0026-895X/89/060917-08$02.00/0

MOLECULAR PHARMACOLOGY, 36:917-924

H-7, a Protein Kinase C Inhibitor, Inhibits Phorbol Ester-Caused Ornithine Decarboxylase Induction but Fails to Inhibit Phorbol Ester-Caused Suppression of Epidermal Growth Factor Binding in Primary Cultured Mouse Epidermal Cells

TERUO NAKADATE,[1] SATOSHI YAMAMOTO, ERIKO AIZU, KIYOTAKA NISHIKAWA, and RYUICHI KATO

Department of Pharmacology, School of Medicine, Keio University, Tokyo 160, Japan

Received April 25, 1989; Accepted September 28, 1989

SUMMARY

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) induced ornithine decarboxylase (ODC) and suppressed ^{125}I-epidermal growth factor (EGF) binding in primary cultured mouse epidermal cells. TPA (30 nM)-caused ODC induction was almost completely blocked by 30 μM H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], a well known protein kinase C inhibitor, but the same concentration of H-7 failed to restore the ^{125}I-EGF binding suppressed by TPA (10 nM). On the other hand, sphingosine, another protein kinase C inhibitor, blocked not only TPA-caused ODC induction but also TPA-caused suppression of ^{125}I-EGF binding. Concentration-response curves of sphingosine for these two TPA-caused cellular responses were almost identical. 1,2-Diacylglycerols such as 1,2-dioctanoylglycerol (30–300 μM) and 1-oleoyl-2-acetylglycerol (OAG) (30–300 μM) mimicked TPA actions. Similar to the case of TPA, suppression of ^{125}I-EGF binding by OAG was barely inhibited by H-7, whereas sphingosine was more effective in inhibiting the OAG-caused suppression of ^{125}I-EGF binding than was H-7. In TPA (50 nM)-pretreated epidermal cells, TPA (10 nM) failed to suppress ^{125}I-EGF binding. H-7 (30 μM) did not affect TPA (30 nM)-caused translocation of protein kinase C. These results clearly demonstrate the differential inhibition by H-7 of the TPA-caused cellular responses and indicate that TPA-caused suppression of ^{125}I-EGF binding to epidermal cells is mediated through protein kinase C function, which is barely inhibited by H-7.

TPA is known to be the most potent skin tumor promoter among various tumor-promoting agents and induces a variety of biochemical changes, such as ODC induction and suppression of ^{125}I-EGF binding in epidermal cells, the main target of two-stage skin carcinogenesis (1–3). Protein kinase C is the receptor of the phorbol ester tumor promoters (4–6) and mediates one arm of the phosphatidylinositol second messenger system, which is involved in signal transduction for a broad array of hormones and cellular effectors including both growth factors and several oncogenes (7, 8).

In fact, several lines of studies demonstrate the participation of protein kinase C in cellular responses induced by TPA in epidermal cells. DG has been considered to be the endogenous activator or ligand of protein kinase C/phorbol ester receptor (4–8). Jeng *et al.* (9) suggested that DGs, which are endogenously generated by the activation of phospholipase C, induce ODC activity and inhibit ^{125}I-EGF binding through the activation of protein kinase C in primary cultured mouse epidermal cells. It has been also demonstrated that synthetic DGs cause ODC induction in both mouse epidermal cells (10, 11) and epidermis (12). Furthermore, it has been recently demonstrated that DG is a second stage tumor promoter in mouse skin (13).

The other conventional way to elucidate the mechanism of TPA action is the use of inhibitors of protein kinase C. For further understanding of the role of protein kinase C in the mechanism of TPA action, we studied the effects of H-7, a well known protein kinase C inhibitor (14), on the TPA-caused ODC induction and the TPA-caused suppression of ^{125}I-EGF binding in primary cultured epidermal cells.

In the present study, we show the differential inhibition by H-7 of the TPA-caused cellular responses that may be mediated through the activation of protein kinase C.

This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan. The work was also supported by a grant from Takeda Science Foundation, Osaka, Japan and a grant from Keio Gijuku, Academic Development Funds, Keio University, Tokyo, Japan.

[1] Present address: Laboratory of Pharmacology, Basic Research Laboratories, Toray Industries, Inc., 1111 Tebiro, Kamakura, Kanagawa 248, Japan.

ABBREVIATIONS: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DG, 1,2-diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol; ODC, ornithine decarboxylase; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; EGF, epidermal growth factor; DOG, 1,2-dioctanoylglycerol; PDBu, phorbol-12,13-dibutylate; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide, PBS(−), phosphate-buffered saline without Ca^{2+} and Mg^{2+}; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Chemicals. The following materials were purchased from the companies indicated: H-7 from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan); DOG, OAG, PDBu, PMSF, and Triton X-100 from Sigma Chemical Co. (St. Louis, MO); Eagle's minimum essential medium (Earle's salts) from GIBCO (Grand Island, NY); fetal calf serum from Hyclone (Logan, UT); DEAE-cellulose (DE52) from Whatman, Ltd. (Maidstone, UK); Chelex 100 from Bio-Rad Laboratories (Richmond, CA); sphingosine from Calbiochem, Behring Diagnostics (La Jolla, CA); and EGF from Toyobo Co., Ltd. (Osaka, Japan). DL-[1-^{14}C]Ornithine (58 mCi/mmol) and ^{125}I-EGF (100 μCi/μg) were obtained from Amersham Laboratories (Buckinghamshire, UK). [^{3}H]PDBu was purchased from New England Nuclear (Boston, MA). The other chemicals used were reagent grade.

Primary culture of mouse epidermal cells. Primary cultured mouse epidermal cells were prepared according to the method described by Yuspa (15) with minor modifications. Epidermal cells were isolated from newborn CD-1 mice (Charles River, Atsugi, Japan) (1–2 days *post partum*), as described previously (10, 15). The cells were plated on 35-mm and 100-mm plastic dishes at an initial density of 3×10^5 cells/cm^2 in Eagle's minimum essential medium supplemented with 10% fetal calf serum, and were cultured at 36° in an atmosphere of 92% air/8% CO_2 for 20 hr. Thereafter, the medium was switched to Ca^{2+}-free Eagle's minimum essential medium supplemented with 10% Chelex-treated (Ca^{2+}-deprived) fetal calf serum and 50 μM $CaCl_2$ (final concentration). The culture medium contained the following antibiotics: 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml fungizone. The medium was changed daily and all experiments were performed with confluent cells at 4 days of cultures, except for the experiments using TPA-pretreated cells (protein kinase C-down-regulated cells). Cell viability was determined by the trypan blue exclusion test. TPA, sphingosine, and H-7 (dihydrochloride salt) were dissolved in DMSO, DMSO, and PBS(−), respectively, and were added to the medium of cell cultures. Final concentrations of DMSO and PBS(−) were 0.1 and 1%, respectively.

ODC induction. Primary cultured epidermal cells in 35-mm dishes were treated with TPA or vehicle on the fourth day of culture in low-Ca^{2+} medium. On the day of experiments, the medium change was not performed, in order to prevent the ODC induction caused by the medium change, although the medium change-caused ODC induction was much less than TPA-caused ODC induction. The cells were incubated for the indicated times at 36° in an atmosphere of 92% air/8% CO_2, in the presence or absence of TPA. H-7 and sphingosine were added to the medium 10 min before the addition of TPA. After the incubation, the cells were washed three times with PBS(−), frozen on dry ice, and stored at −80° 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 were centrifuged at 15,000 × *g* for 30 min. ODC activity of the supernatant was determined by measuring release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine, as described previously (10). H-7 and sphingosine did not directly interfere with the ODC assay system.

