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Molecular cloning and sequencing of the plasma-membrane Ca^{2+} pump of pig smooth muscle

Sabine DE JAEGERE,* Frank WUYTACK, Jan A. EGGERMONT, Hilde VERBOOMEN and Rik CASTEELS
Laboratorium voor Fysiologie, K.U. Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

cDNAs coding for the plasma-membrane Ca^{2+} pump have been isolated from a pig smooth-muscle cDNA library and sequenced. The open reading frame encodes a protein of 1220 amino acids, which corresponds to the one already described in a human teratoma cell line. We demonstrate here that this cDNA probably represents the only isoform of the plasma-membrane Ca^{2+}-transport ATPase expressed in this smooth muscle. There is no evidence for the expression of any other plasma-membrane Ca^{2+}-pump gene, or for the presence of other alternatively spliced isoforms. These results are in apparent contradiction to those obtained on protein levels which demonstrate the reaction of at least two different polypeptides with a panel of antibodies against the plasma-membrane ATPase. It is suggested that these two polypeptides could result from a post-translational modification of one single enzyme.

INTRODUCTION

In concert with the sarcoplasmic/endoplasmic reticulum Ca^{2+} pump, the calmodulin-stimulated plasma-membrane Ca^{2+}-transport ATPase plays an essential role in lowering the cytosolic free Ca^{2+} concentration in most eukaryotic cells [1].

It has recently become clear that the calmodulin-sensitive plasma-membrane Ca^{2+}-transport ATPase belongs to a multigene family, which consists of at least four different genes (giving isoforms PMCA1, PMCA2, PMCA3 and PMCA4). The PMCA1, PMCA2 and PMCA3 cDNAs have been cloned and sequenced in rat brain [2,3], whereas the PMCA4 isoform has been ascribed to the human erythrocyte [4]. Alternative splicing of the primary gene transcript further increases the isoform diversity. For PMCA1, alternative splicing of its pre-mRNA leads to four different isoforms [5]. This process of alternative splicing involves an optional 154 bp exon. Complete inclusion of this exon leads to PMCA1a (1176 aa; M_r 129500), which has been described in rat brain [2], whereas exclusion of this exon results in the isoform PMCA1b (1220 aa; M_r 135000), which has been isolated from a human teratoma library [6]. Finally, inclusion of 87 nucleotides (nt) or 114 nt of the 154 bp exon gives rise to respectively PMCA1c (1249 aa) and PMCA1d (1258 aa), which have both been detected in human skeletal muscle [5].

In view of this plethora of plasma-membrane Ca^{2+} pumps, we have addressed the problem of isoform diversity in pig smooth muscle. Indeed, electrophoresis of affinity-purified plasma-membrane Ca^{2+}-transport ATPase from smooth muscle showed that these preparations consisted of a complex of three bands [7], whereas different antibody preparations directed against the plasma-membrane Ca^{2+} pump recognized the same complex of bands on Western blots of smooth-muscle plasma membranes (F. Wuytack, unpublished work).

To find out whether these different bands can be ascribed to the expression of different PMCA genes and/or to alternative processing of the PMCA transcripts, we have cloned and sequenced the PMCA cDNA clones derived from a pig-stomach smooth-muscle cDNA library. The PMCA mRNAs have also been analysed by means of Northern blotting and a polymerase chain reaction (PCR). However, we could detect only one single cDNA (PMCA1b). This finding contrasts with the appearance of three different bands on Western blots. We therefore suggest that the PMCA1b is post-translationally modified in smooth muscle.

