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Phosphorylation of cytoskeletal proteins tightly associated with the vascular smooth muscle membranes

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SUMMARY A study was performed to determine whether the proteins phosphorylated by cAMP dependent and calmodulin dependent protein kinase in vascular smooth muscle membrane fractions represent integral membrane proteins or are tightly associated cytoskeletal proteins. In the unextracted microsomal fraction cAMP dependent protein kinase phosphorylated proteins of 240K, 105K, and 48K daltons. Similarly, calmodulin dependent protein kinase phosphorylated 65K, 60K, 48K, and 20K dalton bands. The 48K dalton band represented a major protein in the microsomal fraction, and it was a common substrate for both cAMP dependent and calmodulin dependent protein kinase, and the extent of phosphorylation by two kinases was additive. The 48K dalton band showed immunological reaction with monoclonal antibodies raised against human umbilical artery F actin, and it also comigrated with arterial smooth muscle actin on SDS gel electrophoresis.

Plasma membranes prepared from vascular smooth muscle microsomes after extraction with actomyosin extraction buffer consisting of 2 mmol·litre^{-1} TRIS-maleate, pH 6.8, 0.25 mol·litre^{-1} sucrose, 0.5 mmol·litre^{-1} ATP, 0.2 mmol·litre^{-1} $CaCl_2$, 0.1 mmol·litre^{-1} phenylmethylsuphonyl-fluoride, and 1 mmol·litre^{-1} dithiothreitol yielded membranes that were substantially free from cytoskeletal protein contamination. These membranes were enriched 60-80 fold in plasma membrane marker enzymes and showed energy dependent calcium uptake. In the extracted plasma membranes none of the proteins was phosphorylated by cAMP dependent or calmodulin dependent protein kinase. Thus these results show that proteins phosphorylated in the unextracted microsomal fraction or unextracted plasma membranes are not integral plasma membrane proteins but represent tightly associated cytoskeletal proteins.

Membrane phosphorylation has been implicated in the regulation of calcium transport in smooth muscle. It has been shown that calcium uptake is increased by cAMP dependent,[1–4] calmodulin dependent[5–7] protein kinase mediated phosphorylation of microsomes isolated from smooth muscle. The limitations of these studies are that it is not known whether the proteins phosphorylated are integral components of membranes or if they represent the proteins of thin filaments attached to the membranes.

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It has been shown that actin[8 9] and actin binding protein filamin[10 11] are the major constituents of smooth muscle cytoskeleton, and the thin filaments remain attached to the cell membranes.[12 13] Plasma membranes prepared from smooth muscle[14] and platelets[15–18] by sucrose density gradient centrifugation show large amounts of tightly associated actin that cannot be removed by extraction with potassium iodide.[18] Actin[19 20] and actin binding protein filamin[11 12 21] have been shown to be phosphorylated by cAMP dependent protein kinase. It is therefore likely that in the studies in which crude membranes have been used to investigate membrane phosphorylation the proteins phosphorylated may represent contractile proteins tightly associated with the membranes.

The purpose of this study was to investigate the

cAMP dependent and calmodulin dependent phosphorylation of vascular smooth muscle membranes and to characterise the phosphorylated proteins. Our results show that the proteins phosphorylated by cAMP dependent and calmodulin dependent protein kinase are not integral parts of vascular smooth muscle plasma membranes but rather represent cytoskeletal proteins tightly associated with the plasma membranes.

Materials and methods

Bovine brains and carotid arteries used in this study were obtained from FDL Foods, Dubuque, Iowa, USA (γ-^{32}P)-ATP (>10 Ci·mmol^{-1}) was supplied by Amersham Corporation, Arlington Heights, Illinois. [^{45}Ca] was purchased from New England Nuclear (Boston, Massachusetts). EGTA, ATP, and protein standards of known molecular weights, used in gel electrophoresis, were purchased from Sigma Chemical Company. Chemicals for gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemicals used in this study were of analytical grade.

