Stoichiometry of the Retinal Cone Na/Ca-K Exchanger Heterologously Expressed in Insect Cells: Comparison with the Bovine Heart Na/Ca Exchanger[†]

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ABSTRACT: The transport stoichiometry is an essential property of antiporter and symporter transport proteins. In this study, we determined the transport stoichiometry of the retinal cone potassium-dependent Na/Ca exchanger (NCKX) expressed in sodium-loaded cultured insect cells. The Na/Ca and Rb/Ca coupling ratios were obtained by direct measurements of the levels of ^{86}Rb and ^{45}Ca uptake and sodium release associated with reverse Na/Ca exchange. Rb/Ca coupling ratios of 0.98 [standard deviation (SD) of 0.12, 15 observations] and 0.92 (SD of 0.12, 13 observations) were obtained for the chicken and human retinal cone NCKX, respectively. Na/Ca coupling ratios of 4.11 (SD of 0.24, 10 observations) and 3.98 (SD of 0.34, 15 observations) were obtained for the chicken and human retinal cone NCKX, respectively, whereas a lower average coupling ratio of 3.11 (SD of 0.34, 10 observations) was obtained with cells expressing the bovine Na/Ca exchanger (NCX1). These results are consistent with a 4Na/1Ca + 1K stoichiometry for retinal cone NCKX. High Five cells expressing full-length dolphin rod NCKX, *Caenorhabditis elegans* NCKX, or bovine rod NCKX from which the two large hydrophilic loops were removed all showed a significant calcium-dependent ^{86}Rb uptake, whereas no calcium-dependent ^{86}Rb uptake was observed in cells expressing bovine NCX1. The calcium dependence of ^{45}Ca uptake yielded values between 1 and 2.5 μM for the external calcium dissociation constant of the different NCKX proteins studied here.

Members of two gene families of Na/Ca exchangers function as calcium extrusion mechanisms in the plasma membrane of most eukaryotic cells. The Na/Ca exchangers (NCX)[1] are more ubiquitous proteins found in most tissues of mammals and lower organisms (*1*), whereas the potassium-dependent Na/Ca exchangers (NCKX) appear to have a more restricted expression pattern with two distinct gene products expressed in retinal rods and cones, respectively (*2*). Transcripts of a gene product closely related to or identical with the retinal cone NCKX are expressed in various parts of the rat brain (*3*). Analysis of the results of the various genomic sequencing projects suggests the presence of several NCKX paralogs in lower organisms and in prokaryotes; cDNAs of two such NCKX paralogs have been cloned from *Caenorhabditis elegans* (*4*) and *Drosophila* (*5*) and shown to function as potassium-dependent Na/Ca exchangers when expressed in cultured insect cells (*4*, *5*). In situ study of NCKX function has been carried out only in the outer segments of bovine and tiger salamander retinal rod outer segments; rod Na/Ca-K exchange was shown in these two preparations to utilize both the inward sodium gradient and the outward potassium gradient to extrude calcium across the plasma membrane at a stoichiometry of 4Na/1Ca + 1K (*6*, *7*). The bovine rod NCKX cDNA was cloned (*8*), but subsequent expression studies failed to yield consistent results; in some studies, bovine rod NCKX cDNA did not result in a functional protein in heterologous systems (*4*, *9*), whereas in another study, potassium-independent Na/Ca exchange was observed after heterologous expression in HEK293 cells (*10*). The latter study suggested that potassium dependence and potassium transport might be carried out by an accessory protein, as previous studies on the purified rod exchanger had not demonstrated potassium transport nor had the stoichiometry of the purified protein been determined. These studies raise the issue of whether the rod NCKX cDNA alone is sufficient for Na/Ca-K exchange activity observed in situ. Moreover, a recent study shows that potassium sensitivity can be conferred on heart NCX1 by inserting an N-terminal portion of the large cytosolic loop of bovine rod NCKX into the large cytosolic loop of heart NCX1; the authors interpret these data to indicate that the N-terminal portion of the rod NCKX cytosolic loop is the site of potassium transport in bovine rod NCKX (*11*). Significantly, cone NCKX and other NCKX paralogs do not appear to contain a related domain. The various NCKX cDNAs cloned to date encode proteins that show a considerable range in size from 1216 residues (bovine rod) to 661 residues (human cone) or 596 residues (*C. elegans*), and sequence similarity is confined to two sets of proposed

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[1] Abbreviations: NCKX, Na/Ca-K exchanger(s); NCX, Na/Ca exchanger(s); TM, transmembrane-spanning segments; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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transmembrane-spanning segments comprised of ~350 residues in total. The transport stoichiometry (i.e., the Na/Ca and Ca/K coupling ratios) is a fundamental property of Na/Ca(-K) exchangers as it determines the direction of calcium transport as a function of the transmembrane sodium and potassium gradients and membrane potential. In this study, we determined the transport stoichiometry of the retinal cone NCKX heterologously expressed in insect cells. We compared the cone NCKX stiochiometry with that of heart NCX1 by direct measurements of the Na/Ca and Rb/Ca coupling ratios. Rubidium was used as a potassium congener in view of the availability of ^{86}Rb for transport studies.

