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TISSUE LEVELS AND PURIFICATION BY AFFINITY CHROMATOGRAPHY OF THE CALMODULIN-STIMULATED Ca^{2+}-TRANSPORT ATPase IN PIG ANTRUM SMOOTH MUSCLE

GREET DE SCHUTTER, FRANK WUYTACK, JAN VERBIST and RIK CASTEELS

Laboratorium voor Fysiologie, Katholieke Universiteit Leuven, Campus Gasthuisberg, B-3000 Leuven (Belgium)

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The Ca^{2+}-transport ATPase ($(Ca^{2+} + Mg^{2+})$-ATPase) in a plasma membrane-rich fraction of porcine antrum (stomach) smooth muscle, is stimulated 2.9-times by calmodulin in the presence of 0.2 mg/ml saponin and reaches a value of 12.0 ± 2.0 (4) μ mol/100 mg protein (equivalent to 110 g wet tissue) per min at 37°C and 10^{-5} M $[Ca^{2+}]$. Saponin was found to specifically potentiate the calmodulin-$(Ca^{2+} + Mg^{2+})$-ATPase interaction, even in the Triton X-100 solubilized enzyme. The conditions for purification of the $(Ca^{2+} + Mg^{2+})$-ATPase by affinity chromatography on a calmodulin-Sepharose 4B gel were optimized. The purified enzyme has a specific activity of 11.9 μmol/mg protein per min at 37°C, 10^{-5} M $[Ca^{2+}]$, 0.6 μM calmodulin, and shows a double polypeptide band at 140 and 150 kDa. The $(Ca^{2+} + Mg^{2+})$-ATPase can be incorporated in artificial liposomes that thereupon show an ATP-dependent Ca^{2+} uptake (Ca:ATP = 1.0). The magnitude of the calmodulin stimulation of the isolated enzyme depends on its phospholipid environment. When isolated in the presence of phosphatidylserine no calmodulin stimulation is observed. After reconstitution in phosphatidylcholine the calmodulin stimulation amounts to 4.05 ± 0.63 (n = 12) times.

1. Introduction

The decrease in concentration of the ionized calcium in the cytoplasm during relaxation of smooth muscle cells depends on the Ca^{2+} transport systems present in the plasma membrane and in the sarcoplasmic reticulum [1].

Membrane fractions of the porcine coronary artery and stomach (antrum) smooth muscle present a Ca^{2+}-transport ATPase ($(Ca^{2+} + Mg^{2+})$-ATPase) with a high affinity for Ca^{2+} [2,3]. It is, as yet, not clear whether the observed $(Ca^{2+} + Mg^{2+})$-ATPase activity originates only from the plasma membrane or also from the Ca^{2+} transport system in the sarcoplasmic reticulum. The relative importance of both transport mechanisms for smooth muscle relaxation is still unknown.

Some plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPases have recently been shown to bind to and to be regulated by calmodulin [4]. This binding of calmodulin to the $(Ca^{2+} + Mg^{2+})$-ATPase has been used to purify the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocytes [5,6] and of heart sarcolemma [7] by means of calmodulin-affinity chromatography.

In a preliminary report from our laboratory the purification by this method of a functionally active $(Ca^{2+} + Mg^{2+})$-ATPase of smooth muscle microsomes has been described [8]. The present work deals only with this $(Ca^{2+} + Mg^{2+})$-ATPase which has previously been shown [9] to have the functional characteristics of a plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase [4].

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride.

Antibodies against the purified enzyme of porcine erythrocytes were shown to react with the plasma membrane type (Ca^{2+} + Mg^{2+})-ATPase of smooth muscle but not with the sarcoplasmic reticulum type ATPase from skeletal muscle (Ref. 10 and unpublished observations).

In this work we have estimated the total activity of (Ca^{2+} + Mg^{2+})-ATPase present in the membrane fractions from porcine antrum smooth muscle and we have paid special attention to the stimulation of the enzyme by calmodulin. Also the binding of the detergent-solubilized enzyme to the calmodulin affinity gel and the optimal assay conditions of the isolated enzyme have been investigated. It was confirmed that the isolated enzyme is a genuine Ca^{2+} transport enzyme because its incorporation in artificial liposomes induces an ATP-dependent Ca^{2+} accumulation. Furthermore the effect of detergents and of negatively charged phospholipids on the isolated and reconstituted enzyme was investigated.

2. Methods

2.1. Microsomes. Smooth muscle microsomes were prepared from the antral part of the pig stomach as previously described [1] except that the mixed solution was homogenized twice for 20 s with an Ultra Turrax (Type TP 18-10 Janke & Kunkel KG) instead of using a Potter-Elvehjem homogenizer. In the KCl-wash we added 2 mM EGTA to eliminate contaminating calmodulin and 0.5 mM PMSF to inhibit protease activity.

2.2. Solubilization. (Ca^{2+} + Mg^{2+})-ATPase present in the microsomes was solubilized by using Triton X-100 at a detergent/protein (w/w) ratio of 1 : 1 as described in [8].

