Minimal Domain Requirement for Cation Transport by the Potassium-dependent Na/Ca-K Exchanger

COMPARISON WITH AN NCKX PARALOG FROM CAENORHABDITIS ELEGANS*

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The retinal rod Na/Ca-K exchanger (NCKX) is a unique calcium extrusion protein utilizing both inward sodium gradient and outward potassium gradient. Three mammalian rod NCKX cDNAs have been cloned to date, but quantitative analysis of NCKX function in heterologous systems has proven difficult. Here, we describe a simple system for quantitative analysis of NCKX function; stable transformation of cultured insect cells with the novel pEAI153A vector containing NCKX cDNAs was combined with measurements of potassium-dependent 45Ca uptake in sodium-loaded cells. We carried out structure-function studies on NCKX with the following results: 1) two-thirds of the full-length sequence of bovine NCKX could be deleted without affecting potassium-dependent calcium transport and without affecting key properties of the potassium binding site; 2) the affinity of NCKX for potassium was about 10-fold greater in choline medium when compared with lithium medium; this shift was observed in rod outer segments or in cells expressing full-length rod NCKX, the above deletion mutant, or a distantly related NCKX paralog cloned from Caenorhabditis elegans. We conclude that the potassium binding site is highly conserved among members of the NCKX family and is formed by residues located within the two sets of transmembrane spanning segments in the NCKX sequence.

Calcium extrusion across the plasma membrane of cells is vital to all cells, in view of the ubiquitous role of calcium as second messenger and since sustained elevated calcium levels rapidly lead to cell death (1). Calcium extrusion against a large electrochemical calcium gradient is mediated by two classes of plasma membrane proteins, an ATP-driven calcium pump and Na/Ca exchangers. Two groups of plasma membrane Na/Ca exchangers can be distinguished: those that require nor transport potassium (the NCKX family) and those that require and, in the case of the rod photoreceptor NCKX1, have been demonstrated to transport potassium (the NCKX family) (for recent reviews, see Refs. 2 and 3). To date, three NCKX1 cDNAs have been cloned from mammalian rod photoreceptors (4–6) and one NCKX2 cDNA from rat brain (7). Furthermore, several potential NCKX paralogs present in lower organisms have been identified on the basis of analysis of sequences obtained from genomic sequencing projects (2, 8). Studies on functional properties of the “in situ” Na/Ca-K exchanger have been limited thus far to NCKX1 found in the plasma membrane of the outer segments of retinal rod photoreceptors (reviewed in Refs. 9–11). Sequence comparison of the three mammalian NCKX1 orthologs cloned to date reveals a remarkably low sequence identity (~65%) in contrast to sequence identities of >90% observed for other sodium-coupled transporters. We examined functional activity of heterologously expressed dolphin, bovine, and human NCKX1 in several cell systems and only observed consistent and robust functional expression with the dolphin NCKX1 cDNA (6). Comparing the mammalian rod NCKX1 sequences with the sequence from rat brain NCKX2 sequence or with sequences from putative NCKX paralogs in lower organisms reveals high conservation only within the two sets of putative transmembrane-spanning segments and their short connecting loops, whereas very limited or no sequence similarity is apparent between the N-terminal extracellular loops or between the large intracellular loops that bisect the two sets of transmembrane spanning segments between segments 5 and 6. In this study, we tested the hypothesis that the cation binding sites and residues involved in potassium-dependent Na/Ca exchange transport reside within the above two sets of conserved transmembrane spanning segments. First, we tested for potassium dependence in a double deletion construct based on bovine NCKX1, in which the large hydrophilic loops were largely deleted (bNCKX1dd). Second, we cloned the cDNA of an NCKX paralog identified in the Caenorhabditis elegans genomic sequencing project (ceNCKX; GenBank™ accession number AJ005701) and tested for potassium-dependent Na/Ca exchange. Sequence similarity between ceNCKX and its mammalian NCKX paralogs NCKX1 and NCKX2 is limited to the two sets of transmembrane-spanning segments. Potassium-dependent Na/Ca exchange activity was measured in High Five insect cells after stable transformation with the various NCKX cDNAs using a novel expression vector (12). Potassium-dependent Na/Ca exchange transport was observed in cells transformed with bNCKX1dd, in cells transformed with full-length dolphin dNCKX1, and in cells transformed with ceNCKX, whereas no activity was observed in cells transformed with full-length bovine bNCKX1; potassium-independent Na/Ca exchange transport was observed in cells transformed with bovine NCX1. We conclude that stable transformation of insect High Five cells with our novel expression vector is a very useful tool for quantitative studies on members of the NCKX family.

