Molecular Cloning of a Novel Potassium-dependent Sodium-Calcium Exchanger from Rat Brain*

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We have isolated a novel cDNA clone from rat cerebral cortex encoding a protein of 670 amino acids (NCKX2) that has significant similarity to the 1199-amino acid-long Na/Ca-K exchanger of bovine rod outer segment (NCX1). NCKX2 transcripts are 10.5 kilobase pairs in length and are expressed abundantly in neurons throughout the brain and with much lower abundance in selected other tissues. The predicted topology of the rat NCKX2 protein is very similar to that of bovine NCX1, beginning with a solitary transmembrane segment (M0), which is removed as a “signal peptide” in bovine NCX1, an extracellular loop, a cluster of five transmembrane spanning segments (M1 to M5), a long cytoplasmic loop, and a final hydrophobic cluster (M6 to M11). Within the hydrophobic clusters, rat NCKX2 shares 80% identity and 91% similarity with bovine NCX1. The two larger hydrophilic loops are much shorter in rat NCKX2 than in NCX1, accounting for the difference in length between the two proteins, and are dissimilar in sequence except for a 32-amino acid stretch with 69% identity in the cytosolic loop. NCKX2 was epitope-tagged in the extracellular domain and was shown to be expressed at the surface of transfected HEK cells. Analysis of NCKX2 function by fluorescent imaging of fura-2-loaded transfected cells demonstrated that NCKX2 is a potassium-dependent sodium/calcium exchanger.

A plasma membrane sodium-calcium exchange process plays an important role in controlling cytosolic calcium concentrations in a broad number of tissues (1). Detailed functional and structural studies have revealed the existence of two classes of protein that underlie the sodium-calcium exchange process. One class, exemplified by the sodium-calcium exchanger from dog heart, NCX1, catalyzes the exchange of three sodium ions for one calcium ion (2). The other class, exemplified by the sodium-calcium + potassium exchanger of bovine retinal rod outer segments, NCX1, transports four sodium ions in exchange for one calcium and one potassium ion (3).

NCX1 is the most abundantly and widely expressed sodium-calcium exchange gene, with products present in almost every tissue and present at a particularly high level in heart, brain, and kidney. Recent molecular studies have shown that expression of the NCX1 gene is driven by three separate promoters in a tissue specific fashion, giving rise to transcripts with unique 5’-untranslated region exons (4). In addition, there is a region of complex alternative splicing that results in several protein variants with sequence differences in a fairly short region near the carboxyl end of the central cytoplasmic domain of the molecule (5). Two other structurally and functionally homologous gene products have also been identified, NCX2 and NCX3, which are expressed selectively in brain and skeletal muscle, apparently at lower levels than NCX1 (6, 7).

Mechanistically, the NCX1 enzyme is quite similar to NCX1, although coupling of the potassium gradient and the increase in coupling of the sodium gradient to the thermodynamic driving force allow NCX1 to control calcium levels in rods even when the membrane potential and sodium gradient are diminished in darkness (3, 8, 9). Surprisingly, although the proposed overall topology of the molecule is similar—two clusters of five and six hydrophobic helices separated by a central large cytoplasmic loop—there is very little sequence similarity between NCX and NCKX molecules (10). Nevertheless, two short stretches, one in each hydrophobic helix cluster, do show significant similarity. Interestingly, these regions are the sites of highest conservation among sodium-calcium exchangers cloned from various organisms. It was recently noted that the two regions are also similar to one another and thus may have arisen from an ancient gene duplication event, a finding that has led to the speculation that these sites may constitute the ion binding pocket required for transport across the membrane (11, 12).

Although NCX1 expression appears to be restricted to retinal photoreceptors, there is some evidence for a sodium-calcium + potassium exchange process in other tissues. Synchronous plasma membrane preparations from rat brain have been shown to possess sodium-calcium exchange, which is partially dependent upon potassium and is not mimicked by other mono-

1 The abbreviations used are: PCR, polymerase chain reaction; SSCP, standard saline citrate phosphate; BES, N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid.

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valent alkali cations like lithium. In addition, uptake of rubidium (acting as a congener for potassium) was stimulated in the presence of sodium-calcium exchange (15). This potassium-dependent sodium-calcium exchanger has also been shown to possess a sodium-calcium exchange activity that is dependent upon potassium (16).

