Mutually Exclusive and Cassette Exons Underlie Alternatively Spliced Isoforms of the Na/Ca Exchanger*

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We have analyzed the gene structure that gives rise to tissue-specific isoforms of the Na/Ca exchanger. Five distinct isoforms of the Na/Ca exchanger from rabbit brain, kidney, and heart have been identified previously to which we now add a new brain isoform. Reverse-transcribed polymerase chain reaction, library screening, and sequence analysis of cDNA coding regions indicate that the only significant alteration of the Na/Ca exchanger cDNA in rabbit brain, kidney, and heart isoforms is located in the carboxyl end of the putative intracellular loop of the protein, a region recently linked to ionic and metabolic regulation of the Na/Ca exchanger. Additionally, we find that the Na/Ca exchanger isoforms found in lung and skeletal muscle may arise from among these same six isoforms. Examination of the gene structure of the Na/Ca exchanger in rabbit indicates how the single gene that encodes for the Na/Ca exchanger is alternatively spliced to give rise to the five rabbit isoforms. Specifically, sequence analysis of the intron-exon boundaries reveals the presence of two "mutually exclusive" exons in conjunction with four "cassette" exons in the region of the Na/Ca exchanger gene that codes for the carboxyl end of the predicted intracellular loop region. This unusual arrangement of exons in the Na/Ca exchanger gene could allow for the generation of up to 32 different Na/Ca exchanger mRNAs and accounts for the isoforms identified to date.

The sodium-calcium (Na/Ca) exchanger is a transmembrane protein found broadly in animal cells, having been first identified in mammalian heart muscle and squid giant axons (1–3). This electrogenic transporter couples sodium flux to the countertransport of calcium ions with a stoichiometry of three Na ions to each Ca2+ ion (4, 5). In heart the Na/Ca exchanger is responsible for extruding almost all of the calcium that enters the cell during excitation via the sarcocellar calcium current, ICa (6–8). In kidney, this exchanger appears to play an important role in regulating calcium re-absorption in the nephron (9, 10). The Na/Ca exchanger has been found in numerous tissues by immunolocalization and shown to be present in particularly high concentrations in the neuronal synapses (11) and may help to extrude the calcium that enters to activate neurotransmitter release (12). The Na/Ca exchanger is also found in many other cell types, including astrocytes (12), pancreatic β-cells (13, 14), lung (14), liver (14), and skeletal muscle (14–16). With the multiplicity of function of these diverse tissues, it might have been expected that a collection of genes would encode for the different transport proteins. However, examinations of the number of genes coding for the Na/Ca exchanger in human (17, 18) and rat (14) suggest that a single gene encodes this transporter. This finding is consistent with the remarkable similarity of the cDNA sequences that have been reported. Similarities are found both across tissues (heart, kidney and brain) and across species (dog, human, rabbit, rat) (14, 17, 19–23) and raises the possibility that an alternatively spliced single gene product may account for the observed cDNAs.

In the present work focusing particularly on the rabbit, we have investigated the partial gene structure of the Na/Ca exchanger and have also characterized the principal isoforms of the Na/Ca exchanger cDNAs found in rabbit heart, kidney, and brain. Six distinct isoforms have been identified including a novel brain isoform reported here for the first time. We have also found that the Na/Ca exchanger isoforms found in lung and skeletal muscle share common features with these same six isoforms. Our investigation into the structure of the Na/Ca exchanger gene suggests that all of the isoforms identified by us and others can be explained by two mutually exclusive exons and four cassette exons (24) that together could produce up to 32 distinct isoforms.

**EXPERIMENTAL PROCEDURES**

Materials—[α-32P]dCTP, [γ-32P]dCTP, and [α-35S]dATP were purchased from Amersham Corp. Restriction enzymes and reverse transcriptase were purchased from Life Technologies, Inc. T4 kinase, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. Chemicals were molecular biology grade acquired from Sigma and Fischer.

