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Demonstration of two isoforms of the SERCA-2b type Ca^{2+},Mg^{2+}-ATPase in pancreatic endoplasmic reticulum

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An antibody raised against a 12 amino acid peptide corresponding to the C-terminal sequence of the SERCA-2b Ca^{2+},Mg^{2+}-ATPase precipitated Ca^{2+},Mg^{2+}-ATPase activity from pancreatic rough ER. Thapsigargin and vanadate inhibited the activity with the same concentration-dependence as for native ER membranes. Partial purification of Ca^{2+},Mg^{2+}-ATPase using Reactive Dye-agarose affinity chromatography resulted in activation of the enzyme, suggesting the presence of an endogenous inhibitor which was detached by binding to the Reactive Dye. Immunoblots and analysis of immunoprecipitated protein revealed two bands of molecular masses approx. 111 kDa and 97 kDa. It is concluded that pancreatic ER Ca^{2+},Mg^{2+}-ATPase is of the SERCA-2b type and consists of two isoforms.

Introduction

Pancreatic acinar cell ER contains Ca^{2+},Mg^{2+}-ATPase activity which has properties consistent with a role in maintaining the resting cytoplasmic free Ca^{2+} concentration and accumulating internal Ca^{2+} stores from which Ca^{2+} is released upon stimulation of enzyme secretion [1,2]. There is evidence for both Ins(1,4,5)P_3-sensitive and -insensitive Ca^{2+} stores in the pancreatic acinar cell [3–6] although their precise relationship both in location and involvement in Ca^{2+} signalling in response to physiological agonists is not clear.

Three genes encoding intracellular, non-mitochondrial Ca^{2+} (SERCA) pumps have been cloned and sequenced [7–10]. The SERCA 1 gene encodes the skeletal muscle SR Ca^{2+},Mg^{2+}-ATPase [11]. The SERCA-2 gene undergoes alternate splicing to yield SERCA-2a or -2b which are expressed predominantly in slow-twitch skeletal and cardiac muscle or in smooth muscle and several non-muscle tissues, respectively [8,9,12]. Expression of the SERCA-3 gene has been detected in skeletal and heart muscle and in non-muscle tissues [10]. Northern blot and immunological analysis has shown that more than one SERCA gene product, or isoforms of the same product, can be expressed within the same cell type [10,13–15]. Immunolocalisation coupled with single-cell fluorescence imaging of Ca^{2+} in adrenal chromaffin cells [13] suggested that different isoforms are involved in accumulating Ca^{2+} in distinct Ca^{2+} stores.

In the present study, the nature of the Ca^{2+},Mg^{2+}-ATPase of pancreatic ER has been investigated by immunological characterisation and protein purification. The data demonstrate that the Ca^{2+},Mg^{2+}-ATPase is of the SERCA-2b type and suggest the presence of two isoforms.

Materials and Methods

CHAPS, Insoluble Staph A protein (lyophilised crude extract from *Staphylococcus aureus*, Cowan I strain), Reactive Dye-agaroses, aprotinin, PMSF and thapsigargin were from Sigma. 1,2-Phenylenediamine dihydrochloride was from Dakopatts, Glostrup, Denmark and Mimetic Dye-agaroses from Affinity Chromatography, Cambridge, UK. All other reagents were of the highest quality available from BDH.

Preparation of pancreatic rough ER. Pancreas was removed from two overnight-fasted Wistar rats (approx. 250 g weight), trimmed, chopped into small pieces and homogenised in 0.3 M sucrose; 1 mM benzamidine; 5 μg/ml aprotinin; 1 mM phenylmethanesulphonyl fluoride; 2 mM sodium azide; 5 mM dithiothreitol;

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; ER, endoplasmic reticulum; Ins(1,4,5)P_3, inositol 1,4,5-trisphophate; SERCA, sarco-, endoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum; tBHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone.