EGF binding. Primary cultured epidermal cells in 35-mm dishes were treated with TPA or vehicle on the fourth day of culture in low-Ca^{2+} medium. The medium change was not performed on the day of experiments, as in the case of ODC induction. The cells were incubated for the indicated time periods at 36° in an atmosphere of 92% air/8% CO_2, in the presence or absence of TPA. When DG was used instead of TPA, DG was dispersed in a small volume of PBS(−) by sonication and added to the incubation medium. H-7 and sphingosine were added to the medium 10 min before the addition of TPA or DG. After the incubation, the EGF binding assay was performed as reported previously (16–18). In brief, the cells were washed three times with binding assay buffer (minimum essential medium containing 0.1% bovine serum albumin and 20 mM HEPES, pH 7.5) and incubated in the binding assay buffer (1 ml) at 0–4° for 4 hr, in the presence of 50–100 nCi/dish ^{125}I-EGF (approximately 80–160 pM). For the estimation of nonspecific binding, a 1000-fold higher concentration of unlabeled EGF was added to the medium. After the incubation, the cells were solubilized by 1 N sodium hydroxide and the cell-associated radioactivity was counted by γ-counter.

Preparation of TPA-pretreated (protein kinase C-down-regulated) cells. At the time (20 hr after plating) the medium was switched from normal calcium to low Ca^{2+} (50 μM), one group of the cells in 35-mm dishes was treated with 50 nM TPA and the other group was treated with vehicle. The cells were then incubated in the low-Ca^{2+} medium for 48 hr, in the presence or absence of 50 nM TPA. At 24 hr after the start of the TPA or vehicle treatment, medium was changed to the fresh one with or without 50 nM TPA. After 48 hr of TPA treatment, the cells were washed twice with PBS(−) and once with PBS(−) supplemented with 0.1% bovine serum albumin in order to wash out TPA. In order to prepare the cells pretreated with TPA for 14 or 24 hr, TPA was added to the medium 34 or 24 hr after the medium was switched from normal to low Ca^{2+}, respectively. Using these TPA-pretreated cells, the effect of TPA on EGF binding was also examined. TPA-pretreated cells and vehicle-pretreated cells were incubated in fresh medium containing 0.5% bovine serum albumin but without fetal calf serum, in the presence or absence of 10 nM TPA, for 2 hr. After the incubation, the EGF binding assay was performed as described above.

Translocation of protein kinase C. Primary cultured epidermal cells in 100-mm dishes were treated with TPA or vehicle on the fourth day of culture in low-Ca^{2+} medium. The medium change was not performed on the day of experiments. The cells were incubated at 36° for the indicated time periods in an atmosphere of 92% air/8% CO_2, in the presence or absence of TPA. H-7 and sphingosine were added to the medium 10 min before the addition of TPA. After the incubation, the cells were washed three times with PBS(−) and were suspended in 8 ml of ice-cold extraction buffer (buffer A) consisting of 20 mM Tris·HCl buffer (pH 7.4), 2 mM EGTA, 2 mM EDTA, and 2 mM PMSF. Crude preparations of protein kinase C from the cytosol and particulate fractions were obtained as reported previously (19). In brief, the cells were sonicated and centrifuged at 105,000 × *g* for 60 min, and the resultant supernatant was used as the cytosol fraction. Triton X-100 was then added to the cytosol fraction, to a final concentration of 0.3% in order to disturb the adsorption of protein kinase C to the matrix of the column vessel. The 105,000 × *g* precipitate was resuspended, slowly rotated in the original volume of homogenizing buffer containing 0.3% Triton X-100, at 4° for 60 min, and centrifuged at 105,000 × *g* for 60 min. The resultant supernatant was used as the particulate fraction. Both fractions were loaded onto a 0.8 × 3 cm column of DEAE cellulose (DE52) that was preequilibrated with 20 mM Tris·HCl buffer, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, and 50 mM 2-mercaptoethanol (buffer B). After the column was washed with 6 ml of buffer B containing 0.02% Triton X-100, protein kinase C was eluted with 2 ml of buffer B containing 0.02% Triton X-100 and 0.1 M NaCl. The amount of protein kinase C eluted from the DE52 column was determined by the [^{3}H]PDBu binding assay (20). Briefly, 100 μl of sample solution was incubated at 37° for 5 min with 150 μl of reaction mixture containing 66 mM Tris·HCl buffer (pH 7.4), 15 nM [^{3}H]PDBu, 2 mM $CaCl_2$, 100 μg/ml phosphatidylserine, and 4 mg/ml bovine γ-globulin. After the incubation, the reaction mixture was cooled to 4° for 5 min. The bound and free [^{3}H]PDBu were measured using the polyethylene glycol precipitation method. For the estimation of nonspecific binding, 30 μM unlabeled PDBu was added to the reaction mixture. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

Statistical analysis. Statistical analysis was done by *t* test.

Results

Epidermal cells cultured in low-Ca^{2+} (20–50 μM) medium proliferate rapidly as a monolayer and these cells are characterized as basal cells (15). When medium Ca^{2+} was raised to a

normal level, proliferation ceased and squamous differentiation ensued rapidly, as reported previously (15). Therefore, the experiments were performed in low-Ca^{2+} (50 μM) medium.

Treatment of primary cultured mouse epidermal cells with TPA led to a transient induction of ODC activity, and a peak activity was observed 3 hr after TPA treatment (Fig. 1). Treatment of the cells with 30 nM TPA caused a maximal induction of ODC activity. Both time course and concentration responsiveness of TPA-caused ODC induction (Fig. 1) were consistent with previous reports (9, 10, 21).

Incubation of the epidermal cells with TPA caused the reduction of ^{125}I-EGF binding (Fig. 2). Maximum inhibition of ^{125}I-EGF binding was observed by incubating the cells with TPA for 0.5 to 2 hr (data not shown), in agreement with previous reports (17, 22). The effect of TPA was concentration dependent and the maximum inhibitory effect was obtained at 10–30 nM (Fig. 2).

Treatment of the cells with DGs, such as DOG or OAG, for 2 hr at 36° significantly suppressed ^{125}I-EGF binding in a concentration-dependent manner (Fig. 3). According to the trypan blue exclusion test, the concentrations of DGs used in the present study did not induce a toxic effect on the cells, although the concentrations of DGs used were relatively high.

In order to determine whether TPA exerts its inhibitory effect on ^{125}I-EGF binding in TPA-pretreated cells, the following experiments were also conducted. The treatment of epidermal cells with 50 nM TPA for 14 hr caused the reduction of ^{125}I-EGF binding (Fig. 4), consistent with the results shown in Fig. 2. However, in spite of the continuous presence of TPA,

