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CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

V. PREPARATION AND PROPERTIES OF ADENOSINE 3',5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM VARIOUS BOVINE TISSUES*

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SUMMARY

Adenosine 3',5'-monophosphate (cyclic AMP) dependent protein kinases, which catalyze the phosphorylation of proteins by ATP, have been found in each of fifteen bovine tissues examined. The enzymes were partially purified from these tissues, and their properties studied.

Cyclic AMP increased by 5–15-fold the ability of each of these enzymes to phosphorylate histones. The concentration of cyclic AMP required to give half-maximal activation ranged from 30 to 160 nM. The 3',5'-monophosphate derivatives of inosine, guanosine, uridine, and cytidine also activated the enzymes, but only at concentrations considerably higher than those required for cyclic AMP. 2'-Deoxythymidine 3',5'-monophosphate was incapable of stimulating enzyme activity.

Of the proteins tested as substrates, histone was the most effective for all of the enzymes studied. Protamine and casein were also phosphorylated by the enzymes. All of the enzymes had an absolute requirement for a divalent metal. Cyclic AMP stimulated enzyme activity in the presence of Mg^{2+}, Mn^{2+}, or Co^{2+}, whereas it inhibited enzyme activity in the presence of Ca^{2+}.

The concentration of ATP required to give half-maximal velocity was determined for several of the enzyme preparations in the absence and presence of cyclic AMP; in each case, the apparent K_m for ATP was significantly lower in the presence than in the absence of the activator. It was found that adenine, adenosine, AMP, ADP, GDP, riboflavin, FMN, and FAD inhibited enzyme activity to varying degrees.

The fifteen enzyme preparations were found to be generally similar, but some important differences were observed.

Abbreviations: cyclic AMP, adenosine 3',5'-monophosphate; cyclic IMP, inosine 3',5'-monophosphate; cyclic GMP, guanosine 3',5'-monophosphate; cyclic UMP, uridine 3',5'-monophosphate; cyclic CMP, cytidine 3',5'-monophosphate; cyclic dTMP, 2'-deoxythymidine 3',5'-monophosphate.

* Papers III and IV in this series are refs. 6 and 7, respectively.

INTRODUCTION

Adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinases, which catalyze the phosphorylation of casein, protamine, or histone, by ATP, have been found in a variety of vertebrate and invertebrate tissues as well as in bacteria[1–8]. Of these various protein kinases, only that from bovine brain has been extensively purified and characterized[6]. The possibility has been suggested[4,7] that cyclic AMP-dependent protein kinases may mediate all actions of cyclic AMP. Earlier studies on cyclic AMP regulation of phosphorylase and transferase systems of muscle[9,10], liver[11,12], and heart[13] provide support for this unifying hypothesis.

If this hypothesis is correct, then the specificity of the action of cyclic AMP in various tissues can be expected to reside in the nature of the individual protein kinases and/or in the nature of the endogenous substrates for these protein kinases. It therefore seemed of interest to determine the extent to which the different physiological and pharmacological effects of cyclic AMP in various tissues might be reflected in differences in properties of the protein kinases from these tissues. For that reason, we have compared some of the characteristics of partially purified protein kinases from a series of tissues in a single (bovine) species. The properties studied included the apparent K_m for cyclic AMP, metal ion requirements, relative efficacy of various cyclic 3′,5′-mononucleotides to stimulate enzyme activity, ability of various proteins to serve as substrate for the different protein kinases, and effects of inhibitors.

MATERIALS AND METHODS

Materials

Fresh bovine organs were obtained from a local slaughterhouse, frozen in dry ice immediately after removal, and stored at −20° until enzyme purification was started. Chromatographically pure cyclic AMP, cyclic IMP, cyclic GMP, cyclic UMP, cyclic CMP, and cyclic dTMP were obtained from Boehringer Mannheim Corp. The concentrations of the cyclic nucleotides were determined from ultraviolet absorption measurements at their respective λ_{max} at pH 7.0. Published values[14,15] were used for the molar extinction coefficients, except for cyclic IMP which was assumed, at the suggestion of the supplier, to have an extinction coefficient of $12.2 \cdot 10^3$ l · mole^{-1} · cm^{-1} at 248.5 mμ. [γ-^{32}P]ATP was prepared by the method of POST AND SEN[16]. ATP, ADP, AMP, and GDP were purchased from Schwarz BioResearch; adenosine, adenine, FMN, and FAD from Sigma; riboflavin from Nutritional Biochemicals Corp. Commercial sources of protein substrates were as follows: histone "mixture" (calf thymus), "slightly lysine-rich" histone (calf thymus), and "arginine-rich" histone (calf thymus), from Mann Research Laboratories; protamine (salmon sperm) and bovine serum albumin (Fraction V) from Sigma; casein from Nutritional Biochemicals Corporation. DEAE-cellulose (medium mesh, 0.85 mequiv/g) was purchased from Sigma.

