Inhibition of 12-O-Tetradecanoylphorbol-13-acetate-induced Epidermal Ornithine Decarboxylase Activity by Phospholipase A₂ Inhibitors and Lipoygenase Inhibitor

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ABSTRACT

Application of 12-O-tetradecanoylphorbol-13-acetate (TPA; 20 nmol/mouse), a tumor-promoting agent, to mouse skin results in an induction of epidermal ornithine decarboxylase (ODC; EC 4.1.1.17). Induction of ODC by TPA was inhibited by treatment of skin with indomethacin (1.12 μmol/mouse), a cyclooxygenase inhibitor, and the ODC activity suppressed by indomethacin was completely restored by concurrent application of prostaglandin E₂ (PGE₂) (140 nmol/mouse) as described first by Verma et al. (Cancer Res., 40: 308–315, 1980). Treatment of mice with tetracaine (20 and 100 nmol/mouse), a nonspecific phospholipase A₂ inhibitor, inhibited the induction of ODC by TPA. More specific phospholipase A₂ inhibitors, mepacrine (20 μmol/mouse) and p-bromophenacyl bromide (10 μmol/mouse), also inhibited the ODC induction. The TPA-induced ODC inhibited by mepacrine was not restored by the treatment of mice with PGE₂. TPA-induced ODC inhibited by either mepacrine or p-bromophenacyl bromide was partially but significantly restored by treatment with arachidonic acid (1 to 40 μmol/mouse). Neither PGE₂ nor arachidonic acid alone could induce the epidermal ODC. Treatment of mice with nordihydroguaiaretic acid (10 to 90 μmol/mouse), a lipoygenase inhibitor, also inhibited the induction of ODC by TPA. These results strongly indicate that the stimulation of phospholipase A₂ activity is a crucial process in inducing mouse epidermal ODC by TPA and not only cyclooxygenase product(s) but also lipoygenase product(s) are involved in the mechanism of ODC induction. Our present data also suggest that the above arachidonate metabolites are essential but not sufficient factors for the TPA-stimulated induction of ODC.

INTRODUCTION

ODC² (EC 4.1.1.17; L-ornithine carboxy-lyase), the rate-limiting enzyme in the polyamine-biosynthetic pathway, is elevated in various proliferative cell systems and implicated in neoplastic growth (12, 27, 31). In adult mouse epidermis, a rapid, transient induction of ODC is observed by application of TPA, a potent tumor-promoting agent (21, 22). It has been suggested that an induction of ODC is necessary for tumor promotion. However, recent findings indicate that ODC induction is essential but not a sufficient factor for the mechanism of skin tumor promotion (14, 15). It was reported that pretreatment with colchicine or other microtubule-disrupting agents (23), local anesthetics (41), retinoids (11, 31, 34, 35), and putrescine (38) suppressed the TPA-induced elevation of mouse epidermal ODC. The biochemical mechanism of induction of ODC by TPA, however, has not yet been fully understood.

Recently, TPA has been shown to enhance the release of prostaglandins and/or arachidonic acid in dog kidney (MDCK) cells (9, 10, 24), C3H/10T½s mouse and chick embryo fibroblasts (19, 20), rabbit leukocytes (8), Friend erythroleukemia cells (39), epidermal cells (4), and epidermis (1, 3, 30). Verma et al. (30, 33) reported that pretreatment of prostaglandin synthesis inhibitors such as indomethacin, flufenamic acid, or acetylsalicylic acid markedly inhibits the induction of epidermal ODC by TPA. The inhibition of TPA-induced ODC by indomethacin was completely overcome by the treatment with PGE₂. They suggested that PGE₂ may play a crucial role in TPA-induced elevation of ODC activity.

However, our preliminary data demonstrated that TPA-induced ODC activity of mouse epidermis was not restored by PGE₂ when the ODC induction was suppressed by mepacrine, a phospholipase A₂ inhibitor. The above finding prompted us to investigate the possible involvement of phospholipase A₂ activation and arachidonate cascade metabolites in the mechanism of ODC induction by TPA in mouse epidermis.

