Na^+-Ca^{2+}-K^+ Currents Measured in Insect Cells Transfected with the Retinal Cone or Rod Na^+-Ca^{2+}-K^+ Exchanger cDNA

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ABSTRACT The recently cloned retinal cone Na^+-Ca^{2+}-K^+ exchanger (NCKX) was expressed in cultured insect cells, and whole-cell patch clamp was used to measure transmembrane currents generated by this transcript and compare them with currents generated by retinal rod NCKX or by a deletion mutant rod NCKX from which the two large hydrophilic loops were removed. We have characterized the ionic currents generated by both the forward (Ca^{2+} extrusion) and reverse (Ca^{2+} influx) modes of all three NCKX proteins. Reverse NCKX exchange generated outward current that required the simultaneous presence of both external Ca^{2+} and external K^+. Forward NCKX exchange carried inward current with Na^+, but not with Li^+ in the bath solution. The cation dependencies of the three NCKX tested (external K^+, external Na^+, internal Ca^{2+}) were very similar to each other and to those reported previously for the in situ rod NCKX. These findings provide the first electrophysiological characterization of cone NCKX and the first electrophysiological characterization of potassium-dependent Na^+-Ca^+ exchangers in heterologous systems. Our results demonstrate the feasibility of combining heterologous expression and biophysical measurements for detailed NCKX structure/function studies.

INTRODUCTION

Plasma membrane Na^+-Ca^{2+} exchange plays an essential role in Ca^{2+} homeostasis in most cells. Na^+-Ca^{2+} exchange was initially discovered by examining Na^+-dependent ^{45}Ca uptake in a number of different tissues (for review see Blaustein and Lederer, 1999) including retinal rod outer segments (Schnetkamp, 1980). Current changes due to electrogenic Na^+-Ca^{2+} exchange activity were first demonstrated in amphibian retinal rod outer segments (Yau and Nakatani, 1984), and later in cardiac myocytes (Kimura et al., 1987). In outer segments of both rod and cone photoreceptors, this exchanger extrudes Ca^{2+} which has entered via the cGMP-gated channels in darkness; and upon bright light stimulation, the exchanger causes a rapid lowering of cytosolic free Ca^{2+} (Gray-Keller and Detwiler, 1994; Sampath et al., 1998, 1999). Unlike Na^+-Ca^{2+} exchange in most other tissues (in which it is designated NCX), rod photoreceptor Na^+-Ca^{2+} exchange has been shown to require and transport K^+; thus it is designated NCKX (Cervetto et al., 1989; Schnetkamp et al., 1989).

Molecular cloning has revealed two distinct gene families of Na^+-Ca^{2+} exchangers, those related to the heart Na^+-Ca^{2+} exchanger (NCX) and those related to the rod Na^+-Ca^{2+}-K^+ exchanger (NCKX). In situ characterization of NCKX function has been done almost exclusively using retinal rod outer segments. Very little information is available on the properties of Na^+-Ca^{2+}-K^+ exchange in other cell types or tissues. Potassium-dependent Na^+-Ca^{2+} exchange has been demonstrated in human platelets, and it is thought this reflects the rod-type NCKX exchanger (Kimura et al., 1999). A potassium-dependent Na^+-Ca^{2+} exchanger has been cloned from rat brain and its transcripts have been shown to be widely distributed in the brain (Tsoi et al., 1998). Finally, forward Na^+-Ca^{2+} exchange current was demonstrated in retinal cones from the tiger salamander, but no quantitative information on its cation dependence was presented, and the issue of K^+ dependence was not addressed (Nakatani and Yau, 1989). Recently, calcium has been shown to carry a significantly greater fraction of the inward current through cGMP-gated channels in retinal cones compared with retinal rods (Perry and McNaughton, 1991; Frings et al., 1995; Picones and Korenbrot, 1995; Dzeja et al., 1999). Furthermore, the light-induced changes of calcium are significantly larger and faster in retinal cone outer segments compared with rod outer segments (Sampath et al., 1998, 1999). We have recently cloned several mammalian retinal rod and cone NCKX cDNAs, as well as NCKX paralogs from *Drosophila* and *Caenorhabditis elegans*. K^+-dependent Na^+-Ca^{2+} exchange was consistently observed after heterologous expression of these cDNAs in cultured insect cells (Haug-Collet et al., 1999; Szerencsei et al., 2000; Prinsen et al., 2000). In these experiments exchange activity was monitored using ^{45}Ca uptake. Therefore, it remains to be demonstrated that the NCKX cDNAs by themselves code for electrogenic Na^+-Ca^{2+}-K^+ exchangers.

We decided to study potassium-dependent NCKX currents following a report describing K^+-independent Na^+-Ca^{2+} exchange currents in HEK293 cells after transient transfection with bovine retinal rod NCKX1 cDNA (Navangione et al., 1997). We have used whole-cell patch clamp to record NCKX currents in High Five insect cells following stable transfection with cDNAs from retinal rod and cone NCKX that previously were shown to give good functional

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activity in heterologous systems, i.e., dolphin rod NCKX1 (Cooper et al., 1999) and the short splice variant of human or chicken cone NCKX (Prinsen et al., 2000). The short splice variant of cone NCKX lacks a stretch of 17 amino acids in the middle of the large hydrophilic loop that separates two sets of five and six transmembrane spanning segments, respectively. Results obtained with rod and cone NCKX were compared with those obtained with a rod NCKX deletion mutant (Szerencsei et al., 2000). In this deletion mutant we have removed the two large hydrophilic loops, leaving only the two sets of proposed transmembrane spanning segments. This construct allows the hypothesis that the cation binding sites reside within these proposed transmembrane spanning segments to be tested. The dependencies of these three NCKX clones on external Na^+ and external K^+, as well as internal Ca^{2+}, were evaluated. An abstract describing some of these findings has been presented (Sheng et al., 2000).

MATERIALS AND METHODS

NCKX cDNAs and the insect cell expression vector

Bovine heart NCX1 cDNA (gift of Dr. J. P. Reeves), full-length dolphin rod NCKX1 cDNA, the double deletion mutant bovine rod NCKX1 cDNA, and the short splice variants of both the human and chicken retinal cone NCKX cDNAs were cloned into the pIE1/153A insect cell expression vector as described (Prinsen et al., 2000; Szerencsei et al., 2000). Stable High Five cell lines (BTI-TN-5B1-4 cells, High Five TM; Invitrogen, Carlsbad, CA) expressing the above NCX1 or NCKX cDNAs were made as described previously (Prinsen et al., 2000; Szerencsei et al., 2000). Cells were grown and maintained in IPL-41 insect cell medium with 10% fetal bovine serum albumin and an antibiotic-antimycotic mixture (Gibco BRL, Burlington, ON, Canada).

