Biochimica et Biophysica Acta, 302 (1973) 267–281

BBA 66848

ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE OF BOVINE TRACHEAL SMOOTH MUSCLE

HOWARD SANDS, THEODORE A. MEYER AND HOWARD V. RICKENBERG[a]

Division of Research, National Jewish Hospital and Research Center and [a]*Department of Biophysics and Genetics, University of Colorado School of Medicine, Denver, Colo. (U.S.A.)*

(Received October 2nd, 1972)

SUMMARY

An adenosine 3′,5′-monophosphate-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) from bovine tracheal smooth muscle was purified partially and its properties studied. Histone was employed as phosphate acceptor; the apparent K_m for histone was 50 μg/ml and was not affected by adenosine 3′,5′-monophosphate (cyclic AMP). The apparent K_m for ATP ($1.4 \cdot 10^{-5}$ M at 10 mM Mg^{2+}) was also unaffected by cyclic AMP. Cyclic AMP increased the V of the phosphorylation of histone with an apparent $K_{m\ \text{cyclic AMP}}$ of $2.5 \cdot 10^{-8}$ M and a pH optimum of pH 6.5. Activation of the protein kinase by cyclic nucleotides other than cyclic AMP occurred only at unphysiologically high concentrations and the enzyme appeared to be specific for ATP as phosphate donor. A divalent metal ion was required for the reaction; the apparent K_m for Mg^{2+} was $2.5 \cdot 10^{-3}$ M.

Incubation of the partially purified protein kinase (mol. wt approx. 144 500) with cyclic AMP, followed by sedimentation through a sucrose gradient containing cyclic AMP, resulted in the formation of two subunits. A catalytic subunit, the activity of which was not stimulated by cyclic AMP, had a mol. wt of approximately 50 000; a cyclic AMP-binding subunit, devoid of catalytic activity, had a mol. wt of approx. 88 500.

INTRODUCTION

Epinephrine, β-sympathomimetic drugs, and theophylline are used clinically to produce relaxation of bronchial and tracheal smooth muscle during asthmatic attack. They have also been shown to produce bronchiolar and tracheal relaxation *in vitro*[1,2]. It is now assumed that the β-receptor effects of the catecholamines are mediated by stimulation of the activity of adenyl cyclase, and that theophylline inhibits the cyclic AMP phosphodiesterase resulting in an increase in intracellular levels of cyclic AMP. Cyclic AMP and its dibutyryl derivative have been shown to produce relaxation of the smooth muscle of the trachea[3], intestine[4,5] and blood vessels[6]. The mechanism by which cyclic AMP produces relaxation of smooth muscle is not known.

The mode of action of cyclic AMP is now understood in several systems where

interaction of the cyclic nucleotide with protein kinases occurs. For example, the stimulation of glycogenolysis by drugs which exert their effect on the β-receptor is mediated by a cyclic AMP-dependent protein kinase. This enzyme catalyzes the phosphorylation, and concomitant activation, of phosphorylase kinase[7]. Conversely, the activity of glycogen synthetase is inhibited by phosphorylation also catalyzed by a cyclic AMP-dependent protein kinase[8,9]. The occurrence of protein kinases is widespread and has been reported from a variety of tissues as well as phyla, including mammals[10–16], invertebrates[17], bacteria[18] and viruses[19].

The ubiquitous occurrence of cyclic AMP-dependent protein kinases in tissues in which cyclic AMP is the presumed second messenger led to the proposal that a majority of the effects produced by cyclic AMP are mediated by activation of protein kinases[13]. We propose that one step in the production of β-receptor-stimulated relaxation of smooth muscle is the activation of a cyclic AMP-dependent protein kinase. This paper describes the pertinent properties of a protein kinase partially purified from bovine bronchial and tracheal smooth muscle.

METHODS

Materials

Bovine tracheae (from a point below the larynx and including the primary bronchi) were obtained within 0.5 h of slaughter of animals and packed in ice for transport to the laboratory. Smooth muscle layers of the trachea and bronchi were carefully stripped off, cut into small pieces, frozen in dry ice, and stored at −40 °C until purification was started. Cyclic AMP, cyclic IMP, cyclic GMP, cyclic UMP, cyclic CMP, cyclic 2′,3′-AMP, ATP, UTP, GTP*, casein, protamine, bovine serum albumin, tricalcium phosphate gel, lactate dehydrogenase were purchased from Sigma, and calf thymus mixed histones from Schwarz-Mann. DEAE-cellulose (DE-52) was obtained from Reeves Angel and Biogel P-300 from Biorad. Membrane filters, Type B-6 (0.45 μm), were purchased from Schleicher and Schuell. Carrier-free ^{32}P in dilute HCl was obtained from ICN.

Preparation of [γ-^{32}P]ATP

[γ-^{32}P]ATP was prepared from carrier-free ^{32}P using the method of Glynn and Chappell[20].

Assay of protein kinase activity

Protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity was measured at pH 6.5 and 30 °C in an 0.3 ml reaction mixture containing the following: 50 mM sodium glycerol phosphate, 10 mM NaF, 2 mM theophylline, 3.3 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 10 mM $MgCl_2$, 3.3 μM [^{32}P]ATP (containing approx. 10^6 cpm); histone, when present, was used at 100 μg and cyclic AMP at 10^{-6} M. The reaction was started by the addition of ATP and terminated after 7 min by the addition of 0.2 ml of a mixture containing 30% trichloroacetic acid, 0.1 mM KH_2PO_4 and 1 mM non-radioactive ATP. The precipitate was collected on a B-6 membrane filter, washed with 50 ml of 5% trichloroacetic acid–0.1 mM KH_2PO_4, and the radioactivity on the filter was determined in a liquid

* Unless stated otherwise Cyclic nucleotide means Cyclic nucleoside 3′, 5′-monophosphate.

scintillation spectrometer. The scintillation fluid was toluene containing 0.48% 2,5-diphenyloxazole–0.006% 1,4 bis-[2-(5-phenoxazole)]-benzene. Routinely, values for the radioactivity representing the phosphorylation of endogenous protein were subtracted from those obtained in the presence of histone (or other exogenous substrates) when determining the activity of protein kinase with respect to exogenous substrates. The specific activity of the protein kinase is expressed as pmoles of ^{32}P incorporated during 7 min per mg of protein. All assays were performed in duplicate and in the range in which activity was a rectilinear function of protein concentration.

