YM-244769, a Novel Na+/Ca2+ Exchange Inhibitor That Preferentially Inhibits NCX3, Efficiently Protects against Hypoxia/Reoxygenation-Induced SH-SY5Y Neuronal Cell Damage

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ABSTRACT

We investigated the pharmacological properties and interaction domains of N-(3-aminobenzyl)-6-[4-[(3-fluorobenzyl)oxy]phenoxo] nicotinamide (YM-244769), a novel potent Na+/Ca2+ exchange (NCX) inhibitor, using various NCX-transfectants and neuronal and renal cell lines. YM-244769 preferentially inhibited intracellular Na+-dependent 45Ca2+ uptake via NCX3 (IC50 = 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 45Ca2+ efflux via NCX isomers. We searched for interaction domains with YM-244769 by NCX1/NCX3-chimeric analysis and determined that the a-2 region in NCX1 is mostly responsible for the differential drug response between NCX1 and NCX3. Further cysteine scanning mutagenesis in the a-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-nitrobenzoyloxy)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-PK1 cells, which exclusively express NCX1, whereas 2-[4-(4-nitrobenzoyloxy)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 Ca2+ uptake via NCX isomers 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+/Ca2+ exchanger (NCX) can transport Ca2+ 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 Ca2+ (Ca2+2) to outside the cell during repolarization and diastole, which balances Ca2+ entry via L-type Ca2+ 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 Ca2+ 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 Ca2+ 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

ABBREVIATIONS: NCX, Na+/Ca2+ exchanger; XIP, exchanger inhibitory peptide; KB-R7943, 2-[2-[4-(4-nitrobenzoyloxy)phenyl]ethyl]isothiourea methanesulfonate; SEA0400, 2-[4-[(3-fluorophenyl)methoxy]phenoxo]-5-ethoxyanilide; SN-6, 2-[4-(4-nitrobenzoyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester; YM-244769, N-(3-aminobenzyl)-6-[4-[(3-fluorobenzyl)oxy]phenoxo] 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\(^{2+}\)] at the submicromolar level activates NCX activity by promoting the recovery of the exchanger from the Ca\(^{2+}\)-dependent, or “I\(_S\)” inactivation state, whereas high [Na\(^{+}\)] restrains the exchanger by facilitating the entry of the exchanger into the Na\(^{+}\)-dependent, or “I\(_l\)” 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 non-specific 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 benzoyloxyphenyl 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 benzoyloxyphenyl 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 benzoyloxyphenyl derivatives (see Iwamoto, 2005).

YM-244769 (Fig. 1), a highly potent NCX inhibitor, was found by screening newly synthesized benzoyloxyphenyl derivatives for inhibition of Na\(^{+}\)-dependent 45Ca\(^{2+}\) 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\(^{+}\)-dependent 45Ca\(^{2+}\) 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 neonobiliomata 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. Purine 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 NCX2 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 NCX activity were selected by a Ca\(^{2+}\)-killing procedure as described previously (Iwamoto et al., 1998).

**Na\(^{+}\)-Dependent 45Ca\(^{2+}\) Uptake into Cells.** Na\(^{+}\)-dependent 45Ca\(^{2+}\) 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. 45Ca\(^{2+}\) 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 45CaCl\(_2\) (370 kBq/ml) and 1 mM ouabain. After a 30-s
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.

$\text{Na}^+\text{-Dependent }^{45}\text{Ca}^{2+}\text{ Efflux from Cells.}^{45}\text{Ca}^{2+}$ 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 $\mu$M thapsigargin to cause a transient increase in [Ca$^{2+}$]. $\text{Na}^+\text{-Dependent }^{45}\text{Ca}^{2+}\text{ efflux was estimated by subtracting }^{45}\text{Ca}^{2+}\text{ efflux in Ca}^{2+}$- and Na$^+$-free BSS from that in Ca$^{2+}$-free BSS.