Recording of Na^+-Ca^{2+}-K^+ exchange currents

Electrophysiological recordings were carried out using the whole-cell configuration of the patch clamp technique. Pipettes were prepared from glass capillaries (WP Instruments, Sarasota, FL) in the conventional manner and fire-polished to a resistance of 4–6 MΩ when filled with pipette solution. The seal resistance ranged between 5 and 10 GΩ. Currents were recorded with an Axopatch 1D amplifier (Axon Instruments, Inc., Burlingame, CA). Reverse and forward Na^+-Ca^{2+}-K^+ exchange currents were recorded in conventional whole-cell configuration using a holding potential of 0 mV. Current traces were displayed and stored in a computer using Axotape software (Axon Instruments, Inc.). Changes in current were normalized with respect to cell size as judged from cell capacitance; the average capacitance of 70 cells examined in this study was 15.2 pF (SEM = 0.7). To measure the current-voltage relationships the membrane potential was ramped from −60 mV to +60 mV in 500 ms, followed by a return to a holding potential of 0 mV. The protocol was generated with the PCLAMP 5.5 software (Axon Instruments, Inc.). An eight-channel perfusion system (BPS-8, Adams and List Associates, Ltd., Westbury, New York) was used to deliver test solutions to the selected cell. Solution changes could be completed in ~20 ms. All experiments were carried out at room temperature (20–22°C).

Solution composition and experiment protocol

At the start of each experiment, the High Five cells in the recording chamber were superfused at a flow rate of 1–2 ml/min with a solution containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 20 mM Hepes (pH adjusted to 7.4 with arginine), and 10 mM glucose. To activate reverse exchange currents, the bath solution containing 150 mM LiCl, 10 mM sucrose, 1 mM EGTA, and 20 mM Hepes (pH adjusted to 7.4 with arginine) was rapidly changed for one containing 0.1 mM $CaCl_2$ (replacing 1 mM EGTA) and selected concentrations (0–20 mM) KCl, maintaining osmotic strength by replacing LiCl with KCl. The pipette solution contained 130 mM sodium glutamate, 20 mM NaCl, 2 mM MgATP, 20 mM TEA-Cl, 2 mM EGTA, and 20 mM Hepes (pH adjusted to 7.4 with arginine). To activate forward exchange currents, an external solution containing 150 mM LiCl, 10 mM sucrose, 1 mM EGTA, and 20 mM Hepes (pH adjusted to 7.4 with arginine), was rapidly changed for one containing selected concentrations (10–150 mM) of NaCl, maintaining osmotic strength by replacing LiCl with NaCl. In these experiments the pipette solution contained 110 mM potassium glutamate, 20 mM KCl, 2 mM MgATP, 20 mM TEA-Cl, 20 mM Hepes (pH adjusted to 7.4 with arginine), and selected concentrations of free Ca^{2+} obtained with the use of EGTA and 5,5′-Dibromo BAPTA as Ca^{2+} buffers.

Statistical analysis

Data are expressed as mean ± SEM. Differences in current observed for different ionic conditions were analyzed for multiple comparisons using an unpaired *t*-test or one-way ANOVA, followed by the Dunnett's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Outward current generated by the reverse mode of the retinal cone Na^+-Ca^{2+}-K^+ exchanger

The stoichiometry of the Na^+-Ca^{2+}-K^+ exchanger dictates that it is unlikely to mediate Ca^{2+} influx via reverse exchange under any physiological condition. Instead, the Na^+-Ca^{2+}-K^+ exchanger plays a vital role in extruding Ca^{2+} that enters the outer segments of retinal rod and cone photoreceptors via the cGMP-gated channels (Yau and Nakatani, 1984; Nakatani and Yau, 1989). Nevertheless, under experimental conditions in which the transmembrane Na^+ gradient is reversed, the rod Na^+-Ca^{2+}-K^+ exchanger can be made to mediate Ca^{2+} influx in rod outer segments (Schnetkamp, 1986). We have used reverse Na^+-Ca^{2+}-K^+ exchange as a convenient tool to demonstrate functional antiporter activity following stable transfection of High Five cells with retinal cone or retinal rod NCKX cDNA. As indicated in Methods, the shorter splice variants for both human and chicken cone NCKX have been studied. Both lack a nearly identical sequence of 17 amino acids ELGSYGK(R)LKYYDTMTEE at position 360 or 362 of the human and chicken cone NCKX sequence, respectively (in the chicken sequence, R 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. These transcripts con-

sistently yielded higher functional activity compared with transcripts obtained with the full-length cone NCKX when assayed by Na_{in}-dependent ^{45}Ca uptake (Prinsen et al., 2000). We compared the results obtained with cone NCKX with those obtained with dolphin retinal rod NCKX1, as this is the only rod NCKX1 cDNA obtained to date that yields strong functional activity in heterologous systems (Cooper et al., 1999; Szerencsei et al., 2000). Furthermore, we studied membrane currents generated by a mutant bovine rod NCKX1 (*b*NCKX*dd*) from which both large hydrophilic loops were deleted to test the hypothesis that the two sets of predicted transmembrane spanning segments are sufficient for electrogenic Na^+-Ca^{2+}-K^+ exchange. In the *b*NCKX*dd* construct two large stretches of amino acids were deleted, removing ~60% of the full-length bovine NCKX sequence: 1) the first N-terminal 422 residues, thought to form a large hydrophilic extracellular loop; and 2) residues 620–958, which make up the majority of the large cytosolic loop (residues 600-1025). As a positive control for potassium-independent Na^+-Ca^{2+} exchange, High Five cells transfected with bovine heart NCX1 cDNA were studied.

The diagram in Fig. 1 *A* (*top*) illustrates the sequence of rapid solution changes that were applied to untransfected control cells and to cells transfected with the different NCKX cDNAs, or with heart NCX1. Transmembrane current changes were measured with whole-cell patch clamp. The pipette filling solution contained NaCl, because sodium is an essential substrate for this exchanger, and EGTA to prevent rapid increases in intracellular free Ca^{2+}, which develop as a consequence of reverse exchange. The bath control solution contained LiCl *and* EGTA, which prevent Ca^{2+} influx via Na^+-Ca^{2+}-K^+ exchange. To initiate reverse exchange activity, the bath solution was rapidly switched to one containing LiCl and 0.1 mM $CaCl_2$: an outward current was observed in all cells transfected with the bovine heart Na^+-Ca^{2+} exchanger (NCX) cDNA. In contrast, no change in current was observed in untransfected control cells, or in cells transfected with rod or cone NCKX cDNA. In a minority of experiments, cells transfected with the double deletion rod NCKX mutant (*b*NCKX*dd*) cDNA showed small outward currents under this condition, and one such case is illustrated in Fig. 1. Thereafter, the bath control solution was re-applied, and then a solution in which 20 mM LiCl was replaced by 20 mM KCl with EGTA was added. No change in current was observed in cells transfected with any of the three NCKX clones. In the final solution switch, the control bath solution was exchanged for one containing 20 mM KCl and 0.1 mM $CaCl_2$: cells transfected with heart NCX1 cDNA generated an outward current very similar to that observed in the previous solution containing 0.1 mM $CaCl_2$ without K^+. Under these conditions, cells transfected with any of the three NCKX cDNAs also generated an outward current when no change in current was observed without K^+ present. This pattern of results is consistent with the outward current being mediated by the rod or cone

