

Forum Minireview

Functional Proteins Involved in Regulation of Intracellular Ca^{2+} for Drug Development: Pharmacology of SEA0400, a Specific Inhibitor of the Na^+ - Ca^{2+} Exchanger

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Abstract. The Na^+ - Ca^{2+} exchanger (NCX) is involved in regulation of intracellular Ca^{2+} concentration. A specific inhibitor of NCX has been required for clarification of the physiological and pathological roles of NCX. We have developed 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), a highly potent and selective inhibitor of NCX. SEA0400 in the concentration range that inhibits NCX exhibits negligible affinities for the Ca^{2+} channels, Na^+ channels, K^+ channels, noradrenaline transporter, and 14 receptors; and it does not affect the activities of the store-operated Ca^{2+} channel, Na^+ - H^+ exchanger, and several enzymes including Na^+ , K^+ -ATPase and Ca^{2+} -ATPase. Furthermore, recent studies show that SEA0400 attenuates ischemia-reperfusion injury in the brain, heart, and kidney and radiofrequency lesion-induced edema in rat brain. These findings suggest that NCX plays a key role in ischemia-reperfusion injury and may be a target molecule for treatment of reperfusion injury-related diseases.

Keywords: Na^+ - Ca^{2+} exchanger, SEA0400, edema, ischemia/reperfusion injury, apoptosis

Introduction

The Na^+ - Ca^{2+} exchanger (NCX) is involved in regulation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) via the forward mode (Ca^{2+} extrusion) or the reverse mode (Ca^{2+} influx) (1–6). NCX activity is dependent on membrane potential and transmembrane gradients of Na^+ and Ca^{2+} . Elevated intracellular Na^+ concentration, which is observed during ischemia, will cause NCX to function in the reverse mode, resulting in an increase in $[\text{Ca}^{2+}]_i$ (Fig. 1). Because of these properties, NCX is considered to be a promising new target for drugs to reduce ischemic injury. Protocols to inhibit selectively NCX activity are essential for investigating the physiological and pathological roles of NCX. The antisense strategy has been successfully used in vitro, but not in vivo (7–9). NCX-knockout mice were produced, but they died before birth (10). Thus a selective inhibitor of NCX would be extensively useful for studies on the physio-

logical and pathological roles of NCX. A variety of compounds have been reported to inhibit NCX activity, but their use was limited because of lack of selectivity for NCX (1, 4, 5). Several peptides were also reported as potent and specific NCX inhibitors (1). Li et al. (11) synthesized a family of peptides corresponding to the calmodulin-binding autoinhibitory domain of NCX and demonstrated that XIP, one of these peptides, inhibits NCX activity with an IC_{50} value of about 1.5 μM . XIP was the most selective inhibitor of NCX, but it did not appear to permeate through the cell membranes. In the circumstances, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea (KB-R7943) was synthesized as a potent inhibitor of NCX (IC_{50} , about 2 μM) (12). However, the results of previous studies do not support the selectivity of KB-R7943 for NCX (13–15). We have recently found that 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), a newly synthesized compound (Fig. 2), is the most potent and selective inhibitor of NCX so far reported (16). This compound has been identified by screening a compound library

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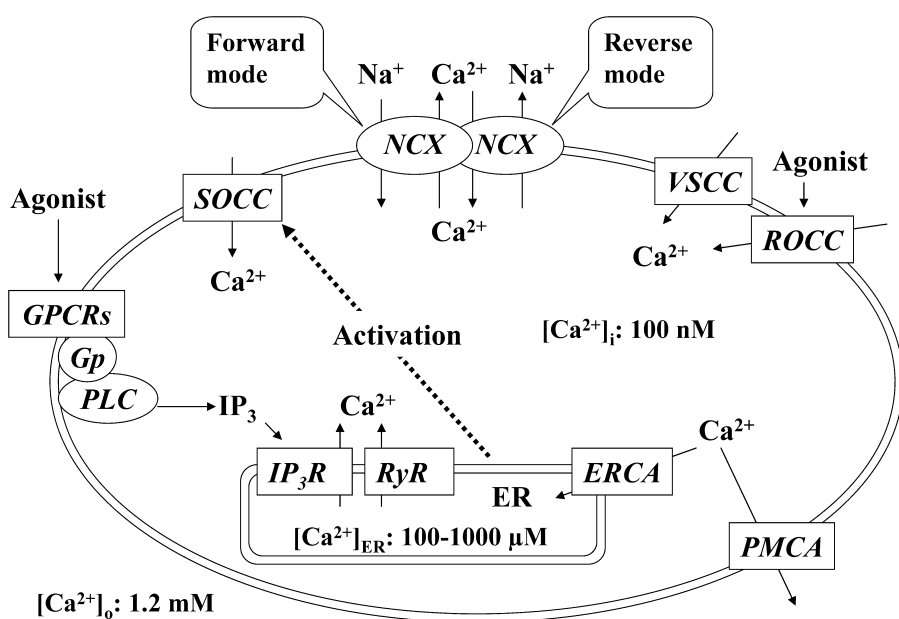


