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MULTIPLE FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN PIG EPIDERMIS

KENJI ADACHI, VICTOR LEVINE, KENNETH M. HALPRIN, HAJIME IIZUKA and KUNIHIKO YOSHIKAWA

Miami Veterans Administration Hospital and Department of Dermatology, University of Miami School of Medicine, Miami, Fla. 33125 (U.S.A.)

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Summary

Pig epidermal cyclic nucleotide phosphodiesterases (EC 3.1.4.16) have been partially purified by DEAE-cellulose column chromatography. At least three different forms of the epidermal phosphodiesterases were identified. They were cyclic GMP-specific, cyclic GMP- and cyclic AMP-hydrolyzing and apparently a cyclic AMP-specific enzyme: the first two forms were soluble and the last was the particulate enzyme. The cyclic GMP-specific soluble fraction had a relatively low K_m, the cyclic GMP- and cyclic AMP-hydrolyzing fraction had a high K_m for the respective substrates and the third particulate enzyme had both high and low K_m values for cyclic AMP. The cyclic GMP-hydrolyzing enzyme was localized almost entirely in the soluble fraction, whereas cyclic AMP-hydrolyzing enzyme was distributed to both soluble and particulate fractions. Thus, our studies show that the multiple forms of pig epidermal enzyme differ distinctly in their substrate affinity, specificity and subcellular distribution.

Introduction

The biological and clinical significance of the cyclic nucleotide (cyclic AMP and cyclic GMP) as controlling signals in the processes of secretion, differentiation, membrane transport, locomotion, immunity and mitosis (and/or cancer) have become ever more obvious. In skin, the diurnal changes in the levels of these cyclic nucleotides have been related to epidermal proliferation [1]. Also in psoriasis, a hyperplastic skin disease, an elevation of cyclic GMP [2], an increase in cyclic AMP [3,4], and a defective adenylate cyclase system have been reported [5,6].

The endogenous levels of these cyclic nucleotides are under the control of both synthetic and degradative enzymes. Data on the degradative enzyme (cy-

clic nucleotide phosphodiesterase) of skin have been less plentiful than the wealth of information for such organs as brain, liver, heart etc. [7,8]. For example there have been only three reports on phosphodiesterase in skin [9—11]. In these studies, only crude homogenates were used as the enzyme source for the kinetic studies. Evidence from several laboratories clearly indicates that multiple forms of cyclic nucleotide phosphodiesterases (EC 3.1.4.16) exist in many tissues, and one would expect the same situation in the skin. In view of the biologically significant role of the cyclic nucleotide phosphodiesterases in controling the intracellular level of cyclic AMP and cyclic GMP, we have attempted to purify and separate the multiple forms of these enzymes in epidermis and to extend the study of their characteristics.

Materials and Methods

Materials. Cyclic [^{3}H] AMP (specific activity 20—40 Ci/mmol) was purchased from New England Nuclear and cyclic [^{3}H] GMP (specific activity 15 Ci/mmol) from Amersham/Searle. The radiochemical purity of the compounds was regularly checked by thin-layer chromatography on cellulose (Analtech, Newark, Del.) or on DEAE-cellulose (Brinkman, Westbury, N.Y.) thin-layer plates, which were developed with 95% ethanol/1 M ammonium acetate (70 : 35, v/v). Unlabelled cyclic AMP and cyclic GMP and other nucleotides were obtained from Sigma (St. Louis, Mo.). All other chemicals were reagent grade or the best commercially available. DEAE-cellulose was obtained from Whatman (DE-52) in a pre-swollen form. Prostaglandin E_2 was generously donated by Dr. John Pike, The Upjohn Co. (Kalamazoo, Mich.).

