Sustained increase in 1,2-diacylglycerol precedes DNA synthesis in epidermal-growth-factor-stimulated fibroblasts

Evidence for stimulated phosphatidylcholine hydrolysis

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A property common to many growth factors is that they must be present for several hours before the commitment to DNA synthesis and cell division occurs. The intracellular signals that are relevant during this period are poorly defined. We examined the formation of 1,2-diacylglycerol in IIC9 fibroblasts after stimulation with epidermal growth factor (EGF), and found that the mass of this lipid remained elevated for at least four hours. The concentration-dependence of EGF-stimulated 1,2-diacylglycerol production and [³H]thymidine incorporation were similar. Studies of phospholipid metabolism strongly suggested that phosphatidylcholine was the source of the 1,2-diacylglycerol generated in response to EGF. EGF did not stimulate the hydrolysis of other phospholipids, including the phosphoinositides, nor did it increase synthesis *de novo* of 1,2-diacylglycerol. This pattern of sustained 1,2-diacylglycerol formation from phosphatidylcholine may be important in the mitogenic signalling of EGF and potentially other growth factors.

INTRODUCTION

One of the biochemical events implicated in transmitting mitogenic signals from the plasma membrane to the nucleus is the elevation of 1,2-diacylglycerol levels. Evidence from this stems from a number of studies in which the addition of mitogens to a variety of cultured cells results in the elevation of 1,2-diacylglycerol, as determined by either metabolic labelling or mass analysis [1–5]. It has also been shown that the addition of exogenous cell-permeant 1,2-diacylglycerols can stimulate a number of mitogen-induced responses [6], including DNA synthesis in some cells [7].

Early studies suggested that receptor-mediated increases in 1,2-diacylglycerol resulted from a stimulated hydrolysis of phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) (reviewed in [8]). However, the stimulated hydrolysis of phosphoinositides cannot account entirely for the generation of 1,2-diacylglycerol in many systems [1,9–11]. For example, insulin has been shown to increase synthesis *de novo* of 1,2-diacylglycerol [12], and several reports have shown that the stimulation of cells with phorbol esters can cause breakdown of phosphatidylcholine, with a rise in 1,2-diacylglycerol [13–17]. Exton and co-workers have demonstrated the agonist-coupled hydrolysis of phosphatidylcholine in hepatocytes [10,18,19]. Recently, similar findings have been reported in lymphocytes stimulated with interleukin-1 [20] and in other agonist–cell systems [21,22]. However, the relationship between the production of 1,2-diacylglycerol from sources other than phosphoinositides and subsequent cellular responses has not been examined.

We have recently shown that the stimulation of cultured fibroblasts with epidermal growth factor (EGF) causes an increase in the mass of 1,2-diacylglycerol [23]. This elevation occurred in the complete absence of (a) stimulated arachidonic acid release or (b) stimulated hydrolysis of phosphoinositides. The source of this 1,2-diacylglycerol and its role in EGF-stimulated mitogenesis have not been established. We now

present evidence that the EGF-stimulated increase in cellular 1,2-diacylglycerol mass is sustained for at least 4 h. Furthermore, this increase is associated with a specific stimulation of phosphatidylcholine hydrolysis. The time course and EGF concentration-dependence of this hydrolysis suggest that it may play a role in the stimulation of mitogenesis by EGF.

EXPERIMENTAL

Materials

BSA (radioimmunoassay grade, fraction V) and highly purified α -thrombin were purchased from Sigma. Media components were obtained from GIBCO. Plastic culture dishes were purchased from Falcon, except for 96-well plates, which were purchased from Costar. The following radioisotopes were purchased from New England Nuclear: [³H]thymidine, 6.7 Ci/mmol; *myo*-[³H]inositol, 10–20 Ci/mmol; [³H]glycerol, 40 Ci/mmol; [³H]serine, 14 Ci/mmol; [³H]choline, 80 Ci/mmol; and [³H]myristic acid, 22.4 Ci/mmol. [³H]Ethanolamine (30 Ci/mmol) was purchased from Amersham. Hydrofluor counting cocktail was purchased from National Diagnostics. Human transferrin was obtained from Calbiochem. EGF was purified from male mouse submaxillary glands (Pel-Freez) by established procedures [24]. T.l.c. plates were obtained from Analtech (Newark, DE, U.S.A.). Organic solvents were purchased from Burdick–Jackson or Aldrich and were of the highest quality obtainable.

