Inhibition of 12-O-Tetradecanoylphorbol-13-acetate-induced Tumor Promotion and Epidermal Ornithine Decarboxylase Activity in Mouse Skin by Palmitoylcarnitine

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ABSTRACT

Palmitoylcarnitine, which has been reported to be an inhibitor of calcium-activated, phospholipid-dependent protein kinase (protein kinase C), inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal ornithine decarboxylase in mouse skin in a dose-dependent manner. Neither acetyl carnitine nor palmitic acid inhibited TPA-caused ornithine decarboxylase induction. In addition, palmitoylcarnitine markedly inhibited skin tumor promotion induced by TPA. Palmitoylcarnitine inhibited epidermal protein kinase C activity which was stimulated by Ca2+ in the presence of phosphatidylserine but failed to inhibit the enzyme activity which was stimulated by TPA in the presence of either phosphatidylserine or Ca2+ plus phosphatidylserine. Therefore, it seems unlikely that the potent anti-tumor-promoting action of palmitoylcarnitine, which is shown in the present study, is explained solely by its effect on protein kinase C.

INTRODUCTION

In mouse epidermis, induction of ODC4 is observed with topical application of TPA (1). It has been suggested that ODC induction is an essential factor for the mechanism of skin tumor promotion (2). Several agents such as steroidal antiinflammatory drugs (3), retinoids (4), difluoromethylornithine (5, 6), and various anticancer drugs (3), retinoids (4), difluoromethylornithine (5, 6), and various anticancer agents (3) inhibit TPA-caused ODC induction or tumor promotion. These studies have aided elucidation of the biochemical mechanism of TPA action in epidermal cells. However, the precise biochemical mechanism of TPA action in mouse skin has not yet been fully understood.

In general, it is considered that the initial event involved in TPA action is the binding of TPA to its plasma membrane-located receptors (12, 13). Recently, it has been reported that TPA directly stimulates protein kinase C (14) and that protein kinase C seems to be identical to the specific binding site for TPA (15-17). Thus, it might be possible that at least a part of TPA action is mediated through the activation of protein kinase C. Palmitoylcarnitine inhibits protein kinase C activity in several tissues, at least in cell free systems (18-21). Recently, we also observed the inhibitory effect of palmitoylcarnitine on TPA action in intact cell systems, i.e., the inhibition of TPA-induced differentiation of HL-60 cells by palmitoylcarnitine (22).

In the present study, we investigated the effect of palmitoylcarnitine on TPA-induced skin tumor promotion and epidermal ODC and on protein kinase C in mouse epidermis.

MATERIALS AND METHODS

Chemicals. TPA, dimethylbenz(a)anthracene, DL-palmitoylcarnitine, DL-acetylcarnitine, palmitic acid, L-α-phosphatidyl-L-serine, ATP, histone (Type III-S), and PMSF were purchased from Sigma Chemical Co., St. Louis, MO. DL-Palmitoylcarnitine was kindly donated by Nippon Kayaku Co. Ltd., Tokyo, Japan. DL-[U-14C]Ornithine (51.3 mCi/mmol) and [γ-32P]ATP (10.5 Ci/mmol) were obtained from New England Nuclear, Boston, MA. [γ-32P]ATP was diluted approximately 10 times with cold ATP before use. The other chemicals used were reagent grade.

Animals and Treatments. Female CD-1 mice (Charles River, Atsugi, Japan), 7-8 weeks of age, were used. Mice were housed in an air-conditioned room (22-23°C) with a light period from 6 a.m. to 6 p.m. Food and water were available ad libitum. The dorsal hair of each mouse was shaved with clippers at least 2 days before use, and only those mice in a resting phase of the hair cycle were used. All chemicals were dissolved in reagent grade acetone: ethanol (1:1, vol/vol) and were applied to the shaved area in a volume of 0.2 ml using a micropipet. Mice were topically treated with vehicle, palmitoylcarnitine, palmitic acid, or acetylcarnitine before, concurrently with, or after TPA application as indicated.

Assay of ODC Activity. Usually 5 h after TPA application, these mice were killed by cervical dislocation, except in the experiment shown in Fig. 1. Epidermis was separated by a brief heat treatment (1), and ODC activities of the soluble epidermal supernatants were determined by measuring the release of 14CO2 from [1-14C]ornithine, as described previously (1, 10). Statistical analysis was done by t-test.

