0026-895X/06/7006-2075–2083$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 6
28464/3155339
Mol Pharmacol 70:2075–2083, 2006
Printed in U.S.A.

YM-244769, a Novel Na^+/Ca^{2+} Exchange Inhibitor That Preferentially Inhibits NCX3, Efficiently Protects against Hypoxia/Reoxygenation-Induced SH-SY5Y Neuronal Cell Damage

Takahiro Iwamoto and Satomi Kita

Department of Pharmacology, School of Medicine, Fukuoka University, Fukuoka, Japan

Received June 30, 2006; accepted September 14, 2006

ABSTRACT

We investigated the pharmacological properties and interaction domains of *N*-(3-aminobenzyl)-6-{4-[(3-fluorobenzyl)oxy]phenoxy} nicotinamide (YM-244769), a novel potent Na^+/Ca^{2+} exchange (NCX) inhibitor, using various NCX-transfectants and neuronal and renal cell lines. YM-244769 preferentially inhibited intracellular Na^+-dependent $^{45}Ca^{2+}$ uptake via NCX3 (IC_{50} = 18 nM); the inhibition was 3.8- to 5.3-fold greater than for the uptake via NCX1 or NCX2, but it did not significantly affect extracellular Na^+-dependent $^{45}Ca^{2+}$ efflux via NCX isoforms. We searched for interaction domains with YM-244769 by NCX1/NCX3-chimeric analysis and determined that the α-2 region in NCX1 is mostly responsible for the differential drug response between NCX1 and NCX3. Further cysteine scanning mutagenesis in the α-2 region identified that the mutation at Gly833 markedly reduced sensitivity to YM-244769. Mutant exchangers that display either undetectable or accelerated Na^+-dependent inactivation, had markedly reduced sensitivity or hypersensitivity to YM-244769, respectively. YM-244769, like 2-[2-[4-(4-nitrobenzyloxyl)phenyl]ethyl]isothiourea methanesulfonate (KB-R7943), protected against hypoxia/reoxygenation-induced cell damage in neuronal SH-SY5Y cells, which express NCX1 and NCX3, more efficiently than that in renal LLC-PK_1 cells, which exclusively express NCX1, whereas 2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester (SN-6) suppressed renal cell damage to a greater degree than neuronal cell damage. These protective potencies consistently correlated well with their inhibitory efficacies for the Ca^{2+} uptake via NCX isoforms existing in the corresponding cell lines. Antisense knockdown of NCX1 and NCX3 in SH-SY5Y cells confirmed that NCX3 contributes to the neuronal cell damage more than NCX1. Thus, YM-244769 is not only experimentally useful as a NCX inhibitor that preferentially inhibits NCX3, but also has therapeutic potential as a new neuroprotective drug.

The Na^+/Ca^{2+} exchanger (NCX) can transport Ca^{2+} either out of cells (i.e., forward mode) or into cells (i.e., reverse mode) in exchange for three Na^+. This exchanger is driven by membrane potential as well as ion gradients through plasma membrane (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000; Shigekawa and Iwamoto, 2001; Annunziato et al., 2004). In cardiac muscle, NCX primarily pumps intracellular Ca^{2+} (Ca_i^{2+}) to outside the cell during repolarization and diastole, which balances Ca^{2+} entry via L-type Ca^{2+} channels during cardiac excitation (Blaustein and Lederer, 1999). Under pathological conditions such as ischemia/reperfusion injury in the heart, brain, and kidney, the exchanger is believed to cause Ca^{2+} overload as a result of elevated intracellular Na^+ concentration ($[Na^+]_i$), leading to cell damage (Blaustein and Lederer, 1999; Annunziato et al., 2004; Iwamoto, 2005; Lee et al., 2005). Recent evidence suggests that Ca^{2+} entry through vascular exchangers is involved in the development of salt-dependent hypertension (Iwamoto et al., 2004c).

Mammalian NCX forms a multigene family encompassing three isoforms: NCX1, NCX2, and NCX3. NCX1 is highly expressed in the heart, brain, and kidney and at much lower levels in other tissues, whereas the expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle

This work was supported by grant-in-aid 18059031 for Scientific Research on Priority Areas (to T.I.) from the Ministry of Education, Sports, Science and Technology of Japan and by grant-in-aid 18590251 for Scientific Research (to T.I.) from the Japan Society for the Promotion of Science.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.028464.

ABBREVIATIONS: NCX, Na^+/Ca^{2+} exchanger; XIP, exchanger inhibitory peptide; KB-R7943, 2-[2-[4-(4-nitrobenzyloxyl)phenyl]ethyl]isothiourea methanesulfonate; SEA0400, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline; SN-6, 2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester; YM-244769, *N*-(3-aminobenzyl)-6-{4-[(3-fluorobenzyl)oxy]phenoxy} nicotinamide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSS, balanced salt solution; LDH, lactate dehydrogenase.

(Quednau et al., 1997; Philipson and Nicoll, 2000). These three isoforms seem to have similar molecular topologies, consisting of nine transmembrane segments and a large central cytoplasmic loop (Nicoll et al., 1999; Iwamoto et al., 2000). The former part, particularly the α-1 and α-2 repeat regions with two opposite re-entrant loops, may participate in ion transport (Nicoll et al., 1996; Doering et al., 1998; Iwamoto et al., 2000); the latter part, possessing the exchanger inhibitory peptide (XIP) region (Li et al., 1991; Matsuoka et al., 1997) and regulatory Ca^{2+} binding sites (Matsuoka et al., 1995), is primarily involved in regulatory properties. NCX1 has been shown to be secondarily regulated by the transport substrates Na^+ and Ca^{2+} (Hilgemann et al., 1992a,b). $[Ca^{2+}]_i$ at the submicromolar level activates NCX activity by promoting the recovery of the exchanger from the $Ca^{2+}{}_i$-dependent, or "I_2," inactivation state, whereas high $[Na^+]_i$ restrains the exchange by facilitating the entry of the exchanger into the Na_i^+-dependent, or "I_1," inactivation state.

A potent and selective NCX inhibitor would be very useful for analyzing the physiological roles and ion transport mechanisms of this exchanger. Moreover, NCX inhibitors might be useful therapeutically for ischemic diseases, arrhythmias, heart failure, and essential hypertension (Annunziato et al., 2004; Iwamoto, 2005; Lee et al., 2005). In 1996, KB-R7943 was introduced as a prototype selective NCX inhibitor (Iwamoto et al., 1996; Watano et al., 1996). This inhibitor was fairly specific to the exchanger but possessed some nonspecific actions against ion channels and receptors (Watano et al., 1996; Pintado et al., 2000; Matsuda et al., 2001). Thereafter, more specific inhibitors such as SEA0400 (Matsuda et al., 2001) and SN-6 (Iwamoto et al., 2004a) were developed from benzyloxyphenyl derivatives; SEA0400 is 80 to 100 times more powerful than KB-R7943 and also has an excellent specificity to the exchanger. However, Reuter et al. (2002) suggested that SEA0400, too, has unknown nonspecific effects.

These benzyloxyphenyl inhibitors have a number of interesting features. All three inhibitors predominantly inhibit the reverse mode of NCX compared with the forward mode (Iwamoto et al., 1996, 2004a, 2004b; Watano et al., 1996; Elias et al., 2001). In addition, KB-R7943 is more inhibitory to NCX3 than to NCX1 and NCX2 (Iwamoto et al., 2001), whereas SEA0400 and SN-6 preferentially block NCX1 compared with NCX2 and NCX3 (Iwamoto et al., 2004a, 2004b). Recent site-directed mutagenesis revealed the important amino acids in NCX1 (Phe213, Val227, Tyr228, Gly833, and Asn839) responsible for inhibition by benzyloxyphenyl derivatives (see Iwamoto, 2005).

