Biochem. J. (1975) **151**, 23–36
Printed in Great Britain

Adenosine 3′:5′-Cyclic Monophosphate-Dependent and Plasma-Membrane-Associated Protein Kinase(s) from Bovine Corpus Luteum

PROPERTIES OF ASSOCIATED ENZYME AND PHOSPHORYLATION OF SPECIFIC PLASMA-MEMBRANE PROTEINS

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(*Received* 11 *January* 1975)

Plasma-membrane fractions FI and FII isolated from bovine corpus luteum by discontinuous sucrose-density-gradient centrifugation, at sucrose-density interfaces of 1.14/1.16 and 1.16/1.18 respectively, contained membrane-associated protein kinases that phosphorylated both the structural proteins of membranes as well as exogenously added protein substrates. Both fractions were characterized with respect to endogenous and exogenous protein substrate specificity, pH-dependence, effect of bivalent metal ions and sensitivity toward cyclic nucleotides. These membrane-associated kinases showed an optimum pH of 6.0 and had an absolute requirement for bivalent metal ions such as Mg^{2+}, Mn^{2+} or Co^{2+} that cannot be replaced by Ca^{2+}. Both the activities were stimulated two- to four-fold by cyclic AMP *in vitro* with an apparent K_m of 83 and 50 nM for fractions FI and FII respectively. Other cyclic 3′:5′-nucleotides were effective only at higher concentrations, but even the most effective, cyclic IMP, showed a stimulation nearly an order of magnitude lower than that of cyclic AMP. In contrast, stimulation by cyclic dTMP and cyclic dAMP was very weak. Cyclic AMP showed no significant effect on the apparent K_m value of both enzymes for histone and $MgCl_2$ but it somewhat decreased the K_m value for ATP. Nucleoside triphosphates like GTP, CTP and UTP inhibited the transfer of [^{32}P]P_i from [γ-^{32}P]ATP into mixed histone catalysed by membrane-associated kinases either in the presence or in the absence of cyclic AMP. In addition to protein kinases, these membrane fractions also possessed cyclic AMP-binding activities. The apparent association constant (K_a) for cyclic AMP binding was 1.0×10^{10} and 2.6×10^{10} M for FI and FII membrane fractions respectively. This binding reaction was specific for cyclic AMP. A 100-fold excess of unlabelled cyclic AMP almost completely (80–90%) inhibited the binding of cyclic [^{3}H]AMP to plasma membranes. Cyclic IMP, 8-bromo cyclic AMP and dibutyryl cyclic AMP were effective competitors at lower concentrations but the extent of inhibition produced by these agents was less than that of unlabelled cyclic AMP. Other cyclic nucleotides were effective only at higher concentrations (10000-fold in excess). The inhibition of cyclic AMP binding by adenosine and 5′-AMP was insignificant even at 1.25×10^5-fold higher concentrations (with 40 nM-cyclic AMP). Fractionation of phosphorylated plasma membranes by hot acid and organic solvents revealed that the major [^{32}P]P_i incorporation was into membrane proteins. Acid hydrolysis of the phosphorylated proteins, followed by high-voltage paper electrophoresis, revealed that the radioactivity was incorporated into phosphoserine and phosphothreonine residues, the extent of incorporation being greater into phosphoserine than into phosphothreonine residues. Eight to nine membrane polypeptides were phosphorylated with [γ-^{32}P]ATP by plasma-membrane-associated protein kinase as resolved by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Of these, two proteins A_1 (mol.wt. 91000) and A_2 (mol.wt. 76000) associated with FI membrane and B_1 (mol.wt. 87000) and B_2 (mol.wt. 77000) proteins associated with FII membranes, whose phosphorylation was specifically stimulated by cyclic AMP, were identified. In conclusion, it appears that the same enzyme was phosphorylating both the endogenous and exogenous substrates; however, the stimulatory effect of cyclic AMP on the extent of protein phosphorylation probably depends on the nature of the phosphate acceptor protein used. In addition, general catalytic properties of these membrane kinases are similar to those of cytosol enzymes, when compared with exogenous protein substrates.

In the bovine corpus luteum cyclic AMP has been shown to be an intermediate in luteinizing hormone-mediated progesterone synthesis (Savard *et al.*, 1965; Marsh *et al.*, 1966; Marsh & Savard, 1966; Marsh, 1970). However, the mechanism by which cyclic AMP brings about this effect is not understood. The wide occurrence of cyclic AMP-dependent protein kinases in a variety of hormone-sensitive tissues has led to the proposal that the physiological effects of cyclic AMP may be mediated through activation of protein kinases (Walsh *et al.*, 1968; Kuo & Greengard, 1969; Greengard & Kuo, 1970; Krebs, 1972; Walsh & Krebs, 1973). We have reported the purification and properties of cyclic AMP-dependent protein kinase from the cytosol fraction of bovine corpus luteum (Menon, 1973). Subsequent studies revealed that a number of ribosomal proteins may serve as a substrate for this purified soluble enzyme (Azhar & Menon, 1974, 1975*b*). In our attempts to understand the regulation of corpus luteum function by gonadotrophins and cyclic AMP, we have studied the properties of plasma membrane-associated protein kinases from this tissue by using both endogenous and exogenous substrates.

The present investigation demonstrates that bound kinase and cyclic AMP-binding activities are present in purified bovine corpus luteum plasma membranes. Endogenous proteins present in the plasma membrane as well as histone, protamine and casein can serve as substrate for these kinases. Further, these membrane-associated protein kinases exhibited catalytic properties comparable with those of cytosol protein kinases when examined with exogenous acceptor proteins as substrates.

Materials and Methods

Materials

Bovine corpora lutea were collected at slaughter and kept in ice-cold 0.9% NaCl. They were processed within 2–3 h or kept frozen at −80°C until further use. Freezing the tissue did not result in any apparent loss of protein kinase activity.

The following chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.: ATP, CTP, GTP, UTP, cyclic AMP, cyclic GMP, cyclic CMP, cyclic UMP, cyclic IMP, cyclic dTMP, cyclic dAMP, 8-bromo cyclic AMP, dibutyryl cyclic AMP, *O*-phospho-DL-serine, DL-*O*-phosphothreonine, casein, protamine, various histone types, catalase and glyceraldehyde 3-phosphate dehydrogenase. Ribonuclease, ovalbumin, chymotrypsinogen and aldolase were obtained from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Carrier-free [^{32}P]P_i was purchased from International Chemical and Nuclear Corporation, Irvine, Calif., U.S.A. Acrylamide and *NN'*-methylenebisacrylamide were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. The following hormones were generously supplied by the Hormone Distribution Program, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md., U.S.A.: bovine follicle-stimulating hormone type B1, porcine follicle-stimulating hormone type P1, bovine luteinizing hormone type B9 and ovine luteinizing hormone type S18. [γ-^{32}P]ATP was prepared as described by Glynn & Chappell (1964).

