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Structural Domains Influencing Sensitivity to Isothiourea Derivative Inhibitor KB-R7943 in Cardiac Na^+/Ca^{2+} Exchanger

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ABSTRACT

KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate) is a potent and selective Na^+/Ca^{2+} exchange (NCX) inhibitor that is 3-fold more inhibitory to NCX3 than to NCX1 or NCX2. Here we searched for amino acid residues that may form the KB-R7943 receptor in the exchanger by analyzing the function of chimeras between NCX1 and NCX3 as well as of their site-directed mutants. We found that the highly conserved α-2 repeat of the exchanger is almost exclusively responsible for the difference in drug response of the isoforms. Such difference was mostly reproduced by single substitutions of residues in the α-2 repeat (V820G or Q826V in NCX1 and A809V or A809I in NCX3), suggesting their importance in drug sensitivity. Cysteine scanning mutagenesis of the α-2 repeat of NCX1 identified one residue (Gly833) that caused a large ($\geq$ 30-fold) reduction in drug sensitivity. We found that the Gly-to-Thr substitution caused even larger reduction in drug sensitivity. Interestingly, extracellularly applied KB-R7943 at 0.8 μM markedly inhibited the whole-cell outward exchange current, whereas the drug applied intracellularly at 30 μM did not. These results suggest that KB-R7943 inhibits the exchanger from the external side in intact cells and that a region of the α-2 repeat of NCX1 containing Gly833 may participate in the formation of the drug receptor. Because we suggested previously that Gly833 is accessible from the inside of a cell, the results raised an interesting possibility that this residue may alter its position during Na^+/Ca^{2+} exchange in such a way that it becomes accessible to external drug.

The mammalian Na^+/Ca^{2+} exchanger forms a multigene family comprising three isoforms (NCX1, NCX2, and NCX3) that shares ~70% identity in the overall amino acid sequences (Nicoll et al., 1990; Li et al., 1994; Nicoll et al., 1996b). NCX1 is highly expressed in heart and brain and at lower levels in many other tissues, whereas expression of the other isoforms is limited mainly to brain. In heart, NCX1 plays the primary role in the extrusion of cytosolic Ca^{2+} from myocytes during each heart beat, whereas the contribution of NCX isoforms in Ca^{2+} handling in other tissues still remains to be precisely defined (Blaustein and Lederer, 1999).

Recent topological studies (Nicoll et al., 1999; Iwamoto et al., 1999a) have suggested that the mature NCX1 protein consists of nine transmembrane segments (TMs) and a large intracellular loop between putative TM5 and TM6, with the N and C termini localized on the extracellular and cytoplasmic sides, respectively (see Fig. 1A). In all known members of the Na^+/Ca^{2+} exchanger family, there exist highly conserved internal repeat sequences designated the α-1 and α-2 repeats in the N- and C-terminal halves of the exchanger molecule, respectively (Schwarz and Benzer, 1997). Similar intragenic repeat structures were found in the $Na^+/Ca^{2+}/K^+$ exchanger (Reiländer et al., 1992; Tsoi et al., 1998) and the recently identified Mg^{2+}/H^+ exchanger (Shaul et al., 1999). In NCX1, the α-1 repeat comprises most of TM2–TM3 and their connecting loop, whereas the α-2 repeat comprises putative TM7 and its C-terminal segment (see Fig. 1A). Our recent study has provided evidence that the putative loop connecting TM2–TM3 forms a re-entrant membrane loop with both ends facing the extracellular side, whereas the α-2 repeat, except for TM7, forms a domain mostly accessible from the cytoplasm (Iwamoto et al., 1999a). Mutational analyses have further suggested that these putative TMs and loops in the α-1 and α-2 repeats are important in the interaction of the exchanger with transport substrates, a nonselective cation inhibitor (Ni^{2+}), and an activator (Li^+) (Nicoll et al., 1996a; Doering et al., 1998; Iwamoto et al., 1999b).

Iwamoto et al. (1996b) and Watano et al. (1996) have

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ABBREVIATIONS: NCX, Na^+/Ca^{2+} exchanger; TM, transmembrane; NCKX, $Na^+/Ca^{2+}/K^+$ exchanger; KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate); Na^+_i, intracellular Na^+; Ca^{2+}_o, extracellular Ca^{2+}; BSS, balanced salt solution.

recently reported synthesis and characterization of a potent inhibitor of the cardiac Na^+/Ca^{2+} exchanger, KB-R7943. This compound is highly selective for the Na^+/Ca^{2+} exchanger, because at up to 10 μM, it exerted little influence, not only on many other ion transporters but also on several cardiac action potential parameters, diastolic $[Ca^{2+}]_i$, and spontaneous beating in cardiomyocytes (Iwamoto et al., 1996b). It is effective in inhibiting all three NCX isoforms (Iwamoto and Shigekawa, 1998). It is now being widely used to study the physiological and pathological roles of the exchanger at the cellular and organ levels. For example, the compound has been shown to efficiently protect the ischemia-associated reperfusion injury of heart (Iwamoto et al., 1996b; Nakamura et al., 1998; Ladilov et al., 1999; Mukai et al., 2000; Satoh et al., 2000), brain (Schröder et al., 1999; Breder et al., 2000), and kidney (Kuro et al., 1999), suggesting its therapeutic potential in this type of cell damage. More recently, however, this compound has been reported to inhibit neuronal nicotinic acetylcholine receptors (Pintado et al., 2000) and *N*-methyl-D-aspartate receptor channels (Sobolevsky and Khodorov, 1999), although evidence has been presented that it does not inhibit the latter directly (Hoyt et al., 1998). An interesting feature of the drug is that it inhibits the Ca^{2+} influx mode (reverse mode) of Na^+/Ca^{2+} exchange much more effectively than the forward mode (Iwamoto et al., 1996b; Watano et al., 1996; Satoh et al., 2000), although the reason for such difference is currently unclear. Another interesting feature is that it is 3-fold more effective on NCX3 than NCX1 or NCX2 (Iwamoto and Shigekawa, 1998). Currently, however, little is known about the molecular mechanism of action for this drug and location of its receptor site in the exchanger molecule.

