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Role of protein kinase C in diacylglycerol-mediated induction of ornithine decarboxylase and reduction of epidermal growth factor binding

(phorbol esters/tumor promotion)

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ABSTRACT **Tumor-promoting phorbol esters induce ornithine decarboxylase (ODCase) activity and reduce epidermal growth factor (EGF) binding in rat tracheal epithelial 2C5 cells. Phorbol esters activate protein kinase C by interacting at the same site as *sn*-1,2-diacylglycerols, the presumed physiological regulators. The effects of added *sn*-1,2-diacylglycerols and those generated by phospholipase C treatment of 2C5 cells on ODCase induction and EGF binding were investigated to establish a role for protein kinase C in these cellular responses. Treatment of 2C5 cells with phospholipase C induced ODCase activity and reduced EGF binding, whereas phospholipases A_2 and D were inactive. When *sn*-1,2-diacylglycerols containing fatty acids 3–10 carbons in length were added to 2C5 cells, those diacylglycerols containing fatty acids 5–10 carbons in length caused ODCase induction and reduction in EGF binding. *sn*-1,2-Dioctanoylglycerol was one of the most active compounds tested. It induced ODCase in a dose- (50–500 μM) and time-dependent manner. The reduction of binding of ^{125}I-labeled EGF by *sn*-1,2-dioctanoylglycerol was also time and dose dependent and appeared to result from a change in EGF affinity and not the number of receptor sites. This series of *sn*-1,2-diacylglycerols showed similar structure–function relationships in their ability to induce ODCase activity, to decrease EGF binding, to stimulate protein kinase C, and to inhibit [^{3}H]phorbol dibutyrate binding to the phorbol ester receptor. These data demonstrate biological activities for a number of diacylglycerols and indicate that protein kinase C activation is implicated in ODCase induction and decreased EGF binding.**

Tumor promoters are compounds that are noncarcinogenic but induce tumors in animals treated with suboptimal doses of chemical carcinogens (1–4). Phorbol esters, the most extensively studied class of tumor promoters, cause profound biological and biochemical alterations in a variety of cells. Two of these alterations, the inhibition of epidermal growth factor (EGF) binding and the induction of ornithine decarboxylase (ODCase) activity, have been widely studied (5–12). A strong correlation exists between the induction of ODCase and the promotion of tumor formation (4, 12–16). Unlike the inhibition of EGF binding (5–8), ODCase induction requires nuclear events (17).

Phorbol ester receptors have been identified in many tissues and cell lines (18, 19). The structure–activity relationship is evidence that these receptors mediate the biological responses of the phorbol esters (20). Nishizuka and coworkers have demonstrated that the Ca^{2+}-phospholipid-dependent, diacylglycerol-stimulated protein kinase, designated protein kinase C, is activated by phorbol esters *in vivo* and *in vitro* (21, 22). This enzyme is thought to be activated *in vivo* by diacylglycerols derived from signal-dependent turnover of phosphatidylinositols (23, 24). Recent evidence indicates that protein kinase C is the phorbol ester receptor (25–29) and that diacylglycerols and phorbol esters interact at a common site on the soluble receptor (25).

In this paper, we investigated the effects of a series of *sn*-1,2-diacylglycerols on ODCase induction, EGF binding, protein kinase C activation, and [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PBt_2) binding in rat tracheal epithelial 2C5 cells. The results suggest that protein kinase C activation is implicated in the cellular responses to diacylglycerols and phorbol esters and demonstrate that diacylglycerols are biologically active. In view of the established correlation between ODCase activity and tumor promotion (10–16), a role for diacylglycerols and protein kinase C in tumor promotion is suggested.

MATERIALS AND METHODS

Cell Culture. Rat tracheal epithelial 2C5 cells were grown as reported (30).

Assay of ODCase Activity. ODCase activity was determined according to Lichti and Gottesman (31).

EGF Binding Assay. Binding of ^{125}I-labeled EGF (^{125}I-EGF) to 2C5 cells was assayed at 0°C for 5 hr as described (32). ^{125}I-EGF (174 μCi/μg, New England Nuclear; 1 Ci = 37 GBq) concentration was 1 ng/ml. The standard error was 5%.

