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THE SECOND MESSENGER SYSTEM OF MOUSE EPIDERMIS I. PROPERTIES AND β-ADRENERGIC ACTIVATION OF ADENYLATE CYCLASE *IN VITRO*

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SUMMARY

1. By a combination of two standard procedures for nucleotide separation, a simple and highly sensitive method has been developed for the assay of adenylate cyclase in homogenates of mouse epidermis incubated with [α-^{32}P]ATP.

2. The enzyme is bound to the particulate fraction sedimenting between 1000 and 15 000 $\times$ *g*. It is activated by fluoride.

3. The activity of the epidermal adenylate cyclase depends on the divalent metal ions Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, with Mn^{2+} being by far the most powerful activator. Ca^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} are inactive or inhibitory.

4. Adrenalin and isoproterenol cause a 20–50% stimulation of cyclase activity whereas noradrenalin has no effect. The activation by catecholamines is prevented by β- but not by α-adrenergic blockers. Under *in vitro* conditions the hormone receptor seems to be partially destroyed.

5. High concentrations of insulin inhibit the formation of cyclic AMP in a dose-dependent manner.

6. No significant effect could be observed with glucagon, porcine growth hormone, epidermal growth factor, serotonin, histamine or prostaglandins E_1 and E_2, incubated in the presence or absence of adrenalin. Skin extracts containing epidermal chalone diminish the yield of cyclic AMP; this does not, however, seem to be a chalone-specific effect.

INTRODUCTION

Adenylate cyclase has been demonstrated in uni- and multicellular organisms and in all tissues so far examined[1,2]. Comparatively little is known about the adenylate cyclase of mammalian skin, in particular that of mouse epidermis. This tissue, however, has become a favoured model for studies on benign and malignant proliferation processes and in the last few years it has served as a main object of research on tissue-

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

specific mitotic regulators or chalones[3]. Therefore, mechanisms by which hormonal and perhaps other regulating influences on skin are transformed into tissue-specific control signals are of special interest. This is especially true for the adenylate cyclase–cyclic AMP system and reactions connected with it.

In this communication we wish to report the demonstration of adenylate cyclase in mouse skin, its basic properties and its stimulation by catecholamines. Further reports on other components of the epidermal second messenger system are in preparation.

MATERIALS AND METHODS

Labelled compounds

[α-^{32}P]ATP (specific activity 0.5–2 Ci/mmole) and cyclic [8-^{3}H]AMP were products of The Radiochemical Centre, Amersham, England. The radiochemical purity of [α-^{32}P]ATP (especially in respect to cyclic AMP) was checked by thin-layer chromatography with the solvent system described below. If necessary, the nucleotide was purified by passing it through a small column of Dowex 50-H^+ previously washed with distilled water.

Other materials

From Boehringer Mannheim the following were obtained: pyruvate kinase (EC 2.7.1.40) from rabbit skeletal muscle; adenylate kinase (EC 2.7.4.3), formerly known as myokinase, from rabbit skeletal muscle; phosphoenolpyruvate (potassium salt); cyclic 3′,5′-AMP. The following products were purchased from Serva, Heidelberg: ATP (disodium salt); cyclic 3′,5′-AMP; L-noradrenalin (bitartrate); DL-isoproterenol·HCl; dithiothreitol; ethyleneglycol-bis-(2-aminoethylether)-*N*,*N*′-tetraacetate (EGTA); insulin (Zn compound, 23 units/mg); histamine·HCl; serotonin (creatinine sulfate complex) and glucagon·HCl. L-Adrenalin (bitartrate) was obtained from Calbiochem, Los Angeles, porcine growth hormone (somatotropin) from Sigma, St. Louis.

We are very much obliged to Dr J. Pike, The Upjohn Company, Kalamazoo, Mich., for supplying us with prostaglandins E_1 and E_2. Propranolol·HCl was a generous gift of the Rhein-Pharma-GmbH, Heidelberg. For Phentolamin we have to thank the Ciba-AG, Wehr, Germany.

Epidermal growth factor was prepared from the salivary glands of male mice according to the method of Cohen[4].

