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Biochimica et Biophysica Acta 1236 (1995) 119–127

BBA Biochimica et Biophysica Acta

Characterisation of endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases in pancreatic β-cells and in islets of Langerhans

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Received 20 September 1994; accepted 6 December 1994

Abstract

We have investigated the plasma membrane (PMCA) and endoplasmic reticulum (SERCA) Ca^{2+}-ATPases involved in active transport of Ca^{2+} in pancreatic β-cell lines (MIN6, HIT T15, RINm5F) and in islets of Langerhans. Under selective membrane phosphorylation conditions (at low ATP concentration, in the presence of Ca^{2+} and La^{3+} and in the absence of Mg^{2+} at 4° C) the only labelled proteins are the phosphoenzyme intermediates of the Ca^{2+}-ATPases. Under these conditions, β-cell membranes incorporated ^{32}P from [γ-^{32}P]ATP into two proteins with molecular mass on acidic SDS-polyacrylamide gels of around 115 and 150 kDa. The 150 kDa band was identified as PMCA (i) by reaction with a monoclonal anti-human erythrocyte plasma membrane Ca^{2+}-ATPase antibody; (ii) by its typical tryptic cleavage pattern which generated an 80 kDa band; (iii) by lack of inhibition of its autophosphorylation by SERCA-specific inhibitors. The 115 kDa band was identified as SERCA (i) by reaction with a polyclonal anti-rat fast skeletal muscle Ca^{2+}-ATPase antibody; (ii) by the concentration-dependent inhibition of its autophosphorylation by thapsigargin and 2,5-di(*t*-butyl)-1,4-benzohydroquinone (tBHQ), which are specific inhibitors of SERCA. The 115 kDa band was further characterised as the SERCA-2b isoform by reaction with a polyclonal rabbit antibody against the 12 C-terminal amino acids of SERCA-2b.

Keywords: Pancreatic β-cell; Beta cell; Islet of Langerhans; ATPase, Ca^{2+}- ; Phosphoenzyme; Antibody

1. Introduction

A rise in intracellular Ca^{2+} is the key trigger to insulin secretion. Stimulation of the pancreatic β-cell by an increase in extracellular glucose concentration causes depolarisation and opening of voltage-sensitive Ca^{2+}-channels; the ensuing influx of Ca^{2+} ions triggers the exocytotic release of insulin. This regulatory effect of glucose on insulin secretion involves intracellular metabolism of the sugar and changes in the probability of opening of ATP-sensitive (K-ATP) channels in the β-cell membrane via an increase in the ratio of [ATP]/[ADP] near the plasma membrane [1]. Although increased influx of Ca^{2+} across the plasma membrane represents the main mechanism whereby glucose elicits insulin secretion, mobilisation of Ca^{2+} from intracellular stores is an important additional process in the response of the β-cell to modulators of secretion such as acetylcholine, which activate phospholipase C and increase the β-cell content of IP_3 [2]. There is also evidence that cyclic ADP ribose may mobilise Ca^{2+} from intracellular stores sensitive to this second messenger [3]. Following its elevation by insulin secretagogues, β-cell Ca^{2+} must be rapidly lowered again by extrusion from the cell and by uptake into the endoplasmic reticulum against steep gradients of Ca^{2+}. In eukaryotic cells, these active movements of Ca^{2+} ions are carried out by Ca^{2+} pumps located in the plasma membranes and subcellular Ca^{2+} stores [4–7].

Abbreviations: SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; PMCA, plasma membrane Ca^{2+}-ATPase; SR, skeletal muscle sarcoplasmic reticulum; HE, human erythrocyte; RE, rat erythrocyte; IL, islets of Langerhans; MIN, MIN6 β-cell line; HIT, HIT T15 β-cell line; RIN, RINm5F β-cell line; EM-2 and EM-3, rabbit polyclonal anti-rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase antibodies; SERCA-2b antibody, rabbit polyclonal anti-peptide antibody raised against the 12 C-terminal amino acids of the SERCA-2b Ca^{2+}-ATPase; 5F10, monoclonal anti-human erythrocyte Ca^{2+}-ATPase antibody (Clone 5F10); tBHQ, 2,5 di(*t*-butyl)-1,4-benzohydroquinone; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Mops, (3-[*N*-morpholino]propane-sulfonic acid); PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid.

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SSDI 0005-2736(95)00103-4

The plasma membrane Ca^{2+}-ATPase exists in a number of isoforms encoded by a multigene family [4]. Screening of diverse mammalian cDNA libraries has indicated the presence at least four gene products (PMCA1-4) whose cDNA sequences have been determined [8–12]; further variability is generated via alternative splicing [13–15]. Isoforms 1 and 4 are transcribed in most tissues in approximately similar amounts, whereas isoforms 2 and 3 are transcribed in appreciable amounts only in specialised tissues (muscle and brain), the gene product 1 being the more abundantly expressed [15]. The pump has a single polypeptide chain of 130–141 kDa [4,16,17].

The Ca^{2+}-ATPase of sarco(endo)plasmic reticulum is an intrinsic membrane protein which has a single polypeptide chain of 110–115 kDa. Five SERCA isoforms have been identified that are derived from three genes — SERCA-1, -2, and -3 [18–20]. Alternative splicing of SERCA-1 and -2 generates additional isoform diversity. SERCA-1a is the major adult fast-twitch skeletal isoform and is expressed at high levels in membranes of striated muscle SR; SERCA-1b is regulated developmentally and is expressed exclusively in neonatal fast-twitch muscle tissues. The SERCA-2 gene also gives rise to two forms: SERCA-2a, found in slow-twitch and cardiac muscle, and SERCA-2b, which has a broader tissue distribution than other isoforms and is considered the major ER-type Ca^{2+} pump of non-muscle cells. The SERCA-3 gene encodes a single transcript which, like SERCA-2b, has a largely non-muscle localisation [21]. Cells may express more than one SERCA gene product or isoforms of the same type [22]. In adrenal chromaffin cells, for example, there is evidence that distinct isoforms mediate Ca^{2+}-uptake into different intracellular Ca^{2+}-stores [23]. Interestingly, acinar pancreas has been shown to contain two species of the SERCA-2b type [24].

