Phosphorylation of calmodulin by plasma-membrane-associated protein kinase(s)

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Plasma-membrane-associated protein kinase(s) from normal rat liver phosphorylates exogenous bovine brain calmodulin in the absence of Ca^{2+} and in the presence of histone or poly(L-lysine). Maximum levels of calmodulin phosphorylation are obtained at a poly(L-lysine)/calmodulin molar ratio of 0.4. Phosphoamino acid analysis revealed that calmodulin is phosphorylated on serine, threonine and tyrosine residues. Endogenous plasma-membrane-associated calmodulin was also phosphorylated by plasma-membrane-associated protein kinase(s) in the absence of added cationic protein or polypeptide. The identity of endogenous phosphocalmodulin was confirmed by immunoprecipitation with a specific anti-calmodulin monoclonal antibody. Ehrlich ascites tumor cell plasma membranes do not contain endogenous calmodulin. However, membrane-associated protein kinase(s) from these tumor cells phosphorylates bovine brain calmodulin in the presence of poly(L-lysine). These data demonstrate that phosphocalmodulin is present in liver plasma membranes and suggest that this post-translational modification could have a physiological role in this location.

Keywords: calmodulin; phosphocalmodulin; protein kinase; plasma membrane; phosphorylation.

A large number of Ca^{2+}-regulated cell functions are mediated by the intracellular Ca^{2+}-receptor protein calmodulin (Means and Dedman, 1980; Manalan and Klee, 1984; Persechini et al., 1989; Bachs et al., 1992). An increase in intracellular free Ca^{2+} concentration results in the reversible formation of a Ca^{2+}/calmodulin complex, that in turn binds to different target proteins. In addition, phosphorylation of calmodulin could be important in the modulation of calmodulin action. For example, it has been demonstrated that calmodulin phosphorylated by casein kinase II has less capacity to activate several calmodulin-target enzymes (Sacks et al., 1992a; Quadroni et al., 1994). Moreover, calmodulin phosphorylated by the insulin-receptor tyrosine kinase exhibits altered interactions with both calmodulin-dependent enzymes and calmodulin antagonists (Williams et al., 1994; Saville and Houslay, 1994).

Calmodulin phosphorylation occurs *in vivo* in different cells and tissues (Plancke and Lazarides, 1983; Fukami et al., 1985; Nakajo et al., 1986; Colca et al., 1987; Sacks et al., 1992b; Joyal and Sacks, 1994; Quadroni et al., 1994). A number of serine/threonine-protein and tyrosine-protein kinases catalyze calmodulin phosphorylation *in vitro*. These include: phosphorylase kinase (Plancke and Lazarides, 1983), the insulin-receptor tyrosine kinase (Häring et al., 1985; Graves et al., 1986; Sacks and McDonald, 1988; Laurino et al., 1988; Sacks et al., 1989a; Benguría et al., 1993; Saville and Houslay, 1994), the epidermal-growth-factor(EGF)-receptor tyrosine kinase (San José et al., 1992; Benguría and Villalobo, 1993; Benguría et al., 1993, 1994), the Src tyrosine kinase from Rous-sarcoma-virus-transformed cells (Fukami et al., 1985), spleen tyrosine protein kinases IIB and III (Meggio et al., 1987), a serine kinase present in plasma membrane fractions obtained from human epidermoid A431 cancer cells (Lin et al., 1986), casein kinase II (Nakajo et al., 1986, 1988; Meggio et al., 1987; Sacks et al., 1992a; Quadroni et al., 1994) and soluble protein kinase(s) from adrenal cortex (Kubo and Strott, 1988).

It has been reported that liver plasma membranes contain two pools of calmodulin, one easily extracted by an EGTA wash, while the other remains tightly bound to the membrane after removal of Ca^{2+} and appears to be associated with cytoskeletal proteins (Gazzotti et al., 1985; Gloor and Gazzotti, 1986). We have previously reported that a protein kinase(s) associated with rat liver plasma membranes phosphorylates an endogenous 16.5-kDa protein, thought to be calmodulin (Ghosh et al., 1988). In this study we show that calmodulin is tightly associated to the plasma membranes and is unequivocally phosphorylated by rat liver plasma-membrane-associated protein kinase(s). We also characterize the phosphorylation of exogenous bovine brain calmodulin by the plasma-membrane-associated protein kinase(s).

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]ATP (triethylammonium salt; 3000–5000 Ci/mmol) and Hyperfilm™-MP X-ray films were purchased from Amersham. X-Omat AR X-ray blue-sensitive films were obtained from Kodak. Hepes and cellulose chromatographic plates were purchased from Merck. Histone (type II-AS) from calf thymus, poly(L-lysine) (38 kDa), calmodulin-dependent cAMP phosphodiesterase from bovine brain, 5′-nucleoti-

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Abbreviations. EGF, epidermal growth factor; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

Enzymes. Protein-tyrosine kinase (2.7.1.112); protein kinase (2.7.1.37); casein kinase II (2.7.1.37); 3′,5′-cyclic-nucleotide phosphodiesterase (3.1.4.17); 5′-nucleotidase (3.1.3.5); alkaline phosphatase (3.1.3.1); trypsin (3.4.21.4).

dase from *Crotalus adamanteus*, alkaline-phosphatase-conjugated goat anti-mouse IgG, Triton X-100, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate and phenylmethylsulfonyl fluoride ($PhMeSO_2F$) were obtained from Sigma Chemical Co. Calmodulin was purchased from Calbiochem. Non-stained and stained molecular-mass standards for electrophoresis, poly(vinylidene difluoride) membranes for immunoblots, Affi-Gel and Affi-Gel Hz hydrazide were purchased from Bio-Rad. Immobilon P^{SQ} was from Millipore. All other chemicals were of analytical grade.

Preparation of liver plasma membrane fractions. Crude and further purified liver plasma membrane fractions from young adult male Sprague-Dawley albino rats (200–250 g) were prepared at 4°C in the presence of 1 mM $PhMeSO_2F$ following the method previously described by us (Church et al., 1988; San José et al., 1993). In most preparations the homogenization in a glass/Teflon homogenizer was increased up to 20 strokes and the 15-s homogenization with the Polytron was omitted. For the preparation of plasma membrane fractions depleted of Ca^{2+}-dependent bound calmodulin, 1 mM EGTA was added to all buffers and sucrose gradient solutions (San José et al., 1992). The membranes were finally resuspended in an EGTA-free buffer.

