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CHARACTERIZATION OF PROTEIN KINASES FROM BOVINE PAROTID GLANDS

THE EFFECT OF TOLBUTAMIDE AND ITS DERIVATIVE ON THESE PARTIALLY PURIFIED ENZYMES

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Summary

1. Four fractions of protein kinase (EC 2.7.1.37) activity (Peak I_H, II_H, III_C and IV_C) have been resolved and partially purified from the 100 000 × *g* supernatant fraction of bovine parotid glands by DEAE-cellulose and phosphocellulose chromatographies.

2. The protein kinases of Peak I_H and II_H were adenosine 3′,5′-monophosphate (cyclic AMP) -dependent and had similar enzymic properties. The enzyme activities of Peak III_C and IV_C were cyclic-AMP independent, but there were some distinct differences between their properties. The protein kinase in Peak III_C was activated by 0.2 M NaCl or KCl and phosphorylated casein preferentially as the substrate, utilizing only ATP as a phosphate donor. On the other hand, the protein kinase in Peak IV_C was inhibited by univalent salts and preferred phosvitin to casein, utilizing either ATP or GTP as a phosphate donor.

3. Tolbutamide increased the K_m value for ATP and the dissociation constant for cyclic AMP, resulting in the inhibition of cyclic-AMP dependent protein kinase activity in the presence of cyclic AMP. Tolbutamide and its carboxy derivative, 1-butyl-3-*p*-carboxyphenylsulfonylurea, exerted almost no inhibitory effect on either the cyclic-AMP dependent protein kinase activities in the absence of cyclic AMP or on the cyclic-AMP independent protein kinase activities.

Introduction

α-Amylase in salivary glands is first synthesized in the microsomes, then rapidly released into the soluble portion of the cell, and subsequently trans-

ferred to the zymogen granules, where it is concentrated and stored [1—4]. The stimulation of β-adrenergic receptor in salivary glands induces potent secretion of the amylase stored in zymogen granules [5]. Adrenergic varicose nerve fibers are located in a typical adrenergic ground plexus closely surrounding the serous acini of submaxillary and parotid glands [6,7].

Bdolah and Schramm [8] have shown that ^{6}N-2-dibutyryl cyclic AMP is as effective as epinephrine in causing the amylase secretion from rat parotid slices, and have suggested that cyclic AMP is an intermediate in the induction of amylase secretion by catecholamines. In salivary glands, thereafter, the existence of adenylate cyclase [9,10] and the elevation of cyclic AMP levels by sympathomimetic drugs [11—14] have been reported. Moreover, Selinger and Schramm [15] showed the existence of cyclic-AMP dependent protein kinase in rat parotid gland.

In a previous report [16], we demonstrated the inhibition of isoproterenol-induced amylase secretion by tolbutamide, which had been reported to inhibit cyclic-AMP dependent protein kinase (EC 2.7.1.37) in vitro, and suggested the participation of this enzyme in the secretion processes. As a preliminary step in clarifying the role of cyclic-AMP dependent protein kinase in amylase secretion, we have partially purified and characterized both cyclic-AMP dependent and independent protein kinases in the 100 000 × *g* supernatant fraction of bovine parotid glands and also examined their subcellular distribution. We have also studied the effects of tolbutamide and its derivative on these partially purified protein kinases.

Materials and Methods

Materials

Histone II, histone III, α-casein, protamine, phosvitin, and ATP were purchased from Sigma, GTP from PL-biochemicals, Inc., cyclic AMP from Daiichi Pure Chemicals Co. Ltd., Tokyo, guanosine 3′,5′-monophosphate (cyclic GMP) from Boehringer Mannheim, DEAE-cellulose from Brown, phosphocellulose (Pll) and glass fiber filter (GF/C) from Whatman, and cellulose ester filters (Cat. No. HAWP-02500) from Millipore Corporation. Tolbutamide and its carboxy derivative, 1-butyl-3-*p*-carboxyphenylsulfonylurea, were kindly supplied by Chugai Pharmaceutical Co. Ltd., Tokyo, and by Yamanouchi Pharmaceutical Co. Ltd, Tokyo, respectively.

[γ-^{32}P] ATP (28—36 Ci/mmol) and [γ-^{32}P] GTP (9—10 Ci/mmol) were purchased from New England Nuclear, and cyclic [^{3}H] AMP (25 Ci/mmol) from the Radiochemical Centre.

Bovine parotid glands were obtained fresh from a slaughterhouse and stored at −80°C before use.

Methods

Subcellular fractionation of bovine parotid glands. Bovine parotid glands were homogenized in a Potter homogenizer in 9 vols. of 0.01 M Tris · HCl (pH 7.6) containing 0.25 M sucrose. The homogenates were filtered twice through two layers of cheesecloth and centrifuged at 700 × *g* for 10 min, at 10 000 × *g* for 20 min, and at 100 000 × *g* for 90 min. Each particulate fraction was

washed twice with the homogenizing medium, and finally suspended in the same medium. 2 mM $CaCl_2$ was added to the medium for washing the 700 × *g* particulate fraction to prevent nuclei from forming a gel.

Resolution and partial purification of protein kinases. The procedure described by Traugh and Traut [17] was slightly modified. Solid ammonium sulfate (32.5 g/100 ml) was added to the 100 000 × *g* supernatant fraction prepared as mentioned above from bovine parotid glands. After stirring for 20 min, the precipitate was collected by centrifugation at 10 000 × *g* for 20 min and dissolved in 5 mM phosphate buffer (pH 7.0) containing 2 mM EDTA (Buffer A). The solution was dialyzed for 24 h against 150 vols. of Buffer A with three changes of buffer, and centrifuged at 10 000 × *g* for 20 min to remove denatured protein. The dialyzed enzyme preparation (130 mg of protein) was applied to a DEAE-cellulose column (2.6 cm × 22 cm), previously equilibrated with Buffer A. The column was washed with 240 ml of Buffer A prior to further elution with a linear gradient of KCl (0—500 mM) in a total volume of 1600 ml of Buffer A. Fractions of 10 ml were collected at a flow rate of 40 ml per h. Fractions corresponding to each peak of protein kinase activity were pooled and applied to a phosphocellulose column (0.6 cm × 7 cm) equilibrated with 50 mM Tris · HCl (pH 7.5) containing 0.25 M NaCl and 0.5 mM dithiothreitol (Buffer B). The fractions which contained more than 0.25 M KCl were dialyzed against Buffer B before loading on the phosphocellulose column. Each column was washed with 12 ml of Buffer B prior to the elution with a linear gradient of NaCl (0.25 to 1.0 M) in a total volume of 70 ml of Buffer B.

