cDNA Cloning and Functional Expression of the Dolphin Retinal Rod Na−Ca+K Exchanger NCKX1: Comparison with the Functionally Silent Bovine NCKX1[†]

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ABSTRACT: cDNAs of human and bovine retinal rod $Na^+-Ca^{2+}+K^+$ exchanger (NCKX1) have previously been cloned, but potassium-dependent Na−Ca exchange activity upon heterologous expression has not been demonstrated. We have cloned NCKX1 cDNA from dolphin, examined function upon transfection in HEK293 cells, and compared the dolphin sequence encoded by the cDNA with those of human and bovine. The dolphin NCKX1 cDNA encodes 1013 amino acid residues. Comparison to bovine and human NCKX1 revealed strong conservation in the transmembrane domains (>95%), but relatively low conservation in the large extracellular (∼50%) and cytosolic (<50%) domains. The dolphin cytosolic domain differs from the bovine sequence by the absence of a stretch of 114 amino acids. HEK293 cells transfected with dolphin NCKX1 cDNA showed K^+-dependent Na−Ca exchange in >95% of the experiments, whereas transfection with bovine NCKX1 yielded no function. The bovine NCKX1 phenotype was imparted on dolphin NCKX1 when the dolphin cytosolic loop was replaced by that from bovine. Conversely, deletion of 114 amino acids from the bovine sequence to match the dolphin sequence resulted in a mutant bovine NCKX1 which performed K^+-dependent Na−Ca exchange. These results suggest that domains within the large cytosolic loop of NCKX1 control functional activity when expressed in heterologous systems.

The $Na^+-Ca^{2+}+K^+$ exchanger (NCKX1)[1] is a protein critical to the transduction of vision in retinal rod photoreceptors *(1, 2)*. In the dark, a large and persistent influx of Ca^{2+} through the cGMP-gated and light-sensitive channels *(3)* is balanced by Ca^{2+} extrusion via NCKX1. NCKX1 couples both the inward Na^+ gradient and the outward K^+ gradient to the extrusion of Ca^{2+} against its gradient *(4)*. Light induces closure of the cGMP-gated channel through a G-protein-coupled cascade *(5)*, but light has no direct effect on NCKX1 function. As a result, light causes a rapid lowering of cytosolic free Ca^{2+}, and this is believed to mediate, at least in part, the process of light adaptation *(6−9)*. Functional properties of NCKX1 in situ have yielded detailed information on the properties of the cation binding sites *(10−12)*, on regulation of the maximal transport rate of the exchanger *(13)*, and on a pronounced and prolonged inactivation of the Ca^{2+} extrusion mode *(14)*. The latter is thought to prevent lowering of cytosolic free Ca^{2+} to values below 1 nM (as predicted from the coupling stoichiometry) during prolonged and bright illumination of rod cells when Ca^{2+} influx through the cGMP-gated channels is abolished.

The bovine and human NCKX1 amino acid sequences have previously been determined by molecular cloning *(15, 16)*. The NCKX1 amino acid sequences show little homology (<4%) with Na^+-Ca^{2+} exchangers (NCX) from other tissues, such as cardiac and kidney, which do not utilize the K^+ gradient to drive Ca^{2+} transport. The moderate degree of identity between human and bovine NCKX1 sequences is in contrast to the high degree of identity (>95%) observed for the amino acid sequence of NCX1 cDNAs obtained from different mammalian species *(17−20)*. Recently, a novel potassium-dependent Na−Ca exchanger cDNA (NCKX2) was cloned from rat brain *(21)*, and was shown to share significant sequence similarity with NCKX1 only in the proposed transmembrane-spanning segments (TMS).

A prerequisite for future studies on the structure−function relationship, regulation, and topology of NCKX1 protein is the availability of a retinal NCKX cDNA that results in robust functional activity upon heterologous expression. Since the cloning of bovine NCKX1 in 1991, only one study has addressed the issue of functional activity upon transfection of cells with NCKX1 cDNA: potassium-independent Na^+-Ca^{2+} exchange activity was described when HEK293 cells were transfected with bovine NCKX1 cDNA *(22)*. We have been unsuccessful in obtaining consistent functional expression of either bovine or human NCKX1 cDNA in a variety of expression systems including Sf9 cells, HEK293 cells, or CHO cells.[2]

Here, we report on cloning, sequencing, and functional expression of NCKX1 from the bottlenose dolphin (*Tursiops*

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[1] Abbreviations: ROS, rod outer segment; NCKX1, retinal rod $Na^+/Ca^{2+}+K^+$ exchanger; NCX, Na^+/Ca^{2+} exchanger; TMS, transmembrane spanning; NCKX2, brain $Na^+/Ca^{2+}+K^+$ exchanger; PCR, polymerase chain reaction; bp, base pair(s); RACE, rapid amplification of cDNA ends.

[2] Szerencsei, R. T., Tucker, J. E., Cooper, C. B., and Schnetkamp, P. P. M., unpublished observations.

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truncatus).[3] We expressed dolphin and bovine NCKX1 cDNA in HEK293 cells under identical conditions, and observed potassium-dependent Ca^{2+} uptake (representing reverse $Na^+-Ca^{2+}+K^+$ exchange) in the case of transfection with dolphin NCKX1 cDNA; no Ca^{2+} uptake was observed when cells were transfected with bovine NCKX1 cDNA. We have focused on the possible role of sequence elements in the N-terminal portion of the large cytosolic loop of bovine NCKX1 in controlling functional activity upon heterologous expression, as we noticed a significant dropout of 114 amino acids in the dolphin sequence as compared with the bovine sequence.