Pig-skin extracts partially fractionated by ethanol precipitation[5] and containing "Epidermal Chalone" were a generous gift of Dr Hondius-Boldingh, N.V. Organon, Oss, The Netherlands. From this material two highly enriched fractions were prepared containing the epidermal G_1-inhibitor and the epidermal G_2-inhibitor as described recently[5,6].

Chrom-AR-sheet, a glass-fiber matrix covered with silicagel, is a product of Byk-Mallinckrodt, Wesel, Germany. Thin-layer foil covered with PEI-cellulose was obtained from Macherey & Nagel, Düren, Germany (Polygram CEL 300 PEI/UV_{254}).

The plates were washed with NaCl solution and water as described by Randerath and Randerath[7] and stored at −25 °C.

Preparation of epidermal homogenates

Epidermal specimens were obtained from female mice (strain NMRI, age 7–8 weeks). The skin on the back of the animals was shaved one week prior to the experiment. Only those mice were used which showed no regrowth of hair. For some experiments hairless mice (Oslo strain) were used.

After dissecting the back-skin and scraping off the subcutaneous tissue carefully with a scalpel, epidermal homogenates were prepared by two different methods:

Method I. The tissue was minced finely with scissors and then homogenized in 1.6 ml of icecold buffer (62 mM Tris·HCl–3 mM Mg^{2+}–0.1 mM EGTA, pH 7.5) using a loosely fitting glass–teflon homogenizer (Potter–Elvehjem) placed in an ice-bath. The homogenate was filtered through a double layer of gauze. The dermal connective tissue which cannot be destroyed by this method of homogenization remained at the filter whereas the filtrate consisted mainly of a homogenate of epidermal cells.

Method II. The skin was fixed on a cork-plate with the epidermis facing upwards. The epidermis was scraped off with a scalpel, suspended in 0.5 ml hypotonic buffer (2.5 mM Tris·HCl–3 mM Mg^{2+}, pH 7.5) and allowed to stand in an ice-bath for 15 min. 0.75 ml of incubation buffer (62 mM Tris·HCl–3 mM Mg^{2+}–0.1 mM EGTA) was added and the mixture was homogenized by 5–10 strokes with a loosely fitting Dounce-homogenizer (Braun-Melsungen, Germany). The homogenate was filtered through a double layer of gauze and the filtrate was used for the experiments.

The DNA content of the homogenates was estimated as described recently[8].

Assay of adenylate cyclase

To measure the activity of the epidermal adenylate cyclase, 50 μl of the epidermal homogenate were added to 50 μl of incubation mixture containing [α-^{32}P]ATP (1–2 μCi), theophylline or cyclic AMP and an ATP-regenerating system. Except where stated otherwise, the final concentrations of the compounds were: 62 mM Tris·HCl buffer (pH 7.5); 3 mM $MgSO_4$, 0.1 mM EGTA, 1.5 mM [α-^{32}P]ATP, 15 mM phosphoenolpyruvate (potassium salt), 0.4 mg/ml pyruvate kinase, 1 mg/ml adenylate kinase and 3 mM cyclic AMP as a "collecting pool" and carrier for cyclic [^{32}P]AMP generated during the reaction. This turned out to be a somewhat more efficient method for protecting labelled cyclic AMP from enzymatic breakdown as compared with the inhibition of phosphodiesterase by theophylline. Without theophylline or the "pool" the yield of cyclic [α-^{32}P]AMP decreased to 20–30% of the control.

After incubating the mixture at 37 °C for a given time the reaction was stopped by placing the incubation vial into a boiling water bath for 3 min. 10 μl of an aqueous solution containing 25 μCi/ml of cyclic [8-^{3}H]AMP were then added. This served as an internal standard during the following purification procedure. To incubations without a "cyclic AMP pool", a further 50 μg of unlabelled cyclic AMP as carrier were added.

Several methods, including the standard procedure of Krishna *et al.*[9], were tried for the separation of labelled cyclic AMP, but no satisfactory results were obtained. Finally a combination of the method of Woods and Waitzman[10] with ion-exchange thin-layer chromatography on PEI-cellulose[7,11] was found to be most suitable. This method leads to highly reproducible results (less than 5% deviation between parallel samples) and is fast enough to process up to 40 samples within one day.

