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

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Received December 14, 2000; accepted March 23, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The effect of the newly synthesized compound 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400) on the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) was investigated and compared against that of 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea (KB-R7943). In addition, the effects of SEA0400 on reperfusion injury in vitro and in vivo were examined. SEA0400 was extremely more potent than KB-R7943 in inhibiting Na\(^+\)-dependent Ca\(^{2+}\) uptake in cultured neurons, astrocytes, and microglia: IC\(_{50}\)s of SEA0400 and KB-R7943 were 5 to 33 nM and 2 to 4 \(\mu\)M, respectively. SEA0400 at the concentration range that inhibited NCX exhibited negligible affinities for the Ca\(^{2+}\) channels, Na\(^+\) channels, K\(^+\) channels, norepinephrine transporter, and 14 receptors, and did not affect the activities of the Na\(^+\)/H\(^+\) exchanger, Na\(^+\),K\(^+\)-ATPase, Ca\(^{2+}\)-ATPase, and five enzymes. SEA0400, unlike KB-R7943, did not inhibit the store-operated Ca\(^{2+}\) entry in cultured astrocytes. SEA0400 attenuated dose-dependently paradoxical Ca\(^{2+}\) challenge-induced production of reactive oxygen species, DNA ladder formation, and nuclear condensation in cultured astrocytes, whereas it did not affect thapsigargin-induced cell injury. Furthermore, administration of SEA0400 reduced infarct volumes after a transient middle cerebral artery occlusion in rat cerebral cortex and striatum. These results indicate that SEA0400 is the most potent and selective inhibitor of NCX, and suggest that the compound may exert protective effects on postischemic brain damage.

The Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) is involved in regulation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) via the forward mode (Ca\(^{2+}\) extrusion) or the reverse mode (Ca\(^{2+}\) influx) (Hryshko and Philipson, 1997; Matsuda et al., 1997). Protocols to inhibit selectively NCX activity are useful for investigating the roles of the exchanger. The antisense strategy has been successfully used in vitro (Lipp et al., 1995; Matsuda et al., 1996; Takuma et al., 1996a; Slodzinski and Blaustein, 1998; Van Eylen et al., 1998; White et al., 1998; Takahashi et al., 1999), but not in vivo. A variety of compounds are reported to inhibit NCX activity, but their use is limited because of lack of selectivity for NCX (Kaczorowski et al., 1989).

Although XIP, a synthetic peptide, is the most selective inhibitor of NCX (Li et al., 1991), it does 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\(_{50}\) for Na\(^+\)-dependent Ca\(^{2+}\) uptake, about 2 \(\mu\)M) (Iwamoto et al., 1996; Watano et al., 1996). Since KB-R7943 at 10 \(\mu\)M did not affect Na\(^+\)/H\(^+\) exchange, dihydropyridine (DHP)-sensitive Ca\(^{2+}\) uptake, passive Na\(^+\) uptake, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, sarcolemmal Ca\(^{2+}\)-ATPase, and Na\(^+\),K\(^+\)-ATPase (Iwamoto et al., 1996), it has been used as a selective inhibitor of NCX. However, it remains to be determined whether KB-R7943 is indeed a selective inhibitor of NCX. Sobolevsky and Khodorov (1999) reported that KB-R7943 blocked N\(-\)methyl-D-aspartate channels in acutely isolated hippocampal

This work was supported by a grant from the Ministry of Education, Sciences, Sports, and Culture of Japan.

ABBREVIATIONS: NCX, Na\(^+\)-Ca\(^{2+}\) exchanger; [Ca\(^{2+}\)]\(_{i}\), intracellular Ca\(^{2+}\) concentration; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea; DHP, dihydropyridine; SOCE, store-operated Ca\(^{2+}\) entry; ROS, reactive oxygen species; SEA0400, 2-[4-(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; H\(_2\)DCF, 2',7'-dichlorofluorescein diacetate; MEM, Eagle’s minimum essential medium; HBSS, Hanks’ balanced saline solution; cNOS, constitutive nitric-oxide synthetase; CPA, cyclopiazonic acid; MCA, middle cerebral artery; NE, norepinephrine; LTB\(_4\), leukotriene B\(_4\); mACh, muscarinic acetylcholine; PAF, platelet-activating factor.
neurons. We have recently found KB-R7943 at 10 μM significantly inhibited the store-operated Ca\(^{2+}\) entry (SOCE; formerly referred to as capacitative Ca\(^{2+}\) entry) (Arakawa et al., 2000). Furthermore, Watano et al. (1999) could not exclude the possibility that the effect of KB-R7943 on ouabain-induced arrhythmias might be mediated by inhibition of voltage-gated Na\(^+\) channels. A more selective inhibitor of NCX is required for studies on physiological and pathological roles of NCX.

