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THE SECOND MESSENGER SYSTEM OF MOUSE EPIDERMIS

IV. CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASE*

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SUMMARY

1. The existence of cyclic nucleotide phosphodiesterase has been demonstrated in homogenates of mouse epidermis by measuring the formation of AMP and GMP from the corresponding cyclic nucleotides.

2. The enzymatic activity is localized almost entirely in the $17000 \times g$ supernatant. The pH optimum is around 8. Kinetic studies show two apparent K_m values for low and high substrate concentrations.

3. Epidermal phosphodiesterase is activated by Mg^{2+} and low concentrations of Mn^{2+} as well as by dithiothreitol and low concentrations of EDTA. At low concentrations of substrate and cation, the hydrolysis of cyclic AMP is stimulated exclusively by Mn^{2+}, whereas the hydrolysis of cyclic GMP is activated exclusively by Mg^{2+}. The reaction with both nucleotides is inhibited by Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} as well as by EDTA in high concentration and by caffeine and theophylline but not by ethyleneglycol-bis-(2-aminoethyl ether)- *N*, *N'*-tetraacetate (EGTA) and 8-hydroxyquinoline. NaF, histamine, serotonine, prostaglandin E_1 and E_2 and pigskin extracts containing "Epidermal Chalone" are without any effect.

4. Although both cyclic nucleotides have almost identical K_m values, the hydrolysis of cyclic AMP cannot be inhibited significantly by cyclic GMP and vice versa. This, together with the specificity with respect to the metal ion, may be taken as evidence for the existence of two epidermal phosphodiesterases, a cyclic AMP phosphodiesterase and a cyclic GMP phosphodiesterase.

INTRODUCTION

Recent observations in several laboratories have provided evidence, that both cyclic AMP and cyclic GMP play an important role in controlling growth and function of epidermis. For example, the well-known antimitotic effect of catecholamines seems to be mediated by a β-receptor–adenylate cyclase–cyclic AMP system [1–7], whereas cyclic GMP is increased in rapidly proliferating psoriatic epidermis [8]. It has been proposed, that in epidermis the equilibrium between proliferation and differentiation

Abbreviation: EGTA, ethyleneglycol-bis-(2-aminoethyl ether)-*N*,*N'*-tetraacetate.

* For Part I, II and III see refs 4, 25 and 31.

is controlled at least partially by the relative concentrations of cyclic AMP and cyclic GMP [8] and that cyclic AMP might be involved in the mechanism of action of local growth inhibitors or chalones [9].

The level of cyclic nucleotides in tissues and organisms can be regulated not only by critical alterations in their rates of formation but also by controlling specifically their degradation (see for example ref. 10).

Therefore, we wish to report on a more detailed study of cyclic nucleotide phosphodiesterase in mouse epidermis.

MATERIALS AND METHODS

Labelled compounds

Cyclic [8-^{3}H]AMP (specific activity > 20 Ci/mmole) and cyclic [8-^{3}H]GMP (> 0,5 Ci/mmole) were obtained from 'The Radiochemical Centre, Amersham, England.' The labelled nucleotides were checked for radiochemical purity by thin-layer chromatography on PEI-cellulose (Macherey and Nagel, Düren, Germany) with 0.3 M LiCl as eluant. If necessary they were purified by thin-layer chromatography on silica-gel-coated glass fiber sheets [11] (Chrom-AR 1000, product of Byk–Mallinckrodt, Wesel, Germany) with 2-propanol– ethylacetate–13.6 M NH_4OH (55:29:16, by vol.) (cyclic AMP) or with 2-propanol–ethylacetate–13.6 M NH_4OH–water (60:20:10:10, by vol.) (cyclic GMP) as eluant. After chromatography the cyclic nucleotides were eluted from the sheets with water.

The labelled compounds were diluted with unlabelled material corresponding to the data given below.

Other materials

Cyclic AMP and cyclic GMP as well as AMP and GMP were products of Boehringer Mannheim. From Serva Heidelberg were obtained: dithiothreitol; ethyleneglycol-bis-(2-aminoethyl ether)-*N*, *N'*-tetraacetate (EGTA); serotonine (creatinine sulfate complex); histamin–HCl.

