A unique combination of plasma membrane Ca^{2+}-ATPase isoforms is expressed in islets of Langerhans and pancreatic β-cell lines

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Changes in free intracellular Ca^{2+} concentration regulate insulin secretion from pancreatic β-cells. The existence of steep Ca^{2+} gradients within the β-cell requires the presence of specialized Ca^{2+} exclusion systems. In this study we have characterized the plasma membrane Ca^{2+}-ATPases (PMCAs) which extrude Ca^{2+} from the cytoplasm. PMCA isoform- and subtype-specific mRNA expression was investigated in rodent pancreatic α- and β-cell lines, and in human and rat islets of Langerhans using reverse-transcription PCR with primers flanking the calmodulin-binding region of rat PMCA. The expression pattern of PMCA 1 and 2 was conserved in different species and islet-cell types since both rat and human islets of Langerhans and all cell lines tested contained the 1b and 2b forms. PMCA 4 isoform subtypes, however, were expressed in a cell-type-specific manner since β-cells expressed PMCA 4b only, whereas in islets of Langerhans, which contain α, β, δ and polypeptide-secreting cells, PMCA 4a and 4b were simultaneously present. No evidence was obtained for the expression of PMCA 3. Characterization of the β-cell Ca^{2+}-pump protein showed that it shared several similarities with the erythrocyte PMCA. It is a P-type ATPase; its phosphorylated intermediate was stabilized by La^{3+}; it reacted with a PMCA-specific antibody; and it was not N-glycosylated. However, the β-cell PMCA had a higher molecular mass than that of the erythrocyte; this difference could be explained by either predominant translation of the PMCA 2 form, which has a molecular mass 3–8 kDa higher than the erythrocyte PMCA 1 and 4 proteins, or by a possible sequence insertion. Thus a unique combination of functionally distinct PMCA isoforms (1b, 2b, 4b) participates in Ca^{2+} homoeostasis in the β-cell.

INTRODUCTION

Stimulation of pancreatic islets or β-cells with glucose causes a series of metabolic and ionic changes leading finally to insulin secretion [1]. These include enhanced oxygen consumption, a rise in the ATP/ADP ratio, closure of ATP-sensitive K^+ channels, membrane depolarization and influx of Ca^{2+} to raise free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). The sudden increase of $[Ca^{2+}]_i$ has a dual effect. On the one hand, it activates a plethora of Ca^{2+}-dependent enzymes and processes mediating the cellular response to the stimulation. It also immediately triggers compensatory mechanisms which aim to reduce $[Ca^{2+}]_i$ back to its resting level. The maintenance of normal Ca^{2+} homoeostasis during sustained cellular activity must involve the ultimate removal of Ca^{2+} from the cell which is accomplished by two main mechanisms: an Na^+/Ca^{2+} exchanger and an ATP-dependent Ca^{2+}-pump [2,3].

The plasma membrane Ca^{2+}-ATPase(s) (PMCAs) belong to the P class of ion-motive ATPases which form a phosphorylated intermediate during the reaction cycle [4]. PMCA is directly regulated by calmodulin which binds to the calmodulin-binding domain near the C-terminus. There is extensive diversity among this family of enzymes. Full-length cDNA encoding four distinct rat PMCA isoforms have been identified [5–7] and additional variability is produced by alternative mRNA splicing at two sites, A and C. Site A includes exons that encode residues in the vicinity of a phospholipid-responsive domain located in the cytoplasmic loop between the second and third transmembrane domains [8]. Splice site C occurs in a region of the pump that binds to calmodulin. Changes in the C-terminal PMCA structure by alternative splicing lead to important regulatory and functional differences between variant isoforms. It has been shown that the b forms (formed by exclusion of the exon at site C) have higher affinity for calmodulin and, as a result, also for Ca^{2+} [9].

In addition to their functional differences, PMCA isoforms show tissue-specific expression [6,7,10–16]. This selective distribution pattern suggests that different cells need specialized Ca^{2+}-pump mechanisms depending on their specific requirements for Ca^{2+} homoeostasis. Functional differences of the Ca^{2+}-transport ATPase(s) in different cell types can be achieved by regulation of the mode of alternative splicing of the gene transcript and expression of the appropriate isoforms [17].

In this study we have investigated the expression pattern of PMCA isoforms and isoform subtypes in β-cell lines and islets of Langerhans from different species. Because of the functional significance of alternative splicing in the calmodulin-binding region, we focused on isoform subtypes in this area. The functional β-cell PMCA proteins were also characterized in comparison with the best-studied erythrocyte PMCA.

EXPERIMENTAL

Materials

Male Wistar rats (200–250 g) were from Harlan UK, Bicester, U.K. Human blood was obtained from the National Blood

Abbreviations used: $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; DTT, dithiothreitol; PMCA, plasma membrane Ca^{2+}-ATPase; RT, reverse transcription; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; tBHQ, 2,5-di(t-butyl)-1,4-benzohydroquinone.

