Analysis of Ion Interactions with the K+-dependent Na+/Ca2+ Exchangers NCKX2, NCKX3, and NCKX4
IDENTIFICATION OF THR-551 AS A KEY RESIDUE IN DEFINING THE APPARENT K+-AFFINITY OF NCKX2*

Frank Visser1, Valeria Valsecchi3, Lucio Annunziato1, and Jonathan Lytton2

From the 1Libin Cardiovascular Institute of Alberta and the Hotchkiss Brain Institute, Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada and the 2Division of Pharmacology, Department of Neuroscience, School of Medicine, Federico II University of Naples, 80131 Naples, Italy

K+-dependent Na+/Ca2+ exchangers (NCKX) catalyze cytosolic Ca2+ extrusion and are particularly important for neuronal Ca2+ signaling. Of the five mammalian isoforms, the detailed functional characteristics have only been reported for NCKX1 and -2. In the current study, the functional characteristics of recombinant NCKX3 and -4 expressed in HEK293 cells were determined and compared with those of NCKX2. Although the apparent affinities of the three isoforms for Ca2+ and Na+ were similar, NCKX3 and -4 displayed ~40-fold higher affinities for K+ ions than NCKX2. Functional analysis of various NCKX2 mutants revealed that mutation of Thr-551 to Ala, the corresponding residue in NCKX4, resulted in an apparent K+ affinity shift to one similar to that of NCKX4 without a parallel shift in apparent Ca2+ affinity. In the converse situation, when Gln-476 of NCKX4 was converted to Lys, the corresponding residue in NCKX2, both the K+ and Ca2+ affinities were reduced. These results indicate that the apparently low K+ affinity of NCKX2 requires a Thr residue at position 551 that may reduce the conformational flexibility and/or K+ liganding strength of side-chain moieties on critical neighboring residues. This interaction appears to be specific to the structural context of the NCKX2 K+ binding pocket, because it was not possible to recreate the K+-specific low affinity phenotype with reciprocal mutations in NCKX4. The results of this study provide important information about the structure and function of NCKX proteins and will be critical to understanding their roles in neuronal Ca2+ signaling.

Transient increases in cytosolic Ca2+ concentrations are a ubiquitous signaling mechanism in animal cells, particularly excitable cells, and subsequent Ca2+ extrusion is mediated by a variety of membrane transporter systems (1, 2). Major contributions to cytosolic Ca2+ clearance are made by two families of Na+/Ca2+ exchangers: those that are independent (NCX)3 or dependent (NCKX) on K+ ions (3–5). Three NCX and five NCKX isoforms have been identified by molecular cloning, and each isoform displays unique tissue and cellular distribution patterns, suggesting that they play specialized, nonoverlapping roles in Ca2+ signaling. NCX1 is predominantly expressed in heart, brain, and kidney but is also present at lower levels in other tissues, whereas expression of NCX2 and -3 is limited to brain and skeletal muscle (6–9). NCKX1 is expressed exclusively in rod photoreceptor outer segments where it plays critical roles in retinal function, whereas NCKX2 expression is limited to neurons, where it has been shown play an important role in synaptic plasticity (10–12) and cone photoreceptors (13). Although they are more broadly expressed, NCKX3 and -4 and -5 are also abundant in the brain but with distinct cellular localization patterns from that of NCKX2 and NCX1 (14, 15). NCKX5 is an exchanger of intracellular membranes that is abundantly expressed in skin and the pigmented epithelium of the eye where it appears to play an important role in pigmentation, although the mechanism is unknown (16).

NCX and NCKX proteins share similar overall topologies with 10 (NCX) or 11 (NCKX) transmembrane-spanning segments (TMs), the first of which (TM 0) is cleaved by a signal peptidase, and the remaining TMs divided into two clusters connected by a large cytoplasmic loop with important regulatory functions (17–21). Amino acid sequence identity between NCX and NCKX proteins is limited to two internally homologous, oppositely oriented regions known as α-repeats 1 and 2, which are found within the N- and C-terminal clusters of TMs, respectively, and have been shown to contain amino acid residues critical for cation liganding and translocation (22–28).

