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Intracellular activation of protein kinase C and regulation of the surface transferrin receptor by diacylglycerol is a spontaneously reversible process that is associated with rapid formation of phosphatidic acid

(receptor endocytosis)

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ABSTRACT **The effect of the synthetic diacylglycerol, *sn*-1,2-dioctanoylglycerol (diC_8), on the expression of the surface transferrin receptor reveals that exogenous diC_8 can act as an intracellular activator of protein kinase C and stimulate both down-regulation and increased receptor phosphorylation in a manner similar to that induced by the active tumor promotor, 4β-phorbol 12,13-dibutyrate. Unlike the spontaneously irreversible effect noted when 4β-phorbol 12,13-dibutyrate is added, this same effect mediated by diC_8 is brief, lasting only minutes, and is spontaneously reversible. The rate of reversibility is dependent on the concentration of diC_8 added, and it is associated with rapid formation of a newly detected intracellular phospholipid that corresponds to *sn*-1,2-dioctanoyl phosphatidic acid. These data, in conjunction with findings that demonstrate that exogenous diacylglycerols (including diC_8) when added to cells do not stimulate cellular phospholipase A_2 or C, argue that protein kinase C is activated only briefly in this system since exogenous diC_8 is subject to rapid intracellular metabolism to phosphatidic acid.**

Phorbol esters are potent tumor-promoting agents that exert pleiotropic effects on cells (1–3). These agents appear to mediate most if not all of their effects by binding and activating their specific intracellular receptor, Ca^{2+} phospholipid-dependent protein kinase (protein kinase C) (4–6). Recently, a primary product of phosphatidylinositol metabolism, 1,2-diacylglycerol, has been found to bind similarly and activate protein kinase C (4, 5, 7–9). This has led to the hypothesis that signal-induced phospholipid turnover with generation of 1,2-diacylglycerols and activation of protein kinase C may be mimicked by addition of phorbol esters (10, 11).

Phorbol esters have been shown to mediate reversible down-regulation (i.e., internalization) of the surface transferrin receptor (12–15). Down-regulation is tightly coupled to increased receptor phosphorylation, while up-regulation is associated with receptor dephosphorylation (13). Although a causal relationship between phosphorylation and surface expression has not been proved, increased phosphorylation of the receptor has been shown recently to be mediated directly by activated protein kinase C (14). Using this model system for the coordinate activation of protein kinase C and regulation of a specific membrane receptor substrate, we have tested the hypothesis that *sn*-1,2-diacylglycerols function as intracellular activators of protein kinase C in intact cells and whether such a response is spontaneously reversible. The latter finding would be predicted to occur if protein kinase C were activated intracellularly only briefly by signal-induced generation of 1,2-diacylglycerol activator(s) whose effect could be reversed rapidly.

EXPERIMENTAL PROCEDURES

Synthetic *sn*-1,2-diacylglycerols were a generous gift of Robert M. Bell (Department of Biochemistry, Duke University Medical Center, Durham, NC) (7, 8). Human ferrotransferrin was purchased from Alpha Therapeutics (Los Angeles). Na[^{125}I]iodide and [^{32}P]orthophosphoric acid were purchased from Atomic Energy of Canada (Ottawa, ON, Canada). Preparation of ^{125}I-labeled ferrotransferrin (^{125}I-transferrin) was as detailed (13). Both 4β- and 4α-phorbol 12,13-dibutyrate (PBt_2) were purchased from Sigma. All other reagents were from commercial sources. Preparation of *sn*-1,2-dioctanoyl phosphatidic acid was as described (16). Growth and maintenance of HL60 human leukemic cells was carried out as described (13).

^{125}I-Transferrin Binding and Affinity Isolation of the Transferrin Receptor. Binding of ^{125}I-transferrin to HL60 cells and phosphorylation and isolation of the transferrin receptor in intact cells was performed as described (13).