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**Experimental Procedures**

**Construction of the Double Deletion Mutant bNCKX1dd**—The large extracellular loop of the rod NCKX1 is preceded by a putative signal peptide, without which proper translation and membrane insertion may not be possible. We therefore removed the extracellular domain of NCKX1, including the putative signal sequence, and replaced it with the signal sequence and much shorter extracellular domain of the bovine cardio cNCKX1 exchange. The 5′ end of the rod exchanger cDNA up to the BglII site was excised. A fragment corresponding to the first amino acid of transmembrane region 1 up to the BglII site was amplified by polymerase chain reaction. A SpeI site was incorporated to the 5′ end. These fragments, when ligated, corresponded to the pure rod cDNA sequence beginning at the first transmembrane amino acid and proceeding through the remainder of the exchanger cDNA. The signal peptide from the bovine cardio cNCKX1 exchanger was then amplified by polymerase chain reaction. This fragment ligated to the rod sequence generated a complete chimeric cDNA. The full-length construct was subsequently digested with NotI and 3′ with EcoRI and cloned into the insect expression vectors. Most of the large cytosolic loop of NCKX1 was removed from the rod exchanger by digestion with AvrII and BanII. In its place, we ligated a short synthetic adaptor, which contained an internal BclI site. The correct sequence of the resulting construct was verified by sequencing and is illustrated in Fig. 1.

**Cloning of C. elegans NCKX**—Primers were designed, based on a putative open reading frame of C. elegans sequence (GenBank™ accession number AJ005701), to amplify the C. elegans exchanger in two pieces. Briefly, the 5′ primer for the amino terminus segment had an Nhel site added just upstream of the ATG initiation codon, and the 3′ primer for the carboxyl terminus overlapping the proposed termination codon with a SpeI site incorporated to the same unique BanHI site and cloned into pcDNA3.1+ (Invitrogen) digested with the same restriction endonucleases. The carboxyl terminus was generated using a 5′ primer just upstream of the same unique BanHI site and a 3′ primer overlapping the proposed termination codon with a BglII restriction site added. This fragment was cloned after digestion with BanHI into likewise digested pcDNA3.1+. Four of each of the two constructs were sequenced and compared with the sequence of the original polymerase chain reaction products from which the clones were constructed. The selected Nhel/BanHI clone was digested with BanHI and the correct BanHI fragment from the carboxyl terminus construct was cloned into it. The resulting clone contained the entire C. elegans exchanger cDNA. The insert was removed by Nhel and NotI digestion, blunted ended with Klenow fragment, and cloned into the Smal site of pE1153A.

**Stable Expression of Recombinant Proteins Using the pE11/153A Vector Containing NCKX cDNAs**—The various NCKX constructs were ligated into the pE11/153A vector using appropriate restriction sites and used to transform DH5α cells, which were plated out overnight at 37°C. Cells were picked and grown over night in L-maltose minimal medium. Plasmid isolation was carried out using the QIAfilter Plasmid Midi Kit (Qiagen, Mississauga, Ontario, Canada). Restriction digestion was used for initial screening of clones. Clones that matched the expected restriction digests then had their 5′ and 3′ ends sequenced. Appropriate clones were then used to transfect High Five™ cells.