In this paper we use the term isoform for the four different gene products and refer to isoform subtypes or splicing variants when we mean the alternatively spliced mRNA molecules.

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Transfusion Centre (Safety Tested, Donor Blood), Oxford, U.K. Human islets of Langerhans were obtained from the Transplant Centre, Churchill Hospital, Oxford, U.K. Cell lines were generously provided by Professor Y. Miyazaki, University of Tokyo (MIN6), the late Professor A. E. Boyd III, Tufts University, Boston (HIT T15), Professor C. B. Wollheim, University of Geneva (RINm5F) and Professor D. Hanahan, Cold Spring Harbour Laboratory, New York (αTC and βTC). Monoclonal anti-(human erythrocyte PMCA) antibody (Clone 5F10) was purchased from Cambridge Bioscience, Cambridge, U.K. Collagenase (Type Ia), anti-(mouse IgG) alkaline phosphatase-labelled antibody and all tissue-culture materials were obtained from Sigma, Poole, Dorset, U.K. Protein molecular-mass markers were from Pharmacia Biosystems, Milton Keynes, U.K. DNA molecular-mass marker (pBR322/*Hae*III + pBR322/*Taq*I) was purchased from Appligene, Durham, U.K. DNA oligonucleotide primers were obtained from Cruachem Ltd., Glasgow, Scotland. N-Glycosidase F (PNGase F) was from Boehringer Mannheim GMBH, Germany. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham, Little Chalfont, U.K.

Cell culture

αTC pancreatic α-cell and RINm5F, MIN6, HIT T15 and βTC pancreatic β-cells were cultured in RPMI 1640 tissue-culture medium containing penicillin (100 units/ml), streptomycin (0.1 mg/ml) and fetal-calf serum (10 %, v/v) at 37 °C in an atmosphere of humidified air (95 %) and CO_2 (5 %) as described previously [18]. Cells were passaged weekly and harvested using trypsin-EDTA. They were seeded in culture flasks at a density of 4×10^7 cells per flasks and cultured for 5 days before RNA or membrane preparation.

Isolation of human and rat islets of Langerhans

The pancreas was removed (with permission) from a heart-beating cadaver of a 62-year-old female, at the same time as retrieval of other organs for organ donation. The pancreas was perfused with cold hypertonic citrate solution and processed for islet isolation using intraductal collagenase distension [19]. The islets were purified using continuous Ficoll density-gradient centrifugation [20]. Following islet purification, the islets were washed in University of Wisconsin solution at 4 °C [21] and left in this solution overnight.

Rat islets were obtained by collagenase digestion of the pancreas [22]. The isolated islets were washed three times in HBSS buffer (pH 7.4) [1.28 mM $CaCl_2$, 0.82 mM $MgSO_4$, 5.5 mM KCl, 0.35 mM K_2HPO_4, 142 mM NaCl, 0.4 mM NaH_2PO_4, 5.5 mM D-glucose, 20 mM Hepes, 0.5 % (w/v) BSA] and selected under a dissecting microscope. For RNA preparation, all the solutions were prepared using diethyl pyrocarbonate-treated water.

RNA preparation

Total RNA was prepared by a single-step method using guanidinium isothiocyanate [23]. The integrity of the RNA was verified by electrophoresis on agarose–formaldehyde gels [24].

Reverse transcription (RT) and PCR

The total RNA (5 μg) was reverse transcribed at 42 °C for 60 min in 50 μl of reaction mixture containing 1 × reverse transcriptase buffer [50 mM Tris/HCl (pH 8.3), 40 mM KCl, 1 mM dithiothreitol (DTT), 6 mM $MgCl_2$], 100 pmol of each 3′-end primer, 0.5 mM of each dNTP, 10 mM DTT, 40 units of rRNasin (Promega Co., Madison, U.S.A.), and 500 units of M-MLV reverse transcriptase (Gibco BRL, Uxbridge, Middlesex, U.K.).

One-tenth of the cDNA was subjected to a PCR containing 1 × PCR buffer [50 mM KCl, 10 mM Tris/HCl (pH 9.0), 5 mM DTT], 1–4 mM $MgCl_2$, 0.1 mM of each dNTP, 40 pmol of each primer and 1 unit of *Taq* DNA polymerase (Promega Co.). PCR was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Essex, U.K.) at 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 10 min. After 30 cycles, a 10 μl aliquot was subjected to electrophoresis in a 1.9 % (w/v) agarose gel containing 0.4 μg/ml ethidium bromide in 1 × TBE buffer. For DNA sequencing, the PCR products were fractionated by electrophoresis in 1.3 % (w/v) low-melting-point agarose gels. The separated bands were isolated and the PCR products were extracted from gel slices by Wizard PCR Preps DNA Purification System (Promega) and then ethanol-precipitated. Specific primer pairs for isoform 1, 2, 3 and 4 mRNAs of rat PMCA flanking the calmodulin-binding region were used to detect the different isoform-specific mRNAs and their splicing variants (Table 1). The sequences were derived from previously published data [5–7,25]. All experiments were repeated at least five times.