Isolation and Identification of ^{32}P-Labeled Phospholipids from Whole Cells. HL60 cells (2×10^7 cells per ml) were incubated at 37°C with [^{32}P]orthophosphoric acid at 1 mCi/ml (1 Ci = 37 GBq) for 2.5 hr as described (13). Cells were then aliquoted and additions were made as indicated in the text. *sn*-1,2-Diacylglycerols were dissolved in chloroform. Chloroform was first removed by evaporation and the lipid residue was dissolved in Me_2SO. Additions in Me_2SO were made as described in the text. The final concentration of Me_2SO in any sample never exceeded 0.5%. After incubation with these agents or with 4β-PBt_2, cells were divided for isolation and identification of [^{32}P]phosphatidic acids (7, 8) and ^{32}P-labeled transferrin receptor as described (13). For ^{32}P phospholipids, 0.5 ml of cells was extracted with 1.8 ml of chloroform/methanol (1:2, vol/vol) as described (7, 8, 17, 18). Phases were then separated by adding 0.6 ml of chloroform and 0.6 ml of water. The lower organic phase was dried under the flow of N_2 and lipids were separated on thin-layer chromatography. Separation of 1,2-dioctanoyl phosphatidic acid from cellular phosphatidic acid was achieved in two different chromatographic systems: (*i*) oxalate-impregnated silica gel G-25 thin-layer chromatography plates were developed in Cl_3CH/MeOH/HCl (174:26:1; vol/vol) (see Fig. 4), and (*ii*) silica gel G-25 thin-layer chromatography plates were developed by using the upper phase from a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10;

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Abbreviations: diC_8, *sn*-1,2-dioctanoylglycerol; PBt_2, 4β-phorbol 12,13-dibutyrate; ^{125}I-transferrin, ^{125}I-labeled ferrotransferrin.

vol/vol). An autoradiograph of this separation is shown in ref. 8.

RESULTS

Effect of *sn*-1,2-Dioctanoylglycerol (DiC_8) and 4β-PBt_2 on Down-Regulation and Phosphorylation of the Surface Transferrin Receptor. The synthetic diacylglycerol, diC_8, can induce down-regulation of the surface transferrin receptor as measured by the decrease in ^{125}I-transferrin binding after incubation of cells with this agent. Down-regulation occurs in a dose-dependent manner when increasing concentrations of diC_8 are added, with the maximal effect occurring between 25 and 50 μM diC_8 (Fig. 1). The amplitude of this effect is similar to that induced by a maximal concentration of 4β-PBt_2 (Fig. 1). The time course for receptor down-regulation is more rapid for diC_8, being maximal by 5 min, while the PBt_2-induced effect is maximal after 15 min (Fig. 1). Furthermore, the diC_8 effect is beginning to reverse itself after 15 min of incubation with the lower concentrations of diC_8 used (i.e., 25–50 μM), and it is fully reversed after 30 min (Fig. 1). Similar results were observed with 50 μM *sn*-1,2-dihexanoylglycerol was substituted for diC_8 (data not shown). Down-regulation is specific for diC_8 since spontaneous reversal can be delayed by the addition of increasing concentrations of diC_8 (i.e., 100 and 200 μM; Fig. 1). By contrast, the PBt_2 effect is not reversible unless this agent is washed free from the cells (Fig. 2) (13). Down-regulation is specific for the active phorbol ester since the inactive tumor promoter 4α-PBt_2 has no effect on expression of the surface receptor (Fig. 1).