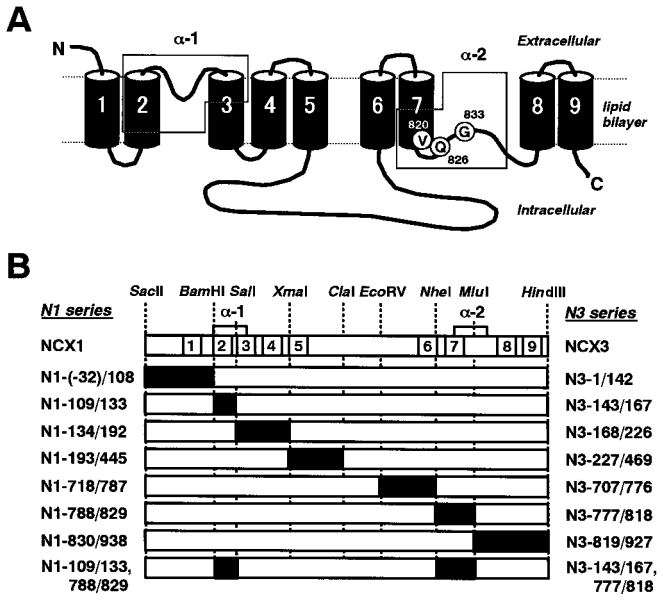

Fig. 1. A topological model of NCX1 (A) and chimeric constructs between NCX1 and NCX3 (B). A, a nine-TM model of NCX1 based on the recent topology analyses (Nicoll et al., 1999; Iwamoto et al., 1999a). The amino acid residues of NCX1 whose mutation alters the KB-R7943 sensitivity are shown. B, two series of chimeras (N1 and N3) were constructed by substituting segments of NCX1 with homologous segments of NCX3, and vice versa. In each series, chimeras are named based on the amino acid numbers for respective isoforms. Broken lines show the restriction enzyme cut sites and the black boxes indicate the substituted segments. The linear model of exchanger is indicated at the top.

In this study, to obtain insights into the mechanism of action of KB-R7943 as well as into the mechanism of Na^+/Ca^{2+} exchange, we constructed a series of chimeric exchangers between NCX1 and NCX3 and searched for the structural domain(s) of the exchanger involved in the determination of sensitivity to KB-R7943. We identified several amino acid residues within the α-2 repeat of the exchanger whose mutations alter apparent affinity of the drug.

Experimental Procedures

Cell Cultures. CCL39 cells and NCX transfectants were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Construction and Stable Expression of Wild-Type, Chimeric, and Mutant Exchangers. We used major splice variants of cDNAs for NCXs and NCKX that are prevalent in dog heart (NCX1.1) and rat brain (NCX2.1, NCX3.3, and NCKX2) (Quednau et al., 1997; Tsoi et al., 1998). NCX cDNAs were cloned into *Sac*II and *Hin*dIII restriction sites in pCRII (Invitrogen, San Diego, CA) or in the mammalian expression vector pKCRH as described previously (Iwamoto et al., 1996a; Iwamoto and Shigekawa, 1998). For isolation of cDNA of NCKX2, total RNA was extracted from rat brain with a TRIzol reagent (Life Technologies, Rockville, MD) and first-strand cDNAs were synthesized using an oligo(dT) primer and SuperScript preamplification system (Life Technologies). cDNAs were amplified by polymerase chain reaction using pairs of sense and antisense primers, 5′-ATATGAATTCACCACCATATCCACCAGAAGATCC-3′ and 5′-ATATGGTACCGTTTAAGATATGGCTTTTCCACTA-3′ [nucleotides −24– −1 and 2011–2034 of NCKX2 (Tsoi et al., 1998)] containing exogenous *Eco*RI and *Kpn*I restriction sites, respectively (underlined). Amplified cDNA insert was cloned into *Eco*RI and *Kpn*I restriction sites in the mammalian expression vector pcDNA3.1 (Invitrogen).

As described in detail previously (Iwamoto et al., 1999b), two series of NCX1/NCX3 chimeras (Fig. 1B) were constructed by exchanging the homologous segments using the newly introduced restriction enzyme sites or endogenous sites (*Sac*II, *Bam*HI, *Sal*I, *Xma*I, *Cla*I, *Eco*RV, *Nhe*I, *Mlu*I, and *Hin*dIII) of the above pCRII clones. Substitution of amino acid residues within the internal α-2 repeat region was performed by site-directed mutagenesis using a polymerase chain reaction-based strategy as described previously (Iwamoto et al., 1999b). Successful construction of the modified cDNAs was verified by sequencing. These cDNAs were transferred into *Sac*II and *Hin*dIII restriction sites in pKCRH.

To stably express wild-type, chimeric, and mutant exchangers, pKCRH or pcDNA3.1 plasmids carrying exchanger cDNAs were transfected in the presence of Lipofectin (Life Technologies) into CCL39 fibroblasts that exhibit little endogenous Na^+/Ca^{2+} exchange activity (Iwamoto et al., 1996a, 1998a,b). Cell clones highly expressing Na^+/Ca^{2+} exchange activity or $Na^+/Ca^{2+}/K^+$ exchange activity were selected by a Ca^{2+}-killing procedure as described previously (Iwamoto et al., 1998b).