PREPARATION OF MICROSOMAL VESICLES

Microsomal vesicles were prepared from the smooth muscle of bovine carotid arteries as described below. Carotid arteries were removed within 30 min after death, cleaned to remove connective tissue, frozen immediately on dry ice and stored at −70°C until used. Approximately 100 g frozen carotid arteries were thawed in 1000 ml of ice cold homogenising buffer containing 20 mmol·litre^{-1} TRIS-maleate, pH 6.8, 0.25 mol·litre^{-1} sucrose, 1 mmol·litre^{-1} dithiothreitol (DTT), 0.1 mmol·litre^{-1} phenylmethylsulphonyl-fluoride (PMSF), 10 mg·litre^{-1} trypsin inhibitor, 0.2 mmol·litre^{-1} benzamidine, and 10 mg·litre^{-1} (TPCK) *N*-tosyl-l-phenylalanine chromethyl ketone. All subsequent steps were carried out at 0-4°C. Arteries were slit open and the adventitia stripped to isolate the media, which was then minced very finely with scissors. The minced tissue was homogenised in a one gallon, stainless steel, Waring blender at maximum speed with two bursts of 30 s each with a 1 min interval between. The homogenate obtained was divided into four equal parts in 500 ml beakers and further homogenised using a Virtis 45 Omni mixer equipped with macro ultra shear. The homogenisation was carried out twice for 15 s with a 1 min interval at a setting of 70. The resulting homogenate was centrifuged twice at 10 000 x *g* for 20 min, and each time the pellet was discarded. The supernatant was filtered through four layers of cheesecloth and centrifuged at 84 000 x *g* for 40 min. The pellet from this step was designated as unextracted microsomes.

EXTRACTION OF MICROSOMES

Microsomes were extracted four times with actomyosin extraction buffer consisting of 2 mmol·litre^{-1} TRIS-maleate, pH 6.8, containing 0.2 mmol·litre^{-1} $CaCl_2$, 0.5 mmol·litre^{-1} ATP, 1 mmol·litre^{-1} DTT, 0.1 mmol·litre^{-1} PMSF, and 0.25 mol·litre^{-1} sucrose at 0°C for 30 min. In some preparations a fifth extraction was carried out overnight. These microsomes were designated as extracted microsomes.

ISOLATION OF PLASMA MEMBRANES FROM MICROSOMES

In the first procedure plasma membranes were isolated from the unextracted microsomes using a discontinuous sucrose density gradient consisting of 10%, 28%, 33%, 37%, and 45% (w/w) sucrose. All sucrose solutions contained 20 mmol·litre^{-1} TRIS-maleate, pH 6.8, 1 mmol·litre^{-1} DTT, 0.1 mmol·litre^{-1} PMSF. The crude microsomes were suspended in 20 ml 10% sucrose (2.5 mg·ml^{-1}) and 10 ml of the suspension was layered on top of each gradient tube which was centrifuged for 90 min at 27 000 rev/min using a Beckman SW 27 rotor. Membranes collecting at the 10-28% sucrose interface were collected and designated as unextracted plasma membranes.

In the second procedure the plasma membranes were prepared from the microsomes extracted with actomyosin extraction buffer using discontinuous sucrose density gradient consisting of 10%, 28%, 33%, 37%, and 45% sucrose (w/w).[22] Briefly, the extracted microsomes were suspended in 14 ml 37% sucrose (3 mg·ml^{-1}) and 7 ml of this suspension was layered on top of 5 ml 45% sucrose in each gradient tube. All these sucrose solutions contained (in mmol·litre^{-1}) 20 TRIS maleate, pH 6.8, 0.6 KCl, 1 DTT, and 0.1 PMSF. The gradients were centrifuged overnight at 27 000 rev/min using a Beckman SW 27 rotor. The membranes collecting at the 10-28% sucrose interface were collected and washed two more times with a buffer containing 20 mmol·litre^{-1} TRIS maleate, pH 6.8, 1 mmol·litre^{-1} DTT, 0.25 mol·litre^{-1} sucrose. These membranes were designated extracted plasma membranes.

MEMBRANE PHOSPHORYLATION

Unless stated otherwise, aliquots of various membrane preparations (25-50 μg protein) were phosphorylated in a 100 μl medium containing (in mmol·litre^{-1}) 40 histidine-HCl buffer, pH 6.8, 10 $MgCl_2$, 10 NaF, and 0.1 (γ^{32}P)-ATP (200-300 cpm·pmol^{-1}) at 30°C. Indicated concentrations of calmodulin, EGTA, EGTA-calcium buffers, trifluoperazine, C subunit of cAMP dependent protein kinase, and protein kinase, inhibitor were included in the assay mixture.

Reactions were terminated after a predetermined time by the addition of 2.5 ml ice cold solution containing 7% perchloric acid and 7% phosphoric acid. About 400 μg bovine serum albumin was added to each tube, vortexed, and centrifuged at 15 000 x *g* for 10 min at 4°C. The pellet was washed two more times with perchloric acid/phosphoric acid mixture. After the final wash the supernatant was completely removed and the pellet was dissolved in 1 ml 0.2 N NaOH, transferred quantitatively into vials containing 10 ml Beckman HP scintillation fluid, and counted for radioactivity using a Beckman LS-300 liquid scintillation spectrometer.