2.3. Calmodulin affinity gel. Calmodulin-Sepharose 4B gel (20 to 30 ml gel containing 2 mg/ml bovine brain calmodulin) was prepared from CNBr-activated Sepharose according to the manufactures instructions. The Triton X-100 solubilized membrane material was added to the calmodulin-Sepharose 4B affinity gel which had been equilibrated with a buffer containing 130 mM KCl, 20 mM K-Hepes (pH = 7.4), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol, 0.4% (w/w) Triton X-100 and 0.05% (w/w) asolectin (crude soybean lipids). This mixture was incubated for 1 h at 4°C in an end-over-end mixer and thereupon the gel was washed on a fritted glass filter with 10 gel volumes of the same buffer to remove the unbound material. The gel was then packed in a chromatography column and a fraction containing (Ca^{2+} + Mg^{2+})-ATPase was released by substituting in the buffer 2 mM K-EGTA for 0.1 mM $CaCl_2$. (Ca^{2+} + Mg^{2+})-ATPase activity was determined immediately after chromatography and active fractions were pooled and stored at −70°C. The calmodulin gel was stored at 4°C for further use in a buffer containing 500 mM NaCl, 50 mM Mops at pH = 7.0, 0.02% NaN_3.

2.4. Reconstitution. The purified (Ca^{2+} + Mg^{2+})-ATPase was incorporated in vesicles by the cholate dialysis method [12].

2.5. Assays. The (Ca^{2+} + Mg^{2+})-ATPase activity was measured by the coupled enzyme system at 37°C [2] at a free Ca^{2+} concentration of 10^{-5} M (0.87 mM $CaCl_2$ and 1 mM EGTA) unless otherwise stated. The Ca^{2+}-uptake in the reconstituted vesicles was estimated with the arsenazo method [13] or with a Ca^{2+}-electrode [14]. The Ca^{2+}-uptake measurements with arsenazo were done in a medium (1 ml) containing: 100 mM KCl, 30 mM imidazole-HCl (pH = 6.8), 5 mM NaN_3, 1 mM $MgCl_2$, 0.02 mM $CaCl_2$, 50 μM arsenazo III and approx. 40 μg/ml protein of reconstituted vesicles. The reaction was started by adding 0.5 mM ATP and the difference in light absorbance between wavelengths 675 and 685 nm was recorded. Protein was determined according to Peterson [15], with bovine serum albumin as standard. To avoid interference by detergents and dithiothreitol, proteins were precipitated by 6% trichloroacetic acid in the presence of 0.012% deoxycholate and pelleted in an Eppendorf centrifuge model 5413 for 5 min. The pellet was washed with 1 ml of a chloroform/methanol (1 : 2, v/v) solution and centrifuged again. Light scattering at 90° was measured with an Aminco SPF-500 fluorescence spectrophotometer (λ = 430 nm, 37°C).

Phospholipids were measured after extraction by the method of Bligh and Dyer [16] followed by ashing and P_i determination as in Ref. 17. SDS-polyacrylamide gel electrophoresis was performed as described before [8]. The calmodulin concentration in the microsomes was estimated (after heat-treatment of the vesicles) by the phosphodiesterase

stimulation [18]. Myosin light-chain kinase activity was measured as described in Ref. 19 with bovine cardiac light chains as substrate.

2.6. Materials. Calmodulin [20] and phosphodiesterase [21], were prepared as described. Bovine cardiac light-chains were kindly provided by Dr. J. DiSalvo (Cincinnati College of Medicine). CNBr-activated Sepharose 4B was purchased from Pharmacia, TLCK (7-amino-1-chloro-3-L-*p*-tosylamidoheptan-2-one) from Serva, dithiothreitol, cholate and deoxycholate from Sigma, PMSF, pyruvate kinase, and lactate dehydrogenase from Boehringer. Phosphatidylserine and phosphatidylcholine (both grade I) from Lipid Products (South Nutfield, Surrey), asolectin from Associated Concentrates (Woodside, L.I., New York), saponin from Merck and A23187 from Calbiochem.

3. Results

3.1. Total ($Ca^{2+} + Mg^{2+}$)-ATPase activity in the microsomes

The total amount of microsomal protein, that can be prepared from 100 g of antrum smooth muscle amounts to 110 mg. Before addition of calmodulin, the total ($Ca^{2+} + Mg^{2+}$)-ATPase activity at 10^{-5} M [Ca^{2+}] amounts to 1.5 μmol P_i/100 mg protein per min. Addition of 1 μg/ml of the Ca^{2+}-ionophore A23187 to the ATPase assay increases this activity to 1.9 μmol P_i/100 mg protein per min (Table I) without affecting the Mg^{2+}-ATPase or ($Na^+ + K^+$)-ATPase activities. Further addition of a saturating concentration of 0.6 μM calmodulin, increases the ($Ca^{2+} + Mg^{2+}$)-ATPase activity to 4.4 μmol P_i/100 mg protein per min. The KCl-extracted microsomes were found still to contain 0.2–1.5 μg calmodulin/mg microsomal protein. These microsomes also develop a total Mg^{2+}-ATPase activity of 9.1 μmol P_i/min and a total ($Na^+ + K^+$)-ATPase activity of 1.7 μmol P_i/min per 100 mg protein. This is not surprising because a large part of the microsomes is of plasmalemmal origin [3]. In order to unmask latent ATPase activity we have disrupted the microsomes with detergent. Addition of 0.1–0.2 mg/ml of saponin doubles the basal ($Ca^{2+} + Mg^{2+}$)-ATPase activity (in the absence of calmodulin) and also the ($Na^+ + K^+$)-ATPase (Fig. 1). The same is true for vesicles preincubated with Triton X-100 or deoxycholate, but neither of these detergents increase the Mg^{2+}-ATPase. Saponin (0.1–0.2 mg/ml) in addition potentiates specifically the stimulation of the ($Ca^{2+} + Mg^{2+}$)-ATPase by calmodulin in the microsomes from 2.2 to 2.9. Deoxycholate and Triton X-100 did not exert such potentiating effect (Fig. 2) but in the ($Ca^{2+} + Mg^{2+}$)-ATPase solubilized by Triton X-

