Biphasic Effects of 12-O-Tetradecanoylphorbol-13-acetate on the Cell Morphology of Low Calcium-grown Human Epidermal Carcinoma Cells: Involvement of Translocation and Down Regulation of Protein Kinase C

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ABSTRACT

The present study was performed to investigate involvement of protein kinase C in the biphasic effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on cell morphology in low calcium (0.07 mM)-grown cells of a human epidermal squamous carcinoma cell line. The low calcium-grown cells formed no desmosomal cell-cell contact and showed roughly circular arrangements of keratin intermediate filaments around the nucleus. Treatment with 10 ng/ml of TPA induced a rapid formation (within 15 min) of cell-cell contact and reorganization of keratin intermediate filaments from a circular organization to a radial arrangement in these low calcium-grown cells. These structural phenomena were associated with a transient increase in membrane-bound protein kinase C activity. However, the prolonged treatment longer than 24 h led to a prominent decrease in the number of cell-cell contacts, that had been once formed, and caused fibroblastic changes of cell morphology in association with a decrease in the membrane-bound protein kinase C activity. Addition of 20 μM 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, a potential inhibitor of protein kinase C, to the medium with TPA blocked the formation of cell-cell contact. Addition of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride alone to normal calcium-grown cell cultures exhibiting cell-cell contact resulted in a decrease in the number of cell-cell contacts and in the fibroblastic morphological changes after 24-h incubation. These results suggest that the effects of TPA are biphasic as follows: the initial stage, inducing cell-cell contact formation associated with the translocation of protein kinase C activity from the cytosol to the membrane; and the late stage, exhibiting a fibroblastic morphological change with a decrease in the number of cell-cell contacts associated with the down regulation of this enzyme activity by TPA.

INTRODUCTION

TPA,1 which is one of the most effective tumor promoters, has long been known to exert profound effects on cultured cells even without pretreatment by carcinogenic agents. TPA acts as a potent modulator of differentiation in epidermal keratinocytes. Actually, this agent induces differentiation in human keratinocytes and SV 40-infected human keratinocytes in parallel with an inhibition of cell growth (1–3), while it stimulates cell growth in cultured mouse epidermal cells (4, 5) after disrupting a population to be programmed to terminally differentiate (6).

In human keratinocytes, TPA treatment induces morphological changes and differentiation (1). Within 2 h of exposure, boundaries between adjacent cells became more distinct, the cells were more angular in appearance, and superficial cells were more bipolar or refractile. By the third day, approximately 50% of cells alter to cornified cells as opposed to 8% in untreated cells (1).

Similarly, in the TPA-treated cultures of SV 40-infected human keratinocytes, the normal polygonal pattern progressively changed to a network of elongated multilayered cell strands surrounding islets of enlarged flattened cells including more keratinized cells. The trend to form cell strands became pronounced with increasing period of exposure to TPA (2). It is of interest to note that mouse keratinocytes also change their morphology to be fibroblastic over 24-h exposure to TPA (4).

Phorbol esters specifically bind to (7–10) and activate protein kinase C, calcium, and phospholipid-dependent protein kinase (11–13). In addition, it has been shown that phorbol esters induce a translocation of protein kinase C from the cytosol to the membrane in various cell types (14–19), and the physiological significance of the translocation attracts much attention.

Mouse epidermal keratinocytes cultured in low calcium (<0.1 mM) do not form desmosome and do not differentiate, but upon raising calcium to the normal level (1 to 2 mM) desmosome formation and differentiation are reported to be initiated (20–23). This is also the case with human keratinocytes (24) and with human epidermal squamous carcinoma cell lines (25, 26).

In the present study, effects of TPA treatment on cell morphology and activity of protein kinase C were examined in a human epidermal squamous cell carcinoma cell line. To examine alterations in cell-cell contact and cell morphology, we observed cytoskeletal organization of KIFs by immunofluorescence microscopy using antibodies to keratin, since KIFs are thought to be one of the fundamental elements to maintain the cell architecture (27) and to bind to sites of cell-cell contact, desmosomes (22).

