ABSTRACT

Compared to 12-O-tetradecanoylphorbol-13-acetate (TPA), the phorbol ester 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate (4-O-methyl-TPA) is only a weak skin irritant and tumor promoter. When topically applied to mouse skin, 400 nmol 4-O-methyl-TPA induce hyperproliferation of epidermis in vivo to approximately the same extent as do 10 nmol TPA. However, in contrast to TPA, 4-O-methyl-TPA-induced proliferation is not followed by sustained epidermal hyperplasia. In addition, the methyl ether of TPA does not cause early prostaglandin accumulation, and its hyperproliferative effect is insensitive to indomethacin inhibition.

Contrary to the action of TPA, 4-O-methyl-TPA induces neither an early increase in the rate of phospholipid turnover nor an early increase in ornithine decarboxylase activity or polyamine accumulation in epidermis and does not desensitize epidermis to the effects of β-adrenergic agonists and epidermal G1 chalone. Finally, 4-O-methyl-TPA is only a weak stimulator of epidermal cyclic adenosine 3',5'-monophosphate phosphodiesterase activity. Consequently, it is concluded that the mechanism of action of 4-O-methyl-TPA as a skin mitogen is different from that of TPA. Whereas TPA evokes prostaglandin-dependent and chalone-insensitive hyperplastic development of epidermis (hyperplastic transformation), i.e., probably a relapse to the neonatal state, 4-O-methyl-TPA apparently stimulates nothing but normal everyday tissue regeneration. In this respect, treatment with 4-O-methyl-TPA resembles a non-damaging stimulus of epidermal cell proliferation (observed, for example, with skin massage), whereas TPA evokes a response which is characteristic for epidermal injury.

Considering these fundamental differences in biological action between TPA and its methyl ether, 4-O-methyl-TPA is concluded to be an unsuitable negative control compound for studies on the biological effects of phorbol ester tumor promoters.

INTRODUCTION

The investigation of the mechanism of action of phorbol ester tumor promoters has recently become a major issue of experimental cancer research. It has been found that these compounds exhibit a wide variety of "hormone-like" effects on different tissues and in vitro systems (3, 7, 15, 36). However, the causal relationship of most of these effects with the phenomenon of tumor promotion has not yet been convincingly established. There are 2 reasons for this discrepancy. (a) With only a few exceptions, most biological systems studied thus far are, at least at the moment, not suitable for accomplishing a clearcut 2-stage carcinogenesis experiment. (b) In mouse skin, the classical target tissue of tumor promotion, all tumor promoters have been found to be irritant mitogens, whereas not every irritant skin mitogen is a tumor promoter (24). This indicates that hyperplasigenic activity is probably a necessary but certainly not a sufficient property of a tumor promoter. The discovery of the 2-stage approach of skin tumor promotion (4, 11, 35) has now unequivocally shown that the promotion-specific effects of a phorbol ester tumor promoter such as TPA3 are different from its pleiotropic effects on cell proliferation. The mitogenic and irritant activities even apparently camouflage the promotion-specific actions. This situation makes it very difficult to decide whether any effect of a phorbol ester observed in a given biological system is related to promotion or merely reflects the pleiotropic efficiency of the compound as a skin mitogen. Even a positive correlation of an observed effect with tumor-promoting efficacy cannot be taken as a convincing proof that this effect is indicative for promotion, since skin mitogenic activity and promoting potency more or less run parallel within the series of phorbol esters.

Since there has been a rapid increase of data on the biological effects of phorbol esters (for reviews, see Refs. 3, 7, 15, and 36), the problem of identifying promotion-specific events and of distinguishing them clearly from those related to pleiotropism has become of outstanding importance for the interpretation of experimental results, the design of theoretical concepts, and the question as to whether a study may provide a better insight into a major problem of experimental cancer research or nothing but new data on basic problems of cell biology.