10 mM Hepes (pH 7.4). Rough ER membranes were then purified as previously described [1,16] and resuspended at a protein concentration of approx. 3 mg/ml in 0.25 M sucrose; 100 mM KCl; 2 mM sodium azide; 5 mM $MgCl_2$; 100 mM imidazole-HCl (pH 6.8). The Ca^{2+},Mg^{2+}-ATPase activity of this preparation was the same in terms of specific activity and dependence on free Ca^{2+} concentration as for membranes purified from isolated pancreatic acini (Brown, Webb and Dormer, unpublished data). Ca^{2+},Mg^{2+}-ATPase, assayed as previously described [1], was defined as difference in ATPase activity in the presence or absence of 0.7 μM free Ca^{2+} (1 mM EGTA/0.5 mM $CaCl_2$ at pH 6.8). Protein was estimated using the Bio-Rad protein assay kit and BSA as standard.

Production of anti-peptide polyclonal antibodies and immunoblotting. The procedure was based on that of Wuytack et al. [17]. A peptide consisting of the 12 C-terminal amino acids of the SERCA-2b Ca^{2+},Mg^{2+}-ATPase was synthesized and coupled to BSA or thyroglobulin (10 mg peptide/8 mg carrier protein) by Cambridge Research Biochemicals, Northwich, Cheshire. The sequence of amino acids was: STDTNFSDMFWS. The preparation of conjugates and injection protocol was as previously described [18]. Antisera were tested for cross-reaction with the peptide or rough ER membranes by ELISA using peroxidase-linked second antibody, colour development with 1,2-phenylenediamine dihydrochloride and detection at 492 nm. Antigens were coupled to plastic microtitre plates by overnight incubation at 4°C in 50 mM carbonate/bicarbonate (pH 9.6) at the concentrations indicated. For immunoblotting, rough ER membranes were solubilised in 30 mM Tris-HCl (pH 6.8) containing 1% w/v SDS, 30 mM dithiothreitol, 5% v/v glycerol and fractionated on 5% SDS-polyacrylamide gels. After electroblotting onto nitrocellulose and blocking with 3% milk powder in 20 mM Tris-HCl, 0.5 M NaCl, 0.02% azide (pH 7.4), blots were incubated overnight at 30°C with primary antibody at 1:500 dilution in blocking buffer containing 1% milk powder, then at 37°C for 2 h with gold-labelled, sheep anti-rabbit antibody (Biocell Research, Cardiff) at 1:100 dilution. Immunoreactive bands were visualised by incubation for 15–30 min at room temperature in silver enhancement reagent (Biocell Research, Cardiff).

Immunoprecipitation. The procedure was based on the method of Doolittle et al. [19]. Rough ER membranes, at a protein concentration of approx. 3 mg/ml, were solubilised by mixing with glycerol (20% v/v) and CHAPS (8 mg per mg protein) on ice for 15 min and centrifuged at 100000 × *g* for 45 min. Solubilised membranes were mixed either with 10 μl antiserum/mg ER protein (final dilution 1:56), preimmune serum or no primary antibody and incubated for approx. 18 h at 4°C. Immune complexes were precipitated by addition of 10 μl (per μl primary antibody) Staph A protein slurry (0.5 mg dry weight/ml insoluble Staph A protein in 0.1 M Tris-HCl (pH 7.5) containing 0.1% w/v *N*-lauryl sarcosine, 0.1% v/v Triton X-100, prepared as described by Doolittle et al. [19]). Following centrifugation for 15 min at 15000 × *g*, pellets were washed once in 0.1 M Tris-HCl (pH 7.5) containing 0.5 M NaCl and twice in 0.1 M Tris-HCl (pH 7.5) containing 0.1% w/v *N*-lauryl sarcosine and 0.15 M NaCl. Immune complexes were dissociated by heating for 5 min at 37°C in 0.1 M Tris-HCl (pH 6.8) containing 2% w/v SDS, 10% v/v glycerol and centrifuging for 5 min at 15000 × *g*. The supernatant was then diluted into the Ca^{2+},Mg^{2+}-ATPase assay as described above.