The prostaglandins E_1 and E_2 were generous gifts of Dr John Pike, The Upjohn Company, Kalamazoo, Mich. We are very much obliged to Dr W. Hondius-Boldingh, N.V. Organon, Oss, The Netherlands, for supplying us with pig-skin extracts partially purified by ethanol precipitation [12] and containing epidermal chalone (G_1- and G_2-inhibitor) [13, 14].

Preparation of epidermal homogenates

Female mice (strain NMRI, age 7–9 weeks) were used for preparation of epidermal specimens. Prior to the experiment, the animals were treated as described recently [4]. The mice were killed by cervical dislocation. The back skin was dissected and fixed on cork plates with the epidermis facing upwards. The epidermis was then scraped off by means of a scalpel and homogenized in 2 ml of ice-cold 0.16 M Tris–HCl buffer (pH 8.0) containing 5 mM Mg^{2+} (if not stated otherwise) using a "Mini-Potter" homogenizer (Braun–Melsungen, Germany).

Assay of phosphodiesterase

A serious drawback of studies with mouse epidermis is the very small amount

of tissue available from one animal. In order to have a reliable routine assay under these conditions a method based on the use of homogenate instead of partially purified enzyme fractions was looked for and found in the procedure of Gulyassy and Oken [15]. In this method enzymatic hydrolysis of cyclic AMP is measured in the presence of an excess of AMP as a trapping agent. The authors have shown that product inhibition is less than 10%, if the incubation time does not exceed 30 min, and that the decay of AMP is below 1% as long as the total metabolism of cyclic AMP is under 20%. Both conditions are observed in our experiments: less than 10% of the cyclic nucleotide were hydrolyzed during an incubation time of 30 min and the formation of the corresponding 5'-nucleotides showed linearity with time (see Fig. 2). Measuring the distribution of radioactivity among the different fractions (5'-nucleotide, cyclic nucleotide, nucleoside and inosine or xanthosine) obtained after incubation of labelled cyclic nucleotide in the presence of rising concentrations of the trapping agent 5'-nucleotide (0–5 mM) we did not observe any product inhibition at all; the degradation of 5'-nucleotide was high in the range of 0–0.1 mM but practically zero with 5 mM of trapping agent. Therefore, the conditions employed in our experiments are suitable for a reliable assay of phosphodiesterase in epidermal homogenates.

The assay may also be applied for determination of the hydrolysis of cyclic GMP. To get a satisfactory separation of GMP from other possible metabolites the concentration of LiCl in the chromatographic system was raised from 0.3 to 0.5 M (R_F values: GTP, 0.0; GMP, 0.15; cyclic GMP, 0.4; guanosine, 0.6; xanthosine, 0.5; inosine, 0.8).

If not stated otherwise, the final concentrations in the incubation mixture (0.3 ml) were: 147 mM Tris–HCl (pH 8.0), 3.3 mM Mg^{2+}, 5 mM AMP or GMP, 40 μM cyclic AMP or cyclic GMP (corresponding to 10 μCi) and 200 μl of epidermal homogenate. The reaction was started by addition of the labelled substrate. After incubation for 20 (or 30) min at 37 °C the vials were placed into an ice bath and the reaction was stopped by adding 25 μl of 50% trichloroacetic acid. The clear supernatant obtained by centrifugation was transferred to a clean tube and extracted three times with 0.5 ml of ether. The aqueous phase was then mixed with 50 μl of "carrier solution" containing 2 mg cyclic nucleotide, 2 mg nucleoside and 2 mg inosine or xanthosine per ml. A 10 μl aliquot was placed as a single spot on to a PEI-cellulose thin layer. The sample was chromatographed, eluted and checked for radioactivity as described recently [4, 15].

The DNA content of the epidermal homogenate was measured as reported previously [4].

