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Primary structure and functional expression of the Na/Ca,K-exchanger from bovine rod photoreceptors

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Complementary DNA encoding the Na/Ca,K-exchanger was isolated from bovine retina cDNA libraries. The complete full-length cDNA is ~4 kb long and contains an open reading frame of 3597 bp. The deduced amino acid sequence corresponds to a protein of 1199 amino acids with a calculated molecular weight of ~130 kDa. Hydrophobicity analysis revealed the presence of two alternating sets of hydrophobic and hydrophilic domains. There also exists a hydrophobic region at the N-terminus which may be part of a cleavable signal peptide. The protein shares limited sequence homology with the Na/Ca-exchanger from cardiac sarcolemma. Northern blot analysis indicates that the ~6 kb transcript is highly specific for retinal tissue. Insect cells infected with recombinant baculovirus bearing the full-length cDNA express a functional Na/Ca,K-exchanger with an apparent relative molecular weight of ~210 kDa, as determined by Western blotting.

Key words: Na/Ca,K-exchanger/phototransduction/retina/rod outer segment

Introduction

Illumination hyperpolarizes vertebrate rod photoreceptors by inducing the closure of cGMP-gated cation channels present in the plasma membrane of the rod outer segment (Fesenko *et al.*, 1985; Stryer, 1986; Yau and Baylor, 1989). In the dark, a significant proportion of these channels exists in the open state, thereby mediating a flow of positive charges (the so-called 'dark current') into the rod outer segment cytosol (Baylor *et al.*, 1979). Although sodium ions are primarily responsible for this influx of positive charges, calcium ions entering the cytosol through the cGMP-gated channel also contribute about 10–15% of the dark current (Capovilla *et al.*, 1983; Yau and Nakatani, 1984a; Hodgkin *et al.*, 1985). These calcium ions are in turn rapidly extruded from the rod outer segment by a highly active exchanger which couples an inwardly directed electrochemical sodium gradient and an outwardly directed electrochemical potassium gradient to the extrusion of calcium ions (Yau and Nakatani, 1984b; Schnetkamp, 1986; Lagnado *et al.*, 1988). It is now known that, under physiological conditions, the efflux of one calcium ion is coupled to the influx of four sodium ions and the efflux of one potassium ion (Cervetto *et al.*, 1989; Schnetkamp *et al.*, 1989).

In previous studies, we have succeeded in purifying and functionally reconstituting the Na/Ca,K-exchanger from bovine rod outer segments (Cook and Kaupp, 1988). We have shown that the purified exchanger retains many of its functional properties such as sodium dependence, electrogenicity and potassium cotransport (Reid *et al.*, 1990; Friedel *et al.*, 1991). The purified protein has an apparent relative molecular mass (M_r) of 220 000 (220 kDa) and is heavily glycosylated (Cook and Kaupp, 1988; Reid *et al.*, 1990). In bovine retina the Na/Ca,K-exchanger is localized exclusively in the plasma membrane of rod outer segments (Reid *et al.*, 1990). We now report the cloning and sequencing of the Na/Ca,K-exchanger complementary DNA together with the deduced amino acid sequence. Homology analysis, tissue specificity and functional expression of the Na/Ca,K-exchanger cDNA are also described.

Results

Cloning of the Na/Ca,K-exchanger cDNA

In order to clone the Na/Ca,K-exchanger cDNA, 2×10^6 plaque forming units of a λgt11 cDNA expression library from bovine retina (Genofit) were screened using polyclonal antibodies raised against the purified protein (Haase *et al.*, 1990). Of 30 plaque-purified clones which scored positive, two λ clones (λ11-1; λ11-7) also showed strong immunoreactivity after rescreening with Na/Ca,K-exchanger specific monoclonal antibody PMe 1B3 (Reid *et al.*, 1990). cDNA inserts of these clones were isolated and recloned into phage M13 (Messing, 1983). DNA sequence analysis showed that the inserts were identical, bearing a repeat sequence corresponding to amino acids 817–873 of the final sequence. Northern blot analysis of bovine retina poly(A)$^+$ RNA revealed that the cDNA inserts hybridized to an mRNA of ~6 kb (data not shown).

In an alternative approach, the Na/Ca,K-exchanger was purified from bovine rod outer segments as previously described (Cook and Kaupp, 1988) and then subjected to SDS electrophoresis. After Coomassie blue staining, the 220 kDa band was excised and exposed to CNBr cleavage. The resulting cleavage products were extracted from the gel, purified by reversed phase HPLC and then subjected to amino acid sequencing (Eckerskorn and Lottspeich, 1989). The amino acid sequences of three oligopeptides (CN-1, CN-2 and CN-3, see Figure 1) were obtained. For N-terminal amino acid analysis, the purified and desialated exchanger was blotted onto activated glass after electrophoresis and then directly sequenced (Eckerskorn *et al.*, 1988).