In detail 50–100 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 as described recently[10]. The spots of cyclic AMP were located under ultraviolet light and cut out with scissors. The nucleotide was eluted with water directly onto a thin-layer plate of PEI-cellulose. The adsorption of cyclic AMP to the ion-exchanger prevented an excessive spreading of the spot even if an excess of water was used for the elution. Thus six spots could be placed on a single plate (20 cm × 20 cm). The progress of the elution was observed under an ultraviolet lamp. After developing the chromatogram ascendingly with 0.3 M LiCl the material containing the cyclic AMP spots was scraped off, placed into a scintillation vial and eluted with 0.5 ml of 0.5 M NH_4OH for 5 min. The radioactivity was then measured according to the method of Hennings *et al.*[8].

RESULTS

Basic conditions for the formation of cyclic AMP in epidermal homogenates

At low concentrations of epidermal homogenate the rate of formation of cyclic AMP was a linear function of time and enzyme concentration, whereas at higher concentrations it decreased due to rapid exhaustion of the substrate ATP *via* fast side reactions (Figs 1 and 2). Correspondingly an ATP-regenerating system (see Methods)was essential only at high homogenate concentrations, whereas at low concentration it only increased the yield of cyclic AMP by some 10%.

Under standard conditions (3 mM Mg^{2+}, 10 mM NaF) and with low homogenate

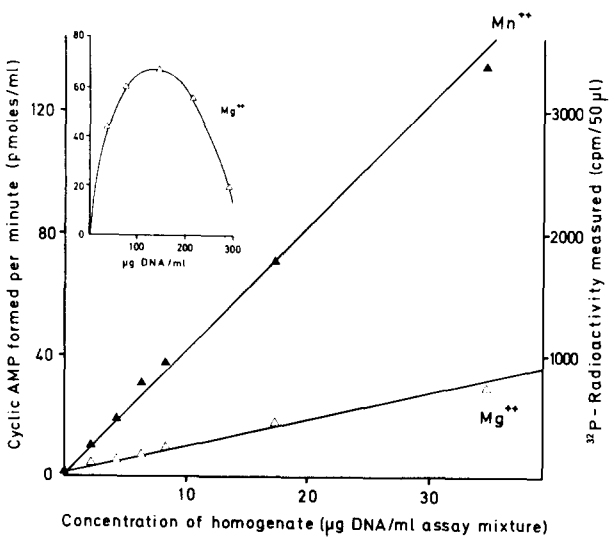

Fig. 1. Formation of cyclic [^{32}P]AMP in epidermis homogenate of mice (NMRI, ♀) as a function of homogenate concentration (expressed as DNA content of the homogenate). The reaction mixture of 0.1 ml contained: 50 µl homogenate, 62 mM Tris·HCl (pH 7.5), 3 mM Mg^{2+} (△) or Mn^{2+} (▲), 0.1 mM EGTA, 3 mM cyclic AMP, 1.5 mM [α-^{32}P]ATP (2 µCi), 15 mM phosphoenolpyruvate (K^+-salt), 40 µg pyruvate kinase, 100 µg adenylate kinase and 10 mM NaF. The incubation time was 30 min, temperature 37 °C. The inset shows the function over a wide range of homogenate concentration. Here the incubation time was 10 min. Left ordinate: amount of cyclic AMP formed in 1 ml of the assay mixture per min; right ordinate: measured radioactivity.