Protein kinase assay. The protein kinase assay was based on the method of Majumder and Turkington [18]. The standard incubation mixture in a total volume of 0.1 ml contained: 50 mM sodium phosphate (pH 6.0), 10 mM magnesium acetate, either 2 μM cyclic AMP or cyclic GMP (as indicated), 2 mM theophylline, 0.2 mM [γ-^{32}P] ATP containing 1.5 · 10^6 cpm, 20 mM sodium fluoride, 0.3 mM EGTA, 0.2 mM EDTA, and 0.3 mg of histone (Sigma, type II). When the assays were carried out by using either casein or phosvitin as substrate, 0.3 mg of histone was replaced by either 0.6 mg of casein or 0.3 mg of phosvitin in 50 mM sodium phosphate (pH 6.9 instead of 6.0). The reaction was usually initiated by the addition of the enzyme fraction and the incubation was carried out for 20 min at 30°C. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. After the addition of 0.1 ml of 0.63% bovine serum albumin, the mixture was allowed to stand at 0°C for at least 30 min. The precipitate was collected by centrifugation, dissolved in 0.1 ml of 1 M NaOH, and reprecipitated by the addition of 1 ml of 10% trichloroacetic acid. Unless otherwise stated, the suspensions were filtered through Whatman GF/C glass fiber discs, and washed with 10 ml of 10% trichloroacetic acid. The discs were dried at 90°C for 10 min, and counted in 7 ml of toluene/0.4% 2,5-diphenyloxazole scintillation fluid. Incubation mixtures without the enzyme fraction served as the blank for the ordinary assay. Assays were all carried out in duplicate.

Binding of cyclic AMP. The incubation mixture in a total volume of 0.3 ml contained: 50 mM sodium phosphate (pH 6.0), 0.3 mM EGTA, 0.2 mM EDTA, 2 mM theophylline, 0.2 μg of bovine serum albumin, and 6.7 nM cyclic [^{3}H]-

AMP (25 Ci/mmol). The reaction was initiated by the addition of the enzyme fraction, and the incubation was carried out for 60 min at 30°C. The protein-bound cyclic [^{3}H]AMP was isolated by Millipore filtration [19]. The filters were washed with 10 ml of 20 mM sodium acetate (pH 6.2) and counted in 10 ml of Bray's scintillation fluid. Assays were all carried out in duplicate.

Protein determination. The protein concentration was measured by the method of Hartree [20], a modified procedure of Lowry et al.

Results

(A) Resolution and partial purification of protein kinase activities

When the column fractions were assayed in the presence of cyclic AMP with histone as substrate, the enzyme preparation from bovine parotid glands was resolved into two distinct peaks of protein kinase activity (Peak I, II) by DEAE-cellulose column chromatography (Fig. 1). In the presence of cyclic GMP, two activity peaks were also observed and coincided with Peak I and II. When the assays were carried out in the absence of cyclic nucleotides with casein as the substrate, two peaks of protein kinase activity (Peak III, IV) were observed, that did not coincide with Peak I and II (Fig. 1).

The major fractions of Peak I, II, III, and IV were pooled separately and chromatographed on phosphocellulose columns. The protein kinase activities specific for histone were not adsorbed onto the phosphocellulose, and therefore were easily resolved from the protein kinase activities specific for casein which were adsorbed on phosphocellulose (Fig. 2). A major peak showing casein-specific protein kinase activity in Peak III and IV was eluted at 0.5 M and 0.6 M NaCl, respectively. In the case of Peak IV, a minor peak specific for casein was eluted just ahead of the major peak. The major peaks of activity for

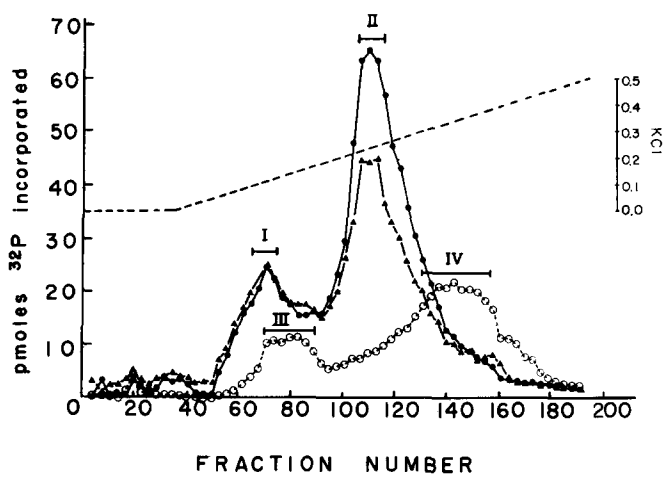

Fig. 1. DEAE-cellulose chromatography of protein kinase from bovine parotid glands. The conditions were described in Methods. Every third fraction was assayed for protein kinase activity in the presence of cyclic AMP (●———●) or cyclic GMP (▲———▲) with histone as the substrate, and in the absence of cyclic nucleotides with casein as the substrate (○———○). The fractions constituting the each peak of protein kinase activity (———) were pooled separately and applied to phosphocellulose column.