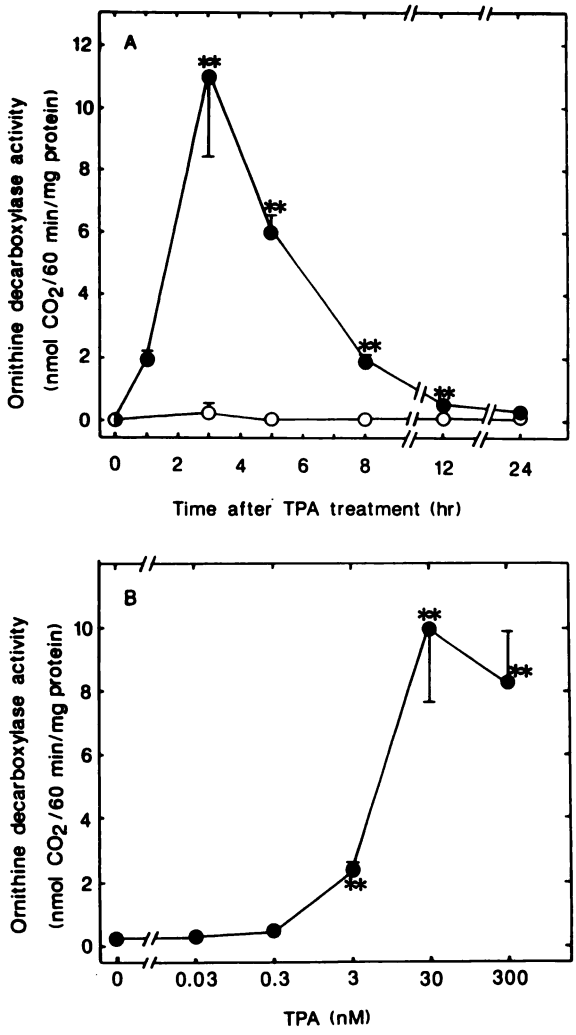

Fig. 1. Time-course and concentration-response relationship of TPA-caused ODC induction in mouse epidermal cells. A, Primary cultured mouse epidermal cells were incubated with 30 nM TPA (●) or vehicle (○) and ODC activity was determined at the times indicated. TPA was added at time 0. Each *point* and *vertical bar* represent mean ± standard error (three or four experiments). $^{**}p < 0.01$ versus vehicle-treated. Similar experiments were repeated twice and the results obtained were reproducible. B, Primary cultured mouse epidermal cells were incubated with 0–300 nM TPA and ODC activity was determined at 3 hr after TPA treatment. Each *point* and *vertical bar* represent mean ± standard error (four experiments). $^{**}p < 0.01$ versus 0. Similar experiments were repeated twice and the results obtained were reproducible.

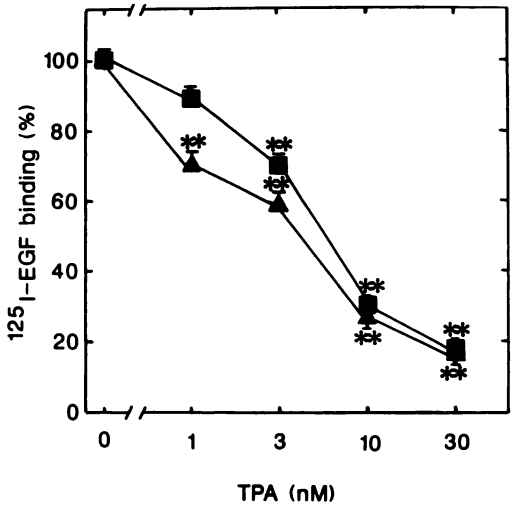

Fig. 2. Effect of TPA on ^{125}I-EGF binding to mouse epidermal cells. Primary cultured mouse epidermal cells were incubated for 30 min (▲) or 2 hr (■) with 0–30 nM TPA. After the incubation, ^{125}I-EGF binding assay was performed as described in Materials and Methods. Data are expressed as percentage of ^{125}I-EGF binding to the vehicle-treated cells (0 nM TPA). ^{125}I-EGF binding to the vehicle-treated cells, i.e., 100%, was 28.6 ± 0.6 cpm/μg of protein (approximately 10700 cpm/35-mm dish). Each *point* and *vertical bar* represent mean ± standard error (three experiments). $^{**}p < 0.01$ versus vehicle-treated. Similar experiments were repeated twice and the results obtained were reproducible.

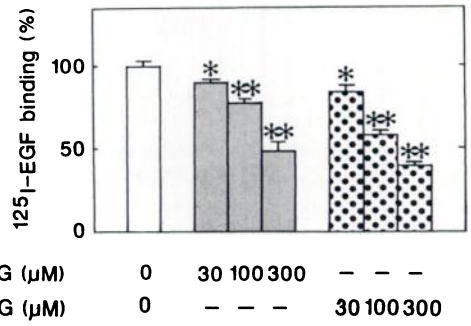

Fig. 3. Effects of DOG and OAG on ^{125}I-EGF binding to mouse epidermal cells. Primary cultured mouse epidermal cells were incubated for 2 hr with either 30–300 μM DOG, 30–300 μM OAG, or vehicle. After the incubation, ^{125}I-EGF binding assay was performed as described in Materials and Methods. Data are expressed as percentage of ^{125}I-EGF binding to the vehicle-treated cells. ^{125}I-EGF binding to the vehicle-treated cells, i.e., 100%, was 35.1 ± 1.0 cpm/μg of protein (approximately 14,000 cpm/35-mm dish). Each *column* and *vertical bar* represent mean ± standard error (three experiments). $^{*}p < 0.05$; $^{**}p < 0.01$ versus vehicle-treated. Similar experiments were repeated twice and the results obtained were reproducible.

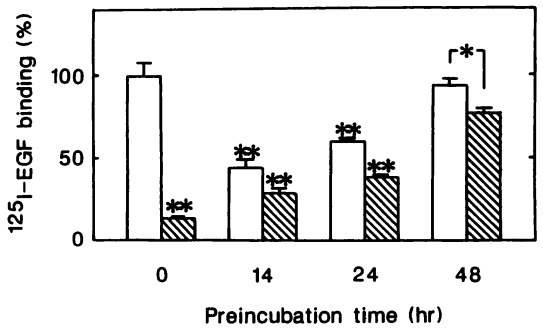

Fig. 4. Effect of TPA on ^{125}I-EGF binding to mouse epidermal cells pretreated with TPA. Primary cultured mouse epidermal cells were preincubated with either 50 nM TPA or vehicle for 0–48 hr. After preincubation, the cells were washed and the medium was replaced with fresh medium containing 0.5% bovine serum albumin but without fetal calf serum. TPA-pretreated and vehicle-pretreated cells were incubated for 2 hr with either 10 nM TPA (▨) or vehicle (□). After the incubation, ^{125}I-EGF binding assay was performed as described in Materials and Methods. Data are expressed as percentage of ^{125}I-EGF binding to the non-TPA-treated cells (0 hr) (□). ^{125}I-EGF binding to the non-TPA-treated cells, i.e., 100%, was 61.2 ± 4.2 cpm/μg of protein (approximately 21,500 cpm/35-mm dish). Each *column* and *vertical bar* represent mean ± standard error (three experiments). $^{**}p < 0.01$ versus non-TPA-treated cells (0 hr); $^{*}p < 0.05$. The ^{125}I-EGF binding activities of the cells pretreated with vehicle for 14, 24, or 48 hr were almost identical to the activity of nonpretreated cells (data not shown). Similar experiments were repeated twice and the results obtained were reproducible.

the ^{125}I-EGF binding activity gradually recovered, when the cells were further incubated with TPA (Fig. 4). After a 48-hr incubation with TPA, the ^{125}I-EGF binding activity recovered to the level of non-TPA-treated cells (Fig. 4). Although TPA caused a significant inhibition of ^{125}I-EGF binding in TPA (50 nM, 48 hr)-pretreated cells, the inhibitory effect was slight and markedly suppressed in these cells (Fig. 4). The amounts of cellular protein per dish of TPA-pretreated cells were similar to those of vehicle-pretreated cells.