Purification on Reactive Dye-agarose columns. Columns were prepared by equilibration of 0.2 g Reactive Dye-agarose with 0.25 M sucrose; 100 mM KCl; 2 mM sodium azide; 5 mM $MgCl_2$; 100 mM imidazole-HCl (pH 6.8) containing 0.01% v/v CHAPS (Buffer A). 0.5 ml of solubilised rough ER membranes (see above), at a protein concentration of 1–3 mg/ml, was applied to the column which was then sealed and incubated at 4°C for approx. 18 h. The column was then eluted with Buffer A, the first two 1-ml fractions collected and a further 5 ml discarded. Elution was continued with Buffer A containing 5 mM ATP and 1 ml fractions collected. Fractions were assayed for Ca^{2+},Mg^{2+}-ATPase activity and protein as described above.

Results

Our previous data showed that solubilisation of active Ca^{2+},Mg^{2+}-ATPase from pancreatic rough ER was achieved using non-ionic or zwitterionic detergents [20]. However, the Ca^{2+},Mg^{2+}-ATPase activity was not stable enough for protein purification. In the present study Ca^{2+},Mg^{2+}-ATPase activity was solubilised from purified membranes by CHAPS, as described in Materials and Methods, with recovery in the soluble fraction of 67.0 ± 10.2% of the protein and 73.1 ± 19.0% of the Ca^{2+},Mg^{2+}-ATPase activity originally present in native rough ER membranes (n = 7). The solubilised Ca^{2+},Mg^{2+}-ATPase retained activity with sufficient stability to enable single-step purification by immunoprecipitation or by Reactive Dye-agarose affinity chromatography.

Antibody characterisation

Polyclonal antibodies raised against a peptide consisting of the 12 C-terminal amino acids of the SERCA-2b Ca^{2+}-ATPase cross-reacted in ELISA with both the peptide and rough ER (Fig. 1). Results are shown for one antibody raised against the peptide coupled to thyroglobulin (THY2) and one against peptide coupled to bovine serum albumin (BSA2). When

