ABSTRACT

The ability of the hyperplasigenic irritant ethyl phenylpropiolate (EPP) to act as a tumor promoter in two-stage carcinogenesis and to stimulate cellular events commonly cited as markers of tumor promoter action was evaluated. Treatment of adult, inbred SENCAR (SSIN) mice, initiated with 7,12-dimethylbenz(a)anthracene, with 5 mg of EPP twice weekly resulted in 100% of the mice developing tumors (4.8 tumors/mouse) after 40 weeks of promotion. Treatment with 3 mg EPP (twice weekly) resulted in 52% of the mice developing tumors (0.9 tumor/mouse). This treatment regimen with EPP produces a sustained epidermal hyperplasia without being overtly toxic. In addition, a 5-mg dose of EPP induced ornithine decarboxylase activity to a level comparable to that induced by the tumor promoter phorbol 12-myristate 13-acetate (PMA): 2.3 nmol CO₂/mg protein/h for EPP versus 4.5 nmol CO₂/mg protein/h for PMA versus 0.04 nmol CO₂/mg protein/h for acetone control. Likewise, the time course of ornithine decarboxylase induction by EPP was the same as seen with PMA (maximum induction at ~6 h). Vascular permeability of the dorsal skin increased significantly in response to EPP (8 times that seen in acetone controls) and exhibited the same kinetics as that seen after exposure to PMA. Activity of protein kinase C (PKC), the cellular receptor for PMA, decreased by 75% to 95% of that in control. This slight decrease in PKC activity is thought to be an indirect effect caused by the hyperproliferative and inflammatory reactions, because EPP was found to be inactive as an in vitro activator of PKC. These results indicate not only that EPP is a good tumor promoter that causes morphological and biochemical responses similar to those induced by PMA, but also that the action of EPP is apparently mediated via a mechanism that does not involve direct interaction with PKC.

INTRODUCTION

The process of multistage carcinogenesis has been extensively studied, particularly in the initiation-promotion model in mouse epidermis (1–4). A number of morphological and biochemical markers are strongly correlated with the tumor promotion process and the development of tumors in this model. Included among these markers are the appearance of "dark cells" (5); the induction of ODC (6) and increased DNA synthesis (6, 7); sustained epidermal hyperplasia (8); the induction of inflammation of the skin (9); and for phorbol ester-like promoters, the activation and subsequent down-regulation of PKC (10, 11). Well-characterized tumor promoters, such as the phorbol ester PMA, have a very strong inductive effect on these events. Similar effects by other compounds are often used as predictive of their tumor-promoting ability. The hyperplasigenic irritant EPP has been reported to induce many of these same changes when applied to the skin of mice. The induction of ODC, epidermal hyperplasia, inflammation and edema of the treated area, and increased numbers of epidermal dark cells have all been reported to occur following EPP treatment (7, 12–15). However, these studies report that EPP was either ineffective or very weak as a tumor promoter (2, 12, 16). The work of Raick (12), in particular, suggests that EPP does have at least minimal tumor-promoting activity. The doses of EPP used in these other studies were found to be extremely toxic to the skin of inbred SENCAR (SSIN) mice when applied using normal tumor promotion protocols: ~10 mg/treatment in experiments reported by Saffiotti and Shubik (16) and 15 mg/treatment in experiments reported by Slaga (2). Therefore, we reduced the dose of EPP and report here results indicating not only that EPP induces many of the biochemical changes mentioned above to a degree comparable to PMA, but also that it acts as an effective tumor promoter when applied after initiation with DMBA.

MATERIALS AND METHODS

Mouse and Tissue Samples. All mice used were female, 6 to 8 week old, inbred SENCAR (SSIN) (17) obtained from the Science Park Veterinary Division. The hair from the dorsal skin was removed by shaving 2 to 3 days before treatment. For skin specimens and cellular isolates, the mice were killed by cervical dislocation, and the dorsal skins were removed. Cellular material for ODC and PKC studies was obtained by placing the skins on ice-cold glass plates and scraping off the epidermis with a razor blade.

Chemicals. DMBA, Evans blue dye, DEAE-cellulose, type II histone, and L-alpha-phosphatidyl-L-serine were purchased from Sigma Chemical Co. (St. Louis, MO). Radioisotopes were obtained from Amersham Corp., Arlington Heights, IL ([3-14C]ornithine hydrochloride; 55 mCi/mmol) and ICN Biomedicals, Inc., Irvine, CA ([gamma-32P]ATP; 25 Ci/mmol). EPP and PMA were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and LC Services (Woburn, MA), respectively. Whatman International Ltd. (Maidstone, England), was the supplier of P81 chromatography paper; reagents for protein determinations were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals and solvents were of the highest quality available.

