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# Epidermal growth factor increases *sn*-1,2-diacylglycerol levels and activates phospholipase D-catalysed phosphatidylcholine breakdown in Swiss 3T3 cells in the absence of inositol-lipid hydrolysis

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Addition of epidermal growth factor (EGF) to quiescent Swiss 3T3 cells resulted in a sustained increase in cellular diacylglycerol (DG) content in the absence of inositol-lipid hydrolysis. In the presence of non-cytotoxic concentrations of butan-1-ol, EGF stimulated the formation of phosphatidylbutanol, indicating that the EGF receptor was able to couple to the activation of phospholipase D (PLD). EGF-stimulated release of choline from Swiss 3T3 cells suggested that the major substrate for this PLD was phosphatidylcholine. Unlike bombesin-stimulated PLD activity, the response to EGF was not inhibited by a selective protein kinase C (PKC) inhibitor (Ro-31-8220), suggesting that it was not dependent on PKC activation. Pre-treatment of Swiss 3T3 cells with the EGF-receptor tyrosine kinase inhibitor AG18 selectively inhibited EGF-stimulated PLD activity; bombesin-stimulated PLD activity was unaffected. Butan-1-ol inhibited phorbol ester- and bombesin-stimulated DG formation suggesting a role for a coupled PLD/phosphatidate phosphohydrolase pathway; in contrast, EGF-stimulated DG formation was unaffected.

## INTRODUCTION

The stimulated hydrolysis of PtdIns(4,5)$P_2$ by phospholipase C (PLC) generates Ins(1,4,5)$P_3$ and *sn*-1,2-diacylglycerol (DG). Both these products are second messengers activating effector systems implicated in controlling many physiological responses, including mitogenesis; Ins(1,4,5)$P_3$ releases $Ca^{2+}$ from an internal store and DG activates protein kinase C (PKC) (Berridge & Irvine, 1989; Kikkawa *et al.*, 1989; Parker *et al.*, 1989). The identification of PKC as the receptor for the DG-mimetic tumour-promoting phorbol esters (Castagna *et al.*, 1982) has underlined the importance of this signal pathway in cell growth.

The stimulated hydrolysis of inositol lipids is not the only pathway of DG formation (Billah & Anthes, 1990; Cook & Wakelam, 1991*a*). The hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D (PLD) has been shown to serve as a pathway for phosphatidate (PtdOH) formation and thence DG via the action of phosphatidate phosphohydrolase (PPH) in fMet-Leu-Phe-stimulated neutrophils (Billah *et al.*, 1989; Bonser *et al.*, 1989). In IIC9 fibroblasts Wright *et al.* (1988) have shown that $\alpha$-thrombin stimulates a biphasic increase in DG, with the second phase being sustained for up to 1 h in the absence of elevated Ins$P_3$ levels, and molecular-species analysis of this DG suggests it is derived from PtdCho (Pessin & Rabin, 1989). Our own studies with Swiss 3T3 cells have shown that bombesin stimulates a biphasic increase in DG mass (Cook *et al.*, 1990); mass analysis reveals that Ins(1,4,5)$P_3$ accumulation is transient, returning to basal levels by 30 s, whereas DG levels remain elevated. Two pieces of evidence suggest that the sustained DG formation may be derived from a non-inositide lipid. First, sustained DG elevation can be mimicked in part by phorbol esters (Cook *et al.*, 1991; Takuwa *et al.*, 1991) at concentrations at which agonist-stimulated inositol lipid hydrolysis is inhibited (Cook & Wakelam, 1991*b*). Second, both bombesin and phorbol 12-myristate 13-acetate (PMA) stimulate the PKC-dependent release of choline (Cho) and phosphocholine (Cho*P*) from Swiss 3T3 cells which appears to precede sustained DG accumulation (Cook & Wakelam, 1989, 1991*b*). Together, these findings suggest that activation of PKC by prior inositol-lipid hydrolysis may serve to stimulate PLD-catalysed PtdCho hydrolysis and subsequent DG formation, which may then further activate PKC.

A number of growth factors which do not stimulate inositol-lipid hydrolysis have been shown to activate PKC, e.g. embryonal carcinoma-derived growth factor (Mahadevan *et al.*, 1987) and colony-stimulating factor-1 (Imamura *et al.*, 1990). In Swiss 3T3 cells EGF has been reported to be without effect upon inositol phospholipid hydrolysis yet the mitogen has been claimed to stimulate PKC activity. Kazlauskas & Cooper (1988), reported epidermal-growth-factor (EGF)-stimulated PKC activation to a similar magnitude to that for PMA or platelet-derived growth factor. Süsa *et al.* (1989) have shown that in Swiss 3T3 cells EGF stimulated a biphasic increase in S6 kinase activity, the second phase of which was inhibited in PKC-depleted cells after prolonged phorbol ester exposure. This suggested that EGF may require a functional PKC to mediate at least some of its effects. However, two reports, utilizing different methods of identification, have failed to demonstrate EGF-stimulated PKC activation (Isacke *et al.*, 1986; Rodriguez-Pena & Rozengurt, 1986). Therefore it is important to determine if EGF stimulates DG generation in Swiss 3T3 cells and by what mechanism should it occur. In this report we show that in Swiss 3T3 cells EGF stimulates PtdCho hydrolysis and DG accumulation, but not inositol-lipid hydrolysis. PtdCho hydrolysis is shown to proceed by a PLD-catalysed route, but this does not appear to be the