We used the following multiple controls to check for possible amplification of contaminant DNA and RNA by PCR: (1) RNA blanks taken throughout the cDNA synthesis step in the absence of reverse transcriptase were used in every PCR reaction, for each set of primers; (2) samples without templates were run for every primer pair for each PCR experiment; and (3) rat brain positive control RNA was run for each experiment.

Sequencing of PCR products

Each non-cloned RT-PCR product was completely sequenced using the Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, OH, U.S.A.). Purified PCR products (1.5–3.0 pmol) were denatured at 95 °C for 3 min in reaction mixture containing a 10-fold molar excess of sequencing primers, and 1 × sequenase buffer [40 mM Tris/HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl]. The primers used for sequencing were either the original primers used to produce the PCR products or internal primers. The above reaction mixture was kept on ice for 10 min, at room temperature for a further 20 min and then sequenced by the dideoxynucleotide chain termination method according to the supplied protocol. All sequencing products were separated on 6 % (w/v) polyacrylamide gels using SequaGel-6 sequencing gel solutions (National Diagnostic Inc., Hessle, U.K.).

Membrane preparation

The membranes from cultured cells and from islets of Langerhans were prepared as described previously [26]. The preparation of human erythrocyte ghosts was performed according to Sarkadi et al. [27].

Formation of the PMCA phosphoenzyme intermediate

For formation of the phosphoenzyme intermediate, 0.5–1.0 mg/ml membrane proteins were resuspended in 30 mM Hepes/KOH (pH 7.0), 75 mM KCl, 50 μM $LaCl_3$ and 20 μM $CaCl_2$ [28]. The membrane suspension was placed on ice, and the reaction was started by the addition of [γ-^{32}P]ATP (0.8 pM final concentration). The reaction was stopped after 1 min by the addition of ice-cold 12 % (w/v) trichloroacetic acid containing

Table 1 Position and sequences of PCR primers used

All primers were based on rat PMCA cDNA sequences (rPMCA 1 and rPMCA 2 [5]; rPMCA 3 [6]; rPMCA 4 [7]). The numbers specify the 5′ position of the primer sequence on the cDNA. For clarity, we number the primers (1–12) and refer to these numbers in the text.

Gene specificity	Direction	Sequence	Starting base	Splicing variants	Length of PCR products	Primer number
PMCA 1	Forward	5′-TAGGCACCTTTGTGGTGCAG-3′	3305	1a	585 bp	1
				1b	431 bp	
				1c	518 bp	
				1d	545 bp	
	Forward	5′-GGGAGTTGCGGCGTGGCCAG-3′	3557	1a	333 bp	2
				1b	179 bp	
				1c	266 bp	
				1d	293 bp	
	Reverse	5′-CGGCTCTGAATCTTCTATCC-3′	3890			3
PMCA 2	Forward	5′-AAGATCCACGGCGAGCGTAAC-3′	3460	2a	787 bp	4
				2b	560 bp	
				2c	732 bp	
	Forward	5′-CACCATCCCTACCAGCAGGCT-3′	3663	2a	584 bp	5
				2b	357 bp	
				2c	529 bp	
	Forward	5′-CATCCATAACTTCATGGCTCA-3′	3906	2a	341 bp	6
				2b	114 bp	
				2c	286 bp	
	Reverse	5′-GCTCGAGTTCTGCTTGAGCGC-3′	4020			7
PMCA 3	Forward	5′-GTCCAATTTGGAGGGAAGCC-3′	3714	3a	646 bp	8
				3b	492 bp	
				3c	579 bp	
	Forward	5′-CAACCGGATCCAGACACAGA-3′	3977	3a	383 bp	9
				3b	229 bp	
				3c	316 bp	
	Reverse	5′-CGTTGTTGTTCTGGTTAGGG-3′	4360			10
PMCA 4	Forward	5′-CTGAACCGGATTCAGACTCA-3′	3456	4a	388 bp	11
				4b	213 bp	
	Reverse	5′-CAGCATCCGACAGGCGCTTG-3′	3844			12

2 mM ATP and 20 mM KH_2PO_4. The denatured proteins were collected by centrifugation (8000 ***g***) for 5 min and washed twice with the above solution. The final precipitates were dissolved in the electrophoresis sample buffer and separated on 7.5 % (w/v) acidic SDS/polyacrylamide gels [29], stained with Coomassie Brilliant Blue, dried, and exposed to Kodak X Omat R film at −70 °C for 24–72 h.

Enzymic deglycosylation of membrane proteins

Membranes prepared from MIN6 β-cells were resuspended in 50 mM Na_3PO_4 buffer (pH 7.0) to a final concentration of 10 mg of protein/ml. The membranes were denatured by boiling in 1 % (w/v) SDS for 10 min and then octyl β-D-glycopyranoside was added to give final concentrations of 2 % (w/v) octyl β-D-glycopyranoside, 0.1 % (w/v) SDS and 1 mg of protein/ml in 50 mM Na_3PO_4 (pH 7.0). The reaction mixture was then incubated at 37 °C with and without 6 units/ml N-Glycosidase F for 4 h and reactions were terminated by boiling in SDS sample buffer [30].