As a consequence of these observations, we have searched for potassium-dependent sodium-calcium exchangers using a homology approach based on the sequence of the cloned bovine NCKX1. We identified and isolated a full-length cDNA, called NCKX2, from rat brain that encodes a novel protein with extensive similarity to bovine NCKX1 in the hydrophobic regions and displays potassium-dependent sodium-calcium exchange activity when expressed in HEK-293 cells.

**MATERIALS AND METHODS**

All molecular procedures were performed essentially according to standard protocols (17, 18) or the directions of reagent manufacturers, unless indicated otherwise. Chemicals were of the highest quality and/or analytical grade available and were obtained from either Fisher, BDH, or Sigma, unless indicated otherwise. Nucleic acid and protein amino acid sequence analyses were performed with the MacVector software package (Oxford Molecular Group), with TopPred II (19), or via Internet sequence analyses were performed with the MacVector software package (Oxford Molecular Group), with TopPred II (19), or via Internet connection to the National Center for Biotechnology Information at the National Institutes of Health.

**Initial Screening by Reverse Transcription-coupled Polymerase Chain Reaction**—Two primers were designed based on sequences of relatively low degeneracy from the end of putative transmembrane span 7 (amino acids 1053–1059, YLMVWWA) and the loop between transmembrane spans 10 and 11 (amino acids 1164–1170, WRMKNKL) of the bovine NCKX1 sequence (10); YYTIATGGTITGGTGGGC (ncke1) and ARIATYTTCTTCACTCROCA (ncke2; standard degenerate nucleotides and amino acids (I = inosine)). DNA was isolated from rat or mouse tissues according to the CsCl centrifugation procedure, was reverse transcribed using oligo(dT) and Superscript II Moloney-murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and amplified with the above primers at an annealing temperature of 42 °C. Products were analyzed on ethidium bromide-stained polyacrylamide gels, and bands of the expected size (351 nucleotides) were excised, subcloned, and sequenced.

**cDNA Library Construction and Screening**—Poly(A)+ mRNA was isolated from rat brain cortical or cerebellum tissue and poly(dT)-cellulose chromatography. An oligo(dT)-primed size-fractionated (>4 kilobase pairs) cDNA library was constructed from this mRNA with Superscript II reverse transcriptase and ligated using Boehringer Mannheim) labeled riboprobes, prepared from the PCR clones described above, essentially as described previously (20). Several positive colonies were purified, and the plasmid DNA containing the longest insert (clone BC-1) was analyzed by restriction endonuclease digestion, Southern blotting, and DNA sequencing. Sequence analysis of this and subsequent clones was obtained on double-stranded templates either with [35S]EdUTP or Sequenase 2.0 (United States Biochemicals) or with the AmpliTaq FS kit from Perkin-Elmer. Fluorescently labeled sequencing reactions were performed at the University of California Core DNA Services facility. The coding and 5’-untranslated regions were sequenced on both strands, whereas the majority of the 3’-untranslated region was sequenced only on one strand.

**Inverse Polymerase Chain Reaction**—The 5′-end of NCKX2 transcripts corresponding to the BC-1 clone was analyzed by using inverse PCR essentially according to published accounts (21). In brief, 5 μg of poly(A)+ mRNA from rat cerebellum cortex was reverse transcribed with Superscript II using the gene-specific primer gsp1 (CTGACTGTCGTTTGAGG) based on the BC-1 sequence. The cDNA was converted to second strand essentially as described previously (20), purified, phosphorylated, and ligated in dilute solution to circularize the cDNA fragments. Circles were then amplified using primers gsp2 (CAGAG-GAGAACCGCAGTGTTGACG) and gsp3 (GGCGCAGTACAAGGCG-

AATTGACTCGG), which are also based on BC-1 sequence upstream from gsp1 and face away from each other. Amplified products were gel purified, subcloned, and sequenced.

