INDUCTION OF CATECHOLAMINE REFRACTORINESS BY ISOPROTERENOL VIA A CYCLOHEXIMIDE-SENSITIVE REACTION IN MOUSE EPIDERMIS IN VIVO

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1. Introduction

The response of cells and tissues to hormones and other regulatory molecules is regulated not only by the availability of the ligand but also by a modulation of the tissue's responsiveness.

In most cases, including the β-adrenergic stimulation of skin [1], temporary subsensitivity (tachyphylaxis) is the response to an excessive stimulation which otherwise would lead to regulatory paralysis [2]. It appears that such an adaptive reaction can be due to several different mechanisms [2] such as a conformational change of the receptor protein, so that it cannot transfer the hormonal signal; 'internalization' of membrane-bound hormone-receptor complexes and subsequent lysosomal degradation in the course of an endocytotic process (down regulation); or an activation of enzymes which destroy the hormone itself or its second messenger, for example cyclic AMP phosphodiesterase. In addition, some evidence exists that β-adrenergic stimulation may give rise to the de-novo synthesis of a short-lived 'refractoriness protein' in the target cell which somehow decreases hormone responsiveness [3]. The latter process seems to be especially interesting since it indicates the existence of a specific and sophisticated regulatory mechanism. As yet, however, it has been demonstrated only with a special tumor cell line in vitro so that its physiological role might be questionable. Here I show that a similar or even identical mechanism exists in a normal tissue in the living animal, i.e. in mouse epidermis.

2. Materials and methods

D,L-Isoproterenol (IPR, isoprenaline) was obtained from Serva (Heidelberg) and 1-methyl-3-isobutyl-xanthine (MIX) from Aldrich Ega-Chemie. Labelled compounds were purchased from Amersham-Buchler (Braunschweig).

Female NMRI-mice (age 7–8 weeks) whose backs had been shaved 4 days earlier [8] were used in all experiments.

For determination of cyclic AMP in epidermis the animals were killed by cervical dislocation and the skin of the back was immediately snap-frozen at −80°C. Then the epidermis was scraped off by means of a pre-cooled scalpel and immediately homogenized in 1.5 ml 3% trichloroacetic acid. Cyclic AMP was isolated from the supernatant as described [4,8] and assayed by means of a commercially available assay kit (Amersham-Buchler, Braunschweig). For determination of DNA see [8]. Under the conditions described no artifactual increase of epidermal cyclic AMP (for example due to ischaemia) was observed.

For determination of protein labelling in epidermis the animals received an i.p. injection of 100 μCi [^{3}H]-amino acid mixture 1 h before they were killed. The epidermis was scraped off as described above and homogenized in 2 ml 0.5 M perchloric acid (PCA). After centrifugation the sediment was washed three times with cold 0.2 M PCA (% 4 ml), suspended in 2 ml 0.5 PCA and heated in a boiling water bath for 10 min. After centrifugation the sediment was washed twice with 2 ml 0.5 M PCA, once with 4 ml ethanol and dried. The residue was suspended in 2.5 ml 0.5 M NaOH, heated at 80°C for 30 min and centrifuged. Then the clear supernatant was used for determination of radioactivity and protein assay according to the Lowry procedure.

The activity of epidermal adenylate cyclase was determined in epidermis homogenate in the presence

of 3 mM $MnCl_2$ [10] using the pooled homogenates of 2 mice (in 2 ml buffer) and [α-^{32}P]ATP as a substrate [10]. The activity of cyclic AMP phosphodiesterase was assayed [9] using the pooled homogenates of 2 mice (in 4 ml buffer) and 20 μM [^{3}H]cyclic AMP as a substrate (high affinity conditions [9]).

3. Results

A single i.p. injection of IPR caused a 10- to 20-fold increase of the level of cyclic AMP in dorsal mouse epidermis within 2.5 min (see also [4]). Between 7 and 10 min the cyclic AMP content began to decline almost reaching control levels after 20 min (fig.1). A maximal response was achieved with 0.25 mg IPR (40 μmol/kg) and no further increase of cyclic AMP production was observed when the IPR-dose was raised to 1 mg (fig.1). Using tritium-labelled IPR the elevation of cyclic AMP content was found not to coincide with the distribution of radioactivity in epidermis in that the former was already terminated before the latter had reached its maximal value. Under the assumption that the radioactivity measured was mainly due to isoproterenol instead of to breakdown products, this observation may be taken as an indication that a catecholamine refractoriness of epidermal cyclic AMP production had developed shortly after treatment.

