Residues Contributing to the Ca$^{2+}$ and K$^+$ Binding Pocket of the NCKX2 Na$^+$/Ca$^{2+}$-K$^+$ Exchanger*

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The Na$^+$/Ca$^{2+}$-K$^+$ exchanger (NCKX) extrudes Ca$^{2+}$ from cells utilizing both the inward Na$^+$ gradient and the outward K$^+$ gradient. NCKX is thought to operate by a consecutive mechanism in which a cation binding pocket accommodates both Ca$^{2+}$ and K$^+$ and alternates between inward and outward facing conformations. Here we developed a simple flurometric method to analyze changes in K$^+$ and Ca$^{2+}$ dependences of mutant NCKX2 proteins in which candidate residues within membrane-spanning domains were substituted. The largest shifts in both K$^+$ and Ca$^{2+}$ dependences compared with wild-type NCKX2 were observed for the charge-conservative substitutions of Glu188 and Asp548, whereas the size-conservative substitutions resulted in nonfunctional proteins. Substitution of several other residues including two proline residues (Pro$^{187}$ and Pro$^{547}$), three additional acidic residues (Asp$^{258}$, Glu$^{265}$, Glu$^{538}$), and two hydroxyl-containing residues (Ser$^{185}$ and Ser$^{545}$) showed smaller shifts, but shifts in Ca$^{2+}$ dependence were invariably accompanied by shifts in K$^+$ dependence. We conclude that Glu$^{188}$ and Asp$^{548}$ are the central residues of a single cation binding pocket that can accommodate both K$^+$ and Ca$^{2+}$. Furthermore, a single set of residues lines a transport pathway for both K$^+$ and Ca$^{2+}$.

The gene families of the SLC8 Na$^+$/Ca$^{2+}$ exchangers (NCX)$^1$ and the SLC24 Na$^+$/Ca$^{2+}$-K$^+$ exchangers (NCKX) comprise proteins that use the inward Na$^+$ gradient (NCX) or both the inward Na$^+$ gradient and the outward K$^+$ gradient (NCKX) to drive Ca$^{2+}$ extrusion from cells that show dynamic Ca$^{2+}$ fluxes (1, 2). The Na$^+$/Ca$^{2+}$-K$^+$ exchanger was originally described as a plasma membrane protein in the outer segments of retinal rod photoreceptors (3, 4), where it is the only export mechanism that drives Ca$^{2+}$ extrusion from cells that show dynamic Ca$^{2+}$ fluxes (1, 2).

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The abbreviations used are: NCX, Na$^+$/Ca$^{2+}$ exchanger; NCKX, Na$^+$/Ca$^{2+}$-K$^+$ exchanger; DMEM, Dulbecco’s modified Eagle’s medium; HEDTA, N-(2-hydroxyethyl)ethylenediamine-N,N’-N’-triacetic acid; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; HEK, human embryonic kidney cells.

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dences of the NCKX2 protein. We developed a sensitive fluorometric assay for NCKX function in mammalian cell lines permitting measurements of cation dependence of NCKX2 mutant proteins with greatly reduced maximal transport rates. Our results show that substitution of several of the residues highlighted in Fig. 1B resulted in parallel changes in K⁺ and Ca²⁺ concentration dependences of the mutant NCKX2 proteins expressed in HEK293 cells.

MATERIALS AND METHODS

Mutagenesis and Protein Analysis— Codons for selected residues were mutagenized in the Myc-tagged short splice variant of human NCKX2 cDNA (AAF25811), and NCKX function of the mutant NCKX2 proteins was analyzed after heterologous expression with the pEIA vector in an insect cell line (High Five cells) as described before (9). Wild-type and mutant NCKX2 proteins expressed in cell lines were detected by Western blotting with the mononclonal Myc antibody (NEB Biolabs, Pickering, Ontario) after separation on SDS-PAGE as described before (20).

Functional Analysis with ⁴⁵Ca Uptake— Insect High Fives were transiently transfected with the various NCKX2 mutants. Na⁺ loaded using the ionophore monensin and washed as described before (9). The final cell leakage limited the amount of time available to sequential measurement of Fluo-3 from the cells. Even in the presence of sulfinpyrazone, dye filters; the ice-cold washing medium contained 140 mM KCl, 80 mM sucrose, 20 mM Hepes, pH 7.4, and 0.05 mM EDTA. NaCl, KCl, LiCl, and choline chloride were all SigmaUltra grade (Sigma-Aldrich). Further details of the ⁴⁵Ca uptake method have been described before (9). Protein content of cell samples was determined with the Bio-Rad protein assay.

Functional Analysis with Fluo-3— Human embryonic kidney (HEK293) cells were subcultured in DMEM (Invitrogen, Burlington, Ontario) supplemented with 10% fetal bovine serum (Invitrogen). HEK293 cells were subcultured in DMEM (Invitrogen, Burlington, Ontario) supplemented with 10% fetal bovine serum (Invitrogen). Protein content of cell samples was determined with the Bio-Rad protein assay.