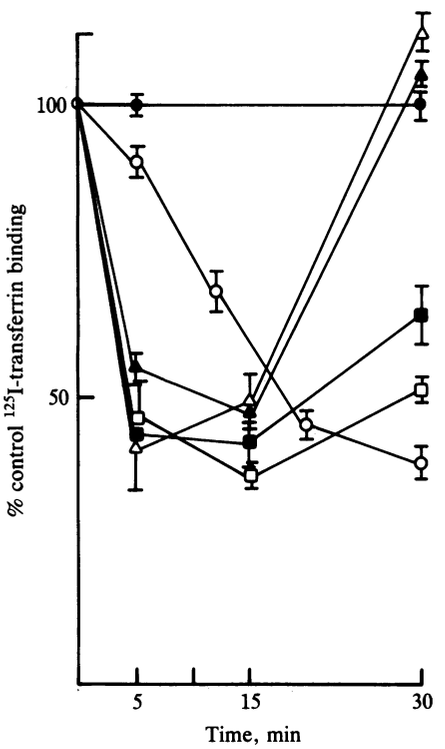

FIG. 1. Concentration and time-dependent effect of synthetic diC_8 on down-regulation of the surface transferrin receptor. Whole cells were incubated with increased concentrations (μM) of diC_8 at 37°C or with 100 nM PBt_2 for the times indicated. ^{125}I-transferrin equilibrium binding was then performed at 4°C as described. ●, Control (4α-PBt_2) binding represents 100% binding and was determined after incubation of cells with 100 nM inactive 4α-PBt_2; ▲, 25 μM diC_8; △, 50 μM diC_8; ■, 100 μM diC_8; □, 200 μM diC_8; ○, 100 nM 4β-PBt_2. Points represent mean ± SEM of triplicate determinations in a representative experiment.

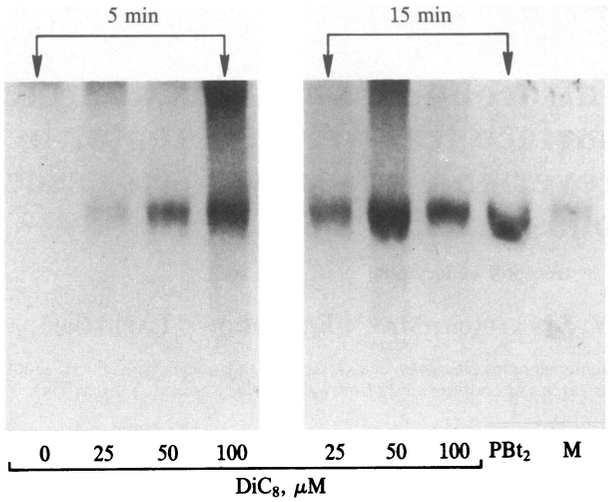

FIG. 2. DiC_8-mediated concentration- and time-dependent phosphorylation of the transferrin receptor. Cells (2×10^7 cells per ml) were incubated with carrier-free [^{32}P]orthophosphoric acid (1 mCi/ml) for 2.5 hr at 37°C. Increasing concentrations of diC_8 (μM) were added to cells at 37°C for the times (min) indicated. Cells were then washed and solubilized, and the transferrin receptor was isolated by affinity chromatography at 4°C (13) as described. After isolation, the receptor was purified by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (13). The resulting autoradiogram is shown. PBt_2 represents the transferrin receptor isolated and purified after incubation of cells with 200 nM PBt_2. M, authentic ^{125}I-labeled transferrin receptor marker used to locate the 94-kDa receptor band as described (13).

Phorbol ester-induced transferrin receptor down-regulation has been demonstrated to be tightly coupled with increased phosphorylation of the receptor (13, 14). Similarly, it can be seen that diC_8 induces transferrin receptor down-regulation as well as increased receptor phosphorylation (Fig. 2). Increased receptor phosphorylation was found to occur by 5 min and is maintained through 15 min when cells were incubated with concentrations of diC_8 ranging from 25 to 100 μM (Fig. 2). Likewise, when PBt_2 is added, increased phosphorylation is observed (Fig. 2).

Synergistic Effect of the Calcium Ionophore A23187 on DiC_8-Induced Down-Regulation of the Surface Transferrin Receptor. We have recently shown that increasing intracellular Ca^{2+}, by incubating cells with the Ca^{2+} ionophore A23187 prior to the addition of phorbol ester, increases the potency and rate of action of the phorbol ester for activating protein kinase C and mediating transferrin receptor down-regulation and phosphorylation (19). Since the synthetic diC_8 can mimic phorbol ester effect(s) on surface transferrin receptor expression, we have tested whether intracellular calcium mobilization can synergize with diC_8 to induce receptor down-regulation. Fig. 3 demonstrates that, although ineffective by itself, addition of 200 nM calcium ionophore A23187 does increase the potency of 5 and 10 μM diC_8 for mediating transferrin receptor down-regulation.