EXPERIMENTAL PROCEDURES

Cloning of the Full-Length Dolphin $Na^+-Ca^{2+}+K^+$ Exchanger cDNA. The full-length dolphin $Na^+-Ca^{2+}+K^+$ exchanger cDNA was cloned by standard molecular techniques. Nucleotides 90 through the stop codon were sequenced, and nucleotides 1465 through the stop codon were cloned, from a dolphin retinal cDNA library. Nucleotides 1 through 1465 were cloned from a fragment generated by PCR on dolphin genomic DNA. The sequence from 214 bp preceding the initiation codon through nucleotide 90 was obtained from a 5′RACE fragment of dolphin retinal cDNA. The full-length clone ligated in pBluescript vector was excised as an *Eco*RI−*Xba*I fragment and subcloned into the eukaryotic expression vector pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA). Sequencing and restriction mapping were carried out on the full-length clone to check for correct ligation, and for PCR errors.

Construction and Nomenclature of Dolphin and Bovine $Na^+-Ca^{2+}+K^+$ Exchanger Mutants and Chimeras. The full-length bovine NCKX1 cDNA was subcloned into pcDNA3.1$^+$, in a manner similar to the full-length dolphin clone, as described above. We deleted a region in the cytosolic domain of the bovine sequence which was found to be absent in the dolphin sequence (see below), from nucleotides 1860−2213 of the bovine NCKX1 sequence. A ~950 bp fragment was synthesized by PCR with an AvrII site at the 5′ end which annealed at nucleotide 1860, and which overlapped at the 3′ end at an *Aat*II site (nucleotide 3130). The *Avr*II−*Aat*II fragment was cloned into the bovine cDNA to generate the "**Bvdel**" mutant. A chimera of the dolphin sequence containing the full bovine cytosolic domain, designated "**Dbv**", was constructed by cloning the bovine *Avr*II−*Aat*II (1860−2177) and *Aat*II−*Aat*II (2177−3130) cytosolic fragments into the dolphin *Avr*II (1878) and *Aat*II (2537) sites. All the constructs were ligated in pBluescript vector, subsequently excised and cloned into the mammalian expression vector pcDNA3.1$^+$, and restriction mapped and sequenced to check for correct orientation and PCR errors.

Transfection and Expression of the Cloned $Na^+/Ca^{2+}+K^+$ Exchanger Constructs in HEK293 Cells. Human embryonic kidney cells (HEK293) were grown at 37 °C, 5% CO_2 in Dulbeco's minimum essential medium (DMEM) (Gibco, Burlington, ON), supplemented with 10% fetal bovine serum (FBS) (Gibco). Cells were seeded onto #1 12 mm glass coverslips (Warner Instruments, Hamden, CT) in 100 mm plates (Becton Dickson, Lincoln Park, NJ) for single-cell imaging as described below. After 12−24 h, transfection was carried out by $Ca(PO_4)_2$ precipitation with 7−10 μg of plasmid DNA as previously described *(23)* or by using 0.5−1 μg of plasmid DNA and 7 μL of Lipofectamine (Gibco) in 12-well plates (Becton Dickson), according to the manufacturer's instructions; 24−72 h after transfection, the cells were assayed for expression. Cells for Western blotting were washed in the 100 mm plates, solubilized in a buffer containing 0.1% Triton X-100, 0.5% deoxycholate, and protease inhibitors, and spin-separated from nuclei before gel loading. The transfers were probed with the PMe 1B3 monoclonal antibody whose epitope has been mapped to the C-terminal portion of the cytosolic loop of bovine NCKX1 *(24)*.

Single-Cell Imaging of Potassium-Dependent Ca^{2+} Influx. Fluorescent dye loading was carried out at 25 °C for 15−30 min in serum-free DMEM containing 5 μM fura-2, AM (Molecular Probes, Eugene, OR). Coverslips were warmed to 37 °C for the duration of the experiments in a PH-3 perfusion chamber (Warner Instruments). The cells were viewed through an Axiovert 135 inverted microscope (Carl Zeiss Canada, Don Mills, ON) equipped with a 20×/0.75 FLUAR objective. Perfusion over the cells was by peristaltic pumping at a rate of ~2 mL/min. Fura-2 fluorescence was generated by illumination at alternating 340/380 nm wavelengths with a DeltaRam High-Speed Illuminator (Photon Technology International, Monmouth Junction, NJ), and was collected at 510 nm (cutoff and band-pass filters were from Chroma Technology Corp., Brattleboro, VT) with an IC-200 intensified CCD (Photon Technology International). Ratios were calculated, and images were analyzed with ImageMaster v1.4 software (Photon Technology International). During Ca^{2+} flux measurements, solutions perfusing over the cells were balanced to 145 mM final salt concentration with combinations of NaCl, KCl, or LiCl, and were buffered to pH 7.4 with 10 mM HEPES−Tris. $CaCl_2$ concentration was 100 μM.

Measurement of Ca^{2+} Flux in Suspensions of Transfected Cells. Cells transfected as above were lifted, loaded with fluo-3 (Molecular Probes), washed, and assayed as previously described *(25)*.

RESULTS

Cloning of the Dolphin NCKX1 cDNA: *Sequence Comparison with Human and Bovine NCKX1*. We screened a dolphin retinal cDNA library with a probe from bovine NCKX1. From two separate screenings, we obtained sequence starting 90 bp downstream of the expected start site through the stop codon. We obtained sequence upstream from nucleotide 90 through the expected start codon to 214 bp preceding the ATG of the longest open reading frame from a fragment generated by 5′RACE on dolphin retinal first-strand cDNA. A TAA stop codon exists in the same frame 12 bases upstream from the designated start site. The coding sequence is 3042 nucleotides long, encoding a protein of 1013 amino acids. Similar to the 2 previously published NCKX1 sequences *(15, 16)*, the Kyte−Doolittle hydropathy analysis revealed 12 hydrophobic segments which form the putative α-helices, designated H0−H11, and 2 large hydrophilic segments, 1 putatively extracellular and preceding H1, the other putatively intracellular and between H5 and H6 (Figure 1). The deduced amino acid sequence of dolphin

[3] The dolphin NCKX1 sequence has been deposited with GenBank, accession number AF059031.

