Biochimica et Biophysica Acta, 693 (1982) 45–52
Elsevier Biomedical Press

BBA 71408

DEMONSTRATION OF THE PHOSPHORYLATED INTERMEDIATES OF THE Ca^{2+}-TRANSPORT ATPase IN A MICROSOMAL FRACTION AND IN A (Ca^{2+} + Mg^{2+})-ATPase PURIFIED FROM SMOOTH MUSCLE BY MEANS OF CALMODULIN AFFINITY CHROMATOGRAPHY

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(Received June 28th, 1982)

Key words: Calmodulin; Ca^{2+} transport; (Ca^{2+} + Mg^{2+})-ATPase; Phosphorylated intermediate; (Porcine smooth muscle)

Ca^{2+}-dependent hydroxylamine-sensitive phosphorylated proteins can be demonstrated in a microsomal fraction of porcine antrum (stomach) smooth muscle and in a Ca^{2+}-transport ATPase ((Ca^{2+} + Mg^{2+})-ATPase) purified from this tissue by means of a calmodulin affinity technique. These phosphoproteins represent the phosphorylated intermediates of the (Ca^{2+} + Mg^{2+})-ATPases. In the (Ca^{2+} + Mg^{2+})-ATPase purified from smooth muscle the phosphorylated intermediate has an M_r of 130 000 corresponding to the value found for erythrocyte (Ca^{2+} + Mg^{2+})-ATPase. In the smooth muscle microsomal fraction this 130 kDa phosphoprotein can also be seen, although its intensity is usually very low compared to a corresponding phosphorylation at M_r 100 000. Including La^{3+} together with Ca^{2+} during phophorylation of the microsomes increased selectively the steady state-level of the 130 kDa phosphoprotein over the value of the 100 kDa one. The 100 kDa Ca^{2+}-dependent phophoprotein could either indicate the presence of a (Ca^{2+} + Mg^{2+})-ATPase of the same type of sarcoplasmic reticulum of skeletal muscle, or it could represent a proteolytic product of the 130 kDa phosphoprotein.

Introduction

The formation of a phosphorylated intermediate is well documeted for the (Ca^{2+} + Mg^{2+})-ATPase of the sarcoplasmic-reticulum of skeletal muscle [1,2] of the cardiac sarcoplasmic reticulum [3] and of erythrocyte membranes [4,5]. Although the (Ca^{2+} + Mg^{2+})-ATPase activity in human erythrocyte membranes is low, it has been possible to demonstrate a Ca^{2+}-dependent phosphorylation of this (Ca^{2+} + Mg^{2+})-ATPase (M_r 130 000) and to differentiate it from a comparable phosphorylated intermediate of the (Na^+ + K^+)-ATPase (M_r 100 000) [4]. Recently a similar phosphorylated intermediate has also been described in smooth muscle microsomes [6].

In our study of the (Ca^{2+} + Mg^{2+})-ATPase purified from smooth muscle, we were also able to demonstrate the presence of a Ca^{2+}-dependent phosphorylated intermediate with an M_r of 130 000 [7]. This (Ca^{2+} + Mg^{2+})-ATPase from smooth muscle was prepared by the calmodulin affinity technique originally designed for erythrocyte (Ca^{2+} + Mg^{2+})-ATPase [8,9]. It is therefore not surprising to find an M_r for the smooth muscle ATPase comparable to that of the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase. This erythrocyte-type (Ca^{2+} + Mg^{2+})-ATPase, characterized by its M_r of 130 000 and by its stimulation by calmodulin, may however not be the only kind of (Ca^{2+} +

Abbreviations: EGTA: ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Mg^{2+})-ATPase in smooth muscle. Because we had observed that in smooth muscle microsomes a protein with M_r 100000 was also phosphorylated in a Ca^{2+}-dependent way, a sarcoplasmic reticulum-type (Ca^{2+} + Mg^{2+})-ATPase, with M_r 100000 might also be present. Alternatively the 100 kDa phosphoprotein might represent a proteolytic product of the 130 kDa protein. We have therefore in this study compared the phosphorylated intermediates in a microsomal fraction of smooth muscle and in the (Ca^{2+} + Mg^{2+})-ATPase purified from smooth muscle microsomes, with the intermediates in erythrocyte membranes and in sarcoplasmic reticulum of skeletal muscle.

Materials and Methods

Preparation of microsomal fractions and of (Ca^{2+} + Mg^{2+})-ATPase. Microsomes from antral smooth muscle of the pig stomach were prepared as reported earlier [10]. Fragmented sarcoplasmic reticulum of skeletal muscle was prepared from white skeletal muscles from the legs and back of the rabbit [11]. Inside-out vesicles of erythrocytes were prepared from human and from porcine blood as described by Steck and Kant [12], but the final Dextran gradient step was omitted. The vesicles were kept in liquid nitrogen until used. In some experiments these vesicles were frozen and thawed three times to break them open. (Ca^{2+} + Mg^{2+})-ATPase was isolated from the pig antrum smooth muscle microsomes by calmodulin affinity chromatography exactly as described in Ref. 7.

