0026-895X/04/6503-802–810$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:802–810, 2004

Vol. 65, No. 3
2914/1132903
Printed in U.S.A.

Effects of SEA0400 on Mutant NCX1.1 Na^+-Ca^{2+} Exchangers with Altered Ionic Regulation

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Received August 25; accepted November 26, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

SEA0400 (SEA) blocks cardiac and neuronal Na^+-Ca^{2+} exchange with the highest affinity of any known inhibitor, yet very little is known about its molecular mechanism of action. Previous data from our lab suggested that SEA stabilizes or modulates the transition of NCX1.1 exchangers into a Na^+_i-dependent (I_1) inactive state. To test this hypothesis, we examined the effects of SEA on mutant exchangers with altered ionic regulatory properties. With mutants where Na^+_i-dependent inactivation is absent, the effects of SEA were greatly reduced. Conversely, with mutants displaying accelerated Na^+_i-dependent inactivation, block of NCX1.1 by SEA was either enhanced or unchanged, depending upon the phenotype of the particular mutation. With mutant exchangers where Ca^{2+}_i-dependent (I_2) inactivation was suppressed, block of exchange currents by SEA was similar to that observed for wild-type NCX1.1. These data strongly support the involvement of I_1 inactivation in the inhibitory mechanism of NCX1.1 by SEA, whereas I_2 inactivation does not seem to serve an important role. The involvement of processes regulated by intracellular Na^+ in the inhibitory mechanism of SEA may prove to be particularly important when considering the potential cardioprotective effects of this agent.

In addition to cotransporting Na^+ and Ca^{2+}, the cardiac plasmalemmal Na^+-Ca^{2+} exchanger (NCX1.1) is autoregulated by the levels of intracellular Na^+ and Ca^{2+} (Hilgemann, 1990; Matsuoka et al., 1995, 1997; Hryshko, 2002), and this regulation may be crucial to exchanger function under both physiological and pathophysiological conditions. Na^+_i-dependent or I_1 inactivation is thought to originate from the three Na^+-loaded state of the exchanger (E_{3ni}), with ion binding sites oriented to the cytoplasmic side of the membrane (Hilgemann et al., 1992b). The basis for this notion is that the extent of I_1 inactivation and the magnitude of outward Na^+-Ca^{2+} exchange currents share a similar Na^+_i dependence. Electrophysiologically, I_1 inactivation results in an exponential decline of current to a steady-state level, analogous to the time-dependent inactivation observed with many voltage-gated ion channels. I_1 inactivation is progressively alleviated by increments of cytosolic (or "regulatory") Ca^{2+}, with a $K_{1/2}$ (substrate concentration required for 50% reaction) of ~3 μM (Hilgemann et al., 1992b).

At low levels of regulatory Ca^{2+}, NCX1.1 can enter into a second inactive state, referred to as Ca^{2+}_i-dependent or I_2 inactivation (Hilgemann et al., 1992a). I_2 inactivation manifests mainly as changes in peak current levels and is relieved by intracellular Ca^{2+} with a $K_{1/2}$ of ~0.3 μM. Because the Ca^{2+}_i-dependent relief of I_2 occurs with an approximately 10-fold higher potency than Ca^{2+}_i-dependent relief of I_1, relatively small increments of cytoplasmic Ca^{2+} increase peak exchange current much more than the corresponding steady-state current component.

Structure-function analyses of NCX1.1 and CALX1.1, a Na^+-Ca^{2+} exchanger from *Drosophila melanogaster*, have provided important information delineating the protein domains involved in I_1 and I_2 inactivation (Matsuoka et al., 1997; Dyck et al., 1998). Regulation of Na^+_i-dependent inac-

This work was supported by operating grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Manitoba. L.V.H. was supported by a Canada Research Chair.

ABBREVIATIONS: SEA, SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); CALX1.1, Na^+-Ca^{2+} exchanger from *Drosophila melanogaster*; I_1, Na^+-dependent inactivation; I_2, Ca^{2+}-dependent inactivation; NCX1.1, wild-type cardiac sarcolemmal Na^+-Ca^{2+} exchanger; RT, room temperature; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea .

tivation is thought to involve amino acids 219 to 238 of NCX1.1, the so-called XIP region, located at the N terminus of the large cytoplasmic loop of the exchanger (Matsuoka et al., 1997). Mutations in this region have been shown to enhance or suppress I_1 inactivation. Similar results have been obtained with CALX1.1 bearing mutations at the analogous positions within its amino acid sequence (Dyck et al., 1998). A high-affinity Ca^{2+} binding site that modulates I_2 inactivation is also located in the large intracellular loop of NCX1.1 and CALX1.1 (Levitsky et al., 1994; Matsuoka et al., 1995; Dyck et al., 1998). Mutations in this region result in substantial alterations in the binding affinity of $^{45}Ca^{2+}$ to NCX1.1 fusion proteins and in the $Ca^{2+}{}_i$-sensitivity of Na^+-Ca^{2+} exchange currents recorded from both NCX1.1 and CALX1.1.

Until recently, there have been very few potent or selective inhibitors of cardiac sarcolemmal Na^+-Ca^{2+} exchange. However, newer studies (Matsuda et al., 2001; Tanaka et al., 2002; Lee et al., 2003) have shown that SEA0400 (SEA) inhibits NCX1.1 with high affinity ($IC_{50} \approx 23$ nM) and confers considerable protective effects against cardiovascular and cerebral ischemia. One potentially important characteristic of SEA action is that it may block Na^+-Ca^{2+} exchange in a mode-selective manner. That is, SEA preferentially inhibits outward versus inward Na^+-Ca^{2+} exchange currents when measured in giant excised membrane patches (Lee et al., 2003). In light of the known involvement of I_1 and I_2 inactivation for these distinct transport modes and their unique dependencies on the prevailing ionic conditions (Hilgemann et al., 1992a,b; Matsuoka et al., 1997), it is reasonable to speculate that SEA targets discrete transport or inactive states of the exchanger, thereby producing an apparent transport mode selectivity. Based on previous data, we have proposed that SEA stabilizes and/or modulates the transition of exchangers from the E_{3ni} state into the I_1 state, with little or no effect on I_2 (Lee et al., 2003). In this study, we have investigated the effects of SEA using mutant NCX1.1 exchangers that exhibit altered ionic regulatory properties. Three distinct categories of mutations were selected for study so that the blocking effects of SEA could be investigated with mutants displaying either enhanced or depressed I_1 inactivation, as well as with a mutant displaying suppressed I_2 inactivation (Matsuoka et al., 1997; Dyck et al., 1998; Maxwell et al., 1999). The data obtained show clearly that SEA blocks NCX1.1 by mimicking or promoting entry of Na^+-Ca^{2+} exchange molecules into the I_1, but not the I_2, inactive state.