The data obtained here showed that TPA treatment of low calcium-grown cells induced a rapid formation of cell-cell contact in association with an increase in protein kinase C activity of the membrane fraction, but the prolonged treatment longer than 24 h resulted in loss of the number of once-formed cell-cell contacts and also in fibroblastic changes in association with a decrease in membrane-bound protein kinase C activity. The pretreatment for 15 min and cotreatment with 20 μM H-7, a potential inhibitor of protein kinase C (28–30), prevented the TPA-induced formation of cell-cell contacts. Furthermore, normal calcium-grown cells, which had many cell-cell contacts with desmosomes, reduced the number of the cell-cell contacts and became fibroblastic by H-7 treatment for 24 h. These TPA-induced changes in cell-cell contact and KIF organization were discussed in terms of the translocation and down regulation of protein kinase C.

MATERIALS AND METHODS

Chemicals

TPA, PDBu, ATP, histone (type III-S), 1,2-diolein, and PS were obtained from Sigma Chemical Co. (St. Louis, MO). DEAE-Sephadex...
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Cell Culture

An isolated cell line from human SCC, DJM-1, which can grow on plastic dishes and the glass coverslips in normal (1.87 mM) or low (0.07 mM) calcium concentration medium without feeder layers at the same growth rate, was used at 25 or 26 passages after isolation of the cell in the present study.

Cells which had been grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 0.4 µg/ml of hydrocortisone, 10 ng/ml of epidermal growth factor, 84 ng/ml of cholera toxin, 100 µg/ml of streptomycin and 100 units/ml of penicillin (31) were treated with a solution of 1000 units/ml of dispase (Godo Shusei Co., Matsudo, Chiba, Japan) for 15 min at 37°C and with 0.25% trypsin containing 0.05% EDTA for 15 min at 37°C so that a free-cell suspension was obtained. Approximately 10^5 cells were inoculated on glass coverslips in a 35-mm plastic dish for the morphological study and 10^4 cells in a 90-mm plastic dish for protein kinase assay in the same medium (1.87 mM calcium concentration, normal calcium medium) as mentioned above or in low-calcium medium (0.07 mM calcium concentration), containing other constituents which were identical to those in normal calcium medium. In order to prepare low-calcium medium with serum, fetal calf serum was treated with 10% Chelex 100 and added to calcium-free medium, respectively, and then appropriate amounts of a 0.3 M CaCl_2.2H_2O stock solution sterilized by filtration were added to the medium to give a final calcium concentration of approximately 0.06 to 0.07 mM according to the method of Hennings et al. (20).

Treatment with TPA and H-7

TPA, which was kept in DMSO at 1 mg/ml of concentration, was dissolved in low-calcium and normal-calcium culture media at a final concentration of 10 ng/ml. PDBu was also dissolved in low-calcium culture media at final concentrations of 10 and 50 ng/ml. A protein kinase inhibitor, H-7 (27-29), was dissolved in low-calcium and normal-calcium culture media at final concentrations of 2, 20 and 100 µM. After cells were grown for 4 to 5 days after plating, the medium was changed with an experimental medium containing one of these agents. The cells were fixed with -20°C methanol after exposure to these agents for 5, 15, 30, 60, and 120 min; and 9, 24, and 48 h. Effects of DMSO were also tested at appropriate concentrations.

Antibodies

Antikeratin. Keratin was extracted from human thigh skin according to the procedures described previously (32). The purified keratin was electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and the polypeptide with a molecular weight of 50,000 was extracted from the gel. This M, 50,000 polypeptide was used to produce antiserum to keratin. As described in an earlier paper (33). The obtained antikeratin antibody reacted with all four major keratins M, 50,000, 56,500, 58,000, and 67,000 extracted from normal human epidermis as examined by immunoblotting and reacted with epidermal cells including basal cells and cultured human keratinocytes (33).