Abbreviations used: DG, *sn*-1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMBH, DMEM containing 20 mM-Hepes, pH 7.4, and 1 % (w/v) BSA; EGF, epidermal growth factor; HBG, Hanks buffered saline containing 10 mM-glucose and 1 % BSA; HBG.Li, HBG containing 10 mM-LiCl; Ins$P_t$, total inositol phosphates; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PPH, phosphatidate phosphohydrolase; PtdOH, phosphatidate; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; Cho, choline; Cho*P*, phosphocholine.

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pathway of DG formation. In addition, we compare aspects of the regulation of PLD by EGF with that for bombesin and PMA. Some of these results have been previously reported in a preliminary form (Wakelam *et al.*, 1991).

## MATERIALS AND METHODS

### Cell culture

Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn-calf serum, 27 mg of glutamine/ml and 250 i.u. of penicillin/ml and 250 $\mu$g of streptomycin/ml at 37 °C in humidified atmosphere of air/$CO_2$ (19:1). All experiments were performed on serum-depleted cells which had been grown to confluency for 48 hours in 1% (v/v) calf serum. For labelling studies the relevant radiochemical (*myo*[$^3H$]-inositol, 1 $\mu$Ci/ml; [*methyl*-$^3$H]choline chloride, 1.5 $\mu$Ci/ml; [9,10(n)-$^3$H]palmitic acid, 2 $\mu$Ci/ml) was included during the final 48 h. In all cases the cells were confluent and quiescent when used.

### Assay of DG mass

Confluent monolayers of Swiss 3T3 cells grown on six-well plates were washed in 1 ml of DMEM/20 mM-Hepes, pH 7.4, containing 1% (w/v) BSA (DMBH) for 60 min at 37 °C before stimulation with EGF, PMA or bombesin in DMBH as indicated. Incubations were terminated by removal of the medium and addition of 0.7 ml of ice-cold methanol, and the lipids were extracted after addition of an equal volume of chloroform. The DG mass in each sample was determined by radio-enzymic assay utilizing phosphatidylserine (6 mol%, 0.288 mM) in Triton X-100 (0.6%, w/v) as a solubilization system as previously described (Paterson *et al.*, 1991). DG mass was expressed as pmol/$10^6$ cells.

### Measurement of total inositol phosphates

Quiescent Swiss 3T3 cells labelled with [$^3$H]inositol were washed in Hanks buffered saline solution, pH 7.4, containing 10 mM-glucose and 1% BSA (HBG) for 5 min at 37 °C. They were then washed in HBG containing 10 mM-LiCl (HBG.Li) for a further 15 min at 36 °C before treatment with the appropriate agent. After this preincubation period, cells were treated with the given concentration of agonist in HBG.Li for the times indicated at 37 °C. Incubations were terminated by aspiration of the medium and addition of 150 $\mu$l of 10% (v/v) $HClO_4$, the cell extracts were neutralized, and total inositol phosphate formation was determined as described previously (Cook & Wakelam, 1991*b*).

### Assays of PLD activity in whole cells

**(1) Assay of [$^3$H]choline release.** Cells grown in 24-well plates and labelled to isotopic equilibrium with [$^3$H]choline were washed in HBG and stimulated as described with agents in HBG. The incubations were terminated by addition of ice-cold methanol, and PtdCho breakdown was determined by isolating [$^3$H]Cho and [$^3$H]Cho*P* from control and stimulated cell extracts by separation on Dowex-50W ($H^+$ form) as previously described (Cook & Wakelam, 1989).

**(2) Assay of PLD phosphatidyltransferase activity.** Swiss 3T3 cells grown in six-well plates were labelled in 2 ml of DMEM + 1% (v/v) newborn-calf serum containing a total of 4 $\mu$Ci of [$^3$H]palmitic acid per well for 48 h. Cells were washed in 1 ml of serum-free DMBH for 20 min at 37 °C before incubation for a further 4 min in 1 ml of DMBH containing 30 mM-butan-1-ol (0.3%, v/v). Incubations were started by replacing the medium with 1 ml of DMBH/0.3% butan-1-ol and the test reagent at the concentrations and for the times indicated. Incubations were terminated by removal of the medium and addition of 0.7 ml of ice-cold methanol; after extraction of lipids in chloroform, [$^3$H]phosphatidylbutanol ([$^3$H]PtdBut) was separated by t.l.c. as described previously (Cook *et al.*, 1991).

### Materials

All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K.; tissue-culture medium and serum were from Gibco/BRL, Paisley, Scotland, U.K. Ro-31-8220 was generously given by Roche Products, Welwyn Garden City, Herts., U.K., and AG18 was generously supplied by A. Levitski, Jerusalem, Israel. All other materials were from previously reported sources.

