Inhibitory Profile of SEA0400 [2-[4-[(2,5-Difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] Assessed on the Cardiac Na\(^{+}\)-Ca\(^{2+}\) Exchanger, NCX1.1

Candace Lee, Neeraj S. Visen, Naranjan S. Dhall, Hoa Dinh Le, Michael Isaac, Platon Choptiany, Gil Gross, Alexander Omelchenko, Toshio Matsuda, Akemichi Baba, Kenzo Takahashi, Mark Hnatowich, and Larry V. Hryshko

Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre, 351 Tache Avenue, Winnipeg, Manitoba, Canada (C.L., N.S.V., N.S.D., M.I., P.C., A.O., M.H., L.V.H.); Division of Cardiology, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada (G.G.); Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka, Suita, Osaka, Japan (T.M., A.B.); and Taisho Pharmaceutical Co., Ltd., Takata 3-Chome, Toshimaku, Tokyo, Japan (K.T.)

Received April 29, 2004; accepted July 1, 2004

ABSTRACT

SEA0400 [2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] has recently been described as a potent and selective inhibitor of Na\(^{+}\)-Ca\(^{2+}\) exchange in cardiac, neuronal, and renal preparations. The inhibitory effects of SEA0400 were investigated on the cloned cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger, NCX1.1, expressed in Xenopus laevis oocytes to gain insight into its inhibitory mechanism. Na\(^{+}\)-Ca\(^{2+}\) exchange currents were measured using the giant excised patch technique using conditions to evaluate both inward and outward currents. SEA0400 inhibited outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents with high affinity (IC\(_{50}\) = 78 ± 15 and 23 ± 4 nM for peak and steady-state currents, respectively). Considerably less inhibitory potency (i.e., micromolar) was observed for inward currents. The inhibitory profile was re-examined after proteolytic treatment of excised patches with \(\alpha\)-chymotrypsin, a procedure that eliminates ionic regulatory mechanisms. After this treatment, an IC\(_{50}\) value of 1.2 ± 0.6 \(\mu\)M was estimated for outward currents, whereas inward currents became almost insensitive to SEA0400. The inhibitory effects of SEA0400 on outward exchange currents were evident at both high and low concentrations of regulatory Ca\(^{2+}\), although distinct features were noted. SEA0400 accelerated the inactivation rate of outward currents. Based on paired pulse experiments, SEA0400 altered the recovery of exchangers from the Na\(^{+}\)-dependent inactive state, particularly at higher regulatory Ca\(^{2+}\) concentrations. Finally, the inhibitory potency of SEA0400 was strongly dependent on the intracellular Na\(^{+}\) concentration. Our data confirm that SEA0400 is the most potent inhibitor of the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger described to date and provide a reasonable explanation for its apparent transport mode selectivity.

In cardiac muscle, Na\(^{+}\)-Ca\(^{2+}\) exchange is the primary mechanism for trans-sarcolemmal Ca\(^{2+}\) removal, a process essential for muscle relaxation (Blaustein and Lederer, 1999; Hryshko, 2002). In general, the Na\(^{+}\)-Ca\(^{2+}\) exchanger removes the same quantity of Ca\(^{2+}\) that enters the myocyte through L-type Ca\(^{2+}\) channels on a beat-to-beat basis (Bers, 2000). As a reversible ion counter-transporter, the exchanger can also serve as a Ca\(^{2+}\) entry mechanism and can potentially contribute to Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (Leblanc and Hume, 1990; Bers, 2000). During ischemia-reperfusion, reverse mode Na\(^{+}\)-Ca\(^{2+}\) exchange is established as an important contributor to cellular injury (Mochizuki and Jiang, 1998; Seki et al., 2002). The general sequelae associated with ischemia-reperfusion injury are as follows: intracellular acidosis occurs during anaerobic metabolism associated with ischemia; upon reperfusion, the Na\(^{+}\)-H\(^{+}\) exchanger (NHE) alleviates the acidosis with concomitant increases in intracellular Na\(^{+}\) ([Na\(^{+}\)])

ABBREVIATIONS: NHE, Na\(^{+}\)-H\(^{+}\) exchanger; [Ca\(^{2+}\)], intracellular Ca\(^{2+}\); KB-R7943, 2-[2-[(4-nitrobenzyl)oxy]phenyl][ethyl]isothiourea methanesulfonate; SEA0400, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline; MES, 4-morpholineethanesulfonic acid; TEA, tetraethylammonium; MOPS, 4-morpholinepropanesulfonic acid; \(\lambda\), rate of current decay; F\(_{SS}\), extent of current inactivation.
favors the occurrence of reverse mode; and intracellular Ca\(^{2+}\) overload and/or cell death. Under these conditions, inhibition of reverse mode Na\(^+\)-Ca\(^{2+}\) exchange represents a promising approach toward attenuating ischemia-reperfusion injury (Nakamura et al., 1998; Ladilov et al., 1999; Mukai et al., 2000; Schafer et al., 2001; Shigekawa and Iwamoto, 2001; Inserte et al., 2002; Seki et al., 2002).