The reason for the use of the unphysiologically low concentration of 3.3 μM ATP was the difficulty in obtaining sufficiently high specific radioactivity, when physiological concentrations of ATP were employed. However the time course of the phosphorylation with histone was rectilinear with time even at the low concentration of ATP (Fig. 3). The rate of histone phosphorylation when 100 μM ATP was employed was approximately 5-fold higher than the rate at 3.3 μM ATP.

Sucrose density gradient centrifugation

The method of Martin and Ames[21] was employed in studies involving sucrose density centrifugation. Centrifugation was conducted in a Beckman SW39 rotor at 39 000 rev./min, 5 °C, and for 28 h. Linear sucrose gradients (5–22%) in 0.02 M Tris–HCl (pH 7.4), 0.1 M KCl, 0.005 M $MgCl_2$, and 0.006 M 2-mercaptoethanol (Buffer A) in the presence or absence of 10^{-6} M cyclic AMP were used. The Biogel P-300 fraction (240–280 μg) in a volume of 0.2 ml was layered over the gradient. Fractions of approximately 0.1 ml were collected from the top of the tubes using an Isco gradient fractionator and assayed for protein kinase activity in the presence or absence of 10^{-6} M cyclic AMP. Horse hemoglobin (mol. wt 64 000) and rabbit muscle lactate dehydrogenase (mol. wt 142 000) were used as standards for the molecular weight calculations[21].

Measurement of binding of cyclic AMP

In order to identify, and to determine the molecular weight of, the cyclic AMP-binding component of the protein kinase, cyclic [^{3}H]AMP (110 μCi, 14 Ci/mmole) was introduced into the gradient in addition to 10^{-6} M unlabeled cyclic AMP. Aliquots (0.01 ml) of each fraction collected were placed on a B-6 membrane filter and washed with 5 ml of Buffer A. The radioactivity remaining on the filter, representing cyclic AMP bound to protein, was determined by liquid scintillation counting using Bray's solution[22].

Other methods

Protein was determined according to the method of Lowry *et al.*[23] using bovine serum albumin as standard. Lactate dehydrogenase was determined by the method described in the *Worthington Enzyme Manual*[24].

RESULTS

Partial purification of cyclic AMP-dependent protein kinase

Preparation of crude homogenate. Frozen bronchial and tracheal smooth muscle (1200–1500 g) was thawed and homogenized in 3 vol. of 4 mM EDTA (pH 7.0) at

16 000 rev./min for 3–4 min in a Servall Omni-Mixer. The homogenate was centrifuged at 15 000 × *g* for 30 min. Homogenization and all subsequent manipulations were carried out at 4 °C.

Acid precipitation. The pH of the supernatant solution was brought to 4.8 by the dropwise addition of 1 M acetic acid with constant stirring. The precipitate was removed by centrifugation and the pH of the supernatant adjusted to pH 6.5 by the dropwise addition of 1 M, pH 7, potassium phosphate buffer.

Fractionation with $(NH_4)_2SO_4$. The supernatant was then saturated to 50% with respect to $(NH_4)_2SO_4$ by the addition of 325 g/l of solid $(NH_4)_2SO_4$. The precipitate containing the protein kinase was collected by centrifugation, redissolved in 100–200 ml of 5 mM potassium phosphate, 2 mM EDTA, pH 7.0 (Buffer B) and dialyzed against two 4-l volumes of the same buffer for 16 h. After dialysis, the solution was centrifuged and a small amount of precipitate discarded.

Fractionation with calcium phosphate gel. 20 g (wet weight) of calcium phosphate gel equilibrated with Buffer B were added to the supernatant solution. The suspension was stirred continuously for 30 min, then centrifuged at 5000 × *g* for 15 min. The gel was then suspended and stirred for 30 min in each of the following series of phosphate buffers composed of dibasic and monobasic potassium phosphate (9:1, v/v) containing 1 mM EDTA: 150 ml of 0.05 M, 40–50 ml of 0.05 M, 40–50 ml of 0.1 M, 40–50 ml of 0.2 M, and 40–50 ml of 0.3 M. After each 30 min of stirring, the gel was collected by centrifugation at 5000 × *g* for 15 min. The enzyme was eluted in the last two or three final washes. These wash fluids were combined and dialyzed against two 4-l volumes of Buffer B for 16 h.

DEAE-cellulose chromatography. The solution containing the enzyme was applied to a column of DEAE-cellulose (DE-52) equilibrated with 5 mM Tris–HCl, pH 7.5 (columns of either 0.9 cm × 15 cm or 2.6 cm × 3.8 cm gave the same elution

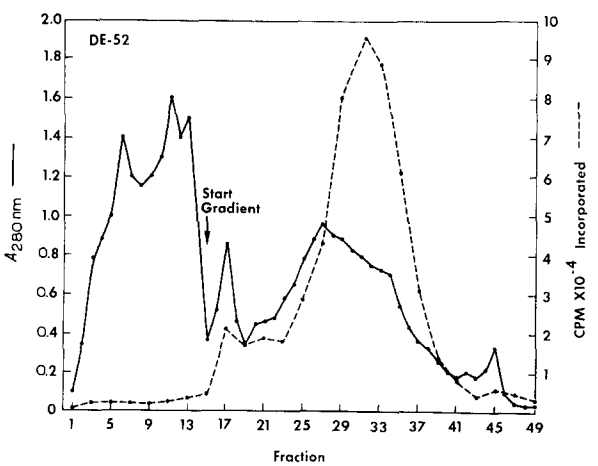


Fig. 1. Chromatography of bovine smooth muscle protein kinase on DEAE-cellulose. Approximately 280 mg of protein obtained from the calcium phosphate gel were applied to a column of DEAE-cellulose (0.9 cm × 15 cm), equilibrated with 5 mM Tris–HCl–1 mM EDTA, pH 7.5. The column was eluted with a linear gradient of 5 mM to 0.5 M Tris–HCl–1 mM EDTA at pH 7.5. Activity was determined in the presence of cyclic AMP and histone; there was no correction for the phosphorylation of endogenous protein. The total gradient volume was 300 ml. Fractions 29–31 were pooled.