Materials and Methods

Oocyte Preparation. Frogs (*Xenopus laevis*) were generally anesthetized in benzocaine for 30 min. Oocytes were removed, follicles teased apart, and oocytes transferred to buffer containing about 16,000 units of collagenase (type II; Worthington Biochemicals, Freehold, NJ), followed by incubation for 1.5 to 2 h at room temperature (RT) with gentle agitation. Oocytes were then defolliculated in 100 mM K_2HPO_4, pH 6.5, for 12 to 20 min with gentle agitation, after which stage V or VI oocytes were selected and maintained at 18°C until injection the following day. Complementary DNAs residing in pBluescript II SK+ (Stratagene, La Jolla, CA) encoding wild-type and mutant NCX1.1 were linearized with HindIII or SpeI and cRNAs synthesized using either mCAP mRNA (Stratagene) or mMessage mMachine (Ambion, Austin, TX) transcription kits. After injection with ~20 to 35 ng cRNA, oocytes were maintained at 18°C for up to 7 days.

Preparation of Mutant Na^+-Ca^{2+} Exchangers. Mutations were generated using the Sculptor in vitro mutagenesis kit (Amersham Biosciences) as described previously (Matsuoka et al., 1997; Dyck et al., 1998). Generally, two types of mutations were examined to determine the involvement of I_1 and I_2 inactivation in block of NCX1.1 currents by SEA. Single amino acid substitutions (F223E, K225Q, K229Q) and multiple amino acid deletions (Δ229–232, Δ680–685) were examined.

Electrophysiological Measurements. Electrophysiological measurements were obtained from days 3 to 7 after injection. Unidirectional outward (reverse) and inward (forward) Na^+-Ca^{2+} exchange current measurements were obtained using the giant excised patch-clamp technique (Hilgemann, 1989), as described previously (Dyck et al., 1998). Before use in voltage-clamp experiments, the vitellin layer of the oocytes was removed by dissection. Oocytes were then placed in a solution containing 100 mM KOH, 100 mM MES, 20 mM HEPES, 5 mM EGTA, 5 to 10 mM $MgCl_2$, pH 7.0, at RT with MES. Gigaohm seals were formed by suction, and inside-out membrane patches were excised by gentle movement of the patch pipette.

Solution changes were accomplished using a computer-controlled, 20-channel solution-switching device. For outward Na^+-Ca^{2+} exchange current measurements, pipette (extracellular) solutions contained 100 mM NMG-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 adjusted to 7.0 at RT with MES. Outward currents were elicited by rapidly switching from Li^+- to Na^+-based bath solutions containing 100 mM [Na^+ + Li^+]-aspartate, 20 mM CsOH, 20 mM MOPS, 20 mM tetraethyl ammonium hydroxide, 10 mM EGTA, 0 to 9.91 mM $CaCO_3$, and 1.0 to 1.5 mM $Mg(OH)_2$; pH adjusted to 7.0 at 30°C with MES or LiOH. For inward Na^+-Ca^{2+} exchange current measurements, the pipette (extracellular) solution contained 100 mM Na-MES, 20 mM CsOH, 20 mM tetraethyl ammonium hydroxide, 10 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 adjusted to 7.0 at RT with MES. Inward currents were activated by switching between Ca^{2+}-free and Ca^{2+}-containing, Li^+-based bath solutions, described above. For brevity, only the Na^+ and Ca^{2+} concentrations of experimental solutions are given under *Results*.

Axon Instruments (Union City, CA) hardware (Axopatch 200a) and software (Axotape) were used for data acquisition and analysis, and Origin software (OriginLab Corp, Northampton, MA) was used for curve-fitting and statistical analyses. Pooled data are presented as mean ± S.E.M. Two-tailed Student's t tests were used for comparison of unpaired data, and $P < 0.05$ was considered significant. Free Mg^{2+} and Ca^{2+} concentrations were calculated using MAXC software (Bers et al., 1994). All experiments were conducted at 30°C.

SEA was dissolved in dimethyl sulfoxide as 20 to 40 mM stocks and diluted directly into bath solutions. After each drug concentration change, at least 32 s were allowed to lapse before re-examining current levels. The concentration of dimethyl sulfoxide never exceeded 0.075% and was without effect on inward or outward Na^+-Ca^{2+} exchange current characteristics. SEA was generously provided by Taisho Pharmaceutical Company, Inc. (Tokyo, Japan).

Results

Outward Na^+-Ca^{2+} Exchange. Outward Na^+-Ca^{2+} exchange current (Ca^{2+} influx) was initiated by switching from a bath (intracellular) solution lacking Na^+ to a solution containing 100 mM Na^+ and variable amounts of Ca^{2+}. The pipette (extracellular) solution contained 8 mM Ca^{2+} and was Na^+-free. Outward Na^+-Ca^{2+} current measurements were typically obtained in the presence of 1 and 10 μM cytosolic Ca^{2+}. This enabled us to study exchange activity with different levels of I_1 and I_2 inactivation, in that the

levels of regulatory Ca^{2+} differentially modulate both of these processes (Hilgemann et al., 1992a,b).

Figure 1, A and B, illustrates the basic characteristics of block by SEA of outward Na^+-Ca^{2+} exchange currents mediated by NCX1.1. Addition of 0.1 μM SEA to the bath solution resulted in a significant inhibition of both peak and steady-state currents activated by 100 mM Na^+_i and 1 μM regulatory Ca^{2+}_i (Fig. 1A). Upon increasing the concentration of regulatory Ca^{2+}_i to 10 μM (Fig. 1B), there was a trend toward alleviation of block by SEA. This achieved statistical significance for peak currents [57 ± 6 (n = 12) versus 31 ± 8% inhibition (n = 8), p = 0.016] but not for steady-state currents [88 ± 5 (n = 10) versus 71 ± 7% inhibition (n = 8), p = 0.059] (Table 1). Previously reported IC_{50} values for inhibition of steady-state and peak outward currents by SEA were 23 and 78 nM, respectively, using the giant excised patch-clamp technique (Lee et al., 2003).

