Chimeric Analysis of Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchangers NCX1 and NCX3 Reveals Structural Domains Important for Differential Sensitivity to External Ni\textsuperscript{2+} or Li\textsuperscript{+}

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Externally applied Ni\textsuperscript{2+}, which apparently competes with Ca\textsuperscript{2+} in all three isoforms of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, inhibits exchange activity of NCX1 or NCX2 with a 10-fold higher affinity than that of NCX3, whereas stimulation of exchange by external Li\textsuperscript{+} is significantly greater in NCX2 and NCX3 than in NCX1 (Iwamoto, T., and Shigekawa, M. (1998) Am. J. Physiol. 275, C423–C430). Here we identified structural domains in the exchanger that confer differential sensitivity to Ni\textsuperscript{2+} or Li\textsuperscript{+} by measuring intracellular Na\textsuperscript{+} with Ca\textsuperscript{2+} uptake in CCL39 cells stably expressing NCX1/NCX3 chimeras or mutants. We found that two segments in the exchanger corresponding mostly to the internal α-1 and α-2 repeats are individually responsible for the alteration of Na\textsuperscript{+} sensitivity, both together accounting for ~80% of the difference between NCX1 and NCX3. In contrast, the segment corresponding to the α-2 repeat fully accounts for the differential Li\textsuperscript{+} sensitivity between the isoforms. The Ni\textsuperscript{2+} sensitivity was mimicked, respectively, by simultaneous substitution of two amino acids in the α-1 repeat (N125G/T127I in NCX1 and G159N/I161T in NCX3) and substitution of one amino acid in the α-2 repeat (V820A in NCX1 and A809V in NCX3). On the other hand, the Li\textsuperscript{+} sensitivity was mimicked by double substitution mutation in the α-2 repeat (V820A/Q826V in NCX1 and A809V/V815Q in NCX3). Single substitution mutations at Asn\textsuperscript{235} and Val\textsuperscript{820} of NCX1 caused significant alterations in the interactions of the exchanger with Ca\textsuperscript{2+} and Ni\textsuperscript{2+}, and Ni\textsuperscript{2+} and Li\textsuperscript{+}, respectively, although the extent of alteration varied depending on the nature of side chains of substituted residues. Since the above four important residues are mostly in the putative loops of the α repeats, these regions might form an ion interaction domain in the exchanger.

The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is an electrogenic transporter that catalyzes exchange of 3 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+} across the plasma membrane of many cell types. Previous studies indicate that it plays a primary role in the extrusion of cytosolic Ca\textsuperscript{2+} from cardiomyocytes, although its contribution in the Ca\textsuperscript{2+} handling in other cell types still remains to be precisely defined (1). The mammalian Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger forms a multigene family comprising three isoforms, NCX1, NCX2, and NCX3, which share ~70% identity in the overall amino acid sequences (2–4). On the basis of the hydropathy analysis, the mature Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger proteins were modeled to consist of 11 TMs and a large central hydrophilic loop between TM5 and TM6, with the N terminus localized on the extracellular side and the C terminus and the large central loop being on the intracellular side of the membrane (4, 5). Recent studies on the topology of the NCX1 polypeptide have produced data that are consistent with the N-terminal half of the 11 TM model (6–10), but these data do not support the C-terminal half of the model (9, 10), indicating that the model requires revision.

Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange occurs almost normally in a mutant exchanger deleted of the large central loop (11–13), indicating that the transmembrane domain alone is sufficient to catalyze ion transport. The large central loop is localized intracellularly (8, 14) and involved in the regulation of the exchanger by cytoplasmic Ca\textsuperscript{2+} (15, 16), cytoplasmic Na\textsuperscript{+} (17), ATP depletion (12, 13), and protein phosphorylation by protein kinase C (13, 18). In the transmembrane domain of all members of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger family, there are two highly conserved internal repeat sequences designated the α-1 and α-2 repeats that comprise most of TMs 2–3 and 8–9 and the loops connecting these TMs, respectively (5, 19). In such repeat sequences, the putative loop regions are more variable than the transmembrane segments. These homologous sequences may be functionally important, because mutations in the putative TMs within the α repeats cause a large reduction in exchange activity or a change in the I-V relationship in NCX1 (5). In addition, the Thr\textsuperscript{103} to Val mutation at the cytoplasmic end of TM2 in NCX1 produces changes in the apparent affinity for intracellular Na\textsuperscript{+} and the selectivity for Li\textsuperscript{+} (7). Therefore these repeat sequences might form part of the structure involved in the ion translocation in the exchanger molecule.