Preparation of plasma membranes

Plasma membranes were isolated by the procedure of Gospodarowicz (1973). The purified FI (sedimented at sucrose *d* 1.14/1.16 interface) and FII (sedimented at sucrose *d* 1.16/1.18 interface) membrane fractions were separately used for the present studies due to their differences in chemical composition (Gospodarowicz, 1973) Purity of membrane fractions was checked by assaying various marker enzymes (Gospodarowicz, 1973; Azhar & Menon, 1975*d*).

Assay of protein kinase activity

The protein kinase activity was assayed by published procedures (Miyamoto *et al.*, 1969; Menon, 1973). The incubation mixture in a final vol. of 0.2 ml contained: 10 μmol of α-glycerophosphate buffer, pH 6.0; 2.5 nmol of [γ-^{32}P]ATP (2.5×10^6 c.p.m.); 2 μmol of KF; 3 μmol of $MgCl_2$; 0.5 μmol of theophylline; 400 μg of calf thymus mixed histone (type IIA); and 10–20 μg of membrane protein. The incubation was carried out at 30°C for 10 min. Assays were performed in duplicate both in the absence and in the presence of cyclic AMP (usually 5 μM) and samples were processed for ^{32}P measurements as described by Menon (1973). Unless otherwise stated, all data on exogenous protein phosphorylation were corrected for endogenous membrane phosphorylation without added substrates (Rubin *et al.*, 1972; Lamay *et al.*, 1974; LaRaia & Morkin, 1974). Enzyme activity is expressed as pmol of [^{32}P]P_i transferred/10 min. Specific activity is expressed as the activity units/mg of protein.

Cyclic AMP-binding assays

Cyclic AMP-binding assays were carried out by a modification of the procedure of Gilman (1970). The assay was performed in a final vol. of 0.2 ml containing: 0.02–35 pmol of cyclic [^{3}H]AMP; 10 μmol of sodium acetate buffer, pH 4.0; 0.5 μmol of 3-isobutyl-1-methylxanthine; and 10–100 μg of membrane protein. The incubations were carried out at 4°C for 2.5 h and then diluted by the addition of 2 ml of cold buffer A (20 mM-potassium phosphate buffer, pH 6.0) and after 5 min the solutions were filtered through Millipore filters (HAWP 02500, 0.45 μm pore size). The filters were then washed with 10 ml of buffer A and dried under an i.r. lamp. The dried filters were then

transferred to scintillation vials and dissolved in 1ml of methylCellosolve (Sargent–Welch Scientific Co., Skokie, Ill., U.S.A.). To these vials was added 10ml of scintillation fluid made up of 3 parts of toluene and 1 part of methylCellosolve containing 4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of fluid. The radioactivity was determined by liquid-scintillation spectrophotometry. The specific binding was calculated by subtracting the radioactivity retained by the filter in the presence of 1mM unlabelled cyclic AMP from that retained in the absence of unlabelled cyclic AMP (MacKenzie & Stellwagen, 1974).

Other enzyme assays

$Na^+ + K^+$- and Mg^{2+}-dependent adenosine triphosphatase, 5′-nucleotidase and glucose 6-phosphatase activities were assayed by the method of Solyom & Trams (1972), NADH–cytochrome *c* reductase by the procedure of Phillips & Langdon (1962), cytochrome *c* oxidase by the method of Cooperstein & Lazarow (1951), succinic dehydrogenase as described by Veeger *et al.* (1969) and glucose 6-phosphate dehydrogenase by the method of Langdon (1966).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

The sodium dodecyl sulphate–polyacrylamide-gel electrophoresis system of Weber & Osborn (1969) was used to determine the molecular weight of phosphorylated plasma-membrane proteins. Samples (250μg) of the phosphorylated plasma-membrane protein were solubilized by incubating for 60min at 37°C in a mixture (0.125ml) of 1% (w/v) sodium dodecyl sulphate, 1% (w/v) 2-mercaptoethanol, 20% (w/v) sucrose and 0.001% Bromophenol Blue in 0.1M-sodium phosphate buffer, pH7.0. The samples (200μg) were subjected to electrophoresis at 5mA/tube in 10% (w/v) polyacrylamide gels (5mm×75mm) in 0.1M-sodium phosphate buffer, pH7.0, containing 0.1% sodium dodecyl sulphate. The electrophoresis buffer was 0.1% sodium dodecyl sulphate in 0.1M-phosphate buffer, pH7.0. After electrophoresis, gels were stained for proteins and glycoproteins by the procedure of Fairbanks *et al.* (1971). For ^{32}P radioactivity determinations, gels were frozen and sliced laterally into 1mm-thick sections by a mechanical slicer. The slices were placed in separate scintillation vials and solubilized by incubating in 0.5ml of H_2O_2 at 40°C overnight. After incubation, 10ml of Herberg's solution (Rapkin, 1964) was added and vials were then counted for radioactivity in a Beckman model LS230 liquid-scintillation spectrometer. Catalase (mol.wt. 60000), ovalbumin (mol.wt. 43000), aldolase (mol.wt. 40000), glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 36000), chymotrypsinogen (mol.wt. 25700) and ribonuclease (mol.wt. 13700) were used as markers for the calculation of molecular weight of the polypeptides (Weber & Osborn, 1969).

Site of phosphate linkage in phosphorylated plasma-membrane proteins

Samples of plasma membranes were self-phosphorylated in the standard protein kinase assay mixture as described above. After 15min of incubation at 30°C, the mixture were treated with 10% (w/v) trichloroacetic acid and centrifuged at 10000*g* for 15min. The sediments containing phosphorylated proteins were washed three times with 10% trichloroacetic acid and twice with a mixture of diethyl ether–ethanol (3:1, v/v). The washed protein precipitates were suspended in 0.4ml of 6M-HCl and hydrolysed in sealed tubes (in an atmosphere of N_2) for 5h at 105°C. The hydrolysates were dried *in vacuo* and redissolved in the electrophoresis buffer. Samples were applied to Whatman 3MM paper together with phosphoserine, phosphothreonine and [^{32}P]P_i standards and electrophoresed in a mixture of 2.5% (v/v) formic acid, 7.8% (v/v) acetic acid (pH1.85) at 2.5kV for 2.5h (Langan, 1969*a*; Kabat, 1970). Amino acids were detected by cadmium–ninhydrin spray (Dryer & Bynom, 1967).

Protein determinations

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Properties of the enzyme

Plasma-membrane fractions FI and FII isolated from bovine corpus luteum were self-phosphorylated

Table 1. *Plasma-membrane-associated protein kinase activity from bovine corpus luteum*

The incubation medium in a final vol. of 0.2ml contained: 2.5nmol of [γ-^{32}P]ATP (2.6×10^{6}c.p.m.); 2μmol of KF; 10μmol of α-glycerophosphate buffer, pH6.0; 3μmol of $MgCl_2$; 0.5μmol of theophylline; 10μg of fragment FI or 22μg of membrane protein and, where required, 400μg of mixed histone with or without cyclic AMP. After incubation at 30°C for 10min the samples were assayed for radioactivity as described in the Materials and Methods section.

