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Expression of cyclic-nucleotide-sensitive and -insensitive isoforms of the plasma membrane Ca^{2+} pump in smooth muscle and other tissues

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cDNA clones encoding the plasma membrane Ca^{2+} pump isoform PMCA1 were obtained from rabbit stomach smooth muscle. The *PMCA1* gene has a 154 base exon which can be alternatively spliced. In splices containing 0, 87 or 114 bases of this exon, the mRNA downstream from this position encodes a protein containing the peptide sequence Lys-Arg-Asn-Ser-Ser (KRNSS), which can be phosphorylated by cyclic-nucleotide-sensitive protein kinase. However, in those splices containing 154 bases, the mRNA encodes a protein that does not contain this sequence. The cDNA clone obtained in this study did not contain the latter exon, and thus it coded for KRNSS. The presence of the various splices of PMCA1 was determined in stomach smooth muscle and other tissues by reverse transcription followed by a polymerase chain reaction. Percentages of transcripts encoding the potentially cyclic-nucleotide-sensitive isoform in various tissues were as follows: liver, 100%; stomach mucosa, 100%; heart, 100%; stomach smooth muscle, 86%; aorta, 83%; brain, 55%. Thus brain was the only tissue which expressed a very high proportion of the isoform of PMCA1 that is insensitive to cyclic-nucleotide-dependent protein kinases.

INTRODUCTION

Smooth muscle and most other mammalian tissues contain two types of Ca^{2+} pumps, one type which is present internally and the other which is present in the plasma membrane (PM), although the relative abundance of these pumps varies considerably in these tissues [1–8]. The two types of pump are distinct in their structure, regulation and immunoreactivity [2,7–9]. The internal Ca^{2+} pump has a subunit size of 100–115 kDa, but the PM pump has a molecular mass of 127–135 kDa [1,2,7,8].

The PM Ca^{2+} pump is activated by calmodulin [1,10]. In some studies it has been reported that the PM Ca^{2+} pump is also activated by cyclic-nucleotide-dependent protein kinases, whereas in others such activation was not observed [5,11–16]. This regulation may be one of the key components in the understanding of the processes involving changes in cyclic nucleotide concentrations, such as the action of adrenergic agents.

Four genes encoding the PM Ca^{2+} pump have been identified [17–20]. Based on Northern blot studies, the product of the PM Ca^{2+} pump gene *PMCA1* is the most widely distributed [18]. The *PMCA1* gene contains a 154 base exon, which was present in the rat brain cDNA clone but absent from the clone reported from human teratoma [19,20]. Consequently, the human teratoma isoform contained a phosphorylation site with the sequence Lys-Arg-Asn-Ser-Ser (KRNSS) for regulation by cyclic-nucleotide-dependent protein kinases, but the rat brain clone did not. By S1-nuclease analysis it was shown that fetal skeletal muscle transcripts contain 114 or 87 bases of this exon, and hence would encode for KRNSS [21]. It is not known, however, how various tissues differ in the cyclic nucleotide sensitivity of their PM Ca^{2+} pump protein.

In this study we report the sequence of a PM Ca^{2+} pump cDNA cloned from rabbit stomach smooth muscle. Using reverse transcription followed by PCR, we report on the nature of the transcripts present in rabbit stomach smooth muscle and mucosa, aorta, liver, heart and brain.

EXPERIMENTAL

Screening of the cDNA library

Approx. 10^6 plaques of a once-amplified rabbit stomach smooth muscle cDNA library in λgt11 [22] were screened using two different cDNA probes, as shown in Fig. 1. Hybridization was performed as previously described [22]. The inserts from these clones were removed by *Eco*RI digestion and subcloned into the *Eco*RI site of Bluescript SK Plus (Stratagene). In order to clone the remaining cDNA, three h.p.l.c.-purified oligodeoxynucleotide primers, i.e. PCRREV, PCR1 and PRC2, were employed. These were based on the sequences of the two clones already obtained by screening with the cDNA probes. The approximate locations of these primers are shown in Fig. 1. PCR2 (GGTCAAGATACTTCTCTGGG) was identical to bases 210–229 of the full sequence; PCR1 (GGGATGATCTTGCAAGTACTCGC) and PCRREV (CTACGAAATGCATTCACCACT) were complementary to bases 2669–2691 and 3740–3760 respectively in the subsequently determined full sequence (see Fig. 2). The first strand was synthesized from rabbit stomach smooth muscle total RNA using PCRREV and amplified by PCR using primers PCR1 and PCR2, as described below. The PCR product was filled using the Klenow fragment of DNA polymerase I, 5′-phosphorylated and ligated into the *Eco*RV site of Bluescript SK Plus [23,24].