RESULTS

Basic conditions for the enzymatic hydrolysis of cyclic nucleotides in epidermal homogenates

The rate of formation of AMP or GMP from the corresponding cyclic nucleotides depended directly on the concentration of homogenate (Fig. 1), on the incubation time (Fig. 2) and on the substrate concentration (Fig. 3). Cyclic GMP was hydrolyzed more than two times faster than cyclic AMP.

With low substrate concentrations the K_m values derived from the Lineweaver–Burk plot were identical for both cyclic nucleotides ($K_m = 4 \cdot 10^{-6}$ M) and the affinity

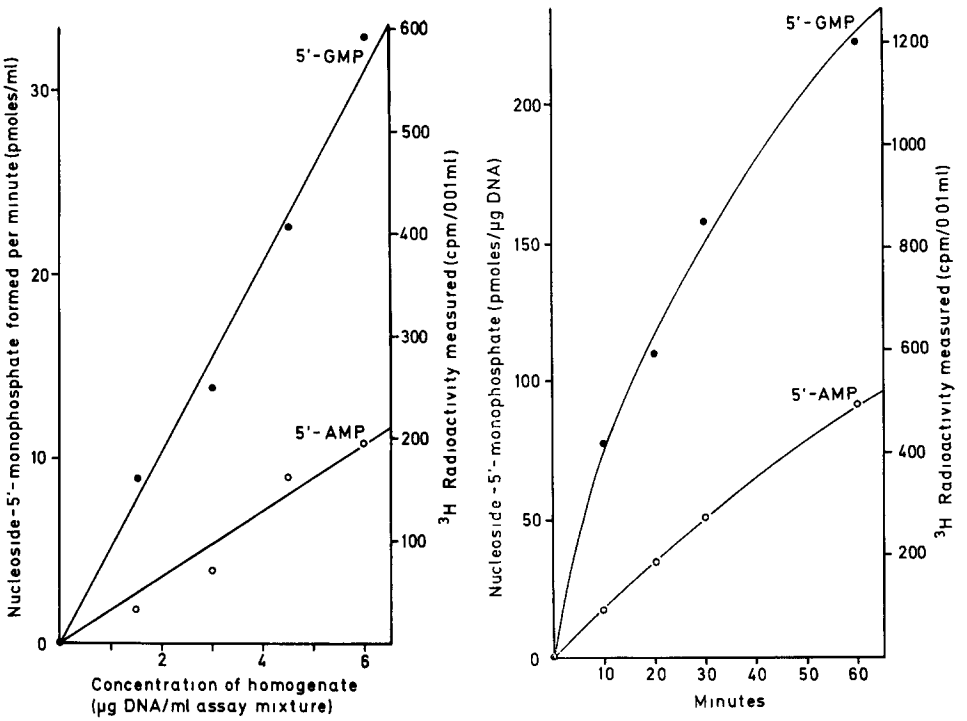

Fig. 1. Formation of [^{3}H]AMP and [^{3}H]GMP from the corresponding cyclic [^{3}H]labelled mononucleotides in epidermal homogenate of female NMRI mice as a function of homogenate concentration (expressed as DNA content of the homogenate). The reaction mixture of 0.3 ml contained Tris–HCl (147 mM), $MgCl_2$ (3.3 mM), AMP or GMP (5 mM) and cyclic [^{3}H]AMP or cyclic [^{3}H]GMP (40 μM). The incubation time was 30 min, temperature 37 °C, pH 8.0. Left ordinate amount of 5'-mononucleotide formed in 1 ml of the assay mixture per min, right ordinate: measured radioactivity

Fig. 2. Time course of the formation of [^{3}H]AMP and [^{3}H]GMP from the corresponding cyclic [^{3}H]labelled mononucleotides in epidermal homogenates of female NMRI mice. The composition of the reaction mixture was the same as described in the legend to Fig. 1.

was higher as with high substrate concentrations (cyclic GMP, $K_m = 3 \cdot 10^{-5}$ M; cyclic AMP, $K_m = 8 \ 10^{-5}$ M; see Fig. 3).

