Inhibitory Regulation of Serum Factor(s)-Caused Ornithine Decarboxylase Induction by the Protein Kinase C System in A431 Human Epidermoid Carcinoma Cells

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ABSTRACT — Replacement of the culture medium with fresh medium containing 10% fetal calf serum caused ornithine decarboxylase (ODC) induction in A431 human epidermoid carcinoma cells. Two peaks of ODC activity were observed at 5 and 14 hr after the medium replacement. The peak activity observed at 5 hr was more prominent than that at 14 hr. The first peak of ODC induction was suppressed by a potent protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), in a concentration-dependent manner. The second peak, however, was not suppressed by TPA. Other potent protein kinase C activators, such as mezerein and 12-O-retinoylphorbol-13-acetate, also suppressed the first peak of ODC induction. Synthetic diacylglycerols, 1,2-dioctanoyl-sn-glycerol and 1-oleoyl-2-acetylglycerol, did not inhibit the serum factor(s)-caused ODC induction. Phorbol-13-acetate, an inactive phorbol ester, also failed to inhibit the ODC induction. The growth of A431 cells was slightly suppressed by TPA. In protein kinase C down-regulated cells, TPA failed to inhibit the serum factor(s)-caused ODC induction. These results suggest that the serum factor(s)-caused ODC induction in A431 cells is negatively regulated by the protein kinase C system, which may not be activated by exogenous diacylglycerols.

Tumor promoter phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) induce various biochemical and biological events, including stimulatory or inhibitory effects on cell growth and differentiation (1, 2). An increased level of intracellular polyamine is observed when the cells respond to growth stimuli. The rate-limiting enzyme of polyamine synthesis is ornithine decarboxylase (ODC), and the induction of this enzyme by growth stimuli is a good index of cell growth (3, 4). We have shown that protein kinase C plays an essential role in TPA-evoked ODC induction in mouse skin and epidermal cells (5–7), targets of skin tumor promotion by this agent. It has been reported that TPA inhibits the growth of A431 human epidermoid carcinoma cells (8). To examine the role of protein kinase C in ODC induction in the cells whose
growth is inhibited by TPA, we examined the effects of TPA and protein kinase C activators on serum factor(s)-caused ODC induction in A431 cells and found that ODC induction is negatively regulated by the protein kinase C system in these cells.

MATERIALS AND METHODS

Materials

The following materials were purchased from the indicated companies: 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (dihydrochloride salt), from Seikagaku Kogyo Co., Ltd., Tokyo, Japan; DEAE-cellulose (DE52), from Whatman, Ltd., Maidstone, UK; TPA, from Chemicals for Cancer Research, Inc., Chicago, IL; 1,2-dioctanoyl-sn-glycerol (DOG), 1-oleoyl-2-acetylglycerol (OAG), mezerein, 12-O-retinoylphorbol-13-acetate (RPA), phorbol-13-acetate and Triton X-100, from Sigma Chemical Co., St. Louis, MO; Dulbecco's modified Eagle's medium (DMEM), from GIBCO, Grand Island, NY; fetal calf serum, from Hyclone Lab., Logan, UT; DL-[1-14C]ornithine (58 mCi/mmol), from Amersham Laboratories, Buckinghamshire, UK; [3H]phorbol-12,13-dibutyrate (PDBu), from New England Nuclear, Boston, MA. R59022 was obtained from Janssen, Beerse, Belgium. The other chemicals used were of reagent grade. A431 cells were kindly donated by Dr. S. Gamou, Department of Molecular Biology, School of Medicine, Keio University.

Culture of A431 cells

A431 cells were plated on 35-mm plastic dish at an initial density of $2 \times 10^5$ cells/dish and were cultured in DMEM (glucose 4.5 mg/ml) supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% air/5% CO₂ without changing the medium. On the third day of culture, experiments were performed. Cell viability was determined by the trypan blue exclusion test. TPA, mezerein, RPA and phorbol-13-acetate were dissolved in dimethylsulfoxide (DMSO) and added to the culture medium. Final concentration of DMSO was 0.1%. 1,2-Diacylglycerols, i.e. DOG and OAG, were dispersed by sonication in a small volume of phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS(−)) and added to the culture medium.

ODC induction

On the day of experiments, the medium of either normal or protein kinase C-down-regulated A431 cells in the 35-mm dish was changed to fresh medium. Then the cells were incubated for the indicated time periods at 37°C in an atmosphere of 95% air/5% CO₂ in the presence or absence of the indicated drugs. Drugs were added to the fresh medium just after the medium-replacement. After the incubation, the cells were washed 3 times with PBS(−), frozen on dry ice and stored at −80°C until assay. The ODC assay was performed as follows: The cells were sonicated in 50 mM sodium phosphate buffer, pH 7.2, containing 200 μM pyridoxal 5'-phosphate and 50 μM EDTA, and centrifuged at 15,000 × g for 30 min. ODC activity of the supernatant was determined by measuring the release of 14CO₂ from DL-[1-14C]ornithine as described previously (6).

