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Epidermal Growth Factor-Induced Hydrolysis of Phosphatidylcholine by Phospholipase D and Phospholipase C in Human Dermal Fibroblasts

GARY J. FISHER,* PATRICIA A. HENDERSON, JOHN J. VOORHEES, AND JOSEPH J. BALDASSARE

Department of Dermatology, University of Michigan Medical Center, Ann Arbor, Michigan (G.J.F., J.J.V.) American Red Cross, St. Louis, Missouri (P.A.H., J.J.B.)

The enzymatic pathways for formation of 1,2-diradylglyceride in response to epidermal growth factor in human dermal fibroblasts have been investigated. 1,2-Diradylglyceride mass was elevated 2-fold within one minute of addition of EGF. Maximal accumulation (4-fold) occurred at 5 minutes. Since both diacyl and ether-linked diglyceride species occur naturally and may accumulate following agonist activation, we developed a novel method to determine separately the alterations in diacyl and ether-linked diglycerides following stimulation of fibroblasts with EGF. Utilizing this method, it was found that approximately 80% of the total cellular 1,2-diradylglyceride was diacyl, the remaining 20% being ether-linked. Addition of EGF caused accumulation of 1,2-diacylglyceride without alteration in the level of ether-linked diglyceride. Thus, the observed induction of 1,2-diradylglyceride by EGF was due exclusively to increased formation of 1,2-diacylglyceride. In cells labelled with [^{3}H]choline, the water soluble phosphatidylcholine hydrolysis products, phosphorylcholine and choline, were increased 2-fold within 5 minutes of addition of EGF. No hydrolysis of phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol was observed. Quantitation by radiolabel and mass revealed equivalent elevations in phosphorylcholine and choline, suggesting stimulation of both phospholipase C and phospholipase D activities. To identify the presence of EGF-induced phospholipase D activity, cells were labelled with exogenous [^{3}H]1-O-hexadecyl, 2-acyl phosphatidylcholine and its conversion to phosphatidic acid in response to EGF determined. Radiolabelled phosphatidic acid was detectable in 15 seconds after addition of EGF and was maximal (3-fold) at 30 seconds. Consistent with the presence of EGF-induced phospholipase D activity, treatment of cells with EGF, in the presence of [^{14}C]ethanol, resulted in the rapid formation of [^{14}C]phosphatidylethanol, the product of phospholipase D-catalyzed transphosphatidylation. The formation of phosphatidylethanol, which competes for the formation of phosphatidic acid by phospholipase D, did not diminish the induction of 1,2-diglyceride by EGF. These data suggest that the phosphatidic acid formed by phospholipase D-catalyzed hydrolysis of phosphatidylcholine is not a major precursor of the observed increased 1,2-diglyceride. Thus, the induction of 1,2-diacylglycerol by EGF may occur primarily via phospholipase C-catalyzed hydrolysis of phosphatidylcholine.

Cellular 1,2-diradylglycerides are composed of a heterogeneous mixture of molecular species, containing carbon side chains of varying lengths and degrees of unsaturation linked by an ester bond to the sn-2 position, and by either an ester (diacyl) or ether (ether-linked) bond to the sn-1 position of glycerol. Recent interest in 1,2-diradylglycerides has focused on their role as second messengers involved in transducing extracellular signals across the cell surface membrane. 1,2-Diacylglycerol is the physiological activator of protein kinase C, which is involved in the regulation of a diverse number of cellular functions (Berridge et al., 1985; Hannun and Bell, 1987). Under appropriate in vitro conditions, naturally occurring ether-linked diglycerides are also able to activate protein kinase C (Ford et al., 1989), although whether they do so in vivo is not known. Agonist-induced accumulation of 1,2-diradylglycerides may arise from phospholipase-catalyzed breakdown of both diacyl and ether-linked species

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*To whom reprint requests/correspondence should be addressed.

Patricia A. Henderson and Joseph J. Baldassare are now at Department of Internal Medicine, School of Medicine, St. Louis University, St. Louis, MO.

of phosphatidylinositol, phosphatidylcholine, or phosphatidylethanolamine (Rider et al., 1988; Kiss and Anderson, 1989; Price et al., 1989; Matozaki and Williams, 1989). Hydrolysis of these phospholipids by phospholipase C directly yields 1,2-diradylglycerides. Alternatively, 1,2-diradylglycerides may be generated by the combined actions of phospholipase D and phosphatidic acid phosphohydrolyase (Cabot et al., 1988; Bocckino et al., 1987a; Nishijima et al., 1989). 1,2-diradylglyceride levels in agonist-stimulated cells may also be elevated as a result of de novo synthesis (Farese et al., 1987). Thus there are complex multiple pathways leading to enhanced formation of 1,2-diradylglycerides in agonist-stimulated cells. In view of the important regulatory role of 1,2-diradylglycerides, the enzymatic pathways that regulate their formation are of great interest.