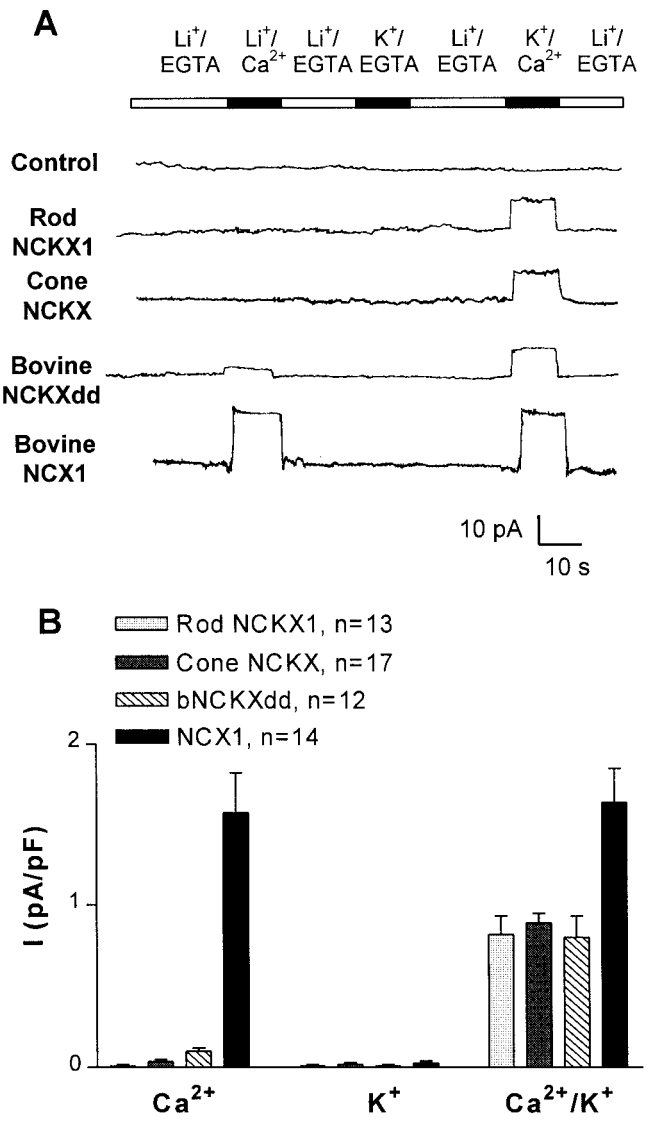

FIGURE 1 Outward Na^+-Ca^{2+}-K^+ exchange currents were measured in untransfected control High Five cells, and in High Five cells expressing dolphin rod NCKX, chicken cone NCKX, the double deletion mutant *b*NCKX*dd*, or bovine heart NCX1. (*A*) The top panel illustrates the sequence of solution changes applied to each cell: the control solution containing LiCl and EGTA was replaced with one containing LiCl and 0.1 mM $CaCl_2$, or one containing LiCl, 20 mM KCl, and 0.1 mM $CaCl_2$ (for detailed solution composition, see Methods). The middle panel illustrates representative current changes in response to these solution changes. (*B*) Peak changes in outward current were normalized with respect to cell size as judged from cell capacitance. Average results (± SEM) are presented for each cell type; *n* denotes the number of cells studied.

Na^+-Ca^{2+}-K^+ exchanger, and suggests that functional activity of these proteins requires the simultaneous presence of both external Ca^{2+} and K^+. Fig. 1 *B* illustrates pooled data obtained using this protocol in 14 cells expressing bovine heart NCX1, 17 cells expressing with chicken cone NCKX, 13 cells expressing dolphin rod NCKX, and 12 cells expressing *b*NCKX*dd*.

Current-voltage relationship of reverse Na^+-Ca^{2+}-K^+ exchange currents

The current-voltage relationships of control High Five cells and of High Five cells transfected with human cone NCKX, with dolphin rod NCKX, or with the double deletion mutant *b*NCKX*dd* are shown in Fig. 2. Very similar current-voltage relationships were observed in media that contained EGTA and either lithium or lithium plus potassium, or in a medium that contained lithium plus calcium but no potassium (represented by the *dotted line*). The results show only very small membrane currents in control (untransfected) High Five cells under our experimental conditions, confirming the absence of functional ion channels. When the superfusate contained lithium, calcium, and potassium an outward current was observed that was very similar in magnitude at all membrane voltages studied (*solid line*). This result is consistent with outward NCKX current being generated by the reverse mode of exchange. The results illustrated in Fig. 2 show recordings obtained from a single cell for each NCKX clone that were representative for at least 10 other cells examined for each NCKX clone.

Forward and reverse Na^+-Ca^{2+}-K^+ exchange currents

We measured the dependence of reverse Na^+-Ca^{2+}-K^+ exchange currents on external K^+ and the dependence of forward exchange Na^+-Ca^{2+}-K^+ exchange currents on external Na^+ in cells transfected with each of the three NCKX cDNAs chosen for detailed analysis. The quantitative relationship describing the dependence on external K^+ was obtained from cells transfected with cone NCKX by using the protocol of solution switches illustrated in the top panel of Fig. 3 *A*. The middle panel shows a typical example (in one cell) of the changes in outward current resulting from the solution changes, while the bottom panel demonstrates the average potassium dependence obtained from recordings on seven different cells expressing cone NCKX. The data were fit by relationship describing a single cation binding site having a single potassium dissociation constant, yielding a value between 2.5 and 5.1 mM $[K^+]_o$ at the 95% confidence level.

Forward exchange Na^+-Ca^{2+}-K^+ current was measured by switching the superfusate containing 150 mM LiCl and 1 mM EGTA to one containing selected concentrations of NaCl (iso-osmotic substitution of LiCl) and 1 mM EGTA. In these experiments the pipette solution contained KCl and 50 μM Ca^{2+}. These solution compositions were based on a well-established property of rod Na^+-Ca^{2+}-K^+ exchange, namely that Li^+ cannot substitute for Na^+ in stimulating Ca^{2+} efflux (Schnetkamp, 1989). When LiCl was replaced by NaCl, an inward current was observed in cells transfected with cone NCKX, but no measurable change in current was observed in untransfected cells (not shown).