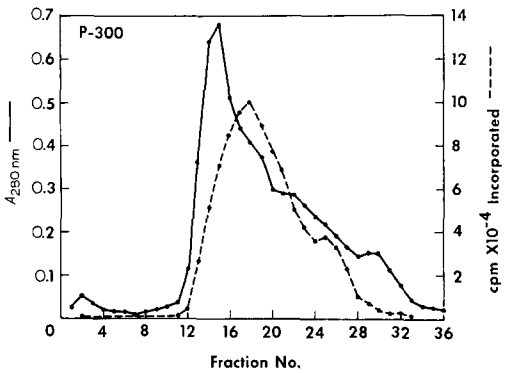


Fig. 2. Chromatography of bovine smooth muscle protein kinase Biogel P-300. Approximately 30 mg of protein were applied to a column of P-300 (2.5 cm × 35 cm) which had been equilibrated with 5 mM potassium phosphate and 2 mM EDTA, pH 7.0. The volume of the protein solution was reduced to 4 ml by vacuum dialysis. Activity was determined in the presence of cyclic AMP and histone; there was no correction for the phosphorylation of endogenous protein. Approximately 10-ml fractions were collected. Fractions 17–19 were pooled.

pattern; however, the latter had a considerably faster rate of flow). The enzyme was eluted at pH 7.5 with 300 ml of 0.005 M to 0.5 M linear Tris–HCl gradient containing 1 mM EDTA. All protein kinase activity adhered to the DEAE. A typical elution pattern is shown in Fig. 1. The enzyme was eluted as a single major peak; fractions with protein kinase activity were pooled and dialyzed against two 4-l volumes of Buffer B. A minor peak of kinase activity was seen in several preparations. Further studies on the minor peak of activity were not conducted.

Chromatography on Biogel P-300. The preparation resulting from the preceding step was reduced in volume to 4–7 ml by vacuum dialysis and placed on a 2.5 cm × 35 cm column of Biogel P-300 which had been equilibrated with Buffer B. The enzyme was eluted with Buffer B. A typical elution pattern is shown in Fig. 2. The enzyme was eluted slightly behind the main protein peak which occurred at approximately the

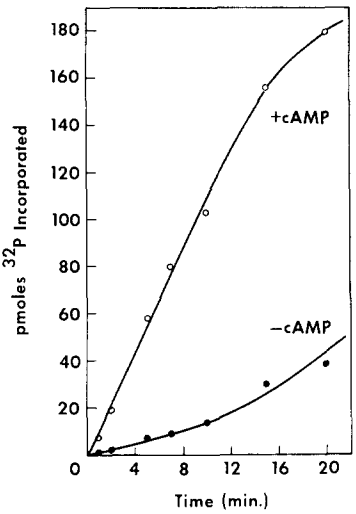
TABLE I

PURIFICATION OF SMOOTH MUSCLE PROTEIN KINASE

Protein kinase was purified as described in the text. Specific activity and total activity were determined in the presence of histone and cyclic AMP after correction for phosphorylation of endogenous protein.

Fraction	*Protein (mg)*	*Specific activity**	*Purification (-fold)*	*Total activity* × 10^{-3}†	*Yield (%)*
Crude homogenate	30 028	162	—	4865	100
pH 4.8 supernatant	15 830	240	1.5	3799	78.1
0–50% saturated $(NH_4)_2SO_4$ precipitate	3 906	374	2.3	1461	30.0
After calcium phosphate gel	288	2662	16	767	15.8
DEAE cellulose	31	6045	37	188	3.9
Biogel P-300	8	10 510	65	84	1.7

* pmoles of ^{32}P incorporated in 7 min per mg of enzyme.
† pmoles of ^{32}P incorporated in 7 min.



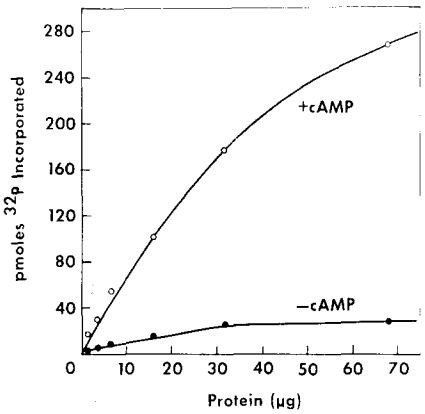


Fig. 3. Time course of phosphorylation of histone by smooth muscle protein kinase in either the presence or absence of 10^{-6} M cyclic AMP. Conditions of incubation were as described under Methods except for the indicated time of incubation. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

Fig. 4. Effect of the concentration of enzyme on activity in either the presence or absence of 10^{-6} M cyclic AMP. Conditions were as described under Methods except for the variation of the concentration of enzyme. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

void volume of the column. The fractions containing protein kinase activity were pooled and frozen in 1–1.5 ml volumes and stored at −40 °C. No change in enzymic activity was seen over a two-month period. A summary of the purification procedure is presented in Table I; similar results were obtained in three separate preparations. The activities shown in Table I were observed in the presence of 10^{-6} M cyclic AMP. The stimulation produced by cyclic AMP was similar throughout the purification. Gel electrophoresis of 80 μg of the P-300 fraction on 0.1% sodium dodecyl sulfate, 10% acrylamide gels showed the presence of five major protein bands.

Properties of protein kinase from tracheal smooth muscle

Time course of phosphorylation of histone. The phosphorylation of histones at 30 °C was rectilinear with time for 10–12 min in either the presence or absence of 10^{-6} M cyclic AMP (Fig. 3). An incubation time of 7 min was chosen for the standard assay of protein kinase activity. It should be stressed here that although histones are a convenient substrate for the assay of protein kinase activity, they probably have little physiological significance with respect to a role of a smooth muscle protein kinase.