Secondary Antibodies. FITC-labeled goat anti-rabbit IgG and FITC-labeled goat anti-human IgG were purchased from Cappel Laboratories (West Chester, PA). These antibodies did not bind to the cells used in this study, when they were applied after incubation in preimmunized rabbit serum and normal human serum.

Immunofluorescence Microscopy

The cells, on the coverslips, were rinsed with phosphate-buffered saline at room temperature and dipped for 7 min in -20°C methanol. The fixed cells on the coverslips were soaked in a solution of 0.5% Triton X-100, 2 mM PMSF, and 2 mM N-tosyl-L-phenylalanine chloromethyl ketone for 10 min at room temperature. The cells were incubated with the rabbit antikeratin antibody for 45 min followed by extensive washing and then incubated with anti-rabbit goat IgG labeled with FITC for 45 min.
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Fig. 1. Normal calcium-grown (1.87 mM) human epidermal SCC cells as visualized by immunofluorescence microscopy using antikeratin antibody and FITC-labeled second antibody. All other figures were also visualized by the same method. KIF bundles are arranged in a radial pattern, and their ends appear to be connected with those of the next cells at cell-cell contacts with desmosomes. The existence of desmosomes in these cells was confirmed by electron microscopy. a, x 320; b, x 1280.

Fig. 2. a, low calcium-grown (0.07 mM) human epidermal SCC cells. Cells do not form cell-cell contact with desmosomes and are scattered evenly on the glass coverslip. KIF bundles are arranged around the nucleus and do not extend into the peripheral cytoplasm. No desmosome formation in these cells was confirmed by electron microscopy. x 1280. b, effects of 15-min treatment with TPA (10 ng/ml) on low calcium-grown SCC cells. Cells gather in groups consisting of 10 to 30 cells. These cells appear to be in contact with each other along a narrow gap between cells (arrows). KIFs in a foamy appearance extend to the peripheral regions of the cell. x 1280. c, effects of 30-min treatment with TPA (10 ng/ml) on low calcium-grown SCC cells. KIFs produce more distinct bundles in a fibrillar appearance. x 1280. d, effects of 60-min treatment with TPA (10 ng/ml) on low calcium-grown SCC cells. Cells form much larger islands consisting of numerous cells and contact each other more tightly. The boundary between cells is unclear because of overlapping of KIF bundles extending into the peripheral cytoplasm. Many KIF bundles appeared to be connected with those of the next cells (arrows). The existence of desmosomes in these cells was confirmed by electron microscopy. x 1280.

stained gaps between cells became unclear by overlapping of KIF bundles, which extended to the cell periphery to form a bridging pattern or roughly radial organization (Fig. 2d). Emitting bundles of KIFs from the juxtanuclear region reached the points of cell-cell contact, probably desmosomes (5, 22, 23), to which KIFs of the next cells were bound (Fig. 2d, arrows). During the next several hours, no remarkable alterations were seen in KIF organization, although arrangement of KIF bundles became more similar to that seen in normal calcium-grown cells.

Twenty-four h after TPA addition, the shape of cells became more angular or bipolar (Fig. 3a). By 48 h, the cell culture was
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Fig. 3. Effects of prolonged treatment with TPA (10 ng/ml) for 24 h (a) and 48 h (b) on low calcium-grown SCC cells. Twenty-four h after TPA addition (a), the shape of cells becomes more angular and bipolar, so that the cellular arrangement alters to become irregular. Forty-eight-h treatment with TPA (b) exerted profound effects on cellular morphology, producing widely spreading cells, smaller cells, and cells with long projections or strands. These cells reduced the number of cell-cell contacts so as to produce an irregular intercellular space. Morphology and cell-cell contact were not recovered even 48 h after replacing the TPA medium with fresh low-calcium medium without TPA. × 320.

