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PLASMA MEMBRANE CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION SYSTEM IN L_6 MYOBLASTS

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Summary

Plasma membranes can be isolated without disruption of cells by the plasma membrane vesiculation technique (Scott, R.E. (1976) Science 194, 743—745). A major advantage of this technique is that it avoids contamination of plasma membranes with intracellular membrane components. Using this method, we prepared plasma membranes from L_6 myoblasts grown in tissue culture and studied the characteristics of the protein phosphorylation system.

We found that these plasma membrane preparations contain protein kinase which is tightly bound to the membrane and cannot be removed by washing in EDTA or in high ionic strength salt solutions. This protein kinase activity can catalyze the phosphorylation of several exogenous substrates with decreasing efficiency as acceptors of phosphate: calf thymus histones f_2b, protamine and caseine. Cyclic AMP causes a dose-dependent stimulation of protein kinase activity; the highest stimulation (4-fold) is achieved at concentration 10^{-5} M cyclic AMP. Cyclic AMP-dependent stimulation can be completely inhibited by heat-stable protein kinase inhibitor isolated from rabbit skeletal muscle. On the other hand, cyclic GMP does not affect the activity of protein kinase.

Plasma membrane-bound protein kinase also catalyzes the phosphorylation of endogenous membrane protein substrates and this is also stimulated by addition of cyclic AMP. Analysis of plasma membrane proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed that specific polypeptides are phosphorylated by cyclic AMP-independent and by cyclic AMP-dependent protein kinase systems.

The results of these studies demonstrate the presence of endogenous cyclic AMP-dependent and -independent protein phosphorylating systems (enzyme activity and substrates) in purified plasma membrane preparations. These data provide a basis for further investigations on the role of plasma membrane

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

phosphorylation as a regulator of membrane functions including those that may control cellular differentiation.

Introduction

Phosphorylation of the plasma membranes of eukaryotic cells may be an important factor which regulates membrane functions that influence many aspects of cell biology [1]. The existence of endogenous protein kinases in the plasma membrane has been postulated by many investigators and in some simple systems, such as erythrocytes, plasma membrane phosphorylation has clearly been demonstrated [1—4]. But in nucleated eukaryotic cells, the evidence for the presence of a plasma membrane-associated protein phosphorylation system is only suggestive. In the majority of studies which employ homogenates of cells and tissues, the highest activity of protein kinase is usually found in soluble fraction with relatively minor activity in particulate fractions, including fractions of plasma membranes [5—9]. In some studies for example highly active protein kinases have been found in intracellular membranes, most prominently in membranes of endoplasmic reticulum [10—12] or sarcoplasmic reticulum [13,14] and in nuclear membranes [15,16]. The presence of protein kinase activity has also been described in the plasma membrane fractions isolated from the homogenates of various cells and tissues [1,5], however, due to inherent methodological difficulties [17] involved in isolation of plasma membranes by the techniques used in such studies, contamination of the plasma membrane fraction with protein kinase-enriched intracellular membranes could not be ruled out.

We have previously reported that plasma membranes from cultured eukaryotic cells can be isolated in the form of vesicles by a procedure which avoids homogenization of cells and which avoids detectable contamination with intracellular membranes [18]. To establish the presence of an intrinsic plasma membrane protein phosphorylating system from eukaryotic cells, we assayed the protein kinase activity and endogenous membrane protein phosphorylation of purified plasma membranes isolated from undifferentiated L_6 myoblasts [19—21]. The data show that such plasma membrane preparations contain an endogenous protein kinase that is sensitive to stimulation by cyclic AMP. This plasma membrane-associated cyclic AMP-dependent protein kinase system is also shown to catalyze the phosphorylation of specific plasma membrane polypeptides.

Materials and Methods

Preparation of plasma membrane vesicles. Cell system: An L_6 myoblast cell line (a gift of Dr. David Shubert) was used [19,20]. The particular L_6 line used in these studies, designated as an L_6 variant, was selected for its ability to proliferate in high density culture. It shows no detectable evidence of morphological differentiation but does show enzymatic differentiation in high density postconfluent cultures as assayed by intracellular creatinine phosphokinase levels.