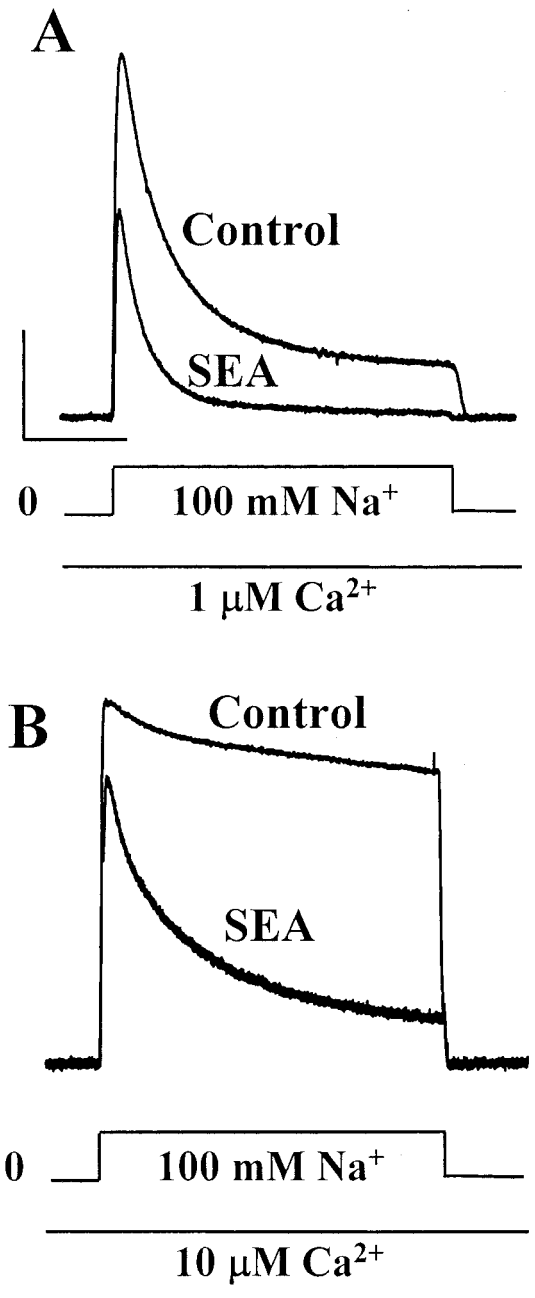

Fig. 1. Inhibition of wild-type NCX1.1 by SEA. A and B show the effect of 0.1 μM SEA on outward Na^+-Ca^{2+} exchange currents mediated by wild-type NCX1.1 in the presence of 1 μM (A) and 10 μM (B) regulatory Ca^{2+}_i on the cytoplasmic side of the patch. Currents were activated by applying 100 mM Na^+ to the cytoplasmic surface of the patch in exchange for 8 mM pipette Ca^{2+}. Scale bars for current (y-axis) and time (x-axis) are 50 pA and 10 s, respectively.

To test the hypothesis that SEA stabilizes or facilitates entry of exchanger molecules into I_1, we studied the effects of SEA on mutant exchangers with altered I_1 and I_2 inactivation profiles. The three distinct categories of mutant exchangers are shown in Fig. 2, A and B, and have been previously characterized (Matsuoka et al., 1997). Group 1 mutants (F223E and K225Q) exhibit an accelerated rate of inactivation (λ) of outward current and either an increase (F223E) or decrease (K225Q) in the extent of I_1 inactivation, as gauged by the ratio of steady-state to peak current (F_{ss}). A representative tracing is shown in Fig. 2A for F223E, which inactivates much faster and to a greater degree than wild-type NCX1.1. For group 2 mutations (K229Q and Δ229–232), the extent of I_1 inactivation is greatly reduced and the residual inactivation is accelerated compared with NCX1.1 (see also Table 3). This is illustrated in Fig. 2A for Δ229–232, where outward currents essentially comprise a square waveform in the absence of Na^+_i-dependent inactivation. This behavior is analogous to that observed for outward Na^+-Ca^{2+} exchange currents mediated by the wild-type exchanger in the presence of high concentrations of regulatory Ca^{2+}. Under these conditions, I_1 inactivation is greatly reduced, but the residual inactivation is accelerated (Hilgemann et al., 1992a). Finally, the Δ680–685 mutation shown in Fig. 2B is characterized by a combination of greatly reduced or absent I_1 and I_2 inactivation. Figure 2B shows overlapping outward currents traces at 0, 1, and 10 μM regulatory Ca^{2+}, illustrating the relative absence of both ionic regulatory processes. Note that the decrease in current magnitude at 10 μM regulatory Ca^{2+}_i primarily reflects competition between intracellular Na^+ and Ca^{2+} for the transport sites of the exchanger. Pooled data for peak and steady-state outward current, F_{ss} and λ under control conditions are given in Tables 1 to 3.

The first set of experiments was conducted on mutants lacking Na^+_i-dependent inactivation. We reasoned that if

TABLE 1
Effect of SEA (0.1 μM) on peak and steady-state outward currents mediated by NCX1.1 and mutant Na^+-Ca^{2+} exchangers

Exchanger Type	I_{peak}(% Inhibition)	n	I_{ss} (% Inhibition)	n
1 μM Ca^{2+}				
WT	57 ± 6	12	88 ± 5	10
F223E	54 ± 4	12	100[a]	11
K225Q	54 ± 8	7	73 ± 9	8
Δ229–232	7 ± 3*	11	20 ± 5*	11
K229Q	22 ± 4*	14	64 ± 6*	14
Δ680–685	32 ± 8*	8	78 ± 3	8
10 μM Ca^{2+}				
WT	31 ± 8	8	71 ± 7	8
F223E	57 ± 6*	10	87 ± 3*	10
K225Q	25 ± 4	7	71 ± 4	7
Δ229–232	9 ± 3*	8	21 ± 10*	8
K229Q	14 ± 5	13	36 ± 3*	13
Δ680–685	31 ± 6	14	65 ± 5	14

I_{peak}, peak outward current; I_{ss}, steady-state outward current; WT, wild-type NCX1.1
[a] Block is to zero current level (see text for details).
* $P < 0.05$ compared with wild type.

SEA blocked Na^+-Ca^{2+} exchange by interacting with the I_1 inactivation process, then block should be suppressed in mutants lacking this inactivation. Indeed, Fig. 3A shows that block of outward current by 0.1 μM SEA is nearly absent in Δ229–232. This weak effect of SEA was observed at both high and low levels of regulatory Ca^{2+}_i (i.e., 1 and 10 μM). Data obtained from the K229Q mutant are illustrated in Fig. 3B. As shown in the left at 1 μM regulatory Ca^{2+}_i, SEA inhibited both peak and steady-state outward currents. The degree of block was intermediate compared with NCX1.1 and Δ229–232, particularly for peak current. When re-examined at a higher regulatory Ca^{2+}_i concentration (Fig. 3B, right), SEA also inhibited currents to an intermediate level between NCX1.1 and Δ229–232. Notably, however, there was a considerable relief of SEA inhibition of steady-state currents in the presence of high regulatory Ca^{2+} (Table 1).

The results obtained with these mutants are of particular interest, because SEA induces I_1-like behavior for K229Q but is nearly without effect for the deletion mutant, Δ229–232. Both of these exchangers seem to lack a functional I_1 inactivation mechanism in the absence of drug (Matsuoka et al., 1997). Furthermore, for K229Q, a significant attenuation of steady-state current inhibition is observed after an increase in the concentration of regulatory Ca^{2+}_i from 1 to 10 μM, indicating that the I_1-like inactivation induced by SEA behaves similarly to the native I_1 inactivation process with respect to regulatory Ca^{2+}_i. Pooled data for inhibition of peak and steady-state current components by SEA in group 2 mutants are provided in Table 1.

