Prostaglandin E-mediated Mitogenic Stimulation of Mouse Epidermis in Vivo by Divalent Cation Ionophore A 23187 and by Tumor Promoter 12-O-Tetradecanoylphorbol-13-Acetate

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ABSTRACT

When applied to mouse skin in vivo, both the strong tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (2 nmol) and the divalent cation ionophore A 23187 (200 nmol) caused the same responses, i.e., skin inflammation and prostaglandin E₂-mediated epidermal hyperplasia. In both cases, these events were accompanied by certain biochemical reactions in the epidermis such as an increase in the biosynthesis of and sensitivity to prostaglandin E₂, increases in ornithine decarboxylase and phosphodiesterase activities, and refractoriness of cyclic adenosine 3':5'-monophosphate production to β-adrenergic stimulation. In contrast to A 23187, TPA did not induce degranulation of mast cells; whereas, in contrast with TPA, A 23187 did not show tumor-promoting activity. These results indicate that the observed biological effects of TPA are no indication of tumor-promoting ability and that, on the other hand, the mitogenic effects of A 23187 are possibly not due to its properties as a calcium ionophore.

INTRODUCTION

Fuller knowledge of the biochemical mechanisms which underlie epidermal hyperproliferation and hyperplasia may lead to a better understanding not only of many skin disorders but also of events related to chemical carcinogenesis, especially of the process of tumor promotion. When applied to mouse skin in vivo, certain agents such as the phorbol ester TPA act as tumor promoters; i.e., they accelerate the development of benign and malignant tumors which had been "initiated" previously by carcinogens in subthreshold doses (45). Although tumor promotion is a long-term process depending on repeated treatments with the promoter, it is generally believed that the responses seen after a single promoter application are indicative of the biological mechanism of promotion. All known tumor promoters induce, for example, both epidermal hyperproliferation and skin inflammation. Consequently, both responses are thought to be necessary conditions for tumor promotion. On the other hand, not every hyperplasigenic irritant possesses tumor-promoting properties. This discrepancy has been interpreted by assuming that cell proliferation and inflammation are not sufficient by themselves to bring about tumor promotion. It should be taken into consideration, however, that skin inflammation and epidermal hyperplasia can be induced and can proceed along different biochemical pathways, some of which may exhibit tumor-promoting activity, while others may not. That the induction of epidermal hyperproliferation may indeed be a rather complex process is supported by the findings that it can be triggered by a wide variety of chemical, physical, or mechanical means; that it is mostly, but not always, accompanied by skin inflammation; and that it is in general, but not necessarily, followed by epithelial hyperplasia (30).

It may not be very useful, therefore, to compare promoting and nonpromoting mitogens and to draw conclusions from such studies on the role of epidermal hyperproliferation in tumor promotion without a detailed knowledge of the intimate biochemical events involved in the hyperproliferative process.

The interpretation of results obtained from studies of the biological effects of tumor promoters would indeed be greatly facilitated if a compound were available which induced skin inflammation and epidermal hyperplasia via essentially the same biochemical pathways as those for tumor-promoting phorbol esters without being a promoter itself. Such a compound would provide an excellent control for all kinds of studies devoted to the problem of tumor promotion and could yield fundamental insights into the problem of the interrelationship between promotion, cell proliferation, and inflammation.

A good candidate for such a role might be the lipophilic antibiotic A 23187 from Streptomyces chartreusis which is known to facilitate the transport of divalent cations across cellular membranes (38). The ionophore A 23187 exhibits many biological activities which are similar to those of the phorbol ester TPA (for a survey, see Ref. 45). Among these are mitogenic activation of lymphocytes (26, 53) and fibroblasts in vitro (2), activation of macrophages (17), stimulation of enzyme release from polymorphonuclear leukocytes (40, 46), induction of platelet aggregation (57), stimulation of endogenous proteases (7, 42) and of prostaglandin biosynthesis (17, 23, 37, 39, 52, 54, 55), and "fluidizing" effects on cell membranes (22).

In this communication, we show that a single application of A 23187 induces in mouse skin in vivo prostaglandin-mediated epidermal hyperplasia, inflammation, and some other responses which are characteristic of TPA treatment. Nevertheless, A 23187 does not show tumor-promoting efficacy while, on the other hand, the phorbol ester does not exhibit a characteristic effect of a calcium ionophore, i.e., degranulation of mast cells.