Expression of bovine NCX1, bovine and zebrafish full-length NCKX1, bNCKX1dd, and NCKX from C. elegans was established after stable transformation of High Five cells with the use of a novel transformed lepidopteran insect cell expression system (12). Transformed cell lines were generated when High Five™ cells were first co-transfected with two vectors, the expression plasmid of interest and a second plasmid conferring resistance to hygromycin B, followed by antibiotic selection in 1 mg/ml hygromycin B and culturing of positive clonal cell lines.

**Protein Concentrations, SDS-Gel Electrophoresis, and Western Blots**—High Five cells were collected and washed twice with 150 mM NaCl, 20 mM Hepes (pH 7.4), 80 mM sucrose, and 200 μM EDTA. The final pellets were resuspended in 200 μl of ice-cold radioimmuno precipitation buffer containing 1% Triton X-100, 0.5% deoxycholate, 140 mM NaCl, 25 mM Tris (pH 7.5), 100 mM EDTA, and a protease inhibitor tablet (Roche Molecular Biochemicals catalog number 1836170), and subsequently incubated on ice for 20 min. The samples were spun down in a microcentrifuge for 5 min at 20,000 × g. Supernatants were removed and assayed for protein concentration using the Bradford dye-binding procedure (Bio-Rad). Bovine serum albumin was used as the standard in all protein assays.

Protein samples were separated on an 8% SDS-polyacrylamide gel and either stained with Gelcode Blue (Pierce) or transferred onto nitrocellulose (Bio-Rad) in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine, 20% methanol, and 0.05% SDS. For Western blotting, the membranes were blocked for 1 h in TBST (10 mM Tris, pH 8.0, 100 mM NaCl, 0.05% Tween 20) and 10% skim milk, briefly rinsed in TBST, and subsequently incubated for 1 h at room temperature with primary antibody (1:200 dilution of PMI-L3B or 10 μg/ml of H2 antibody) in TBST with 1% skim milk added. After washing, the membranes were incubated for 1 h with a 1:15000 dilution of sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) in TBST plus 1% skim and then washed again. Immunodetection was carried out using LumiGlo chemiluminescent reagents (New England Biolabs).

**Northern Blot Analysis**—Total RNA from High Five cells transformed with the different NCKX cDNAs was isolated using the Trizol reagent (Life Technologies, Inc.). Total RNA samples (7.5 μg) were separated on 1% agarose-formaldehyde gels and transferred onto positively charged nylon membrane (Ambion) by capillary transfer. Labeled probes were generated from the cDNA of bNCKX1dd according to the protocol of the manufacturer (Prime I+II; Stratagene).

**45Ca Uptake Experiments**—High Five cells containing the various constructs described above were sodium-loaded by incubation for 15 min in 150 mM NaCl, 80 mM sucrose, 20 mM Hepes (pH 7.4), 0.2 mM EDTA, and 5 μM monensin. Monensin was subsequently removed from the cells by two washes in the above medium without monensin but containing 150 mM LiCl or choline chloride (as indicated), 80 mM sucrose, 20 mM Hepes (pH 7.4), and 0.2 mM EDTA. The final pellet was resuspended in 150 mM LiCl or choline chloride (as indicated), 80 mM sucrose, 20 mM Hepes (pH 7.4), and 0.05 mM EDTA and left at room temperature until use. With a given batch of sodium-loaded cells, 45Ca uptake experiments were completed within 35 min. 45Ca uptake experiments were initiated by a 10-fold dilution in 150 mM LiCl, choline chloride, KCl or NaCl (as indicated), 80 mM sucrose, 20 mM Hepes (pH 7.4), 1 mM FCCP, 35 mM CaCl2, and 1 μCi of 45Ca. At the indicated times, samples were withdrawn, and external 45Ca not taken up by cells was removed by a rapid filtration technique over borosilicate glass fiber filters as described previously (13); the washing medium contained 140 mM KCl, 5 mM MgCl2, 20 mM Hepes (pH 7.4), 80 mM sucrose, and 1 mM EGTA. Sucrose used in the above media was added from a 600 mM stock solution that was passed over a mixed bed ion exchanger to eliminate cation contamination. Experiments on isolated bovine retinal rod outer segments were carried out on calcium-depleted rod outer segments with the sodium loading protocol and media described above; other details concerning such experiments on rod outer segments were as described previously (13, 14).