Most experiments were performed with the light membrane fraction obtained from the second sucrose gradient centrifugation, denoted as purified plasma membranes. When larger quantities of membrane proteins were required, the membrane fraction resulting from the first sucrose gradient was used. This preparation is denoted as crude plasma membranes. The average enrichment in 5′-nucleotidase activity compared to the crude homogenate was approximately 12-fold and 40-fold for the crude and purified plasma membrane fractions, respectively (San José et al., 1993).

Preparation of plasma membrane fractions from tumor cells. Purified plasma membranes from the rat hepatoma AS-30D (ascites cell line) were prepared as previously described (Church et al., 1988; San José et al., 1993), and crude plasma membranes from this tumor cell line were prepared by a modified procedure in which the second sucrose gradient centrifugation was omitted.

Crude plasma membranes from mouse Ehrlich ascites tumor cells were prepared in the presence of 1 mM $PhMeSO_2F$ at 4°C as follows. Tumor cells from 15–20 donor male Swiss albino mice were washed three times in 150 mM NaCl, 5 mM KCl and 20 mM Tris/HCl, pH 7.4. The washed cells were resuspended and homogenized four times for 10 s each using a Polytron homogenizer in a hypotonic buffer containing 15 mM KCl and 5 mM Tris/HCl, pH 8. The crude extract was centrifuged for 15 min at 2000 g_{max}. The pellet was mixed with a solution of 60% (by mass) sucrose, prepared in 5 mM Tris/HCl, pH 8, to obtain a final concentration of 45% (by mass) sucrose. The samples were loaded at the bottom of discontinuous gradients with three atop layers of 41%, 39% and 35% (by mass) sucrose, also prepared in 5 mM Tris/HCl, pH 8. Centrifugation was performed at 141000 g_{max} for 1 h. Membranes migrating at the 35–39% (by mass) sucrose interface were collected with a syringe, diluted 10-fold with 25 mM Na-Hepes, pH 7.4, and centrifuged at 210000 g_{max} for 30 min. The pellet was resuspended in the same buffer, divided into aliquots, frozen in liquid nitrogen and stored at −70°C until use.

Phosphorylation assays. Standard phosphorylation assays, unless indicated otherwise, were performed at 37°C for 1 min in a total volume of 100 µl containing 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 1 mM EGTA, 1.2 µM calmodulin (when added), 0.5 µM poly(L-lysine) (when added), 10 µM (2–20 µCi) [γ-^{32}P]ATP, and 20–100 µg of membrane protein. The reactions were initiated by the addition of radiolabeled ATP, and stopped by the addition of ice-cold 10% (mass/vol.) trichloroacetic acid (final concentration). The supernatant was discarded after centrifugation and the pellet was processed for electrophoresis as described below. The data presented are representative of two or more separated experiments performed under identical or similar conditions.

Immunoprecipitation and immunobloting. The development and properties of the highly specific anti-calmodulin monoclonal antibody used in this study have been previously described (Sacks et al., 1991).

Immunoprecipitation of exogenous bovine brain phosphocalmodulin was performed as follows. Calmodulin was phosphorylated *in vitro* as indicated above, and the reaction was stopped with 10% (mass/vol.) trichloroacetic acid at 4°C. Centrifugation at 10000 g_{max} was performed for 5 min at 4°C and the supernatant was discarded. The pellets were washed with 80% (by vol.) acetone and were suspended in 20 mM NH_4HCO_3 (pH 7.5) containing 0.5% (mass/vol.) Triton X-100. Samples were incubated for 3 h at 4°C with the anti-calmodulin monoclonal antibody cross-linked to an Affi-gel Hz hydrazide matrix as described by the manufacturer, or to an Affi-gel matrix as previously described (Sacks et al., 1992b). Samples were separated by electrophoresis as described below and transferred to a poly(vinylidene difluoride) membrane followed by autoradiography.

Immunoprecipitation of endogenous plasma-membrane-associated phosphocalmodulin was performed as follows. Rat liver plasma membranes were phosphorylated in the absence of exogenous calmodulin or poly(L-lysine) as described above. The reactions were stopped by quick freezing with methanol/solid CO_2 in an equal volume of stop buffer (0.1 mM $PhMeSO_2F$, 0.1 mM vanadate, 5 mM EDTA, and 20 mM sodium pyrophosphate in 150 mM NaCl and 15 mM sodium phosphate, pH 7.4), and samples were stored at −80°C until further processing. The samples were diluted to 1 ml with immunoprecipitation buffer [190 mM NaCl, 6 mM EDTA, 1% (mass/vol.) Triton X-100 and 50 mM Tris/HCl, pH 7.4] and 40 µl of a 1:1 dilution of anti-calmodulin monoclonal antibody linked to Affi-gel in Tris-buffered saline (140 mM NaCl, 2.7 mM KCl and 25 mM Tris/HCl, pH 8) was added. After 3 h at 4°C, washes were carried out as previously described (Sacks et al., 1992b). Following the final wash, proteins were solubilized, separated by electrophoresis and transferred to Immobilon P^{SQ} membranes as previously described (Sacks et al., 1991). This was followed by autoradiography.

Exogenous and endogenous phosphocalmodulin and non-phosphorylated calmodulin were probed with the anti-calmodulin monoclonal antibody and the immune complexes identified using alkaline-phosphatase-labeled goat anti-mouse antibody as described previously (Sacks et al., 1991). The data presented are representative of two or more separate experiments performed under identical or similar conditions.

Other analytical procedures. Slab-gel electrophoresis was performed according to Laemmli (1970) at 12 mA overnight in a linear 5% to 20% (mass/vol.) gradient, or alternatively in 15% (mass/vol.) polyacrylamide gels, in the presence of 0.1% (mass/vol.) SDS at pH 8.3. Gels were stained with Coomassie Brilliant Blue R-250, and after drying under vacuum at 70°C on Whatman 3MM Chr filter paper a blue-sensitive X-ray film was exposed at −20°C or at −70°C for 2–7 days. Quantitative analysis was performed by scanning autoradiographs in a photodensitometer. The photodensitometric intensities of the ^{32}P-labeled bands in the autoradiographs were linearly proportional to the amount of ^{32}P in the bands within the exposure time used.