We have recently found that three mammalian exchanger isoforms have distinct differences in their biochemical and pharmacological properties (20). Divalent cation Ni\textsuperscript{2+} inhibits the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in NCX3 with a 10-fold less affinity than in NCX1 or NCX2. On the other hand, the recently identified inhibitor KB-R7943 is 3-fold more inhibitory

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1 The abbreviations used are: TM, transmembrane helix; I-V, current-voltage; BSS, balanced salt solution; BSA, bovine serum albumin; Na\textsuperscript{+}, intracellular Na\textsuperscript{+}; [Ca\textsuperscript{2+}]\textsubscript{i}, extracellular Ca\textsuperscript{2+} concentration; [Na\textsuperscript{+}]\textsubscript{i}, extracellular Na\textsuperscript{+} concentration; BAPTA, 1,2-bis-(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

2 For the sake of convenience, we use the 11 TM model when we refer to the putative transmembrane helices of the exchanger.
to NCX3 than to NCX1 or NCX2. Furthermore, stimulation of Na\(^+\)/Ca\(^{2+}\) exchange by externally added monovalent cation Li\(^+\) is significantly greater in NCX2 and NCX3 than in NCX1, although these isoforms exhibit low affinity for Li\(^+\). Despite these differences, however, all the NCX isoforms have similar apparent affinities for extracellular transport substrates Ca\(^{2+}\) and Na\(^+\) (20, 21). In this study, taking advantage of high sequence identity in the NCX isoforms, we used chimeric constructs between NCX1 and NCX3 to study the structural domain(s) responsible for the difference in their sensitivity to Ni\(^{2+}\) or Li\(^+\). We identified four amino acid residues within the \(\alpha\)-1 and \(\alpha\)-2 repeats of the exchanger molecule that are predominantly responsible for the observed differential effects of Ni\(^{2+}\) and Li\(^+\).

**Experimental Procedures**

**Cell Cultures—**CCL39 cells (American Type Culture Collection) and their NCX transfectants were maintained in Dulbecco’s modified Eagle’s medium supplemented with 7.5% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin.

**Construction and Stable Expression of Chimeric Exchangers and Mutants—**cDNAs of dog heart NCX1.1 and rat brain NCX3.3 were subcloned into SacI and HindIII sites in pCRII (designated pCRII-NCX1 and pCRII-NCX3, respectively) (18, 20). For construction of chimeras between NCX1 and NCX3, several unique restriction enzyme sites were newly introduced into pCRII-NCX1 and pCRII-NCX3 at analogous positions in the NCX coding regions by site-directed mutagenesis (19). In NCX1, the sites for BamHI, SalI, XmaI, and NheI were created at amino acid positions 108, 133, 192, and 787 (numbers based on Ref. 22), respectively, whereas in NCX3, the sites for BamHI, SalI, XmaI, NheI, and MluI were inserted at positions 142, 167, 226, 776, and 518 (numbers based on Ref. 4), respectively. Generation of these sites preserved the native amino acid sequences. The modified plasmids were designated pCRII-NCX1 and pCRII-NCX3, NCX1/NCX3 chimera, except for N1-718/787 and N3-707/776, were constructed by exchanging homologous segments from pCRII-NCX1 and pCRII-NCX3 using the above newly introduced restriction enzyme sites and endogenous Clal sites (amino acid positions 445 in NCX1 and 469 in NCX3) as shown in Fig. 1. N1-718/787 and N3-707/776 chimeras were constructed after creating new unique sites for EcoRV at positions 718 and 707 in pCRII-NCX1 and pCRII-NCX3, respectively (Fig. 1), which resulted in substitution of one amino acid (V719I in N1-718/787 and V708I in N3-707/776). Successful construction of the modified cDNAs was verified by sequencing (ABI PRISM, Perkin-Elmer). These cDNAs were transferred into SacII and HindIII sites of the mammalian expression vector pKCRH (23).

Substitution of amino acid residues within the internal \(\alpha\) repeat regions was performed by site-directed mutagenesis. In this procedure, DNA fragments were produced by polymerase chain reaction using pCRII-NCX1 or pCRII-NCX3 as a template and following pairs of primers: for mutation in the \(\alpha\)-1 repeat, the sense primers contained an exogenous BamHI site and normal NCX sequences, whereas the antisense primers contained sequences with substituted nucleotides and an exogenous SacII site; for mutation in the \(\alpha\)-2 repeat, the sense primers contained an exogenous Nhel site and normal NCX sequences, while the antisense primers contained an exogenous MluI site and sequences with desired mutations. The polymerase chain reaction products were digested with either BamHI and SalI or NheI and MluI, and inserted into pCRII-NCX1’ or pCRII-NCX3’, and then the full-length mutant cDNAs were transferred into pKCRH. Successful construction was verified by sequencing.