**RNase H Mapping**—The location of the BC-1 clone with respect to its full-length cognate NCKX2 transcript was determined by RNase H digestion of annealed complexes between rat cerebral cortex RNA and antisense primers based on sequence from either end of clone BC-1. Primers used were gsp1 and gsp3 (above), which anneal ~180 and ~30 nucleotides from the 5′-end of clone BC-1, respectively, and gsp4 (TGGGCGGACTGTTTCTA) which anneals about 150 nucleotides from the 3′-end of clone BC-1. Two μg of poly(A)+ mRNA from rat cerebral cortex was reverse transcribed with 200 pmol of each primer in 25 μl of 130 KCl and 13 μM MgCl2, by heating to 65 °C for 5 min and then allowing the mixture to cool slowly to room temperature. Twenty-five μl of 125 mM Tris-HCl, pH 7.8, 10 μM dithiothreitol containing 2 units of RNase H (Pharmacia) was added to each tube, and the digestion was incubated at 37 °C for 20 min. The reaction was then stopped with 2 μl of 0.5 % EDTA, pH 8, the RNA was recovered by ethanol precipitation, and the products were analyzed by Northern blot. Control reactions, one without primer and one without RNase H, were run in parallel.

**Northern Blot Analysis**—Samples of RNA were electrophoresed on 0.8% agarose-formaldehyde gels and transferred to a nylon membranes by capillary diffusion overnight. The UV-cross-linked membranes were hybridized with a digoxigenin-UTP-labeled riboprobe according to the directions of the manufacturer (Boehringer Mannheim) as described previously (20). The probe was a 728-nucleotide HindIII fragment from the 5′-end of clone BC-1.

**In situ Hybridization**—Adult male Wistar rats were perfused transcardially with 4% paraformaldehyde in PBS (130 mM NaCl, 3 mM KCl, 8 mM NaHPO4, 2 mM KH2PO4, pH 7.2, 0.1 mM MgCl2, and 0.1 mM CaCl2), and their brains were removed and embedded in paraffin. Sections of 6–8 μm cut in the horizontal plane were collected on Fisher Plus slides. In situ hybridization was performed essentially as published according to account (22, 23), using digoxigenin-labeled riboprobes that were synthesized from a template corresponding to a 2.3-kilobase pair NsiI fragment derived from the 5′-end of the BC-1 clone. The brain sections were dehydrated in xylenes, hydrated through a graded ethanol/water series, treated with 20 μg/ml of proteinase K in PBS, postfixed with 4% paraformaldehyde in PBS, acylated with acetic anhydride, and then incubated overnight at 68 °C in a hybridization solution composed of 50% formamide, 10% dextran sulfate, 5× SSCP (1× SSCP is 120 mM NaCl, 15 mM sodium citrate, 13 mM KH2PO4, 0.1 mM EDTA, pH 7.2), 0.01% polyvinylpyrrolidone, 0.01% Ficoll, 0.01% bovine serum albumin, 0.25 mg/ml salmon sperm DNA, 0.25 mg/ml yeast RNA, 5% dextran sulfate (200 mg/ml poly A), and 0.5 μg/ml of base-hybridized probe. The slides were then washed at 68 °C, first in 2× SSCP and subsequently in 0.2× SSCP, twice each for 20 min. The hybridized probe was detected using alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:1000, followed by incubation overnight with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate solution.

**Novel Rat Brain Na/Ca-K Exchanger**—Primers flanking the presumed cytoplasmic domain, based on the sequence of BC-1 and including restriction sites and clamps (ncke4, CGTGGAT-TCGTCGACAGTAGAAGGTTGACG), and ncke5, CCGCTCGAGCTGCTTAGGCCGTTTGGTTGACG, were used to amplify oligo(dT)-primed reverse transcribed rat brain RNA. Products were gel-purified, subcloned, and sequenced.

**NCKX2 Expression Construct**—A full-length clone encoding the NCKX2 protein was obtained by combining a fragment based on the inverse PCR product with a fragment from the BC-1 clone as follows. Rat cerebellum RNA was reverse transcribed using the specific primer gsp5 (CTCTTGGGGATCTCCCTG) and amplified using the primers gsp6 (CTAGGGCCCTCCTTGCATACAT), which overlaps the ApaI site at position 229, and ncke6, CCGCTCGAGCTGCTTAGGCCGTTTGGTTGACG, which comprises the initiator methionine codon plus one base of the subsequent codon, 17 nucleotides of 5′-untranslated sequence, and 7 additional nucleotides completing an ApaI site and a clamp sequence. The 257-nucleotide product was digested with ApaI and ligated with Apal-digested 2.3-kilobase pair NsiI 1 subclone (above). The resultant construct, which encoded a full-length NCKX2 open reading frame, was cloned into the 5′-end of an expression vector. The product encoded by this clone could be detected, a FLAG (24) epitope-tag sequence (amino acids DYKDDDDK) was inserted in place of the predicted extracellular sequence found at amino acids 90–97 (DNLDKIRD) using two chimeric primers, each encoding an overlapping segment of the FLAG sequence, as well as upstream fragment NCKX2 sequence (flagnc1: GTGTGGTCCGTCCGTGTGCTCTGAGTTAGTCTTTG) or