Cells and cell culture

IIC9 cells, a subclone of Chinese-hamster embryo fibroblasts [25,26], were grown, maintained, and serum-starved as previously described [23]. Briefly, cultures were grown and maintained in Minimal Essential Medium (Alpha)/Ham's F-12 medium (1:1, v/v) containing 5% (v/v) fetal-calf serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 2 mM-L-glutamine. Sub-confluent cultures were serum-starved by washing three

Abbreviation used: EGF, epidermal growth factor.

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times with Dulbecco's modified Eagle's Medium containing 1 mg of radioimmunoassay-grade BSA/ml, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 2 mM-L-glutamine and 20 mM-NaHepes, pH 7.4. The cultures were then fed with this medium supplemented with 5 μ g of human transferrin/ml (serum-free medium) and incubated for 2 days at 37 °C.

Assay for [³H]thymidine incorporation

Cells were grown and serum-starved in flat-bottom 96-well tissue-cultured clusters (Costar). Cultures were washed twice and then incubated in serum-free medium with or without EGF at the concentrations indicated in Fig. 2. After incubation at 37 °C for 21 h, cultures were pulsed for 2 h with 2 μ Ci of [³H]thymidine/well and harvested with a PHD Cell Harvester (Cambridge Technology).

Mass analysis of 1,2-diacylglycerol levels

Cellular masses of 1,2-diacylglycerol were determined with *Escherichia coli* diacylglycerol kinase (Lipidex, Westfield, NJ, U.S.A.) as described by Preiss *et al.* [27] and as modified in our laboratory [23]. Lipid P was assayed as described by Ames & Dubin [28].

Metabolic labelling of phospholipid head groups

Phosphoinositides were labelled by serum-starving subconfluent cultures for 2 days in an inositol-free medium containing 5 μ g of human transferrin/ml and 5 μ Ci of *myo*-[³H]-inositol/ml [1]. Phosphatidylcholine (and sphingomyelin) and phosphatidylethanolamine were labelled by incubating cultures in serum-free medium for 2 days as described above, except that the medium contained 5 μ Ci of [³H]choline or [³H]ethanolamine/ml respectively. Phosphatidylserine was labelled by incubating cultures with 5 μ Ci of [³H]serine/ml as described above, except that the serum-free medium was Minimal Essential Medium (Eagle) buffered with 25 mM-NaHepes.

Assay for inositol phosphates

Cultures labelled with *myo*-[³H]inositol were treated with and without EGF, and inositol phosphates were extracted and analysed as previously described [1].

Analysis of [³H]choline, [³H]serine and [³H]ethanolamine-containing phospholipid head groups

Serum-starved cultures were washed twice with serum-free medium (as described above) and equilibrated in this medium at 37 °C for at least 30 min. Cultures then received fresh serum-free medium with or without EGF and were incubated at 37 °C as described in the Figure legends. Incubations were terminated by addition of ice-cold methanol, and the lipids and water-soluble components were separated as described by Bligh & Dyer [29]. The organic phase was dried under N₂, and the aqueous phase was dried under a vacuum in a Speedvac Concentrator (Savant).

The water-soluble head groups in the aqueous phase were separated by t.l.c. The dried aqueous phase was dissolved in 50% (v/v) ethanol. A portion of each sample was spotted on to a 500 μ m-thick silica-gel G plate, which was developed in 0.5% NaCl/methanol/NH₃ (50:50:1, by vol.) as described previously [30]. The products were identified by comparison with known standards. After autoradiography, the radioactive spots were scraped into glass vials, and the amount of radioactivity was determined by liquid-scintillation counting. Choline metabolites were quantified on the basis of the specific radioactivity of cellular phosphatidylcholine (determined as described below).

