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

Solubilization and Properties of Solubilized Enzyme

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The solubilization of plasma membrane fractions FI and FII associated protein kinases has been attempted using monovalent salts of high ionic strength and various detergent treatments. Extraction of FI and FII plasma membranes with high ionic strength salt solutions did not release more than 20% of the protein kinase activity. Similarly, monovalent salts released little adenosine 3′:5′-monophosphate (cyclic AMP) binding activity, but after extraction binding capacity of cyclic [^{3}H]AMP to plasma membranes was increased about 150–200%. Triton X-100 was a better solubilizing agent than Lubrol WX or deoxycholate. In addition to solubilization, 0.1% Triton X-100 also stimulated the protein kinase activity 150–200%. The properties of Triton X-100 solubilized FI and FII and purified cytosol KII were characterized with respect to protein substrate specificity, effect of cyclic AMP, cyclic nucleotide specificity, effects of divalent metal ion and gonadotropins. Upon sucrose density gradient centrifugation, FI solubilized protein kinase and cyclic AMP binding activities co-sedimented with a sedimentation coefficient of 6.3 S. The FII solubilized protein kinase sedimented as two components with sedimentation coefficients of 7.7 S and 5.5 S. The cyclic AMP binding activity also sedimented as two components with sedimentation coefficient 6.7 S and 5.5 S. Cyclic AMP caused dissociation of solubilized protein kinase from FI into a single catalytic (4.8 S) and two cyclic AMP binding subunits (8.1 S and 6.7 S). FII solubilized enzyme was dissociated into one catalytic (4.8 S) and one cyclic AMP binding subunit (6.3 S). Fractionation of FI and FII solubilized enzymes on DEAE-cellulose column chromatography resolved them each into two peaks I_a, I_b and II_a, II_b, respectively. Peaks I_b and II_b were more sensitive to cyclic AMP stimulation than I_a and II_a peaks. From these studies it is concluded that the plasma-membrane-associated and cytosol protein kinases have similar catalytic properties but differ in some of their physical properties.

It is now widely accepted that protein kinases dependent on adenosine 3′:5′-monophosphate (cyclic AMP) are the immediate receptors for cyclic AMP in several tissues [1–8]. Cyclic-AMP-dependent protein kinases from several sources including skeletal muscle [6–9], brain [10,11], liver [7,12], heart muscle [13], adipose tissue [8,14,15] and rabbit reticulocytes [16] have been isolated and well characterized. In most cases protein kinases have been shown to consist of two dissimilar functional subunits: a catalytic subunit (C) and a cyclic AMP binding subunit or regulatory subunit (R). The activation of kinase is brought about by the binding of cyclic AMP to the regulatory subunit thereby releasing the fully active catalytic subunit to catalyze the phosphotransferase reaction [17–21].

$$RC + \text{Cyclic AMP} \rightleftharpoons R \cdot \text{Cyclic AMP} + C$$

Although the presence of particulate protein kinases has been shown in some systems [22–28],

Abbreviations. Cyclic AMP, adenosine 3′:5′-monophosphate; cyclic GMP, guanosine 3′:5′-monophosphate; cyclic IMP, inosine 3′:5′-monophosphate; cyclic CMP, cytidine 3′:5′-monophosphate; cyclic UMP, uridine 3′:5′-monophosphate; cyclic dTMP, thymidine 3′:5′-monophosphate; cyclic dAMP, 2′-deoxyadenosine 3′:5′-monophosphate; solubilized FI, solubilized protein kinase from FI plasma membrane; solubilized FII, solubilized protein kinase from FII plasma membrane; cytosol KII, cytosol protein kinase peak II; EGTA, ethyleneglycol bis(2-aminoethyl)-*N*,*N*′-tetraacetic acid.

relatively little attention has been focused on the mechanism of stimulation of these kinases by cyclic AMP [22–28].

Previously we have reported purification and properties of cytosol protein kinases from bovine corpus luteum [29]. About 50% of the total protein kinase activity was found to be associated with the particulate fractions of the cell [29]. More recently we have shown that highly purified bovine corpus luteum plasma membrane fractions, FI and FII, possess cyclic-AMP-dependent protein kinases which can phosphorylate both endogenous and exogenous acceptor proteins [30]. In addition, these plasma membranes also contained gonadotropin-binding and gonadotropin-stimulated adenylate cyclase activities [31,32].

In this communication we describe the solubilization and characterization of protein kinases and cyclic AMP binding activities associated with bovine corpus luteum plasma membrane, the properties of these enzymes are compared with those of the cytosol protein kinase [29]. The results show that plasma-membrane-associated and cytosol protein kinases from bovine corpus luteum have similar catalytic properties but differ in their physical characteristics.

EXPERIMENTAL PROCEDURE

Materials

Bovine corpora lutea were collected from a local slaughterhouse and processed immediately or stored at −80 °C until used. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [33]. The following hormones were generously supplied by the hormone distribution program (National Institutes of Arthritis and Metabolic Diseases, NIH): bovine follicle-stimulating hormone B1, porcine follicle-stimulating hormone P1, bovine luteinizing hormone B8 and ovine luteinizing hormone S18. Histone (type IIA, III, IV, VI, VIII), protamine, casein, albumin, cyclic AMP, cyclic GMP, cyclic dTMP, cyclic UMP, cyclic CMP, and cyclic dAMP were purchased from Sigma Chemical Company. 1-Methyl-3-isobutylxanthine was obtained from Aldrich Chemical Company. ^{32}P-labelled inorganic phosphate (carrier-free) was purchased from International Chemical and Nuclear Corporation and cyclic [^{3}H]AMP (16 Ci/mmol) from Schwarz-Mann. All other chemicals used were of analytical reagent grade.