On the basis of one of the peptide sequences (CN3; amino acid sequence NIFDIT), a degenerate oligonucleotide probe (Ex-N; 5′-GT(G/A/T)AT(G/A)TC(G/A)AA(G/A/T)AT(G/A)TT-3′) was synthesized. 5×10^6 Plaque forming

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                               CACGAGCAGACCCACTCTTTGAGACCTTGATTCTGACAAGTTTCCAGAACTGAGAAGACACCCAGATGTCCAGGTTTGAAGGTTTGTGGTT -120

ACCCTCTGGCTGGACTTCATGGAGGAATCTTCTCTGATATCTAATCTGAATCCCAGAGCATCCCCCATTTTTCTGTGGACAAAATGAGAGGCCTTCTATAGCCAGGGATAACTGGCCATC   -1
                                                                                                                H 0
                                                                                    ======================================
M G K L I R M G A Q E R R S L W P K R L H W S R P L F L L G M L I I G S T Y Q Y   40
ATGGGGAAACTGATCAGGATGGGGGCACAGGAGAGGCGGTCACTCTGGCCAAAGCGACTTCATTGGAGTCGCCCCCTCTTCCTATTGGGAATGTTGATCATAGGTTCCACCTACCAGTAC  120

====================                                                                 ▼ Nterm
L T S P Q G L P T L W A A V S S Q H P V K V A S R AspLeuSerAsnLysGluMetMetMetValSerSerGluThrSer   80
CTGACGAGCCCTCAGGGCCTCCCCACACTGTGGGCAGCAGTCTCTTCCCAACACCCTGTAAAAGTAGCCAGCCGGGACCTTTCCAACAAGGAGATGATGATGGTGAGCAGTGAGACTTCA  240

          ▽
LysSerSerS GluMetGluValE A W A P E A T A G R D G T P P G I A R K N T P S T P R G T A S  120
AAATCTAGCTCTGAAATGGAGGTTGAGGCATGGGCACCGGAAGCCACAGCAGGCAGAGATGGAACACCACCTGGCATAGCCAGGAAGAACACCCCCAGTACACCTAGAGGAACAGCCAGC  360

              *
I T P A I P N N Y S P T P T G T G K V K E D T S A T P S G V L N H Y T Q S R P M  160
ATCACTCCAGCAATACCCAATAACTACAGCCCAACACCAACAGGCACAGGAAAAGTTAAGGAAGACACCTCAGCCACACCCAGTGGAGTACTGAATCACTACACTCAAAGCAGACCAATG  480

V N S Y T R L T A R G E V K S S R P T Q S R G K E E K Y S P S P L G R M V N S Y  200
GTAAATAGTTATACCCGCTTGACAGCCAGGGGAGAAGTAAAGAGCTCCCGCCCAACTCAATCCAGGGGAAAGGAGGAGAAATACTCCCCATCCCCACTTGGTAGAATGGTAAACAGTTAT  600

                                                                               ▽ * CN-1
A P S T L M T M P R S H G I T P R T T V K D R E I M A T K K M LeuAlaT AsnProSerLysArgLeu  240
GCCCCATCCACACTCATGACAATGCCAAGGAGCCATGGCATCACCCCCAGAACAACAGTGAAAGACAGAGAAATTATGGCGACCAAGAAAATGTTGGCGACCAACCCATCCAAGAGACTA  720

      ▽ ▽
ValGluLysT T ProThrProLeuK G I T D N T P T F L L S D L E T D T L T S P R N V V E K K T  280
GTGGAGAAAACCACCCCAACTCCTCTCAAGGGAATAACTGATAACACCCCAACCTTCCTGTTAAGTGACTTAGAAACAGACACCTTGACTTCTCCAAGAAATGTGGTAGAAAAGAAGACC  840

                   *                       *
L T T P R R V D S N S S T N H Q G L V G K N N L T T P Q G M V L E H T A A V S E  320
CTAACTACCCCCAGAAGAGTGGACAGTAATAGCTCAACCAACCACCAGGGGTTGGTGGGGAAAAACAATCTGACAACACCCCAAGGAATGGTCCTGGAGCACACTGCGGCCGTCTCTGAG  960

G Q V T I S T M T R S S P T E T K A S T D A W K V R N P L P R T S A P I I R I S  360
GGGCAAGTGACAATAAGCACCATGACACGCAGCAGCCCAACAGAAACCAAAGCTTCTACTGATGCTTGGAAAGTCAGGAATCCCTTGCCCAGAACCAGTGCACCGATCATCAGAATATCC 1080

                   *                             *
S A T F R G L L K N P S K A P S T P A A P R V R A N P T I Q V R H C L V V E P A  400
TCAGCCACCTTCAGGGGGCTTTTGAAAAACCCTTCCAAAGCACCCAGCACCCCAGCAGCTCCCAGGGTTAGGGCAAATCCGACCATTCAGGTCCGCCACTGCCTGGTGGTGGAGCCAGCC 1200

P V A P T A P S P S W T T A V I P G I P S P S G Q P D L Y P K A E Y P R D L F S  440
CCAGTTGCACCCACTGCCCCCTCCCCTAGTTGGACAACAGCTGTGATCCCAGGGATTCCTAGTCCCTCAGGACAGCCAGACCTGTACCCCAAGGCAGAGTACCCCCGAGACCTGTTCAGT 1320
                                              H 1
                        =================================================
V E E R R Q G W V V L H I F G M M Y V F V A L A I V C D E Y F V P A L G V I T D  480
GTGGAGGAGCGGAGGCAGGGCTGGGTGGTCCTGCACATCTTTGGCATGATGTATGTGTTTGTAGCCTTGGCCATCGTTTGTGACGAGTACTTTGTCCCTGCCCTGGGCGTCATCACAGAC 1440
                                                                H 2
                                       ================================================         ==========
K L Q I S E D V A G A T F M A A G G S A P E L F T S L I G V F I S H S N V G I G  520
AAGCTGCAGATCTCTGAGGATGTGGCAGGCGCTACATTCATGGCTGCTGGAGGCTCTGCCCCCGAGCTCTTCACCTCCCTCATCGGCGTCTTCATCTCCCATAGCAACGTGGGCATTGGC 1560
               H 3
=================================================                    *                ============
T I V G S A V F N I L F V I G T C A L F S R E I L N L T W W P L F R D I T F Y I  560
ACCATAGTGGGCTCTGCGGTGTTCAACATCCTCTTTGTCATTGGCACTTGTGCCCTCTTCTCCCGGGAGATCCTCAACCTCACCTGGTGGCCCTTGTTCCGAGACATCACCTTCTACATC 1680
               H 4                                                       H 5
===============================================       ================================================
F D L M M L I L F F L D S L I A W W E S V L L L L A Y A F Y V F T M K W N Q Q L  600
TTTGACCTAATGATGCTCATCCTCTTCTTCCTGGATAGCCTCATTGCGTGGTGGGAGAGCGTGCTCCTGCTGCTGGCTTATGCCTTCTATGTGTTCACCATGAAGTGGAACCAACAGCTC 1800