Histology. To assess the epidermal response to repeated applications of EPP, the backs of mice were shaved, and the mice were treated with acetone, 5 mg EPP, or 2 µg PMA. All treatments were made in 200-µl volumes. For each compound, two mice were treated one time, and two were treated four times (twice weekly) and killed 48 h after the last treatment. Another two mice were treated with 5 mg EPP 12 times (2 times/week for 6 weeks) and killed 48 h after the final treatment. Residual hair was removed with a depilatory lotion, and portions of the skin were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and mounted on microscope slides. After staining with hematoxylin and cosin, the slides were examined by light microscopy to determine the degree of epidermal hyperplasia.

Tumor Experiments. Mice were shaved 2 days prior to initiation by topical application of DMBA. Tumor promotion was accomplished by twice weekly application of the promoter to the initiated skin of the back. The promotion stage of the experiment was begun 2 weeks after initiation and continued for 40 weeks. Two separate tumor experiments were set up. One experiment included one group (Group A) of 30 mice initiated with 10 nmol of DMBA (2.5 µg in 200 µl acetone) and promoted with 5 mg EPP/treatment twice/week. The second experiment had two groups of 30 mice each, all initiated with 100 nmol of
DMBA; one group (Group B) was promoted with 5 mg EPP, and the second (Group C) with 3 mg EPP. After 40 weeks of promotion, the mice were killed, and the tumors were removed for histological analyses as described above. For comparison, data from a PMA promotion experiment are included (Group D), in which SSIN mice initiated with 10 nmol DMBA were promoted twice weekly with 1 µg PMA.

Ornithine Decarboxylase Activity. The induction of ODC activity was measured using preparations of scraped epidermis from the backs of mice treated with acetone, EPP, or 1 µg PMA. For dose response studies, EPP was applied at 1-, 3-, 5-, or 7-mg doses, and the mice were killed after 6 h. For time course studies, EPP was applied at 5 mg, and the mice were killed at 0, 3, 6, 9, and 15 h. Homogenates of epidermal scrapings were centrifuged at 12,000 x g for 15 min, and aliquots of the supernatant were assayed for ODC activity as described by Weeks and Slaga (18). Briefly, aliquots were incubated at 37°C for 1 h with l-[3H]ornithine, and the liberated 14CO2 was trapped in ethanolamine and counted in a scintillation counter. Total protein added in the reaction was determined according to Bradford (19), and the results were expressed as nmol CO2/mg protein/h. The experiments were carried out at least twice with three to five mice/experimental group.

Vascular Permeability. Vascular permeability, used as an indirect measurement of the degree of inflammation caused by irritants, was measured by slight modifications of the methods of Kata et al. (20) and Young et al. (21). Briefly, treated mice were given injections in the tail vein of 8.3 µl/g body weight of a solution of Evans blue dye (1% w/v in phosphate-buffered saline) 30 min before the mice were killed and the skin was removed. Six 1-cm2 pieces from each skin were homogenized separately in 600 µl of 0.5% NaSO4. The dye was extracted overnight by adding 1.4 ml of acetone. The samples were then centrifuged at 12,000 x g, and the supernatants were removed. The dye concentration in the supernatants was determined by spectrophotometric analysis at 620 nm. Each group contained three to five animals (six determinations/animal) with at least two experiments/comparison.

Protein Kinase C Down-Regulation. Protein kinase C activity was determined in epidermal scrapings from two to three mice/group, 48 h after treatment with acetone, EPP (5 mg), or PMA (2 µg). The buffers used were as follows: Buffer Stock [20 mM TRIZMA base, 2 mM EDTA, and 2 mM ethyleneglycol bis(β-aminomethyl ether)-N,N,N',N'-tetraacetic acid (pH 7.6)]; Buffer A (Buffer Stock plus 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10 µg/ml pepstatin A, 20 µg/ml aprotinin, and 50 µg/ml leupeptin); Buffer B (Buffer Stock plus 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml pepstatin A, 10 µg/ml aprotinin, and 25 µg/ml leupeptin); and Buffer C (Buffer B plus 0.12 mM NaCl). The scrapings were homogenized on ice in 0.75 ml/mouse of Buffer A and centrifuged in a Beckman TL-100 ultracentrifuge (22). After the supernatant was removed, the column was washed with 2 volumes of Buffer B, and the bound proteins were eluted with Buffer C. Fractions (0.5 ml) were collected in polypropylene tubes containing 5 µg pepstatin A, 10 µg aprotinin, and 25 µg leupeptin and stored at 4°C until use. Protein kinase C activity in the fractions was determined as described previously (10, 23).

