A tumour promoter, 12-*O*-tetradecanoylphorbol 13-acetate, increases cellular 1,2-diacylglycerol content through a mechanism other than phosphoinositide hydrolysis in Swiss-mouse 3T3 fibroblasts

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Treatment of Swiss-mouse 3T3 fibroblasts with a tumour-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), increases the absolute amount of 1,2-diacylglycerol (1,2-DG) in a time- and dose-dependent manner. Within 1 min of TPA (0.1 μ M) addition to confluent monolayer cells, cellular 1,2-DG content increases significantly, and reaches a value nearly 4-fold above control at 10 min. The content of 1,2-DG then falls, but still remains at twice the basal value for up to 60 min. The effect of TPA on 1,2-DG content is dose-dependent, with a half-maximal effect obtained with 6 nM. Studies with a series of tumour promoters have revealed that stimulating effect on 1,2-DG formation is closely related to their relative potency as a tumour promoter, as well as their mitogenic effect on 3T3 cells. TPA does not have any stimulatory effect on phosphoinositide turnover, which is a major mechanism of 1,2-DG formation by natural agonists, including a peptide mitogen, bombesin, but significantly inhibits bombesin-induced phosphoinositide turnover. Nevertheless, the content of 1,2-DG is even higher in cells treated with both bombesin and TPA than in cells treated with bombesin alone. In addition, a diacylglycerol kinase inhibitor, R59022, increases 1,2-DG content synergistically with bombesin, but not with TPA. Measurements of the absolute amounts of various phospholipids show that TPA does not increase phosphatidic acid, despite a remarkable increase in 1,2-DG. Further studies on phospholipid metabolism reveal that TPA stimulates metabolic turnover of phosphatidylcholine (PC), without changing the mass of PC. TPA stimulates the incorporation of both [3 H]choline and [32 P]P_i into PC, and the release of [3 H]choline metabolites from prelabelled cells. These findings suggest that TPA increases cellular 1,2-DG content mainly via increased PC turnover and diacylglycerol kinase inhibition. The present study provides evidence for a novel effect of TPA on the metabolism of 1,2-DG in Swiss-mouse 3T3 cells.

INTRODUCTION

A tumour-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), is known to induce diverse biological effects in various types of cells. Several studies have established that TPA directly binds to and activates the Ca²⁺-activated phospholipid-dependent protein kinase (C-kinase), suggesting that the activation of C-kinase is the biochemical basis of the action of TPA [1–4].

In mouse embryo fibroblasts (Swiss 3T3 cells), it has been shown that TPA and a permeant diacylglycerol (DG) analogue, 1-oleoyl-2-acetyl-glycerol, as well as a number of peptide mitogens such as platelet-derived growth factor, fibroblast growth factor, bombesin and vasopressin, stimulate DNA synthesis in a synergistic manner with insulin [5–9]. These mitogens have been demonstrated to stimulate phosphorylation of a common cytosolic 80 kDa protein, a putative substrate for the C-kinase [6,10–12]. The peptide mitogens elicit

phosphoinositide hydrolysis in Swiss 3T3 cells, and one of the resulting products, 1,2-DG, is thought to induce the activation of the C-kinase by enhancing the affinity of this enzyme for Ca²⁺ [3,12,13]. During studies of the effects of these mitogens on the metabolism of 1,2-DG, we unexpectedly found that TPA greatly increases the cellular content of 1,2-DG in 3T3 cells. This effect of TPA is even more marked than that of the maximal dose of bombesin, and its mechanism of action appears to be totally different from that of peptide mitogens which cause phosphoinositide hydrolysis. In the present study, we attempted to characterize this effect of TPA on the metabolism of 1,2-DG and to determine, if possible, the mechanism of TPA action in 3T3 cells.

MATERIALS AND METHODS

Cell culture

Swiss-mouse 3T3 fibroblasts were maintained in Dulbecco's modified minimal essential medium

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DG, diacylglycerol; DMEM, Dulbecco's modified minimal essential medium; TG, triacylglycerol; PDBu, phorbol 12,13-dibutyrate; MEZ, mezerein; DPBA, 12-deoxyphorbol 13-isobutyrate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; SM, sphingomyelin.