Electrophoretic transfer and immunoblot analysis of membrane samples

Electrophoresis was performed on either 1-mm-thick 9 % polyacrylamide gels in the presence of SDS using the buffer system of Laemmli [31], or 1-mm-thick 7.5 % acidic SDS/polyacrylamide gels [29]. Proteins were transferred electrophoretically on to poly(vinylidene difluoride) microporous membrane (Immobilon; Millipore, Bedford, MA, U.S.A.) using an AE-6675 Horizoblot Electrophoretic Transfer Unit with discontinuous buffer system for 2 h at room temperature as recommended by the manufacturer (ATTO, Tokyo, Japan). The Immobilon sheets were blocked overnight at 4 °C with 5 % (w/v) non-fat dry milk in a 1:50 dilution of normal pig serum in PBS. They were then incubated with 5F10 monoclonal anti-(human erythrocyte Ca^{2+}-ATPase) antibody diluted (1:1000) in blocking solution for 12–16 h at 4 °C. After washing twice for 5 min in PBS containing 0.05 % (v/v) Tween-20 and once with blocking solution, the membrane filters were incubated for 3 h at room temperature with alkaline phosphatase-conjugated anti-(mouse IgG) diluted 1:7500 in blocking solution, followed by several rinses with PBS containing 0.05 % Tween-20 for 20 min. The bound antibodies were visualized by addition of substrate solution according to the manufacturer's protocol (ProtoBlot AP; Promega Co.).

RESULTS

Identification of PMCA 1 isoform subtype-specific mRNAs in rodent pancreatic α- and β-cell lines, and rat islets of Langerhans

The splicing process leads to the inclusion or exclusion of one or more complete or partial exons. The spliced forms with full exons have been designated 'a' and those without inserts 'b'. The mRNAs derived from the PMCA 1 gene have four known spliced forms designated PMCA 1a–PMCA 1d (Table 1). Insertion of portions (87 bp or 114 bp) of a single exon of 154 bp with multiple internal donor sites gives rise to isoforms 1c and 1d respectively. Using primers 1 and 3 to amplify PMCA 1 sequences

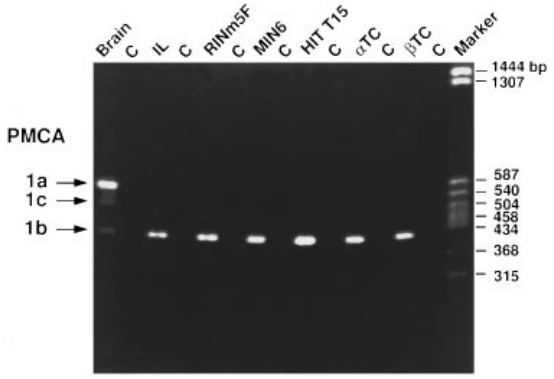

Figure 1 PCR analysis of PMCA 1 isoform subtype-specific mRNAs in pancreatic α- and β-cells and in rat islets of Langerhans using primer pair 1 and 3

Detection in cultured pancreatic α- (αTC) and β-cell lines (RIN5mF, MIN6, HIT T15, βTC) and rat islets of Langerhans (IL) of mRNAs for the PMCA 1 isoform and its splicing variants was performed by RT-PCR as described in the Experimental section. The products of the PCR reaction were separated on a 1.9% (w/v) agarose gel and stained with ethidium bromide. Rat brain, which is known to express PMCA 1a, 1c and 1b forms, was used as the positive control. The 585 bp band corresponds to PMCA 1a which contains a 154 bp alternative exon at the calmodulin-binding site (upper band); PMCA 1c has a 87 bp exon and amplification yielded a 518 bp fragment (middle band); and the 431 bp PCR product (lower band) represented PMCA 1b which does not contain the 154 bp exon. C: RT-PCR control (RNA without reverse transcriptase). Markers: 1444, 1307, 587, 540, 504, 475, 458, 434, 368, 315, 267, 234, 213, 192, 141 bp (identical in all further Figures).

across alternative splice site C in pancreatic cell lines and islets of Langerhans, a single PCR product was obtained (Figure 1). The size of the PCR product (431 bp) was consistent with the form known as PMCA 1b in which the 154 bp exon is completely excluded (Table 1). Rat brain, which is known to express PMCA 1a, 1b and 1c alternative-spliced forms [25], was used as a positive control. In brain the 1a (585 bp) form was the most abundant while 1b (431 bp) and 1c (518 bp) forms were also present, in much lower amounts (Figure 1).