L_6 cells were grown in Dulbecco's minimal essential media containing 10% fetal calf serum without antibiotics in 495 cm^2 roller bottles. These cultures have been shown to be free of mycoplasma contamination by culture (Flow Laboratories, Bethesda, Maryland) and by scanning and transmission electron microscopy. For individual experiments, L_6 cells were grown to 75 percent confluence. At this cell density the cells were undifferentiated. They showed no cell fusion into myotubules and they contain low creatinine phosphokinase activity, i.e. 500–750 munits/mg cell protein. L_6 myoblast monolayer cultures were washed in situ three times in phosphate-buffered saline containing 0.75 mM calcium and 0.5 mM magnesium, pH 7.4. Cell monolayers were then incubated in a plasma membrane vesiculant containing 25 mM formaldehyde/2 mM dithiothreitol/calcium-magnesium phosphate-buffered saline for 2 h at 37°C as described [18]. Plasma membrane vesicles were decanted and sedimented by centrifugation at 30 000 × *g* for 30 min at 4°C and all subsequent steps were also performed at 0–4°C. The pellet was washed three times by recentrifugation in 10 mM Tris-buffered isotonic saline (pH 7.4) to remove reversibly bound formaldehyde and dithiothreitol. After each centrifugation the vesicle pellet was gently resuspended so as to leave any detached cells as a dense white pellet at the bottom of the tube. Plasma membrane fragments were prepared from this washed preparation of plasma membrane vesicles in the following way: they were resuspended in 4 ml of hypotonic 20 mM 2-(*n*-morpholino)ethanesulfonic acid buffer, pH 6.4, and were disrupted by N_2 decompression at 250 lb/$inch^2$ for 5 min. The lysed vesicle suspension was then centrifuged at 250 000 × *g* for 1 h at 4°C. The supernatant, i.e. "soluble", fraction was concentrated by placing the solution in a dialysis bag on Carbowax (Fischer Co., Fair Lawn, N.J.). The concentrate which contained soluble cytoplasmic components was frozen at −80°C in small aliquots for protein kinase assays. The 250 000 × *g* plasma membrane pellet was washed by resuspension in 2-(*n*-morpholino)ethanesulfonic acid buffer and after recentrifugation at $2.5 \cdot 10^5 \times g$ was frozen in small aliquots and stored at −80°C.

Protein kinase assay. Protein kinase activity was assayed using conditions basically as described previously [22–24] with several modifications. The enzyme preparation (10–20 μg/tube) was incubated in a mixture (final volume of 200 μl) containing: 50 mM β-glycerol phosphate buffer (pH 6.5), 10 mM MgCl, 10 mM NaF, 2 mM theophylline, 0.4 mM EGTA and $5 \cdot 10^{-5}$ M [γ^{2}-^{32}P]-ATP ($1 \cdot 10^7$ cpm/tube). Unless otherwise stated, the incubation time was 10 min at 30°C, and 75 μg f_2b calf thymus histones were used as an artifical substrate. The incubation was initiated by addition of ATP and stopped by precipitation with 10% trichloroacetic acid. The incorporation of ^{32}P into proteins was measured by the procedure described previously [22–24].

For measurement of the autophosphorylation of plasma membrane proteins, the membranes (300 μg/tube) were incubated under the similar conditions as for protein kinase assay, but without addition of exogenous substrates, with ATP of comparable or higher specific radioactivity ($1 \cdot 10^7$–$4 \cdot 10^7$ cpm/tube) and for longer incubation intervals (30 min unless otherwise stated). For measurement of the specific activity of endogenous phosphorylation, membranes were processed as described above. When phosphorylated

membranes were to be analyzed by polyacrylamide gel electrophoresis the membranes were washed once in 2-(*n*-morpholino)ethanesulfonic acid buffer (4°C) then solubilized by addition 1/10 volume of a "dissociating solution", i.e. 10% SDS/100 mM dithiothreitol/10 mM EDTA/1.0 mM phosphate, pH 7.4. Mixture of membranes in "dissociating buffer" was heated at 90°C for 5 min, cooled and then applied to 5% polyacrylamide gels.