Preparation of Plasma Membranes

Plasma membrane fractions FI and FII were isolated from bovine corpus luteum according to the method described by Gospodorowicz [31].

Determination of Protein Kinase Activity

Protein kinase activity was determined by previously published procedure [29–34]. The incubation mixture contained in a final volume of 200 µl, 10 µmol glycerol 2-phosphate buffer, pH 6.0, 10 nmol [γ-^{32}P]-ATP (3×10^6 counts/min), 2 µmol KF, 3 µmol $MgCl_2$, 0.5 µmol theophylline, 400 µg calf thymus histone and 10–20 µg enzyme protein. All incubations were carried out at 30 °C for 5 min, in duplicate. Unless otherwise stated, all data on exogenous protein phosphorylation were corrected for membrane phosphorylation without added substrates. Enzyme activity is expressed as picomoles [^{32}P]phosphate transferred/10 min. Specific activity is expressed as the activity units/mg protein.

Cyclic AMP Binding Assays

Cyclic AMP binding assay was carried out by a modification of the procedure of Gilman [35]. The assay was performed in a final volume of 0.2 ml containing 0.02 to 35 pmol cyclic [^{3}H]AMP, 10 µmol phosphate buffer, pH 6.0, 0.5 µmol 1-methyl-3-isobutylxanthine, 2 µmol KF and 50–100 µg membrane protein. The incubations were carried out at 0 °C for 2 h and were terminated by the addition of 2 ml of cold buffer (10 mM Tris · HCl, pH 8.0, containing 40 mM $MgCl_2$) and after 5 min the solutions were filtered through millipore filters (HAWP 025, 0.45 µm). The filters were then washed with 10 ml buffer and dried under an infrared lamp. The dried filters were then transferred to scintillation vials and dissolved in 1 ml of cellosolve. To these vials were added 10 ml of scintillation fluid made up of 3 parts of toluene and 1 part of cellosolve containing 4 g of PPO and 50 mg POPOP per liter 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 1 mM unlabelled cyclic AMP from that retained in the absence of unlabelled cyclic AMP.

Extraction of Plasma-Membrane-Associated Protein Kinase and Cyclic AMP Binding Activities by Monovalent Salts

Plasma membrane fractions FI or FII were suspended (1 mg protein/ml) in buffer A (20 mM Tris · HCl, pH 7.4; 5 mM 2-mercaptoethanol; 20% glycerol, v/v) containing either NaCl, KCl, LiCl or NH_4Cl in a final concentration of 1 M and the suspension was allowed to stand for 60 min at 4 °C and then centrifuged at $150000 \times g$ for 60 min. The supernatant was carefully aspirated and the pellet was washed with the same volume of salt solution in buffer A. After a second centrifugation, the pellet in each case

was again suspended in buffer A. The supernatants and sediments were then dialyzed for 16 h against two changes of buffer A. These fractions were then analyzed directly for protein kinase and cyclic AMP binding activities without further treatment.

Solubilization of Plasma-Membrane-Associated Protein Kinase and Cyclic AMP Binding Activities by Ionic and Nonionic Detergents

Plasma membrane fractions FI and FII were suspended in buffer B (50 mM Tris · HCl, pH 7.4, 100 mM KCl, 1 mM 2-mercaptoethanol) containing different concentrations of various ionic and nonionic detergents and the suspensions were allowed to stand for 60 min at 4 °C and then centrifuged at $150000 \times g$ for 60 min. The supernatants were carefully aspirated and the pellets were washed with the same volume of respective detergent solutions. The sediments in each case, after the second centrifugation, were resuspended in the original volume of buffer B containing various detergents. The addition of detergents to the sediments was necessary since some of the detergent unmasked the kinase activity. The supernatants and sediments were then assayed for protein kinase and cyclic AMP binding activities without further processing.

Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation of detergent-solubilized enzymes were performed by modification of the procedure of Kuczenski [36]. Samples (200 µl) containing 50 – 100 µg protein were layered over 4.8 ml of 5 to 20 % linear sucrose density gradients in buffer C (20 mM Tris · HCl, pH 7.4; 0.1 mM EGTA; 1 mM dithioerythritol; 0.1 % Triton X-100) and centrifugations were performed at 38000 rev./min in Beckman SW 50.1 rotor for 15 h at 4 °C. At the end of the centrifugation the bottom of each tube was punctured and 11-drop fractions were collected. The sedimentation coefficients and molecular weight determinations were carried out according to the method of Martin and Ames [37] with catalase (11.6 S, mol. wt 232000), glyceraldehyde-3-phosphate dehydrogenase (7.7 S, mol. wt 140000) and horse liver alcohol dehydrogenase (5.4 S, mol. wt 84000) as internal markers [11].

Other Methods

Catalase was assayed by the method of Beers and Sizer [38], glyceraldehyde-3-phosphate dehydrogenase by the method of Velick [39] and alcohol dehydrogenase by the method of Vallee and Hock [40]. Protein concentration was measured by the method of Lowry *et al.* [41]. When samples contained more than 0.3 mg of Triton X-100, the detergent was removed by treating the sample with acetone, air-drying the protein precipitate and assaying in the usual manner.