We found that Ca\(^{2+}\) paradox-like phenomenon occurred in cultured rat astrocytes: a persistent increase in [Ca\(^{2+}\)]\(_i\), followed by delayed cell death was observed when the cells were incubated with Ca\(^{2+}\)-containing medium after exposure to Ca\(^{2+}\)-free medium (Matsuda et al., 1996). Furthermore, we have recently shown that the Ca\(^{2+}\) receptor reperfusion induced apoptosis and reactive oxygen species (ROS) production might be involved in the cell death (Takuma et al., 1999). The injury is reduced by heat shock protein (Takuma et al., 1996b), calciurein inhibitors (Matsuda et al., 1998), and anti- ischemic drugs (Takuma et al., 2000a,b). These findings, together with the previous report that a similar paradoxical change in extracellular Ca\(^{2+}\) concentration occurs in ischemic brain tissue (Siemkowicz and Hansen, 1981; Silver and Erecinska, 1992; Kristian et al., 1994), suggest that the Ca\(^{2+}\) paradox-like injury is an in vitro model of cerebral ischemia/reperfusion injury.

We report here that 2-[4-[[2,5-difluorophenyl]methoxy]-phenoxy]-5-ethoxyaniline (SEA0400), a newly synthesized compound, is the most potent and selective inhibitor of NCX so far reported. This compound (Fig. 1) has been identified by screening a compound library for inhibition of Na\(^+\)/K\(^+\)-dependent Ca\(^{2+}\) uptake into isolated cardiac sarcolemmal vesicles and cultured astrocytes. In addition, we demonstrate that SEA0400 protects astrocytes against Ca\(^{2+}\) paradox-like injury and reduces cerebral ischemic damage in rats with a transient middle cerebral artery occlusion.

**Experimental Procedures**

### Materials

Drugs were obtained from the following sources: fetal calf serum (FCS), mouse anti-human glial fibrillary acidic protein monoclonal antibody, isoleucin B\(_2\) (biotin labeled), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Sigma (St. Louis, MO); mouse anti-microtubule-associated protein-2 antibody, Chemicon International, Inc. (Temecula, CA); fluorescein-conjugated goat anti-mouse IgG antibody, Organon Teknika N.V.-Cappel Products (West Chester, PA); 2′,7′-dichlorofluorescein diacetate (H\(_2\)DCF-DA), Molecular Probes, Inc. (Eugene, OR); and Eagle’s minimum essential medium (MEM), Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 45Ca (5-50 mCi/mg) was purchased from Amersham (Tokyo, Japan). SEA0400 and KB-R7943 were synthesized in Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). All other chemicals used were of the highest purity commercially available. In the in vitro studies, SEA0400 and KB-R7943 were dissolved in dimethyl sulfoxide (final concentration 0.1%). In an in vivo experiment, SEA0400 was administered as a lipid emulsion containing 20% soybean oil, and MK-801 was dissolved in saline.

### Cell Culture

Astrocytes were isolated from cerebral cortices of 1-day-old Wistar rats as previously reported (Takuma et al., 1994; Matsuda et al., 1996). Briefly, the tissue was dissociated with dispase and cultured in MEM containing 10% FCS and 2 mM glucose. Cells were placed in 75 cm\(^2\) tissue culture flasks and split once upon confluency (14-21 days). The cells (5 × 10\(^4\) cells/well) were plated on glass coverslips attached to silicon walls and grown for 1 to 2 days in experiments measuring fura-2 fluorescence. In experiments measuring NCX activity, the cells were plated in 24-well plastic tissue culture plates and grown for 14 to 20 days. The astrocytes in the plastic plates consisted of >90% flat polygonal astrocytes (type 1 astrocytes), as confirmed by phase-contrast microscopy and positive immunostaining with anti-glia fibrillary acidic protein antibody, and did not contain neuronal cells, as determined by negative immunostaining with anti-microtubule-associated protein-2 antibody (Sakaue et al., 2000). The dissociated cortical neurons were prepared from 18-day rat fetuses (Sakaue et al., 2000) and seeded onto culture plates. After 48 h, the cells were treated with 10 μM Ara-C for 48 h and cultured in Dulbecco’s modified MEM containing 10% FCS for 6 to 10 days. The cell cultures consisted of more than 90% neurons as determined by positive immunostaining with anti-microtubule-associated protein-2 antibody. Microglia were obtained from cerebral cortices of 1-day-old Wistar rats (Kitanaka et al., 1996; Koyama et al., 2000). Briefly, mixed primary cultures were grown on 75 cm\(^2\) tissue culture flasks in MEM containing 10% FCS and 2 mM glucose. After 10 days in primary culture, microglial cells were harvested by mild shaking (140 rpm, 2 h) and plated on 48-well plastic tissue culture plates (1 × 10\(^3\) cell/well). The cells were incubated for 30 min, washed twice, and further incubated in MEM containing 10% FCS for more than 8 h. The cell cultures consisted of more than 95% microglia as determined by positive isocitoline B\(_4\) staining (Takuma et al., 2000b).