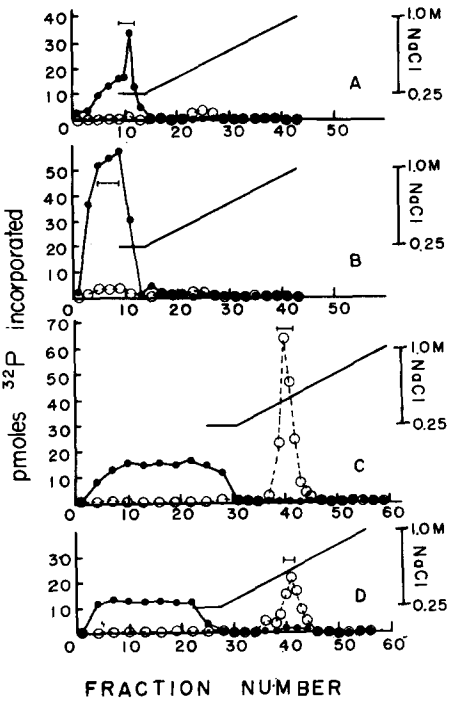

Fig. 2. Phosphocellulose chromatographies of the protein kinase activities in Peak I(A), II(B), III(C), and IV(D) previously resolved by DEAE-cellulose chromatography. The conditions were described in Methods. The amount of each protein applied to phosphocellulose column was as follows: Peak I, 2.5 mg; Peak II, 6.3 mg; Peak III, 4.0 mg; Peak IV, 2.3 mg. Alternate fractions were assayed for protein kinase activity in the presence of cyclic AMP with histone as the substrate (●———●) and in the absence of cyclic nucleotide with casein as the substrate (○- - - - -○). The major peak of activity for histone by phosphocellulose chromatography of Peak I or II protein kinase activity was designated as Peak I_H(A) or II_H(B), respectively, and that of activity for casein resolved by phosphocellulose chromatography of Peak III or IV protein kinase activity was designated as Peak III_C(C) or IV_C(D), respectively. The fractions constituting each peak of protein kinase activity (———) were separately pooled, dialyzed against Buffer A, and used for experiments.

histone, which were resolved by phosphocellulose chromatography of Peaks I or II protein-kinase activity, were designated as Peaks I_H or II_H, respectively; those of activity for casein resolved by phosphocellulose chromatography of Peaks III or IV protein-kinase activity, were designated as Peaks III_C or IV_C, respectively.

Fractions containing the four major peaks of protein kinase activity, purified by phosphocellulose chromatography, were pooled separately and dialyzed against Buffer A before use. Unless otherwise stated, Peaks I_H or II_H protein kinase activity were assayed in the presence of cyclic AMP with histone II as substrate and Peaks III_C or IV_C protein kinase activity were assayed in the absence of cyclic nucleotides with α-casein as substrate.

(B) Properties of protein kinases

Effects of univalent salts and dithiothreitol. The four protein kinase fractions were assayed in the presence of various concentrations of NaCl or KCl. Peak III_C protein kinase activity was stimulated almost 2-fold in the presence

of 0.2 M NaCl and was distinguished from the activities in the other fractions that were inhibited in the presence of NaCl. When NaCl was replaced by KCl, the same effects as with NaCl were observed.

The activity of Peak III_C enzyme was slightly enhanced by 0.5 mM dithiothreitol; 9 and 18 percent increases were found in the absence and presence of 0.2 M NaCl, respectively. The enzyme activity in the other fractions was not affected by dithiothreitol.

In the following studies, Peak III_C protein kinase activity was assayed in the presence of 0.2 M NaCl and 0.5 mM dithiothreitol.

Under the standard assay conditions, the incorporation of radioactive phosphate into protein by the enzymes of the four fractions was linear for at least 20 min during incubation.

pH Optima. The effect of pH on the protein kinase activities in each fraction was studied in a pH range between 5.5 and 7.5, in 50 mM sodium phosphate buffer. As shown in Fig. 3, maximal activities were found with both Peak I_H and II_H enzymes at about pH 6.0 in the presence of cyclic AMP or cyclic GMP, whereas in the absence of cyclic nucleotides the enzyme activity in each fraction was relatively unaffected by pH. Maximal activity was observed with Peak III_C and IV_C enzymes at about pH 6.7 and pH 6.9, respectively.

Effects of cyclic nucleotides. The four protein kinase fractions were assayed in the presence of various concentrations of cyclic AMP or cyclic GMP. As shown in Fig. 4, neither Peak III_C or IV_C enzyme activity was activated by cyclic nucleotides, even at a concentration as high as 10^{-5} M. Both Peak I_H and II_H enzyme activities were stimulated maximally in the presence of cyclic AMP

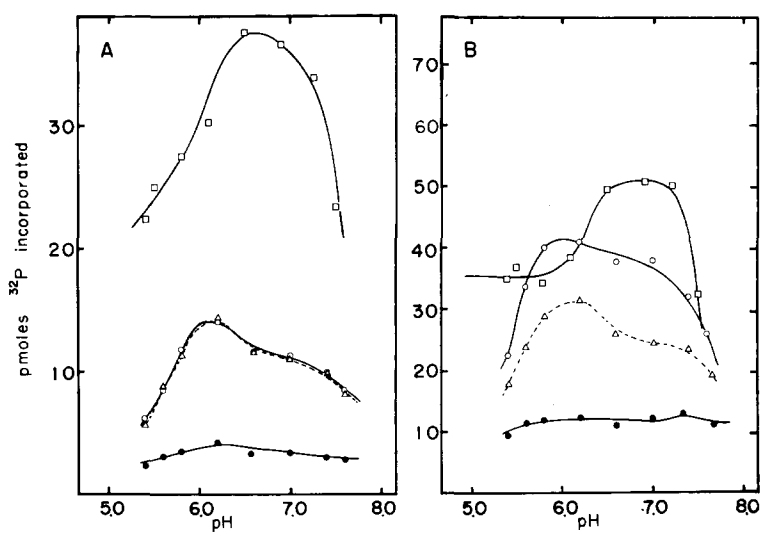

Fig. 3. Effect of pH on the protein kinase activities. In panel A, the protein kinase activities for Peak I_H were assayed at various pH values in the presence of cyclic AMP (○———○) or cyclic GMP (△- - - - -△) and in the absence of cyclic nucleotides (●———●); the enzyme activities for Peak III_C were assayed in the absence of cyclic nucleotides (□———□) with casein as the substrate. In panel B, the protein kinase activities for Peak II_H were assayed at various pH values in the presence of cyclic AMP (○———○) or cyclic GMP (△- - - - -△) and in the absence of cyclic nucleotides (●———●), and the enzyme activities for Peak IV_C were assayed in the absence of cyclic nucleotides (□———□) with casein as substrate.