Indeed, when a second injection of IPR was given

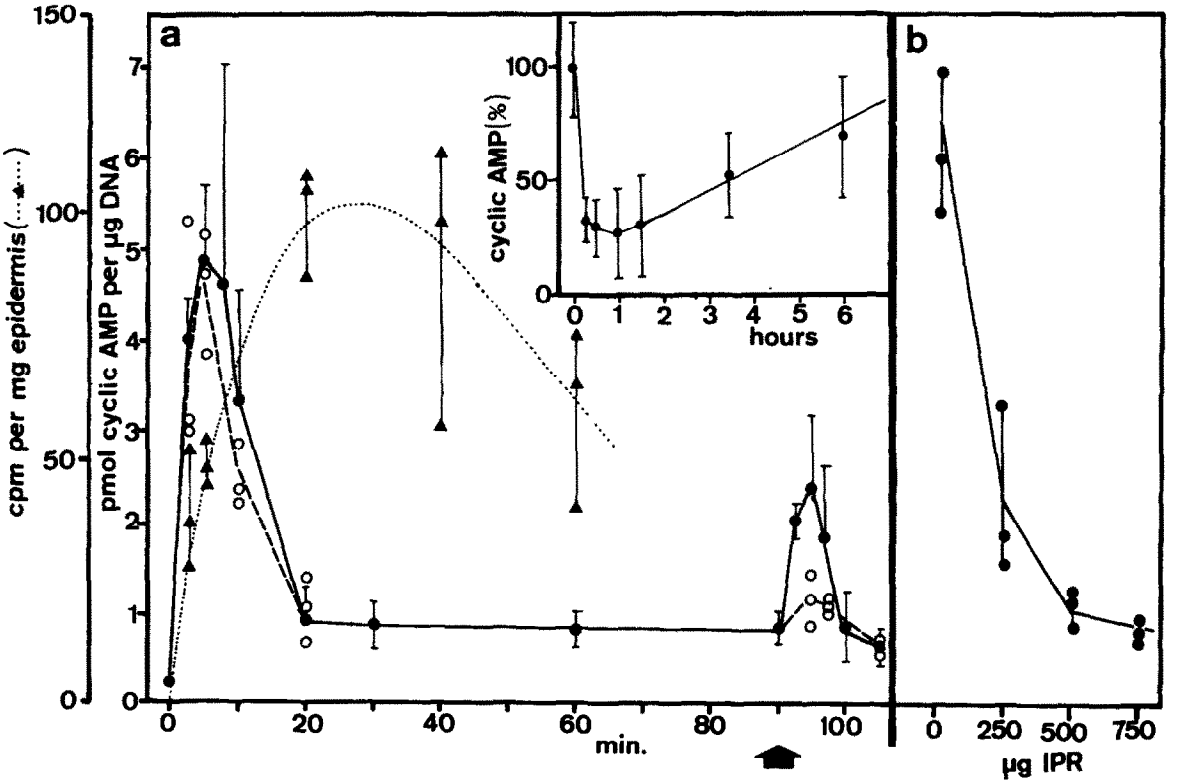

Fig.1. Effect of D,L-isoproterenol (IPR) on cyclic AMP accumulation in dorsal mouse epidermis in vivo. (a) Time course of cyclic AMP accumulation after i.p. injection of either 0.25 mg (black circles, solid line; each point represents the mean of 6 or more experiments (animals) ±S.D.) or 1.00 mg IPR (open circles, broken line; each point represents an experiment with one animal). As indicated by the arrow, a second injection of 0.25 mg IPR was given 90 min after the first injection. The dotted line (black triangles) shows the distribution of radioactivity in epidermis after i.p. injection of 0.1 mg [7-^{3}H]IPR (0.2 μCi). For determination of radioactivity the epidermis was dissolved in 1 ml soluene (60 min, 60°C). Each point represents one animal. (b) Accumulation of cyclic AMP, measured 7 min after a second injection of 0.25 mg IPR at 90 min, as a function of the initial IPR dose (injected at zero time). The insert shows the effect of a second injection of 0.25 mg IPR on cyclic AMP production, measured after 7 min as a function of the time interval between first and second injection (0.25 mg IPR given at zero time; $n \geq 4$, ±S.D.).

90 min after the initial treatment the accumulation of cyclic AMP was found to be greatly diminished (fig.1). The degree of inhibition depended on the initial IPR dose rather than on the amount of cyclic AMP initially generated. Refractoriness could already be observed 20 min after the first injection and lasted for several hours (fig.1).