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(DMEM) (GIBCO) supplemented with 10% (v/v) calf serum (GIBCO) at 37 °C in CO₂/air (1:19) in a subconfluent state with subcultures every 3 days [14]. For experimental purposes, cells were plated in plastic dishes and cultured until confluency, with a change of medium every 3 days. Before each experiment they were made quiescent in DMEM containing 1% platelet-poor plasma [15].

Mitogenesis assay

Quiescent monolayer cells in 24-well plates (Corning, Corning, NY, U.S.A.) were washed once with DMEM and received DMEM containing various concentrations of test substances and 0.5 μ Ci of [*methyl*-³H]-thymidine/ml. After 40 h incubation at 37 °C, the cells were processed as described previously [16], and the incorporation of [³H]thymidine into DNA was measured.

Measurement of phosphoinositide turnover

Confluent monolayers (60 mm dishes) were labelled with *myo*-[2-³H]inositol (8 μ Ci/ml) in DMEM containing 1% platelet-poor plasma for 48 h. The medium was replaced by DMEM containing 10 mM-LiCl and the cells were further incubated for 10 min. Then the cells were treated with either TPA (0.1 μ M) or bombesin (25 nM) and the reaction was stopped by replacing the media with ice-cold 10% (v/v) HClO₄. After 30 min at 4 °C, the HClO₄ solution was collected into glass tubes, neutralized and analysed for inositol phosphates, by Dowex columns [17]. The cells were scraped off the dishes with a rubber policeman, and phosphoinositides were extracted in methanol/chloroform/12 M-HCl (200:100:1, by vol.) [18] and separated on oxalate-impregnated Silica Gel H plates (Supelco, Bellefonte, PA, U.S.A.) in the solvent system chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) [16,19]. Areas corresponding to authentic phospholipids were scraped into vials, and radioactivity was counted in a liquid-scintillation spectrophotometer.

Measurement of the absolute amounts of various lipids

Quantification of 1,2-DG, triacylglycerol (TG) and phosphatidic acid (PA) was performed by using the charring-densitometry technique as described elsewhere [16,19]. The reaction was stopped by quickly washing the cells with ice-cold Hepes-buffered saline solution (145 mM-NaCl, 5 mM-Hepes/NaOH, pH 7.4) followed by addition of 0.1% SDS. The lipids were extracted from the cell lysate by the method of Bligh & Dyer [20] and separated by t.l.c. For quantification of 1,2-DG and TG, samples were applied to heat-activated LK6D plates (Whatman, Clifton, NJ, U.S.A.) and developed either in solvent system benzene/chloroform/methanol (16:3:1, by vol.) (for 1,2-DG) or in light petroleum (b.p. 40–57 °C)/diethyl ether/acetic acid (35:15:1, by vol.) (for TG). The 1,2-DG was also identified by two-dimensional t.l.c. on heat-activated Silica Gel 60 plates (Merck, Darmstadt, West Germany), which were developed in the first direction in the solvent system diethyl ether/hexane/acetic acid (70:30:1, by vol.), air dried for 1 h and then developed in the second direction in the solvent system benzene/diethyl ether/ethanol/conc. NH₃ (sp.gr. 0.880) (500:40:20:1, by vol.). For measurement of PA, samples were applied on heat-activated Silica Gel 60 plates and developed in the solvent system chloro-

form/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.). After separation of lipids, the plates were dried at 100 °C for 30 min, sprayed with 10% (w/v) CuSO₄ in 8% (v/v) H₃PO₄ solution and charred at 170 °C for 15 min. The density of each spot co-migrating with the authentic standards [1,2-dioleoylglycerol, trioleoylglycerol and L- α -phosphatidic acid (dioleoyl) respectively] was measured as described previously [16,19,21]. The standard curves were obtained by using the same standards, and the lipid amount of each sample was calculated as nmol equivalent of the respective standard lipid and expressed on the basis of phospholipid P content. In some experiments, the 1,2-DG content was also determined by an enzymic method described by Preiss *et al.* [22], with *Escherichia coli* diacylglycerol kinase, which is reported to be stereospecific for 1,2-DG, and similar results were obtained.