TABLE I

THE TOTAL ACTIVITY OF THE DIFFERENT ATPases PRESENT IN 100 mg PROTEIN OF KCl-EXTRACTED MICROSOMES AND IN THE MATERIAL SOLUBILIZED FROM THIS AMOUNT OF MICROSOMES BY TRITON X-100 (1:1 WEIGHT RATIO OF TRITON X-100 AND MICROSOMAL PROTEIN)

The values are given as mean ± S.E. The number of observations is given between brackets.

	Total activity (μmol P_i/min) (37°C)				
	Mg^{2+}-ATPase	($Na^+ + K^+$)-ATPase	($Ca^{2+} + Mg^{2+}$)-ATPase		
			1–2 μg A23187 +0 calmodulin	1–2 μg A23187 +0.6 μM calmodulin	Mean stimulation by calmodulin
KCl-extracted microsomes					
100 mg proteins					
0 saponin	9.1 ± 0.66 (5)	1.7 ± 0.15 (3)	1.9 ± 0.25 (5)	4.4 ± 1.20 (4)	2.2 ± 0.30 (4)
200 μg saponin/ml	9.2 ± 0.67 (5)	2.5 ± 0.55 (2)	4.4 ± 0.59 (5)	12.0 ± 2.0 (4)	2.9 ± 0.25 (4)
Triton X-100 solubilized fraction					
36 mg proteins					
0 saponin	3.2 ± 0.24 (4)	1.3 ± 0.26 (4)	2.0 ± 0.67 (5)	2.9 ± 0.40 (5)	1.4 ± 0.02 (5)
200 μg saponin/ml	3.3 ± 0.10 (4)	1.1 ± 0.12 (4)	2.0 ± 0.49 (6)	5.0 ± 0.41 (6)	2.6 ± 0.19 (6)

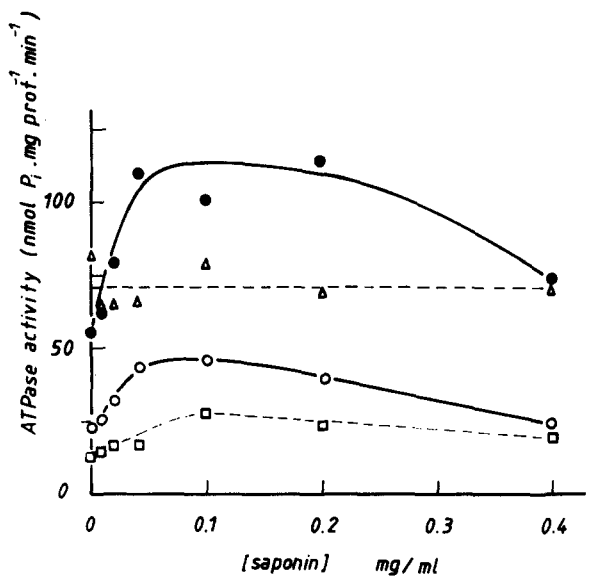

Fig. 1. The effect of different concentrations of saponin (mg/ml) on the ATPase activities (nmol $P_i \cdot mg^{-1} \cdot min^{-1}$) of smooth muscle microsomes (△, Mg^{2+}-ATPase; □, $(Na^+ + K^+)$-ATPase; ○, $(Ca^{2+} + Mg^{2+})$-ATPase without added calmodulin; ●, $(Ca^{2+} + Mg^{2+})$-ATPase + 0.6 μM calmodulin). Saponin was added to the cuvette from a stock solution containing 20 mg/ml in water.

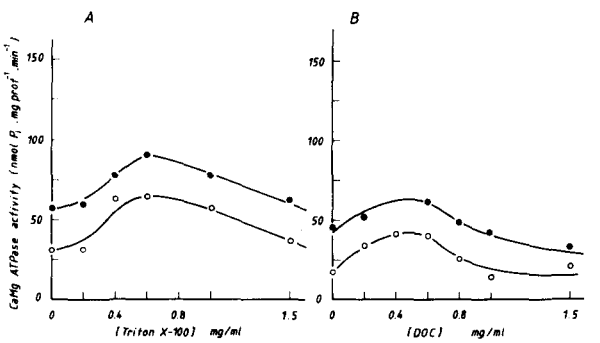

Fig. 2. The effect of different concentrations (mg/ml) of Triton X-100 (A) and deoxycholate (DOC) (B) on the specific $(Ca^{2+} + Mg^{2+})$-ATPase (CaMg ATPase) activity (nmol $P_i \cdot mg^{-1} \cdot min^{-1}$ on the ordinate) measured in the KCl-extracted smooth muscle. The open circles represent the activity without addition of calmodulin, the filled circles after addition of 0.6 μM calmodulin. The microsomes at a protein concentration of 0.5 mg/ml in a medium containing 0.125 M sucrose, 2 mM Tris EDTA, 40 mM imidazole pH 7.1, were preincubated with Triton X-100 or deoxycholate for 20 min on ice. The abscissa indicates the detergent concentrations during preincubation. In the ATPase assay a sample of the preincubated microsomes is diluted 10-fold.