1 This work was supported by grants from the Deutsche Forschungsgemeinschaft and from the Wilhelm-and-Maria-Meyenburg Foundation.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PGE₂, prostaglandin E₂; cyclic AMP, cyclic adenosine 3':5'-monophosphate; MIX, 1-methyl-3-isobutylxanthine; ETPA, 5,8,11,14-eicosatetraynoic acid; ODC, ornithine decarboxylase; PDE, cyclic adenosine 3':5'-monophosphate phosphodiesterase; PGF₂α, prostaglandin F₂α; 4-O-methyl-TPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate.

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MATERIALS AND METHODS

Materials. [2,8-3H]adenosine 3',5'-cyclic phosphate, [methyl-3H]thymidine, [1-14C]ornithine, [5,6,8,11,12,14,15-3H]PGE2, and a cyclic AMP assay kit were obtained from Amersham-Buchler, Braunschweig, West Germany.

Rabbit antisera for PGE2 were products of the Institut Pasteur, Paris, France. Histamine and serotonin were purchased from Serva, Heidelberg, West Germany, and indomethacin and prostaglandins from Sigma, Munich, West Germany. MIX was a product of Aldrich/Ega-Chemie. Vincristine was obtained from Eli Lilly, Bad Homburg, West Germany. ETYA was a generous gift of Hofmann-LaRoche, Grenzach, West Germany.

We are very grateful to Professor E. Hecker, Deutsches Krebsforschungszentrum, Heidelberg, Germany, for supplying us with the phorbol esters; to the Eli Lilly Company for a generous gift of the antibiotic A 23187; and to the Upjohn Company, Kalamazoo, Mich., for 15(S)-15-methylprostaglandin E2.

Animals. Female NMRI mice (age 7 to 8 weeks) were used in all experiments. Details of handling and pretreatment of animals have been described elsewhere (24).

Cyclic AMP Assay. The cyclic AMP content of mouse epidermis was assayed as in Ref. 27 using a highly specific purchasable assay kit based on a protein binding method (49).

Under the assay conditions, no artifactual increase (for example, due to ischemia) of the cyclic AMP level in the tissue and no detectable interference with other compounds (cyclic guanosine 3':5'-monophosphate and other adenosine nucleotides) was observed. The lower limit of detection was approximately 5 pmoles cyclic AMP per ml, whereas the concentration in extracts of untreated epidermis (basal tissue level of cyclic AMP) was approximately 25 pmol/ml. The deviation between assays run in parallel was less than 10%, and the recovery of known cyclic AMP was almost quantitative (90%).

Other Methods. The methods for labeling epidermal DNA and determination of mitotic activity (24), assay of ODC (29), assay of PDE (32), stimulation by isoproterenol of epidermal cyclic AMP production in vivo (19, 27), histamine assay (43, 44), and PGE2 assay (15) have been described in detail elsewhere.

RESULTS

Skin Inflammation and PGE2-mediated Epidermal Hyperplasia Induced by A 23187 or TPA. After a single topical application of the antibiotic A 23187 (200 nmol) to mouse skin in vivo, pronounced skin inflammation with leukocyte infiltration, edema, and epidermal hypertrophy between 2 and 24 h and the development of epidermal hyperplasia between 2 and 4 days were observed (Fig. 1). The latter was preceded by a peak of DNA labeling after 18 hr and a peak of mitotic activity after 26 hr (Chart 2). Only after 6 days did the skin regain its normal state.

Essentially, the same response could be evoked by the phorbol ester TPA (Chart 1) in a 10-fold lower dose (2 nmol/mouse; Fig. 1, Chart 2).

Hyperplasia was completely inhibited when the prostaglandin synthetase inhibitors, indomethacin or ETYA, were applied topically 30 min prior to either A 23187 or TPA (14). When administered 15 min after mitogenic stimulation or later, indomethacin caused no significant inhibition (Chart 3). Conversely, a rapid elevation of the PGE2 level in the epidermis with peaks at 10 min and 1 to 2 hr after application of either TPA or A 23187 was observed (Chart 4). The first peak was not found when the animals were pretreated with indomethacin, as described above (Chart 4).

The inhibitory effect of indomethacin (but not that of ETYA) on a A 23187-stimulated epidermal cell proliferation could be indeed overcome when low doses of PGE2 were applied simultaneously with A 23187. PGE2 did not show such an effect (Chart 5). In contrast with this observation, PGE2 turned out to be only a weak mitogen when applied to untreated mouse skin (Chart 5), and no distinct inhibition by indomethacin of normal epidermal cell proliferation could be observed. Identical observations have been described for TPA (14).

