PREVENTION OF PHORBOL ESTER-INDUCED CATECHOLAMINE REFRACTORINESS BY INHIBITORS OF PROTEIN AND RNA BIOSYNTHESIS IN MOUSE EPIDERMIS IN VIVO

Friedrich MARKS

Deutsches Krebsforschungszentrum (German Cancer Research Center), Institut für Biochemie, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

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1. Introduction

The synthesis of cyclic AMP in mouse epidermis can be stimulated via the β-adrenergic route, for example by means of isoproterenol (IPR) [1,2]. This response has been found to be greatly diminished 1–2 h after a single application of the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and a correlation between this desensitization and tumor-promoting potency has been proposed [3,4]. Although it has been speculated [3] that the β-adrenergic receptor itself is inactivated, the molecular mechanism of this effect remains obscure. There are indeed indications that after TPA-treatment no decrease of binding-sites for the β-adrenergic antagonist [^{3}H]dihydroalprenolol occurs [5] but that TPA 'uncouples' the β-adrenergic receptor from adenylate cyclase [6]. Here I present evidence that the development of TPA-induced catecholamine refractoriness in mouse epidermis in vivo may be dependent on RNA and protein synthesis and, therefore, proceeds via a similar mechanism to catecholamine-induced desensitization of the β-adrenergic response [13].

2. Methods

Female NMRI mice (7–8 weeks) were kept and treated with TPA (local application in 0.1 ml acetone) or D,L-isoproterenol (intraperitoneal (i.p.) injection in 0.25 ml 0.9% NaCl solution) as in [3].

To determine the cyclic AMP level in dorsal epidermis, the animals were killed by cervical dislocation and after rapid dissection the skin was snap-frozen at −70°C. Under these conditions no increase of cyclic AMP due to ischemia was observed. From the deep-frozen tissue the epidermis was scraped off with a pre-cooled scalpel and immediately homogenized in 2.5 ml 5% trichloroacetic acid. After centrifugation the supernatant was extracted 5-times each with 2 ml water-saturated diethyl-ether and lyophilized. The dry residue was then dissolved in 2–5 ml 0.2 M Tris–HCl buffer, containing 4 mM EDTA, and the cyclic AMP assay was carried out with 50 μl aliquots using a commercially available assay kit based on a protein binding method (Amersham-Buchler, Braunschweig). The DNA content of the epidermal homogenate was determined by carrying out the Burton reaction with the sediment of the first centrifugation as in [3]. Adenylate cyclase activity in epidermal homogenates was measured according to [7].

3. Results

A single i.p. injection of isoproterenol caused a 10–20-fold increase of the level of cyclic AMP in dorsal mouse epidermis in <2.5 min. (fig.1). At 7–10 min the cyclic AMP content began to decline, reaching almost control levels after 20 min. The isoproterenol dose employed induced a maximal response and no further increase of cyclic AMP production was observed when the dose was raised to 1 mg/animal.

As shown [1,2], the IPR-induced cyclic AMP synthesis is mediated by a β-adrenergic receptor.

When the animals had been pretreated by local application of the tumor-promoting phorbol ester TPA (20 nmol) the β-adrenergic stimulation was found to be greatly diminished (fig.1b; [3,4]). The catecholamine refractoriness caused by TPA developed with a lag phase of ~1 h (fig.1b). It could not be overcome