## RESULTS

Stimulation of Swiss 3T3 cells with 100 nM-EGF induced an increase in DG mass (Fig. 1). Contrary to that observed in response to bombesin (Cook *et al.*, 1990), there was no EGF-stimulated rapid increase in DG mass at 5 s (Fig. 1*a*). Increases above control were apparent from 30 s onwards, but were statistically significant from 1 min ($P = 0.021$) or 2 min and were maintained at this maximum level for 30 min before declining. Although DG levels were decreased at 60 min, they were still above the corresponding control time point (Fig. 1*b*). The ability of EGF to increase DG levels in Swiss 3T3 cells was dose-

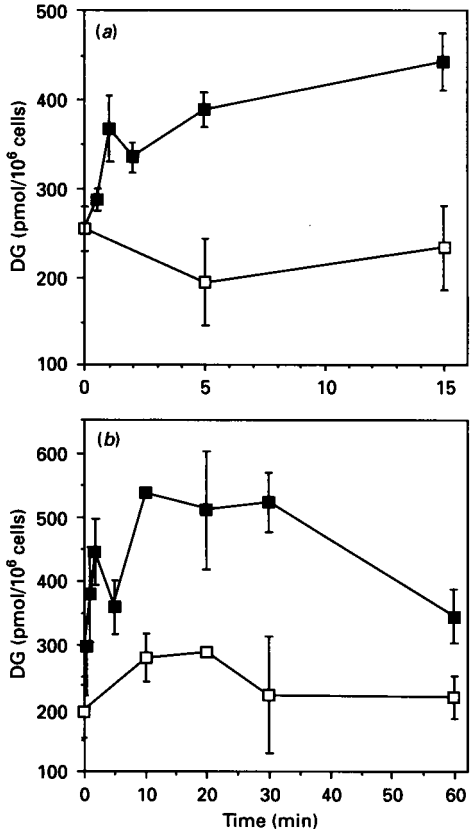


**Fig. 1. EGF-stimulated increases in DG mass**

Confluent and quiescent Swiss 3T3 cells were stimulated for the stated times and DG mass was determined as described in the Materials and methods section. (*a*) and (*b*) demonstrate separate experiments using different time courses; the data are means ± S.D. for $n = 3$ determinations from the separate experiments, and each is typical of three others. Cells: □, control; ■, stimulated with 100 nM-EGF.

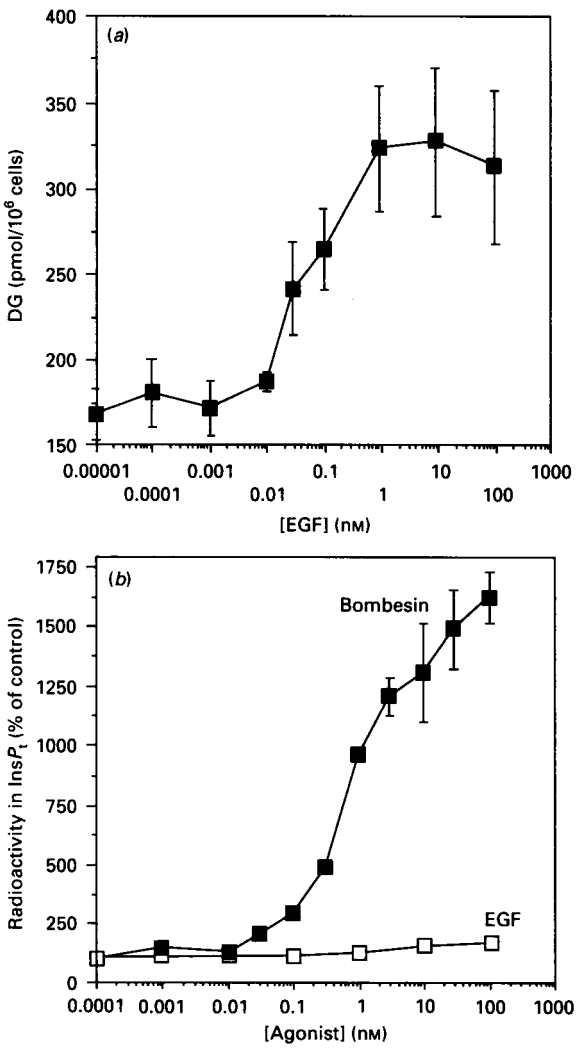


**Fig. 2. Dose-dependence of EGF-stimulated changes in DG and Ins$P_t$**

(*a*) Cells were stimulated with increasing concentrations of EGF for 10 min and increases in DG mass were determined as described in the Materials and methods section. (*b*) [$^3$H]Inositol-labelled cells were stimulated with increasing concentrations of EGF or bombesin for 15 min in the presence of 10 mM-LiCl for 15 min and the generation of inositol phosphates determined as described in the Materials and methods section. The data are means ± S.D. for $n = 3$ determinations from separate experiments typical of three.

dependent (Fig. 2*a*); maximal elevation occurred at approx. 1 nM, with an $EC_{50}$ value of 0.07 ± 0.02 nM. The magnitude of EGF-stimulated increases in DG mass was much smaller than those observed for bombesin or PMA; Table 1 shows the results of an experiment where each stimulant was utilized, allowing a direct comparison. EGF stimulated a net increase of 150 ± 41 pmol of DG/$10^6$ cells (mean ± S.D. of $n = 5$ determinations) representing a 1.81 ± 0.48-fold increase over unstimulated levels (mean ± S.D. from $n = 9$ determinations) at 15 min.