Assay of $^{45}Ca^{2+}$ Uptake. Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing the wild-type or mutated NCXs were assayed as described in detail previously (Iwamoto and Shigekawa, 1998). Briefly, confluent transfectants in 24-well dishes were loaded with Na^+ by the incubation at 37°C for 30 min in 0.5 ml of balanced salt solution (BSS) (10 mM HEPES/Tris, pH 7.4, 146 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, and 0.1% bovine serum albumin) containing 1 mM ouabain and 10 μM monensin. $^{45}Ca^{2+}$ uptake was then initiated by switching the medium to Na^+-free BSS (replacing NaCl with equimolar choline chloride) or to normal BSS, both of which contained 0.1 mM $^{45}CaCl_2$ (1.5 μCi/ml) and 1 mM ouabain. After a 30-s incubation, $^{45}Ca^{2+}$ uptake was terminated by washing cells four times with an ice-cold solution

containing 10 mM HEPES/Tris, pH 7.4, 120 mM choline chloride, and 10 mM $LaCl_3$. Cells were then solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity and protein. When present, KB-R7943 was included in medium at concentrations of up to 100 μM two min before the start of $^{45}Ca^{2+}$ uptake. Assay of Na^+_i/K^+_o-dependent $^{45}Ca^{2+}$ uptake into cells expressing the wild-type NCKX2 was performed under the same condition as that for NCX.

Measurement of Whole-Cell Outward Current. Outward exchange currents from NCX1 transfectants were measured using the whole-cell, patch-clamp technique as described previously (Uehara et al., 1997). Bath solution contained 150 mM LiCl (replacing NaCl), 1 mM $MgCl_2$, 0 or 1 mM $CaCl_2$, 20 μM ouabain, 2 μM nicardipine, 5 μM ryanodine, 0 to 10 μM KB-R7943, and 5 mM HEPES, pH 7.2, whereas pipette solution contained 20 or 100 mM NaOH, 20 mM CsOH, 1.1 mM $MgCl_2$, 20 mM tetraethylammonium chloride, 2 mM MgATP, 2 mM creatine phosphate, 19.8 mM $CaCl_2$, 50 mM EGTA, 0 to 30 μM KB-R7943, and 50 mM HEPES, pH 7.2. The ionized Ca^{2+} concentration in pipette solution was calculated to be 0.16 μM. The outward current was activated by switching bath solution from one without $CaCl_2$ to one with $CaCl_2$. Intracellular application of KB-R7943 was performed by perfusion of pipette solution by the method described by Soejima and Noma (1984). All experiments were performed at about 35°C and the holding and test potentials were −40 mV. All data were acquired and analyzed by the pCLAMP software (Axon Instruments, Foster City, CA).

Statistical Analysis. Data are expressed as means ± S.E. of three independent determinations. Differences for multiple comparisons were analyzed by unpaired *t* test or one-way analysis of variance followed by the Dunnett's test. Values of $p < 0.05$ were considered statistically significant.

Materials. Chinese hamster lung fibroblasts (CCL39) were purchased from American Type Culture Collection (Manassas, VA). KB-R7943 was synthesized by New Drug Research Laboratories, Kanebo (Osaka, Japan). $^{45}CaCl_2$ was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were of the highest grade available.

Results

Chimera Analysis of Effect of KB-R7943. KB-R7943 inhibited the initial rate of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX1-, NCX2-, or NCX3-expressing cells with IC_{50} values of 4.3 ± 0.3, 4.7 ± 0.5, or 1.4 ± 0.2 μM ($n = 3$), respectively (see Fig. 5), confirming our previous report (Iwamoto and Shigekawa, 1998) that NCX3 was about 3-fold more sensitive to KB-R7943 than other isoforms. Taking advantage of the fact that NCX1 and NCX3 exhibit a high sequence homology, we constructed chimeric exchangers between these isoforms to identify region(s) of the exchanger that is important for the interaction with KB-R7943. We constructed two series of chimeras in which one or two segments from NCX3 were transferred into NCX1 in exchange for the homologous segment(s) in the latter (N1 chimeras), and vice versa (N3 chimeras) (Fig. 1B). All these chimeras had exchange activities similar to that of the wild-type NCX1 or NCX3 (see the legend to Fig. 2).

Fig. 2 shows the effect of 3 μM KB-R7943 on the rate of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing individual wild-type and chimeric exchangers. KB-R7943 at this concentration reduced the uptake rate of the wild-type NCX1 or NCX3 by about 25 or 70%, respectively. We found that N1 chimeras (N1–788/829 and N1–109/133,788/829) containing the homologous *Nhe*I-*Mlu*I segment from NCX3 (see Fig. 1B) showed a KB-R7943 sensitivity similar to that of the wild-type NCX3 (Fig. 2A). On the other hand, the N3 chimeras (N3–777/818 and N3–143/167,777/818) containing the *Nhe*I-*Mlu*I segment from NCX1 exhibited a KB-R7943 sensitivity similar to that of the wild-type NCX1 (Fig. 2B). Other N1 and N3 chimeras, however, were not significantly different from the parental NCX1 or NCX3, respectively, indicating that the *Nhe*I-*Mlu*I segment contains residues that are exclusively responsible for the observed differential responses of these isoforms to KB-R7943. The *Nhe*I-*Mlu*I segment contained a large portion of the α-2 repeat, which is highly conserved in all members of the NCX family (see the introduction).

Identification of Residues Influencing KB-R7943 Sensitivity. Fig. 3A shows amino acid sequences of the α-2 repeat regions from NCX isoforms and NCKX2 (Nicoll et al., 1990; Li et al., 1994; Nicoll et al., 1996b; Tsoi et al., 1998). In these regions of NCX1 and NCX3, there are only four residues unique to each isoform. To identify the residues that influence KB-R7943 sensitivity, the unique residues were exchanged between NCX1 and NCX3 or mutated to other amino acids. We found that single mutations Leu808-to-Phe and Thr823-to-Leu in NCX1 and their reciprocal mutations in NCX3 did not produce any change in sensitivity to inhibition by 3 μM KB-R7943 (Fig. 3, B and C). The Val820-to-Ala mutation in NCX1 was also silent, but its reciprocal mutation in NCX3 (N3-A809V) significantly decreased KB-R7943 sensitivity. On the other hand, the Gln826-to-Val mutation in NCX1 increased KB-R7943 sensitivity of the exchanger to a level comparable with that seen in the wild-type NCX3, whereas its reciprocal mutation in NCX3 (N3-V815Q) was

