Depletion of arachidonic acid from GH₃ cells

Effects on inositol phospholipid turnover and cellular activation

David T. DUDLEY,*† Donald E. MACFARLANE† and Arthur A. SPECTOR*†§

Departments of *Biochemistry and †Internal Medicine, University of Iowa, Iowa City, IA 52242, U.S.A.

We have adapted rat pituitary GH₃ cells to grow in delipidated culture medium. In response, esterified linoleic acid and arachidonic acid become essentially undetectable, whereas eicosa-5,8,11-trienoic acid accumulates and oleic acid increases markedly. These changes occur in all phospholipid classes, but are particularly pronounced in inositol phospholipids, where the usual stearate/arachidonate profile is replaced with oleate/eicosatrienoate ($n-9$) and stearate/eicosatrienoate ($n-9$). Incubation of arachidonate-depleted cells with 10 μ M-arachidonic acid for only 24 h results in extensive remodelling of phospholipid fatty acids, such that close-to-normal compositions and arachidonic acid content are achieved for the inositol phospholipids. In comparison studies with arachidonic acid-depleted or -repleted cells, it was found that the arachidonate content does not affect thyrotropin-releasing-hormone (TRH)-stimulated responses measured at long time points, including [³²P]P_i labelling of phosphatidylinositol and phosphatidic acid, stimulation of protein phosphorylation, and basal or TRH-stimulated prolactin release. However, transient events such as stimulated breakdown of inositol phospholipids and an initial rise in diacylglycerol are enhanced by the presence of arachidonate. These results show that arachidonic acid itself is not required for operation of the phosphatidylinositol cycle and is not an obligatory intermediate in TRH-mediated GH₃ cell activation. It is possible that any structural or functional role of arachidonic acid in these processes is largely met by replacement with eicosatrienoate ($n-9$). However, since arachidonate in inositol phospholipids facilitates their hydrolysis upon stimulation by TRH, arachidonic acid apparently may have a specific role in the recognition of these lipids by phospholipase C.

INTRODUCTION

Arachidonic acid is considered to be an essential metabolite in mammals, primarily because of its role as a precursor of prostaglandins, leukotrienes and hydroxylated eicosatetraenoic acid derivatives. In cells, arachidonic acid is mainly found esterified to phospholipids. The inositol phospholipids, phosphatidylinositol (PtdIns) and its phosphorylated derivatives PtdIns4P and PtdIns(4,5)P₂, are particularly enriched in arachidonate (Baker & Thompson, 1972; Holub & Kuksis, 1978; Allan & Cockcroft, 1983; Cockcroft *et al.*, 1984; Dudley & Spector, 1986), and typically 75–85% of these molecules contain this polyunsaturated fatty acid. The inositol phospholipids are involved in transduction of extracellular signals that mobilize intracellular Ca²⁺ (Michell, 1975; Berridge, 1984; Hokin, 1985). In particular, the hydrolysis products of PtdIns(4,5)P₂, inositol trisphosphate (InsP₃) and diacylglycerol, are thought to be intracellular second messengers (Berridge, 1984). InsP₃ has been shown to release Ca²⁺ from intracellular stores (Streb *et al.*, 1983; Gershengorn *et al.*, 1984; Hirata *et al.*, 1984; Joseph *et al.*, 1984), whereas diacylglycerol can activate protein kinase C (Takai *et al.*, 1979; Kishimoto *et al.*, 1980; Kaibuchi *et al.*, 1982; Nishizuka, 1984a,b).

The high content of arachidonic acid in inositol

phospholipids suggests that they may be a source of substrate for eicosanoid synthesis when cells are stimulated. Indeed, turnover of inositol phospholipids has been associated with release of arachidonic acid and its subsequent conversion into eicosanoids (Berridge, 1981), and a pathway has been described by which arachidonic acid is released from inositol phospholipids and their products during stimulated turnover (Bell *et al.*, 1979; Lapetina *et al.*, 1980; Billah *et al.*, 1981; Chau & Tai, 1981; Prescott & Majerus, 1983).

Stimulated turnover of inositol phospholipids does not always result in release of arachidonic acid and eicosanoids, however. In rat pituitary GH₃ cells, TRH stimulates the turnover of inositol phospholipids (Martin, 1983; Rebecchi & Gershengorn, 1983; MacPhree & Drummond, 1984; Dudley & Spector, 1986), but it does not stimulate release of arachidonate or its conversion into eicosanoids (Rebecchi *et al.*, 1983; Kolesnick & Gershengorn, 1984; Dudley & Spector, 1986; see also Berridge, 1984 and Hokin, 1985). Yet in GH₃ cells, the arachidonate-containing (or tetraenoic) PtdIns species rapidly turns over upon stimulation with TRH (Dudley & Spector, 1986). Treatment of GH₃ cells with TRH also stimulates prolactin release (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984) and protein phosphorylation mediated by protein kinase C (Drust & Martin, 1982,

Abbreviations used: TRH, thyrotropin-releasing hormone (thyroliberin); PtdIns, PtdIns4P, PtdIns(4,5)P₂, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate; PtdA, phosphatidic acid; InsP, InsP₂, InsP₃, inositol monophosphate, bisphosphate and trisphosphate; BAC, benzyltrimethyl-n-hexadecylammonium chloride; TPA, 12-O-tetradecanoylphorbol 13-acetate.

† Present address: Department of Pharmacology, Warner-Lambert/Parke-Davis, Pharmaceutical Research Division, Ann Arbor, MI 48105, U.S.A.

§ To whom correspondence and reprint requests should be sent.

1984; Drust *et al.*, 1982). Since these cells exhibit the turnover of arachidonate-containing species but do not release arachidonic acid or convert it into eicosanoids, they are a good model to explore the possibility that arachidonic acid may have other functions, such as an obligatory role in the operation of the PtdIns cycle and stimulus-response coupling. The approach used here was to deplete the cellular lipids of their arachidonate content and determine the effects on TRH-mediated cell activation as measured by stimulated inositol phospholipid turnover, release of prolactin and stimulated protein phosphorylation.

EXPERIMENTAL

Materials

Lipid standards were obtained from Avanti Biochemicals, Birmingham, AL, U.S.A., and Sigma Chemical Co., St. Louis, MO, U.S.A. LK-5D t.l.c. plates were obtained from Whatman Chemical Separation, Clifton, NJ, U.S.A. Radioisotopes were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. Sera were obtained from HyClone Laboratories, Logan, UT, U.S.A., and Falcon tissue-culture flasks from Becton-Dickinson, Oxnard, CA, U.S.A., were used for all cell cultures.