The activity of calmodulin was determined by measuring calmodulin-dependent cAMP phosphodiesterase activity using a coupled assay system with 5′-nucleotidase. The assays were car-

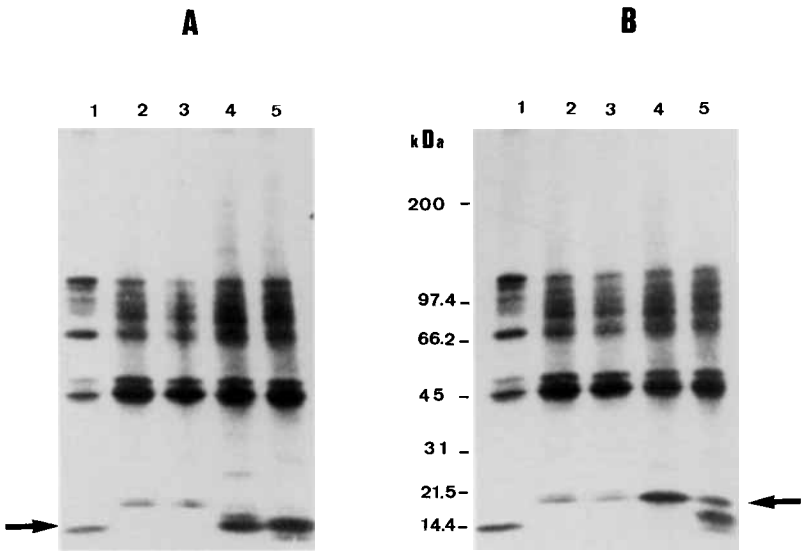

Fig. 1. Phosphorylation of calmodulin by plasma-membrane-associated protein kinase(s). Purified rat liver plasma membranes (42 μg protein) prepared in the absence (lanes 1, A and B), or presence (lanes 2–5, A and B) of EGTA, were incubated for 1.5 min at 37°C in 200 μl of 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 1 mM EGTA, and 20 μg/ml bovine brain calmodulin (lanes 3–5, A and B), 20 μg/ml poly(L-lysine) (lane 4, A and B) and 40 μg/ml histone (lane 5, A and B). Thereafter, 10 μM [γ-^{32}P]ATP was added and the reaction was allowed to proceed for 1 min. The reaction was stopped and the precipitated proteins processed for electrophoresis and autoradiography as described in Materials and Methods. Electrophoreses were run with 10 mM $CaCl_2$ (A), or 10 mM EGTA (B) in the sample buffers. Arrows point to 16.5 kDa (A) and 21 kDa (B).

ried out at 37°C for 30 min in 0.5 ml of a medium containing 50 mM imidazol/HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM cAMP, 100 μM $CaCl_2$, 0.01 U cAMP phosphodiesterase, 0.2 U of 5′-nucleotidase and aliquots of calmodulin extracted from the membranes by EGTA at different temperatures. Bovine brain calmodulin was used as a standard. The phosphate liberated into the medium was measured by the method of Raess and Vincenzi (1980). To quantify the calmodulin extracted from individual samples as described by Pujol et al. (1989), we performed radioimmunoassays using the anti-calmodulin monoclonal antibody following the method of Sacks et al. (1991).

Phosphoamino acid analysis was carried out as described by Hunter and Sefton (1980). The ^{32}P-phosphorylated proteins were cut from the dried gel, rehydrated in 100 mM $(NH_4)HCO_3$, pH 8.3, and digested in two steps with 75 μg tosylphenylalanylchloromethane-treated trypsin for 9 h each at room temperature. The supernatant was lyophilized twice and hydrolyzed with 6 M HCl at 110°C for 2 h. Phosphoamino acids were separated by two-dimensional electrophoresis in thin(0.1 mm)-layer chromatographic cellulose plates as follows. The first dimension was carried out in 2.2% (by vol.) formic acid and 8.7% (by vol.) acetic acid, pH 1.9, and the second dimension was carried out in 0.5% (by vol.) pyridine and 5% (by vol.) acetic acid, pH 3.5. Phosphoserine, phosphothreonine and phosphotyrosine standards were stained with 0.1% (mass/vol.) ninhydrin in ethanol. The plates were dried and autoradiography was performed.

Protein concentrations were determined by the method of Lowry et al. (1951), after precipitating the proteins with 10% (mass/vol.) trichloroacetic acid, using bovine serum albumin as a standard. The concentration of free Ca^{2+} in the assay was determined by a computer program similar to the one described by Goldstein (1979).

RESULTS

A cationic polypeptide is required to phosphorylate bovine brain calmodulin. Incubation of purified plasma membrane fractions with [γ-^{32}P]ATP results in the labeling of a series of polypeptides (Fig. 1). A phosphorylated band of 16.5 kDa was clearly visible (Fig. 1A, lane 1). This band migrates on SDS/PAGE with purified bovine brain calmodulin (Ghosh et al., 1988). When membranes are prepared in EGTA no phosphorylation of the membrane-associated 16.5-kDa polypeptide(s) is observed (Fig. 1A, lane 2). We evaluated the possible phosphorylation of exogenous bovine brain calmodulin by protein kinase(s) present in these EGTA-prepared membranes.

Phosphorylation of calmodulin *in vitro* by the insulin receptor tyrosine kinase (Graves et al., 1986; Laurino et al., 1988; Sacks and McDonald, 1988; Sacks et al., 1989a, 1989b; Fujita-Yamaguchi et al., 1989; Saville and Houslay, 1994), the EGF-receptor tyrosine kinase (San José et al., 1992; Benguría and Villalobo, 1993; Benguría et al., 1993, 1994), and casein kinase II (Meggio et al., 1987; Sacks and McDonald, 1992; Sacks et al., 1992b) requires the presence of a cationic protein or polypeptide. Similarly, phosphorylation of bovine brain calmodulin by plasma-membrane-associated protein kinase(s) requires the presence of poly(L-lysine) (Fig. 1A, lane 4) or histone (Fig. 1A, lane 5). No phosphorylation of calmodulin is detected in the absence of these cationic polypeptides (Fig. 1A, lane 3).

Histone also become phosphorylated in these assays, and there is a partial superimposition of the bands of phosphocalmodulin and phosphohistone (Fig. 1A, lane 5). Therefore, to resolve phosphocalmodulin from phosphohistone we repeated the experiment but altered the electrophoretic mobility of phosphocalmodulin by adding EGTA to the electrophoresis sample buffer (Fig. 1B). EGTA does not alter the electrophoretic mobility of the endogenous membrane-associated 16.5-kDa phosphopolypeptide(s) (compare lanes 1 in Fig. 1A and B). In contrast, bovine brain phosphocalmodulin migrates in these conditions with an apparent molecular mass of 21 kDa (Fig. 1B, lanes 4 and 5). Therefore, phosphocalmodulin can be resolved from phosphohistone when EGTA is present in the electrophoresis sample buffer.