To stably express chimeric and mutant exchangers, pKCRH plasmids carrying NCX cDNAs were transfected in the presence of Lipofectin (Life Technologies, Inc.) into CCL39 fibroblasts that exhibit little endogenous Na\(^+/Ca^{2+}\) exchange activity (13, 20, 24). Cell clones expressing high Na\(^+\)/Ca\(^{2+}\) exchange activity were selected by a Ca\(^{2+}\)-killing procedure as described previously (24).

**Assay of Na\(^+\)-dependent Ca\(^{2+}\) Uptake—**Assay of Na\(^+\)-dependent Ca\(^{2+}\) uptake into cells were described in detail previously (20). Briefly, confluent NCX transfectants in 24-well dishes were loaded with Na\(^+\) by incubation at 37 °C for 30 min in 0.5 ml of 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 mM monensin. 45Ca\(^{2+}\) 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 45CaCl\(_2\) (1.5 µCi/ml) and 1 mM ouabain. After a 30-s incubation, 45Ca\(^{2+}\) 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% NaOH, and aliquots were taken for determination of radioactivity and protein.

**Measurement of Whole Cell Exchange Currents—**Outward and inward currents from NCX transfectants were measured using the whole cell voltage clamp technique as described previously (25). For recording the outward current, the external solution contained 150 mM NaCl, 1 mM MgCl\(_2\), 0 mM CaCl\(_2\), 2 mM ouabain, 2 µM ryanodine, and 5 mM Hepes (pH 7.2), whereas the pipette solution contained 20 mM NaCl, 90 mM CsOH, 40 mM aspartic acid, 3 mM MgCl\(_2\), 10 mM CaCl\(_2\), 5 mM MgATP, 5 mM KCl, 20 mM BAPTA, and 20 mM Hepes (pH 7.2). The ionized Ca\(^{2+}\) concentration in the pipette solution was calculated to be 0.14 µM. The outward exchange current was activated by switching the external solution from one without CaCl\(_2\) to one with CaCl\(_2\). For recording the inward current, the external solution contained 140 mM choline chloride or NaCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 20 mM ouabain, 2 µM ryanodine, 10 µM nicardipine, and 5 mM Hepes (pH 7.2), while the pipette solution contained 30 mM CsCl, 90 mM CsOH, 50 mM aspartic acid, 3 mM MgCl\(_2\), 16 mM CaCl\(_2\), 5 mM MgATP, 5 mM KCl, 20 mM BAPTA, and 20 mM Hepes (pH 7.2). The ionized Ca\(^{2+}\) concentration in the pipette solution was calculated to be 1.75 µM. The inward exchange current was induced by switching the choline chloride-containing external medium to the Na\(^+\)-containing external medium. 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 (Axon Instrument) software.

**Statistical Analysis—**Data are expressed as mean ± S.E. of three to five independent determinations. Differences for multiple comparisons were analyzed by unpaired \(t\) test or one-way ANOVA followed by the Dunnett’s test. Values of \(p < 0.05\) were considered statistically significant.

**Chimeric Analysis of Na\(^+\)/Ca\(^{2+}\) Exchanger Function**

**FIG. 1. Schematic representation of chimeric constructs between NCX1 and NCX3.** Two series of chimeras (N1 and N3 series) 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. The restriction enzyme cut sites are shown by broken lines and the substituted segments were indicated by black boxes. The numbered shaded boxes at the top and bottom show the positions of TMs in the exchanger predicted on the basis of the hydropathy analysis.
RESULTS

Inhibition of Exchange Activities of NCX1 and NCX3 by Ni$^{2+}$—We previously measured the whole cell current from cloned dog cardiac NCX1 expressed in CCL39 cells using a conventional patch-clamp technique (25). Using the same method, we measured the outward and inward currents evoked in NCX1- or NCX3-transfected CCL39 cells by the extracellular application of 2 mM Ca$^{2+}$ or 140 mM Na$^+$ (Fig. 2, A and B). These currents were reproducibly measured; after a recovery interval, a second pulse of Ca$^{2+}$ or Na$^+$ generated the outward or inward current whose peak value was 96–102% of the corresponding first current. However, they were never observed in nontransfected CCL39 cells. Inclusion of Ni$^{2+}$ in the external medium caused inhibition of the outward and inward currents (Fig. 2, A and B). We found that the outward current in NCX1-transfected cells was approximately 10-fold more sensitive to Ni$^{2+}$ than that in NCX3-transfected cells (IC$_{50}$, 39 versus 310 μM) and that the inhibition in these cells reached 92 and 81%, respectively, at 3 mM Ni$^{2+}$ (Fig. 2C). A similar difference in the sensitivity to Ni$^{2+}$ was observed for the inward currents in NCX1- and NCX3-transfected cells (IC$_{50}$, 25 versus >100 μM), although its inhibition by Ni$^{2+}$ occurred at much greater concentrations compared with the outward current (Fig. 2, C and D).

