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PROTEIN KINASE IN THE HAIR FOLLICLES OF THE HUMAN SCALP AND BEARD

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SUMMARY

Protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) which phosphorylates histone but not significant amounts of protamine or casein, was demonstrated in follicular tissues from the human scalp and beard. It was activated up to 6 times by $5 \cdot 10^{-7}$ M cyclic AMP and up to 5 times by the same concentration of cyclic IMP. Cyclic AMP activated the enzyme by dissociating the cyclic AMP receptor subunit from the catalytic subunit. In hair follicles from both scalp and beard the K_m values for cyclic AMP and ATP were about $2.3 \cdot 10^{-8}$ M, and $1.4 \cdot 10^{-5}$ M, respectively, and the optimal pH was 6.7. The biochemical characteristics for the enzymes of the scalp and beard hair follicles did not differ appreciably.

INTRODUCTION

Increasing numbers of studies show that cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) phosphorylates divergent kinds of proteins including histone, protamine, casein and some membrane[1,2] or ribosomal protein[3] and concomitantly activates or inactivates phosphorylase *b* kinase[4], glycogen synthetase I[4] and lipase[5,6] in various mammalian tissues. The biological importance of protein kinase in cell function appears to depend upon its activation by the cyclic AMP system[5–12].

In our focal studies on the pathogenesis of male pattern baldness, we have shown that androgen inhibits adenyl cyclase activity in the human scalp hair follicle[13]. This finding led us to examine the possibility that androgenic action proceeds *via* the adenyl cyclase–protein kinase system, even though steroid hormones generally exert their action *via* a specific receptor protein system. Since androgens clinically cause biphasic results on hair follicles, *i.e.*, a dystrophic effect on scalp hair follicles and a stimulatory effect on beard follicles, we have tried to characterize and compare the protein kinases of these different follicles.

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MATERIALS AND METHODS

Cyclic nucleotides, calf thymus histone, salmon sperm protamine and α-casein were obtained from Sigma; cyclic [^{3}H]AMP was purchased from Schwarz; and [γ-^{32}P]ATP is the product of New England Nuclear.

Hair follicles were obtained from the scalps and beards of three Japanese adult males. Plucked hair follicles were immediately homogenized in a glass homogenizer for 4 min in 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA ((ethylenedinitrilo)-tetraacetic acid) with a ratio of 90 scalp follicles/500 μl and 20 beard follicles/250 μl. After the homogenate had been centrifuged at 10 000 $\times$ g for 30 min, the clear supernatant solution was preincubated with or without $5 \cdot 10^{-7}$ M cyclic AMP at 30 °C for 5 min. An aliquot (200 μl) was layered on the top of 4.8 ml of 5–20% sucrose density gradient solution containing 200 μg/ml of histone or albumin, then centrifuged at 47 000 rev./min for 17 h in a Beckman SW 50 rotor. Without these proteins in the sucrose density gradient solution, the enzyme lost most of its activity during centrifugation. All procedures were carried out at 0–3 °C. Centrifuged solutions were divided into 20 fractions, and protein kinase and the cyclic AMP binding activity were assayed in each fraction.

Protein kinase was assayed by the method of Maeno *et al.*[14] with a modification in the washing step. The standard reaction system contained 5 μmoles of sodium acetate buffer (pH 6.0), 0.1 mg of calf thymus histone, 1 μmole of magnesium acetate, 1 μmole of NaF, 0.2 μmole of theophyline, 0.03 μmole of ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 0.05 nmole of cyclic AMP, 0.5 nmole of [γ-^{32}P]-ATP (product that had a specific activity of 13–16 Ci/mmole was diluted about 30 times with nonradioactive ATP before use), and enzymes for a total volume of 100 μl. Reaction was started by adding ATP after 5 min preincubation at 30 °C, and the entire reaction mixture was incubated for 5 min at the same temperature. Then 1.5 ml of 10% trichloroacetic acid was added to stop the reaction, the aliquot was passed through a millipore filter (HA 0.45 μm), the trapped precipitate was washed with 30 ml of 5% trichloroacetic acid, and radioactivity on the millipore filter was counted in 10 ml of toluene–Omnifluor (New England Nuclear)–Bio-solv (Beckman) counting medium (800 μl of Bio-solv-BBS3 were dissolved in 10 ml of toluene containing 4 g/l of Omnifluor) in the Packard Tricarb liquid scintillation spectrometer. 1 unit of enzyme activity was expressed as pmoles P incorporated into precipitated protein/min. Cyclic AMP binding activity was assayed by the method of Gilman[15] in a total volume of 50 μl. Namely, reaction mixture containing 2.5 μmoles of sodium acetate buffer (pH 4.0), 0.5 pmole of cyclic [^{3}H]AMP (spec. act. 20.8 Ci/mmole), protein kinase inhibitor and enzyme fraction was incubated at 0 °C for 60 min. At the end of incubation period, mixture was diluted with 0.5 ml of chilled 20 mM potassium phosphate buffer (pH 6.0), and passed through Millipore filter with a 0.45-μm pore size. The filter was washed with 20 ml of 20 mM potassium phosphate buffer (pH 6.0) and its radioactivity counted in the same way as for the protein kinase assay. Protein kinase inhibitor prepared from guinea pig skeletal muscle gave, at the optimal concentration, a more than 3-fold increase of binding as compared to assay in its absence. Under the condition described above, the radioactivity (the binding) of cyclic [^{3}H]AMP is linear to the concentration of the