Sequencing

Overlapping deletion mutants were prepared by linearizing the recombinant plasmids with *Apa*I and *Sal*I, followed by digestion

Abbreviations used: PM, plasma membrane; UTR, untranslated region; FITC, fluorescein isothiocyanate.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X59069.

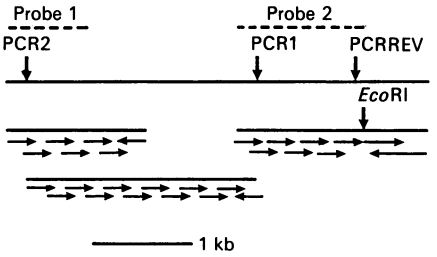


Fig. 1. Strategy for cloning and sequencing of the PM Ca^{2+} pump cDNA

A λgt11 rabbit stomach smooth muscle cDNA library was screened with probes 1 and 2. Probe 2 was a 1.4 kb *Ava*I fragment obtained from the rat PMCA1 clone in pBR322 [19]. Probe 1 was a cDNA fragment from human teratoma PMCA1 [20] and corresponded to bases 497–1198 in Fig. 2. The missing middle piece was obtained by reverse transcription using the primer PCRREV followed by PCR with the primers PCR1 and PRC2, based on the sequences of the other two clones as shown. The cDNA fragments from λgt11 were subcloned into the *Eco*R1 site of Bluescript SK Plus, whereas the fragment from PCR was cloned into the *Eco*RV site of this plasmid. One of the λgt11 clones had an *Eco*RI site in the insert, as shown; in this instance, the two fragments were subcloned separately. Deletion mutants were made as described in the Experimental section and the inserts were sequenced using a universal primer, as shown by horizontal arrows. Undeleted plasmids were sequenced with both T3 and universal primers.

with exonuclease III and S1 nuclease and self-ligation [23,24]. The DNA prepared for the deleted plasmids was sequenced using universal primer, and that for the undeleted plasmids was sequenced using T3 and universal primers by the dideoxy method [25] using a T7 polymerase sequencing kit (Pharmacia).

Reverse transcription

Male albino rabbits weighing 1–2 kg were killed before removing various tissues [26]. From the stomach, two types of tissues were used. First, the mucosa, submucosa and serosa were removed, and the remaining tissue was designated as 'stomach smooth muscle'. Secondly, the mucosal layer was used. Liver, brain, aorta and heart were dissected out as whole organs after removing fatty tissue and blood. All tissues were placed on ice immediately after dissection. Total cellular RNA was prepared from these tissues [27]. A 10 μg portion of total RNA was heated to 70 °C for 5 min and quickly cooled on ice. RNA was annealed with 10 pmol of the PCRREV primer by heating to 68 °C followed by gradual cooling to 42 °C. The final conditions for the transcription reaction were: RNA annealed with primer as described above in 50 mM-Tris/HCl (pH 8.3 at 42 °C)/10 mM-$MgCl_2$/70 mM-KCl/1 mM each of dATP, dCTP, dGTP and dTTP/10 mM-dithiothreitol/20 units of RNA-Guard (Pharmacia)/15 units of AMV reverse transcriptase, in a total volume of 50 μl at 42 °C for 1 h. The reaction was terminated by heating to 90 °C for 10 min [24].

Polymerase chain reaction

PCR was carried out using two primers: PCRREV, as described above for the reverse transcription, and PCRFOR (ATCTTGTGGTTTAGAGGTCTG), corresponding to bases 3696–3716 of the sequence shown in Fig. 2. PCR was carried out under the following conditions: 10 μl of the above first-strand cDNA, 20 pmol of each primer, 200 μM each of dATP, dCTP, dGTP and dTTP, and 3 units of *Taq* polymerase in Promega PCR buffer. PCR consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and reaction at 74 °C for 60 s, for 25 cycles [28]. The samples were electrophoresed in a composite 2% Nusieve plus 1% SeaKem GTG-agarose gel (Mandel Scientific, Canada) in a Tris/acetate/EDTA buffer [24]. The gels were stained with ethidium bromide and photographed on a negative film in the presence of an ultraviolet light source. The relative intensities of the various bands were determined by imaging using an MCID system (Imaging Res.).