Tumor Promotion Experiment. Groups of 20 mice were initiated by applying 200 nmol of dimethylbenz(a)anthracene to the dorsal skin. Promotion with 5 nmol of TPA applied twice weekly was begun 10 days after initiation. Palmitoylcarnitine (0.3, 1, and 3 μmol) was applied concurrently with TPA. These treatments continued for 18 weeks.

Protein Kinase C Activity. The methods of Ashendel et al. (23) and Couturier et al. (24) were modified slightly and used for the present study of protein kinase C activity in mouse epidermis. Epidermis was homogenized in 3 volumes (w/v) of 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA, 2 mM EGTA, and 2 mM PMSF with a polytron homogenizer, and the homogenate was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant obtained was ultracentrifuged at 105,000 x g for 60 min at 4°C. The resultant cytosol fraction was loaded onto a 1.5 x 14 cm column of Sephadex G25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA, 2 mM EGTA, and 2 mM PMSF. The macromolecular fraction (molecular weight, >5000) was used for the assay of protein kinase C activity. Protein kinase activity was determined by incubating 2.5 nmol [γ-32P]ATP (3-5 x 104 cpm), 30 μg histone, 500 nmol magnesium acetate, 6.25 μmol 2-mercaptoethanol, and 15 μl (10-15 μg protein) of partially purified cytosol extract in 125 μl of 20 mM Tris-HCl buffer (pH 7.4) buffer in the presence or absence of palmitoylcarnitine. In Experiment 1 shown in Table 2, 3.1 μg phosphatidyserine and 37.5 nmol CaCl2 were also added to the above incubation mixture, and in Experiment 2, 3.1 μg phosphatidyserine, 125 nmol EGTA, and 3.75 pmol TPA were added. In an experiment shown in Fig. 5, 3.1 μg phosphatidyserine, 0-37.5 nmol CaCl2, and 3.75 pmol TPA were added. In the case of 0 calcium, 125 nmol EGTA were added into the reaction mixtures instead of CaCl2. The reaction was started by the addition of [γ-32P]ATP. Incubation was carried out at 37°C for 3 min; then the reaction was stopped by transferring a 50-μl aliquot of the incubation mixture onto 2.5 cm2 pieces of Whatman cellulose
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phosphate paper (P81). The pieces of paper were washed 4 times with deionized water, twice with acetone, and once with petroleum-ether. The radioactivity on each piece of paper was determined by scintillation counting.

RESULTS AND DISCUSSION

Application of 10 nmol of TPA to mouse skin led to a transient induction of epidermal ODC activity, and a peak activity was observed about 7 h after TPA treatment (Fig. 1). The time course of the enzyme induction is almost in agreement with that reported by O'Brien et al. (1). Application of 2.5 μmol of palmitoylcarnitine to mouse skin inhibited TPA-caused epidermal ODC induction (Fig. 1). Inhibitory effect of palmitoylcarnitine on TPA-caused ODC induction was dose-dependent (Fig. 2). Histological studies showed that the treatment of mice with palmitoylcarnitine at the doses used in the present study did not show obvious pathological changes in epidermal cells (data not shown). As shown in Fig. 3, the inhibition of TPA-caused ODC induction by palmitoylcarnitine was dependent on the time of application of palmitoylcarnitine relative to the time of TPA application. Application of palmitoylcarnitine (2.5 μmol/mouse) to the skin 1 h before or concurrently with TPA treatment elicited significant inhibition of TPA-caused ODC induction by 65 or 85%, respectively. However, application of palmitoylcarnitine (2.5 μmol/mouse) 2 h before or after TPA treatment failed to result in significant inhibition. In addition, palmitoylcarnitine applied 1 h after TPA treatment failed to inhibit TPA-caused ODC induction. Thus, it is unlikely that inhibition of ODC induction is due to the general cell toxicity induced by palmitoylcarnitine. Application of palmitic acid (1 μmol/mouse) and acetylcarnitine (1 μmol/mouse) to the mouse skin 30 min before and concurrently with TPA failed to inhibit TPA-caused ODC induction, while treatment of mice with the same dose of palmitoylcarnitine inhibited it significantly (Table 1, Experiment 1). In addition, treatment of mice with higher doses of palmitic acid and acetylcarnitine also failed to inhibit the TPA-caused ODC induction (Table 1, Experiment II). These results suggest that inhibition of ODC induction is specific for palmitoylcarnitine. Palmitoylcarnitine did not directly interfere with assay for ODC activity (data not shown).