binding protein in the sample analyzed. Protein was measured by the method of Lowry *et al.*[16] with bovine serum albumin as a standard.

RESULTS

Sucrose density gradient centrifugation pattern of the enzyme

When samples (homogenates of scalp hair follicles) were centrifuged after preincubation without cyclic AMP, the curve of protein kinase activity showed a single peak (at 7 S) that was greatly activated by $5 \cdot 10^{-7}$ M cyclic AMP in the assay system. The cyclic AMP binding activity was accompanied to this peak. Centrifuging the samples preincubated with $5 \cdot 10^{-7}$ M cyclic AMP showed a different sedimentation

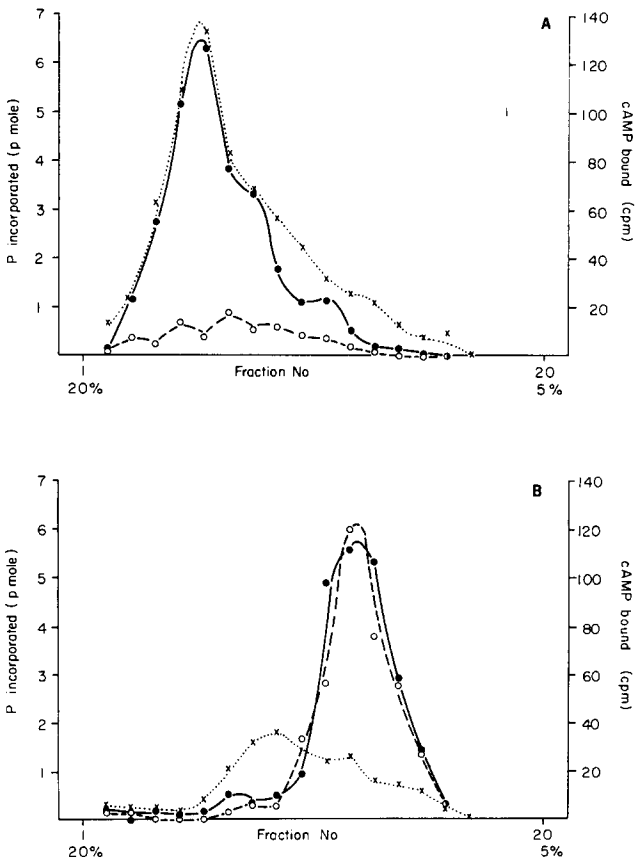

Fig. 1. Sucrose density gradient centrifugation patterns of protein kinase and cyclic AMP binding activity in follicles from scalp hair. Samples were incubated at 30 °C for 5 min with (B) or without (A) $5 \cdot 10^{-7}$ M cyclic AMP before centrifugation. 40 μl of each fraction were used for protein kinase and cyclic AMP binding assay. Protein kinase activity was assayed with $5 \cdot 10^{-7}$ M cyclic AMP (●—●) or without it (○— — —○) at 30 °C for 10 min, omitting preincubation. ×···×, cyclic AMP binding activity. Sucrose did not significantly affect either assay at the concentrations used. Preincubation without cyclic AMP before centrifugation is necessary to diminish endogenous cyclic AMP as well as to standardize the conditions. When the crude homogenate was centrifuged without preincubation, we obtained two almost equal peaks of cyclic AMP binding activity, which were indicative of partial dissociation of the subunits.