**Results**

In this study, we have examined the minimal domain requirement for potassium-dependent Na/Ca exchange of the retinal rod NCKX1 Na/Ca-K exchanger. For this purpose, we generated cloned High Five cell lines after stable transformation with a variety of (mutant) NCKX cDNAs; we used cells transformed with cardiac bovine NCX1 as a positive control for potassium-independent Na/Ca exchange. In a first set of experiments, we compared full-length bovine NCX1 with full-length bovine NCX1 and with our bovine NCX1 double deletion mutant (bNCKX1dd), in which the large N-terminal extracellular loop of 422 amino acids was deleted and replaced with that of bovine NCX1 (69 amino acids) and in which, in addition to the above replacement of the extracellular loop, the majority of the large cytosolic loop (338 of 425 amino acids) was deleted. Fig. 1 illustrates a schematic diagram of the bNCKX1dd construct. We generated a CHO3 cell line after stable transformation with the bNCKX1dd construct and observed no Na/Ca exchange function, either measured with 45Ca uptake or measured by sodium-dependent changes in cytosolic free calcium measured with fluo-3 in an assay we developed earlier (15). In view of these initially negative results, we investigated a novel transformed lepidopteran expression system that yields...
stable transformation of High Five cells (12). This system has proven successful in expressing at high levels a variety of recombinant proteins including secreted glycoproteins, cytoplasmic proteins, nuclear factors, and membrane receptors. Fig. 2 illustrates a Northern blot of CHO or High Five cells expressing either bNCKX1 or bNCKXdd transcripts. The stable CHO cell line expressed somewhat lower levels of transcript when compared with the High Five cells. We generated stable High Five cell lines transformed with bovine cardiac NCX1, bNCKX1, and bNCKXdd and examined protein expression as well as $^{45}$Ca uptake via reverse Na/Ca exchange in sodium-loaded cells. Fig. 3 illustrates protein expression of High Five or CHO cells transformed with bovine cardiac NCX1 and bNCKXdd, respectively, when probed with monoclonal antibody 6H2 against an epitope located on the extracellular loop of bovine NCX1 (16). Protein expression levels of NCX1 in both CHO and High Five cells and expression of bNCKXdd in High Five cells were comparable, while bNCKXdd expression levels in CHO cells were significantly lower.

$^{45}$Ca Uptake in Sodium-loaded High Five Cells Transformed with NCKX cDNAs—Our functional test for NCKX function relies on the well-established property of both classes of proteins to mediate both calcium efflux (forward exchange) and calcium influx (reverse exchange) dependent on the direction of the transmembrane sodium gradient (10). We examined functional expression of NCKX by measuring Na-dependent $^{45}$Ca uptake in High Five cells representing reverse Na/Ca exchange. We loaded High Five cells with high sodium concentration using the alkali cation ionophore monensin as described under “Experimental Procedures” and as used previously by us to examine reverse Na/Ca exchange in retinal rod outer segments (14). In the first set of experiments, we compared full-length bovine rod NCKX1 with full-length bovine cardiac NCX1 and with the double deletion mutant bNCKXdd. $^{45}$Ca uptake was measured in sodium-loaded High Five cells at a low extracellular free calcium concentration of 35 $\mu$M. We measured uptake in four different media as follows. 1) $^{45}$Ca uptake in high sodium medium was used to represent NCKX-independent control uptake, since the high sodium concentration prevents calcium binding to its external transport site at the low extracellular calcium concentration used. 2) $^{45}$Ca uptake in high potassium medium was used for optimal support of reverse Na/Ca-(K) exchange for both NCX1 and NCKX1, since this medium maximizes the outward sodium gradient while satisfying the potassium requirement for NCKX1. 3) $^{45}$Ca uptake in high lithium medium without any potassium present was used to support reverse exchange for NCX1, since reverse exchange via NCX1 does not require any specific external alkali cations. In contrast, lack of external potassium in the lithium medium does not support reverse Na/Ca exchange via NCKX1 despite the large outward sodium gradient. 4) To verify that $^{45}$Ca uptake observed in high potassium medium required the presence of internal sodium, sodium was removed from cells by the reverse of the sodium loading protocol. Sodium-loaded cells were incubated with the alkaloid cation ionophore monensin in the high potassium medium for 2 min just before the start of the $^{45}$Ca uptake experiment. The addition of monensin is expected to mediate a complete replacement of internal sodium by potassium, since the cell volume is only a small fraction of the total volume of the cell suspension.