Preparation of epidermal phosphodiesterases. Since pig epidermis bears a close anatomical resemblance to human epidermis, it was used to prepare the skin phosphodiesterases. The epidermis was removed by a keratome (Storz Instrument, St. Louis, Mo.), with the cutting blade adjusted to 0.2 mm thickness. The following procedures were executed at 3°C. The epidermal slice was homogenized in 20 vols. of double distilled water with a glass homogenizer and then with the "Polytron" (Brinkmann, N.Y.) at maximum speed for a total of 10 min with short interruptions every 2 min. Unbroken cells were precipitated by centrifugation at 500 × *g* for 5 min. The supernatant was centrifuged at 30 000 × *g* for 20 min and the resulting supernatant or soluble fraction was further purified by column chromatography [12]. The particulate fraction was reconstituted with the same amount of water and the suspension was used as the particulate sample. In a second preparation, pig epidermis was homogenized with 0.32 M sucrose instead of water. The soluble fraction obtained by centrifugation at 105 000 × *g* for 60 min was applied to a DEAE-cellulose column. The column chromatography was performed according to the method described by Russell et al. [12].

Phosphodiesterase assay. The enzyme assay system is similar to that described by Scott and Solomon [13]. The procedure involved in the conversion of AMP (or GMP), the product of cyclic AMP (or cyclic GMP) phosphodiesterase reaction, to adenosine (or guanosine) by 5′-nucleotidase and the subsequent separation of the labelled substrate and final product by thin-layer chromatography. Assay mixture contained a final volume of 20 μl: 100

mM glycylglycine, pH 8.0; 5 mM $MgCl_2$; 0.1 μg/μl of snake venom; variable amounts of cyclic AMP (or cyclic GMP) with the labelled cyclic nucleotides (10^5 cpm); and the epidermal enzyme preparation. After incubation at 37°C for an appropriate time, the reaction was arrested in ice water and 5 μl of 2 M HCl were added to each of the tubes. The reaction products were then separated by cellulose thin-layer chromatography. 1 μl of known substances (a mixture containing adenosine, cyclic AMP and AMP, or guanosine, cyclic GMP and GMP, 20 μg/μl, respectively) was spotted on the thin-layer plates and then 1 μl each of the incubated reaction mixtures was spotted. After development with 95% ethanol/1 M ammonium acetate (70 : 35, v/v) for 2 h, spots corresponding to the substrate and the products were scraped into counting vials to which were added 200 μl of 1 M LiCl to elute the nucleotides, and then counted in a medium containing Omnifluor (New England Nuclear, Boston, Mass.) and BBS III (Beckman, Calif.). Protein concentration was measured by either the method of Lowry et al. [14] or a fluorescent method [15].

Results

Different forms of phosphodiesterases from pig epidermis

The elution pattern of the soluble fraction of water-extracted phosphodiesterases through a DEAE-cellulose column showed two major peaks of enzyme activities (Fig. 1); Peak I enzyme was nearly specific for cyclic GMP hydrolysis while Peak II enzyme degraded both cyclic AMP and cyclic GMP. When the soluble fraction (105 000 × *g* supernatant) obtained from the 0.32 M sucrose homogenate was chromatographed with the same system, an identical elution pattern was obtained. Particulate fractions from either water homogenates or sucrose homogenates showed a preference for cyclic AMP as substrate; i.e. the ratio of cyclic AMP/cyclic GMP hydrolysis by the particulate

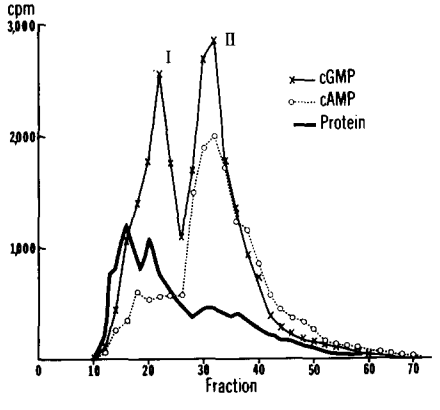

Fig. 1. Separation and purification of phosphodiesterases from pig epidermis. The enzymes were purified by column chromatography with DE-52 (Whatman). The gradient elution to 1 M sodium acetate started at No. 0 fraction. 3 ml per fraction and 90 tubes were collected. The final concentration of cyclic GMP in this assay system was 0.8 μM and that of cyclic AMP 0.3 μM. In the ordinate, enzyme activities are expressed as cyclic nucleotides (cpm) hydrolyzed per 15 min at 37°C. Protein concentration was measured by Farand spectrofluorimeter at 280 nm for the excitation and 350 nm for the absorption [15] and expressed as arbitrary units.

