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THE SECOND MESSENGER SYSTEM OF MOUSE EPIDERMIS

III. GUANYL CYCLASE*

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SUMMARY

1. Guanyl cyclase has been demonstrated in homogenates of mouse epidermis by incubation with [α-^{32}P]GTP. The activity of the enzyme is about four times lower than in kidney and 100 times lower than that of epidermal adenyl cyclase measured under the same conditions.

2. The enzyme is localized almost entirely in the 17 000 $\times$ g supernatant..

3. Epidermal guanyl cyclase has an absolute requirement for Mn^{2+}. Ca^{2+} and Fe^{2+} have a slightly activating effect, whereas Mg^{2+}, Co^{2+} and Zn^{2+} are inactive or inhibitory.

4. The activity of epidermal guanyl cyclase is influenced neither by NaF nor by adrenalin, noradrenalin, isoproterenol, acetylcholine, serotonin, thyroxin, somatotropin or by skin extracts containing epidermal chalone.

INTRODUCTION

Despite its potential importance as a suitable model for studies on growth regulation, differentiation and cancerization, the basic regulatory mechanisms of epidermis are poorly understood. There is some evidence that cyclic nucleotides may be involved in the control of the mitotic and functional homeostasis of epidermis[1,2] and it has been speculated that cyclic AMP may act as a second messenger of epidermis-specific mitotic regulators or chalones[3]. Recently, the formation of cyclic AMP in intact[4,5] and broken[6–8] epidermal cell preparations, as well as under *in vivo* conditions[9], could be demonstrated in several laboratories. However, a significant proof for a connection between cyclic AMP and the chalone system is still lacking. Another possible candidate for mediating tissue-specific control signals might be cyclic GMP, which is the only cyclic nucleotide besides cyclic AMP found in living organisms.

Abbreviation: PEI, polyethylenimine.

* For Part I and II see refs 7 and 9.

Very recently, it has been reported that cyclic GMP might be involved in mitotic regulation[10].

In the following communication the demonstration and some basic properties of guanyl cyclase in epidermal homogenate will be described.

MATERIALS AND METHODS

Labelled compounds

[α-^{32}P]GTP (spec. act. 0.858 Ci/mmole) and cyclic [8-^{3}H]GMP (spec. act. 4.4 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, England. The labelled compounds were diluted with unlabelled material corresponding to the data given below.

Other materials

The following compounds were products of Boehringer Mannheim: pyruvate kinase from rabbit skeletal muscle; phosphoenol pyruvate (potassium salt); 5'-GTP; cyclic 3',5'-GMP. From Serva Heidelberg were obtained: L-noradrenalin (bitartrate) D,L-isoproterenol·HCl; acetylcholine·HCl; serotonin (creatine sulphate complex). L-Adrenalin (bitartrate), L-thyroxin and porcine growth hormone (somatotropin) were delivered by CalBiochem, Los Angeles, U.S.A.

Pig-skin extracts (code number CH 1333) partially purified by ethanol precipitation[11] and containing epidermal chalone (G_1 inhibitor[12] and G_2 inhibitor[11]) were a generous gift of Dr Hondius-Boldingh, N.V. Organon, Oss, The Netherlands.

Chrom-AR-sheet, a glass fiber matrix covered with silica gel, is a product of Byk-Mallinckrodt, Wesel, Germany. Thin-layer foil covered with polethylenimine (PEI)-cellulose was obtained from Macherey and Nagel, Düren, Germany (Polygram CEL 300 PEI/UV_{254}) and treated as described recently.[7]

Preparation of epidermal homogenates

Epidermal preparations were made from the back skin of hairless mice (Oslo strain) by the following method: After killing the animal by cervical dislocation, the back skin was dissected and fixed on a cork plate with the epidermis facing upwards. The epidermis was scraped off with a scalpel, suspended in 0.5 ml buffer containing 62 mM Tris–HCl, 5 mM $MnCl_2$ and 20 mM caffein (pH 7.5), and homogenized using a glass-teflon homogenizer (Potter–Elvejhem) placed in an ice bath. The homogenate was filtered through a double layer of gauze and the filtrate was used immediately.