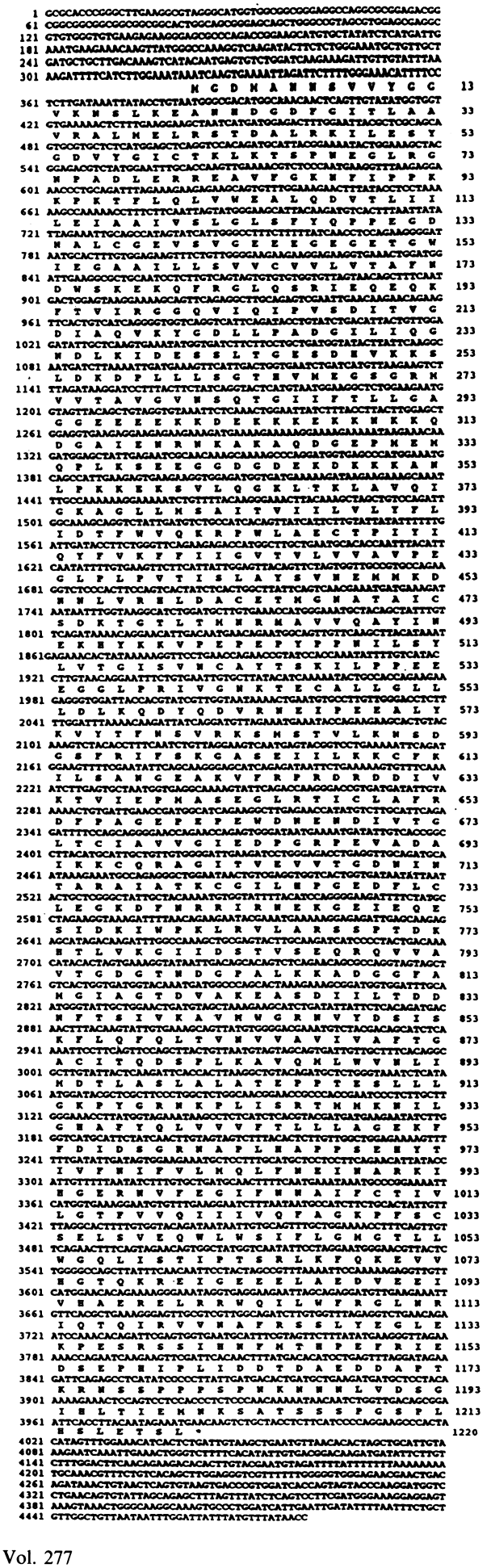
RESULTS

Sequence of the PM Ca^{2+} pump cDNA from stomach smooth muscle

The rabbit stomach cDNA λgt11 library was screened and the two non-overlapping clones shown in Fig. 1 were obtained. Using primers based on the sequences of these clones, the first-strand cDNA was synthesized from rabbit stomach smooth muscle RNA and amplified by PCR, as shown in Fig. 1. The entire cDNA based on these clones was 4482 bases (Fig. 2). It contained an additional 202 bases upstream compared with the cDNA sequence published by Verma *et al.* [20] but lacked 134 bases at the end of the 3'-untranslated region (UTR). Otherwise the two sequences had 96.6% identity. The sequence GGC was repeated seven times, starting at position 61 in the 5'-UTR (Fig. 2). The entire coding region described by Verma *et al.* [20] represents the cDNA bases 385–4044 in Fig. 2. The encoded protein contains 1220 amino acid residues and includes sites for acyl phosphate formation, fluorescein isothiocyanate (FITC) binding and calmodulin binding, as well as the site for phosphorylation by cyclic-nucleotide-dependent protein kinase (KRNSS) (Fig. 2). The sequence identity with the PMCA1 cDNA from rat brain [19] was 86.5%. There was, however, much lower identity with the cDNA sequences of rat brain PMCA2 (59.2%) and with the cDNAs for the internal Ca^{2+} pump proteins (44.2–44.5%) [4,19,22,29–33].