Epidermal growth factor (EGF) is a potent polypeptide mitogen for a wide variety of cell types, including human dermal fibroblasts. The actions of EGF are primarily mediated through its specific cell surface receptor. The EGF receptor possesses intrinsic tyrosine kinase activity, which is stimulated by EGF and required for biological responses to EGF (reviewed in Schlessinger, 1988; Gill et al., 1988). Evidence suggests that activation of the EGF receptor results in rapid stimulation of membrane phospholipid turnover, presumably through modulation of phospholipase activities (Wright et al., 1988). Phosphoinositide-specific phospholipase C-γ has been demonstrated to associate with the EGF receptor and to be phosphorylated by the EGF receptor tyrosine kinase, although the functional consequences of this are not clear (Wahl et al., 1989; Nishibe et al., 1989). In transformed human epithelial cell lines, which overexpress EGF receptors, EGF stimulates phospholipase C-catalyzed phosphoinositide turnover, elevates 1,2-diglyceride content, and activates protein kinase C (Hirai and Shimizu, 1989; Pike and Eakes, 1987; Wahl and Carpenter, 1988). In IIC9 hamster embryo fibroblasts, EGF does not stimulate phosphoinositide breakdown, but does stimulate phosphatidylcholine hydrolysis and accumulation of 1,2-diradylglyceride (Wright et al., 1988). These data suggest that EGF may influence the formation of 1,2-diradylglyceride via several different mechanisms.

To delineate those mechanisms through which EGF regulates 1,2-diglyceride formation in human dermal fibroblasts, we have investigated the kinetics, phospholipid sources, and enzymatic pathways for the formation of 1,2-diacyl and ether-linked glycerides. The data indicate that: 1) EGF stimulates accumulation of 1,2-diacylglyceride, but not ether-linked diglycerides, 2) EGF-induced 1,2-diacylglyceride is formed from phosphatidylcholine not phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol, 3) EGF stimulates phospholipase D-catalyzed formation of phosphatidic acid and phosphatidylethanol from phosphatidylcholine and 4) the major route for EGF-induced 1,2-diacylglyceride formation appears not to be via phospholipase D, but presumably via phospholipase C-catalyzed phosphatidylcholine hydrolysis.

MATERIALS AND METHODS

[^{32}P]ATP, [^{3}H]acetic anhydride, [^{3}H]choline, [^{3}H]inositol, [^{3}H]ethanolamine, [^{3}H]serine, [^{14}C]ethanol, [^{3}H]-0-hexadecyl, 2-hydroxy phosphatidylcholine, and [^{3}H]glycerol were obtained from New England Nuclear (Boston, MA). Purified mouse salivary gland EGF was a generous gift of Daniel Rabin, Johns Hopkins University. Choline, phosphorylcholine, phospholipase C from B. Cereus, 1-0-hexadecyl, 2-oleoyl phosphatidylcholine, choline kinase, and alkaline phosphatase were from Sigma Chemical Co. (St. Louis, MO). Phospholipids and 1,2-dioleoylglycerol were from Avanti Biochemicals (Alabaster, AL). Bovine ethanolamine plasmalogen was from Serdary (Port Huron, MI). All other chemicals were of at least reagent grade.

Cells and cell culture

Human dermal fibroblasts were cultured from keratome biopsies of adult human skin. Dermis was separated from epidermis by trypsinization (0.25% trypsin, 0.1% EDTA) of the tissue for 30 minutes at 37°C in phosphate buffered saline. Once separated the dermis was cut into small pieces (4mm on each side) and placed in a 100mm dish in a minimal amount of media (McCoy's modified 5A containing 10% fetal bovine serum) to prevent the tissue from floating. The tissue was removed after one week, at which time cells were observed to have migrated out of the tissue. When these primary cultures were confluent they were expanded by passage. Cells were used for experiments between passages 3 and 6.

Metabolic labelling of dermal fibroblasts

Subconfluent cultures in 100mm dishes were placed in serum free media and labelled with [^{3}H]choline (5μCi/ml), [^{3}H]ethanolamine (5μCi/ml), [^{3}H]serine (5μCi/ml), or [^{3}H]inositol (5μCi/ml) for two days or with [^{3}H]glycerol (1mCi/ml) for two hours. For incorporation of [^{14}C]ethanol into phosphatidylethanol, [^{14}C]ethanol (2μCi/ml) was added to cells 5 minutes prior to addition of EGF. With the exception of inositol, cells were labelled in McCoy's modified 5A media containing 5μg/ml bovine serum albumin. Cells were labelled with [^{3}H]inositol in inositol free Delbecco's modified Eagle's medium. To measure the conversion of phosphatidylcholine to phosphatidic acid cells were incubated with [^{3}H]1-0-hexadecyl, 2-hydroxy phosphatidylcholine (1μCi/ml) for two hours. Following this, cells were washed to remove unincorporated label, placed in fresh serum free media, and treated with EGF for the indicated times.