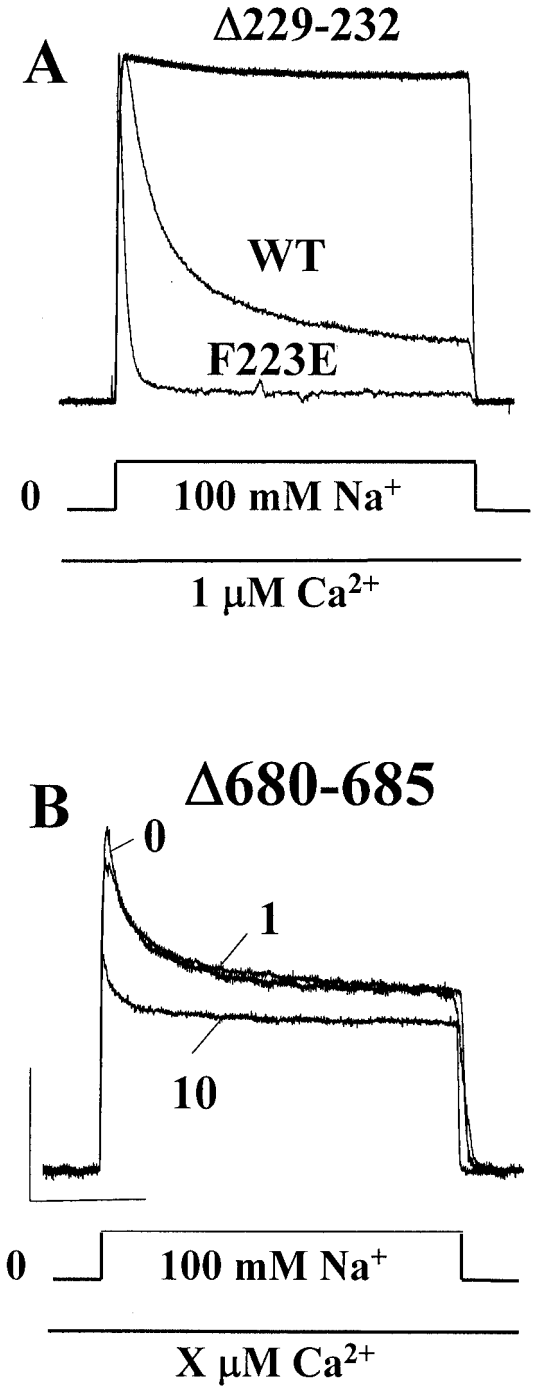

Fig. 2. Electrophysiological profile of wild-type and NCX1.1 mutants. A and B illustrate the three distinct types of mutant NCX1.1 exchangers used in this study to investigate the inhibitory mechanism of SEA. A shows two mutant exchangers with altered Na^+_i-dependent (I_1) inactivation properties. Compared with the NCX1.1 (WT), Na^+-dependent (I_1) inactivation is accelerated and enhanced for F223E and greatly reduced for Δ229–232. B shows the lack of Ca^{2+}_i-dependent regulation in the NCX1.1 mutant, Δ680–685. Currents were activated by 100 mM Na^+ at three different levels of regulatory Ca^{2+}_i, namely 0, 1, and 10 μM. Scale bars for current (y-axis) and time (x-axis) are 50 pA and 10 s, respectively.

TABLE 2
Effect of SEA (0.1 μM) on the extent of Na^+_i-dependent inactivation (F_{ss}) of wild-type and mutant Na^+-Ca^{2+} exchangers

Exchanger Type	Control		SEA	
	F_{ss}	n	F_{ss}	n
1 μM Ca^{2+}				
WT	0.17 ± 0.01	43	0.10 ± 0.03	9
F223E	0.07 ± 0.02*	18	0.06 ± 0.02	11
K225Q	0.24 ± 0.02*	13	0.16 ± 0.04	7
Δ229–232	0.84 ± 0.05*	12	0.69 ± 0.06*	11
K229Q	0.90 ± 0.03*	28	0.38 ± 0.05*	14
Δ680–685	0.63 ± 0.05*	13	0.20 ± 0.04	8
10 μM Ca^{2+}				
WT	0.72 ± 0.05	13	0.27 ± 0.03	12
F223E	0.34 ± 0.04*	17	0.11 ± 0.03*	10
K225Q	0.83 ± 0.03	11	0.37 ± 0.06	7
Δ229- 232	0.94 ± 0.02*	17	0.89 ± 0.04*	9
K229Q	0.94 ± 0.02*	20	0.72 ± 0.03*	12
Δ680–685	0.62 ± 0.05	17	0.32 ± 0.04	13

F_{ss}, I_{ss}/I_{peak}; WT, wild-type NCX1.1.
* $P < 0.05$ compared with wild type.

TABLE 3
Effect of SEA (0.1 μM) on the rate of Na^+_i-dependent inactivation (λ) of wild-type NCX1.1 and mutant Na^+-Ca^{2+} exchangers

Exchanger Type	Control		SEA	
	λ	n	λ	n
1 μM Ca^{2+}				
WT	0.20 ± 0.02	43	0.35 ± 0.05	9
F223E	1.53 ± 0.06*	18	1.78 ± 0.09*	12
K225Q	0.33 ± 0.03*	13	0.56 ± 0.07	7
Δ229–232	1.07 ± 0.24*	10	1.32 ± 0.22*	9
K229Q	0.51 ± 0.08*	15	0.41 ± 0.04	10
Δ680–685	0.34 ± 0.04*	13	0.41 ± 0.02	8
10 μM Ca^{2+}				
WT	0.86 ± 0.22	12	0.20 ± 0.03	12
F223E	1.47 ± 0.15*	17	1.86 ± 0.37*	10
K225Q	1.13 ± 0.25	8	0.49 ± 0.03*	6
Δ229–232	1.57 ± 0.33	11	2.53 ± 0.56*	7
K229Q	0.68 ± 0.32	9	0.51 ± 0.14*	9
Δ680–685	0.32 ± 0.04*	17	0.28 ± 0.04	13

λ (s^{-1}), rate of current inactivation; WT, wild-type NCX1.1.
* $P < 0.05$ compared with wild type.

Figure 4 shows the effect of 0.1 μM SEA on outward Na^+-Ca^{2+} exchange currents for a mutant (F223E), where the rate and extent of I_1 inactivation is enhanced. As demonstrated previously for this mutation of NCX1.1 (Matsuoka et al., 1997), the decay of outward current is substantially accelerated (Fig. 4A), particularly at lower cytoplasmic Ca^{2+} concentrations (Table 1). Consistent with the hypothesis that SEA exerts its blocking actions by modulating I_1 inactivation, inhibition of steady-state outward currents by SEA was considerably enhanced with F223E. Indeed, the extent of block was so pronounced that it was difficult to quantify the percentage inhibition and F_{ss}, as steady-state currents were typically near or at baseline, as shown in Fig. 4, A and B. Peak outward currents remained less affected by SEA in the F223E mutant.

We examined the effects of SEA on a second mutant with enhanced I_1 inactivation, K225Q (not shown). In this mutant, the degree of enhancement of I_1 was considerably less, as shown in Tables 1 to 3. Here, the inhibitory effects of SEA on peak and steady-state outward currents were similar to wild-type at both 1 and 10 μM regulatory Ca^{2+} levels (Table 1). That is, unlike F223E, a clear facilitation of block was not observed for K225Q, possibly reflecting the more subtle nature of this mutation.