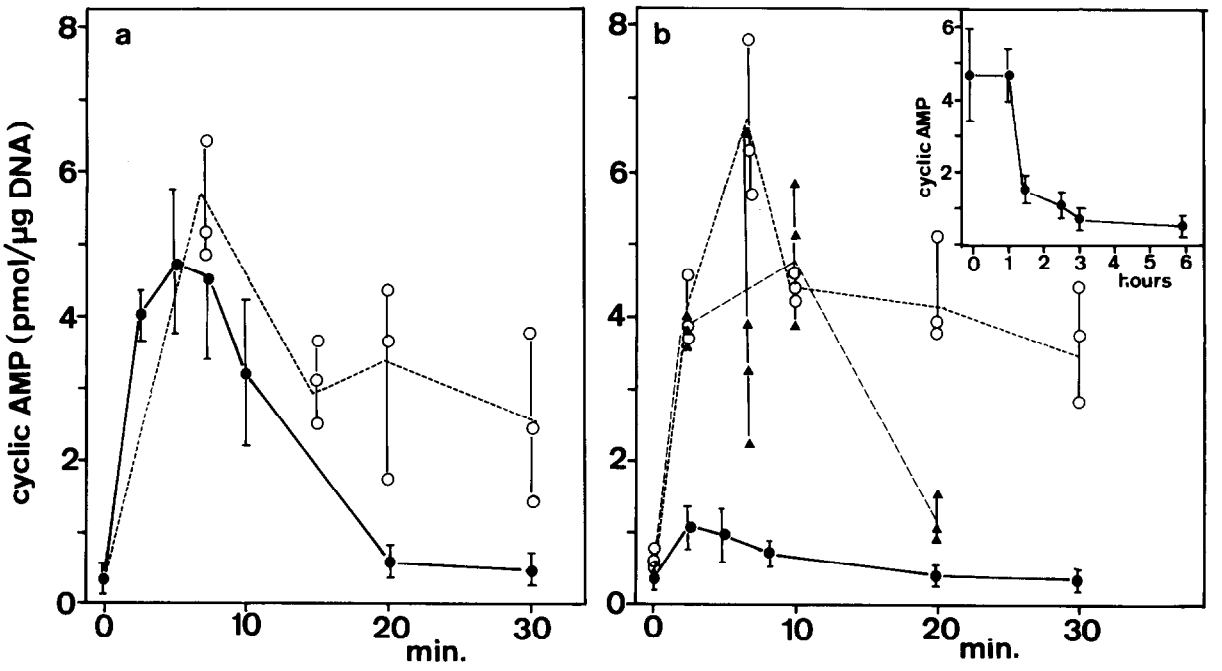

Fig.1. Effect of TPA on isoproterenol-induced cyclic AMP accumulation in dorsal mouse epidermis in vivo. D,L-isoproterenol (IPR, 0.25 mg/animal) was i.p. injected at zero time and the animals were killed at the times indicated. 3 h prior to IPR injection either 0.1 ml acetone (controls, (a)) or 20 nmol TPA in 0.1 ml acetone (b) were topically applied on to the shaved back skin.

(a) The time course of cyclic AMP accumulation in acetone-treated animals after injection of IPR alone (——; $n \geqq 6$, SD) or when 5 mg cycloheximide was i.p. injected 15 min prior to IPR injection (– – –; each point represents an experiment with 1 animal).

(b) The time course of cyclic AMP accumulation in TPA-treated animals. (●——●) Injection of IPR alone ($n \geqslant 4$, SD). (○ · · ○) Two i.p. injections of cycloheximide were given 1 h (5 mg/animal) and 2 h (2.5 mg/animal) after TPA application. (▲– –▲) 2.5 mg Azacyctidine was i.p. injected 20 min prior to TPA application. Each ○ or ▲ represents an experiment with 1 animal.

Insert: The level of cyclic AMP (pmol/µg DNA) in dorsal mouse epidermis as measured 7 min after an injection of 0.25 mg IPR given at different time intervals after local application of 20 nmol TPA at zero time ($n = 6$, SD).

Table 1
Activity of adenylate cyclase in epidermal homogenates from control and TPA-treated mice

Assay conditions	Formation of 5'-AMP (cpm . min^{-1} . mg $protein^{-1}$)					
	Control			TPA-treated		
	I	II	III	I	II	III
Normal	361	310	440	393	330	352
50 mM NaF	1320	1470	1560	1432	1660	1810

The animals were topically treated with either 0.1 ml acetone (controls) or a solution of 20 nmol TPA in 0.1 ml acetone 3 h prior to killing as in fig.1. Enzyme activity was measured according to [7].

by 1-methyl-3-isobutylxanthine (MIX), a powerful inhibitor of epidermal cyclic AMP phosphodiesterase ($K_i \sim 25$ μM). When fig.2 is compared with fig.1 it can be seen that MIX-treatment did not alter the relationship between cyclic AMP accumulation in control and TPA-treated animals. Therefore, the refractoriness cannot be due to enhanced cyclic AMP-phosphodiesterase activity. Desensitization could be entirely prevented when the animals had been treated with cycloheximide (fig.1) in a dose which strongly suppressed epidermal protein biosynthesis in vivo [12]. Best results were obtained when the inhibitor was applied twice 1 h and 2 h after TPA treatment. But also a single dose given either simultaneously or 1 h after TPA application could largely overcome the desensitization of the β-adrenergic response whereas