Treatment of cells and analysis of labelled metabolites

Cultures in serum free media were washed with fresh media and incubated at 37°C for 20 minutes. Cultures were then given fresh media with or without EGF (50ng/ml) and incubated for the times indicated in the text. Incubations were terminated by addition of ice cold methanol (2ml), following aspiration of the media and washing with 2ml phosphate buffered saline (PBS). The cells were scraped from the dish, and the dish was washed with an additional 2ml methanol. The combined methanol fractions were extracted according to the method of Bligh and Dyer (1959). The chloroform and aqueous phases were separated and dried under nitrogen and vacuum, respectively. To analyze incorporation of radioactivity into endogenous phospholipids

the chloroform fraction was subjected to thin layer chromatography (silica gel 60) in chloroform: methanol: 20% methylamine (60:36:10). Metabolism of exogenous [^{3}H]1-0-hexadecyl, 2-hydroxy phosphatidylcholine was analyzed by thin layer chromatography developed in chloroform:methanol:acetic acid (65:30:4). Incorporation of [^{3}H]ethanol into phosphatidylethanol, was determined by thin layer chromatography of the chloroform extract in iso-octane:ethylacetate:acetic acid:water (110:50:20:100) (Pai et al., 1988). Spots were visualized by iodine vapor, identified by co-chromatography with standards, scraped, and counted. [^{3}H]Inositol phosphates in the aqueous fraction were analyzed by Dowex ion exchange chromatography as previously described (Downes et al., 1982). Water soluble metabolites of [^{3}H]ethanolamine and [^{3}H]choline were resuspended in 50% methanol and analyzed by thin layer chromatography according to the methods of Lacal et al. (1987) and Yavin (1985), respectively.

Measurement of choline and phosphorylcholine content and choline kinase activity

Choline and phosphorylcholine content was determined by modifications of the procedures described by Haubrich et al. (1981) and McCaman and Stetzler (1977). Briefly, the aqueous cellular extract from four 100mm dishes was divided into two aliquots and dried under vacuum. For the measurement of choline, one aliquot was redissolved in buffer containing 6×10^5 cpm [^{32}P]ATP (0.35 mM) and 20 units/ml choline kinase. The mixture was incubated at 37°C for one hour and then 100mM phosphorylcholine was added as carrier. [^{32}P]Phosphorylcholine was quantitated by counting following separation from [^{32}P]ATP by Dowex chromatography. For the measurement of phosphorylcholine plus choline, the remaining aliquot was dissolved in buffer at pH 9.2 and treated with alkaline phosphatase (5 units) at 37°C for four hours to convert phosphorylcholine to choline. Following this, alkaline phosphatase was inactivated by boiling the sample for 15 minutes. The pH of the reaction mixture was adjusted to 7.0 with Tris maleate (200mM, pH 6.0) and choline determined as described above. The amount of phosphorylcholine in the original sample was calculated as the difference between the values for choline obtained with and without pretreatment of the sample with alkaline phosphatase.

For the measurement of choline kinase activity, cells in serum-free media were treated with EGF (50ng/ml) for the times indicated in the text and collected by scraping in 50mM tris (pH 8.0). The cells were lysed by brief sonication and a high speed supernatant prepared by contrifugation (100,000 × g for one hour). Choline kinase activity in the supernatant was assayed as described (Weinhold and Rethy, 1974).

Measurement of 1,2-diradylglyceride

Dermal fibroblasts were treated with EGF (50ng/ml) at 37°C for the times indicated in the text. Incubations were terminated by addition of ice cold methanol. 1,2-Diradylglycerides were extracted with chloroform and quantified by conversion to [^{32}P]phosphatidic acid with E. coli diglyceride kinase, according to the procedure of Preiss et al. (1987).

Measurement of 1,2-diacyl- and 1-alkyl, 2-acyl-diglyceride

Alterations in the amounts of 1,2-diacyl and 1-ether, 2-acyl-glycerides in dermal fibroblasts following treatment with EGF were determined by conversion of 1,2-diradylglycerides to radiolabelled 3-acetate derivatives with [^{3}H]acetic anhydride and resolution of the diacyl and ether-linked derivatives by thin layer chromatography (Renkonen, 1966). 1,2-Diradylglycerides were extracted from fibroblast cultures as described above and purified by thin layer chromatography on silica gel 60 developed in benzene:chloroform:methanol (80:15:5). Cellular 1,2-diradylglycerides and standards were dried under a stream of nitrogen and dissolved in 30μl benzene containing 25μg dimethlyaminopyridine. To each sample, 25μCi [^{3}H]acetic anhydride (10μl) and 100μmol unlabeled acetic anhydride (10μl) were added. This was allowed to react for 20 hours at 25°C. Methanol (250μl) was added to destroy excess acetic anhydride and the [^{3}H]diglyceride derivatives extracted by addition of 750μl chloroform/H_2O (2:1). The chloroform extracts were dried and spotted on silica gel 60 TLC plates (Whatman LK6), which were developed in toluene:ether (96:4). 1,2-diacylglycerol acetate (R_f 0.28), 1-alkyl, 2-acyl glycerol acetate (R_f 0.41) and 1-alk-enyl, 2-acyl glycerol acetate (R_f 0.54) were visualized by iodine vapor, identified by comparison to standards, scraped, and counted. The standards utilized were 1,2-dioleoylglycerol, 1-0-hexadecyl, 2-oleoylglycerol, and 1-alk-enyl, 2-acyl glycerol. The later two were prepared by phospholipase C (B. Cereus) digestion of the corresponding synthetic choline and bovine ethanolamine plasmalogen phospholipids, respectively (Conn et al., 1986).

RESULTS

Time course of EGF-stimulated 1,2-diradylglyceride formation

Addition of EGF (50ng/ml) to serum starved human dermal fibroblasts resulted in a rapid increase in 1,2-diradylglyceride. 1,2-Diradylglyceride was maximally elevated (3-fold) within five minutes and remained elevated for at least 120 minutes (Fig. 1). Similar kinetics for EGF-stimulated diglyceride production have been observed in Chinese hamster embryo fibroblasts (Wright et al., 1988).

