Biochem. J. (1986) 240, 633–640 (Printed in Great Britain)

A monoclonal antibody to the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-dependent ATPase from pig stomach smooth muscle inhibits plasmalemmal ($Ca^{2+}+Mg^{2+}$)-dependent ATPase activity

Jan VERBIST,* Frank WUYTACK,* Luc RAEYMAEKERS,* Fred VAN LEUVEN,†
Jean-Jacques CASSIMAN† and Rik CASTEELS*
*Laboratory of Physiology, and †Division of Human Genetics, University of Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

A monoclonal antibody (2B3) directed against the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-dependent ATPase from pig stomach smooth muscle was prepared. This antibody reacts with a 130000-M_r protein that co-migrates on SDS/polyacrylamide-gel electrophoresis with the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase purified from smooth muscle by calmodulin affinity chromatography. The antibody causes partial inhibition of the ($Ca^{2+}+Mg^{2+}$)-ATPase activity in plasma membranes from pig stomach smooth muscle, in pig erythrocytes and human erythrocytes. It appears to be directed against a specific functionally important site of the plasmalemmal Ca^{2+}-transport ATPase and acts as a competitive inhibitor of ATP binding. Binding of the antibody does not change the K_m of the ATPase for Ca^{2+} and its inhibitory effect is not altered by the presence of calmodulin. No inhibition of ($Ca^{2+}+Mg^{2+}$)-ATPase activity or of the oxalate-stimulated Ca^{2+} uptake was observed in a pig smooth-muscle vesicle preparation enriched in endoplasmic reticulum. These results confirm the existence in smooth muscle of two different types of Ca^{2+}-transport ATPase: a calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase located in the plasma membrane and a second one confined to the endoplasmic reticulum.

INTRODUCTION

The experimental evidence for the existence in smooth muscle not only of an ATP-dependent Ca^{2+}-extrusion system in the plasma membrane, but also of an ATP-dependent Ca^{2+}-accumulation system in the endo-(sarco-)plasmic reticulum, has been extended by the demonstration of the corresponding ($Ca^{2+}+Mg^{2+}$)-ATPases. The plasmalemmal ($Ca^{2+}+Mg^{2+}$)-ATPase of smooth muscle has many characteristics in common with plasma-membrane ($Ca^{2+}+Mg^{2+}$)-ATPases of erythrocytes (Niggli *et al.*, 1979; Gietzen *et al.*, 1980), of cardiac muscle (Caroni & Carafoli, 1981), of brain synaptosome plasmalemma (Hakim *et al.*, 1982) and of kidney tubule basolateral membrane (De Smedt *et al.*, 1984) [see also Schatzmann (1982) and Penniston (1983) for reviews]. The major common characteristics are: activation by calmodulin, by acidic phospholipids and by limited trypsin treatment (De Schutter *et al.*, 1984), and formation of a hydroxylamine-sensitive and alkali-labile phosphoprotein of M_r 130000 (Wuytack *et al.*, 1982, 1984). This ($Ca^{2+}+Mg^{2+}$)-ATPase mediates an active Ca^{2+} accumulation in a reconstituted system at a Ca^{2+}/ATP molar ratio of 1 (Verbist *et al.*, 1984).

The ($Ca^{2+}+Mg^{2+}$)-ATPase from the endoplasmic reticulum of smooth muscle, on the other hand, resembles the Ca^{2+}-transport ATPase in sarcoplasmic reticulum from skeletal muscle (Martonosi & Halpin, 1971), i.e. it does not bind calmodulin, it has a similar M_r of 100000 and a similar proteolytic degradation pattern. The Ca^{2+}-dependent phosphorylation is decreased by La^{3+}, whereas the phosphorylation extent of the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase is increased by La^{3+} (Wuytack *et al.*, 1982, 1984; Chiesi *et al.*, 1984; Carsten & Miller, 1984). Furthermore, there seems to be a consistent pattern of immunological similarity between the ($Ca^{2+}+Mg^{2+}$)-ATPases from plasma membranes derived from different tissues and species (Verma *et al.*, 1982; Wuytack *et al.*, 1983; Gietzen & Kolandt, 1985; Verbist *et al.*, 1985). In contrast, the Ca^{2+}-transporting ATPase from plasmalemma is largely immunologically dissimilar from that of sarco-(endo-)plasmic reticulum (Verma *et al.*, 1982; Chiesi *et al.*, 1984; Gietzen & Kolandt, 1985).

Different effects of polyclonal antibodies on plasmalemmal ($Ca^{2+}+Mg^{2+}$)-ATPase activity have been described (Verma *et al.*, 1982; Gietzen & Kolandt, 1985; Verbist *et al.*, 1985). In the present study, the hybridoma technique (Köhler & Milstein, 1975) has been used to produce mouse monoclonal antibodies directed against the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase from pig stomach smooth muscle. An antibody was obtained which inhibited the ($Ca^{2+}+Mg^{2+}$)-ATPase activity and which could be used to study the enzyme in various membrane preparations from smooth muscle and in membranes from pig and human erythrocytes.

EXPERIMENTAL

Preparation of membrane fractions

KCl-extracted smooth-muscle microsomes (microsomal fractions) were prepared from the antral part of

Abbreviations used: ($Ca^{2+}+Mg^{2+}$)-ATPase, ($Ca^{2+}+Mg^{2+}$)-dependent ATPase; ($Na^{+}+K^{+}$)-ATPase, ($Na^{+}+K^{+}$)-dependent ATPase; MAb, monoclonal antibody.

the pig stomach as described by Wuytack *et al.* (1981). Membrane vesicles enriched in endoplasmic reticulum or plasma membrane from pig stomach smooth muscle were prepared as described by Raeymaekers *et al.* (1985). Both pig and human erythrocyte vesicles were prepared as described by Steck & Kant (1974), but the density-gradient step was omitted. Sarcoplasmic reticulum was prepared from pig skeletal fast muscle by the method of Jones & Cala (1981).