In crude homogenates of rat islets, ATP hydrolysis ascribed to the activity of a plasma membrane Ca^{2+}-ATPase has been reported [25–28]. However, in these studies, the membrane preparation used would have contained other Ca^{2+}-ATPases including that of the endoplasmic reticulum and also ectoATPase [29]. Moreover the presence of other cell types besides β-cells further complicates interpretation of these data.

Worley et al. [30] observed that the extent of filling of endoplasmic reticulum Ca^{2+} store regulates the membrane potential of islet β-cells, thereby controlling Ca^{2+} influx and, consequently, insulin secretion. Roe et al. [31] demonstrated direct evidence that abnormalities in the pattern of glucose-stimulated Ca^{2+} signalling are connected with the development of non-insulin-dependent diabetes mellitus (NIDDM). Thus, in an animal model of NIDDM (the *db / db* mouse), the defects in the pattern of glucose-induced changes in the intracellular free Ca^{2+} concentration and insulin secretion coincided with a loss of sarco(endo)plasmic reticulum Ca^{2+}-ATPase activity. It has also been found that the high glucose level characteristic of uncontrolled diabetes probably leads to glycosylation of the plasma membrane Ca^{2+} pump and its inhibition in erythrocyte membranes [7]. Since it has been proposed that disorders of intracellular calcium metabolism play an essential role in the pathophysiology of diabetes and its complications and the associated abnormalities in both action and secretion of insulin [32], molecular characterisation of the β-cell Ca^{2+}-pumping ATPases is an important goal.

In the present study, the nature of the sarco (endo)plasmic reticulum and the plasma membrane Ca^{2+}-ATPases in cultured β-cells and islets of Langerhans has been investigated by autophosphorylation and immunochemical characterisation. The data demonstrate that SERCA-2b and probably the PMCA-1 forms are present.

2. Materials and methods

Male Wistar rats (200–250 g) and New Zealand rabbits were purchased from Harlan U.K., Bicester, UK. Human blood was from National Blood Transfusion Centre (Safety Tested, Donor Blood), Oxford, UK. MIN6, HIT T15 and RINm5F β-cells were generously provided by Prof. Y. Miyazaki, University of Tokyo, Prof. A.E. Boyd III, Tufts University, Boston, and Prof. C.B. Wollheim, University of Geneva, respectively. Monoclonal anti-human erythrocyte plasma membrane Ca^{2+}-ATPase antibody (Clone 5F10) was obtained from Cambridge Bioscience, Cambridge, UK. Rabbit anti-peptide antibody, raised against the 12 C-terminal amino acids of the SERCA-2b Ca^{2+}-ATPase [24], was kindly supplied by Dr R.L. Dormer, University of Wales College of Medicine, Cardiff, UK. The production and characterisation of rabbit anti-rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase antibodies (EM-2 and EM-3) was described previously [33–37]. Tissue culture materials were purchased from Gibco Life Technologies, Paisley, Strathclyde, UK. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham, Little Chalfont, UK. Trypsin, soybean trypsin inhibitor, collagenase (type V), proteinase inhibitors, thapsigargin, goat anti-rabbit IgG and goat anti-mouse IgG alkaline phosphatase labelled antibodies were from Sigma, Poole, Dorset, UK. 2,5-di(*t*-butyl)-1,4-benzohydroquinone (tBHQ) was from Aldrich, Gillingham, Dorset, UK. Molecular weight markers were obtained from Pharmacia Biosystems, Milton Keynes, UK. All other chemicals were of reagent grade.

2.1. Cell culture

MIN6, HIT T15 and RINm5F β-cells were cultured in RPMI 1640 tissue culture medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml) and foetal calf serum 10% (v/v) at 37° C in an atmosphere of humidified air (95%) and CO_2 (5%) as previously described [38]. Cells were passaged weekly and harvested using trypsin-

EDTA. They were seeded in culture flasks at a density of $4 \cdot 10^7$ cells per flasks and cultured for 6 days before membrane preparation.

2.2. Isolation of islets of Langerhans

Islets were obtained by collagenase digestion of rat pancreas [39]. The isolated islets were washed four times in Hepes/Krebs buffer (pH 7.4) (119 mM NaCl, 4.75 mM KCl, 5 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM Hepes-Na, 1 mM PMSF, 1 mM iodoacetamide, 1 mM benzamidine, 2 mM DTT, 1 mM EDTA, 0.1 mg/ml soybean trypsin inhibitor and 10 μM leupeptin) and selected under a dissecting microscope.

2.3. Membrane preparation from cultured β-cells

The membranes from β-cells were prepared as previously described [40]. In brief, after washing twice with phosphate-buffered saline ($7.2 \cdot 10^6$ cell/ml buffer), trypsinised cells were resuspended in hypotonic 5 mM Tris-HCl buffer (pH 8.0) ($5 \cdot 10^7$ cell/ml buffer) containing proteinase inhibitors (1 mM PMSF, 1 mM iodoacetamide, 1 mM benzamidine, 2 mM DTT, 1 mM EDTA, 0.1 mg/ml soybean trypsin inhibitor and 10 μM leupeptin), left for 40 min on ice and then homogenised in a glass homogeniser. The homogenate was centrifuged at 4° C for 10 min at $900 \times g$, then the supernatant was further centrifuged for 40 min at $120\,000 \times g$. The pellets were resuspended at a concentration of 3–6 mg/ml protein in ice-cold 20 mM Mops buffer (pH 7.4) containing the proteinase inhibitors described above.