E L W V K E Q L N K R P V A K V M A L G D L S K P G D G T V V V D E Q Q D N K K  640
GAGCTCTGGGTGAAAGAGCAGCTCAACAAGAGGCCAGTGGCCAAGGTCATGGCCCTAGGGGACCTCAGCAAGCCGGGCGATGGGACAGTTGTGGTGGATGAGCAGCAGGATAACAAGAAG 1920

                  CN-2                 *
L K L S S M LeuThrArgGlySerSerSerAlaSerLeuHisAsnSerThrI R S T I Y Q L M L H S L D P L G E A R  680
CTGAAGCTTTCATCCATGCTGACCCGAGGGAGCAGCTCGGCCTCTCTGCACAACAGCACCATCCGCAGCACCATCTACCAGCTCATGCTCCACAGCCTGGACCCTCTGGGGGAAGCCCGG 2040

P S K D K E E E T L I P E A K A T P Q A K A E S K P E E E P A K L P E V T V T P  720
CCCTCCAAGGACAAGGAGGAGGAGACCTTGATTCCGGAGGCAAAAGCCACGCCTCAGGCCAAAGCAGAGAGCAAACCAGAGGAGGAGCCGGCCAAGCTCCCTGAGGTCACGGTCACACCA 2160

A P A P D V K G D Q E E D P G S Q G V G A E A E N T G E R T G G E A E A P A E G  760
GCCCCTGCTCCAGACGTCAAGGGAGATCAGGAGGAGGATCCTGGCAGTCAGGGAGTCGGGGCCGAAGCTGAGAACACAGGTGAAAGGACAGGTGGAGAGGCGGAAGCTCCTGCTGAAGGT 2280

                                         *                                ...............
E N G E R S G G D A A L G G E S E G K A E N E S E G D I P A E R R G D D E D E G  800
GAAAACGGAGAGCGAAGTGGAGGCGACGCTGCATTAGGAGGTGAAAGTGAAGGAAAAGCAGAGAATGAAAGTGAAGGTGACATCCCGGCAGAAAGGAGAGGAGATGATGAAGACGAAGGT 2400
.........................................................................................
E I Q A E G G E V K G D E D E G E I Q A G E G G E V E G D E D E G E I Q A G E G  840
GAAATCCAGGCAGAAGGTGGTGAAGTGAAAGGTGATGAAGACGAAGGTGAAATCCAGGCAGGAGAAGGTGGGGAAGTGGAAGGTGATGAAGACGAAGGTGAAATCCAGGCAGGAGAAGGT 2520
.........................................................................................
G E V E G D E D E G E I Q A G E A G E V E G D E D E G E I Q A G E A G E V E G D  880
GGGGAAGTGGAAGGTGATGAAGACGAAGGTGAAATCCAGGCAGGAGAAGCTGGGGAAGTGGAAGGTGATGAAGACGAAGGTGAAATCCAGGCAGGAGAAGCTGGGGAAGTGGAAGGTGAT 2640
.........................................................................................
E D E G E I Q A G E G G E V K G D E G E I Q A G E A G E V E G E D G E V E G G E  920
GAAGACGAAGGTGAAATCCAGGCAGGAGAAGGTGGTGAAGTGAAAGGTGATGAAGGTGAAATCCAGGCAGGAGAAGCTGGGGAAGTGGAAGGAGAGGATGGTGAAGTGGAAGGTGGTGAA 2760
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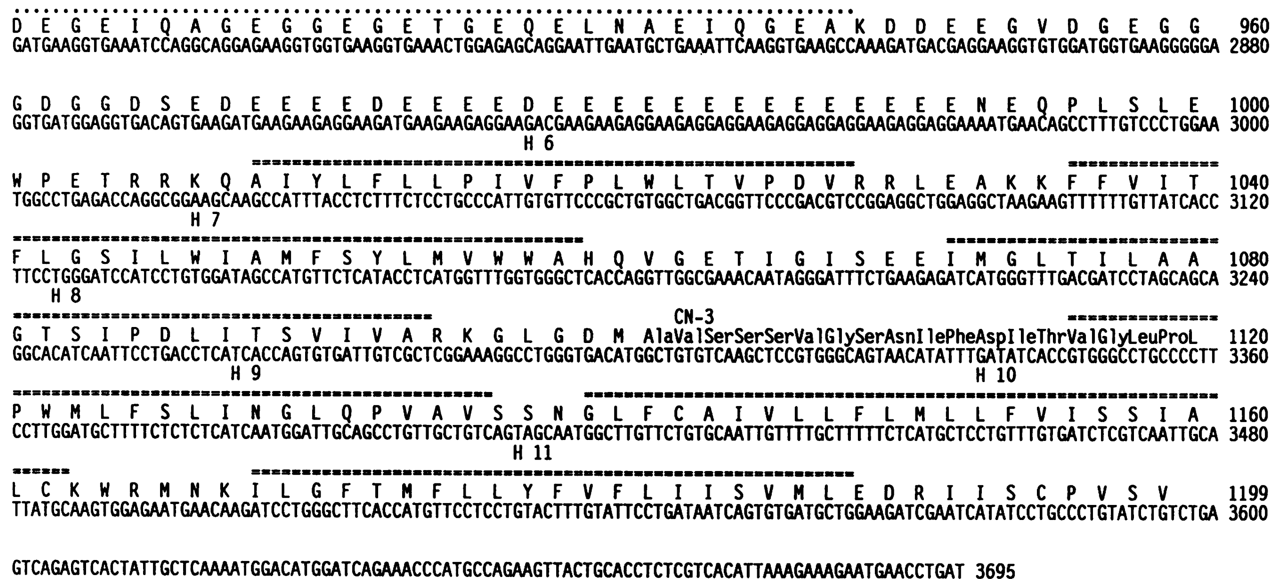