Effect of DiC_8 and 4β-PBt_2 on Formation of *sn*-1,2-Dioctanoyl Phosphatidic Acid. Since the effect on surface transferrin receptor expression stimulated by exogenous diC_8 was found to be rapidly reversible and could be prolonged by addition of higher concentrations of this compound, we examined the possibility that diC_8 was converted rapidly by phosphorylation to the corresponding phosphatidic acid, *sn*-1,2-dioctanoyl phosphatidic acid. When 25–200 μM diC_8 is added to cells equilibrated with [^{32}P]orthophosphoric acid, there is a rapid appearance of a newly phosphorylated phospholipid product that comigrates with authentic *sn*-1,2-dioctanoyl phosphatidic acid (Fig. 4). Formation is maximal by 10 min and is essentially not detected within cells after 30

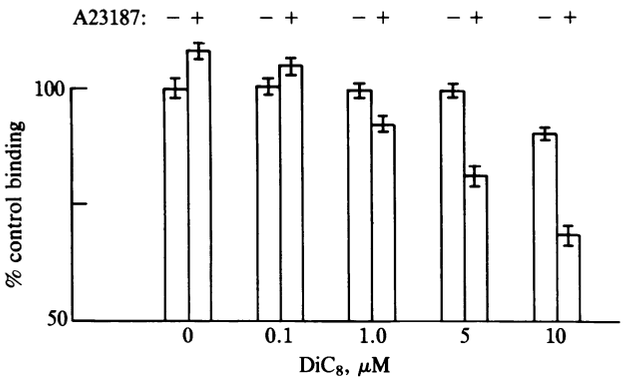

FIG. 3. Effect of A23187 on diC_8-mediated down-regulation of the surface transferrin receptor. Whole cells were prepared as described and suspended in RPMI 1640 medium containing 10 mM Hepes (pH 7.3) and 2% fetal calf serum. (RPMI 1640 medium contains 1 mM $CaNO_3$.) A23187 ionophore was added (+) at 200 nM for 10 min before adding increasing concentrations of diC_8 as shown. After incubation for 5 min at 37°C, cell pellets were collected at 4°C and equilibrium binding with a saturating concentration (21 nM; ref. 13) of ^{125}I-transferrin was performed as described. Results represent triplicate determinations expressed as the mean percent of control binding ± SEM from a representative experiment.

min of incubation at 37°C after addition of 25–100 μM diC_8. There is some residual *sn*-1,2-dioctanoyl phosphatidic acid still observed after 30 min when 200 μM diC_8 is added to cells (Fig. 4). Similar results for diC_8-induced time-dependent appearance of *sn*-1,2-dioctanoyl phosphatidic acid were confirmed in a second solvent system (8) used to separate phosphatidic acids from other phospholipids (Table 1). After addition of 50 μM diC_8 to cells, it was observed that *sn*-1,2-dioctanoyl phosphatidic acid was generated intracellularly within 1 min, was maximal between 5 and 10 min, began to disappear by 15 min, and was not detected after 30 min of incubation (Table 1). By contrast, addition of 100 nM PBt_2 to cells did not induce formation of any newly detected phospholipid metabolites in HL60 cells at a time when the response is maximal (Fig. 4).

DISCUSSION

The effect of the synthetic diacylglycerol, diC_8, on the expression of the surface transferrin receptor revealed that exogenous diC_8 can act as an intracellular activator of protein kinase C and stimulate both down-regulation (i.e., internalization) and increased receptor phosphorylation in a manner similar to that induced by the active tumor promoter PBt_2 (Figs. 1 and 2). While the maximal amplitude of the effect is similar for these two agents, the process is more rapid for diC_8 (Fig. 1). This may occur as a result of a difference in membrane solubility between these two agents. Furthermore, exposure of cells to Ca^{2+} ionophore can increase, in a synergistic manner, the potency of diC_8 for mediating receptor down-regulation (Fig. 3). This finding is consistent with a recent report suggesting that increased intracellular Ca^{2+} may act to recruit protein kinase C to the plasma membrane, thus "priming" the system for activation by a specific activator such as phorbol ester or diacylglycerol (19, 20).