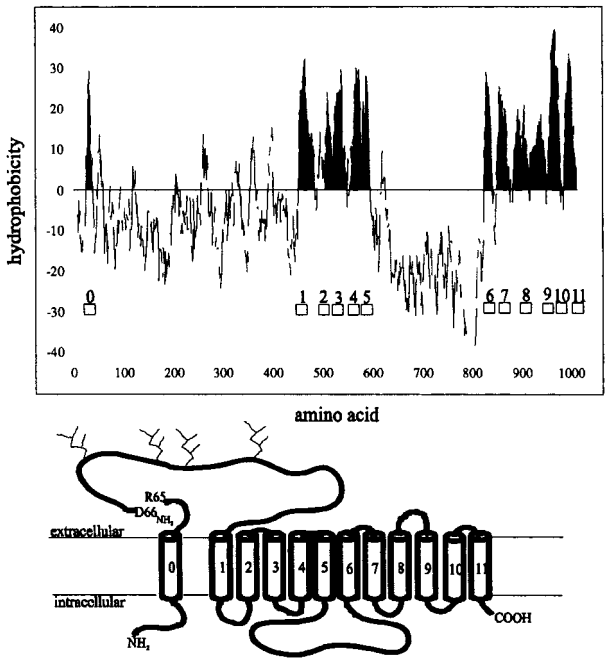

FIGURE 1: Hydrophobicity analysis and cartoon of the proposed topology of the deduced amino acid sequence of the cloned dolphin NCKX1. Hydrophobic segments long enough to form the putative α-helical segments are shaded and designated below by numbers 0 through 11. The proposed signal peptide and cleavage site is indicated in the cartoon. The large extracellular loop precedes H1, and the large cytosolic loop resides between H5 and H6.

NCKX1 is illustrated in Figure 2 and compared with the bovine (as revised by us) and human sequences *(16)*. The dolphin sequence exhibits an overall identity to the bovine sequence of 62%, or 82% identity where only matched positions are counted. The identity between bovine, human, and dolphin is greater than 95% in the TMS regions. The charged residues situated in the hydrophobic segments are conserved between bovine, human, and dolphin NCKX1 sequences. The extracellular domain between H0 and H1 is similar in length in all 3, and is conserved on the order of 50% identity, relatively low in comparison to the TMS domains, and in comparison to the identity observed in other families of 12 TMS domain ion transporters. The large cytosolic loop between H5 and H6 is observed to be the least conserved segment at less than 50% identity when the entire lengths of bovine, human, and dolphin are counted, and with only a 73% identity at matched residues between dolphin and bovine. The first 25 residues of the cytosolic domains of all 3 NCKX1 are highly conserved. Ninety-seven of the next 114 residues of bovine are conserved in the human sequence, but have no match in the dolphin sequence. A high identity is, again, observed between bovine and dolphin for the next 57 residues (38 of which are conserved in human) preceding the repeat region. In dolphin, the putative repeat segment is less well ordered, and is not as long as in bovine, with only four repeat-like stretches. Strong identity picks up at the end of the repeat domain at G951 in bovine aligning to G744 in dolphin, just upstream from the long glutamic acid stretch preceding H6. The glutamic acid stretch is very close in length between all three NCKX1. It is noteworthy that the cytosolic loop of the dolphin NCKX1 cDNA we cloned (220 residues) is considerably shorter when compared to our published human NCKX1 cDNA (283 residues), or with our revised sequence of bovine NCKX1 cDNA (427 residues). The proposed overall topology of all 3 NCKX1 sequences is identical, and similar to that of the family of NCX and other families of 12 TMS domain ion transporters *(26)*.

Functional Expression of Full-Length Dolphin NCKX1 cDNA in HEK293 Cells Measured by Single-Cell Calcium Imaging. We generated a full-length clone by using genomic DNA as a template in PCR to synthesize a fragment, comprising the initiation codon through H1, which we inserted into a partial length clone obtained from cDNA library screening. The full-length clone was subsequently subcloned into the expression vector pcDNA3.1$^+$ for expression in mammalian cells. Other deletion mutants and chimeric dolphin/bovine NCKX1 constructs were made as described under Experimental Procedures (graphically represented in Figure 5). Functional activity of full-length dolphin NCKX1 was assessed after transient transfection into HEK293 cells. Several coverslips from separate transfections, and on each coverslip a field containing 20−60 cells, were analyzed by digital calcium imaging with fura-2. Changes in internal Ca^{2+} over time were measured by a change in the ratio of the intensity of light emitted at 510 nm with excitation at 340 or 380 nm. Our assay is based on the ability of cells successfully transfected with NCX or NCKX proteins to mediate Ca^{2+} influx via reverse exchange when sodium but not calcium in the external medium is removed; Ca^{2+} influx via reverse exchange is expected to require the presence of external potassium in the case of cells transfected with NCKX cDNA, but not in the case of cells transfected with NCX cDNA. The solution switches used (sodium to lithium or sodium to lithium plus 5 mM potassium) are optimal for measuring NCKX1-mediated Ca^{2+} influx via reverse exchange as judged from such experiments in isolated bovine rod outer segments containing NCKX1 *(12)*. We used bovine NCX1 as a positive control for potassium-independent Ca^{2+} influx via reverse exchange.

Functional expression of dolphin NCKX1 in HEK293 cells was examined and compared to functional expression of bovine NCX1 in 20 experiments totaling 48 separate transfections with dolphin NCKX1. Transfections were carried out by either Lipofectamine or calcium phosphate protocols, the latter giving on average a higher successful transfection percentage. Calcium responses of HEK293 cells following transfection could be divided into three groups:

(1) Transfections with control vector gave rise to cells with low and quiescent resting calcium levels; no changes in the cytoplasmic free Ca^{2+} were observed at all upon the solution changes in our experimental paradigm.

(2) Transfections with control vector gave rise to cells with more variable resting calcium levels; random and isolated calcium spikes of 30−60 s duration were observed in some cases; in other cases, synchronous oscillating changes in free Ca^{2+} were observed in clusters of cells (one such case is illustrated in Figure 6, lower panel). In some cases, a slow and delayed rise in free Ca^{2+} was observed upon switching from the sodium−calcium solution to the lithium−calcium solution.