H-7 is a well known protein kinase C inhibitor (14). The effects of H-7 on TPA-caused ODC induction and TPA-caused inhibition of ^{125}I-EGF binding were examined. As shown in Fig. 5B, TPA (30 nM)-caused ODC induction was inhibited by H-7 in a concentration-dependent manner and was almost completely inhibited by 30 μM H-7. H-7 did not shift the time course of TPA-caused ODC induction (data not shown). The same concentration of H-7, however, failed to restore the ^{125}I-EGF binding suppressed by TPA (Fig. 5A). Higher concentration of H-7 (100 μM) only slightly restored the ^{125}I-EGF binding suppressed by TPA. Treatment of the cells with H-7 alone did not affect ODC induction and ^{125}I-EGF binding. Fig. 5C shows the concentration-response curves of H-7 for these two TPA-caused cellular responses, in which the response to TPA in the absence of H-7 is expressed as 100%. An approximately 100-fold difference was observed in the concentration of H-7 needed to inhibit the two TPA-caused cellular responses described above. As described in Materials and Methods, the incubation for the ^{125}I-EGF binding (at 4° for 4 hr) was performed in the absence of TPA and/or H-7. In order to exclude the possibility that the apparent failure of H-7 to inhibit TPA action is due to the absence of H-7 during the binding assay, experiments in which TPA and/or H-7 were added to the incubation mixture for ^{125}I-EGF binding, as well as the preincubation medium (at 36°), were also conducted (Table 1). As clearly shown in Table 1, the addition of the same concentrations of H-7 and/or TPA as in the preincubation medium to the binding assay buffer did not alter the results.

It has been shown that EGF receptors consist of two binding sites, i.e., high and low affinity sites, and that TPA treatment results in the loss of high affinity sites (23). A Scatchard analysis of ^{125}I-EGF binding revealed the reduction of high affinity sites by treatment of the cells with TPA at 36° for 2 hr (Fig. 6A). Treatment of epidermal cells with 30 μM H-7 at 36° for 2 hr did not alter the Scatchard plots, consistent with the data of Fig. 5A (Fig. 6B). Moreover, in the presence of 30 μM H-7, the effect of TPA was still evident and the pattern of Scatchard plots was identical to that in the absence of H-7 (Fig. 6). These results further confirm the inability of H-7 to inhibit the effect of TPA on ^{125}I-EGF binding to epidermal cells.

Staurosporine is known to be a most potent protein kinase C inhibitor and, in fact, it inhibits TPA-stimulated phosphorylation of the 34-kDa protein in intact epidermal cells (24). However, staurosporine (10–100 nM) by itself partially but significantly inhibited ^{125}I-EGF binding. Therefore, the effect of staurosporine on TPA-caused inhibition of ^{125}I-EGF binding could not be estimated (data not shown).

Sphingosine is another type of inhibitor of protein kinase C (25). The effects of sphingosine on TPA-caused ODC induction and TPA-caused inhibition of ^{125}I-EGF binding were also examined. As shown in Fig. 7B, TPA (30 nM)-caused ODC induction was significantly inhibited by 30 μM sphingosine. According to the trypan blue exclusion test, higher concentrations (≧ 60 μM) of sphingosine were apparently cytotoxic. Treatment of epidermal cells with sphingosine alone did not affect ^{125}I-EGF binding to its receptor (Fig. 7A). TPA (10 nM)-caused suppression of ^{125}I-EGF binding was also significantly blocked by 30 μM sphingosine (Fig. 7A). Fig. 7C shows the concentration-response curves of sphingosine for these two TPA-caused cellular responses, in which the response to TPA in the absence of sphingosine is expressed as 100%. These two concentration-response curves of sphingosine were almost superimposable. These two TPA-caused cellular responses were suppressed similarly by 60 and 100 μM sphingosine. However, interpretation of the data is difficult, because sphingosine at these concentrations is apparently cytotoxic and sphingosine by itself reduced ^{125}I-EGF binding (data not shown).

Similar to the case of TPA, OAG (300 μM)-caused suppression of ^{125}I-EGF binding was barely blocked by 30 μM H-7, whereas 30 μM sphingosine reversed OAG-caused suppression of ^{125}I-EGF binding partially but more effectively than H-7 (Table 2).

It has been well established that TPA causes translocation of protein kinase C from cytosol to membranes (26–28). A similar translocation of protein kinase C was also observed in the cultured mouse epidermal cells. Fig. 8A shows the time-dependent changes in the amounts of protein kinase C, which is estimated by [^{3}H]PDBu binding to this enzyme, in either the cytosol or the particulate (membrane) fraction on exposure of the cells to 30 nM TPA. On treatment with TPA, the activity in the cytosol fraction decreased and the activity in the particulate fraction increased simultaneously (Fig. 8A). The maximum effect of TPA was observed 20–30 min after the addition of TPA but an apparent loss of the total activity of protein kinase C, i.e., the sum of the activities in the cytosol and membrane fractions, was not observed. The effects of H-7 and

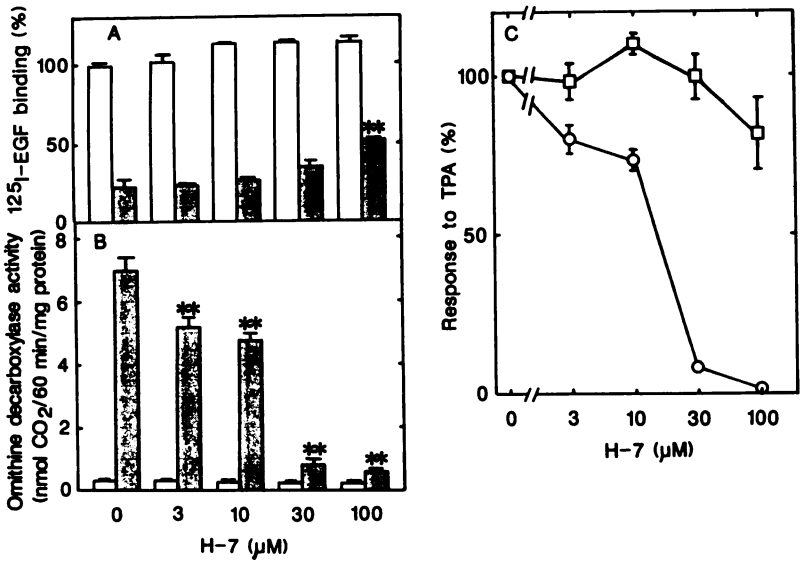

Fig. 5. Effects of H-7 on TPA-caused suppression of ^{125}I-EGF binding and TPA-caused ODC induction in mouse epidermal cells. Primary cultured mouse epidermal cells were pretreated with H-7 or vehicle for 10 min before the addition of TPA. For the experiments of EGF binding (A), the epidermal cells were treated with 10 nM TPA (□) or vehicle (□) for 2 hr; thereafter, ^{125}I-EGF binding assay was performed as described in Materials and Methods. Data are expressed as percentage of ^{125}I-EGF binding to the vehicle-treated cells (no drug treatment). ^{125}I-EGF binding to the vehicle-treated cells (no drug treatment), i.e., 100%, was 40.3 ± 0.8 cpm/μg of protein (approximately 15,500 cpm/35-mm dish). Each *column* and *vertical bar* represent mean ± standard error (three experiments). $^{**}p < 0.01$ versus TPA without H-7. For the experiments of ODC induction (B), epidermal cells were treated with 30 nM TPA or vehicle for 3 hr; thereafter, ODC activity was measured as described in Materials and Methods. Each *column* and *vertical bar* represent mean ± standard error (three or four experiments). $^{**}p < 0.01$ versus TPA without H-7. C, The concentration-response curves of H-7 for two TPA-caused cellular responses (○, ODC induction; □, inhibition of ^{125}I-EGF binding). The data were obtained from A and B and the two other repeated experiments. Response to TPA indicates the difference between the activities observed in the presence and absence of TPA. The response to TPA in the absence of H-7 is expressed as 100%. Each *point* and *vertical bar* represent mean ± standard error (three experiments).