MATERIALS AND METHODS

Preparation of membranes for immunoblot analysis

Tissues were taken from a 20 kg pig. They were dissected, immediately frozen in liquid N_2 and stored at −80 °C. Approx. 5 g of tissue was thawed in 25 ml of 0.25 M-sucrose containing 0.2 mM-phenylmethanesulphonyl fluoride, 1 μM-leupeptin, 1 μM-pepstatin and 0.1 mM-EDTA. The tissues were homogenized by means of an UltraTurrax (Janke and Kunkel, Staufen, Germany) fitted with an N18 shaft and run at maximal speed for 2 × 20 s. The homogenates were spun for 10 min at 1200 $g_{max.}$ in a Sorvall HS-4 rotor, and the supernatants were filtered through gauze and spun for 15 min at 11000 $g_{max.}$ in a Sorvall SS34 rotor. Finally, the supernatants were spun for 60 min at 103000 $g_{max.}$ in a Beckman Ti60 rotor and the pellets were resuspended in a final volume of 1.5 ml of 55% (w/v) sucrose in 0.6 M-KCl.

The 1.5 ml suspension was transferred to a Beckman SW 50.1 tube and overlaid with 1.5 ml of 45% sucrose and 1.5 ml of 33% sucrose, and the tube was filled with 8% sucrose. All of these sucrose solutions were made up in 0.6 M-KCl and were supplemented with the same proteolytic inhibitor cocktail as the homogenizing medium. The SW 50.1 rotor was spun for 17 h at 156000 $g_{max.}$. Material floating upwards and equilibrating at the 8%/33% interface was collected and diluted 5-fold with water supplemented with proteolytic inhibitors, and the membranes were pelleted for 30 min at 224000 $g_{max.}$ in a Beckman Ti75 rotor. The pellets were resuspended in 0.25 M-sucrose. Ghosts were prepared from pig, human and rat blood by the method of Steck & Kant [8]. Proteins were assayed by the enhanced protocol of the bicinchoninic acid method of Pierce (Rockford, IL, U.S.A.) using BSA as a standard [9].

Abbreviations used: PMCA1–PMCA4, plasma-membrane Ca^{2+} pump isoforms 1–4; nt, nucleotide(s); aa, amino acid(s); PCR, polymerase chain reaction.

* To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X53456.

Western blotting

SDS/PAGE electroblotting and immunodetection on Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA, U.S.A.) by means of 4-chloro-1-naphthol were done as described previously [10].

RNA isolation

Total RNA was extracted from pig gastric smooth muscle (antral part) and pig brain according to the Chirgwin procedure [11]. Poly(A)-enriched RNA was prepared with oligo(dT)–cellulose [12] using a spun column procedure (Pharmacia LKB Biotechnology, Uppsala, Sweden).

cDNA synthesis

The synthesis of a λgt 11 oligo(dT)-primed cDNA library derived from pig smooth muscle has been described previously [13]. A λgt 10 random-primed cDNA library from pig antrum was synthesized as follows: 5 μg of poly(A)$^+$ RNA was random-primed and reverse-transcribed with avian-myeloblastosis-virus reverse transcriptase. Second-strand cDNA was synthesized according to the Gubler & Hoffman method [14]. Double-stranded blunt-ended cDNA was ligated to *Eco*RI adaptors and cloned into *Eco*RI-cut λgt 10 arms. The cDNA library was plated on *Escherichia coli* NM514 strain [15]. Approx. 2×10^6 independent clones were obtained from 150 ng of cDNA. The cDNA library was amplified and stored at 4 °C [16].

Screening procedures

The λgt 11 library was screened with an heterologous probe (an internal *Ava*I fragment of the rat-brain plasma-membrane Ca^{2+} pump; nt 2408–3860 of the class 1 cDNA [2]) and with homologous probes. The λgt 10 library was screened with a homologous probe and with deoxyoligonucleotide probes. Double-stranded cDNA probes were nick-translated with [α-^{32}P]dCTP (400 Ci/mmol) up to a specific radioactivity of 10^8 c.p.m./μg of DNA [17]. Filters were prehybridized, hybridized and washed as described previously [13]. Oligonucleotide probes were 5′-end-labelled with T_4 polynucleotide kinase up to a specific radioactivity of 10^8 c.p.m./μg of DNA [16]. Prehybridization, hybridization and washing conditions were as described previously [18], except that the high-stringency wash was done at 65 °C.

