Epidermal growth factor stimulates distinct diradylglycerol species generation in Swiss 3T3 fibroblasts: evidence for a potential phosphatidylcholine-specific phospholipase C-catalysed pathway

Trevor R. PETTITT,* Munir ZAQQA and Michael J. O. WAKELAM*†
Molecular Pharmacology Group, Institute of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Stimulation of 3T3 fibroblasts with epidermal growth factor (EGF) results in an increase in 1,2-diacylglycerol (DAG) mass which is maximal at 25 s, declining at 1 min and returning to basal levels by 30 min. No changes in alkylacylglycerol or alkenylacylglycerol were detected. Three species account for most of this mass increase: 18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9. These species are characteristic of the phosphoinositides; however, previous work failed to detect any EGF-stimulated rise in inositol phosphates in these cells [Cook and Wakelam (1992) Biochem. J. **285**, 247–253]. This ruled out phosphoinositide hydrolysis by phospholipase C, but raised the possibility of phospholipase D/phosphatidate phosphohydrolase-catalysed hydrolysis of phosphatidylinositol. The inclusion of butanol in the incubation medium failed to block the diacylglycerol changes, indicating that the phospholipase D pathway is not involved and that DAG must be derived from another source, probably via phospholipase C-catalysed hydrolysis of a phosphatidylcholine pool that is particularly rich in these species. The tyrosine kinase inhibitor ST-271 almost abolished the elevation in 18:0/20:5,*n*−3, 18:0/20:4, *n*−6 and 18:0/20:3,*n*−9 at 25 s, but only reduced the rise in total DAG mass by about 50%. The protein kinase C (PKC) inhibitor Ro-31-8220 increased DAG levels at all time points but had no effect on the species profiles. This provides additional evidence for PKC-mediated regulation of cell-surface EGF receptors, since the inhibition of PKC would increase the availability and/or ligand binding affinity of receptors at the plasma membrane and hence increase and prolong the response to EGF.

INTRODUCTION

One of the earliest events following cell stimulation by a variety of growth factors, hormones and neurotransmitters is the phospholipase C (PLC)-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2), generating the intracellular messengers diradylglycerol (DRG) and inositol 1,4,5-trisphosphate (InsP_3). Subsequently this DRG can activate protein kinase C (PKC), which then directly or indirectly activates further phospholipases, in particular phosphatidylcholine (PtdCho)-specific PLC and phospholipase D (PLD), while down-regulating PtdInsP_2-specific PLC activity [1–7]. This has the effect of creating a biphasic pattern of DRG generation, the first phase of which mirrors InsP_3 generation (usually peaking within 30 s), and a second sustained phase which mirrors phosphocholine (from the PtdCho-PLC pathway) and/or choline [from the PtdCho-PLD/phosphatidate (PtdOH) phosphohydrolase (PLD/PPH) pathway] generation [8–11]. However, not all agonists show this effect, and instead only stimulate a monophasic production of DRG {e.g. thrombin following chymotrypsin pretreatment, insulin, epidermal growth factor (EGF) and insulin-like growth factor [11–14]}. This suggests a separate, distinct transduction pathway for these growth factor molecules.

EGF, a 54-amino-acid polypeptide, binds to a cell-surface receptor with an intrinsic tyrosine kinase activity that is essential for stimulating an intracellular signalling cascade (reviewed in [15,16]). EGF causes a sustained increase in DRG levels, usually reaching a maximum within 5–10 min, before slowly declining back towards basal levels over the next 60 min [11,14,17,18]. In Swiss 3T3 and IIC9 fibroblasts and BC3H-1 myocytes this occurs in the absence of inositol phosphate generation, indicating that in these cells phosphoinositide-specific PLC remains inactive [11,13,14,17,19]. However, in the HeLa and A431 cell lines inositol phosphate generation is activated [18] suggesting significant differences in the EGF-stimulated lipid signalling pathways between different cell types. In Swiss 3T3 and IIC9 cells, EGF stimulates PtdCho hydrolysis, as demonstrated by the activated increase in [^{3}H]choline and [^{3}H]phosphocholine in prelabelled cells [14,17]. The EGF-stimulated DRG response is unaffected by the inclusion of butanol or ethanol in incubations of all these cell lines, indicating the absence of a coupled PLD/PPH DRG-generating pathway under these conditions. However, the rapid accumulation of phosphatidylalcohol demonstrates that a PLD is indeed activated by EGF [14,18,19]. This PLD functions to generate PtdOH which, presumably either directly or as the lyso derivative, has its own signalling role.