An important aspect of understanding the unique physiological roles of the various NCX and NCKX isoforms is to determine their functional properties. The three NCX isoforms catalyze the exchange of 3 Na+ ions for 1 Ca2+ ion and have been demonstrated to display very similar functional properties with $K_{0.5}$ values of ~100–400 μM for Ca2+ (in the presence of 1 mM Mg2+) and ~10–60 mM for Na+ (29, 30). We have previously

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3 The abbreviations used are: NCX, Na+/Ca2+ exchanger; NCKX, K+-dependent Na+/Ca2+ exchanger; HEK293, human embryonic kidney 293; $K_{0.5}$, concentration for half-maximal Ca2+ uptake; $I_{C50}$, concentration that causes 50% of maximal inhibition; TM, transmembrane segment.
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FIGURE 1. Measurement of recombinant NCKX function in fura-2-loaded HEK293 cells. HEK293 cells grown on coverslips and transfected with expression plasmids for NCKX2, -3, and -4 or empty vector (pcDNA3.1+) were loaded with fura-2 AM and mounted in a perfusion chamber on a microscope stage. A, the ratio of fluorescence emission at 510 nm from excitations at 340 and 380 nm was monitored using a microscope photometer while the cells were perfused alternatively with Na+-containing or Li+-plus K+-containing buffers all in the presence of 0.1 mM Cac2+. B, the data for the first 45 s of each pulse superimposed. C, the initial rate of increase in fura-2 ratio for each pulse. The rate for the first pulse of NCKX2 is not shown, and the dashed line represents the run-down correction based on time-dependent interpolation using the relative rates of pulses 2 and 9. Experiments from three separate coverslips for cells expressing each isoform yielded similar results.

reported the electrophysiological characterization of recombinant rat brain NCKX2 expressed in HEK293 cells (31). NCKX2 was shown to exchange 4 Na+ ions for 1 Ca2+ and 1 K+ with $K_{0.5}$ values of $\sim$1 $\mu$M or 100 $\mu$M for Ca2+ dependence, in the absence or presence of 1 mM Mg2+, respectively, 10 or 35 mM for K+ dependence, in the presence of choline+ or Li+, respectively, and 30 or 60 mM for Na+ dependence or inhibition, respectively. Although NCKX3 and -4 have been demonstrated to function as K+-dependent Na+/Ca2+ exchangers, their detailed functional characteristics are unknown (14, 15). NCKX3 and -4 are closely related, with 71% overall amino acid sequence identity but only 40% identity with NCKX1 and -2, which may underlie functional differences between the isoforms that could have important implications for their roles in Ca2+ signaling.

In the current study, functional characterization of recombinant human NCKX2, -3, and -4 was undertaken by expression in HEK293 cells loaded with fura-2 AM in a perfusion chamber using a microscope photometer. The results of these studies indicated the apparent $K_{0.5}$ for K+ ions of NCKX3 and -4 was 40-fold lower than that of NCKX2. Site-directed mutagenesis of NCKX2 and -4 based on multiple sequence alignments of the $\alpha$-repeat regions was employed to identify the molecular determinants responsible for the apparent differences in K+ affinity. These studies suggest that the identified residues may alter the conformational flexibility and/or liganding strength of key acidic residues in the K+ binding pocket.

EXPERIMENTAL PROCEDURES

All common chemicals and reagents were purchased from Sigma, VWR, or BDH and were minimally of analytical grade. All molecular procedures were performed according to previously established protocols (32) or according to reagent manufacturer’s instructions unless indicated otherwise.

DNA sequencing confirmed that both the long and short splice variants (with or without resides 360–376 in the large cytoplasmic loop, respectively), were obtained and that the sequences were identical to those deposited in GenBankTM for human NCKX2 (12, 13). Subsequent functional analyses suggested that there were no obvious functional differences between the short and long splice variants, so all subsequent experiments were performed using the long splice variant of NCKX2.

Generation of the constructs used for expression of recombinant human NCKX3 and -4 has been described previously (14, 15). PCR was used to insert the FLAG epitope (amino acids DYKDDDDK) into the putative N-terminal extracellular loops of NCKX2 (by replacing residues 89–96), NCKX3 (by insertion after residue 66), and NCKX4 (by insertion after residue 41).