(3) In some experiments, intracellular sodium levels in HEK293 cells were boosted by treatment of transfected cells either with ouabain or with the sodium channel ionophore gramicidin. In most of such experiments, cells transfected

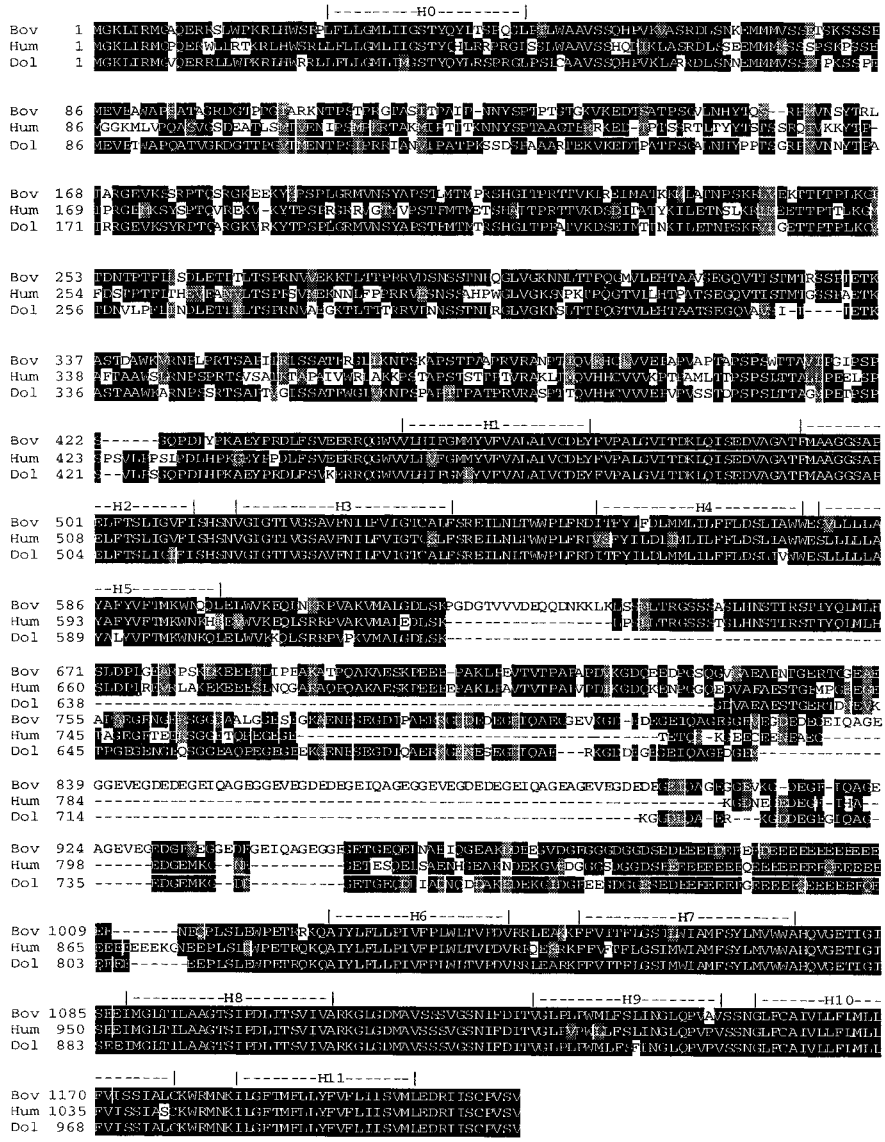

FIGURE 2: Sequence alignment between the previously published NCKX1 and the dolphin NCKX1. Putative transmembrane spanning segments are designated H0 through H11 above the sequence alignment. Greatest identity is observed in the two sets of transmembrane spanning segments, H1−H5 and H6−H11. Greatest sequence divergence is found in the cytosolic loop between H5 and H6. A dropout from the dolphin sequence of 114 amino acids present in the bovine sequence (96 residues of the human) starts at the 24th residue of the cytosolic loop.

with control vector showed a significant (and sometimes delayed) rise in free Ca^{2+} upon switching from the sodium−calcium solution to the lithium−calcium solution that affected the large majority (>80%) of cells on the coverslip, suggesting some mechanism endogenous to HEK293 cells. For this reason, it was rather difficult to interpret the results obtained with sodium-loaded cells when transfected with NCKX1 cDNAs, and results obtained from such experiments are not included here.

In all transfections with bovine NCX1 and in 46 out of 48 transfections with full-length dolphin NCKX1, cells could be identified which gave a strong rise in cytosolic Ca^{2+} (as indicated by the increase in the 340/380 nm ratio of fura-2 fluorescence) when 150 mM NaCl in the external medium was replaced by 145 mM LiCl/5 mM KCl, whereas no such cells could be identified when HEK293 cells were transfected with control vector (Figure 3, upper panel). When the above experiment was repeated with KCl omitted from the lithium medium, only cells transfected with NCX1 showed a strong rise in intracellular Ca^{2+}, whereas cells transfected with dolphin NCKX1 gave no or only a very sluggish response (Figure 3, lower panel) until potassium was added to the medium. When HEK293 cells were transfected either with bovine NCKX1 (see below) or with human NCKX1,[2] Ca^{2+} influx was obtained only in a few cells and not consistently with the above experimental paradigm, suggesting that bovine and human NCKX1 did not show functional activity under the same conditions that consistently permitted us to observe functional activity for dolphin NCKX1.