EXPERIMENTAL PROCEDURES

All NCKX and NCX1 clones used in this study were previously characterized, and cultured High Five cells expressing these NC(K)X proteins were obtained (*2*, *4*). Transformed cells were grown at 27 °C in roller bottles containing 200−600 mL of IPL-41 medium (Canadian Life Technologies Inc., Burlington, ON) to a maximal cell density of 10^6 cells/mL. IPL-41 medium was supplemented with 10% fetal bovine serum, 0.35 g/L sodium bicarbonate, 2.5 g/L tryptose phosphate, 9.0 g/L sucrose, 0.069 mg/L $ZnSO_4 \cdot 7H_2O$, and 7.59 mg/L $AlK(SO_4)_2 \cdot 12H_2O$. Calcium-depleted and sodium-loaded bovine rod outer segments (ROS) were isolated as described previously (*4*).

^{45}Ca and ^{86}Rb Uptake Experiments. High Five cells expressing the various NCKX or NCX1 proteins were sodium loaded with the use of monensin as described previously (*4*). The final washing solution contained 150 mM choline chloride, 80 mM sucrose, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 50 μM EDTA. Cells were resuspended in this medium to a final concentration of 15−30 mg of protein/mL, and the uptake experiments were completed within 15 min. In all experiments, FCCP (2−4 μM) was added to the cell suspension. In some experiments (illustrated in Figures 1−3), ^{45}Ca or ^{86}Rb taken up by cells was separated from external radioisotopes with the use of a rapid filtration technique over borosilicate glass fiber filters as described previously (*4*). In other experiments (illustrated in Figures 4 and 5), NC(K)X-mediated uptake of radioisotopes was terminated by addition of EDTA, followed by rapid sedimentation of cells in an Eppendorf 5417C tabletop centrifuge (1 min at 10 000 rpm). Aliquots of the clear supernatant were counted in a liquid scintillation counter, and the amount of radioactivity was compared with the amount of total radioactivity observed in the cell suspension. Separate aliquots of the supernatant were analyzed for sodium content as described below. A similar protocol was followed to measure the level of ^{45}Ca or ^{86}Rb uptake by bovine ROS; bovine ROS were stored in 600 mM sucrose, 0.05 mM EDTA, and 20 mM Hepes (adjusted to pH 7.4 with arginine) and, immediately, before use diluted 5-fold in the choline chloride/sucrose medium described above.

Atomic Absorption Spectrophotometry. Sodium-loaded High Five cells transformed either with human or chicken cone NCKX or with bovine heart NCX1 were examined for calcium- and potassium-dependent sodium release. High Five cells were sodium loaded as described above. After the final wash, cells were resuspended in 190 mM LiCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 50 μM EDTA to a final concentration of 15−30 mg of protein/mL. In all experiments, FCCP (2−4 μM) was added to the cell suspension. Sodium release was initiated by 2-fold dilution in medium containing (final concentrations) 300 μM $CaCl_2$ with or without 30 mM KCl, or cells were kept in EDTA (control). Calcium-induced sodium release was stopped by addition of 1.5 mM EDTA (final concentration), and cells were quickly (within 5 s) sedimented in an Eppendorf 5417C centrifuge (1 min at 10 000 rpm). Aliquots of the clear supernatant were diluted 20 times with 0.1% (w/v) CsCl to overcome anionic interference, and sodium content was measured in the emission mode at 589 nm using a lean air/acetylene flame in a Pye Unicam SP9 atomic absorption spectrophotometer. In a parallel experiment, the level of calcium uptake was measured under identical experimental conditions except that ^{45}Ca was included in the medium.

RESULTS

The objective of this study was to demonstrate that heterologously expressed Na/Ca-K exchanger (NCKX) proteins transport potassium (or its congener rubidium); furthermore, the short splice variants of the retinal cone NCKX were used to obtain direct measurements of the Na/Ca and Rb/Ca coupling ratios. The shorter splice variants of both human and chicken cone NCKX were used, since cells transformed with these transcripts consistently yielded higher functional activity than cells transformed with the full-length cone NCKX when assayed by Na_{inside}-dependent ^{45}Ca uptake (*2*). The short splice variants lack a nearly identical sequence of 17 amino acids [ELGSYGK(N)LKYYDTMTEE] at position 360 or 362 of the human or chicken cone NCKX sequence, respectively (in the chicken sequence, the N in parentheses replaces K); these 17 amino acids are located in the middle of the large cytosolic loop of cone NCKX that separates the two sets of putative transmembrane-spanning segments (TM's). Figure 1 compares the sequences of the different NCKX cDNAs used in this study. For clarity, the sequences start close to the first set of proposed TM's as no significant sequence similarity is observed between the N-terminal hydrophilic portions of the cone, rod, and *C. elegans* NCKX sequences. As studies on in situ NCKX function have been carried out exclusively in retinal rod outer segments, it is important to include rod-based NCKX clones. Unfortunately, most rod NCKX clones express poorly in heterologous systems and yield little or no Na/Ca-K exchange function, in particular, the bovine rod NCKX (*4*, *9*, *11*). Here, we have used the two NCKX clones (constructs) that gave satisfactory function in heterologous systems, the dolphin rod NCKX and a double deletion mutant based on the bovine rod NCKX (*b*NCKX*dd*, in which the N-terminal domain was replaced with that from bovine NCX1, and in which the large cytosolic loop was deleted) (*4*, *9*). Finally, we used a more distantly related NCKX cDNA cloned from *C. elegans* (*ce*NCKX) (*4*). Figure 1 illustrates that little significant sequence similarity is observed between these different NCKX isoforms outside of the two sets of TM's.