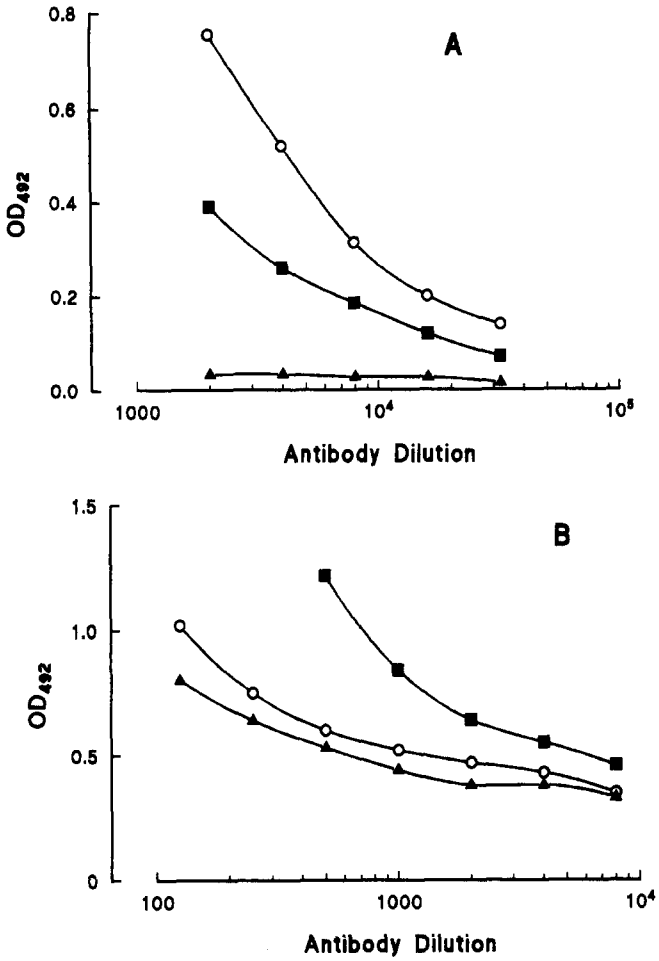

Fig. 1. Cross reaction of anti-peptide antibodies with peptide and pancreatic rough ER. Peptide (A) or rough ER membranes (B) were coupled to plastic multiwell plates at 1 μg/ml or 0.2 μg/ml, respectively. Antibodies to BSA-linked (■) or thyroglobulin-linked (○) peptide or preimmune serum (▲) were diluted as shown, in phosphate-buffered saline containing 0.5% w/v BSA. ELISA's were carried out as described in Materials and Methods.

reacted against peptide, THY2 gave a stronger reaction than BSA2 (Fig. 1A), but the reverse was observed when reacted against rough ER membranes (Fig. 1B). The stronger reaction of BSA2 against rough ER (0.2 μg membrane protein per well) was not due to cross-reaction with BSA since the reaction against BSA (0.1 μg per well) was approx. 10% that of the reaction against rough ER. Furthermore, cross-reaction with rough ER but not BSA was retained after affinity purification of antibodies on peptide-Sepharose (data not shown).

Immunoprecipitation showed that the antibodies bound Ca^{2+},Mg^{2+}-ATPase. Immunoprecipitates were shown to possess Ca^{2+}-activated Mg^{2+}-ATPase activity which was inhibited by vanadate and thapsigargin. Figs. 2 and 3 show inhibition of Ca^{2+}-dependent, Mg^{2+}-ATPase activity as a function of vanadate and thapsigargin concentration for native rough ER membranes

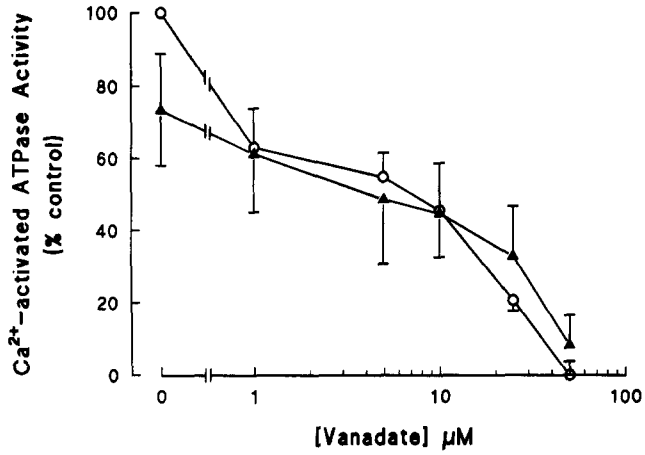

Fig. 2. Inhibition of pancreatic rough ER membrane and immunoprecipitated Ca^{2+},Mg^{2+}-ATPase by vanadate. Solubilised rough ER membranes were immnuoprecipitated with a thyroglobulin-linked peptide antibody, as described in Materials and Methods. Native membranes (○) and immunoprecipitates (▲) were assayed for Ca^{2+},Mg^{2+}-ATPase in the presence or absence of vanadate at the concentrations shown. Points are mean ± S.E. for three determinations with different preparations.

and immunoprecipitated protein. Vanadate completely inhibited activity at 50 μM in both preparations (Fig. 2) and the concentration for half-maximal inhibtion was approx. 7 μM for rough ER membranes. For immunoprecipitated protein, the concentration dependence curve followed that for inhibition of rough ER very closely. The values are similar to those previously reported for inhibition of pancreatic ER Ca^{2+},Mg^{2+}-ATPase by vanadate [21]. As shown in Fig. 3, thapsi-