Quantification of other phospholipids (PI, PS, PE, PC and SM) was performed by measuring phospholipid P after separation of each phospholipid by t.l.c. on Silica Gel 60 plates with the solvent system chloroform/methanol/acetic acid/water (65:43:1:3), as described elsewhere [19].

Measurements of [³²P]P_i and [*methyl*-³H]choline incorporation into phospholipids

Confluent quiescent cells in 60 mm dishes (Corning) were transferred to DMEM with or without TPA (0.1 μ M) and either 50 μ Ci of [³²P]P_i/ml or 2 μ Ci of [*methyl*-³H]choline chloride/ml and incubated for the indicated times. Phospholipids were extracted and separated as described above, and radioactivity in each phospholipid was counted in a liquid-scintillation spectrophotometer.

Measurement of the release of [*methyl*-³H]choline metabolites from prelabelled cells

Confluent cells were labelled with 1 μ Ci of [*methyl*-³H]choline/ml for 48 h in DMEM plus 1% platelet-poor plasma. The cells were washed three times with Hepes-buffered saline solution (37 °C) and incubated in DMEM at 37 °C. After the indicated times, a sample of the medium was placed into a vial and the reaction was stopped. Cellular phospholipids were extracted and separated as described above. Radioactivity in PC, SM and in the medium was counted.

Materials

TPA, 4 β -phorbol, PDBu and phospholipid standards were purchased from Sigma (St. Louis, MO, U.S.A.). MEZ and DPBA were obtained from LC Service (Woburn, MA, U.S.A.). Bombesin and R59022 were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.) and Janssen Life Sciences Products (Beerse, Belgium) respectively. [*methyl*-³H]Thymidine, *myo*-[2-³H]inositol, [³²P]P_i and [*methyl*-³H]choline chloride were purchased from Amersham International (Amersham, Bucks, U.K.).

RESULTS

Effect of TPA and other tumour promoters on cellular 1,2-DG content

Fig. 1(a) shows the time course of changes in the absolute amount of 1,2-DG in 3T3 fibroblasts induced by 0.1 μ M-TPA. Within 1 min after TPA addition, the

Fig. 1. TPA-induced increases in the cellular 1,2-DG content in Swiss-mouse 3T3 fibroblasts

(a) Time course of changes in the 1,2-DG content in response to 0.1 μ M-TPA. (b) TPA dose-response curve for 1,2-DG formation. Cells were treated with various doses of TPA for 10 min. Each value represents the mean $\pm$ S.E.M. for six determinations from two independent experiments.

Fig. 2. Effects of various tumour promoters on the 1,2-DG content of 3T3 fibroblasts

Cells were treated for 10 min with 0.1% dimethyl sulphoxide (control, a) or 0.1 μ M of: 4 β -phorbol (b), DPBA (c), MEZ (d), PDBu (e) and TPA (f). Each value represents the mean $\pm$ S.E.M. for six determinations from two independent experiments.

1,2-DG content increases significantly and continues to rise for up to 10 min, when a nearly 4-fold stimulation over the basal value is observed. The content of 1,2-DG then falls, but still remains at twice the basal value for up to 60 min. The effect of TPA on 1,2-DG formation is dose-dependent and becomes saturated at 0.1–1 μ M, with a half-maximal effect obtained with 6 nM (Fig. 1b). In contrast with the remarkable change in amount of 1,2-DG, TPA does not change 1,3-DG content significantly (results not shown). The content of TG increases slightly after 10 min of TPA (0.1 μ M) treatment (114% of the control value: 0.79 ± 0.01 versus 0.90 ± 0.03 nmol/100 nmol of phospholipid P), then comes down to the basal value by 30 min.

The active tumour promoters PDBu and MEZ induce marked increases in 1,2-DG content to the same extent as TPA does, whereas an inactive compound, 4 β -phorbol, is without effect, and DPBA, which has weak tumour-promoting activity, shows a much smaller effect (Fig. 2).

Interestingly, the relative effect of these compounds on cellular 1,2-DG content is closely related to their mitogenic activity on 3T3 cells (Fig. 3a). Further, comparison of TPA dose-response curves for 1,2-DG formation (Fig. 1b) and [³H]thymidine incorporation (Fig. 3b) reveals that these two temporally separated cellular responses are induced by TPA with a quite similar dose-response relationship (compare Fig. 1b with Fig. 3b).