Phosphorylation. The phosphorylation reactions were performed in Eppendorf 3810 tubes on ice for 20 or 60 s as indicated. The reaction was started by adding [γ-^{32}P]ATP 6 μM at 13 Ci/mmol to a medium containing: 100 mM KCl, 30 mM imidazole-HCl (pH 6.8), microsomes or ATPase and either, $CaCl_2$ 50 μM or K-EGTA 0.5 mM, in a total volume of 200 μl. When indicated 100 μM $LaCl_3$ was added to this medium. The total amount of proteins present was usually 0.2–0.8 mg for smooth muscle microsomes and human or porcine erythrocyte vesicles, 0.4 μg for sarcoplasmic reticulum and 4 μg for purified (Ca^{2+} + Mg^{2+})-ATPase of smooth muscle. The reaction was stopped by 0.5 ml of a stop solution containing: 10% trichloroacetic acid, 50 mM phosphoric acid, 0.5 mM ATP. For studying the phosphorylation of sarcoplasmic reticulum and of purified (Ca^{2+} + Mg^{2+})-ATPase, 0.25 mg bovine serum albumin was also added as carrier protein, because the total amount of protein precipitated by the trichloroacetic acid would otherwise be very small.

The tubes were centrifuged in an Eppendorf centrifuge model 5413 for 5 min ($8000 \times g$) and the pellets were washed three times with 1.2 ml of the stop solution. Between each wash the tubes were again centrifuged for 5 min.

The pellets were dissolved in 100 μl of a mixture containing: 3% sodium dodecyl sulphate, 10% sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 40 mM dithiotreitol and 10 μg/ml pyronin Y. The solubilization was obtained by shaking at room temperature for 10 min. A small methacrylate pestle, moulded to the shape of the Eppendorf tube was used to accelerate the solubilization. Samples of 40 μl were applied on a polyacrylamide gel and the electrophoresis was done as described by Fairbanks [13] using 0.75 mm thick slab gels which contained 5% acrylamide, in the LKB 2001 vertical electrophoresis unit. These gels and the electrode buffer contained 0.1% sodium dodecyl sulphate. The gels were run at 50 mA for 1 h at 12°C. They were then removed and dried for 45 min in a BioRad Model 224 Gel slab dryer. Autoradiography was done on Kodak X-omat R film (XR-1). In an alternative electrophoresis procedure tube gels of 0.7×8 cm with the same composition of gels and electrode buffers were used. Tubes were run at 10 mA/tube for 1 h at 12°C. These gels were then cut in slices of 2 mm width. The slices were incubated overnight at 37°C in 1 ml hydroxyde of hyamine X10 (Packard) before adding 15 ml of scintillation fluid (5.5 g Permablend (Packard)/liter toluene). Polyacrylamide slab gel (0.75 mm thick, 5% acrylamide) electrophoresis was also performed in a buffer at low pH and low temperature [14]. The electrophoresis buffer, adjusted to pH 2.4 contained 94 mM citric acid, 12.4 mM phosphoric acid, 12 mM Tris-base and 0.1% sodium dodecyl sulphate. The gels contained 5% (w/v) polyacrylamide. Polymerization was induced by adding 0.0025% $FeSO_4$, 0.03% H_2O_2 and 0.1% ascorbic acid to the acrylamide solution. Polymerization, preelectrophoresis (1 h,

50 mA/gel) and electrophoresis (3.5 h, 50 mA/gel) were done at 4°C. Samples of 40 μl of the acid precipitated fractions, dissolved in the same sample mixture as described above, were applied on the gels.

The effect of hydroxylamine was studied by incubating the pellets of precipitated phosphorylated protein (after the three washes as indicated above) for 10 minutes at room temperature with 1 ml of a solution containing 150 mM hydroxylamine hydrochloride brought to pH 6.0 with Tris and 150 mM sodium acetate buffer. Controls were treated likewise with a solution of 150 mM Tris-HCl, 150 mM sodium acetate buffer at pH 6.0.

Boehringer Combitek calibration standards were used for estimation of M_r. The part of the slab gels with the M_r reference proteins, were cut off after electrophoresis and stained separately with Coomassie brillant blue. M_r values were obtained from curves relating M_r values of standard proteins to their corresponding R_f values.

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham U.K. It was lyophylized and redissolved in water to remove the ethanol immediately before use.