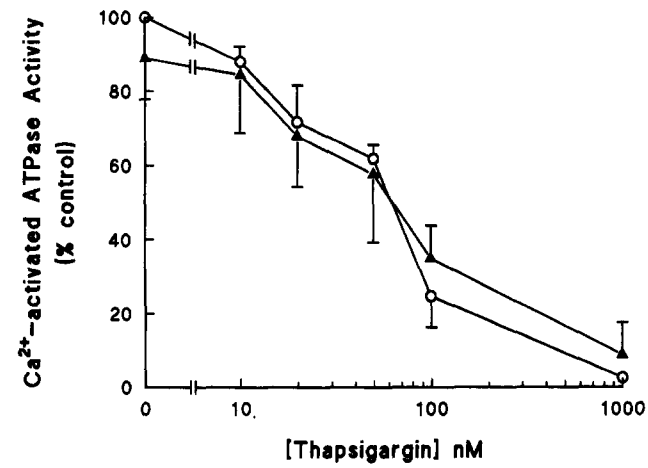

Fig. 3. Inhibition of pancreatic rough ER membrane and immunoprecipitated Ca^{2+},Mg^{2+}-ATPase by thapsigargin. Solubilised rough ER membranes were immnuoprecipitated with a thyroglobulin-linked peptide antibody, as described in Materials and Methods. Native membranes (○) and immunoprecipitates (▲) were assayed for Ca^{2+},Mg^{2+}-ATPase in the presence or absence of thapsigargin at the concentrations shown. Points are mean ± S.E. for three determinations with different preparations.

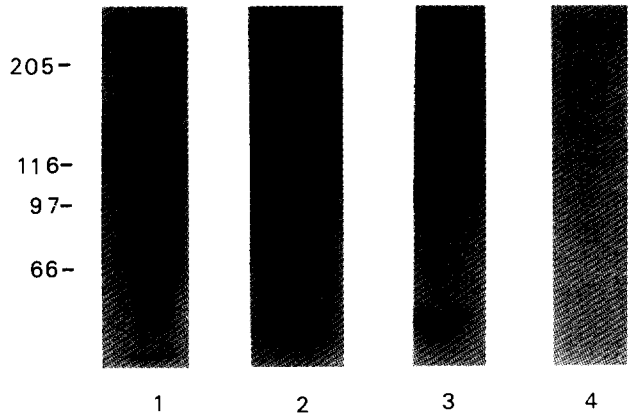

Fig. 4. Immunoblots of pancreatic rough ER membranes with anti-peptide antibodies. Rough ER membranes (20 μg protein) were fractionated on 5% SDS-polyacrylamide gels and immunoblotted as described in Materials and Methods. Lane 1: molecular mass markers stained with Amido black (values in kDa shown on left); lane 2: BSA-linked peptide antibody; lane 3: thyroglobulin-linked peptide antibody; lane 4: preimmune serum.

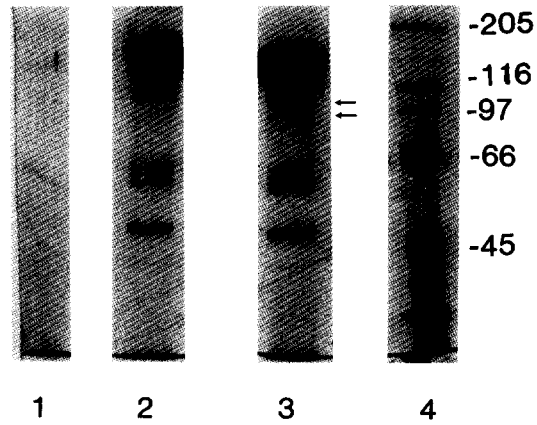

Fig. 5. Purification of pancreatic rough ER Ca^{2+},Mg^{2+}-ATPase by immunoprecipitation. Solubilised rough ER membranes were immunoprecipitated with a thyroglobulin-linked peptide antibody, as described in Materials and Methods. Precipitates were solubilised and fractionated on 7.5% SDS-polyacrylamide gels and silver-stained (lanes 1–3); lane 4: molecular mass markers (values in kDa shown on right). Lane 1: immunoprecipitate without primary antibody; lane 2: immunoprecipitate with preimmune serum; lane 3: immunoprecipitate with anti-thyroglobulin-linked peptide antibody.

gargin inhibition was half-maximal at approx. 60 nM and complete at 1 μM in both preparations.