2.4. Membrane preparation from islets of Langerhans

The freshly selected islets were washed twice in Hepes/Krebs buffer (pH 7.4) containing proteinase inhibitors and homogenised in a glass homogeniser in ice-cold 5 mM Tris-hypotonic buffer (pH 8.0) and incubated for 1 h at 4° C. The homogenate was centrifuged at $900 \times g$ for 10 min to remove nuclei and the supernatant centrifuged at $120\,000 \times g$. The final pellet was resuspended in 20 mM Mops buffer (pH 7.4) at a concentration of 0.5–1 mg protein/ml.

In preliminary studies, islet membranes were prepared by sonication (DAWE Soniprobe, UK) on ice with 75 W for 6×3 s in Mops buffer (pH 7.4) at a concentration of 0.5–1 mg/ml protein.

2.5. Preparation of red blood cell membrane vesicles

Membrane vesicles from human and rat red blood cells were prepared as described by Sarkadi et al. [41]. Briefly, red cell ghosts prepared in hypotonic buffer (10 mM Tris-HCl, 20 mM KCl, 20 mM sucrose, pH 7.4) were washed twice with 40 volumes of 0.5 mM Tris-HCl, 50 μM β-mercaptoethanol, 20 mM Tris-EDTA (pH 8.5) and twice with 10 mM Tris-HCl (pH 7.4). The final pellet was resuspended to a concentration of 4–5 mg/ml membrane protein in 5 mM Tris-HCl, 0.16 M KCl (pH 7.4).

2.6. Preparation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were isolated from predominantly white skeletal muscles of New Zealand rabbits as described by Nakamura et al. [42], and the final pellet was resuspended in 0.3 M sucrose, 10 mM Tris-maleate (pH 7.0) at protein concentration of 30–40 mg/ml.

All the membrane preparations were aliquoted, frozen immediately in liquid nitrogen and stored at −70° C. Protein concentrations were determined using BSA as standard [43].

2.7. Pretreatment of membranes with trypsin, thapsigargin and / or tBHQ

Trypsin treatment was performed at 4° C in 20 μM $CaCl_2$, 50 μM $LaCl_3$, 75 mM KCl, 30 mM KOH-Hepes (pH 7.0) containing 0.5–1.0 mg/ml membrane proteins or 0.12–0.25 μg/ml SR membrane fractions and 10–100 μg/ml trypsin. The reaction was terminated after 2 or 5 min by adding a 20-fold excess of soybean trypsin inhibitor. In tBHQ and thapsigargin inhibition experiments, the untreated or trypsin pre-treated membrane suspensions were incubated for 5 min at 37° C with $5 \cdot 10^{-6}$–10^{-2} M tBHQ or 10^{-7}–$5 \cdot 10^{-5}$ M thapsigargin (added as a 50-fold concentrated solution in dimethyl sulfoxide), placed on ice for an additional 10 min and then the membrane vesicles were processed for Ca^{2+}-pump radiolabelling.

2.8. Phosphorylation of the membrane-bound calcium pump proteins

Phosphorylation was carried out at 4° C in the medium used for proteolysis. The reaction was started by the addition of [γ-^{32}P]ATP (0.8 pM final concentration) with vigorous stirring. The reaction was stopped after 1 min by the addition of ice-cold 12% (w/v) TCA containing 2 mM ATP and 20 mM KH_2PO_4, and the samples were washed twice with the same solution. The final precipitates were dissolved in the electrophoresis sample buffer containing 10 mM EDTA, 60% sucrose, 0.015% Bromophenol blue, 2% SDS and 0.15 M Tris-HCl (pH 6.8). The pH of the samples were adjusted to 7.5–8.0 using 1.7 M Tris-base and incubated for 15 min at room temperature before electrophoresis.

2.9. Acidic SDS-polyacrylamide gel electrophoresis of proteins

Electrophoresis was performed on 1 mm thick acidic SDS-polyacrylamide gels (7.5%) [44]. Membrane samples

(50–100 μg membrane protein or 12.5–25 ng SR) were run in each well along with molecular mass standards (myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; phosphorylase b, 94 kDa; transferrin, 76 kDa; bovine serum albumin, 67 kDa; glutamic dehydrogenase, 53 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa). The gels were run for approx. 3 h with 15 mA constant current (70–120 V) at 4° C on a vertical gel electrophoresis apparatus (AE-6400 Dual Mini Slab Kit, ATTO, Tokyo, Japan). The slabs were either electroblotted onto Immobilon membranes or stained for 1 h in 10% (v/v) acetic acid, 50% (v/v) methanol and 0.05% (w/v) Coomassie brilliant blue R-250. Destaining was carried out in 10% (v/v) acetic acid, 37% (v/v) methanol, 5% (v/v) glycerol for 2 h. Autoradiography of the stained and dried gels was performed with 24–72 h of exposure at −70° C by using x-ray Hyperfilms (Amersham, Little Chalfont, UK) and intensifying screens.

2.10. Electrophoretic transfer for immunoblot analysis of membrane samples

Proteins were transferred electrophoretically onto poly(vinylidene difluoride) microporous membrane (Immobilon; Millipore, Bedford, MA, USA) using an AE-6675 Horizoblot Electrophoretic Transfer Unit with a discontinuous buffer system for 2 h at room temperature as recommended by the manufacturer (ATTO, Tokyo, Japan). Molecular mass markers were stained with 0.01% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid for 5 min and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for 5–10 min.

