24, 26, 27, 31–33, 42, 43). Previously we have observed that KB-R7943 at a low dose (\(< 10 \mu\)M) has virtually no effect on Ca\(^{2+}\) transients corresponding to spontaneous beating in cultured cardiomyocytes (24). In addition, SEA0400 as well as KB-R7943 has been shown to exhibit no or negative effects on heart rate, contractile function, and coronary flow (24, 26, 27, 31, 42, 43). In these cases, these agents should produce Ca\(^{2+}\) accumulation or cardiac positive inotropy if the forward mode of the exchanger (i.e. Ca\(^{2+}\) removal) is blocked by NCX inhibitors. Consequently under physiological conditions SEA0400 and KB-R7943 apparently act on the exchanger as if specifically blocking the reverse mode, although the functional importance of the reverse mode is not very clear.

**Molecular Determinants of NCX1 Inhibition by SEA0400**—As suggested above, SEA0400 selectively blocks NCX1 but has almost no effect on NCX3. Taking advantage of this property, we utilized a chimera strategy to identify critical region(s) of the exchanger involved in the differential response to SEA0400. Analysis with NCX1/NCX3 chimeras revealed that amino acid regions –32 to 108 and 193–445 of NCX1 were responsible for the difference in drug sensitivity between NCX1 and NCX3 (Fig. 3). From further chimaera analysis focused on these regions, we identified two major amino acid regions, 73–108 and 193–230, which accounted for about 50 and 100%, respectively, of the difference in drug sensitivi-
KB-R7943 (16), had no effect on the wild-type NCKX2. To our surprise, however, NCKX1/NCKX2(a-2) exerted the normal sensitivity to SEA0400, as well as KB-R7943, being pharmacologically equivalent to the wild-type NCKX1. From these results, we speculate that the structure of a-2 repeat is not essential in interaction with SEA0400 and KB-R7943. The mutations at Gly-833 in NCKX1 seem to affect drug sensitivity indirectly or allosterically.

**Effects of SEA0400 on NCKX Mutants with Altered Regulatory Properties**—To explore the possible link between NCX inhibitors and ionic regulatory properties, we examined the effects of SEA0400 and KB-R7943 on exchangers with mutated XIP regions, which either have no I1 inactivation (termed XIP-4YW) or accelerated I1 inactivation (termed N1-F223E). Intriguingly, SEA0400 and KB-R7943 were not effective in inhibiting the reverse mode of XIP-4YW, whereas these agents exhibited significant hypersensitivities to N1-F223E compared with the wild-type NCKX1 (Fig. 6B). Consistent with our findings, a more recent analysis of NCKX mutants with altered I1 inactivation kinetics expressed in *X. laevis* oocytes using the giant excised patch technique also reported that the inhibitory effect of SEA0400 was related to the kinetics of I1 inactivation (56). Therefore, there is a possibility that in the I1 inactivation process, the exchanger is preferentially sensitive to inhibition by NCX inhibitors, although little is known about its mechanism. In addition, we have evaluated the effects of SEA0400 on D447VD449H mutants displaying a phenotype for a low regulatory Ca2+ affinity. However, we could not find a significant relationship between drug sensitivity and I1 inactivation, although further study is required to test the details of this relationship.

**Effect of SEA0400 on Renal Hypoxia/Reoxygenation-induced Injury**—SEA0400 and KB-R7943 have been shown to efficiently guard against ischemia/reperfusion injury of the heart (24, 27, 31–33), kidney (34, 35, 44), and brain (36, 37). These results simply suggest that in several organs Na+ adequately guard against ischemia/reperfusion injury of the heart. Injury in original LLC-PK1 cells and cells overexpressing wild-type NCX1 but not in cells overexpressing SEA0400-insensitive NCX1 mutants (N1-F213L, N1-G833C, and XIP-4YW) in cells aggravated hypoxia/reoxygenation-induced cell damage. SEA0400 significantly protected against hypoxia/reoxygenation-induced cell damage in original LLC-PK1 cells and cells overexpressing wild-type NCX1 but not in cells overexpressing SEA0400-insensitive NCX1 mutants. These results suggest that the inhibition of the exchanger by SEA0400 evidently participates in its renoprotective efficacy against hypoxia/reoxygenation-induced cell damage. Thus, this method using SEA0400-insensitive mutants is useful to assess the pharmacological significance of NCX inhibition. It seems that this method can also be widely applied in vivo studies such as genetically engineered mice or adenovirus expression systems.

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