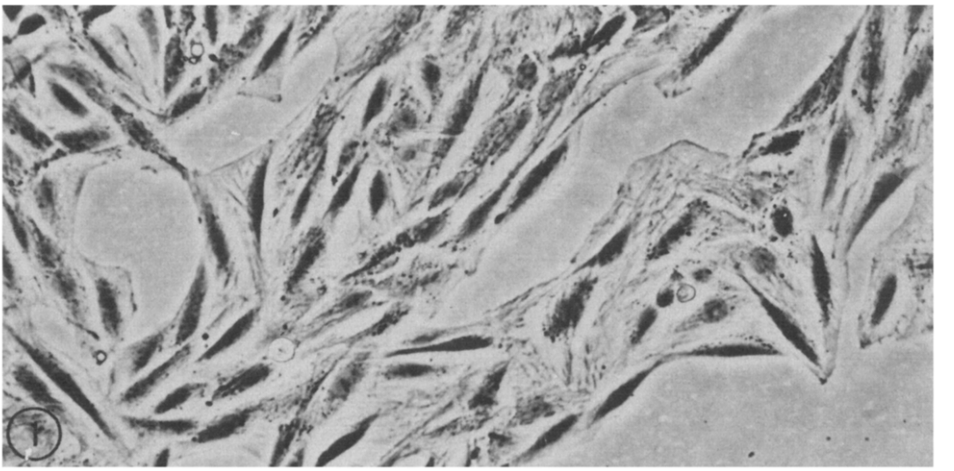
Polyacrylamide gel electrophoresis: 5% polyacrylamide gels were prepared as described by Fairbanks et al. [25]. Samples of dissociated membranes containing 50—100 μg of protein were applied on the top of the gel and subjected to electrophoresis at 8 mA/tube at a field gradient of 200 mV/cm in a buffer containing 0.1% SDS/0.1 M phosphate (pH 7.4). Upon completion of electrophoresis, gels were fixed and stained with Coomassie Blue and destained as described in a previous communication [18,26]. For assessment of molecular weight of individual protein bands, gels were calibrated using the following markers: cytochrome *c*, bovine serum albumin, β-galactosidase. Stained gels were first scanned at A_{660nm} in a Beckman Acta II spectrophotometer equipped with linear transport device and then cut into 1-mm slices. Slices were solubilized in 0.5 ml 0.5 molar H_2O_2 for 4 h at 50°C then cooled and suspended in 3.5 ml of scintillation fluid (Handifluor). ^{32}P incorporation was determined by scintillation counting in Beckman LS250 scintillation counter.

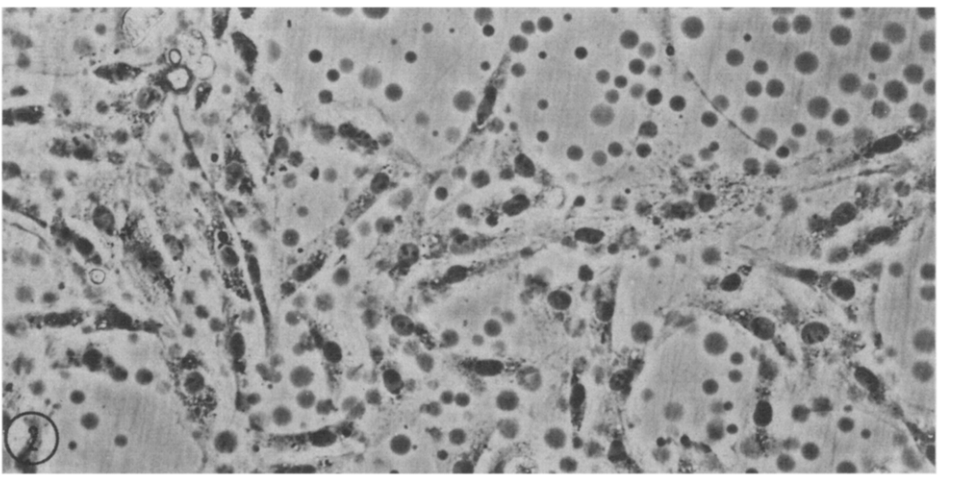
Protein content in individual preparations were determined by the method of Lowry et al. [27] after solubilization in 1% SDS [22—24].

