Inositol phospholipid arachidonic acid metabolism in GH₃ pituitary cells

David T. DUDLEY and Arthur A. SPECTOR

Department of Biochemistry, University of Iowa, Iowa City, IA 52242, U.S.A.

Inositol phospholipids in cultured GH₃ cells, a prolactin secreting, thyrotropin-releasing hormone (TRH) sensitive rat pituitary cell line, exhibit a preferential selectivity for incorporating arachidonic acid. Fatty acid composition data show that all inositol phospholipids are enriched in stearic and arachidonic acids to a much greater degree than other cellular phospholipids. Incubation of GH₃ cells with radioactive stearate, oleate, arachidonate, eicosapentaenoate or docosahexaenoate also showed that much more stearate and arachidonate were incorporated into inositol phospholipids. In short term incubations with tracer amounts of radioactive arachidonate, incorporation was initially into phosphatidylinositol (PtdIns), with phosphatidylinositol 4-phosphate (PtdIns4P), and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] being labelled at later times. During longer incubations, all of the inositol phospholipids reach equilibrium at about 10 h, and the resulting specific activities of the three fractions were similar. These findings suggest that arachidonate is incorporated initially into PtdIns and that PtdIns is then phosphorylated. There was no release of either arachidonate or eicosanoid products when GH₃ cells were incubated with TRH. However, TRH stimulation of ³²P-labelled GH₃ cells resulted in rapid breakdown of PtdIns(4,5)P₂ and PtdIns4P, with concomitant increases in [³²P]phosphatidic acid and [³²P]PtdIns. When the [³²P]PtdIns was further analysed by argentation chromatography to separate PtdIns molecular species, it was found that tetraenoic (stearate/arachidonate) species accounted for 80% of the stimulated labelling. The selectivity for arachidonate incorporation into inositol phospholipids coupled with turnover of the arachidonate-containing molecular species suggests that inositol phospholipids containing arachidonic acid or the diacylglycerol resulting therefrom may play a vital cellular role in GH₃ cells. This role may involve the operation of the PtdIns cycle itself rather than a stimulated release of arachidonate for eicosanoid formation.

INTRODUCTION

Much interest has recently been focused on the inositol phospholipids and their role in transducing the signal of certain extracellular stimuli. In particular, there is now strong evidence for a direct link between inositol lipid hydrolysis and Ca²⁺ mobilization as well as evidence that these lipids participate in mediating secretion events and kinase activation (Berridge & Irvine, 1984; Nishizuka, 1984). Two fatty acids, stearate (C_{18:0}) and arachidonate (C_{20:4}) are especially enriched in inositol phospholipids. While stearate may be synthesized *de novo* by mammalian cells, arachidonate is an essential fatty acid. Consequently, either it or a precursor, linoleic acid (C_{18:2}), must be supplied preformed to the cell. Arachidonic acid is the precursor of prostaglandins, leukotrienes, and hydroxylated eicosatetraenoic acid derivatives. Recently, arachidonate or its metabolites have also been implicated in the secretion process. Results of Canonico *et al.* (1985a) suggest that the diacylglycerol (DAG) resulting from hormone stimulated PtdIns turnover is a source of intracellular unesterified arachidonate and that a non-cyclo-oxygenase metabolite of arachidonate is involved in mediating prolactin release from rat pituitary (Canonico *et al.*, 1985b). Furthermore, use of eicosatetrynoic acid and nordihydroguaiaretic acid to inhibit 12-hydroxyeicosatetraenoic acid synthesis in pancreatic islets resulted in reduction of glucose-stimulated insulin secretion (Turk

et al., 1984). However, addition of 12-hydroxyeicosatetraenoic acid and other oxygenated arachidonate metabolites could not reverse this suppression (Turk *et al.*, 1985).

GH₃ pituitary tumour cells afford a convenient model for studying the interaction of hormone stimulation, transduction events, prolactin secretion and arachidonate metabolism. These cells contain plasma membrane receptors for TRH (Hinkle & Tashjian, 1973; Hinkle *et al.*, 1980), and they release prolactin in response to TRH stimulation (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984). Furthermore, TRH causes rapid breakdown of PtdIns(4,5)P₂ in GH₃ cells, with concomitant generation of inositol trisphosphate and DAG (Martin, 1983; Rebecchi & Gershengorn, 1983; Drummond *et al.*, 1984; MacPhree & Drummond, 1984). However, the role of arachidonate metabolism in this process has not been extensively studied in GH₃ cells.

We report here that the inositol phospholipids of GH₃ cells express a particular selectivity for incorporating arachidonic acid. Furthermore, most of the inositol phospholipid molecules that turn over in response to hormonal stimulation contain arachidonic acid. However, in GH₃ cells this selectivity of inositol phospholipids for arachidonate is apparently separate from any involvement that these lipids may have in serving to supply arachidonate for eicosanoid generation.

Abbreviations used: TRH, thyrotropin-releasing hormone; PtdIns, PtdIns4P, PtdIns(4,5)P₂, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate; PA, phosphatidic acid; DAG, diacylglycerol.