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the Na/Ca,K-exchanger from bovine rod photoreceptors. The nucleotide residues are numbered from the 5′ to the 3′ direction from the first residue of the ATG initiation codon (preceding residues are indicated by negative numbers). The deduced amino acid sequence is shown above the nucleotide sequence. Numbers of the nucleotide and amino acid residues are given at the right hand end of the individual lines. Putative transmembrane segments (H0−H11) are double overlined; their positions were assigned on the basis of hydrophobicity analysis and their termini were tentatively defined. Potential *N*-glycosylation sites are marked with asterisks. The amino acid sequences of oligopeptides purified after cyanogen bromide cleavage (CN-1, CN-2 and CN-3) and the N-terminal (solid triangle) sequence of the purified protein determined by gas phase sequencing are given in the three letter code. Amino acid residues that were not detectable during amino acid sequencing are indicated by open triangles. The repeat region discussed in the text is dotted.

units of a λgt10 cDNA library from photoreceptor poly(A)$^+$ RNA were screened with the 5′-end labelled probe Ex-N under reduced stringency. Positive λ clones were plaque-purified, picked and further analysed for cDNA insert size by PCR using λgt10 primers for amplification (Saiki *et al.*, 1988). Resulting PCR fragments were electrophoresed, blotted onto nylon and hybridized to ^{32}P-labelled Ex-N at 37°C. Increasingly stringent washes and subsequent autoradiography revealed that two cDNA fragments (λgt10N3 and λgt10N7) hybridized strongly. Sequencing of PCR fragments from these two clones using Ex-N with either λgt10 forward primer or λgt10 reverse primer for the PCR, revealed that the clones contained the whole nucleotide sequence coding for peptide CN-3. The cDNA inserts were isolated from the phages and recloned into Bluescript (Stratagene). ^{32}P-labelled transcripts from the extreme N-terminal region of these inserts were used to rescreen the λgt10 library by plaque hybridization. λgt10 clones scoring positive were tested for cDNA insert size as described above and the largest insert was isolated, recloned and sequenced. Thereby the DNA sequence of ~1600 bp coding for the C-terminal part of an open reading frame could be obtained. The ~300 bp cDNA sequence obtained by the antibody screening procedure could be localized within this sequence. In order to obtain the complete protein-coding region, a cDNA library in λZAPII was constructed. Poly(A)$^+$ RNA from bovine retina was internally primed with oligonucleotide Ex-Nt.1 (see Materials and methods) which corresponds to a sequence within the repeat region. After packaging the library was plated and probed with oligonucleotide Ex-Nt.2, the sequence of which is located upstream to oligonucleotide Ex-Nt.1, yielding 18 hybridization positive clones. The cDNA inserts of the two longest clones (λZAP-Nt1, λZAP-Nt2) were rescued with helper phage R408 into Bluescript (Stratagene) and subjected to DNA sequence analysis.

Nucleotide sequence and deduced amino acid sequence of the Na/Ca,K-exchanger

A continuous nucleotide sequence for both DNA strands was reconstituted from overlapping partial sequences determined using the cDNA inserts of the λ phages mentioned below. The coding strand of this sequence and its corresponding amino acid sequence is presented in Figure 1. There is a single open reading frame with an initiating methionine (ATG) starting at position 1 of the nucleotide sequence and a stop codon (TGA) terminating at position 3598. The sequence surrounding the initiating methionine (CCATCATGG) is favourable for functional initiation in eukaryotes (Kozak, 1984, 1987). The open reading frame encodes a protein of 1199 amino acids with a calculated M_r of 129.9 kDa. The amino acid sequences of the three peptides, purified and sequenced after cyanogen bromide cleavage, as well as the N-terminal sequence, could be found and located within the sequence.

The difference between the M_r deduced from the cDNA sequence and that estimated by SDS gel electrophoresis (220 kDa) could be attributable to several factors: (i) the protein has been shown to be heavily glycosylated (Reid *et al.*, 1990); (ii) the protein contains an extremely acidic domain (positions 950−994 in Figure 1) (this may lead to reduced binding of SDS and subsequently to an abnormal migration on SDS electrophoresis); (iii) electrophoretic methods are known to be inherently inaccurate in the determination of the M_r of membrane proteins. That the cloned cDNA does indeed give rise to a mature protein of apparent M_r greater than that deduced from the cDNA sequence is apparent from the expression results presented below.