To gain further insight into the inhibitory effects of SEA, we evaluated its concentration dependence for inhibition of the Δ229–232 and F223E mutants. These particular mutants were selected for further investigation because they displayed the most prominent alterations in their responses to SEA compared with the wild-type exchanger. Pooled results are shown in Fig. 5. For Δ229–232, the inhibitory effects of SEA were evaluated on outward currents activated by 100 mM Na^+_i in the presence of 1 μM regulatory Ca^{2+}. The IC_{50} values for inhibition of peak and steady-state currents were 0.32 ± 0.15 μM and 0.20 ± 0.05 μM (means ± S.E.M.), respectively. In general, these values are ~4 to 10 times higher than those obtained for the wild-type exchanger under identical experimental conditions (Lee et al., 2003). For the F223E mutant, the concentration dependence of SEA was examined on outward currents activated by 100 mM Na^+_i in the presence of 10 μM regulatory Ca^{2+}. These experimental conditions were chosen to reduce the inhibitory potency of SEA and augment the magnitude of steady-state currents. In particular, the F223E mutant shows very small steady-state currents under control conditions, and SEA reduces these even further (Fig. 4). The limited signal-to-noise ratios for these types of measurements make IC_{50} measurements highly prone to error and variability. Nevertheless, within our limited ability to obtain such measurements, IC_{50} values of 65 ± 12 nM and 19 ± 7 nM (means ± S.E.M.) were obtained, for peak and steady-state currents, respectively, under conditions in which I_1 inactivation was reduced by high regulatory Ca^{2+}.

The effect of SEA on a mutant Na^+-Ca^{2+} exchanger with suppressed Ca^{2+}-dependent (I_2) inactivation is shown in Fig. 6. The control data illustrate that steady-state outward currents mediated by Δ680–685 are relatively insensitive to elevation of cytosolic Ca^{2+} compared with NCX1.1 (e.g., Fig. 2B). Irrespective of this loss of Ca^{2+}_i-dependent regulation, the degree of block by SEA of both peak and steady-state current with Δ680–685 was similar to that observed with wild-type (Table 1). These data suggest that block of NCX1.1

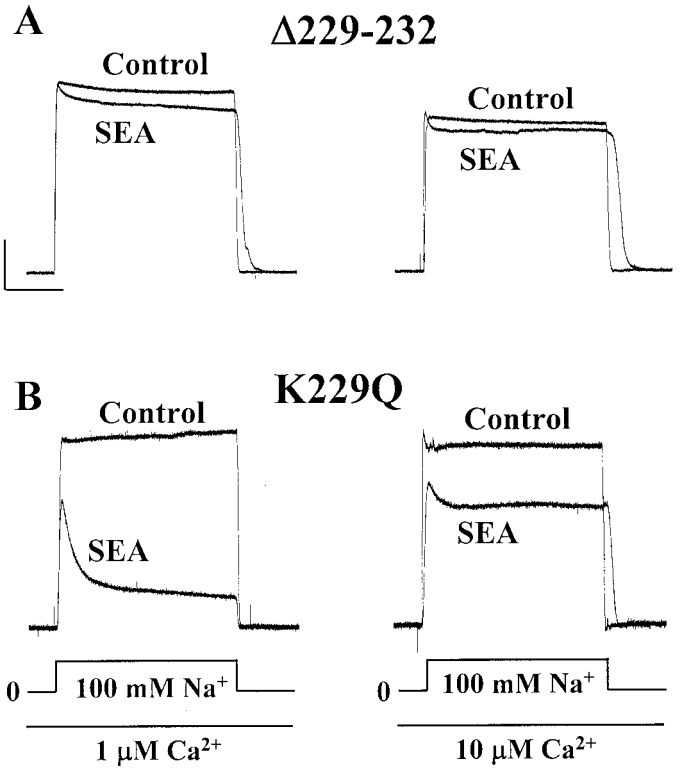

Fig. 3. Inhibitory effects of SEA on outward Na^+-Ca^{2+} exchange currents with mutant exchangers exhibiting suppressed I_1 inactivation. A and B illustrate the effects of SEA (0.1 μM) on outward exchange currents mediated by Δ229–232 (A) and K229Q (B) in the presence of 1 μM (left) and 10 μM (right) regulatory Ca^{2+}. In each, current traces from the same membrane patch have been superimposed to allow direct comparison. Outward currents were generated by rapidly applying 100 mM Na^+ to the cytoplasmic side of the patch in the continuous presence of either 1 μM or 10 μM regulatory Ca^{2+}. Scale bars for current (*y*-axis) and time (*x*-axis) are 50 pA and 10 s, respectively.

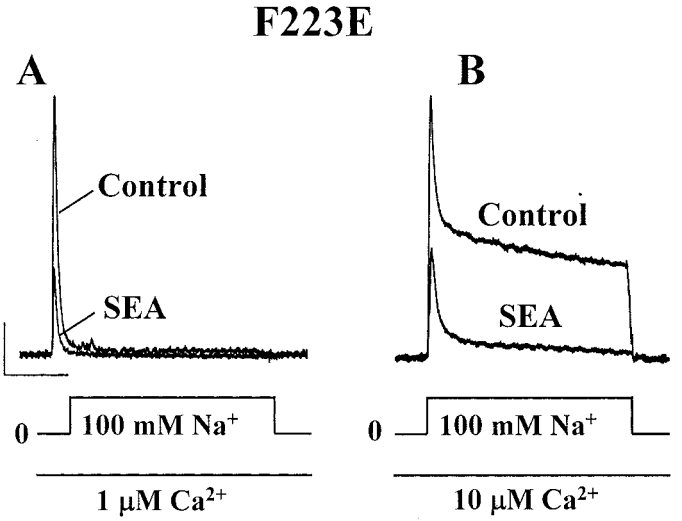

Fig. 4. Effects of SEA on outward Na^+-Ca^{2+} exchange currents mediated by a mutant exchanger exhibiting enhanced I_1 inactivation. A and B illustrate the effects of SEA (0.1 μM) on exchange currents from F223E in the presence of 1 and 10 μM regulatory Ca^{2+}_i, respectively. In each, current traces from the same membrane patch have been superimposed for direct comparison. Outward currents were generated by application of 100 mM Na^+ to the cytoplasmic side of the patch in the continuous presence of either 1 μM or 10 μM Ca^{2+}. Scale bars for current (*y*-axis) and time (*x*-axis) are 25 and 50 pA and 10 s in A and B, respectively.

by SEA may be largely independent of the I_2 inactivation process.

Tables 2 and 3 show F_{ss} and λ data for NCX1.1 and all mutant exchangers in the absence and presence of 0.1 μM SEA. F_{ss} is the ratio of steady-state to peak outward current and represents the fraction of active exchangers generating current at steady-state (Hilgemann et al., 1992b). The measured rate constant for outward current inactivation, λ, is thought to be related to the development of I_1 as follows: $\lambda = \alpha E_{3ni} + \beta$, where α is the rate constant for the transition of exchangers from the E_{3ni} state into I_1, E_{3ni} is the fraction of three Na^+-loaded exchangers oriented to the intracellular surface of the membrane, and β is the rate constant for recovery of exchangers from I_1 to E_{3ni} (Hilgemann et al., 1992b; Omelchenko et al., 1998). In general, λ is a measure of the rate of I_1 inactivation, whereas F_{ss} is an index of the extent of I_1 inactivation.