Phospholipases. Phospholipases C (*Clostridium perfringens*), A_2 (bee venom), and D (peanut) were obtained from Sigma. A unit of activity is as defined by the supplier (Sigma).

[^{3}H]PBt_2 Binding. For [^{3}H]PBt_2 binding to crude membranes, cells were collected, washed in phosphate-buffered saline, and homogenized in 20 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/2 mM $CaCl_2$/2 mM 2-mercaptoethanol/0.01% leupeptin/2 mM phenylmethylsulfonyl fluoride by sonication. The crude membranes were collected by centrifugation at 100,000 × *g* for 60 min. The [^{3}H]PBt_2 binding assay was according to Sando *et al.* (28). [^{3}H]PBt_2 binding took place at 34°C at 10 nM [^{3}H]PBt_2 for 5 min in Waymouth medium without serum. At 5 min binding reached a plateau and unlabeled PBt_2 or other competitors were added. After another 5-min incubation, radioactivity was determined. For measurement of nonspecific binding unlabeled PBt_2 was included from the beginning.

Protein Kinase Assay. Approximately 2 × 10^9 cells were collected from 100 dishes (150 cm). Cells were homogenized

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Abbreviations: EGF, epidermal growth factor; ODCase, ornithine decarboxylase; PBt_2, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; OAG, *sn*-1-oleoyl-2-acetylglycerol; *sn*-1,2-diacylglycerols with identical fatty acids are abbreviated by carbon atom number of the acyl chains and by number of double bonds where appropriate—e.g., diC_8 indicates *sn*-1,2-dioctanoylglycerol.

by sonication and centrifuged at 100,000 × *g* for 2 hr. Protein kinase C was partially purified from the supernatant and its activity was determined as described (33). Phosphatidylserine (Avanti Polar Lipids) and Ca^{2+} concentrations were 20 μg/ml and 5 μM, respectively. The standard error was 2.5% or less.

Diacylglycerols and Analogues. *sn*-1,2-Dioleoylglycerol ($diC_{18:1}$), shorter chain *sn*-1,2-diacylglycerols, the chloro, sulfhydryl, and deoxy analogues of diC_8, and *sn*-1-oleoyl-2-acetylglycerol (OAG) were prepared as described previously (34). 1-Monooleoylglycerol, 2-monooleoylglycerol, and palmityl alcohol were products of Serdary Research Laboratories, London, ON. 1-Octanoylglycerol and 1,3-didecanoylglycerol were obtained from Sigma. Lipids in ethanol were diluted 1:100 into culture medium and sonicated before addition to the cells.

RESULTS

Effect of Phospholipase C. Phorbol esters are effective inducers of ODCase activity in rat tracheal epithelial 2C5 cells (unpublished data). To determine whether protein kinase C activation by diacylglycerols is involved in the induction of ODCase activity, the effect of phospholipase C treatment on 2C5 cells was tested. Rapid degradation of [^{3}H]choline-labeled phosphatidylcholine occurred (data not shown) and an increase in diacylglycerols was presumed (35). Treatment with phospholipase C induced ODCase activity, in agreement with a recent report (36). Phospholipase C was tested at 0.001–1 unit/ml; a maximum effect was observed at 0.01 unit/ml (Table 1). Phospholipases A_2 and D did not induce ODCase activity (Table 1). Phospholipase C treatment reduced EGF binding by about 75% as noted earlier (37), whereas phospholipases A_2 and D were without significant effect.

Induction of ODCase by Diacylglycerol. The phospholipase results suggested a role for diacylglycerols and protein kinase C in ODCase induction and EGF binding, so a direct test of the effects of added diacylglycerols on these parameters and protein kinase C activation was sought. Naturally occurring diacylglycerols that activate protein kinase C *in vitro* (21–26) are without effect when added to cells. Such diacylglycerols are insoluble and cannot be delivered effectively to cells in culture. Nishizuka prepared OAG and demonstrated that it activated protein kinase C in platelets (22). To further investigations of the suggested function of diacylglycerols as bioregulators (22), *sn*-1,2-diacylglycerols containing fatty acids 3–10 carbons in length were prepared along with OAG. When these were tested for their ability to induce ODCase and reduce EGF binding in 2C5 cells, a num-