Purification of $(Ca^{2+}+Mg^{2+})$-ATPase and $(Na^{+}+K^{+})$-ATPase

The calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase from pig stomach smooth muscle was purified as described by De Schutter *et al.* (1984). When the enzyme was required for iodination, the same procedure was applied without adding lipid in the buffers. $(Na^{+}+K^{+})$-ATPase from pig kidney was prepared as described by De Smedt *et al.* (1979).

Immunization, fusion and screening

Female Balb/c mice (10 weeks old) were immunized by intraperitoneal injection of 100 μg of the purified calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase in complete Freund's adjuvant. Subsequent injections of incomplete Freund's adjuvant containing 50 μg of antigen proteins were given 7 and 21 days later.

Spleen cells of immunized mice were fused with Sp 2/0-Ag 14 mouse myeloma cells by using poly(ethylene glycol) 1500 as described by Galfré *et al.* (1977).

Hybridoma colonies were screened for antibody production by a modification of the dot-immunobinding assay (Hawkes *et al.*, 1982) using the Bio-Dot micro-filtration apparatus from Bio-Rad. Approx. 0.5 μg of the purified calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase/well was immobilized on a nitrocellulose membrane (Millipore, type HAHY). After blocking of non-specific binding sites with 1% bovine serum albumin in Tris-buffered saline (130 mM-NaCl/20 mM-Tris, pH 7.3), the 'dots' were incubated first with undiluted culture supernatant for 1 h and then with a 1:50 dilution of peroxidase-conjugated rabbit anti-mouse IgG (also for 1 h). Peroxidase activity was detected by using 4-chloro-1-naphthol (Verbist *et al.*, 1985).

Hybridoma cells from colonies generating 'positive' media in the dot-immunobinding assay were injected into pristane-primed mice in order to generate ascites fluid.

Antibody purification

Antibodies were purified from ascites fluid by anion-exchange chromatography on a 1 ml Mono Q column in the Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). Ascites fluid was pretreated by precipitation with 18% (w/v) Na_2SO_4. After two cycles of centrifugation and wash, the precipitate was redissolved in distilled water in the original volume and dialysed overnight against 0.9% NaCl. The sample was diluted (5-fold) with starting buffer (20 mM-triethanolamine/HCl, pH 7.7) and filtered through a 0.22 μm filter (Millipore). Samples (2.5 ml) were applied with a Superloop. The flow rate was 1 ml/min and the proteins were eluted from the Mono Q column by a linear gradient of 0–0.35 M-NaCl in the starting buffer.

Determination of $(Ca^{2+}+Mg^{2+})$-ATPase activity

The ATPase activity was measured at 37 °C by using the NADH-coupled enzyme assay (Wuytack & Casteels, 1980) at a free Ca^{2+} concentration of 10 μM unless otherwise stated. A 5 μg sample of the purified calmodulin-dependent $(Ca^{2+}+Mg^{2+})$-ATPase or a 20–25 μg sample of vesicular membrane proteins was preincubated in the reaction mixture (in a final volume of 1 ml) with MAb 2B3 for 1 h at room temperature. The reaction was started by adding 0.5 mM-K_2ATP (pH 6.8) (unless otherwise stated). The $(Na^{+}+K^{+})$-ATPase activity was inhibited by 0.1 mM-ouabain; $(Ca^{2+}+Mg^{2+})$-ATPase activity refers to the difference in activity obtained in the presence and absence of Ca^{2+}. In control experiments the effect of non-immune IgG on the $(Ca^{2+}+Mg^{2+})$-ATPase activity was also determined.

Determination of Ca^{2+} uptake in the vesicles

The Ca^{2+} uptake was measured at 37 °C in a medium containing 150 mM-KCl, 5 mM-NaN_3, 30 mM-imidazole/HCl (pH 6.8), 0.5 mM-Tris/ATP, 6 mM-$MgCl_2$, 1 mM-$^{45}CaCl_2$ and 1 mM-EGTA. The membrane protein concentration was 25 μg/ml.

The Ca^{2+} uptake by the endoplasmic-reticulum vesicles was measured with or without 5 mM-potassium oxalate, and the uptake by the plasmalemmal-vesicle preparation with or without 50 mM-potassium phosphate.

The effect of MAb 2B3 on the Ca^{2+}-uptake was measured after a 1 h preincubation with 100 μg of the purified monoclonal antibody at room temperature. The Ca^{2+} uptake was started by adding 0.5 mM-Tris/ATP (pH 6.8). Controls were incubated without ATP to correct for passive Ca^{2+} binding. The vesicles were separated from the solution by Millipore filtration. Thereupon the filters were rinsed and the amount of $^{45}Ca^{2+}$ remaining on them was counted. Controls were done with equal concentrations of non-immune IgG and without IgG.

SDS/polyacrylamide-gel electrophoresis

Laemmli-type slab-gel electrophoresis (Laemmli, 1970) was done on 0.75 mm-thick slab gels with a stacking gel of 3% and a resolving gel of 7.5% acrylamide. Proteins were dissolved in 2% (w/v) SDS/10% (v/v) glycerol/62.5 mM-Tris/HCl (pH 6.8)/1% mercaptoethanol/0.03% Bromophenol Blue and heated for 5 min at 60 °C. This mixture was applied to the gel. Samples of the affinity-purified $(Ca^{2+}+Mg^{2+})$-ATPase were concentrated by precipitation with 6% (v/v) trichloroacetic acid, centrifugation and subsequent resolubilization of the proteins.