When membranes were phosphorylated for SDS polyacrylamide gel electrophoresis the reaction was stopped by the addition of 25 μl·100 μl^{-1} reaction of solubilising solution containing 0.1 mmol·litre^{-1} TRIS-HCl, pH 6.8, 5 mmol·litre^{-1} EDTA, 5% SDS, 25% 2-mercaptoethanol, 50% (w/v) sucrose, and 0.01% bromophenol blue and heated at 100°C for 5 min.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis was carried out using a modified Laemmli discontinuous buffer system[23] on gradient slab gels made up of 5-20% polyacrylamide (acrylamide: bis, 36.5:1) or slab gels consisting of 10% and 4.5% acrylamide in separating and stacking gels respectively. The amounts of protein applied on the gels are given in the figure legends. Low molecular weight and high molecular weight markers (Sigma) were applied together with solubilised membranes. Gels were run at a constant voltage of 170 V for 3.5-4 hours and stained with Coomassie brilliant blue R-250 and destained by diffusion.

AUTORADIOGRAPHY

Slab gels, after destaining, were put in 55% aqueous (v/v) methanol, left overnight, and then sealed in leak proof plastic bags. The gels were then placed in contact with Kodak X-omat-AR *x* ray film using DuPont High Plus intensifying screen. The film was then developed according to the manufacturer's recommendations.

IMMUNOLOGICAL CHARACTERISATION FOR ACTIN CONTAMINATION OF MEMBRANE FRACTIONS

Immediately after SDS gel electrophoresis, proteins were electrophoretically transferred (200 mA, 4½ h) from the SDS gel to nitrocellulose filter paper. The nitrocellulose sheets (0.45 μm obtained from Schleicher and Schwell) were then washed with TRIS buffered saline, pH 7.4, at room temperature for 10 min followed by two washes in 0.3% BSA in TRIS buffered saline, pH 7.4. Incubation with the monoclonal antibodies (obtained from Dr James Lessard, University of Cincinnati, Ohio, USA) raised against smooth muscle F actin, isolated from human umbilical artery, was performed in this buffer for 2 h at room temperature. These antibodies specifically interact with smooth muscle and other muscle type actins but not with non-muscle actins. After washing, the antigen antibody complexes were incubated with goat antimouse IgG and were further incubated with rabbit antigoat IgG coupled to peroxidase (Cappel) and detected with 4-chloro-1-napthol (3 mg·ml^{-1}) in 50 mmol·litre^{-1} TRIS HCl, pH 7.4, containing 200 mmol·litre^{-1} NaCl and 0.02% H_2O_2 for about 5-10 min with gentle shaking. The paper was washed three times with water, dried in the air, and stored in the dark.

Protein kinase inhibitor from rabbit skeletal muscle was prepared essentially by the procedure of Ashby and Walsh[24] through the DEAE cellulose chromatography step. Calmodulin from bovine brain was prepared by the procedure of Sharma and Wang[25]

TABLE 1 *Specificity of calmodulin and cAMP dependent protein kinase mediated phosphorylation of unextracted microsomes. Values are mean(SEM) of three separate experiments*

Additions in the assay	*^{32}P-phosphate (pmol) incorporated·mg^{-1}·min^{-1}*
No addition	226(19)
Protein kinase inhibitor (PKI)	197(15)
EGTA	162(10)
Trifluoperazine (TFP)	182(15)
Calcium	253(23)
Calcium+calmodulin (CM)	316(25)
Calcium+CM+EGTA	158(23)
Calcium+CM+TFP	174(8)
EGTA+C-subunit of cAMP protein kinase (PK)	476(73)
EGTA+C-subunit of cAMP PK+PKI	185(25)
Calcium+CM+C-subunit of cAMP PK	649–(80)
Calcium+CM+C-subunit of cAMP PK+EGTA+PKI+TFP	155(11)

Phosphorylation was carried out for 1 min at 30°C using 25 μg unextracted microsomes. Additions to the assay were: EGTA, 5 mmol·litre^{-1}; calcium 2 μmol·litre^{-1}; cAMP dependent protein kinase inhibitor 2 μg; trifluoperazine 100 μmol·litre^{-1}; calmodulin (CM) 3 μmol·litre^{-1}; catalytic subunit of cAMP dependent protein kinase (C-subunit of PK) 30 units.

and dialysed against 0.1 mmol·litre^{-1} EGTA for three days to remove bound calcium and finally for 24 h against water to remove EGTA. Calmodulin preparation was assayed with calmodulin dependent phosphodiesterase prepared from bovine heart.[25] Protein concentration was determined by the procedure of Bradford[26] with bovine serum albumin as the standard. Reagents for the Bradford assay were purchased from BioRad Laboratories.