RESULTS

Functional Analysis of NCKX2 Mutant Proteins in High Five Cells by ⁴⁵Ca Uptake

In a previous study we carried out scanning mutagenesis of the two α repeats of NCKX2 and identified several residues that are important for NCKX transport function and are thought to be located well within the plane of the lipid bilayer: these include four acidic residues, four hydroxyl containing residues, and two proline residues (20). The results obtained previously for nine of these ten residues (20) are summarized in Fig. 1A. For each residue substitution a set of 2–5 new experiments was added. In addition to the mutant NCKX2 proteins reported before, four new substitutions were analyzed that represent conservative Ser to Thr and Thr to Ser changes. Fig. 1B illustrates the location of these residues within our current model of the NCKX2 topology; the eight residues located in hydrophobic segments H2 and H8 (part of the α1 and α2 repeat, respectively) are conserved between the NCKX and NCX gene families, whereas members of the NCX gene family do not have acidic residues in positions equivalent to the NCKX2 residues Asp⁷⁵⁸ and Asp⁶⁷⁷. The four new mutant NCKX2 proteins represent conservative substitutions of the four critical hydroxyl-containing residues found in all NCX and NCKX proteins. Interestingly, in all three cases (Ser⁴¹⁸⁵, Ser⁴⁴⁵, Ser⁴⁴⁶, Ser⁴⁵⁰), the conservative Ser to Thr substitution resulted in a very strong reduction of mutant NCKX2 transport function compared with wild-type NCKX2, whereas the conservative T544S substitution had much less effect on NCKX2 transport compared with the less conservative T544A and T544C substitutions. When mutant NCKX2 protein samples were analyzed by Western blotting, most showed the 2-band pattern observed for wild-type NCKX2, in which the lower band represents NCKX2 protein from which the signal peptide was cleaved. The SS45T mutant NCKX2 protein consistently showed mostly the upper band representing the uncleaved NCKX2 (Fig. 2). This is a highly uncommon phenotype among the ~150 single residue NCKX2 mutants we have analyzed in the High Five cell system (also observed for the S185C and S185T mutant NCKX2 proteins in some, but not all experiments). As signal peptide cleavage appears essential for plasma membrane targeting (24), strongly reduced transport observed for the SS45T NCKX2 mutant proteins may be caused by the lack of cleaved mutant NCKX2 protein present in the plasma membrane. Surface biotinylation of the SS45T mutant NCKX2 protein (e.g. Ref. 24) confirmed that only the small amount of mutant NCKX2 protein represented by the lower band was present in the plasma membrane (data not shown).

K⁺ Concentration Dependence of Mutant NCKX2 Proteins Determined in High Five Cells by ⁴⁵Ca Uptake

⁴⁵Ca uptake via reverse Na⁺/Ca²⁺–K⁺ exchange was used as a simple quantitative assay for NCKX2 function as illustrated in Fig. 3. Analysis of shifts in K⁺ and Ca²⁺ concentration dependences of NCKX2 mutants requires a sensitive and consistent assay for NCKX function in view of our observation that
maximal NCKX transport activity was strongly reduced upon substitution of residues hypothesized to be involved in cation binding. Only two of the mutant NCKX2 proteins illustrated in Fig. 1 showed sufficient NCKX2 transport activity to allow consistent measurements of the K⁺/H⁴⁺ concentration dependence of ⁴⁵Ca uptake via reverse Na⁺/H⁴⁺/Ca²⁺⁻K⁺ exchange (Fig. 3).

Lowering extracellular K⁺ concentration affected the initial rate and the total amount of ⁴⁵Ca uptake in comparable fash-}

ion, similar as observed before for a variety of different NCKX clones as well as for NCX1 (9, 13, 17). Much higher K⁺ concentrations were required to activate ⁴⁵Ca uptake via reverse Na⁺/Ca²⁺⁻K⁺ exchange in the case of the D548E mutant (apparent K_m ~ 10 mM), whereas the K⁺ dependence of the P187A mutant (apparent K_m ~ 0.8 mM) appeared unchanged when compared with wild-type NCKX2 (apparent K_m ~ 0.7 mM) (apparent K_m values were estimated from an analysis of the initial rates of ⁴⁵Ca uptake). The ability to resolve low NCKX2 activity in these experiments is limited by the nonspecific ⁴⁵Ca uptake observed in high NaCl medium when NCKX2-mediated uptake is completely inhibited. (Note: uptake in NaCl medium was very similar to that observed in cells transfected with empty vector.) In cells expressing wild-type NCKX2, the ratio of specific NCKX2-mediated uptake/nonspecific uptake (i.e. that observed in NaCl medium) was about 10 in this particular experiment, but this ratio had dropped to 2 or less in the case of most of the NCKX2 mutants listed in Fig. 1.

Fluorometric Analysis of NCKX2 Mutant Function in HEK293 Cells

Here we describe a more sensitive assay for NCKX function that measures NCKX2-mediated changes in intracellular free Ca²⁺ in HEK293 cells transfected with (mutant) NCKX2 cDNAs and loaded with the fluorescent Ca²⁺-indicating dye Fluo-3. High Five cells proved to be not suited for this method caused by the fast leakage of Fluo-3 from these cells. The Fluo-3 assay, illustrated in Fig. 4, enabled us to analyze cation dependence for most of the NCKX2 mutant proteins even when maximal transport capacity was severely reduced. HEK293 cells were loaded with Fluo-3 after transfection with either empty vector or vector containing wild-type or mutant human NCKX2 cDNA. About 100,000 of the Fluo-loaded cells were
placed in a cuvette containing 2 ml of a buffered LiCl solution with a final concentration of 100 μM EDTA present. Fluo-3 fluorescence was continuously measured while various additions were made to the cuvette from concentrated solutions under constant stirring (Fig. 4A). First, CaCl₂ was added to a final concentration of 250 μM as indicated by the arrow and by the spike in the trace; the spike was caused by lowering the pipette through the light beam and served as a convenient event marker. The CaCl₂ addition caused a small and instantaneous increase in fluorescence caused by saturation of the small amount of Fluo-3 dye that had leaked out of the cells. No time-dependent increase in Fluo-3 fluorescence was observed indicating the lack of any Ca²⁺ influx in either control or NCKX2-transfected cells under this condition. The second arrow indicates the addition of KCl to a final concentration of 20 mM. This KCl addition caused no change in Fluo-3 fluorescence in control cells, but led to a rapid and large rise in Fluo-3 fluorescence in NCKX2-transfected cells. The fast rise in fluorescence indicates a rapid K⁺-dependent Ca²⁺ influx representing reverse Na⁺/Ca²⁺-K⁺ exchange. The third arrow indicates addition of saponin (final concentration 0.02%) to permeabilize the plasma membrane. Saponin addition caused the release of all Fluo-3 present in the cells, thereby saturating Fluo-3 fluorescence by the high (~150 μM) Ca²⁺ concentration in the medium. Minimum Fluo-3 fluorescence (obtained by addition of saponin in the presence 0.1 mM EDTA) was only slightly lower than fluorescence representing resting Ca²⁺ levels observed in HEK293 cells in the LiCl-EDTA medium at the start of the experiment (i.e. before activating Ca²⁺ influx via the exchanger). The K⁺- and Ca²⁺-dependent increase in Fluo-3 fluorescence observed in cells transfected with wild-type NCKX2 ranged from 30 - 50% of the saponin-induced increase in Fluo-3 fluorescence in different transfection experiments. When NCKX2 cDNA was co-transfected with cDNA encoding green fluorescent protein (GFP) a similar percentage of the transfected cells showed GFP staining indicating the percentage of successfully transfected cells (data not shown). Combined these results suggest that reverse Na⁺/Ca²⁺-K⁺ exchange via wild-type NCKX2 leads to a rapid Ca²⁺ influx that competes effectively with Ca²⁺ clearance mechanisms endogenous to HEK cells and results in a significantly elevated free Ca²⁺ concentration and increased Fluo-3 fluorescence, but only in those cells transfected with NCKX2. Since the fluorescence measurements represent a mixed population of transfected and untransfected cells, we have not attempted to convert fluorescence into free cytosolic [Ca²⁺] for those cells successfully transfected with NCKX2.