To obtain further information on the source and regulation of DRG production in EGF-stimulated Swiss 3T3 mouse fibroblasts, we have examined changes in DRG mass and molecular species profiles at different time points in the presence and absence of butanol, ST-271 (an inhibitor used to block the intrinsic tyrosine kinase activity of the EGF receptor) and Ro-31-8220 [a protein kinase C (PKC) inhibitor]. The results presented here are compared with changes we have observed

Abbreviations used: EGF, epidermal growth factor; DRG, diradylglycerol; DAG, diacylglycerol; PtdOH, phosphatidate; PtdCho, phosphatidylcholine; DNB, 3,5-dinitrobenzoyl (derivative); PLC, phospholipase C; PLD, phospholipase D; PPH, phosphatidate phosphohydrolase; PKC, protein kinase C; HBS, Hanks' buffered saline; PMA, phorbol 12-myristate 13-acetate.

* Present address: University Institute for Cancer Studies, Clinical Studies, Clinical Research Block, Queen Elizabeth Hospital, Birmingham B15 2TH, U.K.
† To whom correspondence should be addressed.

with this cell line when challenged with bombesin [20] and other agents.

MATERIALS AND METHODS

Materials

All solvents were of Analytical or h.p.l.c. grade from Rathburn Chemicals Ltd., Walkerburn, Scotland, U.K. 3,5-Dinitrobenzoyl chloride was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. All water was of deionized quality. Authentic standards were purchased from Sigma Chemical Co., Poole, Dorset, U.K., or Avanti Polar Lipids Inc., Alabaster, AL, U.S.A. Human recombinant EGF was obtained from Gibco BRL, Paisley, Scotland, U.K. The PKC inhibitor Ro-31-8220 was kindly donated by Roche Products (U.K.) Ltd., Welwyn, Herts., U.K., and the tyrosine kinase inhibitor ST-271 was supplied by the Wellcome Foundation, Beckenham, Kent, U.K.

Swiss 3T3 mouse fibroblasts were cultured in 75 cm^2 flasks in Dulbecco's modified Eagle's medium containing glutamine (27 mg/ml), penicillin/streptomycin (250 units/ml and 250 μg/ml respectively), 0.375% (w/v) sodium bicarbonate and 10% (v/v) newborn calf serum at 37 °C in a humidified atmosphere of air/CO_2 (19:1). Upon reaching 90% confluency the medium was replaced with fresh Dulbecco's modified Eagle's medium containing 2% serum and the cells cultured for a further 24 h in order to reach quiescence.

Cell stimulation

Quiescent cultures were washed twice with Hanks' buffered saline (HBS), pH 7.3, pre-incubated in modified HBS [HBS containing 10 mM Hepes, pH 7.3, 10 mM glucose and 1% (w/v) BSA] for 10 min, incubated for a further 15 min in fresh modified HBS (in the presence or absence of inhibitors: 30 mM butanol, 10 μM Ro-31-8220 or 100 μM ST-271) and then stimulated with 100 nM EGF (±inhibitor) in modified HBS for 0 s, 25 s, 1 min, 5 min or 30 min at 37 °C. Optimum concentrations of EGF, butanol, Ro-31-8220 and ST-271 had been determined in previous work [14,21,22]. Incubations were stopped by aspiration of the medium followed by immediate addition of ice-cold methanol. 1,2-12:0/12:0-Diacylglycol (DAG) (1 μg; stock solution; 100 μg/ml in propan-2-ol) was then added as internal standard. Cells were scraped from the flasks, transferred to glass screw-capped tubes and the lipids were extracted using the Folch wash procedure [23]. Briefly, chloroform was added to give a chloroform/methanol ratio of 2:1 (v/v), followed by sufficient 0.88% (w/v) KCl to give a chloroform/methanol/water ratio of 2:1:1 (by vol.). Following vigorous mixing, the phases were allowed to separate; the upper phase was discarded and the lower organic phase was washed with methanol/0.88% (w/v) KCl (1:1, v/v). After drying under a stream of nitrogen, the sample was resuspended in 1 ml of chloroform and stored in a sealed tube at −20 °C until required.