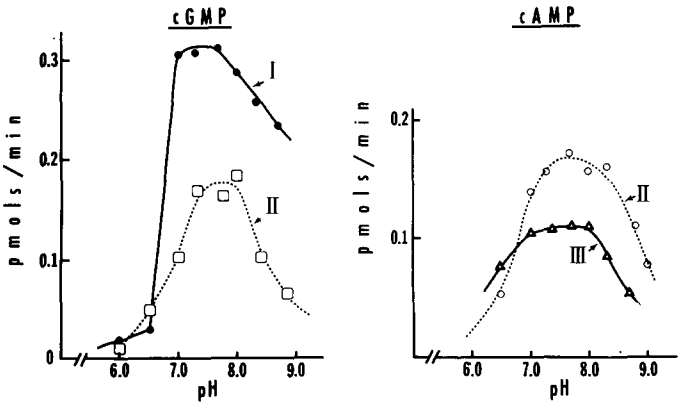

Fig. 2. Effect of pH on hydrolysis of cyclic GMP and cyclic AMP by purified enzymes (Peaks I and II) and a particulate enzyme (III). The enzyme activities are expressed as pmol/min per 10 μl enzymes: protein concentration of each enzyme was 2.4, 1.3 and 12 μg per 10 μl for I, II and III, respectively. Final concentration of cyclic GMP was 0.5 μM and that of cyclic AMP 0.6 μM. Tris · HCl buffer was used except for pH range between 6.0 and 6.5 for which Tris/malate buffer was used instead.

fraction under the same experimental condition shown in Fig. 1 was 3 : 1 whereas these ratios by either total homogenates or supernatant fraction was 1 : 1.

Thus there are at least three different forms of epidermal phosphodiesterases: cyclic GMP-specific, cyclic GMP- and cyclic AMP-hydrolyzing and apparently a cyclic AMP-specific enzyme; the first two forms are soluble and the last is the particulate enzyme. Sonification at maximum speed for 10—15 min did not release the particulate enzyme. Approx. 75% of the cyclic AMP-hydrolyzing enzymes were in the soluble fraction and 25% in the particulate fractions whereas more than 95% of the cyclic GMP-hydrolyzing enzymes were located in the soluble fraction. The maximum purification of Peak I was about 15-fold and that of Peak II 25-fold of the activity with crude homogenate.

Effects of pH on the epidermal phosphodiesterases

Optimal enzyme activities for the three forms of phosphodiesterases are shown in Fig. 2. In all cases, maximum activities were obtained between 7.4 and 8.0; any deviation from this pH range caused marked decreases in the enzyme activities.

Properties of cyclic GMP-hydrolyzing enzymes

Both Peak I and Peak II phosphodiesterases (Fig. 1) hydrolyze cyclic GMP, but only the Peak I enzyme is specific for cyclic GMP. Under the conditions used in Fig. 3A, the cyclic GMP-specific Peak I enzyme has an apparent K_m of $6.7 \cdot 10^{-6}$ M and the cyclic AMP- and cyclic GMP-hydrolyzing Peak II enzyme has a K_m of about $3.3 \cdot 10^{-5}$ M. In our preliminary experiments with crude homogenates of pig epidermis, the presence of two apparent K_m values for cyclic GMP hydrolysis ($5.3 \cdot 10^{-6}$ and $2.6 \cdot 10^{-5}$ M) were demonstrated (Fig. 3B). Obviously, DEAE-cellulose chromatography of the crude supernatant fraction separates these two forms of cyclic GMP-hydrolyzing enzymes into