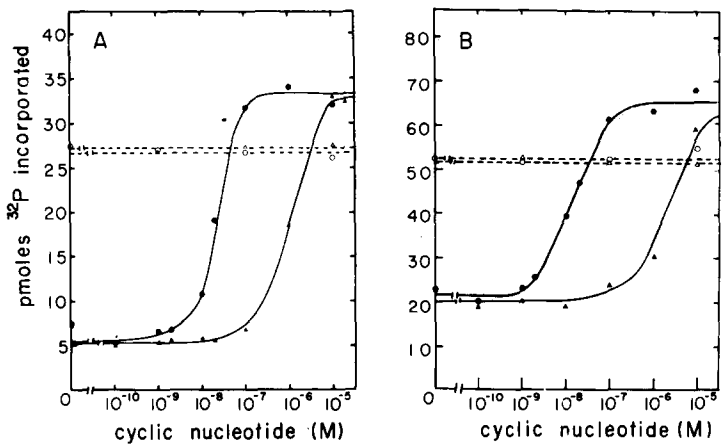

Fig. 4. The effect of various concentrations of cyclic AMP (●———●, ○- - - - -○) or cyclic GMP (▲———▲, △- - - - -△) on the protein kinase activities. In panel A, the protein kinase activities for Peak I_H (●———●, ▲———▲) and Peak III_C (○- - - - -○, △- - - - -△) were assayed with histone and casein, respectively. In panel B, the activity for Peak II_H (●———●, ▲———▲) and Peak IV_C (○- - - - -○, △- - - - -△) were assayed with histone and casein, respectively.

at concentrations greater than $5 \cdot 10^{-7}$ M, and half-maximal stimulations were found with both enzymes at a concentration of approximately $2 \cdot 10^{-8}$ M. Cyclic GMP was able to activate Peak I_H and II_H enzyme activities; however, it required a 100-fold higher concentration than that of cyclic AMP to obtain same activation.

When the reaction was started by the addition of ATP instead of enzyme, the activity of Peak I_H enzyme in the absence of cyclic AMP was markedly enhanced (2-fold), whereas the activity in the presence of cyclic AMP was almost unaffected. This enhancement of activity in the absence of cyclic AMP was not found with Peak II_H enzyme.

Effects of divalent metal ions. The four protein kinase fractions were assayed in the presence or absence of Mg^{2+}, Mn^{2+}, Co^{2+} or Ca^{2+} at concentrations of 2 mM and 10 mM. The maximal activity of each fraction was observed in the presence of 10 mM Mg^{2+}. Co^{2+} was as effective as Mg^{2+} at a concentration of

TABLE I

SPECIFICITY FOR SUBSTRATE PROTEINS

100 μg of each substrate protein was added into 100 μl incubation mixture. The protein kinase activities in Peak I_H and II_H were assayed in the presence of cyclic AMP, and were normalized to the activity with histone II. The activities in Peak III_C and IV_C were normalized to the activity with casein. The numbers in parentheses are pmol of ^{32}P incorporated into each substrate per 20 min.

Substrate protein	Relative protein kinase activity (%)			
	Peak I_H	Peak II_H	Peak III_C	Peak IV_C
Histone II	100(4.7)	100(39.9)	3	9
Histone III (lysine-rich)	53	53	0	5
Protamine	39	49	0	0
Casein	0	2	100(2.7)	100(7.3)
Phosvitin	0	1	37	377

TABLE II

COMPARISON OF THE EFFECTIVENESS OF ATP AND GTP IN PHOSPHOTRANSFERASE REACTIONS

Protein kinase activity was assayed with either [γ-^{32}P] ATP or [γ-^{32}P] GTP in the presence or absence of cyclic AMP with each substrate protein indicated in the second column. Protein kinase activity is given in pmol ^{32}P incorporated per 20 min.

Peak	Substrate	Protein kinase activity				GTP/ATP (+ cyclic AMP)
		ATP		GTP		
		— cyclic AMP	+ cyclic AMP	— cyclic AMP	+ cyclic AMP	
I_H	Histone	2	21	0	0	0.00
II_H	Histone	7	41	0	0	0.00
III_C	Casein	33	33	1	1	0.03
IV_C	Casein	29	29	16	16	0.55
IV_C	Phosvitin	40	43	17	17	0.40

2 mM for the enzyme activity of each fraction, but less effective at a concentration of 10 mM.

When the four protein kinase fractions were assayed with various concentrations of Mg^{2+}, the maximal activity of each fraction was observed at a concentration of 10 mM.

Specificity for substrate proteins. The protein kinase activity of each fraction was assayed with five different proteins as substrates. Table I shows the relative degree of phosphorylation of the various substrates at a concentration of 1 mg/ml. Peak I_H and II_H enzymes had the same substrate specificity with histone II as preferred substrate. Casein was the preferred substrate for Peak III_C enzyme, whereas phosvitin was the preferred substrate for Peak IV_C enzyme.

Specificity for nucleotides. The four protein kinase fractions were assayed with ATP or GTP as phosphate donors. The enzyme in each fraction preferred ATP to GTP (Table II). Only Peak IV_C enzyme was able to utilize GTP effectively, with casein or phosvitin as the substrate. When the activity was assayed at equimolar concentrations of GTP and ATP with casein or phosvitin as substrate, ATP was 2.5 times more effective than GTP with casein, and twice as effective with phosvitin.