Effect of varying concentration of enzyme. The data presented in Fig. 4 show that under standard conditions the phosphorylation of histones was rectilinearly proportional to the concentration of purified enzyme protein up to a concentration of 15 μg of protein per tube (50 μg/ml) in either the absence or presence of 10^{-6} M cyclic AMP. The following assays for the characterization of the enzyme were carried out with 8.4 μg of protein/tube.

pH optimum. Fig. 5 illustrates the effect of pH on the phosphorylation of the

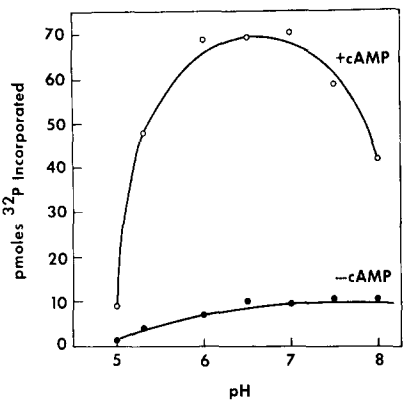


Fig. 5. Effect of pH on phosphorylation of histone by smooth muscle protein kinase in either the presence or absence of 10^{-6} M cyclic AMP. Conditions were as described under Methods except for the variation in pH. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

histone. Maximal activity in the presence of 10^{-6} M cyclic AMP was in the range of pH 6–7. In the absence of cyclic AMP a braoder pH optimum of 6.5–8.0 was observed; cyclic AMP stimulated maximally at pH 6.5.

Substrates of protein kinase. The identity of the "natural" substrate of smooth muscle protein kinase is not known at present. Table II shows that of the proteins tested as substrate, histone was the most effective in both the presence and absence of 10^{-6} M cyclic AMP. Protamine was phosphorylated at about 40% of the rate of histone. Cyclic AMP had a similar stimulatory effect on the phosphorylation of histone and protamine. Casein and bovine serum albumin were phosphorylated at only a fraction of the rate observed with histone. Because of the low extent of phosphorylation, it was difficult to quantitate the stimulatory effect of cyclic AMP on the phosphorylation of casein and bovine serum albumin.

Apparent K_m of protein kinase for histone. In view of the relative effectiveness of histone as a substrate and its use as a substrate in the purification and characterization of the enzyme, it was of interest to determine the apparent K_m of the protein kinase for histone (Fig. 6). Fig. 6A shows that the phosphorylation of histones was linear with increasing histone concentration to approximately 100 μg (330 μg/ml)

TABLE II

SUBSTRATE SPECIFICITY OF SMOOTH MUSCLE PROTEIN KINASE

Conditions were as described under Experimental Procedures except for the variation of substrate. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

Substrate 100 μg	*pmoles ^{32}P incorporated*		*Stimulation by cyclic AMP (-fold)*
	− cyclic AMP	*+ cyclic AMP*	
Histone	27.4	101.9	3.7
Protamine	12.6	42.4	3.6
Casein	0.2	2.1	
Bovine serum albumin	0.0	0.8	

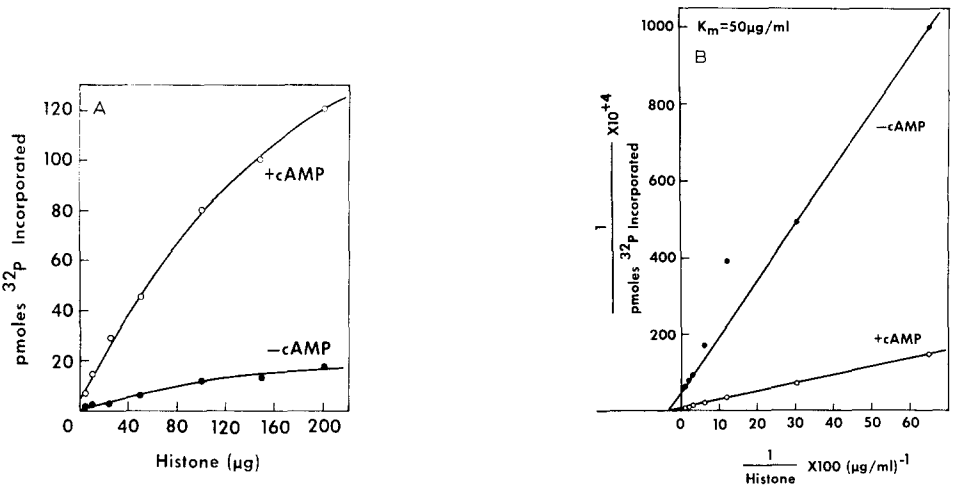


Fig. 6. (A) Effect of histone concentration on the activity of protein kinase in either the presence or absence of 10^{-6} M cyclic AMP. Conditions were as described under Methods except for the variation of the concentration of histone. The reaction mixture contained 8.4 μg of protein from the P-300 fraction. (B) Double-reciprocal plot of the data in Fig. 6A.

regardless of whether cyclic AMP was present or not. Fig. 6B is a double reciprocal plot of the same data. The apparent K_m for histone (50 μg/ml) was not affected by cyclic AMP, whereas the V was increased by the cyclic nucleotide.

Apparent K_m for cyclic AMP. In experiments in which the cyclic AMP concentration was varied, maximal stimulation was found at approximately $2 \cdot 10^{-6}$ M cyclic AMP (Fig. 7A). Increasing the concentration above $5 \cdot 10^{-4}$ M cyclic AMP