Metabolic labelling of cells with [³H]myristic acid

Serum-starved cultures were incubated in serum-free medium containing 5 μ Ci of [³H]myristic acid/ml for 2 h at 37 °C. The medium was then aspirated, and the cells were incubated in serum-free medium with or without EGF (50 ng/ml) for 5 min. Incubations were terminated and extractions were performed as described above for the mass analysis of 1,2-diacylglycerol. The organic phases were dried under a stream of N₂ and redissolved in chloroform/methanol (19:1, v/v). A portion of each sample was spotted on a pre-channelled silica-gel HL plate. The plate was developed in isopropyl ether/acetic acid (24:1, v/v), followed by drying and autoradiography. The 1,2-diacylglycerol spots were scraped and the amount of radioactivity was determined by liquid-scintillation counting. Additional samples were spotted on silica-gel HL plates and were analysed by two-dimensional t.l.c. to determine the distribution of radiolabel [31].

Analysis of *de novo* synthesis of 1,2-diacylglycerol

Cells were grown and serum-starved as described above for the [³H]myristic acid studies. Cultures were labelled with 5 μ Ci of [³H]glycerol/ml for 2 h before stimulation. After washing twice with serum-free medium, the cells were incubated in serum-free medium with or without EGF (50 ng/ml) for 5 min. The incubations were terminated and the amount of radiolabelled 1,2-diacylglycerol was determined as described above for cells labelled with [³H]myristic acid.

Determination of phosphatidylcholine specific radioactivity

This was done by dissolving the dried organic phase (from extracts of [³H]choline-labelled cells, above) in 50 μ l of chloroform/methanol (19:1, v/v) and spotting portions of each sample on two silica-gel HL plates. The plates were developed with chloroform/methanol/NH₃ (13:5:1, by vol.). Phosphatidylcholine was detected by autoradiography and identified by comparison with a known standard. One plate was scraped, and the lipid P was analysed as described in ref. [28]. The phosphatidylcholine on the other plate was scraped into a glass scintillation vial and the amount of radioactivity was quantified by liquid-scintillation counting. The specific radioactivity was then calculated as c.p.m./nmol of lipid P. Under these conditions we did not detect any significant change in the specific radioactivity of phosphatidylcholine over a 2 h period in control and stimulated cultures.

Statistical significance was determined by Student's *t* test.

RESULTS

EGF stimulates a sustained increase in 1,2-diacylglycerol mass

We and others have previously demonstrated that EGF is mitogenic for IIC9 cells [23,25]. It is noteworthy that the stimulation of DNA synthesis by EGF requires the prolonged presence of this mitogen [32]. The reason for this requirement is not clear; however, it suggests that EGF may be coupled to a post-receptor signalling mechanism that must be sustained over a relatively long period of time in order for the commitment to DNA synthesis to occur.

In order to evaluate the relationship between the requirement for the prolonged presence of EGF and 1,2-diacylglycerol levels, we measured the masses of diacylglycerols in IIC9 fibroblasts during a prolonged incubation with EGF. Serum-starved cultures were incubated in the presence or absence of a mitogenic concentration of EGF (50 ng/ml). At various times after the addition of EGF, incubations were terminated and masses of diacylglycerols were determined as previously described [23]. As shown in Fig. 1, stimulation with EGF resulted in a substantial increase in cellular 1,2-diacylglycerol mass. Similar to our pre-

vious findings [23], masses of 1,2-diacylglycerol were maximally elevated 5 min after the addition of EGF, and all appear to contain two ester linkages (1,2-diacylglycerol) (M. S. Pessin, J. J. Baldassare & D. M. Raben, unpublished work). The amount of cellular 1,2-diacylglycerol then declined over the next 55 min, but thereafter remained approx. 30–50 % above control levels for several hours. In cells treated for 4 h with EGF (50 ng/ml), the 1,2-diacylglycerol levels were consistently elevated by $27 \pm 6\%$ above control unstimulated cells (mean $\pm$ S.E.M., $n = 4$, $P < 0.05$). After 8 h, the levels of 1,2-diacylglycerol in EGF-stimulated cultures had declined to control unstimulated values (results not shown).