KIF bundles reached (Fig. 5b), compared with untreated normal calcium-grown cells (Fig. 1). These morphological alterations were reversible. The morphological recovery was clearly demonstrated by replacing H-7-containing medium with the normal fresh medium (Fig. 5c). The recovered cells displayed a characteristic radial KIF organization of normal calcium-grown cells, indicating a large number of cell-cell contacts, probably desmosomes, associated with KIF bundles (Fig. 5c).

When cells were treated with 100 μM H-7, effects similar to those described above were exerted. However, H-7 treatment at 2 μM concentration exhibited almost no detectable morphological changes.

Protein Kinase C Activity. When SCC cells grown in low-calcium medium were treated with TPA for 15 min, the activity of calcium/phospholipid-dependent protein kinase (protein kinase C) increased 3-fold in the membrane fraction, whereas it decreased approximately half in the cytosol fraction. By 3 h after TPA addition, the increased activity in the membrane fraction was reduced nearly to the initial level before addition of TPA, and the activity remained at that level thereafter. In contrast, the kinase activity in the cytosol fraction considerably decreased to 10% of the initial level during 24 h following TPA addition. Subsequently, the activity began to increase and reached 75% of the initial level at 48 h (Fig. 4).

Effects of Protein Kinase C Inhibition on Cell-Cell Contact and KIF Organization. TPA-induced formation of cell-cell contact at the early stage in low-calcium-grown cells was blocked by pre (15 min)- and coinubcation with 20 μM H-7 (data not shown).

When normal calcium-grown cells were treated with H-7 for 24 h, many cells possessing much longer cell processes or strands were observed (Fig. 5a), as seen in the case of long-term treatment of TPA (Fig. 3b). In addition, flattened cells showed a decrease in the number of cell-cell contacts, which composed of three different shapes of cells: widely spreading cells; smaller cells; and cells with long projections or strands (Fig. 3b). The spreading cells revealed well-defined bundles of KIFs. All of these cells appeared to reduce the number of cell-cell contact so that the intercellular spaces were irregularly widened, the pattern of colonies became loose, and the compact arrangement of cells was no longer seen (Fig. 3).

TPA treatment on normal (1.87 mM) calcium-grown cells did not induce any detectable changes in morphology for the first few hours. However, prolonged treatment with TPA over 24 h caused morphological changes to fibroblastic features and formation of long projections similar to those seen in the long-term TPA treatment (24 to 48 h) of low calcium-grown cells (data not shown). These effects of prolonged treatment with TPA were not eliminated even 48 h after the cells were washed and incubated with TPA-free medium.

PDBu treatment on low calcium-grown cells exerted results similar to those obtained by TPA treatment, and these effects were not eliminated 48 h after the cells were washed and incubated with PDBu-free medium.

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Fig. 4. Effects of TPA on protein kinase C activity. Phosphorylation of histone (II-S) by partially purified protein kinase C (the eluate from the DEAE-Sephacel column with 0.15 M NaCl) from the membrane (Θ) and cytosol (○) fractions was measured in cells after different incubation times with 10 ng/ml of TPA. Activity was determined in the presence of calcium, PS, and diolein as described in "Materials and Methods." The actual cpm 2P/5 min/mg protein for membrane fractions before TPA addition were 15,800 with EGTA and 23,100 with calcium, PS, and diolein; those 15 min after TPA addition were 18,000 with EGTA and 37,900 with calcium, PS, and diolein. Points, mean of two different experiments.
DISCUSSION

TPA treatment caused two distinct stages of morphological alterations in low calcium-grown SCC cells: the initial stage, inducing formation of cell-cell contact with desmosomes (Fig. 2); and the second stage, demonstrating fibroblastic changes or formation of long cell projections (strands) with a reduction of the number of cell-cell contacts (Fig. 3).

