Evidence for the presence in smooth muscle of two types of Ca^{2+} -transport ATPase

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Membrane fractions prepared from smooth muscle of the pig stomach (antral part) contain two Ca^{2+} -dependent phosphoprotein intermediates belonging to different Ca^{2+} -transport ATPases. These alkali-labile phosphoproteins can be separated by electrophoresis in acid medium. The 130 kDa phosphoprotein resembles a corresponding protein in the erythrocyte membrane, whereas the 100 kDa protein resembles that of the Ca^{2+} -transport ATPase in sarcoplasmic reticulum from skeletal muscle. These resemblances are expressed in terms of M_r , reaction to La^{3+} and in a similar proteolytic degradation pattern. The presence of the calmodulin-stimulated ATPase in mixed membranes from smooth muscle is confirmed by its binding of calmodulin and antibodies against erythrocyte Ca^{2+} -transport ATPase, whereas such binding does not occur with proteins present in the presumed endoplasmic reticulum from smooth muscle.

Smooth muscle microsomes show an ATP-dependent Ca^{2+} uptake and a corresponding activity of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase that cannot be ascribed to contaminating mitochondria or mitochondrial fragments.

It is now generally accepted that this uptake mirrors the activity of the Ca^{2+} pumps in the plasma membrane and/or in the endo-(sarco)-plasmic reticulum. The ability of the endoplasmic reticulum from smooth muscle to accumulate Ca^{2+} was shown by electronprobe X-ray microanalysis (Somlyo *et al.*, 1979). The dependence of this uptake on ATP was demonstrated by the electron microscopic visualization of calcium oxalate precipitates in these structures *in situ* after presenting Ca^{2+} , oxalate and ATP to smooth muscle cells of which the plasma membranes were rendered permeable to these substrates by saponin treatment (Raeymaekers, 1982).

A calmodulin-stimulated Ca^{2+} -transport ATPase has been purified from smooth muscle by means of affinity chromatography on immobilized calmodulin (Wuytack *et al.*, 1981; De Schutter *et al.*, 1984), but unequivocal evidence for the existence of a separate calmodulin-independent $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in this tissue has not been presented. In the course of our studies of the phosphoprotein intermediates formed during the catalytic cycle of the Ca^{2+} -transport ATPase in

microsomes from antral smooth muscle (Wuytack *et al.*, 1982), we demonstrated, by means of polyacrylamide-gel electrophoresis in acid medium, the presence of two Ca^{2+} -dependent hydroxylamine- and alkali-labile phosphoproteins. One of these phosphoproteins comigrated with the 130 kDa phosphoprotein from erythrocytes and its phosphorylation was enhanced by low concentrations of La^{3+} , whereas the second one had an M_r value similar to that of the 100 kDa phosphoprotein from fragmented sarcoplasmic reticulum of fast skeletal muscle and its phosphorylation was slightly inhibited by La^{3+} .

The presence of the 100 kDa-phosphoprotein could indicate the existence in smooth muscle of a $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of the sarcoplasmic reticulum type, provided one could exclude that this phosphoprotein was a proteolytic product of the calmodulin-stimulated $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of 130 kDa. The present report supports the hypothesis that this 100 kDa phosphoprotein in smooth muscle is an intermediate of a $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase analogous to the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of skeletal-muscle sarcoplasmic reticulum. It is also shown that it is not a proteolytic product of the 130 kDa $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. Furthermore, we provide strong evidence in favour of the hypothesis that this 100 kDa ATPase is confined to the endoplasmic reticulum, whereas the 130 kDa

calmodulin-binding ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase is a plasma membrane component.

Methods

Preparation of the membrane fractions

KCl-extracted microsomes from pig antrum (stomach) smooth muscle were prepared as described by Wuytack *et al.* (1981); pig erythrocyte vesicles and fragmented sarcoplasmic reticulum from pig skeletal muscle were prepared as described by Steck & Kant (1974) and Heilmann *et al.* (1977) respectively.