The critical roles of Na\(^+\)-Ca\(^{2+}\) exchange in both physiological and pathophysiological cardiac function make it an attractive target for pharmacological manipulation (Kusukata et al., 1993; Winslow et al., 1999; Shigekawa and Iwamoto, 2001; Sipido et al., 2002; Pogwizd, 2003). Two agents have recently been described as potent Na\(^+\)-Ca\(^{2+}\) exchange inhibitors. The first compound, 2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate (KB-R7943), was described in 1996 and inhibits the exchanger with micromolar potency in different assay systems (Iwamoto et al., 1996; Watano et al., 1996). KB-R7943 also offers protection against arrhythmogenesis and contractile dysfunction associated with ischemia-reperfusion injury (Ladilov et al., 1999; Mukai et al., 2000; Elias et al., 2001; Schafer et al., 2001; Seki et al., 2002). Mechanistically, most studies have demonstrated that KB-R7943 preferentially inhibits the reverse mode of Na\(^+\)-Ca\(^{2+}\) exchange (Iwamoto et al., 1996; Watano et al., 1996; Elias et al., 2001), although another study has reported that the extent of inhibition is independent of transport direction under bidirectional ionic conditions (Kimura et al., 1999).

However, KB-R7943 also inhibits several unrelated transport systems including Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels, complicating the interpretation of its cardioprotective actions (Sobolovsky and Khodorov, 1999; Arakawa et al., 2000; Pintado et al., 2000). Exemplifying this point, KB-R7943 inhibits Ca\(^{2+}\) transients in myotubes obtained from NCX1.1 knockout mice, where its effects are clearly distinct from any actions on the Na\(^+\)-Ca\(^{2+}\) exchanger (Reuter et al., 2002).

A second inhibitor of the Na\(^+\)-Ca\(^{2+}\) exchanger has recently been described with greatly increased potency (i.e., nanomolar range) compared with KB-R7943 ([Ca\(^{2+}\)]\(_{1}\)). Two agents have been recently been described with greatly increased potency (i.e., nanomolar range) compared with KB-R7943 ([Ca\(^{2+}\)]\(_{1}\)) compared with KB-R7943 (Matsuda et al., 2001; Seki et al., 2002). Complementary DNA encoding NCX1.1, residing in pBluescript II SK\(\ (+\) (Stratagene, La Jolla, CA), was linearized with HindIII (New England Biolabs, Beverly, MA), and cRNA was synthesized using T3 mMessage mMachine in vitro transcription kits (Ambion, Austin, TX) according to the manufacturer’s instructions. Following injection with 2–3 ng of cRNA encoding NCX1.1, oocytes were maintained at 18°C, and electrophysiological measurements were obtained from days 3 to 6 postinjection.

Materials and Methods

Preparation of X. laevis Oocytes and Synthesis of NCX1.1 cRNA. X. laevis oocytes were prepared and stored as previously described (Elias et al., 2001). Complementary DNA encoding NCX1.1 was linearized with HindIII (New England Biolabs, Beverly, MA), and cRNA was synthesized using T3 mMessage mMachine in vitro transcription kits (Ambion, Austin, TX) according to the manufacturer’s instructions. Following injection with 2–3 ng of cRNA encoding NCX1.1, oocytes were maintained at 18°C, and electrophysiological measurements were obtained from days 3 to 6 postinjection.