Analysis of EGF-induced changes in diacyl and ether-linked diglycerides

Recent studies have demonstrated that both 1,2-diacyl and ether-linked diglycerides are present in biological membranes and may accumulate following agonist activation (Rider et al., 1988; Tyagi et al, 1989). We therefore wanted to determine separately the changes in diacyl and ether-linked diglycerides following stimulation of dermal fibroblasts with EGF. Since the enzymatic assay utilized above (Fig. 1) to quantify 1,2-diradylglyceride content does not distinguish between 1,2-diacyl and ether-linked diglycerides (Rider et al., 1988), it was necessary to develop a method to separately quantitate these two diglyceride classes. This was accomplished by converting the 1,2-diradylglycerides to [^{3}H]acetate derivatives and resolving the 1,2-diacyl and ether-linked diglyceride acetates by thin

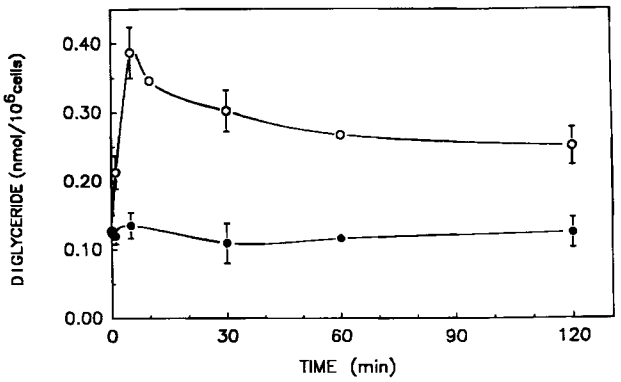

Fig. 1. Stimulation of 1,2-diradylglyceride by EGF. Human dermal fibroblasts were cultured in the absence of serum for two days and placed in serum free media with (○) or without (●) EGF (50ng/ml) for the indicated times. 1,2-diradylglyceride content was determined as described in Materials and Methods. Data points are the means ± S.D. of duplicate determinations from three experiments.

layer chromatography. Utilizing this method, measurement of both diacyl and ether-linked diglyceride acetates was linear between 0.1 and 2.0nmol (Fig. 2).

In unstimulated fibroblasts, approximately 20% of the total 1,2-diradylglyceride was ether-linked (1-O-alkyl, 2-acyl and 1-O-alk-enyl, 2-acyl diglycerides were combined for analysis). Figure 3 demonstrates that the levels of 1,2-diacylglyceride were significantly elevated following addition of EGF. The kinetics and magnitude of change of 1,2-diacylglycerol (Fig. 3) were essentially identical to those observed for total 1,2-diradylglyceride (Fig. 1). In contrast, no change in the level of ether-linked diglycerides was observed in response to EGF. These data demonstrate that the observed increases in 1,2-diglyceride following EGF stimulation are due solely to elevations in 1,2-diacylglycerol content.

Effect of EGF on de novo synthesis of 1,2-diglyceride

To determine whether EGF stimulates de novo synthesis of 1,2-diradylglyceride, serum starved dermal fibroblasts were labelled with [^{3}H]glycerol and treated with or without EGF (50ng/ml) for five or 120 minutes, and radioactivity in 1,2-diradylglyceride was measured. At five minutes, EGF failed to stimulate incorporation of label into 1,2-diradylglyceride (747 ± 201 cpm control, 823 ± 165 plus EGF, n=3). Treatment of cells for 120 minutes with EGF resulted in a 4.8-fold elevation of label in 1,2-diradylglyceride (873 ± 230 cpm control, 4261 ± 309 cpm plus EGF, n=3). These data indicate that the observed elevations in 1,2-diacylglycerol that occur within 5 minutes of EGF addition are not due to de novo synthesis. At 120 minutes, however, de novo synthesis may contribute to the observed accumulation of 1,2-diacylglycerol in response to EGF.

Effect of EGF of phospholipid headgroup turnover

Hydrolysis of inositol, choline and ethanolamine containing phospholipids have all been reported to contribute to 1,2-diglyceride formation. We therefore investigated the effect of EGF on the hydrolysis of different phospholipid headgroup classes in human dermal fibroblasts. Serum deprived cells were incubated with either [^{3}H]choline, [^{3}H]ethanolamine, [^{3}H]serine, or [^{3}H]inositol for 48 hours to metabolically label phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol, respectively. Labelled cells were then exposed to EGF (50ng/ml) for five minutes and radioactivity in the phospholipids and water soluble intracellular metabolites quantitated.

Each of the labelled precursors was readily taken up by the fibroblasts and incorporated predominantly (greater than 90%) into phospholipid. Analysis of cellular phospholipids by thin layer chromatography revealed that each of the radioactive precursors was incorporated almost exclusively into the expected phospholipid headgroup class (data not shown).

Addition of EGF caused a marked increase in total intracellular water soluble radioactivity in choline labelled cells (Table 1). In contrast, no changes in intracellular soluble radioactivity were detected following stimulation with EGF of cells labelled with ethanolamine, serine, or inositol. These data suggest that hydrolysis of phosphatidylcholine is enhanced by EGF.