At the first stage of TPA effects, cell-cell contacts were actively formed within a short period. This phenomenon is very similar to that induced by raising the calcium concentration up to normal level (1.87 mM), as described in our previous experiments (26), and in mouse keratinocytes (20, 22). However, the calcium-induced cell-cell contact and peripheral extension of KIF bundles were more distinct than those seen by 15-min TPA treatment.

It has been shown that addition of calcium to the low-calcium medium activated 4-fold activity of protein kinase C in the membrane fraction when it was assayed at 15 min after the calcium shift, and the increased activity was sustained for at least 3 h. This increase was not associated with a decrease in activity in the cytosol fraction. In the present study, protein kinase C activity was measured and showed a great increase in the membrane fraction and an extreme decrease in the cytosol fraction (Fig. 4). This suggests the translocation of the enzyme from the cytoplasm to the membrane components as seen in other cell types (14–19). These data indicate that an increase in protein kinase C activity of membranes may be involved in TPA induction of cell-cell contact in low calcium-grown cells.

To make a close study of this possibility, the effects of a potential inhibitor of protein kinase C, H-7 were examined for cell-cell contact formation. Concerning the inhibitory specific-
low calcium-grown cells (Fig. 4). This finding leads us to speculate that the reduction of protein kinase C activity may be a trigger to induce the morphological alterations. The reliability of this speculation was supported by the experiment using the protein kinase C inhibitor, H-7, in vivo. The treatment with H-7 longer than 24 h without TPA caused fibroblastic conversion in low calcium-grown cells and a decrease in the number of cell-cell contacts in addition to fibroblastic changes similar to those induced by 24-h treatment with TPA in normal calcium-grown cells (Fig. 5).

In this connection, it is of interest to note that the binding of the agents to the receptors decreases the binding capacity on the subsequent exposure to phorbol esters (down regulation). This has been observed in a variety of cells, including mouse (39) and rat (40) skin keratinocytes. In fetal rat keratinocyte culture, TPA (162 nm) induces a significant increase in protein kinase C activity in the membrane fraction, which reaches a peak in 15 min and then rapidly decreases to about the control level after 120 min. Of equal interest is the finding that low calcium-grown cultured mouse keratinocytes lose the ability of calcium-dependent terminal differentiation by pretreatment with TPA (6). This suggests that the loss of responsiveness to calcium increase is due to a disturbance in signal transduction via protein kinase C which is caused by TPA pretreatment (down regulation). In contrast, it was shown that such down regulation was not observed in normal human epidermal cells (41). However, in the present study, human epidermal SCC cells showed a rapid and transient increase in protein kinase C activity in the membrane fraction by TPA treatment (Fig. 4). Upon TPA additions, protein kinase C activity in the cytosol fraction was rapidly decreased, and it was maintained at the lower level at least for the next 24 h. Therefore this may suggest the existence of down regulation of phorbol ester receptors in our cell system, although the direct binding capacity was not determined. Taking the morphological effects and down regulation into consideration, we would conclude that the biological effects of TPA are biphasic (the initial stage depending on activation and the second stage depending on down regulation of protein kinase C, which may imply an unresponsiveness of the signal transduction system via protein kinase C). The latter is also caused by H-7.

As far as SCC cells used here are concerned, it may be also suggested that activation of protein kinase C has somehow important relevance to formation and stabilization of cell-cell contact with desmosomes, since a rapid effect of TPA (activation of protein kinase C) was formation of cell-cell contact (Fig. 2), and 24-h treatment with H-7 (inhibition of protein kinase C) or TPA (down regulation of protein kinase C) of normal calcium-grown cells, which possessed originally cell-cell contact (Fig. 1), decreased the number of cell-cell contacts (Fig. 5). This was further supported in that calcium addition to low calcium-grown cells induced formation of cell-cell contact within 5 min, and the cell-cell contact was stable thereafter, and that this process was associated with a lasting elevation of protein kinase C activity.  

ACKNOWLEDGMENTS

We thank T. Masuda, N. Ishizaki, and A. Suzuki for expert technical and secretarial assistance.

REFERENCES

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