To confirm the data obtained with primer pair 1 and 3, we re-amplified the above-purified PCR products using PMCA 1-specific primers 2 and 3. Primer 2 is 252 bp downstream from primer 1 (Table 1). In pancreatic cell lines and islets of Langerhans, a single re-amplified product of 179 bp was obtained which was equated with the 1b spliced form. In brain, a 333 bp re-amplified PCR fragment, which corresponded to the 1a form, was again the most dominant.

To further confirm the above data, the non-cloned PCR fragments amplified by the primer pair 1 and 3 were sequenced completely using 1, 2 and 3 primers. The sequence obtained from each PCR product was identical to the published sequence of the PMCA 1b cDNA. Thus, rodent pancreatic α- and β-cell lines and rat islets of Langerhans express only the b spliced form of PMCA 1.

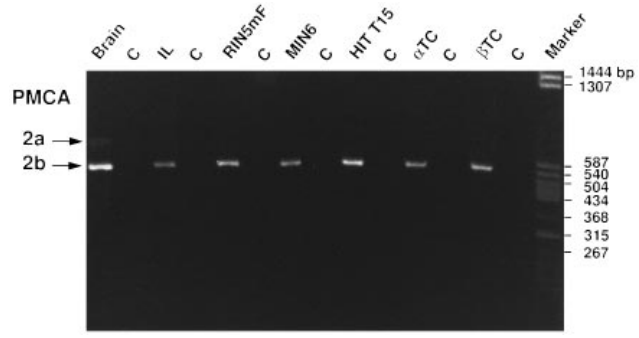

Figure 2 PCR analysis of PMCA 2 isoform subtype-specific mRNAs in pancreatic α- and β-cells and in islets of Langerhans using primer pair 4 and 7

The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. PMCA 2a PCR product at splice site C had a length of 787 bp containing the 227 bp alternative exon (upper band), while the amplification of PMCA 2b which excludes this exon yielded a product of 560 bp (lower band). C: RT-PCR control (RNA without reverse transcriptase). The positions and sizes (in bp) are indicated on the right.

Identification of PMCA 2 isoform subtype-specific mRNA expression in pancreatic cell lines and rat islets of Langerhans

There are two exons (172 bp and 55 bp) flanked by the primer pairs that define the alternative splice site C of PMCA gene 2. The splicing variants are classified as 2a (insertion of the 227 bp sequence), 2b (no insert) and 2c (insertion of the 172 bp exon only). Using primers 4 and 7 to amplify PMCA 2 isoform subtype-specific mRNAs, a 560 bp product was obtained in islets of Langerhans and in all cultured pancreatic cell lines, indicating the presence of the 2b form (Figure 2). There was no evidence from RT-PCR and subsequent analysis of the products for the existence of additional splice forms. In brain also, the splice form 2b was the predominant species, while 2a and 2c were also expressed but in very low amounts (Figure 2).

After re-amplification of the above products using the PMCA 2-specific primer pair 5 and 7 (primer 5 is 203 bp downstream from primer 4, see Table 1), a single product was observed. The size of the fragment (357 bp) was again consistent with the presence of the PMCA 2b form. In brain, PMCA 2b was represented by the 357 bp fragment and the expression of the hardly visible 2a (584 bp) and 2c (529 bp) forms was more obvious after re-amplification.

The PMCA 2 isoform has previously been found to be expressed in a relatively tissue-specific manner [10]. Because of its rare expression outside the central nervous system we used primers 4, 5, 6 and 7 for the complete sequencing of the 560 bp PCR product to confirm its identity in β-cells. The sequences obtained by four separate sequencing reactions were identical to the published sequence of the rat PMCA 2b cDNA. Thus, rat islets of Langerhans, pancreatic α- and β-cells all express the mRNA for the PMCA 2b form.

Analysis of PMCA 1 and 2 isoform expression in human islets of Langerhans

Primers flanking the calmodulin-binding region of rat PMCA were used in each experiment. The cDNA sequences of rat and human PMCA 1 and 2 isoforms are highly homologous [32]. Therefore for the amplification of cDNA from human islets of Langerhans, we were able to use the same PMCA 1 (1 and 3, 2 and 3) and PMCA 2 (4 and 7, 5 and 7) specific primer pairs as we used for rat. Using primers 1 and 3 to amplify PMCA 1 sequence, a 431 bp PCR product was obtained. After re-amplification of this fragment by primers 2 and 3 a single 179 bp product was observed. PCR of PMCA 2 isoform using primers 4 and 7 yielded a 560 bp fragment whose re-amplification by primers 5 and 7 resulted in a 357 bp product (results not shown).