Preparation of delipidated serum

Delipidated serum was prepared as described by Rothblat *et al.* (1976). Fetal-calf serum (100 ml) was slowly added to 1 litre of ice-cold acetone/ethanol (1:1, v/v) and stirred for 4 h. The resulting precipitate was filtered, washed with 250 ml of acetone/ethanol, and finally with 250 ml of diethyl ether. The precipitate was dried and reconstituted in 100 ml of 0.15 M-NaCl buffered with 0.015 M- Na_2HPO_4 , pH 7.4 (phosphate-buffered saline). This solution was dialysed against phosphate-buffered saline, and the insoluble proteins were sedimented by centrifugation at 10000 *g* for 30 min.

Cell culture

GH_3 cells were maintained in monolayer culture in Ham's F10 medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal-calf serum and 50 μg of gentamicin/ml. To establish GH_3 cells deficient in arachidonic acid, they were adapted to growth in Ham's F10 medium supplemented with delipidated 5% fetal-calf serum, 10 mM-Hepes and 50 μg of gentamicin/ml by replacement of half of the medium containing horse/fetal-calf serum with the medium containing delipidated serum. After 4 days, the cells were passed into medium containing only delipidated serum. Cells were passed weekly for 6 weeks, then passed at 2-week intervals, owing to the resulting slowed growth.

Lipids analysis

Cells were harvested by removing the medium, followed by rapid submersion in ice-cold phosphate-buffered saline and addition of 1 ml of methanol to each well. The cells were scraped off and transferred to glass tubes in a total of 2 ml of methanol. Lipids were extracted by addition of 2 ml of chloroform, then 1.6 ml of 0.88% KCl. Phases were separated by centrifugation, the chloroform removed and the upper phase re-extracted with 2 ml of chloroform. The chloroform extracts were combined, dried under N_2 and redissolved in chloroform/

methanol (2:1, v/v). In experiments involving radioactivity, portions were taken for determination of total radioactivity and lipid phosphate.

Polyphosphoinositides were extracted as described by Schacht (1981); 2 ml of chloroform, then 2 ml of 2.4 M-HCl were added, followed by vortex-mixing. The phases were separated by centrifugation at 1000 *g* for 10 min. After the chloroform phase had been removed, the aqueous phase was re-extracted with 2 ml of chloroform. The chloroform phases were combined, washed by addition of 4 ml of methanol/1 M-HCl (1:1, v/v), dried under N_2 , and redissolved in chloroform/methanol (2:1, v/v).

Polyphosphoinositides were separated on oxalate-impregnated LK-5D plates prepared by the method of Jolles *et al.* (1981), and developed in chloroform/methanol/water/ NH_3 (100:70:13:6, by vol.). For separation of PtdIns, PtdA and other phospholipids, t.l.c. was performed on LK-5D plates impregnated with 1.5% boric acid (Fine & Sprecher, 1982) and developed in chloroform/methanol/water/ NH_3 (120:75:6:2, by vol.), or impregnated with 0.5% boric acid and developed in chloroform/methanol/40% methylamine (20:18:3, by vol.). Separation of neutral lipids (mono-, di- and triacylglycerols, cholesterol, cholesterol esters and non-esterified fatty acids) was achieved by using LK-5D plates developed in heptane/diethyl ether/acetic acid (100:25:4, by vol.). A combination of autoradiography using Kodak X-Omat AR film and lipid staining [spraying the plates with 1 mM-8-anilino-1-naphthalene-sulphonic acid and viewing under fluorescent light (Gitler, 1972)] was used to mark the location of various lipids. The phosphate content was measured by the procedure of Chalvardjian & Rudnicki (1970).

To prepare samples for g.l.c. the appropriate bands were scraped off and the lipid extracted from the silica gel by using the above procedures. The extracts were dried and *trans*-esterified by adding 1 ml of 12% BF_3 in methanol (Morrison & Smith, 1964) and heating at 95 °C for 1 h. The reaction was stopped by adding 1 ml of water, and fatty acid methyl esters were extracted with heptane (three 1 ml washes). The heptane phase was dried and dissolved in carbon disulphide. Fatty acid methyl esters were separated by g.l.c. on a 2 mm $\times$ 1.9 m glass column packed with 10% SP2330 or 10% SP2340 on 100/120 mesh Chromosorb W-AW (Supelco, Bellefonte, PA, U.S.A.) with N_2 as the carrier gas and flame-ionization detection (Spector *et al.*, 1983).

Determination of [^3H]inositol phosphates

GH_3 cells were incubated with 3 μCi of [^3H]inositol/ ml in serum-free Ham's F10 medium supplemented with 5 μM bovine serum albumin and 10 mM-LiCl (Berridge *et al.*, 1982). After stimulation, the cells were harvested by addition of 1 ml of methanol. They were scraped into 16 mm $\times$ 100 mm glass tubes and the wells rinsed with 1 ml of water. The methanol was removed by evaporation under N_2 , and the volume adjusted to 2 ml with water. The cells were further disrupted by sonication and the resulting debris removed by sedimentation at 3000 *g*. Portions of the supernatant were chromatographed on 1 ml Dowex (formate form) columns as described by Downes & Michell (1981). Free inositol was eluted with water. InsP , InsP_2 and InsP_3 were eluted with 0.1 M-formic acid containing 0.2 M-, 0.4 M- and 1.0 M-ammonium formate respectively.

Analysis of phosphorylated proteins

GH₃ cells were incubated with 100 μ Ci of [³²P]P_i/ml in Hepes buffer for 1 h. Agonists were present during the final 10 min of incubation. The medium was removed and the cells extracted with the cationic detergent BAC by adding 200 μ l of a solution containing 5% (w/v) BAC, 22.5% (w/v) urea, 5% (w/v) glycerol and 2.5% (v/v) β -mercaptoethanol (BAC buffer). The solubilized proteins were resolved by two-dimensional gel electrophoresis. The first dimension utilized the cationic-detergent system at acid pH (2.0) in 7.5% polyacrylamide gels (Macfarlane, 1983, 1984, 1986), with the cathode in the lower chamber. After electrophoresis, the gel was removed, fixed with a mixture of water/methanol/acetic acid (5:4:1, by vol.), and stained with Coomassie Blue. The gel was then dried and appropriate lanes cut out. These lanes were placed on the stacking gel of a 15%-acrylamide/SDS slab gel (Laemmli, 1970) and overlaid with SDS sample buffer. After electrophoresis, the gel was stained, destained and autoradiographed.