Sample derivatization

Derivatization was based on the modified procedures of Kito and co-workers [24,25] as previously described [20]. Briefly, 0.5 ml of dry pyridine containing 25 mg of 3,5-dinitrobenzoyl chloride was added to each sample; the tube was sealed and heated at 60 °C for 15 min. The reaction was stopped by addition of 2 ml of methanol/water (4:1, v/v) followed by 2 ml of water. This was passed on to a C_{18} Sep-Pak mini-column [Waters-Millipore; previously washed with 10 ml of diethyl ether, 15 ml of methanol and 15 ml of methanol/water (4:1, v/v)], washed with 2 × 15 ml methanol/water (4:1, v/v) and finally eluted with 15 ml of freshly redistilled diethyl ether.

Separation on h.p.l.c.

After drying under nitrogen, the eluant was resuspended in 20 μl of cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.) and the diradyl classes were separated on a Kromasil column (5 μm; 2.1 mm × 250 mm; Hichrom Ltd., Reading, U.K.) using a linear solvent gradient of 90% hexane/cyclohexane/diethyl ether/ethanol (49:49:2:0.1, by vol.) plus 10% cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.), changing to 100% of the second solvent in 30 min at 0.5 ml/min with detection at 250 nm. Each 3,5-dinitrobenzoyl (DNB)-derivatized DRG class was collected, a known amount of 1,2-12:0/12:0-DNB internal standard was added (if required), dried under a stream of nitrogen and resuspended in 20 μl of acetonitrile/propan-2-ol (1:1, v/v). Separation of molecular species was achieved on a Spherisorb S50DS2 column (5 μm; 4.6 mm × 250 mm) using a linear gradient of 100% acetonitrile/propan-2-ol (9:1, v/v) changing to 100% acetonitrile/propan-2-ol (1:1, v/v) in 45 min at 1 ml/min with detection at 250 nm. Identification was by reference to authentic standards and previously obtained relative retention time data [20].

RESULTS AND DISCUSSION

EGF stimulated the rapid elevation of total intracellular 1,2-DAG (but not of 1-alkyl-2-acyl- or 1-alkenyl-2-acyl-glycerol), reaching a maximum of nearly twice the basal level within 25 s, faster than previously reported [11,13,14,18], before declining back to basal by 30 min (Table 1). This increase was concentrated predominantly into three species, 18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9 (Figure 1). This pattern is similar to results we have seen upon stimulation with bombesin [20], prostaglandin $F_{2\alpha}$, phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (T. R. Pettitt, M. Zaqqa and M. J. O. Wakelam, unpublished work) although with these stimulants changes were also detected in certain other (mostly poly-unsaturated) species, probably generated through pathways which remain inactive in response to EGF. The basal profile is slightly different to that seen with our earlier work, probably caused by slight clonal differences together with uncontrollable variability in growth factor and essential fatty acid content between different batches of serum used in cell culture.

18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9 are major species found in PtdIns (see [20]) and probably also in its

Table 1 Changes in total 1,2-DAG mass following stimulation by EGF in the presence and absence of inhibitors

Values are means±S.D. ($n = 6$ in absence of inhibitor and $n = 3$ in presence of inhibitor) and are given relative to basal control values (300–500 pmol of DAG/flask represents 100%). N.D., not determined. *$P < 0.01$ when compared with corresponding stimulation in absence of inhibitor.

	1,2-DAG (% of basal)			
Time	−	+Butanol	+ST-271	+Ro-31-8220
Control	100	96±6	100±5	103±4
25 s	190±12	180±21	153±20*	251±32*
1 min	132±13	117	129	147±27
5 min	95±11	122	N.D.	152±30*
30 min	103±10	94±13	105±14	163±22*