**K⁺ and Ca²⁺ Concentration Dependences of NCKX2 Activity Measured with the Fluo-3 Assay**

Using the above Fluo-3 assay, we measured the K⁺-induced rise in Fluo-3 fluorescence as a function of the external K⁺ concentration (top panels of Fig. 4B). Similar to the pattern observed in the ⁴⁵Ca uptake assay, lowering the external K⁺ concentration below saturating values lowered the initial rate as well as the amplitude of the changes in Fluo-3 fluorescence observed in cells transfected with wild-type NCKX2. The smaller amplitudes of the Fluo-3 signals indicate a lower free [Ca²⁺] in the HEK293 cells caused by a reduced rate of Ca²⁺ influx via NCKX2, while Ca²⁺ clearance via the plasma membrane and endoplasmic reticulum Ca²⁺ pumps remained constant. Thus, the plateau fluorescence reflects the equilibrium free Ca²⁺ concentration that arises from the balance of Ca²⁺ influx via NCKX2 and Ca²⁺ efflux via the Ca²⁺ pump. The K⁺ dependence of the plateau fluorescence levels was very similar.

**Fig. 2. Western blot of serine and threonine NCKX2 mutants.** The indicated NCKX2 mutants were expressed in High Five cells as described before (20). Protein was extracted in a buffer containing 140 mM NaCl, 25 mM Tris (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 mM EDTA, and a protease inhibitor tablet (Roche Applied Science). The extract was spun down (5 min at 20,000 × g), and the supernatant was used. Samples (20 μg protein/lane) were run on SDS-PAGE and analyzed by Western blotting with the monoclonal anti-Myc antibody. control represents cells transfected with empty vector. In most blots wild-type NCKX2 displays a doublet, the lower band representing NCKX2 from which the signal peptide is cleaved (24). In some cases, an additional ghost band is observed at a slightly lower molecular size (e.g. the S94SC mutant NCKX2), probably representing cleaved NCKX2 that was not completely unfolded in the sample buffer. NCKX2-containing samples were only heated for 10 min at 37 °C to avoid aggregation.

**Fig. 3. K⁺ concentration dependence of two NCKX2 mutants analyzed with ⁴⁵Ca uptake.** High Five cells were transfected with cDNA encoding either wild-type NCKX2 (left panel), D548E mutant NCKX2 (middle panel), or the P187A mutant NCKX2 (right panel). ⁴⁵Ca uptake was measured as described under “Materials and Methods” and expressed as nanomoles calcium taken up/mg protein. The uptake medium contained 150 mM LiCl, 80 mM sucrose, 20 mM Hepes, pH 7.4, and uptake was initiated at time 0 by addition of 0.036 mM CaCl₂ plus 1 μCi of ⁴⁵Ca. The indicated K⁺ concentrations were obtained by isotonic substitution of LiCl by KCl: 50 mM KCl (circles), 10 mM KCl (inverted triangles), 1 mM KCl (squares), 0.1 mM KCl (diamonds). Temperature: 25 °C.
to the K+ dependence of the initial rate of the changes in Fluo-3 fluorescence. The observed pattern suggests that both the initial rate and amplitude of the Fluo-3 signal can be used as a semiquantitative indicator of NCKX2 transport function. To ensure that changes in cation dependence were not caused by lower maximal transport activities per se, we compared wild-type NCKX2 with the E651D NCKX2 mutant, previously reported to have about 13% of wild-type activity in the High Five cell system (20). Glu651 is located at the C terminus of NCKX2 (Fig. 1B) and is unlikely to be directly involved in cation trans-