100, saponin increases the calmodulin stimulation from 1.4 to 2.6. However, the potentiating effect of saponin on the calmodulin stimulation was not observed for the $(Ca^{2+} + Mg^{2+})$-ATPase purified by affinity gel chromatography. These results indicate that saponin in some way affects the interaction of calmodulin with the $(Ca^{2+} + Mg^{2+})$-ATPase. The specific activity of the $(Ca^{2+} + Mg^{2+})$-ATPase in the microsomes which is fully activated by the presence of A23187, calmodulin and saponin amounts to 100–110 nmol P_i/mg protein per min. The $(Ca^{2+} + Mg^{2+})$-ATPase in the microsomes was stimulated half maximally by Ca^{2+} at a concentration of $K_{Ca} = 2.2$ μM (in the presence of 0.6 μM calmodulin and 0.2 mg/ml saponin) while calmodulin stimulated the enzyme half maximally at $K_{calm} = 28.2$ nM (in the presence of 0.2 mg/ml saponin and 10 μM Ca^{2+}).

3.2. Solubilization of the $(Ca^{2+} + Mg^{2+})$-ATPase

By treating the microsomes with the non-ionic detergent Triton X-100 at a detergent/protein ratio (w/w) of 1 : 1, (i.e., at 0.4% Triton X-100 and 0.4% microsomal protein), 36 ± 12% (mean ± S.E.) ($n = 8$) of the microsomal proteins were solubilized. The phospholipid/protein ratio (w/w) in the solubilized fraction was found to be close to 1.

The amount of $(Ca^{2+} + Mg^{2+})$-ATPase solubilized by Triton X-100 measured in the absence of calmodulin and in the presence of 2 μg/ml of A23187 and 0.2 mg/ml saponin is about half of the total $(Ca^{2+} + Mg^{2+})$-ATPase activity originally present in the microsomes (Table I). Saponin does not affect the basal activity of the solubilized $(Ca^{2+} + Mg^{2+})$-ATPase but the stimulation of this ATPase by calmodulin is affected differently by the detergents Triton X-100 and saponin. The calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase solubilized by Triton X-100 is decreased from 2.2 to 1.4, but after addition of 0.2 mg saponin/ml this calmodulin stimulation increases again to 2.6.

3.3. Chromatography on a calmodulin-Sepharose 4B gel

Affinity chromatography on a calmodulin-Sepharose 4B gel [8] was used as a further purification procedure. It was observed that to preserve the activity of the enzyme, phospholipids had to be

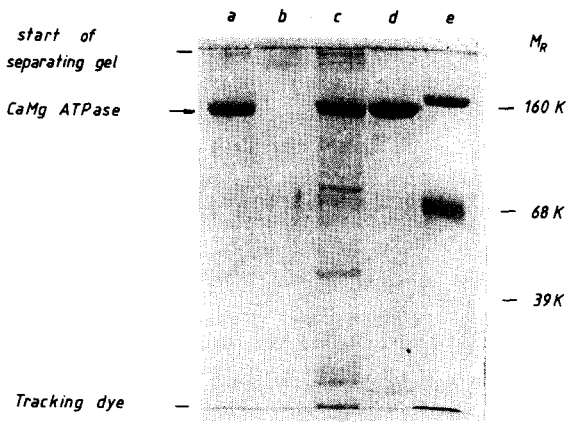

Fig. 3. Laemmli-type SDS-polyacrylamide gel electrophoresis (10% acrylamide in the separating gel) of the calmodulin stimulated $(Ca^{2+} + Mg^{2+})$-ATPase (CaMg ATPase) from smooth muscle (a–c) and from porcine erythrocytes (d). The $(Ca^{2+} + Mg^{2+})$-ATPase of smooth muscle was bound to the calmodulin-Sepharose 4B gel which had been equilibrated with a Ca^{2+}-containing buffer at 0.4% Triton X-100/0.05% asolectin. After binding the gel was divided in three equal parts and eluted with different EGTA containing buffers (a) 0.4% Triton X-100/no asolectin, (b) no Triton X-100/0.05% asolectin, (c) 0.4% Triton X-100/0.05% asolectin. In (a) and (b) no $(Ca^{2+} + Mg^{2+})$-ATPase activity could be determined in the eluates. In (c) the specific activity of the $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of 0.6 μM calmodulin amounted to 6 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$. Lane (d) was the $(Ca^{2+} + Mg^{2+})$-ATPase preparated from porcine erythrocytes with all buffers containing 0.4% Triton X-100/0.05% asolectin. Lane (e) molecular mass standards: 160, 68 and 39 kDa. The gels were stained with Coomassie blue R250.

present continuously. Because the membranes themselves provide sufficient phospholipids after exposure to Triton X-100, it was not necessary to add additional phospholipids during the binding step. However, if no extra phospholipids are added in the washing and elution step, the eluted $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 3) is inactive. As yet we found no procedure to reactivate this inactive enzyme by subsequent addition of phospholipids. We have therefore used in our standard procedure for purification of $(Ca^{2+} + Mg^{2+})$-ATPase, buffers containing 0.4% Triton X-100 and 0.05% asolectin.