Time course of EGF-induced phosphatidylcholine hydrolysis

We next determined the kinetics of accumulation of choline metabolites in response to EGF. Cells were labelled with [^{3}H]choline, stimulated with EGF for various times and intracellular radioactive water soluble products analyzed by thin layer chromatography. In unstimulated cells, approximately 80% of the intracellular soluble radioactivity was found in phosphorylcholine, and choline, in a ratio of 55% to 45%. The remainder of the label was approximately equally distributed among acetylcholine, glycerophosphorylcholine, and CDP-choline. Following addition of EGF (50ng/ml), total intracellular soluble radioactivity was maximally increased (2-fold) within five to fifteen minutes and remained above baseline (1.5-fold elevation) for up to 120 minutes. Analysis of water soluble radioactivity by thin layer chromatography revealed approximately equal elevations (2-fold) in choline and phosphorylcholine. Radioactivity in the other choline metabolites was not altered by addition of EGF (data not shown).

Truett et al. (1989) have reported that the level of phosphorylcholine in stimulated neutrophils determined by labeling with [^{3}H]choline is significantly lower than the mass amount determined by enzymatic assay. This presumably reflects non-uniform labeling of cellular phosphatidylcholine. Whether EGF-induced changes in radiolabelled choline and phosphorylcholine is an accurate measure of alterations in the masses of these compounds, in dermal fibroblasts, is not known. We therefore measured changes in mass of choline and phosphorylcholine in response to EGF (Fig. 4). Choline increased from 3.7nmol/10^6 cells to 5.7nmol/10^6 cells within five minutes after addition of EGF. The amount of choline remained maximally elevated between 5 and 15 minutes and then gradually declined over 120

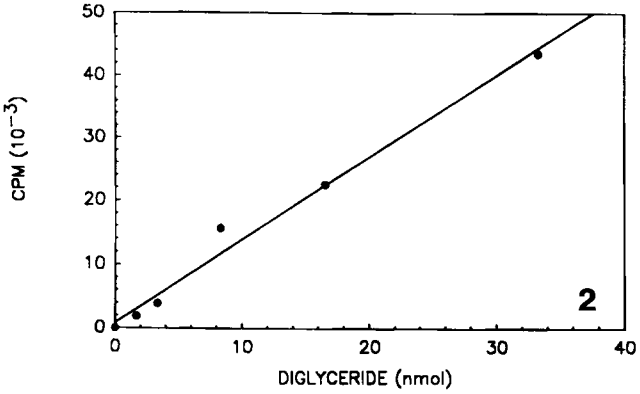

Fig. 2. Quantitative determination of 1,2-diacylglycerol acetate. The indicated amounts of 1,2-dioleoylglycerol were acylated with [^{3}H]acetic anhydride and separated by TLC as described in Materials and Methods. The appropriate spots were scraped from the plate and counted.

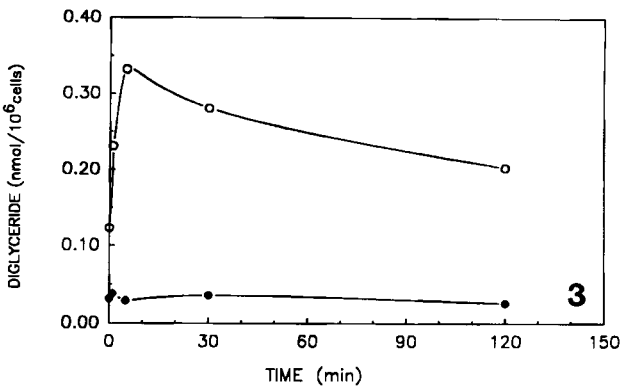

Fig. 3. Time course of EGF-induced alterations in 1,2-diacyl and 1-ether, 2-acyl glycerides. Serum deprived dermal fibroblasts were incubated in serum free media with (○) or without (●) EGF (50ng/ml) for the indicated times. Lipids were extracted and 1,2-diacyl and ether-linked diglycerides quantitated as described in Materials and Methods. Results are the means of duplicate determinations from two experiments.

minutes. Phosphorylcholine increased with kinetics similar to choline, reaching a maximum between 5 and 15 minutes followed by a decline to 50% of maximum over 120 minutes. These data on changes in mass of choline and phosphorylcholine are in good agreement with the data presented above on changes in radiolabelled choline and phosphorylcholine.

Growth factors, including EGF (Warden and Friedkin, 1984), and oncogenic transformation (Macara, 1989) have been reported to induce choline kinase activity in 3T3 fibroblasts. If EGF were to rapidly induce choline kinase activity, this could partially contribute to the observed elevations in phosphorylcholine following stimulation of dermal fibroblasts with EGF. To investigate this possibility, choline kinase activity was assayed in cells following exposure to EGF for 0, 5, and 180 minutes. Treatment with EGF for five minutes did not alter choline kinase activity, compared to control (control, 0.69±0.31; EGF, 0.76±0.25 nmol/min/mg; n=4). A small increase in choline kinase activity was seen following 180 minutes exposure to EGF (1.2 nmol/min/mg). These data indicate that induction of choline kinase activity does not contribute to the increased phosphorylcholine content, that occurs within 5 minutes following EGF treatment.