TABLE 1

Effects of the addition of H-7 and TPA to the binding assay buffer on ^{125}I-EGF binding

Epidermal cells were first treated with vehicle or H-7 and/or TPA for 2 hr at 36°. H-7 was added to the medium 10 min before the addition of TPA. The binding assay was performed in the absence or presence of H-7 and/or TPA at 0–4° for 4 hr. Values are mean ± standard error of three experiments.

Addition		^{125}I-EGF binding activity	
Incubation	Incubation for ^{125}I-EGF binding assay		
		cpm/μg of protein	%
Expt. 1			
Vehicle[a] + vehicle[b]	None	32.8 ± 1.0	100
Vehicle + TPA (10 nM)	None	5.5 ± 0.8	17
H-7 (30 μM) + TPA (10 nM)	None	6.9 ± 0.4	21
H-7 (30 μM) + vehicle	None	30.7 ± 0.5	94
Expt. 2			
Vehicle + vehicle	H-7[c] (30 μM)	35.0 ± 1.3	100
Vehicle + TPA (30 nM)	H-7 (30 μM)	5.3 ± 1.4	15
H-7 (30 μM) + TPA (30 nM)	H-7 (30 μM)	6.1 ± 0.3	20
H-7 (30 μM) + vehicle	H-7 (30 μM)	26.0 ± 1.6	75
Expt. 3			
Vehicle + vehicle	Vehicle	29.9 ± 1.5	100
Vehicle + TPA (10 nM)	TPA (10 nM)	5.2 ± 0.8	17
H-7 (30 μM) + TPA (10 nM)	H-7 (30 μM) + TPA (10 nM)	6.8 ± 0.4	23
H-7 (30 μM) + vehicle	H-7 (30 μM)	31.1 ± 0.3	104

[a] Treatment with H-7 or vehicle for 10 min.
[b] Treatment with vehicle or H-7 and/or TPA for 2 hr at 36°.
[c] Binding assay was performed in the absence or presence of H-7 and/or TPA for 4 hr at 4°.

sphingosine on TPA-caused translocation of protein kinase C were examined. TPA (30 nM)-caused translocation of protein kinase C from cytosol to membranes was not inhibited by 30 μM H-7 (Fig. 8B). Although TPA-caused translocation of protein kinase C tended to be slightly inhibited by sphingosine, the effect was not significant (Fig. 8C).

Discussion

In primary cultured mouse epidermal cells, TPA caused ODC induction as reported previously (10, 11, 21, 29, 30). DGs and phospholipase C mimic TPA action and induce ODC activity in mouse epidermal cells (9–11). A protein kinase C inhibitor, H-7, inhibited TPA-caused ODC induction, consistent with a previous report (31). Furthermore, in the present study, another protein kinase C inhibitor, sphingosine, also inhibited TPA-caused ODC induction. Although H-7 inhibits other protein kinases (14) and sphingosine inhibits some of the calmodulin-dependent enzymes (32), a common mechanism of action of these two compounds is the inhibition of protein kinase C. Therefore, it is highly possible that TPA-caused ODC induction in mouse epidermal cells is mediated by the activation of protein kinase C. Recently, the inhibition by sphingosine of TPA-caused ODC induction in mouse skin has been also reported (33, 34).

The inhibition of ^{125}I-EGF binding to EGF receptors caused by TPA has been reported in various types of cells (18, 35, 36) including mouse epidermal cells (9, 17, 22). The studies using A431 cells and human fibroblasts suggest that the phosphorylation of Thr-654 of the EGF receptor by the activation of protein kinase C plays an important role in the mechanism of the TPA-caused inhibition of ^{125}I-EGF binding (37, 38). In the present study, TPA-caused inhibition of ^{125}I-EGF binding was down-regulated by pretreatment of the epidermal cells with TPA for 48 hr, and DGs such as DOG and OAG mimicked the TPA action. In addition, Jeng *et al.* (9) reported that ^{125}I-EGF binding to epidermal cells was inhibited by treatment of the

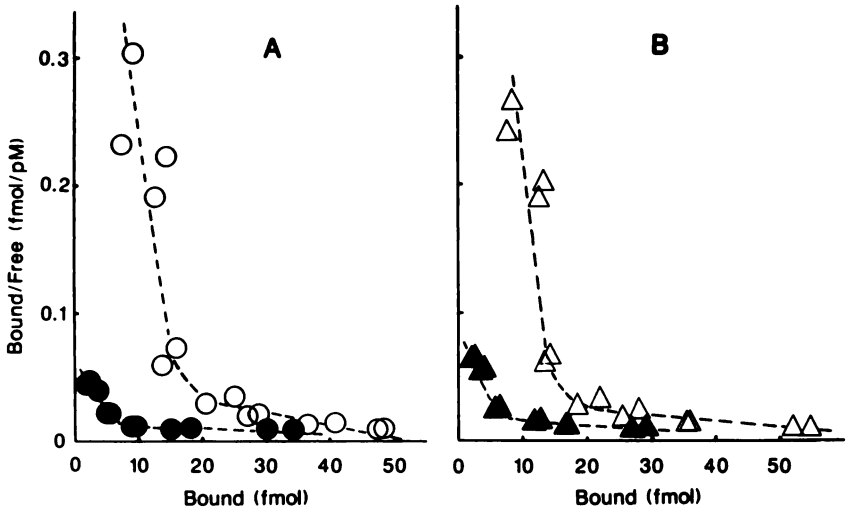

Fig. 6. Scatchard analysis of ^{125}I-EGF binding to mouse epidermal cells treated with or without TPA and/or H-7. Primary cultured mouse epidermal cells were treated with 30 μM H-7 (B) or vehicle (A) at 36° for 10 min. Thereafter, epidermal cells were incubated at 36° for 2 hr in the presence of 10 nM TPA (*closed symbols*) or vehicle (*open symbols*). Cells were then assayed at 4° for ^{125}I-EGF binding, as described in Materials and Methods. K_d and B_{max} values of high and low affinity binding sites, which were estimated from Scatchard plots, were as follows: in A, K_d = 31 pM and 0.94 nM, B_{max} = 53 and 180 fmol/mg of protein for vehicle-treated, and K_d = 250 pM and 2.9 nM, B_{max} = 20 and 170 fmol/mg of protein for TPA-treated; in B, K_d = 25 pM and 1.7 nM, B_{max} = 48 and 230 fmol/mg of protein for vehicle-treated and K_d = 120 pM and 2.8 nM, B_{max} = 25 and 180 fmol/mg of protein for TPA-treated. Similar experiments were repeated twice, and the results obtained were reproducible.