Poly(L)-lysine increases the phosphorylation of calmodulin approximately 12–14-fold (Fig. 2A). However, there appears to be a narrow poly(L-lysine)/calmodulin molar ratio to attain maximum levels of calmodulin phosphorylation. Under our experimental conditions, the optimal poly(L-lysine)/calmodulin molar

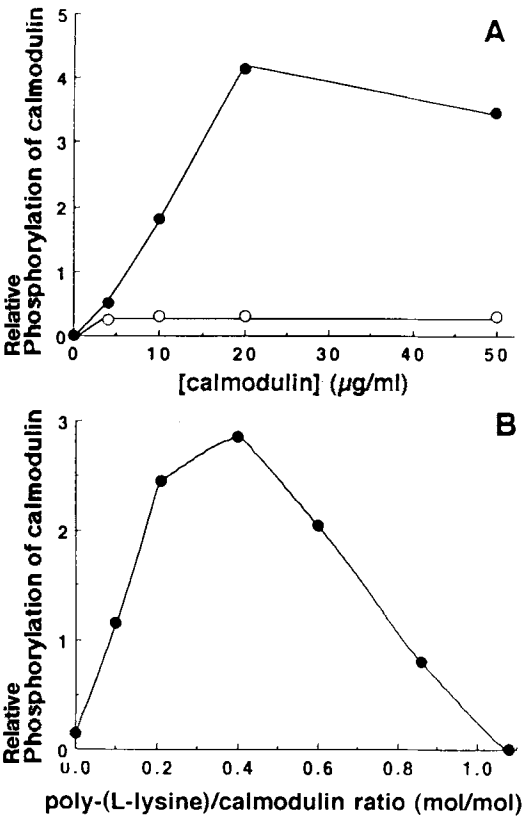

Fig. 2. Effect of poly(L-lysine) on the phosphorylation of exogenous bovine brain calmodulin. (A) Purified rat liver plasma membranes (28 μg protein) prepared in the presence of EGTA were incubated for 40 s at 37 °C in 100 μl of 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 1 mM EGTA, and the indicated concentrations of bovine brain calmodulin. Thereafter, poly(L-lysine) (closed symbols) was added to maintain a constant poly(L-lysine)/calmodulin (mol/mol) ratio of 0.4. 50 s later 10 μM [γ-^{32}P]ATP was added and the reaction was allowed to proceed for 1 min. Control experiments were performed in the absence of poly(L-lysine) (open symbols). (B) Purified rat liver plasma membranes (28 μg protein) prepared in the presence of EGTA were treated as described in (A), except that the poly(L-lysine)/calmodulin (mol/mol) ratio was varied as indicated. The reaction was stopped and the precipitated proteins processed for electrophoresis and autoradiography as described in Materials and Methods. Electrophoresis was run in the presence of 10 mM EGTA in the sample buffer to modify the electrophoretic mobility of phosphocalmodulin to 21 kDa (Fig. 1) to quantify phosphocalmodulin in the scanning photodensitometer.

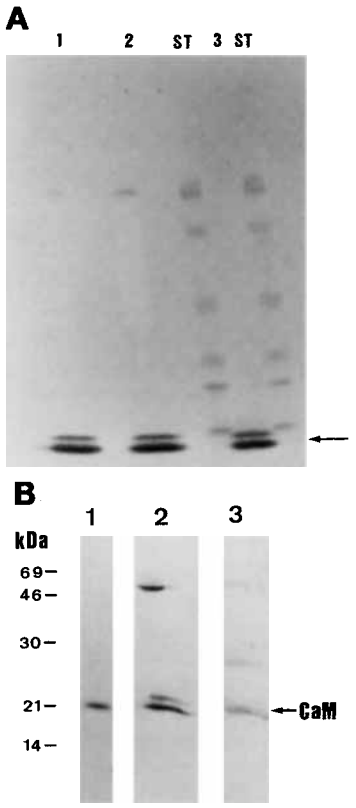

Fig. 3. Immunodetection of calmodulin and exogenous and endogenous phosphocalmodulin. (A) Crude rat liver plasma membranes (50 μg protein) prepared in the presence (lane 1) and in the absence (lane 2) of EGTA, as described in Materials and Methods, and 0.3 μg purified bovine brain calmodulin (lane 3) were processed by electrophoresis in the absence of EGTA in the sample buffer, immunoblotted and probed with the anti-calmodulin monoclonal antibody as described in Materials and Methods. Lanes ST show prestained molecular-mass markers of 106, 80, 49.5, 32.5, 27.5 and 18 kDa. (B) Immunoprecipitation with the anti-calmodulin monoclonal antibody was performed on bovine brain ^{32}P-labeled calmodulin phosphorylated by plasma membranes prepared in the presence of EGTA (lane 1) and on endogenous phosphocalmodulin from ^{32}P-labeled solubilized plasma membranes prepared in the absence of EGTA (lane 2) as described in Materials and Methods. After electrophoresis in the presence of 10 mM EGTA (lane 1) or 10 mM EDTA (lanes 2 and 3) in the sample buffer and transfer to poly(vinylidene difluoride) (lane 1) or to Immobilon P^{SQ} membranes (lanes 2 and 3), autoradiography was performed (lanes 1 and 2). The blot was probed with a specific anti-calmodulin monoclonal antibody and developed with an alkaline-phosphatase-conjugated goat anti mouse IgG (lane 3) as described in Materials and Methods. The position of migration of calmodulin (CaM) is indicated.

ratio is 0.4, with less stimulation detected at higher ratios (Fig. 2B).

Immunoprecipitation of exogenous and endogenous membrane-associated phosphocalmodulin. Immunoblot analysis (Fig. 3A) with an anti-calmodulin monoclonal antibody indicates that the content of calmodulin in the membranes prepared in the presence (Fig. 3A, lane 1) and absence (Fig. 3A, lane 2) of EGTA are virtually identical. A control with bovine brain calmodulin is also presented (Fig. 3A, lane 3). These results were confirmed by radioimmunoassay, which showed a content of 1.3 μg calmodulin/mg membrane protein in both sets of membranes (data not shown).