Enzyme assay

The activity of the cyclic AMP-dependent protein kinase was assayed in an incubation volume of 0.2 ml. The standard assay mixture contained 10 μmole of sodium glycerol phosphate buffer, pH 6.5, 40 μg of histone "mixture", 500 pmole of

[γ-^{32}P]ATP, 2 μmole of $MgCl_2$, 2 μmole of NaF, 0.4 μmole of theophylline, and 0.06 μmole of ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, with or without 1000 pmoles of cyclic AMP.

The reaction was started by the addition of the [γ-^{32}P]ATP. The mixture was incubated at 30° for 5 min, in a shaking water bath, and the reaction was terminated by the addition of 2 ml of 5% trichloroacetic acid. Protein-bound ^{32}P was determined as described for the enzyme from skeletal muscle[1,17], with modification as follows: After the addition of the 2 ml of trichloroacetic acid, 0.2 ml of 0.63% bovine serum albumin was added as carrier protein. Another 2 ml of 5% trichloroacetic acid was added, the mixture was centrifuged, and the supernatant was removed by aspiration. The precipitate was dissolved in 0.1 ml of 1 M NaOH, the protein was reprecipitated with 4 ml of 5% trichloroacetic acid and centrifugation and aspiration were repeated. The precipitate was washed once more, as described above, and then dissolved in 0.1 ml of 1 M NaOH, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation system. The scintillation fluid was prepared by dissolving 8 g of Omnifluor (New England Nuclear) in 1 l of toluene and 1 l of ethylene glycol monomethyl ether. The overall recovery of radioactive protein in the standard assay procedure with histone "mixture" as substrate, both in the absence and presence of cyclic AMP, was 70% of that obtained by the procedure of LANGAN[2]. The data have not been corrected for overall recovery of radioactive protein. One unit of enzyme activity was defined as that amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered protein in 5 min at 30° in the standard assay system. Protein was measured by the method of LOWRY *et al.*[18], using bovine serum albumin as the protein standard.

Enzyme purification

Cyclic AMP-dependent protein kinases from all tissues were purified by the method used for the enzyme from bovine brain[6]. All procedures used for the purification of the enzymes were carried out at 4°. Frozen bovine tissue was thawed, cut into small pieces, and homogenized with 3 vol. of neutral 4 mM EDTA solution for 2 min in a Waring Blendor. The homogenate was centrifuged at 27 000 $\times g$ for 20 min. The supernatant solution was adjusted to pH 4.8 by the dropwise addition of 1 M acetic acid, with stirring. After waiting 10 min, the precipitate was removed by centrifugation at 27 000 $\times g$ for 30 min. The pH of the clear supernatant was then readjusted to 6.5 with 1 M potassium phosphate buffer (pH 7.2). All buffers used in succeeding steps of the purification contained 2 mM EDTA.

Protein kinase activity was precipitated from the neutralized supernatant solution by the addition of solid $(NH_4)_2SO_4$ (32.5 g/100 ml). After stirring for 30 min, the precipitate was collected by centrifugation at 27 000 $\times g$ for 20 min, and dissolved in 6% of the crude extract volume of 5 mM potassium phosphate buffer (pH 7.0). The resulting solution was dialyzed against 20 vol. of the same buffer with two changes of buffer during a 14-h period. After dialysis, the solution was centrifuged at 27 000 $\times g$ for 30 min, and the precipitate was discarded.