2 http://www.ncbi.nlm.nih.gov
downstream flanking NCKX2 sequence (flagnc2: TACAAGGACGACGATGACAAGTACACCCCAGCACA). One ng of the full-length NCKX2 construct (above) was used as template for two PCR reactions: (i) from the M13U primer sequence of the vector (GTAAAACGACGCAGTTG) to the flagnc1 primer; (ii) from the flagnc2 primer to a specific primer just downstream of the XbaI site at position 682 (gsp7: CCAGGTCAAGTTAAGATTTCC). An aliquot of purified product from each of these reactions was combined together with primers M13U and gsp7 to amplify a fragment of about 780 base pairs, which was digested with KpnI and XbaI, subcloned, and sequenced. The KpnI-XbaI fragment was isolated from an appropriate clone and used to replace the corresponding fragment from the full-length NCKX2 construct above, to create a FLAG-tagged full-length NCKX2 clone. The –2.3-kilobase pair FLAG-NCKX2 fragment was then excised by digestion with KpnI and BamH1 and ligated, blunt-ended, into the HindIII-digested mammalian expression vector pRcCMV (Invitrogen).

Expression in HEK-293 Cells—Transfection of Qiagen-purified FLAG-NCKX2 cDNA into HEK-293 cells was performed using a standard calcium-phosphate precipitation protocol with BES buffer essentially as described previously (25). The FLAG-NCKX2 cDNA, cloned in the reverse orientation in the pMTo2 vector (26), was used in control transfections. A clonal derivative of HEK-293 cells, which expresses the SV40 large T antigen, was originally obtained from Ron Kopito (Stanford University). Expression was analyzed 2 days following transfection.

For protein expression studies, postnuclear extracts were prepared by solubilizing transfected cells in 1% Triton X-100, 0.5% deoxycholate, 0.14 M NaCl, 10 mM EDTA, 25 mM TrisCl, pH 7.4, 100 units/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride. Crude microsome preparations were isolated as described previously (27). For immunofluorescence and calcium imaging experiments, cells were plated onto glass coverslips, which had been coated with 1 mg/ml.png

RESULTS

As part of our interest in molecular diversity of sodium-calcium exchangers, we used a reverse transcription-coupled PCR approach to search for novel molecules. Primers were designed from regions of low degeneracy within the carboxyl-terminal cluster of putative transmembrane helices of the bovine retinal rod outer segment Na/Ca-K exchanger, NCKX1. Products were amplified from both rat brain and heart RNA samples and very faintly from heart, but not from any other tissues tested. Cloning and sequencing of these bands revealed two species from eye, one of which was unique (I-14), and the other of which was identical with products in brain and heart (B-2). Comparison of these sequences with the bovine NCKX1 clone suggested that the unique eye clone I-14 was the rat equivalent of NCKX1 and the “brain” clone, B-2, was the product of a novel gene that we call NCKX2.

The novel brain product B-2 was therefore used as a probe to screen at high stringency a cdNA library prepared from rat cerebral cortex mRNA in the vector pdNAI. Several positive clones were isolated, the longest of which was 8.7 kilobase pairs. Partial sequence of this clone (called BC-1) revealed a sequence identical to that of the probe B-2 and a long open reading frame beginning at one end of the clone but without an initiator methionine. Thus, BC-1 did not contain the 5'-end of the transcript, nor was there a poly(A) tail or polyadenylation signal at the 3'-end of the clone. Northern analysis (see below, Fig. 1) indicated that the major transcript corresponding to the BC-1 clone was 10.5 kilobase pairs in length. The position of the BC-1 clone within its cognate transcript was determined using RNase H digestion of complexes formed between rat cerebrum mRNA and antisense primers designed from sequence near either end of the BC-1 clone. Analysis by Northern blot of the digestion products revealed bands of about 9 and 10.2 kb from complexes formed with primers from the 3’- and 5’-ends of BC-1, respectively, compared with the 10.5 kb message found in control reactions (data not shown). This indicated that the BC-1 clone was missing approximately 1.5 kilobase pairs from the 3’-end but only about 300 nucleotides from the 5’-end.