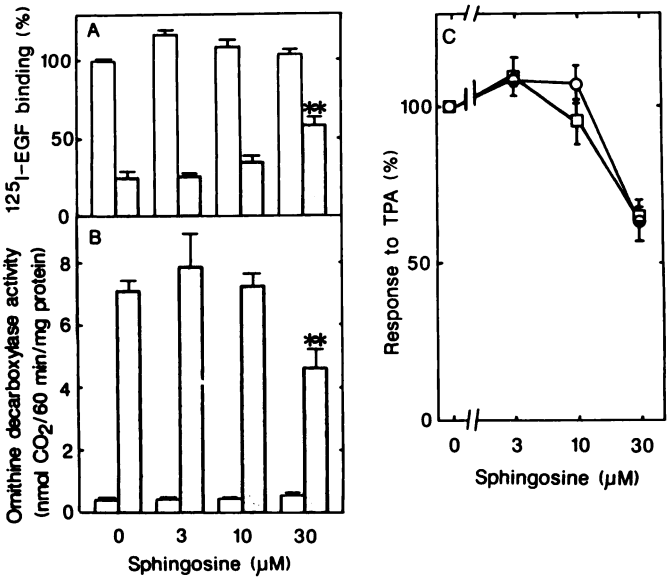

Fig. 7. Effects of sphingosine on TPA-caused inhibition of ^{125}I-EGF binding and TPA-caused ODC induction in mouse epidermal cells. Primary cultured mouse epidermal cells were pretreated with sphingosine or vehicle for 10 min before the addition of TPA. For the experiments of EGF binding (A), the epidermal cells were treated with 10 nM TPA (□) or vehicle (□) for 2 hr; thereafter, ^{125}I-EGF binding assay was performed as described in Materials and Methods. Data are expressed as percentage of ^{125}I-EGF binding to the vehicle-treated cells (no drug treatment). ^{125}I-EGF binding to the vehicle-treated cells (no drug treatment), i.e., 100%, was 19.8 ± 0.3 cpm/μg of protein (approximately 7600 cpm/35-mm dish). Each *column* and *vertical bar* represent mean ± standard error (three experiments). $^{**}p < 0.01$ versus TPA without sphingosine. For the experiments of ODC induction (B), epidermal cells were treated with 30 nM TPA or vehicle for 3 hr; thereafter, ODC activity was measured as described in Materials and Methods. Each *column* and *vertical bar* represent mean ± standard error (three or four experiments). $^{**}p < 0.01$ versus TPA without sphingosine. C, The concentration-response curves of sphingosine for two TPA-caused cellular responses (○, ODC induction; □, inhibition of ^{125}I-EGF binding). The data were obtained from A and B and the two other repeated experiments. Response to TPA indicates the difference between the activities observed in the presence and absence of TPA. The response to TPA in the absence of sphingosine is expressed as 100%. Each *point* and *vertical bar* represent mean ± standard error (three experiments).

TABLE 2

Effects of H-7 and sphingosine on OAG-caused suppression of ^{125}I-EGF binding in mouse epidermal cells

Mouse epidermal cells were pretreated with H-7, sphingosine, or vehicle for 10 min before the addition of OAG (300 μM). Epidermal cells were incubated for 2 hr with OAG or vehicle and, thereafter, ^{125}I-EGF binding assay was performed. Values are mean ± standard error of six experiments.

Addition in incubation	^{125}I-EGF binding activity	
	cpm/μg of protein	%
Expt. 1		
Vehicle + vehicle	34.7 ± 0.8	100
Vehicle + OAG	4.2 ± 0.2	12
H-7 (30 μM) + OAG	6.9 ± 0.3	20
H-7 (30 μM) + vehicle	33.2 ± 0.9	96
Expt. 2		
Vehicle + vehicle	28.8 ± 2.1	100
Vehicle + OAG	4.8 ± 0.3	17
Sphingosine (30 μM) + OAG	10.3 ± 0.4	36
Sphingosine (30 μM) + vehicle	25.3 ± 1.2	88

cells with phospholipase C. All of these results suggest that the suppression of ^{125}I-EGF binding by TPA in mouse epidermal cells is mediated through the activation of protein kinase C.

H-7 is one of the most popularly used protein kinase C inhibitors and inhibits protein phosphorylation through the interaction with the catalytic domain of this enzyme (14, 20). It has been reported that TPA-caused cellular responses are inhibited by H-7 in many type of cells (39–41). The present study clearly shows that H-7 inhibits TPA-caused ODC induction but fails to inhibit TPA-caused suppression of ^{125}I-EGF binding. Such differential inhibition of TPA-caused cellular responses was not observed when sphingosine was used as protein kinase C inhibitor. Sphingosine counteracted the TPA-caused inhibition of ^{125}I-EGF binding with a concentration range similar to that for inhibition of TPA-caused ODC induction. Similar to the case of TPA, suppression of ^{125}I-EGF binding by OAG was also barely inhibited by H-7, whereas sphingosine was more effective in inhibiting the action of OAG than was H-7. It has been reported that TPA-caused suppression of ^{125}I-EGF binding is also counteracted by sphingosine in A431 cells (42). Recently, H-7-uninhibitable cellular responses caused by TPA in Swiss 3T3 fibroblasts have been also reported (43).

Activation of protein kinase C by phorbol esters is associated

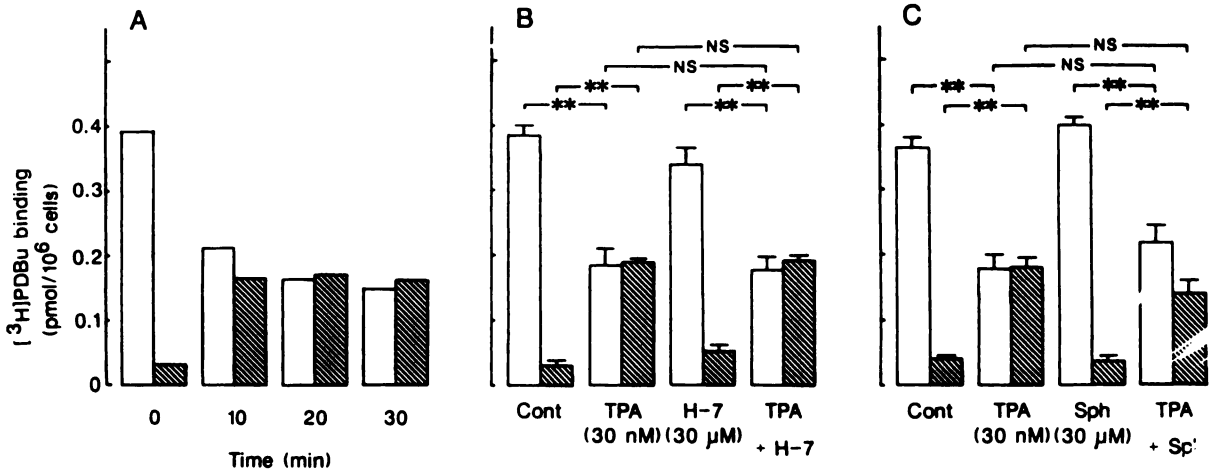

Fig. 8. Effects of H-7 and sphingosine on TPA-caused translocation of protein kinase C from cytosol to membranes. Primary cultured mouse epidermal cells were incubated with 30 nM TPA at 36° for 0–30 min (A). Epidermal cells were incubated at 36° for 10 min with 30 μM H-7 (B), 30 μM sphingosine (C), or vehicle (B and C). Thereafter, the cells were further treated with 30 nM TPA or vehicle at 36° for 20 min (B and C). After the incubation, amounts of protein kinase C in cytosol (□) and particulate (▨) fractions were determined by [^{3}H]PDBu binding to this enzyme in the presence of Ca^{2+} plus phosphatidylserine. Each *column* and *vertical bar* represents mean ± standard error of three experiments. In C, *Sph* indicates sphingosine. $^{**}p < 0.01$; *NS*, not statistically significant.

with translocation of the enzyme from cytosol to membranes (26–28). Following its activation by association with phospholipids and DGs (or TPA) at the membrane, the enzyme is subsequently down-regulated (44, 45). Present results show that treatment of epidermal cells with 30 nM TPA for 20 min effectively translocated protein kinase C from cytosol to membrane. However, the decrease of protein kinase C activity in the cytosol fraction was partial, and down-regulation of protein kinase C was not evident. This may be due to the short exposure time of the cells to a relatively low concentration of TPA. It has been reported that sphingosine reverses TPA-caused translocation of protein kinase C (46) and, subsequently, prevents TPA-caused down-regulation (33, 47). However, in the present study, prevention of translocation of protein kinase C by sphingosine was not clearly observed in primary cultured epidermal cells. Meanwhile, the present results clearly show that H-7 did not block the translocation of protein kinase C. Previously, we reported that H-7 (30 μM) effectively inhibits the TPA-stimulated phosphorylation of the 34-kDa protein in intact epidermal cells (24), indicating that H-7 inhibits protein phosphorylation by acting on the membrane-bound protein kinase C.