Rubidium and Calcium Dependence of Heterologously Expressed NCKX Proteins. ^{86}Rb is a convenient radioisotope to be used for transport studies. We have previously reported that rubidium can replace potassium in activating Na/Ca exchange via bovine rod Na/Ca-K exchange in situ, and that rubidium itself is transported by bovine rod Na/Ca-K

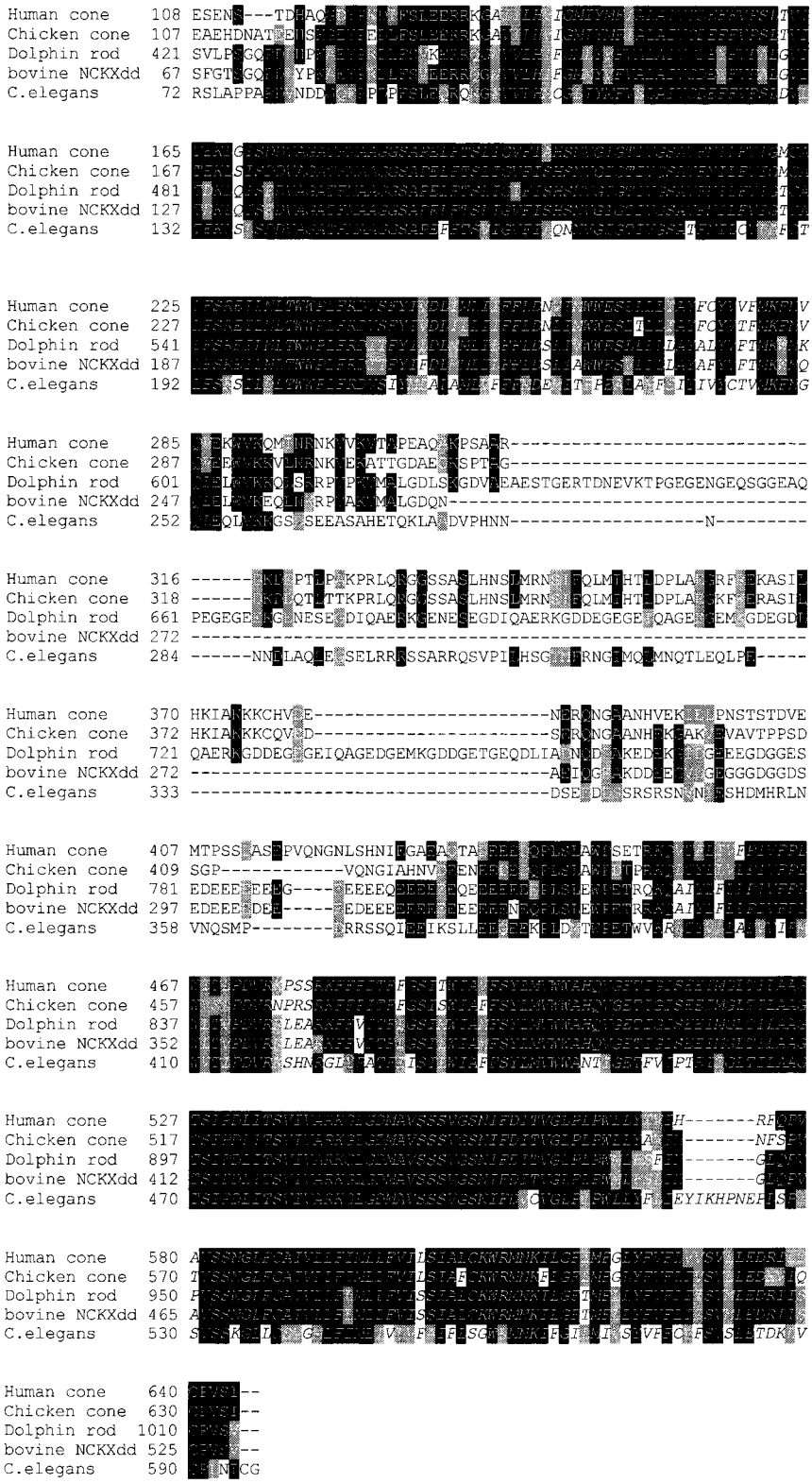

FIGURE 1: Amino acid sequence alignment of human cone NCKX, chicken cone NCKX, dolphin rod NCKX, bovine rod NCKXdd, and *C. elegans* NCKX. The two sets of proposed transmembrane-spanning segments are italicized. The N-terminal hydrophilic loops of the different NCKX proteins are highly variable, both in sequence and in length, and have been omitted for clarity.