profile of protein kinase activity (at 4 S) and cyclic AMP binding activity (5–6 S) with the latter activity peak dissociated from the protein kinase activity peak. This protein kinase shifted to 4 S was not further activated by the presence of $5 \cdot 10^{-7}$ M cyclic AMP (Figs 1A and 1B). Since significant concentrations of free cyclic AMP were not present in this fraction we concluded that the enzyme had been converted into a cyclic AMP-independent form by preincubation with cyclic AMP. These data are consistent with the proposed mechanism for protein kinase activation; that is, cyclic AMP binds to a receptor subunit to dissociate it from a catalytic subunit which is active in its free subunit form. Such an activation mechanism has been proposed for protein kinase from various tissues such as rabbit reticulocyte[7,17], bovine adrenal cortex[8,18], rabbit skeletal muscle[9], bovine heart muscle[19], bovine brain[10], rat liver[20], bovine pineal gland[21], bovine anterior pituitary gland[22], and rabbit red blood cell[12]. Fig. 2 shows the results of an ultracentrifugation study of beard hair follicles. Enzymes from scalp hair follicles and beard follicles did not differ qualitatively in either the sucrose density gradient centrifugation profile or the cyclic AMP activation mechanisms of the protein kinase. However, the follicles

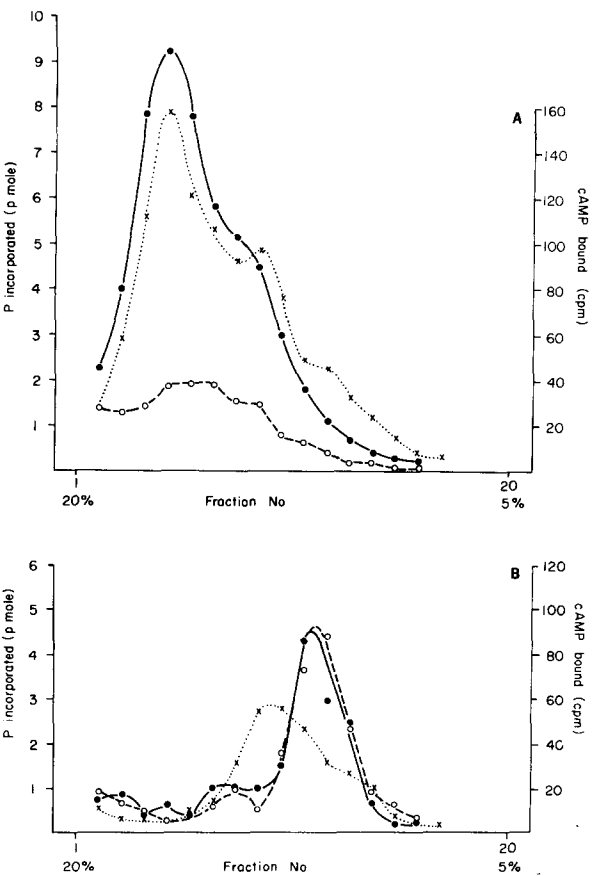

Fig. 2. Sucrose density gradient centrifugation pattern of protein kinase and cyclic AMP binding activity from follicles of beard hair. The condition was the same as that for follicles of scalp hair (Fig. 1).

TABLE I

PROTEIN KINASE ACTIVITY OF CRUDE EXTRACTS AND PARTIALLY PURIFIED ENZYMES

Gross specific activities of partially purified enzyme were calculated on the basis of data on enzyme activity and protein concentration obtained in separate samples, *i.e.*, to measure protein content in each fraction, the centrifugation was performed with the sucrose medium without added protein. Apparently the low purification factor is due to the partial loss of the enzyme activity during centrifugation (the recovery of total enzyme activity is about 30%). Complete inactivation is noted when the homogenate is centrifuged in the sucrose medium without histone or bovine serum albumin.

		Activity/ follicle (pmoles/ follicle per min)	*Specific activity (pmoles/ mg per min)*	*Purification*
Scalp hair follicle	Crude extract	1.32	233	1
	Purified	—	392	1.7
Beard hair follicle	Crude extract	3.37	225	1
	Purified	—	455	2.0

may differ quantitatively in the relative amounts of cyclic AMP receptor and catalytic subunit of protein kinase. Comparing Figs 1B and 2B, we see that the cyclic AMP-binding activity peak of the beard follicle is relatively higher than that of the scalp.