Purification and characterisation of ER Ca^{2+},Mg^{2+}-ATPase

(a) Immunological. Fig. 4 shows examples of immunoblots using antibodies against either BSA- or thyroglobulin-linked peptide. Two major protein bands recognised by the BSA-linked peptide antibody (lane 2) had apparent molecular masses of 107 and 98–100 kDa. The thyroglobulin-linked peptide antibody recognised a broad band (which in some gels resolved clearly into two bands) covering a similar molecular mass range (lane 3). Nine determinations of molecular mass using four different antibodies (two BSA-linked; two thyroglobulin-linked), gave values of 111 kDa (range 107–115 kDa) and 97 kDa (range 92–108).

Immunoprecipitated proteins were also separated by SDS-polyacrylamide gel electrophoresis and silver-stained. Fig. 5 shows a gel on which samples were not reduced so that the majority of the IgG ran at an apparent molecular mass of 150 kDa. The absence of a heavily stained band at this molecular mass in lane 1, which was from an immunoprecipitation carried out in the absence of primary antibody, suggested that the equivalent bands in lanes 2 and 3 were IgG. This was confirmed by subsequent Western blotting using anti-rabbit IgG (data not shown). Two bands (marked by arrows) of molecular masses approx. 106 and 115 kDa were immunoprecipitated using thyroglobulin-linked peptide antibody (lane 3) or BSA-linked peptide antibody (data not shown), but not using preimmune antiserum.

(b) Reactive Dye-Agarose affinity chromatography. Binding to Reactive Red-agarose and elution with ATP has been used in the purification of SR Ca^{2+},Mg^{2+}-ATPase [22]. A range of Reactive- and Mimetic-dyes (Blue, Yellow, Green, Brown and Red) were therefore tested for their use in purifying Ca^{2+},Mg^{2+}-ATPase solubilised from pancreatic rough ER. The most successful were Reactive Brown- and Mimetic Blue I-agarose. Table I shows the amount of solubilised rough ER protein and Ca^{2+},Mg^{2+}-ATPase activity which did not bind to each of the columns (unbound) and the amount which was subsequently eluted from the

TABLE I

Recovery of protein and Ca^{2+},Mg^{2+}-ATPase activity from Reactive Dye-agarose columns

Rough ER membranes were solubilised, applied to Reactive Brown- or Mimetic Blue I-agarose column and eluted with 5 mM ATP as described in Materials and Methods. The first two 1-ml fractions eluted after binding (Unbound) and the first two 1 ml fractions after elution with ATP (ATP-eluted) were combined and assayed for Ca^{2+},Mg^{2+}-ATPase activity and protein as described in Materials and Methods.

Dye	Fraction	Recovery of protein (% total recovered)	Ca^{2+},Mg^{2+}-ATPase	
			% total recovered	% total added
Reactive Brown	unbound	87.1±4.6	74.0± 5.9	603.3±364.7
	ATP-eluted	12.9±4.6	26.0± 5.9	297.4±215.2
Mimetic Blue-I	unbound	94.6±5.4	63.0±11.4	647.1±181.0
	ATP-eluted	5.7±5.3	37.0±11.4	420.8±236.8

columns in the presence of 5 mM ATP (ATP-eluted). Total recoveries of Ca^{2+},Mg^{2+}-ATPase activity were > 100% (last column Table I) and hence amounts were also expressed as a percentage of the total recovered. 74% and 63% of Ca^{2+},Mg^{2+}-ATPase activity did not bind to Reactive Brown and Mimetic Blue I columns, respectively; 90–95% of the total protein was in the unbound fractions.