The enzyme solution was applied to a column of DEAE-cellulose that had been equilibrated with 5 mM potassium phosphate buffer (pH 7.0). The size of the column was roughly proportional to the amount of protein in the enzyme solution (50 g resin per g protein) obtained at the $(NH_4)_2SO_4$ step, and ranged from 1.5 cm $\times$ 20 cm to 4 cm $\times$ 25 cm. After the enzyme had been applied, the column was washed with two

bed volumes of 0.1 M potassium phosphate buffer (pH 7.0); 0.3 M phosphate buffer (pH 7.0) was then applied. In almost all cases, the activity was eluted by the 0.3 M buffer; the exceptions were stomach, in which greater than 98% of the activity was found in the 0.1 M eluate, and duodenum, in which the protein peaks eluted with both the 0.1 and 0.3 M buffers were found to contain high activity.

The active fractions were pooled and dialyzed overnight against 20 vol. of 5 mM potassium phosphate buffer (pH 7.0) with two changes of buffer. These dialyzed enzyme solutions were used for all studies reported in this paper.

RESULTS

Purification of cyclic AMP-dependent protein kinase

The results of the purification of protein kinase from fifteen bovine tissues are summarized in Table I. For the purpose of calculating enzyme activity and the extent of purification of the protein kinase for Table I, the difference between the rate of phosphorylation in the presence and absence of cyclic AMP has been used. The degree

TABLE I

SUMMARY OF PURIFICATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM VARIOUS BOVINE TISSUES

Assay conditions were as described in MATERIALS AND METHODS.

Enzyme source	*Specific activity (units/mg protein)*				*DEAE-cellulose eluate*		
	Crude extract	*pH 4.8-supernatant*	*$(NH_4)_2SO_4$ precipitate*	*DEAE-cellulose eluate*	*Purification (-fold)*	*Recovery (%)*	
						Activity	*Protein*
Brain	13	45	121	1035	76	81	1.02
Pancreas	8	8	14	384	49	17	0.33
Kidney	5	17	30	406	83	8	0.09
Heart	43	44	245	2590	61	32	0.52
Lung	21	45	44	207	10	4	0.37
Liver	2	8	—	751	443	16	0.04
Thyroid	1	28	16	221	258	176	0.68
Adrenal	11	7	43	288	27	6	0.22
Skeletal muscle	12	8	318	1916	156	19	0.12
Testis	45	63	132	1012	23	4	0.18
Ovary	46	45	104	761	16	5	0.28
Stomach	3	11	8	34	13	8	0.62
Duodenum	15	8	44	294	19	12	0.59
Bladder	13	135	140	240	18	25	1.40
Uterus	10	48	28	220	22	13	0.61

of purification of the enzyme varied considerably from tissue to tissue. It seems that the enzyme from some tissues could not be purified efficiently by the procedure developed for the brain enzyme[6]. The high recovery (176%) of thyroid protein kinase activity was due to removal of a particularly potent inhibitor present in the crude extract of the thyroid gland. The presence of material inhibitory to the protein kinase activity, previously reported for brain[3,6] and bacteria[4], was also observed in the crude extracts of several of the tissues examined in the present study.

Effect of varying reaction time and enzyme concentration

Protein kinase preparations from brain, pancreas, kidney, heart, and lung were incubated with and without cyclic AMP (5 μM) for varying amounts of time. In all cases, activity was proportional to incubation time up to 8 min. 5 min was chosen as the standard incubation time for all subsequent studies.

For all enzyme preparations, a linear relationship was found between activity and enzyme concentration, over the range studied. For subsequent studies, solutions of enzymes from different tissues were diluted with 5 mM potassium phosphate buffer (pH 7.0) to give approximately equivalent activity under standard assay conditions.

Effect of varying the concentration of cyclic AMP

The activity of the various enzymes was studied as a function of the concentration of cyclic AMP. The concentration was varied from 0.01 to 50 μM. In all cases, stimulation reached a maximum at about 5 μM cyclic AMP and then decreased at higher cyclic AMP concentrations. The apparent K_m (the concentration of cyclic AMP required to give a half-maximal increase in activity) was found to range from 30 to 160 nM (Table II).

TABLE II

APPARENT K_m VALUES FOR CYCLIC AMP USING PROTEIN KINASE PREPARED FROM VARIOUS BOVINE TISSUES

Assay conditions were as described in MATERIALS AND METHODS except for varying the cyclic AMP concentration.