Determination of prolactin

Prolactin was measured in samples of culture medium by radioimmunoassay using materials supplied by the Rat Pituitary Hormone Distribution Program of the National Institute of Arthritis, Diabetes and Digestive Diseases (NIADDK), as recommended in the kit instructions. Separation of antibody-bound antigen was accomplished with fixed *Staphylococcus aureus* (Gupta & Morton, 1979). Results are expressed in terms of NIADDK prolactin RP-3 standard.

RESULTS

Fatty acyl changes in GH₃ cells

In order to deplete arachidonate from cellular phospholipids, GH₃ cells were grown in medium containing delipidated 5% fetal-calf serum. Table 1 shows the phospholipid fatty acid composition of GH₃ cells grown in medium containing horse and fetal-calf sera (control cells), delipidated fetal-calf serum for 12 weeks (hereafter referred to as 'DL-GH₃ cells'), or delipidated serum for 12 weeks followed by medium supplemented with 10 μ M-arachidonate for 24 h. As compared with the control cells grown in the horse/fetal-calf sera mixture, those grown in medium containing delipidated serum exhibit a markedly different phospholipid fatty acid composition. In these cells, the major fatty acid is oleate. Furthermore, the only polyunsaturated fatty acids present are eicosadienoic acid C_{20:2,n-9} and eicosatrienoic acid C_{20:3,n-9}, and these cells do not contain measurable amounts of either linoleic or arachidonic acid. In the DL-GH₃ cells subsequently enriched with arachidonate, a partial reversion in fatty acid composition towards that found in the control cells occurs. These cells contain up to 13% arachidonate in their phospholipids, slightly more than in the control cells, and a moderate amount of docosatetraenoic acid C_{22:4} accumulates. There is also a decrease in oleate as compared with the DL-GH₃ cells, but the amount remains considerably higher than in the control cells.

The effect of prolonged culture in delipidated medium or repletion with 10 μ M-arachidonate for 24 h on the fatty acid composition of the major phospholipid classes and the inositol phospholipids is presented in Table 2.

Table 1. Fatty acid composition of GH₃ cell total phospholipids

GH₃ cells grown in either medium containing 15% horse serum and 2.5% fetal-calf serum (full serum) or 5% delipidated fetal-calf serum (DL) were harvested, lipids extracted and polar lipids separated by t.l.c. Fatty acid methyl esters were prepared from the polar lipids by *trans*-esterification and analysed by g.l.c. Cultures of DL-GH₃ cells supplemented with 10 μ M arachidonate 24 h before harvest (DL + C_{20:4}) were analysed in the same manner. DL-GH₃ and DL-GH₃ + C_{20:4} samples were pooled from three separate cultures. GH₃ results are mean values from four separate cultures. Abbreviation: N.D., not detected.

Fatty acid	Phospholipid fatty acid composition (%)		
	Full serum	DL	DL + C _{20:4}
C _{16:0}	13.9	11.0	16.4
C _{16:1}	N.D.	2.7	3.4
C _{18:0}	21.8	14.8	19.4
C _{18:1}	22.0	55.2	41.1
C _{18:2}	16.3	N.D.	N.D.
C _{20:2}	N.D.	2.7	N.D.
C _{20:3}	1.7	5.4	1.9
C _{20:4}	10.8	N.D.	13.5
C _{22:4}	0.3	N.D.	2.5
C _{22:5}	4.2	N.D.	N.D.
C _{22:6}	2.3	N.D.	N.D.

The fatty acid profile of PtdIns under usual growth conditions is similar to that reported in numerous other systems (Holub & Kuksis, 1978), consisting primarily of stearic and arachidonic acids. This profile changed drastically in DL-GH₃ cells. The content of arachidonate decreased from about 40% of the total PtdIns fatty acids in control cells to less than 1% in DL-GH₃ cells. It required a minimum of 6 weeks in the delipidated medium to deplete the arachidonate content of PtdIns (results not shown). By contrast, the percentage of stearate decreased only slightly. Fatty acids that increased in PtdIns of DL-GH₃ cells are oleate and eicosatrienoate (*n*-9). PtdIns(4*P*) and PtdIns(4,5)*P*₂ exhibit changes similar to those occurring in PtdIns, and profiles of the three inositol phospholipids are quite similar, supporting the generally held view that they are interrelated metabolically (Holub *et al.*, 1970; Baker & Thompson, 1972; Berridge, 1984).

In contrast with the inositol phospholipids, the major cellular phospholipids, phosphatidylcholine and phosphatidylethanolamine, contain oleate as their most abundant fatty acid in DL-GH₃ cells (Table 2). Phosphatidylethanolamine also accumulates moderate amounts of eicosatrienoate (*n*-9) and stearate. Phosphatidylcholine, however, contains only modest amounts of eicosatrienoate (*n*-9) and stearate in DL-GH₃ cells.

Supplementation of DL-GH₃ cells with 10 μ M-arachidonic acid for 24 h results in large changes in the fatty acid composition of all phospholipid classes. The inositol phospholipids accumulate up to 32% arachidonate, increase their stearate content and decrease their oleate and eicosatrienoate (*n*-9) content. After only 24 h supplementation, the inositol phospholipid fatty acid profiles begin to approach those of the control cells grown in full serum. Phosphatidylcholine and phos-

Table 2. Phospholipid fatty acyl composition of GH₃ cells grown in different media

GH₃ cells grown in the indicated media were harvested and phospholipids extracted and separated by t.l.c. Fatty acid methyl esters were prepared from the separated phospholipids and analysed by g.l.c. Values are the means $\pm$ S.E.M. for samples obtained from a minimum of ten separate passages. Further abbreviations: N.D., not detected. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine. Notes: ^acells grown in Ham's F10 supplemented with delipidated 5% fetal-calf serum; ^bcells grown in delipidated medium were cultured in serum-free Ham's F10 medium containing 5 μ M-bovine serum albumin and 10 μ M-arachidonic acid for 24 h before harvesting; ^ccells were grown in Ham's F10 supplemented with 15% horse serum + 2.5% fetal-calf serum; ^ddata taken from Dudley & Spector (1986).