Fig. 1. Regulation of $[Ca^{2+}]_i$. NCX plays an important role in the regulation of $[Ca^{2+}]_i$; it can mediate not only Ca^{2+} extrusion across the plasma membranes (forward mode) but also Ca^{2+} influx upon reduction of the Na^+ electrochemical gradient (reverse mode). VSCC, voltage-sensitive Ca^{2+} channel; ROCC, receptor-coupled Ca^{2+} channel; GPCRs, G protein-coupled receptor; SOCC, store-operated Ca^{2+} channel; PLC, phospholipase C; Gp, G protein; IP_3R , IP_3 receptor; RyR, ryanodine receptor; ER, endoplasmic reticulum; ERCA, ER Ca^{2+} -ATPase; PMCA, plasma membrane Ca^{2+} -ATPase.

for inhibition of Na^+ -dependent Ca^{2+} uptake into isolated cardiac sarcolemmal vesicles and cultured astrocytes. This review characterizes SEA0400 as a specific inhibitor of NCX and summarizes the recent studies on NCX using SEA0400.

SEA0400 as a specific inhibitor of NCX

SEA0400 is an extremely potent and selective inhibitor of NCX (Table 1). In cultured neurons, astrocytes, and microglia, SEA0400 inhibits Na^+ -dependent $^{45}Ca^{2+}$ uptake with IC_{50} values of 5–33 nM. With respect to selectivity, SEA0400 at 1 μM does not affect L-type Ca^{2+} channel, N-type Ca^{2+} channel, and Na^+ channel. This selectivity seems to be important for studies on the effect on ischemic injury since Ca^{2+} and Na^+ channel inhibitors attenuate cerebral ischemic injury. Furthermore, SEA0400 at 3 μM does not affect other ion channels, receptors, and enzymes that are considered to be involved in key processes in ischemic disease, including Na^+ - H^+ exchange, K^+ channel, adrenoceptors, adenosine receptors, glutamate receptors, muscarinic acetyl-

choline receptors, leukotriene B_4 receptors, platelet activating factor receptors, phospholipases, lipoxygenases, and inducible nitric oxide synthetase. SEA0400 does not affect store-operated Ca^{2+} channels. In addition, Tanaka et al. (17) reported that SEA0400 is a selective inhibitor of NCX current in myocardial preparation. These observations suggest that SEA0400 is a highly specific inhibitor of NCX and that SEA0400 is a valuable new tool for elucidating the pathophysiological roles of NCX. However, Ruter et al. (18) using heart tubes prepared from embryos with NCX knocked out showed that SEA0400 had an effect on Ca^{2+} signal even in the NCX-deficient preparation, although the exact mechanism for the effect on embryonic heart tubes is not known. This result suggests that SEA0400 should be used with caution.

The mammalian NCX forms a multigene family of highly homologous proteins comprising three isoforms, NCX1, NCX2, and NCX3 (1–6). NCX1 is expressed in most tissues, whereas the expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle. There are three isoforms of NCX in the brain, and they show developmental changes: the main isoform of immature rat brain is NCX1, whereas that of adult rat brain is NCX2 (19). SEA0400 inhibits Na^+ -dependent Ca^{2+} uptake into NCX1 and NCX2 transfectants with IC_{50} values of 56 and 980 nM, respectively, but it does not affect the NCX3 transfectant (20). SEA0400 stabilizes or modulates the transition of NCX into a Na^+ -dependent inactive state (21, 22). Furthermore, the inhibitory potency of SEA0400 is dependent on the intracellular Na^+ (21, 22). This inhibitory mechanism

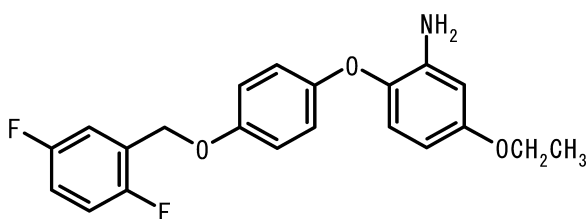


Fig. 2. Chemical structure of SEA0400.