TPA- or A 23187-induced symptoms of skin inflammation such as leukocyte infiltration, edema, and hypertrophy of epidermal cells were not relieved by indomethacin but only by ETYA.

Cyclic AMP Probably Not Involved in PGE2-mediated Epidermal Cell Proliferation. In many tissues including epidermis (1, 58), prostaglandins of the E-series have been found to raise the level of cyclic AMP. We did not observe an increase in cyclic AMP in epidermis 5 to 30 min after treatment with A 23187 or TPA, i.e., at the moment when PGE2 reached its first maximum. In addition, neither PGE2 itself nor its more stable derivative 15(S)-15-methylprostaglandin E2 could elevate the cyclic AMP level in epidermis when applied topically, regardless of whether an i.p. injection of 1 mg MIX, a powerful inhibitor of epidermal PDE (27), had been given 30 min prior to

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F. Marks et al.

Enzyme Induction and Catecholamine Refractoriness after Treatment with TPA. Besides its effects on cell proliferation, TPA has been shown to activate the enzymes ODC (36) and PDE (30, 31) in epidermis via a cycloheximide-sensitive process and to generate refractoriness of epidermal cyclic AMP production to stimulation by β-adrenergic agonists such as isoproterenol (19, 28). The induction of ODC has been found to be inhibited by indomethacin (51). Increase of ODC and PDE activities as well as catecholamine refractoriness were also observed after application of the ionophore A23187 (Charts 6 and 7). The increase in PDE activity and the desensitization of the β-adrenergic effect were found to be insensitive to indomethacin and ETYA treatment under conditions which led to a complete inhibition of stimulated cell proliferation and PGE2 synthesis (data not shown).

Effect of A23187 and of TPA on Mast Cell Degranulation. The degranulation of mast cells is thought to be a Ca2+-dependent exocytotic process (33) which may be taken as a measure of the ability of a given compound to increase the Ca2+ concentration in mast cell cytoplasm, i.e., of its ionophoretic activity.

As shown in Table 1, A23187 did increase considerably the release of histamine from rat peritoneal mast cells in a dose-dependent fashion. Under identical conditions, the phorbol ester TPA did not show such an effect.

Mast cell factors such as serotonin (0.1 μmol) and histamine (1 μmol) did not significantly increase mitotic activity in mouse epidermis when applied topically in methanol solution.

A23187 Is Not a Tumor Promoter. To test the tumor-promoting efficacy of the ionophore A23187, a 2-stage carcinogenesis experiment was performed as described in Table 2. Essentially, no tumor development was observed, and the animals were virtually free of visible symptoms of a toxic effect such as skin necrosis, etc. When the same experiment was carried out with TPA instead of A23187, a strong tumor-promoting effect was seen (Table 2).

Treatment (see below). Furthermore, dibutyl cyclic adenosine 3',5'-monophosphate could not replace PGE2 in overcoming the blockade of cell proliferation by indomethacin when applied simultaneously with A23187 (1 μmol topically or 2 μmol i.p.). Finally, i.p. injection of both β-isoproterenol (1 μmol) and MIX (3 μmol) given together did not cause an increase in mitotic activity in epidermis, although this procedure was followed by a 10- to 20-fold elevation of epidermal cyclic AMP over a period of 30 to 60 min (19, 27).

Chart 4. Level of PGE2 in mouse epidermis after treatment with either 10 nmol TPA (○, ○, ○) or 200 nmol A23187 (●, ●, ●) at 0 time. Control animals received 0.1 ml acetone instead of TPA or ionophore, and the PGE2 level was measured at different time intervals after acetone application. Essentially, no effect of acetone on epidermal PGE2 content was observed over the whole period of the experiment. Each point represents the mean of 5-fold assay. Bars, S.D.

Chart 3. Effect of topical application of indomethacin (——) and ETYA (— — — — ) on a A23187-stimulated cell proliferation in mouse epidermis in vivo. Left, the dose-response curves (the inhibitors were topically applied in acetone solution 30 min prior to treatment with A23187). Right, the relationship between inhibition of mitotic activity by indomethacin (1.1 μmol/mouse) and application scheme. –, application prior to A23187; +, application after A23187. Mitotic activity was determined 27 hr after ionophore application. Each point represents an experiment with one animal (mean of 5-fold assay). Bars, S.D.
The results described above strongly indicate that the tumor-promoting phorbol ester TPA as well as the nonpromoting drug A 23187 (divalent cation ionophore) induce epidermal hyperplasia and inflammation on mouse skin in vivo by means of a similar molecular mechanism.