The amplification by PMCA 1- and PMCA 2-specific primer pairs using cDNAs from different rodent pancreatic α- and β-cell lines, and rat and human islets of Langerhans resulted in products which correspond to PMCA 1b and 2b forms, respectively. There

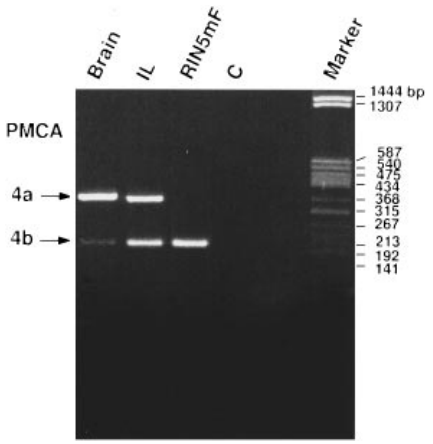

Figure 3 PCR analysis of PMCA 4 isoform subtype-specific mRNAs in RIN5mF β-cells and islets of Langerhans using primer pair 11 and 12

For detection of the mRNAs for the PMCA 4 alternative splice forms, RT-PCR reaction was used as described in the Experimental section. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The 388 bp PCR product represented PMCA 4a mRNA containing the 175 bp alternative exon (upper band). The second splicing variant was PMCA 4b (213 bp, lower band) lacking this exon. C: control (PCR reaction without template).

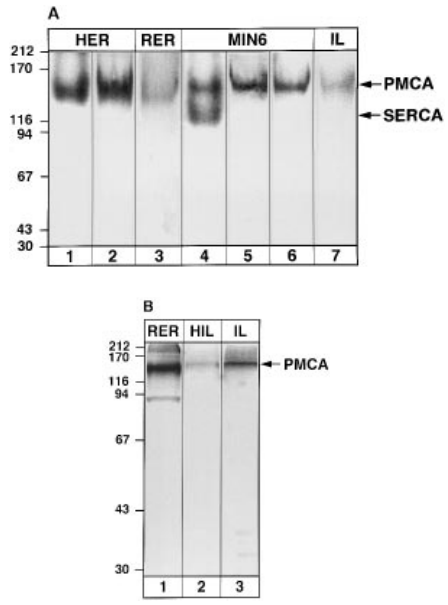

Figure 4 Phosphorylation and immunochemical detection of PMCA in membranes from MIN6 pancreatic β-cell and from human and rat islets of Langerhans

Phosphorylation with [γ-^{32}P]ATP was carried out as described in the Experimental section. Phosphorylated membrane proteins (50 μg) from MIN6 β-cells and human erythrocytes (HER) were separated on acidic SDS/7.5%-polyacrylamide gels and autoradiographed (**A**, lanes 1 and 4). For immunolabelling, membrane proteins (100 μg) from MIN6 β-cells, human (HER) and rat (RER) erythrocytes, human (HIL) and rat (IL) islets of Langerhans were separated by SDS/PAGE on either 7.5% (w/v) acidic gels (**A**) or 9% Laemmli gels (**B**). The gels were electroblotted on to Immobilon membranes and incubated with a 1:1000 dilution of 5F10 monoclonal anti-(human erythrocyte PMCA) antibody. The bound antibody was visualized by reaction with alkaline phosphatase-conjugated anti-(mouse IgG) antibody (1:7500 dilution) (**A**, lanes 2, 3, 5–7; **B**, lanes 1–3). The MIN6 membranes were deglycosylated using N-Glycosidase F and the enzyme-treated products were immunochemically detected using 5F10 antibody (**A**, lane 6). The positions and sizes (in kDa) of the molecular-mass standards are indicated on the left.

was no PMCA 1 and 2 isoform subtype present other than b form in any of the examined cells or tissues. Hence, the expression pattern of PMCA 1 and 2 isoforms in insulin-secreting cells is conserved in different species.

PCR analysis of alternatively spiced PMCA 3 mRNAs at splice site C

For the detection of PMCA 3 mRNAs by RT-PCR, specific primer pairs 8 and 10 and 9 and 10 were used. In brain the PMCA 3a and 3b forms were present (results not shown). However, we were not able to demonstrate the presence of PMCA 3 mRNAs in any of the pancreatic cell lines or islets of Langerhans.

Analysis of alternatively spliced PMCA 4 mRNAs at splice site C in RINm5F β-cells and islets of Langerhans

The splicing pattern of isogene 4 is relatively simple compared with isogene 1 or 2. Only two alternatively spliced variants have been detected, corresponding to the insertion (4a) or exclusion (4b) of the 175 bp exon. Using rat tissues the RT-PCR resulted in two fragments of 388 bp and 213 bp (Figure 3). The 388 bp fragment is consistent with the presence of PMCA 4a, and the 213 bp product corresponds to PMCA 4b (Table 1). In islets of Langerhans, both PMCA 4a and 4b were present in about equal amounts. In RINm5F β-cells, however, PMCA 4b was the only form expressed. The predominant form in brain was PMCA 4a (388 bp), while PMCA 4b (213 bp) was also present but in a much lower amount (Figure 3).

The two PCR products from islets and RINm5F cells were sequenced using specific primers 11 and 12. The sequences of the upper band (388 bp) and the lower band (213 bp) were identical to the published sequences of the rat PMCA 4a and PMCA 4b cDNA respectively. Thus β-cells express the 4b isoform subtype whereas other islet cell types (α, δ and polypeptide-secreting cells) additionally express 4a.