Enzyme source	*Apparent* K_m $\times 10^8$ *(M)*
Brain	11.0
Pancreas	5.3
Kidney	6.6
Heart	4.0
Lung	6.8
Liver	3.2
Thyroid	3.0
Adrenal	10.0
Skeletal muscle	3.3
Testis	8.0
Ovary	14.0
Stomach	16.0
Duodenum	12.0
Bladder	5.4
Uterus	5.5

Effect of metal ions

The effect of varying the concentration of Mg^{2+}, on the enzyme preparations from brain, pancreas, kidney, heart and lung, was studied in the presence and absence of 5 μM cyclic AMP. Maximal stimulation occurred between 10 and 20 mM Mg^{2+}; at higher concentrations of Mg^{2+}, activity decreased markedly. The Mg^{2+} concentration required to give half-maximal velocity varied from 1.6 to 4.5 mM. There was no apparent effect of cyclic AMP on the K_m of the enzyme for Mg^{2+}.

TABLE III

EFFECT OF METAL IONS ON THE ACTIVITY OF PROTEIN KINASE FROM VARIOUS BOVINE TISSUES

Assay conditions were as described in MATERIALS AND METHODS, except for the variation in the kind and amount of each metal ion (added as the chloride salt). Values are given for activity in the absence and presence of 5 μM cyclic AMP (cAMP). All values have been corrected for the traces of activity which occurred in the absence of added metal ion.

Enzyme source	*Protein kinase activity (units)*							
	Mg^{2+}				Mn^{2+}			
	2.5 mM		*10 mM*		*2.5 mM*		*10 mM*	
	−cAMP	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*
Brain	1.0	8.9	2.4	21.1	5.3	11.2	1.5	2.9
Pancreas	1.5	11.6	2.9	21.1	1.8	11.5	2.1	7.8
Kidney	2.0	10.0	4.1	23.1	3.6	11.5	6.4	14.4
Heart	3.0	18.0	8.0	50.8	2.4	26.0	3.4	22.8
Lung	2.9	22.0	6.0	43.7	7.1	30.0	7.6	21.8
Liver	1.6	11.2	1.6	18.1	2.4	5.9	2.9	3.4
Thyroid	2.8	11.2	5.8	23.4	6.7	11.1	4.8	10.6
Adrenal	1.5	4.4	1.1	9.2	1.3	4.2	1.6	3.2
Skeletal muscle	1.2	11.5	3.0	16.8	1.9	4.3	2.0	3.0
Testis	1.0	5.6	2.0	13.0	1.0	4.5	1.6	2.3
Ovary	1.0	4.9	1.8	13.8	0.7	3.4	1.2	1.9
Stomach	1.6	4.0	2.6	8.6	0.7	2.5	1.2	1.4
Duodenum	1.3	5.3	1.8	12.6	1.5	3.4	2.0	2.2
Bladder	3.1	11.6	6.1	25.2	6.2	20.0	3.7	7.1
Uterus	0.8	3.5	1.6	10.1	1.2	7.0	1.7	3.2

All fifteen enzyme preparations had an absolute requirement for a divalent metal. The effect of four different metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}) on the activity of the various protein kinase preparations was studied at metal ion concentrations of 2.5 and 10 mM (Table III). All four of the metal ions tested supported protein kinase activity in the absence of added cyclic AMP. Stimulation by cyclic AMP was greater in the presence of 10 mM Mg^{2+} than of 2.5 mM Mg^{2+}. Mn^{2+} was somewhat less effective, and showed better stimulation at the lower concentration of metal ion. The level of enzyme activity in the absence of cyclic AMP, as well as the stimulation by the cyclic nucleotide, was generally greatest in the presence of Co^{2+}. In the presence of Ca^{2+}, enzyme activity was low, and cyclic AMP inhibited histone phosphorylation.

Analogs of cyclic AMP

The effect of a number of cyclic 3′,5′-nucleotides on the activity of various protein kinase preparations is shown in Table IV. The studies with the cyclic nucleotides did not reveal any marked differences among the protein kinases from various tissues. At low concentrations, cyclic AMP was more effective than any of its analogs in activating the enzymes. At higher concentrations, the other cyclic ribonucleotides were capable of stimulating enzyme activity to near-maximal levels. In contrast, the cyclic deoxyribonucleotide, cyclic dTMP, was incapable of activating the enzyme at any concentration up to 50 μM. In general the order of effectiveness of the activators was cyclic AMP > cyclic IMP > cyclic GMP > cyclic UMP > cyclic CMP > cyclic dTMP.