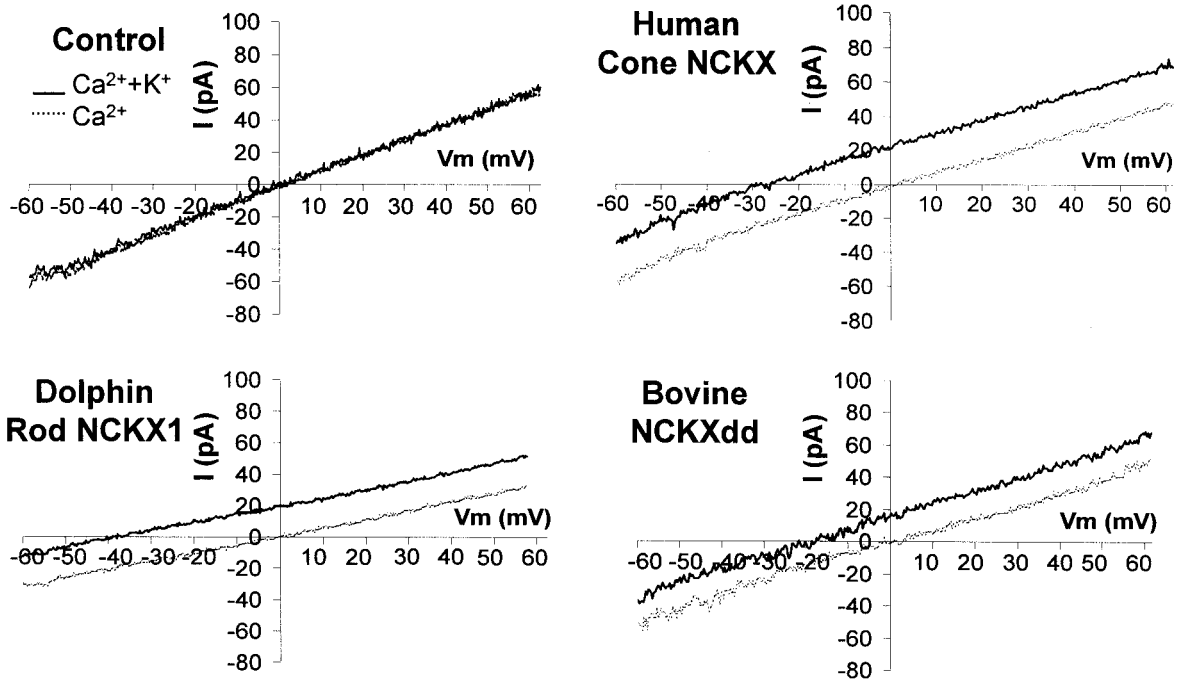

FIGURE 2 Current-voltage relationships of High Five cells expressing different NCKX proteins. Current-voltage relationships were recorded in High Five cells expressing selected NCKX proteins (as indicated) by using the protocol described in Methods. Results are illustrated for two different superfusion solutions: one solution contained 150 mM LiCl, 10 mM sucrose, 0.1 mM $CaCl_2$, and 20 mM Hepes (adjusted to pH 7.4 with arginine) (*dotted line*), while the second test solution contained, in addition, 20 mM KCl (*solid line*).

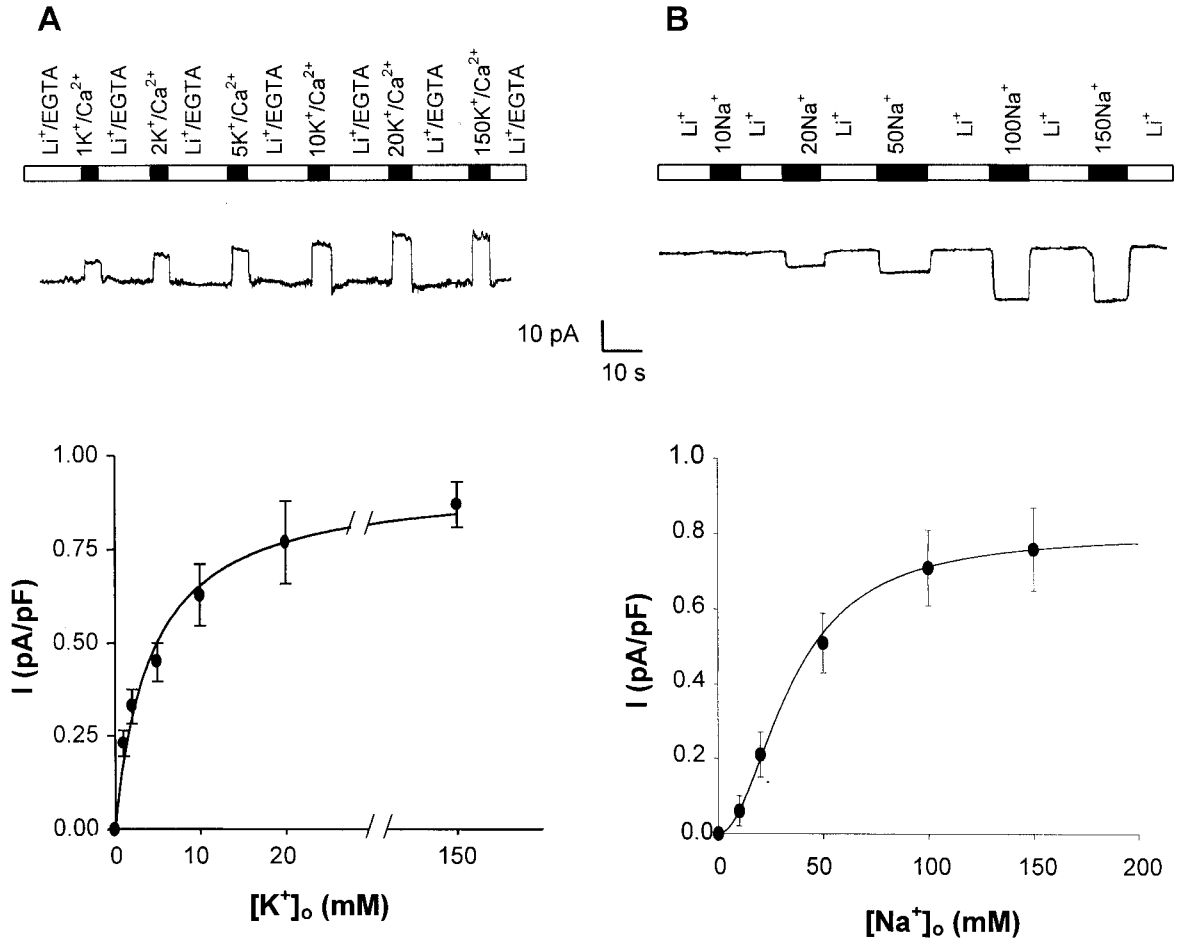

FIGURE 3 Effect of $[K^+]_o$ (*A*) on outward and of $[Na^+]_o$ (*B*) on inward Na^+-Ca^{2+}-K^+ exchange currents in cells transfected with chicken cone NCKX. (*A*) The top panel illustrates the sequence of external solution changes applied; control solution contained LiCl and EGTA, while test solutions contained 100 μM $CaCl_2$, LiCl, and selected concentrations of KCl (in mM) as indicated (for detailed solution composition, see Methods). The middle panel illustrates a representative current record obtained from a High Five cell expressing chicken cone NCKX, while the bottom panel illustrates the average K^+ dependence (± SEM) obtained from 13 cells. (*B*) The top panel illustrates the sequence of external solution changes in this protocol. The control solution contained LiCl and EGTA, while test solutions contained EGTA, LiCl, and the indicated concentrations of NaCl (in mM) (for detailed solution composition, see Methods). The middle panel illustrates a representative current recording obtained from a High Five cell expressing chicken cone NCKX. The bottom panel consists of the concentration response curve, obtained by averaging current recordings (± SEM) from five different cells expressing chicken cone NCKX.