Our previous measurement of Ni$^{2+}$ dependence of Na$^+$-dependent $^{45}$Ca$^{2+}$ uptake gave IC$_{50}$ values of 33 and 343 μM for Ni$^{2+}$ in NCX1- and NCX3-transfected CCL39 cells, respectively (Ref. 20, see also Fig. 3, inset), in agreement with the observed inhibitory potencies of Ni$^{2+}$ on the outward current. Thus both electrophysiological and biochemical data establish that NCX1 and NCX3 exhibit a 10-fold difference in the sensitivity to Ni$^{2+}$, at least when they function in the reverse exchange mode.

NCX Structural Domains Important for Determination of Ni$^{2+}$ Sensitivity—Taking advantage of the fact that NCX1 and NCX3 exhibit high amino acid homology, we used chimeric constructs between the two isoforms to study the structural domain(s) responsible for the difference in their sensitivity to Ni$^{2+}$. Using endogenous or newly introduced common restriction enzyme sites, we constructed two series of chimeric cDNAs in which one or two segments from NCX3 were incorporated into NCX1 in exchange for the homologous segment(s) in the latter (N1 series chimeras), and vice versa (N3 series chimeras) (see Fig. 1). We stably expressed these chimeras in CCL39 fibroblasts. All transfectants, after selection by a Ca$^{2+}$-killing procedure, exhibited exchange activities similar to that of cells expressing the wild-type NCX1 or NCX3 (see the legend to Fig. 3).

We examined the effect of 0.1 mM Ni$^{2+}$ on the initial rate of Na$^+$-dependent $^{45}$Ca$^{2+}$ uptake into cells expressing these chimeric exchangers (Fig. 3). Under the conditions used, Ni$^{2+}$ at this concentration reduced the uptake rate of the wild-type NCX1 or NCX3 by 77 or 24%, respectively. N1 series chimeras N1-109/133, N1-788/829, and N1-109/133,788/829, which contained the homologous BamHI-SalI and/or NheI-MluI segments from NCX3 (Fig. 1), exhibited significantly less sensitivity to inhibition by 0.1 mM Ni$^{2+}$ compared with the wild-type
NCX1 (Fig. 3A). In contrast, N3 series chimeras N3-143/167, N3-777/818, and N3-143/167,777/818 containing the BamHI-SalI and/or NheI-MluI segments from NCX1 (Fig. 1) were more sensitive to inhibition by Ni\textsuperscript{2+} compared with the wild-type NCX3 (Fig. 3B). All other chimeras, however, were not significantly different from the parental NCX1 or NCX3. For some of those chimeras with an altered sensitivity to Ni\textsuperscript{2+}, we determined dose-response profiles for Ni\textsuperscript{2+} by measuring the rate of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (Fig. 4). We next exchanged Leu, Val, Thr, and Gln in the \alpha\textsuperscript{1}- and \alpha\textsuperscript{2}-repeats (see Fig. 1) (19). Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange has been shown to be highly sensitive to mutagenesis in the putative transmembrane helices within the \alpha\textsuperscript{1}-repeats (5), suggesting the functional importance of these regions.

**Amino Acids Residues Involved in Differential Sensitivity to Ni\textsuperscript{2+}**—Fig. 4 shows amino acid sequences of the \alpha\textsuperscript{1} and \alpha\textsuperscript{2} repeats from NCX1, NCX2, and NCX3. Of note, in these regions, there are only a small number of amino acids unique to each isoform, all of which are localized within the BamHI-SalI and NheI-MluI segments. To identify the residues involved in the differential Ni\textsuperscript{2+} sensitivity, these unique residues in the \alpha\textsuperscript{1} repeats were exchanged between NCX1 and NCX3 or mutated to other amino acids (Fig. 5). Three amino acids Val, Asn, and Thr in the \alpha\textsuperscript{1} repeat of NCX1 (corresponding to Val\textsuperscript{118}, Asn\textsuperscript{125}, and Thr\textsuperscript{127}) were replaced with Leu, Gly, and Ile from the same region of NCX3 (corresponding to Leu\textsuperscript{119}, Gly\textsuperscript{126}, and Ile\textsuperscript{161}), respectively, and vice versa. We found that single substitution mutants were not different from the parental NCX1 or NCX3 in their sensitivity to 0.1 mM Ni\textsuperscript{2+} (Fig. 5, A and B). On the other hand, double substitution mutants N1-N125G/T127I and its reciprocal mutant N3-G159N/I161T showed decreased and increased sensitivities to inhibition by Ni\textsuperscript{2+}, respectively, which are comparable to those seen in N1-109/133 and N3-143/167 chimeras (compare Figs. 3 and 5). Double mutants N1-V118L/N125G and N1-V118L/T127I, however, exhibited Ni\textsuperscript{2+} sensitivity similar to that of NCX1 (data not shown). Thus, Asn\textsuperscript{125} and Thr\textsuperscript{127} in the \alpha\textsuperscript{1}-repeat of NCX1 and corresponding Gly\textsuperscript{159} and Ile\textsuperscript{161} in NC3 are responsible for the altered responses of N1-109/133 and N3-143/167 to Ni\textsuperscript{2+}. We also examined the Ni\textsuperscript{2+} sensitivity of NCX1 mutants with Asn\textsuperscript{125} to Cys (N1-N125G) or Thr\textsuperscript{127} to Cys substitution (N1-T127C). Interestingly, N1-N125C, but not T127C, exhibited an increased sensitivity to inhibition by Ni\textsuperscript{2+} relative to that of the parental NCX1 (Fig. 6A).