Measurement of Cytosolic Ca^{2+} Flux in Suspensions of Transfected Cells. Digital calcium imaging of single cells attached to a coverslip samples a limited number of cells. Furthermore, in selecting regions for calculating ratios, there is an inherent bias to choose only positive cells. We, therefore, also examined potassium-dependent Na−Ca exchange in transfected HEK293 cells in suspension, which averages cytosolic calcium flux over a number of cells exceeding 10^5; a cuvette containing a 2 mL suspension of

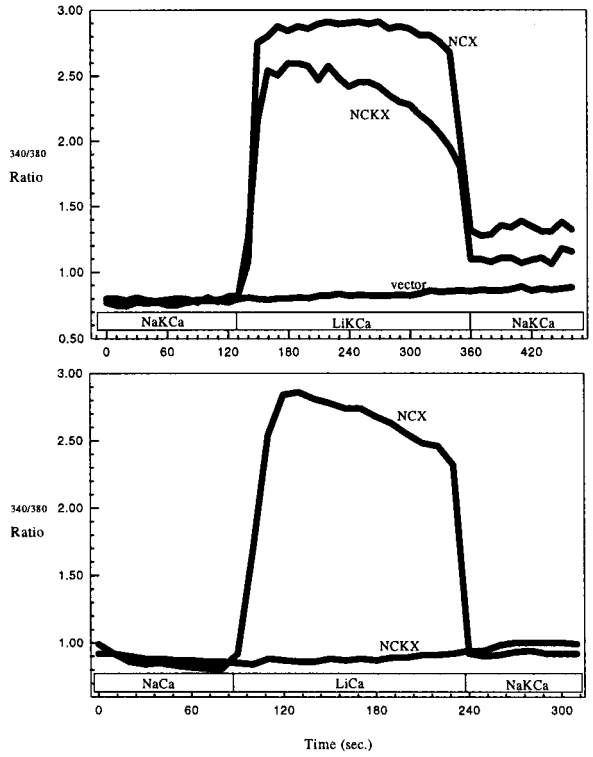

FIGURE 3: Potassium dependence of Ca^{2+} flux in HEK293 cells transfected with bovine NCX1, dolphin NCKX1, or vector DNA. The 340/380 nm ratio of fura-2 fluorescence represents cytosolic Ca^{2+} flux, with the ratio increasing as $[Ca^{2+}]_i$ increases. Upper panel: the response over time of changing the Na^+ gradient from inward to outward, in the presence of 5 mM K^+, by Li^+ replacing Na^+ in the external medium. Lower panel: Ca^{2+} flux in response to the same ion gradient switch, but in the absence of external K^+. All solutions contain 100 μM $CaCl_2$ and are made up to a 145 mM final concentration with Na^+, Li^+, and K^+.

$\sim 2.5 \times 10^5$ HEK293 cells loaded with fluo-3 was placed in a spectrofluorometer. Potassium-dependent Na−Ca exchange was measured by clamping the cytosolic sodium concentration with the ionophore gramicidin as described *(25, 27)*. Even though no sodium gradient across the plasma membrane exists in this condition, a large inwardly directed calcium gradient is expected to drive calcium into the cytosol. This was previously observed in cells transfected with NCX1 *(25)*. In contrast, when cells transfected with the dolphin NCKX1 were tested with this protocol, calcium influx not only required the presence of sodium, but also required the addition of potassium. As shown in Figure 4, cells transfected with dolphin NCKX1 responded to the addition of 20 mM KCl with a large Ca^{2+} influx. Cells transfected with bovine NCKX1, however, showed no response upon the addition of KCl (Figure 4).

Effect of Changes in the Large Cytosolic Loop of NCKX1 on Functional Activity upon Transfection. Significant areas of sequence divergence were noticed when comparing the human, bovine, and dolphin NCKX1 sequences (Figure 2). We focused on a stretch of amino acids in the N-terminal portion of the large cytosolic loop that starts 24 amino acids after the end of H5. Sequence alignment and comparison with the bovine sequence shows a drop out of 18 amino acids in the human sequence, whereas the dolphin sequence shows a drop out of 114 amino acids. To assess the possible role of this domain in controlling functional NCKX1 activity after transfection into cell lines, we deleted the above 114 amino

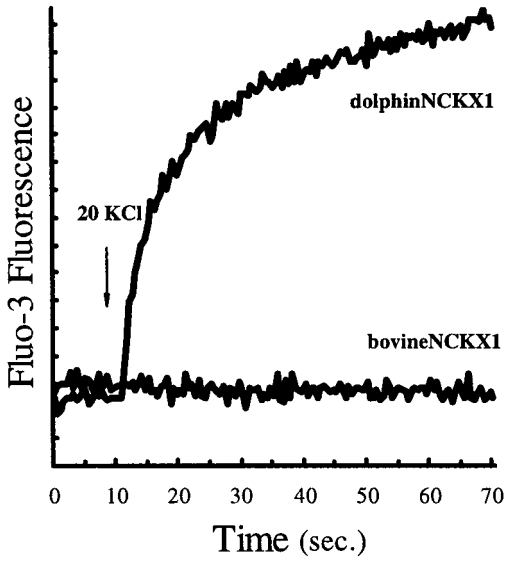

FIGURE 4: Cytosolic Ca^{2+} flux measured by fluo-3 fluorescence in suspensions of bovine or dolphin NCKX1 transfected HEK293 cells. Increase in fluorescence represents increase in concentration of cytosolic Ca^{2+}. 2.5×10^5 cells were suspended in 2 mL of buffer containing 75 mM NaCl, 75 mM LiCl, 200 μM EGTA, 2.5 μM gramicidin. After 2 min, 1 mM $CaCl_2$ was added, followed 10 s later by 20 mM KCl. Upon the addition of external K^+, Ca^{2+} influx was consistently elicited in dolphin NCKX1 transfected cells, whereas no change in cytosolic Ca^{2+} in bovine NCKX1 transfected cells was ever observed. Temperature was maintained at 25 °C.