Iodination of the purified calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase

Iodination of 20–25 μg (in 300 μl) of the $(Ca^{2+}+Mg^{2+})$-ATPase protein prepared in the absence of phospholipids was done as described by Bolton & Hunter (1973). The reaction was stopped by the addition of 500 μl of 0.2 M-glycine in 0.1 M-borate buffer, pH 8.5. Separation of the ^{125}I-labelled protein from the medium was done on a Sephadex G-25 column (1 cm × 20 cm) which had been equilibrated with the Ca^{2+}-free buffer in

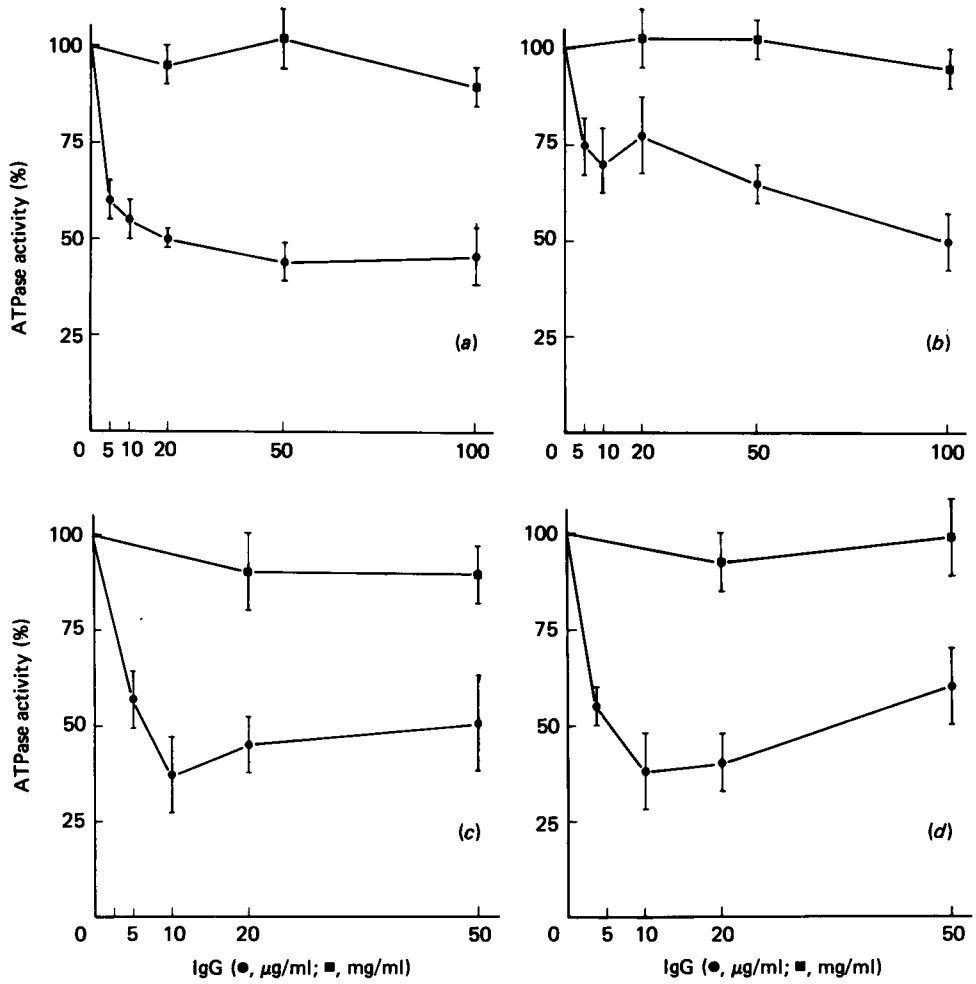

Fig. 1. Effect of MAb 2B3 on ($Ca^{2+}+Mg^{2+}$)-ATPase activities

The effects of the purified MAb 2B3 on ($Ca^{2+}+Mg^{2+}$)-ATPase activity (*a*) in KCl-extracted microsomes, (*b*) in purified calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase from pig stomach (antrum) smooth muscle, (*c*) in pig erythrocyte inside-out vesicles and (*d*) in human erythrocyte inside-out vesicles are shown. The NADH-coupled enzyme assay was used to quantify inhibition of ($Ca^{2+}+Mg^{2+}$)-ATPase activity as described in the Experimental section. A 5 μg portion of the purified calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase or 25–100 μg of membrane proteins was incubated in the reaction medium containing different concentrations of the purified MAb 2B3 for 30 min at room temperature. The reaction was started by adding 0.5 mM-K_2ATP. ■, Non-immune IgG; ●, MAb 2B3. The data are means of six (*a* and *b*) or five (*c* and *d*) separate experiments (vertical bars indicate ± S.E.M.). Maximal ($Ca^{2+}+Mg^{2+}$)-ATPase activity (100%) was measured without the addition of IgG.

which the ($Ca^{2+}+Mg^{2+}$)-ATPase was eluted from the calmodulin affinity column containing 0.25% (w/v) gelatin.