Effect of TPA on phosphoinositide metabolism

In order to explore the mechanism of a TPA-induced increase in the 1,2-DG content, we first examined the effect of TPA on phosphoinositide metabolism. As shown in Table 1, treatment with TPA for 10 min induces neither an increase in inositol phosphates nor a decrease in inositol lipids. Under the same condition, bombesin (25 nM) induces a significant increase in all three inositol phosphates and a decrease in PI, although both PIP₂ and PIP have recovered to the basal value by 10 min [16]. On the other hand, TPA has an inhibitory effect on bombesin-induced phosphoinositide hydrolysis, as reported by other investigators [23]. Pretreatment of cells with TPA (0.1 μ M) for 30 min significantly inhibits bombesin-induced increases in inositol phosphate formation and ⁴⁵Ca²⁺ efflux from prelabelled cells (results not shown). Nevertheless, treatment of cells with a combination of the maximal dose of both bombesin (25 nM) and TPA (0.1 μ M) brings about an even greater increase in cellular 1,2-DG content than either bombesin or TPA alone [0.52 ± 0.01 , 1.52 ± 0.03 , 1.76 ± 0.03 and 2.02 ± 0.06 nmol/100 nmol of phospholipid P in cells treated with 0.1% dimethyl sulphoxide (control), bombesin (25 nM), TPA (0.1 μ M) or bombesin plus TPA respectively]. These findings indicate that the mechanism of a TPA-induced increase in the 1,2-DG content is different from that of bombesin.

To study further the possible differences between the actions of TPA and bombesin, the effect of a DG kinase inhibitor, R59022 [24], on bombesin- or TPA-induced increases in cellular 1,2-DG content was studied. Either R59022 (10 μ M) or a submaximal dose of bombesin (0.2 nM) alone causes a small increase in 1,2-DG content

Fig. 3. Effects of various tumour promoters on DNA synthesis in 3T3 fibroblasts

Quiescent monolayer cells were incubated with [^3H]thymidine ($0.5 \mu\text{Ci/ml}$) and a test substance for 40 h. (a) Comparison of the effects of: a, 0.1% DMSO (control); b, 4β-phorbol; c, DPBA; d, MEZ; e, PDBu; f, TPA. The concentration of the test substance is $0.1 \mu\text{M}$. (b) TPA dose-response curve for [^3H]thymidine incorporation into DNA. Each value represents the mean $\pm$ S.E.M. for three determinations from a typical experiment; 10% foetal bovine serum induced the incorporation of 96600 ± 1600 c.p.m. of [^3H]thymidine per well.

(0.48 ± 0.02 , 0.63 ± 0.11 and 0.68 ± 0.02 nmol/100 nmol of phospholipid P in control cells and cells treated with R59022 and bombesin respectively); however, if these two are combined, then the effect is synergistic, as expected from their different mechanisms of action (1.05 ± 0.08 nmol/100 nmol of phospholipid P). In contrast, there is no such synergism between R59022 and a submaximal dose of TPA (10 nM). These results again indicate that there is a clear difference between the actions of TPA and of bombesin on the metabolism of 1,2-DG.

Effect of TPA on phospholipid metabolism

Since an increase in the 1,2-DG content induced by TPA might derive from phospholipids other than phosphoinositides, we then examined the effect of TPA on the metabolism of these phospholipids. First, the

Table 1. Effects of TPA and bombesin on phosphoinositide metabolism in Swiss-mouse 3T3 cells

Quiescent monolayer cells, which had been labelled with *myo*-[^3H]inositol for 48 h, were washed and stimulated with either TPA ($0.1 \mu\text{M}$) or bombesin (25 nM) for 10 min. The data represent the means $\pm$ S.E.M. for triplicate determinations (c.p.m./dish).

