Antibodies to the calmodulin-binding Ca^{2+}-transport ATPase from smooth muscle

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Antibodies were raised against a calmodulin-binding CaMg-ATPase (Ca^{2+}-transport ATPase) from smooth muscle. The binding of these antibodies to a number of related Ca^{2+}-transport ATPases was studied. Antibodies to the calmodulin-binding ATPase from porcine antrum (stomach) smooth muscle do not only bind to this CaMg-ATPase, but also to the corresponding enzyme in porcine erythrocytes. However, they do not bind to the CaMg-ATPase from sarcoplasmic reticulum of porcine skeletal muscle. The binding of these antibodies to the CaMg-ATPase of smooth muscle, does not inhibit the enzyme activity.

Calmodulin *CaMg ATPase* *Erythrocyte* *Immunochemistry* *Sarcoplasmic reticulum* *Smooth muscle*

1. INTRODUCTION

A calmodulin-binding CaMg-ATPase was purified from smooth muscle by means of affinity chromatography on a calmodulin–Sepharose 4B gel [1]. This CaMg-ATPase activity is the biochemical correlate of a Ca-transport system as can be demonstrated by the presence of an ATP-dependent Ca^{2+} accumulation after incorporation of the ATPase in artificial phospholipid vesicles [2]. This CaMg-ATPase has tentatively been allocated in the plasma membrane mainly in analogy to the localization of its equivalent in the human erythrocyte plasma membrane [3,4].

Studies on the distribution of this enzyme in isolated subcellular fractions prepared by density gradient centrifugation confirm this hypothesis [5]. Both skeletal [6] and heart muscle [7] sarcoplasmic reticulum contain a Ca^{2+}-transport ATPase which differs from the calmodulin-binding ATPase because it shows no direct interaction with calmodulin and because it has a lower M_r: (100000 for the sarcoplasmic reticulum enzymes; 130000–150000 for the calmodulin-binding ATPases).

We now report the production of antibodies to the calmodulin-binding Ca^{2+}-transport ATPase from smooth muscle. These antibodies crossreact with erythrocyte CaMg-ATPase but not with the CaMg-ATPase of sarcoplasmic reticulum from skeletal muscle.

Abbreviations: CaMg-ATPase, (Ca^{2+} + Mg^{2+})-ATPase; SDS, Sodium-dodecylsulphate; IgG, immunoglobulin G

2. METHODS

2.1. *Preparation of the anti-ATPase immunoglobulins*

CaMg-ATPase was prepared from pig antrum (stomach) smooth-muscle membranes [1]. Rabbits were immunized by subcutaneous injections of 150 μg CaMg-ATPase solubilized in 0.5 ml preparation buffer (containing Triton X-100 and Asolectin) and mixed with 0.5 ml Freund complete adjuvant. Booster injections of the same amount of enzyme with Freund incomplete adjuvant were administered after 14 and 28 days and blood was drawn 5 days after the last injection. The immunoglobulin IgG fraction was partially purified

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from the serum [8] and redissolved in phosphate-buffered saline at 17 mg protein/ml.

2.2. *Electrophoresis and electroblotting*

Laemmli-type slab gel electrophoresis was done on 1 mm thick slab gels with a stacking gel of 3% and a resolving gel of 10% acrylamide. Proteins were dissolved in: 2% SDS, 10% glycerol, 62.5 mM Tris–HCl (pH 6.8), 1% mercaptoethanol, 0.03% bromophenol blue, heated for 4 min at 60°C. This mixture (40 μl) was applied to the gel. Samples of the affinity purified CaMg-ATPase were concentrated by precipitation with 6% trichloroacetic acid, and subsequent centrifugation. After washing the pellets with 1 ml of 1:2 (v/v) mixture of chloroform–methanol and centrifugation, the proteins were solubilized in the solubilization mixture.

Electroblotting [9] on nitrocellulose membranes (Millipore HAWP 304 FO) was conducted for 16 h at 30 V (4°C) in a BIORAD Trans blot cell. The transfer buffer contained 190 mM glycine, 25 mM Tris–HCl (pH 8.3), 20% (v/v) methanol. A control gel was stained with Coomassie brilliant blue R 250.