Under low affinity conditions the enzymatic hydrolysis of both nucleotides has a pH optimum around 8 (Fig. 4). The phosphodiesterase activity for both cyclic GMP and cyclic AMP was found to be localized almost entirely in the $17000 \times g$ supernatant.

Effect of divalent cations

At high substrate concentrations (40 μM, $K_m = 3 \ 10^{-5}$–$8 \cdot 10^{-5}$ M), the phosphodiesterase of mouse epidermis was strongly activated by Mn^{2+} and Mg^{2+}. At low concentrations of metal ion the stimulatory activity of both cations was found to be almost the same, whereas above 1 mM Mg^{2+} was the most potent activator (Fig. 5); this is due to an inhibitory effect of high concentrations of Mn^{2+}. Sometimes it was observed that the hydrolysis of cyclic AMP was more activated by Mn^{2+} in concen-

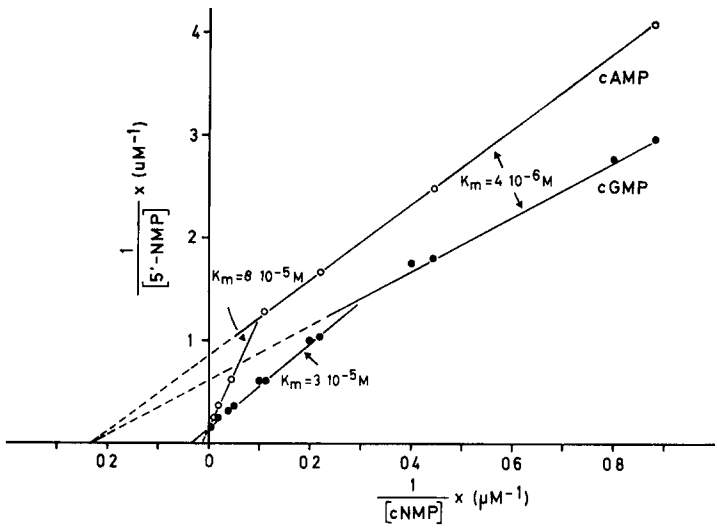

Fig. 3. Modified Lineweaver–Burk plot of the kinetics of the enzymatic hydrolysis of cyclic AMP and cyclic GMP in epidermal homogenates of female mice. With the exception of the substrate concentration, the conditions are the same as described in the legend to Fig. 1.

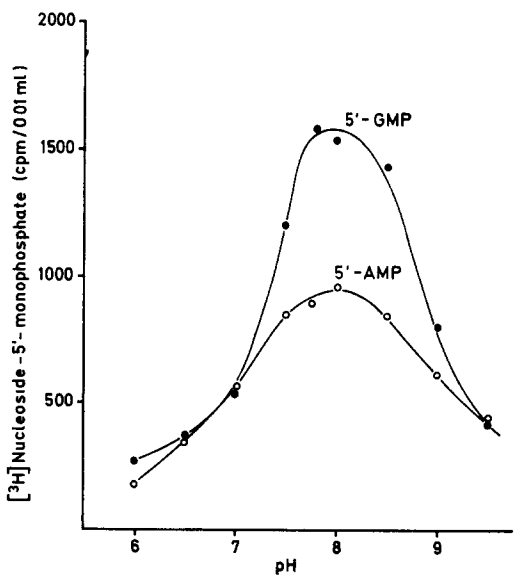

Fig. 4. Formation of [^{3}H]AMP and [^{3}H]GMP from the corresponding cyclic [^{3}H]labelled mononucleotides in epidermal homogenate of female mice at different pH values. The conditions are the same as described in the legend to Fig. 1. The Tris–HCl buffer was replaced, however, in the pH range 6.0–7.0 by sodium cacodylate–HCl buffer and in the pH range 9.0–9.5 by glycine–NaOH buffer.

trations between 10 and 1000 μM than by Mg^{2+}, whereas the reverse was true for cyclic GMP (see for example Table I).