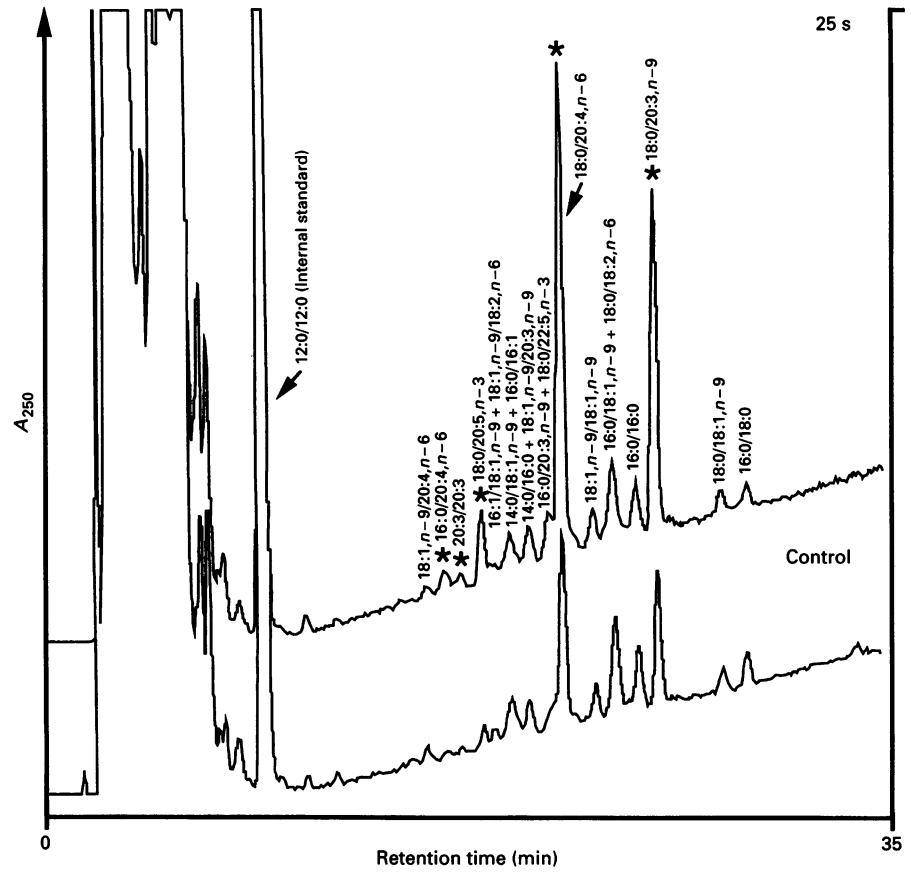

Figure 1 H.p.l.c. of EGF-induced changes in 1,2-DAG profiles

Quiescent Swiss 3T3 mouse fibroblasts were stimulated for 25 s with 100 nM EGF. After stopping the incubation with methanol, the lipids were extracted and derivatized with DNB chloride. The DRG derivatives were then separated on a normal-phase Kromasil column and the individual DAG molecular species were resolved on a reverse-phase Spherisorb S50DS2 column using a gradient of acetonitrile/propan-2-ol (9:1, v/v), changing to 1:1 (v/v) in 45 min at 1 ml/min with detection at 250 nm. *Significantly elevated above basal levels; $P < 0.05$.

polyphosphorylated derivatives (such as PtdInsP_2, which is reported to have a similar profile [26]), suggesting that these lipids may be the source of DAG. This result was surprising since EGF, unlike many other agonists (e.g. bombesin, platelet-derived growth factor, prostaglandin $F_{2\alpha}$, vasopressin), does not cause PLC-catalysed PtdInsP_2 hydrolysis in Swiss 3T3 fibroblasts. Earlier work with this cell line failed to detect any increases in InsP_3 or any other inositol phosphate, ruling out hydrolysis by PLC [14]. However, this raised the possibly that PtdIns was being hydrolysed by PLD, generating PtdOH which was subsequently hydrolysed to DAG by PPH. To examine this, stimulation by EGF was performed in the presence of 30 mM butanol which blocks the PLD/PPH pathway by acting as a better nucleophilic acceptor than water, resulting in the formation of phosphatidylbutanol instead of PtdOH. Phosphatidylbutanol is an unsuitable substrate for PPH, thus preventing DRG formation via this pathway. These experiments showed that butanol had no noticable effect on DAG levels (Table 1) or on DAG species profiles at any of the time points tested (results not shown), indicating that the PLD/PPH pathway makes little or no contribution to EGF-stimulated DRG generation. Our previous work has demonstrated that this PLD/PPH pathway is present in Swiss 3T3 fibroblasts and can be activated by other agonists [21]. Nevertheless, these results are in agreement with previously published data where EGF was found to activate PLD but not PPH [14,19].