Screening of cDNA Library—An adult rabbit brain cDNA library in Ag10 was purchased from Clontech (Palo Alto, CA) and screened by plaque lifting method (26) using a random-primer 32P-labeled human Na/Ca exchanger cDNA fragment (nucleotides 1–1830) (14). The hybridizations were carried out at 42 °C in 50% formamide, 6 x standard sodium phosphate EDTA solution (SSPE) (25), 5 x Denhardt's solution, and 0.5% SDS. The final wash of the filters were at 0.2 x SSPE at 50 °C. The positive plaques were resuspended in 50 μm Tris, pH 7.4, 10 μm MgSO4, 100 μm NaCl, 0.01% gelatin and screened again at lower densities until clonal purity was achieved. Phage lysate DNA was prepared by purification in DEAE-cellulose column (26) and then analyzed by EcoRI restriction digest and electrophoresis in 1% agarose gel.

Polymerase Chain Reaction (PCR)*—Total RNA was isolated from frozen tissues (27) and reverse transcribed in a reaction mixture containing 20 units of RNasin, deoxynucleotide triphosphates to a 1 μmol final concentration, 200 units of Moloney murine reverse transcriptase, and 50 pmol of random hexamer or oligo(dT) primer. The reaction was incubated for 1 h and then phenol/chloroform-extracted followed by a

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair; kb, kilobase.
The genomic fragments are then mapped and sequenced so that very recently from rat brain libraries by Furman et al. (21), and NACA6 isoform (31), NACA4 and NACA5 for the two brain isoforms isolated using the dideoxy chain termination method using T7 DNA polymerase. We have used the terms NACA1 for the cardiac sarcolemmal Na/Ca exchanger NCX1 was to determine the identity and sequence of Na/Ca exchanger cardiac isoform is thus NCX1-NACA1.

Genomic DNA Mapping and Characterization—A rabbit genomic library in AFXII vector was purchased from Stratagene (La Jolla, CA) and screened with a PCR product from rabbit heart encoding amino acids 510-582 (29). Hybridization and washing conditions were similar to described above for cDNA clone isolation. The positive plaques were isolated to clones purified and digested with EcoRI, transferred to a nylon filter by capillary action, and probed with the same PCR product. The fragments that hybridized to this product were then subcloned and sequenced with exon specific oligonucleotides. The sequence of exons and intron-exon boundaries were confirmed in opposite direction by complementary oligonucleotides. The physical map of the genomic fragment was performed by cutting the phage DNA with NotI, followed by partial digestion with EcoRI, Southern transfer, and probing with T7 and T3 oligonucleotide primers. The distance between exons were determined by the restriction digestion digestion and PCR amplification between exons using phage DNA as template.

DNA Sequencing—DNA fragments from the genomic and cDNA libraries were eluted using a DNA purification kit and subcloned in pGEMZII(+) or pBluescript SK(+) or pBluescript KS(II), and sequenced using the dyeoxy chain termination method using T7 DNA polymerase. Sequencing analyses were performed using the PCGene software package (IntelliGenetics, Mountain View, CA).

Nomenclature—The gene designation for the Na/Ca exchanger is NCX1 and is located on the short arm of the human chromosome 2 (18, 30). To uniquely identify the expressed isoforms of the Na/Ca exchanger, we have used the terms NACA1 for the cardiac sarcolemmal Na/Ca exchanger (14, 17, 19, 20, 22), NACA2 for isoform isolated from rabbit kidney cortex by Reilly and Shugrue (23), NACA3 for the new kidney isoform (31), NACA4 and NACA5 for the two brain isoforms isolated very recently from rat brain libraries by Furman et al. (21), and NACA6 for the new brain isoform presented here. The full description of the cardiac isoform is thus NCX1-NACA1.

RESULTS

The first part of the work focuses on the identification of Na/Ca exchanger isoforms in rabbit with emphasis on brain, heart, and kidney. The second part of the work concentrates on the identification of large fragments of genomic DNA that contain all the isoforms specific exons of the Na/Ca exchanger so far described, including the new brain isoform presented here. The genomic fragments are then mapped and sequenced so that all intron/exon boundaries are characterized.