Results

PHOSPHORYLATION OF UNEXTRACTED MICROSOMES

The addition of calcium and calmodulin increased

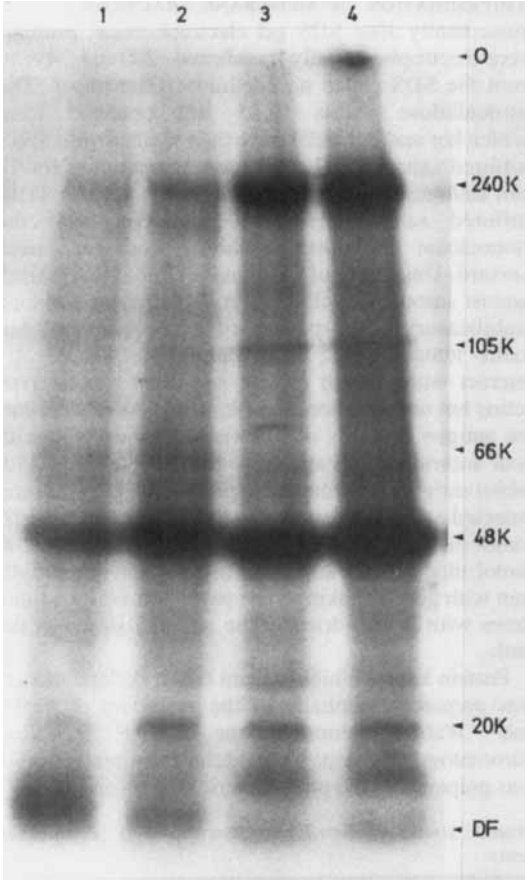

FIG 1 *Autoradiograph of a 0.1% SDS 5-20% gradient polyacrylamide gel electrophoretogram of 35 μg unextracted microsomes phosphorylated under different conditions. Lane 1 = no addition; lane 2 = 1.5 μmol·litre^{-1} calmodulin + 2 μmol·litre^{-1} calcium; lane 3 = 50 units of C-subunit of cAMP dependent protein kinase; lane 4 = 1.5 μmol·litre^{-1} calmodulin + 2 μmol·litre^{-1} + 50 units of C-subunit of cAMP dependent protein kinase. Other assay conditions as described under methods. After completion of phosphorylation the reaction was terminated with 25 μl 5x SDS sample buffer and boiled for 5 min. O = origin; DF = dye front. The molecular weight of phosphorylated proteins was calculated according to the migration of the following markers: myosin, beta galactocidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen, soyabean trypsin inhibitor, and α-lactalbumin.*

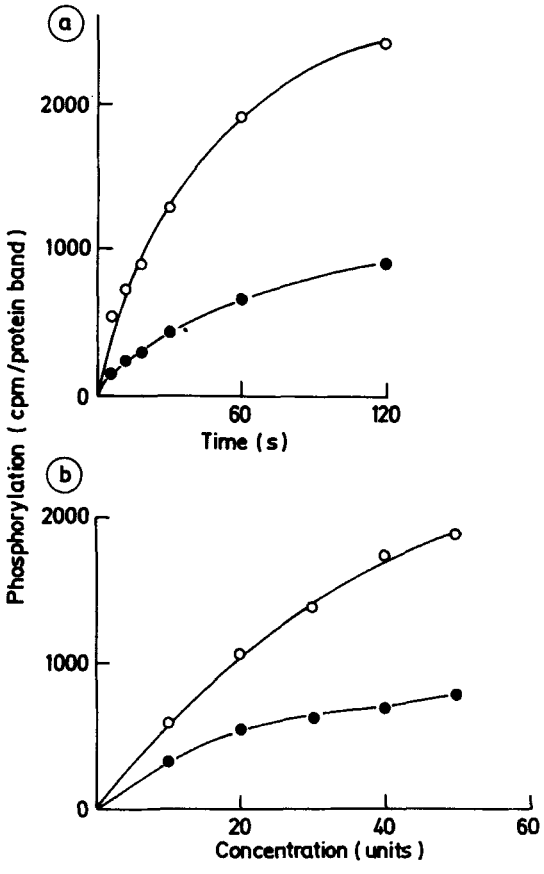

FIG 2 *Phosphorylation of 240K (●) and 48K (○) dalton proteins by C-subunit of cAMP dependent protein kinase as a function of (a) incubation time in the presence of 50 units of C-subunit of cAMP dependent protein kinase and (b) concentration of C-subunit of cAMP dependent protein kinase. Quantification of ^{32}P-phosphate incorporation into the 240K and 48K dalton bands was carried out after localisation of the bands by autoradiography. The radioactive protein bands of 240K and 48K daltons were cut out and solubilised in 30% H_2O_2 by heating overnight at 60°C. Samples were counted in 10 ml Beckman HP scintillation fluid. Phosphorylation carried out in the presence of cAMP dependent protein kinase inhibitor was subtracted from these values. ^{32}P-phosphate incorporation into 240K and 48K dalton bands is given as cpm per protein band.*

^{32}P-phosphate incorporation into unextracted microsomes. Trifluoperazine or EGTA specifically inhibited calcium-calmodulin stimulated phosphorylation (table 1). Similarly, the addition of cAMP dependent protein kinase increased the phosphate incorporation into microsomes which was specifically inhibited by purified cAMP dependent protein kinase inhibitor (table 1). The phosphorylation of microsomes by cAMP dependent and calmodulin dependent protein kinases was additive (table 1).