Preparation of protein kinase C-down-regulated cells

Protein kinase C-down-regulated cells were prepared by treating the cells with 50 nM TPA for 48 hr as described previously (6). The cells were then washed twice with PBS(−) and once with PBS(−) supplemented with 0.1% bovine serum albumin in order to wash out TPA. Using these protein kinase C-down-regulated cells, the effect of TPA on the medium replacement-caused ODC induction was also examined just as described above.

To confirm the down regulation of protein kinase C in the TPA-treated cells, the amount of cellular protein kinase C was determined by [³H]PDBu binding assay just as described previously (6). In brief, the cells were sonicated and centrifuged at 105,000 × g for 60 min. The resultant cytosol and particulate fractions
were treated with Triton X-100. The Triton X-100-treated particulate fraction was centrifuged again at 105,000 × g for 60 min. The resultant supernatant (solubilized particulate fraction) and Triton X-100-treated cytosol fraction were subjected to DEAE cellulose (DE52) column chromatography. The amount of protein kinase C eluted from the DE52 column was determined by [3H]PDBu binding assay. The binding reaction was carried out by incubating the sample solution with 15 nM [3H]PDBu in the presence of Ca$^{2+}$ and phosphatidylserine at 37°C for 5 min. Bound and free [3H]PDBu were separated by the polyethylene glycol precipitation method. For the estimation of non-specific binding, 30 μM unlabeled PDBu was added to the reaction mixture. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

Cell proliferation test

A431 cells (10⁴ cells) placed in the 16 mm-diameter well of plastic multidishes were cultured in the above-mentioned culture medium for 24 hr; then the medium was changed to a fresh one. The cells were further cultured in the presence or absence of 30 nM TPA for 96 hr, and the number of viable cells was counted.

RESULTS

The replacement of culture medium containing 10% fetal calf serum with fresh medium caused ODC induction in A431 cells (Fig. 1). Two peaks of activity were observed 5 and 14 hr after the medium-replacement. The medium containing fetal calf serum was effective for inducing ODC activity, but the medium without fetal calf serum and the conditioned medium were not effective (data not shown). The peak ODC activity observed at 5 hr was more prominent than that observed at 14 hr (Fig. 1). When the cells were treated with 30 nM TPA, the first peak of ODC induction at 5 hr was markedly suppressed (Fig. 1). Although the second peak of ODC induction was apparently not suppressed by TPA, the time of peak activity was shifted from 14 hr to 10 hr. Without medium-replacement, ODC activity was neither altered by vehicle.

**Fig. 1.** Effect of TPA on serum factor(s)-caused ODC induction in A431 cells (time course). The culture medium of A431 cells was replaced with fresh medium at time 0 (circle). The medium-replacement was not performed for some groups of cells (square). The cells were treated with 30 nM TPA (○; ■) or vehicle (□; □) just after the medium-replacement. Then the cells were incubated for the indicated time periods, and cellular ODC activity was determined. Each point and vertical bar represent the mean ± S.E. (n = 3). **P < 0.01, *P < 0.05 vs. medium-replaced plus vehicle-treated cells.
nor by TPA (Fig. 1). The serum factor(s)-caused ODC induction was inhibited by TPA in a concentration-dependent manner (Fig. 2). Phorbol-13-acetate, an inactive phorbol ester (9), failed to inhibit the serum factor(s)-caused ODC induction (Fig. 2). Potent protein kinase C activators, such as mezerein (10) and RPA (11), inhibited the serum factor(s)-caused ODC induction (Fig. 3).

The effect of synthetic 1,2-diacylglycerols, such as DOG and OAG, on the serum factor(s)-caused ODC induction was also examined. These diacylglycerols may activate protein kinase C and mimic TPA actions in various intact cell systems (5, 6, 12, 13). As shown in Fig. 4, DOG (100 μM) failed to inhibit the serum factor(s)-caused ODC induction. Even with 300 μM DOG or 300 μM OAG, the ODC induction was not inhibited (data not shown). Since it cannot be denied that these diacylglycerols are ineffective because of their relatively rapid metabolism compared to TPA, the effects of cumulative addition of DOG and OAG were also investigated. The cells were first treated with 100 μM DOG or 100 μM OAG, and then the same amount of each diacylglycerol was added again to the medium at 2 hr after the first treatment. As clearly shown in Table 1, cumulative addition of diacylglycerols again failed to inhibit ODC induction. Since diacylglycerols are generally phosphorylated into phosphatidic acid by diacylglycerol kinase (12, 13), we also examined the effects of DOG and OAG on the serum factor(s)-caused ODC induction in the presence of the diacylglycerol kinase inhibitor R59022 (14). Although R59022 (10 μM) by itself substantially inhibited (34% inhibition) the serum factor(s)-caused ODC induction, both DOG and OAG did not show any inhibitory effect even in the presence of R59022 (data not shown).