The results presented here suggest that phospholipase A₂ stimulation and resultant arachidonate metabolites, i.e., not only cyclooxygenase product(s) but also lipoygenase product(s), are involved in the mechanism of ODC induction by TPA.

MATERIALS AND METHODS

Female CD-1 mice (Charles River, Kanagawa-ken, Japan), 7 to 8 weeks old, were used. The dorsal hair of each mouse was shaved with clippers at least 2 days before use, and only those mice showing no hair regrowth were used. All animals received water ad libitum throughout the experimental period. Mice were routinely sacrificed between 4 and 6 p.m. All chemicals applied to mouse skin were dissolved in reagent grade acetone except for lysophosphatidylcholine which was dissolved in ethanol:water (5:1:1). The chemicals were applied to the shaved area of individual mice in a total volume of 0.2 ml using a micropipet for each occasion. Five hr after the TPA administration, the mice were sacrificed by cervical dislocation and the skins were excised. The skin was placed in 55°C water for 30 sec, submerged in ice water for at least 30 sec (22), and then placed on an ice-cold plate. Epidermis was removed free from dermis and fat by scraping with a razor blade. The epidermis was immediately refrigerated by liquid nitrogen and stored at −70°C until just prior to homogenization. The epidermal sheets were homogenized in 20 mM Tris-HCl buffer, pH 7.2, in a Polytron PT-10 homogenizer for 10 to 15 sec at 4°C. The supernatant fraction obtained after centrifugation at 50,000 × g for 30 min at 0°C was used for estimation of enzyme activities. In most cases, a soluble epidermal extract was prepared from one mouse. ODC activity was determined by measuring the release of ¹⁴C⁰₂ from [³¹⁴C]ornithine as described by Russell and Snyder (25). In brief, incubation was carried out...
out for 60 min at 37°C in a medium containing 0.4 μmol pyridoxal phosphate, 1.0 μmol dithiothreitol, 0.2 μmol L-ornithine, 30 μmol Tris base (pH 7.2), 0.5 ml epidermal extract, and approximately 0.5 μCi DL-[1-14C] ornithine (51.3 mCi/mmol) in a final volume of 2.0 ml. In order to absorb the generated CO2 and 14CO2, Hyamine 10X was placed in a central well of the incubation flask. The reaction was stopped by the addition of 1.0 ml 2 M citric acid, and incubation was continued for an additional 30 min to ensure complete absorption of 14CO2. Hyamine solution was transferred into a scintillation vial containing 10 ml of a toluene-based scintillation cocktail. Radioactivity was measured in a liquid scintillation counter (Aloka, Tokyo, Japan). Counting efficiency was approximately 87%. All enzyme activities were corrected against the value which was measured in the presence of an excess amount of α-methylornithine (final 5 mM), a reversible inhibitor of ODC. Activity was expressed as nmol CO2 in 60 min per mg protein.

Enzyme activity was proportional to the amount of extract and to the time of incubation within our experimental conditions. None of the drugs which we used (indomethacin, tetracaine, mepacrine, BPB, arachidonic acid, or NDGA) directly interfered with the ODC assay system.

The protein concentration of the epidermal extract was measured by the method of Lowry et al. (13), with bovine serum albumin as standard. TPA, indomethacin, mepacrine, arachidonic acid, NDGA, and α-lysophosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, Mo.; α-methylornithine was from Calbiochem-Behring Corp., La Jolla, Calif.; BPB was from Wako Pure Chemical Industries Ltd., Osaka, Japan. PGE2 was kindly donated by the Research Institute of Toray Co., Kamakura, Japan. DL-[1-14C] Ornithine (51.3 mCi/mmol) was obtained from New England Nuclear, Boston, Mass.