Topology of the Na/Ca,K-exchanger

The deduced amino acid sequence was analysed for regional hydrophobicity (Figure 2A). There are twelve hydrophobic

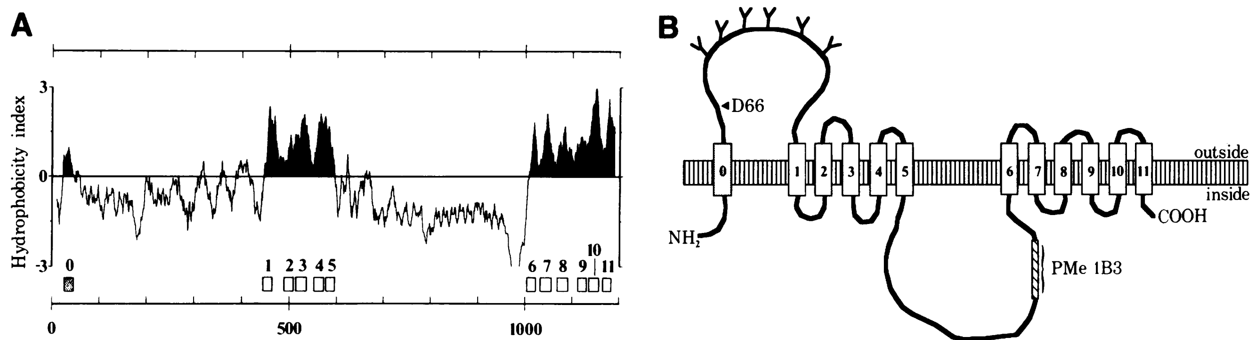

Fig. 2. Hydrophobicity profile (A) and schematic model (B) of the Na/Ca,K-exchanger. (A) Hydropathy analysis was performed using the method of Kyte and Doolittle (1982) with a window setting of 20. The positions of hydrophobic segments (0–11) are indicated by bars. Numbers on the abscissa represent the amino acid number. On the ordinate, hydrophobicity is indicated by positive numbers and hydrophilicity by negative numbers. Calculations were performed using the University of Wisconsin software package (Devereux *et al.*, 1983). (**B**) Proposed transmembrane topology of the Na/Ca,K-exchanger based on the hydrophobicity plot. The presence of 12 putative transmembrane segments (white rectangles) is assumed. Helix 0 may be part of a cleavable signal peptide and is shaded. The binding site of the monoclonal antibody PMe 1B3, and the N-terminus (D-66) of the purified exchanger are indicated. Possible sites for N-linked glycosylation are given only in the extracellular part of the model.

segments that are long enough to form membrane-spanning α helices (referred to as H0–H11 in Figure 1). Several of these putative transmembrane helices contain charged amino acid residues and may therefore be involved in ion transport. H4 and H8 would also be able to form potential amphipathic helices. Amino acid sequence analysis of the purified protein from rod outer segments revealed that the N-terminal amino acid of the purified exchanger is Asp66. Residues 1–65 may therefore constitute a cleavable signal sequence that would subsequently place the N-terminus to the extracellular side of the rod outer segment plasma membrane. Although this sequence is longer than typical signal sequences (von Heijne, 1986), it does possess some of the required characteristics (a cluster of positive charges at the N-terminus followed by a hydrophobic domain and an alanine residue three amino acids before the cleavage site). The presence of arginine one amino acid before the cleavage site is, however, atypical (von Heijne, 1986). Furthermore, we cannot eliminate the possibility that this cleavage phenomenon is simply due to proteolysis during purification of the exchanger protein. The remaining hydrophobic segments exist as two clusters of five (H1–H5) and six (H6–H11) putative transmembrane helices separated by a large hydrophilic domain. This domain was concluded to be cytosolic since it contains the amino acid sequence deduced from cDNA cloned using PMe 1B3 which is known to bind to the cytosolic side of the rod outer segment plasma membrane (Reid *et al.*, 1990).

From this information we can predict the transmembrane topology of the rod photoreceptor Na/Ca,K-exchanger (Figure 2B). From the observation that the 65 N-terminal amino acids could constitute a cleavable signal sequence, it is reasonable to assign the Asp66 N-terminus to the extracellular side of the membrane. This is also suggested by analysis of potential *N*-glycosylation sites, six of which are situated between the N-terminus and the putative transmembrane helix H1. The only other potential *N*-glycosylation sites in the sequence are Asn546 and Asn658. The latter of the two is, however, situated in a region (between putative transmembrane helices H5 and H6) that has been concluded to be cytosolic. Furthermore, we were able to sequence directly Asn658 after peptide purification (CN-2), thereby confirming that this site is not glycosylated. Asn546 is separated from Asn658 by two putative transmembrane helices and is therefore probably also located cytosolically. Since we know that the purified Na/Ca,K-exchanger is extensively glycosylated, some of the first six sites must be glycosylated and are therefore presumably located extracellularly. Interestingly, three of the six potential *N*-glycosylation sites (Asn235, Asn370 and Asn386) exhibit the sequence Asn–Pro–Ser/Thr, which is known to be very rarely *N*-glycosylated (Gavel and von Heijne, 1990). It is also conceivable that the N-terminal region is *O*-glycosylated since the residues Ser84, Thr234, Thr244 and Thr245 could not be detected during amino acid sequencing, possibly due to covalently attached carbohydrate. The proposed topology of the rod Na/Ca,K-exchanger shows remarkable similarity to that of the cardiac Na/Ca-exchanger (Nicoll *et al.*, 1990), i.e. a cluster of five putative transmembrane helices (if the first hydrophobic domains of both proteins are assumed to be parts of signal sequences) followed by a large cytosolic domain and then a cluster of six putative transmembrane helices at the C-terminal. The essential topological difference between the two exchangers would be the extensively elongated N-terminus of the rod photoreceptor protein.