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 Inc., Clifton, NJ, U.S.A. Radioisotopes were purchased from Amersham Corp. Arlington Heights IL., U.S.A. Tissue culture medium was obtained from Gibco Laboratories, Grand Island, NY, U.S.A. Sera were obtained from HyClone Laboratories, Logan, UT, U.S.A. Falcon tissue culture flasks from Becton Dickinson, Oxnard, CA, U.S.A. were used for all cell culture.

Cell culture

GH₃ cells were obtained from the American Type Tissue Culture repository and maintained in monolayer culture using Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal calf serum and 50 µg of gentamicin/ml. For experiments involving radioactivity, cells were seeded into 10 cm² wells and experiments were performed after 1 week. Incubations with radioactively labelled substrates was carried out in either serum-free Ham's F10 containing 5 µM-bovine serum albumin (SFF10) or Hepes buffer (128 mM-NaCl, 5.2 mM-KCl, 2.1 mM-CaCl₂, 2.9 mM-MgSO₄, 5 mM-glucose and 10 mM-Hepes, pH 7.4). For phospholipid assays and fatty acid composition determinations, cells were grown in 175 cm² flasks.

Lipid extraction and chromatography

Cells were harvested by aspirating off 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 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 at 1000 g for 10 min. The lower chloroform phase was transferred to a separate tube, and the upper phase re-extracted with 2 ml of chloroform. The chloroform extracts were combined, dried under N₂ and redissolved in chloroform/methanol (2:1, v/v). In experiments involving radioactivity, aliquots were taken for determination of total radioactivity and lipid phosphate. Polyphosphoinositides were extracted according to Schacht (1981); 2 ml of chloroform and then 2 ml of 2.4 M-HCl were added, followed by vortex-mixing. Phases were separated by centrifugation at 1000 g for 10 min. After the lower chloroform phase was removed, the upper 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 N₂, redissolved in chloroform/methanol (2:1, v/v), and divided into aliquots. Polyphosphoinositides were separated on oxalate-impregnated LK-5D plates prepared by the method of Jolles *et al.* (1981), and developed in chloroform/methanol/water/NH₄OH (50:35:6.5:3, by vol.). For separation of PtdIns, phosphatidic acid (PA) and other phospholipids, t.l.c. was performed on boric acid impregnated LK-5D plates (Fine & Sprecher, 1982) developed in chloroform/methanol/water/NH₄OH (60:37.5:3:1, by vol.). A combination of autoradiography using Kodak X-Omat AR film and lipid staining, by 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.

Separation of molecular species of PtdIns was accomplished by chromatography of isolated PtdIns on LK-5D plates impregnated with 10% AgNO₃, and developed in chloroform/methanol/water (12:7:1, by vol.) (Holub & Kuksis, 1971).

To prepare samples for g.l.c., the appropriate bands were scraped and the lipid extracted from the silica gel using the above procedures. The extracts were dried and transesterified by adding 1 ml of 12% BF₃ 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 three 1 ml heptane washes. The heptane phase was dried, dissolved in carbon disulphide and analysed by g.l.c.

Phosphate assay and determination of phospholipid class content

Phosphates were measured using the procedure of Chalvardjian & Rudnicki (1970). To determine phospholipid class content, lipids were extracted and separated by t.l.c. as described above. The appropriate bands were scraped into glass tubes, 100 µl of HClO₄ was added and the lipids digested and assayed for phosphate. These were found to be: phosphatidylethanolamine, 21.5%; phosphatidylcholine, 48.9%; phosphatidylserine, 4.7%; PtdIns, 7.2%; PtdIns4P, 0.23%; and PtdIns(4,5)P₂, 0.15%.

Determination of prostanoic acid production and release of labelled arachidonate

GH₃ cells were incubated with 5 µM-[1-¹⁴C]arachidonate (59.6 mCi/mmol) and combinations of 1 µM-TRH, 20 µM-ibuprofen or 20 µM-nordihydroguaiaretic acid for 30 min. For t.l.c., the medium was removed, extracted with ethyl acetate and the extract dried under N₂. The extract was redissolved in chloroform/methanol (2:1, v/v) and chromatographed on LK-5D t.l.c. plates in the upper phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10, by vol.). For h.p.l.c., the medium was extracted with chloroform/methanol (2:1, v/v), using 0.9% NaCl containing 1% acetic acid to split the phases. The extract was dried, redissolved in methanol and injected onto a C18 reverse phase Vydac column (Alltech Associates, Deerfield, IL, U.S.A.) and eluted with acetonitrile in water, pH 3.4, using a linear gradient of 27–100% acetonitrile. Detection was by a radioactivity flow monitor (Radiomatic Instruments, Tampa, FL, U.S.A.).

For release experiments, GH₃ cells were labelled for 1 h in Hepes containing 0.5 µCi of [5,6,8,9,11,12,14,15-³H]arachidonate/ml (155 Ci/mmol). The medium was removed and cells washed twice with Hepes containing 50 µM-bovine serum albumin. Fresh Hepes containing 50 µM-bovine serum albumin was then added and cells were stimulated with 1 µM-TRH. After 15 min, the medium was removed, and aliquots were assayed for ³H.

