Structure of the Rat Plasma Membrane Ca$^{2+}$-ATPase Isoform 3 Gene and Characterization of Alternative Splicing and Transcription Products

SKELETAL MUSCLE-SPECIFIC SPlicing RESULTS IN A PLASMA MEMBRANE Ca$^{2+}$-ATPase WITH A NOVEL CALMODULIN-BINDING DOMAIN*

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We have isolated the rat gene encoding isoform 3 of the plasma membrane Ca$^{2+}$-ATPase (PMCA3) and have determined its exon/intron organization. The PMCA3 gene contains 24 exons and spans approximately 70 kilobases. In addition, we have analyzed the splicing and polyadenylation patterns leading to the production of an alternative 4.5-kilobase (PMCA3) skeletal muscle mRNA that differs from the previously characterized 7.5-kilobase brain mRNA (Greeb, J., and Shull, G. E. (1989) J. Biol. Chem. 264, 18569–18576). cDNA cloning, Northern blot hybridization, and polymerase chain reaction analyses of the 4.5-kilobase mRNA demonstrate (i) the inclusion of a novel 68-nucleotide exon (exon 22) that is specific for skeletal muscle and significantly alters the calmodulin-binding domain and (ii) the utilization of an alternative polyadenylation site following exon 23 which eliminates the last coding exon (exon 24) and 3′-untranslated sequence of the 7.5-kilobase mRNA. We have also identified a 42-nucleotide exon (exon 8) that is included in the skeletal muscle PMCA3 mRNAs, but may be either included or excluded in the brain mRNAs. Exon 8 is inserted immediately before the sequence encoding a putative phospholipid binding domain and thus may alter regulatory interactions of the enzyme with acidic phospholipids.

The strict regulation of intracellular free calcium allows calcium to actuate multiple and varied functions within animal cells, including neurotransmitter release, excitation-contraction coupling, secretion, and alterations in cellular metabolism and growth (1). The plasma membrane Ca$^{2+}$-ATPase expels Ca$^{2+}$ from all animal cells and is ultimately responsible for the fine control of intracellular free calcium concentrations. Because the requirements for modulation of intracellular calcium differ among various cell types and within individual cell types under varying physiological conditions, the activity of the plasma membrane Ca$^{2+}$-ATPase must be highly regulated. Direct biochemical regulation occurs through cAMP-dependent phosphorylation and through interactions with calmodulin and acidic phospholipids (2). Plasma membrane Ca$^{2+}$-ATPase activity is regulated genetically by modulation of mRNA levels (3) through tissue-specific expression of at least four separate genes encoding different PMCA1 isoforms (4–7), and by tissue-specific alternative splicing of exons encoding regulatory domains (8–11).

Recent evidence indicates that the genetic regulation of plasma membrane Ca$^{2+}$-ATPase isoform 3 may be particularly interesting with regard to its tissue specificity, developmental regulation, and alternative splicing patterns. In adult rats PMCA3 mRNA expression is virtually restricted to brain and skeletal muscle (6). In contrast, PMCA3 mRNA was not detected in human fetal skeletal muscle (10), suggesting that expression of the PMCA3 gene may be regulated in a developmental stage-specific manner. There is evidence for the existence of a number of alternatively spliced PMCA3 mRNAs. One mRNA, a ~7.5-kb brain transcript encoding PMCA3a, has been characterized by cDNA cloning and sequencing (6). The PMCA3a cDNA contains a 154-nt sequence that is homologous to a 154-nt exon of the PMCA1 gene encoding part of the calmodulin-binding domain. Four variants of PMCA1, termed a, b, c, and d, have been shown to arise by alternative splicing of the 154-nt sequence (4, 8). In a separate study we have demonstrated that the 154-nt exon of PMCA3 (exon 23) is involved in alternative splicing patterns that are analogous to those of PMCA1. In addition to the ~7.5-kb PMCA3 mRNAs, which are expressed in both brain and skeletal muscle, Northern blot analyses demonstrate the presence of an abundant 4.5-kb PMCA3 mRNA in skeletal muscle and a low abundance mRNA in brain which seems to be slightly smaller than the 4.5-kb skeletal muscle mRNA (6). These ~4.5-kb mRNAs do not hybridize with a probe derived from the last coding exon present in the 7.5-kb mRNAs and therefore are likely to encode variant forms of PMCA3.