RESULTS

Extraction of Plasma-Membrane-Associated Protein Kinase by Monovalent Salts and Detergents

To gain information on the mode of association of protein kinase to the plasma membranes, the plasma membrane fraction FI and FII were extracted with monovalent salts of high ionic strength and also with ionic and non-ionic detergents as described in the Experimental section. Following extraction, both phosphotransferase and cyclic AMP binding activities were assayed in the soluble and sediment fractions after separation by centrifugation at $150000 \times g$ for 60 min. A major part of protein kinase and cyclic AMP binding activities were strongly associated with plasma membranes since only minute amounts (15 – 20 %) of these were solubilized by extraction with monovalent salts (NaCl, NH_4Cl, LiCl and KCl) of high ionic strength (1.0 M). Further extraction in media of the same or higher salt concentrations (up to 2.0 M) did not release any additional enzyme. However, extraction of plasma membranes with various monovalent salts resulted in an increase of cyclic AMP binding activity to about 150 – 200 %, compared to untreated membranes. Almost analogous results were obtained when either FI or FII plasma membranes were treated with different monovalent salts.

The solubilization of plasma-membrane-associated protein kinase and cyclic AMP binding activity was also attempted using ionic and nonionic detergents. The results presented in Tables 1 and 2 show that the treatment of FI and FII plasma membrane fractions with sodium deoxycholate, Lubrol WX and Triton X-100 caused the release of protein kinase and cyclic AMP binding activities into the soluble fraction and the amount of the enzyme released varied with the treatment. In addition to solubilization, these agents also unmasked the latent protein kinase activity with an overall increase of 120 to 200 %. Cyclic AMP binding activity was released along with protein kinase by detergents, but at higher detergent concentrations, the binding activity was inhibited by detergent interference with the assay system. Triton X-100 was the better solubilizing agent when compared to other detergents. A final concentration of 0.1 % of Triton X-100 was chosen for all subsequent studies (Tables 1, 2). At this concentration solubilization of plasma-membrane-associated protein kinase was maximum with a negligible interference in cyclic AMP binding assays. Lower concentrations of Triton had little or no effect on the release of the enzyme while higher concentrations were inhibitory. The presence of the

Table 1. *Solubilization of plasma-membrane-associated protein kinases by ionic and non-ionic detergents from bovine corpus luteum*
Plasma membranes were suspended (1 mg protein/ml) in buffer B (50 mM Tris · HCl, pH 7.4, 100 mM KCl, 1 mM 2-mercaptoethanol) containing indicated concentrations of various detergents. After incubation at 4 °C for 1 h, the samples were centrifuged at 150000 × *g* for 60 min. Supernatants and sediments in each case were analyzed for protein kinase activity both in the absence or in the absence of 5 μM cyclic AMP. n.a. = no activity detected

Treatment	[^{32}P]Phosphate transferred			
	sediment		supernatant	
	− cyclic AMP	+ cyclic AMP	− cyclic AMP	+ cyclic AMP
Plasma membrane FI	pmol/mg protein			
None	330	680	n.a.	n.a.
Deoxycholate (0.05%)	140	360	300	1120
Deoxycholate (0.10%)	210	460	160	600
Lubrol WX (0.10%)	100	280	300	1040
Lubrol WX (0.20%)	90	290	320	970
Triton X-100 (0.10%)	300	720	450	1530
Triton X-100 (0.25%)	290	630	510	1320
Triton X-100 (0.50%)	140	390	620	1270
Plasma membrane FII				
None	440	1010	n.a.	n.a.
Deoxycholate (0.05%)	200	440	340	900
Deoxycholate (0.10%)	270	680	180	630
Lubrol WX (0.10%)	150	480	310	1050
Lubrol WX (0.20%)	150	450	320	1010
Triton X-100 (0.10%)	350	680	520	1410
Triton X-100 (0.25%)	320	540	570	1230
Triton X-100 (0.50%)	260	630	400	860

Table 2. *Solubilization of plasma-membrane-associated cyclic AMP binding activity by ionic and non-ionic detergents*
Solubilization of plasma membranes were carried out as described under Table 1. Supernatants and sediments were then assayed for cyclic AMP binding activity as described in the text. n.a. = no activity detected

Treatment	Cyclic [^{3}H]AMP bound			
	plasma fraction FI		plasma fraction FII	
	sediment	supernatant	sediment	supernatant
	counts min^{-1} mg protein^{-1}			
None	7500	n.a.	7800	n.a.
Deoxycholate (0.05%)	6360	6420	5960	6660
Deoxycholate (0.10%)	5620	3540	7760	3960
Lubrol WX (0.10%)	5580	4700	4520	3780
Lubrol WX (0.20%)	6040	2020	4320	2300
Triton X-100 (0.10%)	3450	4800	3420	4350
Triton X-100 (0.25%)	2500	2300	3000	2740
Triton X-100 (0.50%)	1880	1000	3680	2160

detergent was necessary for the maintenance of solubilized protein kinase activity and removal of detergent resulted in loss of activity accompanied by the formation of aggregated proteins (data not shown).

Properties of Solubilized FI, FII and Cytosol Protein Kinase II

The kinetic properties of the solubilized enzyme from plasma membranes FI and FII and cytosol protein kinase II were examined in an effort to elucidate the relationship between the bound form of the enzyme and the soluble enzyme. The phosphorylation of histone by solubilized plasma membrane protein kinase proceeded linearly up to 15–20 min of incubation in the presence or absence of 5 μM cyclic AMP (Fig. 1). Both FI and FII enzyme activities were linear up to 24–36 μg protein per 0.2 ml of incubation medium (Fig. 2) in the presence or absence of cyclic AMP (5 μM). Results presented in Fig. 3 show the binding of cyclic [^{3}H]AMP with increasing amounts of detergent-solubilized protein from plasma membranes. The binding was linear up to 30 μg of protein when assayed in the presence of 10 mM and 40 nM cyclic [^{3}H]AMP concentrations. Binding was also carried out as a function of cyclic AMP concentration and the data thus obtained were used to construct a Scatchard plot [42] which demonstrated two classes of cyclic AMP binding sites with apparent dissociation constants (K_d) of 1.2×10^{-11} M and 2.2×10^{-9} M, respectively (Fig. 4). Similar results were obtained with FII solubilized plasma membrane fraction. The results presented in Table 3 summarize the protein substrate specificity of solubilized FI, FII and cytosol KII. All these enzyme preparations showed a higher degree of activity towards mixed histone/arginine-rich histone than lysine-rich histone, protamine, casein or albumin. Cyclic AMP (5 μM) affected the extent of phosphorylation to a varying degree depending upon the source of protein kinase and the substrate employed (Table 3). All these kinases were stimulated by low concentrations of cyclic AMP (Fig. 5) and the apparent K_a values were in the range of 43 to 47 nM (Table 4). Cyclic GMP and cyclic IMP also stimulated these protein kinases, but K_a values of these nucleotides were much higher (between 1 to 25 μM) than that of cyclic AMP. Other nucleotides such as cyclic UMP, cyclic CMP, cyclic dTMP and cyclic dAMP also stimulated these kinases at higher concentrations (Table 5).