Fig. 3B, lane 1 shows the autoradiograph of ^{32}P-labeled bovine brain calmodulin phosphorylated by rat liver membrane-bound protein kinase(s) and immunoprecipitated with an anti-calmodulin monoclonal antibody. To test whether endogenous membrane-associated calmodulin is also phosphorylated, immunoprecipitation with the same anti-calmodulin monoclonal antibody was performed on plasma membranes prepared in the absence of EGTA and incubated with [γ-^{32}P]ATP. No exogenous calmodulin or cationic polypeptide were added. Plasma membrane-bound ^{32}P-labeled phosphocalmodulin was visible in the autoradiograph of the immunoprecipitated material together with other coimmunoprecipitated phosphopolypeptides (Fig. 3B, lane 2). Moreover, probing the immunoblot with the anti-calmodulin antibody confirmed the identity of the 21-kDa protein as phosphocalmodulin (Fig. 3B, lane 3).

Differential phosphorylation of the 16.5-kDa membrane-associated polypeptide(s) and bovine brain calmodulin. Phosphoamino acid analysis of the endogenous 16.5-kDa phosphopolypeptide(s) revealed phosphorylation exclusively on

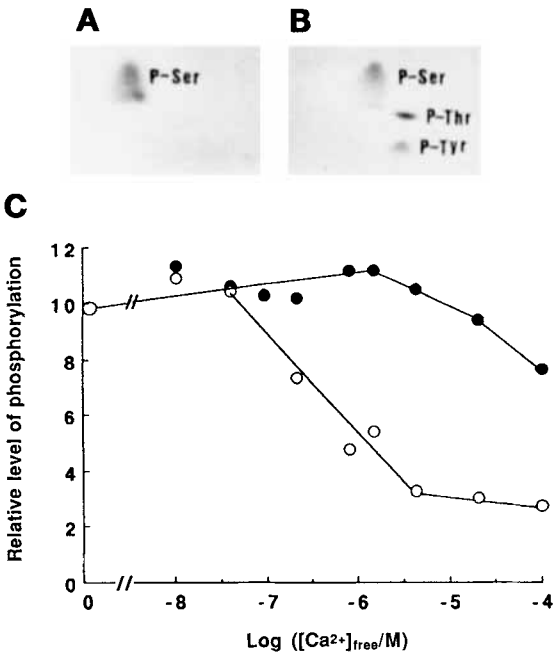

Fig. 4. Phosphoamino acid analysis and effect of Ca^{2+} on the phosphorylation of membrane-associated 16.5-kDa polypeptide(s) and bovine brain calmodulin. (A) Crude rat liver plasma membranes (37 μg protein) prepared in the absence of EGTA were incubated at 37 °C for 1 min in 100 μl of 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 1 mM EGTA and 10 μM [γ-^{32}P]ATP. The samples were boiled for 5 min in the presence of 1% (mass/vol.) Triton X-100 and spun at 15000 *g* for 15 min in a microcentrifuge. The supernatants were treated with 10% (mass/vol.) trichloroacetic acid and the precipitated proteins processed for electrophoresis as described in Materials and Methods. (B) Crude rat liver plasma membranes (36 μg protein) prepared in the presence of EGTA were incubated as above except that the assays were supplemented with 22 μg/ml of calmodulin and 22 μg/ml of poly(L-lysine). After adding 0.5 M NaCl, the samples were boiled, centrifuged, and the supernatant treated with 10% (mass/vol.) trichloroacetic acid. The precipitated proteins were processed for electrophoresis as described for (A). In both cases (A and B) the relevant ^{32}P-labeled bands were cut from the dry gel and phosphoamino acid analysis were carried out as described in Materials and Methods. The positions of migration of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) are indicated. (C) Purified rat liver plasma membranes (26 μg protein) prepared in the absence of EGTA were incubated at 37 °C for 1 min in 100 μl of a medium containing 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, 200 μM EGTA, and different concentrations of $CaCl_2$ to yield the concentrations of free Ca^{2+} indicated (closed symbols). Purified rat liver plasma membranes (26 μg protein) prepared in the presence of EGTA were incubated as above, except that the assay also contained 20 μg/ml bovine brain calmodulin and 20 μg/ml poly(L-lysine) (open symbols). The reaction was stopped and the precipitated proteins processed as described in Materials and Methods. The plot represents the intensity of the ^{32}P-labeled endogenous 16.5-kDa polypeptide(s) (closed symbols) and ^{32}P-labeled bovine brain calmodulin (open symbols).

serine residues (Fig. 4A). In contrast, exogenous bovine brain calmodulin was phosphorylated on serine, threonine and tyrosine residues (Fig. 4B). Therefore, the different phosphorylated residues and/or the degree of phosphorylation could account for the differential electrophoretic behaviour of the endogenous 16.5-kDa phosphopolypeptide(s) and exogenous bovine brain phosphocalmodulin observed in Fig. 1.

A detailed analysis of the effect of Ca^{2+} on the phosphorylation of both the 16.5-kDa polypeptide(s) and bovine brain calmodulin was performed (Fig. 4C). At low free Ca^{2+} concentrations phosphorylation of the 16.5-kDa polypeptide(s) is maximum, and inhibition of approximately 20% is observed at

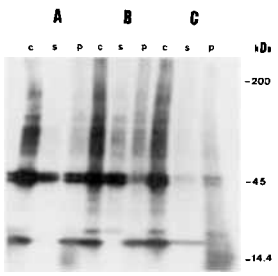

Fig. 5. Solubilization of membrane-associated 16.5-kDa phosphopolypeptide(s). Crude rat liver plasma membranes (60 μg protein) prepared in the absence of EGTA were incubated at 37 °C for 1 min in 200 μl of 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 1 mM EGTA and 10 μM [γ-^{32}P]ATP. An aliquot (80 μl) was removed, precipitated with ice-cold 10% (mass/vol.) trichloroacetic acid and the precipitated proteins processed by electrophoresis and autoradiography (lanes c, A, B and C). A second 100-μl aliquot from the same reaction mixture was incubated in 500 μl of the same medium containing in addition 0.5 mM vanadate, 1 mM $PhMeSO_2F$, and 0.5 M NaCl (A), 6 M urea (B), or 1% (mass/vol.) Triton X-100 (C). The samples were spun for 15 min at 15000 *g* in a microcentrifuge and the supernatants (400 μl) were collected. The residual pellets were washed twice with their respective extraction media. Thereafter, both supernatants (lanes s, A, B and C) and pellets (lanes p, A, B and C) were treated with 10% (mass/vol.) ice-cold trichloroacetic acid and the precipitated proteins were processed by electrophoresis and autoradiography as described in Materials and Methods. The arrow indicates the position of the 16.5-kDa phosphopolypeptide(s).