Fig. 4 illustrates that no sodium-dependent $^{45}$Ca uptake was observed in High Five cells after stable transformation with full-length bovine NCX1; $^{45}$Ca uptake in the four different media was indistinguishable (middle panel), as observed for $^{45}$Ca uptake observed in untransformed cells (see Fig. 6). This indicates the absence of any endogenous Na/Ca or Na/Ca-K exchange activity in High Five cells. Furthermore, cells expressing full-length bovine NCKX1 did not show Na/Ca-K exchange function, consistent with our earlier results obtained in HEK293 cells (6). In contrast, our positive control (High Five cells transformed with bovine NCX1) showed a large amount of $^{45}$Ca uptake in lithium or potassium medium, whereas uptake dropped to levels observed in control cells in either sodium medium or potassium medium with monensin present (right panel). These results demonstrate the presence of a large, potassium-independent reverse Na/Ca exchange activity in cells transformed with NCX1. Next, we examined $^{45}$Ca uptake in High Five cells transformed with the double deletion mutant bNCKX1dd, and observed good uptake, but only in the potassium medium; no uptake greater than uptake in control cells was observed in either sodium medium, lithium medium, or potassium medium with monensin present (D). This result demonstrates potassium-dependent reverse Na/Ca exchange in cells expressing bNCKX1dd. Lack of function was observed in eight separate experiments with cells transformed with full-
length bNCKX1 in which the ratio of $^{45}$Ca uptake observed in potassium medium over that observed in sodium medium ranged between 0.9 and 1.1. In contrast, the ratio of $^{45}$Ca uptake in potassium medium over that observed in sodium medium observed in 25 experiments with cells expressing bNCKX1dd was never less than 2.

Earlier we had cloned the full-length dolphin dNCKX1 in our laboratory and observed strong potassium-dependent Na/Ca exchange as judged from single cell digital calcium imaging on HEK293 cells transfected with dolphin dNCKX1 cDNA (6). This suggests that dNCKX1 is suitable to represent full-length NCKX1 in functional expression studies. Furthermore, we cloned the cDNA of a putative NCKX sequence identified in the C. elegans genomic sequencing project, ceNCKX. The alignment of the bNCKX1dd, full-length dNCKX1, and ceNCKX sequences illustrates that sequence conservation is limited to the N-terminal extracellular loop of NCX1. The alignment of the bNCKX1dd, full-length dNCKX1, and ceNCKX1 sequences illustrates that sequence conservation is limited to the N-terminal extracellular loop of NCX1. The alignment of the bNCKX1dd, full-length dNCKX1, and ceNCKX sequences illustrates that sequence conservation is limited to the N-terminal extracellular loop of NCX1.