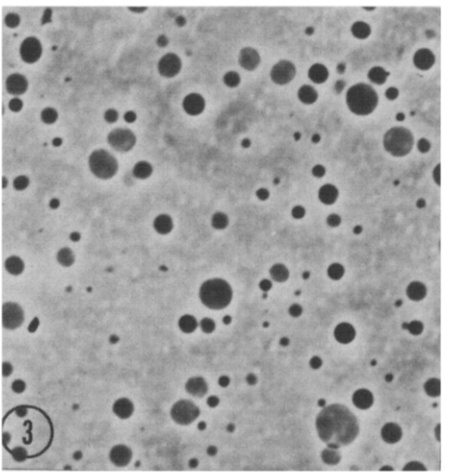
Radioactive [γ-^{32}P]ATP was purchased from ICN, Irvine, Calif. Chemicals and biochemicals, all of the highest purity grade, were purchased from Sigma Co. and other standard suppliers. DNAase-free pancreatic RNAase A (P-L Biochemicals Inc., Milwaukee, Wisc.) was a gift of Dr. William Campbell. Heat-stable protein kinase inhibitor [28] was prepared by methods described in our previous communications [22]. Each experiment was repeated at least three times, unless stated otherwise in text.

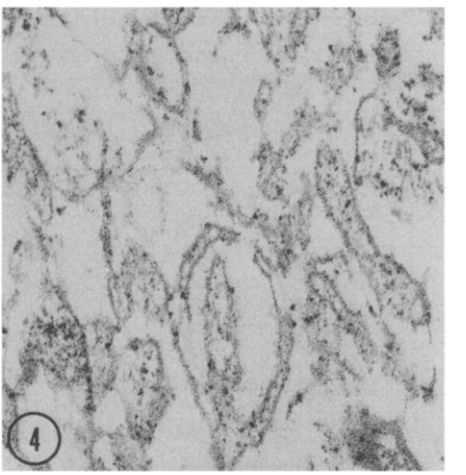
Results

L_6 myoblast cultures (Fig. 1) can be induced to shed plasma membrane vesicles following incubation in a solution of 25 mM formaldehyde/2 mM dithiothreitol (Fig. 2). Plasma membrane vesicles can be easily isolated by decanting and centrifugation (Fig. 3). Washed plasma membrane vesicles were disrupted by decompression and separated into two fractions: (1) 250 000 $\times g$ plasma membrane pellet; and (b) 250 000 $\times g$ soluble supernatant. An electron micrograph of a 250 000 $\times g$ plasma membrane preparation is shown in Fig. 4. Both 250 000 $\times g$ plasma membrane pellet and the 250 000 $\times g$ soluble supernatant fractions from disrupted vesicles contained protein kinase activity stimulated by addition of cyclic AMP and inhibition by heat-stable protein kinase inhibition (Table I). In both preparations, calf thymus histone f_2b type was the most efficient phosphorous acceptor of the three tested proteins. If histone f_2b is taken as 100%, the relative efficiency of artifical substrates for membrane kinase (histone f_2b 100%, protamine 48%, casein 22%) and for the soluble kinase (histone f_2b 100%, protamine 51% and casein 18%) was similar. Also the extent of stimulation by each of the three different substrates was









Figs. 1—4. Phase micrograph of monlayer L_6 myoblast cultures before (1) and after exposure (2) to a plasma membrane vesiculant. Plasma membrane vesicles can be isolated by centrifugation. By phase microscopy they appear as 1—10 μm spheres (3). Purified plasma membrane fragments were isolated by disruption of vesicles and sedimentation at 250 000 $\times g$. They appear as small membrane fragments by electron microscopy (4). Magnification Fig. 1, 200X; Fig. 2, 200X; Fig. 3, 200X; Fig. 4, 90 000X.