Protein Kinase C Activation In Vitro. The ability of PMA to activate PKC in vitro was measured using a modification of the method of Arcocelo and Weinstein (24). Briefly, PKC was partially purified from skin homogenates of untreated mice as described above using DEAE-cellulose chromatography. Using the buffer system described above, the PKC activity was determined in the presence or absence of calcium (2 mM), EPP (0.1, 1, 10, and 100 µg/ml), or PMA (1 µg/ml). The experiment was performed three times.

RESULTS

Histology. The histological response of SSIN mouse skin treated with acetone, EPP, or PMA is illustrated in Fig. 1. Treatment with acetone does not change the appearance of untreated skin; a single layer of basal cells is overlaid with one or two layers of squamous, highly keratinized cells (Fig. 1A).

Four treatments with 2 µg of PMA result in an extremely hyperplastic epithelium with 10 to 15 layers of differentiating suprabasal cells overlaying a well defined basal layer (Fig. 1B). In contrast to PMA treatment, four treatments of EPP (5 mg/treatment) result in hyperplasia that is somewhat less pronounced (Fig. 1C). In this case, the single layer of basal cells is covered by three to five layers of differentiating suprabasal cells. However, when 5 mg of EPP are applied twice weekly for extended periods (6 weeks; 12 treatments), the extent of the hyperplasia is comparable to that produced by PMA with a basal cell layer covered by 10 to 15 layers of suprabasal cells (Fig. 1D).
ETHYL PHENYLPROPIOLATE AND TUMOR PROMOTION

Fig. 1. Hyperplasia in mouse skin treated with EPP or PMA. Mice were treated with acetone, EPP, or PMA and killed 48 h after the last treatment. The skins were processed for histology as described. Test compound and treatments (twice weekly) are: (A) acetone 4 times; (B) PMA (2 μg) 4 times; (C) EPP (5 mg) 4 times; (D) EPP (5 mg) 12 times. A to D, × 1600.

By the end of the experiment, however, many of the papillomas were as large as those that occur with PMA promotion.

Ornithine Decarboxylase Induction. The ability of EPP to induce epidermal hyperplasia suggested that exposure to EPP would likewise produce an increase in ODC activity. As illustrated in Table 1, the level of ODC activity measured in extracts from mouse skin treated with EPP was significantly elevated over that of acetone controls. When measured 6 h after EPP treatment, epidermal ODC activity increased up to 50 times that of control levels. A good dose response increase in activity was observed over the range of 1 to 5 mg of EPP. This increase is similar to that induced by PMA treatment in these mice (~100-fold increase). Time course studies over the period from 3 to 15 h revealed a peak of ODC induction at 6 h under the conditions used here (Fig. 3), a level similar to that seen after PMA treatment under these conditions (data not shown).

Vascular Permeability. To evaluate the effects of EPP on vascular permeability, a component of inflammation, the skin content of Evans blue dye was used as an indicator of these changes. Time course studies showed an increase in the amount of dye recovered from EPP-treated tissues that peaked at 9 h (~12 times that of control) and then declined gradually thereafter (Fig. 4). This time course is the same as that seen following PMA treatment (Ref. 25, and data not shown). The magnitude of the change in permeability 6 h after treatment with EPP is similar to that seen following PMA treatment (Fig. 4 inset; PMA was ~14 times that of the control versus EPP, which was ~8 to 10 times that of the control).

Protein Kinase C. Previous reports have demonstrated the activation and down-regulation of PKC following treatment of mouse skin with PMA (10, 23). Because this event is thought to be the controlling event in PMA-induced tumor promotion, the down-regulation of PKC following EPP treatment was measured. Whereas the application of 2 μg PMA to mouse skin leads to down-regulation of PKC at 48 h (75 to 95% down-regulation; 28 ± 17% of control activity, N = 4), application of 5 mg of EPP applied to the skin results in less than a 20% reduction in PKC activity 48 h after treatment (Fig. 5). The total PKC activity (as percentage of control) seen 48 h after treatment with 5 mg of EPP averaged 82 ± 32% (N = 4). The values obtained for EPP were not significantly different from those for acetone, whereas values for PMA were significantly different from those for acetone and for EPP at P > 0.05 (Fisher's Protected Least Significant Difference). A 24-h time point following EPP treatment also failed to show reduction in PKC activity to the degree seen following PMA treatment (data not shown). No differences in the column elution profiles or peak fractions were observed with epidermal samples from acetone-, EPP-, or PMA-treated mice, with respect to PKC activity (Fig. 5).
ETHYL PHENYLPROPIOLATE AND TUMOR PROMOTION