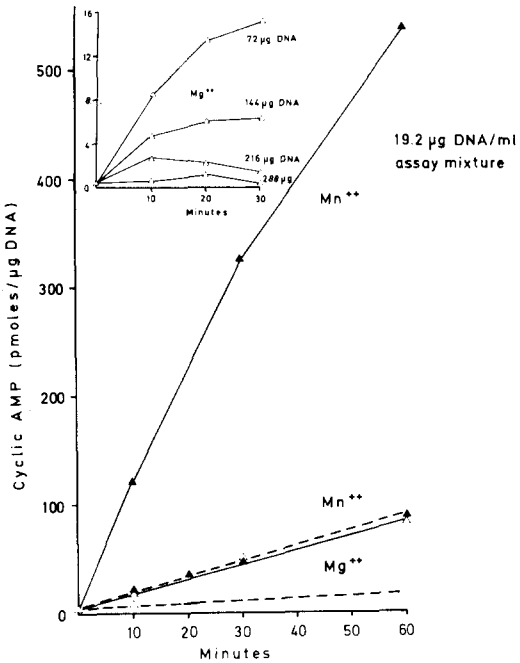

Fig. 2. Time course of the formation of cyclic [^{32}P]AMP in epidermal homogenates of female NMRI mice. The composition of the reaction mixture was the same as described in Fig. 1; — — —, represent experiments carried out in the absence of NaF (basal activity). The homogenate concentration was equivalent to 19 µg DNA/ml assay mixture; the experiment represented by the inset was carried out with homogenates equivalent to 72–288 µg DNA/ml assay mixture.

concentrations (approx. 20–30 µg DNA per ml assay mixture) the activity of the epidermal adenylate cyclase was estimated to be 2.24 ± 0.88 pmoles cyclic AMP formed per min per µg DNA (N = 10, S.D.). The basal activity was on average only 1/7 as high: 0.32 pmoles cyclic AMP formed per min per µg DNA. Thus, referring to the substrate ATP the average yield of cyclic AMP ranged between $8 \cdot 10^{-5}$ % per min per µg DNA in the absence and $6 \cdot 10^{-4}$ % per min per µg DNA in the presence of NaF.

The activity of the epidermal adenylate cyclase depends on the presence of divalent cations with manganese being by far the most potent activator (Figs 1 to 3). Under standard conditions (3 mM divalent metal ions, 10 mM F^-) its effectiveness surpassed that of Mg^{2+} about 6-fold. In the presence of Mn^{2+} the basal activity of the enzyme was frequently as high or even higher than the fluoride-stimulated activity in the presence of Mg^{2+} (see Fig. 2). Under the same conditions, Co^{2+} and Fe^{2+} were at least as active as Mg^{2+}. At concentrations of 10 mM the effectiveness of Co^{2+} decreased whereas the effects of Mg^{2+} and Fe^{2+} increased to the same extent (Fig. 3). Zn^{2+}, Ni^{2+}, Cu^{2+} and Ca^{2+} were inactive or even inhibitory. In order to protect the enzyme from inhibition by Ca^{2+} the incubation mixtures were always supplied with 0.1 mM EGTA.

Like other adenylate cyclases the epidermal enzyme is strongly stimulated by fluoride (Figs 2 and 4) in concentrations above 5 mM. The effect began to level off at 20 mM NaF. This plateau, however, could be shifted to higher concentrations (beyond 100 mM) if the Mn^{2+} concentration was increased to 10 mM. Thus a maximal

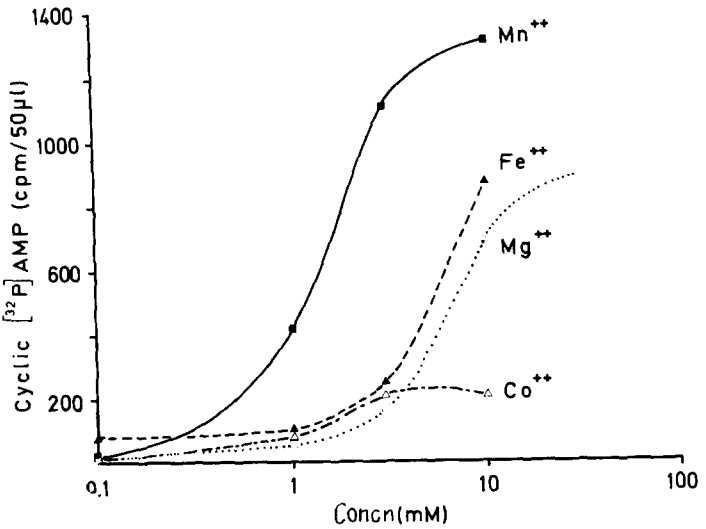

Fig. 3. Formation of cyclic [^{32}P]AMP in epidermal homogenates of female NMRI-mice as function of the concentration of divalent metal ions. Except for divalent metal ions the composition of the incubation mixtures as well as the conditions are the same as those described for Fig. 1. Ordinate: Radioactivity measured per 50 μl of incubation mixture.