TABLE I

SUBSTRATE SPECIFICITY FOR L_6 PLASMA MEMBRANE AND SOLUBLE PROTEIN KINASE

Protein kinase specific activity is expressed as pmol ^{32}P incorporated/min per mg protein ± S.DE.

	Protein kinase activity		
	No additions	$5 \cdot 10^{-6}$ M cyclic AMP	$5 \cdot 10^{-6}$ M cyclic AMP + PKI *
(A) Plasma membrane pellet 250 000 × *g*			
Histone f_2b	11.9 ± 0.4	26.4 ± 0.9	6.6 ± 0.8
Protamine	5.7 ± 0.2	11.8 ± 1.0	—
Caseine	2.6 ± 0.01	3.4 ± 0.4	—
(B) Soluble 250 000 × *g* supernatant			
Histone f_2b	5.5 ± 0.5	15.1 ± 0.8	4.0 ± 0.1
Protamine	2.8 ± 0.2	5.2 ± 0.3	—
Caseine	1.0 ± 0.02	1.2 ± 0.02	—

* PKI, heat-stable protein kinase inhibitor was employed at 235 μg/tube, see Materials and Methods.

similar in membrane and soluble preparations. By contrast the specific activity of protein kinase in the plasma membrane pellet was consistently higher than in the soluble fraction.

Plasma membrane-associated protein activity was linear with time (up to 10 min) and protein content (up to 20 μg/tube) when f_2b histones were used as substrate. Stimulation of protein kinase activity by cyclic AMP both in the membrane and soluble fractions was dependent on concentration of cyclic AMP; maximum stimulation being achieved at 10^{-5} M cyclic AMP (Fig. 5). Neither the protein kinase activity in the soluble or membrane fractions was influenced by the addition of cyclic GMP to our standard incubation mixture. In a further attempt to detect cyclic GMP-sensitive protein kinase activity we retested the enzyme preparations under pH, buffer and substrate conditions reported to be favorable for detection of cyclic GMP-dependent protein kinase

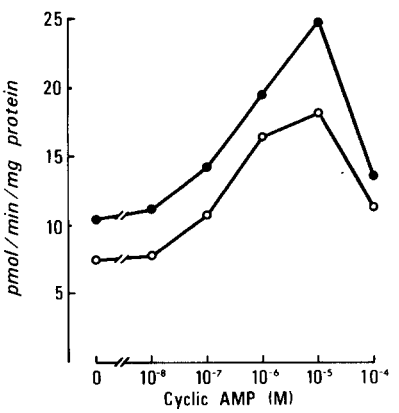


Fig. 5. Plasma membrane (●——●) and soluble (○——○) protein kinase activity in response to addition of cyclic AMP.

activity from other tissues [29]. The incubation mixtures for these assays included 50 mM sodium phosphate/2.5 mM theophylline/40 μg arginine-rich histone/10 mM $MgCl_2$, pH 7.0. Even under these assay conditions cyclic GMP exhibited no effect.

To approach the question whether protein kinase activity associated with L_6 plasma membranes is an intrinsic membrane protein or whether it may be only absorbed on membranes, 250 000 × *g* plasma membrane preparations were washed in 10 mM EDTA and then in 1 M NaCl (Table II). While the specific activity of protein kinase (with or without added cyclic AMP) was lower in washed membranes, substantial activity was retained as well as the ability to be stimulated by cyclic AMP. This finding is interpreted to indicate that at least a majority of the protein kinase activity is membrane bound in plasma membrane preparations isolated by the technique employed in this study [18].