2.3. *Detection of proteins on the nitrocellulose replicates*

After blotting, the protein-binding sites on the nitrocellulose were saturated by incubating the membrane for 4 h with constant agitation at 37°C in a solution A: 0.9% NaCl, 10 mM Na-phosphate (pH 7.0), 0.5% casein. The membrane was then incubated for 1 h in solution A containing 3.4 mg/ml of anti-CaMg-ATPase IgG. Thereupon it was washed 4 times for 10 min with solution A. The first two of these wash solutions contained also 0.05% of Nonidet P-40 (Fluka). This was followed by an incubation with a 1:50 dilution of peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunological Labs, Tilburg) or peroxidase-conjugated swine anti-rabbit immunoglobins (DAKO-immunoglobulins AS, Denmark). The membranes were then washed again, as after incubation with the primary antibody. Peroxidase activity was visualized as a brown precipitate or a bluish–gray reaction product, respectively, after developing the nitrocellulose membrane in a solution containing either 0.01% H_2O_2, 0.5 mg 3,3′-diaminobenzidine/ml in 50 mM Tris–HCl (pH 7.6) or 0.005% H_2O_2, 0.5 mg 4-Cl-1-naphthol/ml in 50 mM Tris–HCl (pH 7.6).

3. RESULTS AND DISCUSSION

3.1. *The binding of antibodies to different Ca^{2+}-transport ATPases*

A Laemmli-type SDS–polyacrylamide gel electrophoresis was used in order to identify and characterize the different CaMg-ATPases. After electrophoresis the proteins were transferred to and immobilized on nitrocellulose membranes by electrophoretic transfer (Western blotting) [9]. The proteins on the nitrocellulose replicates that bind our antibodies against smooth muscle CaMg-ATPase, were visualized by incubation with peroxidase-conjugated secondary antibodies against rabbit immunoglobulins followed by staining for peroxidase activity.

Fig.1 shows an SDS–polyacrylamide gel electropherogram stained with Coomassie brilliant blue, and an electroblot on nitrocellulose of an identical gel. The CaMg-ATPases purified by calmodulin affinity chromatography from porcine erythrocytes and from porcine smooth muscle have a comparable monomer M_r of 140000–150000, which can be differentiated from the lower monomer M_r of 100000 found for the CaMg-ATPase from sarcoplasmic reticulum of porcine skeletal muscle.

The antibodies against smooth muscle CaMg-ATPase not only bound to the CaMg-ATPase from smooth muscle used as antigen for immunization but also to the CaMg-ATPase from porcine erythrocytes. Also in smooth muscle microsomes an intense positive reaction is observed at the same M_r of 140000–150000. Most interestingly however, no binding or only in some cases a low aspecific binding is observed to the CaMg-ATPase of sarcoplasmic reticulum of porcine skeletal muscle even when the gel was deliberately overloaded with this ATPase. Likewise no or a very low binding is observed in the smooth muscle microsomes at M_r 100000. In the smooth muscle microsomes, an intensely staining band is also found at M_r 200000, together with a few fainter ones at 250000, 66000 and 42000. Because the M_r 200000 band is also seen in controls which are incubated with non-immune rabbit antibodies (fig.2) it may result, at least partially,