YM-244769 (Fig. 1), a highly potent NCX inhibitor, was found by screening newly synthesized benzyloxyphenyl derivatives for inhibition of Na_i^+-dependent $^{45}Ca^{2+}$ uptake into NCX1-transfected fibroblasts (Kuramochi et al., 2005a). It has been reported that YM-244769 derivatives are orally bioavailable and efficiently prevent ischemia/reperfusion-induced ventricular tachycardia and fibrillation in rats (Kuramochi et al., 2005b). YM-244769 is thus expected to be a novel anti-ischemic drug. In this study, we investigated the inhibitory properties of YM-244769 by measuring Na_i^+-dependent $^{45}Ca^{2+}$ fluxes, and searched by chimeric and mutational analyses for the structural domains responsible for its inhibition. Moreover, we examined the protective effects of YM-244769 in hypoxia/reoxygenation-induced injury in neuronal SH-SY5Y and renal LLC-PK_1 cells.

Materials and Methods

Cell Cultures. Chinese hamster lung fibroblast CCL39 cells and their NCX transfectants were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum (FCS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) in a humidified incubator gassed with 5% CO_2/95% air at 37°C. Human neuroblastoma SH-SY5Y cells were cultured in DMEM/nutrient mixture Ham's F-12 (1:1) media supplemented with 10% FCS and the antibiotics. SH-SY5Y cells were differentiated into neuron-like cells by treatment with retinoic acid (10 μM) for 2 weeks. Porcine tubular epithelial LLC-PK_1 cells were cultured in DMEM supplemented with 4% FCS and the antibiotics.

Construction and Stable Expression of Wild-Type, Chimeric, and Mutant Exchangers. cDNAs of dog heart NCX1.1 and rat brain NCX2.1 and NCX3.3 were cloned into SacII and HindIII restriction sites in pCRII (Invitrogen, Carlsbad, CA) (Iwamoto et al., 1998). NCX1/NCX3 chimeras, shown in Fig. 3, were constructed as described in detail previously (Iwamoto et al., 2004a). Substitution of amino acid residues in NCX1 was performed by site-directed mutagenesis using a polymerase chain reaction-based strategy as described previously (Iwamoto et al., 2000). 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 (Iwamoto et al., 2001). To obtain stable expression of wild-type, chimeric, and mutant exchangers, pKCRH plasmids carrying exchanger cDNAs were transfected in the presence of Lipofectin (Invitrogen) into CCL39 fibroblasts. Cell clones highly expressing NCX activity or NCKX activity were selected by a Ca^{2+}-killing procedure as described previously (Iwamoto et al., 1998).

Na_i^+-Dependent $^{45}Ca^{2+}$ Uptake into Cells. Na_i^+-dependent $^{45}Ca^{2+}$ uptake into CCL39, SH-SY5Y, or LLC-PK_1 cells was assayed as described in detail previously (Iwamoto et al., 2001). In brief, confluent cells in 24-well dishes were loaded with Na^+ by incubation at 37°C for 30 min in 0.5 ml of balanced salt solution (BSS) (10 mM HEPES/Tris, pH 7.4, 146 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, and 0.1% bovine serum albumin) containing 1 mM ouabain and 10 μM monensin. $^{45}Ca^{2+}$ uptake was then initiated by switching the medium to Na^+-free BSS (replacing NaCl with equimolar choline chloride) or to normal BSS, both of which contained 0.1 mM $^{45}CaCl_2$ (370 kBq/ml) and 1 mM ouabain. After a 30-s

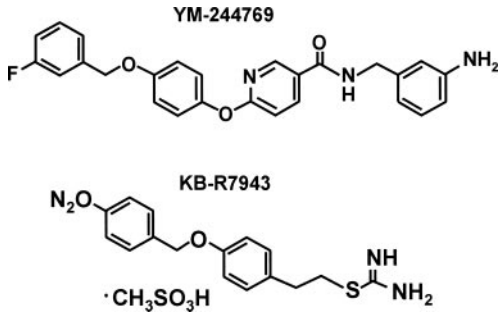

Fig. 1. Chemical structures of benzyloxyphenyl derivatives YM-244769 and KB-R7943. [Reproduced from Kuramochi T, Kakefuda A, Sato I, Tsukamoto I, Taguchi T, and Sakamoto S (2005) Synthesis and structure-activity relationships of 6-{4-[(3-fluorobenzyl)oxy]phenoxy}nicotinamide derivatives as a novel class of NCX inhibitors: a QSAR study. *Bioorg Med Chem* **13:**717–724. Copyright © 2005 Elsevier. Used with permission; and Iwamoto T, Watano T, and Shigekawa T (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem* **271:**22391–22397. Copyright © 1996 American Society for Biochemistry and Molecular Biology.]

or 1-min incubation, $^{45}Ca^{2+}$ 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 $LaCl_3$. Cells were then solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity and protein. When present, NCX inhibitors were included in the medium 10 min before the start of $^{45}Ca^{2+}$ uptake.

Na_o^+-Dependent $^{45}Ca^{2+}$ Efflux from Cells. $^{45}Ca^{2+}$ efflux from CCL39 transfectants cultured in a 35-mm dish was assayed as described previously (Iwamoto et al., 1996). Cells were equilibrated with $^{45}Ca^{2+}$ by incubating them at 37°C for 4 h in 1 ml of BSS containing 740 kBq of $^{45}Ca^{2+}$. After rinsing cells six times with Ca^{2+}- and Na^+-free BSS for 1 min, $^{45}Ca^{2+}$ efflux was measured for 20 s in Ca^{2+}- and Na^+-free BSS or in Ca^{2+}-free BSS; both solutions contained 1 μM thapsigargin to cause a transient increase in $[Ca^{2+}]_i$. Na_o^+-dependent $^{45}Ca^{2+}$ efflux was estimated by subtracting $^{45}Ca^{2+}$ efflux in Ca^{2+}- and Na^+-free BSS from that in Ca^{2+}-free BSS.

Assays of Other Transporters. The activities of Na^+/H^+ exchanger, Na^+,K^+-ATPase, sarcolemmal or sarcoplasmic reticulum Ca^{2+}-ATPases, and L-type Ca^{2+} channel were measured as described previously (Iwamoto et al., 1996).

Hypoxia and Reoxygenation in SH-SY5Y and LLC-PK_1 Cells. SH-SY5Y and LLC-PK_1 cells were grown in 96-well microplates at 2×10^5 cells/well. After 2 days, the medium was changed to HEPES-buffered DMEM without glucose and FCS. The cells were then 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 (Iwamoto et al., 2004a,b). After 8 h of hypoxia for SH-SY5Y cells or 6 h of hypoxia for LLC-PK_1 cells, the cells were put in a humidified incubator gassed with 5% CO_2/95% air for 16 h (SH-SY5Y cells) or for 1 h (LLC-PK_1 cells) in HEPES-buffered DMEM to which glucose was added at the beginning of reoxygenation. After the hypoxia/reoxygenation treatment, lactate dehydrogenase (LDH) activity in the medium was measured using an LDH-Cytotoxic Test kit (Wako Pure Chemicals, Osaka, Japan). NCX inhibitors were added to the medium at the beginning of reoxygenation. The hypoxia/reoxygenation-induced LDH release in the absence of the drug was indicated as 100%.

Treatment of SH-SY5Y Cells with Oligodeoxynucleotides. Antisense and sense phosphorothioate oligodeoxynucleotides, which are highly specific for NCX1 or NCX3, were synthesized as reported previously (Pignataro et al., 2004; Magi et al., 2005). The sequence for each isoform was as follows: NCX1: antisense, 5′-TGAGACTTCCAATTGTT-3′; sense, 5′-AACAATTGGAAGTCTCA-3′; NCX3: antisense 5′-GCCATACACAAGAG-3′; sense, 5′-CTCTTGTGTATGGC-3′. SH-SY5Y cells were incubated with oligodeoxynucleotides (5 μM) and Lipofectamine, according to the manufacturer's protocol. After 4 h, the medium was replaced with DMEM/nutrient mixture Ham's F-12 (1:1) media supplemented with 10% FCS. Control cells were treated with Lipofectamine only. To check the expression levels of NCX isoforms, immunoblot analyses with specific antibodies were performed as described previously (Iwamoto et al., 1998).