Phospholipid	Serum supplement	Fatty acid ...	Fatty acid composition (% of total)							
			C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{20:2}	C _{20:3}	C _{20:4}	C _{22:4}
PtdIns	DL ^a		3.8 $\pm$ 0.5	0.5 $\pm$ 0.2	35.4 $\pm$ 0.9	24.0 $\pm$ 0.8	7.4 $\pm$ 0.5	20.8 $\pm$ 1.5	1.0 $\pm$ 0.3	N.D.
	DL + C _{20:4} ^b		4.6 $\pm$ 0.9	0.5 $\pm$ 0.2	40.5 $\pm$ 0.9	14.6 $\pm$ 2.6	0.2 $\pm$ 0.1	5.1 $\pm$ 0.7	32.0 $\pm$ 0.5	N.D.
	Full ^{c,d}		2.3 $\pm$ 0.1	N.D.	41.5 $\pm$ 0.8	6.9 $\pm$ 0.4	N.D.	N.D.	37.2 $\pm$ 1.4	N.D.
PtdIns4P	DL		12.3 $\pm$ 2.5	1.7 $\pm$ 0.7	33.3 $\pm$ 2.4	26.3 $\pm$ 3.6	2.9 $\pm$ 0.1	11.6 $\pm$ 3.4	0.6 $\pm$ 0.6	N.D.
	DL + C _{20:4}		3.2 $\pm$ 3.2	0.7 $\pm$ 0.7	45.8 $\pm$ 6.0	17.8 $\pm$ 3.2	1.4 $\pm$ 1.4	4.9 $\pm$ 0.9	16.1 $\pm$ 1.7	N.D.
	Full		8.0 $\pm$ 1.5	N.D.	40.8 $\pm$ 0.9	6.4 $\pm$ 0.5	N.D.	N.D.	34.2 $\pm$ 1.0	N.D.
PtdIns(4,5)P ₂	DL		11.6 $\pm$ 1.2	1.3 $\pm$ 0.5	35.4 $\pm$ 3.1	23.0 $\pm$ 2.5	3.4 $\pm$ 0.3	11.1 $\pm$ 1.8	N.D.	N.D.
	DL + C _{20:4}		1.0 $\pm$ 1.0	0.3 $\pm$ 0.3	35.2 $\pm$ 4.5	20.5 $\pm$ 4.0	0.4 $\pm$ 0.4	5.5 $\pm$ 0.4	23.2 $\pm$ 1.1	N.D.
	Full		9.9 $\pm$ 3.3	N.D.	41.2 $\pm$ 1.4	6.2 $\pm$ 0.7	N.D.	N.D.	32.5 $\pm$ 2.4	N.D.
PtdEtn	DL		6.3 $\pm$ 0.8	2.3 $\pm$ 0.2	16.4 $\pm$ 1.4	53.3 $\pm$ 0.7	0.8 $\pm$ 0.3	9.3 $\pm$ 0.8	0.5 $\pm$ 0.3	N.D.
	DL + C _{20:4}		5.9 $\pm$ 0.6	1.0 $\pm$ 0.2	21.4 $\pm$ 1.0	31.9 $\pm$ 0.8	0.2 $\pm$ 0.1	4.5 $\pm$ 0.4	21.7 $\pm$ 1.1	5.1 $\pm$ 1.0
	Full		3.5 $\pm$ 0.1	N.D.	26.4 $\pm$ 1.1	16.4 $\pm$ 1.7	N.D.	N.D.	18.7 $\pm$ 2.7	1.0 $\pm$ 0.4
PtdCho	DL		20.2 $\pm$ 0.3	5.0 $\pm$ 0.2	7.2 $\pm$ 0.7	58.7 $\pm$ 1.1	0.2 $\pm$ 0.1	2.5 $\pm$ 0.4	N.D.	N.D.
	DL + C _{20:4}		21.0 $\pm$ 0.6	4.7 $\pm$ 0.2	9.9 $\pm$ 0.4	44.5 $\pm$ 1.4	N.D.	1.2 $\pm$ 0.2	12.5 $\pm$ 1.1	0.6 $\pm$ 0.2
	Full		18.8 $\pm$ 0.3	N.D.	17.8 $\pm$ 0.4	24.5 $\pm$ 1.5	N.D.	N.D.	11.6 $\pm$ 2.3	0.1 $\pm$ 0.1

Fig. 1. Effect of fatty acid modifications on the TRH-concentration-dependence of [^{32}P]P_i labelling of PtdA and PtdIns

DL-GH₃ cells (○), DL-GH₃ cells repleted with 10 μM-arachidonic acid for 24 h (●) or GH₃ cells grown in a full serum medium (■) were labelled for 50 min in Hepes buffer containing 20 μCi of [^{32}P]P_i/ml. During the final 10 min of incubation, the cells were exposed to the indicated concentrations of TRH. Lipids were extracted and separated by t.l.c. Values are averages for duplicate cultures. Results are expressed as percentage response, 100% being defined as the largest response obtained for each set of cultures.

phatidylethanolamine also undergo rapid remodelling after exposure to arachidonate, resulting in accumulation of arachidonate and decreases in oleate and eicosatrienoate (*n*–9). Phosphatidylethanolamine shows the greatest decrease in oleate content and, additionally, accumulates docosatetraenoic acid, presumably from elongation of the added arachidonate. The fact that the changes observed involve fatty acids that occupy both the *sn*-1 and *sn*-2 positions suggests that certain phospholipid fatty acid profiles may have special functions in the cell.

Growth and morphology of DL-GH₃ cells

Culturing of GH₃ cells in delipidated medium results in much slower growth, as compared with the control cells grown in medium containing horse and fetal-calf sera (results not shown). After an initial lag of about 3 days, the cells started dividing when they were exposed to either medium. When full serum is available, however, the cultures reach much higher densities. Addition of 10 μM-arachidonate to the delipidated medium does not increase the growth rate of DL-GH₃ cells.