Table 1. Effect of phospholipase treatment on ODCase activity and EGF binding in rat tracheal epithelial 2C5 cells

Treatment	ODCase activity		EGF binding	
	nmol CO_2 per hr/mg protein	% of control	cpm/10^6 cells	% of control
Control	0.31 ± 0.01	100	17,430 ± 310	100
Phospholipase C				
0.01 unit/ml	1.19 ± 0.03	384	4,851 ± 58	27.8
0.1 unit/ml	0.72 ± 0.02	232	4,427 ± 104	25.4
Phospholipase A_2				
0.01 unit/ml	0.39 ± 0.01	126	17,252 ± 251	99.0
0.1 unit/ml	0.37 ± 0.01	119	16,515 ± 513	94.8
Phospholipase D				
0.01 unit/ml	0.35 ± 0.01	113	16,804 ± 498	96.4
0.1 unit/ml	0.36 ± 0.02	116	16,288 ± 560	93.4

ODCase activity was determined 5 hr after addition of the phospholipase. For EGF binding, cells were first treated for 1 hr with phospholipase. EGF binding was measured by incubating cells with 1 ng of ^{125}I-EGF per ml for 5 hr at 0°C.

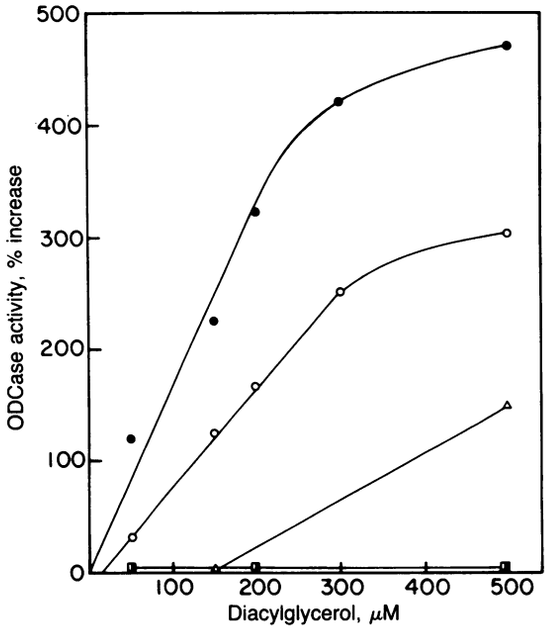

FIG. 1. Action of diacylglycerol derivatives on the induction of ODCase activity as a function of their concentration. Cells were grown in 24-well dishes. At zero time cells were treated with various concentrations of the diacylglycerol. ODCase activity was determined 4 hr after the addition of the lipid. ○, diC_8; ●, diC_{10}; □, diC_8-SH; ■, diC_8-Cl; △, diC_6.

Table 2. Comparison of the action of various lipids on ODCase induction, ^{125}I-EGF binding, [^{3}H]PBt_2 binding, and protein kinase C activity

Lipid	Induction of ODCase, % increase	^{125}I-EGF binding, % decrease	[^{3}H]PBt_2 binding, % decrease	Protein kinase C, % increase
diC_3	0	0	0	NT
diC_4	0	0	10	0
diC_5	63	23	47	NT
diC_6	94	58	84	54
diC_7	119	64	90	NT
diC_8	209	82	>95	135
diC_8-Cl	0	23	37	0
diC_8-SH	0	0	0	0
diC_8-H	0	0	NT	0
diC_9	191	76	>95	NT
diC_{10}	372	74	>95	138
$1,3C_{10}$	97	34	NT	NT
OAG	274	78	>95	138
1-Monooleoylglycerol	0	0	0	0
2-Monooleoylglycerol	0	0	0	NT
1-Octanoylglycerol	NT	NT	NT	0
Palmityl alcohol	0	0	0	NT
$diC_{18:1}$	0	0	NT	151
PMA	522	88	>95	203
PBt_2	271	70	>95	NT
Phorbol	0	0	0	NT

The induction of ODCase was tested at 500 μM, EGF binding at 75 μM, [^{3}H]PBt_2 binding at 250 μM, and protein kinase C at 1 μM diacylglycerol. Phorbol esters were tested at 20 nM except in the [^{3}H]PBt_2 binding assay, in which 20 μM was used. PMA, phorbol 12-myristate 13-acetate. NT, not tested.