The use of an appropriate negative control compound could be of help in escaping this dilemma. Such a control compound must be a nonpromoting irritant skin mitogen, which should induce epidermal hyperproliferation essentially along the same pathways as a promoter. Nonmitogenic compounds such as phorbol or highly toxic irritants such as acetic acid [which kill the initiated cells before they can be promoted (31)] are, of course, quite useless as controls.

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2 To whom requests for reprints should be addressed.
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2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; 4-O-methyl-TPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate; RPA, 12-O-retinoylphorbol-13-acetate; cyclic AMP, cyclic adenosine 3',5'-monophosphate.
Recently, the 4-O-methyl ether of TPA, a weakly promoting skin mitogen (25), as well as the closely related compound 4-O-methylphorbol-12,13-didecanoate have been proposed to be the most appropriate negative control compounds (14) and have been used as such in many laboratories (see, e.g., Refs. 1, 5, 6, 8, 9, 13, 18, 19, 21, 29, 33, 34, 38, 39, and 41). Here we show that the proliferative response of mouse epidermis to 4-O-methyl-TPA is fundamentally different from that to TPA. Therefore, we suggest replacing this phorbol ester by a more suitable negative control compound, i.e., a nonpromoting hyperplasiogenic skin irritant, such as the retinoic acid derivative of TPA (11, 24).

**MATERIALS AND METHODS**

**Chemicals.** The phorbol esters TPA and 4-O-methyl-TPA were generously supplied by Professor E. Hecker, German Cancer Research Center, Heidelberg (Federal Republic of Germany). The synthesis and purification (by preparative high-pressure liquid chromatography) of...
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Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostaglandin E₂ (pg/μg DNA)</th>
<th>DNA labeling (cpm/μg DNA)</th>
<th>Mitotic activity (metaphase figures/visual field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (control)</td>
<td>7.3 ± 2.0 (34)</td>
<td>51 ± 13 (120)</td>
<td>0.21 ± 0.06 (16)</td>
</tr>
<tr>
<td>Acetone + indomethacin</td>
<td>6.9 ± 1.1 (6)</td>
<td>50 ± 16 (10)</td>
<td>0.22 ± 0.05 (16)</td>
</tr>
<tr>
<td>TPA + indomethacin</td>
<td>9.2 ± 2.7 (12)</td>
<td>49 ± 16 (10)</td>
<td>0.16 ± 0.05 (16)</td>
</tr>
<tr>
<td>TPA + prostaglandin E₂</td>
<td>ND (5)</td>
<td>380 ± 55 (10)</td>
<td>ND</td>
</tr>
<tr>
<td>4-O-Methyl-TPA</td>
<td>8.7 ± 3.6 (12)</td>
<td>155 ± 48 (10)</td>
<td>1.24 ± 0.38 (16)</td>
</tr>
<tr>
<td>4-O-Methyl-TPA + indomethacin</td>
<td>ND</td>
<td>144 ± 28 (10)</td>
<td>1.34 ± 0.42 (16)</td>
</tr>
<tr>
<td>4-O-Methyl-TPA + prostaglandin E₂</td>
<td>ND</td>
<td>148 ± 55 (10)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Mean ± S.E.

Details of handling and treatment have been described elsewhere (20).

**Determination of Epidermal Phosphatidylcholine.** For phospholipid analysis, the dissected skin was immediately frozen at −80°C on a cold table (27). Then the epidermis was scraped off and homogenized in 2 ml ice-cold 0.4 M HClO₄. The homogenates were allowed to stand in ice for 30 min and then centrifuged, and the pellet was washed three times with 2 ml cold 0.4 M HClO₄. The lipids were thoroughly extracted 3 times with 2 ml chloroform/methanol (2/1, v/v), each at room temperature. DNA was assayed in the remaining pellet (20). The lipid extract was washed according to the method of Folch et al. (10). The organic solvent was removed by evaporation, and the residue was redissolved in 0.1 ml chloroform/methanol (1/1, v/v) and subjected to chromatographic separation on silica gel using Merck ready-made thin-layer plates, which were developed in pre-equilibrated chambers containing chloroform/methanol/acetate acid/water (75/45/12/3, v/v). The bands were visualized by iodine vapor, and the phosphatidylcholine band was identified by comparison with authentic phosphatidylcholine run on the same plate. The phosphatidylcholine band was scraped off, and the scrapings were used for determination of radioactivity.