Double immunoprecipitation

The ^{125}I-labelled antigen was precipitated by a double-immunoprecipitation technique: 500 μl of 3-day culture medium was added to an equal volume of incubation buffer [100 mM-Tris/HCl (pH 7.6), 0.30 M-NaCl, 2% Triton X-100, 10 mM-EDTA, 2% albumin]; 10 μl of ^{125}I-ATPase (7000 c.p.m.) was incubated with this mixture overnight at room temperature. Then 5 μl of rabbit anti-mouse immunoglobulins and 100 μl of a Protein A–Sepharose suspension (1 mg/ml) in phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.5) were added. After each addition the mixture was incubated at room temperature for 1 h. The final precipitate was washed four times with 2-fold-diluted incubation buffer and solubilized in the sample buffer. Samples (70 μl) were applied to the SDS/polyacrylamide gel for electrophoresis. After electrophoresis the gels were dried and autoradiography was performed with Kodak X-Omat S films for 48 h at −70 °C.

Production of monoclonal-antibody immunoaffinity adsorbent

CNBr-activated Sepharose 4B was reconstituted, washed and then coupled to MAb 2B3 (8 mg of protein/2 g of dry gel) according to the manufacturer's instructions. The gel was stored in a buffer containing 50 mM-Mops (pH 7.0), 500 mM-NaCl and 0.02% NaN_3.

Immunoaffinity purification of the ($Ca^{2+}+Mg^{2+}$)-ATPase

($Ca^{2+}+Mg^{2+}$)-ATPase present in the microsomes was solubilized at a protein concentration of 4 mg/ml in 0.4% Triton X-100/130 mM-KCl/20 mM-potassium Hepes (pH 7.4)/0.5 mM-$MgCl_2$/0.05 mM-$CaCl_2$/0.1 mM-

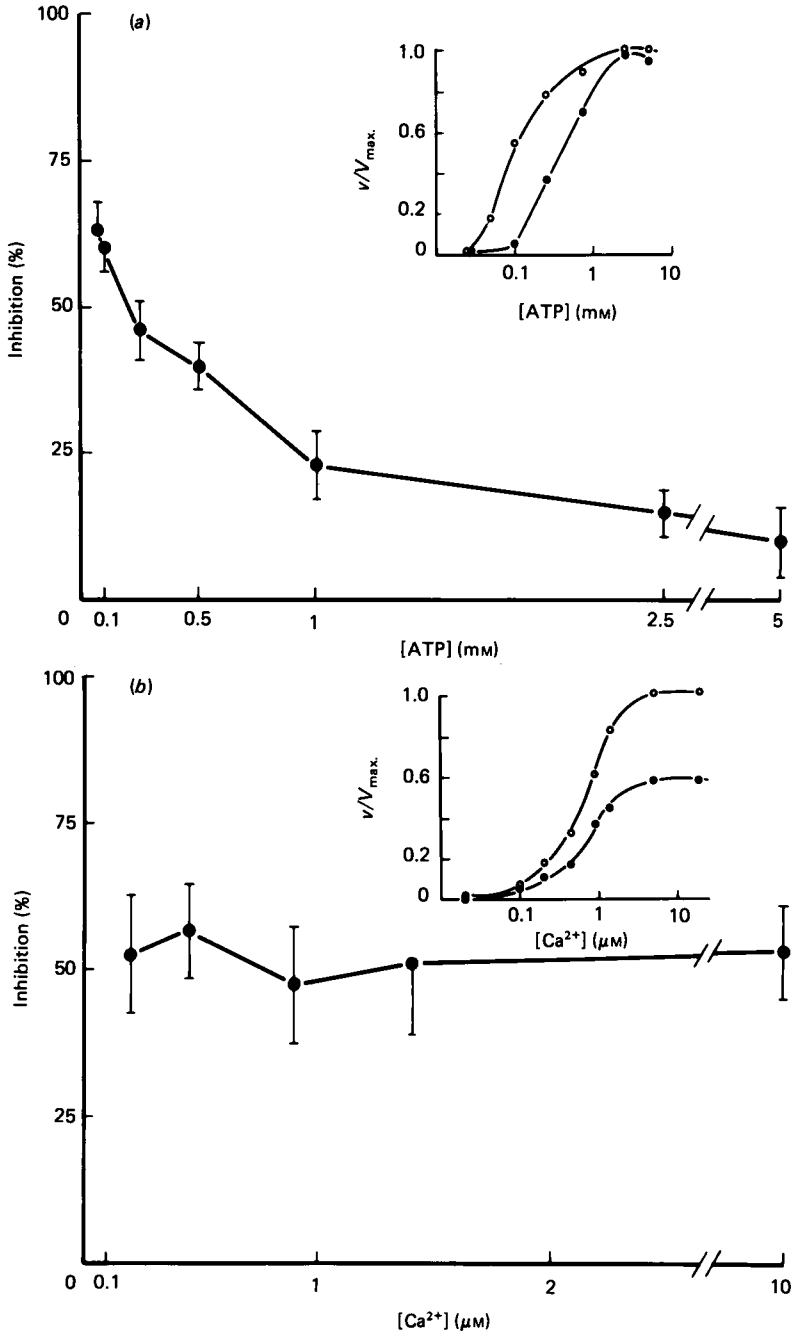

Fig. 2. ATP- and Ca^{2+}-dependence of (Ca^{2+} + Mg^{2+})-ATPase activity in the absence or presence of MAb 2B3