The technique of inverse PCR was used to obtain the missing 5'-end of the NCKX2 transcript. Multiple independently isolated clones all gave rise to the same fragment, extending 226 base pairs further 5' than the BC-1 sequence. The combined sequences of the inverse PCR product and the BC-1 clone code for an NCKX2 cDNA with 148 base pairs of 5'-UTR, an open reading frame of 2090 base pairs, and a 3'-UTR of 6785 base pairs. The initiator methionine of the coding region falls within a sequence that fits the Kozak consensus (28) very well, tC-AGTGA (I-14) to the flagncke1 primer; (ii) from the flagncke2 primer to a downstream site. It is also possible, however, that transcripts corresponding to a low abundance transcript polyadenylated at an upstream site. It is also possible, however, that transcripts visible in tissues outside of the brain may correspond to cross-reaction of the NCKX2 probe with larger amounts of related gene products(s). Note that a faint band (more evident in longer exposures, not shown) at about 6 kilobase pairs was present in

![Fig. 1. Tissue distribution of rat NCKX2 transcripts. Ten ng of total RNA from each of the indicated rat tissues was analyzed by Northern blotting at high stringency using a riboprobe from rat NCKX2. Lanes marked with an asterisk were exposed roughly 10 times longer than the remainder of the blot to emphasize faint bands. These bands were not seen, even at longer exposures, in the other lanes. C, cerebral cortex; Cbl, cerebellum; BS, brainstem; MB, midbrain; Ey, eye; D1a, diaphragm; Ht, heart; Ao, aorta; E0, esophagus; St, stomach; SI, small intestine; LI, large intestine; Li, liver; Lu, lung; Sp, spleen; Thy, thymus; LN, lymph node; Ad, adrenal gland; Kd, kidney cortex. The position of size markers (Life Technologies) is indicated to the left.](http://www.jbc.org/content/4157/14/4157/F1)

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Fig. 2. Neuronal distribution of NCKX2 in rat brain. In situ hybridization using a digoxigenin-labeled NCKX2 antisense riboprobe was performed on horizontal sections from rat brain. Note that in all cases, the sense control resulted in no increase in contrast above the general background observed in the panels of this figure. Panel A, parietal cortex. Numbers to the left indicate the six cortical layers. The scale bar corresponds to 300 μm. Panel B, posterior cerebellum. G, granule cell layer; P, Purkinje neurons; M, molecular layer. The scale bar corresponds to 200 μm. Panel C, hippocampus. The CA1, CA3, and CA4 neurons are indicated. DG, dentate gyrus. The scale bar corresponds to 200 μm. Panel D, stratum (caudate nucleus). LV, lateral ventricle. The areas of faint NCKX2 staining are consistent with the character and location of striosomes. The scale bar corresponds to 200 μm. Panel E, medial geniculate body of the thalamus. The scale bar corresponds to 200 μm.

RNA from eye, which corresponds to cross-reaction with the rat NCKX1 transcript.

The location of NCKX2 transcripts in the brain was determined by in situ hybridization, as shown in Fig. 2. Expression was restricted to neuronal cell bodies in almost all regions of the brain (29, 30). In the neocortex, cells throughout layers II–VI were labeled, with particularly strong expression in layers VI and V. Hippocampal expression was strongest in the CA3 neurons, followed by CA1, then CA4, and then dentate gyrus neurons. Expression was robust throughout the striatum, with clearly observed regional patches of reduced intensity that may correspond to striosomes of the caudate nucleus. Expression was evident in all regions of the thalamus but was particularly strong in the medial geniculate body. In the cerebellum, strong expression was observed in stellate cells of the molecular layer, as well as in neurons of the deep cerebellar nuclei (not shown). Lower expression was also evident in the Purkinje neurons. In addition, expression was observed at high levels in enthorinal cortex, in many pontine nuclei, in the indusium griseum, and at lower levels in mitral and glomerular neurons of the olfactory bulb, and in subependymal neurons of the medial habenula (not shown). Of note, the septal nuclei were negative.