A comparison of the effects of divalent metal ions was also made between the solubilized enzymes FI, FII and cytosol KII. All three enzyme preparations exhibited an absolute requirement for divalent metal ion: Mg^{2+}, Mn^{2+} and Co^{2+} supported protein kinase activity, Zn^{2+} was slightly effective and Ca^{2+} was without effect (Table 6). Maximum activity for all

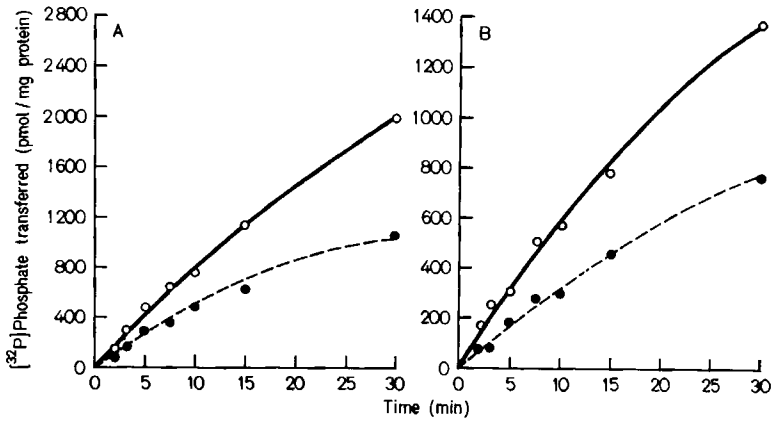

Fig. 1. *Effect of incubation time on the activity of solubilized protein kinase from plasma membrane.* The incubation conditions were similar to that described in the text. (●– – –●) Minus cyclic AMP; (○——○) plus cyclic AMP (5 μM). (A) Solubilized FI; (B) solubilized FII

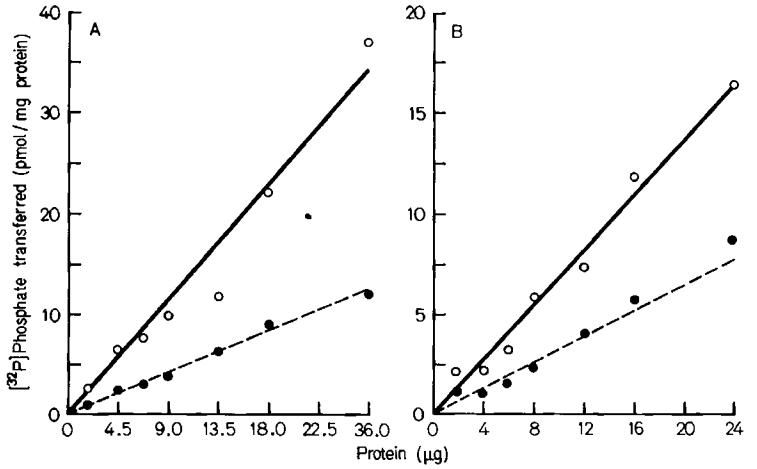

Fig. 2. *Effect of varying the amount of solubilized protein on protein kinase activity.* Assay conditions were similar as described under Materials and Methods except for variation in protein. Incubations were carried out for 5 min. (●– – –●) Minus cyclic AMP; (○——○) plus cyclic AMP (5 μM). (A) Solubilized FI; (B) solubilized FII

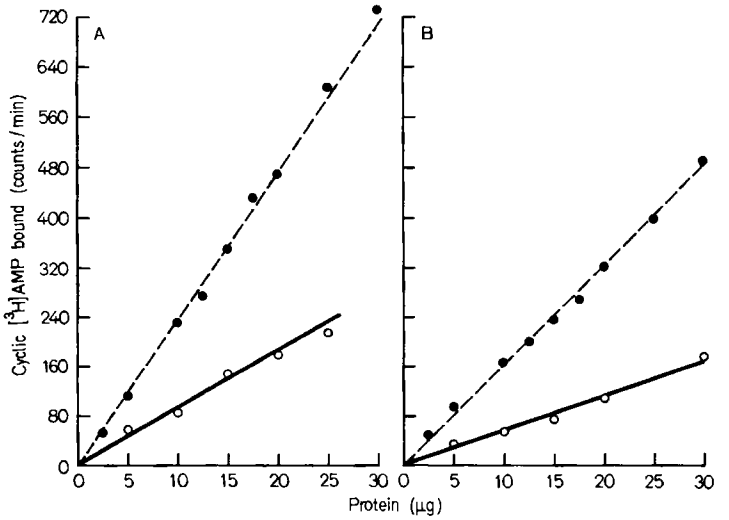

Fig. 3. *Effect of varying the solubilized plasma membrane protein concentration on the cyclic [^{3}H]AMP binding activity.* Cyclic AMP binding activity was carried out as described in the experimental section except that indicated concentrations of membrane proteins were added to the incubation medium. (●– – –●) 10 nM cyclic [^{3}H]AMP; (○——○) 40 nM cyclic [^{3}H]AMP. (A) Solubilized FI; (B) solubilized FII