Mutagenesis—Site-directed mutagenesis to achieve the desired changes in the amino acid sequences was performed using either overlap-extension PCR or megaprimer PCR according to previously established methods (33, 34). The correct sequences of all the constructs was confirmed by DNA sequencing. The amino acid numbering was based on the long splice variants of NCKX2 and NCKX3 and the short splice variant of NCKX4, which corresponds to the versions used in our functional assays.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained in 5% CO2 at 37°C. Transfection of Qiagen-purified expression plasmids into HEK293 cells was performed using a standard Ca2+-phosphate precipitation protocol as previously described (12). Experiments utilizing cells transfected with the parent expression plasmid, pcDNA3.1+ (Invitrogen), were used as negative controls.

Photometry and Data Analysis—Two days after transfection, HEK293 cells on poly-d-lysine-coated coverslips were loaded with 5 $\mu$M fura-2 AM (Invitrogen) and mounted in a perfusion...
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Development of a Functional Assay for Recombinant NCKX2, -3, and -4—HEK293 cells grown on coverslips and transfected with expression constructs for recombinant FLAG-tagged NCKX2, -3, or -4, or pcDNA3.1+ without insert, were loaded with fura-2 AM and mounted in a perfusion chamber on a microscope stage. The ratio of fluorescence emission at 510 nm from excitations at 340 nm and 380 nm with a D-104 microscope photometer using Felix version 1.42 software (Photon Technology International). The perfusion solutions used contained 10 mM D-glucose and 10 mM HEPES-tetramethylammonium, pH 7.4, with either 145 mM NaCl or LiCl and varying concentrations of KCl and/or NaCl to a total of 145 mM, and 0.1 mM CaCl2 or varying concentrations of CaCl2 buffered with 0.5 mM EGTA to the desired final concentration for Ca2+ dependence experiments. The cells were perfused alternatively for 3 min with sodium buffer or 2 min with lithium buffer at a rate of ~3 ml/min in a volume of ~0.5 ml, ensuring complete solution change in ~10 s or less. Ca2+ transport rates for reverse mode NCKX2 activity were determined by linear regression of the initial linear rate of change of the fura-2 ratio. Each coverslip was internally normalized to the average of the rates of the second and last pulses, but if they differed by >20% a correction for the change in maximal rate with time was applied, using a linear interpolation between the second and last pulses to mathematically correct the intervening pulses as illustrated in Fig. 1. All data analysis and curve-fitting were performed using GraphPad Prism version 4.0 software. The Ca2+ and K+ dependence curves and Na+ inhibition curves were fitted using nonlinear regression analysis to the equation: $Y = \frac{\text{Top} - \text{Bottom}}{1 - 10^{(\log(K_{Na} or I_{Ca}) - X)}}$Hillslope, where, with the exception of the NCKX2 K+ dependence curve, the Top was constrained to a value of 100% and the Bottom was constrained to a value of 5%.

RESULTS

Development of a Functional Assay for Recombinant NCKX2, -3, and -4—To assess the apparent affinities of various NCKX isoforms for Ca2+, the initial rates of fura-2 ratio change were determined in HEK293 cells expressing were generally considered significant when the second and last pulses differed more than the variability observed in experiments where no run-down was observed (i.e. >20%). In such cases, a time-dependent correction was applied, based on linear interpolation between pulses two and nine, and used to mathematically correct the intervening pulses (Fig. 1C). It is also worth noting that the amplitude of the Ca2+ responses did not always correlate well with the initial rate of rise (this is seen in the example traces of Figs. 2 and 3). Initial rates were always used for data analysis as illustrated in Figs. 2B and 3B.