Fig. 2. EPP-induced tumor promotion. Groups of 30 inbred SENCAR (SSIN) mice were initiated with DMBA and promoted with EPP or PMA as described. The data are presented as the percentage of mice with tumors (A) and the average number of tumors per mouse (B). Treatment groups are: □, initiation with 10 nmol DMBA and promotion with 5 mg EPP (Group A); △, 100 nmol DMBA for initiation and 5 mg EPP for promotion (Group B); □, 100 nmol DMBA initiation and 3 mg EPP for promotion (Group C); △, 10 nmol DMBA initiation and 1 µg PMA for promotion.

Table 1 ODC induction by EPP and PMA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (µg)</th>
<th>ODC activity (nmol CO₂/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td></td>
<td>0.042 ± 0.031*</td>
</tr>
<tr>
<td>PMA</td>
<td>2</td>
<td>4.519 ± 1.317*</td>
</tr>
<tr>
<td>EPP</td>
<td>1</td>
<td>0.059 ± 0.028</td>
</tr>
<tr>
<td>EPP</td>
<td>3</td>
<td>0.694 ± 0.346</td>
</tr>
<tr>
<td>EPP</td>
<td>5</td>
<td>2.257 ± 0.888*</td>
</tr>
<tr>
<td>EPP</td>
<td>7</td>
<td>2.078 ± 0.860*</td>
</tr>
</tbody>
</table>

* Mean ± SEM, n ≥ 7.
+ Significantly different from acetone (P < 0.001, Fisher's Protected Least Significant Difference).

Phorbol ester tumor promoters also have the ability to replace the Ca²⁺ requirement of PKC for in vitro activation (24). Non-phorbol ester-type promoters do not bind to or activate PKC in vitro (26, 27). The addition of 1, 10, or 100 µg/ml EPP in the absence of Ca²⁺ to in vitro reaction mixtures failed to activate PKC, whereas the addition of 1 µg/ml of PMA (Fig. 6) in the absence of Ca²⁺ resulted in an increase in PKC activity greater than that seen with the addition of 2 mM Ca²⁺ (62.1 ± 7.7 versus 44.0 ± 7.6 pmol/mg protein/10 min for PMA and Ca²⁺, respectively).

DISCUSSION

One approach to elucidating the mechanism of action of tumor promoters, such as PMA, has been to study compounds that have biological activities similar to that of PMA but that lack the ability to promote tumor formation. Such comparisons with complete promoters should allow for the identification of those biological effects induced by promoters that are critical to the tumor promotion process. The hyperplasiogenic irritant EPP was chosen for this study based on reports which considered this compound to be nonpromoting, although it induced many of the morphological and biological effects seen following PMA treatment (12, 16).

Preliminary studies indicated that historical dose levels (10 to 15 mg/treatment) were extremely toxic to mouse skin, as evidenced by severe ulceration and sloughing of the epidermis. The study reported here used reduced levels of EPP, which...
produced no such effects in the skin and allowed sustained epidermal hyperplasia with repeated application. The data from our study support the conclusion that EPP is a moderately effective tumor promoter. Although the number of epidermal tumors (approximately five tumors/mouse) induced by EPP is lower than is seen following PMA promotion, it does equal tumor numbers seen with promotion by other moderate tumor promoters such as benzoyl peroxide (28). Also, the high incidence of tumors, up to 100%, and the fact that the response appears to be dose dependent argues strongly for the complete tumor-promoting activity of EPP. Although these data and their interpretation may appear to vary somewhat from previous reports (2, 12, 16), it should be noted that we have lowered the EPP dose to a relatively nontoxic level and are using mice that were selected for sensitivity to the tumor promotion process. The higher doses used in previous studies may have been toxic, thus negating the tumor-promoting activity of EPP. Additionally, we believe that the studies reported by Raick (12) demonstrate evidence for the complete tumor-promoting activity of EPP in Swiss-Webster ICR mice in that an average of one tumor/mouse was observed.