Despite this increase in cellular DG content, EGF was unable to stimulate a reproducible increase in [$^3$H]inositol phosphate accumulation over controls even in the presence of 10 mM-LiCl. Fig. 2(*b*) shows the dose–response curve for EGF-stimulated changes in [$^3$H]Ins$P_t$ accumulation; a small elevation was sometimes observed at the highest dose (100 nM; Fig. 2*b*), but was not reproducible, and this concentration was at least two orders of magnitude greater than that required for DG accumulation (Fig. 2*a*). The generation of [$^3$H]Ins$P_t$ was negligible in comparison

**Table 1. Effect of butanol on stimulated DG formation**

Swiss 3T3 cells were washed in DMBH for 60 min with the inclusion of 0.3% butan-1-ol for the last 5 min where stated. They were then stimulated for 15 min in the presence or absence of butan-1-ol, and the generation of DG was determined as described in the Materials and methods section. The data are means ± S.D. of one experiment where $n = 3$, typical of two others.

| | DG (pmol/$10^6$ cells) | |
|---|---|---|
| Stimulation | Control | +Butan-1-ol |
| Control | 278 ± 2 | 250 ± 26 |
| 100 nM-bombesin | 948 ± 43 | 728 ± 13 |
| 100 nM-PMA | 824 ± 13 | 406 ± 27 |
| 100 nM-EGF | 374 ± 13 | 382 ± 17 |

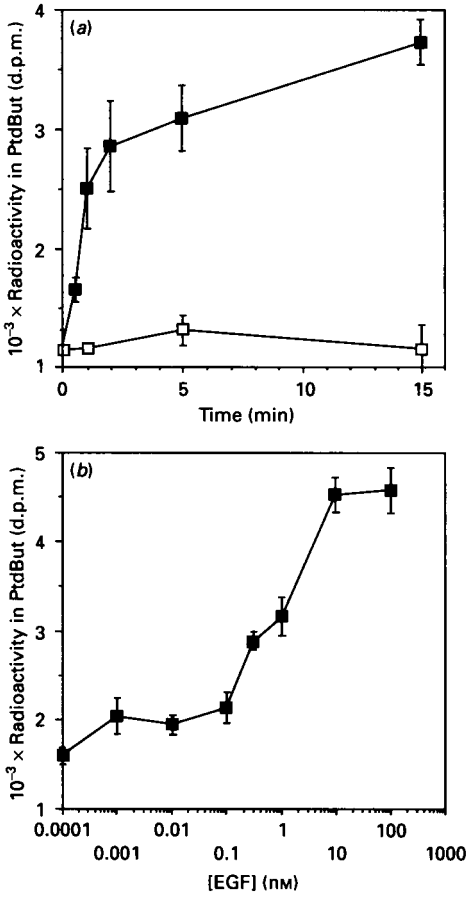


**Fig. 3. EGF-stimulated PtdBut formation**

[$^3$H]Palmitate-labelled cells were stimulated with 100 nM-EGF in the presence of 0.3% butan-1-ol for increasing times (*a*) or with increasing concentrations of EGF for 15 min (*b*), and PtdBut formation was determined as described in the Materials and methods section. The data are means ± S.D. for $n = 3$ determinations from separate experiments typical of three others.

with that stimulated by bombesin (Fig. 2*b*). Furthermore, EGF did not stimulate any apparent increase in Ins(1,4,5)$P_3$ mass up to 5 min when assayed by competitive binding assay (results not shown).

Experiments were performed to investigate if EGF stimulated PtdCho hydrolysis and whether this played a role in DG formation. Fig. 3(*a*) shows the kinetics of EGF-stimulated