Subcloning and sequencing procedures

Positive clones were picked, purified and subcloned into pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.) for sequence analysis. Sets of overlapping cDNA clones were generated with exonuclease III/S1 nuclease (Erase-a-base kit; Promega) [19]. Sequence analysis was performed on double-stranded plasmid DNA [20] using the Sequenase version 2.0 method [21] (Sequenase kit; United States Biochemical Corporation, Cleveland, OH, U.S.A.).

PCR amplification

Oligo(dT)-primed first-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA from pig stomach smooth muscle and pig brain using Moloney murine-leukemia-virus reverse transcriptase (Gibco, Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). Portions corresponding to 1/200th of the first-strand cDNA mix were then PCR-amplified using *Taq* polymerase (Amersham International, Amersham, Bucks., U.K.). The PCR reaction mixture (50 μl) contained 10 mM-Tris/HCl, pH 8.3 (25 °C), 50 mM-KCl, 1.5 mM-$MgCl_2$, 0.01% (w/v) gelatin, 25 pmol of each 5′ and 3′ primer, 0.2 mM of each deoxynucleotide triphosphate and 2.5 units of *Taq* polymerase. The amplification reaction was conducted in a programmable heat block (model TC-1; New Brunswick Scientific, Brussels, Belgium) for 40 cycles, each cycle consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C and 1 min of extension at 72 °C.

Northern blotting

Glyoxylated poly(A)$^+$ RNAs were electrophoresed and transferred to Hybond N$^+$ membranes as described previously [22]. An anti-sense RNA probe, corresponding to nt 1404–1809 of clone PM2-2, was synthesized *in vitro* with SP6 polymerase using a standard protocol [23], except that 120 μCi of [α-^{32}P]CTP (800 Ci/mmol; 20 mCi/ml) per transcription reaction was used and that the total concentration of CTP was adjusted to 20 μM with unlabelled CTP. After digestion with DNAase, the probe was purified on a Sephadex G-50 spun column [16]. It was labelled up to a specific radioactivity of 6×10^8 c.p.m./μg of RNA. Prehybridization, hybridization and washing of the blots were performed as described previously [22].

RESULTS

Immunoblot analysis of plasma-membrane Ca^{2+}-transport ATPases

Fig. 1 shows an immunoblot stained with the monoclonal antibody 4G5 directed against the plasma-membrane Ca^{2+}-transport ATPase of pig antral smooth muscle and compares plasma-membrane-enriched fractions prepared from different tissues. As can be seen from a comparison of lanes 1–4, plasma-membrane fractions from cerebellum, cerebrum and aortic or stomach (antral) smooth muscle all show at least two, and usually three, different immunopositive bands in the M_r range 130000–140000. The relative intensities of these bands depend on the type of tissue. The same staining pattern was obtained with another monoclonal antibody, 2H9, and with a rabbit polyclonal antiserum against the plasma-membrane Ca^{2+} pump of smooth muscle (results not shown).

Only one immunopositive band was observed for the Ca^{2+}-transport ATPase of the erythrocyte ghosts prepared from pig (Fig. 1, lane 8), man (lane 9) or rat (lane 10). The position of this band coincided with the upper band seen in the other tissue

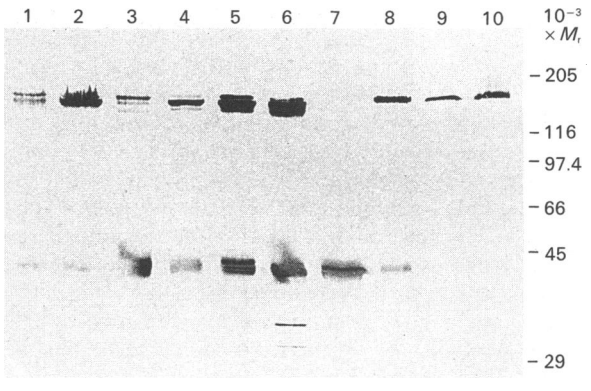

Fig. 1. Comparison of the electrophoretic mobility of the plasma-membrane Ca^{2+}-transport ATPase in different tissues