FIG. 4. Fluo-3 assay of NCKX activity in HEK cells. A, 50 μl of Fluo-3-loaded HEK293 cells (~10^5 cells) transfected with cDNA encoding wild-type NCKX2 or with empty vector (Control) were added to 1.95 ml of a medium containing 150 mM LiCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 100 μM EDTA. Fluo-3 fluorescence was measured as described under “Materials and Methods” and in the text. The arrow at 10 s indicates the addition of CaCl2, to a final concentration of 250 μM; the arrow at 30 s indicates the addition of KCl to a final concentration of 20 mM; whereas the arrow at 90 s indicates the addition of saponin to a final concentration of 0.02%. Fluorescence was normalized to maximal fluorescence observed after addition of saponin. Temperature: 25 °C. B, K+ dependence of reverse exchange was compared in cells expressing wild-type NCKX2 or the E651D NCKX2 mutant. Experimental paradigm was as described above except that the KCl addition was varied to the indicated final concentrations. The initial rate of Ca2+ influx (judged by the fluorescence change observed 5 s after addition of KCl, bottom left) or the amplitude of the fluorescence change (observed 40 s after addition of KCl, bottom right) was averaged for four experiments (error bar represents S.E.). C, Ca2+ dependence of reverse exchange was compared in cells expressing wild-type NCKX2 or the E651D NCKX2 mutant. In the case of the Ca2+ dependence, Ca2+ was added first to the indicated final free concentration of 1.2 (abbr. to 1), 10.8 (abbr. to 10), 100, and 500 μM (see “Materials and Methods”), followed by addition of KCl to a final concentration of 20 mM. The initial rate of Ca2+ influx (judged by the fluorescence change observed 5 s after addition of KCl, bottom left) or the amplitude of the fluorescence change (observed 40 s after addition of KCl, bottom right) was averaged for three experiments (error bar represents S.E.).
port. Consistent with the results obtained with the High Five cell system (20), the maximal amplitude of the Fluo-3 signal observed for the E651D NCKX2 mutant was much smaller than that observed for wild-type NCKX2, indicative of a much reduced maximal transport rate compared with wild-type NCKX2. However, the K+ concentration dependence of the Fluo-3 signals observed in cells expressing the E651D mutant NCKX2 protein appeared very similar to that observed for wild-type NCKX2 (Fig. 4B), demonstrating that reduced maximal transport rate did not itself result in a change in K+ concentration dependence. The patterns illustrated in Fig. 4B were highly reproducible, and for a number of experiments the initial rate of the K+-induced fluorescence changes (change observed after 5 s) and the amplitude of the change in fluorescence change (fluorescence 40 s after addition of KCl) were averaged and normalized with respect to those observed for the highest K+ concentration of 50 mM used. Both analyses yielded very similar K+ concentration dependence for wild-type NCKX2 and the E651D NCKX2 mutant with a half-maximal rate or amplitude observed at ∼1 mM (bottom panels of Fig. 4B). This value is very similar to that observed in Fig. 3 using the 45Ca uptake method. In the same set of experiments, the Ca2+ concentration dependence was measured for wild-type NCKX2 and the E651D NCKX2 mutant at a fixed KCl concentration of 20 mM; the results were analyzed in the same way as shown for the K+ dependence (Fig. 4C). As observed for the K+ dependence, very similar Ca2+ dependence was observed for wild-type NCKX2 and the E651D NCKX2 mutant, both when analyzing the initial rate or the amplitude of the fluorescence signals. Half-maximal rate or amplitude was observed at ∼1 μM free [Ca2+]. Fluo-3 fluorescence does not represent a linear [Ca2+] scale, and some compression should occur at higher fluorescence levels because of saturation of the dye. This could result in an underestimate of apparent Km values. The compression effect should be more obvious for the maximal amplitude of the fluorescence signals and for NCKX2 (mutant) proteins that show high transport rates (fluorescence signals) and for NCKX2 (mutant) proteins that effect should be more obvious for the maximal amplitude of the [Ca2+] change (fluorescence 40 s after addition of KCl) were averaged and normalized with respect to those observed for the highest K+ concentration of 50 mM used. Both analyses yielded very similar K+ concentration dependence for wild-type NCKX2 and the E651D NCKX2 mutant with a half-maximal rate or amplitude observed at ∼1 mM (bottom panels of Fig. 4B). This value is very similar to that observed in Fig. 3 using the 45Ca uptake method. In the same set of experiments, the Ca2+ concentration dependence was measured for wild-type NCKX2 and the E651D NCKX2 mutant at a fixed KCl concentration of 20 mM; the results were analyzed in the same way as shown for the K+ dependence (Fig. 4C). As observed for the K+ dependence, very similar Ca2+ dependence was observed for wild-type NCKX2 and the E651D NCKX2 mutant, both when analyzing the initial rate or the amplitude of the fluorescence signals. Half-maximal rate or amplitude was observed at ∼1 μM free [Ca2+]. Fluo-3 fluorescence does not represent a linear [Ca2+] scale, and some compression should occur at higher fluorescence levels because of saturation of the dye. This could result in an underestimate of apparent Km values. The compression effect should be more obvious for the maximal amplitude of the fluorescence signals and for NCKX2 (mutant) proteins that show high transport rates (e.g. wild-type NCKX2). Two observations presented here suggest that compression is not a major problem. 1) The concentration dependence obtained from the initial rates and from the amplitudes of the fluorescence signals, respectively, was very similar. 2) The K+ and Ca2+ dependences were very similar for the two different NCKX2 proteins illustrated in Fig. 4, although maximal transport activities differed greatly. In previous studies we reported external Km values of −2 μM for Ca2+ and −2 mM for K+ for the wild-type NCKX2 (13, 17, 18).

Four of the NCKX2 mutants listed in Fig. 1A showed no detectable function when analyzed in the HEK293 cell system with Fluo-3 (E188Q, S545C, S545T, D548N), and this was not caused by reduced mutant protein expression or altered plasma membrane targeting as judged by signal peptide cleavage (not shown). The E188Q, S545C, S545T, and D548N mutant NCKX2 proteins appeared to have small, but measurable active activity in the High Five cell system (Fig. 1A). Two possible explanations for this discrepancy are as follows. 1) Several other NCKX clones we have examined show strong function when expressed in High Five cells, but show no (e.g. Drosophila NCKX-X) or no consistent (e.g. chicken rod NCKX1) function when expressed in HEK293 cells (12, 24). 2) High Five cells used in our experiments have high internal Na+ levels after Na+ loading with the ionophore monensin.