RESULTS

Fatty acyl composition of cellular phospholipids

Many tissues exhibit enrichment of PtdIns with stearic and arachidonic acids *in vivo* (Holub & Kuksis, 1978). In the few tissues examined, this is also the case for PtdIns4P

Table 1. Fatty acid composition of GH₃ cell phospholipids

GH₃ cells were harvested after washing with Hepes buffer, and lipids were extracted with chloroform/methanol. Phospholipids were separated by t.l.c. The appropriate bands were scraped, phospholipids were eluted from the silica gel, and dried under N₂. Fatty acid methyl esters were prepared and separated by g.l.c. Data are expressed as means $\pm$ S.E.M. of values obtained from four separate passages of cells. Fatty acids are represented as number of carbon atoms: number of double bonds. Abbreviations: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; LPE, ethanolamine plasmalogen; LPC, choline plasmalogen; N.D., not detected.

Fatty acid	Composition (%)						
	PtdIns	PtdIns4P	PtdIns(4,5)P ₂	PtdEtn	LPE	PtdCho	LPC
C _{16:0}	2.3 $\pm$ 0.1	8.0 $\pm$ 1.5	9.9 $\pm$ 3.3	3.5 $\pm$ 0.1	1.0 $\pm$ 0.1	18.8 $\pm$ 0.3	17.8 $\pm$ 2.1
C _{18:0}	41.5 $\pm$ 0.8	40.8 $\pm$ 0.9	41.2 $\pm$ 1.4	26.4 $\pm$ 1.1	1.5 $\pm$ 0.2	17.8 $\pm$ 0.4	45.3 $\pm$ 2.9
C _{18:1}	6.9 $\pm$ 0.4	6.4 $\pm$ 0.5	6.2 $\pm$ 0.7	16.4 $\pm$ 1.7	24.3 $\pm$ 0.9	24.5 $\pm$ 1.5	18.4 $\pm$ 0.6
C _{18:2}	5.8 $\pm$ 0.7	7.2 $\pm$ 1.0	5.9 $\pm$ 0.7	20.3 $\pm$ 2.1	10.8 $\pm$ 0.4	19.0 $\pm$ 0.4	11.0 $\pm$ 0.3
C _{20:4}	37.2 $\pm$ 1.4	34.2 $\pm$ 1.0	32.5 $\pm$ 2.4	18.7 $\pm$ 2.7	1.5 $\pm$ 0.3	11.6 $\pm$ 2.3	2.2 $\pm$ 0.2
C _{22:4}	N.D.	N.D.	N.D.	1.0 $\pm$ 0.4	1.8 $\pm$ 0.2	0.1 $\pm$ 0.1	N.D.
C _{20:5}	1.3 $\pm$ 0.5	N.D.	N.D.	1.1 $\pm$ 0.4	1.1 $\pm$ 0.3	0.4 $\pm$ 0.2	N.D.
C _{22:5}	1.0 $\pm$ 0.4	N.D.	N.D.	4.5 $\pm$ 0.2	37.0 $\pm$ 1.1	1.0 $\pm$ 0.2	2.5 $\pm$ 0.1
C _{22:6}	N.D.	N.D.	N.D.	2.3 $\pm$ 0.3	16.4 $\pm$ 0.7	0.4 $\pm$ 0.1	1.0 $\pm$ 0.5

and PtdIns(4,5)P₂ (Baker & Thompson, 1972; Allan & Cockcroft, 1983). In order to determine if this profile would extend to a hormone-sensitive, defined cultured cell line, the fatty acyl composition of GH₃ cell phospholipids was determined. Table 1 demonstrates that when these cells are grown in medium containing horse and fetal calf sera, all three inositol phospholipids are markedly enriched in stearate and arachidonate. In contrast, the two most prevalent phospholipids, phosphatidylcholine and phosphatidylethanolamine, contain much more oleic (C_{18:1}) and linoleic acid, and they generally have a more varied fatty acid composition than the inositol phospholipids. Furthermore, the plasmalogen form of ethanolamine phosphatides (LPE), contains a large amount of the long chain polyunsaturated fatty acids of the *n*-3 class, docosapentaenoic (C_{22:5}) and docosahexaenoic (C_{22:6}), but very little arachidonate.

Fatty acid incorporation into cellular phospholipids

To determine if the observed phospholipid fatty acyl composition would be reflected by differences in the incorporation of the respective fatty acids, GH₃ cells were incubated with different 1-¹⁴C-labelled fatty acids for 30 min or 24 h and the pattern of incorporation into phospholipid classes determined. Of the fatty acids tested, stearate and arachidonate were incorporated into PtdIns, PtdIns4P and PtdIns(4,5)P₂ to a considerably greater extent than the other fatty acids (Figs. 1a-1c). This was evident by 30 min (hatched bars), but was more pronounced at 24 h (solid bars). While stearate and arachidonate were also incorporated into phosphatidylcholine and phosphatidylethanolamine, these phospholipids exhibited much less selectivity for these two fatty acids (Figs. 1d and 1e). Oleate was avidly incorporated into phosphatidylcholine, and phosphatidylethanolamine exhibited significant incorporation of eicosapentaenoic acid (C_{20:5}) and docosahexaenoate, as well as oleate. Ethanolamine plasmalogen predominantly incorporated the *n*-3 polyunsaturated fatty acids eicosapentaenoate and docosahexaenoate. Interestingly, the GH₃ cells readily discriminated between arachidonate and eicosapentaenoate even though these

highly polyunsaturated fatty acids differ in only one double bond.