However, unlike the spontaneously irreversible effect noted when PBt_2 is added to HL60 cells, this same effect mediated by diC_8 is brief, lasting only minutes, and is spontaneously reversible (Fig. 1). Full reversibility is dependent on the concentration of diC_8 added, and it is associated with rapid formation of an intracellular phospholipid that corresponds to *sn*-1,2-dioctanoyl phosphatidic acid (Fig. 4). The concentration and time-dependent formation of *sn*-1,2-dioctanoyl phosphatidic acid, when taken together with

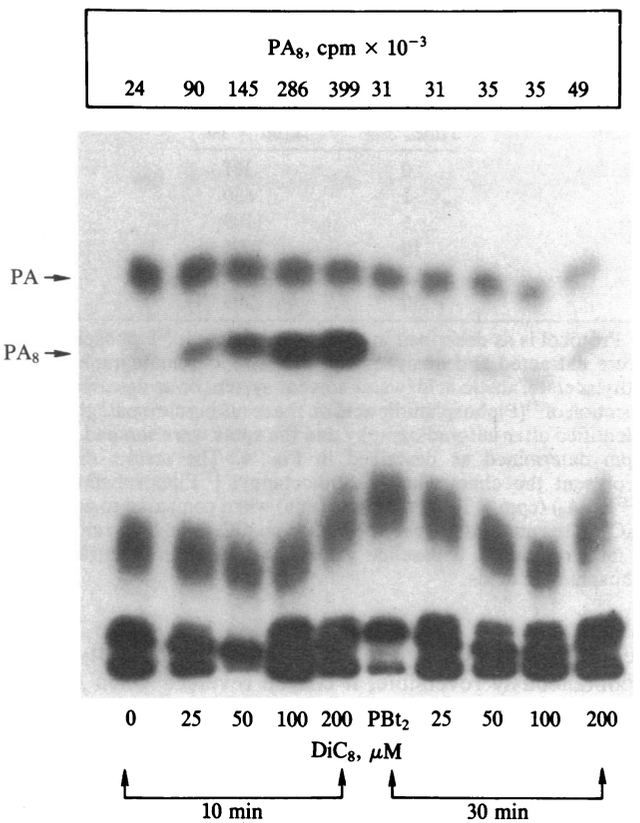

FIG. 4. Effect of concentration and time of formation of *sn*-1,2-dioctanoyl phosphatidic acid by diC_8. Cells (2×10^7 cells per ml) were incubated with [^{32}P]orthophosphoric acid as described in the legend to Fig. 2. Increasing concentrations of diC_8 (μM) were added to cells for 10 and 30 min. ^{32}P phospholipids were extracted and analyzed by thin-layer chromatography in the Cl_3CH/MeOH/HCl solvent system, and the resulting autoradiogram is shown as described. Endogenous phosphatidic acids (PA) (which are esterified with stearic acid in position number 1 and arachidonic acid in position number 2) are separated from the 1,2-didecanoyl phosphatidic (PA_8) acid generated in the presence of exogenous synthetic 1,2-didecanoylglycerol. Authentic unlabeled PA_8 synthesized (7, 18) as described was used as a standard. Spots containing [^{32}P]PA_8 were scraped from the thin-layer plate and counted in a liquid scintillation counter. The cpm corresponding to each lane are displayed in the insert located at the top of the figure.

findings that demonstrate that exogenous diacylglycerols (including diC_8) when added to cells do not stimulate cellular phospholipase A_2 or C (8), argue that protein kinase C is reversibly activated by the exogenous diC_8 (7, 8). While the mechanism by which the diC_8-mediated effect is reversed is not known, at least two likely possibilities exist. First, rapid *sn*-1,2-dioctanoyl phosphatidic acid formation likely results from metabolism of the synthetic diacylglycerol diC_8 (21). Since phosphatidic acid has not been reported to sustain activation of protein kinase C, this finding could explain why down-regulation of the transferrin receptor is not maintained (Fig. 1). Furthermore, phosphatidic acid may actually inhibit activation of the enzyme. Evidence has been published recently that demonstrates that phosphatidic acid newly formed by metabolism of diacylglycerol can dramatically decrease the binding affinity of the protein kinase C–receptor complex for the phospholipid component (22). And it is this phospholipid component that has been shown to specifically affect binding of phorbol esters and competitive agonist activators such as diacylglycerols to the complex (22). Thus, rapid reversibility of diC_8-induced down-regulation of the surface transferrin receptor may result from an inhibitory effect of *sn*-1,2-dioctanoyl phosphatidic acid formation. Second, while the similar effect on transferrin receptor down-