How can we explain the heterogeneity of two separate actions of H-7 on TPA-induced cellular responses? It has been reported that there are several subtypes of protein kinase C, and the functions of these protein kinase C subtypes are suggested to be different (48). A number of proteins are phosphorylated in epidermal cells by TPA treatment (49), suggesting that those proteins are phosphorylated by protein kinase C in these cells. Because H-7 interacts with the catalytic domain of protein kinase C, it also inhibits other kinases such as cyclic nucleotide-dependent protein kinases (14). Therefore, a hypothesis that H-7 has differential inhibitory effects on different subtypes of protein kinase C is unlikely. However, there still exists a possibility that inhibition of protein phosphorylation by H-7 depends on the enzyme-substrate combinations and their intracellular localization. Recently, Kiss *et al.* (50) reported TPA-induced protein phosphorylation that was hardly inhibited by H-7 and/or sphingosine in human leukemic HL60 cells. It has also been suggested that the proteolytic fragments of protein kinase C, i.e., regulatory and catalytic fragments, are generated after the membrane translocation of this enzyme (51–53). A catalytic fragment may be released into the cytosol and a free catalytic fragment has the ability to phosphorylate proteins in a Ca^{2+}- and phospholipid-independent manner (51–53). Protein phosphorylation by this catalytic fragment is also inhibited by H-7 (14, 20). Although we do not know the role of the regulatory fragment remaining on the membrane, a possible participation of any such fragment in certain cellular responses should also be considered.

Blumberg (6) suggested the concept of phorbol ester receptor heterogeneity because all of the behavior of phorbol esters in biological systems cannot be explained by a single homogeneous class of receptors. Moreover, Feuerstein *et al.* (54) and Blenis *et al.* (55) suggested the involvement of kinases other than protein kinase C in the action of TPA. Therefore, we cannot exclude a possibility that the protein kinase C-independent mechanism is involved in some types of cellular responses induced by TPA in primary cultured mouse epidermal cells.

In conclusion, the present study clearly demonstrates the differential inhibition by H-7 of the TPA-caused cellular responses. TPA-caused suppression of ^{125}I-EGF binding in epidermal cells would be mediated by the protein kinase C function that is barely inhibited by H-7.

Acknowledgments

The authors are grateful to Mr. Takaki Kaeriyama and Miss Chino Otsuka for their competent technical assistance.

References

1. Diamond, L., T. G. O'Brien, and W. M. Baird. Tumor promoters and the mechanism of tumor promotion. *Adv. Cancer Res.* **32**:1–74 (1980).
2. Blumberg, P. M. *In vitro* studies in the mode of action of the phorbol ester potent tumor promoters. *Crit. Rev. Toxicol.* **9**:199–234 (1981).
3. Yuspa, S. H. Tumor promotion in epidermal cells in culture, in *Mechanisms of Tumor Promotion* (T. J. Slaga, ed.), Vol. III. CRC Press, Boca Raton, FL, 1–11 (1983).
4. Nishizuka, Y., The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.)* **308**:693–698 (1984).
5. Ashendel, C. L. The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta* **822**:219–242 (1985).