We next exchanged Leu, Val, Thr, and Gln in the \alpha\textsuperscript{2}-repeat of NCX1 (corresponding to Leu\textsuperscript{152}, Val\textsuperscript{169}, Thr\textsuperscript{179}, and Gln\textsuperscript{186}), respectively, with Phe, Ala, Leu, and Val (corresponding to Phe\textsuperscript{155}, Ala\textsuperscript{162}, Leu\textsuperscript{172}, and Val\textsuperscript{189}) of NCX3. Five NCX1 and NCX3 single substitution mutants (N1-L808F, N1-T823L, N1-Q826V, N3-F797L, and N3-L812T) exhibited Ni\textsuperscript{2+} sensitivity not different from the parental NCX1 or NCX3 (Fig. 5, A and B). In contrast, N1-V820A and its reciprocal mutant N3-A809V showed decreased and increased sensitivities to inhibition by 0.1 mM Ni\textsuperscript{2+} compared with NCX1 and NCX3, respectively, whereas N3-V815Q exhibited a slightly increased inhibition by Ni\textsuperscript{2+} relative to NCX3. Double substitution mutants N1-V820A/Q826V and N3-A809V/V815Q, on the other hand, exhibited Ni\textsuperscript{2+} sensitivities similar to single mutants N1-V820A

![Diagram](image-url)
and N3-A809V, respectively. Thus Val820 of NCX1 and Ala809 of NCX3, and probably Val815 of NCX3, are involved in the determination of Ni2+ sensitivity in the α-2 repeat.

We found that the Val820 to Ile substitution in NCX1 (N1-V820I) and the corresponding Ala809 to Ile substitution in NCX3 (N3-A809I) resulted in an increase in the sensitivity to inhibition by Ni2+, whereas substitution of these residues with Gly resulted in a decrease in the Ni2+ sensitivity in N1-V820G, but not in N3-A809G (Fig. 5, A and B). Other mutants N1-Q826E and N1-Q826R and the corresponding mutants N3-V815E and N3-S815K showed the Ni2+ sensitivity not different from that of the parental NCX1 or NCX3.

Structural Domains and Amino Acids Residues Involved in Li+-induced NCX Activation—Monovalent cation Li+ stimulates Na+-dependent ⁴⁴Ca²⁺ uptake by all three NCX isoforms with low affinity, with the extent of stimulation being greater in NCX2 or NCX3 than in NCX1 (20). To obtain insight into the structural domains involved in the Li+-induced NCX activation, we examined the effect of Li+ on the exchange activities of the chimeras and mutants described above. Consistent with the previous result, extracellular application of Li+ caused a dose-dependent increase in the rate of Na+-dependent Ca²⁺ uptake into NCX1- or NCX3-transfected cells, which reached about 145 and 270% at 146 mM Li⁺, respectively, of the control measured in the absence of Li+ (Fig. 6, inset). Fig. 6, A and B, show the extent of stimulation of the uptake by various chimeras and mutants at 146 mM Li⁺. Intriguingly, N1-788/829 and N1-109/133,788/829 containing a major portion of the α-2 repeat from NCX3 produced NCX1 mutants exhibiting Li⁺ sensitivity almost identical to that of the wild-type NCX3. Conversely, the reciprocal mutants N3-777/818 and N3-143/167,777/818 exhibited Li⁺ sensitivity similar to that in the wild-type NCX1. On the other hand, N1 and N3 chimeras were not different from the wild-type NCX1 or NCX3, respectively. Thus the α-2 repeat seems to be exclusively responsible for the differential stimulation by Li⁺.