Results

Demonstration of a Ca^{2+}-dependent phosphoprotein in smooth muscle microsomes

In contrast to what is the case for skeletal muscle, no reports are found in the literature about the M_r of the phosphoprotein intermediate of the Ca^{2+}-transport ATPase of smooth muscle. The phosphoprotein intermediates of all known transport ATPases are acylphosphates and therefore labile in alkaline medium [15]. This is not the case for the phosphoprotein products of protein kinase activity which are alkaline resistant. We have therefore selected for our electrophoresis experiments buffers in the neutral or (in later experiments) in the acid range. An unfortunate consequence of this choice is the lower resolution of these electrophoresis systems compared to the procedure described by Laemmli [16].

The autoradiogram of the phosphorylated proteins of fragmented sacroplasmic reticulum from fast rabbit skeletal muscle and of those of KCl-extracted microsomes from pig antrum smooth

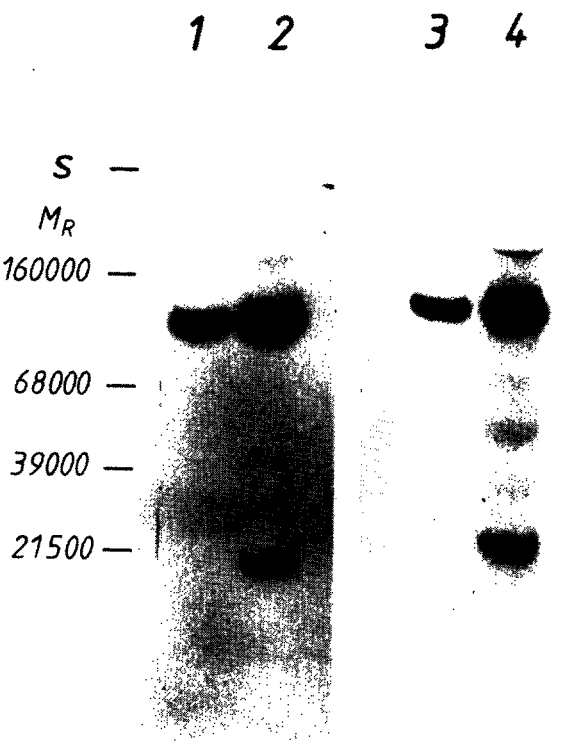

Fig. 1. Autoradiogram of the phosphorylated proteins in the microsomal fraction of porcine antrum smooth muscle and in the sarcoplasmic reticulum of rabbit skeletal muscle. Phosphorylations were done for 20 s at 0°C as indicated in Methods. Polyacrylamide slab gels (5% acrylamide) were run according to Ref. 13 for 1 h at 12°C. Lanes 1 and 3: sacroplasmic reticulum; lanes 2 and 4: smooth muscle microsomes. For lane 1 and 2 no Mg^{2+} was added to the phosphorylation medium while $MgCl_2$ (1 mM) was present for lanes 3 and 4. The positions of start(s) and of some M_r standards are indicated at the left.

muscle are represented in Fig. 1. When the phosphorylation is done in the presence of 1 mM Mg^{2+}, several additional bands of phosphorylated proteins appear in the smooth muscle microsomes, which cannot be observed in the controls containing no extra Mg^{2+}. In fragmented sarcoplasmic reticulum from skeletal muscle as well as in the smooth muscle microsomes however, the darkest band corresponds to an M_r of 100 000 as estimated from the position of marker proteins run on the same gel slab. In most experiments on smooth muscle microsomes some Ca^{2+}-dependent phosphorylation was also observed at M_r 130 000. These two bands can only be clearly discriminated from each other after longer electrophoresis times (see