Statistical Analysis. Data are expressed as means ± S.E. of three or four independent determinations. IC_{50} values were calculated by nonlinear least-squares fits using the program Prism (GraphPad Software, San Diego, CA). Differences for multiple comparisons were analyzed by an unpaired t test or one-way ANOVA followed by Dunnett's test. Values of $p < 0.05$ were considered statistically significant.

Materials. CCL39, SH-SY5Y, and LLC-PK_1 cells were purchased from American Type Culture Collection (Manassas, VA). YM-244769 was synthesized by Astellas Pharm Inc. (Tsukuba, Japan). KB-R7943 and SN-6 were provided by Nippon Organon (Osaka, Japan) and Senju Pharmaceutical Co. Ltd. (Kobe, Japan), respectively. $^{45}CaCl_2$ was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other chemicals were also of the highest grade available.

Results

Inhibitory Properties of YM-244769. We first compared the inhibitory effects of YM-244769 on Na_i^+-dependent $^{45}Ca^{2+}$ uptake (i.e., reverse mode) into CCL39 cells with a stable transfection of NCX1, NCX2, or NCX3. YM-244769 (0.003–1 μM) inhibited dose dependently the initial rates of $^{45}Ca^{2+}$ uptake into NCX1, NCX2, and NCX3 transfectants with IC_{50} values of 68 ± 2.9, 96 ± 3.5, and 18 ± 1.0 nM (n = 4), respectively (Fig. 2), indicating that YM-244769 is approximately four to five times more selective to NCX3 than other isoforms. In NCX3 transfectants, YM-244769 was more than 80 times more inhibitory than KB-R7943 [IC_{50} = 1.5 μM, as reported previously (Iwamoto and Shigekawa, 1998)]. To check whether YM-244769 competes with Ca_o^{2+} for the exchanger, the rate of Na_i^+-dependent $^{45}Ca^{2+}$ uptake into NCX1 transfectants was measured under standard conditions as a function of Ca_o^{2+} concentration (0.06–2 mM) in the presence or absence of 0.05 μM YM-244769. Their double reciprocal plots of uptake rate versus Ca_o^{2+} concentration were linear (data not shown). YM-244769 increased the half-maximal Ca^{2+} concentration (K_{Ca}) value from 0.20 ± 0.02 mM (control) to 0.38 ± 0.03 mM ($p < 0.05$, n = 3) and decreased the corresponding maximal velocity (V_{max}) value from 29 ± 2.2 nmol/mg/30 s (control) to 16 ± 1.5 nmol/mg/30 s ($p < 0.05$), suggesting a type of mixed (competitive and noncompetitive) inhibition.

We next examined the effects of YM-244769 on Na_o^+-dependent $^{45}Ca^{2+}$ efflux (i.e., forward mode) from NCX1, NCX2, or NCX3 transfectants equilibrated with $^{45}Ca^{2+}$ and treated with 1 μM thapsigargin. The rate of Na_o^+-dependent $^{45}Ca^{2+}$ efflux was estimated by subtracting $^{45}Ca^{2+}$ efflux in a Ca^{2+}- and Na^+-free medium from that in a Ca^{2+}-free medium containing 146 mM Na^+. As shown in Fig. 2, YM-244769 at

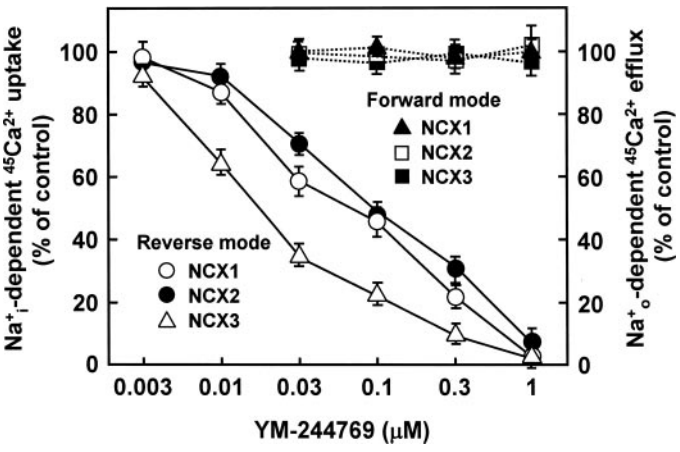

Fig. 2. Dose-response curves for the effects of YM-244769 on Na_i^+-dependent $^{45}Ca^{2+}$ uptake (reverse mode) or Na_o^+-dependent $^{45}Ca^{2+}$ efflux (forward mode) in cells expressing NCX1, NCX2, or NCX3. The initial rates of $^{45}Ca^{2+}$ uptake into cells and $^{45}Ca^{2+}$ efflux from cells equilibrated with $^{45}Ca^{2+}$ were measured in the presence or absence of indicated concentrations of YM-244769 as described under *Materials and Methods*. The uptake rates into NCX1-, NCX2-, and NCX3-transfectants were 11, 6.8, and 9.2 nmol/mg/30 s, respectively. The basal uptake rates into nontransfectants were below 0.05 nmol/mg/30 s. The efflux rates from NCX1-, NCX2-, and NCX3-transfectants were 0.53, 0.33, and 0.48 nmol/mg/20 s, respectively, whereas the rates from nontransfectants were undetectable. YM-244769 was added 10 min before the start of uptake or efflux measurement. Data are presented as a percentage of the control values obtained in the absence of YM-244769. Data are means ± S.E. of four independent experiments.

0.03 to 1 μM did not affect the rate of Na_o^+-dependent $^{45}Ca^{2+}$ efflux from three types of NCX transfectants.

Furthermore, YM-244769 (up to 3 μM) did not significantly influence other Na^+ or Ca^{2+} transport via K^+-dependent Na^+/Ca^{2+} exchanger (NCKX2), Na^+/H^+ exchanger, Na^+,K^+-ATPase, sarcolemmal or sarcoplasmic reticulum Ca^{2+}-ATPases, or L-type Ca^{2+} channel (data not shown), suggesting that YM-244769 has specificity to NCX.

Chimeric Analysis of the Inhibitory Effect of YM-244769. As shown above, NCX1 and NCX3 exhibited different sensitivities to YM-244769 despite the high sequence homology of the two. To identify important region(s) in the NCX1 molecule for the interaction with YM-244769, we performed chimeric analysis between these isoforms. We constructed two series of chimeras in which serial segments from NCX3 were transferred into NCX1 in exchange for the homologous segments (N1 chimeras), and vice versa (N3 chimeras). N1 and N3 chimeras exhibited exchange activities similar to those of wild-type NCX1 and NCX3, respectively (see the legend to Fig. 3).

Figure 3 shows the effects of 0.05 μM YM-244769 on the rates of Na_i^+-dependent $^{45}Ca^{2+}$ uptake into CCL39 cells expressing wild-type or chimeric exchangers. YM-244769 at this concentration reduced the uptake rates of the wild-type NCX1 and NCX3 to approximately 61% and 28% of the control, respectively. Two N1 chimeras, N1–788/829 and N1–109/133,788/829—which contained the common homologous NheI/MluI segment from NCX3—exhibited a YM-244769 sensitivity similar to that of wild-type NCX3 (Fig. 3A). Furthermore, N3 chimeras (N3–777/818 and N3–143/167,777/818) containing the common NheI/MluI segment exhibited a YM-244769 sensitivity similar to that of wild-type NCX1 (Fig. 3B), indicating that this segment is exclusively responsible for the differential drug responses between NCX1 and NCX3.