Endogenous plasma membrane phosphorylation

Next we examined whether protein kinase associated with the plasma membrane phosphorylates specific endogenous plasma membrane proteins. When 250 000 × *g* membrane pellets were incubated without addition of the exogenous artifical substrates, phosphorylation of membrane proteins occurred; however, the extent of ^{32}P incorporation into protein was lower than into the artificial histone substrate and the extent of stimulation by cyclic AMP in terms of percent increase over the basal activity is also less (Table III) than with histone f_2b. This table shows in addition that when endogenous membrane proteins serve as substrate, protein kinase activity was not linear beyond 30 min and that the cyclic AMP sensitivity of the kinase was lost following prolonged incubation, i.e. 60 min. Maximum incorporation of ^{32}P into the L_6 plasma membranes was observed at 30 min at 30°C. Inclusion of protein phosphatase inhibitors such as Zn^{2+} (1 mM) or inhibitors of adenosine triphosphatase, such as ethacrynic acid (10^{-3} M), did not increase the extent of phosphorylation. To determine whether endogenous membrane protein kinase catalyzed the phosphorylation of membrane proteins randomly or whether specific plasma membrane proteins were phosphorylated, we analyzed phosphorylated membranes by gel electrophoresis. L_6 plasma membranes, when

TABLE II

PROTEIN KINASE ACTIVITY IN WASHED AND EXTRACTED L_6 PLASMA MEMBRANES

Plasma membrane preparations washed once in 2-(*n*-morpholino)-ethanesulfonic acid buffer, pH 6.5, and sedimented by centrifugation at 250 000 × *g* for 1 h are designated 'washed'. For this single experiment a plasma membrane preparation was also washed once in the same buffer, then once in 10 mM EDTA/0.1 mM phosphate buffer, pH 7.0; once in 1 M NaCl and finally in same buffer as above. This specimen is designated 'washed and extracted' membranes. Protein kinase specific activity is expressed as pmol ^{32}P incorporated/min per mg protein ± S.E.

	Protein kinase activity	
	No additions	$5 \cdot 10^{-5}$ M cyclic AMP
Washed plasma membranes	10.3 ± 0.2	24.8 ± 0.6
Washed and extracted plasma membranes	8.8 ± 0.1	14.6 ± 0.9

TABLE III

PHOSPHORYLATION OF L_6 PLASMA MEMBRANE PROTEIN BY ENDOGENOUS PROTEIN KINASE

Protein kinase specific activity is expressed of pmol ^{32}P incorporation/min per mg protein ± S.E.

Incubation interval (min)	Protein kinase activity	
	No additions	$5 \cdot 10^{-5}$ M cyclic AMP
15	3.5 ± 0.2	4.8 ± 0.2
30	6.2 ± 0.5	9.5 ± 0.4
60	5.8 ± 0.4	6.4 ± 0.4

analyzed by the electrophoretic system used in this study, contained approx. 25 (15 major and 10 minor) protein bands which were identified by staining with Coomassie Blue. After incubation with [γ-^{32}P]ATP in the absence of exogenous cyclic AMP, only slight incorporation into high molecular weight polypeptides was observed (Fig. 6). In presence of $5 \cdot 10^{-5}$ M cyclic AMP, the extent of phosphorylation of these high molecular weight bands was not significantly intensified, but ^{32}P incorporation into other distinct polypeptides was observed. The most prominent phosphorylation in each of six different experi-

