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DIACYLGLYCEROLS, UNLIKE PHORBOL ESTERS, DO NOT INDUCE HOMOLOGOUS DESENSITIZATION OR DOWN-REGULATION OF PROTEIN KINASE C IN SWISS 3T3 CELLS

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SUMMARY: Addition of 1-oleoyl-2-acetyl-glycerol (OAG), 1,2-dioctanoyl-glycerol (diC8) or phorbol-12,13-dibutyrate (PDBu) to cultures of Swiss 3T3 cells rapidly increases the phosphorylation of the Mr 80,000 protein kinase C (PKC) substrate, inhibits EGF binding and stimulates DNA synthesis. Prolonged incubation (40 h) with PDBu completely blocked these responses to all agents and down-regulated PKC. In contrast, a similar treatment with OAG or diC8, at mitogenic concentrations, neither induced homologous cellular desensitization nor decreased the immunoreactive level or activity of PKC. The results show that PKC down-regulation can be dissociated from PKC-mediated mitogenesis in Swiss 3T3 cells. © 1989 Academic Press, Inc.

Protein kinase C (PKC), which is activated by diacylglycerols and tumor-promoting phorbol esters, plays a major role in the control of a variety of cellular processes (1,2). Considerable evidence implicates PKC as a synergistically acting signal transduction pathway leading to mitogenesis in Swiss 3T3 cells (3), a useful model system for elucidating mechanisms of growth control (4). In these cells, phorbol esters stimulate reinitiation of DNA synthesis in synergy with insulin and other growth factors (5).

Exogenously added cell-permeable diacylglycerols mimic the mitogenic effects of phorbol esters (6). Prolonged exposure to phorbol esters down-regulates PKC (7) and desensitizes the cells to the mitogenic effects of both phorbol esters and diacylglycerols (3,6). Microinjection of purified

Abbreviations: OAG, 1-oleoyl-2-acetyl-glycerol; diC8, 1,2-dioctanoyl glycerol; PDBu, phorbol-12,13-dibutyrate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; R59022, 6-{2-[4-((4-fluorophenyl)phenylmethylene)-1-piperidinyl]ethyl}-7-methyl-5H-thiazolo [3,2a]-pyrimidin-5-one; DMEM, Dulbecco's modified Eagle's medium.

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PKC restores phorbol ester responsiveness to PKC down-regulated 3T3 cells (8).

It is also known that PKC provides feed-back inhibition in cellular signalling (9) including mitogen-induced Ca^{2+} mobilization (10) and transmodulation of EGF receptor affinity (11). Nishizuka (9) suggested that this inhibitory limb of the dual actions of PKC could also play a role in the control of cell proliferation. Specifically, phorbol ester induced PKC down-regulation would remove an inhibitory influence and thereby facilitate the positive effects of synergistic growth factors. A test of this hypothesis requires to determine whether other agents that stimulate mitogenesis via PKC, such as diacylglycerols, also cause PKC down-regulation and hence, desensitization of this pathway.

The effect of prolonged treatment with diacylglycerols on the responsiveness of the PKC mitogenic pathway has not been defined. Huang et al. (12) showed that diacylglycerol greatly increases the conversion of type α PKC from a trypsin insensitive to a sensitive form 'in vitro' and suggested that 1,2-diacylglycerols induce PKC down-regulation 'in vivo'. In contrast, 1,2-dioctanoyl-glycerol (diC8) did not induce down-regulation of PKC in the human breast cancer cell line MCF-7 (13). In this cell line, phorbol esters (14) and diC8 (15) inhibit cell proliferation. Here, we have used Swiss 3T3 cells that express type α PKC (16), to examine whether prolonged exposure to cell-permeable diacylglycerols causes mitogenic desensitization and down-regulation of PKC.

MATERIALS AND METHODS

Culture of Swiss 3T3 cells and measurements of DNA synthesis (17), ^{125}I-EGF binding (18), protein phosphorylation in digitonin-permeabilized cells (19), protein kinase C activity (7) were carried out as previously described unless indicated otherwise. Immunoblotting was performed as described (20). Briefly, the incubations were terminated by rapidly washing the dishes with cold phosphate buffered saline solution (PBS) and adding 300 µl of hot (100°C) sample buffer. The lysates were removed from the dish, boiled for 5 min and then sonicated. Samples for immunoblotting (100 µg protein) were subjected to SDS-PAGE in 8% gels and then transferred to nitrocellulose for 2 h at 200 mA. Nitrocellulose sheets were treated for 5 h at 20°C with 5% dried milk in PBS containing 0.1% Tween 20 and then incubated overnight at 20°C with antibodies at a 1/600 dilution in PBS with 0.1%

Tween 20. Two different monoclonal antibodies were used: one directed against protein kinase C and the other against alpha tubulin. Unbound antibodies were removed by washing the filters three times for 20 min in PBS containing 0.1% Tween 20. Bound antibodies were detected by incubation with ^{125}I-anti-mouse IgG (0.5 µCi/ml) in PBS containing 0.1% Tween 20 for 3 h at 37°C. The filters were washed several times with 1% Tween 20 in PBS and then exposed to X-ray films.