Measurement of Na\(^+\)-Ca\(^{2+}\) Exchange Activity. Na\(^+\)-Ca\(^{2+}\) exchange current measurements were obtained using the giant excised patch-clamp technique (Hilgemann, 1989), as previously described (Elias et al., 2001). Borosilicate glass pipettes were pulled and polished to a final diameter of 20 to 30 μm and coated with a Parafilm/mineral oil mixture to enhance patch stability and reduce electrical noise. The vitellin layer was removed by dissection, and oocytes were placed in a solution containing: 100 mM KOH, 100 mM MES, 20 mM HEPES, 5 mM EGTA, and 5 to 10 mM MgCl\(_2\), pH 7.0 at room temperature (with MES). Gigaohm seals were formed by suction and membrane patches (inside-out configuration) were excised by progressive movements of the pipette tip. Rapid solution changes were accomplished using a computer-controlled, 20-channel solution switcher. Axon Instruments Inc. (Union City, CA) hardware (Axopatch 200A) and software (AxoTape) were used for data acquisition and analysis, and Origin software was used for curve-fitting (e.g., determination of IC\(_{50}\) values) and statistical analyses. A holding potential of 0 mV was employed for all current measurements. For outward Na\(^+\)-Ca\(^{2+}\) exchange current measurements, pipette (i.e., extracellular) solutions contained: 100 mM N-methyl-d-glucamine-MES, 30 mM HEPES, 30 mM TEA-OH, 16 mM sulfamic acid, 8.0 mM CaCO\(_3\), 6 mM KOH, 0.25 mM ouabain, 0.1 mM niflumic acid, and 0.1 mM flufenamic acid, pH 7.0 at room temperature (with MES). Outward currents were elicited by switching from Li\(^+\)-based bath solutions containing: 100 mM [Na\(^+\)-Li\(^+\)]-aspartate, 20 mM CaSO\(_4\), 20 mM MOPS, 20 mM TEA-OH, 10 mM EGTA, 0 to 9.91 mM CaCO\(_3\), and 1.0 to 1.5 mM Mg(OH)\(_2\), pH 7.0 at 30°C (with MES) or LiOH. Free Mg\(^{2+}\) and Ca\(^{2+}\) were adjusted to yield free concentrations of 1.0 and 0 to 30 μM, respectively, using MAXC software (Bers et al., 1994). For inward Na\(^+\)-Ca\(^{2+}\) exchange current measurements, pipettes contained: 100 mM Na-MES, 20 mM CaSO\(_4\), 20 mM TEA-OH, 10 mM EGTA, 10 mM HEPES, 8 mM sulfamic acid, 4 mM Mg(OH)\(_2\), 0.25 mM ouabain, 0.1 mM niflumic acid, and 0.1 mM flufenamic acid, pH 7.0 at 30°C (with MES). Inward currents were activated by switching between Ca\(^{2+}\)-free and -containing, Li\(^+\)-based bath solutions, described above. For combined inward-outward current measurements, pipettes contained: 100 mM Na-MES, 20 mM CaSO\(_4\), 20 mM HEPES, 20 mM TEA-OH, 4 mM sulfamic acid, 2 mM CaCO\(_3\), 0.25 mM ouabain, 0.1 mM niflumic acid, and 0.1 mM flufenamic acid, pH 7.0 at 30°C (with MES). Outward and inward currents were activated using the same solutions as those for initiating pure outward and pure inward currents, described above. Under Results, only the Na\(^+\) and Ca\(^{2+}\) concentrations of experimental solutions are described, for brevity. All experiments were conducted at 30 ± 1°C.

Results

Figure 1A shows a representative trace of Na\(^+\)-Ca\(^{2+}\) exchange currents obtained from a single patch, in the absence and presence of 100 nM SEA0400. For recordings of this type, pipettes contained 2 mM Ca\(^{2+}\) and 100 mM Na\(^+\). Outward

Inhibition of Cardiac Na\(^+\)-Ca\(^{2+}\) Exchange by SEA0400 749

Downloaded from jpet.aspetjournals.org at ASIPE Journals on April 2, 2017
Na\(^+-\)Ca\(^{2+}\) exchange currents were generated by applying 100 mM Na\(^+\) to the cytoplasmic surface of the patch in the presence of 1 mM regulatory Ca\(^{2+}\) i. Inward currents were generated by applying 10 \(\mu\)M Ca\(^{2+}\) to the cytoplasmic surface, in the absence of cytoplasmic Na\(^+\). Typical characteristics of outward and inward currents were observed: outward currents peak and then progressively decay due to the Na\(^+-\)dependent \((I_1)\) inactivation process, whereas inward currents do not show inactivation (Elias et al., 2001). Application of 100 nM SEA0400 led to a marked reduction in the magnitude of outward currents, whereas inward currents were considerably less affected. Pooled data (five patches) treated with 300 nM SEA0400 are shown in Fig. 1B. Here, 300 nM SEA0400 reduced peak and steady-state outward currents by 61 ± 1% and 87 ± 4%, respectively, whereas inward currents were inhibited by 29 ± 4%. Thus, SEA0400 exhibits a preferential inhibition of outward Na\(^+-\)Ca\(^{2+}\) exchange currents, similar to the transport mode selectivity observed with KB-R7943 under these ionic conditions (Elias et al., 2001).

