Differential Regulation of Phosphatidylcholine Biosynthesis by 12-O-Tetradecanoylphorbol-13-acetate and Diacylglycerol in NG108-15 Neuroblastoma x Glioma Hybrid Cells

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Phosphatidylcholine (PtdCho) is quantitatively the most important phospholipid in most mammalian cell membranes (1). Until recently membrane PtdCho was considered to be relatively static and subserving a primarily structural function. More recent studies, however, suggest that a variety of stimuli, including tumor-promoting phorbol esters, can strongly and rapidly affect PtdCho metabolism in a variety of cell types (2-7). We have recently shown that the potent phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates PtdCho metabolism in the neuroblastoma X glioma hybrid cell line NG108-15 (8, 9). The first step in the mechanism of phorbol ester action is thought to involve activation of protein kinase C (10, 11). This Ca2+- and phospholipid-dependent enzyme is believed normally to be activated by diacylglycerol formed in the course of receptor-mediated breakdown of membrane phosphoinositides (11, 12). Cell-permeant diacylglycerols were shown to activate protein kinase C in intact cells (13, 14) and to mimic many of the actions of phorbol esters (e.g., 15-17). The present study was designed to evaluate further the possible involvement of protein kinase C in mediating stimulation of PtdCho biosynthesis by TPA, by comparing its action with that of the synthetic, cell-permeant diacylglycerol sn-1-oleoyl-2-acetylglycerol (OAG) and sn-1,2-dioctanoylglycerol.

MATERIALS AND METHODS

[2H]Choline chloride (80 Ci/mmol) and [32P]ATP (20 Ci/mmol) were purchased from Du Pont-New England Nuclear. 12-O-tetradecanoylphorbol-13-acetate, sn-1-oleoyl-2-acetylglycerol, phosphatidylserine, palmitoylcarnitine, dibucaine, chlorpromazine, retinyl palmitate, sphingosine, polymyxin B, histone-type III, leupeptin, phenylmethylsulfonyl fluoride, Nonidet P-40, and ATP were from Sigma. sn-1,2-Dioctanoylglycerol was from Avanti Polar Lipids, Birmingham, AL. 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was obtained from Seikagaku America, St. Petersburg, FL. Stock solutions of phorbol esters (10 mg/ml in Me2SO) were kept frozen at -20°C. Dicylglycerols were kept as 10 mg/ml solutions in chloroform at -15°C; before experiments, an aliquot was dried under a stream of N2 and the dicylglycerol was reconstituted in 10% Me2SO by sonication and utilized immediately.

Cell culture, labeling, and extraction were performed as previously described (8) and as detailed in the legends to the figures and tables. Under the conditions used, more than 90% of [3H]choline-labeled phospholipids were identified as phosphatidylcholine by thin layer chromatography of the phospholipid fraction on LK6D plates (Whatman) utilizing a mobile phase containing chloroform/methanol/isopropanol 25:25:6 (v/v). In some experiments dicylglycerol-PtdCho was separated from normal cellular PtdCho by thin layer chromatography on C8 reversed phase plates (Whatman) utilizing a mobile phase containing methanol/acetone/triethylamine 25:25:6 aqueous chloroform (90:5:2:5:7). In this system sphingomyelin, PtdCho, lysoPtdCho, and diacylglycerol-PtdCho exhibit Rf values of 0.05, 0.04, 0.30, and 0.47, respectively. Labeled water-soluble choline metabolites present in cell extracts were analyzed by high performance liquid chromatography (HPLC) on a normal phase silica column, as described in detail elsewhere (18), except that ammonium acetate was substituted for sodium acetate in the mobile phase. The retention time of most metabolites is not significantly affected by this modification; the phosphocholine peak, however, is retarded and elutes at 46-47 min. Metabolically labeled peaks were routinely identified by comparing their retention times with those of standards chromatographed under identical conditions.