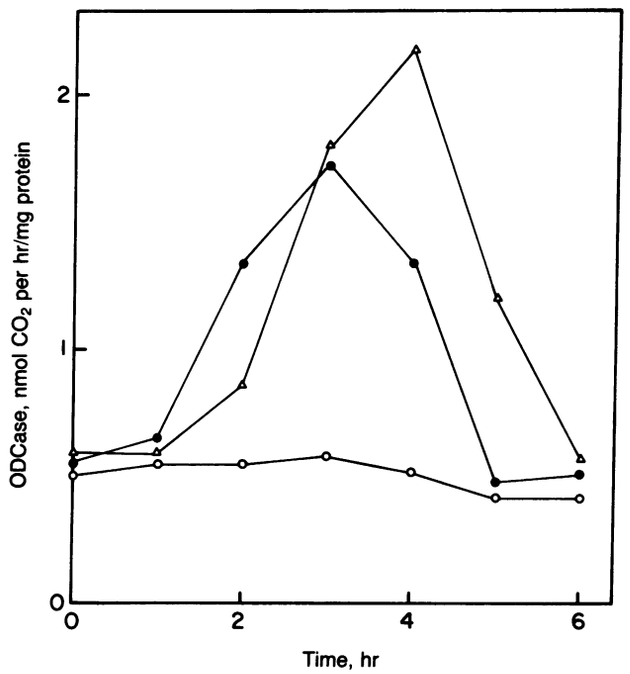

FIG. 2. Time course of the induction of ODCase by the diacylglycerol diC_8 and the phorbol ester PMA. PMA (20 nM) or diC_8 (500 μM) was added at time zero, and at various times ODCase activity was determined. ●, diC_8; △, PMA; ○, control cells receiving only the solvent dimethyl sulfoxide.

ber were active. diC_{10} and diC_8 caused a dose-dependent induction of ODCase activity over the range of 25–500 μM (Fig. 1). diC_5 and diC_6 were less active than diC_8, whereas diC_3 and diC_4 were not active at the levels tested (Table 2). When the hydroxyl moiety of diC_8 was replaced with chloride (diC_8-Cl), sulfhydryl (diC_8-SH), or hydrogen (diC_8-H, deoxy) moieties, these diacylglycerol analogues were inactive, suggesting that the hydroxyl moiety has an important functional role. The time course of ODCase induction by diC_8 and PMA is shown in Fig. 2. The earliest increase in ODCase was detected 2 hr after the addition of the diacylglycerol. ODCase activity reached a maximum at 3 hr and then decreased rapidly thereafter. The time courses of ODCase induction by PMA and diC_8 were similar.

Effect of Diacylglycerol on EGF Binding. Addition of diC_8 to 2C5 cells rapidly reduced ^{125}I-EGF binding. A 10-min exposure decreased EGF binding by approximately 90% (Fig. 3*B*). Inhibition was dependent on diacylglycerol concentration (Fig. 3*A*). Concentrations of diC_8 and diC_{10} as low as 5 μM were effective; maximal reduction of EGF binding was reached at 75 μM. diC_{10} was the most active diacylglycerol tested. diC_8 was nearly as active; diC_6 and diC_4 were less active. The analogues diC_8-Cl, diC_8-SH, and diC_8-H (deoxy) were inactive. To determine whether diC_8 reduced the affinity or number of EGF receptor sites, binding was determined as a function of EGF concentration (Fig. 4). No significant difference in ^{125}I-EGF binding was observed at high concentrations of EGF. Scatchard plot analysis showed that diC_8 did not alter receptor number but caused a change from high- to low-affinity receptor sites. Incubation of the cells at 0°C with diC_8 had no effect on EGF binding.