Mutational Analysis of the Inhibitory Effect of YM-244769. The NheI/MluI segment (i.e., amino acids 788–829 in NCX1) contains a large portion of the α-2 repeat, which is highly conserved in the NCX family (see the Introduction). To identify the critical residues involved in drug sensitivity, we examined the effects of YM-244769 on the rates of Na_i^+-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX1 mutants in which individual residues in the α-2 repeat region were substituted with cysteine (i.e., V804C, F805C, V806C, A807C, L808C, G809C, V812C, P813C, T815C, A817C, S818C, K819C, A821C, A822C, T823C, Q824C, D825C, Q826C, Y827C, A828C, D829C, A830C, S831C, I832C, G833C, N834C, V835C, T836C, G837C, N839C, A840C, N842C, V843C, F844C, and L845C). We found that among these 35 mutations, the G833C substitution almost completely abolished the inhibition by up to 1 μM YM-244769 (Fig. 4), whereas all other substitutions did not significantly alter the drug responses (data not shown).

We further analyzed the effects of YM-244769 on other critical NCX1 mutants that have been shown to exhibit an altered sensitivity to benzyloxyphenyl derivatives, KB-R7943, SEA0400, and SN-6 (Iwamoto et al., 2001, 2004a,b). As shown in Fig. 4, YM-244769 normally inhibited Ca^{2+} uptake by the F213C mutant, which is insensitive to SEA0400 (Iwamoto et al., 2004b), and by the V227M,Y228H double mutant, which has a reduced sensitivity to SN-6 (Iwamoto et al., 2004a), the inhibition in both cases being equal to that by wild-type NCX1 within an IC_{50} range of 70 to 100 nM.

We also examined the effects of YM-244769 on NCX1 mutants, which display altered kinetics of Na^+-dependent inactivation (i.e., I_1 inactivation). As the XIP region (amino acids 219–238 in NCX1) is coupled to the I_1 inactivation (Matsuoka et al., 1997), XIP region mutants XIP-4YW, which was produced by the mutations of Y224W/Y226W/Y228W/Y231W, and F223E exhibit completely eliminated and accelerated I_1 inactivation, respectively (Iwamoto et al., 2004b). XIP-4YW and F223E mutants exhibited a markedly reduced sensitivity and hypersensitivity (approximately 3-fold greater), respectively, to inhibition by YM-244769 (Fig. 4). On the other hand, YM-244769 had a normal sensitivity to the D447V/D498I mutant (Iwamoto et al., 2004b), in which the regulatory Ca^{2+} binding site was mutated to display a phenotype for a low Ca^{2+} affinity (data not shown). In addition, we analyzed the effects of YM-244769 on NCX3 mutant with Δ292-708, in which a large cytoplasmic loop (amino acids 292–708) was deleted to display a deregulated phenotype for both I_1 and I_2 inactivation (Iwamoto et al., 1998). As shown in Fig. 4, this Δ292-708 mutant showed diminished sensitivity to inhibition by YM-244769 (IC_{50} = 0.96 $\pm$ 0.11 μM, n = 3) compared with wild-type NCX3.

Effects of YM-244769 on Neuronal and Renal Hypoxia/Reoxygenation-Induced Injury. We used a human neuroblastoma SH-SY5Y cell line to investigate the neuroprotective effects of YM-244769 and a porcine tubular epithelial LLC-PK_1 cell line to investigate its renoprotective effects. At first, the expression levels of NCX isoforms in these cell lines were analyzed by immunoblot analyses with specific antibodies against three NCX isoforms (Iwamoto et al., 1998). As shown in Fig. 5A, the protein expression of NCX1 and NCX3 was detected in SH-SY5Y cells, which is consistent with their mRNA levels reported previously (Magi et al., 2005). On the other hand, only NCX1 protein expression was observed in LLC-PK_1 cells. In both cell lines, the protein expression levels of NCX isoforms were not modified after 6 or 8 h of hypoxia (data not shown).

In neuronal SH-SY5Y cells, 8 h of hypoxia followed by 16 h of reoxygenation produced a significant LDH release from damaged cells (approximately 18% of total cellular LDH activity), which was accompanied by morphological changes such as bleb formation and breakdown of plasma membrane (data not shown). YM-244769 (0.3 or 1 μM), as well as KB-R7943 (3 or 10 μM) or SN-6 (10 μM), efficiently protected against the hypoxia/reoxygenation-induced LDH release in SH-SY5Y cells (Fig. 5B). We further performed a similar protocol in LLC-PK_1 cells. In the renal cells, 6 h of hypoxia followed by 1 h of reoxygenation produced a significant LDH release from damaged cells (approximately 12% of total cellular LDH activity). YM-244769 (1 μM), KB-R7943 (10 μM), and SN-6 (3 or 10 μM) protected against the hypoxia/reoxygenation-induced LDH release in LLC-PK_1 cells. When the potencies of cell protection by each NCX inhibitor in SH-SY5Y and LLC-PK_1 cells were compared, YM-244769 and KB-R7943 more efficiently suppressed the hypoxia/reoxygenation-induced cell damage in SH-SY5Y cells, whereas SN-6 suppressed the cell damage to a greater degree in LLC-PK_1 cells (Fig. 5B). Furthermore, we measured the effects of NCX inhibitors on the rates of Na_i^+-dependent $^{45}Ca^{2+}$ uptake into SH-SY5Y and LLC-PK_1 cells. As shown in Fig. 6, the inhib-

itory potencies of YM-244769, KB-R7943, and SN-6 for the rates of $^{45}Ca^{2+}$ uptake are very similar to their efficacies for cell protection in SH-SY5Y and LLC-PK_1 cells, suggesting that the hypoxia/reoxygenation-induced cell damage is preferentially mediated by Ca^{2+} overload via NCX isoforms existing in cells.

To confirm the contribution of NCX1 and NCX3 in hypoxia/reoxygenation-induced cell damage in SH-SY5Y cells, these cells were treated with NCX1 and NCX3 antisense oligodeoxynucleotides. As shown in Fig. 7A, the treatment with NCX1 or NCX3 antisense caused a specific reduction in the protein expression of the corresponding NCX isoform (to 10–20% of the control). On the other hand, NCX1 or NCX3 sense oligodeoxynucleotide did not affect the protein expression of NCX isoforms. In SH-SY5Y cells treated with NCX1 or NCX3 antisense, hypoxia/reoxygenation-induced LDH release was significantly attenuated (Fig. 7B): reduction in cell damage was greater in cells treated with NCX3 antisense (by 61%) than in cells treated with NCX1 antisense (by 35%), suggesting that NCX3 contributes to the neuronal cell damage more