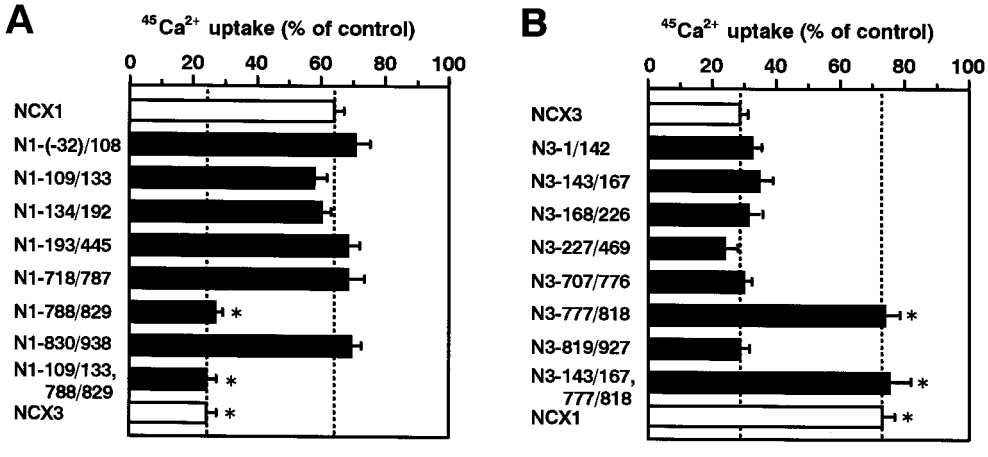

Fig. 2. Effects of KB-R7943 on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing N1 (A) and N3 (B) chimeric exchangers. The initial rate of $^{45}Ca^{2+}$ uptake was measured in the presence or absence of 3 μM KB-R7943 as described under *Experimental Procedures*. The uptake rates of cells expressing these chimeras were 5 to 9 nmol/mg/30 s in the absence of KB-R7943, which were similar to those in cells expressing the wild-type NCX1 or NCX3 (10 and 7 nmol/mg/30 s). Data are presented as percentage of the values obtained in the absence of drug. Data are presented as mean ± S.E. of three independent experiments. *$p < 0.05$ versus NCX1 (in A); *$p < 0.05$ versus NCX3 (in B).

silent. In contrast, simultaneous mutations of Val820 to Ala and Gln826 to Val in NCX1 and their reciprocal mutations in NCX3 mostly reproduced the drug sensitivities seen in the parental NCX3 and NCX1, respectively (Fig. 3, B and C). We found that the Val820-to-Gly mutation in NCX1 and the Ala809-to-Ile mutation in NCX3, but not their reciprocal mutations in the other isoforms, produced alterations in drug sensitivity almost comparable with those seen between the parental exchangers. Other mutations, such as Gln826 to Glu or Arg in NCX1 and their reciprocal mutations in NCX3, were silent. Therefore, residues at positions 820 and 826 of NCX1 are mostly responsible for the differential drug response between NCX1 and NCX3.

To identify other residues in the α-2 repeat that alter drug sensitivity, we examined the effect of 10 μM KB-R7943 on the rates of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX1 mutants in which individual residues in the α-2 repeat region were substituted with cysteine (Fig. 4). We found that the Gly833-to-Cys substitution greatly decreased the extent of inhibition by KB-R7943, whereas all other substitutions did not alter drug response significantly. Similarly, we examined the effects of single cysteine substitutions of 17 residues in the α-1 repeat on the drug sensitivity. These mutants (i.e., L107C, A111C, L115C, S117C, V118C, I119C, E120C, V121C, G123C, H124C, N125C, T127C, A128C, G129C, D130C, S134C, and I136C) did not exhibit altered sensitivity to 10 μM KB-R7943 (data not shown).

We analyzed dose responses of NCX1 mutants N1-V820G, N1-Q826V, and N1-G833C for inhibition of Na^+_i-dependent $^{45}Ca^{2+}$ uptake by KB-R7943 (Fig. 5). The IC_{50} values were estimated to be 1.6 ± 0.3 μM (n = 3) for N1-V820G and 1.7 ± 0.4 μM (n = 3) for N1-Q826V, both of which are comparable with that of the wild-type NCX3 (1.4 μM). In contrast, the dose-response profile could not be obtained with N1-G833C, because it showed only a slight sensitivity to the highest concentration of the drug used here. Figure 6 shows the results for multiple amino acid substitutions at Gly-833. We found that N1-G833T was less sensitive to KB-R7943 than N1-G833C; the drug at 100 μM reduced the uptake rate of N1-G833T by less than 10%, indicating that its IC_{50} value would be much larger than 100 μM. The apparent drug sensitivity of the mutants examined was: NCX1 = N1-G833A = N1-G833D $\gg$ N1-G833S > N1-G833K > N1-G833C > N1-G833T (Fig. 6). Interestingly, Fig. 5 shows that a high dose of KB-R7943 did not affect the rate of Na^+_i-dependent $^{45}Ca^{2+}$ uptake by NCKX2, a K^+-dependent Na^+/Ca^{2+} exchanger that was proposed to have an overall molecular topology similar to NCX despite its low sequence similarity (see Fig. 3A and Tsoi et al., 1998).

We examined the kinetic interactions of NCX1 mutants with transport substrate Ca^{2+}_o in different concentrations of KB-R7943. Table 1 summarizes values for the apparent affinity for $K_{m(Ca)}$ and V_{max} for NCX1, NCX3, N1-V820G, N1-Q826V, and N1-G833C, as estimated from analysis of Ca^{2+}_o concentration-dependence of the rate of Na^+_i-dependent Ca^{2+} uptake with or without drug (data not shown, but see Fig. 3 of Iwamoto and Shigekawa, 1998). We found that these amino acid substitutions, except for N1-G833C, did not alter apparent affinity for Ca^{2+}_o and the mode of interaction of the exchanger with KB-R7943; the drug decreased V_{max} of all these exchangers but increased their $K_{m(Ca)}$ (a mixed type inhibition) (Table 1). The observed absence of the effect on N1-G833C is due most likely to the low affinity of this mutant for the drug (see Figs. 5 and 6).