Ca2+ and K+ Concentration Dependence for Reverse-mode Activity of NCKX2, -3, and -4—To assess the apparent affinities of various NCKX isoforms for Ca2+, the initial rates of fura-2 ratio change were determined in HEK293 cells expressing...
recombinant FLAG-tagged NCKX2, -3, and -4 in buffers containing 135 mM Li⁺/H⁺, 10 mM K⁺, and varying concentrations of Ca²⁺. These experiments were performed using either increasing or decreasing concentrations of Ca²⁺, yielding identical results. Representative experiments in which decreasing concentrations were used are presented in Fig. 2A, whereas representative experiments in which increasing concentrations were used are presented in some of the subsequent figures. Cells transfected with pcDNA3.1+ did not display any notable changes in the fura-2 ratio when perfused with the various buffers. To illustrate the data analysis methods used, the portions of the representative traces shown in Fig. 2A corresponding to the initial rates of fura-2 ratio change for each pulse are presented in Fig. 2B. When the data from four to six coverslips were internally normalized, averaged, and fitted using nonlinear regression analysis, the resulting apparent \( K_{0.5} \) values for NCKX2, -3, and -4 were similar (1.3–1.7 μM), and the Hill slope values ranged from 0.98 to 1.2, suggesting the presence of a single class of Ca²⁺-binding sites (Fig. 2C and Table 1). These data were consistent with the previously reported value of 1.4 μM obtained for recombinant rat brain NCKX2 using electrophysiological methods (31).

**TABLE 1**

| Cation Protein | Apparent affinity | Hill slope | \( n \) |
|----------------|------------------|------------|------|
| Ca²⁺          | NCKX2 1.6 ± 0.2  | 0.98 ± 0.11| 4    |
|               | NCKX3 1.3 ± 0.3  | 1.1 ± 0.2  | 6    |
|               | NCKX4 1.7 ± 0.2  | 1.2 ± 0.2  | 4    |
| K⁺            | NCKX2 40  | ND*        | 7    |
|               | NCKX3 1.3 ± 0.4  | 1.0 ± 0.2  | 4    |
|               | NCKX4 1.1 ± 0.2  | 1.1 ± 0.2  | 7    |
| Na⁺           | NCKX2 34 ± 6    | 1.6 ± 0.4  | 4    |
|               | NCKX3 30 ± 4    | 1.7 ± 0.3  | 4    |
|               | NCKX4 18 ± 3    | 1.8 ± 0.5  | 4    |

*ND, not determined.

**FIGURE 3.** K⁺ concentration dependences of recombinant NCKX2, -3, and -4. The effects of varying K⁺ concentrations on the reverse-mode operation of NCKX2, -3, and -4 in the presence of 0.1 mM Ca²⁺ were determined and analyzed using the functional assay described in the legend to Fig. 2A. Representative data for cells expressing NCKX2, -3, and -4 or transfected with pcDNA3.1+ are presented in panel A. C, data from four to seven coverslips were normalized and averaged. Each point represents the average (± S.E.) of 4 to 14 determinations, and where the point is larger than the S.E., error bars are not shown. The \( K_{0.5} \) values are presented in Table 1.

**FIGURE 4.** Na⁺ inhibition of recombinant NCKX2, -3, and -4. The effects of varying Na⁺ concentrations on the reverse-mode operation of recombinant NCKX2, -3, and -4 in the presence of 10 mM K⁺ and 0.1 mM Ca²⁺ were determined and analyzed using the functional assay described in the legend to Fig. 2A. Representative data for cells expressing NCKX2, -3, and -4 or transfected with pcDNA3.1+ are presented in panel A. B, data from four to seven coverslips were normalized and averaged. Each point represents the average (± S.E.) of 4 to 14 determinations, and where the point is larger than the S.E., error bars are not shown. The IC₅₀ values are presented in Table 1.
Characterization and Mutagenesis of NCKX2, -3, and -4

The K⁺ concentration dependences of the initial rates of fura-2 ratio change in HEK293 cells expressing recombinant NCKX2, -3, and -4 were determined in buffers containing 0.1 mM Ca²⁺ and varying concentrations of K⁺ (Fig. 3). In Fig. 3 (A and B) representative traces and the respective portions of each pulse corresponding to the initial rates of fura-2 ratio change are shown. For all the subsequent figures, only the representative traces are shown. When the average data from four to seven coverslips for the K⁺ concentration dependences were subjected to nonlinear regression analysis, NCKX2 displayed rates that did not saturate within the range of concentrations tested, whereas NCKX3 and -4 displayed apparent Kₐ values of 1.3 and 1.1 mM, respectively, and Hill slope values of 1.0 and 1.1, respectively, consistent with the presence of a single K⁺ binding site (Fig. 3C and Table 1). Because the range of K⁺ concentrations tested did not saturate NCKX2 activity, the data could not be fitted with a high degree of confidence, but an apparent Kₐ of ~40 mM was obtained when the “Top” parameter (i.e. the maximal rate) was unconstrained in the nonlinear regression analysis. This apparent low affinity for K⁺ was consistent with our previously reported apparent Kₐ of ~36 mM for recombinant rat brain NCKX2 obtained using electrophysiology (31). These data indicated that NCKX3 and -4 have ~40-fold higher apparent affinities for K⁺ ions during reverse-mode operation than NCKX2.