exchange in situ (*12*, *13*). To address rubidium transport via heterologously expressed NCKX proteins, sodium-loaded High Five cells stably transfected with either chicken or human cone NCKX were used to measure the level of rubidium-dependent ^{45}Ca uptake via so-called reverse Na/Ca exchange. The rubidium and potassium dependencies of ^{45}Ca uptake in High Five cells expressing either human or chicken cone NCKX were virtually indistinguishable with half-maximal activation of ^{45}Ca uptake observed at 1 mM K^+ or Rb^+ when the major cation in the external medium was choline (not illustrated). Next, we examined the external calcium dependence of the expressed NCKX proteins by measuring the level of potassium-dependent ^{45}Ca uptake as a function of the external free calcium concentration. Figure

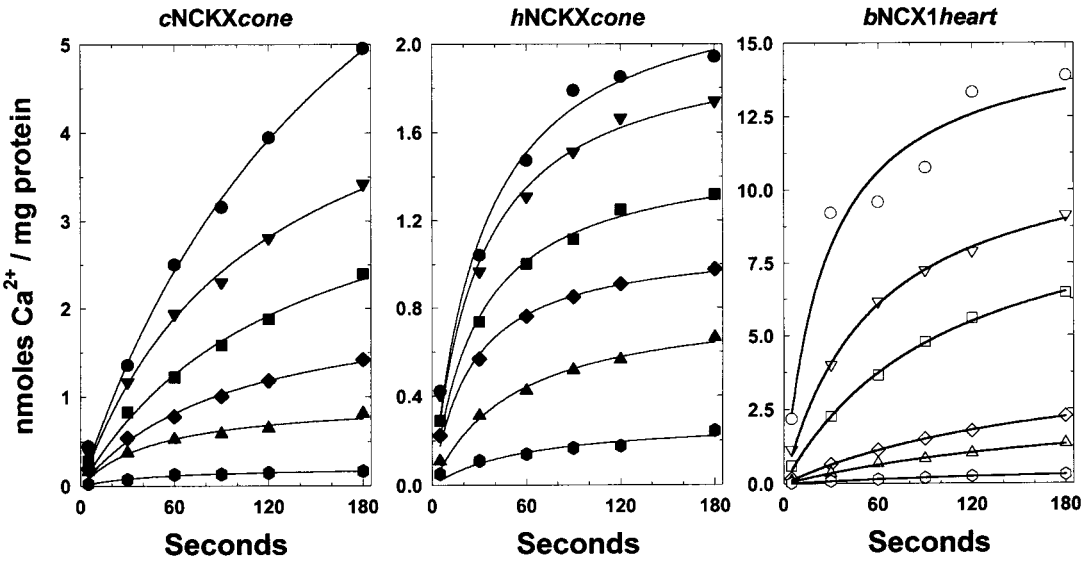

FIGURE 2: Calcium dependencies of ^{45}Ca uptake in High Five cells expressing different NC(K)X proteins. ^{45}Ca uptake was initiated at time zero by addition of ^{45}Ca and 200 μM CaHEDTA to High Five cells in a medium containing 150 mM KCl, 80 mM sucrose, 20 mM Hepes (adjusted to pH 7.4 with arginine), and various concentrations of HEDTA to yield the indicated the following free calcium concentrations (left and center panels): 0.1 (●), 0.5 (▲), 1 (◆), 2 (■), 5 (▼), and 30 μM (●). An apparent calcium dissociation constant of 1.5 μM for the CaHEDTA complex was used: (left) cells expressing chicken cone NCKX, (center) cells expressing human cone NCKX, and (right) cells expressing bovine heart NCX1. In the right panel, the calcium concentrations were as follows: 5 (○), 15 (△), 25 (◇), 50 (□), 100 (▽), and 200 μM (○). In all cases, calcium uptake was corrected for uptake observed in cells in a medium containing 150 mM NaCl instead of KCl; this represented background calcium uptake in High Five cells when reverse Na/Ca(-K) exchange was inhibited. The temperature was 25 °C.

2 illustrates the level of calcium uptake as a function of free external calcium concentration in High Five cells expressing chicken cone NCKX, human cone NCKX, or bovine heart NCX1. Calcium uptake was activated by free calcium concentrations in the low micromolar range in cells expressing the cone NCKX proteins (the range of free calcium concentrations in the left and center panels of Figure 2 is 0.1−30 μM), while a much lower calcium affinity was noted in cells expressing bovine heart NCX1 (the range of free calcium concentrations in the right-hand panel of Figure 2 is 5−200 μM). Eadie−Hofstee plots suggest a single calcium binding site with a K_m for calcium of 2.1 μM [standard deviation (SD) of 0.9, 5 observations] and 2.0 μM (SD of 0.8, 5 observations) for cells expressing chicken and human cone NCKX, respectively. Due to the limited precision of measuring the level of ^{45}Ca uptake at calcium concentrations in the millimolar range, we cannot exclude the presence of a low-affinity calcium transport site on cone NCKX. We also examined calcium uptake as a function of free external calcium concentration in High Five cells expressing the full-length dolphin rod NCKX (*d*NCKX), in cells expressing a double deletion mutant bovine rod NCKX (*b*NCKX*dd*, both large hydrophilic loops were removed), and in cells expressing a Na/Ca-K exchanger cloned from *C. elegans* (*ce*NCKX). The calcium dependencies were very similar to those observed in cells expressing the two cone NCKX proteins. The observed K_m values were 1.1 μM (SD of 0.5, 4 observations), 1.5 μM (SD of 0.7, 3 observations), and 2.7 μM (SD of 2.4, 4 observations) for cells expressing dolphin rod NCKX (*d*NCKX), for cells expressing a double deletion mutant bovine rod *b*NCKX*dd*, and for cells expressing *ce*NCKX, respectively. These values are very similar to K_m values of 0.9−1.1 μM reported for the external calcium dependence of reverse Na/Ca-K exchange of the in situ bovine rod Na/Ca-K exchanger (*14*, *15*).