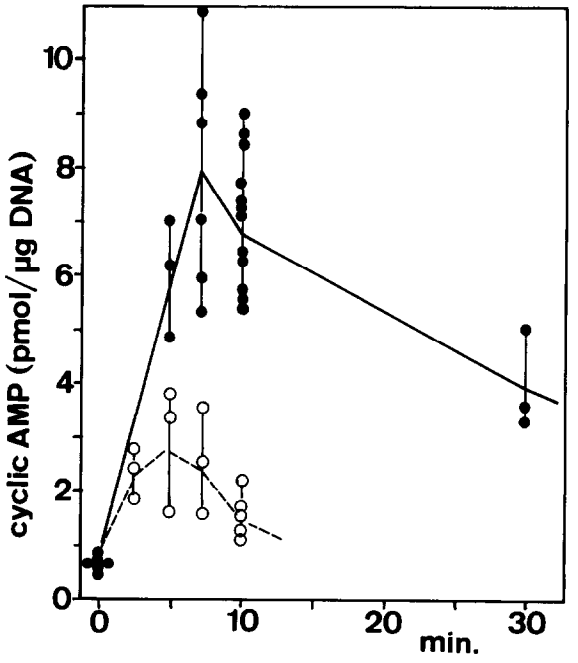

Fig.2. Cyclic AMP accumulation in dorsal mouse epidermis in vivo after combined injection of 1-methyl-3-isobutyl-xanthine (MIX) and D,L-isoproterenol (IPR): effect of pretreatment with acetone (——) or TPA (– – –). The animals which had been pretreated with either acetone or 20 nmol TPA 3 h before (see fig.1) received an i.p. injection of IPR (0.25 mg/mouse) at zero time and were killed at the times indicated. 40 min prior to killing 1.5 mg MIX/0.1 ml acetone was locally applied on to the shaved back skin and 20 min prior to sacrifice an i.p. injection of 1 mg MIX/0.3 ml isotonic NaCl, solution was made. Each point represents an experiment with 1 animal.

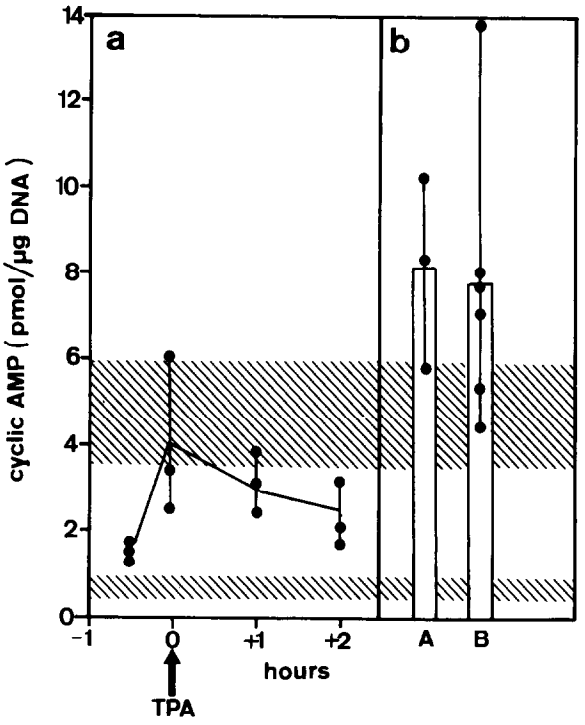

Fig.3. Effect of cycloheximide on IPR-induced cyclic AMP accumulation in dorsal mouse epidermis in vivo after pretreatment with TPA. TPA (20 nmol) was topically applied 3 h prior to IPR injection.

(a) The relationship between cyclic AMP accumulation (as measured 7 min after injection of 0.25 mg IPR) and scheme of cycloheximide injection (5 mg/animal i.p.; –, application prior to TPA, +, application after TPA).