Work with IIC9 fibroblasts showed that increased *de novo* synthesis is unlikely to contribute significantly to the EGF-stimulated elevation in DAG levels [17]. Therefore the data are best explained if DAG generation, particularly at 25 s, occurs via a PLC pathway. In the light of previous data demonstrating generation of choline and phosphocholine in EGF-stimulated Swiss 3T3 cells [14], PLC is presumably acting upon PtdCho. However, the substrate lipid must be in a pool with a very restricted species profile, composed predominantly of 18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9, thus closely resembling that of the phosphoinositides. It should be borne in mind that the published phospholipid species profiles are obtained from total cellular lipid and thus the molecular species profile of plasma membrane PtdCho (where early DAG generation is thought to occur) may be substantially different from that of total PtdCho, with the profile of the former possibly resembling that of the DAG species elevated here. We have previously demonstrated that the species elevated in response to EGF are indeed present in total PtdCho [20]. The idea that PtdCho is the source of DRG is further supported by observations that phosphocholine levels increase following EGF stimulation of IIC9 fibroblasts, whereas ethanolamine, inositol and serine metabolite levels remain unchanged [11,17].

A number of other, but in our opinion less likely, explanations would also fit these data. First, the PLC could be highly specific for lipids with stearate at the *sn*−1 position and a C_{20} poly-

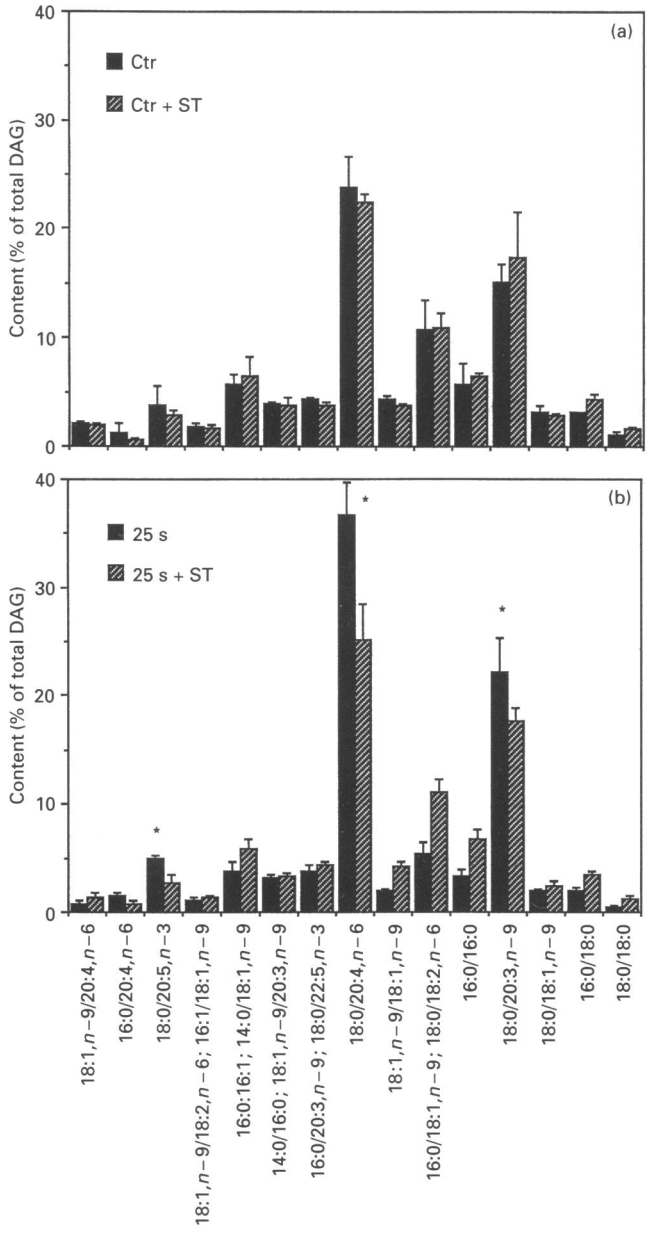

Figure 2 Effect of the tyrosine kinase inhibitor ST-271 on EGF-induced changes in the major 1,2-DAG species

Details were as in Figure 1, except that ST-271 (100 μM) was included in the pre-incubation and incubation media. *Significantly decreased by ST-271; $P < 0.05$. Ctr, control; ST, ST-271. For further details, see the text.