We then evaluated the inhibitory potency of SEA0400 on Na\(^+-\)Ca\(^{2+}\) exchange currents, using conditions where only a single transport mode could occur (i.e., unidirectional ionic conditions). Figure 2A shows a representative trace for outward Na\(^+-\)Ca\(^{2+}\) exchange currents, activated by applying 100 mM Na\(^+\) to the cytoplasmic surface in the continuous presence of 1 mM regulatory Ca\(^{2+}\) i. Pipette Ca\(^{2+}\) was 8 mM and Na\(^+\) i was zero. Inhibitory effects of 0.1 and 1 \(\mu\)M SEA0400 are shown, where SEA0400 was applied to the cytoplasmic surface. SEA0400 potently inhibited both peak and steady-state outward currents. Figure 2B illustrates pooled data over a range of SEA0400 concentrations. These data were obtained by sequential addition of increasing SEA0400 concentrations. As can be seen in both typical traces and pooled data, the inhibitory potency of SEA0400 was greater for steady-state currents (IC\(_{50}\) = 23 ± 4 nM) than for peak currents (IC\(_{50}\) = 78 ± 15 nM). Furthermore, SEA0400 also increased the rate (\(\lambda\)) and extent of current.
To gain insight into the inhibitory mechanism of SEA0400, we conducted a series of experiments to evaluate its interaction with ionic regulatory mechanisms. In the first series, we examined the inhibitory effects of SEA0400 in membrane patches that were treated with 1 mg/ml α-chymotrypsin for 1 to 2 min. This procedure eliminates both Na\(^{+}\)-dependent (I\(_{1}\)) and Ca\(^{2+}\)-dependent (I\(_{2}\)) regulation and leaves the exchanger in a fully activated state (Hilgemann, 1990). Following this treatment, control outward current recordings exhibit a relatively square wave form, and cytoplasmic regulatory Ca\(^{2+}\) is no longer required for current generation. Figure 3A shows representative current traces obtained in the absence and presence of 0.1 and 1.0 μM SEA0400. The extent of current inhibition by SEA0400 was greatly reduced in deregulated as compared with intact exchangers (Fig. 2). Pooled data are shown in Fig. 3B, where an IC\(_{50}\) of 1.2 ± 0.6 μM was estimated. We could not obtain a complete inhibitory profile of SEA0400 because solutions became turbid at 3 μM SEA0400; therefore, we employed this as our highest concentration. These results show that the full inhibitory effects of SEA0400 require intact ionic regulatory mechanisms and/or some structural element that was altered by proteolytic treatment with α-chymotrypsin.

The alteration of inhibitory potency of SEA0400 in deregulated exchangers prompted us to examine the interactions between regulatory Ca\(^{2+}\)- and SEA0400 on the inhibitory process. Figure 4A shows the influence of two regulatory Ca\(^{2+}\) concentrations on outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents, in the absence and presence of 1 μM SEA0400. In control recordings, the prominent influence of regulatory Ca\(^{2+}\)- on current inactivation is evident. At high Ca\(^{2+}\) (e.g., 10 μM), Na\(^{+}\)-dependent (I\(_{1}\)) inactivation is largely eliminated, and the current wave form adopts a square appearance. In contrast, Na\(^{+}\)-dependent inactivation is prominent at 1 μM regulatory Ca\(^{2+}\). This interaction between the I\(_{1}\) and I\(_{2}\) inactivation mechanisms has been well described (Hilgemann et al., 1992a,b; Matsuoka et al., 1995). In the presence of 1 μM SEA0400, inhibition of Na\(^{+}\)-Ca\(^{2+}\) exchange currents is evident at both concentrations of regulatory Ca\(^{2+}\). However, the extent of current inhibition is reduced at 10 μM Ca\(^{2+}\), particularly for peak currents. Moreover, the elimination of I\(_{1}\) inactivation by high regulatory Ca\(^{2+}\) is no longer evident in the presence of SEA0400. This effect is illustrated for pooled data in Fig. 4B for 0.1 and 1.0 μM SEA0400. In Fig. 4C, the influence of regulatory Ca\(^{2+}\) is shown on F\(_{ss}\), the ratio of steady-state to peak currents. In control recordings, 10 μM Ca\(^{2+}\) increases F\(_{ss}\), whereas this was not observed in the presence of SEA0400. This effect of SEA0400 occurred even at the lowest concentrations examined (i.e., 30 nM; data not shown).