Temperature Dependence of NCKX-mediated $^{45}$Ca Uptake—The potassium-dependence of Na/Ca exchange is a key property that distinguishes NCKX from the NCX-type exchangers, which do not require potassium for transport (e.g. Fig. 4). The half-maximal concentration at which potassium activates NCKX1-mediated calcium fluxes in bovine retinal rod outer segments has been shown to vary greatly on the cations present in the medium or in the rod outer segment cytosol (14, 17). For example, in rod outer segments, we observed a large shift toward lower potassium concentrations when the potassium dependence of calcium influx via reverse Na/Ca exchange measured in choline medium was compared with that measured in lithium medium (see Fig. 9). Here, we examined this shift in potassium dependence of Na$_i$-dependent $^{45}$Ca uptake in High Five cells transformed with each of our three functionally active NCKX cDNAs and compared it with the shift observed in isolated bovine retinal rod outer segments. The potassium dependence of Na$_i$-dependent $^{45}$Ca uptake was measured in High Five cells transformed with ceNCKX cDNA in either choline medium (Fig. 8, right panel) or in lithium medium (Fig. 8, left panel). In both media, an increase in external potassium led to an increase in both initial rate and total amount of calcium uptake, but such much lower potassium concentrations were required in choline medium to achieve the same degree of activation when compared with those in lithium medium. Moreover, a significant potassium-independent component of $^{45}$Ca uptake was observed in choline medium in all cases, whereas no potassium-independent component of $^{45}$Ca uptake was observed in lithium medium except a small component in cells transfected with dNCKX1 (not illustrated; also see Ref. 17).

The first three time points of each individual $^{45}$Ca uptake curve were averaged to approximate the initial rate of calcium uptake and the potassium dependence of reverse Na/Ca exchange for retinal rod outer segments and for all three NCKX cDNAs used are shown in Fig. 9. All three NCKX cDNAs yielded potassium-dependent Na/Ca exchangers with a similar potas-
sium dependence with a $K_m$ of about 10–20 mM, while a somewhat lower value of about 8 mM was observed for rod outer segments (Fig. 9, left panel). The external potassium dependence observed in choline medium was shifted to significantly lower values in all cases with $K_m$ values of about 2 mM for $\alpha$NCKX and $\beta$NCKXdd and of 0.6 mM for rod outer segments and for $\delta$NCKX1 (Fig. 9, right panel). Fig. 9 illustrates results for a typical experiment; in 13 experiments with different NCKX, we compared the potassium dependence in lithium medium with that in choline medium and invariably observed a large shift in potassium dependence with an average shift of 10.4-fold (S.E. = 1.9). We conclude that key properties of the

Fig. 5. Amino acid sequence alignment of full-length dolphin $\delta$NCKX1, $\beta$NCKXdd, and full-length $\alpha$NCKX.

Fig. 6. Potassium-dependent $^{45}$Ca uptake in High Five cells transformed with the $\delta$NCKX1 and $\alpha$NCKX cDNAs. $^{45}$Ca uptake was measured in sodium-loaded High Five cells expressing full-length $\delta$NCKX1 or $\alpha$NCKX, or control cells. Incubation media contained 80 mM sucrose, 20 mM Hepes (adjusted to pH 7.4 with arginine), 5 mM EDTA, 35 mM CaCl$_2$, 1 mM $^{45}$Ca, and either 150 mM KCl (filled circles), 150 mM NaCl (inverted triangles), 150 mM LiCl (squares), or 150 mM KCl in the presence of 1 mM monensin (diamonds). Temperature was 25 °C.
FIG. 7. Temperature dependence of potassium-dependent $^{45}$Ca uptake in High Five cells transformed with the dNCKX1 and bNCKXdd cDNAs. $^{45}$Ca uptake was measured in sodium-loaded High Five cells expressing full-length dNCKX1 or bNCKXdd. Incubation media contained 80 mM sucrose, 20 mM Hepes (adjusted to pH 7.4 with arginine), 5 mM EDTA, 35 mM CaCl$_2$, 1 mM MgCl$_2$, and 150 mM KCl or 150 mM NaCl. Left panel, bNCKXdd; right panel, dNCKX1. Temperature was 37 °C (filled circles), 25 °C (inverted triangles), 15 °C (squares), or 0 °C (diamonds).