Na⁺ Inhibition of the Reverse-mode Activity of NCKX2, -3, and -4—To assess the affinities of the various NCKX isoforms for Na⁺ ions, we determined the initial rates of fura-2 ratio change in HEK293 cells expressing recombinant NCKX2, -3, and -4 in perfusion buffers containing 10 mM K⁺, 0.1 mM Ca²⁺, and varying concentrations of Na⁺ (Fig. 4A). HEK293 cells transfected with pcDNA3.1+ did not display any notable fura-2 ratio changes in the presence of the various perfusion solutions. The average data from four separate experiments for each isoform was fitted using nonlinear regression analysis and revealed apparent IC₅₀ values of 34, 30, and 18 mM for NCKX2, -3, and -4, respectively (Fig. 4B and Table 1), which were consistent with the value of 60 mM determined for recombinant rat brain NCKX2 in the presence of 40 mM K⁺ and 1 mM Ca²⁺ using electrophysiology (31). The Hill slope values ranged from 1.6 to 1.8, which was consistent with the presence of multiple classes of Na⁺ binding sites that are either competitive or allosteric (31, 35). These data suggested that NCKX2, -3, and -4 exhibit similar apparent affinities for Na⁺ ions under these conditions of assay.

Multiple Sequence Alignments and Mutagenesis Strategy—In studies on NCX proteins aimed at identifying the amino acid residues responsible for the differential Ni²⁺ and Li⁺ sensitivities of NCX1, -2, and -3, extensive chimeric constructs were generated and tested, revealing that the only regions with any notable effects contained the α-repeat 1 and 2 sequences (36). Furthermore, extensive mutagenesis efforts on NCKX isoforms strongly suggested that the α-repeats contain the structural motifs responsible for cation binding and translocation (22–24). Therefore, to identify the molecular determinants responsible for the apparent differences in K⁺ affinities between...
Characterization and Mutagenesis of NCKX2, -3, and -4

NCKX2 and NCKX3 and -4, we performed multiple sequence alignments containing only the α 1 and 2 repeat regions (Fig. 5). Using this alignment, we identified residues that were different between NCKX2 and NCKX3 and -4 and constructed grouped mutations (M1–M6) that covered the entire lengths of the α-repeat regions, in which the selected residues in NCKX2 were converted to the corresponding residues in NCKX4 and vice versa (Table 2). Because NCKX3 and -4 differ by only eight amino acids in the α-repeat regions and the results presented in Table 1 suggested that these two proteins are functionally very similar, NCKX3 was not subjected to any further analyses. Immunoblotting of postnuclear extracts from HEK293 cells expressing either wild-type or mutant NCKX2 and -4 constructs using anti-FLAG monoclonal antibodies confirmed that all of the generated constructs were expressed at roughly similar levels (data not shown).

K⁺ Affinity Assays for the NCKX2 and -4 Mutant Constructs—
To determine if the introduced mutations resulted in altered K⁺ affinities, the reverse-mode activity of NCKX2 and -4 and their respective mutant constructs were assayed in buffers containing 0.1 mM Ca²⁺ and either 1, 10, or 100 mM K⁺ (Fig. 6). For NCKX2, M1 yielded an apparently decreased affinity for K⁺, whereas M5 was nonfunctional, and M2, M3, and M6 appeared to have no notable effect (Fig. 6A). NCKX2-M4 displayed a dramatically increased affinity for K⁺, so 0.1 mM K⁺ was also tested to examine more thoroughly the K⁺ dependence of this grouped mutant construct (Fig. 6B). These data suggested that one or more of the mutations within M4 conferred increased affinity of NCKX2 for K⁺ ions.