In order to acquire a detailed understanding of the genetic

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mechanisms by which PMCA isoform expression is regulated and the genetic basis for the various alternatively spliced mRNAs, it will be necessary to isolate and characterize the PMCA genes. So far, however, only limited regions of the PMCA genes have been analyzed. In the present study we report the first characterization of the complete exon/intron organization of a plasma membrane Ca\textsuperscript{2+}-ATPase gene. In addition we have determined the structure and genetic basis of the alternative \(-4.5\text{-}kb\) PMCA3 mRNAs of skeletal muscle and brain, and we show that the alternative splicing patterns lead to differences in the putative phospholipid-binding domain, the calmodulin-binding site, and the C terminus.

**EXPERIMENTAL PROCEDURES**

**Isolation of Rat Genomic Clones**—Replica filters of a rat cosmid library (12) were screened with \(^{32}\text{P}\)-labeled restriction endonuclease fragments derived from a rat PMCA3 cDNA clone, RB 7-2 (6). A SacI fragment (nt 259 to 120) was used to isolate cosmid clones A–C (see Fig. 1), and a SacI-HindIII fragment (nt 2249 to 4287) was used to isolate cosmid clones D–H. Filters were prehybridized overnight at 68 °C in 6 \times \text{SSC}, 5 \times \text{Denhardt’s solution}, 0.1% SDS, and 100 \mu\text{g} of denatured salmon sperm DNA/ml (see Ref. 13 for composition of SSC and Denhardt’s solution). Hybridization was performed at 68 °C for 48 h in the same solution (6 \times \text{filter}) containing 1 \times 10\text{^5} \text{cpm/ml} of a \(^{32}\text{P}\)-labeled probe. The probes were labeled with \([\text{\textsuperscript{32}P}]\text{dCTP}\) to a specific activity of 1 \times 10\text{^6} \text{cpm/ug} using the random primer Oligolabeling Kit from Pharmacia LKB Biotechnology Inc. The filters were washed twice in 1 \times \text{SSC}, 0.1% SDS for 30 min at room temperature and then in 0.1 \times \text{SSC}, 0.1% SDS for 60 min at 68 °C. Autoradiography was performed and colonies producing signals on duplicate filters were purified. Plasmid DNA was isolated by the alkaline lysis procedure (13).

**Mapping of Genomic Clones and Analysis of Intron Sizes**—Cosmid DNAs were digested with various restriction enzymes, fractionated by conventional agarose gel electrophoresis through 0.7% agarose, and analyzed by Southern blot hybridization using various oligonucleotide and cDNA probes derived from the PMCA3 cDNA. To resolve large DNA fragments generated by digestion with the rare cutting enzymes, ClaI, SfiI, and Smal, digestion products were fractionated by field inversion gel electrophoresis.

PCR analyses were performed to determine intron sizes. The standard PCR reaction conditions were: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 150 s. Thirty cycles of amplification were carried out using synthetic oligonucleotide primers flanking the XGTlO cloning site. PCR products were subcloned into pBluescript\textsuperscript{TM} II, and dideoxy sequencing was performed. PCR analyses were performed to determine intron sizes. The standard PCR reaction conditions were: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 150 s. Thirty cycles of amplification were carried out using synthetic oligonucleotide primers flanking the XGTlO cloning site. PCR products were subcloned into pBluescript\textsuperscript{TM} II, and dideoxy sequencing was performed.