We then examined how SEA0400 influenced the recovery from Na\(^{+}\)-dependent (I\(_{1}\)) inactivation to test the hypothesis that SEA0400 might stabilize the I\(_{1}\) inactive state. Paired pulse experiments were conducted to determine the rate of recovery from I\(_{1}\) inactivation. Figure 5A illustrates representative current tracings from experiments of this type where outward currents were activated by applying 100 mM Na\(^{+}\) in the presence of 1 μM (upper tracings) or 10 μM regulatory Ca\(^{2+}\) (lower tracings). In the traces shown, a 6.4-s interval was introduced between paired outward current pulses in the absence and presence of 0.1 μM SEA0400. We examined a range of time intervals between paired pulses to obtain information on the rate of recovery. The two graphs in Fig. 5B illustrate how SEA0400 reduced the rate of recovery for peak outward currents at regulatory Ca\(^{2+}\) levels of 3 and 10 μM. Finally, by examining this relationship over a range of regulatory Ca\(^{2+}\) concentrations (i.e., 0.3–10 μM), we were able to identify a clear influence of SEA0400 on this recovery process. Figure 5C shows pooled results obtained from 361 paired pulse comparisons made in 47 patches. In summary, for control patches, increasing regulatory Ca\(^{2+}\) leads to a progressive increase in the rate of recovery from I\(_{1}\) inactiva-

---

**Fig. 3.** A, typical outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents from an excised membrane patch that was deregulated by 1 mg/ml α-chymotrypsin. Currents are shown in the absence and presence of SEA0400 (0.1 and 1.0 μM). B, pooled results (each point represents mean ± S.E obtained from 3–12 patches).
tion. However, the addition of SEA0400 greatly attenuates this effect of regulatory Ca\textsuperscript{2+}/H\textsuperscript{i}, with the most prominent differences observed at the higher levels of regulatory Ca\textsuperscript{2+}/H\textsuperscript{i}. Overall, SEA0400 stabilizes I\textsubscript{1} inactivation in a Ca\textsuperscript{2+}/H\textsuperscript{i}-dependent manner.

Because our data suggested that Na\textsuperscript{+}/H\textsuperscript{i} plays an important role in the inhibitory effects of SEA0400, we tested this directly by comparing its inhibitory profile at two different Na\textsuperscript{+}/H\textsuperscript{i} concentrations. Pooled data are shown in Fig. 6 for outward currents activated by 25 or 100 mM Na\textsuperscript{+}/H\textsuperscript{i} in the continuous presence of 1 \textmu M regulatory Ca\textsuperscript{2+}/H\textsuperscript{i}. Note that the data at 100 mM Na\textsuperscript{+}/H\textsuperscript{i} are identical to that presented in Fig. 2B. The results for peak currents are shown in Fig. 6A and for steady-state currents in Fig. 6B. Note that the potency and efficacy of SEA0400-mediated inhibition of outward currents were greatly reduced at the lower [Na\textsuperscript{+}/H\textsuperscript{i}]. Specifically, when outward currents were activated by 25 mM Na\textsuperscript{+}/H\textsuperscript{i}, we obtained IC\textsubscript{50} values of 500 and 250 nM for peak and steady-state currents, respectively. By comparison, these values were 78 \pm 15 and 23 \pm 4 nM for peak and steady-state currents, respectively, when activated by 100 mM Na\textsuperscript{+}/H\textsuperscript{i}. These data provide compelling support for the idea that the inhibitory effects of SEA0400 are mediated by a Na\textsuperscript{+}/H\textsuperscript{i}-dependent process.

To gain further insight into the Na\textsuperscript{+}/H\textsuperscript{i} dependence of SEA0400 inhibition, the following protocol was examined in the presence and absence of 300 nM SEA0400. Under conditions where both inward and outward currents could be induced, we first generated an outward current, immediately followed by an inward current, and then a second outward was induced (Fig. 7). In the control recordings (Fig. 7A), the rate constant for development of inward current was 0.31 \pm 0.04 s\textsuperscript{-1} (mean \pm S.E., n = 4), primarily reflecting exit from the I\textsubscript{1} inactive state. When SEA0400 was present (Fig. 7B), this rate constant decreased to 0.13 \pm 0.01 s\textsuperscript{-1} (mean \pm S.E., n = 4). Clearly, SEA0400 slows the rate of inward current development, presumably reflecting its enhancement or stabilization of the I\textsubscript{1} inactive state. Moreover, the requirement for Na\textsuperscript{+}/H\textsuperscript{i} in this process is evident when viewing the second outward current pulse. In control recordings, the ratio of peak currents for pulse 2/pulse 1 was 1.03 \pm 0.01 (mean \pm S.E., n = 4), indicating that exchanger availability was nearly identical before and after the intervening inward current. In contrast, after SEA0400 treatment, this ratio increased to 3.07 \pm 0.39 (mean \pm S.E., n = 4). This result indicates that during inward currents, where Na\textsuperscript{+}/H\textsuperscript{i} is absent, the inhibitory effects of SEA0400 are diminished or lost. The augmentation of the subsequent outward current (pulse 2) reflects the increased availability of active exchangers.