TABLE 1. EGF-induced release of phospholipid head groups[1]

	Control	EGF
Choline	8056 ± 485	14,075 ± 1158
Serine	8954 ± 723	9107 ± 682
Ethanolamine	45,265 ± 4806	41,004 ± 4371
Inositol phosphates	21,631 ± 798	16,473 ± 863

[1]Fibroblasts were labelled for two days in serum free media with 5μCi/ml of the indicated tritiated compounds. EGF (50ng/ml) was added for five minutes and intracellular water soluble metabolites analyzed as described in Materials and Methods. Results are means ± S.D. of duplicate determinations from three experiments.

EGF-stimulated formation of phosphatidic acid

The kinetics of phosphatidylcholine turnover as determined by accumulation of choline and phosphorylcholine (Fig. 4) are similar to those observed for formation of 1,2-diacylglycerol (Fig. 2). This suggests that EGF-stimulated 1,2-diacylglycerol formation may result from activation of phospholipase C and/or phospholipase D-catalyzed hydrolysis of phosphatidylcholine.

Pai et al. (1988) have demonstrated agonist activation of phospholipase D activity in HL-60 cells by assessing the conversion of synthetic ether-linked phosphatidylcholine, bearing radiolabel in both the phosphate (^{32}P) and 1-O-alkyl chain (^{3}H), to phosphatidic acid. We have employed a similar procedure to assess whether EGF stimulates phospholipase D activity in human dermal fibroblasts. Cells were incubated with [^{3}H]1-O-hexadecyl-lysophosphatidylcholine (the double-labelled phospholipid was not available to us), treated with EGF for various times, and the radioactive lipids formed analyzed by thin layer chromatography.

Following 2 hour incubation, greater than 90% of the [^{3}H]1-O-hexadecyl-lysophosphatidylcholine taken up by the cells was esterified to phosphatidylcholine. Addition of EGF to the cells resulted in the rapid appearance of [^{3}H]phosphatidic acid (Fig. 5). Increased phosphatidic acid was detectable within 15 seconds and was maximal (3-fold) at 30 seconds, after addition of EGF. The level of [^{3}H]phosphatidic acid returned to baseline within 20 minutes. This may have resulted from dephosphorylation to diglyceride, dealkylation to lyso-phosphatidic acid or reacylation to phospholipid. No accumulation of radioactivity in the neutral lipid fraction, was observed, suggesting that re-esterification to phospholipid may account for the decline in labelled phosphatidic acid. Some metabolism via either dephosphorylation or dealkylation, however, cannot be ruled out.

These data suggest that EGF stimulates phospholipase D-catalyzed hydrolysis of phosphatidylcholine. Although phosphatidic acid may also be formed from phosphatidylcholine by the combined actions of phospholipase C and phosphatidate phosphohydrolyase, the rapid appearance of [^{3}H]phosphatidic acid and the lack

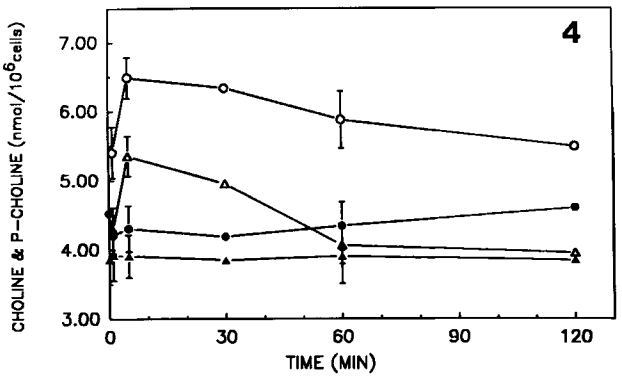

Fig. 4. Time course of EGF-induced accumulation of choline and phosphorylcholine. Dermal fibroblasts in serum free media were incubated in the presence (○, △) or absence (●, ▲) of EGF (50ng/ml) for the indicated times. The cells were scraped from the dish, extracted and choline (▲, △) and phosphorylcholine (○,●) content determined as described in Materials and Methods. The data points are means of duplicate determinations from two or three experiments. Error bars (± S.D.) are shown for points from three experiments.

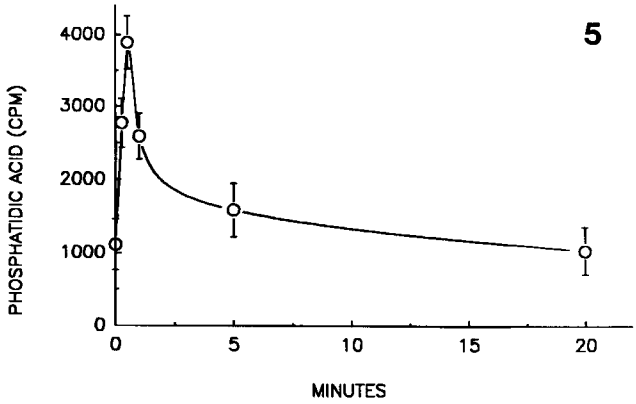

Fig. 5. EGF-induced formation of phosphatidic acid. Serum deprived dermal fibroblasts were incubated with [^{3}H]1-O-hexadecyl, 2-hydroxy phosphatidylcholine (0.5μCi/ml) for 2 hours. The cells were washed to remove unincorporated radioactivity and incubated in serum free media with EGF (50ng/ml) for the indicated times. Cellular phospholipids were extracted and analyzed by TLC as described in Materials and Methods. In the absence of EGF, no change in the amount of [^{3}H]phosphatidic acid was observed during the course of the experiment (data not shown). Results are the means ± S.D. of duplicate determinations from three experiments.

of accumulation of label in the neutral lipid fraction suggest that the majority of the observed increase in [^{3}H]phosphatidic acid in response to EGF is due to activation of phospholipase D.