**RESULTS**

**Organization of the Rat PMCA3 Gene**—A rat genomic library was screened with PMCA3 cDNA probes, and 22 positive clones were identified. The clones were digested with EcoRI, fractionated by agarose gel electrophoresis, and analyzed by Southern blot hybridization. Based on this preliminary analysis, eight overlapping cosmid clones which seemed to contain the entire gene were selected for more detailed analyses. A physical map of these overlapping clones was constructed based on restriction endonuclease mapping data obtained by both conventional agarose gel electrophoresis and by field inversion gel electrophoresis. Exon positions were determined by a combination of restriction endonuclease mapping, Southern blot hybridization, and PCR amplification of the intervening sequences, which allowed an estimation of the distance between exons.

The restriction map and genomic organization of the rat PMCA3 gene is illustrated in Fig. 1. The cosmid clones examined span almost 90 kb, including approximately 7 kb upstream of the gene and 12 kb downstream of the gene. Exon/intron boundaries were determined by DNA sequence analyses of both random and specific subclones, and by comparison of the sequences obtained with known PMCA3 cDNA sequences (6). The nucleotide sequence was determined for analysis of the corresponding genomic sequences.

**Northern Blot Hybridization Analyses**—Northern hybridization analyses were performed exactly as described by Greely and Shull (6). DNA fragments to be used as hybridization probes were produced by PCR amplification of the novel 68- and 88-nt sequences identified in the skeletal muscle cDNAs, which corresponded to exon 22 and the portion of exon 23 beyond the potential splice donor site at position 154. Single-stranded uniformly labeled probes were generated by annealing the antisense PCR primers to the denatured PCR products and extending with the Klenow fragment of DNA polymerase I in the presence of \(\text{o}^-\text{PdCTP}\).

**PCR Analysis of mRNA**—First strand cDNA was synthesized from 3 \mu\text{g} of poly(A)\text{+} RNA from male CD rat brain and hind limb skeletal muscle using Superscript\textsuperscript{TM} reverse transcriptase (GIBCO, Bethesda Research Laboratories) and either oligo-dT and random hexamers or specific oligonucleotide primers. All PCR reactions were performed using Hot Tub\textsuperscript{TM} DNA polymerase (Amersham Corp.). The standard PCR reaction utilized 5% of the total cDNA synthesized, and the standard amplification conditions were: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Thirty cycles of amplification were performed, and the sizes of the PCR products were determined by agarose gel electrophoresis. Southern blot hybridization was performed with internal 5' end-labeled oligonucleotides. Blots were washed for 45 min in 2 \times \text{SSC}, 0.1% SDS at 45 °C. Autoradiography was performed at room temperature for 15 min (Fig. 6) or for 30 min (Fig. 4). PCR products were subcloned into pBluescript\textsuperscript{TM} II, and dideoxy sequencing was performed.

**Fig. 1.** Organization and physical map of the rat PMCA 3 gene. The relative positions of the 24 exons, depicted by vertical bars, are shown on the top line. The scale, in kilobases, is shown below the line and is numbered relative to the translation initiation site. The polyadenylation sites for the 4.5- and 7.5-kb PMCA3 mRNAs are depicted by the boxes "pA" and "pA", respectively. Recognition sites for the restriction enzymes ClaI, SfiI, and Smal are indicated. The relative positions of the cosmid clones used for mapping the gene are indicated by the heavy lines designated Cos A–H.
all exons corresponding to known cDNA sequences as well as for the additional exons (exon 8, exon 22, and the extension of exon 23) that were identified in the studies described below. The coding sequence of the PMCA3 gene exactly matches that of the cDNA except for a single polymorphism in which a C replaces a T at position 2304 in clone RB 7-2. This substitution does not alter the amino acid sequence.