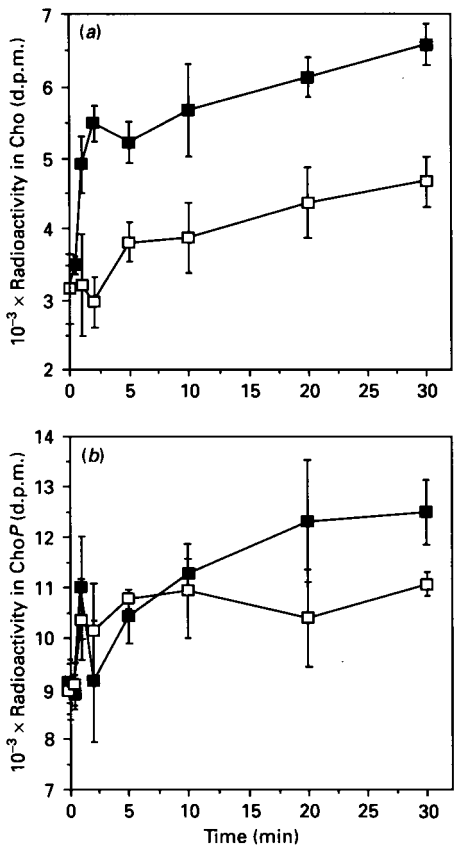


**Fig. 4. EGF-stimulated changes in choline and choline phosphate generation**

Cells prelabelled with [$^3$H]Cho were stimulated with 100 nM-EGF (■) or buffer (□), and the generation of [$^3$H]Cho (*a*) and [$^3$H]Cho-*P* (*b*) was determined as described in the Materials and methods section. The data are means ± S.D. for $n = 3$ determinations from a single experiment typical of three others.

increases in [$^3$H]PtdBut in [$^3$H]palmitate-labelled Swiss 3T3 cells in the presence of 30 mM-butan-1-ol. [$^3$H]PtdBut formation is catalysed by the transferase activity of PLD and serves as a definitive marker for PLD in whole cells, since phosphatidyl-alcohols are not formed by other pathways (Billah & Anthes, 1990). After addition of 100 nM-EGF, increases in [$^3$H]PtdBut were apparent and significant after 30 s ($P = 0.006$) and increased rapidly until 2 min. In all experiments the rate of [$^3$H]PtdBut formation from 2 min onwards was greatly decreased (0–2 min + EGF 854 d.p.m./min; 2–15 min + EGF 65 d.p.m./min; control, no added EGF, 12 d.p.m./min; results from a single representative experiment). This slower increase in [$^3$H]PtdBut was variable; in some cases the 15 min stimulated point was significantly higher than the 5 min stimulated point ($P = 0.032$; Fig. 3*a*), whereas in other experiments the continued rise in [$^3$H]PtdBut was not significant. Fig. 3(*b*) shows the dose–response curve for EGF-stimulated increases in [$^3$H]PtdBut levels in Swiss 3T3 cells; the $EC_{50}$ value was 1.14 ± 0.54 nM (mean ± S.D., $n = 3$ experiments). Thus EGF stimulates PLD activity in a dose-dependent, but transitory, manner.

Over 50% of the incorporated [$^3$H]palmitate is found in PtdCho in Swiss 3T3 cells (Cook & Wakelam, 1991*b*), with less than 10% being found in PtdIns (results not shown). However, to confirm that PLD activity was utilizing PtdCho as a substrate, the effect of EGF on water-soluble choline metabolite levels was examined in Swiss 3T3 cells labelled with [$^3$H]choline chloride. Fig. 4 shows the time course of changes in the radioactivity associated with [$^3$H]Cho and [$^3$H]ChoP in EGF stimulated Swiss 3T3 cells; there was no significant change in [$^3$H]glycerophosphocholine in any experiment performed (results not shown). After addition of 100 nM-EGF, [$^3$H]Cho levels were elevated within 30 s and were significantly above control at 1 min (Fig. 4*a*; $P = 0.025$). Maximal elevation was observed at 2 min, after which the rate of increase slowed, but [$^3$H]Cho levels were maintained above control for at least 30 min and, in some cases, 60 min (results not shown). No significant increases in the radioactivity associated with [$^3$H]Cho*P* were observed in response to EGF until much later time points (typically 30 min; Fig. 4*b*).

We have previously shown that bombesin-stimulated PtdIns(4,5)$P_2$ hydrolysis might lead to activation of PLD via the stimulation of PKC activity (Cook & Wakelam, 1989, 1991*b*). Since EGF was able to increase DG levels and activate PLD without PtdIns(4,5)$P_2$ hydrolysis, this suggested that the EGF receptor might couple to PLD independently of PKC activity. To this end, the effect of the PKC inhibitor Ro-31-8220 (Davis *et al.*, 1989) on EGF-stimulated [$^3$H]PtdBut formation was determined and compared with that on PMA and bombesin stimulation. Fig. 5 shows that in [$^3$H]palmitate-labelled Swiss 3T3 cells, pre-treated for 5 min with increasing concentrations of Ro-31-8220, EGF-stimulated [$^3$H]PtdBut formation was not significantly inhibited. When the results were normalized to 'percentage of maximum' and compared with that for PMA and bombesin, the contrasting effect on the EGF response was clearly observed. PMA-stimulated [$^3$H]PtdBut formation was inhibited by approx. 90%, whereas bombesin-stimulated PLD activity was inhibited by about 50% at 10 μM-Ro-31-8220. In contrast, EGF-stimulated [$^3$H]PtdBut activity was not inhibited, and in some cases there was an enhancement at 3 μM-Ro-31-8220, which was statistically significant ($P = 0.007$) at 10 μM-Ro-31-8220.