For the reciprocal set of mutants in the NCKX4 background, M1, M3, M4, and M6 all displayed apparently decreased affinities for K⁺, whereas M2 and M5 had little effect (Fig. 6, C and D). The observation that many different mutations yielded apparently decreased K⁺ affinities suggested that multiple different structural determinants can directly or indirectly influence this parameter in NCKX4. The NCKX2-M1 grouped mutations, and the reciprocal NCKX4-M1 mutations both resulted in decreased K⁺ affinities, suggesting that these residues were not responsible for the apparent K⁺ affinity differences between NCKX2 and -4 but rather suggested that there was an indirect, nonspecific effect on the tertiary structure of the K⁺-binding pocket. Taken together, the only sets of mutations that resulted in increased apparent K⁺ affinity for NCKX2 and decreased apparent K⁺ affinity for NCKX4 were the respective M4 mutants (Fig. 6). These data suggested that one or more of the amino acid residues mutated in the M4 construct were likely to influence K⁺ affinity directly.

Analysis of NCKX2 Single Residue Mutants—The six residues mutated as a group in the NCKX2-M4 construct (see Table 2) were mutated individually (L549C, I550M, T551A, and K558Q) or in pairs (I546V/V553L), expressed in HEK293 cells and subjected to the same K⁺ affinity assay that was used to test the grouped mutant constructs (Fig. 7).
All of the individual NCKX2 mutant constructs displayed normal activity levels except L549C, which had reduced activity, consistent with previous data on this mutant (22). These constructs all had unaltered \( K^+ / H^+ \) dependences or slightly decreased apparent affinity (I550M) except for the T551A mutant. Strikingly, this single mutant converted NCKX2 to a higher apparent \( K^+ / H^+ \) affinity and suggested that Thr-551 was responsible for the low apparent \( K^+ / H^+ \) affinity of NCKX2 when compared with NCKX4.

Analysis of NCKX4 Single or Combination Mutants—The reciprocal set of mutations in the M4 set to those in NCKX2 were constructed in the NCKX4 background (see Fig. 5 and Table 2) and tested for \( K^+ / H^+ \) affinity (Fig. 8A). Interestingly, the converse of NCKX2-T551A, NCKX4-A469T, did not result in decreased \( K^+ / H^+ \) affinity as might have been predicted from the results of Fig. 7 (Fig. 8, A and C). The only mutation with notably decreased \( K^+ / H^+ \) affinity in NCKX4 was Q476K, the converse of which in NCKX2 (K558Q) had no effect. The NCKX4-M468I mutation appeared to modestly increase the apparent \( K^+ / H^+ \) affinity, which was consistent with the modestly decreased \( K^+ / H^+ \) affinity of the reciprocal mutant NCKX2-I550M (Figs. 7A and 8A). Although NCKX2-L549C displayed severely impaired functional activity, the converse mutation in NCKX4 (C467L) displayed wild-type levels of functional activity (data not shown). We hypothesized that the NCKX4-A469T mutation may confer decreased \( K^+ / H^+ \) affinity in a more “NCKX2-like” structural context and therefore combined each of the mutants in the NCKX4-M4 set together with A469T (Fig. 8B). The only combination mutant that displayed a decreased apparent \( K^+ / H^+ \) affinity was NCKX4-A469T/Q476K, which was similar to the effect of NCKX4-Q476K alone. These data indicated that the reciprocal situation to the NCKX2-T551A mutation could not be reconstructed in the NCKX4 background.