The PMCA3 gene consists of 24 exons that range in size from 42 bp (exon 8) to over 945 bp (exon 24) (Table 1). The known exon sequences total 5.3 kb and are distributed across 70 kb of genomic DNA. The introns range in size from approximately 0.1 to 16.5 kb and have boundaries which conform to the consensus sequence for splice junctions (16). Interestingly, the 5' untranslated sequence is split by two introns which disperse this region across 20 kb of genomic sequence. As discussed below, polyadenylation of PMCA3 transcripts occurs following exon 23 to produce a 4.5-kb mRNA and approximately 2 kb downstream from the known portion of exon 24 to produce a 7.5-kb mRNA. Because we have not yet identified the transcription initiation site or the polyadenylation site for the 7.5-kb mRNA, we cannot rule out the possibility of additional exons upstream and downstream of those identified here.

Characterization of the 4.5-kb PMCA3 mRNAs from Rat Skeletal Muscle and Brain—Previous Northern blot analyses demonstrated a high degree of tissue specificity for expression of the PMCA3 gene and provided evidence for the existence of alternative PMCA3 mRNAs. A probe derived from the 3' end of rat brain cDNA clone RB 7-2 identified an abundant 7.5-kb mRNA in brain and skeletal muscle, whereas a 5' probe detected the same 7.5-kb mRNA but also detected a 4.5-kb mRNA that is abundant in skeletal muscle and present at low abundance in brain (6). The absence of a hybridization signal at 4.5 kb when using the 3' probe suggests that the 4.5-kb mRNA has a divergent 3' end, possibly resulting from alternative splicing, the use of an alternative polyadenylation site, or both.

To investigate the origin of the 4.5-kb PMCA3 mRNA, a rat skeletal muscle cDNA library was screened with a probe from the 3'-coding region of clone RB 7-2. This screening identified four PMCA3 clones which were then purified and analyzed. The nucleotide and deduced amino acid sequence of one clone, RSM-1, is presented in Fig. 2. All four clones began at an EcoRI site, presumably due to EcoRI digestion during preparation of the library, and all ended in a 16-18 nt poly(A) tract. Nucleotide sequence alignment between the skeletal muscle cDNA and the brain PMCA3 cDNA (data not shown) revealed two regions of dissimilarity. The first sequence divergence occurs after the end of exon 21 (nt 3300 of clone RB 7-2) where the skeletal muscle cDNA clone contains an insertion of 68 nt relative to the brain cDNA (Fig. 2, double underline). This 68 nt sequence contains an in-frame stop codon and encodes a novel 15-amino acid sequence that makes the C terminus of the enzyme. To identify the genomic origins of the novel 68- and 88-nt sequences observed in the skeletal muscle cDNA, the corresponding regions of the gene were analyzed. The 68-nt sequence corresponds to an individual exon (exon 22, see Table 1). However the 88-nt sequence preceding the poly(A) tract is not the product of a separate exon. Instead, this 88-nt sequence is an extension of exon 23 and is derived from the genomic DNA immediately downstream of the potential splice donor site at position 154 in exon 23.

To determine the tissue distribution of the novel 68- and 88-nt sequences found in the skeletal muscle clones, PCR-generated probes corresponding to these sequences were used to analyze Northern blots of testis, heart, skeletal muscle, brain, and colon mRNAs. The 68-nt exon 22 probe hybridized with the 4.5-kb skeletal muscle PMCA3 mRNA and with a less abundant 7.5-kb skeletal muscle PMCA3 mRNA, demonstrating that expression of exon 22 is virtually restricted to skeletal muscle and occurs predominantly in the 4.5-kb mRNA species (Fig. 3, left panel). The 88-nt probe hybridized with the 4.5-kb PMCA3 mRNA from skeletal muscle and with the low abundance 4.5-kb mRNA from brain, confirming that the region immediately downstream of the splice donor site at position 154 in exon 23 is expressed in the 4.5-kb mRNA but is not expressed in the 7.5-kb mRNA (Fig. 3, right panel). Northern blot analyses also reveal a diffuse high molecular weight species in brain and in skeletal muscle after a long exposure (data not shown), which may represent processing intermediates.