Additions	$10^{-2}\times$[^{32}P]P_i transferred (pmol/mg of protein)	
	Membrane fraction FI	Membrane fraction FII
None	6.7	3.4
Cyclic AMP (5μM)	7.0	4.3
Mixed histone (400μg)	10.1	7.8
Mixed histone (400μg) +cyclic AMP (5μM)	19.9	15.6

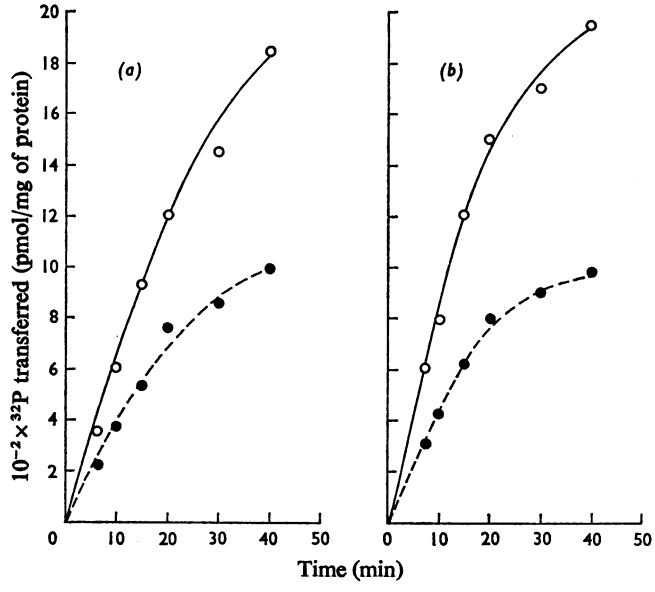

Fig. 1. *Effect of incubation time on the activity of plasma-membrane-associated protein kinases*

The incubation medium in a final vol. of 0.2ml contained: 5nmol of [γ-^{32}P]ATP (1.8×10^6c.p.m.); 2μmol of KF; 10μmol of α-glycerophosphate buffer, pH6.0; 3μmol of $MgCl_2$; 0.5μmol of theophylline; 400μg of mixed histone; 10μg of FI (*a*) or 20μg of FII (*b*) membrane protein and, where required, 1nmol of cyclic AMP. After incubation at 30°C for indicated times the tubes were processed for radioactivity determinations as described in the Materials and Methods section. Values have been corrected for endogenous phosphorylation. ●, Without cyclic AMP; ○, with 5μM-cyclic AMP.

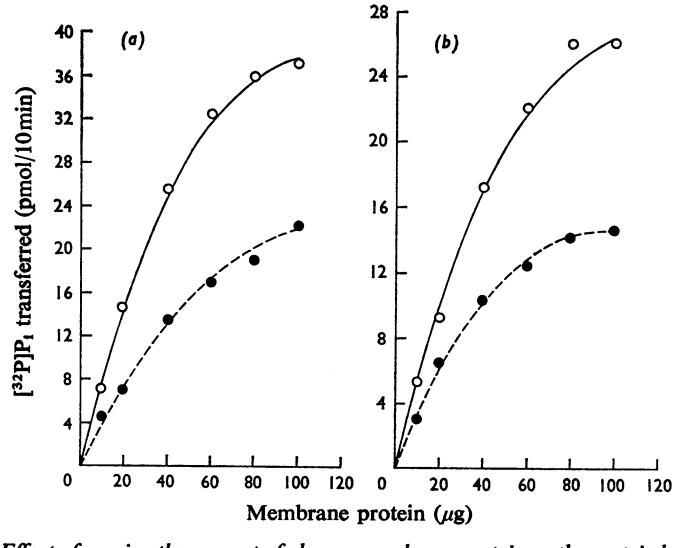

Fig. 2. *Effect of varying the amount of plasma-membrane protein on the protein kinase activity*

The incubation medium in a final vol. of 0.2ml contained: 6nmol of [γ-^{32}P]ATP (2.1×10^6c.p.m.); 2μmol of KF; 10μmol of α-glycerophosphate buffer, pH6.0; 3μmol of $MgCl_2$; 0.5μmol of theophylline; 400μg of mixed histone; the indicated concentrations of membrane protein; and, where required, 1nmol of cyclic AMP. After incubation at 30°C for 10min the tubes were processed for radioactivity determinations as described in the Materials and Methods section. The phosphorylation of endogenous protein substrate was subtracted from these values. (*a*) Protein FI, (*b*) protein FII. ●, Without cyclic AMP; ○, with 5μM-cyclic AMP.

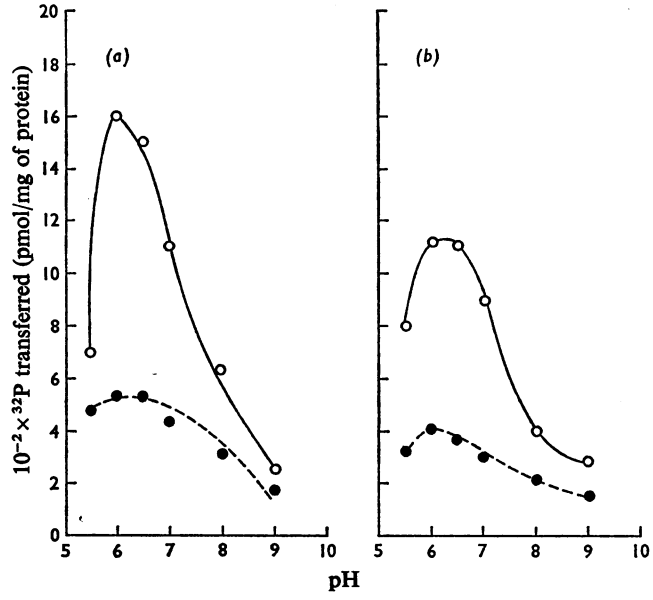

Fig. 3. *Effect of pH on the phosphorylation of histone by plasma-membrane-associated protein kinases*

The incubation medium in a final vol. of 0.2ml contained: 6nmol of [γ-^{32}P]ATP (2.0×10^6 c.p.m.); 2μmol of KF; 10μmol of buffer; 3μmol of $MgCl_2$; 0.5μmol of theophylline; 400μg of mixed histone; 10μg of FI (*a*) or 20μg of FII (*b*) plasma-membrane protein and, where required, 1nmol of cyclic AMP. After incubation at 30°C for 10min the tubes were processed for radioactivity determinations as described in the Materials and Methods section. For pH5.5–7.0, α-glycerophosphate buffer, and, for pH8–9, Tris–HCl buffers were used. Values have been corrected for endogenous phosphorylation. ●, Without cyclic AMP; ○, with 5μM-cyclic AMP.

with [^{32}P]ATP by associated protein kinases (Table 1). This self-phosphorylation was only slightly stimulated by cyclic AMP. In addition, the membrane-associated protein kinases phosphorylated exogenously added histone, which was also stimulated by cyclic AMP (Table 1).

The phosphorylation of histone by plasma-membrane-associated kinase proceeded linearly with time up to 15min of incubation in the presence or the absence of 5μM-cyclic AMP (Fig. 1). Both enzyme activities were linear up to 30–40μg of protein per 0.2ml of incubation medium (Fig. 2) in the presence or the absence of cyclic AMP (5μM). In the presence or the absence of cyclic AMP, the phosphorylation of histone by FI and FII plasma-membrane fractions was maximum at pH6.0 (Fig. 3).