Compared with NCX1.1, F_{ss} was increased for Δ229–232 and K229Q in the absence and presence of SEA, irrespective of the concentration of cytosolic Ca^{2+}. This is consistent with the square-wave current waveform shown in Fig. 3 for Δ229–232, similar to that observed for wild-type NCX1.1 after removal of ionic regulation by limited proteolysis with α-chymotrypsin (Hilgemann, 1990; Matsuoka et al., 1995, 1997; Hryshko, 2002). F_{ss} was reduced and λ was increased for F223E under control conditions, and a further increase occurred in the presence of SEA. The effects of SEA on F223E and Δ229–232 are completely consistent with the notion that SEA exerts its inhibitory effects through interactions with the I_1 inactivation process. For K229Q, SEA seemed to be able to induce an I_1-like inactive state. However, the results with K225Q showed little difference from NCX1.1. We do not have a compelling explanation for this result, but we suggest the following: K225Q shows a modest increase in the rate of inactivation (λ) but an actual decrease in the extent of inactivation (reflected by higher F_{ss} values). This combination of opposing attributes might somehow protect K225Q from increased susceptibility to the inhibitory effects of SEA.

Inward Na^+-Ca^{2+} Exchange. Inward Na^+-Ca^{2+} exchange current (Ca^{2+} efflux mode) was initiated by switching from a Na^+- and Ca^{2+}-free bath solution to a solution containing variable amounts of Ca^{2+}. The pipette (extracellular) solution contained 100 mM Na^+ and was nominally Ca^{2+}-free. Note that I_1 inactivation is undetectable for inward currents as the concentration of Na^+ in the bath solution is

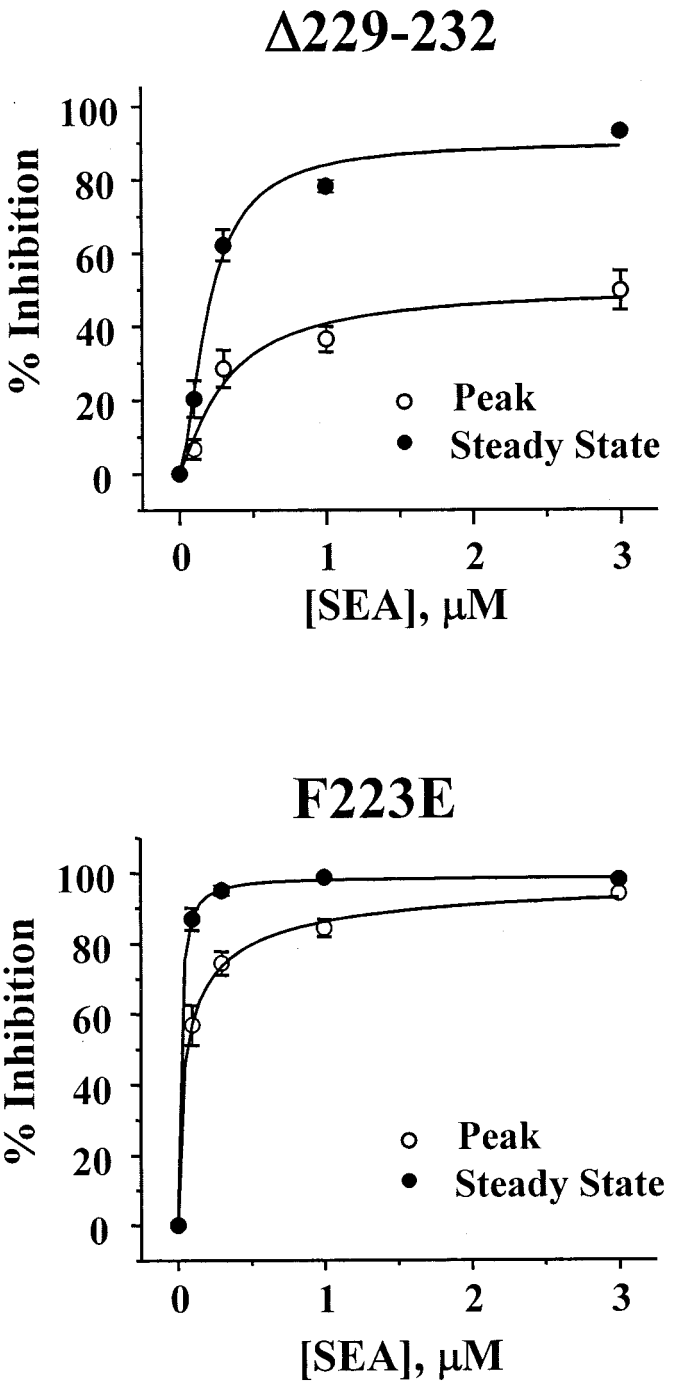

Fig. 5. Concentration dependence of SEA inhibition of outward currents for exchanger mutants, Δ229–232 and F223E. The concentration dependence of SEA inhibition is shown for Δ229–232 in A where outward currents were activated by 100 mM Na^+_i and 1 μM regulatory Ca^{2+}. B, the results are shown for F223E, where outward currents were activated by 100 mM Na^+_i and 10 μM regulatory Ca^{2+}.

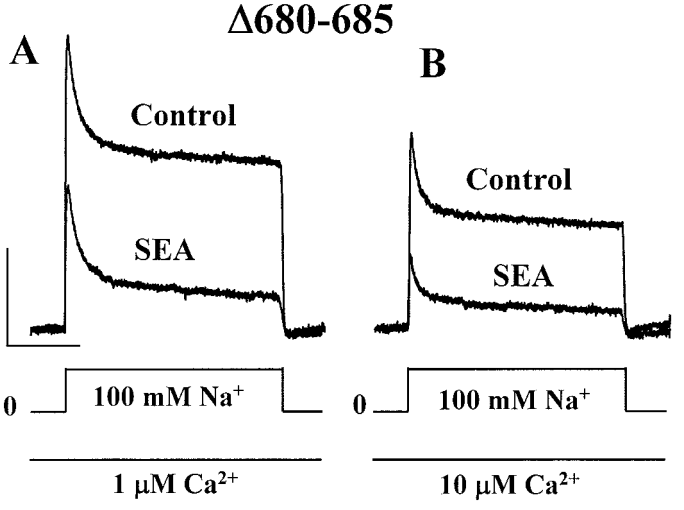

Fig. 6. Inhibitory effect of SEA on a mutant exchanger displaying altered I_1 and I_2 inactivation. A and B illustrate the effects of SEA (0.1 μM) on exchange currents from Δ680–685 in the presence of 1 and 10 μM regulatory Ca^{2+}, respectively. Current traces from the same membrane patch have been superimposed for direct comparison. Outward currents were generated by introduction of 100 mM Na^+ to the cytoplasmic side of the patch in the presence of either 1 or 10 μM regulatory Ca^{2+}. Scale bars for current (y-axis) and time (x-axis) are 40 pA and 10 s, respectively.