The cytosolic domain of the EGF receptor includes a ligand-activated tyrosine kinase catalytic region which is responsible for phosphorylating various key substrates, including effector systems such as PLC-γ1 (Ullrich & Schlessinger, 1990). However, in Swiss 3T3 cells possessing their 'native' receptor complement, EGF does not stimulate inositol-lipid hydrolysis (Fig. 2*b*) or phosphorylation of PLC-γ1 (Meisenhelder *et al.*, 1989). Nevertheless, in order to investigate whether the receptor tyrosine kinase activity was required for EGF-stimulated activation of PLD, Swiss 3T3 cells were pre-treated with the EGF-receptor tyrosine kinase inhibitor AG18 [termed RG50864 in Lyall *et al.* (1989)] for 18 h. When [$^3$H]palmitate-labelled Swiss 3T3 cells were pre-treated with increasing concentrations of AG18, there was a dose-dependent inhibition of subsequent EGF-stimulated [$^3$H]PtdBut formation as shown in Fig. 6. The results were normalized to 'percentage of maximum' to allow comparison with bombesin, which gave a larger response. Maximal inhibition of EGF-stimulated [$^3$H]PtdBut formation was observed at 100 μM-AG18, with an apparent $IC_{50}$ of about 30 μM; an accurate $IC_{50}$ could not be estimated, since the inhibition was not complete. At 100 μM-AG18 the response to EGF was inhibited by approx. 85–90%. In the same experiments in which 100 μM-AG18 exhibited EGF-stimulated [$^3$H]PtdBut formation by 90%, the response to bombesin was completely unaffected (Fig. 6).

Incubation in the presence of primary alcohols allows a preliminary assessment of the role a coupled PLD/PPH pathway plays in DG formation, since it traps phosphatidyl moieties released by PLD activity as the relatively non-reactive phosphatidyl-alcohol (Bonser *et al.*, 1989; Metz & Dunlop, 1991). To determine the contribution made by the PLD/PPH pathway to EGF-stimulated DG formation, the effect of butane-1-ol on EGF-stimulated DG levels was examined. Preincubation of Swiss 3T3 cells with 0.3% butan-1-ol for 5 min resulted in 70% inhibition of PMA-stimulated DG formation and 30% inhibition

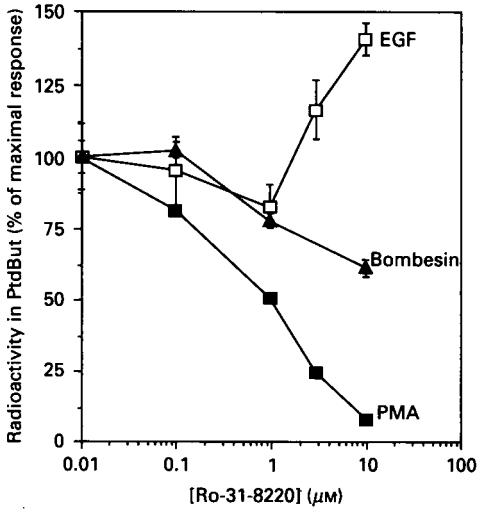


**Fig. 5. Effect of Ro-31-8220 on EGF-stimulated PtdBut formation**

[$^3$H]Palmitate-labelled cells were preincubated for 10 min with increasing concentrations of Ro-31-8220 before being stimulated with 100 nM-EGF, -bombesin or -PMA for 15 min. PtdBut generation was determined as described in the Materials and methods section. The data are from a single typical experiment where the data have been normalized to percentage of maximum response to allow comparison.

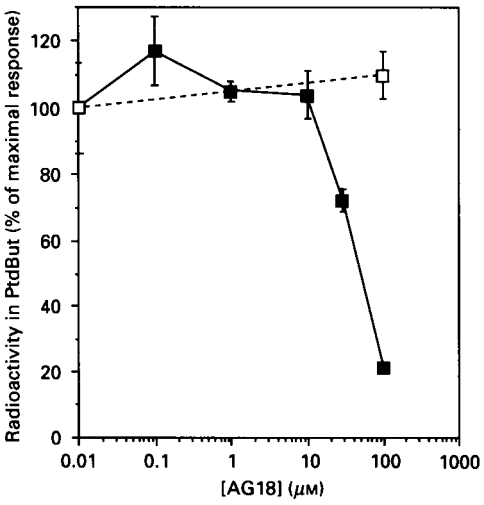


**Fig. 6. Effect of the tyrphostin AG18 on EGF-stimulated PtdBut formation**

Increasing concentrations of AG18 were added to [$^3$H]palmitate-labelled cells from 18 h before stimulation. EGF (100 nM) (■)- and bombesin (100 nM) (□)-stimulated PtdBut formation was determined as in the Materials and methods section. The data are means ± S.D. for $n = 3$ determinations from a single typical experiment.

of bombesin-stimulated DG formation (Table 1). Under the same conditions EGF-stimulated increases in DG formation were unaffected by the presence of butan-1-ol before and during the stimulation (Table 1), suggesting that DG formation did not occur via a PLD/PPH-coupled pathway in response to EGF.