We next tried to examine the effect of H-7, a well-known protein kinase C inhibitor (15), on the TPA-caused inhibition of ODC induction. However, H-7 by itself inhibited the

![Fig. 2. Effects of TPA and phorbol-13-acetate on serum factor(s)-caused ODC induction in A431 cells (concentration-response relationships). TPA (0.03-30 nM), phorbol-13-acetate (3-300 nM) or vehicle was added to the medium just after the medium-replacement. The cells were incubated for 5 hr, and cellular ODC activity was determined. Each column and vertical bar represent the mean ± S.E. (n = 4). **P < 0.01 vs. medium-replaced plus vehicle-treated cells.](image-url)
serum-factor(s)-caused ODC induction: i.e.,
with 10 μM H-7, 46 ± 5% inhibition; with 30 μM H-7, 85 ± 2% inhibition. Therefore, the
effect of H-7 on TPA-caused inhibition could not be determined.

Fig. 3. Effects of mezerein (A) and RPA (B) on serum factor(s)-caused ODC induction in A431 cells. The medium-replacement was performed at time 0. Vehicle (・), 30 nM mezerein (△), 30 nM RPA (■) or 30 nM TPA (●) was added to the medium just after the medium-replacement. The cells were incubated for the indicated time periods, and cellular ODC activity was determined. Each point and vertical bar represent the mean ± S.E. (n = 3). **P < 0.01, *P < 0.05 vs. vehicle-treated cells.

Fig. 4. Effect of DOG on serum factor(s)-caused ODC induction in A431 cells. The medium was replaced with fresh medium at time 0. Vehicle (・), 100 μM DOG (□) or 30 nM TPA (●) was added to the medium just after the medium-replacement. The cells were incubated for the indicated time periods, and cellular ODC activity was determined. Each point and vertical bar represent the mean ± S.E. (n = 3). **P < 0.01, *P < 0.05 vs. vehicle-treated cells.

Table 1. Effects of cumulative addition of DOG or OAG on serum factor(s)-caused ODC induction in A431 cells

| Addition | ODC activity (nmol CO₂/hr/mg protein) |
|----------|-------------------------------------|
| Vehicle  | 2.16 ± 0.26 (n = 4) |
| DOG × 2  | 1.94 ± 0.08 (n = 5) |
| OAG × 2  | 2.37 ± 0.03 (n = 5) |
| TPA × 1  | 0.76 ± 0.02* (n = 4) |

DOG (100 μM) or OAG (100 μM) was added to the medium right after the medium replacement. Two hours after the medium replacement, the same amount of DOG or OAG was cumulatively added to the medium. TPA (30 nM) was added to the medium only right after the medium replacement. Cellular ODC activity was determined 5 hr after the medium replacement. Each value represents the mean ± S.E. *P < 0.01 vs. vehicle

The effect of TPA on the growth of A431 cells was also examined. As shown in Table 2, TPA significantly suppressed the proliferation of A431 cells, but the inhibition was rather
Table 2. Effect of TPA on A431 cell proliferation

|                | Number of viable cells \(\times 10^6\) cells |
|----------------|---------------------------------------------|
|                | 0 hr         | 96 hr          |
| Vehicle (DMSO)| 1.0 ± 0.1    | 76.4 ± 1.2    |
| TPA 30 nM     | 1.0 ± 0.1    | 65.1 ± 1.5*   |

A431 cells were cultured with or without 30 nM TPA for 96 hr, and the number of viable cells was counted. Each value represents the mean ± S.E. \(\bar{n} = 6\). *P < 0.05 vs. vehicle.

The result is consistent with the previous report (8).

In order to investigate whether TPA exerts its inhibitory effect on the ODC induction in protein kinase C-down-regulated cells, the following experiments were also conducted. The cells were first treated with 50 nM TPA for 48 hr in order to down regulate protein kinase C (6), and then the effect of TPA on the serum factor(s)-caused ODC induction was examined. Such a treatment caused a marked down-regulation of protein kinase C in A431 cells (Table 3). As clearly shown in Fig. 5, TPA failed to inhibit the serum factor(s)-caused ODC induction in these cells. The total amounts of cellular protein per dish of TPA-treated (protein kinase C-down-regulated) cells were not significantly different from those of vehicle-treated cells (data not shown).