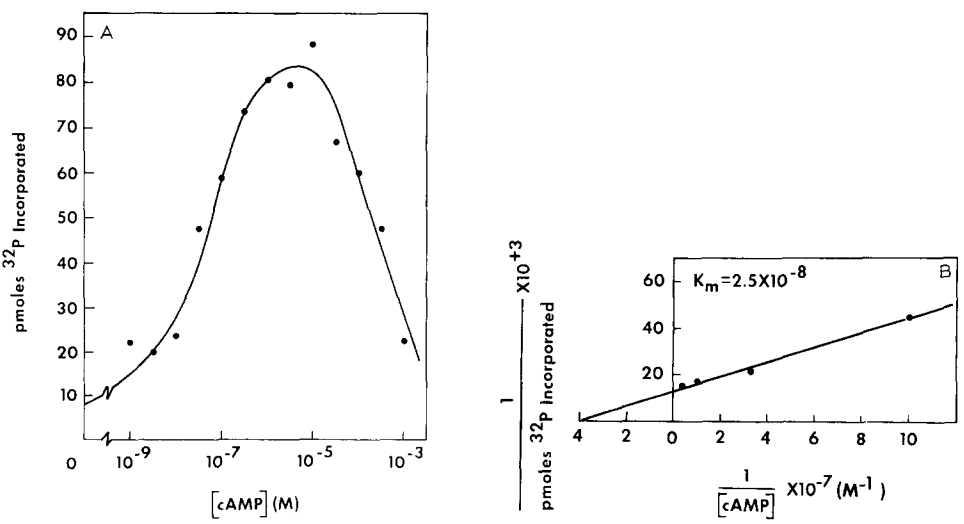
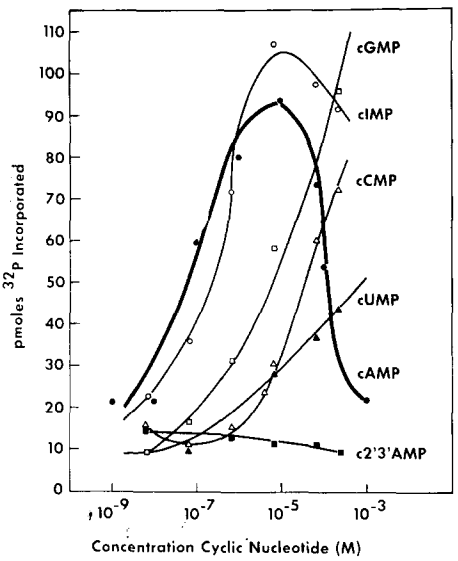


Fig. 7. (A) Effect of increasing concentration of cyclic AMP on the activity of smooth muscle protein kinase. Conditions were as described under Methods except for the varying concentration of cyclic AMP. The reaction mixture contained 8.4 μg of protein from the P-300 fraction. (B) Double-reciprocal plot of the data in Fig. 7A.



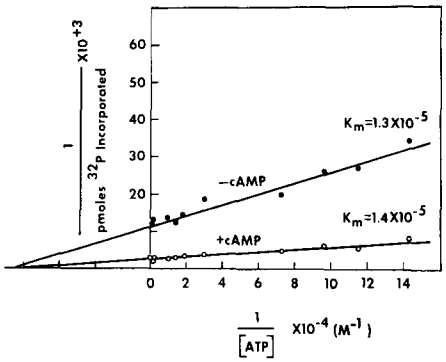


Fig. 8. Effect of concentrations of cyclic nucleotides on the activity of smooth muscle protein kinase. Conditions were as described under Methods except for the cyclic nucleotide and its concentration. The reaction mixture contained 8.4 μg of protein from the P-300 fraction. The activity in the absence of cyclic nucleotides was between 8 and 20 pmoles of ^{32}P incorporated.

Fig. 9. Effect of ATP concentration on the activity of smooth muscle protein kinase. Conditions were as described under Methods except for the varying concentration of ATP. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

resulted in a significant inhibition of activity. The stimulation by cyclic AMP was approximately 10-fold; in other experiments the stimulation of phosphorylation by 10^{-6} M cyclic AMP varied from 3- to 10-fold. A double-reciprocal plot of the data (Fig. 7B) indicates an apparent K_m of $2.5 \cdot 10^{-8}$ M cyclic AMP.

Specificity for cyclic nucleotides. Since cyclic GMP occurs naturally[25,26] and since cyclic GMP-stimulated protein kinases have been reported[27], it was of interest to test whether or not cyclic nucleotides other than cyclic AMP stimulated the smooth muscle protein kinase. The data presented in Fig. 8 show that cyclic GMP stimulated the enzyme to full activity only if used at unphysiologically high concentrations. Cyclic IMP was almost as effective in stimulating the smooth muscle protein kinase as was cyclic AMP, and, in fact, the maximal activity was slightly higher than that achieved in the presence of cyclic AMP. Cyclic CMP and cyclic UMP stimulated the enzyme at very high concentrations. The 2',3'-AMP had no effect on the smooth muscle protein kinase.

Apparent K_m for ATP. The data presented in Fig. 9 indicate that the protein kinase has an apparent K_m of $1.4 \cdot 10^{-5}$ M for ATP in either the presence or absence of cyclic AMP when histone served as the second substrate of the enzyme. Cyclic AMP significantly increased the V of the reaction.

Specificity for nucleoside triphosphate. In experiments designed to determine if nucleoside triphosphates other than ATP could serve as phosphate donors, the protein kinase and histone were incubated either with or without 10^{-6} M cyclic AMP and the standard concentration of [γ-^{32}P]ATP ($3.3 \cdot 10^{-6}$ M). Other non-radioactive nucleoside

TABLE III

NUCLEOSIDE TRIPHOSPHATE SPECIFICITY OF SMOOTH MUSCLE PROTEIN KINASE

Assays were performed as described under Experimental Procedures. Control contained $3.3 \cdot 10^{-4}$ M [γ-^{32}P]ATP only. The same concentration of [γ-^{32}P]ATP was employed in addition to 10^{-4} M of unlabeled nucleoside triphosphate. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

Addition	*pmoles ^{32}P incorporated*		*% of control*	
	− cyclic AMP	*+ cyclic AMP*	*− cyclic AMP*	*+ cyclic AMP*
Control	14.8	114.4	—	—
CTP	14.2	104.7	95.6	91.5
GTP	14.4	92.9	96.8	81.2
UTP	14.3	89.6	96.6	78.3

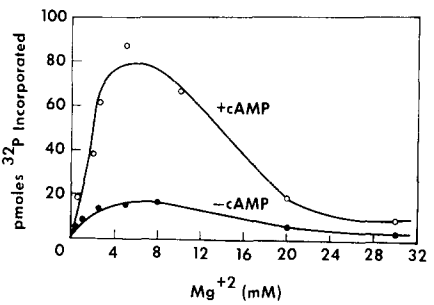


Fig. 10. Effect of Mg^{2+} concentration on the activity of smooth muscle protein kinase in either the presence or absence of 10^{-6} M cyclic AMP. Conditions were as described under Methods except for the variation in Mg^{2+} concentration. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

TABLE IV

EFFECTS OF METAL IONS ON SMOOTH MUSCLE PROTEIN KINASE IN EITHER THE PRESENCE OR ABSENCE OF 10^{-6} M CYCLIC AMP

Conditions were as described under Experimental Procedures except for the variation in the concentration of ion, as indicated. 8.4 μg of the P-300 fraction were used. Data are expressed as pmoles of ^{32}P incorporated per 7 min.