## DISCUSSION

It has previously been reported that EGF does not stimulate the hydrolysis of inositol lipids in Swiss 3T3 cells (Hesketh *et al.*, 1988), and this would be consistent with the reported inability of EGF to stimulate PKC activity in these cells (Isacke *et al.*, 1986; Rodriguez-Pena & Rozengurt, 1986). However, this remains a contentious issue, since two studies have suggested that EGF is able to activate PKC in Swiss 3T3 cells (Kazlauskas & Cooper, 1988; Süsa *et al.*, 1989), which might suggest that EGF can stimulate an increase in DG levels. Here we show for the first time, using a sensitive radioenzymic assay, that EGF is indeed able to stimulate a small, but sustained, increase in DG mass in the absence of inositol lipid hydrolysis in Swiss 3T3 cells (Fig. 1). The $EC_{50}$ for DG formation is similar to that reported for the mitogenic effects of EGF (Wright *et al.*, 1990) and the $K_d$ for EGF binding to Swiss 3T3 cells (Brown *et al.*, 1984). This increase in DG is not due to the hydrolysis of inositol lipids, since EGF failed to stimulate the accumulation of inositol phosphates in the presence of LiCl. The correlation between the ability of bombesin, even at low concentrations, to stimulate [$^3$H]Ins$P_t$ accumulation and DG generation (Cook *et al.*, 1990) suggests that the smaller EGF-stimulated DG response was not simply attributable to a correspondingly smaller inositol phosphate response. Additionally, again in contrast with the response to bombesin (Cook *et al.*, 1990) there was no rapid increase in DG mass at 5 or 10 s. This probably reflects the lack of a rapid activation of PLC-catalysed PtdIns(4,5)$P_2$ hydrolysis; consistent with this, there was no change in Ins(1,4,5)$P_3$ mass over 5 min of stimulation. Indeed, on the rare occasions where EGF did elevate total [$^3$H]Ins$P_t$ over a 20 min stimulation period, it was only at concentrations well in excess of the $EC_{50}$ for DG accumulation; even this response was not reproducible. Thus neither the magnitude nor the dose-dependence of the very variable small inositol phosphate response can account for the observed increases in DG in response to EGF. The ability of EGF to stimulate DG formation in Swiss 3T3 cells is in contrast with the recent report by Bierman *et al.* (1990). Apart from the possibility that this represents clonal differences between different Swiss 3T3 cell lines, differences in sample preparation for the DG mass assay may explain these conflicting results, since Bierman *et al.* (1990) terminated their incubations by aspiration and addition of 10% trichloroacetic acid. Preiss *et al.* (1986) have reported that exposure of *sn*-1,2-DGs to even mild acid or alkali results in acyl-chain migration and loss of the *sn*-1,2-specificity of the assay, leading to an underestimation of increases in DG mass.

The observation that EGF stimulates the formation of [$^3$H]PtdBut (Fig. 3) and the release of [$^3$H]Cho (Fig. 4) with similar kinetics demonstrates that EGF activates a PtdCho-PLD activity in Swiss 3T3 cells. The dose-dependence for [$^3$H]PtdBut formation by EGF gave an $EC_{50}$ of approx. 1 nM; although this was higher than that for DG formation, it was clearly much closer than that for EGF-stimulated inositol phosphate accumulation. This, together with the kinetics of onset of PLD activity, which preceded DG formation, suggested that PtdCho hydrolysis by PLD might serve as the source of DG formation via a coupled PLD/PPH pathway. This would be consistent with the report by Pessin *et al.* (1990), who showed in IIC9 cells that EGF stimulated an increase in DG, with a molecular species profile consistent with it being derived from PtdCho.

The conclusion that PLD-catalysed PtdCho hydrolysis leads to an increase in DG generation is questioned by experiments with butan-1-ol. This primary alcohol 'traps' phosphatidyl moieties as PtdBut and, since the phosphatidyl-alcohols are poor substrates for PPH (Metz & Dunlop, 1991), this allows effective intervention in a putative PLD/PPH pathway (Bonser *et al.*, 1989). Inclusion of 0.3% butan-1-ol in incubations inhibited PMA- and bombesin-stimulated DG formation, suggesting a role for a PLD/PPH pathway. In contrast, 0.3% butan-1-ol did not inhibit EGF-stimulated DG formation (Table 1). Thus,

although PMA, bombesin and EGF (Fig. 4) all activate PLD, the PtdOH produced in this reaction appears not to serve as a precursor for DG via PPH in the case of EGF. The results in Table 1 further suggest that the sustained generation of DG by bombesin is only partly derived through a PLD-catalysed pathway.

In this case, what is the pathway for DG formation in response to EGF? It seems unlikely to be synthesis *de novo* (Wright *et al.*, 1990). EGF stimulated a slow increase in [$^3$H]Cho*P*, which may suggest a PLC pathway for PtdCho-derived DG. PLD activity may make only a small contribution to DG formation at early time points; the effect of butan-1-ol on DG formation was only examined at 15 min, by which time PLD activity was largely desensitized and increases in Cho*P* were apparent. However, under the labelling conditions used there was a high basal level of [$^3$H]Cho*P*, making small increases in radioactivity a relatively insensitive assay for changes in Cho*P* levels. Wright *et al.* (1990) have also observed EGF-stimulated increases in both Cho and Cho*P*, and also find that PLD plays no role in DG formation at all times in the DG time course in IIC9 cells (Wright *et al.*, 1992). Thus a putative PtdCho-PLC pathway may be more important for EGF-stimulated sustained DG formation.

