A TUMOR-PROMOTING PHORBOL ESTER INHIBITS THE CYCLIC AMP RESPONSE OF RAT EMBRYO FIBROBLASTS TO CATECHOLAMINES AND PROSTAGLANDIN E_1

C. ROCHETTE-EGLY and M. CASTAGNA*
Institut de Recherches Scientifiques sur le Cancer Boite Postale no. 8, 94 800 Villejuif, France

Received 25 April 1979

1. Introduction

A group of phorbol diesters has been identified as the active principles in tumor-promoting croton oil [1] among which 12-*O*-tetradecanoyl-phorbol-13-acetate was found to be the most abundant and one of the most active tumor promoters [2]. Tumor-promoting activity is classically tested by the ability to induce malignant epithelioma when applied repetitively to mouse skin pre-treated with a single subthreshold application of a carcinogen [3,4]. Phorbol esters elicit a variety of biological effects that have been observed in many different cell types, in vivo and in vitro. It is considered that TPA reversibly converts a normal phenotype to one that more closely resembles the transformed phenotype. Recent studies in cell culture systems have shown that TPA induces alterations which are currently associated with cell transformation, such as stimulation of glucose uptake [5], induction of plasminogen activator [6], decrease of surface LETS protein [7] or higher levels of saturation density [8].

An impaired hormonal responsiveness of adenylate cyclase has also been associated with transformation [9,10] and the metastatic potential of melanoma cells has been positively correlated with the loss of ability to respond to hormonal stimulation [11]. The adenylate cyclase system of epidermis after TPA applications to mouse skin has been examined [12] and TPA was shown to decrease cyclic AMP accumulation. It was suggested that TPA changed the adrenergic receptors of epidermal cells from β- to α-adrenergic [12].

This study investigated the effect of TPA on the cyclic AMP response of cultured rat embryo fibroblasts after hormonal stimulation. We report that TPA induced a refractory state of a heterologous type, involving response to catecholamines and PGE_1 in these cells. In contrast, results failed to support evidence for a TPA-mediated linkage of adenylate cyclase to α-adrenergic receptor.

2. Materials and methods

PGE_1 was obtained from Sigma (St Louis) and used as solution in 95% ethanol. DL-isoproterenol, L-epinephrine and L-phenylephrine were also purchased from Sigma and used as aqueous solutions in PBS. TPA and MePDD, kindly donated by Professor E. Hecker from Deutsches Krebsforschungszentrum (Heidelberg) were used as solutions in acetone.

The experiments were with secondary cultures of rat embryo cells obtained from day 14 embryos according to [13]. Cells were used 6 days after seeding, when they reached 2×10^5 cells/ml and became confluent. After the addition of PGE_1 or adrenergic agonists to the culture medium, the incubation was continued at 37°C for 5,10 or 30 min in air containing 5% CO_2. At appropriate times, the medium was removed by aspiration and the cells were washed once with ice-cold PBS within seconds. Then 2 ml

Abbreviations: cyclic AMP, cyclic adenosine 3',5'-monophosphate, PBS, phosphate-buffered saline; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; MePDD, 4-*O*-methyl-phorbol-12-13-didecanoate; PGE_1, prostaglandin E_1

* To whom correspondence should be addressed

Elsevier/North-Holland Biomedical Press

cold 6% trichloroacetic acid was added to the culture dishes. The tissue extract was neutralized, concentrated and assayed for cyclic AMP as in [14], whereas the acid-precipitable material dissolved in 0.6 N NaOH was used for protein determination by the Lowry method [15]. In parallel experiments, cells were preincubated for 1,3 or 6 h in the presence of either TPA, MePDD or acetone before the cyclic AMP response to hormones was measured.

3. Results

Figure 1 depicts the accumulation of cyclic AMP in confluent rat embryo cells in response to PGE_1 or catecholamines in relation to time. PGE_1 was the most effective in eliciting cyclic AMP accumulation. The cyclic AMP levels rose very rapidly, reached a value corresponding to 5-times the basal level in < 5 min and returned to the control values by 30 min. Rat embryo fibroblasts were also responsive to L-epinephrine and DL-isoproterenol but to a much lesser extent. In contrast, cells pre-treated with TPA at 100 ng/ml for 9 h were no longer responsive to PGE_1 and β-adrenergic agonists, whereas the basal cyclic AMP levels were not significantly affected.

Similarly, the effect of TPA on the response to phenylephrine was also tested in the rat embryo cell cultures. As shown table 1, TPA-treated cells did not exhibit an enhanced accumulation of cyclic AMP in response to the α-agonist under conditions where the response to β-adrenergic agonists was inhibited.