RESULTS

A single topical application of TPA (20 nmol/mouse) resulted in a substantial and transient increase in mouse epidermal ODC as reported by O'Brien et al. (21, 22). As shown in Table 1, treatment of mouse skin with indomethacin (1.12 μmol/mouse for 2 treatments) resulted in a suppression of ODC induction by TPA. Inhibition of TPA-induced ODC by indomethacin was counteracted by PGE2 (140 nmol/mouse) which was applied concurrently with indomethacin treatments. These results are consistent with those of Verma et al. (30, 33).

When tetracaine (20 and 100 μmol/mouse), a local anesthetic, was applied to the mouse skin, TPA-induced ODC was suppressed dose responsively (Chart 1). As shown in Chart 1, ODC induction by TPA was also inhibited dose responsively by the treatment of mouse skin with mepacrine which has been shown to be a phospholipase A2 inhibitor (2, 16, 17, 29, 40). BPB, another phospholipase A2 inhibitor with an inhibitory mechanism that is different from that of mepacrine (17, 28, 36), also inhibited the TPA-induced increase in epidermal ODC (Chart 1). The inhibition of TPA-induced ODC by mepacrine could not be reversed by treatment of mice with 140 nmol of PGE2 (Chart 2). The above dose of PGE2 completely restored the TPA-induced ODC suppressed by indomethacin (see Table 1). Further increase in doses of PGE2 also failed to counteract the inhibitory effect of mepacrine (Chart 2).

Topical application of arachidonic acid significantly restored the TPA-induced ODC which was suppressed by mepacrine.

![Chart 1](chart1.png)

**Table 1**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>ODC activity (nmol CO2/60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Acetone</td>
<td>0.13 ± 0.06†</td>
</tr>
<tr>
<td>Acetone</td>
<td>TPA</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>TPA + indomethacin</td>
<td>0.41 ± 0.03‡</td>
</tr>
<tr>
<td>Indomethacin + PGE2</td>
<td>TPA + indomethacin + PGE2</td>
<td>1.05 ± 0.21‡</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of individual determinations from 7 or 8 mice.
†p < 0.01 versus TPA.
‡p < 0.01 versus TPA + indomethacin; p is not significant versus TPA.
(20 µmol/mouse), to the extent of 28% with 10 µmol and 34% with 40 µmol of arachidonic acid (Table 2). Further increase in doses of arachidonic acid failed to complete the restoration. Arachidonic acid (1 and 10 µmol/mouse) also partially restored the TPA-induced ODC which was inhibited by BPB (10 µmol/mouse) (Table 2). In addition, topical application of lysophosphatidylcholine (40 µmol/mouse) significantly enhanced the restoring effect of arachidonic acid (10 µmol/mouse) when the TPA-induced ODC was suppressed by BPB and also tended to enhance the restoring effect of arachidonic acid (40 µmol/mouse) when TPA-induced ODC was suppressed by mepacrine (Table 2).

Either PGE2 (140 nmol/mouse) or arachidonic acid (40 µmol/mouse) alone could not induce the epidermal ODC (Table 2). In addition, topical application of lysophosphatidylcholine (20 µmol/mouse) also partially restored the TPA-induced ODC, which was inhibited by BPB (10 µmol/mouse) (Table 2). In addition, topical application of lysophosphatidylcholine (40 µmol/mouse) significantly enhanced the restoring effect of arachidonic acid (10 µmol/mouse) when the TPA-induced ODC was suppressed by BPB and also tended to enhance the restoring effect of arachidonic acid (40 µmol/mouse) when TPA-induced ODC was suppressed by mepacrine (Table 2).

NDGA, an inhibitor of lipooxygenase (7, 18, 37), inhibited the ODC induction by TPA in a dose-dependent manner (Chart 3).