Figure 8A shows the effects of SEA0400 on inward currents for an intact patch and one deregulated by limited proteolysis with 1 mg/ml \alpha-chymotrypsin. Currents were ac-
tivated by applying 10 μM Ca\(^{2+}\) to the cytoplasmic surface in exchange for 100 mM pipette Na\(^{+}\). Notably, the inhibitory potency and efficacy of SEA0400 are considerably less for inward currents compared with outward currents. Following limited proteolysis, we observed a decreased extent of inhibition (Fig. 8B), although this did not achieve statistical significance. For example, at 300 nM SEA0400, inward currents were inhibited by 23 ± 6% (n = 6) and 11 ± 2% (n = 7) for regulated and deregulated patches, respectively (p = 0.067). For intact patches, we estimated an IC\(_{50}\) of ∼3 μM, although a complete concentration-inhibition relationship could not be obtained due to the limited drug solubility. After deregulation, drug potency appeared to increase, although the extent of inhibition was reduced.

We then compared the inhibitory effects of SEA0400 on inward currents activated by different [Ca\(^{2+}\)]\(_i\) (Fig. 8C). Inward currents were activated by 3 or 10 μM Ca\(^{2+}\)\(_i\). Over this ∼3-fold concentration range of activating Ca\(^{2+}\)\(_i\), there was no apparent difference in inhibitory effects of 0.1 and 3 μM of SEA0400. Overall, inward exchange currents are considerably less sensitive to SEA0400 compared with outward currents, similar to our previous observations with KB-R7943 (Elias et al., 2001). Moreover, there is no apparent difference in the inhibitory effects of SEA0400 on inward currents at lower [Ca\(^{2+}\)]\(_i\).

**Discussion**

We characterized the inhibitory effects of the novel Na\(^{+}\)-Ca\(^{2+}\) exchange inhibitor, SEA0400, on the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger. Although SEA0400 interacts with other transport systems (Reuter et al., 2002), its potent inhibition of the Na\(^{+}\)-Ca\(^{2+}\) exchanger warrants further investigation of its mechanism of action. We employed the giant excised patch-clamp technique and studied the cloned Na\(^{+}\)-Ca\(^{2+}\) exchanger, NCX1.1, expressed in X. laevis oocytes. We showed that SEA0400 potently inhibits Na\(^{+}\)-Ca\(^{2+}\) exchange activity, particularly for outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents. We identified prominent interactions of SEA0400 with the ionic regulatory mechanisms of NCX1.1. These data provide an explanation for the apparent mode selectivity of SEA0400 and highlight its potential utility for treating ischemia reperfusion injury.

**Transport Mode Selectivity.** Under conditions where inward and outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents were measured in the same membrane patch, SEA0400 exerted a much greater degree of inhibition for outward currents compared with inward currents. For example, when 300 nM SEA0400 was applied to patches under such conditions, steady-state outward currents were inhibited by ∼90% where inward currents were inhibited by ∼30%. Under con-
ditions where only a single transport mode was examined (i.e., unidirectional conditions), this preferential inhibition of outward currents was retained. Here, an IC\textsubscript{50} of 23 nM was obtained for outward steady-state currents, whereas the IC\textsubscript{50} for inward currents could only be estimated (\textsuperscript{H11011}8\textsuperscript{H9262}M) due to the limited solubility of SEA0400. This preferential inhibition of the reverse mode of Na\textsuperscript{+}/H\textsuperscript{+}-Ca\textsuperscript{2+} exchange by SEA0400 is similar to previous reports for KB-R7943 (Iwamoto et al., 1996; Watano et al., 1996; Elias et al., 2001). In contrast, under “bidirectional” ionic conditions, where membrane voltage controls the transport mode under static ionic conditions, KB-R7943 was reported to be equipo-
tent for either transport direction (Kimura et al., 1999). We have previously suggested that the basis for this discrepancy resides in the different experimental approaches taken (Elias et al., 2001). Specifically, we postulate that both SEA0400 and KB-R7943 favor specific conformations of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, and binding of either compound to this conformation leads to an inactive complex. In this scenario, the enzyme-inhibitor complex is unavailable for transport in any transport direction. However, the proportion of exchangers populating a specific conformation is determined by the prevailing ionic conditions and, therefore, differs considerably under different transport modes (Omelchenko and Hryshko, 1996). Thus, under different ionic conditions, the potency of these two compounds to inhibit Na\textsuperscript{+}/H\textsuperscript{+}-Ca\textsuperscript{2+} exchange will change dramatically, yielding an “apparent” transport mode selectivity. In all of our experiments, SEA0400 was applied to the cytoplasmic surface of the patch. However, similar transport mode selectivity has been observed upon extracellular application of SEA0400 to NCX1 transfected cells (Iwamoto et al., 2004), suggesting a common mechanism of action.