Protein kinase activity (units)							
Co^{2+}				Ca^{2+}			
2.5 mM		10 mM		2.5 mM		10 mM	
−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
1.4	25.3	3.9	29.9	0.26	0.04	0.22	0.10
3.8	36.5	4.4	30.8	0.20	0.15	0.09	0.26
5.6	34.8	8.4	28.6	0.51	0.27	0.27	0.13
3.0	45.3	63.8	59.0	0.47	0.36	0.14	0.15
3.3	52.2	10.3	51.1	0.96	0.27	0.24	0.24
1.2	3.7	3.1	30.0	0.32	0.04	0.23	0.02
4.1	28.1	6.7	36.6	0.28	0.06	0.31	0.02
1.1	13.7	1.6	17.0	0.01	0.02	0.06	0.03
3.0	35.2	2.8	39.7	0.18	0.06	0.16	0.08
1.8	17.0	3.2	22.8	0.22	0.18	0.20	0.14
1.2	15.1	2.2	20.1	0.02	0.00	0.02	0.00
1.8	11.8	1.2	16.1	0.29	0.11	0.20	0.05
1.4	15.4	2.4	20.3	0.09	0.00	0.08	0.00
3.1	22.6	5.3	40.3	0.62	0.25	0.74	0.23
1.8	11.4	1.7	11.9	0.19	0.22	0.49	0.26

Concentration of ATP

The effect of varying the concentration of ATP was studied, using the protein kinase preparations from brain, pancreas, kidney, heart and lung, in the presence and absence of 5 μM cyclic AMP. In the presence of cyclic AMP, double reciprocal plots indicated that the pancreas, kidney, heart and lung enzymes each had a K_m for ATP of 7 μM, while the protein kinase from brain had a K_m of 14 μM. The K_m for the brain enzyme was similar to the K_m (13 μM) obtained with the more highly purified brain enzyme[6].

In the absence of cyclic AMP, double reciprocal plots failed to yield a linear relationship, and the K_m could not be accurately determined. However, it was clear that the concentration of ATP at half-maximal velocity in the absence of cyclic AMP was significantly higher in all cases than the concentration required for half-maximal velocity in the presence of cyclic AMP. In the case of the brain enzyme, there was a 10–15-fold difference; in contrast, in the pancreas, kidney, heart and lung preparations, a difference of approx. 2-fold was noted. Thus, the concentration of ATP required for half-maximal velocity in the absence of cyclic AMP was about 10-fold greater for the brain enzyme than for the other four enzymes.

Protein substrates

A number of proteins were tested (40 μg per assay tube) for their ability to serve as substrates for various cyclic AMP-dependent protein kinases (Table V). For most of the enzymes, histone "mixture" was the best substrate for cyclic AMP-dependent

TABLE IV

COMPARISON OF THE EFFECT OF CYCLIC 3′,5′-MONONUCLEOTIDES ON THE ACTIVITY OF PROTEIN KINASE FROM VARIOUS BOVINE TISSUES

Assay conditions were as described in MATERIALS AND METHODS except for the variation in kind and amount of cyclic nucleotide. Activity for each enzyme preparation is expressed as percentage of activity found for that enzyme preparation in the presence of 5 μM cyclic AMP. All values were corrected for activity in the absence of added cyclic nucleotide.

Enzyme source	*Relative protein kinase activity (%) in the presence of cyclic nucleotides (μM)*																	
	Cyclic AMP			*Cyclic IMP*			*Cyclic GMP*			*Cyclic UMP*			*Cyclic CMP*			*Cyclic dTMP*		
	0.5	*5*	*50*	*0.5*	*5*	*50*	*0.5*	*5*	*50*	*0.5*	*5*	*50*	*0.5*	*5*	*50*	*0.5*	*5*	*50*
Brain	96	100	90	32	82	109	7	33	86	6	12	66	7	15	57	2	2	2
Pancreas	90	100	91	33	87	88	15	35	92	12	38	63	0	8	49	2	0	0
Kidney	98	100	101	39	97	104	7	39	72	13	45	94	0	17	52	0	0	0
Heart	99	100	76	31	84	90	6	43	77	19	62	73	8	35	65	0	0	0
Lung	95	100	83	32	76	89	7	35	78	7	20	58	1	20	55	0	0	4
Liver	94	100	93	66	103	108	16	69	82	5	37	88	10	37	93	1	5	11
Thyroid	93	100	96	65	94	104	5	48	111	4	67	89	1	15	81	0	0	0
Adrenal	80	100	75	33	100	100	8	54	89	3	27	80	12	21	54	1	1	2
Skeletal muscle	97	100	84	45	90	86	21	74	83	3	37	93	1	37	63	4	3	4
Testis	101	100	83	70	90	102	7	40	85	4	20	63	6	28	59	3	0	2
Ovary	104	100	95	34	96	109	3	39	98	3	15	66	6	13	51	0	0	2
Stomach	102	100	95	61	86	100	15	48	80	13	32	97	10	30	97	3	2	10
Duodenum	97	100	88	45	98	106	14	42	103	6	21	85	4	21	53	1	4	4