The cyclic AMP-dependent protein kinase fraction (Figs 1A and 2A) was collected, kept frozen at −20 °C, and used as a partially purified enzyme in the following kinetic studies. Table I summarizes the enzyme activities of crude extracts and partially purified samples. Further purification was not possible because of limited amounts of tissues.

Activation of protein kinase by cyclic AMP

The activity of the partially purified enzyme was assayed with various concentrations of cyclic AMP. It was activated with increasing concentrations of cyclic AMP reaching its maximum activation (3–4 times the activity without cyclic AMP) at a concentration of about $5 \cdot 10^{-7}$ M. Further increase in cyclic AMP concentration

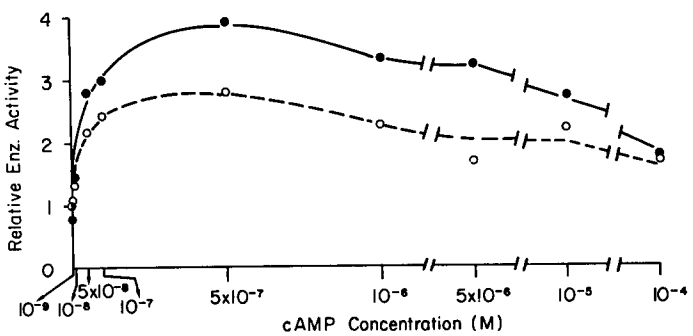

Fig. 3. Cyclic AMP activiation of protein kinase in hair follicles. 30 μl of partially purified enzyme was used for each assay. Relative enzymic activities were calculated on the basis of activity without cyclic AMP. Each point represents the average value obtained by 2–4 different experiments. The scalp (○— — —○) and beard (●—●).

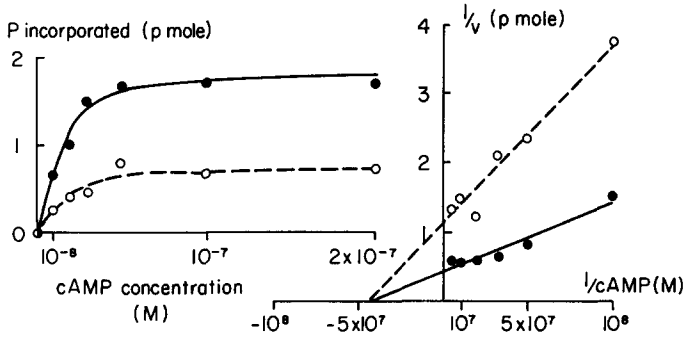

Fig. 4. K_m for cyclic AMP. Data were corrected by the value found when no cyclic AMP was added to the reaction mixture. Each point represents the mean of duplicate assays. Protein kinase of the hair follicles from scalp (○— — —○) and beard (●—●).

inhibited the enzyme activity (Fig. 3). Protein kinase from beard hair follicles appears to be activated more than that from scalp hair follicles. However, the difference is probably not due to an essential qualitative difference between the enzymes from the two tissues as discussed below. K_m value for cyclic AMP is about $2.3 \cdot 10^{-8}$ M for the enzymes from both tissues (Fig. 4).

Effects of other cyclic nucleotides

The results are summarized in Table II. Each cyclic nucleotide was tested at concentrations of $5 \cdot 10^{-7}$ M and 10^{-4} M in the reaction mixture. The enzymes from scalp and beard hair follicles responded in the same manner against these cyclic nucleotide compounds. Cyclic AMP is most effective at the concentration of $5 \cdot 10^{-7}$ M and less effective at 10^{-4} M. Cyclic IMP is nearly 90% as effective as cyclic AMP at $5 \cdot 10^{-7}$ M and is more active at 10^{-4} M than at 10^{-7} M. Cyclic GMP, cyclic UMP

TABLE II

ACTIVATION OF PROTEIN KINASE BY VARIOUS CYCLIC NUCLEOTIDES

Partially purified enzyme was prepared by centrifugation in sucrose density gradient solution containing bovine serum albumin instead of histone. Activity was expressed as pmoles P incorporated into protein/5 min. All cyclic nucleotide solution was prepared fresh.

