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MULTIPLE FORMS OF ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM MONKEY SKIN

RAJ KUMAR[a,b,*] MARIANO TAO[a], RENEA PIOTROWSKI[b] AND LAWRENCE SOLOMON[b]

[a]*Department of Biological Chemistry and the* [b]*Department of Dermatology, University of Illinois College of Medicine, P.O. Box 6998, Chicago, Ill. 60612 (U.S.A.)*

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SUMMARY

Multiple forms of adenosine 3′,5′-cyclic monophosphate-dependent (cyclic AMP-dependent) protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) which catalyze the phosphorylation of various protein acceptors by ATP have been demonstrated in rhesus monkey skin. The enzymes are stimulated most efficiently by cyclic AMP whereas its analogs are less effective. They resemble one another in several of their kinetic parameters and also substrate specificities. The pH optimum for the kinases is between 8.0 and 9.0. The K_m values for cyclic AMP are 1.2 and $2.5 \cdot 10^{-8}$ M for kinases I and III, respectively; and for histones the corresponding values are 0.23 and 0.50 mg/ml. The kinases have similar K_m values for ATP which are not significantly affected by cyclic AMP. The interrelationship between the kinases and their possible clinical significance in skin disorders remain to be established.

INTRODUCTION

Atopic dermatitis is a hereditarily determined predisposition of the skin of certain individuals to have a lowered threshold for itching. A failure or defect in the function of the adenyl cyclase–cyclic AMP system has been postulated as a possible site of abnormality in atopy[1]. A great deal of attention has recently been directed towards cyclic AMP-dependent protein phosphokinases[2–16] in an effort to understand the mechanism of action of cyclic AMP. Our own interests regarding catecholamine receptor activity in atopic dermatitis have prompted us to investigate cyclic AMP-dependent protein kinase in skin[8,9]. Since monkey skin affords a reasonable animal model for our studies on normal human skin, in this communication we report the partial purification and some properties of multiple forms of protein kinase in the skin of rhesus monkey (*Macaca mulatta*). In contrast to other tissues[7,11–15], the elution profile from DEAE-cellulose of monkey skin enzyme resembles that of human skin[9].

* To whom reprint requests should be addressed.

MATERIALS AND METHODS

Rhesus monkey skin (whole body and full thickness) was purchased from Pel-Freez Biologicals, Rogers, Arkansas. [γ-^{32}P]ATP and ^{3}H-labeled cyclic AMP were purchased from New England Nuclear Corp., Cambridge, Mass. The various substrates and cyclic nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo., or P. L. Biochemicals, Milwaukee, Wisc. Analytical grade reagents were used in the studies.

Enzyme isolation

Procedures for enzyme purification and assay of protein kinase and cyclic AMP binding activities have been presented in detail before[8,9]. A brief description of these follows: All operations were carried out at 2–4 °C. Skin devoid of fat, muscle and fascia was removed of hair by means of an Oster animal clipper (blade No. 40) followed by a barber's electric shaver (Electro Tool, Racine, Wisc.). It was then cut into small pieces and pulverized under liquid N_2 in a Wiley Mill. The powder was sonicated (4 times 10 s) in 0.02 M Tris–HCl buffer (pH 7.5), 1 mM dithiothreitol (Buffer A); and the supernatant obtained on centrifugation at 35 000 × *g* was made 1% with respect to streptomycin sulfate. The solution was centrifuged again and the supernatant brought to 50% satn with $(NH_4)_2SO_4$. The precipitate was isolated by centrifugation, dissolved in Buffer A and dialyzed for 15–18 h against this buffer. The enzyme solution was centrifuged at 100 000 × *g* for 90 min. The supernatant obtained was applied to a DEAE-cellulose column (9 cm × 2 cm) previously equilibrated with Buffer A, and eluted with a linear KCl gradient from 0 to 0.3 M. The peak fractions were pooled, dialyzed against Buffer A and concentrated by Diaflo ultrafiltration.