Detection of alternatively spliced transcripts

It has been reported that there is a 154 base exon in the PMCA1 gene [19,20]. However, this exon was absent in the rabbit stomach smooth muscle PMCA1 cDNA clone obtained (Fig. 2). If present, it would start at base position 3735 (Fig. 2). In order to investigate whether this tissue also contained any transcripts retaining this exon, total tissue RNA was reverse-transcribed using the primer PCRREV, which was complementary to base sequence 3740–3760 (Fig. 3), and the resulting first strands were amplified by PCR using PCRFOR and PCRREV. This resulted predominantly in a 65 base PCR product and a lesser amount of a 219 base fragment (Fig. 4), indicating that 86% of the transcripts would encode for KRNSS and 14% would not (Table 1). It was assumed that the intensities in Fig. 4 corresponded to the amounts of DNA formed for each PCR product, and that for each band the amount of DNA was the product of the number of molecules and their size. Therefore, in order to obtain the relative numbers of transcripts, these intensities were divided by the length of each fragment (Table 1). The relative intensities of the bands within each lane were unaffected by the number of PCR cycles used. The stomach mucosa and liver contained the PMCA1 transcripts lacking the 154 base exon. Heart contained mainly transcripts which either did not contain this exon or included 87 bases of it. Either type of mRNA from heart would thus encode for KRNSS (see Fig. 3). In the aorta, 83% of transcripts encoded the phosphorylation site and 17% did not (Table 1). The PCR product using brain cDNA, however, showed a very intense band at 219 bases, a less prominent band at 65 bases and an even fainter one at 152 bases (Fig. 4). Normalization to transcript number revealed that 45% of the brain transcripts were of the cyclic-nucleotide-insensitive isoform (Table 1).



In order to confirm the identity of the transcripts, the DNAs for the 219 base PCR product from brain, the 152 base product from heart and the 65 base band from stomach smooth muscle were subcloned in Bluescript SK Plus and sequenced. The sequences obtained are given in Fig. 5. The 154 base exon in rabbit PMCA1 has 100% identity with the corresponding exons in the human and rat PMCA1 [19,21].

DISCUSSION

It is shown here that rabbit stomach smooth muscle expresses the PM Ca^{2+} pump gene *PMCA1*. Furthermore, the expression of this gene product by stomach mucosa, aorta, liver, heart and brain has been confirmed by PCR, and the tissue selectivity of the alternative splicing results in cyclic-nucleotide-sensitive and -insensitive isoforms of PMCA1. In this Discussion the focus is on the unique features in the primary sequence and on the role of alternative splicing in tissue-selective expression.

Various functional domains of the PM Ca^{2+} pump sequence described previously, namely the acyl phosphate formation site, the FITC binding site, the calmodulin binding site and the potential site for phosphorylation by cyclic nucleotides, were observed [10,14,18,20,21]. Even though there are other potential initiation sites, in all other instances the open reading frames are extremely short to encode for this protein, which is approx. 130 kDa [9]. Additionally, a GGC repeat was observed, starting at position 61. This sequence has not been reported previously for the PM Ca^{2+} pump, since the sequences reported for the PMCA1 cDNA have started downstream from this repeat [19,20]. A GenBank search revealed that there are several other proteins which have GGC repeats in their 5′-UTR. In some of these instances, however, the 5′-UTR is highly enriched in G+C content [34], and hence this repeat would have a high chance of occurring. At present it is not known whether or not the GGC repeat has a role in the regulation of expression of PMCA1. The sequence of the 154 base exon (Fig. 5) which can be spliced alternatively is identical in the rat, rabbit and human, even though the remainder of the rabbit cDNA has only 96.6% and 86.5% identity with the corresponding sequences in human and rat respectively. Whether the 100% identity of the sequence of this exon in the three species is a mere coincidence or has any further biological significance is not known. A cryptic exon has been reported using PCR in pig stomach smooth muscle PMCA1 [35]. The exact structure of this exon was not reported, but from the preliminary results given, this exon in the pig would be larger than 1 kb [35], compared with 154 bases in other species.