The purity of this $(Ca^{2+} + Mg^{2+})$-ATPase obtained by affinity chromatography as observed in the SDS-polyacrylamide gel electrophoresis is increased by prolonged washing of the calmodulin gel with the Ca^{2+}-containing buffers. Laemmli-type SDS-polyacrylamide gel electrophoresis reveals immediately the prominent double band of 140–150 kDa. This band corresponds most likely to the Ca^{2+} transport enzyme because it is the only one which shows the Ca^{2+}-dependent, hydroxylamine sensitive phosphorylation typical for a $(Ca^{2+} + Mg^{2+})$-ATPase activity [9]. Moreover, it has the same molecular weight as the purified $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocytes [23], and antibodies against porcine erythrocyte ATPase crossreact with the present band of 140–150 kDa (Ref. 10 and unpublished observations). In addition, several contaminant polypeptide bands with molecular mass of 75, 42 and 25 kDa are often observed. Only traces of enzyme activity were found for two other calmodulin-binding enzymes which occur in smooth muscle: calmodulin-stimulated phosphodiesterase [24] and myosin light-chain kinase [25]. The specific activity of the purified $(Ca^{2+} + Mg^{2+})$-ATPase measured under optimal conditions in the presence of 0.6 μM calmodulin and at 37°C amounts to 11.9 μmol P_i/mg protein per min at 10^{-5} $[Ca^{2+}]$, a value which is comparable to the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocytes [23]. No ATPase activity could be measured in a solution without Ca^{2+} and containing 1 mM EGTA. The concentrations of Ca^{2+} and calmodulin giving a half-maximum stimulation are, respectively, 5.7 μM and 27.0 nM (in the presence of 0.002% Triton X-100 and 0.005% asolectin). At 0.6 μM calmodulin the K_{Ca} for the ATPase shifts to 4.3 μM. A Hill coefficient (n_H) of 0.99 is calculated for the calmodulin activation of the $(Ca^{2+} + Mg^{2+})$-ATPase.

3.3.1. Optimization of the binding capacity of the calmodulin gel

(a) The concentration of the solubilized $(Ca^{2+} + Mg^{2+})$-ATPase. 5 ml of gel containing a total of 10 mg calmodulin was incubated with an equal volume of Triton X-100 extract of the membranes. The total $(Ca^{2+} + Mg^{2+})$-ATPase activities in the extract was adjusted to values between 0.05 and 5.0 μmol P_i/min. For each condition, 52.0 ± 4.75% ($n = 5$) of the total quantity of enzyme added to the gel, was bound (Table II). Because the ratio of bound/total ATPase remains constant it is likely that the calmodulin affinity gel was not yet

saturated even at the maximal concentration of solubilized $(Ca^{2+} + Mg^{2+})$-ATPase. It was therefore not possible to estimate from our results either the total amount of active calmodulin on the gel or the K_d value for the calmodulin-ATPase equilibrium.

(b) The ratio detergent/phospholipid. The binding of $(Ca^{2+} + Mg^{2+})$-ATPase to the calmodulin affinity gel was studied at a constant asolectin concentration (0.05%) and at different Triton X-100 concentrations. At 0.05% Triton X-100/0.05% asolectin and at 0.9% Triton X-100/0.05% asolectin the amount of bound $(Ca^{2+} + Mg^{2+})$-ATPase is about 4-times less than at the optimal Triton X-100/asolectin concentrations of 0.2%/0.05% (Table II). In our standard enzyme purification conditions we used a calmodulin gel that was preequilibrated with a buffer containing 0.4% Triton X-100/0.05% asolectin. We can calculate that after addition of an equal volume of Triton X-100 extract of the membranes (containing besides detergent also membrane phospholipids) the final Triton X-100/phospholipid ratio must be between 4:1 and 10:1 (i.e., close to its optimal value). It should be pointed out that although an optimal binding of the ATPase to the gel, this Triton X-100 concentration can be lowered to 0.05% (at a constant phospholipid concentration of 0.05%) without releasing ATPase from the gel. Subsequent application of 2 mM EGTA in 0.05% Triton X-100/0.05% phospholipid buffer results in the release of the same total amount of ATPase by elution with EGTA buffer containing 0.4% Triton X-100/0.05% phospholipid. Fig. 3 (lane b) shows that no polypeptides are released when Triton X-100 is completely left out from the elution buffers. It is therefore not possible to incorporate the ATPase in vesicles in the absence of detergent.

3.3.2. The effect of detergent and phospholipid on the specific activity of the purified enzyme

In the $(Ca^{2+} + Mg^{2+})$-ATPase assay the purified ATPase solubilized in the presence of detergent and phospholipids is diluted in the cuvette. The altered detergent/phospholipid concentrations could change the activity of the enzyme. The effect of different detergent/phospholipid compositions on the ATPase activity was therefore studied.