FIG. 8. Potassium dependence of $^{45}$Ca uptake in High Five cells transformed with ceNCKX. $^{45}$Ca uptake was measured in sodium-loaded High Five cells expressing full-length ceNCKX as a function of external potassium concentration. Media contained either 150 mM LiCl (left panel) or 150 mM choline chloride (right panel), or 150 mM NaCl (filled diamonds in both panels). In the left panel, KCl concentration was increased by isosmotic substitution of LiCl to the following final concentrations. Filled circles, 150 mM; open circles, 20 mM; filled inverted triangles, 10 mM; open inverted triangles, 5 mM; filled squares, 1 mM; open squares, no KCl. In the right panel, KCl concentration was increased by isosmotic substitution of choline chloride to the following final concentrations. Filled circles, 150 mM, open circles, 20 mM; filled inverted triangles, 5 mM; open inverted triangles, 1 mM; filled squares, 0.1 mM; open squares, no KCl. Temperature was 25 °C.

site imparting potassium dependence on NCKX are shared between in situ bNCKX1, full-length dNCKX1, the double deletion mutant bNCKXdd, and the C. elegans paralog ceNCKX.

DISCUSSION

The NCKX gene family of potassium-dependent Na/Ca exchangers is a relatively new addition to the pantheon of cation-transporting proteins. Three NCKX cDNAs have been cloned from different mammalian retinal rod photoreceptors (4–6), one from rat brain (7), and several putative members have been identified in lower eukaryotes as well as in several prokaryotes on the basis of sequence similarity within the two sets of transmembrane-spanning segments (2, 8). In situ NCKX protein in retinal rod outer segments was shown to act as a Na/Ca exchanger that both requires and transports potassium; i.e., calcium extrusion is driven by both the inward sodium gradient and the outward potassium gradient at a stoichiometry of 4 Na:1 (Ca + 1 K) (10, 18, 19). NCKX cDNAs of both retinal rod and rat brain were shown to code for potassium-dependent Na/Ca exchangers when expressed in heterologous systems. Potassium-dependent Na/Ca exchange activity was deduced from observations on reverse Na/Ca exchange measured by digital single cell calcium imaging in HEK293 cells transiently transfected with dolphin retinal rod NCKX cDNA or with rat brain NCKX cDNA; reversal of the sodium gradient after replacing external sodium by lithium led only to a rise in cytosolic free calcium when potassium was added to the external medium (6, 7). Transient transfection in HEK293 cells and digital calcium imaging represent a quick and convenient way of assessing NCKX function but are less amenable to rigorous quantitative analysis (6). We believe our study achieved two main objectives: 1) we developed a heterologous expression system amenable to simple and quantitative analysis of NCKX function, and 2) we present the first quantitative structure-function study on NCKX by delineating the domains required for potassium-dependent Na/Ca exchange transport and by comparing rod NCKX with a distantly related paralog cloned from C. elegans with respect to fundamental properties of the potassium binding site.

Stable Transformation of High Five Cells with NCKX cDNAs—Two of the three mammalian rod NCKX1 cDNAs cloned to date did not result in potassium-dependent Na/Ca exchange function after heterologous expression in mammalian cell lines (6). We examined stable transfection of both mammalian CHO cells and insect High Five cells as possible expression systems for NCKX cDNAs. Potassium-dependent Na/Ca exchange function was observed in both systems for the full-length dolphin rod dNCKX1 (Fig. 5) (15). However, our deletion mutant bNCKXdd did not yield function in CHO cells but resulted in strong potassium-dependent Na/Ca exchange activity in High Five cells when compared with CHO cells under our experimental conditions. Moreover, the ability to control the internal cation concentration via appropriate ionophores (e.g., sodium loading via monensin) is a key requirement for studies on NC/KX. Here, we successfully applied a shuttle-type ionophore like monensin to load High Five cells with high internal sodium, and we were able to completely remove monensin by washing with bovine serum albumin-containing medium. On the other hand, the addition of monensin did not cause any significant sodium loading in
CHO or HEK293 cells when applied at the low micromolar concentrations used here for High Five cells (15). Finally, it is easy and economical to grow High Five cells in a homogeneous suspension, suitable for our $^{45}$Ca uptake experiments or for future experiments on NCKX-mediated sodium and potassium fluxes. Combined, we believe the above noted advantages make High Five cells and the novel pIE1/153A expression vector an advantageous system for functional analysis after heterologous expression of members of the NCKX family of potassium-dependent Na/Ca exchangers and, perhaps, for other cation exchangers or cotransporters as well.