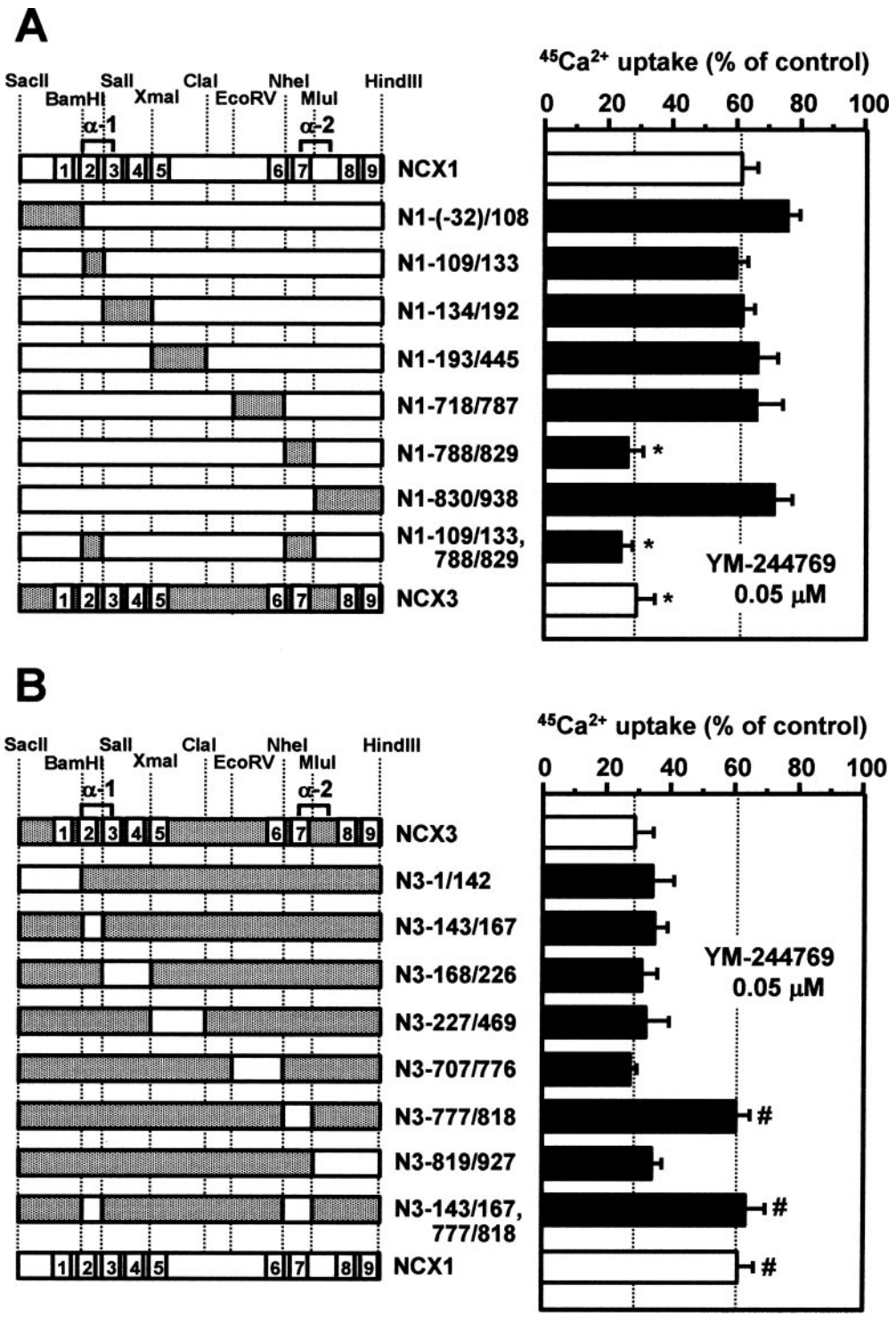

Fig. 3. Effects of YM-244769 on Na_i^+-dependent $^{45}Ca^{2+}$ uptake into cells expressing the wild-type and chimeric exchangers between NCX1 and NCX3. Two series of chimeras (N1 and N3) were constructed by substituting segments of NCX1 with homologous segments of NCX3 (A) and vice versa (B). These chimeras are named based on the amino acid numbers for NCX1. Broken lines show the restriction enzyme cut sites. Hatched boxes indicate the segments substituted with NCX3 sequences. Linear models of the exchangers are indicated at the top and bottom and the numbered open boxes show positions of transmembrane segments. The initial rates of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells were measured in the presence or absence of 0.05 μM YM-244769. The uptake rates in cells expressing these chimeras were 5 to 11 nmol/mg/30 s. Data are presented as a 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; #, $p < 0.05$ versus NCX3.

than NCX1. Consistent with the NCX3-selectivity of YM-244769, this drug (0.3 or 1 μM) efficiently suppressed the hypoxia/reoxygenation-induced cell damage in SH-SY5Y cells treated with NCX1 antisense (i.e., SH-SY5Y cells primarily expressing NCX3) more than in those treated with NCX3 antisense (i.e., SH-SY5Y cells primarily expressing NCX1).

Discussion

A potent and selective NCX inhibitor would be very useful not only for studying the physiological roles of NCX but also for potentially offering a new drug therapy for cardiovascular diseases (Iwamoto, 2005; Lee et al., 2005). YM-244769 is a newly synthesized, potent benzyloxyphenyl NCX inhibitor. In this study, we investigated the pharmacological properties and interaction domains of YM-244769 using CCL39 fibroblasts stably expressing NCX isoforms, NCX1/NCX3-chimeras, and site-directed NCX1 mutants. We show here that YM-244769 is experimentally invaluable as a unique NCX inhibitor that preferentially inhibits NCX3, and has therapeutic potential as a novel neuroprotective drug.

Pharmacological Properties of YM-244769. Our study, using transfectants expressing the NCX family, revealed that YM-244769 inhibits $^{45}Ca^{2+}$ uptake (i.e., reverse mode) via NCX3 approximately four to five times more potently than that via NCX1 or NCX2 (Fig. 2). We have reported previously the isoform selectivity of other benzyloxyphenyl derivatives: KB-R7943 is more inhibitory to NCX3 than to NCX1 and NCX2, whereas SEA0400 and SN-6 predominantly block NCX1 compared with NCX2 and NCX3 (Iwamoto et al., 2001, 2004a,b). KB-R7898, a derivative of KB-R7943, inhibits all three isoforms almost equally (Iwamoto and Shigekawa, 1998). To summarize these isoform selectivities, a graph depicting IC_{50} values for NCX1, NCX2, and NCX3 in benzyloxyphenyl derivatives is shown in Fig. 8. Thus, it is apparent that YM-244769 is a unique NCX inhibitor that preferentially inhibits NCX3. KB-R7943 belongs to the same group as YM-244769, but the former inhibitor is not potent and consequently has some nonspecific actions (Iwamoto et al., 2005). SEA0400, as well as SN-6, is classified as an NCX1 selective inhibitor, whereas KB-R7898 is an all-around inhibitor against the three isoforms (see Fig. 8). Such benzyloxyphenyl derivatives that have different isoform selectivities could be useful for discriminating among the functional characteristics of NCX isoforms.

YM-244769 (up to 1 μM) did not significantly affect the Na_o^+-dependent $^{45}Ca^{2+}$ efflux via the three isoforms (i.e., forward mode), suggesting that YM-244769 possesses reverse mode-selectivity. In existing benzyloxyphenyl NCX inhibitors, reverse mode-selectivity is commonly observed under unidirectional ionic conditions (Iwamoto et al., 1996, 2004a,b; Watano et al., 1996; Bouchard et al., 2004). On the other hand, other data have shown that both SEA0400 and KB-R7943 equally block outward and inward exchange cur-

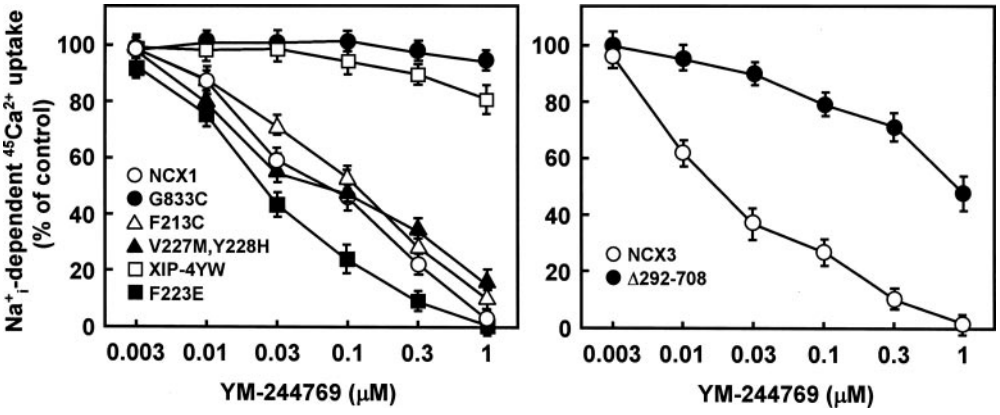

Fig. 4. Effects of YM-244769 on Na_i^+-dependent $^{45}Ca^{2+}$ uptake into cells expressing the wild-type or various mutants of NCX1 and NCX3. The initial rates of $^{45}Ca^{2+}$ uptake were measured in the presence or absence of indicated concentrations of YM-244769. Data are means ± S.E. of three independent experiments.