**Other Methods.** The methods for labelling epidermal DNA in vivo (20), determination of mitotic activity (2, 20), assay of ornithine decarboxylase (25) and cyclic AMP phosphodiesterase activities (26), and assay of prostaglandin E₂ (27) and cyclic AMP (23) have been described in detail elsewhere.

**RESULTS**

**Epidermal Hyperproliferation.** The methylation of the hydroxy group in position 4 of the TPA molecule results in an almost complete loss of the irritant activity and a considerable weakening of the mitogenic potency of the phorbol ester. As shown in Chart 1, 4-O-methyl-TPA had to be used in a dose of at least 40 times that of TPA in order to induce nearly the same proliferative response in the dorsal epidermis of NMRI mice. In contrast to TPA, the methyl ether did not cause an initial depression of DNA labeling.

One of the most striking differences observed was that, while TPA-induced epidermal hyperproliferation was followed by sustained epidermal hyperplasia, i.e., a considerable increase of the number of cells and a doubling of the number of cell layers in interfollicular epidermis, 4-O-methyl-TPA evoked almost no hyperplastic response even when applied in doses up to 800 nmol/animal (Chart 2; Fig. 1). 4-O-Methyl-TPA-induced Epidermal Hyperproliferation Is Not Prostaglandin-mediated. TPA-induced hyperproliferation of mouse epidermis in vivo has been shown to be dependent on a transient accumulation of prostaglandin E₂ immediately after treatment (24, 27). When prostaglandin synthesis was prevented by pretreatment of skin with the cyclooxygenase inhibitor indomethacin, no increase in the rate of cellular proliferation and no hyperplasia could be observed. In contrast, 4-O-methyl-TPA was unable to stimulate epidermal prostaglandin synthesis in vivo (Table 1) or the release of arachidonic acid from prelabeled phospholipids in primary epidermal cell cultures. Consequently, epidermal hyperproliferation induced by the 4-O-methyl ether of TPA was found to be insensitive to indomethacin inhibition (Table 1). This result was confirmed by experiments in which the phorbol ester and prostaglandin E₂ were applied simultaneously. Whereas the stimulatory effect of 4-O-methyl-TPA on DNA labeling remained unchanged, prostaglandin E₂ augmented the TPA-induced DNA synthesis (Table 1).

**Other Effects Possibly Related to Epidermal Hyperproliferation.** TPA and other skin mitogens induce in mouse epidermis ornithine decarboxylase activity within 2 to 6 hr after treatment (25, 32). This effect has been proposed to be related to epidermal cell proliferation and skin tumor promotion (32). No increase of enzyme activity and no accumulation of polyamines were seen after in vivo application of 4-O-methyl-TPA (25).

Another effect possibly linked to the induction of epidermal cell proliferation is an early increase of the rate of phospholipid turnover in vivo. Such an effect could already be clearly observed 1 to 2 hr after TPA treatment, whereas 4-O-methyl-TPA was unable to provoke such an early response. Thus, after at least 3 to 4 hr, the turnover of phosphatidylcholine in 4-O-methyl-TPA-treated epidermis was found to be only slightly increased (Table 2).