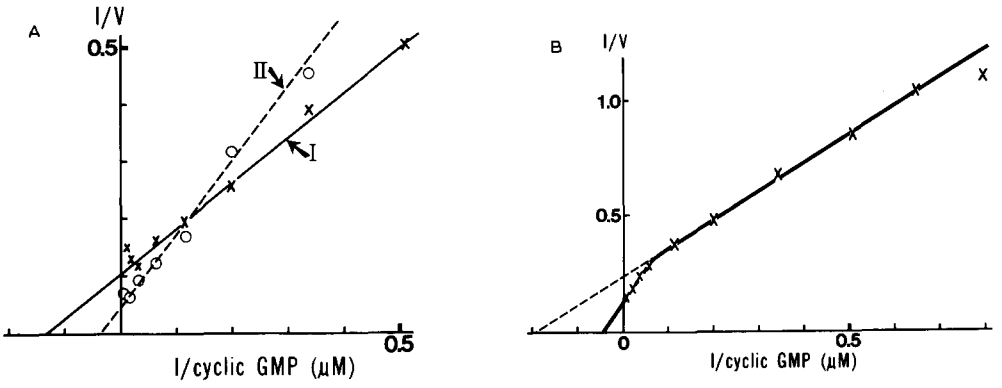

Fig. 3. Cyclic GMP hydrolysis by Peak I and II enzymes. (A) A kinetic analysis with double reciprocal plots (Lineweaver-Burk plot) shows K_m of $6.7 \cdot 10^{-6}$ M for the Peak I and of $2.5 \cdot 10^{-5}$ M for the peak II enzyme, respectively. The assay condition was identical to those described in Fig. 2 except that (1) buffer system was glycylglycine, 100 mM, pH 8.0, and (2) the varied amounts of cyclic GMP were used as the substrate. The cyclic GMP hydrolysis by the crude homogenate of pig epidermis is shown in B. The homogenate (1.25 g wet weight skin/100 ml water) was incubated in the same assay system for 10 min at 37°C.

high and low K_m enzymes. They also differ in response to the addition of cyclic AMP, which competitively inhibits the hydrolysis of cyclic GMP by both Peak I and II enzymes. It requires a much higher concentration of cyclic AMP to inhibit the low K_m (Peak I) enzyme than to do the high K_m (Peak II) enzyme, i.e. K_i for the low and high K_m enzymes were about $2 \cdot 10^{-4}$ and $2 \cdot 10^{-5}$ M, respectively (Figs. 4A and 4B).

Properties of cyclic AMP-hydrolyzing enzymes

Fig. 5 shows double reciprocal plots for the Peak II enzyme in the presence and absence of cyclic GMP at the concentration of 125 and 250 μM. The K_m for cyclic AMP in the absence of cyclic GMP computed from Fig. 5 was $5.6 \cdot 10^{-5}$ M. Cyclic GMP at relatively high concentrations inhibited the cyclic AMP hydrolysis competitively by the Peak II enzyme with a K_i of about $6 \cdot 10^{-5}$ M.

This Peak II enzyme further shows complex kinetics; micromolar concentrations of cyclic GMP (0.6—10 μM thus far tested) stimulated rather than inhibited the hydrolysis of cyclic AMP (Fig. 6). The maximum activation was about 2.5-fold at 3—5 μM of cyclic GMP. An apparent activation constant at this substrate level (cyclic AMP, 0.5 μM) was $7.4 \cdot 10^{-7}$ M. The same concentrations of cyclic GMP, on the other hand, neither stimulated nor inhibited cyclic AMP hydrolysis by the particulate enzyme. We have also tested the effect of micromolar concentrations of cyclic AMP on cyclic GMP hydrolyses by both Peak I and II enzymes and found that the converse was not true; i.e. at a substrate (cyclic GMP) concentration of 0.6 μM, half maximal inhibition (I_{50}) by cyclic AMP was 1 μM and 0.1 μM for the Peak I and II enzymes, respectively.