The low abundance ~4.5-kb mRNA detected in brain appears to be slightly smaller than the one detected in skeletal muscle and does not hybridize with the exon 22 probe. This suggests that the splicing pattern of the 4.5-kb mRNA in brain excludes exon 22, thus leading to a protein having a different C terminus. To investigate the differences in the 3' end of the 4.5-kb mRNAs of brain and skeletal muscle, PCR analyses were performed. First strand cDNAs from brain and skeletal muscle were PCR-amplified using primers from exon 21 and the unique 3' sequence that is specific for the 4.5-kb mRNAs. PCR products were fractionated by agarose gel electrophoresis, and the predicted 463-bp product corresponding to the RSM-1 cDNA sequence was observed in the skeletal muscle sample, in the RSM-1 control, and at barely detectable levels in the brain sample. In addition, a 395-bp product was observed only in the brain sample (Fig. 4, left panel). Southern blot hybridization with an oligonucleotide probe from exon 21 detected both the 463-bp product and the 395-bp product (Fig. 4, center panel), whereas an oligonucleotide from exon 22 detected only the 463-bp product (Fig. 4, right panel). DNA sequence analysis was performed, confirming that the 395-bp PCR product from brain is derived from an alternatively spliced mRNA that lacks exon 22. Thus, these results demonstrate that most of the 4.5-kb PMCA3 mRNA in brain excludes exon 22, whereas exon 22 is always included in the 4.5-kb skeletal muscle mRNA.

Amino Acid Sequence Comparisons—A comparison of the predicted C-terminal amino acid sequences for the PMCA3a and the PCR product of the 4.5-kb mRNA from brain, and the skeletal muscle cDNA (RSM-1) is presented in Fig. 5. The amino acid sequences shown represent translations of the respective cDNA sequences from the beginning of the sequence encoding the calmodulin-binding site in exon 21 to the termination codon. The translation of PMCA3a includes coding sequence from exon 21, the first 154 nt of exon 23, and exon 24; the translation of the PCR product from brain includes sequence from exons 21 and 23; and the translation of the skeletal muscle cDNA includes sequence from exons 21 and 22. As shown in Fig. 5, the predicted protein resulting from the predominant splicing pattern of the 4.5-kb PMCA3
mRNA from brain is almost identical to that of PMCA3a; however, the final 8 C-terminal amino acids derived from exon 24 in PMCA3a are replaced by the 3-amino acid sequence, Ser-Glu-Ser, encoded by the extension of exon 23 that is unique to the 4.5-kb mRNAs. In the skeletal muscle enzyme the first 18 amino acids of the calmodulin-binding site are identical to those of other proteins, but the 15-amino acid sequence encoded by exon 22 forms a novel variable domain of the calmodulin-binding site and a shortened C terminus.

Identification of a Splicing Pattern That Alters the Putative Phospholipid Binding Domain of PMCA3—Recent evidence has indicated that the PMCA2 gene contains three alternatively spliced exons that contribute to the putative phospho-

### Table 1

<table>
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<th>Exon</th>
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<th>Splice donor</th>
<th>Intron size</th>
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* Exon 8 is not present in the RB 7-2 cDNA (6).
* Exon 22 is not present in the RB 7-2 cDNA, and contains an in frame stop codon.
* Exon 23 contains several internal donor sites, as well as an alternative polyadenylation signal.
* Exon 24 is translated in either of two reading frames determined by the location of the upstream splice site.

**Nucleotide sequences around the exon/intron boundaries are presented. Exon sequences are shown in upper case letters and intron sequences in lower case. Amino acid residues encoded by exon sequences flanking the splice junctions are indicated. The location of the splice junctions are given relative to their position in the RB 7-2 cDNA (6). The first base of the ATG initiation codon is designated +1. Approximate intron sizes as determined by restriction mapping and PCR analyses are indicated.**
A novel 68-nt exon found in the skeletal muscle PMCA3 cDNA is marked by a double underline. A single underline marks the 88-nt extension of exon 23 that immediately follows the potential splice donor site at position 154 in exon 23.