To determine further the relative contribution of each phospholipid class to the incorporation of arachidonate, a time course of radioactive arachidonate incorporation into cellular phospholipids was determined. The results expressed as specific activity are shown in Fig. 2. Specific activity determinations are based on the measured amount of each phospholipid as detailed in the Experimental section. This demonstrates that arachidonic acid is preferentially incorporated into inositol phospholipids, even though more of the total arachidonate is channelled into phosphatidylethanolamine and phosphatidylcholine (Fig. 1). By approx. 10 h, incorporation into each of the phospholipids has reached a plateau. Assuming that each phospholipid molecule contains a single arachidonate residue, then roughly 70-80% of the inositol phospholipid pool has been labelled. The fatty acid composition data in Table 1 indicate that 65-75% of the inositol phospholipid molecules contain arachidonic acid, again assuming that there is only one arachidonate residue per phospholipid molecule. Taken together, these data suggest that all of the inositol phospholipid pools have equilibrated with respect to arachidonate in 10 h and, furthermore, that all of the arachidonate contained in the inositol phospholipids can turn over within this time period. The other phospholipid fractions show greatly reduced plateau levels of arachidonate radioactivity relative to their amounts. This is consistent with the fact that arachidonate comprises a lesser percentage of their fatty acids, as indicated in Table 1. The fact that their specific activities also reached plateau values within 10 h suggests that the arachidonic acid contained in these phospholipids also is readily accessible.

The consistency of their fatty acid composition and the arachidonate specific activity results for PtdIns, PtdIns4P and PtdIns(4,5)P₂ suggests that arachidonate is incorporated into PtdIns and that PtdIns is then phosphorylated, as opposed to independent fatty acylation occurring in PtdIns4P and PtdIns(4,5)P₂ themselves. To investigate this further, GH₃ cells were incubated with tracer

Fig. 1. Incorporation of fatty acids into GH₃ cell phospholipids

GH₃ cells were incubated for 30 min (hatched bars) or 24 h (solid bars) with 5 μ M (60 mCi/mmol) ¹⁴C-containing fatty acid in SFF10 medium. At the appropriate time, cells were washed with phosphate-buffered saline containing 50 μ M-bovine serum albumin and lipids were extracted with chloroform/methanol. Phospholipids were separated by t.l.c. Data are expressed as pmol of labelled fatty acid incorporated/nmol of total lipid P, as determined by phosphate assay of aliquots of the extracted lipid. (a) PtdIns, (b) PtdIns4P, (c) PtdIns(4,5)P₂, (d) phosphatidylcholine (PtdCho), (e) phosphatidylethanolamine (PtdEtn), (f) ethanolamine plasmalogen (LPE). Note the difference in ordinate scales.

amounts of [³H]arachidonate for short times and radioactivity in the inositol phospholipids was determined (Fig. 3). PtdIns showed a rapid rise in specific activity. PtdIns4P and PtdIns(4,5)P₂ showed slightly slower labelling kinetics, but reached a similar specific activity as PtdIns by 1 h. However, at all earlier times the specific activity of PtdIns was greater than that of either PtdIns(4,5)P₂ or PtdIns4P. These data also suggest that arachidonate is incorporated primarily into PtdIns and subsequently into PtdIns4P and PtdIns(4,5)P₂, not directly into the polyphosphoinositides.

Determination of prostanoid production and TRH-stimulated arachidonate release

The above data show that in GH₃ cells the inositol phospholipids express a great deal of selectivity and specificity in terms of their fatty acyl constituents. In particular, they readily incorporate and sequester

arachidonate. A possible reason for this would be that upon appropriate stimulation, the inositol phospholipids are the source of arachidonate for conversion to prostanoids or other oxygenated products. However, when GH₃ cells were presented with [1-¹⁴C]arachidonate with or without TRH, they are unable to convert the exogenous [1-¹⁴C]arachidonate to any oxygenated metabolites as determined by either t.l.c. or h.p.l.c. (results not shown). Furthermore, the formation of a lipoxygenase product was not stimulated when a combination of [1-¹⁴C]arachidonate and ibuprofen were added. Likewise, the formation of a cyclo-oxygenase product was not stimulated when a combination of [1-¹⁴C]arachidonate and nordihydroguaiaretic acid were added. This technique, when we apply it to human umbilical cord endothelial or bovine aortic endothelial cells, readily demonstrates the ability of these cell types to produce a variety of prostanoids. Also, when GH₃ cells were labelled with [1-¹⁴C]arachidonate for long periods and then stimulated with TRH, the Ca²⁺ ionophore A23187 or unlabelled arachidonate, they similarly did not produce any oxygenated [1-¹⁴C]arachidonate metabolites (results not shown). Furthermore, when GH₃ cells were labelled with [³H]arachidonate and treated with TRH, there was no stimulated release of [³H]arachidonate when the extracellular medium contained 50 μ M-bovine serum albumin