The top panel of Fig. 3 *B* illustrates the protocol for measuring the Na^+ dependence of forward exchange of cone NCKX. The middle panel illustrates a typical example of the changes in inward current that were observed with this series of solution switches. The relationship between maximum inward current recorded from five different cells and bath Na^+ concentration was sigmoidal (*bottom panel*); the solid line represents the best fit to the Hill equation with a Hill coefficient of 1.8 and a K_m of 38 mM $[Na^+]_o$.

The experiments illustrated in Fig. 3 were carried out with cells transfected with chicken cone NCKX. Similar sets of experiments were carried out with cells transfected with dolphin rod NCKX (Fig. 4) and with cells transfected with the double deletion mutant *b*NCKX*dd* (Fig. 5). For the K^+ dependence of reverse Na^+-Ca^{2+}-K^+ exchange the 95% confidence intervals were 1.6–4.3 mM for dolphin rod NCKX and 1.8–11.7 mM for the double deletion mutant *b*NCKX*dd*.

The Na^+ dependence of forward Na^+-Ca^{2+}-K^+ exchange were fitted with the Hill equation: the best fit yielded Hill coefficients of 1.9 and 1.8 and K_m values for Na^+ of 49 and 45 mM for dolphin rod NCKX and for the double deletion mutant *b*NCKX*dd*, respectively.

Internal Ca^{2+} dependence of forward Na^+-Ca^{2+}-K^+ exchange currents

The dependence of forward exchange activity on $[Ca^{2+}]_i$ was studied in cells transfected with the three different

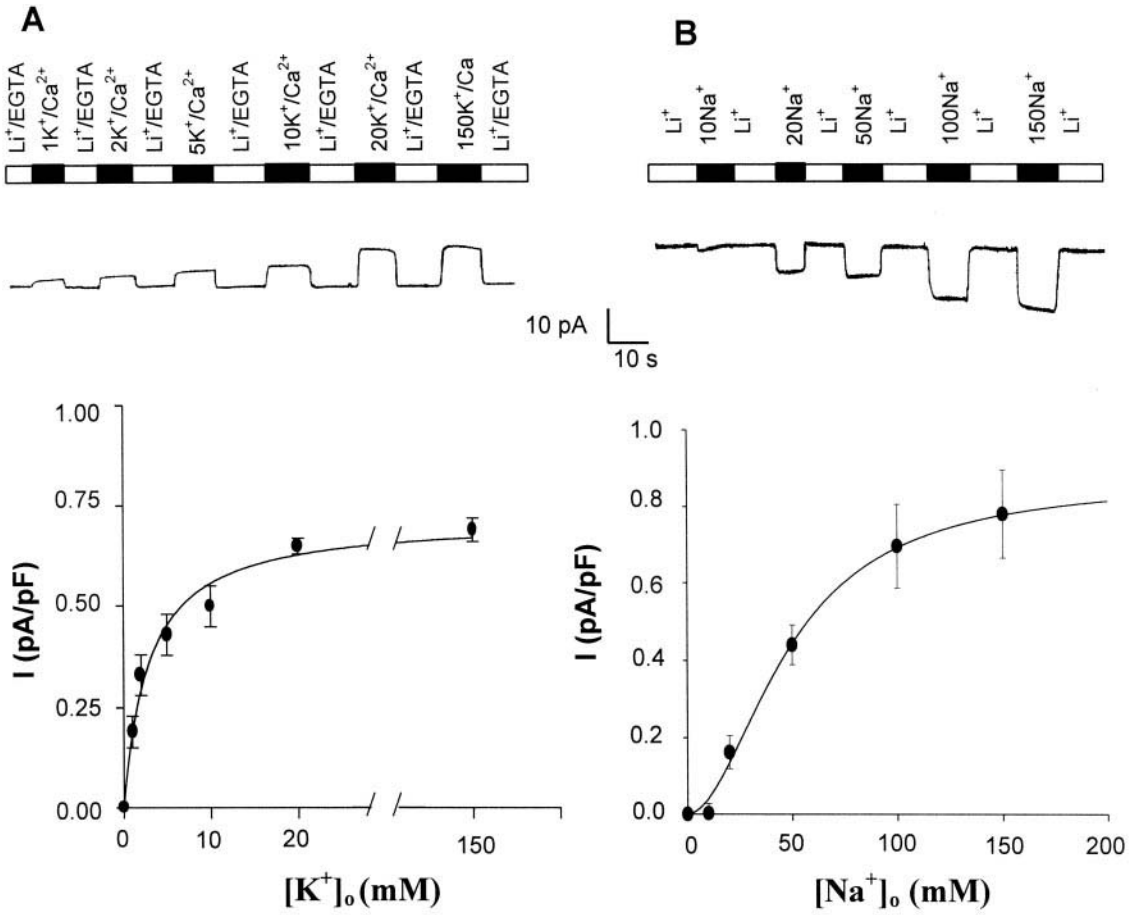

FIGURE 4 Effect of $[K^+]_o$ (*A*) on outward and of $[Na^+]_o$ (*B*) on inward Na^+-Ca^{2+}-K^+ exchange currents in cells transfected with dolphin rod NCKX. Experimental protocols were as described in the legend to Fig. 4. Average results (± SEM) were obtained for seven cells (outward current) and five cells (inward current), respectively.

NCKX cDNAs. An obligatory requirement for internal Ca^{2+} was observed in all three cases. Representative current recordings are illustrated in the top panel of Fig. 6, demonstrating half-maximal stimulation at ~1 μM (Fig. 6 *B*). In these experiments each individual observation was obtained from a separate cell. As high internal Ca^{2+} concentrations of 100 μM or higher often resulted in cell death, the range of internal Ca^{2+} concentrations tested had an upper limit of 50 μM and may not represent true saturation. The apparent dependence on $[Ca^{2+}]_i$ observed in cells transfected with chicken cone NCKX, with dolphin rod NCKX, or with the double deletion mutant *b*NCKX*dd* were very similar, with an apparent K_m value of 0.5–1 μM, comparable to values of 0.9 and 2.3 μM reported before for half-maximal activation of forward exchange by internal Ca^{2+} for the in situ exchanger in bovine and tiger salamander rod outer segments, respectively (Schnetkamp, 1991; Lagnado et al., 1988).