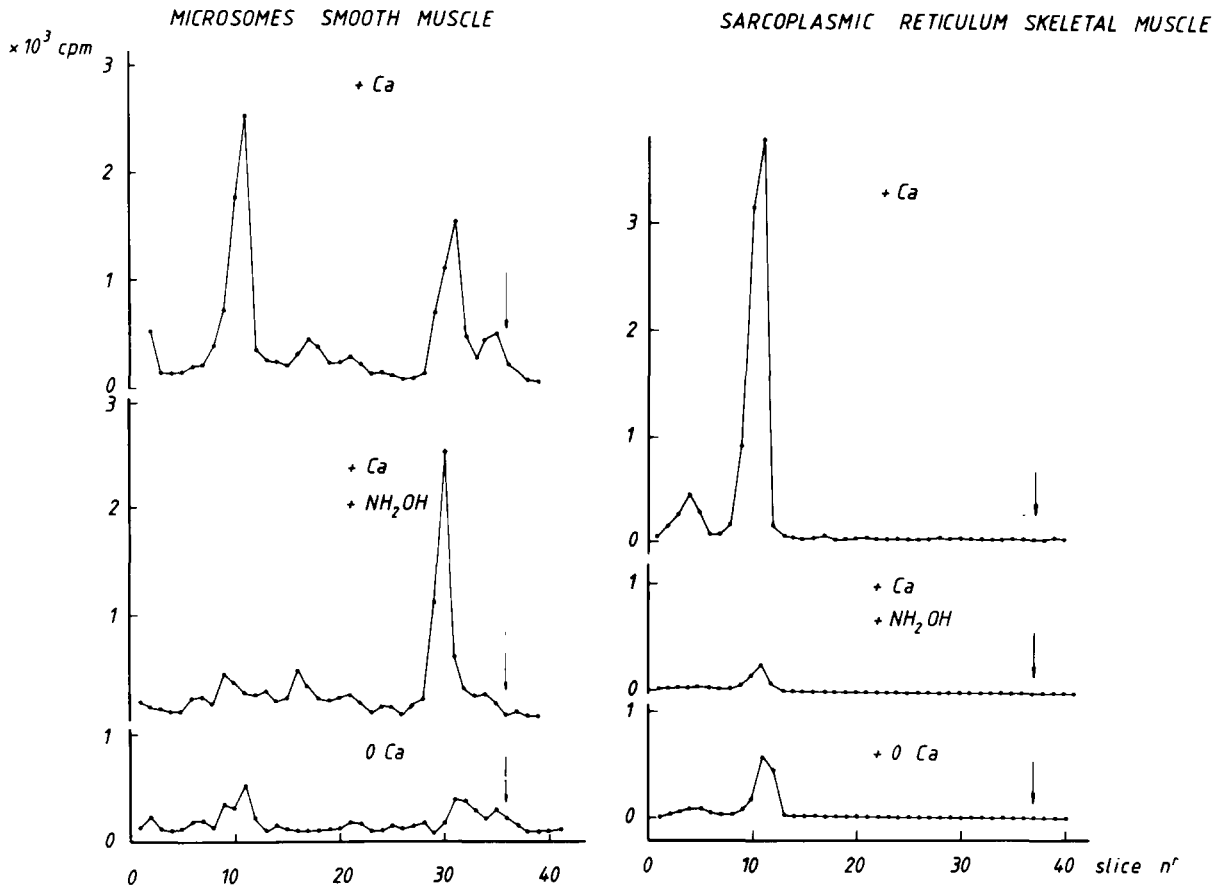

Fig. 2. The effect of hydroxylamine on the phosphorylated proteins in microsomal fractions of porcine antrum smooth muscle and in sarcoplasmic reticulum of rabbit skeletal muscle. Phosphorylations were done for 20 s at 0°C as indicated in Methods. Polyacrylamide tube gels (5% acrylamide) were run according to Ref. 13 for 1 h at 12°C. Gels were cut in slices of 2 mm width. The slices were treated for scintillation counting as described in Methods. The curves represent from top to bottom: phosphorylations in a Ca^{2+}-containing medium, in a Ca^{2+}-containing medium and after hydroxylamine treatment, in the absence of Ca^{2+}. Electrophoresis is from left to right. The arrow indicates the position of the dye front. The M_r 100000 band corresponds approximately to slice No. 10.

Fig. 3). The amount of phosphorylation at M_r 130000 compared to the one at M_r 100000 was however usually low (< 10%) and varied from experiment to experiment. It can be shown (see below) that these proteins with M_r of 100000 and 130000 correspond to the phosphorylated intermediates of the (Ca^{2+} + Mg^{2+})-ATPase while the other bands of phosphorylated proteins, appearing after phosphorylation in the presence of Mg^{2+}, are probably products of protein kinase activities present in the microsomes. It should be pointed out that the experimental conditions used in these phosphorylation experiments (temperature: 0°C, reaction time: 20 s, [ATP] = 6 μM) are not optimal for phosphorylation mediated by protein kinase.

The Ca^{2+}-dependent phosphoproteins are hydroxylamine sensitive

The phosphoproteins in sarcoplasmic reticulum with M_r 100000 and the one present in the microsomes of antral smooth muscle with an M_r of 100000 are both Ca^{2+}-dependent and hydroxylamine-sensitive as shown in Fig. 2. It should be emphasized that the conditions used in Fig. 2 are not optimal to show the 130 kDa band separately from the 100 kDa band in the smooth muscle microsomes. This would require longer electrophoresis times and the inclusion of La^{3+} in the phosphorylation medium (see below). However we would like to point out here already that controls showed that also this 130 kDa Ca^{2+}-dependent

phosphoprotein obtained in the presence of La^{3+} is hydroxylamine sensitive (result not shown). The Ca^{2+} dependency and hydroxylamine sensitivity together with the M_r characterize these proteins as the phosphorylated intermediates of the (Ca^{2+} + Mg^{2+})-ATPases. The phosphoprotein intermediates of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum from skeletal muscle [1] and of erythrocytes [4] are both acylphosphates and therefore hydroxylamine sensitive and labile in alkaline medium [15].