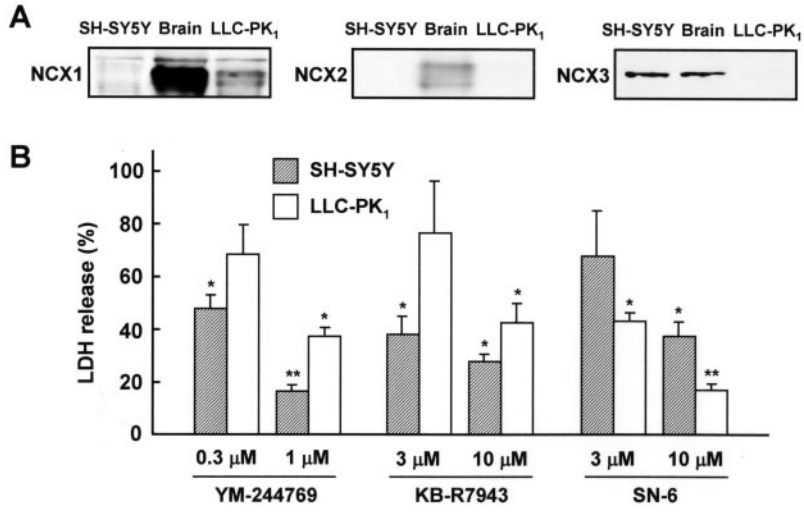

Fig. 5. Expression of three NCX isoforms in SH-SY5Y and LLC-PK_1 cells and neuroprotective and renoprotective effects of NCX inhibitors on hypoxia/reoxygenation-induced cell damage. A, to analyze the expression pattern of NCX1, NCX2, and NCX3 isoforms in SH-SY5Y and LLC-PK_1 cells, microsomes (30 μg) from cells or from mouse brain were subjected to immunoblot analyses with specific antibodies against the isoforms. B, SH-SY5Y cells were exposed to 8 h of hypoxia followed by reoxygenation for 16 h, and LLC-PK_1 cells were exposed to 6 h of hypoxia followed by reoxygenation for 1 h. YM-244769, KB-R7943, or SN-6 was added to the medium at the beginning of reoxygenation. Cell damage was detected by measuring lactate dehydrogenase (LDH) release in the medium. The hypoxia/reoxygenation-induced LDH release in the absence of the drug was indicated as 100%. Data are means ± S.E. of four independent experiments. $*p < 0.05$, $**p < 0.01$ versus hypoxia/reoxygenation-induced LDH release without the drug.

rents under bidirectional ionic conditions (Kimura et al., 1999; Tanaka et al., 2002). The latter result seems reasonable for blockers of a bidirectional transporter. The former result, however, seems to be consistent with in vivo or in vitro pharmacological profiles showing that benzyloxyphenyl NCX inhibitors preferentially protect pathologic Ca^{2+} entry via the reverse mode, despite having minimal effects on normal Ca^{2+} extrusion via the forward mode (Iwamoto et al., 2005).

Structural Domains for NCX1 Inhibition by YM-244769. As described above, YM-244769 is 3.8-fold more effective in inhibiting NCX3 than NCX1. Taking advantage of this property, we employed a chimera strategy to identify critical region(s) of the exchanger involved in the differential response to YM-244769. Analysis using NCX1/NCX3-chimeras revealed that the segment corresponding to amino acids 788 to 829 of NCX1 was primarily responsible for the difference in the drug sensitivity between the two isoforms (Fig. 3). Intriguingly, this segment is the same region involved in the differential response to KB-R7943 between NCX1 and NCX3 (Iwamoto et al., 2001). To further identify critical residues influencing the drug sensitivity, we performed cysteine scanning mutagenesis within the amino acid 804 to 845 region in NCX1, which contains a large portion of the α-2 repeat that is highly conserved in all homologs of the NCX family (Philipson and Nicoll, 2000). We found that the G833C mutation alone exhibited a markedly reduced sensitivity to YM-244769 (Fig. 4). We have reported previously that Gly833 is a critical molecular determinant required for inhibition by KB-R7943, as well as other benzyloxyphenyl derivatives (Iwamoto et al., 2001; 2004a,b). In a topological model of the exchanger, Gly833 is mapped at the re-entrant loop of the α-2 repeat (Iwamoto et al., 2000). To further evaluate the interaction of YM-244769 with other molecular determinants for NCX1 inhibition by SEA0400 or SN-6, we examined the effects of YM-244769 on NCX1 mutants F213C and V227M,Y228H, of which the former is insensitive to SEA0400 and the latter has reduced sensitivity to SN-6 (Iwamoto et al., 2004a,b). YM-244769, like KB-R7943, showed the same inhibitory effect on F213C and V227M,Y228H as on the wild-type NCX1 (Fig. 4). Taken together, consistent with their similarity for NCX3-selectivity, the properties of YM-244769 in various chimeras and mutants are very similar to those of KB-R7943, suggesting that these two inhibitors may have a similar interaction domain in the exchanger molecule.

I_1 Inactivation-Dependent Inhibition by YM-244769. The NCX is regulated by two kinds of inactivation processes, namely I_1 and I_2 inactivation. To explore the possible link between YM-244769 and I_1 inactivation, we examined the effects of YM-244769 on exchangers with mutated XIP regions, which either have no I_1 inactivation (termed XIP-4YW

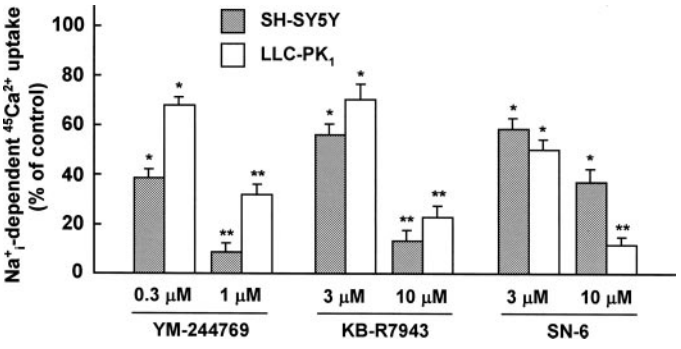

Fig. 6. Effects of NCX inhibitors on Na_i^+-dependent $^{45}Ca^{2+}$ uptake in SH-SY5Y and LLC-PK_1 cells. $^{45}Ca^{2+}$ uptake into Na^+-loaded SH-SY5Y or LLC-PK_1 cells was measured for 1 min in the presence or absence of indicated concentrations of NCX inhibitors as described under *Materials and Methods*. The uptake into SH-SY5Y and LLC-PK_1 cells in the absence of the drug were 1.1 and 0.8 nmol/mg/min, respectively, which are taken as 100% in the figure. Data are means ± S.E. of four independent experiments. $*p < 0.05$, $**p < 0.01$ versus $^{45}Ca^{2+}$ uptake without the drug.

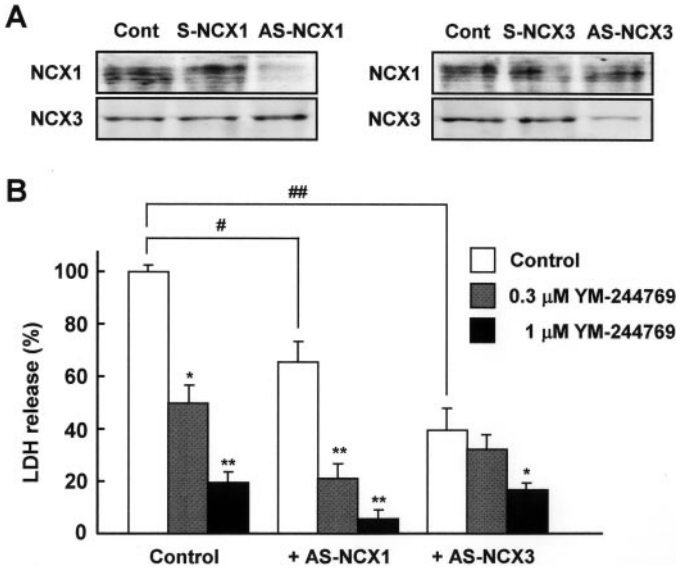

Fig. 7. Effects of NCX1 and NCX3 antisense oligodeoxynucleotides on the expression of NCX isoforms and neuroprotective actions of YM-244769 in SH-SY5Y cells. A, immunoblot analyses of NCX1 and NCX3 proteins in SH-SY5Y cells treated with respective sense (*S*-NCX1 and *S*-NCX3) and antisense oligodeoxynucleotides (AS-NCX1 and AS-NCX3). B, SH-SY5Y cells treated with NCX1 or NCX3 antisense were exposed to 8 h of hypoxia followed by reoxygenation for 16 h. YM-244769 was added to the medium at the beginning of reoxygenation. The hypoxia/reoxygenation-induced LDH release in the absence of the drug and oligodeoxynucleotides indicated as 100%. Data are means ± S.E. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$ versus respective hypoxia/reoxygenation-induced LDH release without YM-244769 in the presence or absence of antisense. #, $p < 0.05$; ##, $p < 0.01$ versus hypoxia/reoxygenation-induced LDH release without YM-244769 and antisense.