2.11. Immunostaining of Immobilon membranes

Before immunostaining, the Immobilon sheets were blocked overnight at 4° C with 5% (w/v) non-fat dry milk and 1:50 dilution of normal swine serum in PBS (blocking solution). The proteins on Immobilon sheets were reacted with different SERCA or PMCA specific antibodies (EM-2 and EM-3 polyclonal antibodies 1:1000, SERCA-2b polyclonal antibody 1:750 and 5F10 monoclonal antibody 1:1000 diluted in blocking solution for 12–16 h at 4° C. After washing twice for 5 min with PBS containing 0.05% (v/v) Tween-20 and once with blocking solution, the membrane filters were incubated for 4 h at room temperature with alkaline phosphatase conjugated anti-rabbit IgG diluted 1:15000 or anti-mouse IgG diluted 1:7500 in blocking solution, followed by several rinses with PBS containing 0.05% (v/v) Tween-20 for 20 min. The bound antibodies were visualized by addition of substrate solution containing 0.46 mM nitroblue tetrazolium, 0.2 mM 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt, 0.1 M Tris-base (pH 9.5), 0.1 M NaCl and 0.05 M $MgCl_2$.

3. Results

3.1. Demonstration of the Ca^{2+}-pumps in β-cell lines and in islets of Langerhans

To identify the types of Ca^{2+}-pump in cultured β-cells and in islets of Langerhans selective protein phosphorylation was used. Under special membrane phosphorylation conditions (at low ATP concentration, in the presence of Ca^{2+} and La^{3+} and in the absence of Mg^{2+} at 4° C) the only labelled proteins are the plasma membrane and endoplasmic reticulum or sarcoplasmic reticulum-type calcium pumps [44]. Using these conditions, membranes from MIN, HIT and RIN β-cells incorporated ^{32}P from [γ-^{32}P]ATP into two proteins forming separate bands on acidic gels with molecular mass around 115 and 150 kDa (Fig. 1). The phosphorylation pattern of islet of Langerhans is also shown in Fig. 1. Preliminary experiments using sonicated islet membrane preparations resulted in at least five phosphorylated bands appearing with molecular mass about 150 kDa, 115 kDa, 68 kDa, 55 kDa and 35 kDa (Fig. 1, IL1). The lower bands (68, 55 and 35 kDa) are likely to be proteolytic fragments of the 150 and 115 kDa proteins. Subsequent experiments used membranes prepared by homogenisation after prior removal of nuclei. With these preparations a large diffuse band appeared on autoradiograms with molecular mass between 110 and 160 kDa (Fig. 1. IL2) which did not resolve clearly into two separate bands. Human erythrocyte and skeletal muscle sarcoplasmic reticulum membranes were used as controls. In the erythrocyte membrane the only protein phosphorylated was the 140 kDa calcium pump, while in the skeletal muscle sarcoplasmic reticulum membrane the

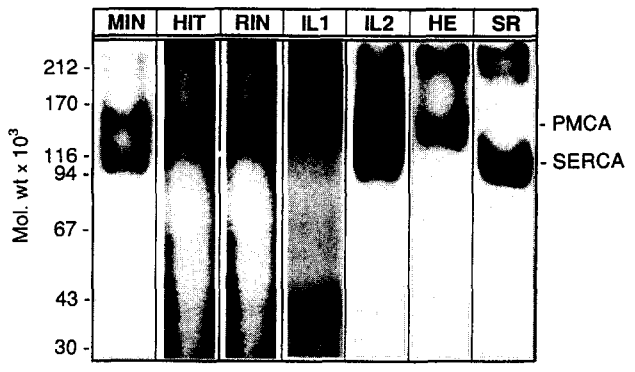

Fig. 1. Detection of the phosphoenzyme intermediates of the Ca^{2+}-ATPases in cultured β-cells, islets of Langerhans, human erythrocytes and sarcoplasmic reticulum. 50 μg of membrane proteins from MIN, HIT and RIN β-cells, islets of Langerhans (IL1 and IL2) and from human erythrocytes (HE) and 12.5 ng sarcoplasmic reticulum (SR) were labelled with [γ-^{32}P]ATP in the presence of 20 μM Ca^{2+} and 50 μM La^{3+} as described under Section 2. Phosphorylated membrane proteins were separated on 7.5% acidic SDS-polyacrylamide gels and autoradiographed. Membranes from islets of Langerhans were prepared by sonication (IL1) or by homogenisation and centrifugation (IL2). The positions of the molecular mass standards are indicated on the left.