KCl-extracted smooth-muscle microsomes (approx. 25 μg of protein/ml) were preincubated without (○) or with (●) 20 μg of MAb 2B3/ml for 30 min at room temperature. The NADH-coupled enzyme assay was used to quantify (Ca^{2+} + Mg^{2+})-ATPase activity. The reaction was started by adding the indicated concentrations of ATP in (*a*) or by adding 0.5 mM-K_2ATP in (*b*). (*a*) shows the percentage inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity as a function of ATP concentration after incubation with MAb 2B3. The data are means for six different experiments (vertical bars indicate ± S.E.M.). The inset in (*a*) shows the ATP-dependence of (Ca^{2+} + Mg^{2+})-ATPase activity (○, without, and ●, with MAb 2B3). The apparent K_m values for ATP obtained were 100 and 350 μM in the absence or presence of MAb 2B3 respectively. (*b*) shows the percentage inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity as a function of Ca^{2+} concentration after incubation with MAb 2B3. Each point is the mean for three different observations (± S.E.M.). The inset in (*b*) shows Ca^{2+}-affinity curves of the (Ca^{2+} + Mg^{2+})-ATPase (○, without, and ●, with MAb 2B3). The apparent K_m for Ca^{2+} was 0.7 μM in the presence or absence of MAb 2B3.

dithiothreitol. The Triton X-100-solubilized material was added to the 2B3–Sepharose 4B affinity gel, which had been equilibrated with a buffer containing 130 mM-KCl, 20 mM-potassium Hepes (pH 7.4), 1 mM-$MgCl_2$, 0.1 mM-$CaCl_2$, 0.1 mM-dithiothreitol and 0.4% Triton X-100. This mixture was incubated for 1 h at 4 °C in an end-over-end mixer, and then the gel was washed on a fritted-glass filter with 100 gel volumes of the same buffer to remove unbound material. The specifically bound material was released by substituting in the buffer

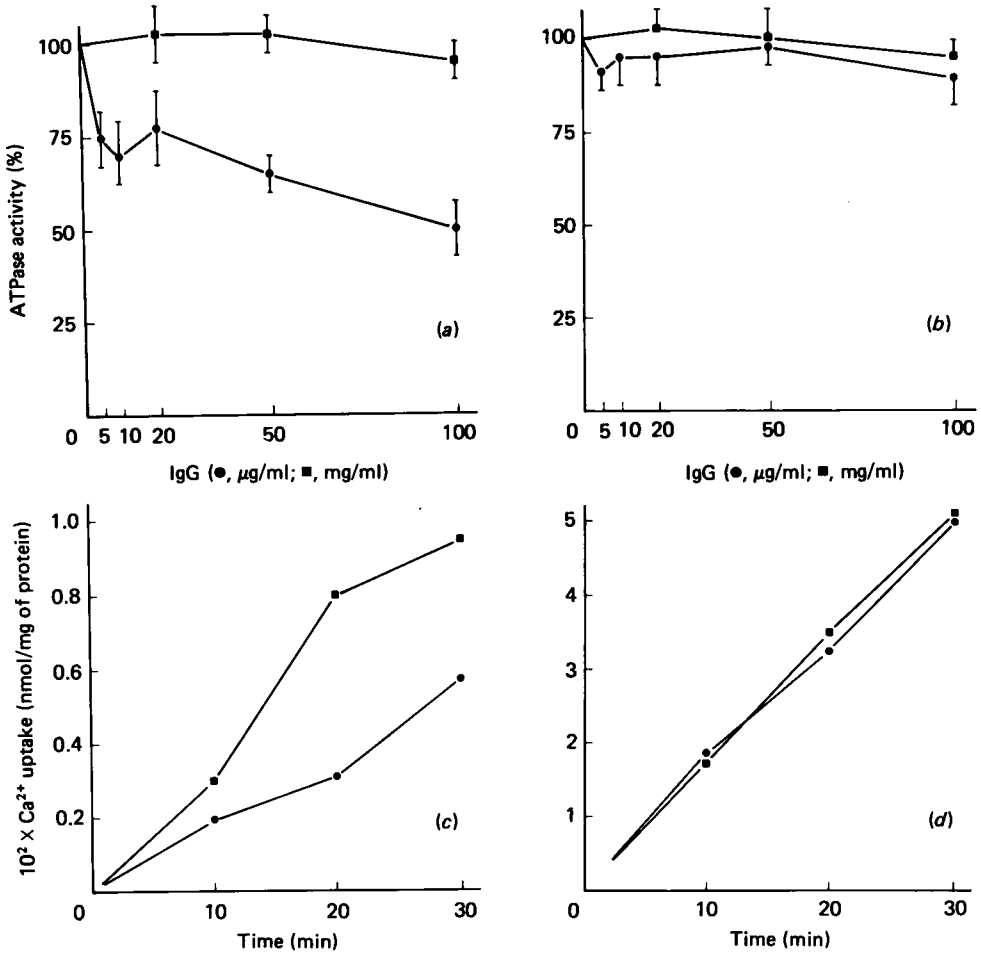

Fig. 3. Effect of MAb 2B3 on ($Ca^{2+}+Mg^{2+}$)-ATPase activities (*a* and *b*) and on Ca^{2+} transport (*c* and *d*) in different pig stomach smooth-muscle membrane preparations

The effects of purified MAb 2B3 on ($Ca^{2+}+Mg^{2+}$)-ATPase activity and on the time course of Ca^{2+} uptake in (*a* and *c*) a membrane fraction enriched in plasma membrane and (*b* and *d*) an endoplasmic-reticulum-enriched membrane fraction are shown. The effects on ($Ca^{2+}+Mg^{2+}$)-ATPase activity were measured as described in Fig. 1 legend. Ca^{2+} uptake was measured as described in the Experimental section. Vesicle protein (25 μg/ml) was preincubated for 1 h at room temperature with 100 μg of purified MAb 2B3/ml. The reaction was started by adding 0.5 mM-Tris/ATP (pH 6.8); ■, non-immune IgG; ● MAb 2B3. Each point represents the mean for six separate determinations (in *a* and *b*) (vertical bars indicate ± S.E.M.); (*c*) and (*d*) show results of one typical experiment (three experiments were performed).