(b) The accumulation of cyclic AMP (as measured 7 min after i.p. injection of 0.25 mg IPR) in animals which had received either 2 (A, 5 mg 1 h after TPA, 2.5 mg 2 h after TPA) or 3 (B, 5 mg each 5 min before and 1 h after TPA, 2.5 mg 2 h after TPA) injections of cycloheximide. Each point represents an experiment with one animal. The hatched zones represent the average standard deviations of the cyclic AMP level as measured 7 min after IPR-injection in either acetone-treated (upper zone, $n = 12$) or TPA-treated mice (lower zone, $n = 12$) which had received intraperitoneal injections of 0.3 ml 0.9% NaCl solution instead of cycloheximide.

injections given either 30 min before or 2 h after TPA were less effective (fig.3). TPA-induced catecholamine refractoriness could be also prevented by azacytidine (fig.1b) in a dose which is known to inhibit epidermal RNA synthesis in vivo [11].

As shown in table 1 the activity of adenylate cyclase as measured in epidermal homogenates was not altered after TPA treatment.

4. Discussion

These results indicate that the TPA-induced catecholamine refractoriness in mouse epidermis in vivo is due to a complex process which can be inhibited by cycloheximide or azacytidine and, therefore, probably involves the de novo synthesis of RNA and protein.

This assumption is consistent with the observed 1 h lag-period of the effect (fig.1b). Such a lag-phase is characteristic for many reactions depending on transcription and translation.

That desensitization is due to a stimulation of cyclic AMP phosphodiesterase or to an inhibition of adenylate cyclase can be ruled out. In addition, the number of β-adrenergic binding sites of the tissue is apparently not diminished [5]. I assume, therefore, that the refractoriness is due to the de-novo synthesis of a short-lived peptidic compound which might perhaps interfere with the receptorcyclase coupling. The existence of such a 'refractoriness protein' has been proposed to explain the cycloheximide-sensitive desensitization of the β-adrenergic response caused by pretreatment of astrocytoma cells in vitro [12] or of mouse epidermis in vivo [13] with isoproterenol. In contrast to TPA-induced desensitization, the agonist-induced refractoriness does not show a distinct lag-phase and can be inhibited by cycloheximide alone but not by azacytidine [13]. Catecholamine refractoriness caused by the phorbol ester TPA has also been observed in fibroblast cell cultures [14]. In contrast to earlier assumptions this reaction is thought to be an expression of growth stimulation rather than a true parameter of tumor-promoting potency [15,16].

References

[1] Marks, F. and Grimm, W. (1972) Nature New Biol. 240, 178–179.
[2] Voorhees, J. J., Duell, E. A., Stawiski, M. and Harrell, E. R. (1974) Adv. Cyclic Nucl. Res. 4, 117–162.
[3] Grimm, W. and Marks, F. (1974) Cancer Res. 34, 3128–3134.
[4] Mufson, R. A., Simsiman, R. C. and Boutwell, R. K. (1977) Cancer Res. 37, 665–669.
[5] Belman, S. and Garte, S. J. (1980) Cancer Res. 40, 240–244.
[6] Garte, S. J. and Belman, S. (1980) Nature 284, 171–173.
[7] Marks, F. and Rebien, W. (1972) Biochim. Biophys. Acta 284, 556–567.
[8] Verma, A. K., Froscio, M. and Murray, A. E. (1976) Cancer Res. 36, 81–87.
[9] Marks, F. and Grimm, W. (1978) Front. Matrix Biol. 6, 152–173.
[10] Mufson, R. A., Simsiman, R. C. and Boutwell, R. K. (1979) Cancer Res. 39, 2036–2040.
[11] O'Brien, T. G., Simsiman, R. C. and Boutwell, R. K. (1975) Cancer Res. 35, 1652–1670.
[12] Terasaki, W. L., Brooker, G., De Vellis, J., Inglish, D., Hsu, C. Y. and Moylan, R. D. (1978) Adv. Cyclic Nucl. Res. 9, 33–52.
[13] Marks, F. (1980) FEBS Lett. 113, 206–210.
[14] Rochette-Egly, C. and Castagna, M. (1979) FEBS Lett. 103, 38–42.
[15] Marks, F., Fürstenberger, G. and Kownatzki, E. (1980) Cancer Res. in press.
[16] Mufson, R. A., Fischer, S. M., Verma, A. K., Gleason, G. L., Slaga, T. J. and Boutwell, R. K. (1979) Cancer Res. 39, 4791–4795.