Metal ion	*Ion concentration*					
	2.5 mM			*10 mM*		
	− cyclic AMP	*+ cyclic AMP*	*Ratio*	*− cyclic AMP*	*+ cyclic AMP*	*Ratio*
No addition	0.2	0.2	1.0	—	—	—
Mg^{2+}	14.1	60.7	4.3	17.3	66.2	3.8
Ca^{2+}	0.4	1.1	2.9	0.6	3.3	5.7
Mn^{2+}	5.2	25.1	4.8	4.6	24.7	5.4
Ba^{2+}	0.7	4.5	6.4	1.0	5.9	5.9
Co^{2+}	14.0	97.0	6.9	13.8	83.0	6.0
Zn^{2+}	0.8	2.2	2.7	1.2	1.9	1.6

triphosphates were added singly at 10^{-4} M (Table III). None of the nucleoside triphosphates significantly inhibited ^{32}P transfer from ATP; the protein kinase appeared to require ATP specifically.

Requirement for divalent cations. Fig. 10 shows the relationship between protein kinase activity and Mg^{2+} concentration. An apparent K_m of approximately 2.5 mM Mg^{2+} was calculated from the data; there was no effect of cyclic AMP on the apparent K_m for Mg^{2+}. Co^{2+} (Table IV) stimulated the protein kinase more effectively than did Mg^{2+}. Other divalent metal ions were less effective than Mg^{2+}, but still permitted stimulation by cyclic AMP. Fig. 11 shows that the observed stimulatory effect of Mg^{2+} on protein kinase activity resulted solely from a decrease in the K_m of the enzyme for ATP. The maximal velocity remained constant when the Mg^{2+} concentration was varied.

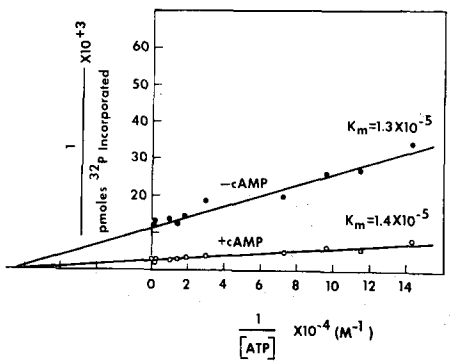


Fig. 11. Effect of increasing concentrations of Mg^{2+} and ATP on phosphorylation of histone by smooth muscle protein kinase. Conditions were as described under Methods except for the variation in Mg^{2+} and ATP concentration. The reaction mixture contained 8.4 μg of protein from the P-300 fraction.

Sedimentation of protein kinase on sucrose density gradient. The sedimentation behavior and molecular weight of the smooth muscle protein kinase were studied using a 5–22% sucrose gradient either with or without 10^{-6} M cyclic AMP. Fig. 12 illustrates the results of such a study. A single peak of protein kinase activity was found when no cyclic AMP was present in the gradient (Fig. 12a). The protein corresponding to this peak had a mol. wt of 144 500 based on calculations using horse hemoglobin (mol. wt 64 000) and rabbit muscle lactate dehydrogenase (mol. wt 142 000) as standards. Cyclic AMP stimulated this protein kinase activity. Preincubation of the enzyme with cyclic AMP and ATP (with concentrations and conditions similar to those used in the assay) followed by sucrose density centrifugation in the presence of 10^{-6} M cyclic AMP resulted in a shift in the molecular weight of the enzyme. A single peak of activity with a mol. wt of approximately 50 000 was obtained and cyclic AMP did not stimulate this activity (Fig. 12b). Between 85 and 100% of the enzymic activity applied to the gradient were recovered. Cyclic AMP binding activity was found in a region with a mean mol. wt of 88 500. If preincubation with cyclic AMP (and ATP) was omitted and the enzyme subjected to sucrose density centrifugation in the presence of 10^{-6} M cyclic AMP, an additional peak of protein kinase activity was found on the shoulder of the peak corresponding to a mol. wt of

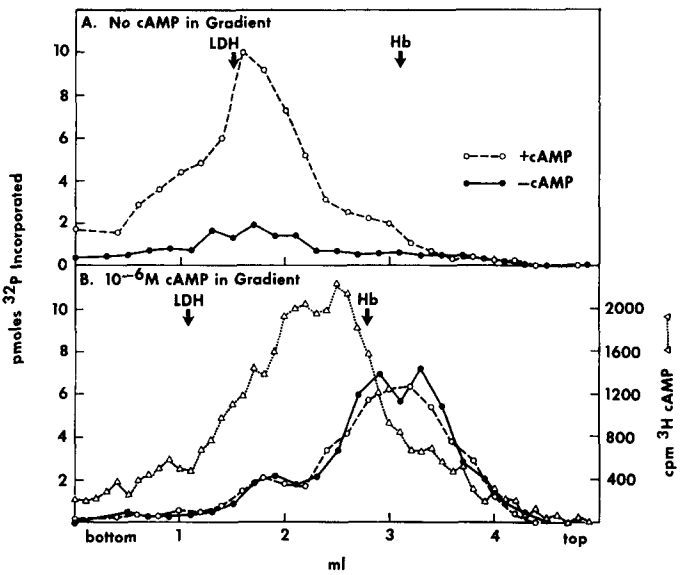


Fig. 12. Sedimentation of smooth muscle protein kinase in a sucrose gradient. Sedimentation conditions were as described under Methods. (A) No cyclic AMP present; (B) preincubation of enzyme with cyclic AMP and ATP at the concentration and conditions of the protein kinase assay; 10^{-6} M cyclic AMP in the gradient (similar findings were obtained when the preincubation with cyclic AMP was carried out in the absence of ATP). ●---●, assayed in the presence of 10^{-6} M cyclic AMP; ●—●, assayed in the absence of cyclic AMP; △—△, cyclic[^{3}H]AMP binding. Hb, horse hemoglobin; LDH, rabbit muscle lactate dehydrogenase.

