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 (diC8), on the expression of the surface transferrin receptor reveals that exogenous diC8 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 promoter, 4β-phorbol 12,13-dibutyrate. Unlike the spontaneously irreversible effect noted when 4β-phorbol 12,13-dibutyrate is added, this same effect mediated by diC8 is brief, lasting only minutes, and is spontaneously reversible. The rate of reversibility is dependent on the concentration of diC8 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 exogenous diacylglycerols (including diC8) when added to cells do not stimulate cellular phospholipase A2 or C, argue that protein kinase C is activated only briefly in this system since exogenous diC8 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, Ca²⁺-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 proven, 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 ferr-transferrin was purchased from Alpha Therapeutics (Los Angeles). Na[125l]iodide and [3H]Jorphosphoric acid were purchased from Atomic Energy of Canada (Ottawa, ON, Canada). Preparation of 125I-labeled ferrtransferrin (125I-transferrin) was as detailed (13). Both 4β- and 4α-phorbol 12,13-dibutyrate (PBTs) 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).

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

Isolation and Identification of 32p-Labeled Phospholipids from Whole Cells. HL60 cells (2 x 10⁷ cells per ml) were incubated at 37°C with [32p]Jorphosphoric 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 Me₂SO. Additions in Me₂SO were made as described in the text. The final concentration of Me₂SO in any sample never exceeded 0.5%. After incubation with these agents or with 4β-PBTc, cells were divided for isolation and identification of [32p]J phosphatidic acids (7, 8) and 32p-labeled transferrin receptor as described (13). For 32p 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 N₂ 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) octylate-impregnated silica gel G-25 thin-layer chromatography plates were developed in Cl₃CH/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/acetonic acid/water (9:5:2:10;

Abbreviations: diC8, sn-1,2-dioctanoylglycerol; PBTc, 4β-phorbol 12,13-dibutyrate; 125I-transferrin, 125I-labeled ferrtransferrin.

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

RESULTS

Effect of sn-1,2-Dioctanoylglycerol (DiC₈) and 4β-PBT₂ on Down-Regulation and Phosphorylation of the Surface Transferrin Receptor. The synthetic diacylglycerol, DiC₈, can induce down-regulation of the surface transferrin receptor as measured by the decrease in ¹²⁵I-transferrin binding after incubation of cells with this agent. Down-regulation occurs in a dose-dependent manner when increasing concentrations of DiC₈ are added, with the maximal effect occurring between 25 and 50 μM DiC₈ (Fig. 1). The amplitude of this effect is similar to that induced by a maximal concentration of 4β-PBT₂ (Fig. 1). The time course for receptor down-regulation is more rapid for DiC₈, being maximal by 5 min, while the PBT₂-induced effect is maximal after 15 min (Fig. 1). Furthermore, the DiC₈ effect is beginning to reverse itself after 15 min of incubation with the lower concentrations of DiC₈ 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-dihexanoyl-glycerol substituted for DiC₈ (data not shown). Down-regulation is specific for DiC₈ since spontaneous reversal can be delayed by the addition of increasing concentrations of DiC₈ (i.e., 100 and 200 μM; Fig. 1). By contrast, the PBT₂ 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-PBT₂ has no effect on expression of the surface receptor (Fig. 1).

![Fig. 1. Concentration and time-dependent effect of synthetic DiC₈ on down-regulation of the surface transferrin receptor. Whole cells were incubated with increased concentrations (μM) of DiC₈ at 37°C or with 100 nM PBT₂ for the times indicated. ¹²⁵I-transferrin equilibrium binding was then performed at 4°C as described. • Control (4α-PBT₂) binding represents 100% binding and was determined after incubation of cells with 100 nM inactive 4α-PBT₂; ▲, 25 μM DiC₈; ●, 50 μM DiC₈; ○, 100 μM DiC₈; △, 200 μM DiC₈; ◇, 100 nM 4α-PBT₂. Points represent mean ± SEM of triplicate determinations in a representative experiment.](image1)

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 DiC₈ 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 DiC₈ ranging from 25 to 100 μM (Fig. 2). Likewise, when PBT₂ is added, increased phosphorylation is observed (Fig. 2).

Synergistic Effect of the Calcium Ionophore A23187 on DiC₈-Induced Down-Regulation of the Surface Transferrin Receptor. We have recently shown that increasing intracellular Ca²⁺, by incubating cells with the Ca²⁺ 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 DiC₈ can mimic phorbol ester effect(s) on surface transferrin receptor expression, we have tested whether intracellular calcium mobilization can synergize with DiC₈ 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 DiC₈ for mediating transferrin receptor down-regulation.

Effect of DiC₈ and 4β-PBT₂ on Formation of sn-1,2-Dioctanoyl Phosphatidic Acid. Since the effect on surface transferrin receptor expression stimulated by exogenous DiC₈ was found to be rapidly reversible and could be prolonged by addition of higher concentrations of this compound, we examined the possibility that DiC₈ was converted rapidly by phosphorylation to the corresponding phosphatidic acid, sn-1,2-dioctanoyl phosphatidic acid. When 25–200 μM DiC₈ is added to cells equilibrated with ⁴¹Porthophosphoric 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. 2. DiC₈-mediated concentration and time-dependent phosphorylation of the transferrin receptor. Cells (2 × 10⁶ cells per ml) were incubated with carrier-free ⁴¹Porthophosphoric acid (1 mCi/ml) for 2.5 hr at 37°C. Increasing concentrations of DiC₈ (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 as described. After isolation, the receptor was purified by NaDodSO₄/polyacrylamide gel electrophoresis as described (13). The resulting autoradiogram is shown. PBT₂ represents the transferrin receptor isolated and purified after incubation of cells with 200 nM PBT₂. M, authentic ¹²⁵I-labeled transferrin receptor marker used to locate the 94-kDa receptor band as described (13).](image2)
There is phospholipid described and of (Fig. 4). phosphatidic sn-1,2-dioctanoyl expression effect addition of to and visualization) may occur as a manner, that may not be irreversible. Thus, rapid sn-1,2-dioctanoyl phosphatidic acid formation likely results from metabolism of the synthetic diacylglycerol diC₈ (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 diC₈-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 \textit{sn},1,2-dioctanoyl phosphatidic acid after addition of 50 \textmu M diC₈ to whole cells

<table>
<thead>
<tr>
<th>Time, min</th>
<th>^[32P]PA₄, \text{ cpm} \times 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>301</td>
</tr>
<tr>
<td>1</td>
<td>630</td>
</tr>
<tr>
<td>5</td>
<td>1109</td>
</tr>
<tr>
<td>10</td>
<td>1065</td>
</tr>
<tr>
<td>15</td>
<td>887</td>
</tr>
<tr>
<td>30</td>
<td>389</td>
</tr>
</tbody>
</table>

Protocol is as described in the legend to Fig. 4. \^[32P] phospholipids were extracted and analyzed by thin-layer chromatography in an ethylacetate/acetic acid/water solvent system as described. The location of \^[32P]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 \textit{sn},1,2-dioctanoyl \^[32P]phosphatidic acid (\^[32P]PA₄) \text{ cpm} \times 10^{-3} with time (min) when compared to no added diC₈. The cpm \times 10^{-3} generated when no diC₈ is added to cells (i.e., 0 min) represents a baseline value for production of intracellular phosphatidic acids.

Endogenous diacylglycerol generation from phospholipid metabolism is usually felt to be accompanied by intracellular Ca\textsuperscript{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 diC₈ 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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