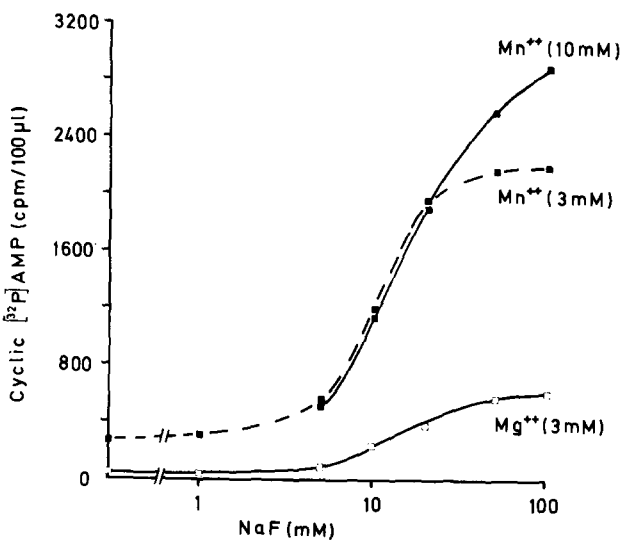

Fig. 4. Effect of NaF upon the formation of cyclic [^{32}P]AMP in epidermal homogenates of female NMRI-mice obtained by *Method I* (see Materials and Methods). The conditions are those described for Fig. 1. The incubations were carried out in the presence of either Mn^{2+} (■) or Mg^{2+} (□).

activation of the epidermal adenylate cyclase was achieved in presence of 10 mM Mn^{2+} and 100 mM NaF. Under these conditions the yield of cyclic AMP was ten to twelve times higher than under standard conditions (3 mM Mg^{2+}, 10 mM NaF).

After centrifugation of the epidermal homogenate at 15 000 × *g* for 20 min some 80–90% of the enzyme activity was found in the sediment indicating that the adenylate cyclase as in other tissues is bound to the particulate fraction of the cell.

Effect of hormones and other potential effectors of adenylate cyclase

The following results were obtained predominantly with epidermal homogenates of hairless mice prepared according to *Method II* (see Materials and Methods) and with 3 mM Mn^{2+} as essential cation, since with homogenates prepared by *Method I* the results were not always reproducible.

As can be seen from Fig. 5 adrenalin and isoproterenol but not noradrenalin exerted a not very prominent but highly reproducible stimulatory effect on epidermal adenylate cyclase when added in concentrations between 0.01 and 0.5 mM. At higher concentrations the enzyme activity decreased rapidly, falling frequently below the control level. Since this inactivation was not observed when the incubation was carried out in the presence of 1 mM ascorbate, it was most probably caused by toxic oxidation products of the catecholamines. Ascorbate did not influence the effect of lower doses of hormone. No quantitative difference between the effects of adrenalin and of isoproterenol could be detected. Both catecholamines constantly stimulated the formation of cyclic AMP over a whole incubation period of at least 30 min. Finally, the relative activation by the hormone was the same whether Mg^{2+} or Mn^{2+} was used as the essential metal ion.