Table 3. Down-regulation of protein kinase C in the TPA-treated A431 cells

|                | Specific \(^{3}H\)PDBu binding (pmol/mg protein) |
|----------------|-----------------------------------------------|
|                | Cytosol | Particulate |
| Control cells  | 4.10    | 9.93       |
| TPA-treated cells | 0.07    | 1.23       |

A431 cells were treated with 50 nM TPA for 48 hr, and the amounts of protein kinase C in the cytosol and particulate fractions were determined by the specific binding of \(^{3}H\)PDBu to this enzyme in the presence of \(\text{Ca}^{2+}\) plus phosphatidylserine. Specific binding was calculated by subtracting the nonspecific binding, which was observed in the presence of 30 \(\mu\)M unlabeled PDBu, from the total binding. Values are the means, of duplicate determinations. Similar experiments were repeated three time, and the results obtained were reproducible.

Fig. 5. Effect of TPA on serum factor(s)-caused ODC induction in protein kinase C-down-regulated A431 cells. A431 cells were preincubated either with vehicle (A) or 50 nM TPA (B) for 48 hr. Thereafter, the cells were washed, and the medium was replaced with fresh medium at time 0. Vehicle-pretreated (A) and TPA-pretreated (protein kinase C-down-regulated) (B) cells were further incubated for the indicated time periods either with vehicle (○) or 30 nM TPA (●). After incubation, cellular ODC activity was determined. Each point and vertical bar represent the mean ± S.E. \(\bar{n} = 3\). **P < 0.01 vs. vehicle-treated cells.
DISCUSSION

TPA as well as RPA and mezerein, all of which are potent protein kinase C activators (9–11), suppressed the first prominent peak of ODC induction caused by medium-replacement, i.e. serum factor(s), in A431 human epidermoid carcinoma cells. Such TPA action disappeared in the protein kinase C-down-regulated A431 cells. Moreover, phorbol-13-acetate, an inactive phorbol ester (9), failed to mimic TPA action. The results clearly indicate the inhibitory regulation by protein kinase C of serum factor(s)-caused ODC induction.

TPA not only inhibited the serum factor(s)-caused ODC induction but also inhibited the growth of A431 cells. However, the inhibitory effect of TPA on A431 cell growth was rather weak. The result is compatible with the previous report that demonstrated, a weak inhibition of A431 cell growth by TPA (8). It has generally been accepted that ODC induction is closely related to cell proliferation. Although TPA did suppress the first peak of ODC induction, its weak inhibitory effect on cell growth may be observed in its failure to suppress the second peak of ODC induction. Alternatively, the first peak of ODC induction is more closely related to the cell proliferation than the second one. The inhibitory actions of TPA on cell proliferation have also been reported in some other types of cells (16, 17). To our knowledge, however, this is the first report that shows the negative modulation of ODC induction by the protein kinase C system.

The serum factor(s)-caused ODC induction was inhibited by H-7, a well-known protein kinase C inhibitor (15). However, the fact that ODC activity was induced by a serum factor(s) even in the protein kinase C-down-regulated cells is incompatible with the idea that the activation of protein kinase C is involved in the ODC induction caused by a serum factor(s) in A431 cells. It is known that H-7 not only inhibits protein kinase C but also inhibits other protein kinases (15). Therefore, it might be possible that another protein kinase(s) is involved in the serum factor(s)-caused ODC induction.

It has been reported that 1,2-diacylglycerol is an endogenous activator of protein kinase C and the synthetic diacylglycerols, such as DOG and OAG, mimic TPA actions in various intact cell systems (5, 6, 12, 13) including the induction of ODC in isolated mouse epidermal cells (5). In A431 cells as well, these diacylglycerols mimic certain TPA actions, such as an inhibition of epidermal growth factor (EGF) binding to its receptors (18–20), stimulation of phosphorylation of serine, threonine residues of EGF receptors (18, 19). In the present study, however, 1,2-diacylglycerols such as DOG and OAG failed to inhibit the serum factor(s)-caused ODC induction in the same A431 cells. It has been reported that 1,2-diacylglycerols do not always mimic TPA actions (21–24). In fact, in intact A65T cells (a mouse thymic leukemia cell line), TPA and DOG mutually stimulate the phosphorylation of a different set of endogenous proteins (24). A functional protein, which negatively regulates ODC induction, may not be a good substrate for the enzyme activated by diacylglycerols. Subcellular distributions of protein kinase C and/or the substrate protein may also contribute to such a phenomenon.

In conclusion, the present study strongly suggests that the serum factor(s)-caused ODC induction in A431 cells is negatively regulated by the protein kinase C system, which is not activated by exogenous diacylglycerols.

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