Apparent K_m values for substrate proteins. The effects of varying concentrations of substrate proteins were studied for the four protein kinase fractions. As shown in Fig. 9, the apparent K_m values for histone II in the presence of cyclic AMP were 0.53 mg/ml with Peak I_H enzyme and 0.39 mg/ml with Peak II_H enzyme. In the absence of cyclic AMP, the K_m values for histone II with Peak I_H and II_H enzymes were 0.71 mg/ml and 0.77 mg/ml, respectively. Cyclic AMP increased the maximal velocity 7.5-fold for Peak I_H enzyme and 4.5-fold for Peak II_H enzyme. The effect of casein concentrations on protein kinase activity in Peak III_C and the effect of phosvitin concentrations on the enzyme activity in Peak IV_C are shown in Fig. 5. In these cases, the enzymes did not exhibit normal Michaelis kinetics. When the effect of casein concentrations on the activity of Peak IV_C enzyme was studied, the enzyme exhibited normal Michaelis kinetics (Fig. 5). The apparent K_m value for casein was 2.9

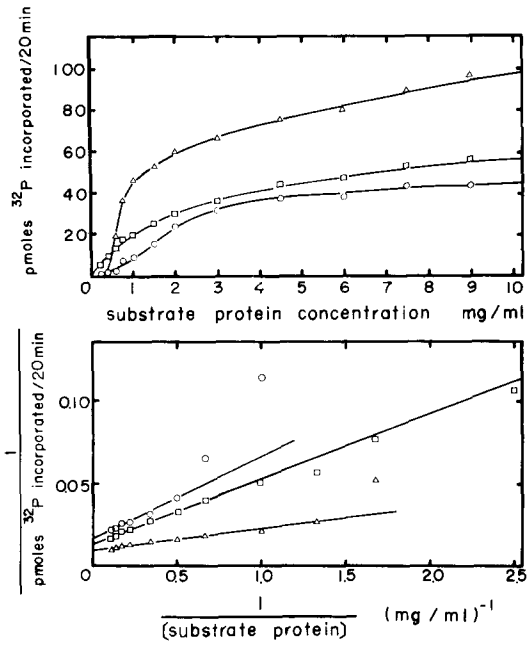

Fig. 5. Effect of the concentrations of substrate proteins on protein kinase activities. Protein kinase activities were assayed in the various concentrations of casein (○———○) for Peak III_C enzyme and of casein (□———□) and phosvitin (△———△) for Peak IV_C enzyme.

mg/ml with Peak III_C enzyme, and the apparent K_m values for phosvitin and casein were 1.3 mg/ml and 2.9 mg/ml, respectively, with Peak IV_C enzyme.

Apparent K_m values for ATP or GTP. The effects of ATP concentrations on protein kinase activities in the four fractions were studied.

As shown in Fig. 10, the apparent K_m values for ATP with Peak I_H and II_H enzymes were not affected by the presence of cyclic AMP, and were 13 μM and 30 μM, respectively. On the other hand, cyclic AMP increased the maximal velocity 7.7-fold for Peak I_H enzyme and 8.9-fold for Peak II_H enzyme.

The apparent K_m value for ATP with Peak III_C enzyme was 300 μM with casein as the substrate. The apparent K_m value for ATP with Peak IV_C enzyme was the same with phosvitin and with casein, i.e. 40 μM.

When the effect of GTP concentrations on protein kinase activities in Peak IV_C enzyme was studied with casein or phosvitin as the substrate, the apparent K_m values for GTP with both substrates were calculated to be 77 μM. The phosphorylation of casein or phosvitin with ATP as a phosphate donor by Peak IV_C enzyme was inhibited competitively by 80 μM GTP (Fig. 6). Similar competitive inhibition by ATP was also observed when the effect of GTP concentrations on Peak IV_C enzyme activity was studied in the presence of 40 μM ATP (Fig. 7).

Dissociation constant for cyclic AMP. Under the experimental conditions described in the Methods, the amount of cyclic AMP bound was maximal after 60 min and was constant for at least the next 30 min. Fig. 11 shows the double reciprocal plots of free cyclic AMP concentrations against bound cyclic

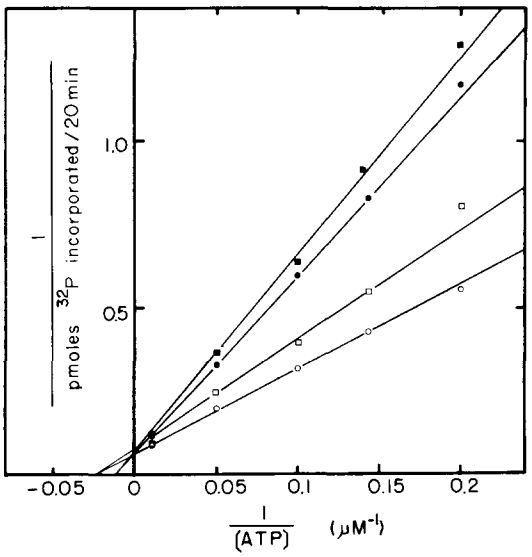

Fig. 6. Effect of GTP on the K_m values for ATP with Peak IV_C enzyme. ^{32}P incorporated into phosvitin from [γ-^{32}P]ATP was determined in the absence (○——○) or presence (●——●) of 80 μM GTP. ^{32}P incorporated into casein from [γ-^{32}P]ATP was determined in the absence (□——□) or presence (■——■) of 80 μM GTP.

AMP with Peak I_H and II_H fractions. The values of dissociation constant with Peak I_H and II_H fractions were 29 nM and 20 nM, respectively.