Apparent K_m values for histone, ATP and $MgCl_2$

The FI plasma-membrane-associated protein kinase exhibited an apparent K_m of 312 and 375μg/ml in the presence or the absence of 5μM-cyclic AMP. Similarly, K_m values for histone by FII plasma-membrane-associated protein kinases were 357 and 416μg/ml in the presence or the absence of cyclic AMP. In contrast with histone, the apparent concentration of ATP needed for half-maximal activity of FI and FII enzyme was slightly decreased by the addition of cyclic AMP. The apparent K_m values of histone, ATP and $MgCl_2$ for FI and FII plasma-membrane enzymes in the presence or the absence of cyclic AMP are given in Table 2.

Effect of cyclic AMP concentration

The stimulation of FI and FII plasma-membrane-associated protein kinase by cyclic AMP is shown in Fig. 4. The concentration of cyclic AMP that gave one-half maximum incorporation of ^{32}P into histone was found to be 83nM for FI and 50nM for FII enzymes respectively (Table 2). The stimulatory effect of cyclic AMP on the activities of these enzymes was always found, but the extent of stimulation ranged from two- to four-fold.

Cyclic nucleotide specificity

The effect of several other 3′:5′-cyclic mononucleotides on the activity of plasma-membrane-associated FI and FII protein kinases is shown in Table 3. At lower concentrations cyclic AMP was most effective in activating FI and FII enzymes. Other nucleotides such as cyclic GMP, cyclic CMP

Table 2. *Apparent K_m values for mixed histone, ATP and $MgCl_2$ for plasma-membrane-associated protein kinase from bovine corpus luteum*

K_m values were determined by measuring the initial reaction velocities and then plotting these values as described by Lineweaver & Burk (1934).

	Apparent K_m values			
	Plasma-membrane fraction FI		Plasma-membrane fraction FII	
Substrates	Minus cyclic AMP	Plus cyclic AMP (5 μM)	Minus cyclic AMP	Plus cyclic AMP (5 μM)
Mixed histones (type IIA)	257 μg/ml	312 μg/ml	416 μg/ml	375 μg/ml
ATP	88 μM	56 μM	71 μM	52 μM
$MgCl_2$	3.5 mM	3.5 mM	3.0 mM	3.0 mM
Cyclic AMP	83 nM		50 nM	

Table 3. *Effect of cyclic nucleotides on plasma-membrane-associated protein kinase from bovine corpus luteum*

The incubation conditions were similar to Fig. 4 except that the indicated concentrations of cyclic nucleotide were also included. Results are means of three separate experiments. Values for endogenous phosphorylation have been subtracted.

		$10^{-2} \times [^{32}P]P_i$ transferred (pmol/mg of protein)			
		Membrane fraction FI		Membrane fraction FII	
Cyclic nucleotides	Concn. of nucleotides ...	5 μM	0.1 mM	5 μM	0.1 mM
None		4.7	4.7	3.1	3.4
Cyclic AMP		12.3	11.8	9.1	7.3
Cyclic CMP		5.5	11.5	3.9	7.1
Cyclic dTMP		4.6	5.4	4.1	3.8
Cyclic IMP		10.7	12.6	7.7	9.9
Cyclic UMP		6.0	11.1	4.0	7.3
Cyclic GMP		7.5	12.8	5.3	7.9
8-Bromo cyclic AMP		11.8	8.1	8.8	6.2
Cyclic dAMP		4.7	6.3	3.4	4.2

and cyclic UMP stimulated the protein kinase activities at higher concentrations. In contrast, cyclic IMP and 8-bromo cyclic AMP showed significant stimulation even at lower concentrations. Cyclic dTMP and cyclic dAMP were practically ineffective in stimulating the membrane-associated protein kinases.

Effect of bivalent metal ions

The plasma-membrane-associated protein kinases showed an absolute requirement for bivalent metal ions and more specifically for Mg^{2+}. The apparent K_m for Mg^{2+} was 3.5 mM in the case of FI enzyme and 3.0 mM for FII enzyme. Maximum activity of these two enzymes was observed at Mg^{2+} concentrations of 10–15 mM. Cyclic AMP had no effect on the apparent K_m values of either of these enzymes for Mg^{2+} (Table 2). The effect of other bivalent metal ions is shown in Fig. 5. At a lower concentration (2 mM) Co^{2+} was the most effective activator for both enzymes either in the presence or the absence of cyclic AMP (5 μM). However, the maximum effect of cyclic AMP was observed in the presence of 10 mM-Mg^{2+}. Mn^{2+} was also stimulatory but was slightly less effective than Mg^{2+}. Zn^{2+} and Fe^{2+} were the least effective metal ions. No activity could be detected in the presence of 2 or 10 mM-Ca^{2+} either in the presence or the absence of 5 μM-cyclic AMP.

Substrate specificity of acceptor proteins

Mixed histone, arginine-rich histone and slightly lysine-rich histone were preferred substrates for plasma-membrane-associated protein kinases with or without 5 μM-cyclic AMP (Table 4). In contrast, protamine, lysine-rich histone, casein and albumin were poor ^{32}P acceptors and their phosphorylation was not affected by cyclic AMP (Table 4).

Effect of nucleoside triphosphates on histone phosphorylation by membrane protein kinases

The effect of nucleoside triphosphates on cyclic AMP-stimulated phosphorylation of mixed histone by plasma-membrane-associated kinases is shown in Table 5. Both in the presence or the absence of 5 μM-

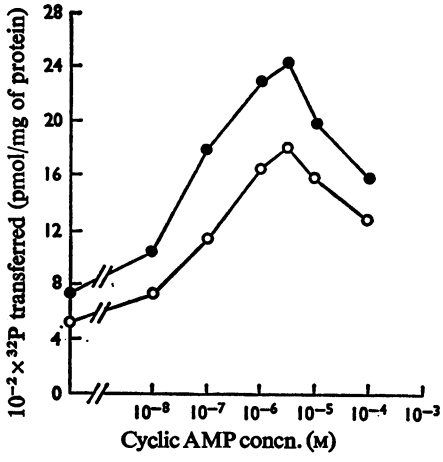

Fig. 4. *Effect of increasing concentrations of cyclic AMP on plasma-membrane-associated protein kinase activity*

The incubation medium in a final vol. of 0.2ml contained: 5nmol of [γ-^{32}P]ATP (2.6×10^6c.p.m.); 2μmol of KF; 10μmol of α-glycerophosphate buffer, pH6.0; 3μmol of $MgCl_2$; 0.5μmol of theophylline; 400μg of mixed histone; 10μg of FI (●) or 20μg of FII (○) membrane protein and the indicated concentrations of cyclic AMP. After incubation at 30°C for 10min, the tubes were processed for radioactivity determinations as given in the Materials and Methods section. Values have been corrected for endogenous phosphorylation.