The erythrocyte PM Ca^{2+} pump is phosphorylated by cyclic-AMP-dependent protein kinase and the peptide sequence surrounding the labelled residue is APTKRNSS*PPPSPD [14]. Apart from a substitution of Asn for Asp, this sequence is identical to amino acid residues 1171–1184 of the rabbit stomach PMCA1 reported in Fig. 2. The synthetic peptide

Fig. 2. cDNA and deduced protein sequence of the PM Ca^{2+} pump

The sequences comprising bases 1–1296 and 2575–4482 are for clones from the cDNA library, and that of bases 210–2691 is for the PCR clone. The sequences from overlapping clones were identical. Sequences 1–209, 1297–2574 and 2692–4482 were from single clones. The functional amino acid sequences present in this sequence and identified previously for PMCA1 are: acyl phosphate residue Asp at 465, FITC binding region at 598–609, calmodulin binding domain at 1098–1115 bp, and a potential cyclic-nucleotide-dependent protein kinase phosphorylation site KRNSS at 1174–1178 [20]. The 5′-UTR contains a GGC repeat at 61–82. A 154 base exon reported to be included in some splices is absent, but if present it would start at base no. 3735.

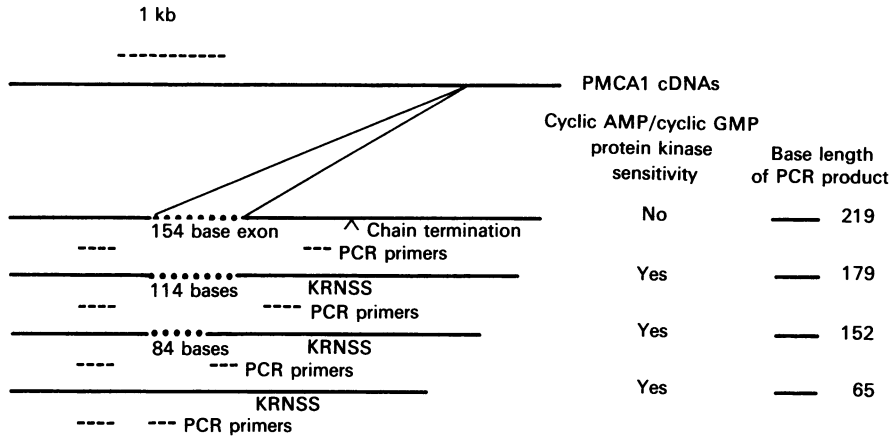


Fig. 3. Strategy for the detection of alternative splices of PMCA1

The 154 base exon (•••••) reported to be present in some splices and not in others, if present, would start at position 3735 in Fig. 2. Alternative splicing can potentially result in inclusion of 154, 114, 87 or 0 bases of this exon [21]. Inclusion of 114, 87 or 0 bases would result in the downstream mRNA encoding the potential phosphorylation site KRNSS as in Fig. 2, but inclusion of all 154 bases would result in a frame shift and hence no coding for KRNSS, as well as in an earlier chain termination. Thus reverse transcription using the primer (----) PCRREV which was complementary to bases 3740–3760, followed by PCR using the same primer and PCRFOR (----), which was identical to the bases 3696–3716 in Fig. 2, would result in products of 219, 179, 152 and 65 bases respectively when 154, 114, 87 and 0 bases of the exon are included. Only the 219 base product would correspond to transcripts encoding the cyclic-nucleotide-insensitive PM Ca^{2+} pump. All the others would correspond to the transcripts encoding the cyclic-nucleotide-sensitive isoform.

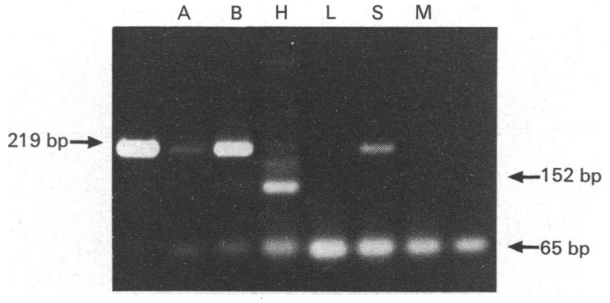


Fig. 4. PCR detection of alternative splices in rabbit stomach smooth muscle (S), mucosa (M), aorta (A), brain (B), liver (L) and heart (H)

cDNA from PMCA1 of rat brain containing the 154 base exon and cDNA of PMCA1 of rabbit stomach smooth muscle were amplified using the same primers and used as molecular size markers of 219 and 65 bp. The identity of the 152 base fragment was established by subcloning and sequencing (see Fig. 3 for strategy of this experiment).