The NCKX2 transcript encodes a protein with a predicted size of 74,651 Da and the sequence shown in Fig. 3. Hydrophathy analysis suggests 12 transmembrane segments resulting in a protein with a topology analogous to both NCX1 and NCKX1, as shown in Fig. 4. Both a single consensus glycosylation site and a potential cleavage site for signal peptidase are present on the extracellular loop between M0 and M1, in positions analogous to the known sites of NCX1 (31, 32). In addition, a sequence weakly resembling a calmodulin binding motif (33) is present at the beginning of the cytoplasmic loop following M5, analogous to the location of the XIP region of NCX1 (34). Analysis of NCKX2 transcripts from rat brain RNA by PCR has also revealed the presence of a slightly shorter species, apparently as a consequence of alternative splicing, which encodes a protein lacking a 17-amino acid stretch within the large cytoplasmic loop, as shown in Figs. 3 and 4. The location of several potential sites for modification by a variety of protein kinases is also illustrated in Fig. 3.

The similarity between rat NCKX2 and bovine NCKX1 is shown in Fig. 5. The two proteins share an overall amino acid identity of 55% (and a similarity of 67%). If only the two regions encompassing the clusters of transmembrane spans M1–M5 were negative.
and M6–M11 are considered, then the identity rises to 80% (91% similarity). Strikingly, within the two α-repeat regions, first recognized by Schwarz and Benzer (11) and thought to possibly constitute the ion transport binding sites, NCKX2 and NCKX1 share almost 100% identity (excepting an A for an S in the loop between M2 and M3), and there is a stretch of 72 consecutive identities from M7 to M9. Outside these regions, in the hydrophilic loops, there is very little similarity between NCKX2 and NCKX1 except for a stretch of 32 amino acids within the cytoplasmic loop (NCKX2 329–360; immediately preceding the site of alternative splicing) that shares 69% identity (and 84% similarity).

Because the sequence similarity between NCKX2 and NCKX1 is so high within the membrane regions thought to form the ion transport portion of the molecule, it seemed likely that NCKX2, like NCKX1, would operate as a Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchanger (35, 36). To test this hypothesis, a FLAG tag was first introduced into the extracellular domain of NCKX2 (see Figs. 3 and 4), and the resulting molecule was expressed in HEK-293 cells. Immunoblots performed on fractions derived from transfected cells using the M2 anti-FLAG antibody showed a strong band at about 76 kDa, which was enriched in membrane fractions compared with whole-cell lysates and was absent from control-transfected cells (Fig. 6A). Immunofluorescence analysis of paraformaldehyde-fixed transfected cells revealed a relatively high transfection efficiency (generally in the range of 20–50%). As shown in Fig. 6B, nonpermeabilized transfected cells showed a strong staining on the cell surface, presenting as punctate clusters, particularly visible in the focal plane of the membrane at the top or bottom of the cell. The surface accessibility of the FLAG epitope is consistent with its expected location in the large extracellular loop of NCKX2, as well as with surface delivery of the molecule. Cells permeabilized with Triton X-100 show the same punctate surface pattern, as well as perinuclear and reticular patterns probably corresponding to NCKX2 protein either retained in, or in transit through, the endoplasmic reticulum and golgi network. Control transfected cells showed no significant fluorescence with the M2 antibody under these conditions.

Having established that NCKX2 is expressed at the surface of a large fraction of the transfected cells, we used fluorescent calcium ratio imaging with fura-2 to examine the transport function of the expressed protein, as illustrated in Fig. 7. Fura-2-loaded transfected cells on coverslips were mounted in a perfusion device on the microscope stage at room temperature. The cells were first incubated in either a medium lacking...
potassium (Fig. 7A) or a medium containing ouabain (Fig. 7B) to raise sodium levels within the cell. The bathing medium was then changed from a 145 mM sodium-containing to a 145 mM lithium-containing solution in the absence of potassium. This maneuver will reverse the sodium gradient and remove sodium competition at the outwardly facing calcium binding sites, and it should therefore favor calcium entry, employing the reverse mode of the exchanger. If NCKX2 function requires the cotransport of potassium with calcium, however, there should be little change in intracellular calcium until potassium is present in the bathing medium. In the transfected cells, and sometimes in the control transfected cells, the sodium-to-lithium solution switch elicited a small increase in the fura-2 fluorescence. A subsequent solution switch to 5 mM potassium/140 mM lithium caused a pronounced increase in fura-2 fluorescence only in transfected cells, which then declined slowly with time. Switching back to a 5 mM potassium/140 mM sodium-containing medium caused a very rapid reduction in fluorescence. These changes were repeatable and were generally not enhanced if a solution switch to 145 mM potassium was used instead of to 140 mM lithium/5 mM potassium, as shown in Fig. 7. Thus, it appears that NCKX2 expresses a strong dependence on potassium for function. In control experiments using the bovine cardiac exchanger clone p17 (data not shown), no such potassium dependence was observed, and equivalent changes in fura-2 fluorescence were seen on solution switches from sodium to either lithium- or potassium-containing media.