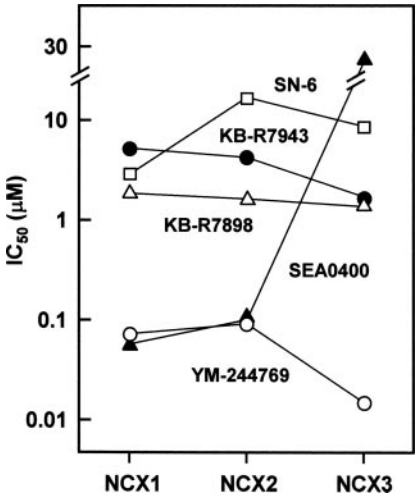

Fig. 8. Comparison of the IC_{50} values of benzyloxyphenyl NCX inhibitors. The IC_{50} values of KB-R7943, KB-R7898, SN-6, and SEA0400 for the Na_i^+-dependent $^{45}Ca^{2+}$ uptake into NCX1-, NCX2-, and NCX3-transfectants are taken from previous data (Iwamoto and Shigekawa, 1998; Iwamoto et al., 2004a,b).

mutant) or accelerated I_1 inactivation (termed F223E mutant) (Iwamoto et al., 2004b). It is intriguing that YM-244769 had a reduced sensitivity to the XIP-4YW mutant, whereas this drug exhibited significant hypersensitivity to the F223E mutant (Fig. 4), suggesting that the inhibitory effect of YM-244769 is related to the kinetics of I_1 inactivation. Similar reduction in the drug sensitivity was observed in NCX3 mutant with Δ292-708, a deregulated phenotype for both I_1 and I_2 inactivation (Fig. 4). Furthermore, we evaluated the effects of YM-244769 on NCX1 mutant (i.e., D447V/D498I) displaying a phenotype for a low regulatory Ca^{2+} affinity, but we could not detect a significant relationship between the drug sensitivity and I_2 inactivation. These properties have also been observed in other benzyloxyphenyl derivatives (Bouchard et al., 2004; Iwamoto et al., 2004a,b).

We speculate that the interaction of benzyloxyphenyl derivatives with the exchanger probably stabilizes the I_1 inactive state or accelerates the rate of entry into an I_1 inactive state. Such inhibitory mechanisms would help to explain why benzyloxyphenyl NCX inhibitors selectively block the reverse mode compared with the forward mode. Under unidirectional ionic conditions, the reverse mode is induced when $[Na^+]_i$ is high, whereas the forward mode is generated when $[Na^+]_i$ is reduced. NCX1 molecules thus tend to undergo I_1 inactivation in experimental conditions for the reverse mode, suggesting an apparent, but not substantial, reverse mode-selectivity. This suggests that benzyloxyphenyl derivatives may be relatively dormant under normal conditions (low $[Na^+]_i$), but become effective under pathological conditions (high $[Na^+]_i$). This should be an ideal profile for therapeutic agents against Na_i^+-dependent diseases, such as myocardial ischemia/reperfusion injury (Nakamura et al., 1998; Takahashi et al., 2003) and salt-sensitive hypertension (Iwamoto et al., 2004c).

Neuronal and Renal Protection by YM-244769. Under pathological conditions, such as ischemia/reperfusion injury in various organs, the NCX is believed to cause Ca^{2+} overload as a result of elevated $[Na^+]_i$, leading to cell damage (Blaustein and Lederer, 1999; Annunziato et al., 2004). Indeed, KB-R7943, SN-6, and SEA0400 have been shown to efficiently guard against ischemia/reperfusion injury in the heart (Nakamura et al., 1998; Elias et al., 2001; Takahashi et al., 2003), kidney (Ogata et al., 2003; Yamashita et al., 2003), and brain (Schröder et al., 1999; Matsuda et al., 2001). On the other hand, it has been reported that during permanent cerebral ischemia, the inhibition of NCX1 and NCX3 aggravates brain injury (Pignataro et al., 2004), suggesting that the roles of NCX should be differentiated in the phases of ischemia and reperfusion.

YM-244769 is the most potent NCX3 inhibitor among benzyloxyphenyl derivatives. As a model experiment, we verified the pharmacological efficacy of YM-244769 against hypoxia/reoxygenation-induced injury in human neuroblastoma SH-SY5Y cells expressing NCX1 and NCX3 isoforms. The treatment with YM-244769 at the beginning of reoxygenation markedly protected against hypoxia/reoxygenation-induced neuronal cell damage (Fig. 5B). Similar effects were observed in the treatment with KB-R7943 or SN-6, but YM-244769 showed the highest efficiency among them for neuronal protection. To evaluate the tissue specificity, we further analyzed the efficacy of YM-244769 against hypoxia/reoxygenation-induced injury in renal LLC-PK_1 cells exclusively expressing NCX1 isoform. YM-244769 also potently protected against the hypoxia/reoxygenation-induced renal cell damage compared with KB-R7943 and SN-6. Very interestingly, when the potencies of cell protection by each NCX inhibitor in SH-SY5Y and LLC-PK_1 cells were compared, YM-244769 and KB-R7943, which preferentially inhibit NCX3, more efficiently suppressed the neuronal cell damage, whereas SN-6, which preferentially inhibits NCX1, suppressed the renal cell damage to a greater extent (Fig. 5B). These protective potencies consistently correlated well with their inhibitory efficacies for Na_i^+-dependent Ca^{2+} uptake into the corresponding cell lines, although the inhibitory efficacies of NCX inhibitors were weaker than those in CCL39 overexpressing NCX isoforms, probably because of the different kinetics of I_1 inactivation. These results suggest that the hypoxia/reoxygenation-induced injuries in SH-SY5Y and LLC-PK_1 cells are predominantly mediated by Ca^{2+} overload, via both NCX1 and NCX3 in the former and via NCX1 in the latter. In fact, antisense knockdown of NCX1 and NCX3 in SH-SY5Y cells confirmed that NCX3 contributes to the neuronal cell damage to a greater degree than NCX1 (Fig. 7). Thus, it seems advantageous to clinically develop YM-244769, which preferentially inhibits NCX3, as a new neuroprotective drug.

Acknowledgments

We are grateful to Dr. S. Naganuma (Astellas Pharm Inc., Tsukuba, Japan) for providing YM-244769.

References

Annunziato L, Pignataro G, and Di Renzo GF (2004) Pharmacology of brain Na^+/Ca^{2+} exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* **56:**633–654.

Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* **79:**763–854.

Bouchard R, Omelchenko A, Le HD, Choptiany P, Matsuda T, Baba A, Takahashi K, Nicoll DA, Philipson KD, and Hnatowich M, et al. (2004) Effects of SEA0400 on mutant NCX1.1 Na^+-Ca^{2+} exchangers with altered ionic regulation. *Mol Pharmacol* **65:**802–810.