Phosphorylation of unextracted microsomes in the presence of cAMP dependent protein kinase resulted in phosphate incorporation into two major bands of 240K and 48K daltons. Several other minor protein bands of 105K, 20K, and 8K daltons were also phosphorylated (fig 1). The 48K dalton protein band was also the major protein band phosphorylated by calcium and calmodulin (fig 1). In addition, in the presence of calcium and calmodulin several minor protein bands of 65K, 60K, and 20K daltons were also phosphorylated.

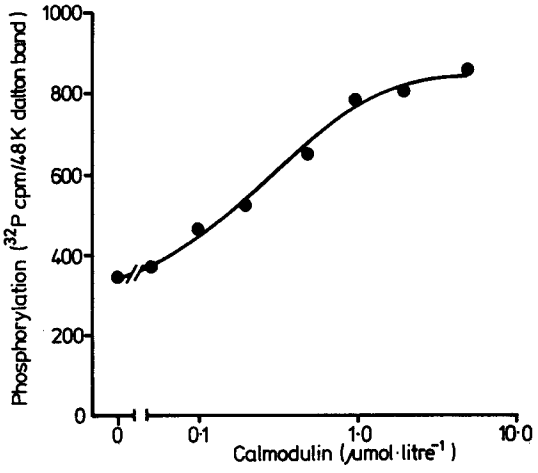

FIG 3 *Calmodulin dose dependence of phosphorylation of 48K dalton protein. Phosphorylation of 35 μg unextracted microsomes in the presence of 2 μmol·litre^{-1} calcium and varying concentrations of calmodulin was carried out for 1 min (see methods). Quantification of ^{32}P-incorporation into 48K dalton band was carried out as for fig 4.*

PHOSPHORYLATION OF 240K AND 48K DALTON BANDS

Cyclic AMP dependent protein kinase mediated phosphorylation of 240K and 48K dalton bands present in the unextracted microsomes showed that the reaction was time dependent and reached maximum in approximately 2 min (fig 2a). The phosphorylation of these two proteins was dependent on the concentration of C subunit of cAMP dependent protein kinase in the assay (fig. 2b). The ^{32}P-phosphate incorporated into the 48K dalton band was greater than that incorporated into the 240K dalton band (fig 2), which could be due to different quantities of these proteins present in the microsomes. The phosphorylation of 48K dalton band by cAMP dependent and calmodulin dependent protein kinases was additive (table 2). The cAMP dependent protein kinase mediated phosphorylation was completely inhibited by the addition of 2-3 μg purified cAMP dependent protein kinase inhibitor isolated from rabbit skeletal muscle (data not given).

Calmodulin increased phosphorylation of 48K dalton protein in the unextracted microsomes in a dose dependent manner, and the half maximal phosphorylation was achieved at 0.35 μmol·litre^{-1} calmodulin and maximum phosphorylation at approximately 2 μmol·litre^{-1} calmodulin (fig 3).

CHARACTERISATION OF 240K AND 48K DALTON BANDS

The contamination of unextracted and extracted microsomes and unextracted and extracted plasma membranes with cytoskeletal proteins was examined by SDS polyacrylamide gel electrophoresis. The unextracted plasma membranes were substantially free from contamination with 240K and 200K dalton bands but that for 48K daltons was still the major band (fig 4, lane C). These results show that fractionation of unextracted microsomes on sucrose gradients is not sufficient to remove tightly associated cytoskeletal proteins from the plasma membranes. Extraction of microsomes with actomyosin extraction buffer for 30

TABLE 2 *Phosphorylation of 48K dalton band by calmodulin and cAMP dependent protein kinase. Values are mean(SEM) of eight gels*

Addition	*^{32}P-phosphate incorporation (cpm/48K dalton band)*
No addition	917(38)
Calcium+calmodulin (CM)	1624(103)
EGTA+C-subunit of cAMP protein kinase (PK)	2520(125)
Calcium+Cm+C-subunit of cAMP PK	3789(312)

50 μg unextracted microsomal vesicles were phosphorylated in the presence of calmodulin, C-subunit of cAMP dependent protein kinase for 1 min at 30°C. After SDS gel electrophoresis the 48K dalton band was cut, solubilised in 30% H_2O_2, and counted. Additions in the assay were: calcium 2 μmol·litre^{-1}; calmodulin (CM) 3 μmol·litre^{-1}; C-subunit of cAMP dependent protein kinase 50 units; EGTA 5 mmol·litre^{-1}.