The DNA content of the homogenate was estimated as described recently[7].

Assay of guanyl cyclase

To measure the activity of the epidermal guanyl cyclase, 50 μl of the epidermal homogenate were added to 50 μl of incubation mixture containing [α-^{32}P]GTP (1–2 μCi), cyclic GMP as a "collecting pool" and as carrier for cyclic [^{32}P]GMP generated during the reaction and a GTP-regenerating system[24]. The final concentrations of the components were: 62 mM Tris–HCl, 5 mM $MnCl_2$, 20 mM caffein, 5 mM cyclic 3',5'-GMP, 1 mM [α-^{32}P]GTP, 60 mM KCl, 15 mM phosphoenol pyruvate and 0.4 mg/ml pyruvate kinase. The final pH was 7.5.

Except where stated otherwise, the incubation was carried out at 37 °C for

30 min. The reaction was terminated by adding 10 μl of an aqueous solution of cyclic [8-^{3}H]GMP (25 μCi/ml) as an internal standard and placing the incubation vial into a boiling water bath for 3 min.

Cyclic [^{32}P]GMP was separated quantitatively from other labelled metabolites of GTP by a modification of the precedure described recently for the purification of cyclic [^{32}P]AMP[7]. For this purpose, 70 or 100 μl of the clear supernatant obtained by centrifugation of the incubation mixture were spotted onto Chrom-AR sheets as a streak (0.5 cm × 3 cm) and chromatographed ascendingly with isopropanol–ethyl-acetate–conc. ammonium hydroxide–water (6:2:1:1, by vol.). The cyclic GMP was then transferred to PEI-cellulose thin-layer plates, rechromatographed with 0.3 M

TABLE I

R_F values of possible radioactive metabolites of [α-^{32}P]GTP in the chromatographic System I (Chrom-AR, 2-propanol–ethyl acetate–13.6 M ammonium hydroxide–water (6:2:1:1, by vol.) and in System II (PEI-cellulose, 0.3 M LiCl).

	System I	*System II*
5′-GTP	< 0.1	< 0.1
5′-GMP	< 0.1	0.2
2′(3′)-GMP	< 0.1	0.2
5′-dGMP	< 0.1	0.2
Cyclic 3′,5′-GMP	0.6	0.5
5′-ATP	< 0.1	< 0.1
5′-ADP	< 0.1	< 0.1
5′-AMP	0.1	0.3
2′(3′)-AMP	0.3	0.2
2′,5′-ADP	< 0.1	< 0.1
Cyclic 3′,5′-AMP	0.7	0.5
5′-IMP	< 0.1	0.4
5′-XMP	< 0.1	< 0.1

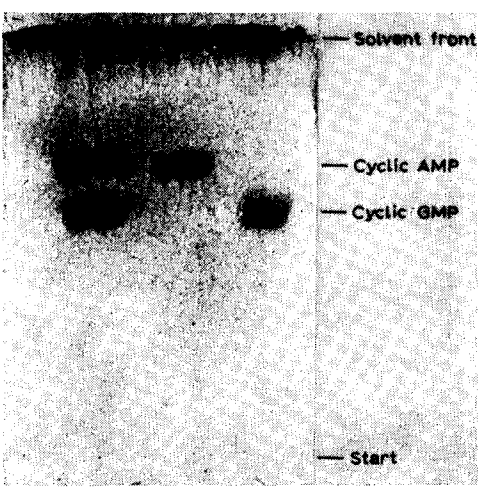

Fig. 1. Separation of cyclic GMP (R_F = 0.6) from cyclic AMP (R_F = 0.7) by chromatography on Chrom-AR with 2-propanol–ethyl acetate–13.6 M ammonium hydroxide–water (6:2:1:1, by vol.). 50 μl of a 5 mM solution of each nucleotide were spotted on to the sheet. The chromatogram was photographed under ultraviolet light (254 nm).