Fig. 2. Time course of arachidonic acid incorporation into GH₃ phospholipids

GH₃ cells were incubated with 5 μ M extracellular [1-¹⁴C]arachidonate (58 mCi/mmol) in SFF10 for the indicated times. Cells were washed and lipids extracted with chloroform/methanol. Data are expressed as pmol of arachidonate incorporated/nmol of each phospholipid. The specific activity was determined by taking into account the amount of arachidonate present in the cultures. Error bars represent S.E.M. for three separate cultures.

Fig. 3. Time course of arachidonate incorporation into GH₃ inositol phospholipids

GH₃ cells were washed with Hepes buffer containing 50 μ M-bovine serum albumin, then twice with Hepes buffer alone. Labelling was initiated by addition of Hepes buffer containing 2 μ Ci of [³H]arachidonate/ml (155 Ci/mmol). Cells were harvested by aspirating off the medium followed by washing with Hepes buffer containing 50 μ M-bovine serum albumin, then scraping into 1 ml of methanol. Chloroform was added to the cells, lipids were extracted and separated by t.l.c. ○, PtdIns; □, PtdIns4P; ■, PtdIns(4,5)P₂.

Fig. 4. Effect of TRH on long-term [³²P]P_i-labelled GH₃ cell phospholipids

GH₃ cells were labelled in SFF10 medium containing 30 μ Ci of [³²P]P_i/ml for 24 h. Cells were then stimulated by addition of 1 μ M-TRH. At the appropriate time, cells were harvested, lipids extracted with chloroform/methanol and separated by t.l.c. Error bars indicate S.E.M. for three separate cultures. ●, PA; ○, PtdIns; □, PtdIns4P; ■, PtdIns(4,5)P₂.

as a fatty acid trap (results not shown). These results are similar to those reported by Rebecchi *et al.* (1983) and Kolesnick & Gershengorn (1984), and show that, in GH₃ cells, release of arachidonate from inositol phospholipids and subsequent oxygenation does not play a role in TRH-mediated cellular activation.

Fig. 5. Effect of TRH on [³²P]P_i-pulse-labelled GH₃ cell phospholipids

GH₃ cells were labelled for 40 min in Hepes buffer containing 10 μ Ci of [³²P]P_i/ml. Cells were then stimulated by addition of 1 μ M-TRH and harvested at the times shown. Lipids were extracted by chloroform/methanol and separated by t.l.c. Values are averages of duplicate cultures. ●, PA; ○, PtdIns; □, PtdIns4P; ■, PtdIns(4,5)P₂.

Hormone-stimulated turnover of inositol phospholipids

It has been reported that TRH causes turnover of PtdIns in GH₃ cells (Martin, 1983; Rebecchi & Gershengorn, 1983; Drummond *et al.*, 1984). Fig. 4 shows that, when GH₃ cells are labelled with [³²P]P_i for long periods and then treated with TRH, there is a rapid decrease in the amount of radioactive PtdIns4P and PtdIns(4,5)P₂, with a concomitant increase in radioactive PA. Radioactive PtdIns levels decrease only slightly in this time frame, while other cellular phospholipids were unaffected. This suggests that upon receptor stimulation PtdIns(4,5)P₂ and possibly PtdIns4P are being hydrolysed. If cells are pulse-labelled with [³²P]P_i and stimulated with TRH, a large increase in radioactivity in PA and PtdIns is seen (Figs. 5 and 6a). During the first 5 min, more radioactivity is contained in PA than in PtdIns; subsequently, PtdIns becomes more heavily labelled. Radioactivity in PtdIns4P and PtdIns(4,5)P₂ also increased after the first 1 min, while other cellular phospholipids were unaffected. Under these conditions, the phosphate specific activity of the lipids is quite low, and the experiment is primarily measuring resynthesis. These results suggest that an initial event in TRH action is receptor-initiated hydrolysis of PtdIns(4,5)P₂ and possibly PtdIns4P, resulting in formation of DAG and inositol phosphates. The DAG is subsequently phosphorylated to PA, which is converted to PtdIns, resulting in the well-known 'PtdIns cycle'.