Rubidium-Dependent Na/Ca Exchange via NCKX Proteins Is Accompanied by Rubidium Transport. The calcium dependencies reported above suggest that submillimolar external calcium concentrations should be sufficient to examine whether the rubidium dependence of ^{45}Ca uptake via reverse Na/Ca exchange reflects cotransport of calcium and rubidium. Figure 3 illustrates that addition of calcium to the incubation medium caused a large increase in the level of ^{86}Rb transport into cells expressing either human or chicken retinal cone NCKX. No calcium-dependent ^{86}Rb transport was observed in control untransfected High Five cells (not illustrated). Reverse Na/Ca exchange via retinal cone NCKX carries an outward current (*16*), and calcium-dependent ^{86}Rb transport observed here could conceivably represent a compensating inward rubidium current via a cation channel endogenous to High Five cells. To minimize such compensating currents via endogenous channels, in all experiments a high concentration of the electrogenic protonophore FCCP (2−4 μM/mg of protein) was added to the cells to provide an electrical shunt. To further address the possibility that ^{86}Rb transport might reflect a compensating current via an endogenous cation channel, the level of calcium-dependent ^{86}Rb transport was measured in High Five cells expressing bovine heart NCX1; the level of ^{86}Rb uptake was not increased when calcium was added to the medium to drive reverse Na/Ca exchange, and ^{86}Rb uptake levels were identical to those observed in the EDTA controls of cells expressing the retinal cone NCKX proteins (Figures 3C and 4). In contrast, the level of ^{45}Ca uptake in cells expressing NCX1 exceeded by ~10-fold that observed in cells expressing the retinal cone NCKX under the conditions used here. To avoid lowering the amount of specific radioactivity of ^{86}Rb, a relatively low external concentration of 0.4 mM Rb was used; this did not affect NCX1-mediated reverse Na/Ca exchange, but NCKX-mediated reverse Na/Ca exchange

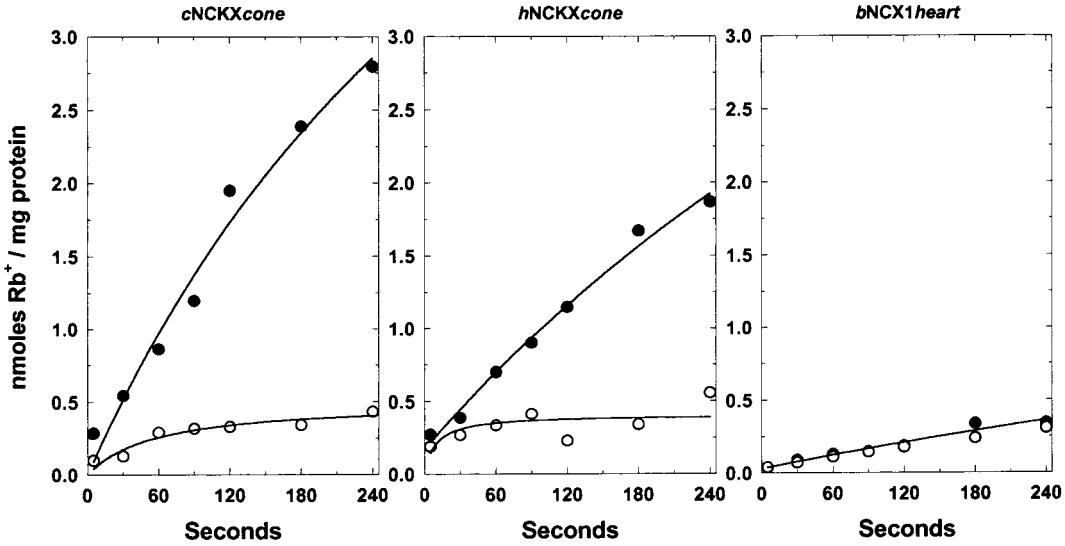

FIGURE 3: Calcium-dependent ^{86}Rb uptake in High Five cells transformed with different cone NCKX or heart NCX1 cDNAs. ^{86}Rb uptake was initiated at time zero by addition of ^{86}Rb, 0.4 mM RbCl, and either 0.4 mM $CaCl_2$ (●) or 0.4 mM EDTA (○) to a suspension of sodium-loaded High Five cells. High Five cells expressed chicken retinal cone NCKX (left), human retinal cone NCKX (center), or bovine heart NCX1 (right). The suspension medium contained 150 mM choline chloride, 80 mM sucrose, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 0.05 mM EDTA. The temperature was 25 °C.