100 μM free Ca^{2+}. In contrast, the phosphorylation of bovine brain calmodulin is significantly inhibited at lower concentrations of free Ca^{2+} (0.1−1 μM), reaching approximately 70% inhibition at 10 μM free Ca^{2+}.

The membrane-associated 16.5-kDa phosphopolypeptide(s) requires detergents for extraction. The membrane-associated 16.5-kDa phosphopolypeptide(s) are not present in the EGTA-prepared membranes (Fig. 1A and B, lanes 2). However, they are not extracted by a simple EGTA wash (data not shown), suggesting that they may be tightly bound to the membranes. Harsh extraction procedures such as high salt, urea, and Triton X-100 treatments were performed (Fig. 5). Plasma membranes prepared in the absence of EGTA were incubated under standard assay conditions in the presence of [γ-^{32}P]ATP to phosphorylate the membrane-associated 16.5-kDa polypeptide(s). The phosphorylated membranes were subsequently incubated with 0.5 M NaCl (Fig. 5A), 6 M urea (Fig. 5B), or 1% (mass/vol.) Triton X-100 (Fig. 1C). The supernatants (Fig. 5, lanes s) and particulate fractions (Fig. 5, lanes p) were separated by centrifugation and processed by electrophoresis and autoradiography. The phosphorylated 16.5-kDa polypeptide(s) were detected predominantly in the particulate fractions (Fig. 5, lanes p), rather than in the supernatants (Fig. 5, lanes s) of the NaCl-treated (Fig. 5A) or urea-treated (Fig. 5B) membranes. In contrast, the 16.5-kDa phosphopolypeptide(s) was detected in the supernatant (Fig. 5C, lane s) of Triton X-100-treated membranes. Lanes c (Fig. 5) present controls of phosphorylated membranes before the NaCl (Fig. 5A), urea (Fig. 5B) or Triton X-100 (Fig. 5C) treatments.

The 16.5-kDa phosphopolypeptide(s) remained in the supernatant and did not become denatured or precipitated upon boiling (data not shown). Subsequent experiments revealed that efficient extraction of the 16.5 kDa phosphopolypeptide(s) from the membrane occurs at concentrations of Triton X-100 as low as 0.1% (mass/vol.) (data not shown).

Analysis of calmodulin in plasma membrane fractions from tumor cells. Fig. 6A depicts the phosphorylation patterns of

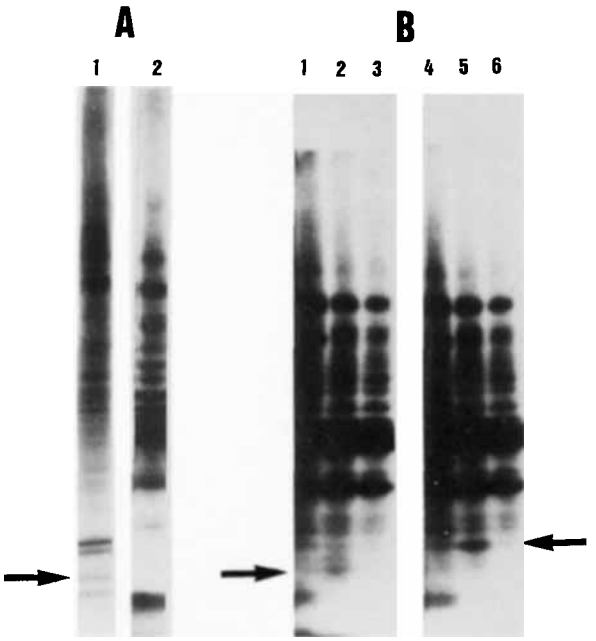

Fig. 6. Protein kinase(s) associated with plasma membranes from Ehrlich ascites tumor cells phosphorylate bovine brain calmodulin. (A) Purified plasma membranes (40 μg protein) from rat ascites hepatoma AS-30D cells prepared in the absence of EGTA were incubated at 37°C for 1 min in 100 μl of 17.5 mM Na-Hepes, pH 7.4, 7 mM $MgCl_2$, 0.5 mM EGTA, and 5 μM [γ-^{32}P]ATP (lane 1). Crude plasma membranes (100 μg protein) from mouse Ehrlich ascites tumor cells prepared in the absence of EGTA were incubated at 37°C for 1 min in 200 μl of 15 mM Na-Hepes, pH 7.4, 6 mM $MgCl_2$, 2 mM EGTA, 0.08% (mass/vol.) Triton X-100, and 10 μM [γ-^{32}P]ATP (lane 2). (B) Crude plasma membranes (100 μg protein) from mouse Ehrlich ascites tumor cells prepared in the absence of EGTA were incubated as above except that 20 μg/ml calmodulin (lanes 1, 2, 4 and 5) and 20 μg/ml poly(L-lysine) (lanes 2, 3, 5 and 6) were added to the assay system. In both cases the reactions were stopped, and the precipitated proteins were processed by electrophoresis and autoradiography as described in Materials and Methods. In (B) the samples were run in the presence of 10 mM $CaCl_2$ (lanes 1–3) or 10 mM EGTA (lanes 4–6) in the electrophoresis sample buffer. Arrows point to 16.5 kDa (left side, A and B) and to 21 kDa (right side, B).

plasma membrane proteins from rat ascites hepatoma AS-30D cells (Fig. 6A, lane 1) and mouse Ehrlich ascites tumor cells (Fig. 6A, lane 2) prepared in the absence of EGTA. The 16.5-kDa phosphorylated band was detected in only trace amounts, if at all, in these samples. Incubation of isolated membranes with alkaline phosphatase before phosphorylation did not result in the appearance of any phosphorylated band corresponding to the 16.5-kDa polypeptide(s) (data not shown). This indicates that the absence of a significant ^{32}P-labeled 16.5-kDa band in the tumor membranes is not due to prephosphorylation of these polypeptide(s) in intact cells.