LiCl, eluted and checked for its radioactivity as described recently[7]. The R_F values of cyclic GMP and other possible metabolites of [α-^{32}P]GTP are shown in Table I.

Though there is apparently no dramatic difference between the R_F values of cyclic GMP and cyclic AMP, both nucleotides were clearly separated by chromatography on Chrom-AR (Fig. 1). In order to control the separation procedure, a small amount of "carrier" cyclic AMP was always added to the solution to be chromatographed.

The absence in the cyclic GMP spot of cyclic AMP, possibly generated *via* the pathway GTP → ATP → cyclic AMP, is further indicated by the fact that the radioactivity of the cyclic GMP fraction was not increased at all by carrying out the incubation in presence of NaF (see Results), which has been shown to be a powerful activator of epidermal adenyl cyclase[7].

The detection limit for cyclic GMP depends on the specific radioactivity of the substrate [α-^{32}P]GTP. Under the conditions described above, it was estimated to be in the range of between 3 and 5 pmoles.

RESULTS

Basic conditions for the formation of cyclic GMP in epidermal homogenates

As shown by Figs 2 and 3, the formation of cyclic [^{32}P]GMP from [α-^{32}P]GTP can be demonstrated with mouse epidermis homogenate. Under the conditions used in our experiments, the accumulation of the cyclic nucleotide was a linear function of the amount of homogenate up to concentrations equivalent to 100 μg of DNA per ml assay mixture (Fig. 2) and of time (Fig. 3). At higher homogenate concentrations the reaction became non-linear.

The average activity of the epidermal guanyl cyclase was estimated to be

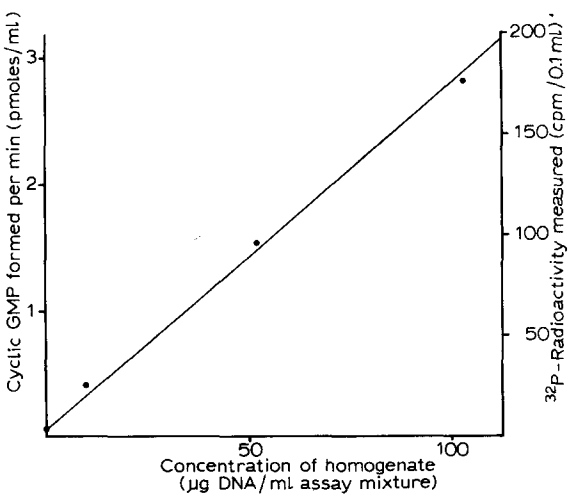

Fig. 2. Formation of cyclic [^{32}P]GMP in epidermis homogenate of hairless mice as a function of homogenate concentration (expressed as DNA content of the homogenate). The reaction mixture of 0.1 ml contained: 62 mM Tris–HCl (pH 7.5), 5 mM Mn^{2+}, 5 mM cyclic GMP, 1 mM [α-^{32}P]GTP ($17 \cdot 10^6$ cpm/ml), 20 mM caffein, 60 mM KCl, 15 mM phosphoenol pyruvate and 0.4 mg/ml pyruvate kinase. The incubation time was 30 min at a temperature of 37 °C.

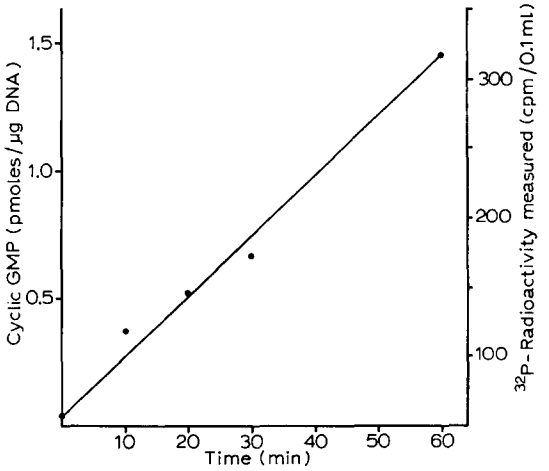

Fig. 3. Time course of the formation of cyclic [^{32}-P]GMP in epidermal homogenates of hairless mice. The composition of the reaction mixture was the same as described in Fig. 1. The homogenate concentration was equivalent to 108 μg DNA/ml assay mixture.