The particulate enzyme predominantly hydrolyzed cyclic AMP. Double reciprocal plots showed the presence of two apparent K_m values; high and low, of approx. $6 \cdot 10^{-5}$ and $6 \cdot 10^{-6}$ M, respectively (data not shown). The effect of cyclic GMP on cyclic AMP hydrolysis by the particulate enzyme is complex and non-linear (Fig. 7), but it was clear that the effect was inhibitory at

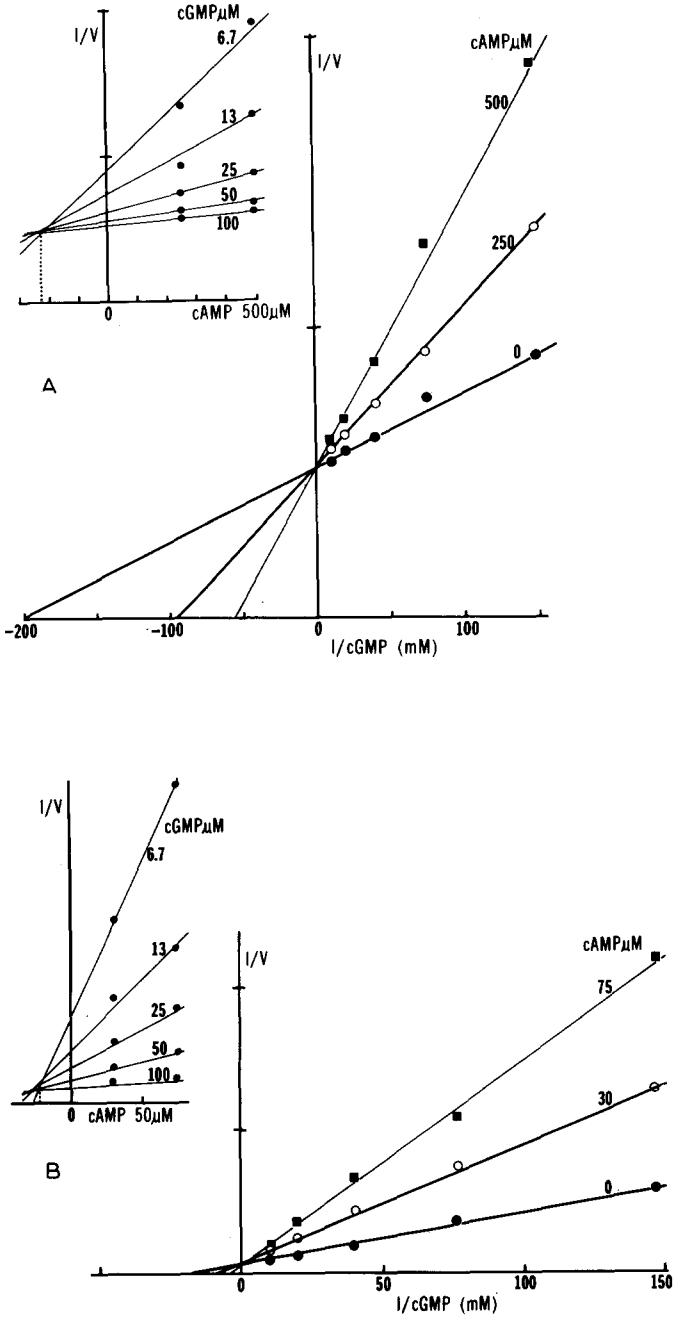

Fig. 4. Effects of cyclic AMP on cyclic GMP hydrolysis by the Peak I (A) and the Peak II (B) enzymes. The experimental conditions were identical to those shown in Fig. 3 except that different amounts of cyclic AMP were added as indicated in the figures. Both A and B graphs show the double reciprocal plots intercept on y axes to indicate the inhibition by cyclic AMP being "competitive". The inserted smaller graphs were replottings of the double reciprocal plots and used for the graphical computation of inhibition constants (K_i).