The effect of palmitoylcarnitine on the incidence of TPA-induced skin papilloma formation was examined. As shown in Fig. 4, treatment of initiated mice with 5 nmol of TPA alone resulted in 29.9 papillomas/mouse 18 weeks after the start of promotion. Application of palmitoylcarnitine (1–3 μmol) concurrently with TPA resulted in 63–84% inhibition in the number of papillomas per mouse compared with mice receiving only TPA (at the 18th week). The percentage of tumor-bearing mice was also markedly reduced by the treatment with palmitoylcarnitine (Fig. 4). Body weight gain of palmitoylcarnitine-treated mice was not significantly different from that of the non-treated group (data not shown). Therefore, the present results clearly show that palmitoylcarnitine inhibited not only TPA-caused
Table 1 Effect of palmitoylcarnitine, acetylcarnitine, and palmitic acid on TPA-caused epidermal ODC induction

Mice were treated with vehicle, palmitoylcarnitine, acetylcarnitine, or palmitic acid 30 min prior to the application of TPA (10 nmol/mouse). Each inhibitor was applied again to the mouse skin concurrently with TPA. Mice were killed 5 h after TPA treatment for the determination of ODC activity.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>ODC activity (nmol CO2/60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Experiment I</td>
<td>Vehicle</td>
<td>0.07 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>1.21 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>TPA (10 nmol)</td>
<td>0.64 ± 0.15b</td>
</tr>
<tr>
<td></td>
<td>Palmitoylcarnitine (1 µmol)</td>
<td>1.18 ± 0.14c</td>
</tr>
<tr>
<td></td>
<td>(6 µmol)</td>
<td>1.83 ± 0.36d</td>
</tr>
<tr>
<td></td>
<td>Acetylcarnitine (1 µmol)</td>
<td>1.58 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>(6 µmol)</td>
<td>0.41 ± 0.09c</td>
</tr>
<tr>
<td></td>
<td>Palmitic acid (1 µmol)</td>
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</tr>
<tr>
<td></td>
<td>(6 µmol)</td>
<td>1.51 ± 0.26c</td>
</tr>
<tr>
<td>Experiment II</td>
<td>Vehicle</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>1.58 ± 0.13</td>
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<tr>
<td></td>
<td>TPA (10 nmol)</td>
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<td>Palmitoylcarnitine (6 µmol)</td>
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</tr>
<tr>
<td></td>
<td>(6 µmol)</td>
<td>1.51 ± 0.26c</td>
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</tbody>
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* Mean ± SE of individual determinations from 5 mice.
† P < 0.05 versus TPA.
‡ Not significantly different from TPA.
§ P < 0.01 versus TPA.

TPA-caused epidermal ODC induction but also TPA-induced tumor promotion in mouse skin.

Recently, we showed that palmitoylcarnitine fails to inhibit [3H]TPA binding in HL-60 cells (22). Therefore, it seems unlikely that the potent anti-tumor-promoting action of palmitoylcarnitine is due to the inhibition of TPA binding by this agent. Meanwhile, at least in a cell-free system, palmitoylcarnitine is shown to be an inhibitor of protein kinase C (18–21). We further studied the effect of palmitoylcarnitine on protein kinase C activity in mouse epidermis.

Partially purified cytosol fraction of epidermal homogenate was used for the assay of protein kinase activity. Protein kinase activity was measured using [γ-32P]ATP and histone as substrates. Protein kinase C activity was detected in the presence of Ca2+ (12.5–37.5 nmol/tube) plus phosphatidylserine (Table 2 and Fig. 5). This is consistent with previous reports (23, 25). Protein kinase activity was also stimulated by the addition of TPA, phosphatidylserine, and EGTA but not Ca2+. The stimulatory effect of TPA on protein kinase activity, which was observed in the presence of phosphatidylserine, was evident only under the addition of none or a small amount of CaCl2 (3.8 nmol/tube). When a larger amount of CaCl2 (12.5–37.5 nmol/tube) was added to the incubation mixture, the stimulatory effect of TPA became diminished. The reason for this is that the enzyme activity was stimulated solely by Ca2+ in the presence of phosphatidylserine (Fig. 5). That is to say, in the presence of phosphatidylserine, neither the protein kinase activity stimulated by Ca2+ nor the activity stimulated by TPA showed additive effects. Couturier et al. (24) also observed the protein kinase activity in crude 100,000 × g supernatant of brain homogenate in the presence of phosphatidylserine, TPA, and 0.5 mM EGTA but in the absence of added Ca2+. Donnelly and Jensen (26) also found that TPA is able to stimulate partially purified liver protein kinase C in the presence of phosphatidylserine and EGTA but without Ca2+. They claimed that TPA activates protein kinase C by a mechanism that is independent of Ca2+. Couturier et al. (24), however, reported that in the presence of 50 mM EGTA, protein kinase activity stimulated by TPA plus phosphatidylserine is no longer observed. Based on their data, they claimed that tumor promoter does not substitute for Ca2+ but decreases Ca2+ requirements far below those which can be measured using available techniques at the present time. Therefore, at present, we cannot draw a conclusion as to whether TPA plus phosphatidylserine can activate protein kinase C in the complete absence of Ca2+.