TABLE V

COMPARISON OF ABILITY OF SOME PROTEINS TO SERVE AS SUBSTRATE FOR VARIOUS PROTEIN KINASES

Assay conditions were as described in MATERIALS AND METHODS, except for the variation in the kind and amount of proteins used as substrate. 40 μg of substrate were added per assay except where indicated. All values are corrected for activity in the absence of added protein substrates. The concentration of cyclic AMP (cAMP) was 5 μM.

Enzyme source	*Protein kinase activity (units)*											
	Histone-mixture		*Histone, lysine-rich*		*Histone, arginine-rich*		*Protamine*		*Casein*		*Casein (600 µg)*	
	−cAMP	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*
Brain	0.9	12.3	0.5	7.8	0.3	6.5	1.1	3.5	0.4	0.8	0.2	2.7
Pancreas	1.5	9.0	0.9	11.7	0.5	2.7	0.9	2.5	0.1	0.6	0.1	1.9
Kidney	1.0	10.8	0.7	7.5	0.8	5.6	0.9	1.5	0.2	0.8	0.2	1.9
Heart	3.3	22.3	2.6	24.1	1.0	13.6	1.4	6.0	0.1	0.8	0.1	1.6
Lung	3.3	14.4	1.5	7.7	0.9	7.7	2.1	4.6	0.1	0.9	0.2	0.9
Liver	1.2	12.9	0.5	8.5	0.5	5.8	0.6	3.3	0.0	0.3	0.1	3.4
Thyroid	1.9	11.7	0.7	6.8	0.6	4.7	0.8	1.5	0.1	0.7	0.1	2.6
Adrenal	1.3	11.7	1.3	7.9	1.0	6.1	1.4	3.4	0.0	0.3	0.3	3.9
Skeletal muscle	1.2	12.4	0.8	8.3	0.4	5.5	1.6	2.8	0.1	0.6	0.1	3.6
Testis	1.3	13.5	0.7	6.2	0.9	5.2	2.8	4.1	0.1	0.6	0.4	2.6
Ovary	1.3	11.2	0.4	4.0	0.5	4.4	1.7	2.8	0.2	0.4	0.3	1.9
Stomach	2.8	11.0	1.2	4.0	1.0	3.3	0.9	2.3	0.2	0.5	0.4	1.8
Duodenum	1.5	10.4	0.7	5.2	0.6	3.4	1.0	1.7	0.1	0.2	0.3	2.2

TABLE VI

INHIBITION BY ADENOSINE, FMN AND AMP OF PROTEIN KINASE PREPARATIONS FROM VARIOUS BOVINE TISSUES

Assay conditions were as described in MATERIALS AND METHODS, except for the presence of inhibitors, where indicated. The concentration of cyclic AMP (cAMP) was 5 μM. The data have been corrected for zero time control values. The concentration of inhibitors was 50 μM.