Protein kinase assay

Protein kinase activity was measured by the incorporation of [γ-^{32}P]ATP into histones. The assay mixture consisted of 0.1 M Tris–HCl buffer (pH 8.5), 4 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, 1.8 mg/ml of histone, 5 μM cyclic AMP and enzyme protein. After incubation at 37 °C for 5 min, the reaction was terminated by addition of 10% trichloroacetic acid. The precipitate was filtered through GF/C glass fiber filter, washed with 10% trichloroacetic acid and counted in Bray's solution[17]. Specific activity of the enzyme was expressed as pmoles of ^{32}P incorporated per mg of protein per 5 min incubation at 37 °C. Protein concentration was determined by the method of Lowry *et al.*[18] using bovine serum albumin as the standard.

Cyclic AMP binding assay

Cyclic AMP binding was determined by a Millipore filtration technique using ^{3}H-labeled cyclic AMP[7]. The binding mixture contained 0.02 M Tris–HCl buffer (pH 7.5), 4 mM $MgCl_2$, 0.5 μM ^{3}H-labeled cyclic AMP and enzyme. After incubation at 37 °C for 3 min, the reaction was chilled in ice, diluted with cold 0.02 M Tris–HCl buffer (pH 7.5), containing 4 mM $MgCl_2$ and filtered through Millipore filters (HA 0.45 μm pore size). The filters were washed repeatedly with the same buffer and counted in Bray's solution.

RESULTS

DEAE-cellulose chromatography

The elution profile (Fig. 1) indicated the presence of three kinase activities emerging at approx. 0.05, 0.15, and 0.2 M KCl, respectively. All three forms of the enzyme not only bound cyclic AMP strongly but were also activated by low concen-

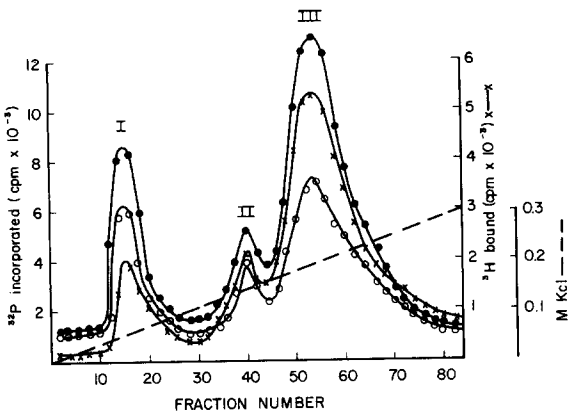

Fig. 1. Separation of monkey skin protein kinases on DEAE-cellulose. Approx. 600 mg of protein obtained from the 100 000 × *g* centrifugation were applied to a column of DEAE-cellulose (9 cm × 2 cm) equilibrated with 0.02 M Tris–HCl buffer (pH 7.5), containing 1 mM dithiothreitol. The column was eluted with a linear gradient of 0–0.3 M KCl in a total volume of 200 ml of the same buffer. Fractions of 2.5 ml each were collected and aliquots (50 μl) of alternate fractions assayed for kinase activity in the presence (●—●) and absence (○—○) of cyclic AMP. Cyclic AMP binding activity (×—×) was also measured in the same fractions.

trations of the cyclic nucleotide. While kinase I was different from kinase III based on elution characteristics, at present our data do not define whether kinase II was a distinct entity or an altered form of either of the other two species.

Effects of varying enzyme concentration

Since kinase II was not available in sufficient quantity, only kinase I and kinase III were studied in more detail. The phosphorylation of histones by kinase I and kinase

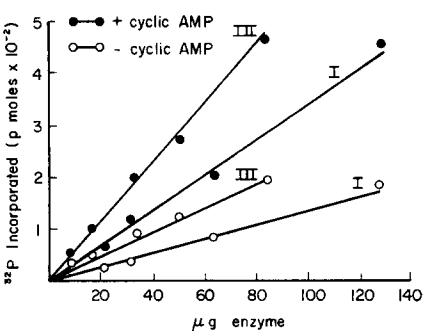

Fig. 2. Relationship between protein concentration and histone phosphorylation. Different amounts of protein were used in each incubation. Specific activity of [γ-^{32}P]ATP was 28 cpm/pmole. Enzyme activity was assayed as described.