Assays of Other Transporters. The activities of Na$^+$/H$^+$ exchanger, Na$^+$/K$^+$-ATPase, sarcosmemal 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 $2 \times 10^4$ 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 (Milton Healthcare, Ingamshire, UK). All other chemicals were also of the highest grade available.

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$^+$-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 $\mu$M) 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$_{50} = 1.5$ $\mu$M, as reported previously (Iwamoto and Shigekawa, 1998)). To check whether YM-244769 competes with Ca$^{2+}$ for the exchanger, the rate of Na$^+$-dependent $^{45}$Ca$^{2+}$ uptake into NCX1 transfectants was measured under standard conditions as a function of Ca$^{2+}$ concentration (0.06 - 2 mM) in the presence or absence of 0.05 $\mu$M YM-244769. Their double reciprocal plots of uptake rate versus Ca$^{2+}$ concentration were linear (data not shown). YM-244769 increased the half-maximal Ca$^{2+}$ concentration (K$_{50}$) 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$^+$-dependent $^{45}$Ca$^{2+}$ efflux (i.e., forward mode) from NCX1, NCX2, or NCX3 transfectants equilibrated with $^{45}$Ca$^{2+}$ and treated with 1 $\mu$M thapsigargin. The rate of Na$^+$-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 the concentration of 1 $\mu$M inhibited $^{45}$Ca$^{2+}$ efflux by 30% in NCX1, 45% in NCX2, and 42% in NCX3 transfectants. The initial rates of $^{45}$Ca$^{2+}$ efflux decreased in a concentration-dependent manner. The IC$_{50}$ values were 0.80, 2.9, and 96 $\mu$M for NCX1, NCX2, and NCX3 transfectants, respectively. These results indicate that YM-244769 inhibits $^{45}$Ca$^{2+}$ efflux in a concentration-dependent manner.
0.03 to 1 μM did not affect the rate of Na⁺-dependent ⁴⁵Ca²⁺ efflux from three types of NCX transfectants.

Furthermore, YM-244769 (up to 3 μM) did not significantly influence other Na⁺ or Ca²⁺ transport via K⁺-dependent Na⁺/Ca²⁺ exchanger (NCKX2), Na⁺/H⁺ exchanger, Na⁺/K⁺-ATPase, sarcolemmal or sarcoplasmic reticulum Ca²⁺-ATPases, or L-type Ca²⁺ 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⁺-dependent ⁴⁵Ca²⁺ 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 NCX1 and NCX3, respectively (see the legend to Fig. 3).

Figure 4, this double mutant, which has a reduced sensitivity to SN-6 (3 or 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-PK₁ 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), 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). 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-PK₁ 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-PK₁ cells. When the potencies of cell protection by each NCX inhibitor in SH-SY5Y and LLC-PK₁ 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-PK₁ cells (Fig. 5B). Furthermore, we measured the effects of NCX inhibitors on the rates of Na⁺-dependent ⁴⁵Ca²⁺ uptake into SH-SY5Y and LLC-PK₁ cells. As shown in Fig. 6, the inhib-
itory potencies of YM-244769, KB-R7943, and SN-6 for the rates of $^{45}\text{Ca}^{2+}$ uptake are very similar to their efficacies for cell protection in SH-SY5Y and LLC-PK1 cells, suggesting that the hypoxia/reoxygenation-induced cell damage is preferentially mediated by $\text{Ca}^{2+}$ 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.
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 $^{45}$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$_{+}$-$^{45}$Ca$^{2+}$ uptake into cells expressing the wild-type or various mutants of NCX1 and NCX3.](image)

![Fig. 5. Expression of three NCX isoforms in SH-SY5Y and LLC-PK1 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-PK1 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-PK1 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.](image)
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 benzoxoxyphenyl 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 benzoxoxyphenyl 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 various chimera mutants (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 region) or I\(_1\) 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 region) or I\(_1\) Inactivation-dependent inhibition by YM-244769.
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 \( \Delta 292-708 \), 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 \( Na^+ \)-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 (Blauster 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 (Schroeder 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-PK1 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-PK1 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 \( Na^+ \)-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-PK1 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.

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References


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