We observed a maximal activity of the purified $(Ca^{2+} + Mg^{2+})$-ATPase at a final Triton X-100 concentration in the enzyme assay medium of 0.01–0.02% (w/v), and at 0.0025% asolectin (Fig. 4A). At 0.0025% Triton X-100/0.0025% asolectin, the specific activity of the $(Ca^{2+} + Mg^{2+})$-ATPase was about half the value obtained under the optimal conditions. The addition of Triton X-100 up to a concentration of 0.01 or 0.02% increased the

TABLE II

THE EFFECT OF DIFFERENT EXPERIMENTAL CONDITIONS ON THE BINDING CAPACITY OF THE CALMODULIN-SEPHAROSE 4B GEL

Binding of the ATPase as obtained in different systems. In A the total amounts of solubilized $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of 0.4% Triton X-100/0.05% asolectin (w/w) was varied and added to 5 ml of gel. In B the same amount of solubilized $(Ca^{2+} + Mg^{2+})$-ATPase (2.5 μmol P_i/min) was again added to 5 ml of gel, but the Triton X-100/asolectin ratio in the incubation step was varied. In the two types of experiments the solubilized $(Ca^{2+} + Mg^{2+})$-ATPase is presented to the gel in a volume of 5 ml. In the ATPase assays the Triton X-100/asolectin ratio was always the same (10:1). In the assays for the experiment displayed in A, 0.6 μM calmodulin was added.

A. Total activity (nmol P_i/min/ml gel)				
Added to	227	368	557	1012
Bound and eluted	125	188	301	486
Fraction bound	0.55	0.51	0.54	0.48
B. % Triton X-100/% asolectin (w/w) in the incubation	0.05/0.05	0.2/0.05	0.5/0.5	0.9/0.05
Total activity (nmol P_i/min/ml gel) bound and eluted	100	321	232	104
Fraction bound	0.20	0.64	0.46	0.21

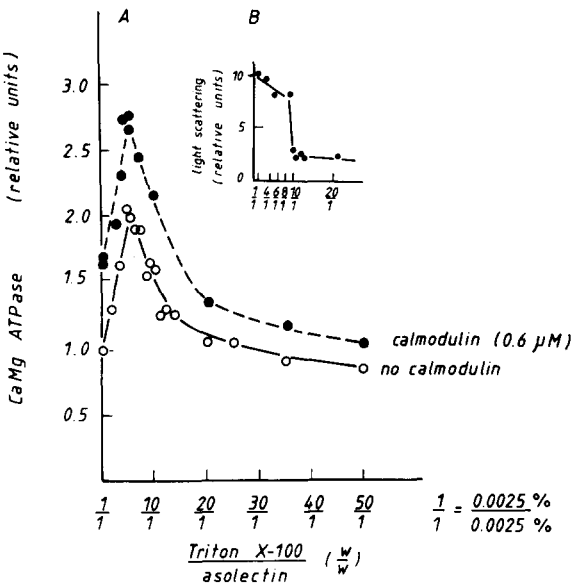

Fig. 4. The effect of the ratio Triton X-100/asolectin in the enzyme assay medium: (A) on the activity of the purified $(Ca^{2+} + Mg^{2+})$-ATPase of smooth muscle without addition of calmodulin (○), in the presence of 0.6 μM calmodulin (●) (B) on the light scattering of the assay medium ($\lambda = 430$ nm, 37°C). The $(Ca^{2+} + Mg^{2+})$-ATPase activities and light scattering are given on the ordinate in relative units against the Triton X-100/asolectin ratio on the abscissa. 1/1 corresponds to 0.0025% Triton X-100/0.0025% asolectin in the assay.

ATPase activity again. The lower activity of the ATPase at low detergent/phospholipid ratios is the result of vesicle formation as is indicated by the observations that only at a Triton X-100/phospholipid ratio below 2:1 the activity of the purified enzyme can be increased by 1 μg/ml A23187 and that at Triton X-100/phospholipid ratios above 10:1 the light scattering suddenly decreases (Fig. 4B).

Increasing the Triton X-100 concentration in the assay medium from 0.0025% to 0.025% at a constant asolectin concentration of 0.0025% decreases the calmodulin stimulation by about 20%, but a further increase of the Triton X-100 concentration does not result in a lower calmodulin stimulation. This could be due to the fact that Triton X-100 interferes with calmodulin activation if present as monomers but not if it is present as micelles.

3.3.3. The effect of the nature of the phospholipids on the specific activity of the purified ATPase

When the $(Ca^{2+} + Mg^{2+})$-ATPase from antrum is purified in the presence of asolectin, calmodulin increases the activity of the soluble enzyme by a factor of 1.4 ± 0.03 ($n = 45$). When the enzyme is eluted from the calmodulin gel in a medium containing phosphatidylcholine, the calmodulin activation amounts to 2.3 ± 0.13 ($n = 15$) while in the presence of phosphatidylserine, the ATPase is not affected by calmodulin. The total activity of enzyme bound to and released from the affinity gel in the presence of phosphatidylcholine (measured with 0.6 μM calmodulin) amounts to about 3/4 of the activity recovered in the presence of asolectin and to about 1/2 of the total activity obtained in the presence of phosphatidylserine. These findings suggest that, although there is no stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase by calmodulin in the presence of phosphatidylserine, calmodulin still binds to the ATPase under these conditions. These observations are similar to the ones made on $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes [26].