The accumulation of cyclic AMP in epidermis could be considerably prolonged when cycloheximide was i.p. injected 15 min prior to isoproterenol (fig.2). When a rechallenge with IPR was made 90 min after the first treatment no refractoriness of cyclic AMP production could be observed in cycloheximide-treated animals (fig.2). Azacytidine, which has been shown to prevent in epidermis events due to RNA biosynthesis [5], did not show such an effect.

The accumulation of cyclic AMP in epidermis could also be prolonged when the animals were treated with MIX, a powerful inhibitor of cyclic AMP phosphodiesterase, prior to IPR injection (fig.2). Therefore, the refractoriness could perhaps be due to an increased activity of this enzyme in IPR-treated skin. This possibility was, however, ruled out because no increased phosphodiesterase activity was observed in

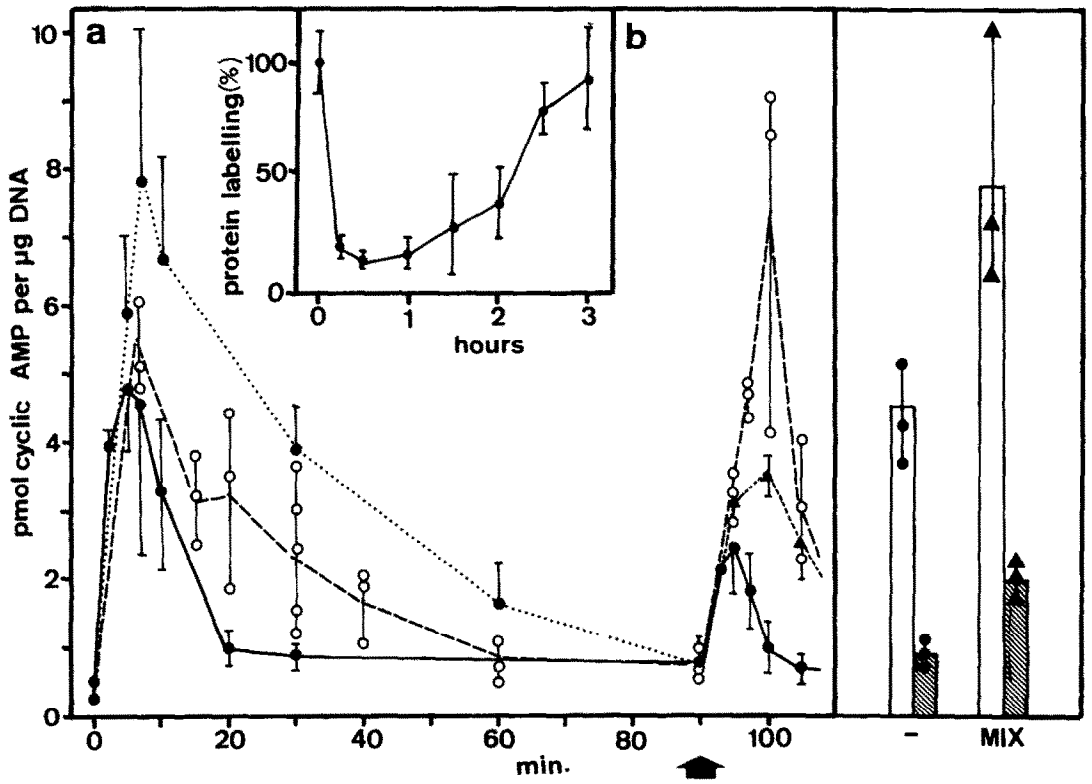

Fig.2. Effect of cycloheximide and 1-methyl-3-isobutyl xanthine (MIX) on IPR-induced accumulation of cyclic AMP in dorsal mouse epidermis in vivo. The animals were injected i.p. twice with 0.25 mg IPR each at zero time and 90 min later (arrow). (a) Black circles, solid line: control animals (see fig.1). Open circles, broken line: 5 mg cycloheximide were injected i.p. 15 min prior to the *first* IPR treatment. Black circles, dotted line: 1.5 mg MIX (dissolved in acetone-ethanol) was topically applied 40 min prior and 1 mg MIX injected i.p. 5 min prior to the *first* IPR treatment. Black triangles, dotted line, the animals were treated with MIX as described above prior to the *second* IPR injection. For further details see fig.1. (b) Effect of four injections of either NaCl-solution (controls: open columns) or 0.25 mg IPR each (hatched columns) given at 0, 30 and 60 and 90 min on the accumulation of cyclic AMP as measured 7 min after the fourth injection (circles: no pre-treatment; triangles: treatment with MIX as described above prior to the *fourth* IPR injection). Each point represents one animal. The insert shows the effect of cycloheximide on protein labeling in mouse epidermis in vivo. 0.9% NaCl solution (controls) or cycloheximide (5 mg) was injected at zero time and the animals were killed at the times indicated. 1 h prior to killing 100 µCi [^{3}H]amino acid mixture (Amersham-Buchler) was injected i.p. Each point represents the mean value of 5 or more experiments (animals) ±S.D. Ordinate, cpm per mg protein expressed in % of the control.

Table 1
Activities of adenylate cyclase (AC) and cyclic AMP-phosphodiesterase (PDE) in epidermal homogenates from control and IPR-treated mice

Enzyme	Assay conditions	IPR dose (mg)	Enzyme activity (cpm/min per mg protein)					
			Control			IPR-treated		
			Exp. I	II	III	Exp. I	II	III
AC	normal	1	510	904	532	598	899	502
AC	50 mM NaF	1	1897	2870	2756	2702	3462	2765
PDE	normal	0.25	1266 ± 133			1431 ± 202		
PDE	50 μM $CaCl_2$	0.25	1303 ± 235			1233 ± 167		
PDE	normal	1	1898 ± 267			1067 ± 159		
PDE	normal[a]	0.25	2065 ± 231			1704 ± 198		

[a] 5 mg cycloheximide were i.p. injected 15 min prior to IPR-injection

The animals were injected with either 0.9% NaCl solution (controls) or DL-isoproterenol 90 min prior to killing as described in fig.1. For phosphodiesterase mean values of 5 assays (±S.D.) are given