**Shifts in External K+ and Ca2+ Dependencies of Reverse Na+/Ca2+/K+ Exchange in NCKX2 Mutants**

**Acidic Residues within Membrane Helices: Glu188, Asp258, and Asp548**—First we focused on the two acidic residues conserved among members of the NCX and NCKX gene families: Glu188 and Asp548 are thought to be located well within the plane of the lipid bilayer in strategic positions in helices H2 and H8, respectively (Fig. 1B). The external Ca2+ and K+ concentration dependences of mutant NCKX2 proteins carrying the charge-conservative substitutions E188D or D548E, respectively, were compared with those of wild-type NCKX2 as illustrated in Fig. 5. When the initial rates of the fluorescence signals were analyzed, the E188D and D548E mutant NCKX2 proteins showed considerable shifts to lower affinities for both K+ and Ca2+ dependences when compared with wild-type NCKX2 (Fig. 5), and these shifts were the largest observed for the various NCKX2 mutants examined in this study. Very similar results were obtained when the amplitudes of the fluorescence signals were analyzed (not shown). The shift in apparent Ca2+/Km was from a value of ∼1 μM for wild-type NCKX2 to a value of close to 100 μM for both the E188D and D548E mutant NCKX2. The shift in apparent K+/Km was from a value of ∼1 mM for wild-type NCKX2 to a value of >10 mM for both the E188D and D548E mutant NCKX2. No NCKX activity was observed in HEK293 cells transfected with either the E188Q or the D548N NCKX2 mutants in which the charge was removed (not shown), consistent with their importance as key residues in Ca2+ binding.

Asp258 and Asp577 are the other acidic residues that are thought to be located deep within membrane helices of NCKX2 and important for its function (Fig. 1A). Mutagenesis of the Asp577 residue results in a complex phenotype that has been reported elsewhere (32). Both D258N and D258E mutant NCKX2 proteins showed K+-dependent Na+/Ca2+ exchange function when expressed in HEK293 cells. Similar to the results observed in the High Five cell system (Fig. 1A), the size-conservative D258N showed higher maximal activity than the charge-conservative D258E substitution as judged by the amplitudes of the fluorescence signals observed (data not shown, see also Fig. 9). When the initial rates of the fluorescence signals were analyzed, the D258N mutant showed K+ and Ca2+ concentration dependences that were not or only slightly shifted compared with those of wild-type NCKX2, whereas the D258E mutants consistently showed shifts in both K+ and Ca2+ dependences although the shifts observed were considerably smaller than those observed in Fig. 5 for the E188D and D548E mutants (apparent Km values of ∼10 μM for Ca2+ and ∼3 mM for K+) (Fig. 6, two top panels). Very similar results were obtained when the amplitudes of the fluorescence signals were analyzed (not shown).

**Proline Residues Preceding Glu188 and Asp548**—The two α repeats contain two highly conserved proline residues, Pro187 and Pro547, that precede Glu188 and Asp548, respectively. The bottom panels of Fig. 6 illustrate the K+ and Ca2+ concentration dependences of mutant NCKX2 proteins in which Pro187 or Pro547 is replaced by either alanine or cysteine. When the initial rates of the fluorescence signals were analyzed, the P187A and P547C NCKX2 mutants showed a small shift in both K+ and Ca2+ dependences, whereas the P187C and P547A mutants showed larger shifts to lower apparent affinities for both K+ and Ca2+. Very similar results were obtained when the amplitudes of the fluorescence signals were analyzed (not shown).

**Acidic Residues Located in Short Connecting Loops**—The two sets of transmembrane regions of NCKX2 contain a number of acidic residues, thought to be located at the membrane/water interface or in the short connecting loops rather than well within the membrane (20). We examined K+ and Ca2+ dependences for substitutions of most of these acidic residues. For example, Glu265 is located in the very short (3 residues) H4-H5...
The E265D and in particular the E265Q mutant NCKX2 proteins showed small shifts in both K<sup>+</sup> and Ca<sup>2+</sup> dependences when compared with wild-type NCKX2 (Fig. 7). Likewise, Glu<sup>651</sup> is conserved in all NCKX sequences, but the E651D mutation showed wild-type K<sup>+</sup> and Ca<sup>2+</sup> dependences even though the maximal transport rate was strongly reduced (Fig. 4). Four additional acidic residues were examined as they are located near membrane helices H2 and H8, which contain the two critical acidic residues Glu<sup>188</sup> and Asp<sup>548</sup> discussed above (Figs. 1 and 5). Asp<sup>172</sup> and Asp<sup>173</sup> are thought to be located at the cytoplasmic interface of helix H2, while Glu<sup>532</sup> and Glu<sup>533</sup> are thought to be located at the extracellular interface of helix H8. These could be strategic positions controlling access to the cation binding site(s) located within the membrane, and, hence, the effect of the charge-eliminating but size-conservative substitutions for these four acidic residues were examined. We previously showed that these size-conservative substitutions had only minor effects on maximal transport activity (20). Fig. 7 shows that three of these four mutant NCKX2 proteins showed wild-type cation dependence, while only the E533Q NCKX2 mutant protein showed significant shifts in both Ca<sup>2+</sup> (apparent K<sub>m</sub> ~ 10 μM) and K<sup>+</sup> (apparent K<sub>m</sub> ~ 10 mM) dependences when compared with wild-type NCKX2. Fig. 7 shows an analysis of the initial rates of the fluorescence signals. Very similar results were obtained when the amplitudes of the fluorescence signals were analyzed (not shown).

Thus, 7 of 13 NCKX2 mutants, illustrated in Figs. 5–7, show changes in Ca<sup>2+</sup> concentration dependence, which were always compared with wild-type NCKX2 (Fig. 7). Likewise, Glu<sup>651</sup> is conserved in all NCKX sequences, but the E651D mutation showed wild-type K<sup>+</sup> and Ca<sup>2+</sup> dependences even though the maximal transport rate was strongly reduced (Fig. 4). Four additional acidic residues were examined as they are located near membrane helices H2 and H8, which contain the two critical acidic residues Glu<sup>188</sup> and Asp<sup>548</sup> discussed above (Figs. 1 and 5). Asp<sup>172</sup> and Asp<sup>173</sup> are thought to be located at the cytoplasmic interface of helix H2, while Glu<sup>532</sup> and Glu<sup>533</sup> are thought to be located at the extracellular interface of helix H8. These could be strategic positions controlling access to the cation binding site(s) located within the membrane, and, hence, the effect of the charge-eliminating but size-conservative substitutions for these four acidic residues were examined. We previously showed that these size-conservative substitutions had only minor effects on maximal transport activity (20). Fig. 7 shows that three of these four mutant NCKX2 proteins showed wild-type cation dependence, while only the E533Q NCKX2 mutant protein showed significant shifts in both Ca<sup>2+</sup> (apparent K<sub>m</sub> ~ 10 μM) and K<sup>+</sup> (apparent K<sub>m</sub> ~ 10 mM) dependences when compared with wild-type NCKX2. Fig. 7 shows an analysis of the initial rates of the fluorescence signals. Very similar results were obtained when the amplitudes of the fluorescence signals were analyzed (not shown).