Morphologically, cells grown in medium containing

Table 3. Effect of repletion with arachidonic acid on [^{32}P]P_i incorporation into DL-GH₃ lipids in response to TRH

DL-GH₃ cells ('DL') or DL-GH₃ cells supplemented with 10 μM-arachidonate ('DL + C_{20:4}') for 24 h were incubated with 20 μCi of [^{32}P]P_i/ml for 50 min. 1 μM-TRH was present during the final 1 ('1 min TRH') or 10 min ('10 min TRH') of the incubation. Results are expressed as a percentage of non-TRH-treated controls and are the means ± S.E.M. for seven separate cultures. Further abbreviations: PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine.

Lipid	[^{32}P]P _i incorporation (% of values in unstimulated cells)			
	1 min TRH		10 min TRH	
	DL	DL + C _{20:4}	DL	DL + C _{20:4}
PtdA	256 ± 28	297 ± 51	537 ± 48	468 ± 32
PtdIns	98 ± 4	116 ± 10	545 ± 42	563 ± 65
PtdCho	103 ± 5	115 ± 4	113 ± 8	124 ± 8
PtdEth	122 ± 8	117 ± 18	132 ± 10	125 ± 9

delipidated serum are very similar to those grown in medium containing full serum. No changes were noted when cultures depleted or enriched with arachidonic acid were compared with control cells, as assessed by scanning or transmission electron microscopy (results not shown).

Effect of fatty acyl changes in PtdIns turnover

GH₃ cells respond to TRH by generating a 'PtdIns cycle' response (Martin, 1983; Rebecchi & Gershengorn, 1983; MacPhree & Drummond, 1984; Dudley & Spector, 1986). This is due to receptor-stimulated hydrolysis of PtdIns(4,5)P₂ to diacylglycerol and InsP₃. It is possible, therefore, that these responses might be affected by the changes in inositol phospholipid fatty acid composition that result from growing the cells in medium containing delipidated serum. In order to determine if the altered GH₃ cells would still generate a PtdIns response, DL-GH₃ cells were pulse-labelled with [^{32}P]P_i for 50 min and stimulated with TRH during the final 10 min of incubation. Fig. 1 shows the effect of this treatment upon the stimulated labelling of PtdIns and PtdA with [^{32}P]P_i, as a function of TRH concentration. For comparison, results with DL-GH₃ cells supplemented with 10 μM-arachidonic acid for 24 h, and control GH₃ cells grown in medium containing full serum are also shown. As in the control cells, stimulation with TRH results in large increases in the amount of [^{32}P]P_i incorporated into PtdIns and PtdA in DL-GH₃ cells, indicating that they still retain a stimutable PtdIns cycle. In all three culture conditions, the TRH-concentration-dependence of both PtdA and PtdIns labelling is essentially identical. A maximal effect is obtained in each case with 1 μM-TRH; consequently, this concentration was used in further experiments.

To assess the time-dependence of stimulated labelling of PtdIns-cycle intermediates, DL-GH₃ cells were labelled with [^{32}P]P_i as described in Fig. 1 and then stimulated for 1 or 10 min with 1 μM-TRH. Table 3 shows that TRH

Table 4. Effect of repletion with arachidonic acid on TRH-stimulated hydrolysis of long-term [32 P] P_i -labelled DL-GH $_3$ cells

At 24 h before an experiment, DL-GH $_3$ cells were fed with serum-free Ham's F10 medium containing 20 μ Ci of [32 P] P_i /ml and 5 μ M-bovine serum albumin with either no added fatty acid ('DL') or 10 μ M-arachidonate ('DL + C $_{20:4}$ '). Cells were stimulated by adding 1 μ M-TRH and harvested either 15 ('15 s') or 30 s ('30 s') later. Lipids were extracted and separated by t.l.c., and the radioactivity in each fraction determined. Results are expressed as a percentage of non-TRH-treated controls and are the means $\pm$ S.E.M. for seven separate cultures. Control values (c.p.m./nmol of lipid P_i): for PtdIns(4,5) P_2 , 106; for PtdIns4 P , 81; for PtdIns, 1026; and for PtdA, 143. These did not differ significantly in DL or DL + C $_{20:4}$ cells. There was approx. 35 nmol of lipid P_i in each culture. Statistical significance of difference from corresponding DL value: *0.01 > P > 0.001; †0.02 > P > 0.01; ‡0.001 > P . Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

Lipid	[32 P] P_i incorporated (% of values in unstimulated cells)			
	15 s		30 s	
	DL	DL + C $_{20:4}$	DL	DL + C $_{20:4}$
PtdIns(4,5) P_2	67.4 $\pm$ 3.0	55.1 $\pm$ 1.2*	67.3 $\pm$ 5.1	49.4 $\pm$ 3.3†
PtdIns4 P	78.4 $\pm$ 2.8	58.7 $\pm$ 1.3‡	64.4 $\pm$ 3.7	49.7 $\pm$ 2.1*
PtdIns	98.5 $\pm$ 3.8	102.3 $\pm$ 4.0	99.3 $\pm$ 5.0	94.9 $\pm$ 2.8
PtdA	111.4 $\pm$ 8.5	113.6 $\pm$ 14.3	120.8 $\pm$ 8.0	120.1 $\pm$ 12.1
PtdCho	103.3 $\pm$ 2.4	100.4 $\pm$ 3.0	106.3 $\pm$ 2.1	96.3 $\pm$ 2.6
PtdEtn	97.1 $\pm$ 2.3	98.5 $\pm$ 3.3	103.2 $\pm$ 2.4	97.7 $\pm$ 3.5

stimulation results in increases in [32 P] P_i radioactivity in PtdA within 1 min and an even greater increase by 10 min. Increases in [32 P]PtdIns radioactivity are also apparent by 10 min, but not at 1 min. Phosphatidylcholine and phosphatidylethanolamine are only minimally affected at both times. To determine if repletion of arachidonate in cellular phospholipids would alter this response, DL-GH $_3$ cells were incubated with 10 μ M-arachidonate for 24 h, then labelled with [32 P] P_i and stimulated with TRH. There is no significant difference in the stimulated labelling of any phospholipid fraction as a result of arachidonate enrichment.