An immunoblot is shown after reaction of the monoclonal antibody 4G5 against the pig antrum plasma-membrane Ca^{2+}-transport ATPase. SDS/PAGE of a plasma-membrane-enriched fraction was done on a 7.5% slab gel. Lanes received the following types and amounts of membrane protein: 1, pig cerebellum (58 μg); 2, pig cerebrum (96 μg); 3, pig aorta (36 μg); 4, pig antrum (12 μg); 5, pig antrum control for lane 6 (32 μg); 6, pig antrum (43 μg) with provoked proteolysis; 7, pig liver (97 μg); 8, pig erythrocyte ghosts (29 μg); 9, human erythrocyte ghosts (36 μg); 10, rat erythrocyte ghosts (50 μg).

membranes. These different migration patterns are also observed upon Coomassie Brilliant Blue staining of gels of the ATPase purified by means of calmodulin-affinity chromatography or immunoaffinity chromatography using another monoclonal antibody, 2B3 [7].

Fig. 1 (lanes 5 and 6) shows the effect of *post mortem* proteolysis on the ATPases of the antrum. The membranes in lane 5 were prepared taking the utmost care to prevent any artefactual proteolysis, i.e. we used freshly prepared tissue which had been quickly frozen in liquid N_2, and we homogenized the tissue and prepared the membranes under full protection of the proteolysis inhibitors: phenylmethanesulphonyl fluoride and leupeptin against serine and thiol proteinases, pepstatin against acid proteinases and EDTA against metalloproteinases. For the sample in lane 6, we deliberately tried to induce severe *post mortem* autolysis by incubating the tissue for 2 h at 37 °C and omitting the proteolytic protecting agents from the homogenizing medium and fractionating buffers. Nevertheless, an almost identical banding pattern of the ATPase was observed as that in lane 5, and there was no increase in the smaller components of the ATPase complex. Autolysis did however cause a dramatic change in redistribution of proteins during subcellular fractionation. It can therefore be proposed that *post mortem* proteolysis does not contribute to the plasma-membrane Ca^{2+} pump diversity observed on Western blots, and that the different bands may represent distinct plasma-membrane Ca^{2+}-pump isoenzymes. This would be compatible with the demonstration of at least four genes and several alternative splicing modes for the plasma-membrane Ca^{2+} pump.

Isolation of cDNA clones

An oligo(dT)-primed pig smooth-muscle cDNA library was initially screened with a rat PMCA1 cDNA probe (nt 2408–3860 of clone RB 5–10 [2]) which resulted in ten positive clones. One of them, clone PM1-2 (863 bp), contained a short open-reading frame (nt 1–702) encoding 235 amino acids which was almost identical with the 235 *C*-terminal portion of PMCA1b [6]. In order to extend the sequence towards the 5′-terminus of the messenger, we rescreened the same library with PM1-2, which yielded four positive clones. Three of them were 2783 bp in length and contained a 3′-terminus which was identical with that of clone PM1-2. One clone, called PM2-2, was analysed in detail and its reading frame was found to be open up to the 5′-end of the cDNA sequence. The encoded peptide corresponded to aa 346–1220 of PMCA1b, which indicated that the cDNA sequence coding for the *N*-terminus was still lacking. We rescreened the λgt 11 library with a probe corresponding to the 5′-terminus of PM2-2 (an *Nco*I–*Nco*I fragment; nt 1404–1809; Fig. 2). However, all of the positive clones proved to be the same as clone PM2-2. From these results, we concluded that the 5′-terminus of the mRNA encoding the plasma-membrane Ca^{2+} pump from smooth muscle was probably absent from our oligo(dT)-primed library. This could be due either to the presence of a secondary structure in the mRNA, or to the presence of an A-rich region in the mRNA. Interestingly, all of the PM2 clones stopped in an A-rich region corresponding to nt 900–1080 (see Fig. 2). Annealing of the oligo(dT) primer to such an internal A-rich region would result in a premature stop of the first-strand cDNA primed at the 3′ poly(A) tail. To circumvent this problem, we constructed a hexanucleotide random-primed library.