Under our experimental conditions, the fractional distribution of exchangers within a specific transport conformation differs when recording pure inward, pure outward, or combined inward and outward currents. This is readily apparent when considering Na\textsuperscript{+}-dependent (I\textsubscript{1}) inactivation, a prominent characteristic of outward currents that is completely absent for inward currents. If SEA0400 targets the Na\textsuperscript{+}-dependent inactive state or some prominent precursor, its potency would be greatest for outward currents. Because this state(s) is less populated for inward currents, the potency of SEA0400 to block inward currents would be reduced. This

---

Fig. 6. Influence of Na\textsubscript{i} concentration on the inhibitory potency of SEA0400 for peak (A) and steady-state (B) outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents. Data points show % inhibition of peak and steady-state currents in mean ± S.E. format obtained from 5 to 7 and 3 to 13 individual patches for 25 and 100 mM Na\textsuperscript{+}\textsubscript{i}, respectively. Solid lines represent the best least squares fit.

Fig. 7. A three-pulse protocol was used to evaluate the effects of SEA0400 on exchanger recovery kinetics. Two outward currents were examined with an intervening inward current between these pulses. Representative tracings are shown in the absence (upper) and presence (lower) of 0.3 \textmu M SEA0400.
differs from voltage ramps experiments conducted under static ionic conditions. Here, the distribution of exchanger states would be pre-established by the prevailing ionic conditions prior to the voltage ramps. Therefore, the targets for drug binding are similar, and no transport mode selectivity would be anticipated, as was demonstrated (Kimura et al., 1999). Moreover, the intermediate potency (between unidirectional inward and outward transport modes) observed under bidirectional conditions is predicted by this scenario. Notably, neither giant excised patches nor whole-cell voltage-clamp experiments reflect the physiological operation of the Na$^{+}$/H$^{+}$-Ca$^{2+}$/H$^{+}$ exchanger. It remains our contention that the potency of these inhibitors varies depending upon experimental conditions, with the greatest potency observed for conditions approaching the unidirectional outward transport mode.

**Inhibitory Mechanism of SEA0400.** The cardiac Na$^{+}$/Ca$^{2+}$ exchanger exhibits two prominent auto-regulatory mechanisms referred to as Na$^{+}$-dependent (I$_{1}$ inactivation) and Ca$^{2+}$-dependent (I$_{2}$ regulation) (Hilgemann et al., 1992a,b). Both of these allosteric mechanisms prominently alter the activity of Na$^{+}$/Ca$^{2+}$ exchangers, presumably by altering the fraction of active transporters, and both mechanisms are readily apparent when examining outward Na$^{+}$/Ca$^{2+}$ exchange currents. Upon application of cytoplasmic Na$^{+}$, outward currents peak and then gradually decay, reflecting entry into the Na$^{+}$-dependent or I$_{1}$ inactive state (Hilgemann et al., 1992b). In the absence of cytoplasmic regulatory Ca$^{2+}$, exchangers appear to reside in the I$_{2}$ inactive state and generate little to no outward current despite ionic gradients favoring transport (Hilgemann et al., 1992a). As regulatory Ca$^{2+}$ is progressively increased, exchange activity increases despite the fact that this intervention decreases the electrochemical gradients favoring exchange.

We show a prominent interaction between SEA0400 and the intrinsic ionic regulatory mechanisms of NCX1.1. Deregulation of the exchanger with α-chymotrypsin eliminates both I$_{1}$ and I$_{2}$ regulation and leaves the exchanger in a fully active state (Hilgemann, 1990). This intervention produced a substantial reduction in the inhibitory effects of SEA0400 (Figs. 3 and 8, respectively). For example, outward currents became approximately 50-fold less sensitive and inward currents were nearly insensitive to SEA0400 after proteolysis. At present, we cannot exclude the possibility that proteolytic treatment affects structural domains involved in SEA0400 binding and/or inhibition because our study does not provide insight into the specific site of action of SEA0400. Nevertheless, several lines of evidence indicate a direct functional involvement of ionic regulatory mechanisms in the inhibitory effects of SEA0400.