0.1 mM-glycine/HCl (pH 2.8) for 20 mM-potassium Hepes (pH 7.4). The eluted proteins were analysed by electrophoresis.

Materials

Sp 2/0-Ag 14 mouse myeloma cells were provided by G. Köhler (Schulman *et al.*, 1978). Dulbecco's Modified Eagle Medium and HAT-medium were from GIBCO, Grand Island, NY, U.S.A. Poly(ethylene glycol) 1500 was from Boehringer, Mannheim, Germany, and pristane was from Aldrich. Rabbit anti-mouse and peroxidase-conjugated rabbit anti-mouse immunoglobulins were supplied by DAKO-Patts, Copenhagen, Denmark. Freund's complete and incomplete adjuvants were from Difco, Detroit, MI, U.S.A. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer. The Bolton–Hunter reagent and $^{45}CaCl_2$ were from Amersham. Protein A–Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia. Nitrocellulose membranes for blotting were from Millipore, and M_r standards for gel electrophoresis were from Sigma.

RESULTS

Screening

Supernatants from hybridoma cultures were assayed for anti-($Ca^{2+}+Mg^{2+}$)-ATPase antibodies by dot-immunobinding assay (Hawkes *et al.*, 1982). Of 33 wells examined, 11 were positive. These were propagated intraperitoneally in Balb/c mice and the antibody-containing ascites fluid was collected 2–3 weeks later. The purified antibodies were assayed for anti-($Ca^{2+}+Mg^{2+}$)-ATPase activity by using the NADH-coupled enzyme assay. Only one of the antibodies (designated '2B3', of the IgG_1 subclass) inhibited the enzyme activity.

None of the purified antibodies reacted in the immunoblotting assay of the enzyme after SDS/poly-

acrylamide-gel electrophoresis and transfer on to nitrocellulose membranes. Dot-immunobinding of MAb 2B3 to the purified calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase spotted on nitrocellulose membranes revealed that denaturation of the $(Ca^{2+}+Mg^{2+})$-ATPase with SDS prevents subsequent binding of the antibody (results not shown).

MAb 2B3 cross-reacted with vesicles from pig erythrocytes spotted on nitrocellulose membranes (results not shown), but did not cross-react with vesicles from sarcoplasmic reticulum of pig skeletal muscle, nor with the purified $(Na^+ + K^+)$-ATPase from pig kidney.

Effect of MAb 2B3 on $(Ca^{2+}+Mg^{2+})$-ATPase activity

Fig. 1 shows the effect of different concentrations of MAb 2B3 on the $(Ca^{2+}+Mg^{2+})$-ATPase activity (Fig. 1*a*) in KCl-extracted smooth-muscle microsomes (Wuytack *et al.*, 1981) and (Fig. 1*b*) in an enzyme preparation purified from the same tissue by calmodulin affinity chromatography. MAb 2B3 was found to inhibit the $(Ca^{2+}+Mg^{2+})$-ATPase activity by about 50–60% (in the presence of 0.5 mM-ATP) both in the microsomes and in the purified enzyme. The enzyme activity was inhibited to the same extent in the absence or presence of calmodulin. The anti-catalytic effect was not tissue- or species-specific. The $(Ca^{2+}+Mg^{2+})$-ATPase activity was also inhibited in pig and human erythrocyte membrane vesicles (Figs. 1*c* and 1*d*). IgG prepared from non-immune mouse serum had no effect on $(Ca^{2+}+Mg^{2+})$-ATPase activity.

MAb 2B3 did not affect the $(Ca^{2+}+Mg^{2+})$-ATPase activity in sarcoplasmic reticulum from pig skeletal muscle, nor did it inhibit the $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities measured in different membrane preparations.

Effect of MAb 2B3 on [ATP]- and $[Ca^{2+}]$-dependence of $(Ca^{2+}+Mg^{2+})$-ATPase activity

As shown in Fig. 2(*a*), the inhibitory effect of MAb 2B3 depends on the ATP concentration. The enzyme's apparent K_m value for ATP increased from 100 μM in the absence to 350 μM in the presence of the antibody. MAb 2B3 lowered the $V_{max.}$ for ATP by less than 10%.

Fig. 2(*b*) shows that the inhibitory effect of MAb 2B3 is not Ca^{2+}-dependent. The apparent K_m value for Ca^{2+} was 0.7 μM-Ca^{2+} in both the presence and the absence of MAb 2B3. The $V_{max.}$ for Ca^{2+} was lowered by 40% by MAb 2B3 at an ATP concentration of 0.5 mM.

Effect of MAb 2B3 on $(Ca^{2+}+Mg^{2+})$-ATPase activity and Ca^{2+} transport in subcellular membrane fractions from pig stomach smooth muscle

MAb 2B3 inhibits the $(Ca^{2+}+Mg^{2+})$-ATPase activity (Fig. 3*a*), and decreases the rate of Ca^{2+} uptake measured in the presence of phosphate in a membrane fraction from pig antrum smooth muscle enriched in plasmalemma (Fig. 3*c*). In the endoplasmic-reticulum-enriched fraction, however, the antibody does not significantly inhibit the $(Ca^{2+}+Mg^{2+})$-ATPase activity (Fig. 3*b*), nor does it slow down the oxalate-stimulated Ca^{2+} uptake (Fig. 3*d*).