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accompanied by changes in K\(^+\) concentration dependence. The other six NCKX2 mutants showed no clear shifts in either K\(^+\) or Ca\(^{2+}\) dependence. For the two mutants (D548E and P187A) that could be analyzed with the 45Ca uptake assay (Fig. 3), the results were consistent with those obtained with the Fluo-3 assay.

Serine and Threonine Residues—Membrane-spanning helices H2 and H8 contain four hydroxyl-containing residues, substitutions of which had a strong effect on NCKX2 function (Fig. 1). The S185A, S185T, S552C, S552T, T544A, T544S, and S545A (the latter not in all experiments) mutant NCKX2 proteins all gave measurable NCKX2 function in HEK293 cells, whereas the S545T and S545C mutant NCKX2 proteins showed no NCKX2 function when analyzed with the Fluo-3 assay. Compared with the other amino acid substitutions studied here, the substitution of the serine and threonine residues resulted in mutant NCKX2 proteins that showed more variable cation dependence as evidenced by the often larger error bars. Shifts in both K\(^+\) and Ca\(^{2+}\) concentration dependences were observed for the S545A and S185T NCKX2 mutant (Fig. 8, top panel), although it should be pointed out that the S545A mutant showed very low transport activity as judged by the very small amplitudes of the Fluo-3 signals observed, and no activity was observed in some experiments. The shift in K\(^+\) concentration dependence observed for the S185T NCKX2 mutant was small but a significant difference in the normalized rate was observed at a K\(^+\) concentration of 1 mM (\(p < 0.005\) in the Student's t test). More anomalous patterns were observed for the Thr544 and Ser552 mutants. The two Thr544 mutants did not show a clear correlation between shifts in K\(^+\) dependence and shifts in Ca\(^{2+}\) dependence (Fig. 9, middle panel); the two Ser552 mutants showed no shift in K\(^+\) dependence, but consistently showed an anomalous Ca\(^{2+}\) dependence in which the higher Ca\(^{2+}\) concentrations of 100 and 500 \(\mu\)M appeared to be some-
what inhibitory (Fig. 8, bottom panel). This was not observed for any of the other NCKX2 mutants reported here.

Summary of the Analysis of NCKX2 Mutant Proteins with the Fluoro-3 Assay

The maximal amplitude of the fluorescence signals observed for the various NCKX2 mutant proteins in different transfection experiments was normalized with respect to that observed for wild-type NCKX2 in each experiment as summarized in Fig. 9. The results are broadly consistent with those described before using an assay based on 45Ca uptake in the High Five cell system (20), although some differences should be noted. For example, the E265Q, D258E, and D258N mutant NCKX2 proteins appeared to have higher activity when expressed in HEK293 cells compared with expression in High Five cells. It also should be noted that NCKX2 mutant protein expression levels in HEK293 cells were not quite as uniform as observed in the High Five cell system. The High Five cell system would appear to be a better system to evaluate changes in maximal transport rate for the various NCKX2 mutants, while the HEK293 cell system allows the evaluation of cation dependence for each individual mutant.

DISCUSSION

This is the first study to identify residues of the NCKX Na+/Ca2+/K+ exchanger that contribute to the Ca2+ and K+ binding pocket as assessed by changes in Ca2+ and K+ concentration dependences of transport via NCKX mutant proteins in which selected residues were replaced. A simple and sensitive fluorometric assay using the fluorescent Ca2+-indicating dye Fluo-3 was developed to measure the Ca2+ or K+ concentration dependence of reverse Na+/Ca2+/K+ exchange observed in HEK293 cells after transient transfection with cDNA encoding wild-type human NCKX2 or different NCKX2 mutants. The fluorescence signals had sufficient resolution to permit analysis of cation dependence of NCKX mutant proteins, even for those with greatly reduced maximal transport rates. Twenty-six different NCKX2 mutants proteins were analyzed as summarized in Fig. 9: four showed no measurable function, nine showed no clear changes in Ca2+ or K+ dependence, while thirteen of the NCKX2 mutant proteins showed clear changes in Ca2+ and/or K+ dependence. There was no correlation between a change in maximal transport rate (as judged by the maximal amplitude of the fluorescence signals) and a change in cation dependence for the various NCKX2 mutant proteins, showing that a reduction in maximal transport rate is not necessarily accompanied by a change in cation affinities.

Two groups of residues in the human NCKX2 protein were targeted. First, eight residues were selected that are conserved between all members of the mammalian NCX and NCKX gene families; substitution of these eight residues was previously found to greatly reduce maximal transport activity in both NCX1 (21, 22) and NCKX2 (20) precluding a detailed kinetic analysis. In the human NCKX2 clone used here, these residues are located in membrane helices H2 (Ser185, Pro187, Glu188) and H8 (Thr544, Ser545, Pro547, Asp548, Ser552) as illustrated in Fig. 1B. Second, as Ca2+ binding sites in proteins invariably contain negatively charged residues, an additional seven acidic residues were selected, based on their location in the two sets of membrane-spanning segments (Fig. 1B) as well as on the high degree of conservation of these residues in different mammalian NCKX isoforms and in NCKX from lower organisms. Here, we propose different roles for these residues based on their location and based on the effect of substitution on transport properties: (1) three acidic residues (Glu188, Asp258, Asp548) within the horizontal mid-plane of the membrane; (2) acidic residues at the interface between membrane helices and the extracellular medium; (3) hydroxyl-containing residues that line the hydrophilic surface of membrane helices H2 and H8, and which are located one helical turn away from residues E188 and D548, respectively. Two novel findings from this study are: (1) substitutions in the NCKX2 protein that caused changes in Ca2+ concentration dependence invariably caused changes in K+ concentration dependence; if the shift in Ca2+ concentration dependence was large, the shift in K+ dependence was large as well. As the residues involved are thought to be located at quite different positions relative to the membrane surface, this suggests strongly that Ca2+ and K+ share a common transport pathway; (2) Glu188 and Asp548 are critical residues lining a common Ca2+ and K+ binding pocket.