**Effects of arachidonic acid and lysophosphatidylcholine on inhibition of TPA-induced epidermal ODC activity by mepacrine and BPB**

Mice were treated with acetone (vehicle), mepacrine (20 µmol), or BPB (10 µmol) 30 min prior to the topical application of TPA (20 nmol) or TPA plus arachidonic acid (1 to 40 µmol). The same amounts of mepacrine (20 µmol) or BPB (10 µmol) were applied again to the mice skin concurrently with TPA. When mice were treated with lysophosphatidylcholine, either lysophosphatidylcholine or vehicle (ethanol/water, 5:1) was applied on mouse skin in a volume of 100 µl 15 to 30 min after TPA. Mice were sacrificed for the determination of ODC activity 5 hr after TPA treatment.

**Table 2**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>n</th>
<th>ODC activity (nmol CO2/60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Acetone</td>
<td>2</td>
<td>0.06 ± 0.05a</td>
</tr>
<tr>
<td>Acetone</td>
<td>TPA</td>
<td>5</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>Mepacrine</td>
<td>TPA + mepacrine</td>
<td>5</td>
<td>0.09 ± 0.01e</td>
</tr>
<tr>
<td>Mepacrine</td>
<td>TPA + mepacrine + arachidonic acid (10 µmol)</td>
<td>5</td>
<td>0.40 ± 0.05c</td>
</tr>
<tr>
<td>Mepacrine</td>
<td>TPA + mepacrine + arachidonic acid (40 µmol)</td>
<td>4</td>
<td>0.56 ± 0.06c</td>
</tr>
<tr>
<td>Mepacrine</td>
<td>TPA + mepacrine + arachidonic acid (10 µmol) + lysophosphatidylcholine (20 µmol)</td>
<td>4</td>
<td>0.65 ± 0.17</td>
</tr>
<tr>
<td>BPB</td>
<td>TPA + BPB</td>
<td>4</td>
<td>0.16 ± 0.03b</td>
</tr>
<tr>
<td>BPB</td>
<td>TPA + BPB + arachidonic acid (1 µmol)</td>
<td>4</td>
<td>0.34 ± 0.05d</td>
</tr>
<tr>
<td>BPB</td>
<td>TPA + BPB + arachidonic acid (10 µmol)</td>
<td>4</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>BPB</td>
<td>TPA + BPB + arachidonic acid (10 µmol) + lysophosphatidylcholine (20 µmol)</td>
<td>5</td>
<td>0.52 ± 0.06a</td>
</tr>
</tbody>
</table>

- *Mean ± S.E. of individual determinations from 4 to 5 mice.
- a p < 0.05 versus TPA.
- b p < 0.01 versus TPA + BPB.
- c p < 0.01 versus TPA + mepacrine.
- d p < 0.05 versus TPA plus BPB.
- e p < 0.01 versus TPA plus BPB + arachidonic acid (10 µmol).

**Effect of PGE2 and arachidonate on epidermal basal ODC activity**

Mice were treated with acetone (vehicle), TPA (20 nmol), PGE2 (140 nmol), or arachidonic acid (40 µmol) 5 hr before sacrifice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC activity (nmol CO2/60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.07 ± 0.12a</td>
</tr>
<tr>
<td>TPA</td>
<td>1.15 ± 0.13</td>
</tr>
<tr>
<td>PGE2</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1.12 ± 0.03</td>
</tr>
</tbody>
</table>

- *Mean ± S.E. of individual determinations from 6 mice.

**DISCUSSION**

Our present data clearly show that the TPA-induced ODC suppressed by indomethacin, a cyclooxygenase inhibitor, was completely restored by the concurrent application of PGE2 with indomethacin. The results are consistent with those of Verma et al. (30, 33). TPA has been shown to enhance the release of prostaglandins, such as PGE2, in various cell types including epidermal cells (4) and epidermis (1, 3, 30). Thus, we confirmed the previous findings (30) that PGE2 plays an essential role in the mechanism of ODC induction by TPA in mouse epidermal cells.