The first response seen is an increase in PGE₂ synthesis in the epidermis within 10 min, which can be suppressed by topical application of the cyclooxygenase inhibitor indomethacin; the level of PGF₂α is not increased (15). In addition, indomethacin treatment prevents the whole sequence of events finally (i.e., after 2 to 5 days) leading to epidermal hyperplasia provided that the drug has been given not later than 15 min after TPA (13-15) or ionophore application. This together with the observation that indomethacin inhibition can be overcome by application of PGE₂ but not by PGF₂α (15) shows that the early increase in PGE₂ is an event obligatory for epidermal hyperproliferation. This conclusion is confirmed by the fact that a hyperproliferative response which is not preceded by an early PGE₂ peak is insensitive to indomethacin inhibition (13). Elevated prostaglandin levels seen at later times after stimulation (3, 4, 51) are obviously less important for the hyperplastic response.

The immediate stimulation of PGE₂ synthesis must be considered, therefore, to be involved in the trigger event for a certain type of epidermal hyperplasia (probably for that which is generally accompanied by skin inflammation). It was shown recently in epidermal cell cultures that TPA-sensitive prostaglandin synthesis is located in the epidermis rather than in other parts of the skin (16). This means that E-series prostaglandins or closely related compounds constitute an integral part of the endogenous growth control mechanism of the tissue. Thus, for the first time, the stimulatory effect of TPA on pros-

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**DISCUSSION**

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of animals</th>
<th>Rate (%)</th>
<th>Yield (%)</th>
<th>Rate (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
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<tr>
<td>Acetone</td>
<td>28 (28)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A 23187</td>
<td>15 (14)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPA</td>
<td>29 (28)</td>
<td>14</td>
<td>0.25</td>
<td>57</td>
<td>2.71</td>
</tr>
</tbody>
</table>

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**Table 2**

**Effect of A 23187 or TPA on the release of histamine from rat peritoneal mast cells**

Aliquots of a suspension of peritoneal cells in Hanks' balanced salt solution containing 30,000 mast cells/0.3 ml were incubated at 37° for 7 min, and the histamine released into the medium was determined as described (43, 44) by means of a Technicon autoanalyzer. The total release measured after heating at 100° for 7 min was 3.7 μg/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histamine release (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.1</td>
</tr>
<tr>
<td>A 23187 (0.4 μM)</td>
<td>0.1</td>
</tr>
<tr>
<td>A 23187 (0.6 μM)</td>
<td>0.6</td>
</tr>
<tr>
<td>A 23187 (0.8 μM)</td>
<td>2.6</td>
</tr>
<tr>
<td>TPA (0.1–10 μM)</td>
<td>0.1</td>
</tr>
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</table>
acin, normal skin is somewhat insensitive to PGE2 stimulation. It must be emphasized that the molecular mechanism of this effect is entirely obscure.

Although indomethacin is considered to be an antiinflammatory drug, it does not relieve the symptoms of TPA- or ionophore-induced skin irritation. This result indicates that in contrast to epithelial hyperproliferation the accompanying irritation is not mediated by prostanoids. Both induced cell proliferation and inflammation can be prevented, however, when the “‘wrong arachidonic acid’” ETYA is applied. This compound is thought to inhibit not only prostaglandin biosynthesis but also the competing pathway of unsaturated fatty acid metabolism, i.e., lipoxygenation (48). It might be proposed, therefore, that lipoxygenase products, such as 12-hydroxycosatetraenoic acid, which have been shown to be chemotactic for polymorphonuclear leukocytes (18, 35, 50) are somehow involved in the inflammatory response.

During the hyperproliferative response of mouse skin to TPA or A 23187, certain biochemical effects are observed in epidermis the physiological role of which is still not understood. One of these, the induction of ODC, is also sensitive to indomethacin inhibition and is therefore probably mediated by PGE2 (51). Two other reactions, namely, the activation of PDE and the development of catecholamine refractoriness, can be inhibited neither by indomethacin nor by ETYA. This would mean that TPA and A 23187 are also able to induce reactions which are clearly not mediated via the arachidonic acid cascade.