Phosphorylation of the membranes

Phosphorylation was conducted as described by Wuytack *et al.* (1982). The reaction medium contained 30 mM-imidazole/HCl (pH 6.8), 100 mM-KCl, 50 μM - CaCl_2 and 0.5 mg of membrane protein/ml. In some experiments also 50 μM - LaCl_3 was added. The reaction was started by the addition of 6 μM -ATP containing [γ - ^{32}P]ATP (New England Nuclear; Tricine-stabilized, product NEG-002A) at 13 $\mu\text{Ci}/\text{mmol}$. After 20 s at 0°C the reaction was stopped and samples were prepared for electrophoresis as described earlier.

Partial proteolysis before phosphorylation

For the experiments in which membrane fractions were treated with trypsin, 1 mg of protein/ml of membranes was suspended in 0.25 M-sucrose. To 100 μl of this suspension were added 20 μl of 1 M-KCl and 20 μl of 300 mM-imidazole/HCl (pH 6.8). After mixing with 10 μl of trypsin (1 mg/ml), proteolysis was allowed to proceed for 15 min at 0°C. The reaction was stopped by 10 μl of soya bean trypsin inhibitor (2 mg/ml). After addition of 20 μl of 0.5 mM- CaCl_2 or 20 μl of 0.5 mM- $\text{CaCl}_2/0.5$ mM- LaCl_3 , phosphorylation was started by 20 μl of the ATP stock solution as indicated above.

Electrophoresis

Gel electrophoresis was conducted at 4°C and pH 2.4 in 0.75 mm thick gels (Wuytack *et al.*, 1982). Polyacrylamide-gradient gels were used (De Smedt *et al.*, 1984) in which the polyacrylamide concentration was increased non-linearly with the distance, from 4% at the top to 15% at the bottom. A gradient former was used with two open compartments. The mixing chamber, with a diameter of 2 cm, contained initially 22.7 ml of electrophoresis buffer containing 15% (w/v) acrylamide, 0.8% bisacrylamide, 15% (w/v) sucrose and a catalyst mixture consisting of 0.00025% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$,

0.01% ascorbic acid and 0.03% H_2O_2 . The reservoir chamber, with a diameter of 3 cm, contained 50.9 ml of buffer and catalysts only. A total of 34 ml of gradient solution was pumped simultaneously into two gel moulds. Electrophoresis was run for 4 h at 100 mA in electrophoresis buffer (93.8 mM-citric acid/12.4 mM-phosphoric acid/12 mM-Tris base/0.1% sodium dodecyl sulphate) adjusted to pH 2.4. Gels were quick-dried and autoradiographed as described by Wuytack *et al.* (1982).

Calmodulin binding

Proteins were separated on 7.5% slab gels by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis according to Laemmli (1970) and transferred to nitrocellulose sheets (Millipore HAWP 304 FO) by means of a Bio-Rad transblot cell at 40 V for 16 h at 10°C as described by Wuytack *et al.* (1983).

After blotting, the nitrocellulose membranes (maximum 8 cm $\times$ 12 cm) were incubated twice for a total of 3–4 h at 37°C in 20 ml of a quench buffer containing 150 mM-NaCl, 0.1 mM- CaCl_2 , 20 mM-Tris/HCl (pH 7.4), 0.5% casein and 0.5% bovine serum albumin. The blots were then incubated for 30 min at room temperature with 5 ml of the quench buffer containing in addition 0.15 nmol of ^{125}I -calmodulin at 5×10^7 c.p.m./nmol. The unbound ^{125}I -calmodulin was washed away by two washes with 50 ml of quench buffer followed by two washes with quench buffer without bovine serum albumin and finally two washes with 50 ml of quench buffer without albumin and casein. Each of these washes lasted for 10 min and was done at 0°C. The blots were then dried under an i.r. lamp and autoradiographed at -70°C .

Calmodulin (0.1 mg), prepared from bovine brain according to Sharma & Wang (1979), was iodinated according to the manufacturers instructions with the '[^{125}I]-Radioiodination System' from New England Nuclear. After stopping the iodination reaction ^{125}I -calmodulin was separated from $^{125}\text{I}^-$ on a column (0.7 cm $\times$ 10 cm) of Sephadex G-25M. The column was equilibrated and eluted with 10 mM-sodium phosphate buffer (pH 7.0) and 0.8 ml fractions were collected. Bovine serum albumin (20 mg/ml) was added to the fraction containing the iodinated calmodulin to reduce radiation decomposition. The final product was stored in small aliquots at -20°C .