Doering AE, Nicoll DA, Lu Y, Lu L, Weiss JN, and Philipson KD (1998) Topology of a functionally important region of the cardiac Na^+/Ca^{2+} exchanger. *J Biol Chem* **273:**778–783.

Elias CL, Lukas A, Shurraw S, Scott J, Omelchenko A, Gross GJ, Hnatowich M, and Hryshko LV (2001) Inhibition of Na^+/Ca^{2+} exchange by KB-R7943: transport mode selectivity and antiarrhythmic consequences. *Am J Physiol* **281:**H1334–H1345.

Hilgemann DW, Collins A, and Matsuoka S (1992a) Steady-state and dynamic properties of cardiac sodium-calcium exchange. Secondary modulation by cytoplasmic calcium and ATP. *J Gen Physiol* **100:**933–961.

Hilgemann DW, Matsuoka S, Nagel GA, and Collins A (1992b) Steady-state and dynamic properties of cardiac sodium-calcium exchange. Sodium-dependent inactivation. *J Gen Physiol* **100:**905–932.

Iwamoto T (2005) Sodium-calcium exchange inhibitors: therapeutic potential in cardiovascular diseases. *Future Cardiol* **1:**519–529.

Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, and Katsuragi T (2004a) The exchanger inhibitory peptide region-dependent inhibition of Na^+/Ca^{2+} exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol* **66:**45–55.

Iwamoto T, Kita S, Uehara A, Imanaga I, Matsuda T, Baba A, and Katsuragi T (2004b) Molecular determinants of Na^+/Ca^{2+} exchange (NCX1) inhibition by SEA0400. *J Biol Chem* **279:**7544–7553.

Iwamoto T, Kita S, Uehara A, Inoue Y, Taniguchi Y, Imanaga I, and Shigekawa M (2001) Structural domains influencing sensitivity to isothiourea derivative inhibitor KB-R7943 in cardiac Na^+/Ca^{2+} exchanger. *Mol Pharmacol* **59:**524–531.

Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, and Katsuragi T (2004c) Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med* **10:**1193–1199.

Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, and Shigekawa M (1998) Protein kinase C-dependent regulation of Na^+/Ca^{2+} exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* **37:**17230–17238.

Iwamoto T and Shigekawa M (1998) Differential inhibition of Na^+/Ca^{2+} exchanger isoforms by divalent cations and isothiourea derivative. *Am J Physiol* **275:**C423–C430.

Iwamoto T, Uehara A, Imanaga I, and Shigekawa M (2000) The Na^+/Ca^{2+} exchanger NCX1 has oppositely oriented reentrant loop domains that contain conserved

aspartic acids whose mutation alters its apparent Ca^{2+} affinity. *J Biol Chem* **275:**38571–38580.
Iwamoto T, Watano T, and Shigekawa T (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem* **271:**22391–22397.
Kimura J, Watano T, Kawahara M, Sakai E, and Yatabe J (1999) Direction-independent block of bi-directional Na^+/Ca^{2+} exchange current by KB-R7943 in guinea-pig cardiac myocytes. *Br J Pharmacol* **128:**969–974.
Kuramochi T, Kakefuda A, Sato I, Tsukamoto I, Taguchi T, and Sakamoto S (2005a) Synthesis and structure-activity relationships of 6-{4-[(3-fluorobenzyl)oxy]phenoxy}nicotinamide derivatives as a novel class of NCX inhibitors: a QSAR study. *Bioorg Med Chem* **13:**717–724.
Kuramochi T, Kakefuda A, Yamada H, Tsukamoto I, Taguchi T, and Sakamoto S (2005b) Discovery of an *N*-(2-aminopyridin-4-ylmethyl)nicotinamide derivative: a potent and orally bioavailable NCX inhibitor. *Bioorg Med Chem* **13:**4022–4036.
Lee C, Dhalla NS, and Hryshko LV (2005) Therapeutic potential of novel Na^+-Ca^{2+} exchange inhibitors in attenuating ischemia-reperfusion injury. *Can J Cardiol* **21:**509–516.
Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, and Philipson KD (1991) Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J Biol Chem* **266:**1014–1020.
Magi S, Castaldo P, Carrieri G, Scorziello A, Di Renzo G, and Amoroso S (2005) Involvement of Na^+-Ca^{2+} exchanger in intracellular Ca^{2+} increase and neuronal injury induced by polychlorinated biphenyls in human neuroblastoma SH-SY5Y cells. *J Pharmacol Exp Ther* **315:**291–296.
Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, and Ota T, et al. (2001) SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* **298:**249–256.
Matsuoka S, Nicoll DA, He Z, and Philipson KD (1997) Regulation of cardiac Na^+-Ca^{2+} exchanger by the endogenous XIP region. *J Gen Physiol* **109:**273–286.
Matsuoka S, Nicoll DA, Hryshko LV, Levitsky DO, Weiss JN, and Philipson KD (1995) Regulation of the cardiac Na^+-Ca^{2+} exchanger by Ca^{2+}. Mutational analysis of the Ca^{2+}-binding domain. *J Gen Physiol* **105:**403–420.
Nakamura A, Harada K, Sugimoto H, Nakajima F, and 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.
Nicoll DA, Hryshko LV, Matsuoka S, Frank JS, and Philipson KD (1996) Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J Biol Chem* **271:**13385–13391.
Nicoll DA, Ottolia M, Lu L, Lu Y, and Philipson KD (1999) A new topological model of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J Biol Chem* **274:**910–917.
Ogata M, Iwamoto T, Tazawa N, Nishikawa M, Takaoka M, Yamashita J, and Matsumura Y (2003) A novel and selective Na^+/Ca^{2+} exchange inhibitor, SEA0400, improves ischemia/reperfusion-induced renal injury. *Eur J Pharmacol* **478:**187–198.
Philipson KD and Nicoll DA (2000) Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* **62:**111–133.
Pignataro G, Gala R, Cuomo O, Tortiglione A, Giaccio L, Castaldo P, Sirabella R, Matrone C, Canitano A, and Amoroso S, et al. (2004) Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia. *Stroke* **35:**2566–2570.
Pintado AJ, Herrero CJ, Garcia AG, and 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.
Quednau BD, Nicoll D, and Philipson KD (1997) Tissue specificity and alternative splicing of the Na^+/Ca^{2+} exchanger isoforms NCX1, NCX2 and NCX3 in rat. *Am J Physiol* **272:**C1250–C1261.
Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS, Goldhaber JI, and Philipson KD (2002) Knockout mice for pharmacological screening: testing the specificity of Na^+-Ca^{2+} exchange inhibitors. *Circ Res* **91:**90–92.
Schröder UH, Breder J, Sabelhaus CF, and Reymann KG (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.
Shigekawa M and Iwamoto T (2001) Cardiac Na^+-Ca^{2+} exchange: molecular and pharmacological aspects. *Circ Res* **88:**864–876.
Takahashi K, Takahashi T, Suzuki T, Onishi M, Tanaka Y, Hamano-Takahashi A, Ota T, Kameo K, Matsuda T, and Baba A (2003) Protective effects of SEA0400, a novel and selective inhibitor of the Na^+/Ca^{2+} exchanger, on myocardial ischemia-reperfusion injuries. *Eur J Pharmacol* **458:**155–162.
Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, and Shigenobu K (2002) Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* **135:**1096–1100.
Watano T, Kimura J, Morita T, and 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.
Yamashita J, Kita S, Iwamoto T, Ogata M, Takaoka M, Tazawa N, Nishikawa M, Wakimoto K, Shigekawa M, and Komuro I, et al. (2003) Attenuation of ischemia/reperfusion-induced renal injury in mice deficient in Na^+/Ca^{2+} exchanger. *J Pharmacol Exp Ther* **304:**284–293.

Address correspondence to: Dr. Takahiro Iwamoto, Department of Pharmacology, School of Medicine, Fukuoka University, 7-45-1 Nanakuma Jonan-ku, Fukuoka 814-0180, Japan. E-mail: tiwamoto@cis.fukuoka-u.ac.jp