Table 1. Time course of formation of *sn*,1,2-dioctanoyl phosphatidic acid after addition of 50 μM diC_8 to whole cells

Time, min	$[^{32}P]PA_8$, cpm $\times 10^{-3}$
0	301
1	630
5	1109
10	1065
15	887
30	389

Protocol is as described in the legend to Fig. 4. ^{32}P phospholipids were extracted and analyzed by thin-layer chromatography in an ethylacetate/acetic acid/water solvent system or as described. The location of ^{32}[P]phosphatidic acid on the resulting chromatogram was identified after autoradiography and the spots were scraped and the cpm determined as described in Fig. 4. The results displayed represent the change in *sn*-1,2-dioctanoyl $[^{32}P]$phosphatidic acid ($[^{32}P]PA_8$) (cpm $\times 10^{-3}$) with time (min) when compared to no added diC_8. The cpm $\times 10^{-3}$ generated when no diC_8 is added to cells (i.e., 0 min) represents a baseline value for production of intracellular phosphatidic acids.

regulation induced by the active phorbol ester PBt_2 is not spontaneously reversible, it can be reversed when PBt_2 is washed free from the cells (13). This finding demonstrates that removal of the activator of protein kinase C can lead to reversal of the effect. Thus, if the diC_8 agonist is rapidly metabolized by the cells, this might effectively remove enough of the agent to allow for rapid reversibility of receptor down-regulation. Indeed, other investigators have demonstrated that diC_8 needed to be added to cells at bihourly intervals to observe an effect on HL60 cellular differentiation that mimicked that of phorbol 12-myristate 13-acetate (16). Presumably, this process of phorbol ester-induced differentiation requires the sustained activation of protein kinase C (23), which can be mediated with phorbol esters but not with single administrations of diacylglycerols since these agents can be rapidly metabolized, as we and others (21) have noted.

Under the currently held model for extracellular signal-induced activation of inositol phospholipid turnover, specific endogenous diacylglycerol activators of protein kinase C may be generated (10, 11). It is the activation of this enzyme that is postulated to mediate the specific effect, at least in part, related to the extracellular signal. Since synthetic *sn*-1,2-diacylglycerols, including diC_8, have been demonstrated to activate partially purified protein kinase C *in vitro* (4, 5, 8, 14), data presented here suggest that exogenous diC_8 permeates the cell membrane to activate intracellular protein kinase C. However, since *sn*-1,2-diacylglycerols, whether exogenous or specifically generated within the membrane may be metabolized rapidly to phosphatidic acid, as we have observed with diC_8, activation of protein kinase C may not be sustained. Thus, responses that are likely dependent on the activated state of protein kinase C, such as phorbol ester-induced down-regulation of the surface transferrin receptor (12–14), may be rapid and spontaneously reversible when induced by diacylglycerols. Such rapid reversibility would represent a convenient method of intracellular regulation of this important enzyme.

Endogenous diacylglycerol generation from phospholipid metabolism is usually felt to be accompanied by intracellular Ca^{2+} mobilization (10, 11). A dissimilarity between processes activated by exogenous diacylglycerol addition and the physiological (i.e., endogenous) generation of this agent may, however, exist. Whether or not such a brief and spontaneously reversible effect mediated by diC_8 actually mimics a similar physiologic activation of intracellular protein kinase C and the effector pathway is not clear, but it represents the simplest explanation for these data. However, one possible consequence of sustained stimulation of this crucial intracellular enzyme by any nonmetabolized or slowly metabolized agent(s) such as phorbol esters may account, at least in part, for their observed effects on growth control such as tumor promotion and induction of cellular differentiation and/or proliferation (1, 2, 11, 15, 16).

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