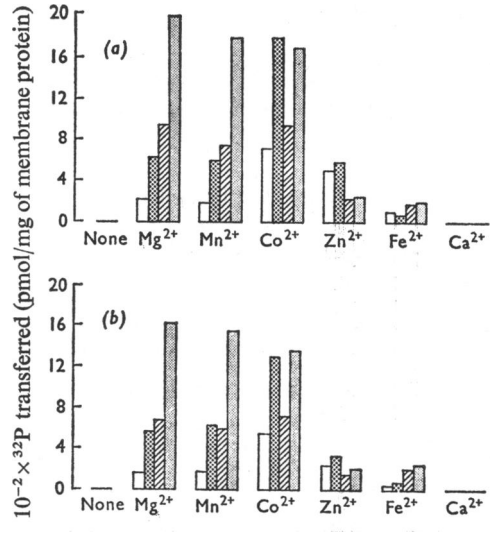

Fig. 5. *Effect of various bivalent metal ions on plasma-membrane-associated protein kinase activity*

Incubation conditions were similar to those in Fig. 4 except that indicated concentrations of various bivalent metal ions were included into the incubation medium. □, 2mM Cation; ▨, 2mM cation+5μM-cyclic AMP; ■, 10mM cation; ▩, 10mM cation+5μM-cyclic AMP. (*a*) FI enzyme; (*b*) FII enzyme.

Table 4. *Substrate specificity of plasma-membrane-associated protein kinase from bovine corpus luteum*

The incubation conditions were similar to those in Fig. 4 except that different protein substrates were added. Each of the different protein substrates was present in equal concentrations by weight (400μg). The phosphorylation of endogenous protein substrate was subtracted.

	$10^{-2} \times [^{32}P]P_i$ transferred (pmol/mg of protein)			
	Plasma-membrane fraction FI		Plasma-membrane fraction FII	
Substrate	Minus cyclic AMP	Plus cyclic AMP (5μM)	Minus cyclic AMP	Plus cyclic AMP (5μM)
Mixed histone (type IIA)	6.4	13.0	4.4	11.3
Arginine-rich histone (type IV)	4.4	11.4	4.1	8.5
Arginine-rich histone (f3) (type VIII)	6.6	10.0	4.6	9.1
Lysine-rich histone (type III)	2.1	2.2	1.5	0.7
Slightly lysine-rich histone (f2a) (type V)	8.7	17.7	5.7	8.5
Casein	0.9	1.7	1.5	1.0
Protamine	2.4	3.7	3.1	3.0
Albumin	1.0	1.3	1.6	1.0

cyclic AMP, GTP, CTP and UTP inhibited the incorporation of ^{32}P from [γ-^{32}P]ATP into histone (Table 5).

Lack of gonadotrophin effect

Menon (1973) reported that the cytosol protein kinase purified from bovine corpus luteum was directly stimulated by luteinizing hormone in addition to its stimulation by cyclic AMP. We have therefore also tested the effect of various gonadotrophins such as follicle-stimulating hormone, luteinizing hormone and human chorionic gonadotrophin on plasma-mem-

Table 5. *Effect of nucleoside triphosphates on the activity of plasma-membrane-associated protein kinase from bovine corpus luteum*

The incubation conditions and other details were the same as described in Fig. 4 except that the indicated nucleoside triphosphates were present in a final concentration of 0.1 mM.

Nucleoside triphosphate (0.1 mM)	$10^{-2}\times$ [^{32}P]P_i transferred (pmol/mg of protein)		
	Minus cyclic AMP	Plus cyclic AMP (5 μM)	Ratio
Plasma-membrane fraction FI			
No addition	6.5	20.0	3.2
UTP	2.2	7.6	3.4
CTP	3.3	9.5	2.8
GTP	3.0	9.2	3.0
Plasma-membrane fraction FII			
No addition	6.0	19.3	3.4
UTP	2.2	7.3	3.8
CTP	2.0	9.0	4.3
GTP	2.3	9.1	3.9

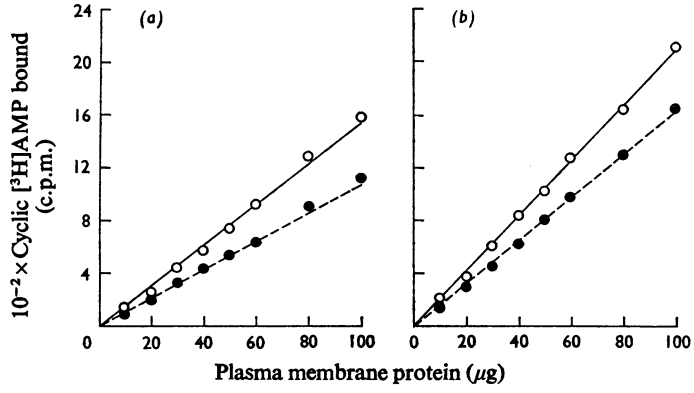

Fig. 6. *Effect of increasing plasma-membrane protein concentrations on cyclic AMP-binding activity*

The incubation medium in a final vol. of 0.2 ml contained: 10 μmol of sodium acetate buffer, pH 4.0; 4 or 8 pmol of cyclic [^{3}H]AMP and indicated amounts of plasma-membrane protein. After incubation at 0°C for 2.5 h the contents of the tube were diluted with 1 ml of 20 mM potassium phosphate buffer, pH 6.0, and then filtered through Millipore filters (0.45 μm pore size). Further details are given in the Materials and Methods section. Values are means of duplicate determinations. (*a*) FI and (*b*) FII enzymes; ●, 20 nM-cyclic [^{3}H]AMP; ○, 40 nM-cyclic [^{3}H]AMP.

brane-associated protein kinases. In contrast with cytosol protein kinase these gonadotrophins had no effect on FI and FII enzymes (results not given).

Cyclic AMP-binding to bovine corpus luteum plasma membranes

As several cyclic AMP-dependent cytosol protein kinases (Brostrom *et al.*, 1970; Gill & Garren, 1970; Tao *et al.*, 1970; Kumon *et al.*, 1972; Corbin *et al.*, 1972; Miyamoto *et al.*, 1973; Traugh & Traut, 1974; Azhar & Menon, 1975*a*) can be separated into catalytic and regulatory subunits, the binding of the cyclic AMP to corpus luteum plasma membranes was tested. The binding measured at 20 and 40 nM-cyclic AMP was linear with increasing concentrations of plasma membranes up to 100 μg of protein as shown in Fig. 6. The cyclic AMP-binding was specific for this nucleotide, as indicated in Fig. 7. A 100-fold higher concentration of unlabelled cyclic AMP almost completely inhibited (80–90%) the binding of cyclic [^{3}H]AMP to the plasma membranes. At a 100-fold higher concentration only cyclic IMP, dibutyryl cyclic AMP and 8-bromo cyclic AMP competed effectively with cyclic AMP. Competition by other cyclic nucleotides was very weak at lower concentrations (0.5 μM) and required 10000-fold molar excess

Table 6. *Effect of cyclic nucleotides on specific binding of cyclic AMP to bovine corpus luteum plasma membranes*

The incubation medium in a final vol. of 0.2ml contained: 10μmol of sodium acetate buffer, pH4.0; 8pmol of cyclic [^{3}H]-AMP; 40μg of membrane proteins; and the indicated concentrations of various cyclic nucleotides or nucleosides. After incubation at 0°C for 2.5h the contents were diluted with 1ml of 20mM-potassium phosphate buffer, pH6.0, and then filtered through Millipore filters (0.45μm pore size). Further details are given in the Materials and Methods section. Values are means of triplicate determinations. The values in parentheses represent relative activities taking the activity in the absence of test substances as 100%.