Subcellular distribution. The protein kinase activity in each fraction obtained by differential centrifugation, was assayed in various experimental conditions with histone, casein, or phosvitin as substrate and with GTP or ATP as a phosphate donor. These results are shown in Table III. When ATP was used as a phosphate donor, approximately 4/5 of the total kinase activity that phos-

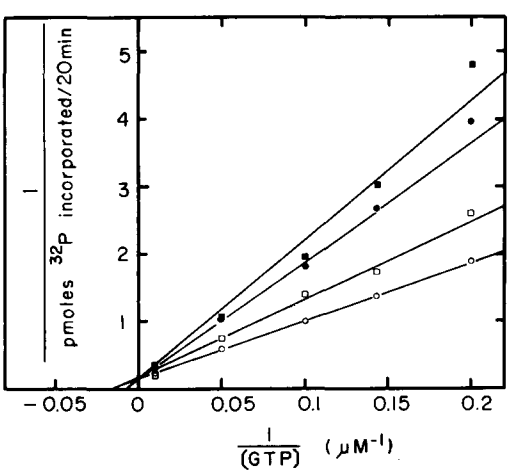

Fig. 7. Effect of ATP on the K_m values for GTP with Peak IV_C enzyme. ^{32}P incorporated into phosvitin from [γ-^{32}P]GTP was determined in the absence (○——○) or presence (●——●) at 40 μM ATP. ^{32}P incorporated into casein from [γ-^{32}P]GTP was determined in the absence (□——□) or presence (■——■) of 40 μM ATP.

TABLE III

SUBCELLULAR DISTRIBUTION OF PROTEIN KINASE ACTIVITY IN BOVINE PAROTID GLANDS

The procedure for subcellular fractionation was described in Methods. Protein kinase activity for each fraction was assayed with [γ-^{32}P] ATP or [γ-^{32}P] GTP as a phosphate donor. When assays were carried out with ATP as a phosphate donor, histone, casein or phosvitin were used as substrates. Histone phosphorylation was examined in the presence or absence of cyclic AMP, and casein phosphorylation was studied in the presence or absence of NaCl. With GTP as a phosphate donor, casein or phosvitin was used as substrate. Incubation mixtures without substrate served as the blank in these assays. The blank values with crude enzyme preparations tended to be high. Therefore, in these assays, the dissolution by NaOH and the reprecipitation by trichloroacetic acid in the standard assay described in the text were repeated once more. The numbers in parentheses indicate the percentage of total activity in each fraction. The protein in each fraction obtained from 1 g of tissue was as follows: 700 × *g* precipitate, 9.40 mg; 10 000 × *g* precipitate, 3.74 mg; 100 000 × *g* precipitate, 3.14 mg; and 100 000 × *g* supernatant, 21.5 mg. Protein kinase activity is given in pmol ^{32}P incorporated/min/g gland tissue.

Fraction	ATP					GTP	
	Histone		Casein		Phosvitin	Casein	Phosvitin
	+ cyclic AMP	— cyclic AMP	+NaCl	—NaCl			
700 × *g* precipitate	87(1.6)	55(3.7)	964(27.4)	671(10.7)	542(10.8)	68(3.5)	37(2.9)
10 000 × *g* precipitate	272(5.0)	87(5.8)	219(6.2)	155(2.5)	153(3.0)	38(2.0)	26(2.0)
100 000 × *g* precipitate	409(7.5)	186(12.4)	290(8.3)	220(3.5)	170(3.4)	9(0.5)	9(0.7)
100 000 × *g* supernatant	4693(85.9)	1176(78.2)	2040(58.1)	5203(83.3)	4160(82.8)	1819(94.1)	1223(94.4)

phorylated histone in the presence of cyclic AMP was observed in the 100 000 × *g* supernatant fractions, whereas the kinase activity that phosphorylated casein and was enhanced in the presence of 0.2 M NaCl was found in the particulate fractions. When GTP was used as a phosphate donor, approximately 94% of the total kinase activity that phosphorylated casein or phosvitin was recovered in the 100 000 × *g* supernatant fraction. Subcellular distributions of protein kinase activity similar to those shown in Table III were found in another preparation.

(C) Studies on the effect of tolbutamide and its carboxy derivative on protein kinase

Effect of tolbutamide and its derivative on protein kinase activity. The protein kinase activity of each fraction was assayed in the presence of various concentrations of tolbutamide or its carboxy derivative, 1-butyl-3-*p*-carboxyphenylsulfonylurea. As the concentration of tolbutamide was increased, it inhibited the enzyme activity in Peak I_H or II_H in the presence of cyclic AMP, whereas it slightly activated Peak I_H enzyme and had almost no effect on Peak II_H enzyme in the absence of cyclic AMP (Fig. 8). On the other hand, the carboxy derivative of tolbutamide showed no effect or a slight inhibition on Peak I_H or II_H enzymes in the presence and absence of cyclic AMP.

When the effect of various concentrations of tolbutamide or its carboxy derivative on the activity of Peak III_C or IV_C enzymes was studied, both