The high recoveries of Ca^{2+},Mg^{2+}-ATPase activity suggested activation during binding and elution from the Reactive Dyes. Activation could not be achieved by dialysis of solubilised rough ER against column elution buffer (Rathbone and Dormer, unpublished data) suggesting that the effect was not due to dilution of CHAPS or removal of a low molecular mass contaminant. The increases in activity might, therefore, reflect removal of an endogenous inhibitor which became bound to the column. However, following binding of solubilised rough ER to Mimetic Blue I columns and elution in the presence of 5 mM ATP, further fractions were eluted in the presence of high salt (2 M KCl), high pH (8.0), glycerol or guanidinium HCl and added back to activated fractions in the Ca^{2+},Mg^{2+}-ATPase assay. In no case could an inhibitory activity be demonstrated (Rathbone and Dormer, unpublished data).

Owing to its activation on Reactive Dye columns, it was not possible to assess purity of the eluted Ca^{2+},Mg^{2+}-ATPase by calculating specific activity. In addition, the very low amounts of protein eluted by ATP (less than 3 μg/ml from Mimetic Blue I columns), hampered determination of purity of fractions on SDS polyacrylamide gels. Silver-stained gels (Fig. 6) showed that CHAPS solubilised a range of proteins of molecular masses between 20 and 150 kDa (lane 6) and confirmed that the majority of proteins applied to the column did not bind (lanes 4 and 5). In the first fraction eluted by ATP (Fig. 6, lane 2), an extremely faint protein band in the same order of molecular mass as demonstrated immunologically (approx. 105 kDa), was observed. The readily observable bands, of approx. 70 kDa and 125 kDa molecular mass, consistently copurified in these fractions. Measurements of activity eluted from the Mimetic Blue I column (Table I) indicated that the Ca^{2+},Mg^{2+}-ATPase of pancreatic rough ER comprises less than 0.1% of the total protein and that Reactive-Dye affinity chromatography purified the Ca^{2+},Mg^{2+}-ATPase by at least 300-fold.

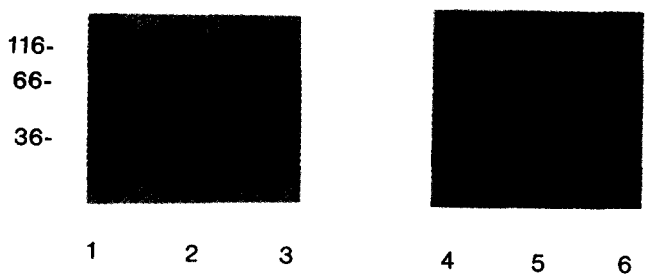

Fig. 6. Purification of pancreatic rough ER Ca^{2+},Mg^{2+}-ATPase by Reactive Dye affinity chromatography. Rough ER membranes were solubilised, applied to a Mimetic Blue I-agarose column and eluted with 5 mM ATP as described in Materials and Methods. Fractions were solubilised and fractionated on 7.5% SDS-polyacrylamide gels and silver-stained. Lane 1: Molecular mass markers (values in kDa shown on left); lanes 2 and 3: first two fractions eluted by ATP (approx. 100 ng and 20 ng protein, respectively; lanes 4 and 5: first two unbound fractions (0.6 μg and 6 μg protein, respectively; lane 6: solubilised rough ER proteins (7 μg).

Discussion

The immunological data presented, demonstrate that the Ca^{2+},Mg^{2+}-ATPase of pancreatic ER is of the SERCA-2b type by virtue of possessing the C-terminal extension differentiating it from all other types [8,9,12]. The data further indicated that the Ca^{2+},Mg^{2+}-ATPase is present in two isoforms. Wuytack et al. [17] first described an antibody against a peptide in the unique C-terminal sequence which differentiated the 2b form in smooth muscle, liver and kidney. In the present study, immunoprecipitation of Ca^{2+}-activated ATPase activity, which was inhibited by vanadate and thapsigargin (Figs. 2 and 3), demonstrated that antibodies raised against the 12 amino acid peptide recognised SERCA-type ATPases. The membrane fraction from which Ca^{2+},Mg^{2+}-ATPase has been purified was predominantly rough ER as demonstrated by enrichment of rough ER markers and the absence of significant amounts of either plasma or Golgi membranes [16].