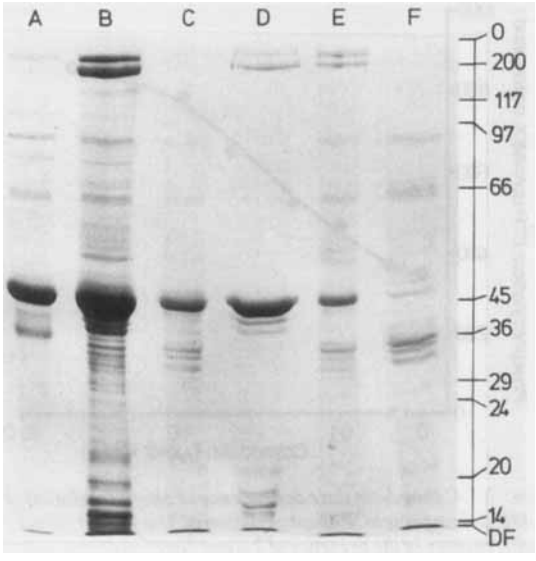

FIG 4 *Coomassie blue staining of a 0.1% SDS 10% polyacrylamide gel electrophoretogram of various membrane fractions. Membranes, 25 μg in 0.1 ml SDS buffer (except for unextracted microsomes, which were 50 μg in 0.1 ml SDS buffer), were boiled for 5 min and chromatographed as described. Lane A = crude actin isolated from bovine carotid artery acetone powder; lane B = unextracted microsomes; lane C = unextracted plasma membranes; lane D = proteins extracted fom microsomes by actomyosin extraction buffer; lane E = extracted microsomes; lane F = extracted plasma membranes. The molecular weight markers were the same as for fig 2.*

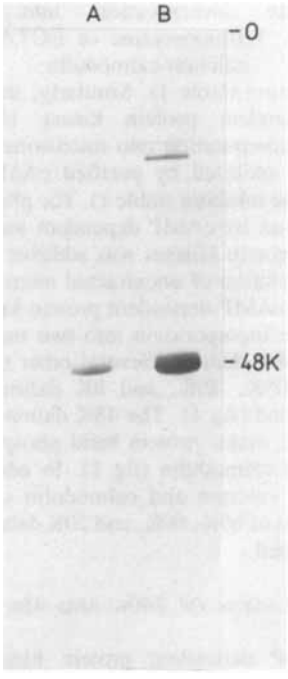

FIG 5 *Immunological localisation of actin in unextracted microsomes and extracted plasma membranes using smooth muscle actin specific monoclonal antibodies. 10 μg unextracted microsomes (lane B) and 10 μg extracted plasma membranes (lane A) were electrophoresed on 0.1% SDS 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose paper and reacted with specific antibodies (see methods).*

min at 0°C, however, extracted almost 50% of the proteins present. The extracted microsomes were substantially less contaminated with 240K, 200K, and 48K dalton proteins (fig 4, lane E) than the unextracted microsomes (fig 4, lane B). Repeated extraction of crude microsomes with actomyosin extraction buffer up to five times including one overnight extraction did not result in complete removal of actin (fig 4, lane E) suggesting that actin is tightly associated with membranes. The major bands present in the unextracted microsomes (fig 4, lane B), extracted microsomes (fig 4, lane E), and the proteins extracted from microsomes by actomyosin extraction buffer (fig 4, lane D) were of 240K, 200K, and 48K daltons. That the 48K dalton band present in the microsomes represents actin was confirmed by the immunoprecipitation technique using specific antibodies raised against smooth muscle actin (fig 5). The unextracted microsomes (lane B) showed a significant amount of contamination with actin, whereas the plasma membranes (lane A) prepared from the extracted microsomes were substantially free of actin contamination. The plasma membranes prepared from the extracted microsomes were, however, almost completely free of 240K, 200K, and 48K dalton proteins (fig 4, lane F). These results show that actin remains tightly associated with smooth muscle plasma membranes; however, with the procedure described here a plasma membrane fraction can be prepared that is substantially free from actin contamination.