3.4. Reconstitution

Asolectin vesicles in which the purified $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle was incorporated [12] presented immediately upon addition of ATP a high rate of both Ca^{2+} uptake and ATPase activity. At the time when net Ca^{2+} accumulation ceased, this ATPase activity declined to about one-fifth of the initial rate (Fig. 5). On addition of the Ca^{2+}-ionophore A23187 at this stage, Ca^{2+} is released from the vesicles and the ATPase activity is reactivated by a factor of 6.5 ± 1.6 ($n = 9$). These experiments show clearly that the $(Ca^{2+} + Mg^{2+})$-ATPase activity is tightly coupled to Ca^{2+} transport. During the initial phase of Ca^{2+} uptake, the ratio of Ca^{2+} accumulated to ATP hydrolyzed was 1.04 ± 0.15 ($n = 16$). Phosphatidylcholine vesicles containing ATPase accumulated less Ca^{2+} than similar asolectin vesicles and the $(Ca^{2+} + Mg^{2+})$-ATPase in these vesicles was stimulated by the ionophore only by a factor of 1.73 ± 0.13 ($n = 6$) indicating a greater leakiness of these vesicles for Ca^{2+}.

The stimulation by calmodulin of $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in asolectin vesicles

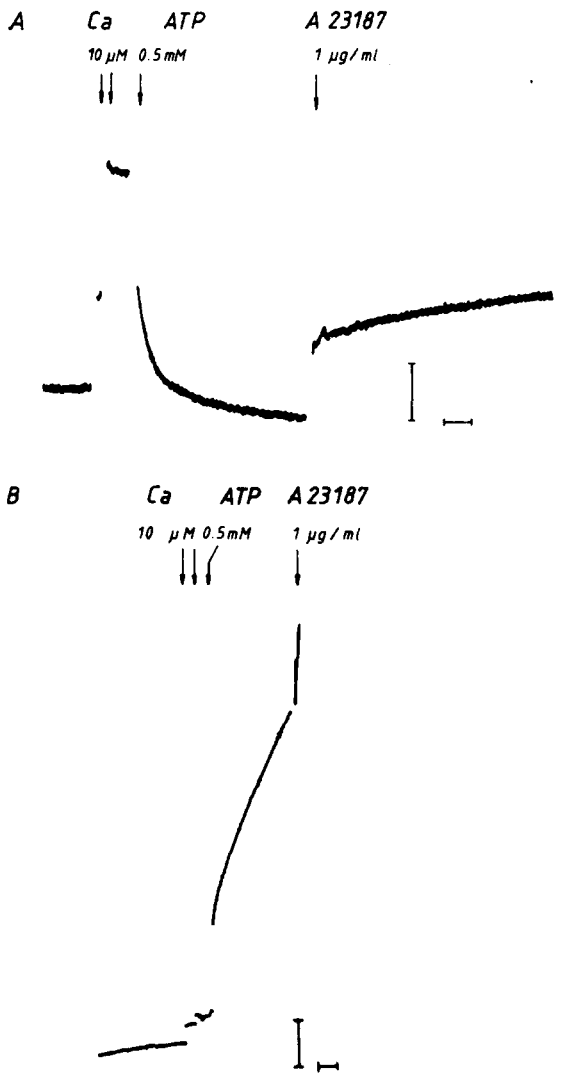

Fig. 5. Ca uptake (A) and corresponding ATPase activity (B) for $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle reconstituted in asolectin vesicles at 28°C. Ca uptake was measured by the arsenazo III method and the ATPase activity by the coupled enzyme system as indicated in the methods section. The two experiments were done in parallel. After two additions of 10 μM Ca, the uptake was started by adding 0.5 mM ATP. After approx. 5 min, 1 μg A23187 was added. In (A) an upward deflection indicates an increased amount of Ca in the medium (vertical calibration bar 0.005 *A* units, horizontal bar 1 min). In (B) the ATPase activity can be calculated from the (upward) slope of the curve, (vertical calibration bar 0.05 *A* units, horizontal bar 1 min).

amounted to 1.25 ± 0.09 ($n = 24$) while in phosphatidylcholine vesicles it was 4.05 ± 0.63 ($n = 12$).