epidermal homogenates made from IPR-treated animals as compared with control mice, and pretreatment of the animals with cycloheximide did not show any effect on phosphodiesterase activity either in control or in IPR-treated mice (table 1). Furthermore, under conditions (50 μM Ca^{2+}) which have been shown to lead to a maximal activation of the calcium-dependent regulator protein (CDR) of PDE [6] no difference could be seen between IPR-treated and control animals thus ruling out the possibility that CDR rather than PDE was activated (table 1). Finally, refractoriness could not be overcome by an excessive dose of MIX administered prior to the last IPR injection. As shown in fig.2 an increase of cyclic AMP level of only 150–200% was observed, which occurs also in control animals. As shown in table 1 there was also no difference in basal or fluoride-stimulated adenylate cyclase activity in epidermal homogenates of IPR-treated animals as compared with control mice.

4. Discussion

The results show that a cycloheximide-sensitive desensitization of β-adrenergic cyclic AMP production which has been recently observed with C6-2B astrocytoma cells in vitro [3] can also occur in mouse epidermis in vivo. Therefore, this autoregulatory process may be considered to be physiologically significant.

To explain the cycloheximide-sensitivity a 'refractoriness protein' with a high turnover rate has been postulated. This protein was assumed to inhibit the interaction of the hormone-receptor complex with adenylate cyclase without reducing the number of receptor molecules [3]. Due to technical difficulties a reliable determination of the receptor content of mouse epidermis in vivo has not yet been achieved. Nevertheless, a similar interpretation might be applicable for the observations described here, especially since neither an activation of cyclic AMP phosphodiesterase nor an inactivation of adenylate cyclase was observed in subsensitive mouse epidermis and because it is well known that endocytotic down regulation of receptor sites does not depend on protein synthesis [2].

The time course of the events indicates that the synthesis of putative refractoriness protein starts shortly after catecholamine injection. Thus, by using this mechanism the cell is able not only to adapt to subsequent hormonal challenge but, in addition, to terminate – together with cyclic AMP-PDE – even the response to the initial stimulation. This seems to be especially remarkable since in most cases studied thus far a prolonged exposure to the hormone was necessary to achieve significant subsensitivity.

It is not clear whether or not cyclic AMP is involved in the synthesis of the inhibitor. After all, a clear-cut correlation between cyclic AMP level and subsequent desensitization was not observed, and comparing the temperature sensitivity of cyclic AMP production with that of refractoriness in C6-2B astrocytoma cells Nickols and Brooker [7] also came to the conclusion that cyclic AMP is not directly involved in the development of refractoriness.

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