nominally zero. Inward Na^+-Ca^{2+} exchange currents were measured in the presence of both low (3 μM) and high (10 μM) cytosolic Ca^{2+} concentrations. Figure 7A shows that inward currents mediated by NCX1.1 are relatively insensitive to block by SEA, compatible with the notion that SEA requires interaction with the Na^+_i-dependent inactivation process. The effect of SEA on inward exchange currents generated by Δ229–232 is shown in Fig. 7B, and a similar insensitivity was obtained. However, when F223E was examined (Fig. 7C), SEA produced a significant block of inward current. A similar tendency was observed with K225Q (Table 4). These results show that SEA is capable of exerting significant inhibitory effects on mutant exchangers with enhanced I_1 inactivation, even under conditions in which this regulatory mechanism is typically minimal or absent. The greater block of inward currents by SEA for F223E compared with K225Q corresponds to the results obtained for outward currents.

Discussion

The objective of these experiments was to test the hypothesis that SEA inhibits NCX1.1 by enhancing Na^+_i-dependent (I_1) inactivation. This hypothesis was tested using NCX1.1 and mutant exchangers with altered ionic regulatory properties. We found that inhibition of exchange currents by SEA was greatly suppressed in mutants with suppressed I_1 inactivation (Δ229–232, K229Q) compared with the wild-type exchanger. This was particularly evident for the deletion mutant, Δ229–232, whereas SEA seemed to induce an I_1-like inactivation in the point mutant, K229Q. For the two mutants with accelerated I_1 inactivation (F223E, K225Q), block of outward current by SEA was facilitated only for the mutant with increased inactivation (F223E) and was without significant effect on the point mutation (K225Q). Finally, Δ680–685, which lacks Ca^{2+}_i-dependent (I_2) inactivation, behaved similarly to wild-type with respect to inhibition by SEA, suggesting that I_2 inactivation may not play a major role in the inhibitory action of this agent. Our data provide further evidence supporting a prominent role for Na^+_i-dependent inactivation in the inhibitory action of SEA.

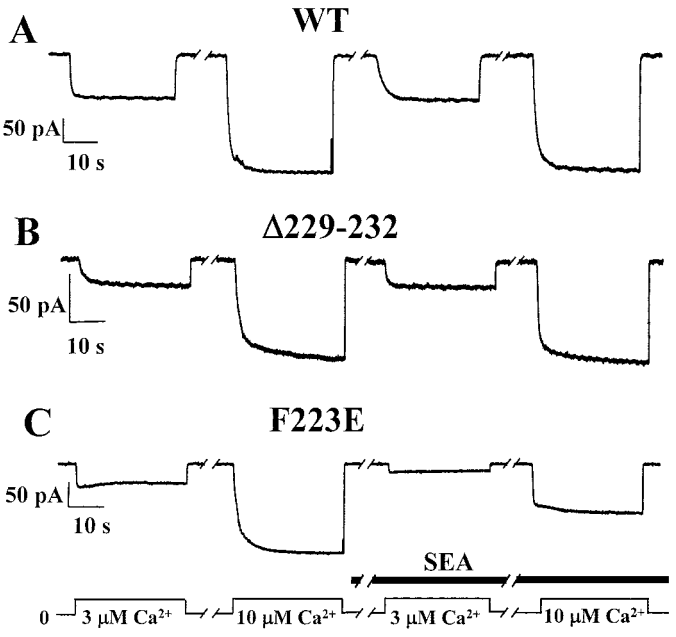

Fig. 7. Effects of SEA on inward Na^+-Ca^{2+} exchange currents mediated by wild-type and mutant NCX1.1 exchangers exhibiting depressed and enhanced I_1 inactivation. A to C, representative tracings from NCX1.1 (A), Δ229–232 (B), and F223E (C) in the absence (left) and presence (right) of 0.1 μM SEA. Inward Na^+-Ca^{2+} exchange currents were generated by rapidly applying either 3 or 10 μM Ca^{2+} to the cytoplasmic side of the patch in exchange for 100 mM pipette Na^+.

Role of I_1 Inactivation in SEA Inhibition. Ionic regulation of NCX1.1 involves protein domains within the large intracellular loop of the exchanger between transmembrane segments 5 and 6 (Philipson and Nicoll, 2000; Hryshko, 2002; Quednau et al., 2004). The so-called XIP region, which comprises amino acids 219 to 238, seems to be involved in Na^+_i-dependent inactivation because mutations in this region affect both the rate and degree of inactivation (Matsuoka et al., 1997). Similar data have been reported for CALX1.1 when mutations were made at the analogous positions of its amino acid sequence (Dyck et al., 1998). Given our previous observations (Lee et al., 2003) of 1) an apparent transport mode-specific block of NCX1.1 exchangers by SEA, 2) a substantial relief of inhibition in de-regulated exchangers after α-chymotrypsin treatment, 3) a reduction of block after elevation of cytosolic Ca^{2+} concentration, which typically relieves I_1 inactivation, and 4) a prominent intracellular Na^+_i-dependence for the inhibitory potency of SEA, we postulated that inhibition of NCX1.1 by SEA was state-dependent and that SEA stabilized or promoted entry of exchange molecules into the I_1 inactive state. Several lines of evidence in the present work directly support this hypothesis.

The strongest evidence favoring a state-dependent blocking mechanism by SEA is the data obtained with Δ229–232 and F223E. The ability of SEA to inhibit outward or inward Na^+-Ca^{2+} exchange currents was greatly reduced with Δ229–232, a mutant in which I_1 inactivation seems to be absent (Matsuoka et al., 1997). Similarly, the degree of inhibition was increased for F223E, a mutant showing an increase of ~2-fold in the rate and extent of I_1 inactivation compared with NCX1.1. Indeed, exposure of F223E to SEA resulted in a nearly complete inhibition of steady-state outward current in the presence of 1 μM Ca^{2+}_i. Notably, when complete concentration dependence profiles were obtained for these two mutants, a substantial decrease in the apparent IC_{50} was obtained for Δ229–232 whereas only a slight increase (not significant) in potency was observed for F223E compared with the wild-type exchanger (Lee et al., 2003).

TABLE 4
Effect of SEA (0.1 μM) on steady-state inward currents mediated by NCX1.1 and mutant Na^+-Ca^{2+} exchangers

Exchanger Type	I_{ss} (% Inhibition)	n
3 μM Ca^{2+}		
WT	3 ± 1	6
F223E	52 ± 4*	6
K225Q	15 ± 5	10
Δ229–232	13 ± 3*	4
K229Q	5 ± 2	12
10 μM Ca^{2+}		
WT	3 ± 1	15
F223E	28 ± 5*	10
K225Q	10 ± 5	8
Δ229–232	2 ± 1	8
K229Q	7 ± 2	10

I_{ss}, steady-state inward current; WT, wild-type NCX1.1.
* $P < 0.05$ compared with wild type.