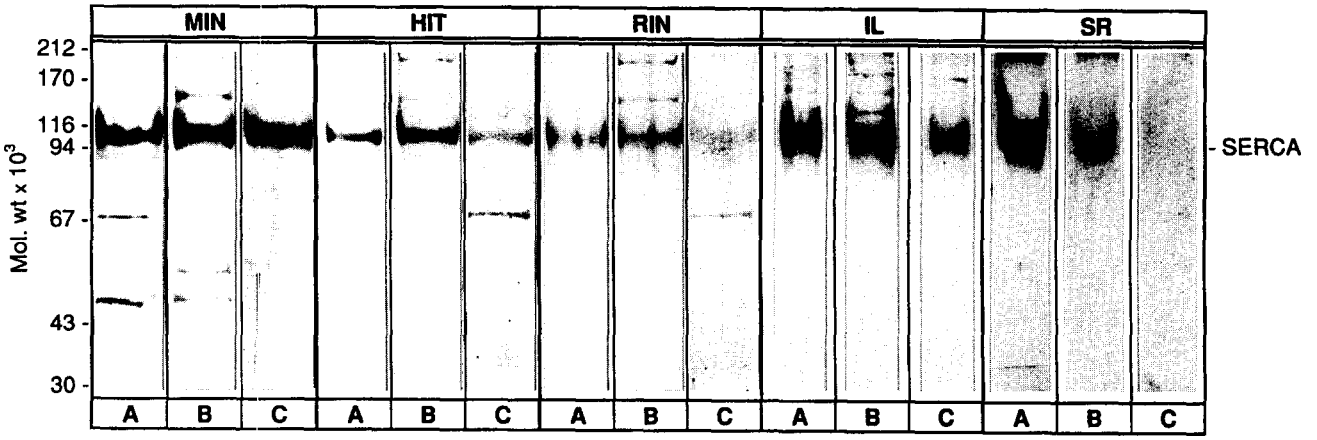

Fig. 2. Immunolabelling of sarco(endo)plasmic reticulum Ca^{2+}-ATPase in cultured β-cell lines, islets of Langerhans and sarcoplasmic reticulum using polyclonal antibodies for SERCA and SERCA-2b. Membrane samples prepared from MIN, HIT and RIN β-cell lines, islets of Langerhans (IL) and sarcoplasmic reticulum (SR) were immunolabelled with rabbit anti-rat skeletal muscle SR Ca^{2+}-ATPase antibodies: EM-2 (lanes A), EM-3 (lanes B) and a rabbit anti-peptide antibody, raised against the 12 C-terminal amino acids of the SERCA-2b Ca^{2+}-ATPase: SERCA-2b (lanes C). 25 ng of SR and 100 μg of membrane proteins were applied to each lane of 7.5% acidic SDS-polyacrylamide gels and then transferred onto Immobilon membranes. For immunoblot analysis, EM-2, EM-3 antibodies at 1:1000 and SERCA-2b antibody at 1:750 dilutions were used. The bound antibodies were visualised by alkaline phosphatase-conjugated anti-rabbit IgG (1:15000 dilution).

phosphorylated protein was the 105 kDa calcium pump (Fig. 1, HE and SR).

3.2. Immunological detection of SERCA and PMCA in β-cell lines and in islets of Langerhans

The EM-2 and EM-3 antibodies, which recognise all the known isoforms of SERCA [33–37], reacted with a 115 kDa protein in all samples from cultured β-cells and from islets of Langerhans. Both of the antibodies labelled the 105 kDa band in SR prepared from skeletal muscle (Fig. 2, lanes A and B). It seems that the molecular mass of the SERCA protein in β-cells and in islet of Langerhans is slightly higher than in skeletal muscle. This observation is in accordance with the previous phosphorylation results in indicating that β-cells contain a 115 kDa SERCA isoform.

The SERCA-2b antibody labelled a protein of similar molecular mass in β-cells and islets of Langerhans as that labelled by the EM-2 and EM-3 antibodies. However, no immunoreaction was seen with SR prepared from skeletal muscle (Fig. 2, lane C). This antibody was raised against the last 12 C-terminal amino acids of the SERCA-2b Ca^{2+}-ATPase [24] which is unique for this isoform. In accordance with the specificity of the SERCA-2b antibody, we did not observe any cross-reactivity with the SERCA-1 skeletal muscle SR Ca^{2+}-ATPase. Thus the β-cells contain the SERCA-2b isoform.

The 5F10 monoclonal antibody, which was raised against the human erythrocyte PMCA, reacted with a 150 kDa protein in β-cells, in islets of Langerhans and with the 140 kDa band in human and rat erythrocytes which were used as controls (Fig. 3). The estimated molecular masses are slightly higher than those expected from published values because of the different gel electrophoresis system used. We therefore identify the 150 kDa species as a β-cell PMCA.

3.3. Specific inhibition of Ca^{2+}-pump phosphoenzyme intermediate formation by thapsigargin and tBHQ

To further confirm that the 115 kDa species is a SERCA isoform we studied the effects of specific SERCA in-

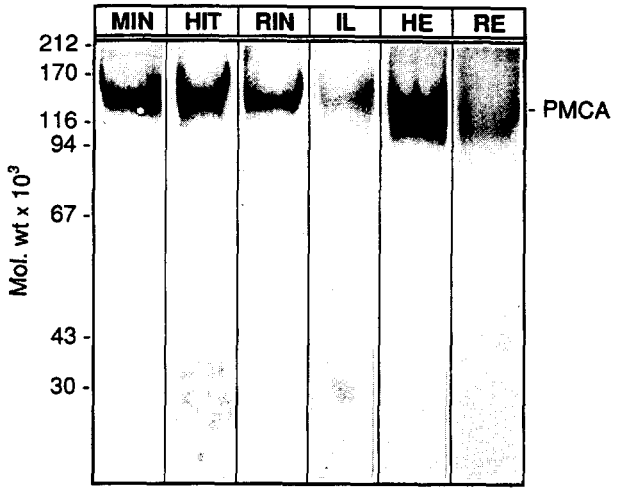

Fig. 3. Immunological detection of plasma membrane Ca^{2+}-ATPase in cultured β-cell lines, islets of Langerhans and in human and rat erythrocytes using monoclonal anti-human erythrocyte plasma membrane Ca^{2+}-ATPase antibody (Clone 5F10). MIN, HIT and RIN β-cells, islets of Langerhans (IL), human erythrocyte (HE) and rat erythrocyte (RE) membrane preparations were separated by SDS-PAGE on 7.5% acidic gels. 100 μg of membrane proteins were applied to each lane. The gels were electroblotted onto Immobilon membranes and incubated with 1:1000 dilution of 5F10 monoclonal antibody. The bound antibody was detected by reaction with alkaline phosphatase-conjugated anti-mouse IgG antibody (1:7500 dilution) as described under Section 2.