Effect of ethanol on EGF-stimulated 1,2-diglyceride formation

In the presence of ethanol, phospholipase D-catalyzed hydrolysis of phosphatidylcholine results in transphosphatidylation of phosphatidylcholine to phosphatidylethanol. Transphosphatidylation of exogenous alcohol into phosphatidylcholine has been shown to be a reliable measure of agonist-induced phospholipase D activity (Bocckino et al., 1987b; Billah et al., 1989a; Randall et al., 1990). To further substantiate the presence of EGF-stimulated phospholipase D activity in dermal fibroblasts, cells were treated with 0.5% [^{14}C]ethanol for five minutes prior to addition of EGF (at time 0) for five minutes, and the amount of [^{14}C]phosphatidylethanol determined. Analysis of cellular phospholipids by thin layer chromatography revealed that EGF significantly stimulated formation of [^{14}C]phosphatidylethanol within five minutes. A small increase over background in formation of [^{14}C]phosphatidylethanol was also observed when the [^{14}C]ethanol was added for 10 minutes, 110 minutes after addition of EGF (Fig. 6A). These data indicate that phospholipase D activity in human dermal fibroblasts is stimulated within minutes of addition of EGF, and remains elevated for two hours.

To assess the relative contribution of phospholipase D activation to EGF-induced accumulation of 1,2-diglyceride, the effect of ethanol on 1,2-diglyceride formation was determined. If the phosphatidic acid formed from hydrolysis of phosphatidylcholine by phospholipase D is a major precursor for 1,2-diglyceride formation, than diversion of phosphatidic acid to phosphatidylethanol should result in diminished accumulation of 1,2-diglyceride. This has been shown to occur in f-met-leu-phe-stimulated human neutrophils (Billah et al., 1989b).

As expected, EGF alone induced a 3-fold increase in 1,2-diglyceride content within five minutes (Fig. 6B). Treatment of cells with ethanol five minutes prior to addition of EGF for five minutes, under identical conditions shown to result in phosphatidylethanol formation (Fig. 6A), had no significant effect on EGF-induced formation of 1,2-diglyceride (Fig. 6B). Similarly, ethanol added for 10 minutes, 110 minutes after treatment of cells with EGF, was without effect on the increased 1,2-diglyceride observed at 120 minutes after addition of EGF. These data suggest that although EGF rapidly stimulates phospholipase D activity, the phosphatidic formed may not be a major source of 1,2-diglyceride formed in response to EGF in human dermal fibroblasts.

DISCUSSION

The above data demonstrate that EGF stimulates rapid and sustained accumulation of 1,2-diacylglycerol. Although 20% of the total 1,2-diglyceride in quiescent cells was ether-linked, no increase in ether-linked diglycerides was detected following stimulation with EGF. Pessin and Raben (1989) also found in IIC9 cells that the 1,2-diglyceride induced by α-thrombin was entirely diacyl. Kennerly (1987) also has observed that activation of mast cells by IgE receptor cross-linking resulted in accumulation of only 1,2-diacylglycerides. In contrast, significant elevations in both diacyl and ether-linked diglycerides have been reported to occur in TPA-primed human neutrophils stimulated with f-met-leu-phe (Rider et al., 1988).

Although EGF has been demonstrated to stimulate phosphoinositide turnover in A-431 cells and other transformed cell types, it did not in normal human