Protein kinase C activity was assayed essentially according to Kraft and Anderson (19). The reaction mixture contained (in a final volume of 250 ml): 5-10 µg of enzyme protein, 6 µmol of Tris-HCl, pH 7.2, 5

12-O-Tetradecanoylphorbol-13-acetate (TPA), a tumor promoter and potent activator of protein kinase C, stimulates [3H]choline incorporation into phosphatidylcholine (PtdCho) in NG108-15 cells (Liscovitch, M., Freese, A., Blusztajn, J. K. and Wurtman, R. J. (1986) J. Neurochem. 47, 1936-1941). In the present study we demonstrate that two cell-permeant diacylglycerols, sn-1-oleoyl-2-acetylglycerol and sn-1,2-di-octanoylglycerol, also stimulate [3H]choline incorporation into PtdCho. However, the effect of diacylglycerol is additional to that produced by a maximally effective concentration of TPA (0.5 µM), suggesting that the two agents may not act via the same mechanism. In addition, the protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (at 200 µM) inhibits the action of TPA by 59% while not affecting that of diacylglycerol. Finally, preincubation of the cells with TPA (0.1 µM) for 24 h reduces protein kinase C activity in the cells and completely abolishes the effect of additional TPA on choline incorporation. In contrast, diacylglycerol-induced stimulation of PtdCho-biosynthesis was not inhibited in the cells that were desensitized to TPA. These results suggest that the effect of the two-cell-permeant diacylglycerols on PtdCho biosynthesis either is not mediated by protein kinase C activation, or, is mediated by a TPA-insensitive isoenzyme of protein kinase C.
with OAG or sn-1,2-dioctanoylglycerol was significantly higher than choline incorporation (p < 0.01). The stimulation by TPA together with phospholipids in the presence of TPA and OAG was evident. The cells were then extracted and 0.1 % Me,SO (vehicle), and increasing concentrations of diacylglycerol dissolved in H,O and 20 μl were analyzed by HPLC (18).

RESULTS

In the course of 1 h of incubation of NG108-15 cells with [3H]choline, OAG stimulated [3H]choline incorporation into PtdCho by 17–60%, in a dose-dependent manner (Fig. 1A). Addition of TPA alone (at 0.5 μM) stimulated the incorporation by 107%; when the cells were incubated in the presence of both OAG (500 μM) and TPA (0.5 μM), choline incorporation was stimulated in an additive manner (Fig. 1A). Similar results were obtained when another synthetic diacylglycerol, sn-1,2-dioctanoylglycerol, was utilized (Fig. 1B). Since the concentration of TPA used in these experiments exceeds the concentration at which the TPA maximally stimulates choline incorporation (8), the additional effects of OAG and sn-1,2-dioctanoylglycerol suggest that diacylglycerols may affect phosphatidylcholine biosynthesis via a different mechanism. The possibility that diacylglycerols used in this study stimulate [3H]choline incorporation into PtdCho by serving as substrates for the CDP-choline:diacylglycerol phosphocholine transferase was evaluated by measuring the formation of [3H]dihexanoyl-PtdCho in sn-1,2-dioctanoylglycerol-treated, [3H]choline-labeled cells. Dihexanoyl-PtdCho was separated from other species of PtdCho by thin layer chromatography on CN reversed-phase plates (as described under "Materials and Methods"). [3H]Dihexanoyl-PtdCho was found to consist of no more than 1–2% of total [3H]choline-labeled PtdCho in the sn-1,2-dioctanoylglycerol-treated cells (results not shown).

We have previously shown that, while stimulating [3H]choline incorporation into PtdCho, TPA causes a parallel decrease in the amount of [3H]phosphocholine present in cell extracts (8). Both OAG and sn-1,2-dioctanoylglycerol also decreased the amount of water-soluble radioactivity extracted from the cells; HPLC analysis of labeled water-soluble choline metabolites extracted from control and sn-1,2-dioctanoylglycerol-treated cells indicated that most of the water-soluble radioactivity present in [3H]choline-labeled cells could be identified as [3H]phosphocholine (Fig. 2). sn-1,2-Dioctanoylglycerol substantially decreased the labeling of the [3H]choline and [3H]phosphocholine peaks, while increasing that of [3H]glycerophosphocholine (Fig. 2). These effects are very similar to those produced by treatment of the cells with TPA (8), and suggest that like TPA, diacylglycerols activate PtdCho biosynthesis at a step distal to choline uptake and phosphorylation.