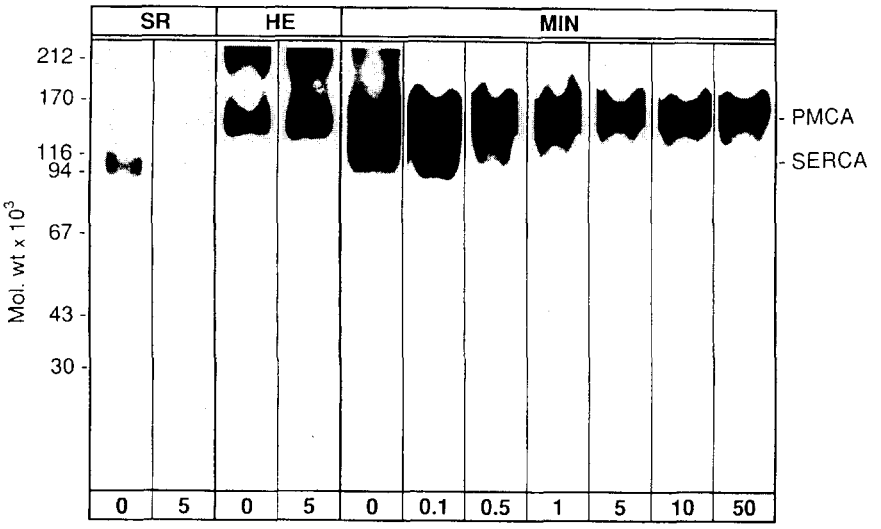

Fig. 4. Inhibition of the phosphoenzyme intermediate formation of Ca^{2+}-ATPases by thapsigargin. Ca^{2+}-pumps in membrane proteins from MIN β-cells, human erythrocytes (HE) and from sarcoplasmic reticulum (SR) were labelled with [γ-^{32}P]ATP in the absence or the presence of various concentration of thapsigargin. 50 μg of MIN and HE membrane proteins and 12.5 ng of SR proteins were used. The phosphorylated proteins were resolved by SDS-PAGE and autoradiographed. The concentrations of thapsigargin were: 0 and 5 μM in SR and HE samples, 0, 0.1, 0.5, 1, 5, 10 and 50 μM in MIN samples.

hibitors on the formation of the phosphoenzyme intermediate. When the Ca^{2+}-pumps in MIN β-cell membranes were labelled with [γ-^{32}P]ATP at increasing thapsigargin concentrations, the autophosphorylation of the 115 kDa Ca^{2+}-pump was inhibited, while no effect was observed on phosphorylation of the 150 kDa pump protein. Inhibition occurred with 0.5–1.0 μM thapsigargin and above 1 μM thapsigargin the inhibition was complete (Fig. 4). Skeletal muscle SR and human erythrocyte membranes were used as controls. The phosphorylation of the 105 kDa SR Ca^{2+}-ATPase protein was inhibited in the presence of 5 μM thapsigargin, but the same thapsigargin concentration in erythrocyte membrane samples did not affect phosphoenzyme intermediate formation of the 140 kDa PMCA (Fig. 4).

Phosphorylation of the 115 kDa SERCA protein in MIN β-cell membranes was also inhibited by tBHQ. The inhibition was complete at 0.1 mM tBHQ concentration. The inhibitor had no effect on PMCA phosphorylation, even at a high (10 mM) tBHQ concentration (Fig. 5).

3.4. Trypsin proteolysis of Ca^{2+}-pumps

As no specific inhibitors of PMCA are available, we sought to confirm our identification of the 150 kDa species as PMCA by carrying out limited tryptic digestion which yields different characteristic cleavage products of PMCA and SERCA [33]. Following tryptic cleavage of the Ca^{2+}-pumps in MIN β-cell membranes, the resulting fragments were phosphorylated (Fig. 6A, lanes 1–8). After exposure to 100 μg/ml trypsin for 2 and 5 min (Fig. 6A, lanes 3 and 4), two bands appeared with molecular masses of about 80 and 55 kDa. The 55 kDa fragment probably arose from the 115 kDa SERCA protein as it was also obtained after tryptic cleavage of skeletal muscle SR Ca^{2+}-ATPase as detected either by phosphorylation (Fig. 6A, line 10) or by immunoreaction with EM-3 antibody (Fig. 6A, lane 12). To confirm the origin of the 55 kDa fragment, we also tried to use immunolabelling, but the EM-2, EM-3 antibodies did not show detectable reaction with the tryptic fragments of SERCA in MIN β-cell membranes under these conditions. The SERCA-2b antibody raised against a C-terminal oligopeptide, as expected, did not label the phosphorylated 55 kDa fragment which contains the N-terminal half of the enzyme (results not shown). The 80 kDa band

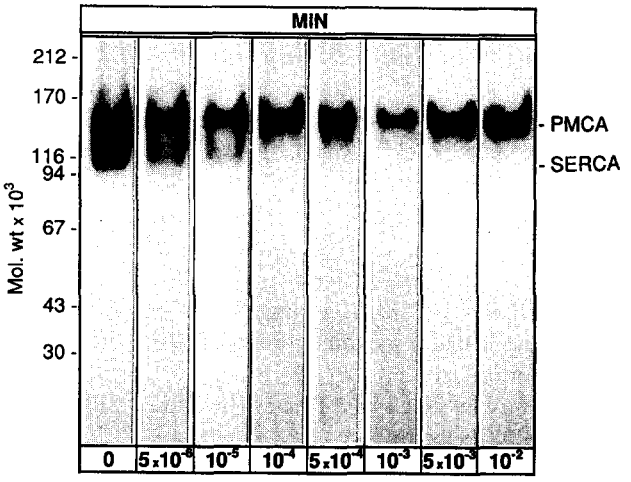

Fig. 5. Inhibition of the phosphoenzyme intermediate formation of Ca^{2+}-ATPases in MIN β-cells by tBHQ. The membranes were preincubated with $5 \cdot 10^{-6}$–10^{-2} M tBHQ for 5 min at 37° C and then radiolabelled with [γ-^{32}P]ATP at 4° C in medium containing 20 μM $CaCl_2$, 50 μM $LaCl_3$, 75 mM KCl, 30 mM Hepes (pH 7.0) and 0.5 mg/ml protein. The proteins were separated on 7.5% acidic SDS-polyacrylamide gels and autoradiographed.