Protein Kinase C Activation and Inhibition of [^{3}H]PBt_2 Binding by Diacylglycerols. The ability of diacylglycerols to activate 2C5 cell protein kinase C activity *in vitro* is shown in Fig. 5. diC_{10}, diC_8, OAG, and $diC_{18:1}$ stimulated protein kinase C activity at 0.1 μM, whereas 1-monooleoylglycerol and 1-octanoylglycerol were barely active. diC_8-SH was not active and diC_8-Cl was a poor activator at 100 μM. At high concentrations, diC_8-H inhibited protein kinase C activity.

Diacylglycerol derivatives compete for binding of [^{3}H]-PBt_2 to brain cytosolic fractions (25). The ability of this series of diacylglycerols to inhibit [^{3}H]PBt_2 binding to isolated membrane fractions prepared from 2C5 cells was tested. diC_{7-10} and OAG were effective inhibitors of [^{3}H]PBt_2 binding. Those with shorter acyl chains (diC_{4-6}) inhibited less well. diC_3 was inactive at the concentrations tested. Similar results were obtained when [^{3}H]PBt_2 binding to intact cells was investigated, with the exception of diC_{10}, which inhibited binding only poorly (data not shown).

Structure–Activity Relationships. The abilities of diacylglycerols and diacylglycerol analogues to induce ODCase activity and reduce EGF binding were compared with their capacities to activate protein kinase C and to inhibit [^{3}H]PBt_2 binding to the phorbol ester receptor (Table 2). diC_{10}, diC_9, and OAG were the most active in inducing ODCase and decreasing EGF binding. These compounds were also the most effective in activating protein kinase C and inhibiting [^{3}H]-PBt_2 binding. The activity of diacylglycerols decreased with shorter acyl chain lengths. diC_3 and diC_4 were essentially inactive at the concentrations tested in inducing ODCase, reducing EGF binding, activating protein kinase C, or inhibiting [^{3}H]PBt_2 binding. Replacement of the hydroxyl group of diacylglycerol with a chloride- sulfhydryl, or hydrogen moiety rendered the molecules essentially inactive. 1,3-diC_{10}

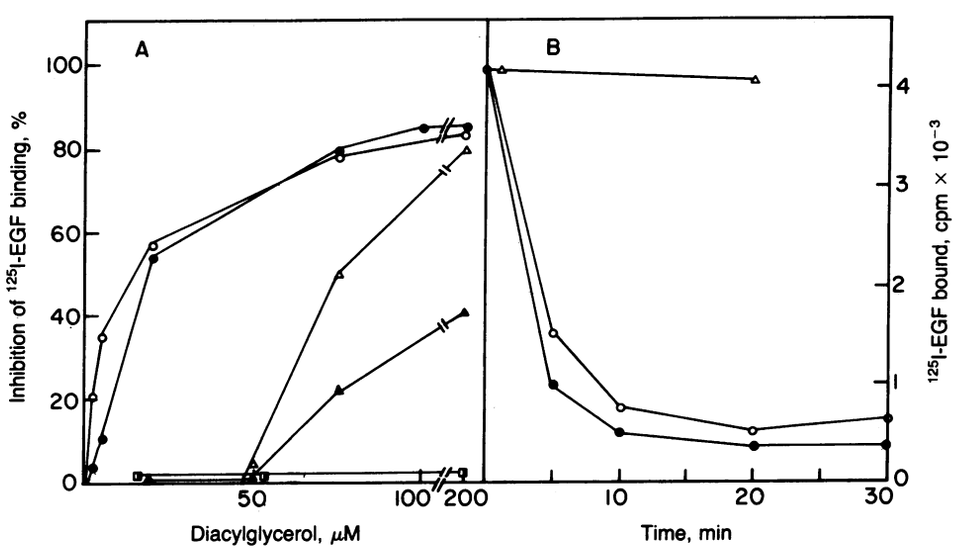

FIG. 3. Inhibition of binding of [^{125}I]EGF to tracheal epithelial cells by diacylglycerol. (*A*) Inhibition of EGF binding as a function of diacylglycerol concentration. Cells were pretreated with diacylglycerol for 1 hr. ^{125}I-EGF binding was determined at 0°C for 5 hr. ○, diC_{10}; ●, diC_8; △, diC_6; ▲, diC_5; □, diC_8-SH; ■, 1-monooleoylglycerol. (*B*) EGF binding as a function of incubation time. ○, 200 μM diC_8; ●, 20 nM PMA; △, control.