DISCUSSION

We have identified a novel Na/Ca exchanger molecule expressed in rat brain that shares a high degree of sequence similarity with the Na/Ca-K exchanger of bovine retinal rod outer segments (NCKX1) and requires potassium for function. We have, therefore, called this new molecule NCKX2. An Internet BLASTP search (37) of rat NCKX2 against the nonredundant data base collection (GenBank CDS translations, Protein Data Bank, SwissProt, and PIR; built August 4, 1997) reveals a large collection of highly significant matches, including molecules from various mammals, various bacteria, Caenorhabditis elegans, yeast, Drosophila, and Arabidopsis. These can be divided into three groups based on the similarity scores. The first (highest scoring) group includes the bovine NCKX1 gene product (gi 108825), a partial clone from what is presumably rat NCKX1 (gi 1222549), and sequences derived from C. elegans cosmids F35F12.2 (gi 1813931) and C35A5 (gi 1279245; this sequence was actually derived from a TBLASTN search). These proteins share a high degree of similarity over a large portion of the NCKX2 sequence (although restricted largely to the regions encompassing the two main clusters of transmembrane spanning segments). It seems reasonable to conclude that they are likely to be functional homologs. In all other matching sequences, the region of similarity are largely confined to the two α-repeats. The next highest scoring set includes five sequences from different bacteria, four C. elegans sequences, one yeast sequence, and one plant sequence. No function has been ascribed to any of these molecules, although it should be pointed out that they are more similar to NCKX1 or NCKX2 than they are to NCX1. The third (and lowest) scoring set includes all mammalian members of the NCX1, NCX2, and NCX3 families, as well as homologs from Drosophila, C. elegans, yeast, and bacteria.

Thus, the sequence composing the α-repeats appears to have an ancient origin and has adapted to a role in various membrane proteins, which all may exchange calcium for other cations. If, as suggested by Schwarz and Benzer (11), the α sequence forms the cation binding site(s) for transport, then a comparison of these regions between the potassium-transporting (NCKX) and nontransporting (NCX) classes might prove informative in identifying potential liganding residues. Fig. 8 shows an alignment of α1- and α2-repeats between rat NCKX2 and NCX1. Although the similarity in these segments is evident, there are sufficient number of differences involving potential oxygen-containing liganding residues. It is therefore difficult to make clear predictions. Two regions do, however, stand out: a cluster of three serine residues at 575–577 and an aspartic acid at position 584, both within the second transmembrane domain of the NCKX2 α2-repeat. These residues are conserved in bovine NCKX1, rat NCKX2, and the C. elegans homolog from cosmid F35F12.2 but not in any of the other aligned sequences.