Materials: Phorbol 12,13 dibutyrate (PDBu), diacylglycerols, bovine serum albumin and digitonin were obtained from Sigma. All radiochemicals, nitrocellulose and monoclonal antibodies were purchased from Amersham International.

RESULTS AND DISCUSSION

Addition of the cell permeable diacylglycerols, OAG and diC8 to cultures of Swiss 3T3 cells in the presence of insulin caused a potent dose-dependent stimulation of DNA synthesis (Fig. 1A). Maximal effects, obtained at 100 µg/ml, were comparable to those induced by PDBu and insulin or fresh serum.

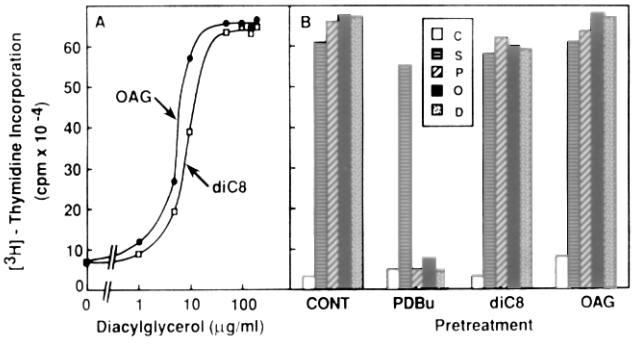

Figure 1 Diacylglycerols do not induce homologous mitogenic desensitization in Swiss 3T3 cells. A Effect of varying concentrations of OAG or diC8 on [^{3}H]-thymidine incorporation. Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated in 2 ml DMEM/Waymouth's medium containing [^{3}H]-thymidine, insulin at 1 µg/ml, bovine serum albumin at 1 mg/ml and various concentrations of OAG or diC8. The incorporation of radioactivity into acid-insoluble material was measured after a 40 h incubation. In this experiment, addition of 10% fetal bovine serum or 100 ng/ml PDBu together with 1 µg/ml insulin produced respectively 58 x 10^4 cpm and 60.5 x 10^4 cpm incorporated into acid-insoluble material at 40 h. B Effect of prolonged treatment with PDBu or diacylglycerols on the subsequent stimulation of [^{3}H]-thymidine incorporation by Swiss 3T3 cells. Quiescent cells were incubated for 40 h in their own conditioned medium, in the absence (Cont) or presence of PDBu (200 ng/ml), OAG (100 µg/ml) or diC8 (100 µg/ml). At this time, cells were washed three times in DMEM containing 1 mg/ml bovine serum albumin, incubated in this medium for 20 min at 37°C, followed by two additional washes to remove residual compounds. The cultures were then incubated in the absence (C, open) or presence of 10% fetal bovine serum (S, horizontal), 100 ng/ml PDBu (P, diagonal), 100 µg/ml OAG (O, closed) or 100 µg/ml diC8 (D, dotted) as described in Fig. 1A.

To determine whether prior exposure of 3T3 cells to either OAG or diC8 can influence the subsequent mitogenic response to these agents, quiescent cultures of these cells were treated with OAG or diC8 at 100 µg/ml in the absence of insulin for 40 h. The cultures were then washed extensively to remove residual diacylglycerols and the stimulation of DNA synthesis in response to these agents together with insulin was measured. Fig. 1B shows that prior exposure to either OAG or diC8 did not decrease the mitogenic responsiveness to these agents or to PDBu. In contrast, prior treatment of