Table 1. Characterization of SEA0400

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| Inhibition of NCX by SEA0400 |
| Na ⁺ -dependent Ca ²⁺ uptake in cultured rat neurons: IC ₅₀ = 33 nM (16) |
| Na ⁺ -dependent Ca ²⁺ uptake in cultured rat astrocytes: IC ₅₀ = 5.0 nM (16) |
| Na ⁺ -dependent Ca ²⁺ uptake in cultured rat microglia: IC ₅₀ = 8.3 nM (16) |
| Na ⁺ -dependent Ca ²⁺ uptake in canine cardiac sarcolemmal vesicles: IC ₅₀ = 90 nM (29) |
| Na ⁺ -dependent Ca ²⁺ uptake in rat cardiomyocytes: IC ₅₀ = 92 nM (29) |
| The inward NCX current in guinea pig cardiomyocytes: IC ₅₀ = 40 nM (17) |
| The outward NCX current in guinea pig cardiomyocytes: IC ₅₀ = 32 nM (17) |
| The functional proteins that are not affected by SEA0400 |
| Ion transporters: Na ⁺ -H ⁺ exchanger, Na ⁺ ,K ⁺ -ATPase, Ca ²⁺ -ATPase (16) |
| Ion channels and transporters: L-type Ca ²⁺ channel, N-type Ca ²⁺ channel, Na ⁺ channel, K ⁺ channel, store-operated Ca ²⁺ channel, noradrenaline transporter (16) |
| Myocardial ionic currents: Na ⁺ current, L-type Ca ²⁺ current, delayed rectifier K ⁺ current and inwardly rectifying K ⁺ current (17) |
| Receptors: adenosine receptors (A ₁ , A ₂), adrenergic receptors (α ₁ , α ₂ , β ₁ , β ₂), glutamate receptors (AMPA, kainite, NMDA), muscarinic acetylcholine receptor, bradykinin receptors (B ₁ , B ₂), leukotriene B ₄ receptor, platelet-activating factor receptor (16) |
| Enzyme: phospholipase A ₂ , phospholipase C, 5-lipoxygenase, inducible nitric oxide synthase, constitutive nitric oxide synthase (16) |

seems to be an ideal attribute when considering its potential utility against ischemia-reperfusion injury.

The protective effect of SEA0400 against reperfusion injury

Previous studies showed that the NCX inhibitor KB-R7943 attenuated ischemic injury in cardiac (23) and renal (24) preparations. However, it is obscure whether NCX is involved in the injury, since the specificity of the inhibitor is questionable. We found that SEA0400 attenuated Ca²⁺ paradox-like injury in cultured astrocytes (16). This Ca²⁺ paradox-like injury is mediated by an increase in [Ca²⁺]_i, resulting in production of reactive oxygen species (ROS), and SEA0400 blocks Ca²⁺ paradox-induced ROS production, resulting in an antiapoptotic effect (25–28). Furthermore, SEA0400 is effective in reducing infarct volume in the cerebral cortex after middle cerebral artery (MCA) occlusion. The finding suggests that NCX is involved in reperfusion injury in a MCA occlusion model, although the precise mechanism is not known. The similar protective effect of SEA0400 has been reported in *in vitro* and *in vivo* injury models of cardiac (29–31) and renal (32) preparations. Furthermore, Luo et al. (33) have recently shown that SEA0400 prevented kainate-induced death of adult mouse retinal neurons, suggesting that the lethal Ca²⁺ entry was via reverse operation of NCX.

Pharmacological effects of SEA0400

Disruption of Ca²⁺ homeostasis in endothelial cells and astrocytes is considered to be responsible for

dysfunction of blood-brain barrier permeability (34). We have found that administration of SEA0400 (3 and 10 mg/kg, *i.v.*) significantly attenuated the increase in brain water content and disruption of blood-brain barrier permeability after radiofrequency lesion in rats (35). The findings suggest that NCX plays a role in the generation of vasogenic edema accompanied by disruption of the blood-brain barrier. Iwamoto et al. (36) have recently found a new vascular effect of SEA0400. *In vivo* administration of SEA0400 suppressed the developmental hypertension and organ damage in an experimental hypertensive model. Furthermore, they showed using genetically NCX1-engineered mice that NCX1 contributes to hypertension. These findings suggest that NCX is a target molecule for treatment of some types of hypertension.