To determine if the altered fatty acid compositions would affect TRH-stimulated hydrolysis of PtdIns(4,5) P_2 , DL-GH $_3$ cells were labelled with [32 P] P_i for 24 h and then exposed to TRH. By 24 h the inositol phospholipids have reached equilibrium with respect to [32 P] P_i (results not shown); therefore, changes in radioactivity under these conditions reflect changes in inositol phospholipid mass. Table 4 shows that, within 15 s after exposure to 1 μ M-TRH, the cellular content of radioactive PtdIns(4,5) P_2 and PtdIns4 P decreases to about 70% of the initial levels. These lower values are maintained at 30 s, but generally return to control levels by 10 min (results not shown). The amount of radioactive PtdIns does not change within these short times, consistent with the view that TRH-receptor activation results primarily in hydrolysis of PtdIns(4,5) P_2 and possibly PtdIns4 P , but not of PtdIns. PtdA shows slight increases in radioactivity at 15 s and 30 s, but phosphatidylcholine and phosphatidylethanolamine are unaffected. When DL-GH $_3$ cells are enriched with arachidonate and treated in a similar manner, there are, likewise, decreases in radioactive PtdIns(4,5) P_2 and PtdIns4 P and moderate increases in PtdA radioactivity. However, the decreases in PtdIns4 P and PtdIns(4,5) P_2 are significantly greater than in DL-GH $_3$ cells at both 15 and 30 s after stimulation with 1 μ M-TRH. As in DL-GH $_3$ cells, there is no appreciable change in PtdIns, phosphatidylethanolamine and phosphatidylcholine radioactivity after TRH stimulation when the cells are enriched with arachidonate.

Table 5. Effect of repletion with arachidonic acid on incorporation of [3 H]inositol into DL-GH $_3$ -cell phospholipids

DL-GH $_3$ cells were incubated with serum-free Ham's F10 medium containing 5 μ M-bovine serum albumin, 3 μ Ci of [3 H]inositol/ml and 10 mM-LiCl ('DL') or this medium supplemented with 10 μ M-arachidonic acid ('DL + C $_{20:4}$ ') for 24 h. The medium was removed, and the cells washed with phosphate-buffered saline and extracted with chloroform/methanol. Phospholipids were resolved on oxalate-impregnated t.l.c. plates. Bands corresponding to unlabelled standards were counted for 3 H. Values are averages $\pm$ S.E.M. for three separate cultures and are expressed as [3 H]inositol incorporated/nmol of total lipid P present in the sample.

Lipid	Incorporation of [3 H]inositol (d.p.m./nmol of lipid P)	
	DL	DL + C $_{20:4}$
PtdIns	10337 $\pm$ 308	10448 $\pm$ 336
PtdIns4 P	337 $\pm$ 32	302 $\pm$ 27
PtdIns(4,5) P_2	276 $\pm$ 3	277 $\pm$ 31

Similar enhanced decreases in PtdIns(4,5) P_2 and PtdIns4 P in DL-GH $_3$ cells repleted with arachidonate were noted when the cells were stimulated with 500 nM-TRH for 30 s [79.4 as against 53.6% of control for PtdIns(4,5) P_2 , depleted and repleted respectively; 58.8 as against 43.7% of control for PtdIns4 P].

The finding of increased degradation of PtdIns4 P and PtdIns(4,5) P_2 on stimulation with TRH when the cellular lipids are repleted with arachidonic acid suggests that, in this situation, there should be increased formation of Ins P_3 and diacylglycerol. To investigate this, DL-GH $_3$ cells and those repleted with arachidonic acid were labelled with [3 H]inositol for 24 h in the presence of 10 mM-LiCl. Table 5 shows that there are equivalent

Fig. 2. Effect of repletion with arachidonic acid on production of [³H]inositol phosphates after TRH stimulation of DL-GH₃ cells

DL-GH₃ cells were incubated with serum-free Ham's F10 medium containing 5 μ M-bovine serum albumin, 3 μ Ci of [2-³H]inositol/ml and 10 mM-LiCl (▨) or this medium supplemented with 10 μ M-arachidonic acid (■) for 24 h. Cells were then stimulated with 1 μ M-TRH for 15 (a) or 30 s (b). [³H]inositol phosphates were determined by chromatography on Dowex anion-exchange columns. Values are percentages of control from unstimulated cultures, and the error bars indicate the S.E.M. ($n = 4$). Control values were: InsP, 30593 c.p.m./culture; InsP₂, 3699 c.p.m./culture; InsP₃, 2514 c.p.m./culture.

amounts of [³H]inositol incorporated into the inositol phospholipids of both types of cells. Other cultures of the labelled cells were stimulated with TRH and the amount of water-soluble [³H]inositol phosphates determined. Fig. 2 shows that there is an increase in [³H]InsP₂ and [³H]InsP₃ within 15 and 30 s after stimulation. DL-GH₃ cells repleted with arachidonic acid accumulated greater amounts of [³H]InsP₃ and [³H]InsP₂ than did DL-GH₃ cells. These results are consistent with the observation made with [³²P]P_i-labelled cells that TRH causes greater decreases in PtdIns4P and PtdIns(4,5)P₂ when these lipids contain esterified arachidonic acid.

The change in diacylglycerol levels upon TRH stimulation was determined in DL-GH₃ cells labelled with [³H]glycerol for 96 h before the experiment. For comparison, cultures labelled in an identical manner were supplemented with 10 μ M-arachidonic acid during the final 24 h. Fig. 3 shows that, at very early times after stimulation with TRH, there is an enhanced accumulation of [³H]diacylglycerol in DL-GH₃ cells that had been repleted with arachidonic acid. However, by 10 min,

Fig. 3. Effect of repletion with arachidonic acid on production of [³H]diacylglycerol after TRH stimulation of DL-GH₃ cells

DL-GH₃ cells were supplemented with 10 μ Ci of [³H]-glycerol (33.7 Ci/mmol)/ml for 96 h. Identical cultures were additionally supplemented with 10 μ M-arachidonic acid during the final 24 h. The medium was then removed and the cells washed with phosphate-buffered saline. Cells were then stimulated with 1 μ M-TRH. At appropriate times, cells were harvested, lipids extracted and analysed by t.l.c. Results are shown as percentages of non-stimulated controls and are averages for duplicate experiments. ○, DL-GH₃ cells; ●, DL-GH₃ cells repleted with 10 μ M-arachidonic acid.

the levels of [³H]diacylglycerol had become equal in the two cell treatments. This is consistent with results described above, and suggests that initially there is an enhanced degradation of inositol phospholipids containing arachidonate. Nevertheless, in cells depleted of arachidonate, there is still substantial degradation of these lipids, and at later times (e.g. 10 min) no difference is apparent.