The purity of the preparation was ascertained by Laemmli (1970) gel electrophoresis and autoradiography.

Immunoblotting

Immunoblotting techniques were conducted as described earlier (Wuytack *et al.*, 1983).

Results and discussion

Effect of limited proteolysis on the phosphorylation intermediates

Figs. 1 and 2 show the different Ca^{2+} -dependent phosphoproteins which are formed before and after partial proteolysis of the membranes. These phosphoproteins represent the catalytic intermediates of the Ca^{2+} -transport ATPases (Wuytack *et al.*, 1983; De Smedt *et al.*, 1983).

Limited proteolysis by trypsin before phosphorylation strongly diminishes the 100 kDa phosphoprotein from skeletal muscle sarcoplasmic reticulum and results in the appearance of two phosphorylated low- M_r breakdown products, one with an M_r of 50 kDa and one with an M_r of 31 kDa (Fig. 1, track 5; Fig. 2, tracks 3 and 4). This observation is in agreement with the work of

Stewart *et al.* (1976) and that of Dux & Martonosi (1983). The presence of these two bands can be explained by the cleavage of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase molecule by trypsin into fragments A and B, followed by a second cleavage of the A fragment into A_1 and A_2 fragments. The phosphorylation site is localized in the A_1 fragment. The 100 kDa phosphoprotein band of smooth muscle microsomes is similarly degraded by trypsin (Fig. 1, tracks 7 and 8). Because we have previously shown that La^{3+} increases the phosphorylation levels in erythrocyte $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, whereas it slightly lowers the phosphorylation levels in sarcoplasmic reticulum from skeletal muscle, we have also in the present work studied the effect of La^{3+} on the phosphorylation of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase fragments. La^{3+} slightly inhibits the phosphorylation of the breakdown products from

Fig. 1. Autoradiogram of a polyacrylamide quick-dried slab gel showing the phosphoprotein intermediates of several Ca^{2+} -transport ATPases separated by electrophoresis

Phosphorylation was performed in the presence of $50 \mu\text{M}$ - Ca^{2+} either without (tracks 1–4) or after (tracks 5–8) limited trypsin digestion as described in the Methods section. Tracks 1 and 5, fragmented sarcoplasmic reticulum of rabbit fast skeletal muscle; tracks 2 and 6, membrane vesicles from pig erythrocytes; tracks 3 and 7, KCl-extracted microsomes from smooth muscle; tracks 4 and 8, same as 3 and 7 but phosphorylation in the presence of $50 \mu\text{M}$ - Ca^{2+} and $50 \mu\text{M}$ - La^{3+} . Track 9 is a mixture of $[^{14}\text{C}]$ methylated proteins (Amersham International). The polyacrylamide concentration of the slab gel increased non-linearly with the distance, with a progressively larger increase in polyacrylamide concentration from top (4%) to bottom (15%).

Fig. 2. Autoradiogram of a polyacrylamide quick-dried slab gel showing the phosphoprotein intermediates of the Ca^{2+} -transport ATPases of fragmented sarcoplasmic reticulum of rabbit fast skeletal muscle (tracks 1–4) and of vesicles from pig erythrocytes (tracks 5–8)

Tracks 1, 2, 5 and 6 are unproteolysed controls. Tracks 3, 4, 7 and 8 are from vesicles phosphorylated after limited tryptic digestion. Vesicles in tracks 1, 3, 5 and 7 were phosphorylated in the presence of $50 \mu\text{M-Ca}^{2+}$, those in tracks 2, 4, 6 and 8 in the presence of $50 \mu\text{M-Ca}^{2+}$ and $50 \mu\text{M-La}^{3+}$ (see the Methods section).

skeletal muscle membranes (Fig. 2, cf. tracks 3 and 4) and smooth-muscle microsomes (Fig. 1, cf. tracks 7 and 8). The similarity in breakdown patterns of both the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of skeletal muscle sarcoplasmic reticulum and of the 100 kDa smooth-muscle $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, suggests that these enzymes could be homologous.