Minimal Domain Requirement and Properties of the Potassium Binding Site—In situ properties of potassium-dependent Na/Ca exchange have so far been characterized only in the outer segments of retinal rod photoreceptors (reviewed in Refs. 10, 11, and 20). A recent study on the proteolysis of the native exchanger from bovine rod outer segments has shown that potassium-dependent Na/Ca exchange was still observed after epipodes for several monoclonal antibodies located on the two large hydrophilic loops were removed, suggesting that large portions of these loops may not play a significant role in cation transport per se (21). The full-length dolphin $dNCKX1$ (6) and the rat brain NCKX2 (7) were both shown to be potassium-dependent Na/Ca exchangers, although they share only limited sequence similarity within the large hydrophilic loops, again suggesting that these loops may not play a significant role in cation binding and cation transport. We have extended the above observations significantly by demonstrating potassium-dependent Na/Ca exchange transport in our $bNCKXdd$ construct, which lacks the two large hydrophilic loops, and in cells transformed with $cNCKX$, in which sequence similarity with the mammalian NCKX proteins is limited to two sets of transmembrane-spanning segments (Fig. 5).

Characterization of the Potassium Binding Sites in Different NCKX Proteins—Requirement for and transport of potassium constitute the major functional differences between members of the NCX and NCKX families of Na/Ca exchangers. Therefore, we have focused here on characterizing some of the properties of this unique potassium site in our heterologously expressed NCKX proteins. Earlier studies from our laboratory reported a significant Na$_i$-dependent reverse Na/Ca exchange in isolated bovine rod outer segments when uptake was measured in buffered sucrose media without added potassium, whereas no reverse exchange was observed in lithium medium without added potassium (17). Here, we measured the potassium dependence of reverse Na/Ca exchange in lithium medium and in choline medium and observed in all cases a significant potassium-independent component of reverse Na/Ca exchange in choline but not in lithium medium. The three NCKX/mutant NCKX proteins tested here as well as in situ NCKX in isolated bovine retinal rod outer segments showed a characteristic 5–10-fold increase in potassium affinity when measured in choline medium ($K_m = 0.6–3$ mM) compared with when measured in lithium medium ($K_m = 10–20$ mM) (Figs. 8 and 9). Values measured in bovine rod outer segments were about 2-fold lower than those observed for the NCKX proteins expressed in High Five cells (Fig. 9). Very similar shifts in potassium affinity were recently observed in another study in which the same expression system was used for the retinal cone NCKX proteins from human and chicken.3 In all cases, a significant (20–25%) potassium-independent reverse Na/Ca exchange was observed in choline medium but not in lithium medium (not illustrated). From the above observations, we conclude that amino acids making up the potassium binding site are located somewhere within the two sets of TM1–TM5 and TM6–TM11.

Temperature Dependence of Potassium-dependent Na/Ca Exchange—We observed that the temperature dependences of NCKX-mediated reverse Na/Ca exchange observed for cells expressing full-length dolphin $dNCKX1$ and for cells expressing $bNCKXdd$ were very similar. Since $bNCKXdd$ lacks both large hydrophilic loops, this result suggests that the temperature dependence of NCKX-mediated transport originates from the ion translocation event and that putative regulatory features imposed on cation transport by the large hydrophilic loops are unlikely to play a major role.

NCKX-mediated calcium uptake in High Five cells ranged between 0.6 and 0.8 nmol of calcium/mg of protein for the three different NCKX proteins tested here, whereas NCX1-mediated calcium uptake in High Five cells was about 12 times greater; a similar number was obtained when comparing initial rates of uptake (see Figs. 4 and 6). The amount of $bNCKXdd$ protein expressed was very similar to that expressed for NCX1 (Fig. 3). The difference noted here between NCX-mediated calcium uptake and rod NCKX-mediated calcium uptake is consistent with the difference in turnover numbers reported for NCX

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We should point out that we are uncertain how much of the NCX or NCKX protein was present in the plasma membrane.

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Domain Requirement for Na/Ca-K Exchanger Cation Transport