This unamplified random-primed library was screened with the above-mentioned *Nco*I–*Nco*I fragment, and this resulted in the detection of 20 positive clones. After rescreening these with two oligonucleotide probes (corresponding to nt 114–130 and nt 860–885 of the human PMCA1b cDNA [6]), only four clones which reacted with both probes were retained. One clone (PM4-14) was analysed in detail (Fig. 2). It was 1765 bp in length and was found to overlap with clone PM2-2 at its 3′-end over a length of 312 nucleotides.

Sequence analysis was performed on clones PM2-2 and PM4-14 as indicated in Fig. 2. The compiled nucleotide sequence and the deduced amino acid sequence obtained using the two overlapping clones are represented in Fig. 3. The open reading frame encoding the enzyme is 3660 nt in length and is preceded by 415 nt of 5′-untranslated region. It probably begins at the first in-frame methionine residue, as previously suggested by Verma *et al.* [6] for the teratoma clone. The 161 nt 3′-untranslated sequence terminates in an A-rich region but does not contain an upstream polyadenylation signal. The absence of a poly(A) tail probably resulted from oligo(dT) priming to this A-rich region. Therefore the complete 3′-untranslated region of the PMCA1 smooth-muscle mRNA is still lacking.

The isoform described here encodes a protein of 1220 aa (M_r 135000) and is 98% identical to the human PMCA1b isoform [6]. In line with the PMCA nomenclature, we propose that our clone be called pig PMCA1b.

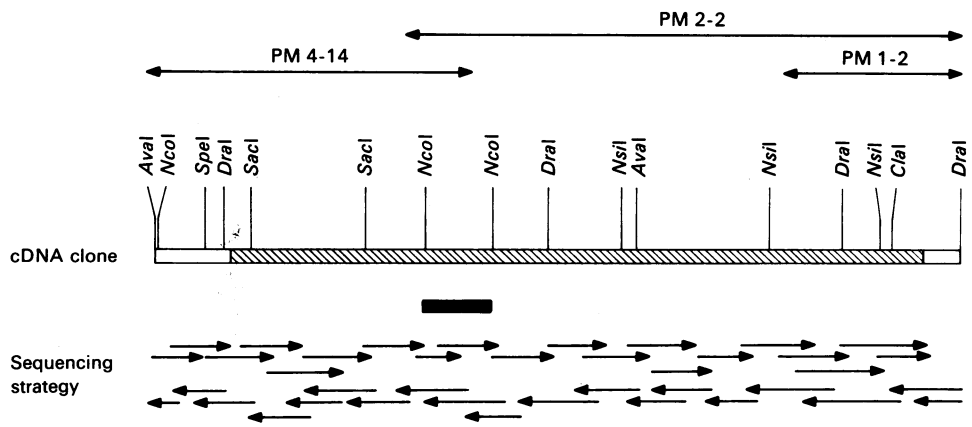

Fig. 2. Partial restriction map and sequencing strategy of the cDNA clones PM1-2, PM2-2 and PM4-14

cDNA clones PM1-2, PM2-2 and PM4-14 are shown schematically. The hatched box indicates the open reading frame and the open box indicates the untranslated region. Sequencing has been performed on clones PM2-2 and PM4-14 in both directions. The most important restriction sites are indicated with vertical lines. The *Nco*I–*Nco*I fragment (nt 1404–1809) used as a probe during different screenings is represented by a black box.