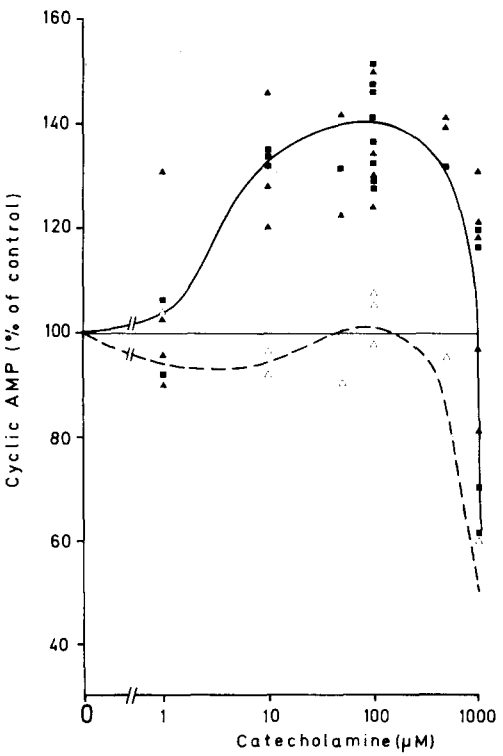

Fig. 5. Effect of adrenalin (▲), isoproterenol (■) and noradrenaline (△) upon the formation of cyclic [^{32}P]AMP in epidermal homogenates of hairless mice obtained by *Method II* (see Materials and Methods). The reaction mixture (0.1 ml) contained: 50 μl homogenate (equivalent to 1.5–2.5 μg DNA), 62 mM Tris·HCl, pH 7.5, 3 mM $MnCl_2$, 0.1 mM EGTA, 3 mM cyclic AMP, 1.5 mM [α-^{32}P]ATP (1–2 μCi), 15 mM phosphoenolpyruvate (potassium salt), 40 μg pyruvate kinase, 100 μg adenylate kinase. The mixtures were incubated at 37 °C for 30 min. Ordinate: Percent of the control (without catecholamine, =100%). Abcissa: Concentration of catecholamine.

After preincubation of the epidermal homogenate with 1 mM propranolol (0 °C, 15 min), a β-adrenergic blocker, the effect of adrenalin or isoproterenol was completely abolished. No such inhibition was observed after preincubation with 1 mM phentolamine, an α-adrenergic blocker (Fig. 6). This result indicates that the catecholamine receptor of the epidermal adenylate cyclase is of the β-adrenergic type.

The small effects exerted by the catecholamines *in vitro* are in sharp contrast to the strong β-adrenergic elevation of the level of intraepidermal cyclic AMP observed *in vivo*[46]. This leads to the conclusion that the hormone receptor system of the epidermal adenylate cyclase is partially destroyed under *in vitro* conditions. Since no difference was found regarding the responsiveness of the enzyme to adrenalin after mild homogenization with a loosely fitting Dounce-homogenizer as compared to strong homogenization with a tightly fitting Potter homogenizer, the hormone receptor does not seem to be destroyed by mechanical treatment alone. Preincubation of the "Dounce-homogenate" for 30 min at 37 °C and pH 7.5, however, not only caused a 40–50% decrease in the basal activity but completely abolished the hormone dependent activation. Thus, the inactivation of the β-receptor seems to be an autolytic effect due to the liberation of degrading enzymes during the maceration and homogenization of the epidermis.

Apart from catecholamines, several other potential effectors of adenylate cyclase were tested. Under the conditions used here no significant stimulatory or inhibitory effect could be observed with glucagon (1–10 μM), serotonin (0.1 mM), histamine (0.1 mM), epidermal growth factor (0.3 mg/ml), prostaglandin E_1 (1–50 μM), prostaglandin E_2 (1–50 μM) or porcine growth hormone (0.1–1 mg/ml) tested alone or with adrenalin (0.1 mM).