### Na\(^{+}\)/Ca\(^{2+}\) Exchange Activity

Na\(^{+}\)/Ca\(^{2+}\) exchange activity was determined by assaying Na\(^{+}\)-dependent 45Ca\(^{2+}\) uptake as reported previously (Takuma et al., 1994). Briefly, the cells were preincubated in Hanks’ balanced saline solution (HBSS) for 20 min, and the medium was switched to HBSS containing 45Ca\(^{2+}\) and incubated for 5 min. To increase intracellular Na\(^+\) concentration, 1 mM ouabain plus 20 μM monensin (for astrocytes and microglia) and 10 μM monensin (for neurons) were used. Monensin was added simultaneously with the isotope. Ouabain was added 5 min before monensin in astrocytes and microglia. NCX inhibitors were added 5 min before monensin and present during 45Ca\(^{2+}\) uptake reaction.

### Binding Assays to Various Receptors, Ion Transporters, and Ion Channels

The binding assays were performed as a contract study by CERE (Celle l’Evescaut, France). The assays were performed under experimental conditions as shown in Tables 1 and 2. Na\(^{+}/H^+\) exchange (Jean et al., 1986), Na\(^{+}\), K\(^+-\)ATPase (Fiske and Subbarow, 1925), Ca\(^{2+}\)-ATPase (Jean and Klee, 1986), phospholipase A\(_2\) (Katsumata et al., 1986), phospholipase C (Nakanishi et al., 1985), 5-lipoxygenase (Coffey et al., 1992), inducible nitric-oxide synthetase (Estrada et al., 1992), and constitutive nitric-oxide synthetase (cNOS) (Bredt and Snyder, 1990) activities were determined as reported previously.

### SOCE

SOCE was determined by measuring cyclopiazonic acid (CPA)-induced change in [Ca\(^{2+}\)]\(_i\), in cultured astrocytes (Arakawa et al., 2000). [Ca\(^{2+}\)]\(_i\) was determined as reported previously (Takuma et al., 1994; Sakaue et al., 2000). Briefly, astrocytes were perfused with HBSS consisting of (in mM): NaCl, 137; KCl, 5.4; CaCl\(_2\), 1.3; MgCl\(_2\), 0.49; MgSO\(_4\), 0.406; Na\(_2\)HPO\(_4\), 0.335; KH\(_2\)PO\(_4\), 0.44; NaHCO\(_3\), 4.17; and glucose, 5.55. Fura-2 fluorescence (510 nm emission excited by 340 and 380 nm illumination) from cells, as well as background fluorescence, was imaged using an ARGUS-HISCA image processor (Hamamatsu, Japan). Since CPA-induced Ca\(^{2+}\) signal varied from cell to cell, the effect of drug on SOCE was assessed in each cell; the

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**Fig. 1. Chemical structure of SEA0400.**
were washed and exposed to Earle’s solution (control) and Ca\(^{2+}\) using confluent astrocytes in fetal calf serum-free medium. The cells were incubated at 37°C for 30 min in normal Earle’s solution for 3 days (for MTT assay) and 5 days (for DNA analysis). In an experiment, the cells were treated with thapsigargin at 100 nM for 3 days. Cell viability was measured by a colorimetric assay using MTT (Matsuda et al., 1996). MTT reduction activity was found to be proportional to the number of cells (Matsuda et al., 1996; Takuma et al., 1999). In a later experiment, the cells were treated with thapsigargin at 100 nM for 3 days and then incubated with 10 nM CGP 12177, an inhibitor of Ca\(^{2+}\)-ATPase. The cells were then rinsed twice with normal HBSS to remove excess dye. The cells were reperfused in normal HBSS for 1 h, and the conversion of H\(_2\)DCF-DA to its fluorescent product dichlorofluorescein by ROS, presumably H\(_2\)O\(_2\) and hydroxyl radical, was determined with excitation at 485 nm and emission at 535 nm using a Wallac Multilabel counter (Behl et al., 1994; Takuma et al., 1999).