The upper left panel shows the hybridization pattern with a 68-nt probe corresponding to exon 22 (probe A). The upper right panel shows the hybridization pattern with an 88-nt probe (probe B). The bottom center panel shows the hybridization pattern with a 7.5-nt probe corresponding to exon 23 (probe C). The predicted location of in-frame termination codons (TAA and TGA).

Amino acid sequence comparisons of the calmodulin-binding domain and C terminus of alternative PMCA3 proteins. Partial deduced amino acid sequences for the PMCA3a cDNA (6) and the 4.5-kb mRNAs from brain and skeletal muscle (SK. MUS.) are presented. The sequences shown extend from the beginning of the calmodulin-binding domain (consisting of the 18-amino acid constant domain followed by the 10-amino acid variable domain) to the C terminus. Gaps delineate sequences encoded by separate exons or by the extension of exon 23 (in the case of the brain mRNA, see text).

DISCUSSION

In this study we have determined the exon/intron organization of the gene encoding isofrom 3 of the plasma membrane Ca"+-ATPase. In addition, we have determined the structures of the ~4.5-kb PMCA3 mRNAs of skeletal muscle and brain, and show that they arise from alternative splicing and polyadenylation patterns that differ from those observed previously for other PMCA mRNAs (4, 8, 10, 11). Of particular interest is the inclusion of a novel 68-nt exon in the skeletal muscle mRNA, which significantly alters both the variable region of the calmodulin-binding domain and the C terminus.

The PMCA3 gene spans approximately 70 kb and consists of at least 24 exons, three of which are involved in alternative splicing. In contrast, the genes for other P-type ATPases, which contain 22-23 exons, span 13-40 kb (12, 20-22).
Although the PMCA3 gene has about the same number of exons as the other P-type ATPase genes, there is little apparent similarity in the location of the exon/intron boundaries. Contributing to the size of the PMCA3 gene are two large introns that separate the 5′-untranslated sequence into 3 exons dispersed over 20 kb of genomic sequence. The reason for these large introns in the 5′-untranslated sequence is unclear, but an interesting possibility is that they may contain elements that regulate PMCA3 gene expression.

It is possible that the PMCA3 gene contains additional exons besides those identified here. The previously characterized PMCA3 cDNA, clone RB 7-2 (6), which was derived from the 7.5-kb brain mRNA, lacked approximately 2 kb of 3′-untranslated sequence. Whether this sequence is part of exon 24 or is split by one or more introns has not been determined. It is also possible that additional exons occur at the 5′ end of the gene. The genomic DNA extending upstream of the 5′ end of clone RB 7-2 contains a sequence consisting of ACC trinucleotide repeats interspersed with regions of TCC trinucleotide repeats, which extend for over 200 nt of genomic sequence. The reason for these large introns in the 5′-untranslated sequence is unclear, but an interesting possibility is that they may contain elements that regulate PMCA3 gene expression.

The observation that the 3′ end of the alternative 4.5-kb skeletal muscle mRNA was determined by direct cDNA cloning and confirmed by Northern blot and PCR analyses. As expected from the previous Northern hybridization data (6) the 4.5-kb mRNA lacks exon 24, which is included in the 7.5-kb mRNAs. In place of exon 24 the 4.5-kb skeletal muscle mRNA contains a unique 88-nt sequence derived from the genomic DNA immediately following the potential splice donor site at position 154 in exon 23. Northern blot and PCR analyses show that the 4.5-kb brain mRNA also contains this sequence. Although the skeletal muscle cDNAs terminated with a poly(A) tract, they did not contain the canonical AAATAAA polyadenylation signal (24). However, the sequences GAATAA and CATAAA occur 42 and 63 nt upstream from the poly(A) tract, and one or both of these sequences may be responsible for directing cleavage and polyadenylation of the 4.5-kb PMCA3 mRNAs. These sequences have been shown to stimulate cleavage and polyadenylation, although with reduced efficiency compared to that achieved with the AAATAAA sequence (17). It is unlikely that the poly(A) tract observed in the skeletal muscle cDNAs arose as a result of nonspecific priming by oligo-dT because the genomic sequence at the apparent site of polyadenylation consists of a CA dinucleotide followed a pyrimidine-rich region, which appears to provide an appropriate context for cleavage and polyadenylation (17, 25) and would be unlikely to form a hybrid with oligo-dT. Thus, formation of the 4.5-kb PMCA3 mRNAs appears to involve a variant polyadenylation signal that is utilized efficiently in skeletal muscle, but is used in only a small proportion of the brain mRNAs.