We examined the effect of substitution of four unique amino acids in the α-2 repeat on the response of NCX1 or NCX3 to 146 mM Li⁺ (Fig. 6). Single substitution mutants N1-L808F, N1-V820A, N1-T823L, N1-Q826V, N3-F797I, N3-A809V, N3-L812T, and N3-V815Q exhibited Li⁺ sensitivity not different from that of each parental exchanger (Fig. 6, A and B). On the other hand, double substitution mutant N1-V820A/Q826V and its reciprocal mutant N3-A809V/V815Q showed Li⁺-dependent stimulation similar to those of the wild-type NCX3 and NCX1, respectively. Thus both Val820 and Gln826 of NCX1 and Ala809 and Val815 of NCX3 are responsible for the different response of the exchanger to Li⁺.

Interestingly, 146 mM Li⁺ decreased Na⁺+-dependent ⁴⁴Ca²⁺ uptake by N1-V820I to a level below that of the parental NCX1 without Li⁺ (Fig. 6A). The corresponding NCX3 mutant N3-A809I also exhibited significantly less stimulation by Li⁺ than NCX3 (Fig. 6B). In contrast, the uptake rate was stimulated to a greater extent in N1-V820G and the corresponding N3-A809G than the parental NCX1 or NCX3 (Fig. 6, A and B).

Kinetic Properties of NCX1 α Repeat Mutants—In Figs. 5 and 6, we screened for the α-repeat mutants exhibiting altered sensitivity to a single dose of Ni²⁺ (0.1 mM) or Li⁺ (146 mM). In Fig. 7, A and B, we measured the dose-response profiles for Ni²⁺ or Li⁺ of NCX1 α-repeat mutants N1-N125C, N1-N125G/T127I, N1-V820I, and N1-V820A/Q826V in the presence of 0.1 mM Ca²⁺. The IC₅₀ values for Ni²⁺ were estimated to be 98 ± 6 μM for N1-N125G/T127I, 82 ± 2 μM for N1-V820A/Q826V, 33 ± 3 μM for NCX1, 15 ± 3 μM for N1-V820I, and 10 ± 1 μM for N1-N125C (Fig. 7A). Thus, Ni²⁺ was more inhibitory to N1-N125C and N1-V820I, but was less inhibitory to N1-N125G/T127I and N1-V820A/Q826V, than to NCX1. We found that the dose-response profile of N1-V820A/Q826V for Ni²⁺ was very similar to that of N1-V820A (data not shown). Li⁺, on the other hand, stimulated Ca²⁺ uptake by all these mutants with half-maximal stimulation at about 30 mM, except for N1-V820I (Fig. 7B). The dose-response profiles of N1-N125C and N1-N125G/T127I for Li⁺ were of a NCX1 type, whereas the response of N1-V820A/Q826V to Li⁺ was of a NCX3 type. The dose-response relation of N1-V820A for Li⁺ was of a NCX1 type (data not shown). Interestingly, in N1-V820I, Li⁺ exerted dual effects, slightly stimulating at low concentrations (10–30 mM) and inhibiting at high concentrations (146 mM) (Fig. 7B).

We measured the initial rates of Na⁺+-dependent ⁴⁴Ca²⁺ uptake by NCX1 α-repeat mutants as a function of [Ca²⁺]o. Double-reciprocal plots of uptake rates versus [Ca²⁺]o were all linear for the wild-type NCX1, N1-N125C, N1-N125G/T127I, N1-V820I, N1-V820A/Q826V, and the wild-type NCX3 in the presence or absence of Ni²⁺, Na⁺, or Li⁺ (Fig. 8A, and data not shown), suggesting preservation of a single Ca²⁺-binding site in each mutant under the conditions used. Values of Km(Ca) and...
Fig. 7. Dose-response curves for the effect of Ni2+ or Li+ on Na+-dependent 45Ca2+ uptake into cells expressing several important NCX1 mutants. The initial rates of Na+-dependent 45Ca2+ uptake were measured in the presence or absence of the indicated concentrations of Ni2+ (A) or Li+ (B). In B, the uptake was measured in Na+-free medium in which the LiCl concentration was varied while maintaining LiCl plus choline chloride at 146 mM. Data are presented as percentage of the values obtained in the absence of Li+. Data are means of three independent experiments.

Fig. 8. Kinetic analyses of the effect of Ni2+ on [Ca2+]o-dependence of Na+-dependent 45Ca2+ uptake into cells expressing wild-type NCX1 or N1-N125C. A, initial rates of Na+-dependent 45Ca2+ uptake into cells expressing wild-type NCX1 or N1-N125C were measured in the presence of 0.067 to 2 mM CaCl2 and in the absence of Ni2+ (○) or presence of 30 (●) or 60 μM (△) Ni2+. The data are shown in a double-reciprocal plot. B, the data in A are presented in the Dixon plot. The CaCl2 concentrations used were 0.067 (○), 0.1 (●), 0.2 (△), and 0.4 mM (▲). Straight lines were drawn using the least-square method.