Concentration-dependence of EGF-stimulated DNA synthesis and 1,2-diacylglycerol production

The above results suggest that the increased 1,2-diacylglycerol

Fig. 1. Time course of EGF-stimulated diacylglycerol production

Serum-starved cultures of IIC9 cells were incubated in the absence (□) or presence (■) of EGF (50 ng/ml) at 37 °C. At the indicated times, incubations were terminated and cellular levels of diacylglycerol and organic P were analysed as described in the Experimental section. Data are expressed as nmol of diacylglycerol/100 nmol of lipid P (mol%). Each point represents the average of duplicate samples. Vertical bars represent the range of values. Similar results were obtained in two additional experiments.

levels, because they are sustained, may be related to EGF-stimulated DNA synthesis. In order to examine this relationship further, we determined the concentration-dependence of EGF-stimulated DNA synthesis and compared it with that of EGF-stimulated 1,2-diacylglycerol production.

Serum-starved cultures were incubated in the presence or absence of various concentrations of EGF as indicated in Figs 2(a) and 2(b). The masses of 1,2-diacylglycerol in IIC9 cells were measured (as above) after 5 min incubation at 37 °C. Cells were stimulated under similar conditions, and DNA synthesis was determined by the measurement of [³H]thymidine incorporation as described in the Experimental section. We found that EGF stimulated a concentration-dependent increase in 1,2-diacylglycerol production (Fig. 2a) and DNA synthesis (Fig. 2b). Both responses were maximally stimulated at 10 ng of EGF/ml. The concentration of EGF that produced a half-maximal response of both parameters was 0.1 ng/ml. These data further support the correlation between 1,2-diacylglycerol production and the stimulation of DNA synthesis.

Source of EGF-stimulated 1,2-diacylglycerol

We previously demonstrated that EGF stimulated an increase in 1,2-diacylglycerol in the apparent absence of a stimulated hydrolysis of phosphoinositides [23]. Several other investigators have also found that EGF stimulates mitogenesis without phosphoinositide turnover [33,34]. Two other potential mechanisms of stimulated 1,2-diacylglycerol formation modulated by other agonists are: (a) the stimulation of synthesis *de novo* [12]; and (b) the increased hydrolysis of other phospholipids [19–21,35]. We examined these two mechanisms to determine whether they are involved in the increased 1,2-diacylglycerol mass present in EGF-stimulated cells. We initially chose to study the sources of EGF-stimulated 1,2-diacylglycerol after 5 min of incubation. This is the time at which EGF-stimulated masses of 1,2-diacylglycerol are at a maximum, and presumably the contributions from minor as well as major sources may be evident. Also, since these studies involved metabolically labelled cells, short stimulations would minimize the possibility of missing a source owing to a redistribution of the radiolabel between hormone-sensitive and hormone-insensitive pools.

The stimulation of synthesis *de novo* was assayed as described by Farese *et al.* [12]. Serum-starved cultures were incubated with serum-free medium containing 5 μ Ci of [³H]glycerol/ml for 2 h.

Fig. 2. Concentration-dependence of EGF-stimulated diacylglycerol production and thymidine incorporation

(a) Serum-starved cultures were incubated at 37 °C for 5 min in serum-free medium alone or supplemented with the indicated concentrations of EGF. Incubations were terminated and diacylglycerol levels were analysed as described in the Experimental section. Data are expressed as the percentage increase in diacylglycerol in EGF-stimulated cultures above control values. Each point represents the mean $\pm$ S.E.M. ($n \geq 3$). (b) Serum-starved cultures were washed twice and then received serum-free medium alone or supplemented with the indicated concentrations of EGF. The amount of [³H]thymidine incorporated into DNA during a 2 h pulse (21–23 h after addition of EGF) was analysed as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. ($n = 6$).