Glu188 and Asp548 Are Key Residues in the NCKX Cation Binding Pocket—Our results suggest strongly that Glu188, Asp548, and perhaps Asp258 are pivotal residues lining a single K+ and Ca2+ binding pocket: 1) Eliminating the charge on either Glu188 or Asp548 by size-conservative substitutions resulted in nonfunctional NCKX2 mutant proteins; Glu188 and Asp548 are the only two acidic residues for which this is true among the twenty found within the transmembrane spanning...
domains of NCKX2. 2) The charge-conservative E188D and D548E substitutions resulted in the largest shifts in the dependences for both K⁺ and Ca²⁺ observed here for the various NCKX2 mutant proteins (Fig. 5). 3) The pH and Mg²⁺ dependences of Ca²⁺ transport via NCKX1 have features similar to Ca²⁺ binding to EGTA, suggesting the involvement of multiple carboxylate moieties (25). 4) Proline residues are often found in the middle of membrane helices where they produce a kink in the helix and a region of increased flexibility (26). Substitutions of the highly conserved proline residues Pro¹⁸⁷ and Pro⁵⁴⁷, respectively, resulted in marked shifts in both K⁺ and Ca²⁺ dependences (Fig. 6). 5) Glu¹⁸⁸ and Asp⁵⁴⁸ were found to be in close proximity as judged by site-directed disulfide mapping. 6) The D258E mutation showed clear shifts to lower affinities for both K⁺ and Ca²⁺, suggesting it could be a contributing residue of the K⁺ and Ca²⁺ binding pocket (Fig. 6). However, the importance of Asp⁵⁴⁸ is clearly less established than that of Glu¹⁸⁸ and Asp⁵⁴⁸.

Iwamoto et al. (22) reported that substitution of several aspartate residues in the transmembrane spanning segments of NCX1 affected the affinity toward Ca²⁺ and suggested these residues are important in Ca²⁺ liganding, whereas substitution of Glu¹¹³ and Asp¹¹⁴, the conserved equivalent of NCKX2 residues Glu¹⁸⁸ and Asp⁵⁴⁸ in NCX1, resulted in mutant NCX1 proteins with insufficient activity to assess Ca²⁺ affinity. Distinct from our results with the NCKX2 residues Glu¹³⁸ and Asp⁵⁴⁸, charge-eliminating substitutions (e.g. to cysteine) of the above aspartate residues in NCX1 did not result in nonfunctional NCX1 proteins. Therefore, we believe that Glu¹³⁸ and Asp⁵⁴⁸ are the central Ca²⁺ liganding residues in NCKX2, and we believe the equivalent residues Glu¹¹³ and Asp¹¹⁴ play this role in NCX1. It should be pointed out that the external Kₘ of wild-type NCX1 is about 200 μM Ca²⁺ (22), while the external Kₘ of the various NCKX isoforms is 1–3 μM Ca²⁺ (13, 14, 18). A peculiar aspect then is that large shifts in K⁺ concentration dependence are observed in NCKX2 for the charge-conservative substitutions of Glu¹³⁸ and Asp⁵⁴⁸, residues conserved in NCX1 that does not transport K⁺. Earlier experiments showed that alkali cations such as Li⁺ and K⁺ could stimulate Ca²⁺:Ca²⁺ self exchange mediated by NCX1 in cardiac sarcolemma vesicles and could inhibit Na⁺:Ca²⁺ exchange via NCX1 suggesting that alkali cations can bind near the cation binding pocket and affect transport without being transported themselves (reviewed in Refs. 4 and 27). Perhaps other residues in NCX1, not found in NCX1, enable K⁺ not only to bind to the cation binding pocket, but also to be transported. We have identified Asp⁷⁵⁵ as one such residue (32). Clearly, other residues influence the apparent Kₘ of Ca²⁺ transport, perhaps by increasing the local Ca²⁺ and K⁺ concentration through electrostatic effects. For example, the aspartate residues identified by Iwamoto et al. in NCX1 (see above) may function this way, and in NCKX2 residues Glu⁶⁵⁶ and Glu⁶⁵⁸ could play this role.