Amplification of mRNAs from mouse cell lines (αTC, βTC, MIN6), a hamster cell line (HIT T15) and human islets of Langerhans failed to produce PCR fragments which could have corresponded to any of the PMCA 4 alternatively spliced forms. These results are consistent with the known species variability of PMCA 4 at splice site C [7].

Characterization of PMCA protein

The properties of pancreatic β-cell PMCA protein were analysed and compared with the well-characterized erythrocyte PMCA. The Ca^{2+}-transport ATPases in MIN6 β-cell membranes were selectively labelled with [γ-^{32}P]ATP using special conditions for phosphorylation (see the Experimental section). To prevent the hydrolysis of the acyl phosphate intermediates, the phosphorylated proteins were separated on acidic gels [26,29]. Membranes from erythrocytes were used as controls because the only Ca^{2+}-transport ATPase they contain is the PMCA protein. A radioactive band of 140 kDa was observed in human erythrocytes (Figure 4A, lane 1), whereas two bands of 115 and 145 kDa were detected in MIN6 β-cell membranes (Figure 4A, lane 4). The lower band, which was absent in the erythrocytes, corresponds to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump since, as we showed previously [26], its phosphoenzyme intermediate formation was inhibited by SERCA-specific inhibitors [thapsigargin and 2,5-di(t-butyl)-1,4-benzohydroquinone (tBHQ)] and it cross-reacted with SERCA-specific antibodies.

To confirm that the 145 kDa phosphorylated protein is PMCA, immunochemical detection was used. The 5F10 antibody, which was raised against the human erythrocyte PMCA, reacted with the 145 kDa protein in MIN6 cells (Figure 4A, lane 5), human (Figure 4B, lane 2) and rat islet of Langerhans (Figure 4A, lane 7 and Figure 4B, lane 3) and with the 140 kDa band in human (Figure 4A, lane 2) and rat erythrocytes (Figure 4A, lane 3).

The molecular mass of the β-cell and islet PMCA enzyme consistently exceeded that of the erythrocyte pump by 5 kDa. This difference was observed when either acidic [29] or Laemmli [31] gel electrophoresis was used (Figures 4A and 4B). This altered molecular mass can not be explained by species differences because the 5F10 antibody cross-reacted with human and rat islets of Langerhans (Figure 4B, lanes 2 and 3) and also with erythrocyte membranes from both species (Figure 4A, lanes 2 and 3). The antibody labelled identical bands in the same tissue independently of its species origin. To investigate the possibility that glycosylation contributed to the molecular mass difference we used enzymic deglycosylation, which specifically removes N-linked sugars. As the result shows (Figure 4A, lane 6) the β-cell PMCA was not N-glycosylated. The efficiency of our enzymic deglycosylation procedure has been verified in parallel studies characterizing ionotropic glutamate receptor subunits in MIN6 membranes in which we observed a positive deglycosylation reaction [30].

DISCUSSION

The expression pattern of PMCA isoform and spliced variant mRNAs was analysed in islets of Langerhans and β-cell lines by RT-PCR. The data obtained indicate that β-cells express a unique combination of PMCA isoform subtypes. We found that all different rodent pancreatic α- and β-cell lines, human and rat islets of Langerhans expressed PMCA 1b and 2b forms. Thus, the expression pattern of these isoforms is conserved in different species and islet cell types. We could find no evidence for the presence of PMCA 3 isoforms in either pancreatic cell lines or in islets of Langerhans. In the pancreatic β-cell line RINm5F we found PMCA 4b mRNA. However, in islets of Langerhans, which in addition to β-cells also contain α-, δ- and pancreatic polypeptide-containing cells, we detected simultaneous expression of PMCA 4a and 4b mRNAs. There are two possible explanations for this. The first is that the insulinoma cell line (RINm5F) expresses a different PMCA 4 spliced form compared with the normal β-cell. The second, which is more likely, is that the non-insulin-containing cells of the islets of Langerhans express PMCA 4a and 4b forms simultaneously whereas β-cells contain solely the PMCA 4b form. The second explanation is supported by a recent study in which the distribution of PMCA 4 mRNAs was examined in human brain regions; it was found that hypothalamus, inferior olive, olfactory bulb and substantia nigra expressed the 4b form and all other brain regions contained 4a and 4b; 4a alone was never observed [33]. This clearly shows that functionally distinct tissue regions or cells can express different PMCA isoform subtypes from the neighbouring cells.

The four PMCA genes are not equally expressed in all tissues. PMCA 2 and 3 forms are more specialized and are expressed almost exclusively in the central nervous system [6,12–14]. PMCA 1 has been found to be transcribed in all tissues examined in approximately equivalent amounts [10–12]. PMCA 4 mRNAs are also expressed in most tissues [14,15] and it has been suggested that PMCA 4 may serve a housekeeping function [12]. However, in some recent studies substantial variations in PMCA 4 mRNA levels in adult rat tissues were found [7,16], indicating that variants of PMCA 4, at least in rat, might be involved in more specialized functions. There is little information about the distribution of different isoforms at the single cell level although a few reports suggest the spatial polarization of pump distribution. In hepatocytes, the pump is localized predominantly in the sinusoidal pole [34], and in pancreatic acinar cells different kinetic and functional properties of PMCA activity were found at the two poles of these cells [35]. A recent study on regional and cellular distribution of PMCA 1 mRNAs in rabbit intestinal tissues suggests region-specific transcriptional regulation of the PMCA 1 gene [36].