Conclusion

NCX may be one of molecular targets for improvement of Ca²⁺-mediated pathology. The most interesting point at the beginnings of NCX research was that NCX might be responsible for the pharmacological effect of cardiac glycosides, and its inhibitors had a positive inotropic effect in isolated guinea pig atria (37, 38). Although there is no inhibitor of NCX that is clinically used, the recent studies using SEA0400 suggest that the inhibitor will be used for treatment of ischemic events such as heart attack, stroke, and brain edema and hypertension. We hope that SEA0400 further contributes to clarification of the physiological and pathological roles of NCX.

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References

- Matsuda T, Takuma K, Baba A. Na^+ - Ca^{2+} exchanger: physiology and pharmacology. *Jpn J Pharmacol.* 1997;74:1–20.
- Hryshko LV, Philipson KD. Sodium-calcium exchange: recent advances. *Basic Res Cardiol.* 1997;92 Suppl 1:45–51.
- Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev.* 1999;79:763–854.
- Philipson KD, Nicoll DA. Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol.* 2000;62:111–133.
- Shigekawa M, Iwamoto T. Cardiac Na^+ - Ca^{2+} exchange. Molecular and pharmacological aspects. *Circ Res.* 2001;88:864–876.
- Akabas MH. Na^+ / Ca^{2+} exchange inhibitors: potential drugs to mitigate the severity of ischemic injury. *Mol Pharmacol.* 2004;66:8–10.
- Lipp P, Schwaller B, Niggli E. Specific inhibition of Na-Ca exchange function by antisense oligodeoxynucleotides. *FEBS Lett.* 1995;364:198–202.
- Matsuda T, Takuma K, Nishiguchi E, Hashimoto H, Azuma J, Baba A. Involvement of Na^+ - Ca^{2+} exchanger in reperfusion-induced delayed cell death of cultured rat astrocytes. *Eur J Neurosci.* 1996;8:951–958.
- Takuma K, Matsuda T, Hashimoto H, Kitanaka J, Asano S, Kishida Y, et al. Role of Na^+ - Ca^{2+} exchanger in agonist-induced Ca^{2+} signaling in cultured rat astrocytes. *J Neurochem.* 1996; 67:1840–1845.
- Wakimoto K, Kobayashi K, Kuro-o M, Yao A, Iwamoto T, Yanaka N, et al. Targeted disruption of Na^+ / Ca^{2+} exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. *J Biol Chem.* 2000;275:36991–36998.
- Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, et al. Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+ - Ca^{2+} exchanger. *J Biol Chem.* 1991; 266:1014–1020.
- Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+ / Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem.* 1996;271: 22391–22397.
- Sobolevsky AI, Khodorov BI. Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the Na^+ / Ca^{2+} exchange inhibitor KB-R7943. *Neuropharmacology.* 1999;38: 1235–1242.
- Arakawa N, Sakaue M, Yokoyama I, Hashimoto H, Koyama Y, Baba A, et al. KB-R7943 inhibits store-operated Ca^{2+} entry in cultured neurons and astrocytes. *Biochem Biophys Res Commun.* 2000;279:354–357.
- Pintado AJ, Herrero CJ, Garcia AG, Montiel C. The novel Na^+ / Ca^{2+} exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol.* 2000; 130:1893–1902.
- Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, et al. 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.* 2001;298:249–256.
- Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effects of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol.* 2002;135:1096–1100.
- Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS, et al. Knockout mice for pharmacological screening testing the specificity of Na^+ - Ca^{2+} exchange inhibitors. *Circ Res.* 2002;91: 90–92.
- Sakaue M, Nakamura H, Kaneko I, Kawasaki Y, Arakawa N, Hashimoto H, et al. Na^+ - Ca^{2+} exchanger isoforms in rat neuronal preparations: different changes in their expression during postnatal development. *Brain Res.* 2000;881:212–216.
- Iwamoto T, Kita S, Uehara A, Imanaga I, Matsuda T, Baba A, et al. Molecular determinants of Na^+ / Ca^{2+} exchange (NCX1) inhibition by SEA0400. *J Biol Chem.* 2004;279:7544–7553.
- Bouchard R, Omelchenko A, Le HD, Choptiany P, Matsuda T, Baba A, et al. Effects of SEA0400 on mutant NCX1.1 Na^+ - Ca^{2+} exchangers with altered ionic regulation. *Mol Pharmacol.* 2004; 65:802–810.
- Lee C, Visen NS, Dhalla NS, Le HD, Isaac M, Choptiany P, et al. Inhibitory profile of SEA0400 [2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] assessed on the cardiac Na^+ - Ca^{2+} exchanger, NCX1.1. *J Pharmacol Exp Ther.* 2004;311:748–757.
- Ladilov Y, Haffner S, Balser-Schafer C, Maxeiner H, Piper HM. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na^+ / Ca^{2+} exchanger. *Am J Physiol.* 1999; 276:H1868–H1876.
- Kuro T, Kobayashi Y, Takaoka M, Matsumura Y. Protective effect of KB-R7943, a novel Na^+ / Ca^{2+} exchange inhibitor, on ischemic acute renal failure in rats. *Jpn J Pharmacol.* 1999; 81:247–251.
- Takuma K, Lee E, Kidawara M, Mori K, Kimura Y, Baba A, et al. Apoptosis in Ca^{2+} reperfusion injury of cultured astrocytes: roles of reactive oxygen species and NF- κ B activation. *Eur J Neurosci.* 1999;11:4204–4212.
- Takuma K, Mori K, Lee E, Enomoto R, Baba A, Matsuda T. Heat shock inhibits hydrogen peroxide-induced apoptosis in cultured astrocytes. *Brain Res.* 2002;946:232–238.
- Takuma K, Kiri M, Mori K, Lee E, Enomoto R, Baba A, et al. Roles of cathepsins in reperfusion-induced apoptosis in cultured astrocytes. *Neurochem Int.* 2003;42:153–159.
- Takuma K, Baba A, Matsuda T. Astrocyte apoptosis: implications for neuroprotection. *Prog Neurobiol.* 2004;72:111–127.
- Takahashi K, Takahashi T, Suzuki T, Onishi M, Tanaka Y, Hamano-Takahashi A, et al. Protective effects of SEA0400, a novel selective inhibitor of the Na^+ / Ca^{2+} exchanger, on myocardial ischemia-reperfusion injuries. *Eur J Pharmacol.* 2003; 458:155–162.
- Magee WP, Deshmukh G, DeNinno MP, Sutt JC, Chapman JG, Tracey WR. Differing cardioprotective efficacy of the Na^+ / Ca^{2+} exchanger inhibitors SEA0400 and KB-R7943. *Am J Physiol Heart Circ Physiol.* 2003;284:H903–H910.
- Yoshiyama M, Nakamura Y, Omura T, Hayashi T, Takagi Y, Hasegawa T, et al. Cardioprotective effect of SEA0400, a selective inhibitor of the Na^+ / Ca^{2+} exchanger, on myocardial ischemia-reperfusion injury in rats. *J Pharmacol Sci.* 2004;95: 196–202.