Table 3. *Comparative substrate specificity of detergent-solubilized plasma membrane protein kinases and soluble protein kinase II from bovine corpus luteum*
Incubation conditions were similar as described under Materials and Methods. Each of the different protein substrates were present in equal concentrations by weight (400 μg). When present, cyclic AMP concentration was 5 μM

Substrate	[^{32}P]Phosphate transferred					
	solubilized FI		solubilized FII		soluble protein kinase II	
	− cyclic AMP	+ cyclic AMP	− cyclic AMP	+ cyclic AMP	− cyclic AMP	+ cyclic AMP
	pmol/mg protein					
Mixed histone	360	1400	270	1370	1900	11630
Arginine-rich histone	330	1340	180	840	2080	14680
Arginine-rich histone (f3)	300	960	200	710	1030	5350
Lysine-rich histone (f1)	170	180	30	340	340	490
Slightly-lysine-rich histone (f2a)	20	80	60	320	490	3620
Protamine	1000	1530	470	810	840	4830
Casein	400	410	200	200	1440	1410
Albumin	130	90	60	30	70	30

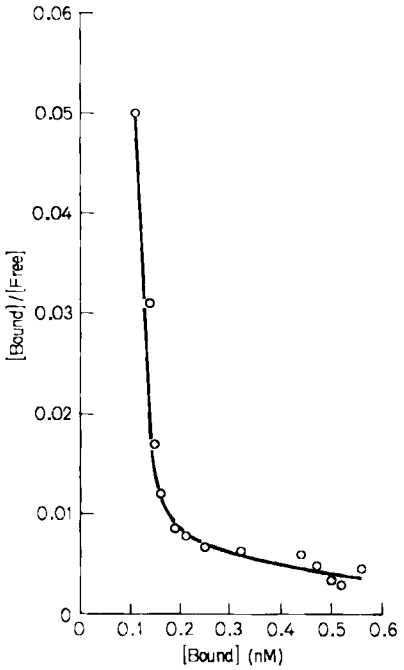

Fig. 4. *Scatchard plot for the binding of cyclic [^{3}H]AMP to detergent-solubilized FI plasma membrane fraction.* The cyclic AMP concentration utilized were in the range of 0.1 nM to 175 nM. [Bound] = concentration of bound cyclic [^{3}H]AMP; [Free] = concentration of free [^{3}H]AMP

these enzymes preparations was observed in the presence of 10 mM Co^{2+}, but maximal cyclic AMP stimulatory effect was observed in the presence of 10 mM Mg^{2+}. Follicle-stimulating hormone, luteinizing hormone and human chorionic gonadotropin did not shown any stimulatory effect on solubilized FI and FII protein kinases. In contrast to these observations cytosol protein kinase II has been shown to be stimulated by luteinizing hormone [29, 43, 51].

Table 4. *Apparent K_a values of cyclic nucleotides for solubilized plasma-membrane-associated protein kinase from bovine corpus luteum*
The apparent K_m for activation (K_a) of cyclic nucleotides were calculated according to Lineweaver and Burk [50] from the data shown in Fig. 5

Enzyme	K_a values		
	cyclic AMP	cyclic GMP	cyclic IMP
	nM	μM	
Solubilized FI	47	25	1.3
Solubilized FII	43	10	1.5
Cytosol KII	20[a]	20	3.0

[a] Taken from [29].

Sucrose Density Gradient Centrifugation

In an effort to determine whether the FI and FII enzymes solubilized with Triton X-100 exhibit any gross differences in physical structure from the cytosol enzyme (KII), aliquots of the cytosol KII and solubilized enzymes were separately applied on linear sucrose density gradients and centrifugations were carried out as described under the experimental section. The results are presented in Fig. 6. The detergent-solubilized FI enzyme sedimented as a single species with a position corresponding to a sedimentation coefficient of 6.3 S (mol. wt 126000). Cyclic AMP binding activity also sedimented in the same position with a sedimentation coefficient of 6.3 S (mol. wt 126000).

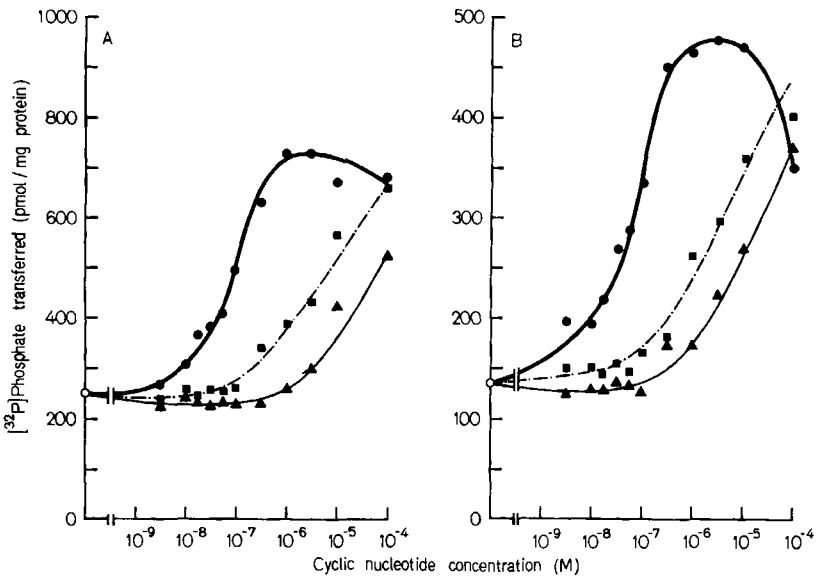

Fig. 5. *Effect of increasing concentrations of cyclic AMP, cyclic GMP and cyclic IMP on solubilized FI, FII and cytosol KII protein kinases.* Incubation conditions were similar to that described under Experimental Procedure except for the variation in the concentration of the indicated cyclic nucleotides. (●—●) Cyclic AMP; (▲—▲) cyclic GMP; (■·-·-·■) cyclic IMP. (A) Solubilized FI; (B) solubilized FII