Partial proteolysis of pig erythrocyte vesicles reduces the M_r of the phosphoprotein intermediate of the Ca^{2+} -transport ATPase from 130 kDa to about 110 kDa (Fig. 1, cf. tracks 2 and 6), while the stimulatory effect of La^{3+} on the phosphorylation is retained (Fig. 2, cf. tracks 7 and 8). The 130 kDa

phosphoprotein from smooth-muscle microsomes reacts similarly to proteolysis, both with respect to the decrease of its M_r and its reaction to La^{3+} (Fig. 1, tracks 7 and 8). The decrease of M_r of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase by about 30 kDa units had already been observed in human erythrocytes by Enyedi *et al.* (1980) and by Taverna & Hanahan (1980).

The 100 kDa sarcoplasmic reticulum-type phosphoprotein described above can unambiguously be discriminated from the 110 kDa proteolytic breakdown product of the erythrocyte-type 130 kDa protein. First, there is the small

difference in M_r . Second, the phosphorylation of the 100kDa phosphoprotein observed before proteolysis is inhibited by La^{3+} , whereas the phosphorylation of the proteolytic product found at a slightly higher position is stimulated by La^{3+} . Third, both the 110kDa phosphorylated proteolysis products which are readily formed by trypsinization from the 130kDa phosphoprotein in erythrocyte vesicles and smooth muscle membranes are remarkably resistant to further degradation. Under our experimental conditions, even after prolonged trypsin treatment there was no phosphorylation of proteolytic products from erythrocyte vesicles with M_r below 100kDa. A similar absence of phosphorylated fragments with M_r below 100kDa has been reported by Enyedi *et al.* (1980) for human erythrocyte membranes. However, Zurini *et al.* (1984) recently obtained from purified $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of human erythrocytes a proteolytic fragment of M_r 76000–81000 which forms an acylphosphate intermediate. But even these phosphorylated tryptic fragments have a M_r which is clearly different from that of the tryptic fragments of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase from the skeletal-muscle sarcoplasmic reticulum.

Subcellular distribution of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPases in smooth muscle

The above data indicate the presence in smooth muscle of two types of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. In this section we present data on their subcellular distribution. We have therefore tried to identify the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPases present in two different populations of membranes from smooth muscle obtained on the basis of a difference in permeability to oxalate. As in skeletal muscle, also for smooth muscle the stimulation of Ca^{2+} accumulation by oxalate is a property of the endoplasmic reticulum (Raeymaekers, 1982). Hence a selective loading of the vesicles derived from the endoplasmic reticulum with calcium oxalate was used for the purification of these structures (Raeymaekers *et al.*, 1983).

Three different observations indicate that the erythrocyte-type $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase is not present in the smooth-muscle endoplasmic reticulum membranes. First, ^{125}I -calmodulin overlay, as illustrated in Fig. 3, indicates the absence of calmodulin-binding sites in the oxalate-loaded fraction. By treating nitrocellulose electroblots of

Fig. 3. ^{125}I -Calmodulin binding to nitrocellulose blots of polypeptides separated by sodium dodecyl sulphate/polyacrylamide (7.5%) slab gel electrophoresis

Track 1, microsomes from pig erythrocyte membranes; track 2, fragmented sarcoplasmic reticulum of pig skeletal muscle; track 3, microsomes from pig smooth muscle; track 4, same as track 3 but the blot was autoradiographed for a shorter time; track 5, another preparation of the smooth muscle microsomes; track 6, a subfraction of smooth muscle microsomes prepared by isopycnic equilibration between 20 and 25% (w/w) sucrose; track 7, a subfraction prepared from that in track 6 by calcium oxalate loading the vesicles according to Raeymaekers *et al.* (1983). Note the absence of ^{125}I -calmodulin binding to the polypeptides in skeletal-muscle sarcoplasmic reticulum and in the calcium oxalate-loaded fraction from a smooth muscle. S indicates the start position; F, the dye front. The arrow indicates the position of the calmodulin-binding $(\text{Ca}^{2+} + \text{Mg}^{2+})$ ATPase.