0.040 $\pm$ 0.017 pmole cyclic GMP formed/min per μg DNA ($n = 5$; S.D.). This is about 100 times lower than the activity of epidermal adenyl cyclase measured under the same conditions[7] (in presence of 5 mM Mn^{2+} and without NaF). Also, as compared with other tissues, the epidermal guanyl cyclase has a very low activity, which was found to be approximately one quarter of that in mouse kidney (0.165 pmole cyclic GMP/min per μg DNA) and 1/30 of that in mouse lung (1.17 pmoles cyclic GMP/min per μg DNA).

Epidermal guanyl cyclase has an absolute requirement for Mn^{2+} in concentrations above 1 mM (Fig. 4), which cannot be replaced by Mg^{2+}, Co^{2+} or Zn^{2+} (Table II). Ca^{2+} (and perhaps Fe^{2+}) exhibited a much smaller but reproducible

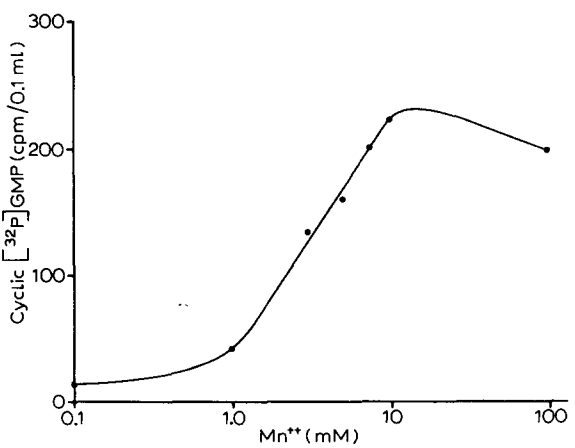

Fig. 4. Formation of cyclic [^{32}P]GMP in epidermal homogenates of hairless mice as a function of the Mn^{2+} concentration. The assay conditions correspond to those described for Fig. 1. Ordinate: Radioactivity measured per 0.1 ml of incubation mixture.

TABLE II

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF EPIDERMAL GUANYL CYCLASE
The assay conditions correspond to those described in Fig. 2 (epidermal homogenate equivalent to 160 μg DNA/ml, $8{,}8 \cdot 10^6$ cpm/ml [α-^{32}P]GTP). The concentration of each metal ion was 5 mM.

Me^{2+}	[^{32}P] cyclic GMP (cpm/0.1 ml)
None	6
Mn^{2+}	166
Ca^{2+}	26
Mg^{2+}	6
Co^{2+}	7
Fe^{2+}	29
Zn^{2+}	0
Mn^{2+} + Ca^{2+}	198
Mn^{2+} + Mg^{2+}	129
Mn^{2+} + Co^{2+}	26
Mn^{2+} + Zn^{2+}	1

stimulatory effect (Table II). In the presence of Mn^{2+} the formation of cyclic GMP was slightly inhibited by Mg^{2+} and abolished by Co^{2+} and Zn^{2+} but activated by Ca^{2+} (Table II). In contrast to adenyl cyclase[7], the activity of guanyl cyclase was not enhanced by NaF (1–10 mM).

After centrifugation of the epidermal homogenate at 17 000 $\times$ g for 20 min, approx. 90% of the cyclase activity was found in the supernatant, whereas the sediment was almost inactive.