Enzyme source	*Protein kinase activity (units)*									
	Expt. 1						*Expt. 2*			
	None		*Adenosine*		*FMN*		*None*		*AMP*	
	−cAMP	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*
Brain	1.9	11.3	1.3	9.1	1.1	0.7	1.9	23.7	2.0	21.9
Pancreas	2.1	23.8	1.3	15.3	1.8	0.2	2.1	11.3	2.1	10.7
Kidney	3.4	28.0	2.2	15.9	1.2	0.3	3.0	22.9	2.9	19.7
Heart	5.2	62.5	4.7	50.8	3.1	0.4	8.3	43.4	5.9	37.8
Lung	9.6	55.4	9.9	67.4	4.7	1.2	6.3	41.5	6.3	32.6
Liver	1.6	14.5	0.8	9.7	1.6	0.2	1.1	15.8	1.7	13.2
Thyroid	3.1	9.7	1.3	7.5	0.8	0.2	2.2	12.2	1.7	9.8
Adrenal	1.6	13.5	1.0	9.3	1.1	0.5	0.8	9.3	1.3	9.7
Skeletal muscle	1.0	17.0	0.5	9.0	0.6	0.1	1.8	14.9	1.3	12.1
Testis	2.1	13.9	1.1	8.0	1.0	0.7	1.6	10.7	1.3	12.4
Ovary	1.2	7.7	0.7	3.3	0.5	0.2	2.1	10.5	1.5	10.9
Stomach	2.9	12.6	2.6	11.6	0.8	3.4	3.0	9.6	0.9	2.6
Duodenum	1.0	10.7	1.1	10.1	1.0	3.5	2.7	14.0	1.5	7.8

kinase activity. Effectiveness of the substrates was generally in the order: histone "mixture" > "lysine-rich" histone > "arginine-rich" histone > protamine > casein. Bovine serum albumin, in amounts of 40 μg per assay tube, showed slight or no ability to serve as substrate for any of the protein kinases. Casein was also tested at a level of 600 μg per assay. At this concentration it was significantly more effective than at 40 μg; it was still inferior, however, to the lower concentration of all three histones.

The effect of varying the concentration of histone "mixture" or "arginine-rich" histone on enzyme activity was studied in preparations from brain, pancreas, kidney, heart and lung. The concentration at half-maximal velocity for all preparations with either substrate, in the presence or absence of 5 μM cyclic AMP, was in the range of 10–40 μg of protein. No significant differences between enzyme preparations were observed.

Effects of inhibitors

Eight compounds (adenine, adenosine, AMP, ADP, GDP, riboflavin, FMN and FAD) were tested for their ability to inhibit protein kinase activity. The inhibitors were present at a concentration of 50 μM in the assay system, both with and without 5 μM cyclic AMP. Adenosine inhibited, to varying degrees, the activity of protein kinase from ten of the thirteen tissues examined. Exceptions were the enzymes from lung, stomach and duodenum, which were not inhibited by adenosine (Table VI). AMP was a weak inhibitor in most cases, but did inhibit stomach and duodenum preparations significantly. For all enzymes, adenine and GDP were weak inhibitors while ADP inhibited enzyme activity about 50%. FMN was the most potent of the compounds studied and inhibited enzyme activity in all cases. In eleven of the enzyme preparations, cyclic AMP inhibited protein kinase activity in the presence of FMN. In the enzymes from stomach and duodenum, cyclic AMP stimulated activity, although at a decreased level, when FMN was present.

The effect of varying the concentration of FMN was studied using the protein kinase preparation from heart. The concentration of FMN required to give half-maximal inhibition was 60 μM in the absence and 12 μM in the presence of 5 μM cyclic AMP. In the same preparation, it was found that FAD and riboflavin were inhibitory, but had less than one-tenth the potency of FMN, on a molar basis. As in the case of FMN, the concentration required for half-maximal inhibition by FAD and riboflavin was less in the presence than in the absence of cyclic AMP.

DISCUSSION

Cyclic AMP-dependent protein kinases have been found in fifteen bovine tissues, partially purified, and characterized. The results reported in this paper indicate that the various cyclic AMP-dependent protein kinases have many properties in common. Thus, various divalent metal ions, analogs of cyclic AMP, and protein substrates affected the activity of the enzyme from most of the sources in a similar manner. Moreover, the concentrations of histone and Mg^{2+} required for half-maximal velocity were approximately the same for all of the enzymes studied.

Some significant differences were evident, however. Thus, in thirteen of the fifteen tissues examined, protein kinase activity was eluted by 0.3 M potassium

phosphate buffer in the course of the chromatographic purification on the DEAE-cellulose column. In contrast, more than 98% of the activity from stomach lining was eluted by the 0.1 M potassium phosphate buffer, while the activity from duodenum was distributed approximately equally between the 0.1 and the 0.3 M fractions. In order to exclude the possibility of an artifact being responsible for the difference in chromatographic properties between the stomach enzyme and other enzymes, the enzyme preparations from the brain and stomach were rechromatographed on DEAE-cellulose columns, as described above, both separately, and after pooling the enzymes from the two sources. The stomach enzyme was again eluted in the 0.1 M potassium phosphate buffer fraction and the brain enzyme in the 0.3 M fraction, in the case of the individual enzymes as well as of the pooled samples.