PHOSPHORYLATION OF PURIFIED PLASMA MEMBRANES

The extracted plasma membranes were phosphorylated in the presence of C subunit of cAMP dependent protein kinase and in the presence of calmodulin. In the presence of C subunit of cAMP dependent protein kinase a very minor band in the 48K dalton region was phosphorylated (fig 6, lane B). This phosphorylation appears to be due to the phosphorylation of actin tightly associated with the extracted plasma membranes (fig 4, lane F, and fig 5

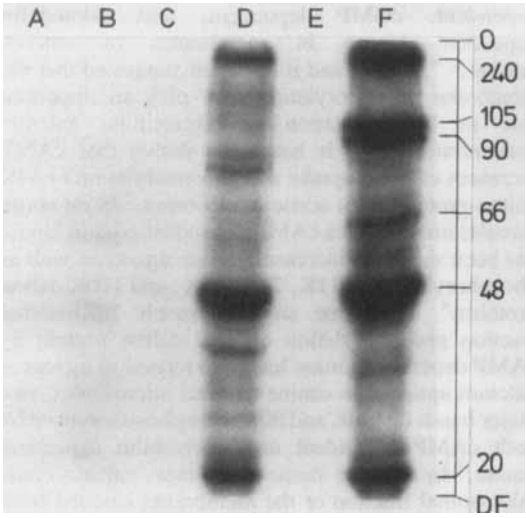

FIG 6 *Autoradiograph of a 0.1% SDS 10% polyacrylamide gel electrophoretogram of extracted plasma membranes (lanes A and B); proteins extracted from microsomes with actomyosin extraction buffer (lanes C and D); crude actin extracted from bovine carotid artery acetone powder (lanes E and F). 25 μg protein from each fraction was phosphorylated in the absence (lanes A, C, and E) and presence (lanes B, D, and F) of 50 units of C-subunit of cAMP dependent protein kinase for 1 min at 30°C (see methods). Molecular weight markers are as for fig 2.*

lane A). On the other hand, in the presence of calmodulin there was no phosphorylation of extracted plasma membranes (data not shown). This lack of phosphorylation could be due either to lack of cytoskeletal proteins that are removed by extraction or to removal of calmodulin dependent protein kinase during extraction. The 48K dalton band together with several other proteins was also phosphorylated by cAMP dependent protein kinase in crude actin isolated from bovine carotid artery acetone powder (fig 6, lane F). Similarly, proteins extracted from microsomes by actomyosin extraction buffer were also phosphorylated by cAMP dependent protein kinase (fig 6, lane D). Our results would indicate that the phosphorylation of smooth muscle membranes by cAMP dependent and calmodulin dependent kinases is due to the phosphorylation of contractile proteins associated with the membranes and not to an integral protein of plasma membranes.

Plasma membrane vesicles prepared by the procedures developed in our laboratory were enriched 60-80 fold in (Na^+-K^+)-ATPase, 5'-nucleotidase, and phosphodiesterase I activities. The plasma membrane vesicles accumulated calcium in the presence of ATP, which was increased by calmodulin and phosphate.[22]

Discussion

These data show that the unextracted microsomal fraction can be phosphorylated by cAMP dependent and calmodulin dependent protein kinase. The proteins phosphorylated are not, however, of membrane origin but represent actomyosin and actin binding proteins tightly associated with the membranes that can be extracted using actomyosin extraction buffer. We have shown that, out of many proteins present in the unextracted microsomal fraction, the 48K and 240K dalton proteins are the major proteins that are phosphorylated by cAMP dependent protein kinase. Several lines of evidence presented below suggest that the 48K dalton band present in microsomes represents tightly associated actin: (*a*) it comigrates with actin isolated from vascular smooth muscle acetone powder on SDS polyacrylamide gel (fig 4), (*b*) it can be extracted from microsomes using actomyosin extraction buffer at 0°C (fig 4), and (*c*) it immunoreacts with smooth muscle specific actin antibodies (fig 5). The 240K dalton band appears to be actin binding protein filamin because, firstly, it extracts along with actin by extraction with actin extraction buffer (fig 4), and, secondly, its molecular weight is similar to that reported for membrane associated filamin of vas deferens.[12]

The present data also show that the membrane associated 48K dalton band characterised as actin is a substrate for both cAMP dependent and calmodulin dependent protein kinase. The phosphorylation of actin by cAMP dependent and calmodulin dependent kinase was additive (table 2). It would appear that the two kinases phosphorylate actin at two different sites for the following reasons: (*a*) only one protein band was obtained under different conditions of SDS polyacrylamide gel electrophoresis and (*b*) when the phosphorylation by one kinase was complete the addition of a second kinase always increased phosphorylation. In addition to the membrane associated actin, actin extracted from the acetone powder of vascular smooth muscle, and that extracted from crude microsomes by actomyosin extraction buffer, were also phosphorylated by cAMP dependent protein kinase. The significance of actin phosphorylation in smooth muscle function is not known. The phosphorylation of actin by cAMP dependent protein kinase has also been shown in vivo using S-49 lymphoma cells.[19 20].