Sidedness of Effect of KB-R7943. Fig. 7, A and B, shows the results from electrophysiological measurements in which sidedness of action of KB-R7943 was examined. We measured whole-cell outward exchange current evoked by the extracellular application of 1 mM Ca^{2+} in cells expressing the wild-type NCX1 preincubated with or without external 0.8 μM KB-R7943 or perfused internally with or without 30 μM KB-R7943. These outward currents were never observed in nontransfected CCL39 cells (Uehara et al., 1997). When applied externally for 6 min, 0.8 μM KB-R7943 significantly

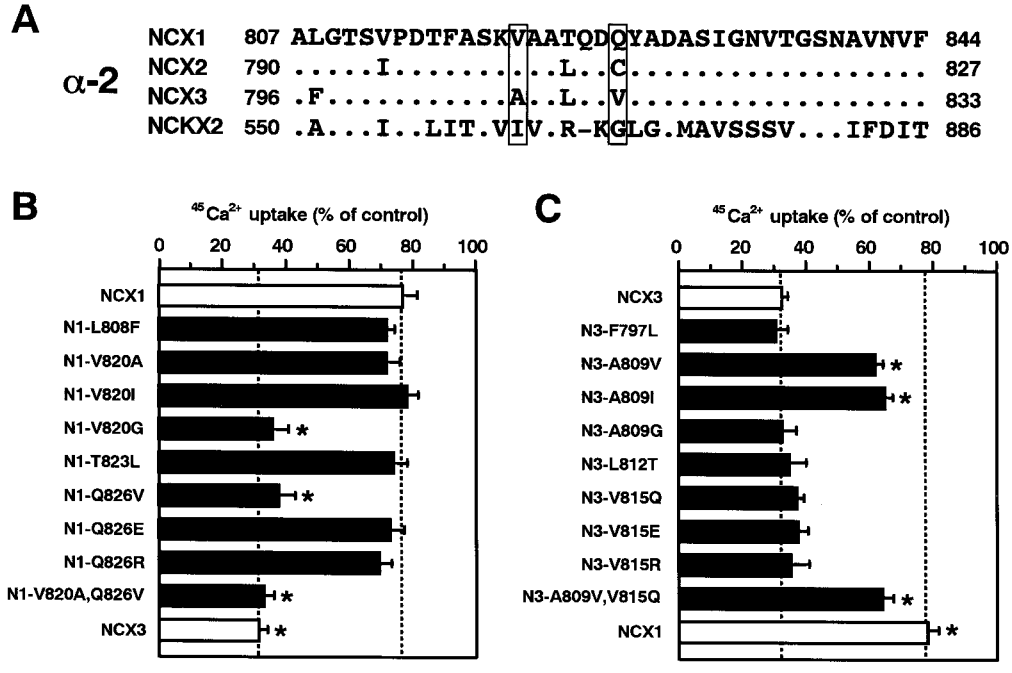

Fig. 3. Amino acid alignment of α-2 repeat regions in NCX isoforms and NCKX2 (A) and effects of KB-R7943 on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX variants mutated in the α-2 repeat (B and C). A, conserved amino acids among exchangers are indicated with dots and alignment gaps with dashes. Boxes show the amino acid positions of NCX1 and NCX3 whose mutation alters the KB-R7943 sensitivity. B and C, we constructed mutants in which the amino acid residues in the α-2 repeat unique to each NCX isoform were exchanged between NCX1 and NCX3 or mutated to other residues. The initial rates of $^{45}Ca^{2+}$ uptake into cells expressing NCX1 mutants (B) or NCX3 mutants (C) were measured as described in Fig. 2. The uptake rates in these mutants were 3 to 9 nmol/mg/30 s in the absence of KB-R7943. Data are presented as percentage of the values obtained in the absence of drug. Data are presented as means ± S.E. of three independent experiments. *$p < 0.05$ versus NCX1 (in B); versus NCX3 (in C).

reduced the current; its initial peak was reduced to 39 ± 2.3% (n = 3) of the control (Fig. 7A). When cells were washed with drug-free solution, the current was recovered to the control level. In contrast, KB-R7943 produced no significant inhibition of the outward current when it was applied intracellularly for 15 min (Fig. 7B). These results suggest that KB-R7943 inhibits the exchanger from the extracellular side. The IC_{50} value of the wild-type NCX1 for external KB-R7943 obtained from these electrophysiological measurements was 0.89 ± 0.23 μM (n = 4), which is significantly lower than that from $^{45}Ca^{2+}$ uptake measurements (Fig. 5). A similarly low IC_{50} value was reported by Watano et al. (1996), who used electrophysiological methods. The reason for such difference in the IC_{50} is not clear, but could be caused by differences in the conditions used, such as the preincubation time.

In Fig. 7C, we evoked a series of outward exchange currents in the same cell, first by application of 1 mM Ca^{2+} and then by application of 1 mM Ca^{2+} plus 10 μM KB-R7943 to see how fast the drug works from the external medium and how fast it washes out. The cell was washed with drug-free solution for 30 sec during the interval between the current inductions. As seen in the control trace in which Ca^{2+} alone was applied, the current rapidly reached a peak and then decayed gradually to a steady state, with the extent of the decay being 42 ± 6.9% (n = 3) at 20 sec. In the second current trace, in which Ca^{2+} and the drug were applied together, the peak was decreased slightly [by 15 ± 1.1% (n = 3)] but the current at the steady state was reduced markedly [by 83 ± 5.2% (n = 3) at 20 s] compared with that of the control current. The change in current decay was clearly recognizable within 5 s after the induction of the outward current. In the third current trace, in which Ca^{2+} and the drug were applied for the second time, the peak was decreased to about half of that in the previous current trace, whereas the current at 20 s was reduced slightly. In the third and fourth applications of Ca^{2+} plus KB-R7943, the current traces were essentially the same as the third current trace (data not shown). Finally, when the cell was washed with drug-free solution for 5 min, the peak current was recovered to 80 to 90% of the original control, although the steady-state current remained somewhat more inhibited (Fig. 7C). Thus, the expression of the drug effect was much faster than its washout. The observed fast onset of drug action is consistent with the view that KB-R7943 inhibits the exchanger from the extracellular side.