Table 5. *Effect of certain nucleotides on detergent-solubilized plasma-membrane-associated protein kinases and cytosol protein kinase from bovine corpus luteum*
Standard assay conditions were employed except for the addition of indicated concentrations of other cyclic nucleotides

Cyclic nucleotide	(concn)	[^{32}P]Phosphate transferred		
		solubilized FI	solubilized FII	cytosol KII
		pmol/mg protein		
None		770	350	1340
Cyclic AMP	(1 μM)	1690	1130	5970
Cyclic AMP	(5 μM)	1640	1180	5140
Cyclic CMP	(5 μM)	1050	770	–
Cyclic CMP	(0.1 mM)	1470	1110	5760
Cyclic UMP	(5 μM)	900	860	3290
Cyclic UMP	(0.1 mM)	1390	1060	5170
Cyclic dTMP	(5 μM)	730	520	1130
Cyclic dTMP	(0.1 mM)	950	690	1860
Cyclic dAMP	(5 μM)	830	640	2110
Cyclic dAMP	(0.1 mM)	1250	940	5110

Solubilized FII enzyme sedimented as one minor and one major component with sedimentation coefficients of 7.7 S (mol. wt 156000) and 5.5 S (mol. wt 110000), respectively. Cyclic AMP binding activity also sedimented as two components with sedimentation coefficients of 6.7 S (mol. wt 134000) and 5.5 S (mol. wt 110000), respectively. The cytosol protein kinase II, on the other hand, sedimented as a single peak with a sedimentation coefficient of 7.4 S (mol. wt 144000). Cyclic AMP binding activity also sedimented with a sedimentation coefficient of 7.4 S.

Table 6. *Effect of divalent metal ion on detergent-solubilized plasma-membrane-associated and cytosol protein kinase KII from bovine corpus luteum*
The incubation conditions were similar to that described in the text except indicated concentrations of various divalent metal ions were also included in the incubation mixture. Cyclic AMP concentration (when present) was 5 μM

Divalent metal ion (M^{2+})	[^{32}P]Phosphate transferred at			
	2 mM M^{2+}		10 mM M^{2+}	
	– cyclic AMP	+ cyclic AMP	– cyclic AMP	+ cyclic AMP
Solubilized FI	pmol/mg protein			
None	0	0		
Mg^{2+}	460	1050	850	1960
Mn^{2+}	540	1000	780	1860
Co^{2+}	790	1520	1000	1570
Zn^{2+}	230	180	–	–
Ca^{2+}	0	0	0	0
Solubilized FII				
None	0	0		
Mg^{2+}	190	520	370	930
Mn^{2+}	170	480	360	900
Co^{2+}	390	740	530	880
Zn^{2+}	90	80	90	90
Ca^{2+}	0	0	0	0
Cytosol KII				
None	500	580		
Mg^{2+}	1050	6670	2210	13400
Mn^{2+}	920	7500	2030	15000
Co^{2+}	2050	12840	2250	10600
Zn^{2+}	350	700	160	210
Ca^{2+}	90	70	80	60

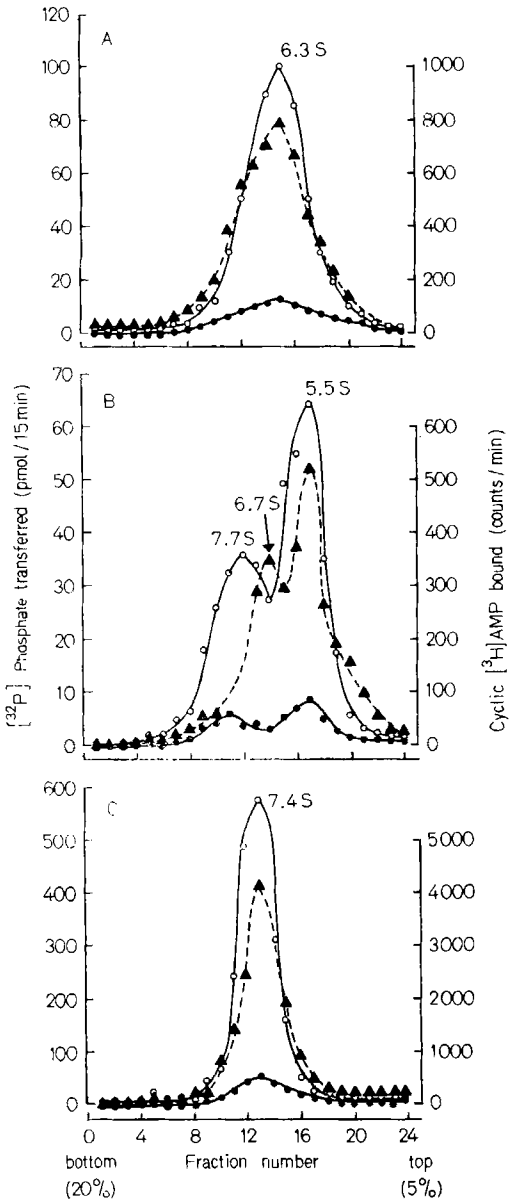

Fig. 6. *Sucrose density gradient centrifugation of (A) solubilized FI, (B) solubilized FII and (C) cytosol KII protein kinases.* Aliquots of solubilized FI (100 μg), FII (100 μg) and cytosol KII (80 μg) in 0.2 ml of 20 mM Tris · HCl, pH 7.4, 0.1 mM EGTA, 1 mM dithioerythritol and 0.1% Triton X-100 were layered in separate centrifuge tubes, onto 4.8 ml of 5–20% sucrose density gradient containing the same concentration of Tris, EGTA, Triton X-100 and dithioerythritol. After centrifugation, fractions from each tube were assayed for kinase activity in the presence (○——○) or absence (●——●) of 5 μM cyclic AMP. Cyclic AMP binding activities were also determined in each fraction (▲ ▲)