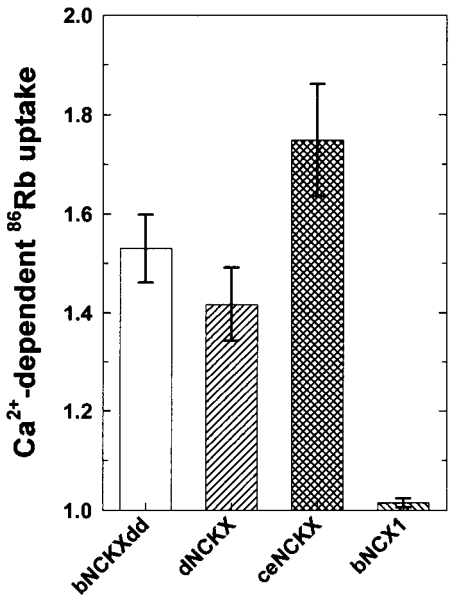

FIGURE 4: Calcium-dependent ^{86}Rb uptake in High Five cells transformed with different NCKX or NCX cDNAs. ^{86}Rb uptake was assessed in the presence of 0.4 mM $CaCl_2$ or 0.4 mM EDTA as described in the legend of Figure 3. Data are represented as the ratio of uptake observed in calcium-containing medium over that observed in EDTA-containing medium. In control untransfected cells, a ratio of 1 was observed (i.e., no calcium-dependent ^{86}Rb uptake, not shown). High Five cells were used expressing bovine retinal rod NCKX from which the two large hydrophilic loops were removed (*b*NCKX*dd*), dolphin retinal rod NCKX (*d*NCKX), an NCKX paralog from *C. elegans* (*ce*NCKX), or cells expressing the bovine heart Na/Ca exchanger (*b*NCX1). Average ratios (± standard deviation) are illustrated representing eight (*b*NCKX*dd*), four (*d*NCKX), six (*ce*NCKX), or seven (*b*NCX1) independent experiments. The temperature was 25 °C.

operated at about 30% of the rate observed at a high rubidium concentration.

The retinal cone NCKX proteins expressed in High Five cells were previously shown to give ~4-fold higher functional activity than rod NCKX proteins or the *C. elegans* NCKX paralog (*ce*NCKX) (*2*, *4*). Consistent with this, calcium-dependent ^{86}Rb transport was considerably more modest in High Five cells expressing the full-length dolphin rod NCKX (*d*NCKX), in cells expressing a double deletion mutant bovine rod NCKX (*b*NCKX*dd*), and in cells expressing *ce*NCKX. Nevertheless, a consistent calcium-dependent ^{86}Rb uptake was observed in cells expressing these different NCKX proteins (Figure 4, a 40−60% increase over the level of ^{86}Rb uptake observed in EDTA). In contrast, no calcium-dependent ^{86}Rb uptake at all was observed in cells expressing bovine heart NCX1, although calcium uptake levels in the latter were about 50 times higher than uptake levels observed with the NCKX clones illustrated in Figure 4.

Kinetic Correlation between Potassium-Dependent Calcium Uptake and Sodium Release. The most direct method for quantifying potassium- and calcium-dependent sodium release from cells is to measure changes in the extracellular sodium concentration by means of atomic absorption spectrophotometry. Reverse Na/Ca exchange in sodium-loaded High Five cells expressing the retinal cone NCKX was initiated by addition of potassium and calcium; the reaction was stopped by addition of excess EDTA, and the sodium content was determined in the clear supernatant after sedimentation of cells. Figure 5 (●) illustrates that the combined addition of potassium and calcium caused a time-dependent release of sodium from High Five cells expressing cone NCKX. No time-dependent release of sodium was observed in control cells, or in cells expressing cone NCKX when either potassium or calcium was omitted. Moreover, addition of both potassium and calcium in all our experiments resulted in a very similar time-dependent ^{45}Ca uptake level as measured with the same protocol in separate aliquots of the same suspension of High Five cells expressing cone NCKX [Figure 5 (○)] (no time-dependent ^{45}Ca uptake was observed in control cells or in cells expressing cone NCKX when potassium was omitted). Note that the abscissa for sodium uptake is scaled up 4-fold compared to the abscissa for calcium uptake, demonstrating that the Na/Ca coupling ratio in this experiment was ~4.