### TABLE 1

<table>
<thead>
<tr>
<th>Ion transporters</th>
<th>Substrate, Tracer of Ligand</th>
<th>SEA0400 (µM)</th>
<th>KB-R7943 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^{+}/)K(^{-}) exchanger</td>
<td>A7r5 cells</td>
<td>(^{22})Na (0.2 µM)</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Na(^{+}), K(^{-})-ATPase</td>
<td>Dog kidney</td>
<td>ATP (2 nM)</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase (pump)</td>
<td>Rat liver membrane vesicles</td>
<td>ATP (1 mM), (^{45})Ca(^{2+})</td>
<td>&gt;30</td>
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</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Origin</th>
<th>SEA0400 (µM)</th>
<th>KB-R7943 (µM)</th>
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</thead>
<tbody>
<tr>
<td>Adenosine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A(_1)</td>
<td>2-Chloro-N(_6)-[(^{3}H)]cyclopentyladenosine</td>
<td>96</td>
<td>94</td>
</tr>
<tr>
<td>A(_2)</td>
<td>[(^{3}H)]CGS 21680</td>
<td>84</td>
<td>73</td>
</tr>
<tr>
<td>Adrenergic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha)</td>
<td>[(^{3}H)Prazosin]</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>(\beta)</td>
<td>[(^{3}H)]RX 821002</td>
<td>106</td>
<td>123</td>
</tr>
<tr>
<td>(\beta)</td>
<td><a href="-">(^{3}H)</a>-CGP 12177 + 10 nM ICI 118551</td>
<td>95</td>
<td>91</td>
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<tr>
<td>Glutamate</td>
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<td></td>
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<td>AMPA</td>
<td>[(^{3}H)]AMPA</td>
<td>87</td>
<td>76</td>
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<tr>
<td>Kainate</td>
<td>[(^{3}H)]Kainic acid</td>
<td>107</td>
<td>91</td>
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<tr>
<td>NMDA</td>
<td>[(^{3}H)]CGP 39653</td>
<td>87</td>
<td>93</td>
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<tr>
<td>mACH</td>
<td>[(^{3}H)]QNB</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>Bradykinin</td>
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</tr>
<tr>
<td>B(_1)</td>
<td>[(^{3}H)]desArg(^{15})-KD</td>
<td>79</td>
<td>92</td>
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<tr>
<td>B(_2)</td>
<td>[(^{3}H)]Bradykinin</td>
<td>97</td>
<td>100</td>
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<tr>
<td>LTB(_4)</td>
<td>[(^{3}H)]LTB(_4)</td>
<td>94</td>
<td>45</td>
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<td>PAF</td>
<td>[(^{3}H)]C(_{18})-PAF</td>
<td>103</td>
<td>81</td>
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<tr>
<td>Transporter</td>
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<tr>
<td>NE transporter</td>
<td>[(^{3}H)]Nisoxetine</td>
<td>84</td>
<td>26</td>
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<td>Phospholipase A(_2)</td>
<td>Porcine pancreas</td>
<td>79</td>
<td>77</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>Rat brain</td>
<td>108</td>
<td>139</td>
</tr>
<tr>
<td>Lipoxygenase</td>
<td>Differentiated HL-60 cells</td>
<td>91</td>
<td>62</td>
</tr>
<tr>
<td>iNOS</td>
<td>RAW 264-7 cells</td>
<td>102</td>
<td>90</td>
</tr>
<tr>
<td>cNOS</td>
<td>Rat cerebellum</td>
<td>102</td>
<td>80</td>
</tr>
</tbody>
</table>

AMP, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; QNB, quinuclidinyl benzilate; iNOS, inducible nitric-oxide synthetase.

Change in [Ca\(^{2+}\)], after drug application was measured as previously reported (Arakawa et al., 2000).