\( K^+ / H^+ \) and \( Ca^{2+} / H^+ \) Concentration Dependences for Reverse-mode Activity of NCKX2-T551A—To characterize the NCKX2-T551A mutant in more detail, HEK293 cells expressing this mutant were assayed for \( Ca^{2+} / H^+ \) and \( K^+ / H^+ \) affinities using the same experimental protocol as was used in Figs. 2 and 3 for the wild-type exchangers (Fig. 9). The \( K^+ \) concentration dependence of NCKX2-T551A yielded a \( K_{0.5} \) value of 0.86 ± 0.12 mM, which was similar to the value obtained for NCKX4 and dramatically lower than that of the wild-type protein (Fig. 9, A and B). In contrast, the \( Ca^{2+} / H^+ \) dependence of NCKX2-T551A yielded a \( K_{0.5} \) value of 0.74 ± 0.14 \( \mu \)M, which was 53% of the wild-type NCKX2 value (Fig. 9, C and D). These data suggested that a single mutation, T551A, selectively increased the apparent \( K^+ \) affinity of NCKX2 to a value similar to that of NCKX4, without a concurrently dramatic shift in \( Ca^{2+} / H^+ \) affinity.

\( K^+ \) and \( Ca^{2+} / H^+ \) Concentration Dependences for Reverse-mode Activity of NCKX4-Q476K—As for NCKX2-T551A, full \( K^+ \) and \( Ca^{2+} / H^+ \) concentration dependence experiments were performed...
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for NCKX4-Q476K (Fig. 10). NCKX4-Q476K displayed an apparent $K_{0.5}$ value for $K^+$ of $13 \pm 2$ mM, which was 12-fold higher than the value obtained for wild-type NCKX4 but ∼3-fold lower than that of NCKX2 (Fig. 10, A and B). The apparent $K_{0.5}$ of NCKX4-Q476K for $Ca^{2+}$ was $5.6 \pm 1.4$ μM, which was 3.3-fold higher than that of wild type (Fig. 10, C and D). These results suggested that this mutation reduced the apparent affinity of NCKX4 for both ions, although the effect on $K^+$ interactions was somewhat larger.

**DISCUSSION**

In the current study we have developed a functional assay, which has allowed us to characterize the properties of recombinant NCKX3 and -4 and demonstrated that, although their apparent affinity parameters for $Ca^{2+}$ and $Na^+$ were similar to those of NCKX2, they displayed a dramatically higher apparent affinity for $K^+$ (Figs. 2–4 and Table 1). The high $K^+$ affinity suggests that NCKX3 and -4 may be uniquely subject to regulation by $K^+$ channels in close proximity, which could increase the local external $K^+$ concentration to a level that would saturate the external-facing $K^+$ binding site and hence inhibit normal $Ca^{2+}$ extrusion. NCKX2 activity would be much less affected in this context, because the external $K^+$ sites have low affinity and would not be saturated.

The apparent low affinity of NCKX2 for $K^+$ obtained in the current study was strikingly similar to those independently reported for recombinant rat brain NCKX2 expressed in HEK293 cells using electrophysiological methods and recombinant human NCKX2 heterologously expressed in insect cells obtained by $^{45}Ca^{2+}$ uptake assays (13, 31). Kinetic studies on bovine rod NCKX1, which has a high degree of amino acid sequence identity with NCKX2 within the α-repeats, originally indicated an apparent affinity for $K^+$ in the 1–2 mM range (37, 38). Those studies utilized preparations of bovine rod outer segments and were done under entirely different conditions from our studies. Subsequently, reports, using conditions similar to those employed here to assess apparent $K^+$ affinity of NCKX1 in bovine outer rod segments and recombinant bovine NCKX1 expressed in insect cells, demonstrated a nonsaturating low apparent affinity for $K^+$ ions indistinguishable from that of recombinant human
NCKX2, and very similar to the value we obtained (13, 39). Furthermore, assays of K\(^{+}\)-dependent Na\(^{+}\)/Ca\(^{2+}\) exchange activity, likely to be NCKX2-mediated, in acute preparations of neurohypophysial axon terminals suggested an apparent K\(^{+}\) affinity of \(\sim 30 \text{ mM}\) for the forward mode of the exchanger, which was consistent with the “low affinity” phenotype observed in our recombinant assay system (40).