The enzymes from stomach and duodenum were further differentiated from the others by inhibitor studies. In the presence of FMN, cyclic AMP stimulated the activity of the enzyme preparations from stomach and duodenum, but inhibited activity in all of the other enzyme preparations. Moreover, the enzyme preparations from stomach and duodenum were inhibited more by AMP and less by adenosine than were the other kinases. The only exception was the enzyme from lung, which was not inhibited by adenosine. The identification of the brain and stomach enzymes recovered after chromatography of the pooled samples, discussed above, was aided by their relative sensitivity to inhibitors.

It is unlikely that the enzymes from stomach and duodenum arose from bacteria in the gastro-intestinal tract. First, the stomach and duodenum were washed throughly before freezing. Secondly, the properties of the protein kinase from *Escherichia coli*, the only bacterial protein kinase studied to date[4], are quite different from those of the mammalian system, the bacterial enzyme being totally inactive in the presence of Mg^{2+}.

Another tissue-specific property was observed in the studies on the apparent K_m of the enzymes for ATP. In the absence of cyclic AMP, the apparent K_m of the brain enzyme for ATP was an order of magnitude greater than that of the enzymes from pancreas, kidney, heart and lung. Furthermore, cyclic AMP had a far greater effect on the apparent K_m of the brain enzyme for ATP than it did for any of the other four enzymes. Some small differences were also noted in studies on the apparent K_m of the various enzymes for cyclic AMP. Thus, the concentration of cyclic AMP required for half-maximal stimulation varied from 30 to 160 nM, *i.e.* over about a 5-fold range. Moreover, there was some variation among the enzymes in their sensitivity to various cyclic 3',5'-nucleotides.

An interesting species difference was found in the sensitivity of skeletal muscle enzyme to inhibition by AMP. It was previously reported that the cyclic AMP-dependent protein kinase from rat skeletal muscle was almost completely inhibited ($>$ 98% inhibition) by 50 μM AMP (ref. 7). This was confirmed in the present investigation, and contrasts with the ineffectiveness of AMP in inhibiting the enzyme from bovine muscle. Thus, in one experiment in the present study, in which the enzymes from rat and beef muscle were directly compared, 50 μM AMP inhibited the enzyme from rat muscle by 90% and the enzyme bovine muscle by only 18%. A dramatic difference in the sensitivity of the two muscle enzymes also exists with respect to inhibition by GDP: the rat muscle enzyme was inhibited over 80% (ref. 7), whereas, in the present study, the bovine muscle enzyme was inhibited only 12% by 50 μM GDP.

It is of interest that conditions have been found in which the effect of cyclic AMP on protein kinase is to inhibit rather than stimulate enzyme activity. Thus, in the presence of either Ca^{2+} or FMN, the stimulatory effect of cyclic AMP is reversed. It seems quite possible, therefore, that certain of the effects of cyclic AMP might, in some *in vivo* situations, be mediated by inhibition rather than activation of protein kinase activity.

In whole blood, only slight cyclic AMP-dependent protein kinase activity was observed. This finding indicates that protein kinase activity in other tissues was not due to contaminating blood in the tissues. Cyclic AMP-dependent protein kinase activity was found in spleen; however, the activity was lost during chromatography on DEAE-cellulose, so that results for spleen have not been included in the present report.

It is possible that some of the similarities and differences in properties of the enzymes from various sources reported here are the result of having used enzymes which were not completely purified. However, the results obtained in the present study with the partially purified enzyme from brain agree well with the results obtained previously with a more highly purified enzyme from bovine brain[6].

The results reported in this paper demonstrate many similarities among the cyclic AMP-dependent protein kinases from a variety of bovine tissues, indicating that the tissue specificity of cyclic AMP-mediated systems is probably determined primarily by variations in the substrates for the kinases. Differences in the properties of the protein kinases do exist, however, and may play some important role in the specificity mechanisms.

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