**4-O-Methyl-TPA Does Not Induce G₁, Chalone Refractoriness.** Hyperplasiogenic agents such as TPA have been shown to desensitize mouse epidermis to the inhibitory effect of epidermal G₁, chalone, a tissue-specific endogenous inhibitor of the G₁/S transition (2, 20). This effect has been proposed to be causally related to the development of the hyperplastic state. Epidermis retained its chalone sensitivity when DNA labeling was measured 18 hr after application of different doses of 4-
O-methyl-TPA (Chart 3) or between 16 and 24 hr after 400 nmol O-methyl-TPA (data not shown).

4-O-Methyl-TPA Does Not Exhibit Pronounced Effects on the Epidermal Cyclic AMP System. TPA has some effects on the cyclic AMP system in epidermis in vivo. Thus, it leads to a limited accumulation of cyclic AMP after 1 to 2 hr (13), to a considerable stimulation of cyclic AMP phosphodiesterase activity (26, 37) and to a pronounced and long-lasting desensitization of cyclic AMP formation to β-adrenergic stimulation (13, 30). In contrast, 4-O-methyl-TPA has almost no influence on the β-adrenergic effect (Chart 4) and induces only a rather moderate and short-lived increase of phosphodiesterase activity (Chart 5).

Tumor-promoting Efficacy of 4-O-Methyl-TPA. We have shown recently that a 2-stage carcinogenesis experiment performed with 4-O-methyl-TPA as a promoter (400 nmol/appli-

Table 2

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Acetone</th>
<th>TPA</th>
<th>4-O-Methyl-TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.8 ± 6.3*</td>
<td>23.9 ± 5.9</td>
<td>18.1 ± 4.9</td>
</tr>
<tr>
<td>2</td>
<td>16.9 ± 4.6</td>
<td>48.6 ± 12.8</td>
<td>16.4 ± 5.4</td>
</tr>
<tr>
<td>3</td>
<td>15.3 ± 5.7</td>
<td>43.8 ± 13.5</td>
<td>24.9 ± 5.6</td>
</tr>
<tr>
<td>4</td>
<td>17.1 ± 2.0</td>
<td>38.7 ± 6.1</td>
<td>28.2 ± 4.0</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 6 mice.

Chart 4. Effect of an i.p. injection of DL-isoproterenol on the accumulation of cyclic AMP in acetone (---), TPA (○○○○), or 4-O-methyl-TPA-treated (-----) mouse epidermis in vivo. Isoproterenol (1 μmol/0.25 ml 0.9% NaCl solution) was injected at 0 time, and the animals were killed at the times indicated. Phorbol esters (20 nmol TPA; 400 nmol 4-O-methyl-TPA) were topically applied 6 hr before isoproterenol injection. The 4-O-methyl-TPA curve shows individual points, whereas the other points are mean values of at least 6 experiments (animals). Bars, S.D. of the cyclic AMP level in the epidermis of control animals (no phorbol ester treatment and no isoproterenol, n = 20). Inset, cyclic AMP accumulation 7 min after isoproterenol injection in TPA (---) and 4-O-methyl-TPA (-----)-treated animals as a function of the time interval between phorbol ester application and isoproterenol injection (abscissa). To diminish a possible effect of cyclic AMP phosphodiesterase on cyclic AMP accumulation, these experiments were carried out with animals which had been pretreated with 1-methyl-3-isobutylxanthine (1.5 mg topically applied in 0.1 ml acetone 40 min and 1 mg/0.3 ml 0.9% NaCl solution injected i.p. 15 min prior to isoproterenol administration). Number of animals, ≥5. Lower bars, average S.D. of the cyclic AMP level in control animals; no isoproterenol, and received acetone instead of phorbol ester (n ≥ 20). Upper bars, average S.D. of the cyclic AMP level 7 min after i.p. injection of isoproterenol into 1-methyl-3-isobutylxanthine-treated animals which received acetone instead of phorbol esters (n ≥ 20).