The dishes were then washed and incubated at 37 °C with serum-free medium or medium supplemented with either 50 ng of EGF/ml or 5% fetal-calf serum. The incubations were terminated after 5 min, the time at which EGF maximally elevated 1,2-diacylglycerol levels (Fig. 1). The lipids were extracted and separated by t.l.c. as described in the Experimental section. The amount of radiolabel incorporated into 1,2-diacylglycerol was quantified by liquid-scintillation counting. As shown in Table 1, although serum stimulated a 2.4-fold increase in labelled diacylglycerol, EGF failed to stimulate an increase in the amount of label incorporated into this lipid. These data indicate that an increase in synthesis *de novo* does not contribute significantly to the elevation of 1,2-diacylglycerol levels observed in response to EGF.

Table 1. Effect of EGF and serum on synthesis of diacylglycerol *de novo*

Serum-starved cultures were incubated in serum-free medium containing 5 μ Ci of [3 H]glycerol/ml for 2 h at 37 °C. Cultures were then incubated for 5 min at 37 °C in fresh serum-free medium with or without EGF (50 ng/ml) or 5% fetal-calf serum. Incubations were terminated and the amount of [3 H]diacylglycerol was analysed as described in the Experimental section. The data represent means $\pm$ S.E.M. of two experiments performed in duplicate.

Addition	[3 H]Diacylglycerol (c.p.m./dish)
None	573 $\pm$ 65
EGF	514 $\pm$ 22
Serum	1400 $\pm$ 16

Several reports have suggested that some stimulated diacylglycerols are derived from the hydrolysis of non-inositol-containing phospholipids (e.g. phosphatidylcholine) in a manner which results in the generation of water-soluble head groups and 1,2-diacylglycerols [13–16,19–21]. In order to investigate phospholipid hydrolysis as a potential source of stimulated diacylglycerols in EGF-stimulated cells, cultures were serum-starved and metabolically labelled for 48 h with [3 H]inositol, [3 H]choline, [3 H]serine, or [3 H]ethanolamine as described in the Experimental section. The cultures were then incubated in serum-free medium in the presence or absence of EGF (50 ng/ml) at 37 °C, and the generation of water-soluble products was analysed. As shown in Table 2, EGF stimulated an increase in intracellular as well as extracellular [3 H]choline metabolites. In contrast, EGF did not cause significant changes in the water-soluble head group products in cells prelabelled with inositol, serine, or ethanolamine.

The choline metabolites released in response to EGF may have been derived from the hydrolysis of phosphatidylcholine and/or sphingomyelin. To examine the latter possibility, we quantified the amount of sphingomyelin in cells labelled with either [3 H]choline (which labels the head group of sphingomyelin) or [3 H]serine (which labels the backbone of sphingomyelin). After 5 min with or without EGF (50 ng/ml) the cellular lipids were extracted and the distribution of radiolabel was determined by t.l.c. as described in the Experimental section. We found no difference in labelled sphingomyelin in control or EGF-stimulated cells (results not shown). These data suggest that sphingomyelin is not a major source of the EGF-stimulated [3 H]choline metabolites.

Table 2. Release of 3 H-labelled water-soluble metabolites

Cultures were serum-starved and labelled with [3 H]serine, [3 H]ethanolamine, [3 H]choline or *myo*-[3 H]inositol for 48 h. Cultures were then incubated in the absence or presence of EGF (50 ng/ml) for 5 min at 37 °C. The medium was removed and incubations were terminated by the addition of cold methanol. The amount of water-soluble radioactivity associated with the cells and released into the medium was analysed as described in the Experimental section. The data represent means $\pm$ S.E.M. of at least two experiments performed in duplicate. Numbers in parentheses indicate the percentages of control values. * $P < 0.05$; ** $P < 0.001$; other values are not statistically significantly different from control. N.D., not determined.