A Single Set of Residues of NCKX2 Lines a Common Transport Pathway for K⁺ and Ca²⁺—Previous kinetic studies on an in situ NCX1 (28–30) and wild-type NCX1 or NCKX2 expressed in cell lines (13, 18) suggest that Ca²⁺ and K⁺ each bind to a separate single occupancy binding site. The NCKX Na⁺/Ca²⁺-K⁺ exchanger found in the outer segments of rod photoreceptors has been shown to carry out both countertransport of 4 Na⁺/(1 Ca²⁺ + 1 K⁺) and self-exchange of (Ca²⁺ + K⁺)/Na⁺ (Ca²⁺ + K⁺)/Ca²⁺ (Ca²⁺ + K⁺) dependent on the type of cations present on both sides of the outer segment plasma membrane (4). This is strongly suggestive of a consecutive model of transport in which the NCKX protein must simultaneously bind both 1 Ca²⁺ and 1 K⁺ to a single (set of) binding site(s) that can alternate between an inward facing and an outward facing conformation. This raises the question whether Ca²⁺ and K⁺ use the same transport pathway or separate pathways. In this study, shifts in cation dependence were found not only for residues thought to be lining a binding pocket in the center of membrane helices (e.g. Glu¹³⁸, Asp¹⁴⁸, Asp⁵⁴⁸, see above), but also for 2 (of a total of 16) residues thought to be in a more peripheral location at the membrane/external solution interface (Glu⁶⁵⁶ and Glu⁶⁵⁸, Figs. 1 and 7). Furthermore, substitution of hydroxyl residues Thr³⁴⁴ and Ser³⁴⁵ along helix H8 could affect cation dependence as well (Fig. 8). As the boundaries of helix H8 appear well defined by a pair of acidic residues at the extracellular interface (Glu⁶⁸ and Glu⁶⁸) and by a pair of basic residues (Arg³⁷⁷ and Lys⁵⁵⁸) at the cytosolic interface, it appears rather unlikely that H8 unfolds into a binding pocket in which all the above residues are in close proximity. We suggest that residues lining a common pathway for Ca²⁺ and K⁺ to traverse the membrane can contribute to the apparent Kₘ values. In a previous study we suggested a role for helices H2 and H8 in cation transport as cysteine residues inserted near the extracellular surface of these two helices resulted in strong inhibition of NCKX transport by thiol modification with the positively charged MTSET reagent, in particular for G536C and L540C in H8 (31). This suggests that these inserted cysteine residues line an aqueous pathway that provides access to the cation binding pocket located further inside the membrane. Fig. 10 shows a helical wheel representation of helix H8 that contains all the residues modification of which could affect cation dependence, starting with Glu⁶⁵⁸ at the extracellular interface, followed by Glu⁶⁵⁶ Leu⁶⁴⁰, the Thr³⁴⁴ and Ser³⁴⁵ pair until one reaches Asp³⁴⁸. Our results strongly suggest a common transport pathway for Ca²⁺ and K⁺ as changes in residues that caused a shift in Ca²⁺ concentration dependence with respect to wild-type NCKX2 invariably caused a shift in K⁺ concentration dependence as well. Thus, large shifts in both K⁺ and Ca²⁺ dependences were observed for the E188D and D548E mutants (Fig. 5), while smaller shifts were observed for some of the Pro¹⁸⁷, Pro⁴⁷, Glu⁶⁵⁶, Glu⁶⁵⁸, and Asp⁵⁴⁸ mutant NCKX2 proteins (Figs. 6 and 7). For example, the P187C mutation resulted in a marked shift in K⁺ and Ca²⁺ concentration dependences, while this dependence were shifted much less in the P187A mutant (Fig. 6).

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² T. G. Kinjo, K.-J. Kang, R. T. Szerenceei, and P. P. M. Schnetkamp, submitted manuscript.
the P547A mutant resulted in a marked shift in both K\(^+\) and Ca\(^{2+}\) concentration dependences, while those dependences were shifted much less in the P547C mutant.

The Importance of the Hydroxyl-containing Residues in Helices H2 and H8—Helices H2 and H8 contain three serine residues (Ser\(^{185}\), Ser\(^{545}\), Ser\(^{552}\)) and one threonine residue (Thr\(^{544}\)) that are important for efficient NCKX2 transport (Fig. 1), probably by lining a hydrophilic interface at the membrane/aqueous environment (e.g. Asp\(^{173}\) and Glu\(^{333}\), see above). Three additional observations underline the importance of the three serine residues, in particular. 1) These residues may also provide coordination sites for Ca\(^{2+}\) and K\(^+\) binding, as several replacements of the hydroxyl-containing residues resulted in altered cation dependence (e.g. S185T, S545A, and T544S, Fig. 8) although the results showed greater variance when compared with the other residues tested. 2) The serine residues Ser\(^{185}\), Ser\(^{545}\), and Ser\(^{552}\) share with the acidic residues Asp\(^{575}\) and Asp\(^{556}\) (20) the property of conservative replacement with a residue that merely extends the side chain by a single methylene group was poorly tolerated (i.e. Ser to Thr and Asp to Glu, respectively) (Fig. 1). This suggests severe steric constraints at these residue positions. The latter appears particularly true for the S545T substitution (but not the S545A and S545C substitutions in which the size of the side chain is not increased), which displays trafficking defects, a rather uncommon phenotype for the many NCKX2 mutants generated in our laboratory (Fig. 2). 3) The serine residues located in an area of increased flexibility as site-directed disulfide mapping showed that these serine residues are in close proximity to the key Ca\(^{2+}\) liganding residues Glu\(^{188}\) and Asp\(^{548}\), and to each other.

In addition to contributing to the cation binding site, the placement of the serine and threonine residues one helical turn away from the plane defined by residues Glu\(^{188}\) and Asp\(^{548}\) suggests they may control access to Glu\(^{188}\) and Asp\(^{548}\) in the inward and outward facing conformations of NCKX. In the outward facing conformation H2 and H8 might be tilted in such a way that Glu\(^{188}\) and Asp\(^{548}\), Ser\(^{545}\)/Thr\(^{544}\) and perhaps Ser\(^{192}\), 2 contribute to coordinating Ca\(^{2+}\) binding, while Ser\(^{185}\) and Ser\(^{552}\) are tilted toward each other and impede cation passage. The subsequent conformational change that results in the inward facing conformation of NCKX changes the tilt in H2 and H8 in such a way that Ser\(^{185}\) and Ser\(^{552}\) move apart and now contribute with Glu\(^{188}\) and Asp\(^{548}\) to coordinating Ca\(^{2+}\) and allow passage to the intracellular space. This conformational change could cause a tilt of Ser\(^{545}\)/Thr\(^{544}\) toward Ser\(^{192}\) and block cations from exiting to the extracellular space. We propose that pairs of hydroxyl-containing residues on helices H2 and H8 (and possibly additional ones as well) function as gates that control the direction of transport and ensure the obligatory coupling of cation fluxes observed for NCKX. Future work will be directed toward addressing this issue.

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