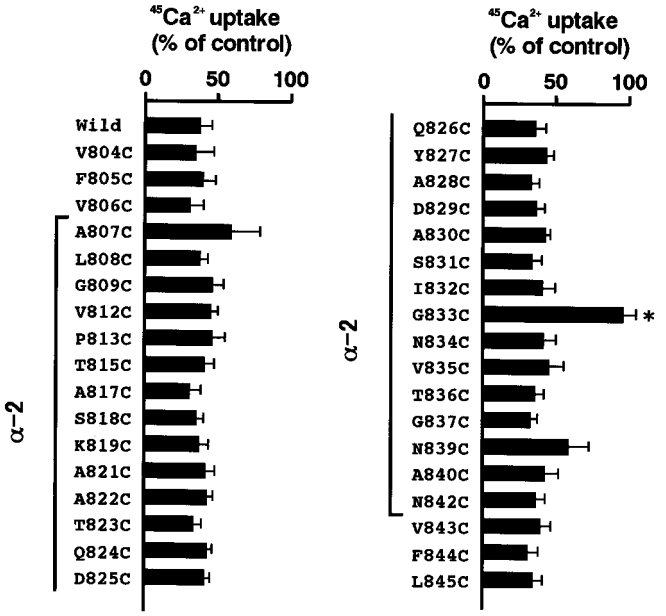

Fig. 4. Effect of KB-R7943 on activities of NCX1 mutants carrying single cysteine substitution in the α-2 repeat. Initial rates of Na^+_i-dependent $^{45}Ca^{2+}$ uptake were measured in the presence or absence of 10 μM KB-R7943; otherwise, details as in Fig. 2. Data are presented as mean ± S.E. (n = 3). *$p < .05$ versus wild-type NCX1.

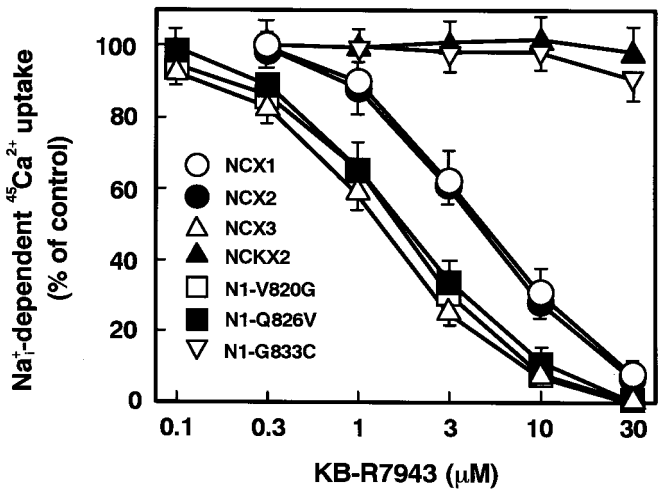

Fig. 5. Dose-response curves for the effects of KB-R7943 on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing wild-type NCX isoforms, several important NCX1 mutants, and NCKX2. The initial rates of $^{45}Ca^{2+}$ uptake were measured in the presence or absence of indicated concentrations of KB-R7943. Data are means ± S.E. of three independent experiments.

Discussion

In this study, taking advantage of our previous observation that KB-R7943 is 3-fold more effective in inhibiting NCX3 than NCX1 or NCX2 (Iwamoto and Shigekawa, 1998), we initially employed a chimera strategy to identify structural domain(s) of the exchanger responsible for the difference in

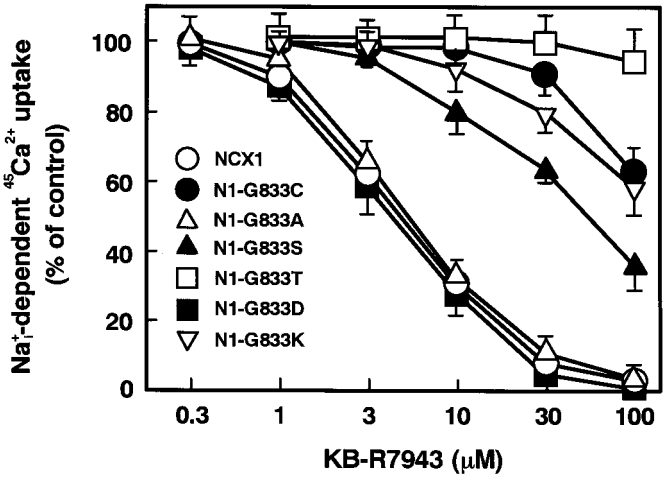

Fig. 6. Effects of multiple amino acid substitutions at Gly833 of NCX1 on dose-responses for KB-R7943. The initial rates of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing wild-type NCX1 and substitution mutants were measured in the presence or absence of indicated concentrations of KB-R7943; otherwise, details as in Fig. 2. Data are presented as means ± S.E. of three independent experiments.

the drug sensitivity of isoforms. Analyses with N1 and N3 chimeras, in which homologous segments were transferred individually or in combination from NCX3 into NCX1 and vice versa, revealed that a *Nhe*I-*Mlu*I segment corresponding to amino acids 788–829 of NCX1 or 777–818 of NCX3 was fully responsible for the difference in drug sensitivity between the two isoforms (Fig. 2). This segment contains a large fraction of the α-2 repeat that is highly conserved in all homologs of the Na^+/Ca^{2+} exchanger family (Schwarz and Benzer, 1997).