50 000. The activity in this peak (mol. wt greater than 50 000) was stimulated by the addition of cyclic AMP to the kinase assay and was observed in several experiments. The location of the cyclic AMP binding activity within the gradient was the same as that shown for the preincubated enzyme.

DISCUSSION

The widespread distribution of cyclic AMP-dependent protein kinases led Kuo and Greengard[13] to propose that the effects of cyclic AMP in general might be mediated by protein kinases. The role of protein kinase in the activation of glycogen phosphorylase of skeletal muscle is well established[28]. The activities of glycogen synthetase[9] and of the triglyceride lipase of adipose tissue[29] are also regulated by the phosphorylation of the respective enzymes catalyzed by cyclic AMP-stimulated protein kinases. It has been suggested that the regulation of transcription in eukaryotes[30], of synaptic transmission[31], of vasopressin-induced changes in the permeability of the medullary membranes of the kidney[32], of the action of hypophysiotropic hormones[16], the activity of the lacrymal gland[33], regulation of the activity of the mammary gland[34], and the control of diurnal rhythm *via* the pineal gland[15] are mediated by protein kinases. In this paper we describe certain properties of a partially purified protein kinase isolated from bovine bronchial and tracheal smooth muscle.

The procedure which we employed led to the isolation of one major species of

protein kinase (a small secondary peak of kinase activity was seen after DE-52 column chromatography of several preparations). This is in contrast with findings made employing the pituitary[16] and skeletal muscle[36] as sources of protein kinase. In the case of the pituitary, cyclic AMP-dependent and cyclic AMP-independent forms were observed after SE-Sephadex C 50 chromatography; whereas in the case of skeletal muscle two large and distinct peaks of cyclic AMP-dependent protein kinase activity were found after DE-52 chromatography. Multiple forms of rat skeletal muscle protein kinase have been reported recently[37].

It is of interest to compare some of the properties of the bovine tracheal smooth muscle protein kinase with those of protein kinases from other tissues. Although the assay conditions differed, the pH optimum of 6.5 to 7 reported here for smooth muscle protein kinase is similar to that of the enzyme from the pituitary[16], brain[38], and skeletal muscle[36], and differs from the pH optimum of 8.5 to 9.0 reported for rabbit reticulocytes[39], that of frog bladder epithelium[40] of pH 7.5, and trout testes[41] also of pH 7.5.

Stimulation of the tracheal smooth muscle protein kinase was maximal at a cyclic AMP concentration of 10^{-6} M and the apparent K_m for cyclic AMP was $2.5 \cdot 10^{-8}$ M. Similar K_m values were reported for the protein kinases of the pituitary[16], skeletal muscle[36], and other tissues[12]. Protein kinases obtained from the brain, adrenal, ovary, stomach, and duodenum had apparent K_m values of $1.1 \cdot 10^{-7}$–$1.6 \cdot 10^{-7}$ cyclic AMP[12]. The stimulation of protein kinase activity by high concentrations of cyclic nucleotides other than cyclic AMP observed in the present study has also been reported for other protein kinases[16,36,38]. Cyclic GMP, which occurs naturally, stimulated the activity of the bovine tracheal smooth muscle protein kinase only at unphysiologically high concentrations. A smooth muscle protein kinase stimulated by physiological concentrations of cyclic GMP occurs in lobster smooth muscle and various tissues of other arthropods[17].

The bovine tracheal smooth muscle protein kinase appears to be specific for ATP as donor of the γ-phosphate; other nucleoside triphosphates did not inhibit the transfer of phosphate from ATP. The apparent K_m of the protein kinase for ATP was $1.4 \cdot 10^{-5}$ M in the presence of 10 mM Mg^{2+} and was not affected by cyclic AMP. As the Mg^{2+} concentration was lowered to 1 mM, the apparent K_m increased, whereas the V was unaffected. Similar findings, and an explanation for them, have been reported by Reimann *et al.*[36] for the protein kinase of skeletal muscle. In the case of the bovine pituitary protein kinase, lowering the Mg^{2+} concentration affected both the K_m for ATP and the V of the reaction[16]. The apparent K_m of $1.4 \cdot 10^{-5}$ M ATP determined in the present study for the protein kinase of bovine tracheal smooth muscle was unaffected by cyclic AMP, and is similar to the $K_{m\ ATP}$ reported for several other tissues and species[16,36]. The protein kinase of bovine brain[38] which had an apparent K_m of $1.3 \cdot 10^{-4}$ M in the presence of cyclic AMP and of $2.2 \cdot 10^{-4}$ M in its absence, and the protein kinase of rabbit reticulocyte[39] which showed a K_m of $1.7 \cdot 10^{-4}$ M in the presence and $2.5 \cdot 10^{-4}$ M in the absence of cyclic AMP, seem to be exceptional. Our data show that in the case of bovine tracheal smooth muscle protein kinase, cyclic AMP activates by increasing the V rather than by affecting the K_m for the substrates of the reaction.

Studies of the protein kinase of rabbit reticulocytes[42] as well as of other species and tissues[16,36] suggest that the enzyme is a dimer composed of a catalytic subunit (mol. wt of 40 000–60 000) and of an inhibitory subunit (mol. wt of approximately

80 000). The dimer is inactive; the binding of cyclic AMP to the inhibitory subunit causes it to dissociate from the catalytic subunit which becomes enzymically active. In the case of the bovine tracheal smooth muscle protein kinase a single peak of cyclic AMP-dependent activity occurred when the preparation was sedimented in the absence of cyclic AMP (Fig. 12a); the mol. wt of this fraction was approximately 144 500. When the preparation was incubated with cyclic AMP prior to sedimentation, a peak of cyclic AMP-independent protein kinase activity of mol. wt 50 000 and a peak of cyclic AMP-binding activity of a mol. wt of approximately 88 500 were found. No attempt was made to reconstitute the cyclic AMP-dependent protein kinase by recombination of the catalytic and regulatory components. It appears, however, that unless the enzyme is preincubated with cyclic AMP, complete dissociation does not occur. In the absence of preincubation with cyclic AMP a peak of kinase activity slightly heavier than the 50 000 mol. wt enzyme was found; the protein of this fraction showed cyclic AMP-stimulated protein kinase activity. The 50 000 mol. wt cyclic AMP-independent catalytic unit and the cyclic AMP binding activity in the region of 85 000 mol. wt were also formed under these conditions. The significance of this observation is not clear. A similar finding, however, was made by Miyamoto *et al.*[43] with respect to the behavior of bovine brain protein kinase. The cyclic AMP-stimulated form of that enzyme had a mol. wt of 140 000. When it was centrifuged through a gradient containing either cyclic AMP or histone, a cyclic AMP-stimulated component of mol. wt 80 000 was formed. A cyclic AMP-independent form of the enzyme occurred, when the gradient contained both cyclic AMP and histone. Both the 140 000 and 80 000 mol. wt proteins bound cyclic AMP.