The 240K dalton band present in the microsomes appears to be a specific substrate for cAMP dependent protein kinase mediated phosphorylation. Similarly, the phosphorylation by cAMP dependent protein kinase of a 240K dalton band characterised as filamin has been shown in guinea pig vas deferens smooth muscle,[12] chicken gizzard,[21] fibroblasts,[11] and

macrophages and platelets.[27] Similar to our observations, in these studies filamin was not phosphorylated by intrinsic calmodulin dependent kinase.

In addition to 240K and 48K dalton proteins several other proteins were phosphorylated in the unextracted microsomal fraction by cAMP dependent and calmodulin dependent protein kinase. Calmodulin dependent protein kinase increased the phosphorylation of 65K, 60K, and 20K dalton proteins, whereas cAMP dependent protein kinase increased the phosphorylation of 130K dalton proteins. Myosin light chain kinase isolated from vascular smooth muscle has been shown to be a 130K dalton protein that is phosphorylated by cAMP dependent protein kinase.[28] The 20K dalton band most likely represents myosin light chains that are phosphorylated by calmodulin dependent myosin light chain kinase.[29] The identification of other proteins needs further experimentation. None of the proteins phosphorylated by cAMP dependent and calmodulin dependent protein kinase in the unextracted microsomal fraction could be phosphorylated in the plasma membranes prepared from the microsomes extracted with actomysin extraction buffer followed by density gradient centrifugation in the presence of 0.6 mol·litre–1 KCl, suggesting that the proteins phosphorylated in the microsomes are not integral membrane proteins and may represent contractile proteins tightly associated with the membranes. Alternatively, it is also possible that during the extraction procedure calmodulin dependent protein kinase is removed, resulting in the lack of phosphorylation of plasma membrane proteins in the presence of calmodulin. Nevertheless, this seems unlikely for two reasons: (*a*) the protein bands that are phosphorylated by both cAMP dependent and calmodulin dependent protein kinase were extracted by actomyosin extraction buffer and (*b*) the addition of cAMP dependent protein kinase did not phosphorylate plasma membrane proteins. Although the exact role of cytoskeletal protein phosphorylation in the smooth muscle function is not known, it has been shown that in cultured smooth muscle cells the addition of isoproterenol leads to reversible disappearance of microfilament bundles and cells start to become rounded.[30] It is likely therefore that phosphorylation of cytoskeletal proteins of microfilament bundles may lead to their depolymerisation resulting in the altered cell morphology and function. The reversible phosphorylation of actin and actin binding proteins, which remain associated with plasma membranes, may have an important role in the control of smooth muscle function.

Several investigators have studied the endogenous substrate proteins for phosphorylation by cAMP dependent, cGMP dependent, and calmodulin dependent kinase in membranes of smooth muscle,[1–4, 7 30–33] and it has been suggested that the membrane phosphorylation may play an important role in the regulation of intracellular calcium concentration.[1–4 7] It has been shown that cAMP increases calcium uptake and phosphorylation of 44K dalton protein in rat aortic microsomes.[2] In rat aortic sarcolemmal vesicles cAMP dependent protein kinase has been shown to increase calcium uptake as well as phosphorylation of 11K, 21K, 44K, and 110K dalton proteins.[4] In uterine smooth muscle microsomal fraction phosphorylation of 48K dalton protein by cAMP dependent kinase has been related to increased calcium uptake.[1] In canine tracheal microsomes, two major bands of 130K and 80K were phosphorylated by both cAMP dependent and calmodulin dependent kinase.[5] In all these studies, however, either a crude microsomal fraction or the membranes isolated from crude microsomes by density gradient centrifugation were used. The present studies have conclusively shown that extraction of microsomes with buffer containing 0.6 mol·litre^{-1} KCl followed by density gradient centrifugation cannot eliminate contractile protein contamination of plasma membranes. Similarly, it has been shown that platelet membranes isolated by density gradient contain significant amounts of actin[15–18] that is not removed by extraction with 0.6 mol·litre^{-1} potassium iodide (KI),[18] suggesting that actin remains tightly associated with the membranes.

We have presented evidence that actin and actin binding proteins, which may represent cell cytoskeleton, remain tightly associated with the membranes and are not removed by extraction with high ionic strength buffers or by sucrose density gradient centrifugation. Therefore, our observations will raise questions regarding the origin of different proteins using unextracted membrane fractions which have been shown to be phosphorylated by various kinases and claimed to have a role in calcium transport.

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