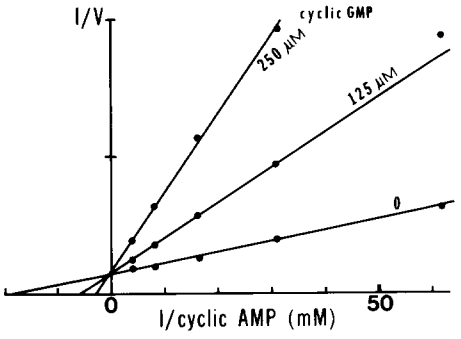

Fig. 5. Cyclic GMP (125 and 250 μM) inhibition of cyclic AMP hydrolysis by the Peak II enzyme. Double reciprocal plots in the presence and absence of cyclic GMP as indicated in the figure.

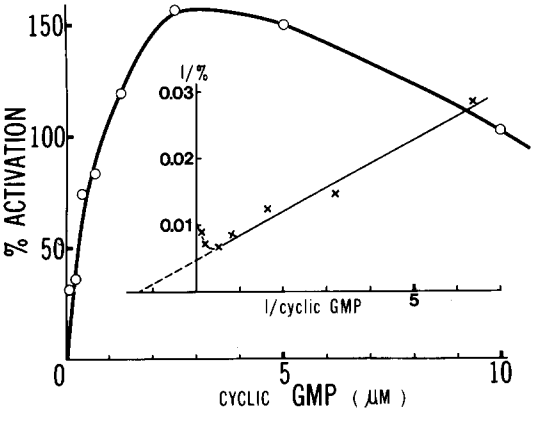

Fig. 6. Cyclic GMP (0.6—10 μM) activation of cyclic AMP hydrolysis by the Peak II enzyme. Final cyclic AMP concentration in the reagent mixture was 0.5 μM.

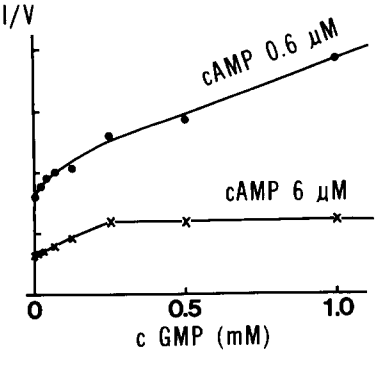

Fig. 7. Cyclic AMP hydrolysis by the particulate enzyme. The substrate (cyclic AMP) level was 0.6 and 6.0 μM. The concentration of the inhibitor (cyclic GMP) was varied from 16 μM to 1 mM. The particulate enzyme was prepared fresh each time before the experiments.

TABLE I

COMBINATION OF PEAK II ENZYME AND PARTICULATE ENZYME

The substrate (cyclic [^{3}H] AMP) concentration was 0.5 μM. Cyclic GMP was added as an activator of the Peak II enzyme at the final concentration of 2 μM. A half volume each of the Peak II and the particulate enzyme was used for the combined fraction. Cyclic AMP hydrolysis was expressed arbitrarily as cpm. Values are an average of triplicate data.

	Peak II enzyme	Particulate enzyme	Combined
Control	800	3200	2100
Cyclic GMP added	1850	3300	2000

the concentration range tested (16 μM to 1 mM). Although the particulate enzyme is predominantly cyclic AMP specific, a weak cyclic GMP-hydrolyzing activity was also detected in this fraction. A kinetic analysis indicated that the K_m for the cyclic GMP hydrolysis by the particulate enzyme was $2.9 \cdot 10^{-5}$ M, which corresponds roughly to the Peak II enzyme ($K_m = 3.3 \cdot 10^{-5}$ M).