When PtdIns from [³²P]P_i-pulse-labelled GH₃ cells was further analysed by AgNO₃ t.l.c., a procedure that separates on the basis of double bond content, it was found that all of the PtdIns fractions showed an increase in labelling after the cells were treated with TRH (Fig. 6b). Furthermore, the tetraenoic PtdIns fraction accounted for 80% of the total turnover (Table 2). This fraction consists predominantly of PtdIns molecules containing stearate and arachidonate, as indicated by the fatty acid analysis obtained from the isolated material. Since

Fig. 6. Separation of [^{32}P]P₁-pulse-labelled GH₃ cell phospholipids and of PtdIns molecular species

GH₃ cells were pulse-labelled for 40 min with SFF10 medium containing 30 μCi of [^{32}P]P₁/ml. Half of the cells were then stimulated with 1 μM -TRH. After 10 min stimulation, cells were harvested, lipids extracted and separated by t.l.c. (a) Autoradiograph of the initial separation. The small band running below PtdIns represents a small amount of PtdIns that is retained at the interface of the pre-absorbent layer and the silica gel. The PtdIns band was eluted from the silica gel and re-chromatographed on AgNO₃-impregnated silica gel plates. (b) Autoradiograph of the AgNO₃ separation of PtdIns molecular species.

65–75% of the PtdIns molecules are of the tetraenoic species, this suggests that there is little selectivity, in terms of fatty acyl composition, for turnover. However, this result clearly shows that tetraenoic PtdIns is the major species undergoing turnover in response to TRH stimulation.

DISCUSSION

PtdIns is reported to have an unusual fatty acyl profile, containing predominantly stearate and arachidonate (Holub & Kuksis, 1978). This pattern occurs in a large

variety of tissues and therefore appears to be a general phenomenon. Studies also indicate that this unusual profile extends to the polyphosphoinositides. Although not as extensively studied as PtdIns, reports indicate that brain (Holub *et al.*, 1970; Baker & Thompson, 1972) and erythrocyte (Allan & Cockcroft, 1983) PtdIns4P and PtdIns(4,5)P₂ are also highly enriched in stearate and arachidonate. We have extended this to include a hormone-sensitive, continuously cultured rat pituitary cell line, in which case the fatty acid compositions of PtdIns, PtdIns4P and PtdIns(4,5)P₂ are almost identical. Furthermore, we have examined radioactive fatty acid incorporation into the phosphoinositides and find the same pattern in each of the three inositol phospholipid fractions. This suggests that the fatty acids are being incorporated into PtdIns and that PtdIns is then being phosphorylated to form the polyphosphoinositides, for it seems unlikely that acyl transferases would exhibit the same specificities toward the three different phosphoinositides. In addition, short-term incubations with tracer amounts of [^3H]arachidonate shows PtdIns being labelled first, with PtdIns4P and PtdIns(4,5)P₂ lagging behind and eventually reaching similar specific activities. This is the expected pattern for a precursor-product relationship, with arachidonate being incorporated primarily into PtdIns rather than directly into PtdIns4P or PtdIns(4,5)P₂. Furthermore, in long-term incubations with [$1\text{-}^{14}\text{C}$]arachidonate, the specific activities of the three phosphoinositides reach similar plateau values, a finding that is also consistent with these lipids being metabolically linked.

There has been much controversy regarding the biological importance of the observed fatty acyl profile of inositol phospholipids. Several workers have suggested that PtdIns is the source of arachidonate for prostanoid synthesis (Bell & Majerus, 1980; Hong & Deykin, 1981; Marshall *et al.*, 1980; Rittenhouse-Simmons, 1981), accounting for the greater incorporation and storage of arachidonate in this fraction. Although this is an attractive hypothesis, it is difficult to demonstrate conclusively and unequivocally. Also, other investigators have evidence that lipids such as phosphatidylethanolamine and phosphatidylcholine can serve as arachidonate sources (Chilton *et al.*, 1984; Thomas *et al.*, 1984; McKean *et al.*, 1981), with little or no involvement of PtdIns (for review see Irvine, 1982). Such discrepancies could be due to different stimuli having separate coupling

Table 2. Stimulated labelling and fatty acid composition of PtdIns molecular species

PtdIns from GH₃ cells pulse-labelled with 30 μCi of [^{32}P]P₁/ml for 40 min and treated with 1 μM -TRH (or control) for 10 min was isolated and subjected to AgNO₃ t.l.c. as detailed in Fig. 6. Radioactivity present was determined for the separated bands and is expressed as c.p.m./nmol of lipid P₁ present in the lipid extract. Values are mean $\pm$ S.E.M. for three separate cultures. The fatty acid composition was determined by eluting PtdIns from the separated bands, followed by transesterification of the fatty acyl residues and analysis by g.l.c. Abbreviation: N.D., not detected.

Band	^{32}P			Fatty acid composition (%)				
	Control	TRH	(% of total labelling)					
	(c.p.m./nmol)	(c.p.m./nmol)		C _{16:0}	C _{18:0}	C _{18:1}	C _{20:3}	C _{20:4}
Di/trienoic	33 $\pm$ 1	166 $\pm$ 13	7.4	6.3 $\pm$ 0.6	41.9 $\pm$ 1.7	14.0 $\pm$ 2.3	28.8 $\pm$ 2.1	N.D.
Tetraenoic	136 $\pm$ 8	1568 $\pm$ 129	80.0	1.5 $\pm$ 0.9	48.4 $\pm$ 2.1	1.6 $\pm$ 0.9	0.9 $\pm$ 0.5	43.0 $\pm$ 2.9
Polyenoic	24 $\pm$ 2	117 $\pm$ 25	5.2	N.D.	12.9 $\pm$ 0.7	33.3 $\pm$ 0.1	N.D.	44.1 $\pm$ 2.8

mechanisms, and in fact there may be several hormone-sensitive arachidonate pools either in the same or different types of cells.