DISCUSSION

In an appropriate dose, the almost nonpromoting phorbol ester 4-O-methyl-TPA induces hyperproliferation of mouse epidermis along a pathway which does not involve early prostaglandin synthesis and phospholipid turnover and development of G ß-chalone and catecholamine refractoriness. Induction of
ornithine decarboxylase activity is also not observed under in vivo conditions (25), whereas in epidermal cell cultures a stimulation of this enzyme by 4-O-methyl-TPA was found (40). An explanation for this discrepancy is not at hand. 4-O-Methyl-TPA-induced epidermal hyperproliferation in vivo is neither accompanied by skin inflammation nor followed by pronounced hyperplasia. Our results clearly distinguish the mechanism of action of 4-O-methyl-TPA from that of the tumor promoter TPA and lend additional support to the concept of "hyperplastic transformation" of mouse epidermis in vivo. The term was introduced by us in order to characterize a special kind of proliferative response of epidermis to exogenous stimulation. Furthermore, it has been shown that this transformation is triggered by a synergistic action of the stimulus together with endogenously generated prostaglandin E2 and involves the temporary breakdown of an endogenous control device, i.e., the G1 chalone mechanism, as well as the induction of ornithine decarboxylase, finally leading to a rather sudden development of hyperplasia (24). Hyperplastic transformation seems to be always accompanied by skin inflammation, and frequently a concurrent desensitization of epidermis to β-adrenergic stimulation is observed.

The whole process of hyperplastic transformation is certainly not due to an overshooting of epidermal cell proliferation but is the result of a specifically induced (by prostaglandin synthesis) fundamental change of the homeostatic equilibrium of the tissue. It may be interpreted as a transient relapse of the adult epidermis into a state of differentiation and proliferation which resembles neonatal epidermis (24). One reason to call this response "hyperplastic transformation" was the necessity to distinguish it explicitly from any stimulation of normal cell proliferation in mouse epidermis. The first evidence that such a differentiation has indeed to be made came from studies on mechanical stimulation of epidermal cell growth (2). It was shown that, whereas epidermal damage (by means of sandpaper rubbing) induced the whole sequence of events characteristic for the development of sustained hyperplasia (with the exception of catecholamine refractoriness), a nondamaging stimulus such as skin massage caused neither prostaglandin synthesis (12), induction of ornithine decarboxylase (25), catecholamine (28), and chalone refractoriness nor skin inflammation (2). Although the mitotic response seen after massage was almost indistinguishable from that after epidermal damage, damage led to epidermal hyperplasia whereas massage did not (2). It was concluded, therefore, that hyperplastic transformation is the characteristic response of adult mouse epidermis to

![Chart 5. Effect of TPA and 4-O-methyl-TPA on the activity of cyclic AMP phosphodiesterase in mouse epidermis in vivo. The phorbol esters (O-O, 10 nmol TPA; O-O, 20 nmol TPA; •-•, 400 nmol 4-O-methyl-TPA) were dissolved in 0.1 ml acetone each and topically applied. The animals were killed at the times indicated, and the enzyme activity was assayed in epidermis homogenate according to the procedure in Ref. 26. Points mean values of at least 9 experiments (animals). Bars, S.D. & average S.D. of the controls, which received acetone instead of phorbol ester (24 ±7 pmol 5'-AMP formed per mg protein per min; n = 34).](chart5.png)