Combination of the Peak II and the particulate enzymes

Table I summarizes the effect of a small amount of cyclic GMP (2 μM) on the combined enzymes. The Peak II enzyme is clearly activated by the addition of 2 μM cyclic GMP, whereas the particulate enzyme is neither activated nor inhibited by cyclic GMP at this concentration. When the Peak II and the particulate enzymes are combined together, the positive effect of cyclic GMP no longer prevails. In the control group, the sum of a half each of the Peak II and particulate enzymes (800 × 0.5) + (3200 × 0.5), equaled the value obtained by the reconstituted enzyme or 2100 cpm. However, when cyclic GMP was added, the sum of a half each, (1850 × 0.5) + (3300 × 0.5) = 2600 cpm, far exceeds the experimental value (2000); this indicates the loss of cyclic GMP activation of the Peak II enzyme after the recombination.

Discussion

In the present communication, we have shown that epidermal phosphodiesterases, like those from other organs, have multiple forms. These include the cyclic GMP-specific form (soluble), the cyclic AMP-specific form (particulate), and a soluble enzyme which can hydrolyze both cyclic AMP and cyclic GMP. These features are analogous to those of liver phosphodiesterases, as reported by Russell et al. [12]. However, the details of the kinetic parameters are not exactly the same. For example, the liver Peak I enzyme (cyclic GMP specific) was not affected by the addition of cyclic AMP whereas our epidermal enzyme was inhibited at high cyclic AMP concentrations; the liver particulate enzyme (cyclic AMP specific) was easily solubilized by sonification, but our epidermal particulate enzyme was not.

It is clear from the above kinetic data that the epidermal phosphodiesterases, like the enzymes from other organs, have a complex regulatory mechanism. Of interest is the activation of cyclic AMP hydrolysis by the Peak II-soluble enzyme. Micromolar concentrations of cyclic GMP did not affect the crude epi-

dermal homogenate enzyme (refs. 10 and 11 and confirmed by us). Also in the present work cyclic GMP activation did not occur with particulate enzyme nor the soluble Peak II enzyme combined with the particulate enzyme. Thus if activation of cyclic AMP hydrolysis by micromolar concentrations of cyclic GMP is of significance in vivo it appears to act in only a limited compartment of the cell, namely the cytoplasm.

The Fact that phosphodiesterase activities far exceed adenylate cyclase activities in all tissues strongly suggests that at least some of the phosphodiesterases exist in a latent or inactive form. Russell et al. [12] found that the particulate enzyme (a low K_m, high affinity, cyclic AMP specific) was activated by trypsin treatment or aging in vitro. This finding suggested the latent nature of this phosphodiesterase and also its physiological significance [12]. We have carried out a similar experiment with epidermal slices, but the results did not clearly show any activation by trypsin. Alternatively, one might propose that the activation and inactivation mechanism of the Peak II enzyme is by release from and binding to the particulate enzyme. The finding that the cyclic GMP Peak II enzyme had a similar K_m to the cyclic GMP phosphodiesterase in the particulate fraction was favourable to this concept. Our preliminary experiment to test this hypothesis, however, failed; i.e. the incubation of epidermis with epinephrine caused a marked increase in intracellular cyclic AMP but did not change the ratio of soluble vs. particulate enzyme. Thus the mechanism for the physiological regulation of skin phosphodiesterases still remains unclear.

It appears that the high and low affinity enzymes are under different genetic controls in many tissues. For example, adrenocorticotropin or epinephrine can activate only the high affinity enzyme in fat and hepatoma [16,17]. Whether or not the different forms of phosphodiesterase become abnormal in psoriasis, a hyperplastic skin disease, or in skin cancer is of great interest and the current investigation may be looked upon as a basic road map for studying the "pathology" of phosphodiesterases in skin.

Acknowledgement

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