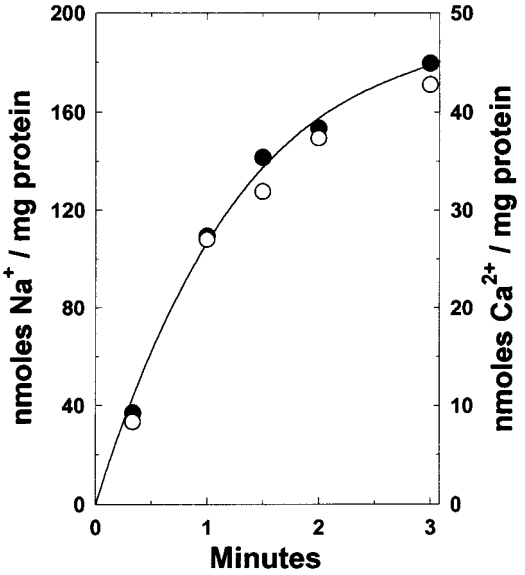

FIGURE 5: Kinetic correlation between potassium-dependent calcium uptake and potassium-dependent sodium release in High Five cells expressing retinal cone NCKX. ^{45}Ca uptake and sodium release were initiated at time zero by diluting separate aliquots of High Five cells with a medium containing 220 mM LiCl or 190 mM LiCl in addition to 30 mM KCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 0.3 mM $CaCl_2$ (plus ^{45}Ca in the ^{45}Ca uptake measurement). Samples were rapidly sedimented at the indicated times, and changes in ^{45}Ca or sodium content were measured in the clear supernatant. No time-dependent ^{45}Ca uptake or sodium release was observed in the 220 mM LiCl control medium (sodium content measured in this medium was subtracted as background). The temperature was 25 °C.

Rb/Ca and Na/Ca Ratios of Reverse Na/Ca Exchange via Retinal Cone NCKX. The simple protocol described above for the sodium release experiment was adopted to measure the Rb/Ca and Na/Ca transport ratios of reverse Na/Ca exchange carried out by both human and chicken retinal cone NCKX expressed in High Five cells. Separate aliquots of High Five cells (at 10 mg of protein/mL) were incubated with ^{86}Rb or ^{45}Ca or without added radioisotopes but the same amount of "cold" rubidium or calcium added. NCKX-mediated reverse Na/Ca exchange was stopped after 2−3 min by addition of excess EDTA, and cells were immediately (within 10 s) sedimented in a tabletop centrifuge. Clear samples of the supernatant were counted for ^{86}Rb or ^{45}Ca or analyzed for sodium. Figure 6A illustrates that the Rb/Ca transport ratio obtained for the chicken and human cone NCKX was close to 1, while no rubidium transport accompanied calcium uptake via bovine heart NCX1. The Rb/Ca coupling ratio observed in bovine ROS was also close to 1. Figure 6B illustrates that the average Na/Ca transport ratio obtained for the human and chicken cone NCKX was close to 4, while the average Na/Ca transport ratio obtained for bovine heart NCX1 was close to 3.

DISCUSSION

In this study, we measured the Rb/Ca and Na/Ca transport coupling ratios of the retinal cone NCKX expressed in High Five cells. The results demonstrate that both chicken and human retinal NCKX cDNAs yield proteins that cotransport calcium and rubidium in exchange for sodium when transfected into High Five cells. The observed average Rb/Ca coupling ratio was close to 1, while the observed average Na/Ca coupling ratio was close to 4 (Figure 6). The simplest overall transport stoichiometry consistent with our results is 4Na/1Ca + 1Rb, as has been reported previously for the rod Na/Ca-K exchange activity in situ (*6*, *7*). Our results demonstrate unambiguously that the single polypeptide encoded by NCKX cDNA is sufficient for Na/Ca-K exchange activity. We previously determined a K/Ca coupling ratio of 1 for forward Na/Ca exchange of bovine rod Na/Ca-K exchange activity in situ (*6*); here, a Rb/Ca coupling ratio