The phosphorylated intermediate of the (Na^+ + K^+)-ATPase is also an acylphosphoprotein of M_r 100000 [17]. However our phosphoprotein of M_r 100000 in smooth muscle microsomes is not the intermediate of the (Na^+ + K^+)-ATPase because the phosphorylation is not only Ca^{2+}-dependent, but it also occurs in the absence of Na^+, while it is known that the presence of Na^+ is essential for the phosphorylation of (Na^+ + K^+)-ATPase [4].

In smooth muscle microsomes we also observed a band with an M_r 20000 which is hydroxylamine insensitive. Because this band in not present in skeletal muscle sarcoplasmic reticulum and because myosin is a known contaminant of smooth muscle microsomes [10] it most likely corresponds to the 20 kDa light chain of smooth muscle myosin.

A comparison of Ca transport ATPase intermediates in different systems

Because the Ca^{2+}-transport ATPases have all an M_r of 100000 or higher, we have, in order to enhance the resolution in the high M_r range, increased the duration of the electrophoresis from 1 h in the experiments depicted in Figs. 1 and 2, to 3.5 h in the experiments of Figs. 3 and 4. As a consequence, the low M_r proteins which are less interesting for our purpose, migrated off the gel.

We have also preferred to run the electrophoresis in the following experiments at pH 2.4 and at 4°C in order to increase the stability of the phosphoprotein intermediates. This decrease in both pH and temperature of the electrophoresis buffer did however not result in any obvious change of distribution pattern of the phosphorylated intermediates on the autoradiograms.

Fig. 3 compares the phosphorylation in the high M_r range of microsomes from smooth muscle, of

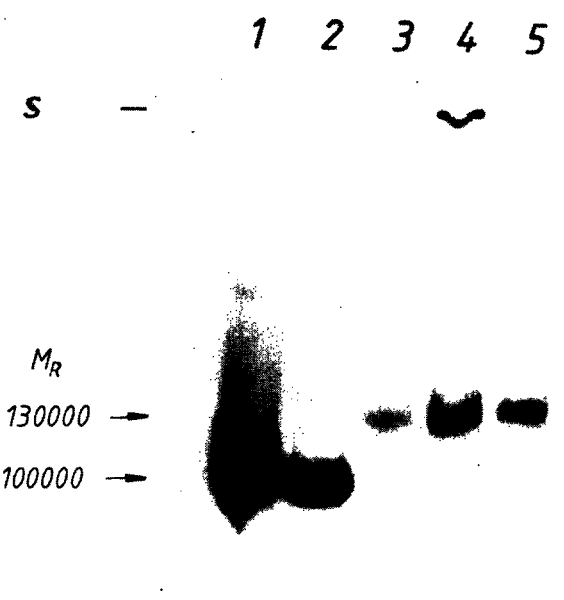

Fig. 3. Autoradiogram of the phosphorylated proteins in the microsomal fraction from porcine antrum smooth muscle, in sarcoplasmic reticulum of rabbit skeletal muscle, in inside-out vesicles of porcine and human erythrocytes and in (Ca^{2+} + Mg^{2+})-ATPase purified from smooth muscle by calmodulin affinity chromatography. Phosphorylations in presence of Ca^{2+} were done for 60 s at 0°C as indicated in Methods. Polyacrylamide slab gels (5% acrylamide) were run according to Ref. 13 for 3.5 h at 12°C. The duration of the electrophoresis was longer than that in Figs. 1 and 2. The low molecular weight proteins migrated off the plate. The positions of start (s) and of M_r 130000 and 100000 are indicated at the left. The spot observed at the start position of lane 4 is due to incomplete solubilization of the human erythrocyte vesicles. Lane 1: porcine antrum smooth muscle microsomes; lane 2: rabbit skeletal muscle sarcoplasmic reticulum; lane 3: porcine inside-out erythrocyte vesicles; lane 4: human inside-out erythrocyte vesicles; lane 5: (Ca^{2+} + Mg^{2+})-ATPase purified from porcine antrum smooth muscle by calmodulin affinity chromatography.

sarcoplasmic reticulum from skeletal muscle, of inside-out vesicles from procine erythrocytes and from human erythrocytes and of the smooth muscle (Ca^{2+} + Mg^{2+})-ATPase which has been purified by means of the calmodulin affinity chromatography. Most of the phosphorylated proteins of the smooth muscle microsomes occurred in the M_r 100000 band, but also some phosphorylation can be seen at M_r 130000. However, (Ca^{2+} + Mg^{2+})-