Dissociation and Activation of Solubilized FI and FII Protein Kinases by Cyclic AMP

Cyclic AMP stimulates all cytosol protein kinases including corpus luteum cytosol KII [43], by binding of the nucleotide to the regulatory subunit resulting in the release of the catalytic moiety [17–21]. We have tested this mechanism for solubilized FI and FII plasma membrane enzymes. When preincubated with 5 μM cyclic AMP and then centrifuged in the presence of cyclic AMP (1 μM), both FI and FII enzymes were dissociated into catalytic (phototransferase) and cyclic AMP binding subunits. FI enzyme was dissociated into a catalytic moiety with a sedimentation coefficient of 4.8 S (mol. wt 96000) and two cyclic AMP binding moieties with sedimentation coefficients of 8.1 S (mol. wt 168000) and 6.7 S (mol. wt 134000), respectively. Similarly, solubilized FII was also dissociated by cyclic AMP into a catalytic moiety and cyclic AMP binding moiety with sedimentation coefficients of 4.8 S (mol. wt 96000) and 6.3 S (mol. wt 124000).

DEAE-Cellulose Column Chromatography of Solubilized FI and FII Enzyme

Since soluble protein kinases have been shown to be present in more than one isoenzymic form [9, 11–16], we have tested whether the solubilized FI and FII exist in more than one such form. Results presented in Fig. 7 show that DEAE-cellulose chromatography of solubilized enzymes resulted in the separation of two peaks. Solubilized FI was resolved into I_a and I_b. Peak I_a was practically insensitive to cyclic AMP, but I_b was greatly stimulated by cyclic AMP (3–5-fold). Only one cyclic AMP binding activity was detected which was associated with I_b peak. Similar elution profile was also found with FII solubilized enzyme. Using this procedure, some purification of these enzymes (5–8-fold) was also achieved.

DISCUSSION

The results presented here demonstrate the solubilization and characterization of plasma-membrane-associated protein kinases from bovine corpus luteum. Protein kinase associated with plasma membranes was strongly attached to the plasma membrane matrix, and could be solubilized either by ionic or nonionic detergent treatments. Extraction of plasma membranes with monovalent salts of high ionic strength resulted in the release of a very small amount of protein kinase activity, further suggesting their strong association with the membrane constituents. This strong association could be due to the presence of large amount of lipids and phospholipids in the plasma membrane from bovine corpus luteum [31]. Bovine corpus luteum plasma membrane fractions FI and FII have been shown to contain about 0.66 mg and 0.37 mg of protein, respectively [31]. In a preliminary experiment, it was found that treatment of plasma membranes with *n*-butanol or acetone resulted