unsaturated fatty acid at the *sn*−2 position. This would generate the observed changes in DAG composition. However, until the relevant phospholipase is isolated and characterized, this hypothesis can only remain speculation. Secondly, the EGF-stimulated hydrolysis could be of a PtdIns-glycan rich in these three molecular species. Support for this idea comes from observations of PtdIns-glycan hydrolysis which seemed to mirror DAG production and show a similar myristate content in BC3H-1 myocytes stimulated with insulin, insulin-like growth factor I or EGF [27,28], although the contribution that this made to total DAG generation was unclear and there is no evidence for such a pathway in Swiss 3T3 cells. A further possibility is that the cells contain a DAG kinase activity selective for C_{20} species which is inhibited by tyrosine phosphorylation. However, no such entity has been demonstrated to date. Schaap et al. [29] have demonstrated that the 80 kDa form of DAG kinase can be phosphorylated on tyrosine residues when the cDNA for the enzyme is co-transfected into COS-7 cells together with that for the EGF receptor. Whether such a phosphorylation has any effect upon enzyme activity is unclear; indeed, it is not clear if such a phosphorylation occurs in a cell expressing physiological levels of the receptor. In addition the 80 kDa form of DAG kinase has not been demonstrated to show any substrate selectivity. Finally, it cannot be completely ruled out that PtdIns hydrolysis is being stimulated, but it would have to be at an extremely low level.

Mutagenesis experiments have shown that the intrinsic tyrosine kinase activity of the EGF receptor is essential for EGF-stimulated cellular responses, although ligand binding is unaffected by loss of kinase function [15]. To confirm that this kinase activity is indeed required for DRG generation, 3T3 fibroblasts were stimulated with EGF in the presence of the tyrosine kinase inhibitor ST-271. These experiments showed that this compound caused a significant difference at 25 s, reducing the rise in total DAG mass by about 50% (Table 1) and almost completely abolishing the elevation of the 18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9 species (Figure 2). This could suggest that approx. 50% of the elevated DAG is made up of these three species, with the remaining proportion being a general increase in DAG levels, i.e. small increases in a large number of species. Incomplete inhibition of the EGF-receptor tyrosine kinase activity, together with a non-specific down-stream effect of ST-271, would then explain the discrepancies seen between data for reductions in total DAG mass and those for individual DAG species. It is possible that the activated EGF receptor can activate further tyrosine kinase activities, only some of which may be ST-271-sensitive. Thus it is possible that there are two EGF-activated DAG-generating pathways, one of which that is very sensitive to ST-271 inhibition of tyrosine kinase (responsible for the generation of 18:0/20:5,*n*−3, 18:0/20:4,*n*−6 and 18:0/20:3,*n*−9 species) and another being rather less sensitive to this inhibitor, generating DAG with a profile largely indistinguishable from the basal pattern (hence only detectable by changes in total DAG mass).

The involvement of PKC in regulating DRG production was investigated by stimulating the fibroblasts with EGF in the presence of Ro-31-8220, a PKC inhibitor. These experiments showed that while Ro-31-8220 had no obvious effect on DAG species profiles (Figure 3), total DAG levels were significantly elevated in the presence of the inhibitor (Table 1). This provides strong supporting evidence for the idea that PKC regulates cell-surface EGF function [30,31]. The EGF receptor is phosphorylated on Thr-654 by PKC [32], causing a reduction in ligand-binding affinity and hence a decrease in signalling capacity [30]. This PKC-mediated phosphorylation acts as a negative feedback mechanism, preventing sustained stimulation by EGF. By blocking this desensitization, more receptors remain in the high-affinity state, hence enhancing and prolonging the response. Since Ro-31-8220 enhanced the DAG response at 25 s, desensitization is clearly very rapid, normally commencing within 25 s. In support of this possibility we have previously observed a similar potentiation of EGF-stimulated PLD activation in Ro-31-8220-treated cells [14].

Thus, in summary, EGF stimulates both PtdCho-specific PLD and probably PtdCho-specific PLC activities in Swiss 3T3 fibroblasts. However, it is only the PtdCho-PLC pathway which is involved in generating DAG and thus presumably activating PKC. The pool of PtdCho hydrolysed by PLC appears to be a