DISCUSSION

We report the first direct demonstration of K^+-dependent Na^+-Ca^{2+} exchange currents, measured following expression of three different NCKX cDNAs in a heterologous system. As no specific inhibitors have been identified for the Na^+-Ca^{2+}-K^+ exchanger in retinal rod outer segments, current changes due to NCKX were identified based on their absolute requirement on internal Ca^{2+} for generation of inward current (Fig. 6), and on both external Ca^{2+} and external K^+ for generation of outward current (Fig. 1). Both retinal rod and cone NCKX, and a double deletion mutant rod NCKX construct, were expressed in insect cells and generated both outward current (due to Ca^{2+} influx via reverse exchange) and an inward current (Ca^{2+} efflux via forward exchange), and this was dependent on the direction of the transmembrane Na^+ gradient.

We initially focused on the characterization of the cone Na^+-Ca^{2+}-K^+ exchanger that was cloned in our lab. There were two reasons for this: 1) very little information is available on exchanger activity in retinal cones, as a result of the technical difficulties of recording from isolated cone outer segments; 2) the dynamics of light-induced changes in cytosolic free Ca^{2+} in retinal cone outer segments are considerably faster compared with those observed in retinal

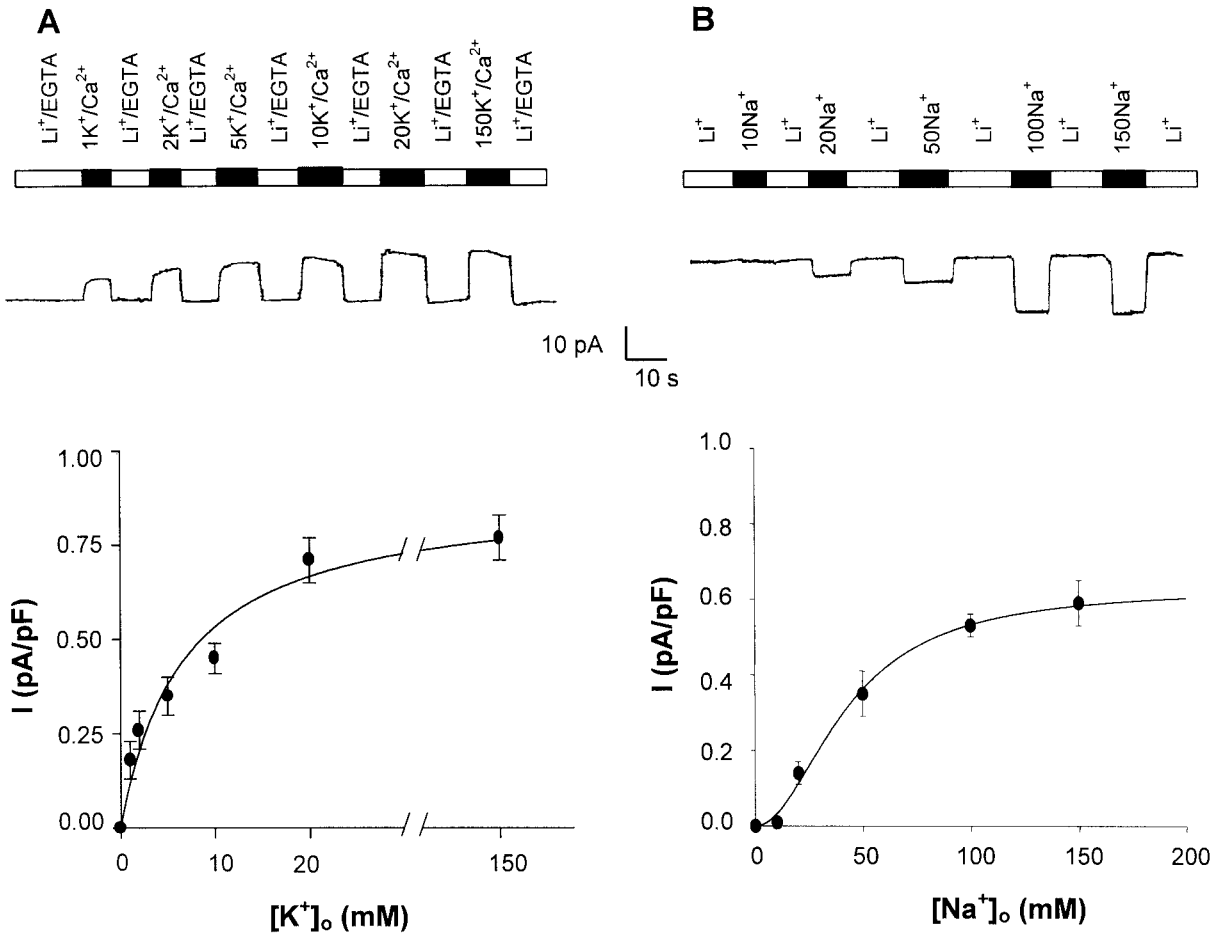

FIGURE 5 Effect of $[K^+]_o$ (*A*) on outward and of $[Na^+]_o$ (*B*) on inward Na^+-Ca^{2+}-K^+ exchange currents in cells transfected with the double deletion mutant *b*NCKX*dd*. Experimental protocols were as described in the legend to Fig. 4. Average results (± SEM) were obtained for seven cells (outward current) and five cells (inward current), respectively.

rod outer segments (Gray-Keller and Detwiler, 1994; Sampath et al., 1998, 1999). We subsequently carried out a comparative set of experiments with two rod-based NCKX cDNAs expressed in the same heterologous system. The results obtained in this study demonstrate that all three NCKX cDNAs tested code for K^+-*dependent* and electrogenic Na^+-Ca^{2+} exchangers when expressed in insect cells. These results are in contrast with those obtained by Navangione et al., who described K^+-*independent* Na^+-Ca^{2+} exchange currents after transient transfection of HEK293 cells with full-length bovine rod NCKX cDNA (Navangione et al., 1997). In our hands, transfection of HEK293 cells or High Five cells with full-length bovine rod NCKX1 did not yield detectable Na^+-Ca^{2+} exchange function, measured either by whole-cell patch clamp (results not illustrated), with ^{45}Ca uptake (Szerencsei et al., 2000), or with Ca^{2+}-indicating dyes (Cooper et al., 1999). As a result, we cannot address possible reasons for this difference.