Recently, Fürstenberger et al. (4) reported that TPA stimulates the release of arachidonic acid as well as prostaglandins from epidermal cells prelabeled with [3H]arachidonic acid. The above findings indicate that as with other types of cells, also in epidermal cells, TPA releases arachidonic acid by stimulating phospholipase A2. If that is the case, phospholipase A2 inhibitors should block the ODC induction by TPA. Our present data clearly demonstrate the inhibition of TPA-caused ODC induction by relatively specific phospholipase A2 inhibitors, i.e., mepacrine (2, 16, 17, 29, 40) and BPB (17, 28, 36). Tetracaine, one of the local anesthetics which has been shown to inhibit phospholipase A2 (26), also inhibits the ODC induction by TPA. These results strongly suggest that phospholipase A2 stimulation is involved in the mechanism of ODC induction by TPA.

When ODC induction by TPA was inhibited by mepacrine, PGE2 failed to overcome the inhibitory effect of mepacrine (Chart 2). This finding confirms the idea that PGE2 is an essential factor but not one sufficient to induce ODC activity by TPA (30). The fact that PGE2 alone failed to stimulate ODC induction (Table 3; Ref. 30) supports the above contention. When TPA enhances the release of arachidonic acid by stimulating phospholipase A2, part of the released arachidonic acid would be oxidized through the cyclooxygenase pathway to prostaglandin derivatives or through the lipoxygenase pathway to hydroperoxy or hydroxy derivatives of fatty acids. Partial restoration of TPA-stimulated ODC induction which was inhibited by phospholipase A2 inhibitors, by exogenous arachidonic acid indicates the possible involvement of an unknown arachidonate metabolite(s) other than PGE2 in the mechanism.
of ODC induction by TPA. PGE$_2$ fully restored the TPA-caused ODC induction inhibited by indomethacin but failed to restore the ODC induction inhibited by mepacrine. Thus, it is likely that the unknown arachidonate metabolite(s) (other than PGE$_2$) is a product(s) of the lipooxygenase pathway rather than of the cyclooxygenase pathway. Moreover, the present studies demonstrated the partial but significant inhibition of TPA-caused ODC induction by NDGA, which inhibits lipooxygenase (7, 18, 37) without inhibiting cyclooxygenase (18). These results strongly indicate the involvement of a lipooxygenase product(s) in the mechanism of ODC induction by TPA.

Arachidonic acid could partially but not completely overcome the inhibitory effect of phospholipase A$_2$ inhibitors, i.e., mepacrine or BPB. When TPA stimulates phospholipase A$_2$, lysophospholipids would be accumulated in the tissue with a concurrent release of arachidonic acid. Therefore, it is possible that a certain type of lysophospholipid plays a significant role in the mechanism of ODC induction by TPA. Recently, Verma and Boutwell (32) reported that extracellular calcium is required for the ODC induction by TPA. A certain type of lysophospholipid plays a significant role in calcium movement through the biological membrane (5). Our preliminary data showed that lysophosphatidylcholine could only slightly enhance the restorative effect of arachidonic acid. However, at this moment, it is too early to draw a conclusion concerning the role of lysophospholipids in the mechanism of ODC induction by TPA. It cannot be denied that some nonspecific toxic effects of mepacrine and BPB prevented the full restoration of ODC activity by arachidonic acid.

The fact that arachidonic acid alone failed to induce ODC activity indicates that arachidonate metabolites are necessary but not sufficient for the induction of ODC. Recently, it was reported that 1,2-diacylglycerol is generated in plasma membranes of chick embryo myoblasts by TPA (6). Therefore, in addition to the stimulation of phospholipase A$_2$, TPA may cause a variety of alternations in lipid metabolism. Thus, some other factor(s) such as 1,2-diacylglycerol, calcium ion, or other unknown intracellular second messenger(s), in addition to phospholipase A$_2$ products, may play an important role in the mechanism of ODC induction by TPA.

REFERENCES

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