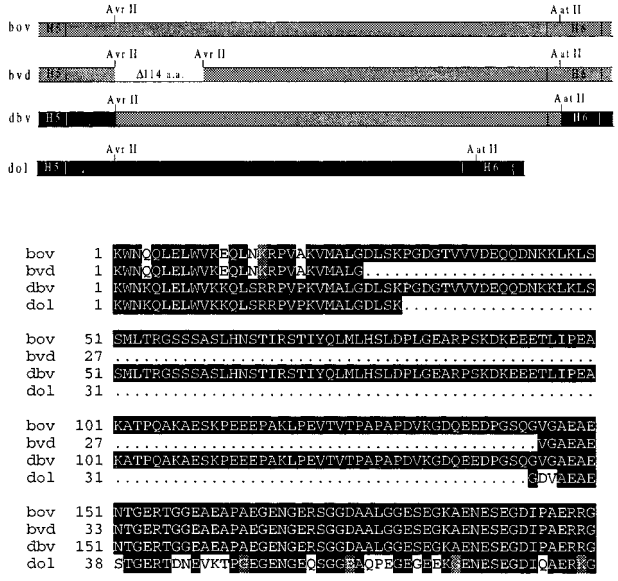

FIGURE 5: Graphical representation and sequence alignment through the cytosolic loop of the mutant NCKX1 constructs. **Bov** and **Dol** are the full-length bovine and dolphin NCKX1 sequences. **Bvdel** is the bovine sequence less the 114 amino acid stretch dropped out of the dolphin sequence. **Dbv** is the dolphin sequence with the cytosolic domain of the bovine replacing the native sequence. Mutations were carried out at mutual *Avr*II and *Aat*II sites as described.

acids from the bovine sequence (**Bvdel**) and then examined the functional activity of this construct after transient transfection into HEK293 cells. Conversely, we replaced the cytosolic loop in the dolphin NCKX1 with the cytosolic loop from bovine NCKX1 (**Dbv**). The above constructs are illustrated in Figure 5. Full-length dolphin NCKX1 and the **Bvdel** construct showed the characteristic potassium-dependent rise in free Ca^{2+} (as illustrated in Figure 3) when

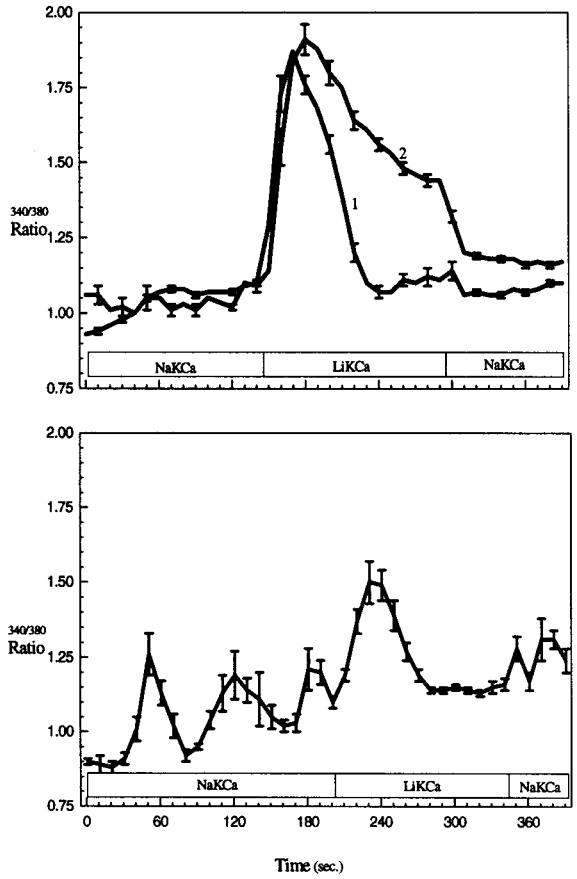

FIGURE 6: Variability in the Ca^{2+} flux response of transfected HEK293 cells to Na^+ gradient changes. Traces shown are averages of several single cells on the same coverslip from a single transfection. Measurements were taken, and solutions were made as in Figure 4. Upper panel: cells transfected with **Dbv** (1, n = 18) or **Bvdel** (2, n = 10) constructs. Cells transfected with **Bvdel** followed the characteristic Ca^{2+} flux of NCKX1. In some experiments, cells transfected with **Dbv** tended to exhibit a sharp, but short duration Ca^{2+} flux coincidental with solution changes. Lower panel: in some experiments, cells transfected with vector alone (n = 8) exhibited spontaneous transient fluxes unrelated to solution changes. In some cases, as shown, the solution change would elicit a sharp, short duration flux, followed by a quiescence, as in **Dbv** (1) above.

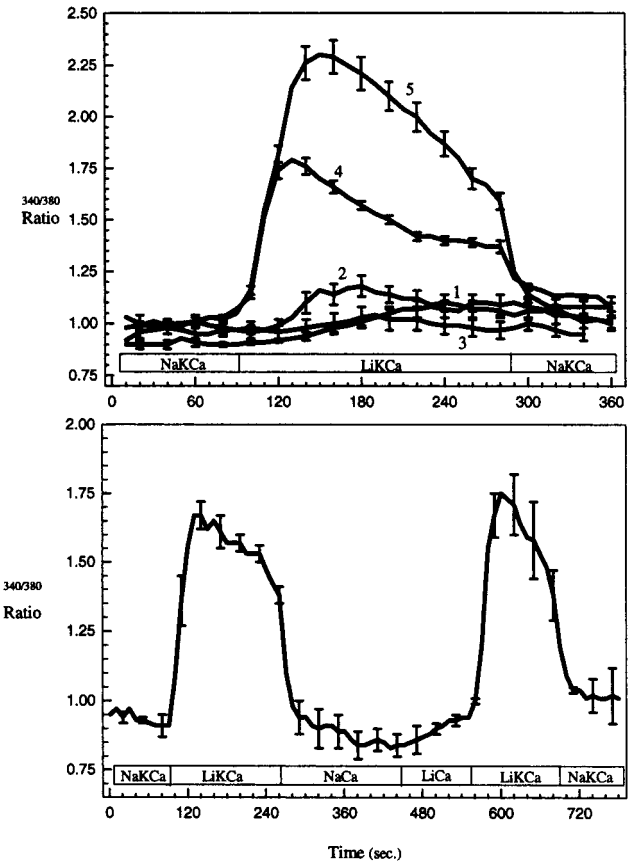

FIGURE 7: Ca^{2+} flux in HEK293 cells transfected with mutant constructs of NCKX1. Measurements and solutions were as in Figure 3. Each trace is the average of several single cells from different coverslips from 6−10 different experiments. Upper panel: 1, vector (n = 55); 2, bovine (n = 39); 3, **Dbv** (n = 25); 4, **Bvdel** (n = 17); 5, dolphin (n = 32). Lower panel: potassium dependence in the **Bvdel** construct is shown by the absence of Ca^{2+} flux when K^+ is not present in the external medium (average of three cells from a single transfection).