**Ca\(^{2+}\) Paradox-Like Injury.** Reperfusion experiments were carried out using confluent astrocytes in fetal calf serum-free medium. The cells were washed and exposed to Earle’s solution (control) and Ca\(^{2+}\)-free Earle’s solution (Ca\(^{2+}\) paradox) for 30 min, and then incubated with normal Earle’s solution for 3 days (for MTT assay) and 5 days (for DNA ladder and Hoechst 33342 staining) (Matsuda et al., 1996; Takuma et al., 1999). In an experiment, the cells were treated with thapsigargin at 100 nM for 3 days. Cell viability was measured by a colorimetric assay using MTT (Matsuda et al., 1996). MTT reduction activity was found to be proportional to the number of cells (Matsuda et al., 1996). Measurement of ROS Production. Cells, plated in 96-well plastic tissue culture plates, were incubated at 37°C for 30 min in normal or Ca\(^{2+}\)-free HBSS containing 10 µM H\(_2\)DCF-DA and 0.25 µg/ml Cremophor EL, and then rinsed twice with normal HBSS to remove excess dye. The cells were reperfused in normal HBSS for 1 h, and the conversion of H\(_2\)DCF-DA to its fluorescent product dichlorofluorescin by ROS, presumably H\(_2\)O\(_2\) and hydroxyl radical, was determined with excitation at 485 nm and emission at 535 nm using a Wallac Multilabel counter (Behl et al., 1994; Takuma et al., 1999). ROS production is expressed as a percentage of control cells. The linearity and sensitivity of ROS assay were confirmed using H\(_2\)O\(_2\) prior to the experiment.

**Analysis of the DNA Ladder.** The cells collected by centrifugation were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 0.5% N-lauroylsarcosine, and 0.2 mg/ml proteinase K, and incubated at 37°C overnight. DNA was extracted by phenol/
cholorform (1:1; v/v) and precipitated with ethanol. The pellet was dissolved in the Tris-EDTA buffer containing 0.5 mg/ml RNase A and incubated at 37°C for 30 min to digest RNA. Equal amounts of DNA samples were subjected to 1.8% agarose gel electrophoresis. DNA in the gel was stained with ethidium bromide and photographed (Takuma et al., 1999).

**Hoechst 33342 Staining.** The cells, plated on a chamber slide, were fixed with 10% formaldehyde and stained with Hoechst 33342 as previously reported (Takuma et al., 1999). An inverted microscope (Olympus, IX70) equipped with a reflected fluorescence illuminator (Olympus, IX-FLA) and a 40× objective lens was used to visualize individual nuclei.

**Focal Cerebral Ischemia.** The effect of SEA0400 on ischemia/ reperfusion injury was performed as a contract study by Panapharm Laboratories Co., Ltd. (Uto, Kumamoto, Japan). Male Sprague-Dawley rats, weighing 290 to 320 g, were used for the study. Temporary focal ischemia was induced by intraluminal vascular occlusion with a suture as described previously (Longa et al., 1989; Kuge et al., 1995). Rats were anesthetized with isoflurane in a mixture of 70% N₂O and 30% O₂, and mechanically ventilated during surgery. Rectal temperature was controlled at 37°C throughout the experiment with a feedback-regulated heating lamp. The femoral vein was cannulated to infuse drugs. A 19-mm length of 4-0 surgical monofilament nylon suture with its tip rounded by heating and coated with silicone was inserted into the external carotid artery stump and carefully advanced from the carotid bifurcation to occlude the origin of the right middle cerebral artery (MCA). During MCA occlusion, the rats were without anesthesia. After 2 h of MCA occlusion, the suture was withdrawn to allow reperfusion. Thirty minutes after MCA occlusion, the neurological status (failure to extend forepaw) of the rats was assessed. Rats were sacrificed at 22 h after reperfusion. The brains were quickly removed and coronally sectioned in 2-mm-thick slices. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride and photographed. The infarct volumes were expressed in slices. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride, and these were quickly removed and coronally sectioned in 2-mm-thick slices. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride and photographed. The infarct volumes were expressed in slices.