Effect of hormones and epidermal chalone

Hormone-active compounds such as adrenalin, noradrenalin, isoproterenol, acetylcholine, L-thyroxin, serotonin (all 0.1 mM) and somatotropin (0.5 mg/ml) did not show any significant stimulatory or inhibitory effect on the formation of cyclic GMP in epidermal homogenates. The same was true for pig-skin extract containing epidermal chalone[11–13] (G_1 and G_2 inhibitor), which was incubated in concentrations between 0.1 and 1 mg per ml in the absence and in the presence of 1 or 5 mM Mn^{2+}, 5 mM Ca^{2+} or in the presence of both 5 mM Ca^{2+} and 1 or 5 mM Mn^{2+}.

DISCUSSION

Guanyl cyclase, which has been found in all tissues examined so far, could be demonstrated in epidermis for the first time. This tissue is of particular interest because it is a favoured model for studies on tissue-specific growth regulation by chalones[11–14] and on differentiation, as well as on benign and malignant proliferation processes, as for example represented by the two-stage mechanism of chemical carcinogenesis[15]. Therefore, the knowledge of mechanisms by which hormonal and other regulatory influences on epidermis are transformed into tissue-specific control signals is urgently wanted.

Recent studies in several laboratories, including our own, have shown that epidermis contains a complete "second messenger system", including adenyl cyclase[4–8], cyclic nucleotide phosphodiesterase (refs 1 and 16, and Marks, F. and Raab,

I., unpublished) cyclic AMP-dependent protein kinase[1,18], and (as described in this paper) guanyl cyclase. The *in vitro* activity of epidermal guanyl cyclase is about 100 times lower than that of the corresponding adenyl cyclase. On the other hand, epidermal phosphodiesterase has the same affinity to both cyclic GMP and cyclic AMP (Marks, F. and Raab, I., unpublished). Therefore, the level of cyclic GMP in epidermis may be expected to be extremely low. Similar observations have been made by other authors: with a few exceptions the tissue content of cyclic GMP is always lower than that of cyclic AMP[19–21].

Although cyclic GMP seems to occur in living systems as frequently as cyclic AMP, only little is known about its physiological function. While there is indeed some evidence that the nucleotide may be involved in cholinergic neurotransmission[26,27], a role as a second messenger-like cyclic AMP could not as yet be demonstrated, especially because all attempts to demonstrate a hormone-dependent stimulation of guanyl cyclase *in vitro* have thus far been without any success[22–25]. This is also true for epidermis. It should be considered, however, that the properties of cyclases studied in broken cell preparations may differ entirely from those observed *in vivo* or in the intact tissue. For example, the β-adrenergic receptor of epidermal adenyl cyclase is nearly 90% destroyed during homogenization and incubation[7]. It may therefore be suggested that under pure *in vitro* conditions, hypothetic guanyl cyclase-receptor complexes are completely inactivated or at least uncoupled. The observation that after perfusion of the rat heart or incubation of brain and heart slices, the level of cyclic GMP was increased by acetylcholine and decreased by isoproterenol and noradrenalin[26–28] may be taken as an evidence for this assumption, since similar effects have never been found with homogenates. Thus studies with more or less artificial *in vitro* systems, like that described in this paper, should be regarded only as a first step in elucidating the biological role of cyclic GMP in a given tissue.

The basic *in vitro* properties of epidermal guanyl cyclase are quite similar to those reported for the enzyme in other tissues and differ considerably from those of adenyl cyclase[21–29]. Thus epidermal guanyl cyclase is stimulated by Mn^{2+}, and to a much smaller extent by Ca^{2+} and Fe^{2+}, but not by F^-, and is inhibited by Mg^{2+} and Co^{2+}. In contrast, epidermal adenyl cyclase is activated by Mn^{2+}, Mg^{2+}, Fe^{2+} and Co^{2+} and especially by F^-, but is strongly inhibited by Ca^{2+} (ref. 7). Furthermore, epidermal guanyl cyclase seems to be mainly a soluble enzyme, whereas adenyl cyclase is bound to the particulate fraction of the cell[7]. With some exceptions, also in other tissues, guanyl cyclase has been found predominantly in the supernatant[21]; these experiments do not exclude, however, the possibility that in the intact cell the enzyme is bound loosely to structural elements.

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