Na/Ca Exchanger Isoforms in Rabbit Brain—Our first goal was to determine the identity and sequence of Na/Ca exchanger message(s) in brain tissue. Thus, a rabbit brain cDNA library was screened at moderate stringency with a DNA fragment derived from the human cardiac Na/Ca exchanger clone (14), resulting in the identification of two full-length clones with deduced open reading frames of 941 and 934 amino acids. Sequence comparison of the two clones showed that the translated protein of both clones would be identical with the exception of an insertion of 21 nucleotides encoding the sequence ALLNLNE (Fig. 1). The deduced polypeptide has a predicted mass of 105 kDa and a characteristic leader peptide at the amino-terminal end. Hydrophathy analysis (32) indicates 11 potential membrane-spanning segments and a large hydrophilic domain between membrane-spanning segments 5 and 6, a structure similar to the one proposed for the cardiac Na/Ca exchange protein (19, 29). The large hydrophilic region is presumed to be located on the intracellular side of the membrane and is thought to be involved in the regulatory aspects of the exchanger function (33). With this membrane topology, the 21-nucleotide insertion would occur in the carboxyl end of the intracellular loop between amino acids 603-609.

Recently, Furman et al. (21) published two Na/Ca exchanger isoforms from rat brain libraries. One clone is nearly identical to the RB20 clone and is probably the rat homolog of this brain isoform from rabbit. The second one, isolated only from a rat hippocampal cDNA library, was unique as it contained an insertion of 23 amino acids not seen in both rabbit brain full-length clones (as discussed below). The sequence obtained in clone RB20 was shadowed, and the amino acid changes compared with the rabbit kidney Na/Ca exchanger (23) are marked with asterisks. The two rabbit brain isoforms were strikingly similar to the rabbit kidney exchanger cloned recently by Reilly and Shugrue (23), with overall 97% identity at amino acid level. Most of the amino acid substitutions between the brain and kidney clones were located in the carboxyl end of the putative intracellular loop as indicated in Fig. 1.

PCR Amplification from Distinct Tissues—The results obtained from our rabbit brain library screening suggest that the difference between the rabbit brain Na/Ca exchanger cDNAs identified above is produced by the insertion or deletion of a small 21-bp exon in the putative intracellular loop. In order to investigate the presence of additional isoforms in this “variable” region of the Na/Ca exchanger in brain and other tissues, we performed a reverse-transcribed PCR analysis on first strand of cDNA made from rabbit kidney, heart, brain, skeletal muscle, and lung. The primers were designed to flank the area of sequence divergence between clones RB11 and RB20, as shown in Fig. 1, and expected to generate a amplification product of 450 and 429 bp, respectively, with brain rabbit cDNA if these were the only Na/Ca exchanger isoforms present in brain tissue. The results obtained with the PCR amplification, at high stringency conditions, are shown in Fig. 2. Amplification using rabbit brain cDNA as template yielded a dominant band of about 420 bp and several other minor bands of larger size. Likewise, parallel amplification with rabbit cardiac and renal
isoforms of the Na/Ca exchanger identified to date together. Another plausible explanation requires the transcription of additional genes, but this seems unlikely given the high degree of sequence conservation noted in the full-length isoforms and also the presence of single bands in Southern blots of rat genomic DNA digested with several restriction enzymes (14). Additionally, localization of the human Na/Ca exchanger gene indicates that it resides at a single location on chromosome 2 (18, 30).

Genomic Basis for Alternatively Spliced Na/Ca Exchanger mRNAs—To examine the possibility that alternative splicing gives rise to the diversity of Na/Ca exchanger isoforms, we began to characterize the structure of the rabbit gene fragments that contained the exons encoding the identified isoforms. We have attempted to determine the minimum number of exons, their position within the genome, and their reading frame. The work described here identifies the genomic organization needed to explain the known six isoforms and provide the foundation for predictions of other possible isoforms. The predictions are based on the sequenced cDNAs and the gene map presented below.