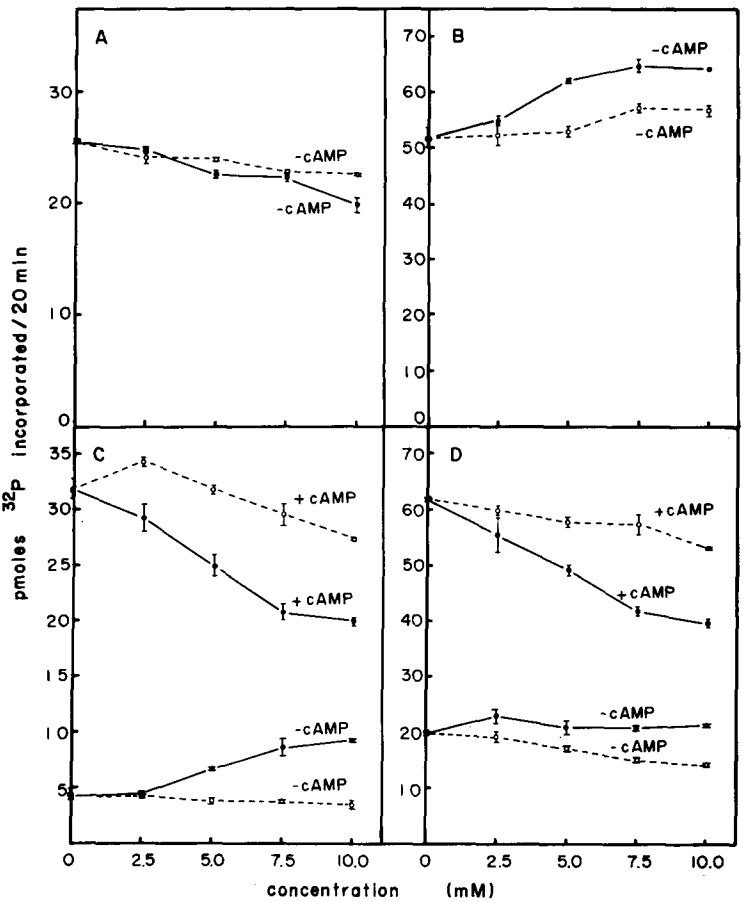

Fig. 8. Effect of various concentrations of tolbutamide (●——●) or its carboxy derivative (○- - - - -○) on the protein kinase activities in the presence or absence of cyclic AMP. Casein was used as substrate for Peak III_C enzyme (A), phosvitin for Peak IV_C enzyme (B), and histone for Peak I_H enzyme (C) and Peak II_H enzyme (D).

compounds slightly inhibited Peak III_C enzyme and slightly activated Peak IV_C enzyme.

Effect of tolbutamide on the apparent K_m value for histone. The effect of histone concentrations on protein kinase activity in Peak I_H or II_H was studied in the presence of 7.5 mM tolbutamide. In the presence of cyclic AMP, tolbutamide had almost no effect on the K_m value for histone with Peak I_H or II_H enzymes. However, the maximal velocity was slightly decreased (Fig. 9).

Effect of tolbutamide on the apparent K_m value for ATP. The effect of ATP concentrations on protein kinase activity in Peak I_H or II_H was studied in the presence of 7.5 mM tolbutamide. In the presence of cyclic AMP, tolbutamide increased the K_m value for ATP 5-fold for Peak I_H enzyme and 2.5-fold for Peak II_H enzyme. In each experiment, the maximal velocity was not affected (Fig. 10).

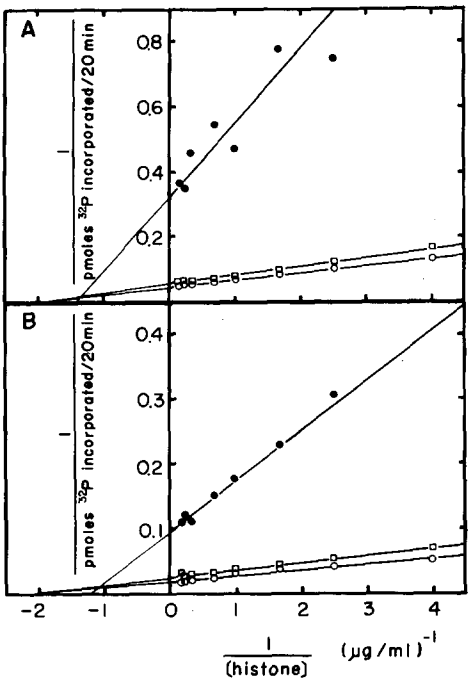

Fig. 9. Double reciprocal plots illustrating the effect of histone concentrations on the rate of phosphorylation by Peak I_H enzyme (A) and by Peak II_H enzyme (B). The protein kinase activity in each fraction was assayed in the presence (○———○) or absence (●———●) of 2 μM cyclic AMP, and in the presence of 7.5 mM tolbutamide with 2 μM cyclic AMP (□———□).

Effect of tolbutamide on the dissociation constant for cyclic AMP. In the presence of 7.5 mM tolbutamide, the cyclic AMP bound to the Peak I_H or II_H fractions was assayed with various concentrations of free cyclic AMP (Fig. 11). Under these conditions, tolbutamide increased the dissociation constant for cyclic AMP 1.4-fold for Peak I_H fraction and 1.7-fold for Peak II_H fraction. In addition, tolbutamide reduced the number of binding sites for cyclic AMP to three quarters for Peak I_H fraction and to two-thirds for Peak II_H fraction.

Discussion

In the present studies, four fractions of protein kinase activity were partially purified from bovine parotid glands and characterized. In accordance with the previous findings on multiple forms of cyclic-AMP dependent protein kinase in various tissues [21,22], the enzymes in two fractions (Peak I_H, II_H) among these four fractions were cyclic-AMP dependent and had similar enzymic properties except for the effect of preliminary incubation with histone. It has been already demonstrated that the preliminary incubation of cyclic-AMP dependent protein kinase with casein or histone decreased the dependence of the enzyme activity on cyclic AMP [22,23] and that the presence of histone or protamine was capable of leading to dissociation of the catalytic subunit from the regulatory subunit [24,25]. Therefore, this effect of preliminary incubation seems to