Effect of fatty acyl changes on stimulated protein phosphorylation

TRH stimulates protein phosphorylation in GH₃ cells (Drust & Martin, 1982, 1984; Drust *et al.*, 1982). This is partially due to activation of protein kinase C by the diacylglycerol formed after receptor-stimulated hydrolysis of PtdIns(4,5)P₂. To determine if the fatty acyl changes produced by growth of GH₃ cells in delipidated medium would affect stimulated protein phosphorylation, two-dimensional polyacrylamide-gel electrophoresis was carried out on extracts of GH₃ cells that were stimulated with TRH for 10 min after labelling with [³²P]P_i. Fig. 4 shows autoradiograms of extracts from DL-GH₃ cells or DL-GH₃ cells repleted with arachidonic acid. Many proteins are labelled under these conditions. Upon stimulation with TRH for 10 min, however, only two proteins had consistent increases of radioactivity (Fig. 4; spots B and E; approx. 33 and 22 kDa respectively). This occurred both in DL-GH₃ cells and in those enriched with arachidonic acid. To quantify these increases, the spots marked in Fig. 4 were excised from the gels and assayed for radioactivity (Table 6). Proteins

Fig. 4. Effect of fatty acid modification on TRH-stimulated protein phosphorylation

DL-GH₃ cells or DL-GH₃ cells enriched with 10 μ M-arachidonic acid (C_{20:4}) were labelled for 1 h in Hepes buffer containing 100 μ Ci of [³²P]P_i/ml. During the final 10 min of labelling, the cells were exposed to 1 μ M-TRH. The medium was aspirated and cells solubilized by adding 200 μ l of BAC buffer. The solubilized cellular proteins were separated by two-dimensional gel electrophoresis, and autoradiograms were obtained. The horizontal axis is electrophoresis in the first dimension (BAC), with migration running from left to right. The migration of M_r standards is indicated on the vertical (SDS) axis. These were: phosphorylase B (M_r 92000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000) and soybean trypsin inhibitor (M_r 21500). (a) DL-GH₃; (b) DL-GH₃ + TRH; (c) DL-GH₃ + C_{20:4}; (d) DL-GH₃ + C_{20:4} + TRH.

A, C and D were chosen as representative control spots because they were in the proximity of spots B and E, contained roughly the same amount of radioactivity as B and E, and did not exhibit stimulated phosphorylation

when the cells were treated with TRH. In DL-GH₃ cells treated with TRH, there is an approx. 1.4-fold increase in radioactivity in both spots B and E. Similar increases occurred in the DL-GH₃ cells enriched with arachidonate.

Table 6. Stimulated protein phosphorylation in DL-GH₃ cells

DL-GH₃ cells ('DL') or DL-GH₃ cells enriched with 10 μ M-arachidonic acid for 24 h ('DL+C_{20:4}') were labelled with 100 μ Ci of [³²P]P_i/ml for 1 h in Hepes buffer. TRH was present at 1 μ M in half of the cultures during the final 10 min of incubation. [³²P]P_i-labelled proteins were extracted and separated on two-dimensional polyacrylamide gels as described in Fig. 4. The indicated spots were excised from the gel, digested with tissue solubilizer and [³²P]P_i radioactivity was determined in a liquid-scintillation counter. Spots A and C typically contained 400–600 c.p.m. of [³²P]P_i, spot D 800–1000 c.p.m. of [³²P]P_i and spots B and E 200–400 c.p.m. of [³²P]P_i. Results are expressed as the ratio of stimulated over control cells, and are the average for two separate experiments.

Band	Labelling (fold)	
	DL	DL+C _{20:4}
A	1.12	0.85
B	1.37	1.38
C	1.00	1.00
D	1.14	1.02
E	1.43	1.33

For comparison, GH₃ cells grown in full serum were labelled in a similar manner and then stimulated with TRH or TPA (results not shown). There was an approx. 2-fold increase in the radioactivity content of spots B and E when the cells were stimulated with either TRH or TPA. Many effects of TPA are thought to be mediated through protein kinase C (Castagna *et al.*, 1982; Nishizuka, 1984a,b). The similarity of the responses suggests that the TRH-stimulated labelling of spots B and E may also be due to activation of protein kinase C.

Effect of fatty acyl changes on prolactin release

As a consequence of TRH stimulation, GH₃ cells release prolactin (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984). To determine whether arachidonic acid depletion would affect prolactin release, cells were incubated with either 1 μ M-TRH or 80 nM-TPA for 3 h. There was no significant difference in the amount of prolactin spontaneously released from DL-GH₃ cells either depleted (2.31 ± 0.15 ng/well, average $\pm$ S.E.M. from four separate cultures) or enriched with arachidonic acid (1.94 ± 0.24). TRH stimulates prolactin release, but there is no difference in the amount released by DL-GH₃ cultures or DL-GH₃ cultures repleted with arachidonate (4.49 ± 0.49 and 4.46 ± 0.64 respectively). TPA also stimulates prolactin release, but again there is no difference in the amount released from cells either depleted or enriched with arachidonic acid (3.95 ± 0.37 and 4.01 ± 0.32 respectively). These results suggest that arachidonic acid is not, in itself, required in GH₃ cells for the process of prolactin release.

DISCUSSION

Growth of GH₃ cells in delipidated medium resulted in depletion of the *n*–6 polyunsaturated fatty acids linoleate and arachidonate, and a buildup of the *n*–9

unsaturated fatty acids oleate and eicosatrienoate. These are the same changes as occur in essential-fatty-acid deficiency (Holman, 1968). Eicosatrienoate (*n*–9) is derived from oleic acid by 6-desaturation, followed by chain elongation and 5-desaturation (Mead & Slaton, 1956; Fulco & Mead, 1959). This is the same pathway through which linoleic acid is converted into arachidonic acid (Klenk, 1965; Mead, 1968), and eicosatrienoic acid (*n*–9) differs from arachidonate only in that it lacks a double bond at the 14,15-position. Under conditions where *n*–6 fatty acids are present, they are the preferred substrates for this pathway, and oleic acid elongation and desaturation products do not accumulate (Dhopeswarkar & Mead, 1961; Peluffo *et al.*, 1963). When *n*–6 fatty acids are absent or deficient, however, oleic acid becomes a substrate for the 6-desaturase, and eicosatrienoate (*n*–9) accumulates (Lundberg, 1980). Although fatty acid compositional changes occurred in all of the phospholipid classes examined (Table 2), the accumulation of eicosatrienoate (*n*–9) was particularly pronounced in the inositol phospholipids PtdIns, PtdIns4P and PtdIns(4,5)P₂. In PtdIns, the appearance of eicosatrienoate (*n*–9) paralleled the decrease in arachidonic acid content (results not shown), suggesting that it was replacing arachidonate. Additionally, there was a tendency for stearic acid to decrease and be replaced by oleate, resulting in maintenance of a tetraenoic species in the inositol phospholipids.