We undertook the isolation of a rabbit genomic fragment harboring the variable segment of the Na/Ca exchanger gene by plating and screening a rabbit genomic library. The initial screening at high stringency conditions was performed with the heart PCR product (see above) yielding several positive clones. One clone L211 with a total insert size over 15 kb contained nine EcoRI fragments and was further characterized by restriction site analysis. The resulting physical map of the partial Na/Ca exchanger gene and the relationship with the brain cDNA clone RB11 are shown in Fig. 4. This large gene fragment contains only a small fraction of the Na/Ca exchanger coding sequence with very large intervening intronic areas. However, we cannot discard the possibility that additional exons will be found in these areas upon further sequencing.

The exons encoding the variable part of the Na/Ca exchanger message were found in this large genomic fragment by Southern blot of the EcoRI fragments and probing with exon specific oligonucleotides. Three EcoRI genomic fragments (1.8-, 2-, and 3.3-kb fragments) were isolated and the sequence of intron-exon boundaries determined as shown in Fig. 5. The two exons (A and B) were found in the 1.8-kb genomic fragment with the exon denoted A of 108 bp encoding amino acids 568-602 of the brain and cardiac exchanger and a second exon B of 105 bp encoding amino acids 568-601 of the kidney isoforms.

Two interesting features of these exons are that they encode polypeptides with conserved amino acids and that the first amino acid in both exons (lysine) is in frame with the common 5' exon. If both exons were to be spliced together in
a single message, the deduced polypeptide would be in a different reading frame. Indeed, in all CDNA encoding the Na/Ca exchanger, so far described, one and only one of these exons (A or B) is present.

Additionally four exons were found in the 2- and 3.3-kb fragments (Fig. 4). They encode for the amino acids ALLMLN (exon C), GGPTT (exon D), GKLYL (exon E), GQVLRKVHARDHVPVSTVITA (exon F) and are also flanked by the consensus intron-exon boundary sequences (Fig. 5). These four exons are in frame with exons A or B at the 5' end and in frame with the downstream conserved exon in the 3' end. Indeed, any one of them could be included in the Na/Ca exchanger message and still maintain the original open reading frame of the translated polypeptide. The exons C, D, E, and F in conjunction with exons A and B can account for all the isoforms observed by library screening and by reverse-transcribed PCR amplification (Fig. 6).

**DISCUSSION**

The Na/Ca exchanger has been studied in detail in recent years and is the best characterized antiport mechanistically. The stoichiometry, mechanism of reaction, turnover rate, and intracellular modulation has been investigated with increasingly elegant electrophysiological and biochemical techniques (see Ref. 3). These studies were carried out mainly in two types of preparations: cardiac myocytes and giant cells of invertebrates, primarily due to high density of Na/Ca exchangers in cardiac membrane and the excellent control of extracellular and intracellular environment in squid giant axons or barnacle muscle fibers. In other cell types, such as mammalian neurons, skeletal muscle fibers, epithelia from distal and proximal nephron, and pancreatic beta cells, the exchanger has been detected but not as well characterized. Biochemical and flux measurements are consistent with the notion that distinct forms of the Na/Ca exchanger isoforms are present in the central and peripheral nervous system (34), kidney (35), and in platelets (36).

Our previous work with the cloned human cardiac Na/Ca exchanger has started to address the issue of localized differences of the Na/Ca exchanger in diverse tissues (14). We found that transcripts encoding a homologous forms of the exchanger were present in high levels in lung, brain, and kidney when examined in Northern Blots. Furthermore, restriction fragment analysis of genomic DNA suggested that only a single gene encodes this family of plasma membrane transporter (14, 17).