Cyclic nucleotides	*Scalp hair follicle*		*Beard hair follicle*	
	Activity	*Activation*	*Activity*	*Activation*
o	0.27	1	0.44	1
Cyclic AMP $5 \cdot 10^{-7}$ M	1.72	6.27	2.28	5.21
$1 \cdot 10^{-4}$ M	1.46	5.32	1.53	3.49
Cyclic IMP $5 \cdot 10^{-7}$ M	1.51	5.52	2.04	4.65
$1 \cdot 10^{-4}$ M	1.81	6.61	2.12	4.85
Cyclic GMP $5 \cdot 10^{-7}$ M	0.75	2.74	0.83	1.89
$1 \cdot 10^{-4}$ M	1.72	6.27	2.15	4.91
Cyclic CMP $5 \cdot 10^{-7}$ M	0.61	2.21	0.60	1.37
$1 \cdot 10^{-4}$ M	1.91	6.95	2.21	5.04
Cyclic UMP $5 \cdot 10^{-7}$ M	0.58	2.11	0.60	1.37
$1 \cdot 10^{-4}$ M	1.53	5.56	2.22	5.07

and cyclic CMP are much less effective at the concentration of $5 \cdot 10^{-7}$ M, although they fully activate the enzyme at 10^{-4} M.

The rates of activation in this experiment were much higher than those in the previous experiment (Fig. 3) because albumin was used instead of histone as a stabilizer in the sucrose density gradient solution. As reported earlier[10], histone added to the sucrose density gradient solution causes partial dissociation of the receptor subunit from the catalytic subunit at the preincubation step of the enzyme assay and possibly during other procedures. This partial dissociation activates the enzyme without cyclic AMP; thus the activation rates by cyclic AMP are lower with the protein kinase prepared in the histone medium. Activation by protamine has also been reported recently[23].

Effect of ATP concentration on the enzyme activity

The change of ATP concentration in the assay system caused the same response in the protein kinase from both scalp and beard hair follicles. The enzyme activity was increased in proportion to the increase in ATP concentration. The K_m value for ATP is about $1.4 \cdot 10^{-5}$ M for the enzymes from both tissues (Fig. 5).

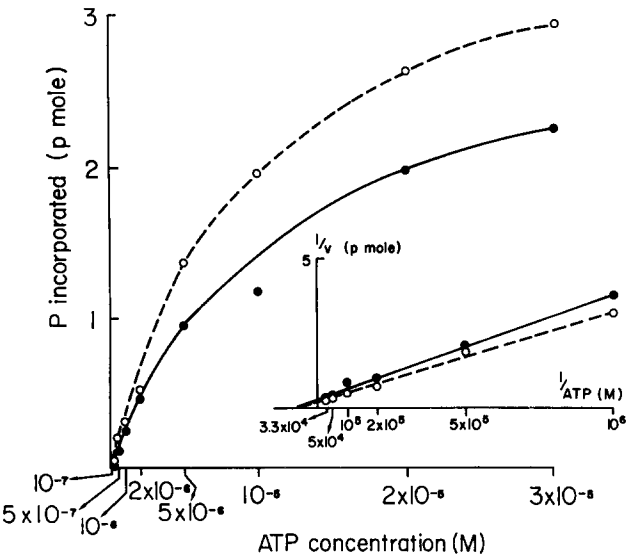

Fig. 5. Effect of ATP concentration on the protein kinase activity. 20 μl of partially purified enzyme from scalp hair follicle (○— — —○) and 10 μl of that from beard hair follicle (●—●) were assayed.

Proteins as a phosphate acceptor

Calf thymus histone, salmon sperm protamine and α-casein were tested as phosphate acceptors in the protein kinase reaction (Table III). Histone was the best substrate for the enzymes from both tissues. Except for a slight phosphorylation of casein by the crude extract of hair follicles, little or no phosphorylation was noted when protamine or casein was used as a substrate.

TABLE III

PHOSPHORYLATION OF VARIOUS PROTEINS BY PROTEIN KINASE

The enzyme preparation was the same as that in Table II. Assays were carried out for every substrate protein (0.1 mg/100 μl of reaction system) in duplicate with duplicate blanks. The blank tubes were incubated without substrate protein which was added after the incubation. Endogenous soluble protein phosphorylation, in the case of crude extract, was not noted in any significant amount at this protein concentration (scalp hair follicle (0.8 μg), beard hair follicle (1.1 μg) in 100 μl reaction system). Protein phosphorylation was expressed as pmoles P incorporated/mg enzyme protein per min .