By exchanging four different amino acid residues of the α-2 repeat between NCX1 and NCX3 (Fig. 3A) and testing the resulting mutants for altered drug sensitivity, we found that NCX1 mutants N1-Q826V and N1-V820A/Q826V and NCX3 mutants N3-A809V and N3-A809V/V815Q were able to reproduce ~70 to 85% of the difference in KB-R7943 response observed between the parental exchangers (Fig. 3, B and C). This suggests that Val820 and Gln826 of NCX1 and the corresponding Ala809 and Val815 of NCX3 play predominant roles in generating the differential drug responses of exchangers. On the other hand, although other mutants N1-V820G and N3-A809I exhibited similar alterations in drug sensitivity, N1-V820A, N1-V820I, N3-A809G, and N3-V815Q-A were silent. Because these mutations were silent and because the effective mutations produced only a small (3-fold) change in drug sensitivity, it seems likely that Val820 and Gln-826 of NCX1 (and the corresponding residues in NCX3) do not interact directly with KB-R7943 with their mutations allosterically influencing binding of the drug to its receptor.

To identify other residues that possibly interact with KB-R7943 but cannot be detected in the chimera study, we screened cysteine-substituted NCX1 mutants of most residues in the α-2 repeat and about half of residues in the α-1 repeat for altered drug sensitivity. We found that N1-G833C alone exhibited a much-reduced affinity for the drug compared with the wild-type NCX1 (Fig. 4). We made multiple amino acid substitutions at this position with relatively small residues except Lys (Fig. 6). We found that Thr produced the largest effect on the IC_{50} value, increasing it by much more than 30-fold. Lys was almost as effective as Cys in reducing drug sensitivity, although Asp did not produce any effect. Interpretation of these data is not simple, because single factors, such as the residue size, do not seem to be able to explain them. The observed difference between Lys and Asp may suggest the involvement of electrostatic interaction and may be consistent with our previous report that the positive charge on the isothiourea moiety of KB-R7943 is important for the high inhibitory potency (Iwamoto et al., 1996b). The marked reduction in KB-R7943 sensitivity caused by mutation at Gly833 suggests that this residue may participate in the formation of the drug receptor.

Fig. 7 has provided strong pieces of evidence that KB-R7943 inhibits Na^+/Ca^{2+} exchanger only from the external side in intact CCL39 cells. We confirmed the same side-dependent effect using the *Xenopus laevis* oocyte expression system, in which up to 100 μM KB-R7943 was injected into oocytes or added extracellularly to examine its effect on Na^+_i-dependent $^{45}Ca^{2+}$ uptake (S. Kita, T. Iwamoto, and M. Shigekawa, unpublished observations). Such absence of effect of intracellular KB-R7943 was also observed in cardiomyocytes by Watano et al. (1996) (see under *Discussion* in this reference). However, we also suggested previously that the drug may inhibit the exchanger from the cytoplasmic side, because Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cardiac sarcolemmal vesicles with predominantly (~70%) inside-out orientation was inhibited almost completely by KB-R7943 (Iwamoto et al., 1996b). Consistent with the latter finding, the exchange current of NCX1 expressed in *X. laevis* oocytes, as measured using the giant excised patch technique, was reported to be inhibited by KB-R7943 applied to the cytoplasmic surface (Elias et al., 2000). Why the exchanger is inhibited from the external side in intact cells is unclear at present. One possibility could be that intimate interactions between the membrane and submembrane cytoskeleton existing in intact cells are disrupted in isolated membrane or excised patch preparations, which might result in alteration of the cytoplasmic surface of the exchanger, thereby exposing a binding site for the drug. Another possibility is that the intracellular drug concentration may not reach a level sufficient to inhibit the exchanger, possibly because of binding or sequestration of the drug by cytoplasmic proteins. Because

TABLE 1

Kinetic parameters as determined from double-reciprocal plot analyses of Ca^{2+}-dependences of the rate of Na^+_i-dependent Ca^{2+} uptake into cells expressing NCX1, NCX3, or NCX1 mutants in the presence or absence of KB-R7943.

The initial rates of $^{45}Ca^{2+}$ uptake were measured in the presence of 0.067 to 2 mM $CaCl_2$ and at 0 to 10 μM KB-R7943 and analyzed as shown previously (Iwamoto and Shigekawa, 1998). Values are presented as means ± S.E. (n = 3).

	Treatment	$K_{m(Ca)}$	V_{max}
		mM	*nmol/mg/30 s*
NCX1	Control	0.19 ± 0.03	20 ± 1.2
	KB-R7943 (3 μM)	0.23 ± 0.03	13 ± 1.0*
	KB-R7943 (10 μM)	0.38 ± 0.03*	9 ± 0.5*
N1-V820G	Control	0.21 ± 0.02	15 ± 0.7
	KB-R7943 (1 μM)	0.24 ± 0.02	11 ± 0.8*
	KB-R7943 (3 μM)	0.33 ± 0.03*	8 ± 0.7*
N1-Q826V	Control	0.22 ± 0.01	13 ± 1.0
	KB-R7943 (1 μM)	0.25 ± 0.04	10 ± 1.1
	KB-R7943 (3 μM)	0.31 ± 0.04*	7 ± 0.3*
N1-G833C	Control	0.20 ± 0.01	14 ± 0.8
	KB-R7943 (3 μM)	0.23 ± 0.04	12 ± 1.0
	KB-R7943 (10 μM)	0.21 ± 0.02	13 ± 0.5
NCX3	Control	0.18 ± 0.02	15 ± 1.3
	KB-R7943 (1 μM)	0.20 ± 0.03	10 ± 0.8*
	KB-R7943 (3 μM)	0.26 ± 0.02*	6 ± 0.4*

* $p < 0.05$ vs. control.

we perfused the cell interior with high concentrations of drug, we feel that the latter possibility is unlikely.