The variety of iso- and alternatively spliced forms is likely to be associated with functional differences. Hofmann et al. [37] have identified two Ca^{2+}-binding sites in the 1b form which lie in the region that is influenced by alternative splicing at site C. Inclusion of the exon leading to form 1a abolishes these Ca^{2+}-binding sequences. Another functional consequence of the splicing is the absence of the consensus sequence for protein kinase A phosphorylation in the PMCA 1a variant [38]. The 4a form has about a 10-fold lower affinity for Ca^{2+}-calmodulin than the 4b form. This reduced calmodulin affinity caused a reduced affinity for Ca^{2+} [9,39]. It was also observed that alternative splicing confers pH dependence to the regulation by calmodulin of the activity of PMCA 1 isoform subtypes [40]. The Ca^{2+}-dependent ATPase activity of the PMCA 2 pumps has a 5–10-fold higher affinity for calmodulin and ATP than the PMCA 4 pump [41].

In β-cells, the entry of Ca^{2+} that precedes exocytosis is unevenly distributed over the cell and is concentrated to the region with the highest density of secretory granules. In this region, the $[Ca^{2+}]_i$ is 5–10-fold higher than elsewhere in the cytosol, reaching concentrations of several micromolar; single-channel recordings confirm that the L-type Ca^{2+} channels are clustered in the part of the cell membrane adjacent to this region [42,43]. Because of this polarization the different regions of the β-cell probably require different PMCA activities. This could be achieved by either expressing functionally distinct PMCA isoforms or by differential cellular distribution of their expression. The expression of such forms (1b, 2b and 4b) strongly suggests functional specialization of PMCAs in meeting the physiological requirements of the β-cell. It is also possible that, similarly to the L-type Ca^{2+} channels, these functionally different PMCA isoforms display a differential distribution within the cell, but this needs further investigation.

It is likely that each of the PMCA transcripts characterized here will result in a functional protein since it has been shown that the mRNA expression pattern of the isoforms and spliced forms agrees reasonably well with the data derived by using isoform-specific antibodies [44]. The molecular masses of islet and β-cell PMCAs exceeded that of the erythrocyte PMCA by 5 kDa. This difference was not due to N-glycosylation. One possible explanation is that the dominantly translated form in β-cells is the PMCA 2 protein, which has a molecular mass 3–8 kDa higher than the 1 and 4 forms [44]. In human and rat erythrocytes those two forms are expressed, but PMCA 2 is not present. The possibility of a sequence insertion outside the calmodulin-binding region in the β-cell PMCA could also explain the molecular mass difference. The properties of PMCA protein of the pancreatic β-cell share several similarities with PMCAs of other eukaryotic cells. The β-cell pump is evidently a P-type ATPase, its phosphorylated intermediate is stabilized by La^{3+}, and the autophosphorylation reaction is insensitive to thapsigargin and tBHQ. The limited proteolysis reaction showed a typical tryptic cleavage pattern and both the full enzyme and its proteolytic fragment cross-reacted with a PMCA-specific antibody [26].

In vivo, differential splicing patterns allow fine-tuning of the

pump activity since the enzyme isoforms have variable sensitivity towards different regulators. Such specialization could underlie the ability of PMCA to meet the specific cellular requirements for Ca^{2+} homoeostasis in a tissue-selective manner. Because the PMCA plays an important role in the control of intracellular calcium it can be considered as a potential site for altered ionic regulation in disease states. It has been proposed that abnormal intracellular calcium metabolism causes insulin resistance and impaired insulin secretion and may be a common denominator for the constellation of type-2 diabetes, hypertension, obesity and vascular disorders [45–47]. Since PMCA, as shown here, displays a high isoform variability in β-cells and islets of Langerhans, it is an important candidate to be examined for its spatial distribution, changes in isoform expression pattern and potential role in diabetes.

We thank Professsor Y. Miyazaki (University of Tokyo), Professor C. B. Wollheim (University of Geneva), and Professor D. Hanahan (Cold Spring Harbour Laboratory, New York) for providing cell lines, and Mr. D. W. Gray and A. Cahill (The Transplant Centre, Churchill Hospital, Oxford) for providing human islets of Langerhans. We are grateful to Dr. Virginia Urquidi for advice. These studies have been supported by the British Diabetic Association and the Wellcome Trust. A. V. is an R. D. Lawrence Fellow of the British Diabetic Association.

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Received 17 August 1995/30 October 1995; accepted 3 November 1995