At low substrate concentrations (2 μM, $K_m = 4 \cdot 10^{-6}$ M) the enzymatic hydrolysis of cyclic AMP was much more strongly stimulated by low concentrations of

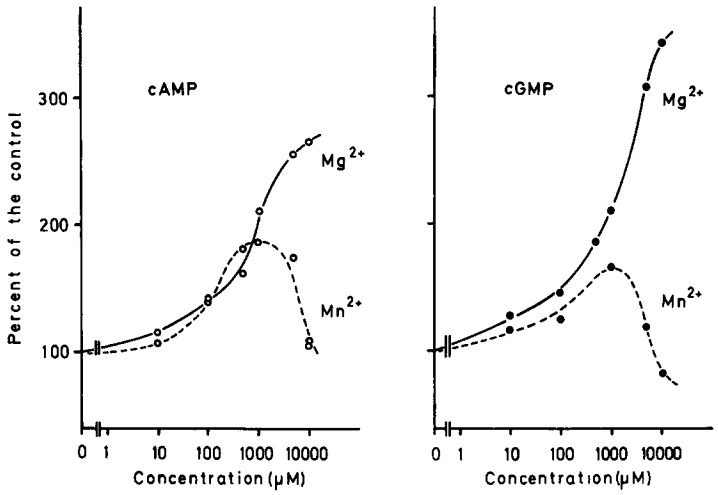

Fig. 5. Stimulation by Mg^{2+} and Mn^{2+} of the enzymatic hydrolysis of cyclic AMP and cyclic GMP in epidermis homogenates of female mice under "low affinity conditions" (substrate concentration 40 μM). Except for the metal ions, the conditions of the assay are the same as described in the legend to Fig. 1. Abscissa: concn of metal ion.

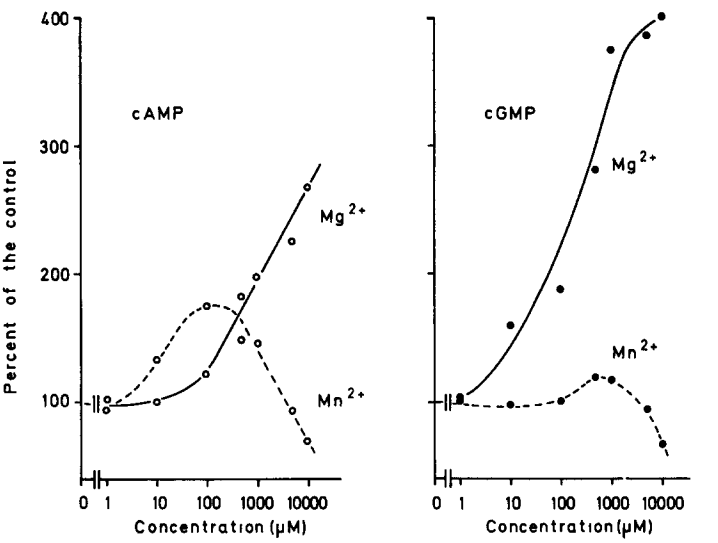

Fig. 6. Stimulation by Mg^{2+} and Mn^{2+} of the enzymatic hydrolysis of cyclic AMP (left) and cyclic GMP (right) in mouse epidermal homogenate under "high affinity conditions" (substrate concentration 2 μM). The other conditions are the same as described in the legend to Fig. 1. Abscissa: concn of metal ion.

Mn^{2+} than by Mg^{2+}. In contrast, the hydrolysis of cyclic GMP was exclusively stimulated by Mg^{2+}, with Mn^{2+} showing no significant effect (Fig. 6).

Several other metal ions were more or less inhibitory (Table I). Since the activity of epidermal phosphodiesterase was increased by dithiothreitol (Table I), inactivation of essential sulfhydryl groups may be the reason for the inhibition by heavy metals.

TABLE I

EFFECT OF METAL IONS, CHELATING AGENTS AND DITHIOTHREITOL ON THE ACTIVITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN MOUSE EPIDERMIS HOMOGENATES.

With the exception of the Mg^{2+} content, the assay conditions are the same as those described in the legend to Fig. 1.