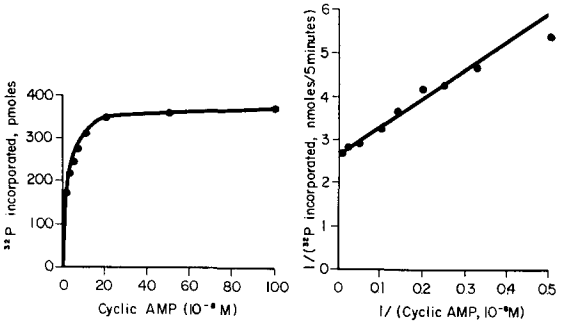

Fig. 3. Activity of kinase I measured against different concentrations of cyclic AMP. 63 μg of enzyme was used per incubation. Specific activity of [γ-^{32}P]ATP was 18 cpm/pmole. A double reciprocal plot of the data is shown on the right hand side.

III was proportional to the quantity of enzyme added (Fig. 2), and the reaction was stimulated by cyclic AMP. Both species of enzymes were optimally active between pH 8.0 and 9.0 (data not shown).

Kinase activity as a function of the concentration of cyclic AMP and its analogs

The effect of varying the concentration of cyclic AMP on the activity of kinase I is shown in Fig. 3. Kinase I and kinase III were maximally stimulated at concentrations of about 10^{-7} and 10^{-6} M of cyclic AMP, respectively. Higher cyclic AMP concentration ($>10^{-5}$ M) inhibited the enzymes. An apparent K_m for cyclic AMP of $1.2 \cdot 10^{-8}$ M for kinase I and $2.5 \cdot 10^{-8}$ M for kinase III were obtained.

Uridine 3',5'-monophosphate, guanosine 3',5'-monophosphate and cytidine 3',5'-monophosphate were less effective than cyclic AMP in stimulating the kinases. The K_m values of the two kinases for these cyclic nucleotides which were higher than for cyclic AMP, ranged between $1.5 \cdot 10^{-6}$ and $2.9 \cdot 10^{-6}$ M.

Apparent K_m for ATP

The activities of kinases I and III as a function of ATP concentration both in the presence and in the absence of cyclic AMP are given in Fig. 4. The apparent

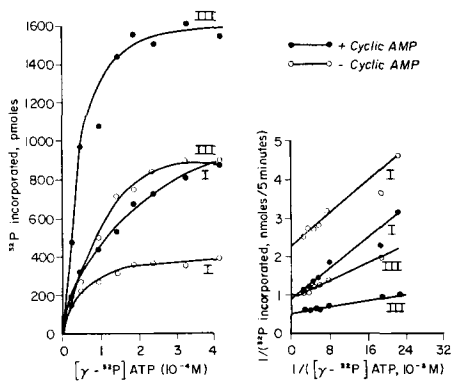

Fig. 4. The effect of varying ATP concentration on kinase activity. 63 μg of each enzyme was used per incubation. Specific activity of [γ-^{32}P]ATP was 26 cpm/pmole.

TABLE I

EFFECT OF ATP ANALOGS ON MONKEY SKIN KINASE ACTIVITY

Experimental procedures are described in the text. The effect of various nucleotides was determined at a final concentration of 0.2 mM in the presence of cyclic AMP. 63 μg of each kinase was used per incubation. Enzyme activity is expressed as pmoles of ^{32}P incorporated per 5 min incubation. Specific activity of [γ-^{32}P]ATP was 19 cpm/pmole.

	Kinase I		*Kinase III*	
	^{32}P incorporated (pmoles)	*% control*	*^{32}P incorporated (pmoles)*	*% control*
Control	260	100	805	100
+ ADP	143	55	584	73
+ AMP	193	74	474	59
+ GTP	162	63	543	67
+ GDP	175	77	654	81
+ CTP	94	36	541	67
+ UTP	199	76	585	73

K_m values (for ATP) for kinase I were 4.5 and $10.5 \cdot 10^{-5}$ M in the absence and presence of cyclic AMP, respectively, and for kinase III values of $6.8 \cdot 10^{-5}$ and $4.2 \cdot 10^{-5}$ M were obtained under the same conditions. Cyclic AMP stimulated the enzyme activity considerably and also increased the maximum velocity of the enzyme reaction.