	Intracellular metabolites (c.p.m.)		Metabolites in medium (c.p.m.)	
	Control	EGF	Control	EGF
Serine	14 574 $\pm$ 693	14 194 $\pm$ 948 (97)	12 508 $\pm$ 1587	13 086 $\pm$ 1046 (105)
Ethanolamine	50 2836 $\pm$ 26071	42 3518 $\pm$ 29 444 (84)	20 698 $\pm$ 2004	22 732 $\pm$ 3371 (110)
Choline	8123 $\pm$ 398	10 760 $\pm$ 806 (132)*	6069 $\pm$ 365	8126 $\pm$ 468 (122)**
Inositol phosphates	20 775 $\pm$ 402	19 538 $\pm$ 966 (94)	N.D.	N.D.

Fig. 3. EGF-stimulated release of total choline metabolites

Cultures were serum-starved and labelled with 5 μ Ci of [3 H]choline/ml for 48 h. The cells were then washed and incubated in fresh serum-free medium alone or supplemented with EGF (50 ng/ml) at 37 °C. At the indicated times, medium was removed, and the amount of cell-associated water-soluble choline metabolites (a) and the amount of metabolites released into the medium (b) were analysed as described in the Experimental section. Each point represents the specific release of choline metabolites in response to EGF (stimulated – control). The data represent means of two experiments each performed in duplicate.

Fig. 4. EGF-stimulated release of choline and phosphocholine

Cultures were labelled with 5 μ Ci of [3 H]choline/ml for 48 h and incubated for various times at 37 $^{\circ}$ C in the absence or presence of EGF (50 ng/ml) as described in the legend to Fig. 3. The water-soluble products, [3 H]choline (a) and [3 H]phosphocholine (b), were isolated by t.l.c. and the amount of radioactivity was determined by liquid-scintillation counting. The amount of each metabolite was quantified based on the specific radioactivity of phosphatidylcholine determined as described in the Experimental section. The data represent means of two experiments each performed in duplicate. All stimulated values were significantly increased above control values ($P < 0.05$). The levels of choline and phosphocholine in quiescent unstimulated cells were 110 ± 7 and 104 ± 6 pmol/dish (mean $\pm$ S.E.M., $n = 4$) respectively.

It has recently been reported that under short-term labelling conditions phosphatidylcholine is preferentially labelled with [3 H]myristic acid [12,17]. We utilized this method to assess further whether the hydrolysis of phosphatidylcholine was responsible for the elevation of 1,2-diacylglycerol mass. Consistent with previous reports, in cells labelled for 2 h with 5 μ Ci of [3 H]myristic acid/ml, phosphatidylcholine represented $\geq 90\%$ of the radiolabel incorporated into phospholipids, without appreciable incorporation into other phospholipids, including phosphatidylinositol-glycans ([12], and results not shown). In [3 H]myristic acid-labelled cells, EGF stimulated a $24 \pm 3\%$ rise in [3 H]1,2-diacylglycerol above control values at 5 min (mean $\pm$ S.E.M., $n = 4$, $P < 0.001$). Together with the results of the [3 H]choline-labelling experiments, these data indicate that phosphatidylcholine is a major source of the EGF-stimulated 1,2-diacylglycerol. It remains possible that additional sources of 1,2-diacylglycerol may contribute to the later sustained elevation. However, as shown below, the hydrolysis of phosphatidylcholine exceeds the increase in the amount of 1,2-diacylglycerol for at least 2 h.

Analysis of EGF-stimulated choline metabolites

The choline metabolites generated in response to EGF were analysed by t.l.c. as previously described [30]. Analysis of intracellular [3 H]choline-labelled water-soluble products revealed that choline and phosphocholine were the only metabolites to change as a result of EGF stimulation. We detected no changes in the amount of labelled CDP-choline or glycerophosphocholine (results not shown).