Discussion

The calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity

We have used in this work a microsomal fraction enriched in plasma membranes from porcine antrum smooth muscle. In previous reports [8,11] we have described in this fraction a $(Ca^{2+} + Mg^{2+})$-ATPase which is stimulated by calmodulin by a factor of 1.6 to 1.9. This is only a moderate stimulation as compared to the 7.14 ± 0.80 ($n = 5$) fold stimulation that we observed under the same experimental conditions for porcine erythrocyte vesicles. In contrast the $(Ca^{2+} + Mg^{2+})$-ATPase activity of sarcoplasmic reticulum either from porcine or from rabbit skeletal muscle was not stimulated by calmodulin. The $(Ca^{2+} + Mg^{2+})$-ATPase in the membranes used in this study was stimulated by calmodulin by a factor of 3 with a K_m of 28.2 nM at $[Ca^{2+}] = 10^{-5}$ M. The higher value of this calmodulin stimulation as compared to the values we have reported previously, is partially due to the presence of 2 mM EGTA in the KCl-extraction step used in the preparation of the microsomes. Hereby more calmodulin is removed from the membranes although 12 to 90 pmol calmodulin/mg microsomal protein remains firmly bound to or entrapped in the vesicles. It is not clear whether this remaining calmodulin can still interact with the $(Ca^{2+} + Mg^{2+})$-ATPase. However because the amount of microsomal protein in the ATPase assay is approximately 50 μg/ml the calmodulin concentration contributed by the microsomes to the test solution ranges between 0.6 and 4.5 nM. This is well below the K_m of 28.2 nM. The higher calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase in microsomes in the present study is also partially the result of the addition of saponin in the ATPase assay, because this substance potentiates the calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase present in the membrane as well as in the solution but not of the purified ATPase. Such a potentiating effect on the stimulation of an enzyme by calmodulin that is not accompanied by a concomitant decrease in the basal enzyme activity in the absence of calmodulin, has to our knowledge, not been reported in the literature.

It is well known that the nature of lipid environment strongly affects the magnitude of the

calmodulin stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes [6,26]. This also applies for the $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle. A calmodulin stimulation of 4.05-times was found for $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in phosphatidylcholine whereas in asolectin this stimulation amounts only to 1.25-times the basal activity. The above value of 4.05 is similar to that obtained for purified $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte reconstituted in phosphatidylcholine [4], but it is lower than the value found in erythrocyte membranes in situ. These observations suggest that the $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle has the same intrinsic possibility to be stimulated by calmodulin as the enzyme in erythrocytes. The difference in lipid environment of the $(Ca^{2+} + Mg^{2+})$-ATPase in the membranes from smooth muscle and from erythrocytes might be responsible for the smaller stimulation of the enzyme by calmodulin in the smooth muscle membranes.

Properties of the purified $(Ca^{2+} + Mg^{2+})$-ATPase

Laemmli-type gel electrophoresis of the affinity-purified $(Ca^{2+} + Mg^{2+})$-ATPase reveals two closely spaced polypeptide bands with a molecular mass of 140 and 150 kDa as has been also reported for the $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes [23]. One or both of them correspond to the $(Ca^{2+} + Mg^{2+})$-ATPase.

Because this $(Ca^{2+} + Mg^{2+})$-ATPase is a membrane protein detergents are required for its isolation whereas phospholipids must be present to preserve its enzymatic activity. This creates some particular problems. In order to solubilize the membranes the weight ratio of Triton X-100/membrane phospholipids should be larger than 1, furthermore to obtain an optimal binding to the calmodulin gel a ratio Triton X-100/phospholipid between 4 to 8 was required. At a lower detergent/phospholipid ratio less ATPase is bound to the gel, probably as a result of the formation of liposomes or aggregates of lipids incorporating ATPase molecules. However, at Triton X-100/phospholipid ratios larger than 8, binding of the ATPase was also decreased because the detergent also binds to the calmodulin on the gel. Once the $(Ca^{2+} + Mg^{2+})$-ATPase is bound to the calmodulin gel we could decrease the Triton X-100/phospholipid ratio to 1 without loosing enzyme activity. The concentrations of phospholipid and detergent in the assay medium are also very important. When a sample of the purified ATPase is diluted in the assay medium, this may result in the formation of vesicles with the ensuing decrease in specific activity. We found it necessary to have at least 0.01–0.02% Triton X-100 in the assay medium provided the Triton X-100/phospholipid ratio is between 4 : 1 and 10 : 1.

Calmodulin stimulates the purified smooth muscle $(Ca^{2+} + Mg^{2+})$-ATPase half maximally at a concentration of $K_{0.5} = 27.0$ nM. The total amount of protein in the ATPase assay is about 6 μg. Assuming that 50% of this amount is $(Ca^{2+} + Mg^{2+})$-ATPase and the remainder contaminant proteins, an ATPase concentration of 20 nM would be reached for a molecular mass of the $(Ca^{2+} + Mg^{2+})$-ATPase of 150 kDa. For a 1 : 1 interaction of calmodulin with the ATPase one can calculate a dissociation constant for the calmodulin-enzyme of $K_d = 17$ nM by applying the formula $K_d = K_{0.5} - 0.5[\text{ATPase}]_{\text{total}}$ [27]. The Hill coefficient (n_H) calculated for the calmodulin activation of the $(Ca^{2+} + Mg^{2+})$-ATPase of 0.99 might indicate such 1 : 1 interaction of $(Ca^{2+} + Mg^{2+})$-ATPase and calmodulin. A value for n_H for human erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase close to 1 is also given by Cox et al. [28].

Our reconstitution experiments clearly show that the calmodulin-dependent $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle is a Ca^{2+}-transport ATPase. We obtain a stoichiometric ratio (Ca^{2+} transported: ATP hydrolysed) of 1 in the asolectin vesicles. This value was also reported for the erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in asolectin [12].

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