Further sequence analysis revealed a remarkable series of eight repeats in the amino acid 796–948 region, based around the sequence GEVEGDE**DEGEIQA**GEGGEVE-GDE, where the bold amino acids are completely conserved in all eight repeats. The codon usage within the repeat region was also found to be highly conserved. Although the repeats show some similarity to the EF-hand motif of calcium-binding proteins (Tufty and Kretsinger, 1975; Moncrief *et al.*, 1990), we are unable to speculate on their function. A further interesting feature of the sequence is a stretch of acidic amino acids in the 950–994 region. Interestingly, two other calcium-transporting proteins, i.e. the ryanodine receptor (Takeshima *et al.*, 1989) and the cardiac sarcolemma Na/Ca-exchanger (Nicoll *et al.*, 1990), also possess such acidic stretches.

Comparision with the cardiac Na/Ca-exchanger

Surprisingly, the rod photoreceptor Na/Ca,K-exchanger sequence shows very little homology with that of the cardiac Na/Ca-exchanger. We did, however, detect two regions of significant similarity (Figure 3). These two regions are situated within the putative transmembrane clusters of both exchangers and may therefore be directly involved in ion transport. Interestingly, similarity was found in regions predicted from topological models to occur in similar

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                 H2                              H3
 487 DVAGATFMAAGGSAPELFTSLIGVF...ISHSNVGIGTIVGSAVFNILFVIGTCALFSRE  543  retina
 130 TVSNLTLMALGSSAPEILLSVIEVCGHNFTAGDLGPSTIVGSAAFNMFIIIALCVYVVPD  189  cardiac muscle
                H2                                H3

      H7              H8
1058 WAHQVGETIGISEEIMGLTILAAGTSIPDLITSVIVARKG.LGDMAVSSSVGSNIFDI 1114  retina
 818 LASHFGCTIGLKDSVTAVVFVALGTSVPDTFASKVAATQDQYADASIGNVTGSNAVNV  875  cardiac muscle
      H7          H8
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Fig. 3. Amino acid sequence comparison between two regions of the retinal Na/Ca,K-exchanger and the cardiac sarcolemma Na/Ca-exchanger. Homology analysis was carried out according to Gribskov *et al.* (1987). Alignment of the amino acid residues is presented in single letter code. Residue numbers are shown at both sides of the alignment. Identical residues are marked by vertical lines and conservative substitutions with colons. Gaps were introduced to maximize homology. Putative transmembrane segments (the first of which in both proteins is assumed to be part of a cleavable signal peptide) are indicated by numbered bars.

locations. This is also the case for the above mentioned acidic stretches which occur in both proteins at the C-terminal end of the large cytoplasmic loop between the putative transmembrane helices five and six.

Tissue distribution of Na/Ca,K-exchanger mRNA

We examined expression of the Na/Ca,K-exchanger in various bovine tissues by Northern blot analysis. The results (Figure 4) revealed that the Na/Ca,K-exchanger cDNA hybridized to a mRNA of ~6 kb that is produced exclusively in the retina. Other tissues (heart and brain) known to exhibit high Na/Ca-exchange activity showed no cross-hybridization, even though there is some evidence that brain synaptic plasma membrane vesicles may have some potassium-cotransporting Na/Ca-exchange activity (bands at ~5 kb in all of the lanes represent nonspecific hybridization of the riboprobe to 28S ribosomal RNA) (Dahan *et al.*, 1991).

Functional expression of the Na/Ca,K-exchanger in Sf9 insect cells

In order to establish if the cDNA alone encodes the functional Na/Ca,K-exchanger, we constructed an expression plasmid (pVL-Ex) which carries the entire protein coding region of the exchanger cDNA under the control of the polyhedrin promoter of the baculovirus *Autographa california* (AcMNPV). Insect cells (Sf9) were cotransfected with pVL-Ex and wild-type AcMNPV DNA and recombinant virus was isolated. Western blot analysis of infected cells with the monoclonal antibody PMe 1B3 revealed the presence of a diffuse immunoreactive band of M_r ~210 kDa (Figure 5A). Uninfected Sf9 cells or cells infected with wild-type virus were found to be devoid of both Na/Ca,K-exchanger function and immunoreactivity (data not shown).

For functional studies, we employed a previously described reconstitution procedure (Cook and Kaupp, 1988) based on the detection of Na^+-induced release of calcium from proteoliposomes using the metallochromic dye arsenazo III. In order to enrich Na/Ca,K-exchange activity, solubilized membranes from infected insect cells were carried through the first step of our previously described purification procedure (Cook and Kaupp, 1988). As shown in Figure 5B, Na^+ was found to specifically release Ca^{2+} in the presence of K^+ from proteoliposomes prepared with the expressed Na/Ca,K-exchanger. Other alkali cations tested did not induce Ca^{2+} release. In Figure 5C, we investigated the effects of K^+ gradients on the Na^+-induced Ca^{2+} release from such proteoliposomes. Favourable gradients (i.e. where intraliposomal K^+ was greater than extraliposomal) stimulated Na^+-induced Ca^{2+} release, whereas unfavourable gradients (i.e. where intraliposomal K^+ was less than extraliposomal) inhibited the rate of transport. These results are consistent with the notion that K^+ is cotransported in the same direction as Ca^{2+} during Na/Ca-exchange, and confirm that we are indeed dealing with the Na/Ca,K-exchanger.

Fig. 4. High stringency RNA blot analysis of bovine tissues for the presence of Na/Ca,K-exchanger mRNA. Molecular size markers at the left are from a commercially available RNA ladder (BRL) that was run on the same gel.