The potassium requirement for the Na/Ca-K exchanger of rod outer segments was originally thought to be a unique adaptation to the unusual ionic environment of the vertebrate eye. In the dark, the cyclic-nucleotide-gated channels of rod outer segments are chronically open, resulting in a depolarized membrane and high intracellular concentrations of both sodium and calcium. The extra thermodynamic driving force imparted by the potassium and extra sodium coupling allows calcium to be extruded via the exchanger even under these
conditions (3). The identification of NCKX2 from rat brain and the finding of at least two highly related sequences in the C. elegans genome, however, suggest the existence of a gene family with origins that predate the evolution of vertebrate vision. Presumably the products of genes such as NCKX2 will be found in cells in which plasma membrane calcium flux is high and where calcium homeostasis must be maintained in the face of a dramatically reduced membrane potential and sodium gradient. Alternatively, it is also possible that NCKX2 operates in a cellular environment in which calcium concentrations must be kept extremely low. Only an exchanger of the NCKX type could harness sufficient thermodynamic driving force to achieve such levels. In this regard, it is interesting to compare the neuronal pattern of expression of NCKX2 with NCX1 (38). In neocortex and hippocampus, there is a large degree of overlap in the cellular expression of these two molecules. NCKX2 appears to be more abundant in the deeper cortical layers (VI compared with V) and more abundant in CA3 than in CA1 hippocampal neurons when compared with the pattern of NCX1 expression. In other regions of the brain, however, there is an almost mutually exclusive pattern of expression. So, for example, NCX1 is expressed at very high levels in the septal nuclei, where NCKX2 is absent; NCKX2 is expressed throughout the striatum at high levels (excluding striosomes of the caudate nucleus), whereas only occasional cells express NCX1; thalamic expression of the two molecules appears to be concentrated in distinct nuclei; and in cerebellum, NCKX2 is present at high levels in stellate cells that lack NCX1. A detailed comparison of both transcripts and protein for NCKX2 and NCX1 is currently in progress.

The inclusion of an epitope tag in the large amino-terminal extracellular loop of NCKX2 has allowed us to demonstrate conclusively the surface location of this molecule when expressed in HEK cells. The punctate pattern that we observed appears similar to the distribution of the Na/Ca exchanger, NCX1, observed in smooth muscle, neurons, and astrocytes (39). It has been suggested that this pattern of expression reflects a physical connection between surface calcium transporters and the underlying endoplasmic (or sarcoplasmic) reticulum (39, 40). Whatever the cause for such a surface distribution, it is clear that both NCKX2 and NCX1 must share the attributes that result in the punctate appearance.

Both canine NCX1 and bovine NCX1 are known to be glycosylated in the first, extracellular, loop and to have their first hydrophobic transmembrane span proteolytically removed by what is presumably a signal peptidase (10, 31, 32). The rat NCKX2 molecule possesses sites for both of these putative processing steps. Analysis of the protein expressed in HEK cells demonstrated a band that runs very close to the predicted size of the protein (75 kDa). The slightly diffuse nature of the major band, as well as the appearance of a second, doublet band, suggests that these processing steps may indeed take place with NCKX2. More careful in vitro studies combined with mutagenesis will be necessary to clarify this issue, however.

The rat NCKX2 protein expressed in HEK cells clearly shows a strict potassium dependence for transport function. Because this dependence is satisfied by 5 mM K+ in the presence of 140 mM Li+, it is rather similar to the requirements of the rod outer segment NCX1 molecule (3) but quite distinct from the well-characterized monovalent cation requirement of the NCX1 (14, 15). Bovine NCX1 has been demonstrated to both depend upon and transport K+ in rod outer segments (35, 36), and the purified protein is also clearly dependent upon potassium for function (41). These properties have not, however, been demonstrated clearly for the cloned enzyme (10, 42). Our data are thus the first to demonstrate the potassium ion requirement for the function of a cloned NCX-type of Na/Ca exchanger. Compared with bovine cardiac NCX1, however, the magnitude of the changes in intracellular free calcium seen with the rat NCKX1 expressed in HEK cells were much smaller and less consistent. Antibody staining data indicate that these two molecules are both expressed in a large fraction of cells at high density. This suggests that there is a difference in the efficiency of calcium transport between the two molecules. Estimates of the turnover number for cardiac NCX1 are in the range of 2500–5000 s−1 (43, 44), whereas those of the rod outer segment NCX1 vary from 2 to 115 s−1 (41, 45). Thus, rat NCKX2 may...
Fig. 8. Alignment of the α-repeats for rat NCKX2 and rat NCX1. Alignment of the α-repeat regions was performed with the ClustalW algorithm using MacVector. Residues identical in at least three of the four sequences are shown in boldface letters and highlighted with light shading. A consensus motif is shown below the aligned sequences. The amino acid coordinates (with the initiator methionine = 1) of the different sequences are indicated at each side. The location of the putative transmembrane segments (TMS) predicted for these regions are overlined. Dashes indicated gaps introduced to maximize the alignment.

The identification and cloning of rat NCKX2 provides a system with which to investigate further the structure, function, and regulatory requirements of the family of potassium-dependent Na/Ca exchangers.

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