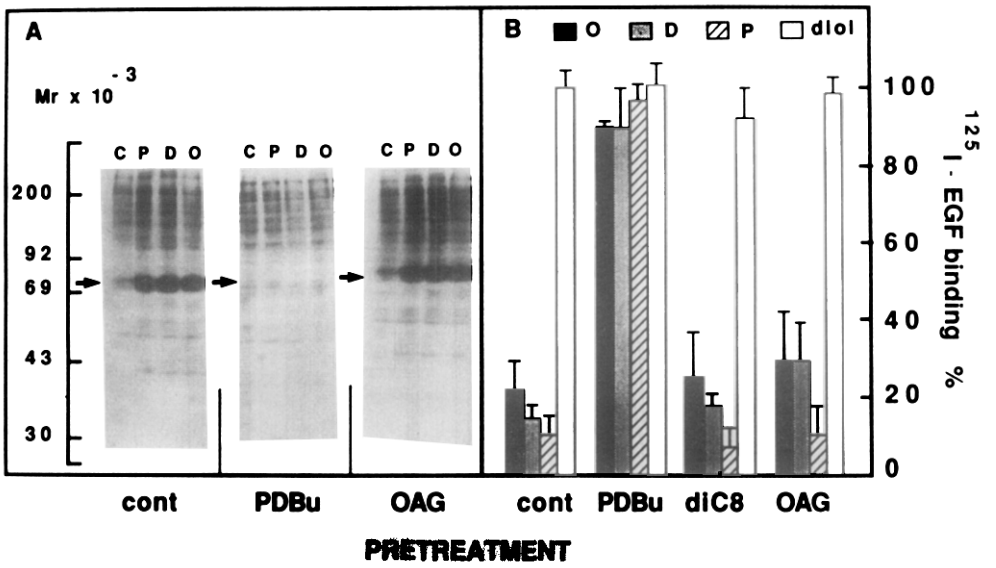

Figure 2 Effect of pretreatment with PDBu or diacylglycerols on the subsequent stimulation of Mr 80,000 protein phosphorylation by OAG, diC8 or PDBu (A) or inhibition of ^{125}I-EGF binding (B). Panel A Quiescent cells were incubated for 40 h in the absence (Cont) or presence of PDBu (200 ng/ml) or OAG (100 µg/ml) as described in Fig. 1B. The cultures were then washed twice with isotonic KCl salt solution (19) and exposed to 100 ng/ml PDBu (P), 20 µg/ml diC8 (D), 24 µg/ml OAG (O) or no addition (C) in permeabilisation medium (19) containing 10 µM [γ-^{32}P]ATP. Reactions were terminated after 1 min, and samples were analysed by SDS-polyacrylamide gel electrophoresis. The arrow indicates the position of the Mr 80,000 protein band. Panel B Quiescent Swiss 3T3 cells were incubated for 40 h in the absence (Cont) or presence of PDBu (200 ng/ml), diC8 (100 µg/ml) or OAG (100 µg/ml). After extensive washing, cells were then incubated for 1 h at 37°C in the absence or presence of 100 µg/ml OAG (O, closed), 100 µg/ml diC8 (D, dotted), 100 ng/ml PDBu (P, diagonal) or 100 µg/ml diolein (diol, open). The cultures were washed and incubated for 2 h at 4°C in 1 ml of binding medium containing 2 ng/ml (110,000 cpm/ng) ^{125}I-EGF. Cell-associated radioactivity was then determined as described in Materials and Methods. Results were expressed as the percentage of the control value (mean ± SD, n = 3 to 5). 1,2 diolein (diol, open) which is not able to stimulate protein kinase C in intact fibroblasts (6) and failed to induce any significant inhibition of ^{125}I-EGF binding to Swiss 3T3 cells is shown as an additional control.

parallel cultures with PDBu completely blocked the subsequent mitogenic response induced by PDBu, OAG or diC8 (Fig. 1B). Thus, exogenously added diacylglycerols differ strikingly from phorbol ester in their ability to induce mitogenic desensitization of the PKC pathway. To substantiate this conclusion, we measured early events elicited via PKC.

Activation of PKC rapidly increases the phosphorylation of a Mr 80,000 cellular protein in intact and digitonin-permeabilized 3T3 cells (19) and causes inhibition of specific ^{125}I-labelled EGF binding (11). Fig. 2 shows that addition of OAG, diC8 or PDBu stimulated Mr 80,000 phosphorylation in permeabilized 3T3 cells and inhibited specific binding of ^{125}I-EGF. As expected, prolonged incubation (40 h) with PDBu completely blocked the effect of OAG, diC8 and PDBu on both early responses. In contrast, a similar long incubation with 100 µg/ml OAG did not prevent the stimulation