We have characterized three fundamental properties of the cation binding site(s) on the three Na^+-Ca^{2+}-K^+ antiporters tested: their dependence on external Na^+ and K^+, and on internal Ca^{2+}. Each of these parameters was found to be, within experimental error, very similar among the three different NCKX clones tested, and also very similar to those reported previously for the in situ rod Na^+-Ca^{2+}-K^+ exchanger. The dependence on external Na^+ of NCKX activity expressed in insect cells could be fitted reasonably well by a Hill equation with a Hill coefficient of 1.8–1.9 and a K_{Na} of 38–48 mM (Figs. 3–5), similar to a Hill coefficient of 2 and K_{Na} of 35 mM reported previously in situ for the bovine rod Na^+-Ca^{2+}-K^+ exchanger (Schnetkamp, 1991). Both sets of values were obtained in the absence of external Ca^{2+}; increasing external Ca^{2+} causes a progressive increase in K_{Na} without affecting the Hill coefficient (Schnetkamp, 1991). Reverse NCKX currents required external K^+, with half-maximal concentrations of 2–7 mM (Fig. 3–5). These values can be compared with values of 5–10 mM obtained when measuring ^{45}Ca fluxes with the same cell lines expressing the NCKX clones or with bovine rod outer segments under very similar experi-

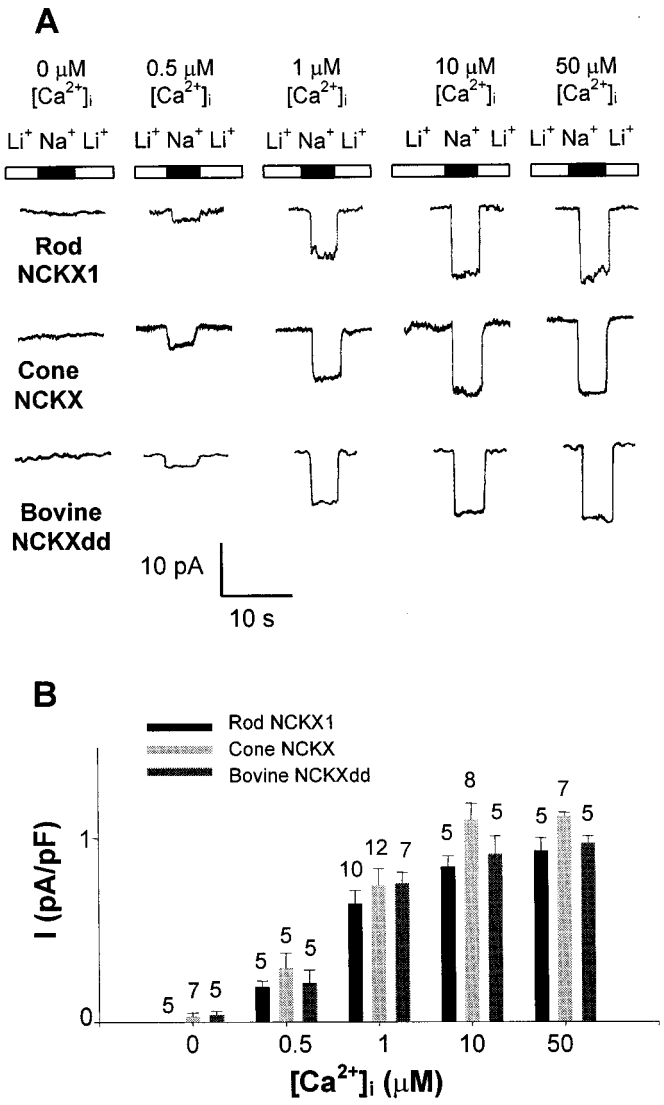

FIGURE 6 Effect of $[Ca^{2+}]_i$ on inward Na^+-Ca^{2+}-K^+ exchange currents. (*A*) Representative current records of inward Na^+-Ca^{2+}-K^+ exchange currents were obtained from High Five cells expressing chicken cone NCKX, dolphin rod NCKX1, or the double deletion mutant *b*NCKX*dd*. An inward current was recorded when the external solution was switched from one containing LiCl and EGTA to one containing NaCl and EGTA, as indicated by the top bar (see Methods for detailed solution composition). The pipette solution contained either 50 μM added $CaCl_2$ or Ca^{2+} buffers (EGTA or 5,5′-Dibromo BAPTA), yielding free Ca^{2+} concentrations of 0, 0.5, 1, and 10 μM, respectively. Free Ca^{2+} concentrations in the solutions used were verified with fluo-3 (measurements not shown). (*B*) Inward Na^+-Ca^{2+}-K^+ exchange currents obtained from cells expressing chicken cone NCKX, dolphin rod NCKX1, and the double deletion mutant *b*NCKX*dd*, respectively, were averaged (± SEM) from the indicated number of cells and presented as a bar diagram.

mental conditions (Prinsen et al., 2000; Szerencsei et al., 2000). It should be noted that the K^+ dependence of in situ rod NCKX has been shown to vary considerably, dependent on the precise ionic composition of the medium (e.g., Schnetkamp et al., 1995), and a similar observation was made with NCKX clones expressed in insect cells (Prinsen et al., 2000; Szerencsei et al., 2000). The current-voltage relationships were found to be very similar for the three NCKX clones studied; very little voltage dependence was observed (Fig. 2), consistent with results obtained in situ for the Na^+-Ca^{2+}-K^+ exchanger in rod outer segments isolated from tiger salamander retina (Lagnado et al., 1988).

The dependence of forward NCKX current on internal Ca^{2+} was found to be in the low (0.5–1) micromolar range (Fig. 6), comparable to values of 0.9 and 2.3 μM reported previously for half-maximal activation of forward exchange by internal Ca^{2+} for the in situ exchanger in bovine and tiger salamander rod outer segments, respectively (Schnetkamp, 1991; Lagnado et al., 1988). Considering the experimental error in our measurements and given the complexities of obtaining cation binding constants for a multiple cation transporter, we cannot exclude minor differences between the cation binding constants for the three different NCKX proteins tested here. However, the significant similarities suggest strongly that the fundamental properties of the NCKX cation binding sites are shared among rod and cone NCKX, and are located within the amino acid sequences that make up the two sets of highly conserved transmembrane spanning segments. This is consistent with the high degree of conservation observed at the amino acid level for the two sets of transmembrane spanning segments and short connecting loops of both rod and cone NCKX (82% identity between human rod and cone NCKX, 84% identity between chicken rod and cone NCKX; Prinsen et al., 2000). In a previous study we demonstrated that the two sets of proposed transmembrane spanning segments and short connecting loops were sufficient for K^+-dependent Na^+-Ca^{2+} exchange transport (Szerencsei et al., 2000); these results are corroborated and significantly extended by the present results (Figs. 2–6) obtained here with the double deletion rod *b*NCKX*dd* construct in which we removed the two large hydrophilic loops comprising ~60% of the rod NCKX sequence. It is clear from our results that fundamental properties of the cation binding sites, as well as the electrogenicity and voltage dependence of the NCKX exchanger, were retained in this double deletion mutant. Interestingly, sequence identity for the rod and cone NCKX paralogs is very low when examining the large hydrophilic loops, which may suggest very different regulatory features associated with these two types of NCKX proteins. Ongoing studies are directed toward characterizing regulatory features of both rod and cone NCKX, using site-directed mutagenesis.