Effect of nucleotide analogs

Protein kinase activity was measured in the presence of several nucleotides other than ATP at a concentration of 0.2 mM. The results shown in Table I indicate that all the nucleotides tested inhibited the activity of the kinases from 30 to 40%.

Specificities of acceptor proteins

The two species of kinases studied exhibited maximum acceptor activity against

TABLE II

PROTEIN KINASE ACTIVITY MEASURED AGAINST VARIOUS PROTEIN ACCEPTORS

Assays were performed as described in the text except for addition of different protein acceptors to the appropriate tubes. 63 μg of each of kinase I and kinase III were used per incubation. Kinase activity is expressed as pmoles of ^{32}P incorporated per 5 min incubation. Specific activity of [γ-^{32}P]ATP was 20 cpm/pmole.

Substrate	*Kinase I*			*Kinase III*		
	(−)Cyclic AMP	*(+)Cyclic AMP*	*Stimulation (%)*	*(−)Cyclic AMP*	*(+)Cyclic AMP*	*Stimulation (%)*
Histone	131	218	67	202	415	105
Lysine-rich histone	255	511	100	482	834	73
Arginine-rich histone	96	156	62	158	257	63
Albumin	6	15	150	38	35	—
Protamine	25	13	—	38	26	—
Casein	7	13	86	—	8	—

lysine-rich histones. "Mixed" histones and arginine-rich histones also served as substrates but to a lesser degree (Table II), while albumin, protamine, and casein were poor phosphate acceptors. The K_m values of histones for kinase I were 0.15 mg/ml in the absence of cyclic AMP and 0.23 mg/ml in its presence, whereas those for kinase III were 0.42 mg/ml and 0.50 mg/ml under similar conditions. The lack of cyclic AMP effect on some of the substrates like protamine may be related to the ability of the substrate to activate the enzyme by releasing the catalytic subunit[10].

DISCUSSION

The elution profile of DEAE-cellulose chromatography of monkey skin kinase closely resembled that of the human skin enzymes[9]. Both showed three peaks of enzyme activity although we are still examining the possibility whether kinase II is a distinct form or is derived from kinase I or III. Many other tissues examined such as reticulocytes[7], erythrocytes[11], muscle[12] and liver[13] revealed either two species or in certain instances[12–14] a third component as well, believed to be the catalytic subunit(s) of the enzyme(s). All three forms of the skin enzyme were stimulated by cyclic AMP suggesting that they consist of both catalytic and regulatory subunits[7]. Several examples have been cited in the literature which indicate that the multiplicity of forms may be attributed to aggregation–disaggregation or proteolysis of a parent type[11–13,19–21].

The other chemical properties of monkey skin kinases were comparable to those of kinase from several other tissues[2–16]. The pH optima of kinases I and III were not markedly different from each other nor did cyclic AMP significantly alter the pH optima. The behavior of monkey skin enzymes towards various cyclic nucleotides was also similar, but cyclic AMP was the most efficient activator. Cyclic AMP did not alter the K_m value of ATP for histone for the kinase reaction but appreciably increased the V of the reaction. When compared to human skin enzymes, the degree of stimulation of the monkey skin kinases by cyclic AMP was lower for different substrates (Table II), but their substrate specificity was similar[9]. Since kinases I and III reported here resemble each other in many of their properties, it would be premature to attempt an hypothesis about their specific physiological functions. However, the remarkable congruency in the properties of the kinases from the skin of human and monkey will enable us to employ the monkey as a suitable animal model in our future studies on skin.

The kinase variants reported here were isolated from whole skin and therefore may be located either in epidermis or dermis or both. Furthermore, since skin is made up of a heterogeneous population of cells, these multiple forms may reside in different cells or cell organelles of a particular cell type. Protein phosphokinases may be involved in modulation of gene activity by virtue of their ability to phosphorylate the histone of chromatin[4]. These enzymes may, therefore, play a yet undefined role in carcinoma of the skin as well as in disorders such as psoriasis and ichthyosis where altered cellular kinetics have been observed[22–24]. In addition, the altered catecholamine disposition in the skin of patients with atopic dermatitis[25] may also be related to an unknown aspect of the cyclic AMP-stimulated protein kinase cascade. Several of these questions, including the nature of the subunit structure of the enzymes, are currently under investigation.

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