**Blood Pressure and Regional Cortical Blood Flow.** Male Sprague-Dawley rats, weighing 289 to 325 g, were anesthetized with 1 to 2% halothane. A catheter was inserted into the femoral artery and connected to a pressure transducer to record blood pressure. Regional cortical blood flow was measured by a laser Doppler flowmeter (Neuroscience, Tokyo, Japan; FLO-N1), with probe placement at 2 mm posterior and 6 mm lateral to the bregma, as previously reported (Toung et al., 1999). SEA0400 or its vehicle with an equivalent volume was i.v. injected at 3 mg/kg immediately after MCA occlusion, and then infused at 1 and 3 mg/kg/h for 2 h. MK-801 was i.p. injected at 3 mg/kg immediately after MCA occlusion.

**Fig. 3.** Effect of SEA0400 on SOCE in cultured astrocytes. Left, a typical CPA-induced Ca²⁺ signal. SEA0400 at the indicated concentrations was added 5 min before CPA. Results are means ± S.E. of 4 wells (A), 4 to 12 wells (B), and 14 to 15 wells (C).

**Results**

**Effects on NCX Activity.** Figure 2 shows the effects of SEA0400 and KB-R7943 on Na⁺-dependent ⁴⁵Ca²⁺ uptake in cultured neurons, astrocytes, and microglia. IC₅₀ values of SEA0400 were 33 nM (neurons), 5.0 nM (astrocytes), and 8.3 nM (microglia), and those of KB-R7943 were 3.8 μM (neurons), 5.0 nM (astrocytes), and 8.3 μM (microglia). In these cells, SEA0400 was >100 times more potent than KB-R7943 in inhibiting the NCX activity.

**Selectivity.** We have recently reported that KB-R7943 inhibited not only NCX, but also SOCE in cultured astrocytes (Arakawa et al., 2000). Figure 3 shows the effect of SEA0400 on SOCE in cultured astrocytes. Left, a typical CPA-induced Ca²⁺ signal in astrocytes, and the horizontal bar indicates the presence of 10 μM CPA. Right, dose response of the effect of SEA0400 on the Ca²⁺ signal. SEA0400 at the indicated concentrations was added 5 min after addition of CPA. Results are means ± S.E. of 25 to 38 cells. **P < 0.01, compared with the control (Dunnett’s test).**

**Fig. 2.** Effects of SEA0400 and KB-R7943 on Na⁺-dependent ⁴⁵Ca²⁺ uptake in cultured neurons, astrocytes, and microglia. A, neurons; B, astrocytes; C, microglia. SEA0400 (open symbols) and KB-R7943 (closed symbols) at the indicated concentrations were added 5 min before ⁴⁵Ca²⁺. Results are means ± S.E. of 4 wells (A), 4 to 12 wells (B), and 14 to 15 wells (C).

The effects of SEA0400 and KB-R7943 on ion transporters and ion channels are summarized in Table 1. Both compounds did not affect the Na⁺/H⁺ exchange, Na⁺,K⁺-
ATPase, Ca\(^{2+}\)-ATPase, K\(^{+}\) channel, and N-type Ca\(^{2+}\) channel. SEA0400 inhibited the DHP site of the L-type Ca\(^{2+}\) channel but not the verapamil site, and conversely, KB-R7943 inhibited the verapamil site of the L-type Ca\(^{2+}\) channel but not the DHP site. Both compounds inhibited the diltiazem site of the L-type Ca\(^{2+}\) channel and site 2 of the Na\(^{+}\) channel, but SEA0400 was 7 and 15 times less potent than KB-R7943, respectively. Table 2 shows the effects of SEA0400 and KB-R7943 on 14 receptors, the NE transporter, and five enzymes. SEA0400 at 3 \(\mu\)M did not affect receptors, the NE transporter, and enzymes tested here, although at 30 \(\mu\)M it inhibited \(^{3}\text{H}\)leukotriene B\(_4\) (LTB\(_4\)) and \(^{3}\text{H}\)nisoxetine binding. On the other hand, KB-R7943 at 3 \(\mu\)M inhibited muscarinic acetylcholine (mACH), LTB\(_4\), and platelet-activating factor (PAF) receptors and NE transporter. In addition to these receptors, KB-R7943 at 30 \(\mu\)M affected the binding to \(\alpha\), \(\beta\), and bradykinin B\(_1\) receptors and enzyme activities of phospholipase A\(_2\), phospholipase C, 5-lipoxygenase, and cNOS.