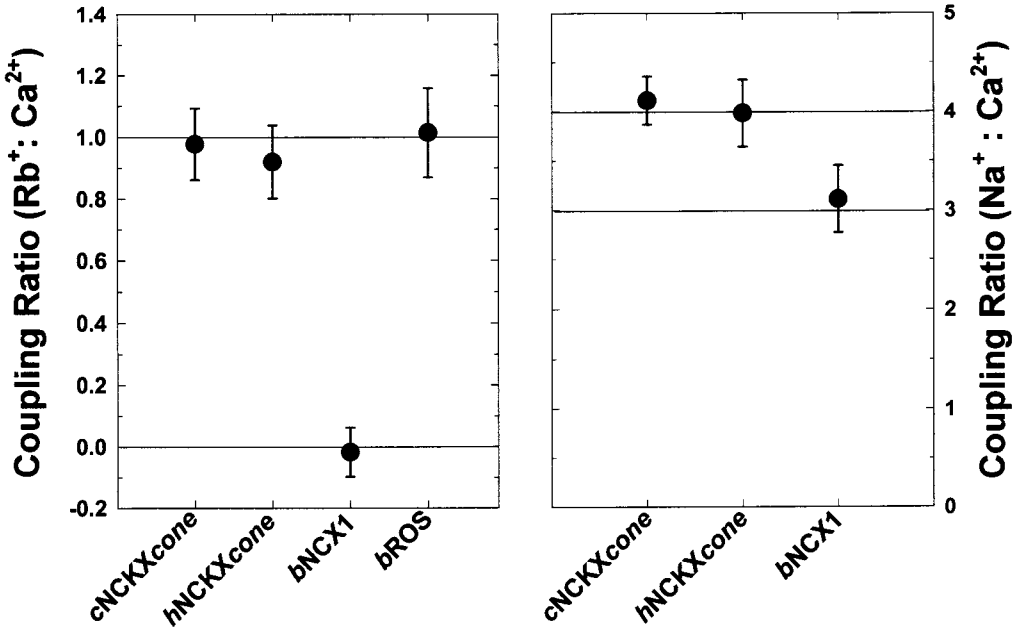

FIGURE 6: Rb/Ca and Na/Ca coupling ratios of the retinal cone NCKX. The Rb/Ca and Na/Ca coupling ratios were measured as described in the text in High Five cells expressing the human or chicken retinal cone NCKX, or expressing bovine heart NCX1. In the left panel, average values ± standard deviation are illustrated for 15 experiments with chicken cone NCKX, 13 experiments with human cone NCKX, 9 experiments with bovine heart NCX1, and 8 experiments with bovine retinal rod outer segments. In the right panel, average values ± standard deviation are illustrated for 10 experiments with chicken cone NCKX, 15 experiments with human cone NCKX, and 10 experiments with bovine heart NCX1. The temperature was 25 °C.

of 1 was found for reverse Na/Ca exchange carried out by the endogenous bovine rod Na/Ca-K exchanger (Figure 5), indicating that the stoichiometries observed for forward and reverse Na/Ca-K exchange are the same. The results presented here confirm that the heart Na/Ca exchanger does not transport potassium or rubidium, while a Na/Ca coupling ratio of close to 3 was obtained for NCX1 (Figure 6). This result is consistent with earlier studies (*1*), but differs from a Na/Ca ratio of 4, reported in a recent study presenting careful measurements of the reversal potential of NCX1 currents in large inside-out patches under conditions in which sodium and calcium are present on both sides of the membrane (*17*). It should be pointed out that our stoichiometry measurements are carried out under unidirectional conditions to prevent self-exchange fluxes, i.e., calcium (and potassium) present on one side of the membrane and sodium on the other.

Three factors were instrumental in obtaining direct measurements of the transport stoichiometry of the retinal cone NCKX. (1) Cone NCKX cDNAs, in particular, the short splice variants, gave rise to considerably higher functional activity compared with other NCKX cDNAs. (2) High Five cells were grown in roller bottles, and large volumes containing high cell counts containing large amounts of NCKX protein could be obtained economically. (3) The background level of ^{86}Rb uptake (as measured in untransfected control cells or as measured in cells expressing cone NCKX when suspended in a medium with a high sodium concentration) was greatly reduced by the efficient monensin-mediated replacement of internal alkali cations (i.e., potassium) with sodium (similar attempts in mammalian cell lines were considerably less successful). Cells transformed with the dolphin rod NCKX, the double deletion bovine rod NCKX, and the *C. elegans* NCKX cDNAs lacked sufficient functional activity to permit quantitative measurements of the Rb/Ca and Na/Ca transport ratios with sufficient precision. Nevertheless, High Five cells expressing these three NCKX proteins yielded consistent calcium-dependent ^{86}Rb transport associated with reverse Na/Ca exchange (Figure 4); no calcium-dependent ^{86}Rb uptake was observed in all experiments with control untransfected cells or with cells transformed with bovine heart NCX1, despite the fact that the latter showed by far the highest ^{45}Ca uptake levels. The only domains that the four different NCKX proteins (rod, cone, *C. elegans*, and double deletion bovine rod NCKX) used in this study have in common are two sets of five and six proposed transmembrane-spanning segments, respectively (Figure 1). No significant sequence similarity is obvious in the N-terminal extracellular loops. The full-length rod and cone sequences contain a conserved stretch of 30 amino acids located in the N-terminal part of the proposed large cytosolic loop that bisects the two sets of proposed transmembrane-spanning segments. This conserved stretch is present in the cone NCKX splice variant used here, but not in the *C. elegans* and dolphin rod NCKX cDNAs used here [dolphin rod NCKX cDNA lacks 114 residues in the N-terminal portion of the cytosolic loop, most likely representing a splice variant missing exons 3−6 (*18*)]. Combined, the results presented here do not support a recent model in which a domain within the N-terminal part of the large cytosolic loop of bovine rod NCKX (residues 601−997) imparts potassium sensitivity and potassium transport (*11*). Furthermore, the double deletion mutant bovine rod NCKX (containing only residues 601−621 of the N-terminal part of the large cytosolic loop of bovine rod NCKX) consistently showed calcium-dependent ^{86}Rb uptake associated with reverse Na/Ca exchange. We conclude that residues involved in potassium (rubidium) binding and potassium (rubidium) transport mediated by NCKX proteins are exclusively located within the two sets of proposed transmembrane-spanning domains. Likewise, the similarity in the calcium dissociation constants, observed here for reverse Na/Ca-K exchange in cells expressing the different NCKX proteins, implies that residues involved in calcium binding are located within the two sets of proposed transmembrane-spanning domains without involvement of residues located in the two large hydrophilic loops, not conserved among the NCKX proteins studied here.

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