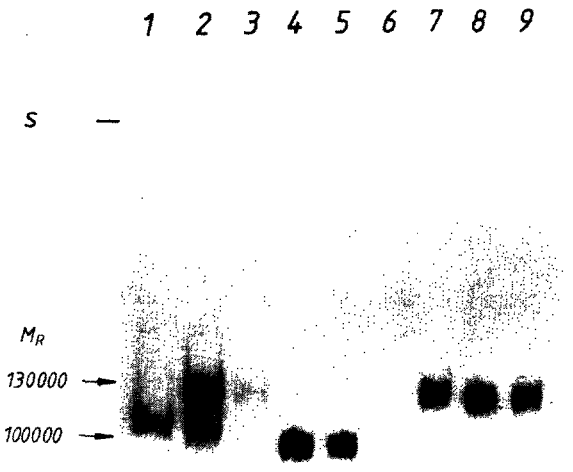

Fig. 4. Autoradiogram of the phosphorylated proteins in the microsomal fractions of porcine antrum smooth muscle, sarcoplasmic reticulum of rabbit skeletal muscle and inside-out vesicles of porcine erythrocytes. Phosphorylations were done for 60 s at 0°C as indicated in Methods. The phosphorylations in lanes 1, 4 and 7 were done in the presence of 50 μM Ca^{2+}, those in lanes 2, 5 and 8 in the presence of 50 μM Ca^{2+} and 100 μM La^{3+}, and those in lanes 3, 6 and 9 in the presence of 100 μM La^{3+}. Lanes 1–3: smooth microsomes; lanes 4–6: skeletal muscle sarcoplasmic reticulum; lanes 7–9: inside-out vesicles of porcine erythrocytes. Polyacrylamide (5%) slab gels were run according to Ref. 14 for 3.5 h at 4°C. Note the longer electrophoresis duration compared to Figs. 1 and 2. The low molecular weight proteins migrated off the plate. The positions of start (s) and of M_r 130000 and 100000 are indicated at the left.

ATPase of smooth muscle purified by means of the calmodulin affinity technique presents only phosphorylation at the M_r 130000 band, i.e. at the same M_r as the phosphorylated proteins from porcine or human erythrocyte vesicles. These results show that the $(Ca^{2+} + Mg^{2+})$-ATPase purified from smooth muscle by means of the calmodulin affinity technique, has the same M_r as the corresponding enzyme in human and porcine erythrocytes. They further show that the presence of the 130 kDA protein can be demonstrated in the smooth muscle microsomes by means of its phosphoprotein intermediate. But an important open question remains why most of the phosphoprotein in these microsomes has an M_r of 100000.

La^{3+} increases selectively the 130 kDa Ca^{2+}-dependent phosphoprotein level

It has been shown that La^{3+} increases the steady state level of phosphorylation in the erythrocyte microsomes [18,19], probably by decreasing the rate of dephosphorylation of the intermediate in the normal forward running mode of the ATPase. Such an increase in steady-state level of phosphorylation by La^{3+} has not been described for sarcoplasmic reticulum of skeletal muscle. Because of the discrepancy between the M_r of the main phosphorylated components in the microsomal fraction from smooth muscle and in the $(Ca^{2+} + Mg^{2+})$-ATPase purified from this fraction, we have investigated the action of La^{3+} on these different phosphorylations. The effect of La^{3+} on the phosphorylation of proteins of smooth muscle microsomes of porcine erythrocyte vesicles and of skeletal muscle sarcoplasmic reticulum are represented in Fig. 4 and in Table I. The Ca-dependent

TABLE I

THE EFFECT OF La^{3+} ON THE TOTAL AMOUNT OF Ca^{2+}-DEPENDENT PHOSPHORYLATION IN THE MICROSOMAL FRACTIONS FROM SMOOTH MUSCLE, FROM PORCINE ERYTHROCYTES AND IN THE SARCOPLASMIC RETICULUM FROM SKELETAL MUSCLE

The values represent the ratio of peak areas integrated from densitometric scans of the autoradiography plates by means of the LKB 2202 Ultro Scan Laser densitometer. To obtain the ratios, the peak areas of the total amount of Ca dependent phosphorylated proteins obtained in the presence of 50 μM Ca^{2+} and 100 μM La^{3+} were divided by the corresponding areas obtained in the presence of 50 μM Ca^{2+} but without La^{3+}. Both in the presence and absence of La^{3+}, this Ca^{2+}-dependent phosphorylation is represented by a single peak for the sarcoplasmic reticulum (M_r 100000) and erythrocyte vesicles (M_r 130000), but by the sum of two peaks (M_r 130000 and 100000) in case of the smooth muscle microsomes. Phosphorylation and electrophoresis conditions were the same as in Fig. 4.