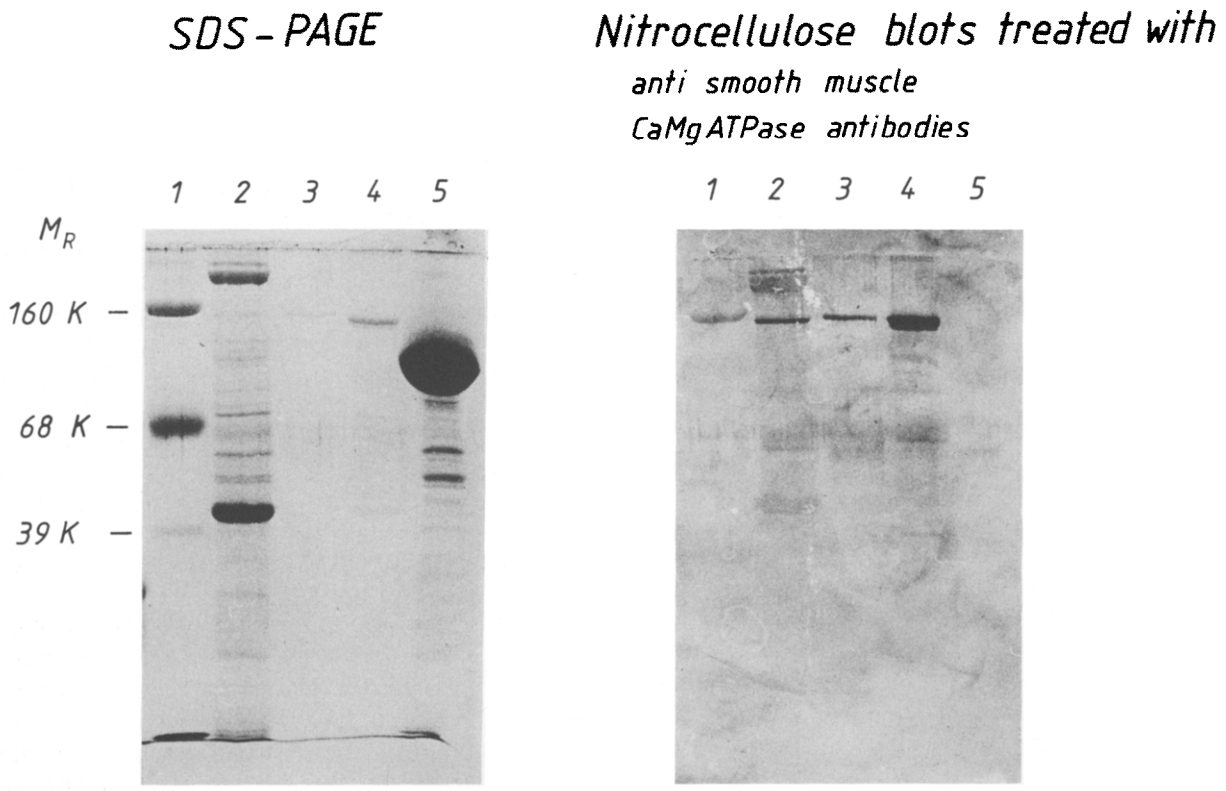

Fig.1. SDS–polyacrylamide gel electrophoretogram stained with Coomassie brilliant blue (left) and a nitrocellulose electroblot of a similar gel treated with antibodies against smooth muscle CaMg-ATPase (right). The binding of these primary antibodies was visualized by a brown precipitate of diaminobenzidine using peroxidase conjugated goat-anti rabbit IgG as a secondary antibody. Following fractions were electrophoresed: (1) M_r standards; (2) microsomes from porcine antrum smooth muscle, consisting mainly of plasma membrane fragments [5]; (3) calmodulin-binding CaMg-ATPase from porcine antrum smooth muscle; (4) calmodulin-binding CaMg-ATPase from porcine erythrocytes; (5) sarcoplasmic reticulum vesicles from porcine skeletal muscle.

from an aspecific interaction with the secondary antibody or staining system. The same controls indicate that the faintly staining bands of M_r 250000, 66000 and 42000 are due to a specific binding of the primary antibodies. These bands could be dimers or proteolytic products of the calmodulin-binding CaMg-ATPase or they could represent distinct proteins, unrelated to the ATPase. An aspecific staining is also observed for the M_r 160000 standard (subunit of RNA-polymerase of *E. coli*) (fig.2).

Our experimental results cannot be explained by the presence of antibodies to calmodulin in our IgG fraction. Such antibodies could bind to calmodulin–CaMg-ATPase complexes on the nitrocellulose replicates. This possibility can be excluded because there was no reaction of our antibodies with bovine brain calmodulin which was either electrophorized and transferred (20 μg) to nitrocellulose or spotted directly (5–0.08 μg) on nitrocellulose.

3.2. *The effect of the antibodies on the CaMg-ATPase activity*

The binding of our antibodies to the CaMg-ATPase of smooth muscle did not inhibit the CaMg-ATPase activity of the purified CaMg-ATPase nor that of the microsomal fraction from porcine antrum smooth muscle. These ATPase tests were done with the coupled enzyme system [1] in the presence of 10^{-5} M Ca^{2+} and with or without 10 μg calmodulin/ml. The tests were performed with either 3 μg purified ATPase or 100 μg microsomal protein and 0.84 mg anti-CaMg-