	Radioactivity (c.p.m./dish)		
	Control	TPA	Bombesin
IP ₁	11600 $\pm$ 350	11500 $\pm$ 740	49130 $\pm$ 1440
IP ₂	970 $\pm$ 80	980 $\pm$ 70	4690 $\pm$ 490
IP ₃	940 $\pm$ 80	890 $\pm$ 170	2340 $\pm$ 170
PI	115500 $\pm$ 960	125110 $\pm$ 3170	106410 $\pm$ 670
PIP	11990 $\pm$ 250	12860 $\pm$ 310	11920 $\pm$ 180
PIP ₂	7730 $\pm$ 110	8870 $\pm$ 380	7320 $\pm$ 280

Table 2. Effect of TPA on ^{32}P incorporation into phospholipids

Quiescent cells were incubated with ^{32}P ($50 \mu\text{Ci/ml}$) in the presence or absence of $0.1 \mu\text{M}$ -TPA for the indicated times. Data represent the means $\pm$ S.E.M. for triplicate determinations.

	^{32}P incorporated (c.p.m./dish)			
	30 min		60 min	
	Control	TPA	Control	TPA
PC	590 $\pm$ 35	2290 $\pm$ 75	2135 $\pm$ 105	16040 $\pm$ 1195
PI	595 $\pm$ 20	2105 $\pm$ 35	2720 $\pm$ 55	5455 $\pm$ 270
PE	75 $\pm$ 15	135 $\pm$ 5	400 $\pm$ 50	1570 $\pm$ 60
PA	310 $\pm$ 30	835 $\pm$ 30	715 $\pm$ 20	2330 $\pm$ 140

cellular contents of PC, PS, PI, PE and PA were quantified. The control values in untreated cells are: PC, 32.3 ± 2.8 ; PS, 7.5 ± 0.9 ; PI, 5.5 ± 0.2 ; PE, 21.8 ± 0.5 ; PA, 0.24 ± 0.01 (nmol/100 nmol of phospholipid P; means $\pm$ S.E.M. for six determinations). None of these phospholipids shows any significant change in response to TPA ($0.1 \mu\text{M}$) for up to 60 min (results not shown). It is noteworthy that PA, which could be either a direct precursor or a phosphorylated product of 1,2-DG, does not show any significant change despite the dramatic change in 1,2-DG content.

In order to evaluate whether TPA has any effect on the metabolic turnover of these phospholipids, we examined the effect of TPA on ^{32}P incorporation into these phospholipids (Table 2). Treatment of cells with $0.1 \mu\text{M}$ -TPA significantly stimulates ^{32}P incorporation into PC, PI, PA and PE after either 30 or 60 min. The degree of stimulation by TPA is variable among these phospholipids, especially at 60 min. The stimulatory effect of TPA is most prominent on incorporation of ^{32}P into PC (8-fold over the control value at 60 min). The other phospholipids show 2–4-fold increases in ^{32}P incorporation. PS and SM do not incorporate appreciable amounts of ^{32}P in either the presence or the absence of TPA (results not shown). In contrast with the above results, when [^3H]inositol is employed as a tracer,

Table 3. Effect of TPA on [³H]choline incorporation into phosphatidylcholine

Quiescent 3T3 cells were incubated in DMEM containing 2 μ Ci of [³H]choline/ml with or without 0.1 μ M-TPA for the indicated times, and radioactivity incorporated into PC was determined. Data represent the means $\pm$ S.E.M. for triplicate determinations. Incorporation of [³H]choline into SM was negligible (results not shown).

	[³ H]Choline incorporated (c.p.m./dish)	
	30 min	60 min
Control	1390 $\pm$ 110	4200 $\pm$ 320
TPA	3840 $\pm$ 180	10350 $\pm$ 780

Table 4. Effect of TPA on the release of [³H]choline metabolites from prelabelled cells

Quiescent 3T3 cells, prelabelled with [³H]choline for 48 h, were washed three times and incubated with or without TPA (0.1 μ M) for the indicated times. Radioactivities released into media (medium) and those in phosphatidylcholine (PC) and sphingomyelin (SM) were determined. Each value represents the mean $\pm$ S.E.M. for triplicate determinations.