Number of expts.	Smooth muscle microsomes	Porcine erythrocyte microsomes	Skeletal muscle sarcoplasmic reticulum
171181	3.83	1.17	0.77
191181	3.00	1.36	1.15
251181	6.30	1.60	0.92
81281	–	1.50	0.89

phosphorylation is stimulated to the largest extent by La^{3+} in the smooth microsomes. Most of the extra phosphorylation due to the presence of La^{3+} is accounted for by an increase of the phosphorylation of the 130 kDa band. Under these conditions the phosphorylation in the 130 kDa band is much higher than in the 100 kDa band. The stimulation of the phosphorylation in microsomes of erythrocytes is also localized at M_r 130000, but its increase is appreciably less than in the smooth muscle microsomes. Finally in the sarcoplasmic reticulum of skeletal muscle the phosphorylated band remains completely localized at M_r 100000 in the absence as well as in the presence of La^{3+} and the presence of this ion does not increase the phosphorylation level but rather decreases it. Fig. 4 shows also the phosphorylations obtained in the presence of 100 μM La^{3+} without added Ca^{2+} (lanes 3, 6, 9). Under these conditions a weak phosphorylation is observed in the smooth muscle microsomes at M_r 130000, no phosphorylation is found at the M_r 100000 position of smooth muscle microsomes and skeletal muscle sarcoplasmic reticulum, whereas in the porcine erythrocyte vesicles a considerable phosphorylation is observed. If both Ca^{2+} and La^{3+} are absent no phosphorylation is observed at M_r 130000 or 100000. The La^{3+} stimulated phosphorylations are hydroxylamine sensitive (results not shown).

These findings suggest that the extra phosphorylation which appears in the microsomal fraction from smooth muscle by simultaneous addition of Ca^{2+} and La^{3+} might be due to the stimulation of the Ca-dependent phosphorylation by La^{3+} rather than to a La^{3+}-dependent phosphorylation. A La^{3+}-dependent phosphorylation does certainly not occur in the sarcoplasmic reticulum from skeletal muscle. Because the usual Ca^{2+} chelators (EGTA or EDTA) have an even greater affinity for La^{3+} than for Ca^{2+}, we cannot make use of those substances to chelate all Ca^{2+} contaminating the microsomal fractions while leaving the 100 μM La^{3+} free in solution.

Discussion

The Ca^{2+}-dependent, hydroxylamine-sensitive phosphorylation of proteins can be considered as a marker for the $(Ca^{2+} + Mg^{2+})$-ATPases in the different tissues. Two types of $(Ca^{2+} + Mg^{2+})$-ATPase have been studied rather intensively by a number of groups i.e. the one present in sarcoplasmic reticulum isolated from skeletal muscle [1,2] and that present in erythrocyte membranes [4,5]. These ATPases can be distinguished on the basis of their molecular weight and their sensitivity to calmodulin. Because of our complete lack of knowledge on the $(Ca^{2+} + Mg^{2+})$-ATPase in smooth muscle it seemed an acceptable hypothesis to consider those two $(Ca^{2+} + Mg^{2+})$-ATPases as a model of transport molecules which might also occur in smooth muscle cells. We have therefore studied the phosphorylated intermediates of the $(Ca^{2+} + Mg^{2+})$-ATPases of different tissues and under different experimental conditions by comparing their position in the electrophoretograms.

The present results demonstrate that also in smooth muscle phosphorylated intermediates of the Ca^{2+}-transport ATPases are formed. These phosphorylated intermediates are not only found in the purified $(Ca^{2+} + Mg^{2+})$-ATPase, but they can also be observed in the microsomal fraction of the same tissue.

This is, to the best of our knowledge, the first report showing that the phosphoprotein intermediate of the $(Ca^{2+} + Mg^{2+})$-ATPase purified from smooth muscle by means of the calmodulin affinity technique has the same electrophoretic mobility as the corresponding phosphoprotein isolated from erythrocyte membranes. Both $(Ca^{2+} + Mg^{2+})$-ATPases have an M_r of 130000. The position of these two proteins in the electrophoretograms can easily be distinguished from that of the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcoplasmic reticulum which has an M_r of 100000.