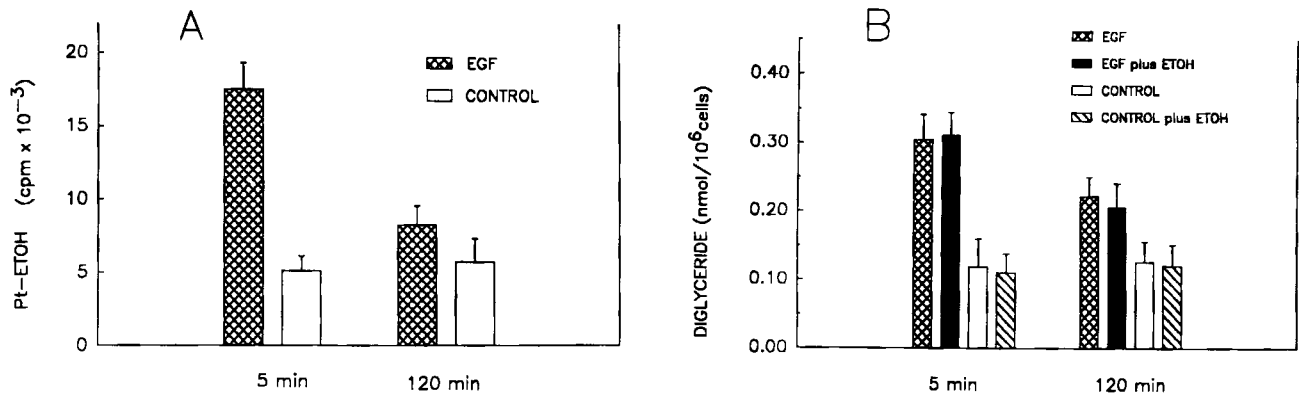

Fig. 6. Effect of ethanol on EGF-stimulated phosphatidylethanol and 1,2-diglyceride formation. **A:** Serum deprived dermal fibroblasts were incubated in serum free media with (▩) or without (□) EGF (50ng/ml). [^{14}C]Ethanol (0.5%) was added 5 minutes prior to EGF (left bars) or 110 minutes after addition of EGF (right bars). At the indicated times cellular phospholipids were extracted and [^{14}C]phosphatidylethanol determined as described in Materials and Methods. **B:** Dermal fibroblasts were treated with (▩, ■) or without (□, ▨) EGF as in A in the presence (□, ▨) or absence (▩, □) of 0.5% ethanol. Cellular lipids were extracted and analyzed for 1,2-diglyceride content as described in Materials and Methods. Results are the means ± S.D. of duplicate determinations from three experiments.

dermal fibroblasts. Similarly, EGF did not stimulate phosphatidylethanolamine turnover as has been reported for TPA in several cell types (Kiss and Anderson, 1989). Wright et al. (1988) were also unable to detect EGF-stimulated phosphatidylinositol or phosphatidylethanolamine hydrolysis in hamster embryo fibroblasts.

In contrast, EGF caused significant elevations in water soluble metabolites of phosphatidylcholine. Both choline and phosphorylcholine, but not CDP-choline or acetylcholine, increased rapidly following exposure of dermal fibroblasts to EGF. These data are consistent with EGF stimulating breakdown of phosphatidylcholine via activation of phospholipase C and/or phospholipase D. The kinetics of accumulation of choline, phosphorylcholine and 1,2-diacylglyceride were similar, suggesting that increased amounts of 1,2-diacylglyceride arise through EGF-stimulated hydrolysis of phosphatidylcholine.

EGF markedly stimulated phospholipase D-catalyzed transphosphatidylation of ethanol into phospholipid within five minutes. This did not, however, reduce EGF-induced formation of 1,2-diglyceride. This is in contrast to studies in human neutrophils, in which ethanol, at the concentration employed in our study, significantly inhibited 1,2-diglyceride formation (Billah et al., 1989b). Assuming that the transphosphatidylation reaction catalyzed by phospholipase D in human dermal fibroblasts occurs to the same extent as that observed in human neutrophils, the lack of effect of EGF-induced phosphatidylethanol formation on EGF-induced 1,2-diglyceride accumulation suggests that the phosphatidic acid that is formed by the action of phospholipase D is not a major source of 1,2-diglyceride. If this is the case, then phospholipase C-catalyzed phosphatidylcholine hydrolysis may be the predominant pathway for formation of 1,2-diacylglycerol in EGF-stimulated dermal fibroblasts. The observed increase in phosphorylcholine following EGF treatment is consistent with the activation of this pathway, although it does not provide direct proof, since phosphorylcholine may be formed from choline by choline kinase. Choline kinase activity was detected in human dermal fibroblasts, however, EGF treatment for five minutes did not alter its activity. The elevations in phosphorylcholine content, which were observed within five minutes of EGF stimulation, are therefore not due to increased choline kinase activity. Several studies have inferred from indirect evidence that agonist-induced 1,2-diglyceride formation occurs primarily through phospholipase C-catalyzed phosphatidylcholine hydrolysis (Besterman et al., 1986; Diaz-Meco et al., 1989; Larrodera et al., 1990).

In contrast, studies using hepatocytes (Bocckino et al., 1987a), neutrophils (Billah et al., 1989b) and REF52 cells (Cabot et al., 1988) have concluded that agonist-induced formation of 1,2-diglyceride from phosphatidylcholine occurs through sequential activation of phospholipase D and phosphatidate phosphohydrolyase. This conclusion is based on preferential accumulation of choline over phosphorylcholine, the kinetics of formation of phosphatidic acid and 1,2-diacylglycerol and inhibition of 1,2-diglyceride formation by the phosphatidate phosphohydrolyase inhibitor propranolol. Much of these data, however, have been obtained using the active phorbol ester TPA as an agonist and may therefore reflect mechanisms different from those elicited by physiological agonists such as EGF. TPA, unlike physiological agonists, directly activates protein kinase C, bypassing 1,2-diacylglyceride formation. Thus TPA-induced activation of phospholipase D and 1,2-diacylglyceride formation may be a consequence of protein kinase C activation. Physiological agonists, conversely, stimulate phospholipid hydrolysis, resulting in formation of 1,2-diglyceride, prior to activation of protein kinase C. Thus the mechanisms through which TPA and physiological agonists stimulate 1,2-diglyceride formation may differ. Vasopressin, however, sim-

ilar to TPA, has been reported to induce formation of 1,2-diglyceride in hepatocytes through activation of phospholipase D (Bocckino et al., 1987a). Thus depending on the cell type and agonist, 1,2-diglyceride may be generated from phosphatidylcholine by either phospholipase C or phospholipase D.

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