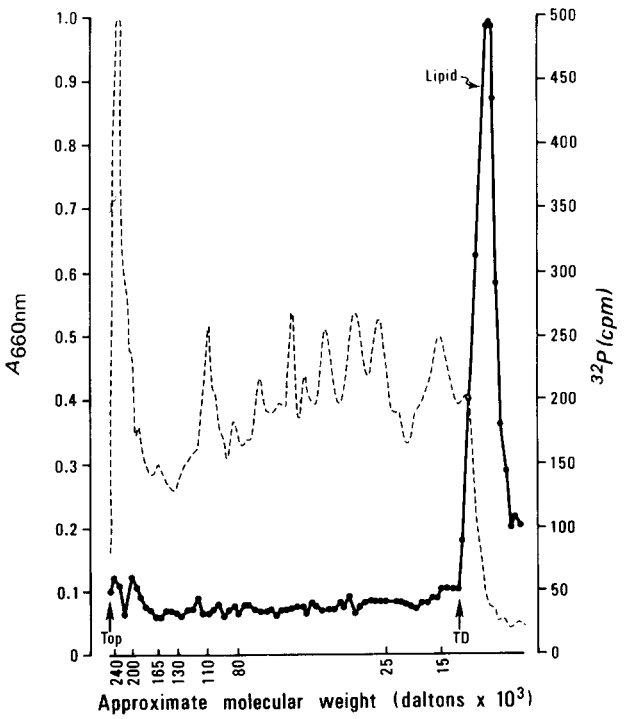


Fig. 6. Analysis of L_6 plasma membrane preparation by polyacrylamide electrophoresis. Approx. 25 polypeptides are evident as Coomassie Blue-stained bands (- - - - -). When incubated in the absence of cyclic AMP only two polypeptides with high molecular weights are phosphorylated as is lipid which migrates beyond the tracker dye (TD).

ments was observed in two polypeptides labeled P_1 and P_2 (Fig. 7). P_1 has an approximate molecular weight of 130 000 and P_2 of 110 000. Slight phosphorylation of other minor polypeptides was also observed in some experiments. In all analyses ^{32}P was incorporated into a region of the gel that migrated just beyond the tracker dye. This material represents plasma membrane lipid; it can be removed by extraction of phosphorylated membrane with methanol/chloroform prior to electrophoresis. This material also stains as lipid with oil red "0". ^{32}P incorporation into membrane phospholipids has also been previously described and characterized in human red blood cell membranes [2].

To establish further the nature of the plasma membrane phosphoprotein bands, several additional experiments were performed similar to those previously described [30]. Phosphorylated plasma membrane preparations were suspended in 4 ml of 1 mM hydroxylamine in 20 mM 2-(*n*-morpholino)-ethanesulfonic acid buffer, pH 6.5, and incubated at 30°C for 30 min. The membranes were then collected by centrifugation at 250 000 × *g* and then analyzed by gel electrophoresis. In other experiments, phosphorylated membranes were subjected to incubation with DNAase-free pancreatic ribonuclease A at 30°C for 30 min prior to electrophoresis. Such treatments did not affect the major

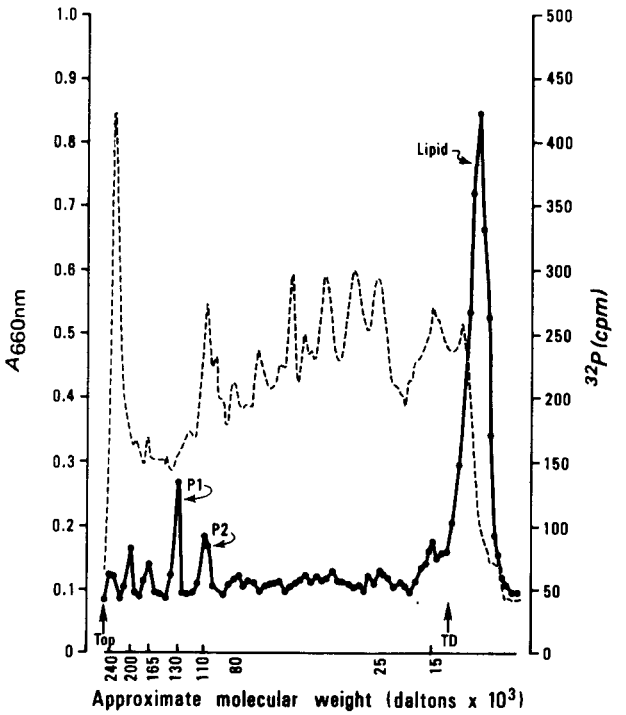


Fig. 7. L_6 plasma membranes incubated under identical conditions as in Fig. 6 but in the presence of $5 \cdot 10^{-5}$ M cyclic AMP. Phosphorylation was most prominent in polypeptides P_1 (130 000 daltons) and P_2 (110 000 daltons). Less prominent phosphorylation was also observed in other polypeptides. ^{32}P which migrates in the region of the gel beyond the tracker dye (TD) is thought to represent phosphorylated lipid.

phosphorylated protein bands which are observed either in the presence or absence of cyclic AMP. Extraction with chloroform/methanol mixture also failed to remove ^{32}P from phosphorylated polypeptides.