A second major difference between the 4.5-kb skeletal muscle mRNA and the 7.5-kb brain mRNAs is the inclusion of exon 22 in the skeletal muscle message. Northern blot and PCR analyses show that this 68-nt exon is expressed almost exclusively in skeletal muscle and that it encodes part of an alternative calmodulin-binding site and a C terminus that differ substantially from those identified in other PMCA isoforms. The amino acid sequence encoded by this exon includes the sequence KKMLRTT, which resembles some of the sequences that serve as phosphorylation sites for cGMP-dependent protein kinase and protein kinase C (26).

The calmodulin-binding site of all known PMCA isoforms consists of a perfectly conserved 18-amino acid sequence followed by a variable region consisting of approximately 10 amino acids (27). The conserved 18-amino acid sequence is encoded by the 3′ end of exon 21 in the PMCA3 gene and by the homologous exons of the genes encoding isoforms 1, 2, and 4. The variable 10-amino acid sequence is encoded by the
exon immediately following the exon encoding the conserved region. Thus, depending on the splicing pattern, within a given gene different mRNAs can encode the variable region. The sequence encoded by PMCA3 exon 23 (or the homologous exon from PMCA 1, 2, or 4) produces a more acidic calmodulin-binding site (A form), while the variable region encoded by PMCA3 exon 24 (or the homologous exon from PMCA 1, 2, or 4) yields a more basic calmodulin-binding site (B form) (discussed in Ref. 27). The variable region of the calmodulin-binding site for the A and B forms of each isoform are similar in that they all have the consensus sequence -VV-F--- (27). In each protein the first amino acid of this variable sequence is hydrophobic (either a Met or an Ile residue) and the second amino acid is charged (Asp or Glu in the A forms, and Arg or Lys in the B forms). An even greater degree of sequence conservation is observed among the A forms as a group and the B forms as a group. In contrast, the variable region of the calmodulin-binding site encoded by exon 22 bears no resemblance to the consensus sequence of the A or B forms.

Enyedi et al. (27) have demonstrated that the variable region of the calmodulin-binding site influences both the affinity for calmodulin and the autoinhibitory activity of the calmodulin-binding domain. The B form of the calmodulin-binding domain exhibits a 10-fold higher affinity for calmodulin and is 15 times more potent as an autoinhibitor of pump activity than the A form. It has been suggested that these functional differences may be related to differences in the net charge of the two classes of calmodulin-binding domain and that the greater positive charge of the B forms contributes to the higher calmodulin affinity and autoinhibitory activity (27). Because the variable region encoded by exon 22 differs substantially from those of the A and B forms it is difficult to predict what effect it may have on the regulation of Ca2+-extrusion by PMCA3 in skeletal muscle, although the net positive charge suggests that it might increase the calmodulin binding affinity and autoinhibitory activity. It will be of interest to determine the effect of this alternative sequence on calmodulin affinity and autoinhibitory activity, and whether its potential regulatory activity can be modulated by phosphorylation.