Addition	10^{-2} × Cyclic [^{3}H]AMP bound (c.p.m./mg of protein)	
	Plasma-membrane fraction	
	FI	FII
No addition	198.0 (100)	246.2 (100)
Cyclic AMP (0.5μM)	33.5 (16.8)	35.4 (14.4)
Cyclic GMP (0.5μM)	180.5 (91.2)	228.5 (92.8)
Cyclic GMP (50μM)	79.0 (40.0)	71.2 (28.9)
Cyclic IMP (0.5μM)	117.3 (59.2)	140.7 (57.1)
Cyclic IMP (50μM)	38.0 (19.2)	37.6 (15.3)
Cyclic UMP (0.5μM)	183.3 (92.6)	242.4 (98.4)
Cyclic UMP (50μM)	82.0 (41.4)	93.7 (38.2)
Cyclic CMP (0.5μM)	175.0 (88.4)	232.4 (94.4)
Cyclic CMP (50μM)	90.5 (45.7)	108.1 (43.9)
Cyclic dTMP (0.5μM)	194.3 (98.1)	237.9 (96.7)
Cyclic dTMP (50μM)	184.0 (92.9)	234.3 (95.2)
Cyclic dAMP (0.5μM)	194.8 (98.4)	234.2 (95.1)
Cyclic dAMP (50μM)	141.0 (71.2)	149.2 (60.6)
8-Bromo cyclic AMP (0.5μM)	109.5 (55.3)	138.0 (56.1)
8-Bromo cyclic AMP (50μM)	33.0 (16.6)	26.3 (10.7)
Dibutyryl cyclic AMP (0.5μM)	115.5 (58.3)	125.4 (50.9)
Dibutyryl cyclic AMP (50μM)	30.8 (15.5)	32.0 (13.0)
5′-AMP (500μM)	197.0 (99.5)	234.8 (95.4)
5′-AMP (5mM)	181.5 (91.7)	214.2 (87.0)
Adenosine (500μM)	197.8 (99.9)	226.8 (92.1)
Adenosine (5mM)	187.5 (94.7)	207.3 (84.2)

Table 7. *Apparent equilibrium association (K_a) and dissociation (K_d) constants for cyclic AMP binding to purified plasma membranes from bovine corpus luteum*

Constants were calculated from the results shown in Fig. 7.

Plasma membrane	Equilibrium association constant (K_a)	Equilibrium dissociation constant (K_d)
Fraction FI	1.9×10^{10}M	0.52×10^{-10}M
Fraction FII	2.6×10^{10}M	0.38×10^{-10}M

to achieve inhibition comparable with that of unlabelled cyclic AMP (Table 6). The inhibition of binding by adenosine and 5′-AMP was insignificant, even at 1.25×10^5-fold higher concentration. Analysis of the binding reaction by the method of Scatchard (1949) demonstrated non-cooperativity and one class of cyclic AMP-binding sites. The apparent association constant (K_a) and dissociation constant (K_d) for FI and FII membrane fractions are presented in Table 7.

Self-phosphorylation of plasma membranes

In the absence of added acceptor proteins, there was extensive self-phosphorylation of plasma-membrane constituents by membrane-associated protein kinases (100–500pmol of [^{32}P]P_i was incorporated/10min per mg of protein). The initial rate of phosphorylation was rapid, especially by FII plasma membranes, and reached a maximum within 15 to 20min of incubation. The endogenous phosphorylation was slightly enhanced by cyclic AMP (Table 1). This observed stimulation by cyclic AMP was found to be due to increased phosphorylation of two specific proteins in membranes (results are presented below).

Characterization of the phosphorylated products

To characterize the phosphorylated products, the identity of the labelled reaction product was established by the procedure described in the Materials and Methods section. The results presented in Table 8 show that only 30–35% radioactivity could be released from phosphorylated plasma membranes with hot trichloroacetic acid and organic solvents.

Table 8. *Characterization of phosphorylated components from plasma membranes of bovine corpus luteum*

In Expt. 1, a total of 200μg of FI or 310μg of FII plasma-membrane (proteins) fractions were self phosphorylated with [γ-^{32}P]ATP (1×10^7c.p.m.) for 15min at 30°C by membrane-associated protein kinases. The acid-insoluble residue was then extracted with trichloroacetic acid at 90°C for 30min. After centrifugation, the residue was extracted with organic solvents (Hajra *et al.*, 1968). The fractions including final residue were then counted for radioactivity. In Expt. 2, 15μg of FI or 20μg of FII plasma-membrane (protein) fraction was used to phosphorylate mixed histone with [γ-^{32}P]ATP (1.2×10^6c.p.m.) by membrane-associated protein kinases. After hot trichloroacetic acid and organic solvent extraction (as described in Expt. 1), the residues were treated with 1M-NaOH at 37°C for 18h. The proteins were then reprecipitated with trichloroacetic acid and radioactivity was then determined both in the soluble fractions as well as in protein residues. Results represent the mean of two separate determinations.

	Radioactivity in plasma-membrane fractions			
	FI		FII	
Treatments	($10^{-2}\times$c.p.m.)	(%)	($10^{-2}\times$c.p.m.)	(%)
Expt. 1				
Solubility characteristics				
1. 10% (w/v) Trichloroacetic acid extraction at 90°C for 30min	14.9	13.8	9.7	9.5
2. Organic solvent extraction	21.3	20.7	18.4	18.2
3. Final protein residue	71.5	65.5	72.8	72.3
	Soluble fraction (c.p.m.)	Residue (c.p.m.)	Soluble fraction (c.p.m.)	Residue (c.p.m.)
Expt. 2				
Alkali-labile radioactivity				
1. None	172	8	201	9
2. Cyclic AMP (5μM)	203	15	275	10
3. Mixed histones (400μg)	301	31	401	41
4. Mixed histones (400μg) + cyclic AMP (5μM)	625	33	840	46

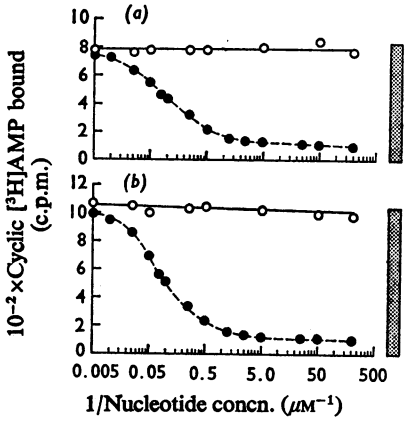

Fig. 7. *Effect of unlabelled cyclic AMP and 5'-AMP on the binding of cyclic [^{3}H]AMP to plasma membranes of bovine corpus luteum*

The incubation medium in a final vol. of 0.2ml contained: 10μmol of sodium acetate, pH4.0; 8pmol of cyclic [^{3}H]AMP; 40μg of membrane protein and indicated concentrations of unlabelled cyclic AMP or 5'-AMP. Other details were similar to those described in Fig. 6. The stippled bar represents the binding of cyclic [^{3}H]AMP to plasma membrane without any unlabelled nucleotide. (*a*) FI and (*b*) FII enzyme. ●, Unlabelled cyclic AMP; ○, 5'-AMP.