6. Blumberg, P. M. Protein kinase C as the receptor for the phorbol ester tumor promoters. *Cancer Res.* **48**:1–8 (1988).
7. Berridge, M. J. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**:345–360 (1984).
8. Berridge, M. J. Inositol lipids and cell proliferation. *Biochim. Biophys. Acta* **907**:33–45 (1987).
9. Jeng, A. Y., U. Lichti, J. E. Strickland, and P. M. Blumberg. Similar effects of phospholipase C and phorbol ester tumor promoters on primary mouse epidermal cells. *Cancer Res.* **45**:5714–5721 (1985).
10. Sasakawa, N., K. Ishii, S. Yamamoto, and R. Kato. Induction of ornithine decarboxylase activity by 1-oleoyl-2-acetyl-glycerol in isolated mouse epidermal cells. *Biochem. Biophys. Res. Commun.* **128**:913–920 (1985).
11. Verma, A. K., R.-C. Pong, and D. Erickson. Involvement of protein kinase C activation in ornithine decarboxylase gene expression in primary culture of newborn mouse epidermal cells and in skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res.* **46**:6149–6155 (1986).
12. Smart, R. C., M.-T. Huang, and A. H. Conney. *sn*-1,2-Diacylglycerols mimic the effects of 12-*O*-tetradecanoylphorbol-13-acetate *in vitro* by inducing biochemical changes associated with tumor promotion in mouse epidermis. *Carcinogenesis (Lond.)* **7**:1865–1870 (1986).
13. Verma, A. K. The protein kinase C activator L-α-dioctanoylglycerol: a potent stage II mouse skin tumor promoter. *Cancer Res.* **48**:1736–1739 (1988).
14. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. Isoquinolinesulfonamides: novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**:5036–5041 (1984).
15. Yuspa, S. H. Methods for the use of epidermal cell culture to study chemical carcinogenesis, in *Methods in Skin Research* (D. Skerrow and C. J. Skerrow, eds.). John Wiley & Sons, New York, 213–249 (1985).
16. Sako, T., A. I. Tauber, A. Y. Jeng, S. H. Yuspa, and P. M. Blumberg. Contrasting action of staurosporine, a protein kinase C inhibitor, on human neutrophils and primary mouse epidermal cells. *Cancer Res.* **48**:4646–4650 (1988).
17. Strickland, J. E., A. M. Jetten, H. Kawamura, and S. H. Yuspa. Interaction of epidermal growth factor with basal and differentiating epidermal cells of mice resistant and sensitive to carcinogenesis. *Carcinogenesis (Lond.)* **5**:735–740 (1984).
18. Wattenberg, E. V., H. Fujiki, and M. R. Rosner. Heterologous regulation of the epidermal growth factor receptor by palytoxin, a non-12-*O*-tetradecanoylphorbol-13-acetate-type tumor promoter. *Cancer Res.* **47**:4618–4622 (1987).
19. Aizu, E., S. Yamamoto, T. Nakadate, I. Kiyoto, and R. Kato. Palmitoylcarnitine reverses 12-*O*-tetradecanoylphorbol-13-acetate-induced refractory state for the TPA-caused ornithine decarboxylase induction in mouse skin. *Carcinogenesis (Lond.)* **9**:309–313 (1988).
20. Nakadate, T., A. Y. Jeng, and P. M. Blumberg. Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochem. Pharmacol.* **37**:1541–1545 (1988).
21. Yuspa, S. H., U. Lichti, T. Ben, E. Patterson, and H. Hennings. Phorbol esters stimulate DNA synthesis and ornithine decarboxylase activity in mouse epidermal cell cultures. *Nature (Lond.)* **262**:402–404 (1976).
22. Locker, J. M., G. T. Bowden, L. M. Matrisian, and B. E. Magun. Tumor promoter-induced inhibition of epidermal growth factor binding to cultures of mouse primary epidermal cells. *Cancer Res.* **41**:2308–2314 (1981).
23. Magun, B. E., L. M. Matrisian, and G. T. Bowden. Epidermal growth factor: ability of tumor promoter to alter its degradation, receptor affinity and receptor number. *J. Biol. Chem.* **255**:6373–6381 (1980).
24. Yamamoto, S., I. Kiyoto, E. Aizu, T. Nakadate, Y. Hosoda, and R. Kato. Differential inhibition by staurosporine, a potent protein kinase C inhibitor, of 12-*O*-tetradecanoylphorbol-13-acetate-caused skin tumor promotion, epidermal ornithine decarboxylase induction, hyperplasia and inflammation. *Carcinogenesis (Lond.)* **10**:1315–1322 (1989).
25. Hannun, Y. A., C. R. Loomis, A. H. Merrill, Jr., and R. M. Bell. Sphingosine inhibition of protein kinase C activity and of phorbol dibutylate binding *in vitro* and in human platelets. *J. Biol. Chem.* **261**:12604–12609 (1986).
26. Kraft, A. S., W. B. Anderson, H. L. Cooper, and J. J. Sando. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J. Biol. Chem.* **257**:13193–13196 (1982).
27. Kraft, A. S., and W. B. Anderson. Phorbol ester increases the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond.)* **301**:621–623 (1983).
28. Guy, G. R., J. Gordon, L. Walker, R. H. Michell, and G. Brown. Redistribution of protein kinase C during mitogenesis of human B lymphocytes. *Biochem. Biophys. Res. Commun.* **135**:146–153 (1986).
29. Chida, K., and T. Kuroki. Inhibition of DNA synthesis and sugar uptake and lack of induction of ornithine decarboxylase in human epidermal cells treated with mouse skin tumor promoters. *Cancer Res.* **44**:875–879 (1984).
30. Fischer, S. M., G. Furstenberger, F. Marks, and T. J. Slaga. Event associated with mouse skin tumor promotion with respect to arachidonic acid metabolism: a comparison between SENCAR and NMRI mice. *Cancer Res.* **47**:3174–3179 (1987).
31. Kiyoto, I., S. Yamamoto, E. Aizu, and R. Kato. Staurosporine, a potent protein kinase C inhibitor, fails to inhibit 12-*O*-tetradecanoylphorbol-13-acetate-caused ornithine decarboxylase induction in isolated mouse epidermal cells. *Biochem. Biophys. Res. Commun.* **148**:740–746 (1987).
32. Jefferson, A. B., and H. Schulman. Sphingosine inhibits calmodulin-dependent enzymes. *J. Biol. Chem.* **263**:15241–15244 (1988).
33. Gupta, A. K., G. J. Fisher, J. T. Elder, B. J. Nickoloff, and J. J. Voorheer. Sphingosine inhibits phorbol ester-induced inflammation, ornithine decarboxylase activity, and activation of protein kinase C in mouse skin. *J. Invest. Dermatol.* **91**:486–491 (1988).
34. Enkvetchakul, B., A. H. Merrill, Jr., and D. F. Birt. Inhibition of the induction of ornithine decarboxylase activity by 12-*O*-tetradecanoylphorbol-13-acetate in mouse skin by sphingosine sulfate. *Carcinogenesis (Lond.)* **10**:379–381 (1989).
35. Lee, L., and I. B. Weinstein. Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. *Science (Wash. D. C.)* **202**:313–315 (1978).
36. Jetten, A. M., B. R. Ganong, G. R. Vandenbark, J. E. Shirley, and R. M. Bell. Role of protein kinase C in diacylglycerol-mediated induction of ornithine decarboxylase and reduction of epidermal growth factor binding. *Proc. Natl. Acad. Sci. USA* **82**:1941–1945 (1985).
37. Hunter, T., N. Ling, and J. A. Cooper. Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature (Lond.)* **311**:480–483 (1985).
38. Davis, R. J., and M. P. Czech. Tumor-promoting phorbol diesters cause the phosphorylation of epidermal growth factor receptors in normal human fibroblasts at threonine-654. *Proc. Natl. Acad. Sci. USA* **82**:1974–1978 (1985).
39. Matsui, T., Y. Nakao, T. Koizumi, Y. Katakami, and T. Fujita. Inhibition of phorbol ester-induced phenotypic differentiation of HL-60 cells by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine. *Cancer Res.* **46**:583–587 (1986).
40. Sha'afi, R. I., T. F. P. Molski, C.-K. Huang, and P. H. Naccache. The inhibition of neutrophil responsiveness caused by phorbol esters is blocked by the protein kinase C inhibitor H-7. *Biochem. Biophys. Res. Commun.* **137**:50–60 (1986).
41. Thueson, D. O., J. A. Kennedy, C. D. Wright, and M. C. Conroy. Specific inhibition of phorbol ester and diC_8-induced histamine release from human basophils by the protein kinase C inhibitors, H-7 and H-9. *Biochem. Biophys. Res. Commun.* **144**:732–740 (1987).
42. Faucher, M., N. Girones, Y. A. Hannun, R. M. Bell, and R. J. Davis. Regulation of the epiderml growth factor receptor phosphorylation state by sphingosine in A431 human epidermoid carcinoma cells. *J. Biol. Chem.* **263**:5319–5327 (1988).
43. Burch, R. M., A. L. Ma, and J. Axelrod. Phorbol ester and diacylglycerols amplify bradykinin-stimulated prostaglandin synthesis in Swiss 3T3 fibroblasts: possible independence from protein kinase C. *J. Biol. Chem.* **263**:4764–4767 (1988).
44. Solanki, V., and T. J. Slaga. Specific binding of phorbol ester tumor promoters to intact primary epidermal cells from Sencar mice. *Proc. Natl. Acad. Sci. USA* **78**:2549–2553 (1981).
45. Chida, K., N. Kato, and T. Kuroki. Down-regulation of phorbol diester receptors by proteolytic degradation of protein kinase C in a cultured cell line of fetal rat skin keratinocytes. *J. Biol. Chem.* **261**:13013–13018 (1986).
46. Kolesnick, R. N., and S. Clegg. 1,2-Diacylglycerols, but not phorbol esters, activate a potential inhibitory pathway for protein kinase C in GH_3 pituitary cells. *J. Biol. Chem.* **263**:6534–6537 (1988).
47. Grove, D. S., and A. M. Mastro. Prevention of the TPA-mediated down-regulation of protein kinase C. *Biochem. Biophys. Res. Commun.* **151**:94–99 (1988).
48. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.)* **334**:661–665 (1988).
49. Wirth, P. J., S. H. Yuspa, S. S. Thorgeirsson, and H. Hennings. Induction of common patterns of polypeptide synthesis and phosphorylation by calcium and 12-*O*-tetradecanoylphorbol-13-acetate in mouse epidermal cell culture. *Cancer Res.* **47**:2831–2838 (1987).
50. Kiss, Z., E. Deli, and J. F. Kuo. Phorbol ester stimulation of sphingomyelin synthesis in human leukemic HL60 cells. *Arch. Biochem. Biophys.* **265**:38–42 (1988).
51. Lee, M.-H., and R. M. Bell. The lipid binding, regulatory domain of protein kinase C. *J. Biol. Chem.* **261**:14867–14870 (1986).
52. Huang, K.-P., and F. L. Huang. Conversion of protein kinase C from a Ca^{2+}-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. *Biochem. Biophys. Res. Commun.* **139**:320–326 (1986).
53. Pontremoli, S., E. Melloni, M. Michetti, O. Sacco, F. Salamino, B. Sparatore, and B. L. Horecker. Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca^{2+}-requiring proteinase. *J. Biol. Chem.* **261**:8309–8313 (1986).
54. Feuerstein, N., A. Sahai, W. B. Anderson, D. S. Salomon, and H. L. Cooper. Differential phosphorylation events associated with phorbol ester effects on acceleration versus inhibition of cell growth. *Cancer Res.* **44**:5227–5233 (1984).
55. Blenis, J., J. G. Spivack, and R. L. Erikson. Phorbol ester, serum, and Rous sarcoma virus transforming gene product induce similar phosphorylations of ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **81**:6408–6412 (1984).

Send reprint requests to: Dr. Satoshi Yamamoto, Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.