Northern blot and PCR analyses show that the ~4.5-kb brain mRNA encodes a PMCA3 variant with a calmodulin binding domain and C terminus that differ from those encoded by the 4.5-kb skeletal muscle mRNA. In the brain mRNA exon 22 is excluded and exon 21 is spliced to exon 23, thereby encoding the same calmodulin-binding domain as that of PMCA3a. However, as in the case of the 4.5-kb skeletal muscle mRNA, the potential donor splice site of exon 23 is not used, and the 88-nt extension of exon 23, with the alternative polyadenylation site, is included in the mRNA. The beginning of the 88-nt extension of exon 23 encodes an alternative C-terminal sequence, Ser-Glu-Ser, which replaces the eight amino acid C-terminal sequence of PMCA3a that is encoded by exon 24. Whether this alternative C terminus has functional significance is unclear; however, it should be noted that alternative C termini occur in other Ca2+-pumps, including SERCA1 (20), SERCA2 (28, 29), and the mammalian homolg of the yeast secretory pathway Ca2+-ATPase (30).

Also, we have observed seemingly minor alterations in the C termini of PMCA1 and PMCA2 as a result of alternative splicing.2 It will be of interest to determine, by means of in situ hybridization, whether the 88-nt sequence is broadly distributed in brain, as would be expected if it were due to a low level functionally insignificant use of the alternative polyadenylation site or if this sequence is restricted to a specific subset of cells. The latter possibility would be more consistent with a significant function for the alternative C-terminal sequence.

In addition to the calmodulin-binding domain, three other regions of PMCA isoforms have been proposed as possible sites of alternative splicing (reviewed in Refs. 18 and 31). These include a CAMP-dependent protein kinase site near the C terminus of PMCA1, the exon encoding putative transmembrane domain 10 of PMCA4, which corresponds to exon 20 of PMCA3, and the exons encoding part of the putative phospholipid-binding region. Analysis of the PMCA1 gene2 shows that the 33-nt sequence encoding the CAMP-dependent phosphorylation site, which was deleted in a single cDNA (4), is not a separate exon, but rather is part of the exon corresponding to exon 24 of PMCA3. Thus, the original evidence indicating that this region might be involved in alternative splicing was apparently the result of a cloning artifact. We used PCR analyses to examine the possibility that exon 20 of PMCA3 was excluded in some mRNA species but found no evidence for such a splicing product in brain or skeletal muscle (data not shown). Although it is possible that this splicing pattern does occur in PMCA4, an alternative possibility is that the PMCA4 cDNA in which this exon was excluded may have been derived from an aberrantly processed mRNA.

Alternative splicing of exons encoding part of the putative phospholipid-binding domain (19) was recently demonstrated for PMCA2 mRNAs. Adamo and Penniston (11) identified four different splicing patterns in this region of the PMCA2 mRNA, and Carafoli (18) reported that these alternative splicing patterns are due to the use of three separate exons, of 60, 33, and 42 nt in length, which can be entirely excluded or used in combinations that insert 14, 31, or 45 amino acids into the PMCA2 protein. For PMCA3 we observed a similar, but less complex splicing pattern, in which a single 42-nt exon (exon 8) was either included or excluded from the mRNA (Fig. 6). Exon 8 was included in all of the skeletal muscle mRNAs and in the majority of the brain mRNAs. The amino acid sequence encoded by exon 8 is closely related to that encoded by the 42-nt exon of PMCA2. In addition, like the alternatively spliced exons of PMCA2, the first 6 amino acids encoded by exon 8 are partially conserved with the first 6 amino acids encoded by exon 9. This implies a common function for these sequences, and it seems reasonable to predict that the inclusion or exclusion of exon 8 would modulate this function. It has been proposed that the enzyme variants resulting from these alternative splicing patterns in PMCA2 may differ in their sensitivity or specificity for acidic phospholipids (11). The occurrence of similar splicing patterns in PMCA3 mRNAs suggests that interactions with acidic phospholipids may serve an important regulatory role in this isoform as well.

REFERENCES

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**Rat Plasma Membrane Ca²⁺-ATPase Isoform 3 Gene**