In view of the absence of the 16.5-kDa phosphopeptides, plasma membranes from Ehrlich ascites tumor cells were evaluated for their ability to phosphorylate exogenous bovine brain calmodulin (Fig. 6B). Addition of either calmodulin (Fig. 6B, lanes 1 and 4) or poly(L-lysine) (Fig. 6B, lanes 3 and 6) in the phosphorylation assay did not result in significant phosphorylation of the 16.5-kDa polypeptide(s). In contrast, addition of both calmodulin and poly(L-lysine) produced a significant phosphorylation of a protein that exhibited the Ca^{2+}-induced electrophoresis mobility shift characteristic of calmodulin, migrating at 16.5 kDa in the presence of Ca^{2+} (Fig. 6B, lane 2), and at 21 kDa in the presence of EGTA (Fig. 6B, lane 5).

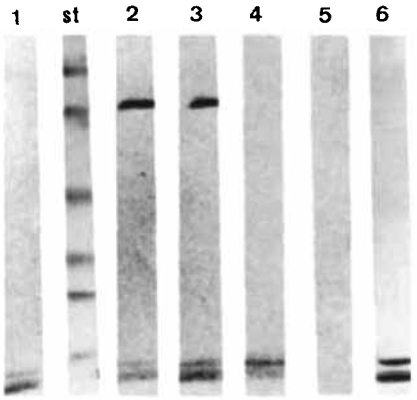

Fig. 7. Calmodulin is absent from membrane fractions from Ehrlich ascites tumor cells. Bovine brain calmodulin (0.5 μg; lane 1), purified rat liver plasma membranes (50 μg protein; lane 2), crude rat liver plasma membranes (50 μg protein; lane 3), supernatant of the Triton X-100-extracted material from purified rat liver plasma membranes (50 μg protein; lane 4), Ehrlich ascites tumor cell crude plasma membranes (50 μg protein; lane 5), and AS-30D rat hepatoma cell crude plasma membranes (50 μg protein; lane 6) were probed with the anti-calmodulin monoclonal antibody as described in Materials and Methods. All plasma membrane fractions were prepared in the absence of EGTA. Prestained molecular-mass markers of 106, 80, 49.5, 32.5, 27.5 and 18 kDa are also shown (lane st).

Calmodulin was detected as a double band by immunoblot analysis in purified (Fig. 7, lane 2), and crude (Fig. 7, lane 3) liver plasma membranes, and in the Triton X-100-solubilized material from the purified membranes (Fig. 7, lane 4). Plasma membranes isolated from the AS-30D hepatoma cells also contained calmodulin (Fig. 7, lane 6). In contrast, no calmodulin was seen in the plasma membranes isolated from Ehrlich ascites tumor cells (Fig. 7, lane 5). A control with purified bovine brain calmodulin is also presented (Fig. 7, lane 1). The non-specific 80-kDa band observed in the membrane fractions (Fig. 7, lanes 2 and 3) appeared when probed with the antibody against mouse IgG coupled to alkaline phosphatase even in the absence of the anti-calmodulin monoclonal antibody (data not shown).

EGTA extracts of membrane fractions were examined for their calmodulin content with a calmodulin-dependent cAMP phosphodiesterase assay (Table 1). Addition of increasing amounts of EGTA extract from normal rat liver plasma membrane fractions resulted in an essentially linear increase in calmodulin-dependent cAMP phosphodiesterase activity. Addition of 100 μl of EGTA-extracted membrane proteins produced an activity of 999 ± 116 nmol · min^{-1} · mg $protein^{-1}$ and 1050 ± 50 nmol · min^{-1} · mg $protein^{-1}$ when extracted at 37°C and 100°C, respectively. In contrast, 100-μl EGTA extracts from Ehrlich ascites tumor plasma membranes failed to activate cAMP phosphodiesterase. These results confirmed the lack of calmodulin detectable by immunobloting in membrane fractions from Ehrlich ascites tumor cells prepared in the absence of EGTA, as shown in Fig. 7. Phosphodiesterase activity reached 966 ± 33 nmol · min^{-1} · mg $protein^{-1}$ in the presence of 12.1 nM purified calmodulin (Table 1).

DISCUSSION

We have shown that rat liver plasma membranes contain a set of endogenous, tightly-bound 16.5-kDa phosphopolypeptides which were extracted by Triton X-100 and did not exhibit the Ca^{2+}-induced electrophoretic mobility shift observed in calmodulin (Burgess et al., 1980). However, phosphocalmodulin is a

Table 1. Calmodulin-dependent activity in plasma membrane fractions from normal rat liver and Ehrlich ascites tumor cells. Crude plasma membranes (100 μg protein) prepared in the absence of EGTA were incubated for 2 min at 100°C or 5 min at 37°C in 1 ml of 15 mM Na-Hepes, pH 7.4, 1 mM EGTA and centrifuged at 15000 *g* for 15 min at 4°C. $CaCl_2$ was added to the supernatants to neutralize the EGTA and aliquots were assayed for stimulation of bovine brain cAMP phosphodiesterase activity as described in Materials and Methods. The mean ± range of the calmodulin-dependent cAMP phosphodiesterase activity of two separate experiments is presented.

Addition	Extraction procedure	[Calmodulin]	Volume	Calmodulin-dependent cAMP phosphodiesterase activity
		nM	μl	nmol · min^{-1} · mg protein^{-1}
None				0
Bovine brain calmodulin		1.2		1657
		2.4		233 ± 16
		4.8		567 ± 100
		7.2		716 ± 33
		12.1		966 ± 33
Rat liver membrane extract	EGTA (37°C)		10	100 ± 83
			30	333
			50	533 ± 66
			100	999 ± 116
	EGTA (100°C)		10	158 ± 25
			30	516 ± 33
			50	725 ± 58
			100	1050 ± 50
Ehrlich tumor cell membrane extract	EGTA (37°C)		100	0
	EGTA (100°C)		100	0

component of this endogenous 16.5-kDa mixture of phosphopolypeptides since it is immunoprecipitated with a highly specific anti-calmodulin monoclonal antibody. In contrast to exogenous calmodulin, phosphorylation of endogenous plasma-membrane-associated calmodulin takes place in the absence of added cationic protein or polypeptide. This suggests that an endogenous physiological factor, that replaces poly(L-lysine), could be present in the membranes to enable the phosphorylation of endogenous calmodulin. Alternatively, the involvement of different protein kinase(s) in the phosphorylation of endogenous and exogenous calmodulin could explain the differential requirement for the exogenous polycation.