$V_{\text{max}}$ estimated from these plots are summarized in Table I. An important finding is that in the absence of Ni2+, Na+, or Li+, the $K_{m(\text{Ca})}$ values in these mutants were not different from that of the wild-type NCX1, except for N1-N125C. Other mutants such as N1-N125G, N1-T127C, N1-V820A, and N1-Q826V also had $K_{m(\text{Ca})}$ values similar to that of the wild-type NCX1 (data not shown). In N1-N125C, however, the $K_{m(\text{Ca})}$ increased 3-fold ($p < 0.05$) compared with NCX1 (Table I).

External Na+ increased $K_{m(\text{Ca})}$ values in these NCX1 α repeat mutants (Table I), consistent with the fact that Na+ is transported by the exchanger as a substrate, although like Li+, it also functions as a stimulating monovalent cation at relatively low concentrations (20, 26–28). Since N1-N125C exhibited a decreased affinity for Ca2+ compared with NCX1 (see above), we measured the inward exchange currents evoked in N1-N125C-transfected cells as a function of [Na+]i. We found a 1.4-fold increase ($p < 0.05$) in the $K_{m(\text{Na})}$ in N1-N125C compared with the wild-type NCX1 (Fig. 9).

Application of Ni2+ increased $K_{m(\text{Ca})}$ without influencing $V_{\text{max}}$ in the NCX1 α repeat mutants (Fig. 8A, Table I, and data not shown). We used the Dixon plot to identify the type of inhibition for Ni2+ and determine its intrinsic inhibition constant ($K_{i(Ni)}$). The obtained profiles of Dixon plots were consistent with the view that Ni2+ competes with Ca2+ for the same site (Fig. 8B and data not shown). $K_{i(Ni)}$ values calculated from the Dixon plots are summarized in Table II. N1-N125C and N1-V820I showed a 3-fold decrease in the $K_{i(Ni)}$ compared with wild-type NCX1, whereas N1-N125GT127I and N1-V820A/Q826V exhibited a 4-fold increase. We found that N1-V820A had a $K_{i(Ni)}$ value (40 μM) similar to that for N1-V820A/Q826V.

Li+ at 146 mM, on the other hand, did not significantly affect $K_{m(\text{Ca})}$ in NCX1, N1-N125C, N1-N125GT127I, N1-V820A, or N1-Q826V, and NCX3, whereas it increased $V_{\text{max}}$ for these exchangers by 35, 48, 35, 119, and 143%, respectively (Table I). In N1-V820I, however, Li+ increased $K_{m(\text{Ca})}$ by 5-fold and $V_{\text{max}}$ by 44%. In this mutant, therefore, Li+ markedly decreased the apparent Ca2+ affinity, although the Li+ stimulation remained similar to that in NCX1.

**DISCUSSION**

Previous studies have demonstrated that external divalent and trivalent cations inhibit Ca2+ transport by Na+/Ca2+ ex-

| Cations          | $K_{m(\text{Ca})}$ (μM) | $V_{\text{max}}$ (nmol/mg/30 s) |
|------------------|-------------------------|---------------------------------|
| NCX1             | Control                 | 0.20 ± 0.03                      | 23 ± 1.2  |
|                  | Ni2+ (60 μM)            | 0.74 ± 0.02                      | 20 ± 1.5  |
|                  | Na+ (50 mM)             | 0.41 ± 0.03                      | 25 ± 1.5  |
|                  | Li+ (146 mM)            | 0.27 ± 0.05                      | 31 ± 2.9  |
| N1-N125C         | Control                 | 0.58 ± 0.05                      | 25 ± 1.8  |
|                  | Ni2+ (60 μM)            | 4.35 ± 0.50                      | 25 ± 1.5  |
|                  | Na+ (50 mM)             | 1.66 ± 0.15                      | 25 ± 2.8  |
|                  | Li+ (146 mM)            | 0.70 ± 0.09                      | 34 ± 2.6  |
| N1-N125GT127I    | Control                 | 0.23 ± 0.02                      | 17 ± 1.5  |
|                  | Ni2+ (60 μM)            | 0.38 ± 0.03                      | 18 ± 1.1  |
|                  | Na+ (50 mM)             | 0.42 ± 0.04                      | 19 ± 2.1  |
|                  | Li+ (146 mM)            | 0.27 ± 0.09                      | 23 ± 1.8  |
| N1-V820I         | Control                 | 0.24 ± 0.02                      | 16 ± 1.0  |
|                  | Ni2+ (60 μM)            | 1.45 ± 0.07                      | 14 ± 1.1  |
|                  | Na+ (50 mM)             | 1.06 ± 0.08                      | 16 ± 2.2  |
|                  | Li+ (146 mM)            | 1.46 ± 0.10                      | 23 ± 1.9  |
| N1-V820A/Q826V   | Control                 | 0.18 ± 0.05                      | 16 ± 2.0  |
|                  | Ni2+ (60 μM)            | 0.35 ± 0.04                      | 15 ± 1.7  |
|                  | Na+ (50 mM)             | 0.38 ± 0.03                      | 18 ± 2.3  |
|                  | Li+ (146 mM)            | 0.20 ± 0.03                      | 35 ± 3.1  |
| NCX3             | Control                 | 0.15 ± 0.02                      | 14 ± 1.3  |
|                  | Ni2+ (60 μM)            | 0.24 ± 0.05                      | 15 ± 1.5  |
|                  | Na+ (50 mM)             | 0.53 ± 0.06                      | 17 ± 2.3  |
|                  | Li+ (146 mM)            | 0.19 ± 0.02                      | 34 ± 1.6  |