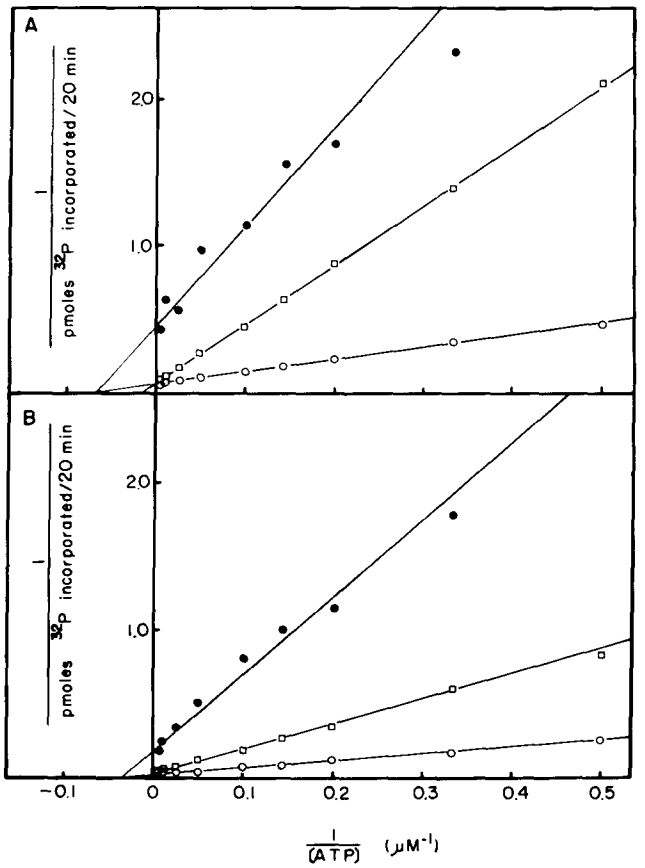

Fig. 10. Double reciprocal plots illustrating the effect of ATP concentrations on the rate of histone phosphorylation by Peak I_H enzyme (A) and by Peak II_H enzyme (B). The protein kinase activity in each fraction was assayed in the presence (○——○) or absence (●——●) of 2 μM cyclic AMP, and in the presence of 7.5 mM tolbutamide with 2 μM cyclic AMP (□——□).

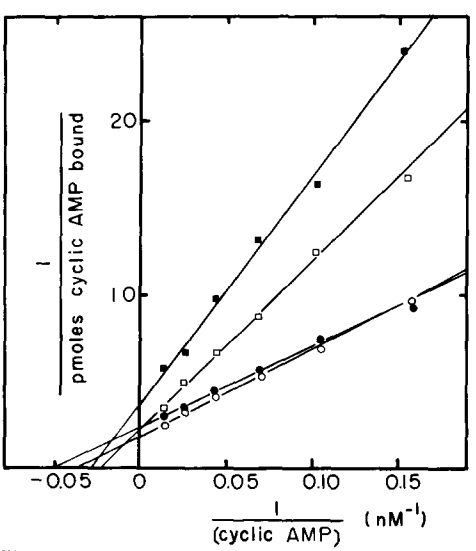

Fig. 11. Double reciprocal plots illustrating the effect of cyclic AMP concentrations on the amount of cyclic AMP bound to Peak I_H fraction or II_H fraction. The amount of cyclic AMP bound to each fraction was determined in the absence (○——○: Peak I_H fraction, ●——●: Peak II_H fraction) or presence (□——□: Peak I_H fraction, ■——■: Peak II_H fraction) of 7.5 mM tolbutamide.

be derived from the dissociation of the catalytic subunit. As it has been reported that cyclic AMP does not change the K_m value of cyclic-AMP dependent protein kinase for ATP except for the enzyme in brain [21], cyclic AMP does not change the K_m value of the enzyme from parotid gland for ATP, either.

The enzyme activity of two other fractions (Peak III_C, IV_C) was cyclic-AMP independent, but there were some distinct differences between their enzymic properties. The protein kinase activity in Peak III_C was activated by 0.2 M univalent salts with casein as a preferential substrate and with ATP only as a phosphate donor. On the other hand, the activity in Peak IV_C was inhibited by univalent salts with phosvitin as a preferential substrate and with either ATP or GTP as a phosphate donor. The protein kinases which are activated by univalent salts and utilize GTP as a phosphate donor have been already reported by Rodnight and Lavin [26] and Traugh and Traut [17]. However, the protein kinase in Peak III_C seems to be different from previously reported enzymes, in that it is not able to utilize GTP as a phosphate donor, even though it is activated by univalent salts. The kinetic results shown in Figs. 6 and 7 suggest that there is a single protein kinase in Peak IV_C which utilizes either ATP or GTP as a phosphate donor and both casein and phosvitin as protein substrates.

Wray and Harris [27] demonstrated that tolbutamide inhibited cyclic-AMP dependent protein kinase in adipose tissue. We tried to use this compound as an inhibitor of cyclic-AMP dependent protein kinase in slice experiments on rat parotid gland; these showed that tolbutamide also inhibited the enzyme activity in rat parotid gland and that amylase secretion was markedly inhibited by this compound [16]. In the present experiments, the effects of tolbutamide and its derivative on partially purified protein kinase were studied. Tolbutamide inhibited the cyclic-AMP dependent protein kinase activities in the presence of cyclic AMP, but had almost no inhibitory effect on cyclic-AMP independent protein kinase activities. A carboxy derivative of tolbutamide, 1-butyl-3-*p*-carboxyphenylsulfonylurea, exerted no inhibitory effect on cyclic-AMP dependent and independent protein kinase activities. The presence of tolbutamide or its carboxy derivative had a rather stimulating effect on the enzyme activity in Peak IV_C. In order to clarify the inhibitory mechanism of tolbutamide on cyclic-AMP dependent protein kinase, the apparent K_m for ATP, the apparent K_m for histone and dissociation constant for cyclic AMP have been determined in the presence of tolbutamide. Tolbutamide increased the K_m value for ATP and the dissociation constant for cyclic AMP, but exerted almost no effect on the K_m values for histone.

The results obtained up to now have suggested that only the amylase stored in zymogen granule might be released into lumen during the secretion processes [2,28]. By secretory stimuli, the zymogen granule membrane fuses with the luminal membrane and the content is released into the lumen [29]. If cyclic-AMP dependent protein kinase truly participates in amylase secretion, it seems likely that it may act on this fusion process. Attention has been payed to the microtubule system, as the subcellular structure that plays an important role in the movement of these granules [30,31]. In fact, Butcher and Goldman [32] showed that colchicine, which disrupts microtubules, interfered with the secre-

tion of amylase from parotid tissue. On the other hand, several papers have accumulated correlating protein kinase with the presence of microtubules [31,33] since Goldman et al. [34] presented the first paper. Though these reports agree with the phosphorylation of microtubule subunits by protein kinase, the physiological significance of the phosphorylation is not yet clear. There remains to be found the intrinsic substrate for protein kinases in salivary gland.

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