From substituted cysteine scanning analysis of NCX1 topology using the inhibition of exchange activity by thiol-modifying probes as the marker for accessibility (Karlin and Akabas, 1998), we found that cysteine at position 833 was accessible to internally but not externally applied 2-trimethylammonioethylmethane thiosulfonate, suggesting that Gly833 is exposed on the cytoplasmic side of the membrane (see Iwamoto et al., 1999a and also Fig. 1A). Val820 and Gln826 also seem to be located on the cytoplasmic side, because cysteines incorporated at positions 818, 821, 825, and 827 of NCX1 were accessible to internal 2-trimethylammonioethylmethane thiosulfonate (Iwamoto et al., 1999a). In our more recent study, we have found that D825C and N842C are also accessible to external smaller probes, transitional metal ions and 2-aminoethylmethane thiosulfonate, respectively (Iwamoto et al., 2000). It seems, therefore, that the C-terminal region of α-2 repeat is part of a reentrant membrane loop with both ends facing the cytoplasmic side. We speculate that the segment containing Gly833 undergoes alternate exposure to cytoplasmic or extracellular medium via the conformation change of the exchanger during Na^+/Ca^{2+} exchange. On the basis of a large reduction of drug sensitivity in N1-G833C or N1-G833T (Fig. 6) and the above topological consideration, we hypothesize that the segment containing Gly833 forms part of the drug receptor site or at least is near it. At present, however, we cannot rule out the possibility that mutations at Gly833 located on the cytoplasmic side may allosterically alter the conformation of the external KB-R7943 binding site, thereby reducing its affinity for the drug.

NCX isoforms exhibit difference in other pharmacological properties; divalent cation Ni^{2+} inhibits exchange activity of NCX1 or NCX2 10-fold more potently than that of NCX3, whereas stimulation of exchange by monovalent cation Li^+ is significantly greater in NCX2 and NCX3 than in NCX1 (Iwamoto and Shigekawa, 1998). Ni^{2+} and Mg^{2+} inhibit Na^+/Ca^{2+} exchange competitively with extracellular Ca^{2+} for the transport site (Kimura, 1996; Iwamoto and Shigekawa, 1998). Li^+, on the other hand, seems to accelerate the exchange by increasing the V_{max} (Iwamoto and Shigekawa, 1998). In our previous study, in which we used the same chimera strategy as that used here, we showed that residues corresponding to Asn125 and Thr127 in the α-1 repeat and Val820 in the α-2 repeat of NCX1 are involved in the generation of most of the difference in sensitivity to Ni^{2+} between NCX isoforms (Iwamoto et al., 1999b). In the same study, we also showed that residues corresponding to Val820 and Gln826 of NCX1 are responsible for the differential sensitivity of NCX isoforms to Li^+. Because Val820 and Gln826 of NCX1 were shown here to alter KB-R7943 affinity, it is intriguing to note that double substitution at positions 820 and 826 of NCX1 with corresponding residues from NCX3 (i.e., N1-V820A/Q826V) is able to generate simultaneous changes in responses to different modifiers.

As discussed briefly above, our recent topological study of NCX1 has provided evidence suggesting that the loop connecting the putative TM2 and TM3 in the α-1 repeat forms a re-entrant membrane loop, with both ends facing the extracellular side, whereas the C-terminal portion of the α-2 repeat also forms a reentrant membrane loop domain largely accessible from the cytoplasm (Iwamoto et al., 1999a, 2000) (see Fig. 1A). We have recently observed that site-directed mutations of highly conserved aspartic acids localized in the loop domains of α repeats (Asp130 in the α-1 repeat and Asp825 and Asp829 in the α-2 repeat) cause up to 6-fold reduction in the apparent affinity of NCX1 for the transport substrate extracellular Ca^{2+} (Iwamoto et al., 2000). Therefore, the important residues whose mutations alter apparent affinities of the exchanger for inhibitors (Ni^{2+} and KB-R7943), an activator (Li^+), and the transport substrate are all located in the putative loop domains of the α-1 and α-2 repeats. It seems, then, that the putative loop regions of α repeats may form parts of the ion translocation pathway of the exchanger. KB-R7943 seems to block this pathway by interacting with a receptor site in the α-2 repeat loop domain.

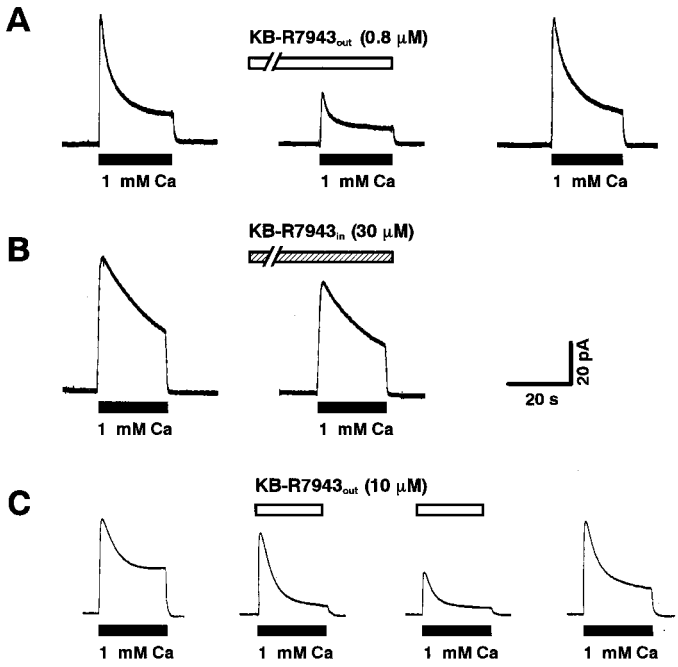

Fig. 7. Effect of externally or internally applied KB-R7943 on the whole-cell exchange current from cells expressing the wild-type NCX1. A, the outward current was evoked successively in the same cell by external application of 1 mM Ca^{2+}; the cell was placed in drug-free bath solution and then in bath solution containing 0.8 μM KB-R7943 for 6 min, followed by washing for 6 min with drug-free solution. B, a cell was perfused internally with drug-free pipette solution and then with 30 μM KB-R7943-containing solution for 15 min by the method of Soejima and Noma (1984). C, the outward current was evoked by 1 mM external Ca^{2+} for 20 s. At intervals of 30 s, the current was successively evoked by external application of 1 mM Ca^{2+} plus 10 μM KB-R7943. After washing for 5 min, the current was evoked again by 1 mM external Ca^{2+}. Similar results were obtained from three to five independent experiments.

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