Discussion

The primary structure of the rod photoreceptor Na/Ca,K-exchanger has been deduced by cloning and sequencing its cDNA. The predicted topology consists of a long

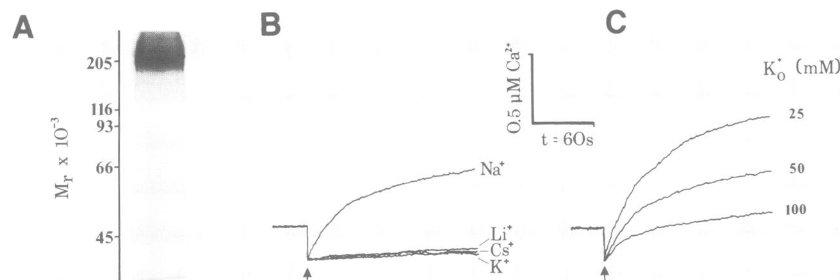

Fig. 5. Expression of the cloned cDNA encoding the bovine Na/Ca,K-exchanger in insect cells infected with a recombinant baculovirus. **(A)** Western blot analysis of the Na/Ca,K-exchanger expressed in Sf9 insect cells using monoclonal antibody PMe 1B3 and alkaline phosphatase-coupled second antibody. **(B)** Alkali cation-induced Ca^{2+} release from proteoliposomes containing the Na/Ca,K-exchanger at 50 mM K^+ (intra- and extraliposomal). **(C)** Effects of transliposomal K^+ gradients on Ca^{2+} release from proteoliposomes containing the Na/Ca,K-exchanger at 50 mM intraliposomal K^+ (K_0^+ refers to the extraliposomal K^+ concentration).

glycosylated N-terminus on the extracellular side of the membrane followed by a cluster of five putative transmembrane helices. This is followed by a large cytosolic loop and another cluster of six transmembrane helices at the C-terminus. This topography is strikingly similar to that of the cardiac Na/Ca-exchanger, which exhibits a much shorter N-terminal region. Although there is essentially little sequence homology between the rod photoreceptor Na/Ca,K-exchanger and the cardiac Na/Ca-exchanger, there are two regions that show significant similarity. Since these two regions are situated within the putative transmembrane helix clusters of both exchangers, they may be directly involved in ion transport. At this stage it is not possible to ascribe the K^+-cotransporting property of the rod photoreceptor exchanger to any particular region.

Interestingly, there are other examples of transporter proteins that exhibit a very similar topology, i.e. a cluster of six (or five in the case of transporters where the first putative transmembrane helix is part of a cleavable signal peptide) putative transmembrane helices followed by a large cytoplasmic domain and then another cluster of six putative transmembrane helices. These include (besides the cardiac Na/Ca-exchanger) for example the multidrug resistance gene product (Gros *et al.*, 1986), the cystic fibrosis gene product (Riordan *et al.*, 1989) and adenylate cyclase (for which a transport has been speculated) (Krupinski *et al.*, 1989). It remains to be seen if this motif will be found in other as yet unsequenced transport proteins, thereby alluding to the existence of a 'superfamily' of transporters with structural similarities.

Materials and methods

RNA isolation

Total RNAs were prepared from tissue by the guanidium thiocyanate method (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was purified from total RNA by two passages of affinity chromatography on oligo(dT)−cellulose (Pharmacia/LKB, Freiburg, FRG) according to standard procedures (Ausubel *et al.*, 1989; Sambrook *et al.*, 1989).

Isolation of cDNA clones

A λgt11 cDNA expression library from bovine retina (Genofit) was screened with poly- and monoclonal antibodies according to standard procedures (Huynh *et al.*, 1985; Snyder *et al.*, 1987; Sambrook *et al.*, 1989). A retinal cDNA cloned into the *Eco*RI site of λgt10 vector was kindly provided by Dr Jeremy Nathans. Phages were plated with host strain C600 and plaques were transferred to nitrocellulose. Hybridization with oligonucleotide Ex-N to plaques on the filter was performed as described by Ausubel *et al.* (1989). Hybridization utilising radioactive transcripts were performed as described below.

PCR amplification of λgt10 clones

Single plaques from positive λgt10 clones were picked and cDNA inserts were amplified using oligonucleotides 5'-CTTTTGAGCAAGTTCA GCCTGGTTAAGTCC-3' (λgt10for) and 5'-AGAGGTGGCTTATGAG TATTTCTTCCAGGG-3' (λgt10rev) for 30 cycles at 94°C for 2 min, 50°C for 2 min and 72°C for 10 min (Saiki *et al.*, 1988). 10 µl aliquots of the resulting PCR products were size fractionated on a 1% agarose gel and transferred to a nylon membrane for subsequent Southern analysis. The blots were probed with 5' ^{32}P-labelled oligonucleotide Ex-N. Blots were washed starting at a temperature of 37°C for 5 min, subsequently raising the temperature for 2°C after every autoradiography.

cDNA library construction

Poly(A)$^+$ RNA was prepared from bovine retina as described above and 5 µg were reverse transcribed with murine Moloney leukemia virus reverse transcriptase (SuperScript RNase H$^-$ reverse transcriptase, BRL) using 3 µg primer Ex-Nt.1 (5'-AACTAGT**CTCGAG**GCCTGGATTTCACCTTC -3'; the region complementary to the sequence is underlined, the *Xho*I site used for cloning is in bold) corresponding to the repeat region of the amino acid sequence of the Na/Ca,K-exchanger (amino acids 796−948 in Figure 1). cDNA was synthesized using the λ-ZAP cDNA synthesis kit (Stratagene) and cloned into λZAP-XR following the procedure given by the manufacturer (Stratagene). After packaging (Gigapack Gold, Stratagene) the library was plated on the host strain PLK-F' (2.5 × 10^4 p.f.u.) and screened with 5' end-labelled probe Ex-Nt.2 (5'-TCACCTGTGTTCTCAGC-3'). Two positive clones (λZAP-Nt1 and λZAP-Nt2) out of 18 positives bearing the longest cDNA inserts were used for further manipulations.