Depletion of arachidonate in GH₃ cells did not result in loss of TRH-sensitivity. A classic PtdIns response was still maintained in the depleted cells, in that PtdIns(4,5)P₂ and possibly PtdIns4P are hydrolysed. The similarity of the TRH-concentration-dependence in the three types of GH₃ cells (Fig. 1) suggests that these lipid modifications do not affect the affinity of the receptor for TRH. Furthermore, no effect of the fatty acid alterations on stimulated PtdIns and PtdA labelling was detected (Table 3), indicating that the diacylglycerol formed by the initial hydrolysis is readily converted into PtdIns. These results indicate that inositol phospholipids do not have to contain arachidonic acid for the PtdIns cycle to be operative. The close structural similarity, however, may allow eicosatrienoate (*n*–9) to substitute effectively for arachidonate in many of the reactions involving the inositol phospholipids and their intermediates.

As compared with DL-GH₃ cells, somewhat greater decreases in PtdIns4P and PtdIns(4,5)P₂ occurred upon TRH stimulation when the cells were repleted with arachidonic acid (Table 4). This suggests that arachidonate may play a role in the recognition of these lipids by phospholipase C. The greater hydrolysis of inositol phospholipids when they are enriched with arachidonic acid is consistent with the increased InsP₂ and InsP₃ formation, as well as that of diacylglycerol, that is observed upon short-term TRH stimulation (Figs. 2 and 3). However, it should be emphasized that considerable hydrolysis of inositol phospholipids occurs even when the esterified arachidonate is replaced with eicosatrienoate (*n*–9).

TRH stimulates the phosphorylation of several proteins in GH₃ cells (Drust & Martin, 1982, 1984; Drust *et al.*, 1982), some of which are mediated by protein kinase C (Drust & Martin, 1984). We also find TRH-stimulated phosphorylation of at least two proteins. Since the same protein phosphorylations were stimulated by TPA, it appears that they are mediated by protein

kinase C. Whereas the apparent M_r values for these phosphorylated proteins do not correspond to those reported by Drust *et al.* (1982), the separation procedures employed were markedly different. The higher- M_r proteins found to be phosphorylated by Drust *et al.* (1982) may not have been resolved by our procedure, and, conversely, the procedure of Drust *et al.* (1982) probably did not resolve the lower- M_r proteins found to be phosphorylated here. Since the diacylglycerol produced in the DL-GH₃ cells will have a markedly different fatty acyl composition, the possibility that activation of protein kinase C might be affected was examined. Our results indicate that this kinase is equally activated, however, whether the diacylglycerol contains stearate/arachidonate or oleate/eicosatrienoate ($n-9$). Furthermore, even though greater amounts of diacylglycerol are present after a 15 or 30 s stimulation with TRH, no difference in protein kinase C activation was observed. The protein phosphorylation studies required a 10 min period of TRH treatment to obtain enough radioactivity for measurement. Fig. 3 suggests that, by this time, there are similar levels of diacylglycerol present in DL-GH₃ cells and DL-GH₃ cells repleted with arachidonate. Therefore, the failure to detect differences in protein phosphorylation in DL-GH₃ cells and those repleted with arachidonic acid could be an artefact of the relatively long time period needed for this assay, as well as the insensitivity of the method to small changes.

The present data also show that arachidonic acid is not required for TRH-stimulated prolactin release in GH₃ cells. This is in contrast with results obtained by Canonico *et al.* (1985a,b), indicating that arachidonate release from lipid precursors and its subsequent conversion into lipoxygenase or epoxigenase products is an important event in prolactin release. These workers, however, utilized primary pituitary cultures or hemipituitary glands *in vitro* and, consequently, their preparations contained a variety of cell types. It is possible that the effects seen by Canonico *et al.* (1985a,b) involve complex interactions amongst the cell types present and that the arachidonic acid metabolites are not formed within the hormone-secreting cells themselves.

Many of the pathological aspects of essential fatty acid deficiency are attributed to the inability of affected tissues to produce appropriate prostaglandins (Laposata *et al.*, 1982). Although eicosatrienoate ($n-9$) can be converted into hydroxy fatty acids and to leukotrienes (Jakschik *et al.*, 1980; Hammarstrom, 1981), it is not a substrate for cyclooxygenase (Ziboh & Hsia, 1972). Consequently, many studies have focused on the effect of eicosatrienoate ($n-9$) accumulation on prostaglandin formation (Christ & Nugteren, 1970; Vincent & Zijlstra, 1978; Bailey, 1980; Laposata *et al.*, 1982). The present results suggest that eicosatrienoate ($n-9$) may also help fulfill membrane structural requirements or other functional roles of the membrane phospholipids (Lundberg, 1980), such as the coupling process of TRH stimulation. However, replacement with eicosatrienoate ($n-9$) apparently does not completely satisfy the lipid structural specificity required by phospholipase C. It is possible that the stearate/arachidonate fatty acid composition of inositol phospholipids may allow them to be positioned in the membrane in such a conformation that they are more readily available for hydrolysis by phospholipase C. Stoffel & Pruss (1969) and Ghosh *et al.* (1973) have shown that the molecular area of phospho-

lipids and diacylglycerols depends on both the composition of the fatty acid chains and the polar head groups. Increasing unsaturation and increasing polarity of the head group increases the molecular area. Although inositol phospholipids were not studied, it may be inferred that highly polar species, such as PtdIns(4,5) P_2 , would be loosely packed in a membrane lipid bilayer, perhaps facilitating access by phospholipase C. Thus it is possible that, because of stringent physiochemical constraints, the phospholipase C reaction requires that the inositol phospholipids contain stearic and arachidonic acids to operate optimally. It appears that substitution of eicosatrienoate ($n-9$) cannot completely satisfy these structural requirements.

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