Ca\(^{2+}\) Paradox-Like Injury. In cultured astrocytes, SEA0400 attenuated Ca\(^{2+}\) paradox-like injury at concentrations more than 0.1 \(\mu\)M, although it did not affect thapsigargin-induced cell injury (Fig. 4). Similar protection was observed by KB-R7943 at the concentrations more than 1 \(\mu\)M (data not shown). SEA0400 reduced paradoxical Ca\(^{2+}\) challenge-induced ROS production in a dose-dependent manner (Fig. 5). Figures 6 and 7 show the effect of SEA0400 on paradoxical Ca\(^{2+}\) challenge-induced apoptosis in cultured astrocytes. SEA0400 decreased the DNA ladder formation in a dose-dependent manner (Fig. 6). Hoechst 33342 staining showed that SEA0400 at 1 \(\mu\)M blocked almost completely nuclear condensation (Fig. 7).

**Fig. 4.** Effect of SEA0400 on Ca\(^{2+}\) paradox-like injury (A) and thapsigargin-induced injury (B) in cultured rat astrocytes. Open and closed circles indicate control and Ca\(^{2+}\) paradox- or thapsigargin-treated cells, respectively. The indicated concentrations of drugs were added at 10 min before Ca\(^{2+}\) reperfusion or thapsigargin treatment and present until assay. Results are means \(\pm\) S.E. of eight wells obtained from four separate experiments. **P < 0.01, significant from control; ††P < 0.01, significant from Ca\(^{2+}\) paradox without SEA0400 (Dunnett’s test).

**Fig. 5.** Effect of SEA0400 on Ca\(^{2+}\) reperfusion-induced ROS production in astrocytes. ROS was measured in the cells reperfused for 1 h after exposure to normal (control, open circles) or Ca\(^{2+}\)-free Earle’s solution (Ca\(^{2+}\) paradox, closed circles). SEA0400 at the indicated concentrations was added 10 min before Ca\(^{2+}\) reperfusion and present until assay. Results are means \(\pm\) S.E. of 10 wells obtained from two separate experiments. **P < 0.01, significant from control; ††P < 0.01, significant from Ca\(^{2+}\) paradox without SEA0400 (Dunnett’s test).
Cerebral Ischemia. SEA0400 did not affect the mean blood pressure, the regional cortical blood flow during the 2-h observation period (Table 3), and body temperature (data not shown). Infarcts were assessed by 2,3,5-triphenyltetrazolium chloride staining 24 h after MCAo of the cerebral cortex and striatum. Infarcts were more predominant in the cerebral cortex than in the striatum.

Figure 8 shows the effects of SEA0400 and MK-801 on the infarct volume in the cerebral cortex and striatum. SEA0400 (3 mg/kg bolus i.v. + 3 mg/kg/h for 2-h continuous infusion) attenuated the infarct volume in the cerebral cortex and striatum. In contrast, MK-801 (3 mg/kg i.p.) showed a protective effect only in the cerebral cortex.

Discussion

The present study shows that SEA0400 is an extremely potent and selective inhibitor of NCX. SEA0400 inhibited NCX activity in neurons, astrocytes, and microglia, with IC50 values of 5 to 33 nM. We have found in the separate experiment that SEA0400 also inhibited NCX activity in dog sarcolemmal vesicles and cultured rat myocytes: the IC50 values were 90 and 92 nM, respectively. These results indicate that SEA0400 inhibits markedly NCX activity in cells and cell-free systems. In view of the previous reports (Iwamoto and Shigekawa, 1998; Sakaue et al., 2000), a slight difference in the potency of SEA0400 among preparations may be due to that in the sensitivity of NCX isoforms to the inhibitor. With respect to selectivity, SEA0400 showed a slight affinity for the Na1 channel site 2, the Ca2+ channel DHP site, and the Ca2+ channel diltiazem site, but their IC50 values were 360 times greater than that of NCX. This selectivity seems to be important for studies on the effect on ischemic injury, since Ca2+ and Na+ channel inhibitors attenuate cerebral ischemic injury (Gemba et al., 1993; Taylor and Meldrum, 1995). Furthermore, SEA0400 at the concentrations up to 3 μM did not affect other ion channels, receptors, and enzymes that were considered to be involved in key processes in ischemic diseases, including Na+/H+ exchange (Phillis et al., 1996), K+ channels (Bari et al., 1996; Fink et al., 1996), adrenergic receptors (Zhu et al., 1996; Semkova and Kriegstein, 1999), adenosine receptors (Von Lubitz, 1999), glutamate receptors (Barone et al., 1992; Bonventre et al., 1997), PAF receptors (Bonventre et al., 1997), phospholipases (Bonventre et al., 1997; Farooqui et al., 1997), lipoxigenase (Rao et al., 1999), and inducible nitric-oxide synthetase (Del Zoppo et al., 2000). In contrast, KB-R7943 affected mACh, LTB4, and PAF receptors, and NE transporter at 3 μM, corresponding to the IC50 value for NCX inhibition. In addition, the previous study showed that KB-R7943 inhibited SOCE (Arakawa et al., 2000), whereas SEA0400 did not affect SOCE. Taken together, the present study suggests that SEA0400 is a valuable new tool for elucidating the pathophysiological roles of NCX.