Summarizing our results, we conclude that as far as hyperplasogenic activity is concerned, TPA and A 23187 do have a common mechanism of action. The fact that A 23187, in contrast with TPA, is not a tumor promoter and that it is nontoxic in long-term experiments makes the substance an ideal control for all kinds of studies on the biological effects of tumor promoters. It may be superior to all negative controls used thus far, which are either nonhyperplasogenic agents such as phorbol, or highly toxic irritants such as acetic acid (which probably kill the tumor cells before they can be promoted), or weak promoters such as 4-O-methyl-TPA (29), the unsaturated TPA analog T8 (29), or the diterpene ester mezerein (34). Some of those compounds such as 4-O-methyl-TPA even have a mechanism of mitogenic action which is quite different from that of TPA or A 23187 (13, 19, 29). In addition, it is now definitely proven that all reactions observed after a single TPA treatment such as inflammation, prostaglandin-mediated hyperplasia, ODC and PDE induction, and catecholamine refractoriness are by no means sufficient conditions for tumor promotion. They might nevertheless be necessary inasmuch as enhanced cell proliferation is clearly necessary for but not the only cause of tumor promotion. This view is consistent with the finding that TPA-induced tumor promotion in mouse epidermis can be modulated by such prostaglandin synthesis inhibitors as indomethacin (51) as well as by prostaglandins themselves (11).

Our results might lead to the proposal that the phorbol ester, like A 23187, is a divalent cation ionophore. This is, however, not consistent with the fact that, on contrast with A 23187, TPA cannot induce the degranulation of mast cells, which is thought to be absolutely dependent on calcium ions (33). Recently, TPA has been shown even to decrease the Ca2+ influx and the intracellular Ca2+ level in cultured chick myoblasts (41).

Why then do both compounds evoke identical responses in epidermis? One possible explanation might be that the mitogenic effect of A 23187 is not mediated by calcium ions but is due to some other property of the antibiotic. There are several effects of A 23187 which are assumed but not proved because of its ionophoretic properties. This is especially true for such multistep processes as induced cellular proliferation. If such a sequence of events does not proceed in the absence of calcium ions, as has been frequently observed, this does not necessarily mean that calcium is involved in the A 23187-induced triggering process but that it may be essential for later steps in the course of the cellular response. The role of Ca2+ ions as triggers of lymphocyte transformation has recently been seriously questioned by showing that A 23187 in an amount which is optimal for stimulation does not cause a measurable calcium influx into the cytoplasm as compared with untreated cells (20). On the other hand, the ionophore has been shown to disorder lipid structure and to disturb lipid-protein interactions in lymphocyte membranes by a mechanism which does not depend on calcium or magnesium ions but which may solely involve an interaction of the lipophilic drug with membranous lipoprotein complexes leading to a “‘fluidizing’” effect (22). A similar effect has been reported for the phorbol ester TPA (6, 12). The decrease in membrane microviscosity that is caused by either TPA (6, 12) or A 23187 (22) is followed by a rapid increase in the rate of phospholipid turnover which may perhaps give rise to an activation of phospholipase A2 and prostaglandin synthesis. The stimulation of phospholipid metabolism by TPA in lymphocytes is indeed inhibited by ETYA (56), as is the proliferative response of epidermis to A 23187.

Such a more general “membrane perturbation” (which may be, nevertheless, quite specific) as the primary event in TPA- or A 23187-induced epidermal hyperproliferation may be offered as an alternative explanation to the assumption of a specific receptor interaction of the mitogen. The observations cited above as well as our own results make it indeed rather difficult to postulate an interaction of TPA or A 23187 with a single cellular binding site, especially when the profound structural differences of the 2 compounds are considered. In particular, the ionophore lacks the long-chain fatty acid residue which is of paramount importance for the biological activity of TPA. The existence of 2 different “‘receptors”’ the activations of which lead to identical responses can not, of course, be ruled out. It must be emphasized, furthermore, that possible arguments against a phorbol ester receptor will be valid only as far as the hyperplasogenic response to a single treatment is concerned. A binding site for phorbol esters which is perhaps exclusively involved in mediating tumor promotion has been recently demonstrated in different cell types (8-10), and there seems to exist a positive correlation between binding affinity and tumor-promoting efficacy. However, as long as no reliable and measurable biochemical parameters of tumor promotion are known, it may be difficult or even impossible to draw final conclusions about the physiological role of such binding sites.

ACKNOWLEDGMENTS

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F. Marks et al.


Fig. 1. Effect of A 23187 and TPA on mouse skin. a, untreated; b, 16 hr after topical application of 200 nmol A 23187 (in 0.1 mol acetone); c, 48 hr after topical application of 200 nmol A 23187 (in 0.1 mol acetone); d, 16 hr after topical application of 2 nmol TPA (in 0.1 mol acetone); e, 48 hr after topical application of 2 nmol TPA (in 0.1 mol acetone). Note the leukocyte infiltration and epidermal hyperplasia in b and d and the epidermal hyperplasia in c and e.
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