Added	Concentration (mM)	Relative activity with substrate	
		Cyclic AMP	Cyclic GMP
Control		100	100
Mg^{2+}	0.1	96	115
Mg^{2+}	5.0	345	294
Mn^{2+}	0.1	150	90
Mn^{2+}	5 0	169	91
Ca^{2+}	0.1	84	84
Ca^{2+}	5.0	41	59
Co^{2+}	0 1	92	74
Co^{2+}	5.0	90	71
Ni^{2+}	0 1	74	82
Ni^{2+}	5.0	41	81
Cu^{2+}	0.1	59	41
Cu^{2+}	5 0	17	38
Zn^{2+}	0 1	32	33
Zn^{2+}	5 0	14	26
Fe^{2+}	5 0	76	35
EDTA	0.1	140	159
EDTA	1 0	21	13
EGTA	0 1	98	94
EGTA	1 0	93	99
8-Hydroxyquinoline	5.0	98	116
Dithiothreitol	10 0	100	162

The enzymatic activity measured in the absence of exogeneous metal ion was activated by low and inhibited by high concentrations of EDTA, but was not influenced by either EGTA or 8-hydroxy-quinoline (Table I).

Substrate specificity

The presence of one of the cyclic nucleotides in the incubation mixture could not significantly inhibit the hydrolysis of the other. This is shown in Fig. 7 for several sets of conditions at low substrate concentration (2 μM). Similar results have been obtained also at high substrate concentrations (40 μM). It may be concluded, therefore, that epidermis contains at least two rather specific phosphodiesterases catalyzing the degradation of either cyclic AMP or cyclic GMP.

In contrast to observations made with several other tissues [16–19], the epidermal cyclic AMP phosphodiesterase was not stimulated by cyclic GMP in low concentrations and vice versa (Fig. 7).

Other potential effectors

Like phosphodiesterase in other tissues, the epidermal enzymes were strongly inhibited by methylxanthines such as theophylline and caffeine. The hydrolysis of cyclic GMP was less affected than the hydrolysis of cyclic AMP (Fig. 8).

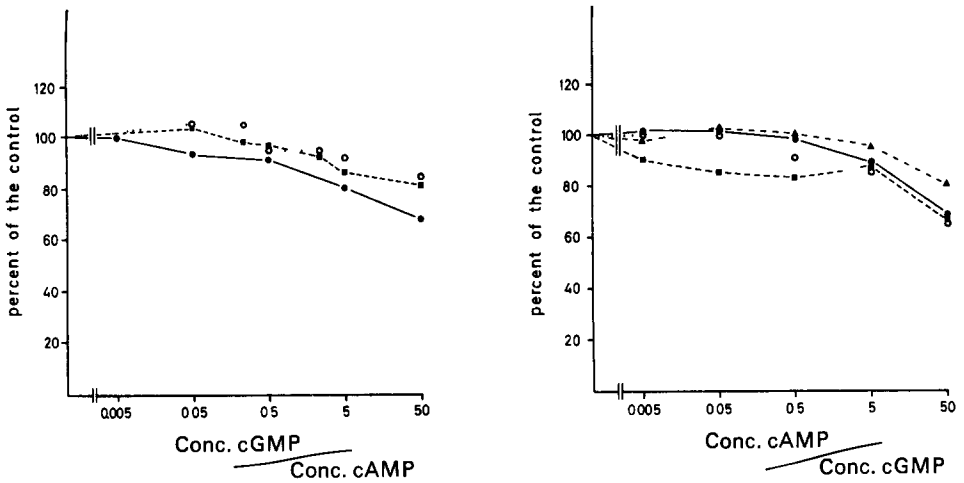

Fig. 7. Effect of cyclic GMP on the enzymatic hydrolysis of cyclic AMP (left) and effect of cyclic AMP on the enzymatic hydrolysis of cyclic GMP (right) in mouse epidermal homogenate under "high affinity conditions" (substrate concentration 2 μM). ●—●, without exogeneous metal ion; ■—■, plus 0.1 mM Mn^{2+}; ○ . ○, plus 0.1 mM Mg^{2+}; ▲-.-.-▲, plus 5.0 mM Mg^{2+}.