However, the results for F223E were obtained under conditions in which inhibitory potency was reduced (i.e., at high regulatory Ca^{2+}) to obtain measurable signals, making such direct comparisons difficult. Moreover, analysis of this type does not permit any conclusions to be derived regarding the site of action of SEA because the apparent IC_{50} values obtained do not necessarily provide information on the drug binding affinity. Specifically, our results indicate that I_1 inactivation is involved in the inhibitory effects of SEA. Whether this relationship involves direct interactions with the XIP region of the exchange molecule or occurs via long range interactions and/or stabilization of distinct exchanger states cannot be determined by such analyses.

Interestingly, an intermediate response was observed for a second mutant lacking I_1 inactivation, K229Q. No obvious I_1 inactivation was observed for K229Q under control conditions. However, SEA clearly induced an I_1-like inactivation of outward current for this mutant. Moreover, as with the native I_1 inactivation process, increasing regulatory Ca^{2+} substantially alleviated the extent of this inhibition. We speculate that the subtler point mutation retains the capability of transitioning from the E_{3ni} state into the I_1 inactive state, although the I_1 state may be very unstable in this mutant. In the current study, SEA seemed to stabilize the I_1 state. In contrast, with the larger deletion mutant (Δ229–232), we postulate that the I_1 state is nearly unable to form and therefore SEA loses most of its effectiveness. We also observed that treatment of patches with α-chymotrypsin to remove ionic regulation greatly reduced the inhibitory effects of SEA for wild-type NCX1.1 (Lee et al., 2003) and for all mutants investigated here (data not shown).

SEA produced a substantial inhibition of inward currents with F223E, even though I_1 inactivation is not normally observed for these currents. Although we cannot fully account for this behavior, a plausible explanation is that the I_1 state is clearly facilitated in F223E, reflected by a greatly reduced steady-state current and an increased rate of current inactivation. Typically, the I_1 state, which originates from the E_{3ni} transport state of the exchanger, has a far greater likelihood of developing for outward currents because cytoplasmic Na^+ is high. Because the subsequent occlusion and reorientation of exchanger binding sites to the extracellular surface is rate-limiting (Hilgemann et al., 1991), exchangers can accumulate in E_{3ni} from where they partition into I_1 or undergo ion translocation. In contrast, for inward currents, the only source of E_{3ni} is from exchangers reorienting from the extracellular side with bound Na^+ (i.e., from the E_{3no} state). Here, unbinding of Na^+ is the most favored transition because there is no Na^+ on the intracellular surface. Because exchangers are far less likely to accumulate in E_{3ni} during inward currents, I_1 inactivation is normally not observed.

Based on the foregoing, SEA is far more effective at inhibiting outward currents compared with inward exchange currents. However, in the case of F223E, the I_1 state is considerably augmented. Possibly, this enhancement may be sufficient such that I_1 can also occur during inward current, albeit to a lesser extent. If SEA further stabilized this small population of I_1 inactivated exchangers, its inhibitory effects could be explained. The somewhat diminished effects of SEA on K225Q inward currents offer general support for this possibility. Clearly, other potential explanations exist, although we presently favor the above, based on the similarities between K225Q and F223E.

Notably, the inhibitory actions of SEA were also apparent for peak outward Na^+-Ca^{2+} exchange currents, albeit to a lesser extent than that observed for steady-state currents. Therefore, it seems reasonable to postulate that the inhibitory effects of SEA include additional actions beyond that of facilitating I_1 inactivation. In general, peak outward current magnitude is thought to reflect exchange activity before the development of I_1 inactivation. However, caution is also required for this interpretation, because the magnitude of peak current reflects the balance between its rate of development (limited largely by solution switching time) opposed by the rate at which I_1 inactivation develops. Thus, under conditions at which I_1 inactivation is greatly facilitated, either by drugs and/or mutagenesis, it remains possible that this process could influence the magnitude of peak currents. At present, we lack the temporal resolution to distinguish between these two possibilities. The greatly diminished effects of SEA for inward currents at which I_1 inactivation is absent and the coincident changes in inhibitory potency with the preponderance of the I_1 inactive state do not exclude either possibility.

Given the $Ca^{2+}{}_i$-dependence of both I_1 and I_2 inactivation, it is also possible that SEA exerts some of its blocking action via modulation of I_2 inactivation. We addressed this possibility by investigating the effects of SEA on a deletion mutant, Δ680–685, where I_2 inactivation is significantly reduced (Maxwell et al., 1999). Indeed, block of peak and steady-state outward currents by SEA for the Δ680–685 mutant was very similar to that of wild-type NCX1.1. However, involvement of I_2 inactivation cannot be completely ruled out, because previous work has shown that both I_1 and I_2 inactivation are altered with Δ680–685 (Maxwell et al., 1999).

It is interesting to compare our results with those previously obtained with KB-R7943, a less potent and less selective Na^+-Ca^{2+} exchange inhibitor first described in 1996 (Iwamoto et al., 1996; Watano et al., 1996). Using a variety of techniques, KB-R7943 was also found to exhibit transport mode selectivity, whereby inhibition of the reverse transport mode was favored (Iwamoto et al., 1996; Watano et al., 1996; Elias et al., 2001; but see Kimura et al., 1999). The site of action of KB-R7943 remains unknown, although amino acid residues (particularly Gly833) within the α-2 repeat region of the exchanger have been reported to markedly affect drug sensitivity (Iwamoto et al., 2001). These authors have suggested that the site of KB-R7943 is on the external side of intact cells, because drug sensitivity was greatest when applied extracellularly, whereas drug applied intracellularly was largely without effect (Iwamoto et al., 2001). At present, the site of action of SEA must be considered unknown. Based on our current study and our previous work with KB-R7943, we find that intracellular application of these agents produces profound inhibitory effects on the reverse transport mode of exchange current. Moreover, the inhibitory potencies and characteristics are nearly identical to those reported in intact cells. Although our experimental results favor an intracellular or intramembrane site of action, we cannot exclude the possibility of an external site of action. Finally, the identified interaction between SEA and the intrinsic I_1 inactivation mechanism does not imply a direct physical interaction with the XIP domain of the exchanger.

In summary, our data provide a plausible explanation for the apparent mode selectivity of SEA. That is, the major effect of SEA is to enhance Na^+_i-dependent inactivation. The effectiveness of inhibition of Na^+-Ca^{2+} exchange current by SEA generally follows the prevalence of the I_1 inactive state, whether this is manipulated by mutagenesis, as in this study, or by altering the ionic transport conditions (Lee et al., 2003). Recognition of this molecular mechanism of action of SEA should prove beneficial toward facilitating the development and pharmacology of Na^+-Ca^{2+} exchange inhibitors. Moreover, the enhanced inhibitory potency of SEA under conditions of elevated intracellular Na^+ seems to be an ideal attribute when considering its potential utility against ischemia-reperfusion injury.

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