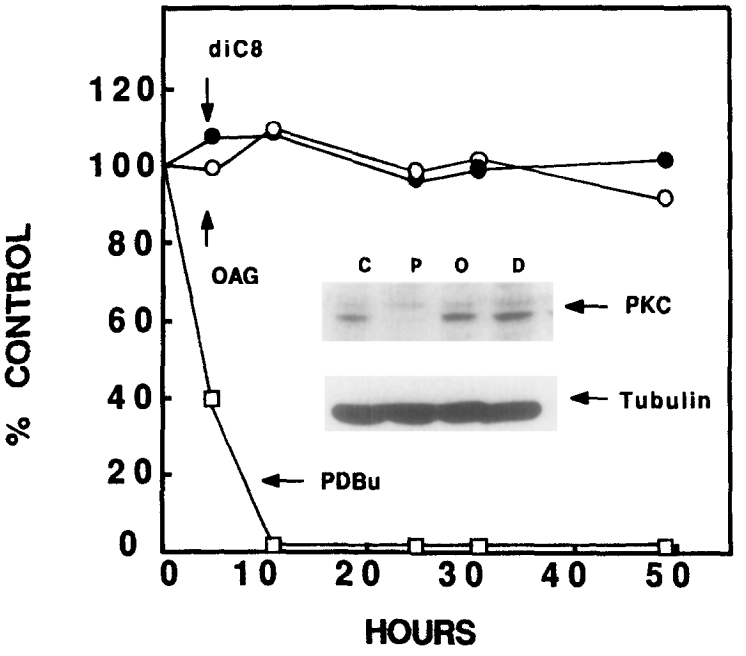

Figure 3 Immunoblot analysis of detergent-solubilized extracts from Swiss 3T3 cells. Quiescent cultures of Swiss 3T3 cells grown in 90 mm Nunc dishes were treated without (Control) or with 200 ng/ml PDBu (P), 100 µg/ml OAG (O) and 100 µg/ml diC8 (D) for varying times. The results of the densitometric scanning are plotted as percent of the value for control cells. The inset shows the bands corresponding to PKC (80 kDa) and to tubulin (55 kDa) after 30 h of treatment. Tubulin expression served as a control for the amount of total cellular protein loaded in each gel track within an individual experiment.

of Mr 80,000 phosphorylation or the inhibition of ^{125}I-EGF binding produced by the subsequent addition of OAG, diC8 or PDBu (Fig. 2). In all preceding experiments the cultures were pretreated with diacylglycerols for 40 h. In other experiments, we monitored the inhibition of ^{125}I-EGF binding by OAG or diC8 (at 100 µg/ml) as a function of time (0.5 - 24 h). We verified that these diacylglycerols did not induce transient desensitization at earlier times.

The results shown in Figs. 1 and 2 strongly suggest that cell-permeable diacylglycerols stimulate mitogenesis via PKC without causing either transient or persistent down-regulation of the enzyme. To test directly this possibility we measured PKC activity and the amount of PKC molecule by immunoblot analysis (20). We used a monoclonal antibody that recognizes a sequence conserved in PKC α and β. Swiss 3T3 cells are known to express exclusively type α PKC (16). Treatment with either OAG or diC8 at 100 µg/ml did not cause any decrease in immunoreactive PKC (Fig. 3) or in

TABLE 1

Effect of prolonged pretreatment with PDBu, diC8 or OAG on PKC activity

Pretreatment	PKC activity (pmole/min)
—	9.6 ± 0.5
PDBu	0.2 ± 0.01
OAG	8.3 ± 0.6
diC8	8.7 ± 0.6

The cultures were pretreated for 40 h without or with 200 ng/ml PDBu, 100 µg/ml OAG, or 100 µg/ml diC8 as indicated. Then, the cultures were washed and the cells were scraped off the dishes. Detergent-solubilised cell extracts for determination of PKC activity were prepared as previously described (7). Incorporation of ^{32}P from [γ-^{32}P]-ATP into histone H1 was assayed in the presence or absence of phosphatidylserine (80 µg/ml), $CaCl_2$ (5 x 10^{-4} M) and phorbol ester (100 ng/ml) for 3 min at 30°C. The extract from control or treated cells contained 50 µg of protein. Mean ± SD of triplicate determinations.

PKC activity (Table 1). In contrast, PDBu caused striking down-regulation in parallel cultures. Similar results were obtained when the diacylglycerols were added either at 150 µg/ml or in the presence of 5 µM R59022, a potent diacylglycerol kinase inhibitor which is biologically active in 3T3 cells (21; data not shown).

The results presented here demonstrate that cell-permeable diacylglycerols, at mitogenic concentrations, neither induce desensitization of the PKC signal transduction pathway nor cause down-regulation of the enzyme. Since PKC down-regulation can be dissociated from PKC mediated mitogenesis, the removal of the inhibitory effects of PKC cannot be crucial for stimulation of the cell cycle in 3T3 cells. It is conceivable that down-regulation of PKC may play a role in the expression of other long-term biological responses evoked by phorbol esters (e.g. cell differentiation) in other cell types. A comparison of the effects of phorbol esters with those of cell-permeable diacylglycerols may provide an approach to distinguish between stimulation or down-regulation of PKC as a mediator of the biological actions of phorbol esters.

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