transfected in cells characteristic of group 1 experiments (i.e., low and quiescent calcium levels in cells transfected with control vector), whereas no functional activity was observed with full-length bovine NCKX1 or with the **Dbv** construct. When group 2 experiments (see above) were considered as well, the following results were obtained with the full-length bovine NCKX1 and with the **Dbv** construct. In 3 out of 14 transfections with **Dbv**, cells could be identified that showed a marked but transient increase in cytosolic free Ca^{2+} when sodium was replaced by lithium in the perfusion medium; in 1 particularly clear case, nearly all cells (>90%) on the coverslip responded but the rise in cytosolic free Ca^{2+} was transient and free Ca^{2+} had returned to base line well before the solution switch back to the sodium medium (Figure 6, upper panel). This pattern is similar to that described above for some of the transfections with vector alone, although in this case it affected a much greater percentage of cells. In 8 out of 16 transfections with the **Bvdel** construct, cells could be identified that showed a marked increase in cytosolic free Ca^{2+} when sodium was replaced by lithium in the perfusion medium. In this case, however, the pattern of changes in calcium was very similar to that observed for the dolphin NCKX1: the rapid rise upon switching to the lithium plus potassium medium was followed by a slow sag in free Ca^{2+}, and a rapid and abrupt return to base line Ca^{2+} levels was observed on switching back to the sodium solution (cells from one coverslip are illustrated in Figure 6, upper panel, representing two clusters of cells responding in this fashion, while other clusters of cells either did not respond or showed synchronous Ca^{2+} oscillations as illustrated for one cluster of cells in Figure 6, lower panel).

Figure 7 (upper panel) illustrates the average calcium response of a large number of cells from different transfection experiments when sodium in the perfusion medium was replaced by lithium plus potassium. As described above, it was straightforward to select cells that appeared to be transfected "successfully" in the cases of bovine NCX1, dolphin NCKX1, and **Bvdel** constructs; clumps of cells showed functional activity as illustrated in Figure 3, whereas other clumps of cells on the same coverslip either were quiescent or showed calcium oscillations with little or no correlation to the solution switches. In the case of the **Bvdel** construct, Ca^{2+} uptake was clearly dependent on potassium ions (Figure 7, lower panel). Transfections with vector control, with bovine NCKX1, or with **Dbv** did not yield cells that showed clear calcium responses such as those illustrated

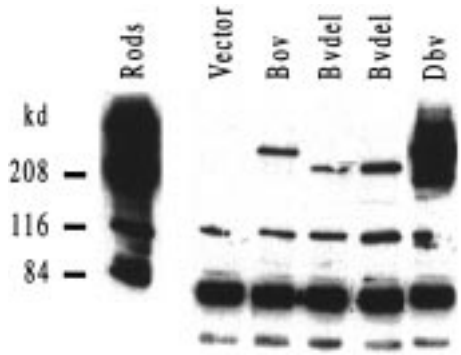

FIGURE 8: Western blot of protein isolated from HEK293 cells transfected with NCKX1 constructs. 50 μg of protein was run per lane, and the PMe 1B3 antibody against the cytosolic domain of NCKX1 was used in immunoblotting. Isolated bovine retinal rod outer segments were used as a positive control, showing bands at and above 200 kDa as the native bovine NCKX1. Vector controls used as negatives to differentiate background antibody binding lack bands above 120 kDa.

in Figure 3. In these cases, we selected "positive cells" as those that showed any calcium response at all, typically a delayed rise in calcium as described above (giving rise to the delayed little hump seen in Figure 7).

Protein Expression of Different NCKX1 Constructs Transfected into HEK293 Cells. Protein expression of NCKX1 constructs in HEK293 cells was examined by Western blotting with the monoclonal antibody PMe 1B3 as illustrated in Figure 8. The epitope of PMe 1B3 has been localized to residues 756−856 of bovine NCKX1 *(24)*; bovine NCKX1 as well as our **Dbv** and **Bvdel** constructs contains this epitope. The Western blot illustrated here came from two experiments in which the vector control transfections yielded cells with quiescent calcium levels without oscillating calcium transients observed in some other experiments (see above). No correlation was observed between protein expression as judged from the Western blot and functional activity when assayed in single cells with digital calcium imaging. For example, the **Dbv** construct in this as well as in other experiments gave rise to, by far, the largest amount of protein expression (Figure 8, far right lane), although no functional expression was observed with this construct. The full-length bovine NCKX1 and the **Bvdel** construct gave comparable, but lower protein expression levels, as compared to **Dbv**. Strong potassium-dependent Na−Ca exchange activity was observed in one of the two **Bvdel** transfections (the protein sample presented in the second lane from the right), whereas no functional activity was observed in the other **Bvdel** transfection or in the transfection with full-length bovine NCKX1.

DISCUSSION

Sequence Comparison. In this study we cloned the cDNA of the retinal rod Na−Ca+K exchanger (NCKX1) from the bottlenose dolphin, *Tursiops truncatus*, and we examined functional expression upon transfection into HEK293 cells. We previously observed a surprisingly large sequence variability between the human rod NCKX1 recently cloned by us *(16)* and the bovine rod NCKX1 cloned earlier by Reiländer et al. *(15)*. In particular, the two large domains predicted to be located in the extracellular space (between H0 and H1) and cytoplasm (between H5 and H6) were poorly conserved across species compared to similar domains in NCX1 when compared across species. The surprising sequence variability was also manifest when comparing the dolphin NCKX1 sequence with those of both human and bovine NCKX1 (Figure 2). In addition, the dolphin NCKX1 cDNA we cloned showed the absence of a long segment coding for 114 or 96 amino acids in the N-terminal portion of the large cytosolic loop when compared with the bovine and human sequences, respectively (Figure 2). Examination of the intron−exon boundaries of the human NCKX1 gene *(28)* suggests that the different cDNAs represent different splice variants, and, indeed, RT-PCR amplification of cDNA derived from retinal RNA has revealed the presence of such alternative splice variants in rat[4] and human.[5] We have not carried out genomic studies on the dolphin NCKX1, although we suspect a similar case where the clone we isolated is missing a splice cassette for the 114 amino acid segment.