To evaluate further the relationship between EGF-stimulated 1,2-diacylglycerol production and phosphatidylcholine hydrolysis, we examined the kinetics of EGF-stimulated release of choline metabolites. The addition of EGF to quiescent cultures resulted in an increase in intracellular and extracellular choline metabolites for at least 2 h (Fig. 3). The stimulated level of intracellular choline metabolites reached a peak at 30 min and declined to an elevated steady-state level between 60 min and 2 h (Fig. 3a). EGF stimulated an increase in the release of choline metabolites into the medium that was linear for the first 60 min and which continued for at least 2 h (Fig. 3b). As shown in Fig. 4(a), after the addition of EGF to serum-starved cultures, intracellular free choline was maximally increased after incubation for 5 min and then began to decline toward control levels.

Fig. 5. Time course of EGF-stimulated phosphatidylcholine hydrolysis and diacylglycerol production

Serum-starved cultures were incubated in the absence or presence of EGF (50 ng/ml) for the indicated times. The amount of phosphatidylcholine hydrolysed (○) was calculated based on the total (intracellular and medium) stimulated release of choline metabolites and the specific radioactivity of phosphatidylcholine at each time point. Diacylglycerol production (●) was analysed as described in the legend to Fig. 1 and is expressed as the amount of diacylglycerol produced in response to EGF (stimulated – control).

In contrast, intracellular phosphocholine continued to increase until it was maximally elevated 30 min after the addition of EGF, followed by a gradual decline (Fig. 4b). Analysis of the extracellular metabolites revealed that [3 H]choline was the only product released at 15 min; however, after 2 h incubation with EGF, a small amount of stimulated phosphocholine release into the medium was observed (13% of released radioactivity at 2 h).

Comparison of EGF-stimulated 1,2-diacylglycerol production and phosphatidylcholine hydrolysis

The data indicate that EGF stimulates an elevation of 1,2-diacylglycerol mass and an increase in phosphatidylcholine hydrolysis. We calculated the amount of phosphatidylcholine hydrolysed, based on the generation of choline metabolites and

the specific radioactivity of cellular phosphatidylcholine (Fig. 5). It should be noted that these values represent a minimum amount of phosphatidylcholine hydrolysed, since it is not possible to account for the re-incorporation of the head-group products into phosphatidylcholine. Shown also in Fig. 5 is the net increase in 1,2-diacylglycerol in cells stimulated with EGF. After 5 min of stimulation, the estimated amount of phosphatidylcholine hydrolysed and the amount of 1,2-diacylglycerol produced were similar (120 pmol/dish). Further incubation resulted in the continued hydrolysis of phosphatidylcholine, whereas 1,2-diacylglycerol levels gradually declined and then remained at a plateau for hours. These findings suggest that, in addition to the regulation of 1,2-diacylglycerol production from phosphatidylcholine, there may also be regulation of 1,2-diacylglycerol metabolism in EGF-stimulated cells.

DISCUSSION

Knowledge of the mechanism(s) by which cell growth is regulated is of prime importance to our understanding of biological processes such as embryogenesis, wound healing and neoplasia. A significant effort has therefore been directed toward discerning the mechanisms by which growth factors modulate cell division. It is becoming increasingly clear that the intracellular signalling in response to growth factors is complex: some mitogens stimulate a number of pathways simultaneously (reviewed in [36]), and the signalling mechanisms utilized are sometimes cell-type dependent. With regard to EGF, there is recent evidence that the tyrosine kinase activity of the EGF receptor may be involved in mitogenic signalling [37]. It has also been shown that stimulation with EGF, similar to a number of other mitogens, leads to protein kinase C-mediated phosphorylation [38]. In a recent study, Susa *et al.* [39] reported that EGF stimulated the prolonged activation of S6 kinase that was dependent on protein kinase C. In the present paper we have demonstrated the sustained increase in 1,2-diacylglycerol in EGF-stimulated fibroblasts and have examined its potential relationship to the stimulation of DNA synthesis. In addition, we have investigated the source of the 1,2-diacylglycerol generated in response to EGF.