Previous studies showed that the NCX inhibitor KB-R7943 attenuated ischemic injury in cardiac (Nakamura et al., 1998; Ladilov et al., 1999) and renal (Kuro et al., 1999) preparations. However, it is obscure whether NCX is involved in the injury, since the specificity of KB-R7943 for NCX is questionable as described above. We previously used an antisense strategy to study the involvement of NCX in Ca2+ paradox-like injury in cultured astrocytes (Matsuda et al., 1996). In the present study, we further examined whether the selective NCX inhibitor SEA0400 attenuates Ca2+ paradox-like injury in astrocytes. SEA0400 attenuated Ca2+ reperfusion injury in a dose-dependent manner, although the concentration required for the protective effect was 10 times higher than that required for

![Fig. 6. Effect of SEA0400 on Ca2+ reperfusion-induced DNA fragmentation in astrocytes. DNA was extracted from the cells reperfused for 5 days after exposure to Ca2+-free medium. SEA0400 at the indicated concentrations was added 10 min before Ca2+ reperfusion and present until assay. A typical experiment is shown. M, 100-base pair marker.](image)

![Fig. 7. Effect of SEA0400 on Ca2+ reperfusion-induced nuclear condensation in astrocytes. Nuclear condensation was examined by Hoechst 33342 staining. Cells were exposed to normal (A, C) or Ca2+-free Earle's solution (B, D) for 30 min, and incubated with Earle's solution for 5 days. SEA0400 at 1 μM was added 10 min before Ca2+ reperfusion and present for 5 days (C, D). A typical experiment is shown.](image)
NCX inhibition. We have recently shown that Ca\(^{2+}\) paradox-like injury is mediated by an increase in [Ca\(^{2+}\)], resulting in ROS production (Takuma et al., 1999). The present study showed that SEA0400 blocked ROS production in a dose-dependent manner. The significant effect of SEA0400 was observed in DNA ladder formation and Hoechst 33342 staining, suggesting an antiapoptotic effect of the compound. It is likely that the compound inhibits the NCX-mediated Ca\(^{2+}\) influx, but not Ca\(^{2+}\)-mediated processes, resulting in cell toxicity since SEA0400 does not affect thapsigargin-induced cell toxicity. It is considered that NCX inhibition further increases [Ca\(^{2+}\)]\(_i\) in thapsigargin-treated cells, since the forward mode of NCX contributes to a recovery of the increased level of [Ca\(^{2+}\)]\(_i\) to the resting level. However, SEA0400 did not aggravate thapsigargin-induced cell toxicity. The exact reason for the apparent discrepancy is not known. Iwamoto et al. (1996) reported that KB-R7943 selectively inhibits the reverse mode of NCX, and it has a beneficial effect in in vitro and in vivo models of cerebral ischemic injury implies that glial mechanism may be involved at least partly in the in vivo effect of SEA0400. It is also considered that SEA0400 may affect indirectly the cerebral circulation after MCA occlusion. The precise mechanism underlying the protective effect of SEA0400 on the cerebral ischemic injury is not known. Studies on the therapeutic time window of SEA0400 in this model and the effects in permanent MCA occlusion are in progress.

In conclusion, the results of the present study demonstrate that SEA0400 is the most potent and selective inhibitor of NCX, and it has a beneficial effect in vitro and in vivo models of cerebral ischemic injury.

References
Buri P, Louis TM, Meng W and Busija DW (1996) Global ischemia impairs ATP-

Behl C, Davis JB, Lesley R and Schubert (1994) Hydrogen peroxide mediates amyo-


Bredt DS and Snyder SH (1990) Isolation of nitric oxide synthase, a calmodulin-


Coffey M, Peters-Golden M, Fantone JC and Sporn PHS (1992) Membrane associa-
tion of a novel neuronal two P domain K⁺ channel to phosphatidylinositol 4ul.

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