Incubation of ^{32}P-labelled proteins with 1M-NaOH at 37°C for 18h released almost all of the radioactivity into the medium, suggesting that the phosphate was linked to proteins by a covalent linkage (Table 8). These results also suggest that the major [^{32}P]P_i incorporated was into membrane protein itself. Additionally, acid hydrolysis and subsequent high-voltage paper electrophoresis of the phosphorylated product revealed that the radioactivity was incorporated into phosphoserine and phosphothreonine residues (Table 9). Approximately 90% of the radioactivity was incorporated into phosphoserine and 10% into phosphothreonine residues (Table 9).

Electrophoretic separation of phosphorylated plasma-membrane proteins

To examine the qualitative and quantitative pattern of protein phosphorylation, plasma membranes phosphorylated with [γ-^{32}P]ATP in the presence and the absence of 5μM-cyclic AMP were solubilized with 1% sodium dodecyl sulphate–1% 2-mercaptoethanol. The individual polypeptides were resolved by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and the gels were either sliced and counted for radioactivity or stained and scanned in a spectrophotometer. Nine distinct phosphorylated bands were

Table 9. *Relative labelling of phosphothreonine and phosphoserine residues from plasma-membrane proteins phosphorylated by membrane-associated protein kinase*

A total of 210μg of FI and 300μg of FII plasma-membrane fractions (protein) were self-phosphorylated for 15min with 10μCi of [γ-^{32}P]ATP as described in the Materials and Methods section. The trichloroacetic acid-insoluble residue was resuspended in 0.4ml of 6M-HCl at 105°C for 5h in sealed ampoules filled with N_2. After drying *in vacuo*, the samples were dissolved in electrophoresis buffer and phosphorylated amino acids were separated by high-voltage paper electrophoresis. Radioactivity corresponding to phosphoserine and phosphothreonine was determined by direct paper-strip counting in a Beckman LS-230 liquid-scintillation spectrometer. Results represent means of duplicate determinations. The ratio is expressed as the radioactivity of phosphoserine over that of phosphothreonine.

	Radioactivity in plasma-membrane fractions					
	FI			FII		
Phosphorylated amino acids	(10^{-2}×c.p.m.)	(%)	Ratio	(10^{-2}×c.p.m.)	(%)	Ratio
Phosphoserine	101	91.5	10.6	96	90.9	10.0
Phosphothreonine	9.4	7.5		9.6	9.1	

identified (Fig. 8*a*) in FI plasma-membrane fractions, and cyclic AMP specifically enhanced the phosphorylation of two peaks designated A_1 (mol.wt. 91000) and A_2 (mol.wt. 76000) of approximately 30–40%. Similarly phosphorylated FII plasma-membrane proteins were resolved into eight distinct bands and, like FI, the phosphorylation of two protein bands, namely B_1 (mol.wt. 87000) and B_2 (mol.wt. 77000), was enhanced by cyclic AMP (Fig. 8*b*).

Discussion

In the present study we sought to examine the properties of bovine corpus luteum-associated protein kinases by using endogenous and exogenous acceptor proteins. In general, catalytic properties of these membrane-associated protein kinases seem to resemble those described for the cytosol enzyme from bovine corpus luteum (Menon, 1973) and from other mammalian tissues (Miyamoto *et al.*, 1969; Corbin & Krebs, 1969; Jard & Bastide, 1970; Jergil & Dixon, 1970; Tao *et al.*, 1970; Reimann *et al.*, 1971; Kumon *et al.*, 1972; Rubin *et al.*, 1972; Miyamoto *et al.*, 1973; Traugh & Traut, 1974). The kinetics of the phosphorylation reaction revealed that cyclic AMP, although it stimulated the phosphotransferase activity, had negligible effect on the apparent K_m values for histone and $MgCl_2$. However, cyclic AMP did slightly decrease the K_m value for ATP.

The activation of the plasma-membrane protein kinases by cyclic AMP is similar to that observed with most cytosol protein kinases. Other 3′:5′-cyclic nucleotides also stimulated, but much higher concentrations were needed except for cyclic IMP. This effect could be due to the structural similarity between cyclic AMP and cyclic IMP (Reimann *et al.*, 1971; Walsh & Krebs, 1973). With regard to the protein substrate specificity, like the enzyme from liver (Langan, 1969*b*), brain (Miyamoto *et al.*, 1969), trout testis (Jergil & Dixon, 1970), heart (Brostrom *et al.*, 1970) or skeletal muscle (Reimann *et al.*, 1971), these enzymes also phosphorylated histones more readily than casein and protamine. In general, both the membrane fractions (FI and FII) did not exhibit considerable variations in their catalytic properties. However, solubilized plasma-membrane protein kinases derived from these two membrane fractions showed considerable differences in their physical properties from each other as well as from the soluble kinases (S. Azhar & K. Menon, unpublished observation).

Beside the phosphorylation reaction, the binding of cyclic AMP to proteins has also been characterized in isolated plasma-membrane fractions FI and FII from bovine corpus luteum. The results of these studies indicate that high-affinity binding and linear Scatchard (1949) binding plots can be demonstrated for both plasma-membrane fractions. The binding activity studied was highly selective for cyclic AMP as shown by competition with related compounds. Since plasma membranes are known to be a rich source of cyclic AMP phosphodiesterases (Russell & Pastan, 1974) and 5′-nucleotidase (Solyom & Trans, 1972) we eliminated the possibility that hydrolysis products of cyclic AMP (instead of cyclic AMP itself) were being bound by showing ineffectiveness of 5′-AMP and adenosine to compete for cyclic AMP binding. Further, the breakdown of cyclic AMP was minimized by using 3-isobutyl-1-methylxanthine and employing low pH for binding reaction (Gilman, 1970; MacKenzie & Stellwagen, 1974).