Functional Studies. A second major impetus for the present study was that structure−function, regulation, and topology studies of the retinal NCKX protein require the availability of NCKX1 cDNA that results in robust functional activity upon heterologous expression. Earlier, we had not been successful in obtaining either consistent or strong functional expression of either the bovine or the human NCKX1 cDNAs upon heterologous expression in cell lines.[2] In contrast, the dolphin NCKX1 cDNA, cloned in this study, showed consistent and robust functional expression of potassium-dependent Na−Ca exchange in two different experimental paradigms: functional expression of NCKX1 cDNA was tested in single cells by the ability of cells to increase cytosolic free calcium when the inward sodium gradient was reversed by replacing external sodium with lithium (Figure 3), or in $>10^5$ cells in suspension by addition of potassium under conditions of sodium-clamp (Figure 4). Single-cell imaging of dolphin NCKX1 transfected cells gave positive results for >95% of transfections, and measurements in suspension gave strong potassium-dependent calcium influx in all cases. As opposed to single-cell imaging, suspension measurements control for differential expression levels and transport rates in individual cells by measuring an averaged calcium flux over the large number of cells in suspension.

Our results on HEK293 cells transfected with NCKX1 cDNA from dolphin are in contrast with those of Navangioni et al. *(22)*, who report functional activity, but no potassium dependence, in HEK293 cells transfected with bovine NCKX1. In our hands, transfection with bovine NCKX1 only occasionally resulted in weak responses in a few cells when analyzed by single-cell imaging. By Ca^{2+} measurements averaged over a large number of cells in suspension, the bovine NCKX1 showed no calcium flux in response to the addition of potassium (Figure 4).

Our observations are consistent with those from the recently cloned potassium-dependent Na−Ca exchanger from rat brain (NCKX2) *(21)*. Overall sequence alignment gives ~30% identity between NCKX1 and NCKX2 proteins. An overall sequence alignment is, however, misleading: NCKX2 is 670 amino acids long, which is considerably shorter than

[4] Poon, S., Tucker, J. E., Leach, S., Schnetkamp, P. P. M., and Lytton, J., in preparation.

[5] Tucker, J. E., and Schnetkamp, P. P. M., unpublished observations.

either dolphin or bovine NCKX1, which both exceed 1000 amino acids in length. Within the transmembrane spanning segments of the proteins, identity is strong, on the order of 80%, suggesting that a similar ion transport mechanism may take place. The large cytosolic and extracellular domains, however, are not conserved. Observations by others[4] indicate that modest functional activity was seen in the full-length NCKX1 clone derived from rat eye, when transfected in HEK293 cells and examined by single-cell imaging. In cells transfected with dolphin NCKX1, calcium influx required the presence of external potassium in a very similar fashion as observed for native NCKX1 in rod outer segments *(12)*. Our results indicate that this cDNA clone provides an excellent tool for future studies on structure−function relationships, regulation, and topology of NCKX1. Together, these functional comparisons, and the sequence alignments of our cDNAs, also suggested to us that a domain within the large cytosolic loop of NCKX1 plays a role in inhibiting the functional activity of bovine NCKX1 we observed.

Regulatory elements of NCX1 have been demonstrated to be located on the large cytosolic loop that bisects the two sets of TMS domains *(29)*. We, therefore, examined the effect of sequence elements, where the three NCKX1 sequences diverge in the large cytosolic loop of NCKX1, on functional expression in HEK293 cells. We generated two constructs which match the different splice variants mentioned above. **Bvdel** eliminates 114 amino acids from the bovine sequence to match the splice variant represented by our dolphin NCKX1 cDNA, while in **Dbv** the cytosolic loop is removed from dolphin NCKX1 and replaced by that from bovine NCKX1. It is apparent from our results that deleting the 114 amino acid segment from the bovine NCKX1 generated a cDNA which showed clear potassium-dependent Na−Ca exchange under the conditions of our experimental paradigm (Figure 7). Transfection of HEK293 cells with **Bvdel** yielded functional expression very similar to that observed with the dolphin NCKX1 cDNA cloned by us, albeit at a lower success rate (∼50% as opposed to >95%). Conversely, by constructing the **Dbv** mutant of the dolphin NCKX1 which contained the entire cytosolic loop of bovine NCKX1 including the 114 amino acid segment, we generated a clone which produced no function, despite the fact that this mutant cDNA consistently yielded the largest amount of protein (Figure 8). In addition, although protein expression was on the same order of magnitude for full-length bovine NCKX1 as for the **Bvdel** construct (Figure 8), only the transfected **Bvdel** construct resulted in functional activity. Our observations suggest involvement of the large cytosolic loop in the ability of NCKX1 clones to yield functional expression when expressed in cell lines, which prohibits effective study of the protein. Perhaps, lack of functional activity of bovine NCKX1 in cell lines may indicate that an inactivation mechanism previously observed for bovine NCKX1 in situ *(14, 30)* is inadvertently and permanently engaged. On the other hand, we cannot exclude that the 114 amino acid segment, or another inherent property of the bovine NCKX1, leads to incorrect protein folding or targeting, which may contribute to diminished or absent function. As the full-length splice variant was the predominant species found in bovine retinal RNA,[5] we consider it likely that functional activity, or lack thereof, as observed upon heterologous expression in cell lines, is a peculiarity of heterologous expression and does not pertain to the native NCKX1 protein expressed in rod outer segments.

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