- 32 Ogata M, Iwamoto T, Tazawa N, Nishikawa M, Yamashita J, Takaoka M, et al. A novel and selective $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor, SEA0400, improves ischemia/reperfusion-induced renal injury. *Eur J Pharmacol.* 2003;478:187–198.
- 33 Luo X, Baba A, Matsuda T, Romano C. Susceptibilities to and mechanisms of excitotoxic cell death of adult mouse inner retinal neurons in dissociated culture. *Invest Ophthalmol Visual Sci.* 2004;45:4576–4582.
- 34 Brown RC, Davis TP. Calcium modulation of adherens and tight junction function: a potential mechanism for blood-brain barrier disruption after stroke. *Stroke.* 2002;33:1706–1711.
- 35 Koyama Y, Matsui S, Itoh S, Osakada M, Baba A, Matsuda T. The selective $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor attenuates brain edema after radiofrequency lesion in rats. *Eur J Pharmacol.* 2004;489:193–196.
- 36 Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, et al. Salt-sensitive hypertension is triggered by Ca^{2+} entry via $\text{Na}^+/\text{Ca}^{2+}$ exchanger type-1 in vascular smooth muscle. *Nature Med.* 2004;10:1193–1199.
- 37 Blaustein M. The cellular basis of cardiotonic steroid action. *Trends Pharmacol Sci.* 1985;6:289–292.
- 38 Luciani S, Floreani M. $\text{Na}^+/\text{Ca}^{2+}$ exchange as a target for inotropic drugs. *Trends Pharmacol Sci.* 1985;6:316.