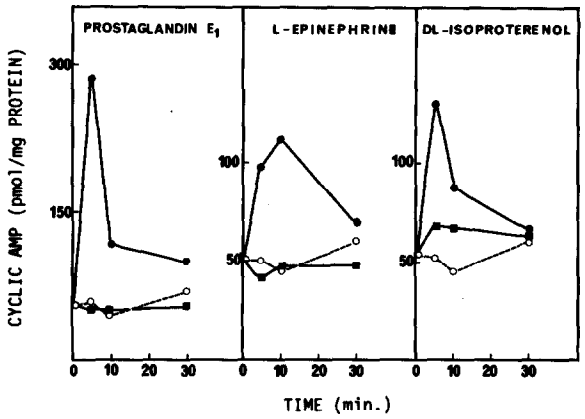

Fig.1. Cyclic AMP levels of rat embryo fibroblasts after various periods of exposure to PGE_1, L-epinephrine or DL-isoproterenol. Hormones were added at 10 μM either to cells pretreated with TPA at 100 ng/ml for 9 h (–■–) or to untreated cells (–●–). Unstimulated controls were run with PGE_1 or catecholamine solvents (–○–).

The degree of refractoriness depended on dose and duration of the treatment with the tumor promoter. Figure 2 shows the relative effects of both parameters on the cyclic AMP accumulation in response to PGE_1. TPA-mediated inhibitory effect was detectable at as low as 1 ng/ml for a 9 h pretreatment, whereas at 100 ng/ml a 3 h pretreatment was sufficient for eliciting the loss of responsiveness to PGE_1.

MePDD was similarly tested. After a 9 h pretreatment, the TPA derivative, which is devoid of tumor-

Table 1
Effect of TPA on the accumulation of cyclic AMP following exposure to phenylephrine in rat embryo fibroblasts

Treatment	Phenylephrine (μM)	Cyclic AMP (pmol/mg protein)			
		0 min	5 min	10 min	30 min
Controls	1	44.8 ± 1.6	39.2 ± 3.2	42.8 ± 1.1	31.1 ± 1.8
	10	45.3 ± 2.1	47.7 ± 1.0	42.2 ± 2.2	35.6 ± 0.8
Acetone-pre-	1	43.7 ± 0.7	43.3 ± 1.3	40.6 ± 1.9	39.9 ± 2.3
treated cells	10	39.7 ± 3.2	40.8 ± 1.7	39.9 ± 2.8	42.1 ± 1.5
TPA-pretrea-	1	45.4 ± 4.3	44.2 ± 0.5	42.8 ± 2.8	37.8 ± 2.5
ted cells	10	43.7 ± 2.2	45.4 ± 2.0	39.2 ± 3.7	40.7 ± 2.9

Cells were preincubated for 9 h in presence of TPA at 100 ng/ml prior to exposure to the α-agonist. Results represent mean values of 4 determinations ± SE

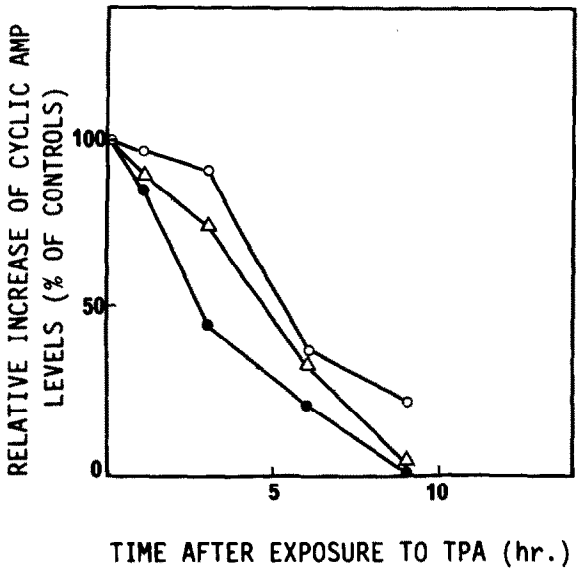

Fig.2. Cyclic AMP response to PGE_1 of rat embryo fibroblasts pretreated for various periods of time with graded doses of TPA: 1 ng/ml (–○–); 10 ng/ml (–△–); 100 ng/ml (–●–). The cyclic AMP levels were measured 5 min after the addition of 10 μM PGE_1. Control values represent the increase in the cyclic AMP content of untreated cells which were exposed for 5 min to 10 μM PGE_1.