The occurrence of a cyclic AMP-dependent protein kinase in tracheal smooth muscle suggests that the enzyme may play a role in the relaxation of the muscle brought about by cyclic AMP. An attempt to identify the endogenous substrate(s) of the enzyme in smooth muscle is now in progress in this laboratory.

ACKNOWLEDGMENTS

This work was supported by Research Grants RR-05474-10 and HL-14964-01 from the National Institutes of Health, United States Public Health Service and Research Grant GB 8292 from the National Science Foundation.

REFERENCES

1 Guigis, H. M. (1969) *Arch. Int. Pharmacodyn.* 182, 147–160
2 Blumenthal, M. N. and Brody, T. M. (1969) *J. Allergy* 44, 63–69
3 Moore, P. F., Iorio, L. C. and McManus, J. M. (1968) *J. Pharm. Pharmacol.* 20, 368–372
4 Takagi, K., Takayanagi, I. and Tomiyama, A. (1971) *Jap. J. Pharmacol.* 21, 271–273
5 Kawasaki, A., Kashimoto, T. and Yoshida, H. (1969) *Jap. J. Pharmacol.* 19, 494–501
6 Rubin, B., O'Keefe, E. H., Waugh, M. H., Kotler, D. G., DeMaio, D. A. and Horovitz, Z. P. (1971) *Proc. Soc. Exp. Biol. Med.* 137, 1244–1248
7 Walsh, D. A., Perkins, J. P. and Krebs, E. G. (1968) *J. Biol. Chem.* 243, 3763–3774
8 Schlender, K. K., Wei, S. H. and Villar-Palasi, C. (1969) *Biochim. Biophys. Acta* 191, 272–278
9 Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkler, F. L., Walsh, D. A. and Krebs, E. G. (1970) *J. Biol. Chem.* 245, 6317–6328
10 Langan, T. A. and Smith, L. K. (1967) *Fed. Proc.* 26, 603
11 Langan, T. A. (1968) *Science* 162, 579–580
12 Kuo, J. F., Krueger, B. K., Sanes, J. R. and Greengard, P. (1970) *Biochim. Biophys. Acta* 212, 79–91

13 Kuo, J. F. and Greengard, P. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 1349–1355
14 Rubin, C. S., Erlichman, J. and Rosen, O. M. (1972) *J. Biol. Chem.* 247, 36–44
15 Gill, G. N. and Garren, L. D. (1970) *Biochem. Biophys. Res. Commun.* 39, 335–343
16 Labrie, F., Lemaire, S., and Courte, C. (1971) *J. Biol. Chem.* 246, 7293–7302
17 Kuo, J. F., Wyatt, G. R. and Greengard, P. (1971) *J. Biol. Chem.* 246, 7159–7167
18 Kuo, J. F. and Greengard, P. (1969) *J. Biol. Chem.* 244, 3417–3419
19 Stand, M. and August, J. T. (1971) *Nature New Biol.* 233, 137–140
20 Glynn, I. M. and Chappell, J. B. (1964) *Biochem. J.* 90, 147–149
21 Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372–1379
22 Bray, G. A. (1960) *Anal. Biochem.* 1, 279–285
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
24 *Worthington Enzyme Manual* (1972), p. 7, Worthington Biochemical Corp., Freehold, New Jersey
25 Hardman, J. G., Davis, J. W. and Sutherland, E. W. (1966) *J. Biol. Chem.* 241, 4812–4815
26 Goldberg, N. D., Dietz, S. B. and O'Toole, A. G. (1969) *J. Biol. Chem.* 244, 4458–4466
27 Kuo, J. F. and Greengard, P. (1970) *J. Biol. Chem.* 245, 2493–2498
28 Walsh, D. A., Perkins, J. P., Bronstrom, C. O., Ho, E. S .and Krebs, E. G. (1971) *J. Biol. Chem.* 246, 1968–1976
29 Corbin, J. D., Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1970) *J. Biol. Chem.* 245, 4849–4851
30 Langan, T. A. (1970) *Adv. Biochem. Psychopharmacol.* 3, 307–323
31 Maeno, J. H., Johnson, E. M. and Greengard, P. (1971) *J. Biochem.* 246, 134–142
32 Dousa, T. P., Sands, H. and Hechter, O. (1972) *Endocrinol.* 91, 757–763
33 Takats, A., Farago, A. and Antoni, F. (1972) *Biochim. Biophys. Acta* 268, 77–81
34 Majumder, G. and Turkington, R. (1971) *J. Biol. Chem.* 246, 5545–5554
35 Fontana, J. A. and Lovenberg, W. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 2787–2790
36 Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971) *J. Biol. Chem.* 246, 1986–1995
37 Zapf, J. and Froesch, E. R. (1972) *FEBS Lett.* 20, 141–144
38 Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) *J. Biol. Chem.* 244, 6395–6402
39 Tao, M. (1971) *Arch. Biochem. Biophys.* 143, 151–157
40 Jard, S. and Bastide, F. (1970) *Biochem. Biophys. Res. Commun.* 39, 559–566
41 Jergil, B. and Dixon, G. H. (1970) *J. Biol. Chem.* 245, 425–434
42 Tao, M., Salas, M. L. and Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 408–414
43 Miyamoto, E., Petzold, G. L., Harris, J. S. and Greengard, P. (1971) *Biochem. Biophys. Res. Commun.* 44, 305–311