To assess further the involvement of protein kinase C in the actions of TPA and diacylglycerol, we tested the efficacy of several protein kinase C inhibitors in blocking these actions. Palmitoylcarnitine, dibucaine, chlorpromazine, retinal, and sphingosine (all at a concentration of 10 μM) did not affect TPA-induced stimulation of choline incorporation (data not shown); at higher concentrations all these agents were evidently cytotoxic, causing visible morphological changes and detachment of the cells from the plates. Polymyxin B (at 0.5 mM) was also ineffective in inhibiting the action of TPA. We next tested the action of H-7, an isoquinolinesulfonil derivative, which has been reported to be a...
relatively potent protein kinase C inhibitor (21). At a concentration of 200 μM (at which the inhibitor had no apparent cytotoxic action), H-7 inhibited the action of TPA on choline incorporation by 59% but did not affect the action of OAG (Table I). Similarly, H-7 had no effect on the stimulation of choline incorporation by sn-1, 2-dioctanoylglycerol (results not shown). Although, among various protein kinase inhibitors, H-7 exhibited the highest inhibitory potency toward protein kinase C (21), it is not a selective inhibitor of protein kinase C and, at the high concentration used in our study, it may have inhibited other protein kinases as well. These findings indicate that the mechanisms through which TPA and diacylglycerol affect PtdCho biosynthesis differ in their susceptibility to protein kinase inhibition and suggest that the action of TPA may involve a protein phosphorylation step.

Prolonged treatment of various cell types with TPA renders the cells insensitive to subsequent treatment with this agent (reviewed in Ref. 22). This desensitization is accompanied by, and presumably results from, a decrease in the amounts of protein kinase C enzyme protein (as determined by enzymatic activity (22) and immunoreactivity (23)). In similar experiments we have treated NG108-15 cells with TPA in order to render the cells unresponsive to a subsequent challenge with the phorbol ester. Such cells could then be used to investigate the possible involvement of protein kinase C in the action of TPA and diacylglycerol. As shown in Table II, NG108-15 cells

**TABLE I**

Effect of H-7 on the stimulation by TPA and OAG of [3H]choline incorporation into choline phospholipids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Without H-7</th>
<th>With H-7 (200 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me2SO, 0.1%</td>
<td>44.6 ± 2.0</td>
<td>46.3 ± 2.7</td>
</tr>
<tr>
<td>TPA, 0.1 μM</td>
<td>81.4 ± 5.6*</td>
<td>60.1 ± 7.2*</td>
</tr>
<tr>
<td>OAG, 500 μM</td>
<td>73.2 ± 3.2*</td>
<td>77.7 ± 4.1*</td>
</tr>
</tbody>
</table>

*Significantly different from Me2SO-treated cultures, p < 0.01.

**TABLE II**

Characterization of protein kinase C activity in NG108-15 cells

Cells were incubated for 1 h at 37 °C in 0.5 ml of growth medium containing 0.5 μCi [3H]choline, Me2SO (vehicle), and TPA or OAG in the absence or presence of H-7. The cells were then extracted (6) and radioactivity present in the lipid extract was quantitated. Results are expressed as the mean ± S.D. of quadruplicate cultures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fraction</th>
<th>Protein kinase C activity</th>
<th>Control</th>
<th>TPA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cytosolic</td>
<td>518 ± 30</td>
<td>176 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Particulate</td>
<td>519 ± 49</td>
<td>291 ± 65</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Cytosolic</td>
<td>1251 ± 53</td>
<td>173 ± 105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Particulate</td>
<td>1065 ± 249</td>
<td>236 ± 110</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE III**

Effect of a 24-h incubation with TPA on protein kinase C activity in NG108-15 cells

Cells were incubated for 24 h in the presence of TPA (100 nM) or its vehicle (0.1% Me2SO). The cells were then homogenized and the homogenate was ultracentrifuged as described in the legend to Table II. Protein kinase C present in the particulate fraction was extracted from the 120,000 × g pellet by homogenization in a 20 mM Tris-HCl buffer, pH 7.2, containing 2 mM Na-EDTA, 0.5 mM Na-EGTA, 350 μg/ml phenylmethylsulfonyl fluoride, and 1% Nonidet P-40, followed by ultracentrifugation (120,000 × g, 60 min). Both soluble and detergent-extracted fractions were then partially purified on DE52 columns and assayed as described in the legend to Table II and under “Materials and Methods." Results are expressed as mean ± S.D. (half the range) of duplicate cultures, each assayed in triplicate.