		10 ⁻³ $\times$ Radioactivity (c.p.m./dish)		
		10 min	30 min	60 min
Medium	Control	11.1 $\pm$ 0.5	13.0 $\pm$ 0.2	18.1 $\pm$ 0.5
	+ TPA	16.4 $\pm$ 0.2	23.7 $\pm$ 0.5	35.1 $\pm$ 1.0
PC	Control	97.9 $\pm$ 1.0	97.7 $\pm$ 3.0	100.6 $\pm$ 2.0
	+ TPA	97.2 $\pm$ 2.7	93.9 $\pm$ 2.2	92.6 $\pm$ 2.3
SM	Control	15.8 $\pm$ 0.3	16.8 $\pm$ 0.9	17.4 $\pm$ 0.7
	+ TPA	15.2 $\pm$ 0.0	17.2 $\pm$ 1.8	18.8 $\pm$ 0.1

TPA does not stimulate incorporation of ³H into PI [3440 $\pm$ 350 and 3450 $\pm$ 260 c.p.m./dish at 60 min, in control- and TPA (0.1 μ M)-treated cells respectively].

To evaluate further the effect of TPA on PC metabolism, incorporation of [³H]choline into PC and its release from prelabelled cells were studied. As shown in Table 3, TPA (0.1 μ M) significantly stimulates the incorporation of [³H]choline into PC, at both 30 min and 60 min (3 and 2.5 times the control value respectively). The stimulatory effect is not due to increased uptake of [³H]choline into the cells, since there is no difference between the amount of radioactivity recovered in the water-soluble fractions from control and TPA-treated cells (results not shown). In contrast, incorporation of [³H]choline into SM is negligible in both control and TPA-treated cells (results not shown). Table 4 shows the effect of TPA on [³H]choline release from cells prelabelled with [³H]choline to a steady state. TPA (0.1 μ M) significantly stimulates the release of water-soluble [³H]choline metabolites into media at 10, 30 and 60 min after the addition of TPA. In the corresponding TPA-treated cells, [³H]choline radioactivity in PC, but

not in SM, gradually decreases. These findings indicate that TPA stimulates both synthesis and degradation of PC, i.e. PC turnover, which is analogous to agonist-induced phosphoinositide turnover.

DISCUSSION

The results in the present study demonstrate for the first time that TPA induces an increase in the absolute amount of 1,2-DG in Swiss-mouse 3T3 fibroblasts. The effect is time- and dose-dependent (Figs. 1a and 1b), and even greater than that induced by the maximal dose of bombesin, which is a potent mitogen thought to be acting through phosphoinositide hydrolysis in 3T3 cells. This effect is shared with other active tumour promoters, PDBu and MEZ, but not with an inactive compound, 4 β -phorbol (Fig. 2). Moreover, with these tumour promoters there is a close correlation between their effect on 1,2-DG formation and their mitogenic activity in 3T3 cells over the same dose range (Figs. 3a and 3b). These findings indicate that this effect of tumour promoters on 1,2-DG metabolism represents a novel aspect of cellular actions common to tumour promoters of both the phorbol ester and non-phorbol classes. It is not known at present how this effect of TPA on 1,2-DG formation contributes to the activation of the C-kinase by TPA.

In various types of cells, it has been established that a certain class of hormones induces hydrolysis of plasma-membrane phosphoinositides to produce two intracellular messengers, inositol 1,4,5-trisphosphate and 1,2-DG. The former induces intracellular Ca²⁺ mobilization and the latter activates C-kinase, thereby mediating diverse actions of these hormones [3,25,26]. In 3T3 cells we have observed that bombesin induces a prompt and sustained increase in the cellular 1,2-DG content through hydrolysis of phosphoinositides [16]. In contrast, TPA does not induce either breakdown of phosphoinositides or production of inositol phosphates (Table 1). Moreover, TPA inhibits bombesin-induced phosphoinositide hydrolysis [23]. Nevertheless, a combination of the maximal doses of bombesin and TPA shows an even greater effect on 1,2-DG content than that induced by bombesin alone. These findings indicate that the mechanism of action of TPA on 1,2-DG metabolism is different from that of bombesin and is not through hydrolysis of phosphoinositides.