Binding of iodinated $(Ca^{2+}+Mg^{2+})$-ATPase to MAb 2B3

$(Ca^{2+}+Mg^{2+})$-ATPase purified from smooth muscle by calmodulin affinity chromatography was labelled by the Bolton & Hunter (1973) technique. The distribution of radioactivity in the labelled enzyme preparation and in the material bound to MAb 2B3 and precipitated by the double-immunoprecipitation technique (see the Experimental section) was investigated by SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 4). In the ^{125}I-labelled enzyme preparation, radioactivity was incorporated into one major band of M_r 130000–140000, and into four minor bands of M_r 45000, 63000, 74000 and 90000. MAb 2B3 precipitated only the protein that migrates on electrophoresis with the same apparent M_r as the calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase (i.e. 130000–140000; Wuytack *et al.*, 1982).

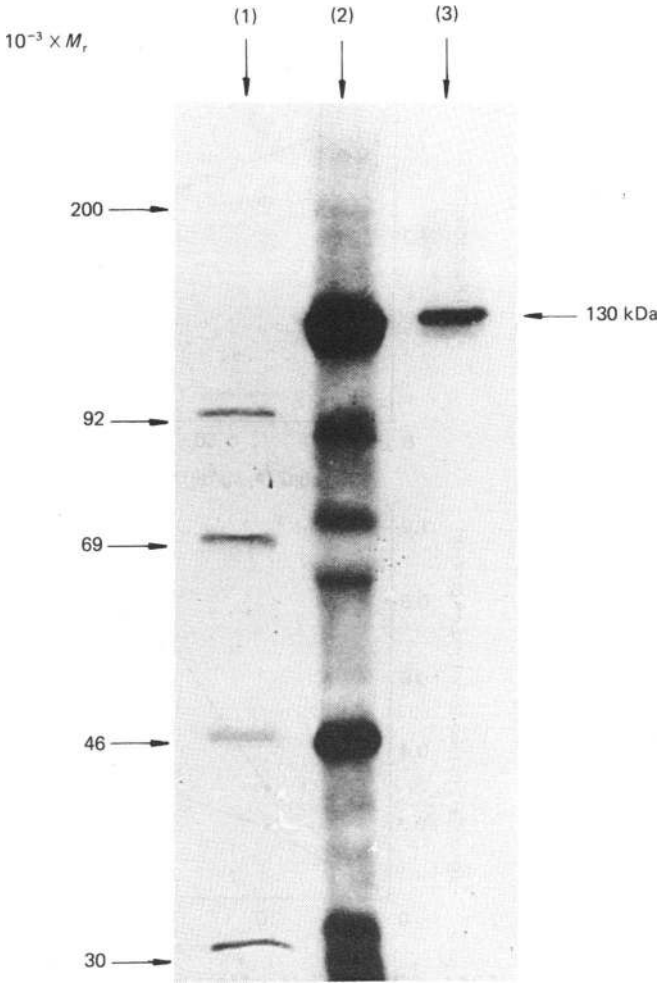

Fig. 4. Double immunoprecipitation of ^{125}I-labelled $(Ca^{2+}+Mg^{2+})$-ATPase by MAb 283

Autoradiogram of a polyacrylamide gel: (1) M_r standards; (2) ^{125}I-labelled calmodulin-binding $(Ca^{2+}+Mg^{2+})$-ATPase purified from smooth muscle; (3) ^{125}I-labelled antigen precipitated by double immunoprecipitation as described in the Experimental section. The iodinated $(Ca^{2+}+Mg^{2+})$-ATPase preparation was incubated first with hybridoma culture supernatant 2B3 and then with rabbit anti-(mouse IgG) antibodies. This complex was bound to Protein A–Sepharose beads. After the beads were washed, the immunoprecipitate was solubilized with SDS.

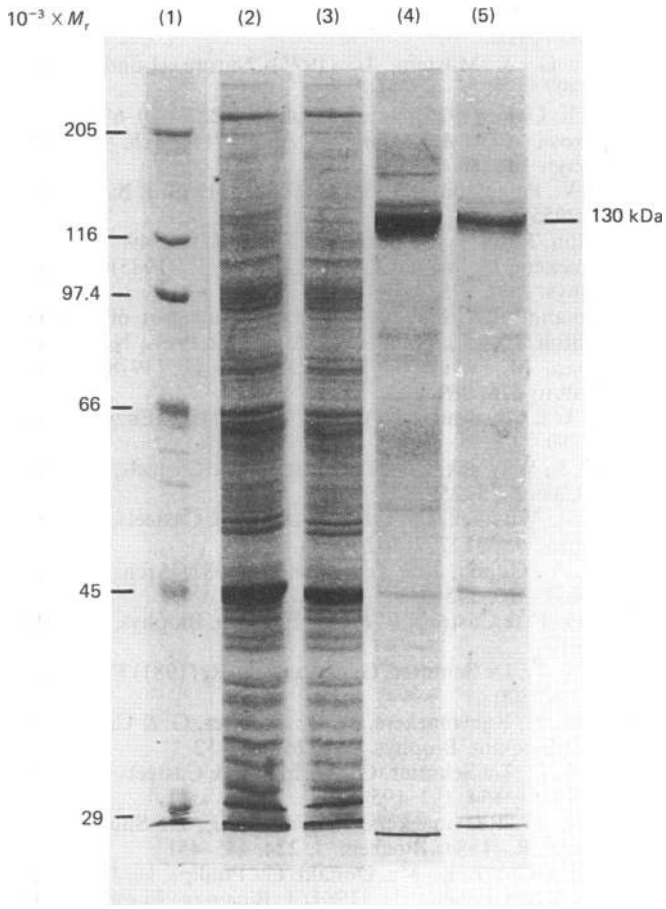

Fig. 5. Purification of ($Ca^{2+}+Mg^{2+}$)-ATPase from a detergent-solubilized extract of crude microsomes from pig stomach smooth muscle by immunoaffinity chromatography

An SDS/polyacrylamide-gel electrophoretogram stained with Coomassie Brilliant Blue is shown. (1) M_r standards; (2) Triton-X-100-solubilized microsomes; (3) fraction not bound to MAb 2B3–Sepharose 4B immunosorbent; (4) eluate from MAb 2B3–Sepharose 4B immunosorbent; (5) eluate from calmodulin–Sepharose 4B column.