Bombesin-stimulated PLD activity is preceded by the rapid formation of Ins(1,4,5)$P_3$ and DG; 'down-regulation' of PKC and inhibitors, such as Ro-31-8220, inhibit bombesin- and PMA-stimulated PLD activity, consistent with PLD being regulated by PKC and, in the case of bombesin, dependent on prior inositol-lipid hydrolysis (Cook & Wakelam, 1989, 1991*b*; Cook *et al.*, 1991). In contrast, EGF-stimulated PLD activity was not inhibited by Ro-31-8220 (Fig. 5), consistent with the observation that EGF did not stimulate inositol-lipid hydrolysis. Thus activation of PLD by EGF in Swiss 3T3 cells does not require activation of PKC or prior inositol-lipid hydrolysis. In some cases there was significant enhancement of the EGF response at higher concentrations (10 $\mu$M) of the drug. The EGF receptor is subject to a PKC-dependent phosphorylation of Thr-654 (Hunter *et al.*, 1984), resulting in a shift to a lower-affinity binding form with decreased signalling capacity (Cochet *et al.*, 1984). This can be mimicked in whole cells by agents which activate PKC, e.g. PMA, bombesin and PDGF (Rozengurt, 1986; Wright *et al.*, 1990). Thus inhibition of PKC by Ro-31-8220 could account for the potentiation of EGF-stimulated PLD activity by preventing transmodulation and thereby allowing the response to persist.

The ability of the tyrphostin AG18 (100 $\mu$M) to inhibit EGF-stimulated PLD activity by up to 90% clearly indicates that the response requires a receptor-stimulated tyrosine-phosphorylation event. At 100 $\mu$M-AG18, bombesin-stimulated PLD activity was unaffected, ruling out a non-specific cytotoxic effect of the compound. Thus it is possible that an isoenzyme of PLD may be a substrate for the EGF-receptor tyrosine kinase in a manner analogous to PLC-$\gamma$1 in A431 cells (Meisenhelder *et al.*, 1989), or a PLD-activating component may be regulated by tyrosine phosphorylation.

The function of the EGF-stimulated rise in DG is unclear. The simplest role would be to activate PKC, and this might account for the observed inhibition of sustained S6 kinase activation in PKC-depleted Swiss 3T3 cells stimulated with EGF (Süsa *et al.*, 1989). However, there is considerable debate as to whether EGF is able to activate PKC in Swiss 3T3 cells (Isacke *et al.*, 1986; Rodriguez-Pena & Rozengurt, 1986; Kazlauskas & Cooper, 1988). It is possible that DG produced from PtdCho hydrolysis in the absence of Ins(1,4,5)$P_3$ may activate $Ca^{2+}$-independent forms of PKC such as PKC-$\epsilon$ (Schaap & Parker, 1990) or PKC-L (Bacher *et al.*, 1991). In addition, it is now quite clear that PKC is not the only intercellular phorbol ester/DG receptor (Ahmed *et al.*, 1990); DG may regulate other non-protein kinase targets which play roles in mitogenic competence.

Activation of PLD appears to be a common response to a variety of mitogenic and non-mitogenic stimuli, but, as demonstrated in this paper (Table 1), may not always function as a pathway for DG formation; thus PLD activation, and in particular its product PtdOH, may serve other distinct functions. Various roles have been proposed for PtdOH. In neutrophils the kinetics of PtdOH formation, produced largely by a PLD pathway (Billah *et al.*, 1989; Cockcroft, 1984), correlate with enzyme secretion (Cockcroft, 1984), suggesting a role for PtdOH in membrane fusion and secretion events. The exogenous addition of PtdOH and lyso-PtdOH to fibroblasts results in inositol-lipid hydrolysis, activation of PKC, inhibition of adenylate cyclase and stimulation of DNA synthesis (Moolenaar *et al.*, 1986; Van Corven *et al.*, 1989), suggesting that the two agents may act as growth factors. PtdOH is also able to inhibit p21$^{ras}$ GTPase-activating protein (*ras* GAP) (Tsai *et al.*, 1989) and stimulate a putative *ras* GTPase-inhibiting protein (Tsai *et al.*, 1990) *in vitro*. Also it is reported that PtdOH may activate kinases in a manner analogous to DG (Bocckino *et al.*, 1991). Thus it seems likely that PtdOH may serve a role as a second messenger in its own right, with perhaps diverse intracellular and extracellular targets, as well as, under certain circumstances, being a precursor of DG. Thus EGF appears to stimulate the hydrolysis of PtdCho by both a PLC and a PLD, with the two lipid products, DG and PtdOH, being potentially important in mediating the mitogenic response.

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