On the other hand, the present study demonstrates that TPA stimulates metabolic turnover of PC in Swiss 3T3 cells, without changing the total mass of this phospholipid (Tables 2–4). Thus TPA stimulates the incorporation of ³²P and [³H]choline into PC (Tables 2 and 3), with a concomitant release of [³H]choline radioactivity from prelabelled cells, which is associated with a decrease in the radioactivity from PC (Table 4). Although the water-soluble [³H]choline metabolites released by the action of TPA have not been identified in this study, the finding that the cellular content of PA does not increase along with this PC turnover strongly suggests that TPA stimulates a reaction catalysed by a phospholipase C, rather than by phospholipase D. Since the cellular content of PC is about 2 orders of magnitude greater than that of 1,2-DG in the basal condition, an increased turnover of only a small pool of PC would be enough to serve as a source for a 4-fold increase in the cellular 1,2-DG content, without changing the mass of PC. There are a few reports in other cell types showing

a stimulatory effect of TPA on PC turnover and relating this to 1,2-DG formation [27–30]. Besterman *et al.* [30] have reported that PDBu and platelet-derived growth factor induce rapid PC turnover in pre-adipocyte cell line 3T3-L1. They have also shown that PDBu induced a prompt increase in diacylglycerol in [^{14}C]oleate-labelled cells; however, their period of observation was limited to 75 s. The results in the present study confirm and extend these previous findings by demonstrating a sustained elevation in actual 1,2-DG content for up to 60 min. It is likely that these effects of TPA are universal among various types of cells.

TPA also increases incorporation of ^{32}P into PI, PE and PA (Table 2), without changing the mass of these lipids. However, when [^3H]inositol is employed as a tracer, TPA does not stimulate its incorporation into PI. It should also be noted that the degree of TPA-induced stimulation of incorporation of radioactive tracers into PC is quite different for ^{32}P (Table 2) and [^3H]choline (Table 3). Therefore TPA-induced stimulation of ^{32}P incorporation into these phospholipids may not reflect the net increase in the rate of their synthesis. It might be possible that TPA alters the specific radioactivity of the cellular ATP pool, since TPA is known to stimulate glucose transport in 3T3 cells [16,31], which is presumably followed by a change in ATP metabolism. It is also possible, however, that the turnover of PE and PA is stimulated secondarily to PC turnover, by increased availability of 1,2-DG, as has been suggested by others [27].

Although the stimulation of PC turnover seems to serve as a major source of TPA-induced 1,2-DG formation, the present study suggests another interesting mechanism by which TPA may increase the cellular 1,2-DG content: an inhibition of the diacylglycerol kinase. Results obtained with a DG kinase inhibitor, R59022, raise this possibility. As expected, the effect of the submaximal dose of bombesin in 1,2-DG formation is synergistically enhanced by the concomitant presence of R59022. In contrast, R59022 has no further effect on TPA-induced 1,2-DG formation. Moreover, the measurement of the cellular PA content indicates that the mass of PA does not change significantly for up to 60 min of TPA treatment, despite a marked increase in the mass of 1,2-DG (Fig. 1a). These results are compatible with the notion that TPA inhibits the diacylglycerol kinase activity, resulting in an inhibition of conversion of 1,2-DG into PA, which may contribute to a sustained increase in 1,2-DG. Since there is a report that TPA is a relatively poor substrate for partially purified DG kinase *in vitro* [32], it is possible that TPA inhibits this enzyme through an indirect mechanism.

Besides these two mechanisms, TPA may increase the 1,2-DG content through other mechanisms. As suggested in rat hepatocytes [33], TG could also serve as a source of 1,2-DG, although a small increase in the TG content observed in the present study is coincident with the peak of 1,2-DG, favouring the view that it is derived from 1,2-DG rather than serving as a precursor for it. On the other hand, another study has shown that TPA inhibits diacylglycerol lipase activity *in vitro* [34], suggesting that this action may lead to an increase in the cellular 1,2-DG content.

It has been reported that substantial portions of cellular PC are 1-alkyl-2-acyl compounds in rat platelets [35] or rabbit alveolar macrophages [36]. These findings

raise the possibility that treatment of cells with TPA increases 1-alkyl-2-acylglycerol in addition to 1,2-diacylglycerol. This view is supported by a study in MDCK cells [29]. Little is known about the physiological significance of this class of compounds. Further studies are required to reveal the metabolism and the physiological role of this class of compounds in Swiss-mouse 3T3 cells.

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