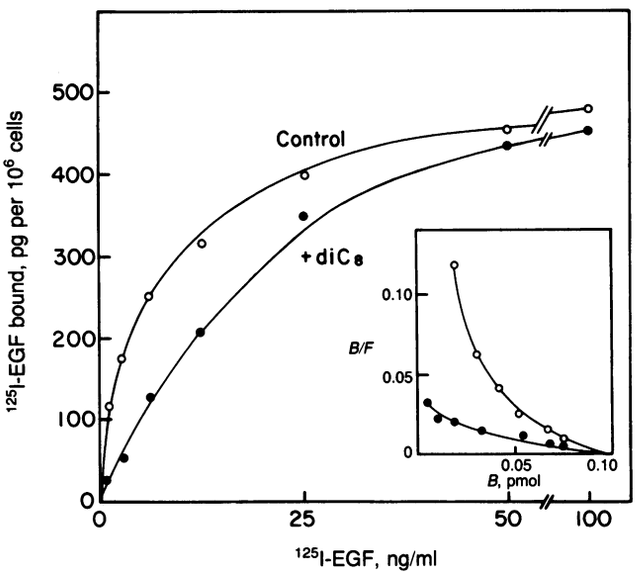

FIG. 4. Effect of diC$_8$ on the binding of ^{125}I-EGF to tracheal epithelial cells as a function of ^{125}I-EGF concentration. Cells were preincubated for 1 hr without diC$_8$ (○) or with 200 μM diC$_8$ (●). Then ^{125}I-EGF binding was determined after 5-hr incubation at 0°C at the indicated concentrations. (*Inset*) Scatchard plot analysis of the data. *B*, ^{125}I-EGF bound; *F*, ^{125}I-EGF free.

was less active than 1,2-diC$_{10}$. Furthermore, 1-monooleoylglycerol and 2-monooleoylglycerol were essentially inactive. These results indicate a specificity for 1,2-diacylglycerols. A good correlation between the biological activity of the diacylglycerols tested and their ability to activate protein kinase C and inhibit [^{3}H]PBt$_2$ binding was observed.

DISCUSSION

Diacylglycerols produced in rat tracheal epithelial 2C5 cells by phospholipase C treatment (Table 1) or added to cell cultures (Figs. 1–4, Table 2) are biologically active. They cause

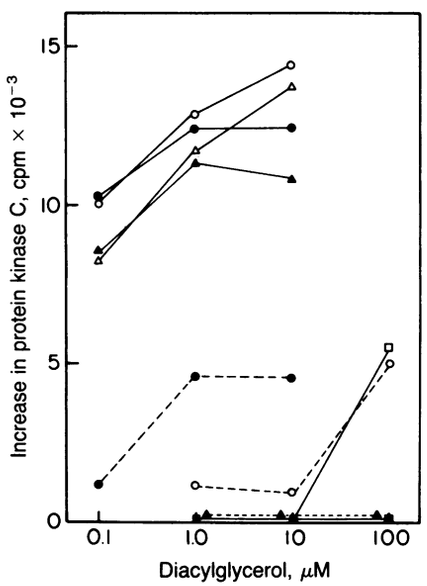

FIG. 5. Stimulation of protein kinase C by different diacylglycerol derivatives. The activity of protein kinase C in the absence of diacylglycerol was 8522 cpm. ●—●, diC$_{10}$; ○—○, diC$_{18:1}$; △—△, diC$_8$; ▲—▲, OAG; ●---●, diC$_6$; ○---○, diC$_8$-Cl; □—□, diC$_4$; △---△, diC$_8$-SH; ▲---▲, 1-monooleoylglycerol; and ■—■, diC$_8$-H.