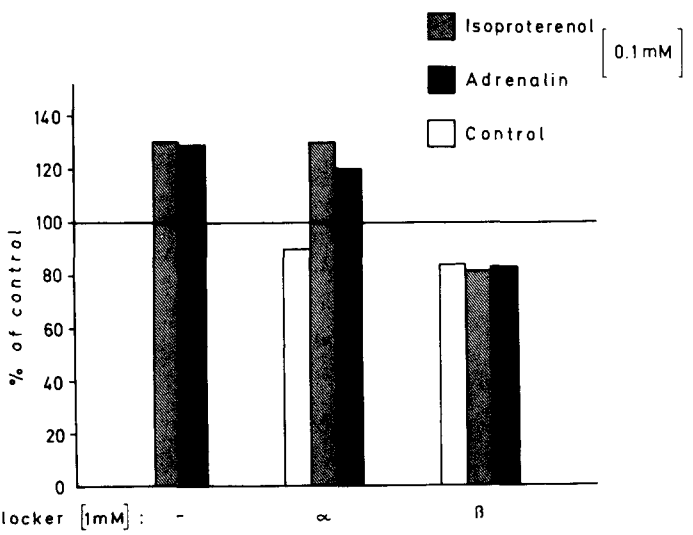

Fig. 6. Effect of adrenergic blockers (1 mM) upon the stimulation of epidermal adenylate cyclase by adrenalin (0.1 mM; black columns) and isoproterenol (0.1 mM; hatched columns). The homogenates were preincubated with either phentolamine (α-blocker) or propranolol (β-blocker) at 0 °C for 15 min. The empty columns represent the basal activity (without catecholamine). The conditions are those described for Fig. 5. Ordinate: Percent of the control (without catecholamine or blocker, = 100%).

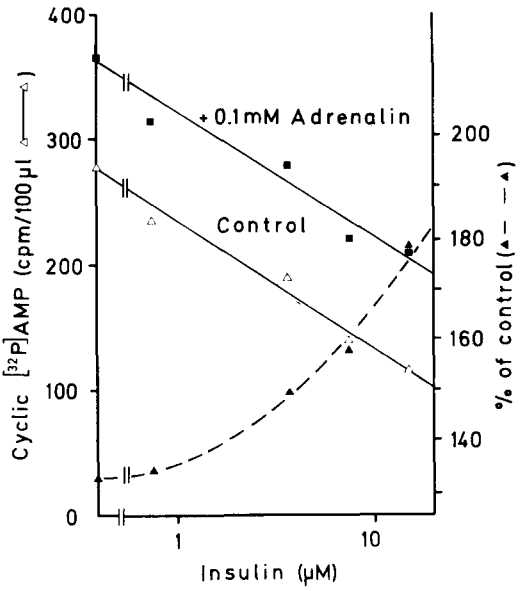

Fig. 7. Inhibition by insulin of the basal (△) and the adrenalin-stimulated (■) formation of cyclic [^{32}P]AMP in epidermal homogenates of hairless mice. The conditions are those described for Fig. 5. ▲—▲ represents the degree of the adrenalin-dependent stimulation as a percent of the control (without adrenalin, right ordinate) as a function of the insulin concentration.

Insulin, however, exerted a dose-dependent inhibition of cyclic AMP formation both in the absence and in the presence of 0.1 mM adrenalin (Fig. 7). The activation by adrenalin expressed as a percentage of the control (without adrenalin) increased linearly with rising concentrations of insulin. This indicates that the basal reaction, but not the catecholamine stimulated activity, was inhibited by insulin.

Pig-skin extracts containing epidermal growth inhibitors (chalone) strongly decreased the yield of cyclic AMP in experiments with and without adrenaline. Since this inhibition was observed with fractions obtained by gel chromatography and having no chalone activity[6], as well as with highly enriched G_1- and G_2-inhibitors[5,6], it remains doubtful that it is really a result of chalone activity.

DISCUSSION

The mitotic and functional homoeostasis of the epidermis is thought to be controlled by a tissue-specific regulator, called "chalone"[3]. Epidermal chalone has been shown to inhibit epidermal mitosis in the G_2-phase[3]; recently another specific inhibitor for the G_1-phase has been isolated from pig-skin[6]. *In vitro*, the inhibitory effect on the G_2-phase is augmented by adrenalin[3]; similar observations have been made with several other tissues[12–19]. Thus it has been postulated that each tissue contains a specific G_2-inhibitor and that adrenaline plays the role of an essential "cofactor" for such regulatory substances[3]. Since adrenalin generally acts *via* the cyclic AMP system, it has been further proposed that the hypothetic "adrenalin–chalone com-

plex" causes a specific stimulation of adenylate cyclase in its target tissue[21]. In this hypothesis cyclic AMP is thought to be the second messenger of tissue-specific signals regulating cell proliferation.