cDNA cloning and cDNA sequencing

DNA fragments from the positive λgt11 or λgt10 clones were isolated and recloned into pBluescript II SK+ or KS+ (Stratagene) or M13mp18 (Messing, 1983; Yanisch-Perron *et al.*, 1985). The insert cDNA of the bacteriophage clones λZAP-Nt1 and λZAP-Nt2 were rescued from the λ phage with helper phage R408 as described by the manufacturer (Stratagene). Progressive unidirectional deletions of the cDNA inserts were obtained using the ExoIII/mung bean nuclease method (Henikoff, 1984). Deletion plasmids were purified by the method described by Del Sal *et al.* (1988). Plasmids were used for restriction analysis and double-stranded templates for sequencing by the dideoxy termination procedure (Sanger *et al.*, 1977), using T7 DNA polymerase. The cDNA clones used for nucleotide sequence analysis are as follows: λgt11-1 and λgt11-7 (carrying nucleotides 2399−2657); λgt10N3 and λgt10N7 (carrying nucleotides 2829 extending beyond the sequence shown); λgt10T28 (carrying nucleotides 2122−3695); λZAP-Nt1 (carrying nucleotides 650−2400); λZAP-Nt2 (carrying nucleotides −210−2430). The sequence presented in Figure 1 was determined on both strands.

Northern blot hybridization analysis

RNA samples (15 µg) were denatured with glyoxal, size fractionated on a 1% agarose gel and transferred onto nylon membrane (Biodyne A, Pall) by vacuum blotting. After prehybridization for 4 h, hybridization was performed at 42°C overnight in 50% formamide, 50 mM Na-phosphate, 5 × Denhardt's solution, 0.1% SDS and 250 µg heat-denatured herring sperm DNA/ml using an *in vitro* synthesized antisense RNA of the complete Na/Ca,K-exchanger full-length cDNA (pKSEx; as described below) as the radioactive probe. The membrane was washed subsequently four times in 2 × SSC, 0.1% SDS at room temperature for 5 min and then twice in 0.1 × SSC, 0.1% SDS at 50°C for 15 min. A final high stringency wash

was performed twice for 15 min at 65°C with 0.1 × SSC, 0.1% SDS. The blot was then analysed by autoradiography.

Cell culture techniques

The insect cell line Sf9 (*Spodoptera frugiperda*; ATCC accession under CRL 1711) was propagated at 27°C in TNM-FH medium supplemented with 5% fetal calf serum as described (Summers and Smith, 1987). Wild-type AcNPV and plasmid pVL1392 were generously given by Dr Max Summers of A and M College, Texas. The insect baculovirus was propagated on monolayer or suspension cultures of Sf9 cells.

Construction and functional expression of the full-length cDNA in Sf9 insect cells

A full-length cDNA encoding the entire open reading frame for the Na/Ca,K-exchanger was constructed as follows: the cDNA insert of λgt10T28 cloned into pBluescript KS+ (C-terminal region of the Na/Ca,K-exchanger coding region) was digested with *Eco*NI (single restriction site at position 2268 in the sequence shown in Figure 1) and *Xba*I (single restriction site in the vector) and the plasmid bearing the C-terminal sequence was isolated from the agarose gel. The rescued pBluescript SK-Nt2 from λZAP-Nt2 clone was also digested with *Eco*NI and *Xba*I and the *Eco*NI−*Xba*I fragment encoding for the N-terminal region was isolated. The two fragments were ligated to yield pKSEx. The full-length cDNA fragment of pKSEx was isolated after digestion with *Xba*I and *Kpn*I and cloned into the appropriately digested baculovirus transfervector pVL1392 under the control of the polyhedrin promoter (pVL-Ex). Recombinant baculovirus was produced by cotransfecting Sf9 cells with 1 μg of genomic AcMNPV DNA and 10 μg of plasmid pVL-Ex. Screening for recombinant virus was performed by limiting dilution and DNA dot blot hybridization using *in vitro* synthesized labelled transcripts from plasmid pSK-Ex (Fung *et al.*, 1988). Putative recombinant virus was further analysed by visual plaque screening (Summers and Smith, 1987) and Southern hybridization of appropriately digested DNA isolated from cells infected with recombinant virus. The total absence of any wild-type AcMNPV was ensured by PCR analysis of the culture supernatant of infected cells (Vasudevan *et al.*, 1991). For functional studies Sf9 cells were grown in cell culture flasks or suspension culture, infected with recombinant baculovirus at a m.o.i. ~5−10 and harvested 3 days after infection. SDS electrophoresis on 9% polyacrylamide gels and Western blotting were carried out using the monoclonal antibody PMe 1B3 and alkaline phosphatase-coupled second antibody as previously described (Haase *et al.*, 1990).

Solubilization and functional reconstitution of the Na/Ca,K-exchanger expressed in Sf9 insect cells

Sf9 cells infected with recombinant baculovirus (~30 mg protein) were hypotonically washed and then solubilized in 0.15 M KCl buffer and subjected to DEAE chromatography exactly as previously described (Friedel *et al.*, 1991). The column was eluted with 0.7 M KCl buffer yielding a fraction containing the Na/Ca,K-exchanger. This fraction was then concentrated and reconstituted into calcium-containing asolectin liposomes using a previously described CHAPS dialysis procedure (Cook *et al.*, 1986) at a phospholipid concentration of 10 mg/ml and a total protein concentration of 0.2 mg/ml. Proteoliposomes contained 10 mM HEPES−arginine, pH 7.4, 100 mM choline−Cl, 50 mM KCl (all inside and outside) and 4 mM $CaCl_2$ (inside only). Calcium release was spectroscopically monitored using the metallochromic dye Arsenazo III as described elsewhere (Friedel *et al.*, 1991).

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