Also, the application of EPP to mouse skin produces morphological and biochemical changes similar to those observed following treatment with other known tumor promoters. These events include sustained hyperplasia, the induction of ODC activity, and increased vascular permeability.

Histological comparison of mouse skin exposed four times to EPP or PMA showed that both compounds elicited a hyperplastic response, with PMA producing a more pronounced hyperplasia than EPP. However, the dose of PMA (2 µg) was slightly higher than that normally used for tumor promotion in this strain of mouse and may have accounted for some of the difference. After treatments with EPP, the skin exhibited a degree of hyperplasia comparable to that produced by PMA (single or multiple PMA treatments). This delayed response to EPP may explain the delayed appearance of tumors in EPP-treated mice compared with PMA-treated animals (11 to 13 weeks for EPP versus 5 to 6 weeks for PMA). These results are in agreement with previous work that documented the ability of EPP to induce epidermal hyperplasia (12, 16, 29). The literature also indicates that EPP treatment results in an increased number of dark keratinocytes in the epidermis, although the number is smaller than is seen following PMA treatment (5, 15, 29).

The induction of ODC activity is considered to be an essential event in tumor promotion and is characteristic of all tumor promoters. ODC induction by EPP follows a time course very much like that produced by PMA, with a peak of activity between 6 and 9 h. The extent of ODC induction (~50 times that of acetone controls) was fully one-half that produced by PMA and is in concordance with its promoting activity when compared with PMA. Previous studies have reported either no induction (30) or ODC induction of 6, 28, and 30 times that of controls (7, 31, 32).

Our study also demonstrated the ability of EPP to induce vascular permeability as measured by dye leakage in treated skin. These observations are in agreement with previous reports of increased permeability (13) and increased dermal cellular infiltrates (15) following EPP treatment. There are other reports that indicate that EPP produces responses similar to PMA, although to a smaller extent. These include increased protein synthesis (33), lipid peroxidation (34), sister chromatid exchange (35), and xanthine oxidase levels (36).

The phorbol ester-type tumor promoters act through a cellular receptor, PKC (10, 11). Other, non-phorbol ester promoters, such as palytoxin and thapsigargin, do not appear to interact with PKC (26, 27), nor do they induce ODC activity. EPP may be a tumor promoter of a type different from either the phorbol ester type or the non-phorbol esters such as palytoxin or thapsigargin, since it induces ODC activity but does not down-regulate PKC. Donnelly et al. (37) have previously reported a lack of effect by EPP on PKC down-regulation. The possibility cannot be ruled out, however, that EPP effects are mediated via only one or more isozymes of PKC, or that EPP may bind to PKC with very low affinity and thus produce only minimal activation. Either of these could account for the observation in this study that EPP treatment resulted in a 20% down-regulation of PKC (~80% for PMA) and a 50-fold increase in ODC activity (100-fold for PMA). Overall, it appears that EPP works through a mechanism independent of PKC and that the small amount of apparent down-regulation is not significant.
and is a reflection of the hyperplastic state of the epidermis, as has been indicated for benzyl peroxide (37). It is possible that the activation of certain cellular processes could result in the indirect activation of PKC. Fatty acids, including arachidonic acid and its metabolites, have been shown to activate PKC (38, 39). By stimulating the production of such products, EPP could indirectly cause some activation of PKC, thus accounting for the 20% down-regulation.

A number of other reports indicate that the mechanism of action of EPP is different from that of PMA. EPP does not alter yolk sac morphology, as does PMA (40), it has no effect on terminal differentiation in mouse keratinocytes (41), it does not induce terminal differentiation in human keratinocytes (42), and EPP-induced ODC activity is not inhibited by indomethacin or enhanced by prostaglandin E₂, as is induction by PMA (43). These reports, along with the data presented here, indicate that EPP probably operates through a mechanism different from PMA and may have a mechanism of action different from other non-phorbol ester promoters such as palytoxin and thapsigargin.

The study presented here demonstrates the ability of EPP to promote tumors in the initiated skin of SSIN mice and to cause the morphological and biochemical processes that are necessary for tumor promotion to occur, in a manner comparable to that of PMA. The mechanism of action of EPP does not appear to be through direct interaction with PKC, the cell receptor for PMA. Further studies now in progress to examine more closely the effects of EPP on PKC should provide a clearer understanding of the mechanisms involved.

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Tumor-promoting Activity of Ethyl Phenylpropiolate

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