The presence of this 130 kDa phosphoprotein can also be demonstrated in the microsomes from smooth muscle, but in the absence of La^{3+} the phosphorylation is usually much lower than that observed at M_r 100000. La^{3+} however considerably and selectively enhances the phosphorylation level of the 130 kDa band, which then becomes the more intensive one of the two bands. Such an increase of phosphorylation is not observed for the 100 kDa band of smooth muscle microsomes, nor for the phosphoprotein band in sarcoplasmic reticulum. This observation indicates that it is difficult to draw a firm conclusion on the relative

amounts of proteins at M_r 130000 and 100000 which can be phosphorylated. Where La^{3+} does not exert an effect (at M_r 100000) we measure the steady-state level of phosphorylation. However if La^{3+} blocks the dephosphorylation (at M_r 130000) an equilibrium level of phosphorylation is obtained. Both such a steady-state level and an equilibrium level of phosphoproteins are underestimates of the total amount of proteins which can be phosphorylated. It is nevertheless clear that for smooth muscle microsomes in the presence of La^{3+} a considerable phosphoprotein level is observed at M_r 130000 that is not apparent when phosphorylation is done in its absence.

From other observations we know that a large part of the Ca^{2+}-transport ATPase in smooth muscle microsomes is of the erythrocyte type. First the $(Ca^{2+} + Mg^{2+})$-ATPase activity is stimulated by calmodulin by a factor of 3 to 4 (at $[Ca^{2+}] = 10^{-5}$ M) in microsomes carefully washed in the presence of EGTA (unpublished observation). Such a large stimulation by calmodulin would not be present if most of the ATPase were of the calmodulin independent sarcoplasmic reticulum-type. Second, when during purification of the $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle, care was taken not to overload the calmodulin affinity gel with detergent solubilized ATPase, all of the $(Ca^{2+} + Mg^{2+})$-ATPase was bound to the gel and no unbound calmodulin-independent component of the $(Ca^{2+} + Mg^{2+})$-ATPase was observed.

The Ca^{2+}-dependent phosphorylation of a protein with M_r 100000 in the smooth muscle microsomes can be explained in two ways. Because it has an M_r of 100000, its phosphorylation is not stimulated by La^{3+} and because it does not bind to a calmodulin affinity gel, it might correspond to the sarcoplasmic reticulum-type Ca^{2+}-transport ATPase. It cannot be excluded however that this phosphoprotein is a proteolytic product of the erythrocyte-type ATPase. It has been shown that when erythrocyte microsomes are treated with trypsin an M_r 100000 product is formed which still presents $(Ca^{2+} + Mg^{2+})$-ATPase activity and which can still be phosphorylated, but becomes insensitive to calmodulin [20]. It should be pointed out that we have included several proteinase inhibitors during preparation of the smooth muscle microsomes.

The method to demonstrate the presence of $(Ca^{2+} + Mg^{2+})$-ATPases by means of their acylphosphoprotein intermediates proves to be a very sensitive and powerful one. This technique can certainly be applied to many other physiological systems.

Acknowledgment

This work was supported by grant No. 3.0087.74 of the Fonds voor Wetenschappelijk Geneeskundig Onderzoek, Belgium.

References

1 Makinose, M. (1969) Eur. J. Biochem. 10, 74–82
2 Yamamoto, T. and Tonomura, Y. (1967) J. Biochem. (Tokyo) 62, 558–575
3 Suko, J. and Hasselbach, W. (1976) Eur. J. Biochem. 64, 123–130
4 Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 324–336
5 Rega, A.F. and Garrahan, P.J. (1975) J. Membrane Biol. 22, 313–327
6 Raeymaekers, L. and Hasselbach, W. (1981) Eur. J. Biochem. 116, 373–378
7 Wuytack, F., De Schutter, G. and Casteels, R. (1981) FEBS Lett. 129, 297–300
8 Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958
9 Gietzen, K., Tejcka, M. and Wolf, H.U. (1980) Biochem. J. 189, 81–88
10 Wuytack, F., De Schutter, G. and Casteels, R. (1981) Biochem. J. 198, 265–271
11 Heilmann, C., Brdiczka, D., Nickel, E. and Pette, D. (1977) Eur. J. Biochem. 81, 211–222
12 Steck, T.L. and Kant, J.A. (1974) in Methods in Enzymology, Vol. 31 (Fleischer, S. and Packer, L., eds.), pp. 172–180, Academic Press, New York
13 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2616
14 Lichtner, R. and Wolf, H.U. (1980) Biochim. Biophys. Acta 598, 486–493
15 Lipmann, F. and Tuttle, C. (1945) J. Biol. Chem. 159, 21–28
16 Laemmli, U.K. (1970) Nature 227, 680–685
17 Bastide, F., Meissner, G., Fleischer, S. and Post, R.L. (1973) J. Biol. Chem. 248, 8385–8391
18 Schatzmann, H.J. and Bürgin, H. (1978) Ann. N.Y. Acad. Sci. 307, 125–147
19 Szasz, I., Hasitz, M., Sarkadi, B. and Gardos, G. (1978) Mol. Cell Biochem. 22, 147–152
20 Enyedi, A., Sarkadi, B., Szasz, I., Bot, G. and Gardos, G. (1980) Cell Calcium 1, 299–310