Purification of ($Ca^{2+}+Mg^{2+}$)-ATPase by use of a MAb 2B3–Sepharose 4B immunoadsorbent

Purification of the enzyme by monoclonal-antibody immunoadsorbent was achieved by adding the immunoadsorbent to KCl-extracted and Triton X-100-solubilized crude microsomes from pig stomach smooth muscle and eluting the adsorbed enzyme from the gel batchwise with 0.1 M-glycine/HCl (pH 2.8) at 4 °C. This single immunoaffinity step resulted in approximately the same purification as the calmodulin affinity chromatography described by De Schutter *et al.* (1984). This immunopurified enzyme no longer had any enzymic activity, but it showed, like the enzyme purified by calmodulin affinity chromatography, a major band of apparent M_r 130000 after SDS/polyacrylamide-gel electrophoresis (Fig. 5). In addition, both purification procedures yield also a minor band of M_r 45000. This M_r-45000 protein might be actin, since antibodies against actin from rabbit skeletal muscle (Miles Laboratories) bind to the purified ($Ca^{2+}+Mg^{2+}$)-ATPase preparation spotted on nitrocellulose membranes.

DISCUSSION

In this study we report the isolation and partial characterization of a monoclonal antibody to the calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase from pig stomach (antrum) smooth-muscle plasma membranes. Our results show that we have obtained a monoclonal antibody that binds specifically to the ($Ca^{2+}+Mg^{2+}$)-ATPase of M_r 130000 from smooth muscle. This antibody is probably directed against an antigenic determinant in a functionally important site of this plasmalemmal Ca^{2+}-transport ATPase, since it inhibits the enzyme activity; or inhibition could be due to secondary effects caused by steric hindrance or conformational changes. The antibody appears to act as a reversible competitive inhibitor of ATP, but the inhibition is non-competitive with respect to Ca^{2+} in both the presence and the absence of calmodulin.

The antigenic determinant of the smooth-muscle Ca^{2+}-transport ATPase recognized by MAb 2B3 seems not to be tissue- and species-specific, since MAb 2B3 binds to pig and human erythrocyte inside-out vesicles and also inhibits their ($Ca^{2+}+Mg^{2+}$)-ATPase activity. MAb 2B3 not only inhibits the ($Ca^{2+}+Mg^{2+}$)-ATPase activity but also significantly slows down the Ca^{2+} uptake in a plasma-membrane-enriched vesicle preparation from smooth muscle. In contrast, the ($Ca^{2+}+Mg^{2+}$)-ATPase activity and the oxalate-dependent Ca^{2+} uptake by a membrane fraction enriched in endoplasmic reticulum from the same tissue were not significantly inhibited by the antibody.

These results confirm the existence in smooth muscle of two different types of Ca^{2+}-transport ATPase: a calmodulin-binding ($Ca^{2+}+Mg^{2+}$)-ATPase located in the plasma membrane, and a second one which is confined to the endoplasmic reticulum (Wuytack *et al.*, 1984; Raeymaekers *et al.*, 1985; Verbist *et al.*, 1985). The relative ease with which milligram quantities of antibody can be produced in ascites fluid has allowed us to prepare an immunoaffinity gel for purification of plasmalemmal ($Ca^{2+}+Mg^{2+}$)-ATPases. This gel can be used for the purification of the enzyme from tissues for which calmodulin affinity chromatography has failed so far.

This monoclonal antibody is, to the best of our knowledge, the first one directed against a plasma-membrane Ca^{2+}-transport ATPase. MAb 2B3 has the advantage that it also recognizes plasmalemmal Ca^{2+}-transport ATPases of other animal species, but has the drawback that it does not recognize denaturated proteins, thus precluding its use for Western-type immunoblot detection. Monoclonal antibodies binding to other Ca^{2+}-transport ATPases of the endo-(sarco)-plasmic reticulum have been described (Goldin *et al.*, 1983; Zubrzycka-Gaarn *et al.*, 1984), but none of these was found to inhibit the ATPase and transport activity. A monoclonal antibody inhibiting Ca^{2+} accumulation and skeleton formation in cultured embryonic cells of the sea urchin *Strongylocentrotus purpuratus* has been described (Carson *et al.*, 1985). Although that antibody reacted with a 130000-M_r cell-surface protein, it is unlikely that this protein corresponds to a calmodulin-stimulated Ca^{2+} pump, because it was only detected on 5% of the cells and it also occurred in the extracellular material associated with the spicule.

This work was supported by grant no. 3.0042.83 from the F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk

Onderzoek), Belgium. We thank L. Mekers and I. Willems for excellent technical assistance and M. Vander Aerschot for typing the manuscript. J.V. is a research assistant of the N.F.W.O. (Nationaal Fonds voor Wetenschappelijk Onderzoek), Belgium.

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Received 21 April 1986/25 July 1986; accepted 18 August 1986