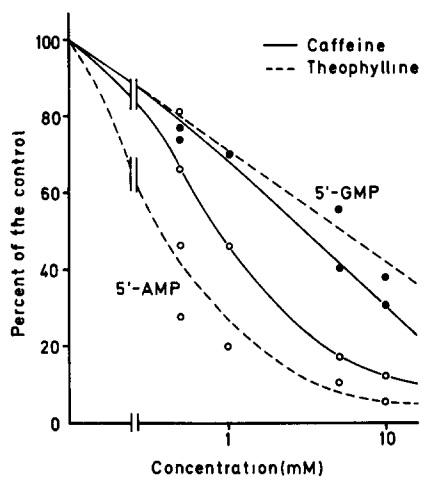

Fig. 8. Formation of AMP and GMP from the corresponding cyclic nucleotides in the presence of methylxanthines. The assay conditions are the same as described in the legend to Fig. 1. Abscissa: concn of methylxanthine.

NaF (0.1–10 mM), a strong stimulator of epidermal adenylate cyclase [4], was without any effect on cyclic AMP phosphodiesterase. The same was true for insulin (0.1–100 munits per assay), histamine (0.01–10 mM), 5-hydroxytryptamine (0.01–10 mM), prostaglandin E_1 and E_2 (1–100 μm) as well as for pig-skin extracts containing epidermal chalone (G_1- and G_2-inhibitor; 0.1–1 mg per assay). The latter was tested either at low (2 μM) or at high (40 μM) concentrations of substrate (cyclic AMP or cyclic GMP) and either in the absence or in the presence of Mg^{2+} or Mn^{2+}.

DISCUSSION

Data presented in this paper provide evidence that mouse epidermis contains at least two phosphodiesterases, specific either for cyclic AMP or for cyclic GMP. Both enzymes are very similar in their pH dependency, intracellular distribution and inhibition by methylxanthines. They differ, however, considerably in their dependency on activating metal ions: whereas Mg^{2+} is more efficient in stimulating cyclic GMP phosphodiesterase, cyclic AMP phosphodiesterase has a pronounced preference for Mn^{2+}. These findings are quite similar to those reported previously on phosphodiesterase partially purified from bovine brain [20].

The kinetics of the enzymatic hydrolysis of both cyclic nucleotides are characterized by two apparent K_m values showing high affinity at low and low affinity at high substrate concentrations. The resulting non-linearity of the Lineweaver–Burk plots seems to be typical for phoshodiesterases in many tissues (see for example refs 17–23).

Recently multiple forms of phosphodiesterase have been demonstrated [19, 26–28] and evidence has been provided that the low-affinity enzymes might be generated by dimerization or aggregation of the high-affinity enzymes in the presence of high concentrations of Mg^{2+} or substrate [27].

The "K_m values" measured in our experiments are remarkably consistent with those reported for cyclic AMP phosphodiesterase from whole skin [29]. However, the values (see also ref. 29) are obtained under the assumption that at high substrate concentrations the low-affinity enzymes do not contribute appreciably to the kinetics of the high-affinity enzymes and vice versa, which has been shown to be an oversimplification [30]. Therefore, they only roughly approximate the real Michaelis constants.

The occurrence in epidermis of at least two phosphodiesterases specific either for cyclic AMP or for cyclic GMP may be taken as evidence that the levels of the two cyclic nucleotides are regulated independently especially since both nucleotides are generated by separate cyclases [4, 31]. This might be of considerable importance since it has been proposed that the nucleotides may have antagonistic effects in such a way that cyclic AMP inhibits cell proliferation and concurrently enhances functionalization whereas cyclic GMP is involved in triggering cell proliferation [8]. However, convincing experimental support for this hypothesis is still lacking. It may be concluded, therefore, that although a complete second messenger system has been shown to exist in epidermis, much work remains to be done in order to clarify its physiological role.

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