We find that GH₃ cells do not exhibit an accelerated release of arachidonate when stimulated with TRH, and they do not convert arachidonate to prostanoids or other oxygenated eicosanoid products. This is in agreement with the findings of Rebecchi *et al.* (1983) and Kolesnick & Gershengorn (1984) and supports the idea that in GH₃ cells, endogenous eicosanoid products do not mediate cellular activation or secretion. This is also consistent with the findings of Turk *et al.* (1985) who reported that addition of oxygenated arachidonate metabolites could not overcome eicosatetrynoic acid- or nordihydroguaiaretic acid-inhibited secretion of insulin in pancreatic islets. Yet, our results indicate that GH₃ cells exhibit preferential handling of arachidonic acid by inositol phospholipids. Therefore, the GH₃ cell affords a convenient model to examine the possibility that arachidonate contained in the phosphoinositides has other metabolic roles besides eicosanoid production. These cells respond to TRH by generating a classic PtdIns response, hydrolysis to DAG followed by resynthesis to PA and PtdIns. This suggests that arachidonate may be serving a role in the operation of the PtdIns cycle itself. Our analysis of PtdIns species turning over in response to TRH showed that approx. 80% were tetraenoic. Since up to 75% of the PtdIns pool consists of tetraenoic species, there apparently is no enzymic selectivity, with regard to fatty acyl composition, in this segment of the pathway. However, the enrichment of PtdIns with arachidonic acid may ensure that upon stimulation, it is the tetraenoic (or arachidonate containing) species that undergo turnover.

Several other workers have examined the labelling of PtdIns molecular species. Waku *et al.* (1982), utilizing Ehrlich ascites tumour cells, found that [³²P]P_i and [³H]glycerol were primarily incorporated into dienoic PtdIns. This probably represents synthesis *de novo* in this rapidly growing cell, and fractionation of the molecular species involved in turnover in stimulated cells was not attempted. In contrast, Luthra & Sheltawy (1976), using guinea pig cerebral hemispheres, found that tetraenoic PtdIns was more highly labelled than other PtdIns molecular species with [³²P]P_i during a 5 min incubation, similar to our findings in GH₃ cells. Likewise, Sugiura & Waku (1984) observed enhanced [³²P]P_i labelling of tetraenoic PtdIns and PA in pig lymphocytes stimulated with concanavalin A. Sasaki & Hasegawa-Sasaki (1985), using a human T-cell leukaemia line labelled to equilibrium with [³H]glycerol, also noted an increase in [³H]PA and [³H]DAG species containing stearate/arachidonate upon stimulation with phytohaemagglutinin. Although no change was noted in [³H]PtdIns, stimulation was for only 2 min and under the labelling conditions employed any increased tetraenoic species would have been masked by the large background of labelled tetraenoic PtdIns. Assuming that PtdIns is similarly enriched with arachidonic acid, the above work suggests that while there may be no selectivity for tetraenoic PtdIns *per se*, the major species turning over is tetraenoic PtdIns, in agreement with our results in GH₃ cells.

Mahadevappa & Holub (1983) reported a preferential hydrolysis of arachidonate-containing species upon thrombin stimulation in platelets. Their data showed that 81% of the degraded PtdIns was tetraenoic. However, at

least 71% of platelet PtdIns is tetraenoic (Mahadevappa & Holub, 1982). Therefore, it appears that little actual selectivity occurred, similar to our findings in GH₃ cells. Furthermore, purified phospholipase C from platelet cytosol does not discriminate between PtdIns containing [³H]oleate or [1-¹⁴C]arachidonate (Chau & Tai, 1982). Studies *in vitro* at other points along the PtdIns cycle also show little selectivity for the tetraenoic species. Holub & Piekarski observed no preferential conversion of DAG into PA in rat brain (Holub & Piekarski, 1978) or guinea pig brain (Holub & Piekarski, 1979) microsomes when the DAG contained stearate/arachidonate as opposed to other fatty acids. Early work showed that CDP-DAG containing unsaturated fatty acid in the *sn*-2 position enhanced inositol incorporation into PtdIns (Bishop & Strickland, 1970). However, Holub & Piekarski (1976) subsequently found that newly synthesized [¹⁴C]CDP-DAG formed from endogenously labelled [¹⁴C]PA closely resembled the unsaturation pattern of the [¹⁴C]PA, and in particular, only a very slight enrichment of tetraenoic CDP-DAG was observed.