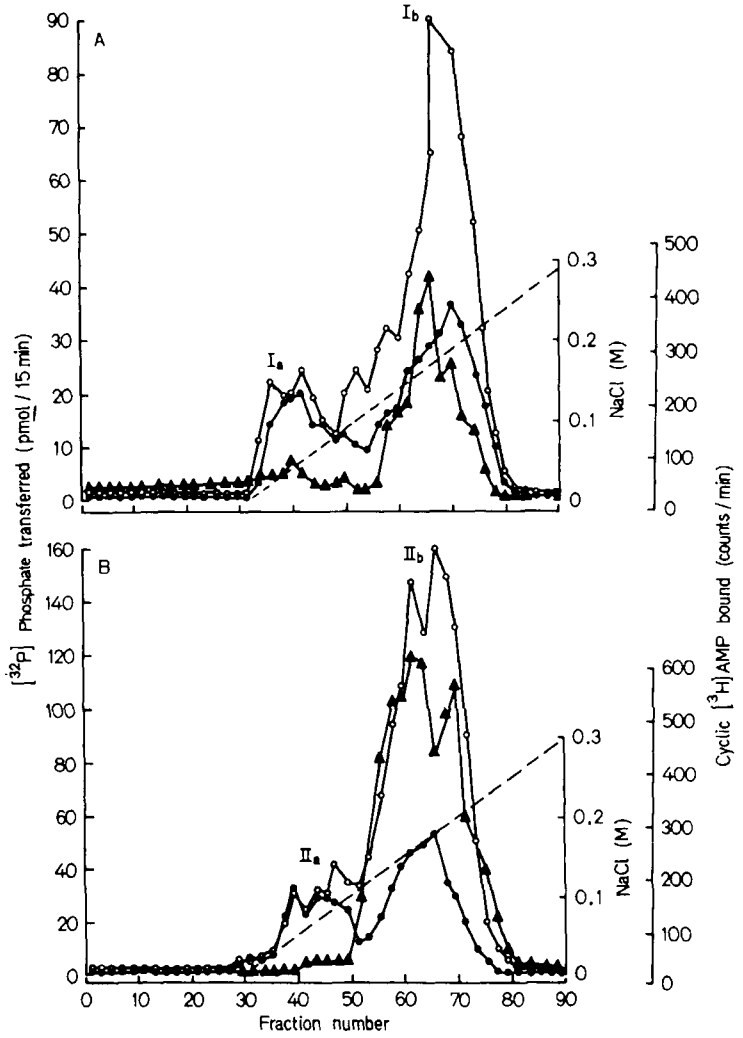

Fig. 7. *DEAE-cellulose column chromatography of (A) solubilized FI and (B) solubilized FII protein kinases.* Solubilized FI (1 02 mg protein, 0.0602 units) and FII (1.64 mg protein and 0.084 units) were applied on DEAE-cellulose column (11 × 1.5 cm) previously equilibrated with buffer D (50 mM Tris HCl, pH 7.4, 1 mM 2-mercaptoethanol and 0.1 % Triton X-100). The column was washed with 20 volumes of buffer D and then protein kinase and cyclic AMP binding activities were eluted with a linear gradient of 0.05 M to 0.45 M NaCl (35 ml each in buffer D). 1-ml fractions were collected and protein kinase activity was determined in alternate fractions both in the presence (○——○) and absence (●——●) of 5 μM cyclic AMP. Cyclic AMP binding activity was also determined in alternate fractions (▲——▲). (----) NaCl concentration

in the major loss of protein kinase and cyclic AMP binding activities. Both of these procedures resulted in the removal of large proportions of lipids. Solubilization with Triton X-100 also removed phospholipids but the enzymic activity was not lost even when solubilized enzyme was subjected to DEAE-cellulose chromatography. In this procedure, although the lipids may still be removed the detergent may maintain the activity by providing the necessary structural requirements for stabilization of the enzyme. Removal of Triton X-100 resulted in the loss of enzyme activity accompanied by the formation of aggregated protein. Such aggregation phenomenon has also been observed upon reduction of the detergent concentrations in detergent-solubilized membrane preparation from a variety of sources [44–47], although it is not necessarily accompanied by inactivation of enzymic activity [47].

The catalytic and cyclic AMP binding components of plasma-membrane-associated protein kinases seem to be strongly bound together and both activities were released together when either salt or detergent extraction procedures were employed. In contrast, Rubin *et al.* [24] have shown that erythrocyte membrane-bound protein kinase could be dissociated by high salt extraction into a catalytic activity with concomitant loss of cyclic AMP dependence and a cyclic AMP binding activity which remained associated with the membrane. In the same system Triton X-100 failed to solubilize protein kinase activity completely. The bovine adenohypophyseal plasma-membrane-associated protein kinase has been shown to

be solubilized equally both by NH_4Cl or Triton X-100 treatments [28]. Although the solubilized enzymes prepared by either procedure were responsive to cyclic AMP, quantitative data were not available on the solubilization of cyclic AMP binding activity [28]. Thus the plasma-membrane-associated protein kinases from different sources seem to have different characteristics, which may be in part dependent upon the nature of their association with membrane components.

The solubilized protein kinases exhibit the usual catalytic properties of protein kinases. These kinases displayed a maximum activity with histone in the presence of cyclic AMP and were completely dependent on divalent metal ions. These solubilized enzymes seem to be identical in their properties to cytosol KII. Both types of enzymes were activated by cyclic AMP at much lower concentrations than other cyclic nucleotides. In addition, both solubilized and cytosol K II [29] could be resolved into two peaks by ion-exchange chromatography. Upon elution from DEAE-cellulose column, the solubilized FI and FII protein kinases possessed one major peak with cyclic AMP binding and protein kinase activities and a minor kinase peak almost independent of cyclic AMP. However, different molecular species of both binding and kinase activities were demonstrable by a sucrose density gradient centrifugation (Fig. 2). These differences in the molecular size of the protein kinases and cyclic AMP binding activities under different experimental conditions could be due to aggregation, dissociation or proteolysis [9]. The presence of multiple forms of protein kinase of our plasma membrane fractions are consistent with similar reports in the literature concerning soluble protein kinases [9, 11–13, 16, 27]. Protein kinase from adrenal cortex has been shown to consist of a separate cyclic AMP binding and kinase subunits in addition to a cyclic-AMP-dependent protein kinase [17]. In liver Kumon *et al.* [12] have identified two protein kinases with slightly different concentration dependence on cyclic AMP for activity, which on dissociation produced physically different binding subunits but indistinguishable kinase subunits. The bovine corpus luteum cytosol protein kinase II has been found to consist of a single protein kinase and cyclic AMP binding subunit [43]. In muscle, a common kinase subunit appeared to be complexed with more than one cyclic AMP binding activity giving rise to several cyclic-AMP-dependent protein kinases with different sedimentation and chromatographic properties [9]. Recently Yuh and Tao [48] have described the purification and properties of two cyclic AMP binding proteins with a higher sedimentation coefficient (9.3 S). These cyclic AMP binding proteins have been shown not to be derived from cyclic-AMP-dependent protein kinases since they have no inhibitory action on the catalytic moiety of protein kinase. The presence of cyclic AMP binding activity with a higher sedimentation value in solubilized bovine corpus luteum plasma membranes may suggest the same possibility, but that remains speculative at this time.

The cyclic AMP stimulation of plasma protein kinases follows the same mechanism similar to that found for cytosol protein kinases [4–21]. Dissociation of the solubilized FI and FII enzymes by cyclic AMP resulted in the formation of identical catalytic subunits with dissimilar cyclic AMP binding activities. It is possible that the multiple form of protein kinases reported here might be formed by association of identical subunits with heterogenous regulatory subunits [24, 49]. In conclusion, many of the characteristics of protein kinases from plasma membranes and cytosol do seem reasonably consistent except for some variation in their physical properties.

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