Here we extended these initial observations by first cloning the rabbit brain isoforms of the Na/Ca exchanger using the human cardiac isoform as a probe. Sequence analysis showed the complete conservation of the rabbit brain Na/Ca exchanger in comparison with rabbit kidney exchanger (33) with the marked exception of the carboxyl end of the putative intracellular loop where there is only limited homology with the renal exchanger. The extensive amino acid conservation between the brain and cardiac sarcolemmal exchangers are consistent with the immunological studies by Yip et al. (37) reporting the similarity in size for the Na/Ca exchanger protein in canine heart and in rat brain. In previous reports the Na/Ca exchanger obtained from synaptic membrane preparations was found to be smaller (38) than reported here. This may be due to proteolysis.
Furthermore, for the first time, we provided evidence that suggests a mechanism by which the mRNA for the specific Na/Ca exchanger isoforms is generated. The isolation of rabbit genomic clones shows that at least six distinct exons are encoding the variable part of Na/Ca exchanger message and these exons, some of them as short as 15 bp, are separated by long intronic sequences. Sequence analysis of the intron-exons boundaries reveals the unusual structure of these exons encoding the carboxyl end of the Na/Ca exchanger intracellular loop. One pair (exons A and B) appear to be probably mutually exclusive (24). By this we mean that in order to maintain the open reading frame of the coding polypeptide, one member of the pair must always be spliced into the mRNA but that both cannot be spliced together nor can both be skipped. Structurally the polypeptide encoded by both exons is similar with several acid and basic amino acids in homologous positions. The exon B contains an extra arginine which confers a more basic character to this polypeptide.

Even more interesting is the genomic structure of the other 4 exons located downstream from exon A and exon B. The amino acid sequences coded by these small exons are in frame with exon A and B and also in frame with each other. Indeed, these exons could be classified as cassette type exons (24), as individual exons can be included or excluded independently while still maintaining the open reading frame. This intron/exon arrangement could theoretically allow the generation of up to 32 Na/Ca exchanger isoforms. In our study in rabit we isolated five of the six distinct isoforms with distinct distribution among different tissues. The isoform isolated from rat hippocampal library (22) that was not seen in this study can be also explained by the exon-intron arrangement presented here.

This complex genomic organization of the Na/Ca exchanger gene is strikingly similar to the exon-intron arrangement of troponin-T gene where the hypervariability within the amino-terminal region of troponin-T region is based on the presence of five cassette exons in conjunction with two mutually exclusive exons (39). In this case it has been shown that differences in the splicing pattern in this region of the troponin-T gene have specific effects upon the interaction of tropinin with tropomyosin (40).

What is the likely function of these tissue-specific splicing variants in Na/Ca exchanger intracellular loop? Recently, Matsuoka et al. (33) investigated the role of the cardiac sarcomeral Na/Ca exchanger intracellular loop by deletion mutagenesis and giant patch analysis. Mutants with a deletion in almost the whole intracellular loop (amino acids 240-679) still exhibited exchange activity but without the characteristic regulation by intracellular Ca2+ or inhibition by the exchanger inhibitory peptide ("XIP") observed in the wild type exchanger (41). Smaller deletions indicated that the important domain involved in intracellular calcium regulation and exchanger inhibitory peptide interaction was actually between amino acids 562-685. The splicing variants are located in the region corresponding to amino acids 561-645. Hence, the isoform diversity observed in this current study could well be concerned with regulatory aspects of the exchanger function, namely by intracellular calcium and exchanger inhibitory peptide. The in vitro expression of the different brain, kidney, and heart isoforms will allow a direct test of the intriguing possibility that alternative splicing of the Na/Ca exchanger message generates distinct isoforms with distinct intracellular calcium modulation.

In summary we have presented evidence for an unexpected degree of heterogeneity of the Na/Ca exchanger message. Several isoforms, differing in the carboxyl end of the intracellular loop, are generated by alternatively splicing of common exons in a tissue-specific manner. The unusual intron-exon arrangement of the Na/Ca exchanger gene encoding this area of the intracellular loop could potentially lead to as many as 32 isoforms.