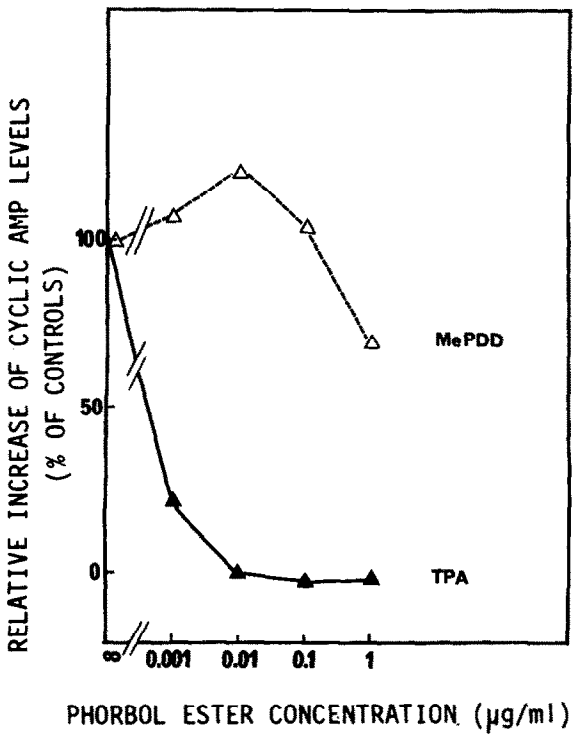

Fig.3. Responsiveness of rat embryo fibroblasts to PGE_1 following a 9 h pretreatment with either the tumor-promoting TPA (–▲–) or the inactive derivative MePDD (–△–). The intracellular cyclic AMP content was measured 5 min after exposure to 10 μM PGE_1.

promoting activity, was found to be $> 10^3$-fold less effective than TPA in inducing refractoriness to PGE_1 of rat embryo fibroblasts (fig.3).

It should be stressed that acetone, the phorbol ester solvent, did not affect, at the concentrations used ($\leq 0.1\%$), either basal or stimulated levels of cyclic AMP.

4. Discussion

We report here that TPA causes the refractoriness of the adenylate cyclase system to β-adrenergic agonists in cultured rat embryo fibroblasts. These results confirm the decrease in the responsiveness to β-adrenergic agonists observed [12] following topical applications of TPA on mouse epidermis. However, no evidence was found to confirm the suggestion, [12], that TPA caused the change from β- to α-adrenergic receptors since phenylephrine did not elevate intracellular cyclic AMP of either untreated or treated cells. A similar conclusion was drawn [16] as a result of a study on mouse skin.

This study also provides evidence for a heterologous type of refractoriness to hormones following TPA treatment since the tumor promoter also mediates a time-dependent loss of responsiveness of rat embryo fibroblasts to PGE_1. The TPA derivative MePDD, inactive as tumor promoter, was much less effective.

Although it was reported [17] that TPA caused the induction of low-affinity cyclic nucleotide phosphodiesterase activity in mouse epidermis, the possibility that an increased breakdown of cyclic AMP could account for the observed lack of responsiveness seems unlikely. Recently it has been indeed reported that injections of isobutyl-methylxanthine, a potent cyclic nucleotide phosphodiesterase inhibitor, did not overcome the TPA-mediated suppression of

hormonal response in mouse skin [18]. Similarly we failed to restore the normal response to hormones in TPA-treated fibroblasts receiving 1 mM aminophylline (unpublished results). Further, the possibility has been raised that the induction of cyclic nucleotide phosphodiesterase observed in mouse skin was unrelated to the induction of the refractory state since it seems to a result of the various treatments, such as skin depilation and stripping, preceding the determination of cyclic AMP content of mouse skin. Those treatments did not interfere significantly with the accumulation of cyclic AMP after β-adrenergic stimulation [18].

An alternative candidate for the site of TPA-mediated refractoriness would be the hormone-sensitive adenylate cyclase system.

Studies conducted in secondary cultures of rat embryo cells have shown that the tumor promoter stimulated cell proliferation and released confluent cells from quiescence [19]. As cells progressed into the pre-replicative phase of the mitotic cycle, the basal levels of cyclic AMP and adenylate cyclase activity rose from 2–6 h after the TPA treatment and returned to control values by 9 h, at the onset of DNA synthesis. Levels of cyclic AMP content and adenylate cyclase activity were similar to those of serum-stimulated cells. Further, the fluoride stimulation of adenylate cyclase activity was found unchanged in TPA-treated cells [20]. As a consequence, it can be assumed that the induced refractory state to hormones is not due to a temporary inactivation of adenylate cyclase.

The enzymatic portion of adenylate cyclase and hormone-receptor proteins are different entities [21]. The possibility that a TPA mediates the alteration of the latter components leading to the loss of receptor sites or/and the uncoupling from the adenylate cyclase should be explored. It is worth noting that TPA is an amphiphatic compound which alter phospholipid and cholesterol metabolism [22,23] as well as lipid microviscosity [23]. Several reports have provided demonstrations of the role of selective perturbations in the lipid environment of hormone-sensitive adenylate cyclase [24,25], thereby favoring the hypothesis that TPA may uncouple the system.

It is well substantiated that cells may regulate the levels of cyclic AMP through the process of cyclic AMP release, as reviewed in [26]. Although the basal levels of cyclic AMP are unaffected, the possibility that TPA may enhance the efflux of hormone-elevated intracellular cyclic AMP cannot be ruled out.

It may be worth stressing that the presented characteristics of the TPA-induced refractoriness are common to those of the agonist-induced loss of responsiveness to hormones. Both processes did not abolish basal and fluoride-stimulated activities, did not seem to involve cyclic nucleotide phosphodiesterase and required a lag phase before being expressed thereby suggesting that TPA interferes with the mechanism by which cells did not loose their responsiveness to their agonists.

Acknowledgements

The authors wish to express their thanks to Dr I. Chouroulinkov for supplying us with cell cultures.

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