sodium dodecyl sulphate/polyacrylamide gels with ^{125}I -calmodulin, we have demonstrated the binding of calmodulin to polypeptides from erythrocyte membranes (Fig. 3, track 1), to polypeptides from a mixed population of smooth muscle microsomes (Fig. 3, tracks 3, 4 and 5), as well as to polypeptides of a subfraction of smooth muscle membranes that has been enriched in plasma membranes (Fig. 3, track 6). We could not observe a binding of ^{125}I -calmodulin to polypeptides from the skeletal muscle sarcoplasmic reticulum (Fig. 3, track 2) nor to the polypeptides from the smooth muscle oxalate-loaded fraction (Fig. 3, track 7). ^{125}I -calmodulin, besides binding to the calmodulin-sensitive $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (indicated in Fig. 3 by the arrow), also binds to some polypeptides of unspecified nature in smooth muscle and erythrocyte microsomes. All the ^{125}I -calmodulin bindings were Ca^{2+} -dependent and did not occur if the nitrocellulose blots were incubated with ^{125}I -calmodulin in the presence of 1 mM-EDTA instead of 0.1 mM- CaCl_2 (see under 'Methods'). Moreover, the radioactive ^{125}I -calmodulin bands could be washed off the blots by three 10 min washes at room temperature with a medium containing 150 mM- NaCl , 20 mM-Tris/HCl (pH 7.4) and 1 mM-EGTA.

Secondly, we have demonstrated by means of immunoblot-binding procedures that antibodies against erythrocyte $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase bind to the calmodulin-stimulated $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase present in a mixed smooth-muscle membrane preparation, but not to the enzyme of the membrane fraction obtained after oxalate-loading (Fig. 4). A further argument in favour of the hypothesis is that whereas the erythrocyte-type $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase is stimulated by calmodulin, neither the oxalate-stimulated Ca^{2+} -accumulation, nor the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity in the oxalate-loaded fraction from smooth muscle, is affected by this activator. In contrast both of these activities are increased in plasma-membrane-enriched fractions from smooth muscle (Raeymaekers *et al.*, 1983). It should however be pointed out that in principle the absence of such stimulation of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in the oxalate-loaded fraction might also be due to the lipid environment of the ATPase in these membranes. An enrichment in negatively charged lipids (Niggli *et al.*, 1981; Gietzen *et al.*, 1982) can also in smooth muscle augment the activity of the calmodulin-binding $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase and thereby prevent a further stimulation by calmodulin of the enzyme without affecting its binding to the activator (De Schutter *et al.*, 1984). Unfortunately the very low solubility of lanthanum oxalate precludes the use of La^{3+} to enhance the phosphorylation of a 130 kDa phosphorylation product, if it

Fig. 4. Binding of immunoglobulins against the calmodulin-stimulated $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase from pig erythrocytes to a blot obtained from a parallel electrophoresis to the one depicted in Fig. 3

Tracks 1, 2 and 3 correspond to respectively tracks 5, 6 and 7 from Fig. 3. The immunoblotting procedure was executed as described by Wuytack *et al.* (1983). Whole antiserum of rabbits immunized against purified $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase from pig erythrocytes was used as primary antiserum. Staining for peroxidase activity was done with 4-chloro-1-naphthol. S indicates the start position; F, the dye front. The arrow indicates the position of the calmodulin-binding $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase.

were present in the oxalate-loaded membranes. The free La^{3+} concentration in the assay would not reach a sufficient value to affect phosphorylation if oxalate were not completely removed. Because of the risk of false negative results, La^{3+} was not used to test the absence of a 130 kDa $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in the calcium oxalate-loaded endoplasmic reticulum fractions from smooth muscle.

It is therefore concluded that the membranes from antral smooth muscle contain, besides the calmodulin-stimulated $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase that has many characteristics in common with the erythrocyte $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, also a second $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase that has the same M_r as

the corresponding enzyme in skeletal muscle sarcoplasmic reticulum, shows a similar breakdown pathway by trypsin and does not bind calmodulin. In smooth muscle the latter enzyme seems to be confined to the endoplasmic reticulum.

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