Reactive Dye affinity chromatography has been used to purify the Ca^{2+},Mg^{2+}-ATPase from SR [22]. The inefficiency of binding of the pancreatic ATPase to Reactive Dyes (see Table I) may reflect a difference in conformation of the ER ATPase around the nucleotide binding site. Alternatively, since solubilisation of SR Ca^{2+},Mg^{2+}-ATPase was shown to reduce its affinity for Reactive Dyes [23], a more marked effect on affinity of the ER ATPase could explain the lack of binding. Activation of the Ca^{2+},Mg^{2+}-ATPase after passage through Reactive Dye columns suggested that ER membranes possess an endogenous inhibitor (see Results). Dissociation of an inhibitor could be an explanation for the persistent activation of the Ca^{2+},Mg^{2+}-ATPase after isolation of rough ER from stimulated pancreatic acinar cells [1].

The two Ca^{2+},Mg^{2+}-ATPase isoforms visualised by immunoblotting and immunoprecipitation (Figs. 4 and 5) ran at apparent molecular masses in the range 107–115 kDa and 92–108 kDa. Proteins of apparent molecular mass of the order of 125 kDa and 105 kDa were observed in fractions purified by Reactive Dye affinity chromatography (Fig. 6). Immunoblotting of SERCA-2b Ca^{2+},Mg^{2+}-ATPase expressed in COS-1

cells [24], showed a major protein at approx. 115 kDa as predicted by the cDNA sequence. In addition, a minor band at slighly lower molecular mass was noted [24] and this could correspond to the second form seen in the present study. Northern blotting of pancreatic tissue has demonstrated mRNA for SERCA types 2b and 3 [10]. However, the antibody used in the present study would not be expected to recognise type 3 since this form does not possess the C-terminal extension to which the peptide sequence corresponds [10]. Platelets also express mRNA for the 2b form and immunoblotting with a 2b-specific antibody showed a single protein band of approx. 100 kDa [15,25]. The presence of a 100 kDa protein, suggested to be a Ca^{2+},Mg^{2+}-ATPase on the basis of Ca^{2+}-dependent autophosphorylation, was previously reported in pancreatic ER [21]. The band shown was, however, very diffuse over the region from approx. 100–120 kDa and may have consisted of more than one protein.

The significance of the presence of different isoforms of ER Ca^{2+},Mg^{2+}-ATPases in the same cell is not yet clear. It may be that different isoforms possess targetting information to direct them to distinct intracellular compartments. This possibility was discussed when differential splicing of the SERCA-2 gene was first described [9,12]. Evidence that different Ca^{2+},Mg^{2+}-ATPase isoforms may account for Ca^{2+} uptake into different Ca^{2+} stores was obtained in adrenal chromaffin cells [13]. Platelets have also been shown to possess two Ca^{2+},Mg^{2+}-ATPase isoforms of apparent molecular masses 100 and 97 kDa [15,25]. The 97 kDa form is apparently not a 2b-type Ca^{2+},Mg^{2+}-ATPase and was recognised by a non-specific anti-SR Ca^{2+},Mg^{2+}-ATPase antibody. Furthermore, it was shown to be less sensitive to thapsigargin, but more sensitive to tBHQ than the 2b form [15,26]. It may be relevant that in adrenal chromaffin cells, evidence suggested that tBHQ-sensitve stores are a subset of the thapsigargin-sensitive stores [27].

In pancreatic acinar cells, therefore, where there is considerable evidence for the presence of Ins(1,4,5)-P_3-sensitive and -insensitive Ca^{2+} stores [3–6], it will be important to purify the two Ca^{2+},Mg^{2+}-ATPase isoforms demonstrated in the present study and determine whether they possess different properties and cellular localisation.

Acknowledgements

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