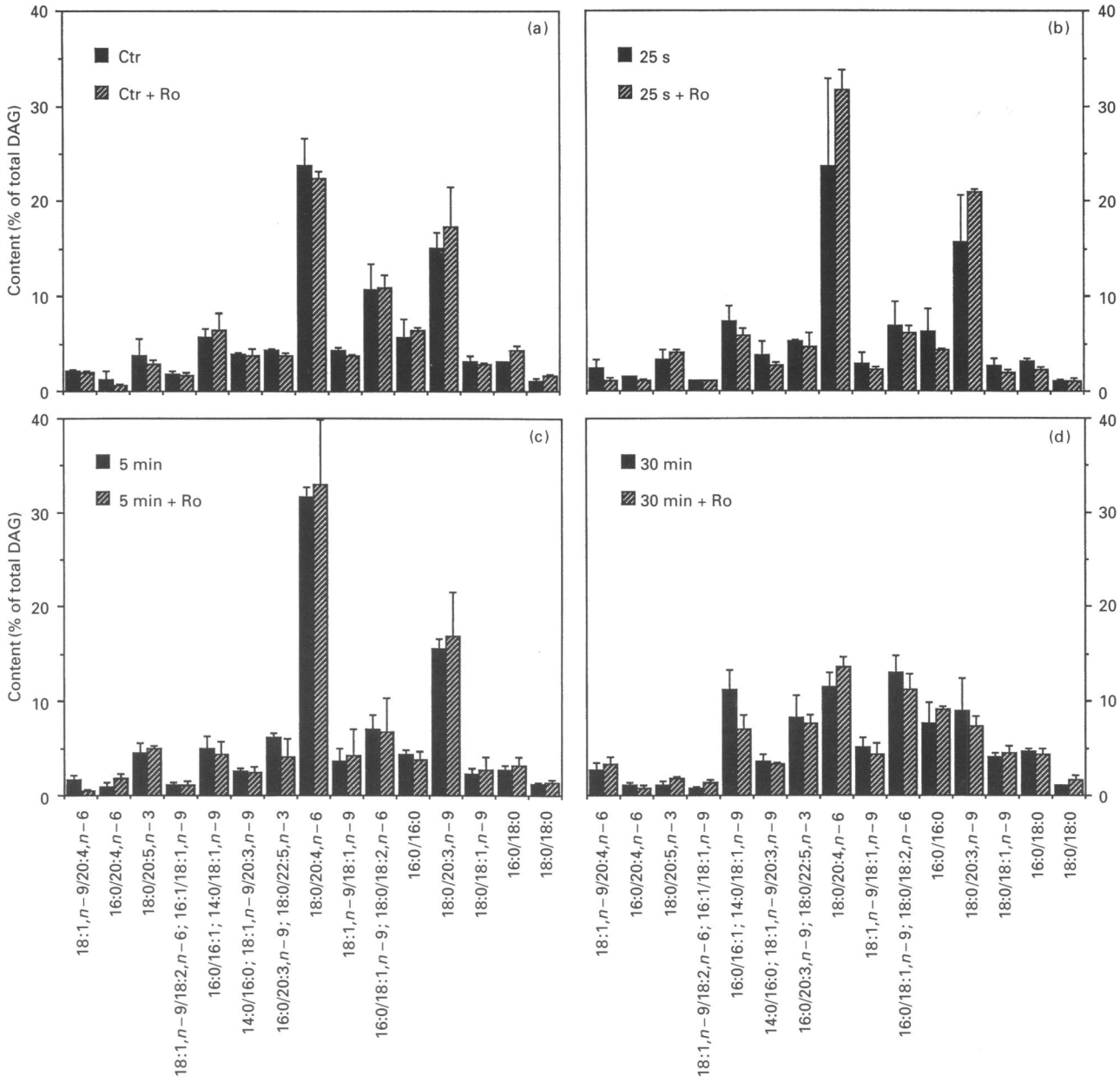

Figure 3 Effect of the PKC inhibitor Ro-31-8220 on EGF-induced changes in the major 1,2-DAG species

Details were as in Figure 1, except that Ro-31-8220 (10 μM) was included in the pre-incubation and incubation media. Ctr, control; Ro, Ro-31-8220. For further details, see the text.

minor fraction of the total cellular PtdCho content and to have an acyl structure more typical of the inositides. These data also raise questions about the correct identification of PtdInsP_2 as the sole early source of DAG in previous work, based on similarities in the kinetics of inositol phosphate generation and on similar molecular species profiles. The work presented here indicates that another hydrolysable phospholipid pool, probably plasma membrane PtdCho, also has a species profile similar to that of early DAG, and thus two PLC-catalysed pathways may be responsible for the rapid generation of DAG. Further work will seek to clarify this situation.

This research was supported by a grant from the Wellcome Foundation.

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Received 5 August 1993/8 October 1993; accepted 12 October 1993