The interaction between SEA0400 and ionic regulation is apparent from the kinetic characteristics of control versus
drug-treated outward currents. In the presence of SEA0400, λ and the extent of experimental inactivation were approximately doubled for outward currents (Fig. 2). Alterations in both of these parameters suggest a pronounced enhancement of I1 inactivation. SEA0400 also impaired the alleviation of I1 inactivation that routinely occurs in the presence of elevated cytosolic Ca2+ (Hilgemann et al., 1992a,b). Pronounced I1 inactivation was still apparent at high regulatory Ca2+ levels, whereas control currents normally adopt a square appearance (Fig. 4). Regulatory Ca2+ normally accelerates the rate of recovery from I1 inactivation, and this effect was greatly reduced in the presence of SEA0400 (Fig. 5). In Fig. 7, we showed a prominent recovery of the exchanger population when outward currents are separated by an intervening inward current. That is, when Na+ is absent, the inhibitory effects of SEA0400 are greatly reduced. All of these results strongly support the notion that the major effect of SEA0400 is facilitation of I1 inactivation.

Finally, the most compelling evidence favoring an involvement of I1 inactivation in the inhibitory mechanism of SEA0400 is the observation that its inhibitory potency is dramatically altered by the concentration of Na+, used to activate outward currents. Specifically, the potency of SEA0400 decreased by ~10-fold for a 4-fold decrease in Na+. Notably, the extent of I1 inactivation is also directly controlled by the [Na+]i (Hilgemann et al., 1992b). Based on all of these data, we propose that SEA0400 directly facilitates I1 inactivation of the cardiac Na+/Ca2+ exchanger. At present, we cannot distinguish whether this occurs by directly targeting the I1 state or a transport state from which I1 inactivation originates. Speculatively, however, the most likely candidate state would be the three Na+-loaded configuration of the exchanger with intracellularly oriented ion binding sites (Omelchenko and Hryshko, 1996). We cannot and do not exclude the possibility that SEA0400 has additional effects beyond that of I1 stabilization. In particular, this agent exerts low potency inhibition of inward currents as well as for outward currents from deregulated exchangers. Peak outward currents are also inhibited despite the fact that these are thought to reflect exchange activity prior to the development of I1. Arguably, however, a pronounced enhancement of I1 inactivation could affect peak currents if this developed at a sufficient rate to overlap with current development. Given our current temporal resolution, we cannot distinguish between these possibilities. Overall, the major inhibitory effects of SEA0400 require the presence of intracellular Na+ and generally follow the preponderance of the I1 inactive state. Similar conclusions have been reached based on the evaluation of mutant Na+/Ca2+ exchangers with altered I1 inactivation (Bouchard et al., 2004; Iwamoto et al., 2004).

**Relevance to Ischemia-Reperfusion Injury.** The most obvious application for Na+/Ca2+ exchange inhibitors relates to their potential as adjunctive therapy in ischemia-reperfusion injuries (Magee et al., 2003). Here, the major goal is to eliminate or reduce the Ca2+ overload associated with reperfusion. Interestingly, various NHE inhibitors have undergone clinical trials with the identical target of minimizing reperfusion-associated Ca2+ overload (Avkiran and Marber, 2002). Although experimental studies with NHE inhibitors have shown tremendous benefit, results from clinical trials have been considerably less positive (Theroux et al., 2000; Avkiran and Marber, 2002). In view of the preliminary experimental evidence showing similar or superior cardioprotective effects of NCX inhibition as compared with NHE inhibition (Matsumoto et al., 2002; Magee et al., 2003; Takahashi et al., 2003), we feel that the therapeutic potential of SEA0400 warrants further investigation.

Our study revealed several aspects of SEA0400 action that show great promise for this class of agent. First, SEA0400 should have minimal actions on physiological Na+/Ca2+ exchange function because [Na+]i, is typically very low under these conditions (e.g., ~10 mM). Under pathophysiological conditions, however, the potency of SEA0400 would progressively increase with increasing [Na+]i, as occurs during ischemia-reperfusion injury. Clearly, initial results for SEA0400 in the setting of ischemia-reperfusion injury strongly support its efficacy (Matsumoto et al., 2002; Magee et al., 2003; Takahashi et al., 2003), although all conclusions must be tempered by the demonstration that SEA0400 exerts additional effects beyond that of Na+/Ca2+ exchange inhibition (Reuter et al., 2002). Because the therapeutic goal would be to preferentially block reverse mode Na+/Ca2+ exchange activity, but not the requisite forward mode, this apparent mode selectivity of SEA0400 appears to be an optimal attribute worthy of further investigation for adjunctive or prophylactic use against ischemia-reperfusion injury.

**References**


Hilgemann DW (1990) Regulation and deregulation of cardiac Na+/Ca2+ exchange in giant excised membrane patch [[nature (Lond) 342]:242–245.