In addition to exogenous phosphorylation, these membrane-bound protein kinases also phosphorylated the plasma-membrane proteins. Differential solvent extractions revealed that major [^{32}P]P_i incorporation was into the protein components. Susceptibility to hot alkali suggested that the product was a phosphoprotein. The identification of [^{32}P]-

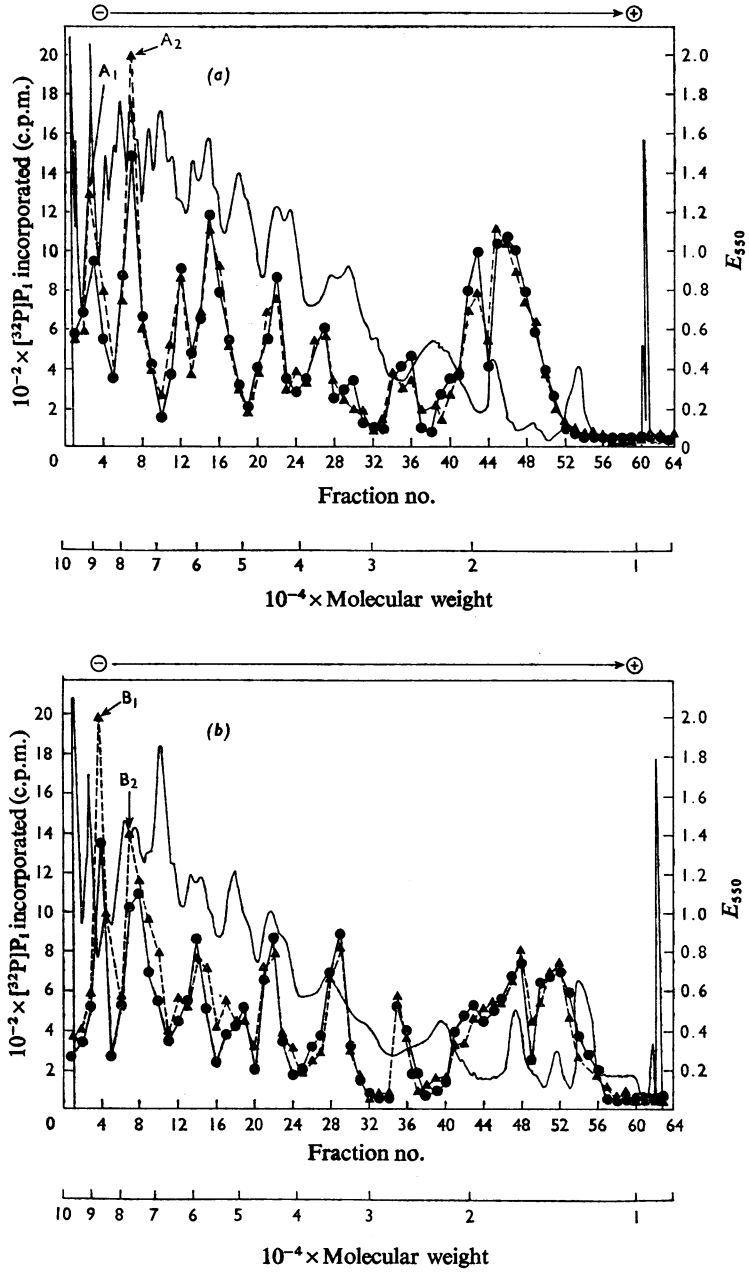

Fig. 8. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of phosphorylated plasma-membrane proteins*

Plasma membranes were phosphorylated in 0.2ml of a mixture containing: 10μmol of α-glycerophosphate buffer, pH6.0; 3μmol of $MgCl_2$; 2μmol of KF; 0.5μmol of theophylline; 250μg of membrane protein; 105nmol of [γ-^{32}P]ATP (7.5×10^7 c.p.m.); and, where required, 1nmol of cyclic AMP. After incubation at 30°C for 10min the reaction was stopped by the addition of 2ml of 7.5% (w/v) trichloroacetic acid. The pellet was washed three times at 0°C by resuspension in 7.5% trichloroacetic acid. The final pellet was washed once with ether. The pellet in each case was solubilized by incubating for 60min at 37°C in a mixture (0.125ml) of 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol, 20% (w/v) sucrose and 0.001% Bromophenol Blue in 0.1M-sodium phosphate buffer, pH7.0. The samples (0.1ml; 200μg of protein) were subjected to electrophoresis as described in the Materials and Methods section. (*a*) FI protein, (*b*) FII protein. ●, Without cyclic AMP; ▲, with 5μM-cyclic AMP; ——, E_{550}.

phosphothreonine and [^{32}P]phosphoserine residues in phosphorylated proteins further suggested that the product was not a phosphopeptide intermediate that is formed in the reaction of membrane-bound adenosine triphosphatase (Hokin *et al.*, 1965; Roses & Appel, 1973).

In the absence of cyclic AMP eight to nine polypeptides of bovine corpus luteum plasma membranes were selectively phosphorylated by membrane-associated protein kinase. However, in the presence of low concentrations (5μM) of this cyclic nucleotide the phosphorylation of two specific proteins was greatly enhanced (Figs. 8*a* and 8*b*). It is possible from these observations and those obtained from exogenous substrates, to ascribe the cyclic AMP-dependent phosphorylation reaction to the stimulation of the activity of protein kinase. The relatively high molecular weight of the two specifically phosphorylated membrane components agrees with the relative high molecular weight of endogenously phosphorylated membrane proteins that have been reported in other systems (Bacalao & Rieber, 1973; Rieber & Bacalao, 1973; Ueda *et al.*, 1973; Roses & Appel, 1973). In synaptic membranes two cyclic AMP-dependent proteins are phosphorylated by endogenous protein kinases (Ueda *et al.*, 1973). The cyclic AMP-dependent endogenous phosphorylation of human erythrocyte ghosts involves proteins of higher molecular weights (Rubin & Rosen, 1973; Roses & Appel, 1973). Rieber & Bacalao (1973) have reported the phosphorylation of high-molecular-weight proteins from chinese-hamster ovarian cells. Cyclic AMP, however, was essentially without effect on the phosphorylation of these ovarian plasma membrane proteins. Chang *et al.* (1974), on the other hand, have described the cyclic AMP-stimulated endogenous phosphorylation of two specific rat adipocyte plasma-membrane proteins whose molecular weights were less than 30000.

The significance of such membrane phosphorylation in terms of regulation of cellular function(s) is currently unknown. Several investigators have proposed the possible regulatory roles of plasma-membrane phosphorylation in modifying cellular functions (Guthrow *et al.*, 1972; Roses & Appel, 1973; Ueda *et al.*, 1973; Chang *et al.*, 1974) and membrane-bound adenylate cyclase responsive to hormone and F^- (DeLorenzo *et al.*, 1973; Najjar & Constantopoulos, 1973; Schmidt *et al.*, 1974). From present studies it is conceivable that protein kinase(s) associated with the plasma membrane and the processes of phosphorylation and dephosphorylation of two plasma-membrane proteins may have regulatory functions at the level of the cell membrane in the intact luteal cell. In this context we have reported that the plasma membranes from bovine corpus luteum possess gonadotrophin-stimulated adenylate cyclase and gonadotrophin-binding activities (Menon & Kiburz, 1974). However, the role of membrane-associated protein kinases in the modification of corpus luteum function remains merely speculative.

We thank Mrs. Kathie Nosal for excellent technical assistance and Mrs. Joanne Robinson and Mrs. Cindy Giraud for expert secretarial assistance. This work was supported in part by Grant no. HD06656 from NIH and the Population Council. S. A. is a Population Council Postdoctoral Fellow.

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