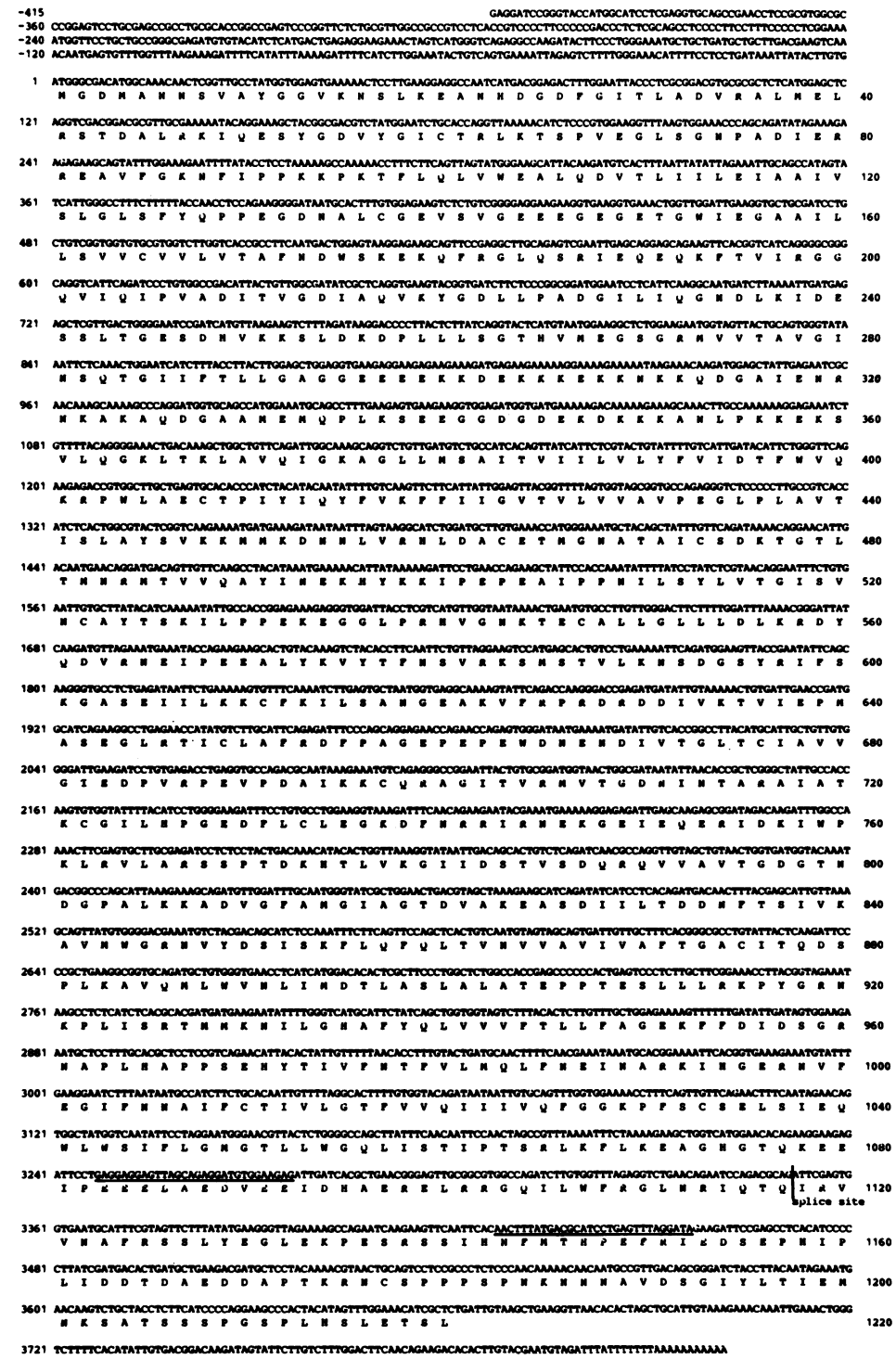

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the pig plasma-membrane Ca^{2+}-pump cDNA

The compiled nucleotide sequence and derived amino acid sequence of the two overlapping clones PM2-2 and PM4-14 are shown continuously. Nucleotides are numbered on the left side, with nucleotide 1 being the A of the putative ATG start codon. Amino acids are numbered on the right side. The splice site involved in the alternative splicing process of PMCA1 is indicated by a vertical line. Sequences corresponding to both primers used in the PCR experiment are underlined.