EDSEPHIPLIDDTDAEDDAPTKRNSSPG, which resembles amino acid residues 1153–1179 (Fig. 2), except for a substitution of Asn for Gly, has also been shown to be an excellent substrate for the cyclic-AMP-dependent protein kinase [14]. In erythrocytes, phosphorylation at this site increases the initial velocity of the Ca^{2+} pump and its affinity for Ca^{2+} [14]. Thus this KRNSS sequence is the site required for the activation of the PM Ca^{2+} pump by cyclic-AMP-dependent protein kinase, and it is encoded by some splices of PMCA1 and not by others. There have been several biochemical studies on various tissues in which activation and/or phosphorylation of the PM Ca^{2+} pump by cyclic-nucleotide-dependent protein kinase has been reported, but in others the results were negative [11–16]. The alternative splicing of the PMCA1 mRNA to encode proteins with or without KRNSS now provides a plausible explanation for the discrepancies in these studies. However, more importantly, it establishes a basis for another means of differential regulation of this pump activity in a tissue-dependent manner. In several instances in which, during development or under pathophysiological conditions, tissues undergo changes which may influence

Table 1. Proportions of transcripts encoding for the sequence KRNSS in various tissues

Relative intensities (R.I.) of bands were determined for the various bands in each lane as percentages of the total intensity in that lane. For computing numbers of transcripts, the intensity of each transcript was divided by its length. This value was then converted to a percentage of the total transcripts in that lane. Values are means (±S.D.) of three experiments.

	R.I. (% of total)				Transcripts (% of total)				Transcripts endcoding for KRNSS
Transcript ...	219	179	152	65	219	179	152	65	(%)
Aorta	40	0	5	55	17	0	3	80	83±3
Brain	70	0	5	25	45	0	3	52	55±7
Heart	0	0	56	44	0	0	32	68	100±11
Liver	0	0	0	100	0	0	0	100	100±1
Stomach smooth muscle	33	0	4	63	14	0	2	84	86±1
Stomach mucosa	0	0	0	100	0	0	0	100	100±1

219
ATCTTGTGGTTTAGAGGTCTGAACAGAATCCAAACACAGATGGATGTAGT
GAATGCTTTCCAGAGTGGAAGTTCCATTCAGGGGGCTCTAAGGCGGCAAC
CCTCCATCGCCAGCCAGCATCATGATGTAACAAATATTTCTACCCCTACA
CATGTAGTGTTTTCCTCTTCTACTGCTTCTACTACTGTGGGGTATTCGAG
TGGTGAATGCATTTCGTAG

152
ATCTTGTGGTTTAGAGGTCTGAACAGAATCCAAACACAGATGGATGTAGT
GAATGCTTTCCAGAGTGGAAGTTCCATTCAGGGGGCTCTAAGGCGGCAAC
CCTCCATCGCCAGCCAGCATCATGATATTCGAGTGGTGAATGCATTTCGT
AG

65
ATCTTGTGGTTTAGAGGTCTGAACAGAATCCAAACACAGATTCGAGTGGT

Fig. 5. Sequences of the PCR products

PCR products of 219 bp obtained in Fig. 4 from brain, 152 bp from heart and 65 bp from stomach were cloned and sequenced. The sequences underlined represent the full or partial segments of the 154 base exon included in these splices.

the cellular cyclic nucleotide levels, the function of the PM Ca^{2+} pump may thus also be altered.

The expression of the PM Ca^{2+} pumps at the protein and mRNA levels has been shown to be much greater in brain than in other tissues examined so far [6,18]. At the mRNA level the brain not only expresses the various PM Ca^{2+} gene products, but also elicits the highest level of PMCA1 mRNA [18]. Here we demonstrate that the brain also differs from other tissues in that it contains a large amount of the PMCA1 splice with the 154 base inclusion. Whether or not alternative splicing in itself is important for the greater abundance of this mRNA in the brain is not known. Furthermore, whether or not a partial retention of this exon, as in the heart, affects the expression of this protein is also unknown. The internal Ca^{2+} pump mRNA is also alternatively spliced, so that one isoform is expressed in the cardiac muscle and another in the smooth muscle and non-muscle tissues [4,6,22,29–33]. In this instance the cardiac muscle, which predominantly expresses one splice, has a much higher abundance of this protein than the various tissues that express predominantly the other splice [26]. Thus alternative splicing may play a role not only in the type of Ca^{2+} pumps expressed in various tissues but also in the level of their expression.

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