Table 2 Protein kinase activity in the cytosolic fraction of mouse epidermal homogenate in the presence of either Ca2+ plus phosphatidylserine or TPA plus phosphatidylserine and the effects of palmitoylcarnitine on these activities

Cytosol fraction of epidermal homogenate was partially purified by a gel filtration (Sephadex G25), and the macromolecular fraction was used as a crude enzyme preparation. Protein kinase activity was determined as described in "Materials and Methods."
which is stimulated by TPA plus phosphatidylserine is probably due to protein kinase C.

The effect of palmitoylcarnitine on protein kinase C activity in mouse epidermis was also examined. As shown in Table 2, palmitoylcarnitine inhibited protein kinase activity stimulated by Ca\(^{2+}\), indicating that this compound inhibits Ca\(^{2+}\)-dependent protein kinase(s), such as calmodulin-dependent kinase (19). Palmitoylcarnitine also inhibited protein kinase activity stimulated by Ca\(^{2+}\) plus phosphatidylserine (Table 2 and Fig. 5). The inhibitory effect of palmitoylcarnitine on protein kinase C stimulated by Ca\(^{2+}\) plus phosphatidylserine has been reported previously (18–21). As far as we know, there is no report concerning whether palmitoylcarnitine inhibits protein kinase C activity stimulated by TPA. As shown in Table 2 and Fig. 5, palmitoylcarnitine failed to inhibit protein kinase C activity stimulated by TPA irrespective of any Ca\(^{2+}\) concentration present in the medium. Similar results were obtained using partially purified enzyme preparation of mouse brain homogenate (data not shown). Thus it is highly possible that palmitoylcarnitine cannot inhibit TPA-stimulated protein kinase C activity in an intact cell system, i.e., epidermis.

At present, the mechanism involved in the anti-tumor-promoting action of palmitoylcarnitine is unclear. Recently, it has been reported that the Ca\(^{2+}\)-calmodulin system may be involved in the mechanism of the actions of tumor promoters, such as teleocidin (27) and TPA (28), and that palmitoylcarnitine also inhibits calmodulin-sensitive, Ca\(^{2+}\)-dependent protein kinase at relatively high concentrations (19). Although calmodulin-dependent actions are more potently inhibited by W-7 than palmitoylcarnitine (19, 29), TPA-caused epidermal ODC induction and mouse skin tumor promotion were less potently inhibited by W-7 than palmitoylcarnitine (30). Thus, it seems unlikely that an anti-calmodulin action of palmitoylcarnitine plays a major role in the mechanism of the anti-tumor-promoting activity of this agent. It has also been reported that a relatively high concentration of palmitoylcarnitine inhibits lysophospholipase (31), Na\(^{+}\), K\(^{+}\)-ATPase (32, 33), or Ca\(^{2+}\)-ATPase (32). However, it also seems unlikely that the above-mentioned effects of palmitoylcarnitine play a major role in the mechanism of the anti-tumor-promoting activity of this agent, because only at relatively high concentrations is palmitoylcarnitine able to inhibit the above-mentioned enzymes. However, we cannot completely exclude the involvement of inhibiting these enzyme systems in the mechanism of the anti-tumor-promoting action of palmitoylcarnitine.

In summary, the present results clearly show the anti-tumor-promoting action of palmitoylcarnitine. However, it seems unlikely that the anti-tumor-promoting action of palmitoylcarnitine is explained solely by its effect on protein kinase C activity. Further investigation is now under way.

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REFERENCES

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