Protein	*Scalp hair follicle*		*Beard hair follicle*	
	Crude extract	*Purified*	*Crude extract*	*Purified*
Protamine	0	0	0	28
α-Casein	21	0	36	0
Histone	231	491	274	1050

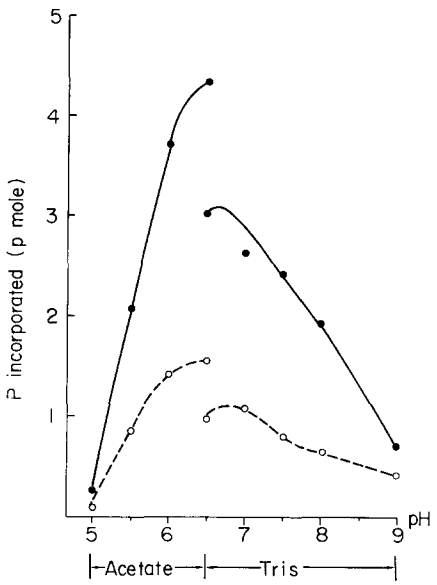

Fig. 6. Effect of pH on protein kinase activity. 30 μl of aliquots of the cyclic AMP-dependent protein kinase fraction of scalp hair follicle (○— — —○) and beard hair follicle (●—●) were assayed. Each point represents the mean value of duplicate assay.

The effect of pH

The maximum enzyme activity was obtained at a pH between 6.5 and 7.0, and the enzyme was relatively sensitive to pH. It was more active in acetate buffer than in Tris buffer (Fig. 6). Optimal pH appears to be the same for the enzymes from both tissues.

DISCUSSION

Active protein kinase was noted in hair follicles from the human scalp and beard. Crude extract from a single hair follicle is sufficient for this assay system, and

easily plucked hair follicles provide fresh and convenient tissue samples when needed.

Although many studies on this enzyme have shown histone rather than protamine or casein to be the preferred nonspecific phosphate acceptor, the opposite results have been found with enzymes from the bovine heart[19] and pineal gland[21]; thus, the substrate specificity varies with different so-called protein kinases. Histone is a good substrate for enzyme from human hair follicle while protamine is not significantly phosphorylated by either crude extract or partially purified enzyme. The slight phosphorylation of casein by crude extract seems to have been due to another enzyme such as phosvitin kinase. This kinase which appears to be in human epidermis phosphorylates casein as well as phosvitin but little histone[24].

The activation mechanism by cyclic AMP for follicular protein kinase is considered to be the same as that reported for kinases from various mammalian tissues. Although only 2 distinct peaks of protein kinase activity were noted in our sucrose density gradient centrifugation experiments, the small shoulder peak between cyclic AMP-dependent and independent protein kinase activity suggests either another form of this enzyme[7,18,20,25,26], or a dimerous component as shown in bovine brain protein kinase[10] where two main activity peaks are interpreted as tetramers (composed of 2 catalytic and 2 receptor subunits) and monomers of catalytic subunits, respectively.

The preliminary values for the cyclic AMP content of scalp and beard hair follicles obtained with the method of Gillman[15] were 0.04 and 0.16 pmole per follicle, respectively. If we assume that the volumes of these hair follicles were 0.5 μl and 2 μl, the gross concentration of cyclic AMP in these tissues was $0.8 \cdot 10^{-7}$ M, which is enough to regulate the activity of protein kinase in hair follicles *in vivo* (the K_m and maximal activation concentrations are $2.3 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M, respectively).

Thus all the data, including the K_m value for ATP, pH dependency, and nucleotide specificity show essentially the same characteristics for protein kinase from the scalp and beard hair follicles. The protein kinase of human hair follicles closely resembles that of the bovine anterior pituitary gland[22] and rabbit skeletal muscle[25] and also, with a few differences, is similar to the protein kinases of rat liver, rat fat pad, and bovine brain[27-29]. The data suggest the presence of similar protein kinases in a wide variety of mammalian tissue. Protein kinase of human skin, recently reported by Kumar *et al.*[30,31] also has essentially the same characteristics as hair follicle protein kinase except that the optimal pH is in the alkaline range.

Adenyl cyclase has previously been shown in human hair follicle[13]; in this report we have shown the presence of cyclic AMP and cyclic AMP-dependent protein kinase. Together these data appear to substantiate the presence of the adenyl cyclase–cyclic AMP–protein kinase system in tissue from human hair follicles. The different effects of androgenic hormone on the hair follicles of the scalp and beard must be explained by some other differences than those in the characters of the protein kinases themselves.

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