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REFERENCES

Blaustein, M. P., and W. J. Lederer. 1999. Sodium/calcium exchange: its physiological implications. *Physiol. Rev.* 79:763–854.

Cervetto, L., L. Lagnado, R. J. Perry, D. W. Robinson, and P. A. McNaughton. 1989. Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature.* 337:740–743.

Cooper, C. B., R. J. Winkfein, R. T. Szerencsei, and P. P. M. Schnetkamp. 1999. cDNA-cloning and functional expression of the dolphin retinal rod Na^+-Ca^{2+}-K^+ exchanger NCKX1: comparison with the functionally silent bovine NCKX1. *Biochemistry.* 38:6276–6283.

Dzeja, C., V. Hagen, U. B. Kaupp, and S. Frings. 1999. Ca^{2+} permeation in cyclic nucleotide-gated channels. *EMBO J.* 18:131–144.

Frings, S., R. Seifert, M. Godde, and U. B. Kaupp. 1995. Profoundly different calcium permeation and blockage determine the specific function of distinct cyclic nucleotide-gated channels. *Neuron.* 15:169–179.

Gray-Keller, M. P., and P. B. Detwiler. 1994. The calcium feedback signal in the phototransduction cascade of vertebrate rods. *Neuron.* 13: 849–861.

Haug-Collet, K., B. Pearson, S. Park, S. Webel, R. T. Szerencsei, R. J. Winkfein, P. P. M. Schnetkamp, and N. J. Colley. 1999. Cloning and characterization of a potassium-dependent sodium/calcium exchanger in *Drosophila. J. Cell Biol.* 147:659–670.

Kimura, J., E. M. Jeanclos, R. J. Donnelly, J. Lytton, J. P. Reeves, and A. Aviv. 1999. Physiological and molecular characterization of the Na^+/Ca^{2+} exchanger in human platelets. *Am. J. Physiol. Heart Circ. Physiol.* 277:H911–H917.

Kimura, J., S. Miyamae, and A. Noma. 1987. Identification of Na^+-Ca^{2+} exchange current in single ventricular cells of guinea-pig. *J. Physiol.* 384:199–222.

Lagnado, L., L. Cervetto, and P. A. McNaughton. 1988. Ion transport by the Na/Ca exchange in isolated rod outer segments. *Proc. Natl. Acad. Sci. USA.* 85:4548–4552.

Nakatani, K., and K.-W. Yau. 1989. Sodium-dependent calcium extrusion and sensitivity regulation in retinal cones of the salamander. *J. Physiol.* 409:525–548.

Navangione, A., G. Rispoli, N. Gabellini, and E. Carafoli. 1997. Electrophysiological characterization of ionic transport by the retinal exchanger expressed in human embryonic kidney cells. *Biophys. J.* 73:45–51.

Perry, R. J., and P. A. McNaughton. 1991. Response properties of cones from the retina of the tiger salamander. *J. Physiol.* 443:561–587.

Picones, A., and J. I. Korenbrot. 1995. Permeability and interaction of Ca^{2+} with cGMP-gated ion channels differ in retinal rod and cone photoreceptors. *Biophys. J.* 69:120–127.

Prinsen, C. F. M., R. T. Szerencsei, and P. P. M. Schnetkamp. 2000. Molecular cloning and functional expression the potassium-dependent sodium-calcium exchanger from human and chicken retinal cone photoreceptors. *J. Neurosci.* 20:1424–1434.

Sampath, A. P., H. R. Matthews, M. C. Cornwall, J. Bandarchi, and G. L. Fain. 1999. Light-dependent changes in outer segment free-Ca^{2+} concentration in salamander cone photoreceptors. *J. Gen. Physiol.* 113: 267–277.

Sampath, A. P., H. R. Matthews, M. C. Cornwall, and G. L. Fain. 1998. Bleached pigment produces a maintained decrease in outer segment Ca^{2+} in salamander rods. *J. Gen. Physiol.* 113:267–277.

Schnetkamp, P. P. M. 1980. Ion selectivity of the cation transport system of isolated cattle rod outer segments: evidence of a direct communication between the rod plasma membrane and the rod disk membranes. *Biochim. Biophys. Acta.* 598:66–90.

Schnetkamp, P. P. M. 1986. Na^+-Ca^{2+} exchange in the outer segments of bovine rod photoreceptors. *J. Physiol.* 373:25–45.

Schnetkamp, P. P. M. 1989. Na^+-Ca^{2+} or Na^+-Ca^{2+}-K^+ exchange in the outer segments of vertebrate rod photoreceptors. *Prog. Biophys. Mol. Biol.* 54:1–29.

Schnetkamp, P. P. M. 1991. Optical measurements of Na^+-Ca^{2+}-K^+ exchange currents in intact outer segments isolated from bovine retinal rods. *J. Gen. Physiol.* 98:555–573.

Schnetkamp, P. P. M., D. K. Basu, and R. T. Szerencsei. 1989. Na^+-Ca^{2+} exchange in the outer segments of bovine rod photoreceptors requires and transports potassium. *Am. J. Physiol. Cell Physiol.* 257:C153–C157.

Schnetkamp, P. P. M., J. E. Tucker, and R. T. Szerencsei. 1995. Ca^{2+} influx into bovine retinal rod outer segments mediated by Na^+-Ca^{2+}-K^+ exchange. *Am. J. Physiol. Cell Physiol.* 269:C1153–C1159.

Sheng, J.-Z., C. F. M. Prinsen, R. B. Clark, W. R. Giles, and P. P. M. Schnetkamp. 2000. Na^+-Ca^{2+}-K^+ currents measured in insect cells transfected with the retinal cone NCKX cDNA *Biophys. J.* 78:53*a*. (Abstr.).

Szerencsei, R. T., J. E. Tucker, C. B. Cooper, R. J. Winkfein, P. J. Farrell, K. Iatrou, and P. P. M. Schnetkamp. 2000. Minimal domain requirement for cation transport by the potassium-dependent Na^+-Ca^{2+}-K^+ exchanger: comparison with a NCKX paralog from *Caenorhabditis elegans. J. Biol. Chem.* 275:669–676.

Tsoi, M., K. H. Rhee, D. Bungard, X. F. Li, S. L. Lee, R. N. Auer, and J. Lytton. 1998. Molecular cloning of a novel potassium-dependent sodium-calcium exchanger from rat brain. *J. Biol. Chem.* 273:4155–4162.

Yau, K.-W., and K. Nakatani. 1984. Electrogenic Na^+-Ca^{2+} exchange in retinal rod outer segments. *Nature.* 311:661–666.