$a p < 0.05$ versus control.
experiments. Data were fitted using Hill equation, which gave \( K_m \) for the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \) in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.

Inward currents in cells expressing wild-type NCX1 or N1-N125C. Inward currents from cells expressing the wild-type NCX1 (○) or N1-N125C (●) were induced by external application of different concentrations (8–140 mM) of \( \text{Na}^+ \), in the presence of nominally free \( \text{Ca}^{2+} \). The peak amplitudes of the measured currents were normalized to those induced by 140 mM \( \text{Na}^+ \). Each point is the mean ± S.E. of three or four independent experiments. Data were fitted using Hill equation, which gave \( K_m \) values and Hill coefficients of 41 ± 0.79 mM and 1.9 ± 0.08 for NCX1 and 57 ± 2.1 mM and 1.6 ± 0.08 for N1-N125C, respectively.

**Table II**

|               | K\(_{i(Ni)}\)   |
|---------------|---------------|
| NCX1          | 13 ± 1.2      |
| N1-N125C      | 5.3 ± 0.78    |
| N1-N125G/T1271| 51 ± 3.6      |
| N1-V820I      | 4.0 ± 0.15    |
| N1-V820A/Q826V| 46 ± 2.4      |
| NCX3          | 125 ± 9.1     |

\(^* p < 0.05\) versus NCX1.
increased 3–4-fold in these mutants compared with NCX1 (Table II and “Results”). In contrast, among these mutants, N1-V820A/Q826V alone exhibited an enhanced response to Li+ (Fig. 7B and “Results”). Taken together, the results indicate that in NCX1, the Asn125 to Gly substitution produced no evidence for the involvement of Asn125 and Thr127 in the absence of Li+. In contrast, Glu826 to Val mutation was able to enhance Li+ stimulation when Val820 was simultaneously mutated to Ala (Figs. 6A and 7B). In addition, Glu826 could potentially influence Ni2+ affinity, since the NCX3 single mutant N3-V815Q exhibited a slightly but significantly higher sensitivity to inhibition by Ni2+ relative to the wild-type NCX3 (Fig. 5B). Val815 in NCX3 is equivalent to Glu826 in NCX1.

All these results show that in NCX1, mutations of Asn125 and Thr127 in the α-1 repeat and of Val820 and Glu826 in the α-2 repeat are able to cause alteration in the interaction of the exchanger with Ca2+, Ni2+, and Li+, respectively. Since all these residues do not appear to serve as direct ligands for cations (see above) and since the nature of the side chains of substituted residues appears to determine the type of the cation affected, it is likely that mutations at these residues are able to induce conformational changes in the neighboring cation-binding sites, thereby influencing the interaction of the latter with externally applied cations. However, involvement of a long-range allosteric effect also cannot be ruled out.

At present, we have little information about the location of the binding sites for Ca2+, Ni2+, and Li+, and the amino acids directly liganding these cations. Interestingly, the four amino acid residues identified here are mostly localized in the putative loops in the α repeats. By substituted cysteine accessibility mapping of the NCX1 polypeptide using the inhibition of exchange activity of the mutants by membrane-impermeant SH-labeling reagent as a marker, we have recently provided evidence suggesting that the loop in the α-1 repeat forms a re-entrant membrane loop with both ends facing the extracellular side and at least one of the residues (Asn125) being accessible from the inside, while the corresponding region in the α-2 repeat is mostly exposed on the cytoplasmic side (10). The opposite membrane orientation of the α-1 and α-2 repeats has also been suggested by Nicoll et al. (9). The same latter group has recently reported that Cys151 and Thr815 (or Ala821) within the α-1–2 repeats are localized close to each other in space in NCX1 (32). These structural data appear to be consistent with a hypothesis that the putative loop region of each α repeat has an opposite orientation and is relatively close to each other within the membrane bilayer, possibly forming an ion interaction domain within the ion transport pathway of the exchanger molecule.

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Chimeric Analysis of Na\(^+\)/Ca\(^{2+}\) Exchanger Function

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