A survey of the previously published NCX and NCKX structure-function studies indicates that four key hydrophilic residues, one in the middle of each of the four TM s in the \(\alpha\)-repeats and conserved between NCX and NCKX isoforms are likely to be involved in cation liganding (Fig. 5). Glu-188 and Asp-548, in TM s 2 and 7, respectively, of NCKX2 have been demonstrated to be critical determinants of both K\(^{+}\) and Ca\(^{2+}\) affinities and are largely irreplaceable, whereas mutations of the corresponding residues in NCX1 resulted in nonfunctional proteins (22, 23, 28). Mutation of Asp-575 in TM 8 of NCKX2 to the corresponding residue in NCX (N842) resulted in a protein that exhibited Na\(^{+}\)/Ca\(^{2+}\) exchange function that was K\(^{-}\)-independent, suggesting an important role for this residue in forming the K\(^{+}\) binding site (24). Evidence based on disulfide cross-linking studies has suggested that Glu-188, Asp-548, and Asp-575, although far apart in the primary sequence, are close together in the folded protein where they could form the cation binding pocket of NCKX2 (Fig. 5) (41). Lastly, it was recently shown that Asn-143 in TM 3 of NCX1 could only be functionally replaced by an Asp side chain, although the importance of its counterpart in NCKX2, Asn-215, has not been investigated (26). Taken together, these studies suggest that Glu-188, Asn-215, Asp-548, and Asp-575 are key liganding residues that form the Ca\(^{2+}\)/K\(^{+}\) binding site of NCKX2.

The NCKX2-T551A mutant displayed a unique phenotype, because it displayed a large shift in apparent K\(^{+}\) affinity that was not paralleled by a similar shift in apparent Ca\(^{2+}\) affinity. Thr-551 of NCKX2 is on the same side of the TM7 helix as Asp-548 and therefore also in close proximity to Asp-575, a critical determinant of the K\(^{+}\) binding site (24, 41). Previous studies have demonstrated that many NCKX2 mutants with altered K\(^{+}\) affinities often displayed concurrent shifts in Ca\(^{2+}\) affinities, which argues strongly in favor of a model in which both Ca\(^{2+}\) and K\(^{+}\) share a common binding pocket (23). Because both K\(^{+}\) and Ca\(^{2+}\) must be bound to initiate transport, the binding mechanism is likely to be ordered and cooperative, and at least some of the atoms involved in liganding are likely to be exclusive to each cation. Thr-551, which lies cytoplasmic of the critical liganding residues in the most current topology model (19, 20), is unlikely to play a direct role in liganding of K\(^{+}\) ions (because removal of this potential liganding residue actually increases K\(^{+}\) affinity) but rather may alter the flexibility, orientation, and/or strength of one of the side-chain oxygen atoms involved in liganding, resulting in a specific effect on K\(^{+}\) interactions. This model suggests that it is possible to specifically alter the affinity of one cation and not the other, even though they likely bind to the same pocket, by means of subtle conformational influences on specific liganding moieties.

The observation that the reciprocal mutation to NCKX2-T551A in NCKX4 (A469T) had no effect on apparent K\(^{+}\) affinity suggests that a combination of additional molecular determinants, present only in NCKX2, are required to obtain the structural configuration necessary for Thr-551 to have its influence on K\(^{+}\) affinity. However, the Q476K mutation in NCKX4, which corresponds to K558Q in NCKX2, resulted in decreased affinities for both K\(^{+}\) and Ca\(^{2+}\) (Fig. 10). Based on the current topology models of NCKX2, this residue is predicted to lie at the cytosolic end of TM 7 and is unlikely to directly influence cation liganding (19, 20). Therefore, the observed effects were likely due to an indirect conformational influence of the introduced positively charged Lys residue on the NCKX4 protein. NCKX2 normally possesses a Lys residue at the equivalent position, substitution of which with Gln does not affect K\(^{+}\) affinity, suggesting that the structural context of this residue is also different in NCKX4 from that in NCKX2.

This study has provided novel functional information regarding NCKX3 and -4, which will be valuable in determining the specific role of these two isoforms in Ca\(^{2+}\) signaling. It has been demonstrated that NCKX proteins make substantial contributions to Ca\(^{2+}\) clearance in neurons, but it has thus far not been possible to determine which specific isoforms contribute in various subcellular signaling contexts (10, 40). The large apparent differences in K\(^{+}\) affinities demonstrated in this study may serve as a means to dissect the relative contributions of NCKX2 versus NCKX3 and -4. Future functional studies on in situ NCKX proteins in acute brain preparations and/or cultured neurons will be aimed at addressing these questions.

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