**DISCUSSION**

These data indicate that phorbol esters and diacylglycerols can both stimulate PtdCho biosynthesis in NG108-15 cells. Cell-permeant diacylglycerols were previously shown to stimu-
ulate PtdCho biosynthesis also in the human promyelocytic leukemia cell line HL-60 (24) and in the mouse epidermal cell line HEL-37 (25). In the present study we demonstrate that the stimulation elicited by diacylglycerol is additional to that obtained with a maximally effective concentration of TPA. In addition, while stimulation of [3H]choline incorporation by TPA can be inhibited by H-7, the effect of diacylglycerol is completely insensitive to this inhibitor. Finally, in cells that are no longer capable of responding to TPA with increased PtdCho biosynthesis (due to a preincubation with this agent), diacylglycerols can still stimulate synthesis of this phospholipid. These observations strongly suggest that diacylglycerol and TPA affect PtdCho biosynthesis by different mechanisms. This conclusion is consistent with a previous report that, in HL-60 cells, OAG stimulated choline incorporation into PtdCho and sphingomyelin, but, unlike TPA, failed to increase choline incorporation into lysophosphatidylcholine (24).

Taken together, the evidence reported here and in our previous work (8) indicates that TPA exerts its action on PtdCho biosynthesis in NG108-15 cells by activating protein kinase C. It may thus be concluded that diacylglycerol either failed to activate protein kinase C, or that its mode of activation was not identical with that of TPA. Recent evidence indeed suggests that TPA and diacylglycerol are not totally equivalent activators of protein kinase C, causing nonidentical patterns of cellular protein phosphorylation (24, 26, 27). This difference may underlie the failure of diacylglycerol to mimic the effects of phorbol esters on HL-60 cell differentiation (24, 28), granulosa cell maturation (29), platelet activation (30), and granulocyte superoxide production (31), and could be related to the inability of exogenous diacylglycerol to cause the stable association of soluble protein kinase C with cell membranes (29, 32). Diacylglycerol-induced stimulation of PtdCho biosynthesis may then be explained either by its direct interaction with CTP:phosphocholine cytidylyltransferase (33), which is the rate-limiting enzyme in the pathway of PtdCho biosynthesis (34), or by its degradation into free fatty acids, which also can activate the cytidylyltransferase directly (35).

Alternatively, the difference in the mechanism through which TPA and diacylglycerol stimulate PtdCho biosynthesis may be accomplished by activation of different protein kinase C isoforms, which recently have been recognized to exist in the brain (36-38). The response of these isoenzymes to phorbol esters and diacylglycerol may not be identical. It should be noted that NG108-15 cells are somatic hybrids of neuroblastoma and glioma cells, and thus could conceivably express at least two protein kinase C isozymes, a neuronal type and a glial type, respectively. Such a hypothesis could explain why, in TPA-desensitized cells, further activation by TPA is completely abolished (Table IV) although some protein kinase C activity is still present (Table III). Another explanation for the complete desensitization to TPA, in spite of the significant residual levels of protein kinase C, is that pretreatment with TPA induces an esterase that quickly hydrolyzes the phorbol ester.

The difference in the mode of action of TPA and diacylglycerol lies probably in early activation steps; both TPA (8) and diacylglycerol (Fig. 2) decrease cellular [3H]phosphocholine, suggesting that they both activate a final enzymatic step in the pathway of PtdCho biosynthesis that is distal to choline transport and phosphorylation. This final common step could be the reaction catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), or, alternatively, the last enzymatic step in the CDP-choline pathway which is catalyzed by CDP-choline:1,2-diacylglycerol phosphocholine transferase (EC 2.7.8.2). The cytidylyltransferase step is rate limiting for PtdCho biosynthesis through the Kennedy pathway in a variety of vertebrate cells (34), and TPA has been shown to increase cytidylyltransferase activity in HeLa cells (39) and in a line of rat skeletal myoblasts (40). Similarly, phospholipase C treatment, which would result in formation of endogenous diacylglycerol, stimulated [3H]choline incorporation and cytidylyltransferase activity in Chinese hamster ovary cells (41, 42). Direct phosphorylation of the cytidylyltransferase by phorbol ester-activated protein kinase C is, however, unlikely (43), suggesting that one or more intermediate steps exist in the cascade leading from protein kinase C activation to increased PtdCho biosynthesis.

In conclusion, the present results suggest the existence of multiple pathways for regulating PtdCho biosynthesis in NG108-15 cells.

REFERENCES


Phosphatidylcholine Biosynthesis in NG108-15 Cells