Indeed, it has been recently shown that under *in vitro* conditions cyclic AMP[22], as well as its dibutyryl derivative[23,47], causes a block in the G_2-phase in the epidermis of adult mice[22] and newborn rats[23,47], leading to potent inhibition of mitosis in this tissue. Similar results were described for other normal and malignant tissues as well as for cell cultures[24–32]. Consequently, it was attempted to detect a catecholamine-dependent stimulation of the epidermal adenylate cyclase. Recently Brønstad *et al.*[33] succeeded in demonstrating a strong increase in the formation of cyclic AMP from labelled precursors after incubating pieces of hamster skin with adrenalin. Similar results were obtained by Voorhees *et al.*[23,34] using epidermal explants of newborn rats and isoproterenol; in these experiments adrenalin exhibited only a very small stimulatory effect. Finally, the level of cyclic AMP in mouse epidermis has been measured under *in vivo* conditions; it has been found to be elevated 4- to 12-fold following intraperitoneal injection of adrenalin or isoproterenol[46]. As was observed in *in vitro* experiments, this increase was prevented by β-adrenergic but not by α-adrenergic blockers.

With epidermal homogenates, however, the catecholamine-dependent activation of the adenylate cyclase turned out to be much smaller[35] or even absent[36]. As indicated by the results presented in this paper, this is most probably due to some kind of autolytic inactivation of the adrenalin receptor during the preparation of the epidermal homogenate. Generally speaking the catecholamine receptor seems to be rather susceptible to damage, thus the stimulatory effects of hormones in homogenates are in most cases much smaller than *in vivo* or in the intact tissue[37].

Nevertheless there is now accumulating evidence that the adenylate cyclase of the epidermis is coupled to a β-adrenergic receptor. It may, therefore, be proposed with some degree of confidence that the inhibitory effect of catecholamines on the mitotic activity of epidermis is mediated by cyclic AMP. The role played by the epidermal "G_2-chalone" in this system is still not clear. Even with the most highly purified preparations of chalone available at this moment, no stimulatory effect on the formation of epidermal cyclic AMP could be observed *in vivo* or *in vitro*.

Among the other possible biological effectors of adenylate cyclase tested in our experiments, only relatively high concentrations of insulin exhibited a distinct effect on the enzyme. Since this hormone seems to inhibit the basal activity but not the adrenalin-elevated reaction it may be speculated that epidermal homogenates contain two different enzymes, one of which is connected to a β-adrenergic receptor and not influenced by insulin, whereas the other has no catecholamine-receptor but is inhibited by insulin. A selective inhibition of the adrenalin-stimulated formation of cyclic AMP by insulin or prostaglandin E_2 as described recently for human epidermis[38] could not be detected. Although it is well known that insulin counteracts the accumulation of cyclic AMP induced by glucagon or catecholamines in liver[39] and adipose tissue[40,41], the interpretation of these effects is still controversial. It is not clear whether the hormone acts upon adenylate cyclase[42,43] directly, or upon phosphodiesterase[44,45], or both. In the case of mouse epidermis we favour the idea of an insulin effect on adenylate cyclase. In order to be able to say whether the effect is a pure *in vitro* effect or whether it has some physiological significance, further experimental investigations must be carried out.

The general properties of the adenylate cyclase of mouse epidermis as described in this paper for the first time are similar not only to those reported for the enzyme in guinea-pig skin[36] and rat epidermis[23,35], but also to those described for other tissues. Although in many tissues Mn^{2+} are more potent activators than Mg^{2+}, the marked preference of the epidermal enzyme for Mn^{2+} seems, however, to be rather unique.

Under the conditions normally used in adenylate cyclase assays (Mg^{2+}:ATP = 2:1; 10 mM NaF *versus* 3 mM Mg^{2+}), the activity of the epidermal enzyme was far from maximal. It could be increased considerably, either by raising the metal ion concentration (and using Mn^{2+} instead of Mg^{2+}), or by increasing the amount of F^- in the assay mixture. An inhibitory effect of high concentrations of Mn^{2+}, Mg^{2+} and F^-, as is sometimes observed in other tissues, could not be detected.

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