ODCase induction (Figs. 1 and 2) and reduce EGF binding (Figs. 3 and 4) in a saturable, dose-dependent, and time-dependent manner, mimicking the effects of phorbol esters (5–12). These *sn*-1,2-diacylglycerols activate protein kinase C (Fig. 5) and inhibit [^{3}H]PBt$_2$ binding to the phorbol ester receptor (Table 2) of 2C5 cells, in agreement with the concept that the binding sites of phorbol esters and diacylglycerols are identical (22, 25). The structure–function correlations for various diacylglycerols and analogs indicate that compounds that are most active in stimulating protein kinase C activity and inhibiting [^{3}H]PBt$_2$ binding are also the most effective in inducing ODCase activity and decreasing EGF binding, implicating a role for protein kinase C in these responses.

Although protein kinase C appears to be involved in these responses, the mechanism by which they are affected is not identical. ODCase induction involves *a nuclear event*, whereas reduction of EGF binding does not (5–7, 17). We hypothesize that activation of protein kinase C transmits a signal to the nucleus, which results in enhanced transcription of the ODCase gene and increased ODCase synthesis. This could occur via the phosphorylation of proteins, such as histones, that regulate gene expression (38). In the case of the EGF receptor, protein kinase C may phosphorylate the receptor directly, resulting in a reduction of its affinity for EGF. Phorbol ester- and diacylglycerol-induced phosphorylation of the EGF receptor at serine and threonine residues (39–41), the specificity of protein kinase C for serine and threonine, and the structure–activity relationships of diacylglycerols (Table 2) support this concept.

The *sn*-1,2-diacylglycerols demonstrated to be biologically active in causing ODCase induction and reduction of EGF binding are physically able to become associated with cell membranes, undergo transmembrane movement, and activate cytosolic protein kinase C. Diacylglycerols such as dioleoylglycerol are effective in activating protein kinase C *in vitro* (23) but are not active when added to cells. The physical properties of such diacylglycerols favor phase separation from aqueous media and preclude effective delivery. Studies of the diacylglycerol dependence of microsomal glycerolipid enzymes (42, 43) suggested that diacylglycerols containing short chain fatty acids were easier to deliver to membranes and hence, to cells. Clearly this was the case. Not all *sn*-1,2-diacylglycerols containing short-chain fatty acids (diC$_3$ and diC$_4$) are active, suggesting that protein kinase C activation is dependent on chain length or that partitioning of diacylglycerols into membrane bilayers is necessary for activation. The structure–function relationships observed are consistent with both of these mechanisms. The most effective diacylglycerols appear to be balanced in their solubility, ability to associate with bilayers, and decreased phase separation. Specificity for *sn*-1,2-diacylglycerols is apparent from the reduced activity noted with *sn*-1,3-diC$_{10}$, and inactivity of monooleoylglycerols and chloro, sulfhydryl, and deoxy analogs of diC$_8$.

Published biological activities for diacylglycerols include studies by Nishizuka and colleagues (22, 44), who employed OAG to activate protein kinase C in platelets and cause 40-kDa peptide phosphorylation, and studies of OAG activation of neutrophils (45) and stimulation of secretion in platelets (46). In that OAG could have been pharmacologically related to phorbol esters (22) and not typical of *sn*-1,2-diacylglycerols, the data presented demonstrating biological activity for numerous diacylglycerols strengthen the emerging role of diacylglycerols as bioregulators produced from phosphatidylinositol in response to extracellular signals. Clearly, unsaturated fatty acids are not a structural requirement, as was suggested earlier (22). Recently, diC$_8$ was shown to promote differentiation of HL60 cells into macrophages (34). Generalizations of whether 1,2-diacylglycerols function as intracel-

lular bioregulators await testing in numerous systems. The set of diacylglycerols employed in these studies should facilitate such studies.

Given these results and the strong correlation that exists between ODCase induction and tumor promotion (11–13), a role for diacylglycerols and protein kinase C in tumor promotion is suggested. This opens the possibility that other classes of tumor promoters could function by enhancing the production of diacylglycerols, thereby activating protein kinase C indirectly rather than directly, just as phorbol esters do. Similarly, enhanced synthesis or activation of protein kinase C by an oncogene may function in tumor promotion in carcinogenesis (22). The activities of diacylglycerols as tumor promoters can now be tested in two-stage carcinogenesis protocols.

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