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 12 wk</td>
</tr>
<tr>
<td></td>
<td>Rate</td>
</tr>
<tr>
<td><strong>A.</strong> TPA (4x; 10 nmol)</td>
<td>TPA (32x; 10 nmol)</td>
</tr>
<tr>
<td>TPA (4x; 10 nmol)</td>
<td>TPA (32x)</td>
</tr>
<tr>
<td>TPA (4x; 10 nmol)</td>
<td>4-O-Methyl-TPA (32x; 400 nmol)</td>
</tr>
<tr>
<td><strong>B.</strong> 4-O-Methyl-TPA (4x; 400 nmol)</td>
<td>4-O-Methyl-TPA (32x; 400 nmol)</td>
</tr>
<tr>
<td>4-O-Methyl-TPA (4x; 400 nmol)</td>
<td>Acetone (32x)</td>
</tr>
<tr>
<td>Acetone (4x)</td>
<td>4-O-Methyl-TPA (32x; 400 nmol)</td>
</tr>
<tr>
<td>4-O-Methyl-TPA (4x; 400 nmol)</td>
<td>RPA (32x; 10 nmol)</td>
</tr>
<tr>
<td><strong>C.</strong> TPA (4x; 10 nmol)</td>
<td>Acetone (32x)</td>
</tr>
<tr>
<td>Acetone (4x)</td>
<td>RPA (32x; 10 nmol)</td>
</tr>
<tr>
<td>TPA (4x; 10 nmol)</td>
<td>RPA (32x; 10 nmol)</td>
</tr>
</tbody>
</table>

* Percentage of tumor-bearing animals per group.

b Number of tumors per animal.
injury (probably counteracting the damaging influence by building up a thicker epithelium and more resistant horny layer), whereas a nondamaging stimulus increases nothing but the rate of normal chalone-controlled everyday tissue regeneration (24).

Within the framework of this concept, irritant skin mitogens such as TPA may be regarded as compounds which either somehow damage the tissue or, more probably, mimic tissue damage, whereas the nonirritant mitogen 4-O-methyl-TPA apparently resembles a nondamaging stimulus ("chemical massage"). Whether hyperplasia and skin inflammation are causally related remains an open question. The fact that hyperplasia can be prevented by cyclooxygenase inhibition while the symptoms of inflammation remain unchanged is at least inconsistent with such an assumption. Thus, skin inflammation may be nothing more than an accompanying symptom of hyperplastic transformation.

The observations described in this paper may also have important implications for the chalone concept (for details, see Ref. 17), since chalone-controlled epidermal hyperproliferation (which does not result in hyperplasia), previously induced only by skin massage, has now been repeated by means of quite a different mitogenic stimulus. This supports the idea of a distinct physiological role of epidermal G1 chalone, i.e., as a regulatory factor which helps to maintain the special (nonhyperplastic) morphology and growth behavior of normal adult mouse epidermis. As yet, no exception seems to exist to the rule that hyperplastic transformation of the epidermis is preceded by chalone refractoriness, so that a causal relationship between both events may exist.

As far as we know, all skin tumor promoters induce hyperplastic transformation of epidermis, whereas not every hyperplastic transformant is a skin tumor promoter. Thus, hyperplastic transformation may be regarded as necessary but certainly not a sufficient condition of tumor promotion. As already emphasized, a suitable negative control compound for studies on phorbol ester tumor promotion should be a nonpromoting hyperplastic transformant. The fact that 4-O-methyl-TPA does not induce hyperplastic transformation invalidates its role as a negative control compound.

In addition, 4-O-methyl-TPA is neither a potent first-stage nor a second-stage tumor promoter when tested in combination with either the second-stage promoter RPA or TPA. The observations of a weak activity of 4-O-methyl-TPA as a first-stage promoter in Sencar mice (a strain highly susceptible for 2-stage carcinogenesis) indicates that some strain differences may exist in this respect (35). Since RPA is a strong hyperplasticogenic transformant, it may be concluded that hyperplastic transformation is obligatory for the second stage of promotion, probably to make the reversibly growing benign tumors generated in the classical 2-stage carcinogenesis experiment visible to the naked eye (24).

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REFERENCES

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Effects of the Phorbol Ester 4-O-Methyl-12-O-Tetradecanoylphorbol-13-acetate on Mouse Skin in Vivo: Evidence for Its Uselessness as a Negative Control Compound in Studies on the Biological Effects of Phorbol Ester Tumor Promoters

Gerhard Fürstenberger, Hartmut Richter, Thomas S. Argyris, et al.


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