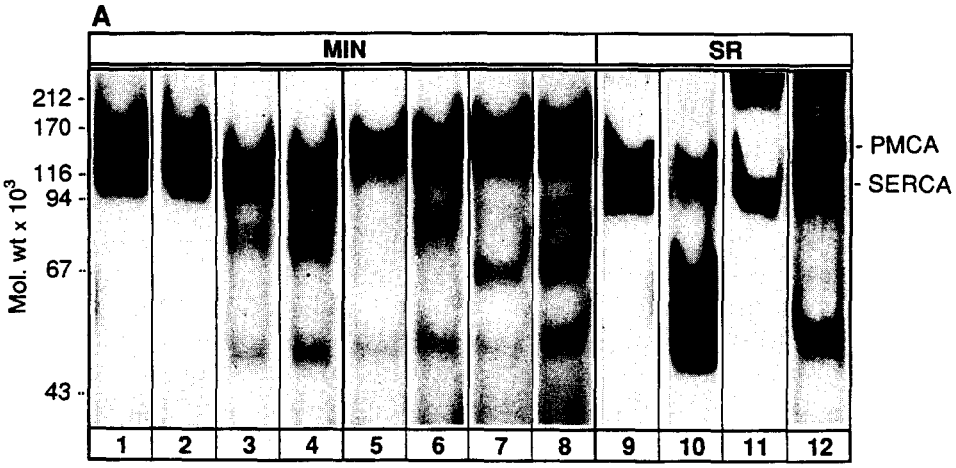

Fig. 6. Tryptic digestion of Ca^{2+}-ATPases in MIN β-cell, sarcoplasmic reticulum and human erythrocyte membranes. (A) MIN β-cell (lanes 1–8) and sarcoplasmic reticulum (SR) (lanes 9–12) proteins were partially digested with trypsin as described under Section 2. Aliquots were taken before the addition of trypsin (lanes 1, 9, 11) and after 2 min (lanes 3, 5, 7) and 5 min (lanes 4, 6, 8, 10, 12). The reaction was terminated by the addition of 20-fold excess of soybean trypsin inhibitor. The proteolysis products were either labelled with [γ-^{32}P]ATP (lanes 1–10) or with EM-3 antibody (lanes 11, 12) in the presence of 0.5 μM (lines 5, 6) or 2 μM (lanes 7, 8) thapsigargin. Lane 2, trypsin and soybean trypsin inhibitor were added simultaneously to the sample. (B) Membrane proteins (1 mg/ml) from MIN6 β-cells and human erythrocytes (HE) were partially digested with 100 μg/ml trypsin at 4° C for 0 min (lanes 1, 4), for 2 min (lanes 2, 5) and for 5 min (lanes 3, 6). The products of proteolysis were separated by SDS-PAGE on 7.5% acidic gels, transferred to Immobilon membranes, and then incubated with a 1:1000 dilution of the 5F10 antibody, as described under Section 2. Each sample contained 100 μg of membrane proteins. The bound antibody was detected by reaction with alkaline phosphatase-conjugated anti-mouse IgG antibody at 1:7500 dilution.

was recognised by 5F10 antibody (Fig. 6B, lines 2 and 3) and is therefore a proteolytic fragment of the PMCA. After proteolytic treatment, a band with molecular mass around 76 kDa was labelled by 5F10 antibody in human erythrocyte membranes, and is therefore a proteolytic fragment of PMCA.

The effects of thapsigargin on tryptic proteolysis are also shown in Fig. 6A (lanes 5–8). In contrary to the intact enzyme, thapsigargin had no effect on the phosphorylation of the 55 kDa tryptic fragment derived from SERCA. In the presence of 0.5 or 2 μM thapsigargin a new 68 kDa fragment appeared, which is probably either a different proteolytic fragment or a further degradation product of the 80 kDa fragment which disappeared under these conditions. It seems that thapsigargin affects the proteolytic cleavage pattern of the PMCA protein, but does not inhibit the ability of the tryptic fragments of the SERCA to autophosphorylate.

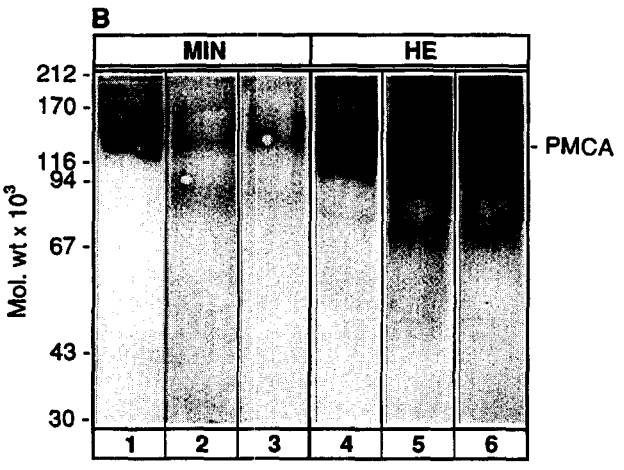

Fig. 6 (continued).