PMCA1b is the only product of gene 1 which is expressed in smooth muscle

The PMCA1 pre-mRNA can be spliced in four different ways [5]. The splicing pattern in pig smooth muscle was analysed by means of a PCR experiment. For this purpose, two primers were synthesized corresponding to the sequence 5′-GAGGAGGAG-TTAGCAGAGGATGTGGAAGAG-3′ (nt 3662–3691) and the inverted complementary sequence 5′-TATCCTAAACTCAGG-ATGCGTCATAAAGTT-3′ (nt 3842–3871) of the pig PMCA1b clone. These primers encompass the site at which the 154 bp exon

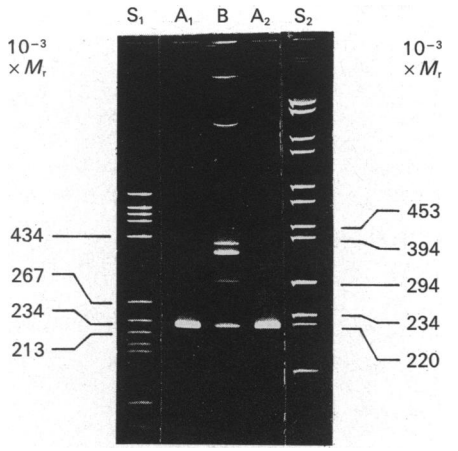

Fig. 4. PAGE of the PCR reaction products

A 6% polyacrylamide gel was run at 250 V for 105 min. Each lane contains 10 μl of PCR mixture. Poly(A)$^+$ RNA of pig antrum (lane A_1), of pig brain (lane B) and the cDNA clone PM2-2 (lane A_2) have been used as templates. M_r markers V and VI from Boehringer (Mannheim, Germany) were used as standards (S_1 and S_2 respectively).

is included totally or partially. PCR amplification of oligo(dT)-primed first-strand cDNA from pig antrum resulted in only one band (Fig. 4, lane A_1). A DNA fragment of the same size was obtained when the cDNA clone PM2-2 was amplified with the same primers (lane A_2). In contrast, six different DNA bands could be amplified using pig brain poly(A)$^+$ RNA as template (lane B). Brain RNA was used as a positive control because it is known that different isoforms of PMCA1 are expressed in this organ [2,3]. According to their length (approx. 220, 298, 350 and 390 bp), the four lower bands amplified from brain mRNA could correspond to the four differently spliced isoforms of PMCA1 described by Strehler *et al.* [5]. However, further analysis is necessary to confirm this.

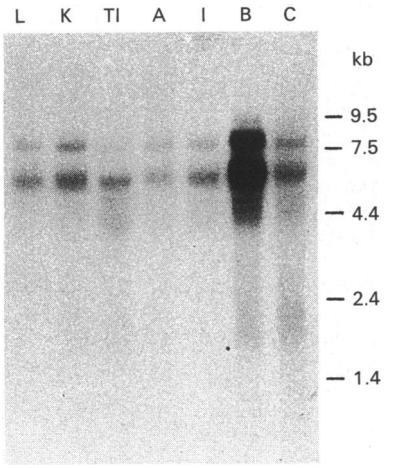

Fig. 5. Northern blot analysis of PMCA1 in different tissues

An anti-sense probe corresponding to the *Nco*I–*Nco*I fragment indicated in Fig. 2 was used to screen the blot containing poly(A)$^+$-enriched RNA of different tissues (5 μg each): liver (L), kidney (K), total intestine (TI), antrum (A), ileum (I), brain (B) and cerebellum (C). The blot was exposed for 7 days at −70 °C with an intensifying screen.