Calmodulin phosphorylated by the insulin-receptor tyrosine kinase does not exhibit the characteristic Ca^{2+}-induced electrophoretic mobility shift (Laurino et al., 1988; Saville and Houslay, 1994), as is the case of chicken brain calmodulin phosphorylated on serine residues (Plancke and Lazarides, 1983). In contrast, calmodulin phosphorylated on tyrosine by Src exhibits less electrophoretic mobility than the non-phosphorylated species (Fukami et al., 1985), and calmodulin phosphorylated by the EGF-receptor tyrosine kinase exhibits identical Ca^{2+}-induced electrophoretic mobility shift to non-phosphorylated calmodulin (San José et al., 1992; Benguría et al., 1994). The different electrophoretic behaviour of exogenous bovine brain phosphocalmodulin and the 16.5-kDa phosphopolypeptide(s) could be due to its differential phosphorylation. Therefore, the absence of a Ca^{2+}-induced electrophoretic mobility shift does not exclude the presence of phosphocalmodulin in the mixture of 16.5-kDa phosphopolypeptides present in the plasma membranes. Direct proof of its presence was demonstrated by immunoprecipitation with a highly specific monoclonal antibody (Fig. 3B).

Phosphorylation of calmodulin *in vitro* by the insulin-receptor tyrosine kinase (Sacks and McDonald, 1988; Sacks et al., 1989a) and the EGF-receptor tyrosine kinase (San José et al., 1992; Benguría and Villalobo, 1993; Benguría et al., 1993, 1994) is inhibited by Ca^{2+}. Similarly, physiological Ca^{2+} concentrations attained in the cytosol of activated cells (1−10 μM) inhibits the phosphorylation of bovine brain calmodulin by plasma membrane-associated protein kinase(s). In contrast, supra-physiological Ca^{2+} concentrations (100 μM) only partially inhibited the phosphorylation of other membrane-associated 16.5-kDa polypeptide(s). The phosphorylation site(s) of calmodulin could be occluded upon binding of Ca^{2+}, partially preventing the action of the protein kinase(s). Alternatively, Ca^{2+} may induce a conformational change in calmodulin, preventing access of the protein kinase(s) to the phosphorylation site(s). It should be noted, however, that the presence of high concentrations of Mg^{2+} in the assay system (required for the protein kinase activity) could partially mask the inhibitory effect of Ca^{2+} on the phosphorylation of calmodulin, since Mg^{2+} at high concentrations may bind to the Ca^{2+}-binding sites of calmodulin (Tsai et al., 1987).

Phosphorylation of calmodulin *in vitro* has been observed on tyrosine (Fukami et al., 1985; Häring et al., 1985; Colca et al., 1987; Sacks and McDonald, 1988; Laurino et al., 1988; Sacks et al., 1989a; Benguría et al., 1994) as well as on serine and threonine (Planck and Lazarides, 1983; Fukami et al., 1985; Lin et al., 1986; Nakajo et al., 1988; Kubo and Strott, 1988). Moreover, calmodulin is phosphorylated in intact hepatocytes on serine, threonine and tyrosine residues (Sacks et al., 1992b) by both casein kinase II and the insulin-receptor kinase (Joyal and Sacks, 1994). Similarly, our results show that exogenous bovine brain calmodulin is phosphorylated on serine, threonine and tyrosine residues by membrane-associated protein kinase(s). Nevertheless, we did not detect any stimulatory effect of insulin or EGF on the phosphorylation of calmodulin in the membranes (data not shown). The absence of insulin-induced or EGF-induced phosphorylation of calmodulin could be due to the presence of tyrosine phosphatases present in the plasma membranes (Gruppuso et al., 1991). However, phosphorylation of calmodulin on tyrosine residues by non-receptor tyrosine kinase(s), and/or dual specificity kinase(s) are possibilities that cannot be eliminated.

Plasma membranes interact with the cytoskeletal network (Niggli and Burger, 1987; Carraway and Carraway, 1989) at spe-

cific anchoring points, and calmodulin has been reported to bind to certain plasma-membrane-associated cytoskeletal proteins (Gazzotti et al., 1985; Gloor and Gazzotti, 1986). Moreover, disruption of membranes is required to extract tightly bound calmodulin (Manalan and Klee, 1984; Anderson and Gopalakrishna, 1985). Although our results clearly show that washing the membranes with EGTA released calmodulin which activated cAMP phosphodiesterase, we also observed that the amount of immunodetectable calmodulin did not significantly change by preparing the membranes in the absence or presence of EGTA. This was demonstrated by two different techniques, namely immunoblotting and radioimmunoassay after extraction of calmodulin from the membranes by heating at 95°C for 3 min as described earlier (Sacks et al., 1991). These results suggest that the amount of EGTA-extractable calmodulin represents a minor pool, and that the major calmodulin pool is tightly associated with the membranes. It has been demonstrated that calmodulin binds very efficiently to gangliosides in a Ca^{2+}-dependent manner (Higashi et al., 1992). Therefore, it should be of interest to explore the possibility of the existence of Ca^{2+}-independent tight interactions of calmodulin with other membrane components.

We have also observed that the endogenous 16.5-kDa phosphopolypeptide(s) from rat liver plasma membrane migrates as a high-molecular-mass complex in non-denaturing gel electrophoresis (data not shown), suggesting that they may be associated with high-molecular-mass plasma-membrane-bound cytoskeletal proteins or alternatively they could form oligomers. However, this high-molecular-mass phosphorylated complex is not recognized by the anti-calmodulin antibody on immunoblot (data not shown).

The disorganization of the cytoskeleton observed in highly undifferentiated tumor cells (Ben-Ze'ev, 1985) could explain the absence of 16.5-kDa phosphopolypeptide(s) in the plasma membrane preparations from rat ascites hepatoma AS-30D and mouse Ehrlich ascites tumor cells. Furthermore, the membrane fractions from Ehrlich ascites tumor cells, but not the membrane fractions from the AS-30D rat hepatoma, are devoid of calmodulin. However, calmodulin-devoid plasma membranes from Ehrlich ascites tumor cells contain the protein kinase(s) responsible for the phosphorylation of exogenous bovine brain calmodulin in the presence of poly(L-lysine).

Our results clearly demonstrate that both endogenous plasma-membrane-associated calmodulin and exogenous bovine brain calmodulin are phosphorylated by plasma-membrane-bound protein kinase(s). Further work is required to identify the kinase(s) involved and to elucidate the role of plasma-membrane-bound phosphocalmodulin in the organization of the cytoskeleton and other cellular processes.

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