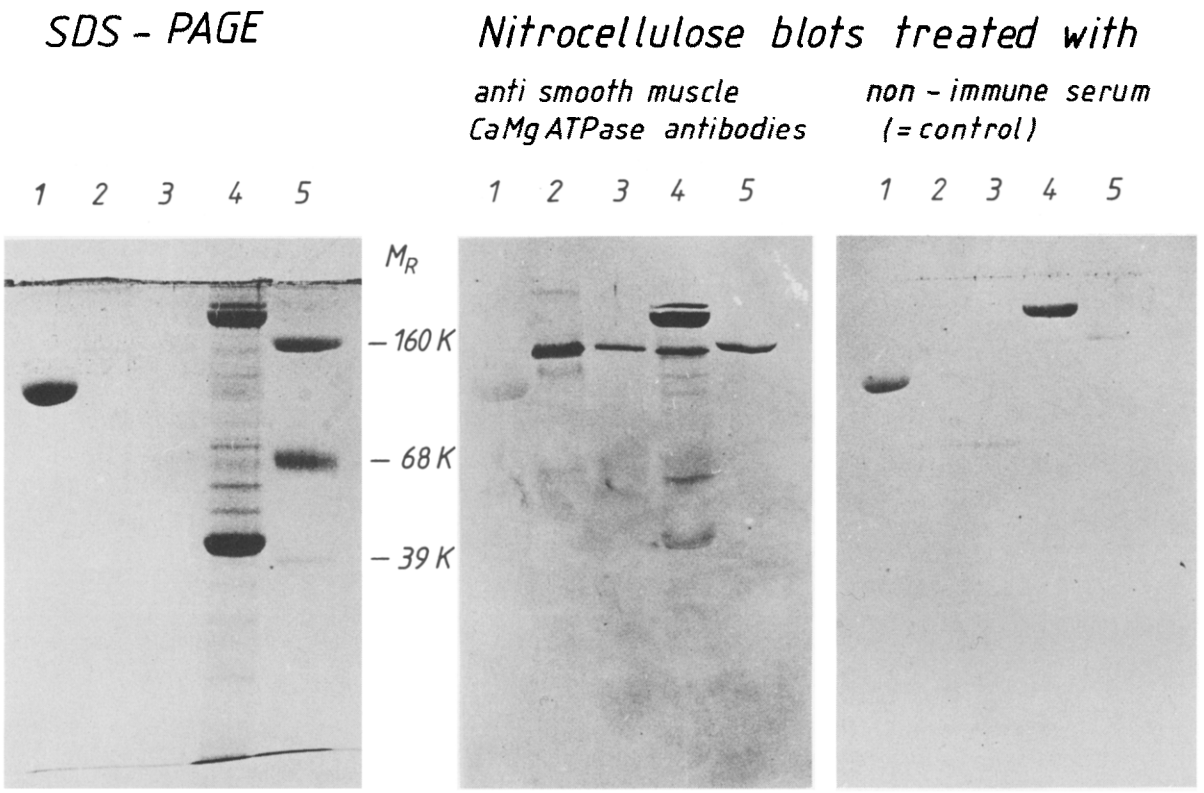

Fig.2. SDS–polyacrylamide gel electrophoretogram stained with Coomassie brilliant blue (left) and nitrocellulose electroblots of a similar gel treated with antibodies against smooth muscle CaMg-ATPase (middle) or with non-immune rabbit antibodies (right). The binding of these primary antibodies was visualized as a bluish-gray reaction product of 4-Cl-1-naphthol using peroxidase conjugated swine–anti-rabbit IgG as a secondary antibody. Following fractions were electrophoresed: (1) sarcoplasmic reticulum vesicles from porcine skeletal muscle; (2) calmodulin-binding CaMg-ATPase purified from porcine antrum smooth muscle; (3) calmodulin-binding CaMg-ATPase purified from porcine erythrocytes; (4) microsomes from porcine antrum smooth muscle consisting mainly of plasma membrane fragments [5]; (5) M_r standards.

ATPase IgG.

A different experimental approach (i.e., a competitive radioimmunoassay test for cross-reactivity) has shown that antibodies against human erythrocyte CaMg-ATPase crossreact with solubilized membrane proteins of other tissues like rat corpus luteum and rat brain synaptic membranes, but not with CaMg-ATPase from rabbit skeletal muscle sarcoplasmic reticulum [10].

We report here the comparable observation that antibodies against CaMg-ATPase from smooth muscle bind to the corresponding CaMg-ATPase from porcine erythrocytes but not to the ATPase from sarcoplasmic reticulum of porcine skeletal muscle. However, at variance with the observations in [10], we could not detect an inhibitory effect of our anti-smooth muscle CaMg-ATPase antibodies on the CaMg-ATPase activity.

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REFERENCES

[1] Wuytack, F., De Schutter, G. and Casteels, R. (1981) FEBS Lett. 129, 297–300.
[2] Wuytack, F., De Schutter, G., Verbist, J. and Casteels, R. (1983) Arch. Int. Physiol. Biochem., in press.
[3] Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9918.
[4] Gietzen, K., Tejcka, M. and Wolf, H.U. (1980) Biochem. J. 189, 81–88.
[5] Raeymaekers, L., Wuytack, F., Eggermont, J., De Schutter, G. and Casteels, R. (1983) Biochem. J. 210, 315–322.
[6] Martonosi, A. and Halpin, R.A. (1971) Arch. Biochem. Biophys. 144, 66–77.
[7] Suko, J. and Hasselbach, W. (1976) Eur. J. Biochem. 64, 123–130.
[8] Steinbuch, M. and Audran, R. (1969) Rev. Franc. Clin. Biol. 14, 1054–1058.
[9] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Verma, A.K., Gorski, J.P. and Penniston, J.T. (1982) Arch. Biochem. Biophys. 215, 345–354.