4. Discussion

The presence of PMCA in islets of Langerhans has previously been inferred from studies of Ca^{2+} fluxes. Attempts to detect plasma membrane Ca^{2+}-ATPase activity have also been reported [25–28,45–47]. However, these data suffer from two limitations. Firstly, the presence of membrane from other cellular sites in the putative plasma membrane fractions used can not be excluded. Secondly, we have found (Ashcroft, S.J.H., unpublished observations) that although rat islet membrane preparations possess a very high Ca^{2+}-dependent ATPase activity, this is not attributable to a Ca^{2+}-pumping ATPase since it is as active in the presence of Mg^{2+} as in the presence of Ca^{2+}. Moreover, this Ca-Mg-ATPase activity is primarily located extracellularly since intact islets or β-cells are also highly active in Ca-Mg-dependent ATP hydrolysis and therefore this β-cell ATPase is an ectoenzyme, possibly similar to liver ectoATPase [48]. Previous studies on the ATPase activity in homogenates of islets of Langerhans have failed to take account of the presence of Ca-Mg-ectoATPase in their preparations.

The present study provides clear evidence for the presence of PMCA in β-cells. Under conditions that exclude the possibility of protein kinase-catalysed phosphorylation,

we demonstrated the formation of a 150 kDa phosphoprotein in β-cell membranes. The identification of this band as a phosphoenzyme intermediate of PMCA was confirmed by the immunolabelling of a band of identical mobility using an antibody (5F10) which cross-reacts with the Ca^{2+}-ATPases of other plasma membranes [49] and does not distinguish between the different isoforms of PMCA. Moreover, in MIN6 β-cell membranes, upon limited trypsin proteolysis, the disappearance of the 150 kDa band was accompanied by formation of an 80 kDa autophosphorylatable Ca^{2+}-pump fragments which also reacted with 5F10 anti-PMCA antibody.

Although 5F10 antibody labelled a 150 kDa protein in β-cells and in islets of Langerhans, a slightly smaller 140 kDa band was recognised in erythrocytes. Similar to our results, it has been found that the 5F10 antibody labelled a 145 kDa protein in liver [50,51], which exceeded the molecular mass of the erythrocyte PMCA by 5–10 kDa. The possibility of different glycosylation or a sequence insertion in the pump molecule could account for the molecular mass difference, but no conclusive explanations for the difference can be offered currently. The 1 and 4 isoforms of PMCA are transcribed in most of the tissues tested [10,15], with the isoform 1 being the more abundantly expressed. PCR analysis has shown that the rat exocrine pancreas expresses PMCA-1 mRNA predominantly [52]. Similarly to the liver [50,51] and to the small intestine [53], the β-cells and islets of Langerhans probably express the PMCA1 isoform. Further studies are required to resolve this point.

There is considerable evidence for a functionally important Ca^{2+}-store in the endoplasmic reticulum of β-cells [54,55]. The presence of a SERCA can be inferred from the increase in β-cell intracellular Ca^{2+} elicited by thapsigargin [30,31,56]. Uptake and release of Ca^{2+} from microsomal preparations derived from β-cells has also been documented [54,57,58]. The Ca^{2+}-ATPase activity of β-cell microsomal preparations has been ascribed to the activity of SERCA, but in our view these studies suffer from the same limitations as those that have sought to measure the ATPase activity of PMCA. In one study more direct evidence for the presence of SERCA was provided by immunolabelling Western blots of islets of Langerhans with an anti-SERCA antibody [31]. The present study extends this latter observation in several ways. We demonstrated that β-cell membranes, under conditions where protein kinases are inactive, incorporate radioactivity from [γ-^{32}P]ATP into a 115 kDa band. Confirmation that this species is the phosphoenzyme intermediate of SERCA came from inhibitor studies. Thapsigargin, as well as tBHQ, selectively inhibits the enzymes of the SERCA family [59–61] and has no effect on the activity of PMCA [62]. We showed that phosphorylation of the 115 kDa SERCA protein in β-cell membranes was inhibited in a dose-dependent manner by both these inhibitors and no effect was observed on phosphorylation of the 150 kDa PMCA protein. Immunological studies further supported this identity. Thus, anti-SERCA antibodies (EM-2 and EM-3) reacted with a species of identical mobility on Western blots of β-cells to that obtained by radiolabelling. Using an isoform-specific antibody, we demonstrated that the isoform of SERCA present in cultured β-cells and islets of Langerhans is of the SERCA-2b type. In contrast to the two Ca^{2+}-ATPase isoforms which were labelled (111 kDa and 97 kDa) in exocrine pancreatic endoplasmic reticulum [24], the SERCA-2b (and also the EM-2 and EM-3) antibodies labelled just one 115 kDa band in endocrine pancreatic β-cells.

Papp et al. [63] observed that La^{3+} abolished the inhibition by thapsigargin of the SERCA pumps and phosphorylation of its proteolytic fragments even at 2 μM thapsigargin concentration, which gave complete inhibition in the absence of La^{3+}. However, we found that inhibition of the SERCA-2b protein was complete with 1 μM thapsigargin in the presence of La^{3+}. Thapsigargin did not inhibit autophosphorylation of any of the trypsinised fragments in the presence of La^{3+}; however, the tryptic cleavage pattern of PMCA was changed by thapsigargin in a dose-dependent manner. The reason for this is unknown, but may indicate that thapsigargin is able to interact with PMCA without causing functional inhibition. The nature of the binding site for thapsigargin has not been established [21].

In conclusion, our results demonstrate that two separate calcium transport ATPases, SERCA-2b and PMCA (probably PMCA1), are present in cultured β-cells and islets of Langerhans.

Acknowledgements

We are grateful to Prof. Y. Miyazaki, University of Tokyo, Prof. A.E. Boyd III, Tufts University, Boston, and Prof C.B. Wollheim, University of Geneva, for providing MIN6, HIT T15 and RINm5F β-cells, respectively. The SERCA-2b antibody was a generous gift from Dr R.L. Dormer, University of Wales College of Medicine, Cardiff, UK. These studies have been supported by grants from the British Diabetic Association, the Medical Research Council and the Wellcome Foundation.

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