Discussion

These results demonstrate the presence of protein kinase activity in L_6 myoblast plasma membranes obtained under conditions which did not involve homogenization or other mechanical disruption of cells. With this method of preparation contamination with intracellular membranes [17] appears to be avoided. Observations show that membranes prepared using this procedure contain an active protein kinase which can be stimulated by the addition of cyclic AMP. The observation that such protein kinase activity cannot be removed by extraction of membrane preparations under conditions which are known to remove peripheral membrane proteins suggests that the kinase is tightly bound to the plasma membrane in our preparations. Moreover the protein kinase activity in the plasma membrane fraction sedimenting at 250 000 $\times g$ is consistently higher than that detected in the soluble supernatant fraction. Since the 250 000 $\times g$ supernatant contains the remnants of cytoplasm of the parent cell, this finding supports the notion that protein kinase is endogenous to the membrane at least in plasma membrane preparations isolated by the techniques employed in our studies.

In its basic properties the protein kinase activity in L_6 plasma membrane preparations does not appear to be different from protein kinase present in other soluble and membrane preparations isolated from numerous eukaryotic cells and tissues [1,5—9,22—24]. This is true in terms of its sensitivity to stimulation by cyclic AMP, inhibition by protein kinase heat-stable inhibitor [28] as well as with respect to its specificity towards series of artificial substrates.

It is of interest that the protein kinase activity contained in L_6 plasma membranes phosphorylates endogenous membrane proteins with apparent specificity. Only high molecular weight polypeptides are phosphorylated in the absence of cyclic AMP. In the presence of cyclic AMP different polypeptides are phosphorylated. The most prominent cyclic AMP-dependent phosphoproteins have molecular weight sof 130 000 (polypeptide P_1) and 110 000 (polypeptide P_2). These two polypeptides were observed to represent the major phosphoproteins in each of six different analyses. The ^{32}P incorporated into these membrane proteins is thought to represent ^{32}P covalently bound to amino acids of specific polypeptides by phosphoester bonds. The ^{32}P radioactivity was not removed by extraction with lipid solvent [1,2,30], by incubation with ribonuclease A nor by repeated precipitation with trichloroacetic acid [5,30]. That ^{32}P is bound as a phosphoester that is not an acyl phosphate is also supported by its resistance to treatment with hydroxylamine [30,31].

It is tempting to speculate on the biochemical characteristics of plasma membrane phosphoproteins and on their functional significance. In red blood cell membranes phosphoproteins appear to represent either cytoskeleton proteins like spectrin [2] or transmembrane proteins which are thought to act as specific receptor and transport molecules, i.e. glycophorin [32] and band 3

[2]. The phosphorylation of glycophorin, band 3 and band 4.5 in red blood cells is selectively stimulated by cyclic AMP whereas the phosphorylation of spectrin is not. By contrast in thyroid membranes no selective cyclic AMP-dependent membane protein phosphorylation has been observed [33]. L_6 myoblast cyclic AMP-dependent phosphoproteins might represent proteins similar to glycophorin, band 3 or band 4.5 which are thought to function as receptor or transport molecules [34]. If so, modulation in plasma membrane protein phosphorylation-dephosphorylation by intracellular or extracellular stimuli mediated by physiological concentrations of cyclic AMP could serve a bioregulatory function. This hypothesis provides a basis for our future studies to investigate in more detail the role of modulation of cyclic AMP-dependent plasma membrane protein phosphorylation in the control of cellular growth and differentiation.

Acknowledgements

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