Northern blotting

Fig. 5 shows a Northern blot hybridized with an anti-sense RNA probe derived from pig PMCA1b. For each tissue analysed, we could detect the presence of two mRNAs of approx. 5.5 and 7.5 kb. The PMCA1b cDNA described here is only 4236 nt in length and obviously lacks a considerable 3′-end tail. Within this section which is lacking, two polyadenylation sites might be present, as has been suggested earlier [3].

Low-stringency screening

In order to find out whether other gene products are expressed in smooth muscle in addition to the PMCA1 isoform, we screened our cDNA libraries at different stringency levels. However, low-stringency screening gave no additional signal as compared with the high-stringency screening, indicating that the PMCA1 gene product is probably the only one expressed in smooth muscle.

DISCUSSION

Antibodies (both polyclonal and monoclonal) directed against the plasma-membrane Ca^{2+}-transport ATPase recognize at least two, and usually three, polypeptides in plasma membranes from smooth muscle and other tissues. Furthermore, the Ca^{2+}-transport ATPase purified from smooth muscle, either by calmodulin-affinity chromatography or by means of immunoaffinity chromatography, stained as a double protein band on polyacrylamide gels [7]. These results are reminiscent of those described previously by Sweadner [24] for the α-subunit of the Na^+/K^+-ATPase in the brain tissues of different mammals. Electrophoretic analysis of the α-subunit revealed two closely spaced bands on polyacrylamide gels, which was interpreted as being indicative of the presence of at least two α-subunit isoforms. This has been corroborated by the detection of three different cDNAs encoding the α-subunit of the Na^+/K^+ pump (α_1, α_2 and α_3) [25].

Before considering multigene expression and/or alternative splicing as an explanation for the generation of multiple Ca^{2+}-transport ATPase bands, one should exclude an artefactual *post mortem* proteolysis. This is certainly indicated for the Ca^{2+}-transport ATPase, as many calmodulin-binding proteins are preferential calpain substrates [26]. However, our results suggest that the different immunoreactive polypeptides observed in plasma-membrane fractions from smooth muscle and most other tissues are not the result of a *post mortem* proteolysis artefact, as they are observed equally with and without careful protection against proteolysis. In the light of recent evidence in favour of the expression of two pump genes in the erythrocyte [4], it is remarkable that only one band is observed in this tissue, and this band does not co-migrate with the main band observed in the other tissues.

PMCA isoform diversity has also been analysed at the mRNA level. Repetitive screening of two pig smooth-muscle cDNA libraries with a combination of probes at high and low stringency yielded only PMCA1-derived cDNA clones. As the PMCA1 transcript is subject to alternative splicing, the splicing pattern in smooth muscle was analysed by a PCR. However, only one spliced variant was detected, indicating that only one plasma-membrane Ca^{2+} pump exists at the mRNA level in antral smooth muscle. The smooth-muscle plasma-membrane Ca^{2+} pump is 98% identical with the human PMCA1b isoform and 93% identical with the rat PMCA1a isoform. The PMCA1a and PMCA1b isoforms are almost identical up to amino acid 1117 (Fig. 3), after which they diverge. The PMCA1b isoform is characterized by an alternative calmodulin-binding B domain and by the presence of a putative phosphorylation site for cyclic AMP-dependent protein kinase [6,27].

Northern blot analysis showed the presence of two PMCA1 messengers in the different tissues. This is probably due to alternative polyadenylation of one pre-mRNA. In order to confirm this, further investigation at the genomic level will be necessary. mRNA diversity based upon alternative cleavage/polyadenylation and/or splicing has also been described for the sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump expressed in slow skeletal muscle, cardiac muscle, smooth muscle and non-muscle tissues [22].

In conclusion, immunoblot analysis of the plasma-membrane Ca^{2+} pump in smooth muscle revealed three different immunoreactive peptides. However, only one plasma-membrane Ca^{2+}-pump cDNA could be detected. The encoded isoform corresponds to the PMCA1b type, which is also present in other tissues and which has, because of its ubiquitous expression, been designated as the 'housekeeping' ATPase [3]. The two enzymes observed at the protein level could be due to post-translational modification of one protein.

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