It is known that for EGF, as well as other growth factors, the prolonged presence of the agonist is required for the commitment to DNA synthesis [32,40]. It is therefore likely that the second messenger(s) generated in response to these agonists is (are) sustained. In keeping with this premise, we have found that EGF induces a sustained elevation of 1,2-diacylglycerol mass in IIC9 fibroblasts that persists for at least 4 h (Fig. 1). Furthermore, α -thrombin, which also stimulates DNA synthesis in these cells, similarly stimulates a sustained increase in 1,2-diacylglycerol (L. A. Rangan, T. M. Wright & D. M. Raben, unpublished work). These data support the hypothesis that one of the relevant signals leading to stimulated DNA synthesis in response to mitogens is the sustained elevation of cellular 1,2-diacylglycerol. Additional evidence in favour of this hypothesis is our finding that EGF stimulated DNA synthesis and the elevation of 1,2-diacylglycerol mass with similar concentration dependencies ($ED_{50} = 0.1$ ng/ml) (Fig. 2). These results are at present only correlative, and additional studies are necessary to define further the relationship between the generation of 1,2-diacylglycerol and subsequent cellular events such as DNA synthesis.

In order to gain insight into the mechanism(s) by which EGF stimulates an increase in 1,2-diacylglycerol in fibroblasts, we examined the potential source(s) of this lipid. Consistent with other studies of cells which respond mitogenically to EGF [33,34], we did not find a stimulated hydrolysis of the phosphoinositides ([23], and Table 2). Examination of other potential

phospholipid sources revealed that EGF stimulated the hydrolysis of phosphatidylcholine, as evidenced by the stimulated release of choline and phosphocholine (Fig. 4) and the generation of 1,2- $[^3H]$ diacylglycerol from cells which had been acutely labelled with $[^3H]$ myristic acid. We did not detect the stimulated hydrolysis of phosphatidylethanolamine or phosphatidylserine in response to EGF (Table 2). Similar to the formation of 1,2-diacylglycerol, the generation of choline and phosphocholine continued for at least 2 h (Figs. 3 and 4). These results provide an explanation for the previously reported findings that EGF stimulation results in activation of protein kinase C [38,39] in the apparent absence of phosphoinositide turnover [23,33,34].

The enzymes involved in the EGF-stimulated hydrolysis of phosphatidylcholine in IIC9 fibroblasts have not as yet been identified. Our data suggest two possible pathways: (A) the activation of phospholipase D, generating choline and phosphatidic acid, followed by the hydrolysis of phosphatidic acid to 1,2-diacylglycerol by phosphatidate phosphohydrolase; and (B) the activation of a phosphatidylcholine-specific phospholipase C, generating phosphocholine and 1,2-diacylglycerol. A possible explanation for our observation that choline release precedes the release of phosphocholine is that choline, generated via pathway (A), may be subsequently phosphorylated to phosphocholine. Alternatively, phosphocholine generated via pathway (B) may be rapidly metabolized until the rate of phosphocholine generation exceeds the rate of metabolism. Preliminary results from our laboratory indicate that EGF stimulates a phospholipase D-mediated hydrolysis of phosphatidylcholine (pathway A).

The mechanism(s) by which the prolonged generation of second messengers (e.g. 1,2-diacylglycerol) affects subsequent cellular processes such as DNA synthesis is (are) unknown. It has recently been appreciated that, after stimulation of quiescent cells with growth factors, there is a sequential expression of many genes [41–45]. The initial phase of 'immediate early' transcription occurs in the absence of new protein synthesis [46,47], and presumably results from the distal effects of receptor-mediated signalling on pre-existent transcription-regulatory factors. Some of the immediate early genes have been found to encode transcription factors [48–50] whose effects *in vivo* may be dependent on post-translational modification resulting from continued transmembrane signalling. Less is known about the identity and function of many proteins expressed later in the course of the cellular response to growth factors. Sustained second-messenger generation may be important in the functional regulation of these proteins.

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