The above results *in vitro* are consistent with our findings in GH₃ cells that the enzymes of the PtdIns cycle are not selective for fatty acid composition. However, our data show that arachidonic acid is preferentially incorporated into inositol phospholipids, leading to their enrichment with arachidonic acid as is widely observed in other systems (Holub & Kuksis, 1978). This process probably occurs via an acyltransferase mechanism apart from the PtdIns cycle itself. The preferential incorporation and enrichment with arachidonate is then apparently responsible for the stimulated production of DAG species highly enriched in arachidonate, and the subsequent reincorporation of these tetraenoic species into inositol phospholipids. In GH₃ cells, the turnover of these tetraenoic species occurs without the generation of unesterified intracellular arachidonate or eicosanoid production. This suggests that turnover of tetraenoic PtdIns, or the tetraenoic DAG that are formed in this process, may have other vital cellular functions.

These studies were supported by grants AM 28516 and HL 14230 from the National Institutes of Health.

REFERENCES

- Albert, P. R. & Tashjian, A. H. Jr. (1984) *J. Biol. Chem.* **259**, 5827–5832
- Allan, D. & Cockcroft, S. (1983) *Biochem. J.* **213**, 555–557
- Baker, R. R. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270**, 489–503
- Bell, R. L. & Majerus, P. W. (1980) *J. Biol. Chem.* **255**, 1790–1792
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Bishop, H. H. & Strickland, K. P. (1970) *Can. J. Biochem.* **48**, 269–277
- Canonica, P. L., Cronin, M. J. & MacLeod, R. M. (1985a) *Life Sci.* **36**, 997–1002
- Canonica, P. L., Judd, A. M., Koike, K., Valdenegro, C. A. & MacLeod, R. M. (1985b) *Endocrinology (Baltimore)* **116**, 218–225
- Chalvardjian, A. & Rudnicki, E. (1970) *Anal. Biochem.* **36**, 225–226
- Chau, L. Y. & Tai, H. H. (1982) *Biochim. Biophys. Acta* **713**, 344–351
- Chilton, F. H., Ellis, J. M., Olson, S. C. & Wykle, R. L. (1984) *J. Biol. Chem.* **259**, 12014–12019

- Drummond, A. H., Bushfield, M. & MacPhree, C. H. (1984) *Mol. Pharmacol.* **25**, 201–208
- Fine, J. B. & Sprecher, H. (1982) *J. Lipid Res.* **23**, 660–663
- Gershengorn, M. C. & Thaw, C. (1983) *Endocrinology* (Baltimore) **113**, 1522–1524
- Gitler, C. (1972) *Anal. Biochem.* **50**, 324–325
- Hinkle, P. A. & Tashjian, A. H. Jr. (1973) *J. Biol. Chem.* **248**, 6180–6186
- Hinkle, P. A., Lewis, D. G. & Greer, T. L. (1980) *Endocrinology* (Baltimore) **106**, 1000–1005
- Holub, B. J. & Kuksis, A. (1971) *J. Lipid Res.* **12**, 510–512
- Holub, B. J. & Kuksis, A. (1978) *Adv. Lipid Res.* **16**, 1–125
- Holub, B. J. & Piekarski, J. (1976) *Lipids* **11**, 251–257
- Holub, B. J. & Piekarski, J. (1978) *J. Neurochem.* **31**, 903–908
- Holub, B. J. & Piekarski, J. (1979) *Lipids* **14**, 309–311
- Holub, B. J., Kuksis, A. & Thompson, W. (1970) *J. Lipid. Res.* **11**, 558–564
- Hong, S. L. & Deykin, D. (1981) *J. Biol. Chem.* **256**, 5215–5219
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Jolles, J., Zwiers, H., Dekker, A., Wirtz, K. W. A. & Gispén, W. H. (1981) *Biochem. J.* **194**, 283–291
- Kolesnick, R. N. & Gershengorn, M. C. (1984) *J. Biol. Chem.* **259**, 9514–9519
- Luthra, M. G. & Sheltawy, A. (1976) *J. Neurochem.* **27**, 1503–1511
- McKean, M. L., Smith, J. B. & Silver, M. J. (1981) *J. Biol. Chem.* **256**, 1522–1524
- MacPhree, C. H. & Drummond, A. H. (1984) *Mol. Pharmacol.* **25**, 193–200
- Mahadevappa, V. G. & Holub, B. J. (1982) *Biochim. Biophys. Acta* **713**, 73–79
- Mahadevappa, V. G. & Holub, B. J. (1983) *J. Biol. Chem.* **258**, 5337–5339
- Marshall, P. J., Dixon, J. F. & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3292–3296
- Martin, T. F. J. (1983) *J. Biol. Chem.* **258**, 14816–14822
- Morrison, W. R. & Smith, L. M. (1964) *J. Lipid Res.* **5**, 600–608
- Nishizuka, Y. (1984) *Nature* (London) **308**, 693–698
- Rebecchi, M. J. & Gershengorn, M. C. (1983) *Biochem. J.* **216**, 287–294
- Rebecchi, M. J., Kolesnick, R. N. & Gershengorn, M. C. (1983) *J. Biol. Chem.* **258**, 227–234
- Rittenhouse-Simmons, S. (1981) *J. Biol. Chem.* **256**, 4153–4155
- Sasaki, T. & Hasegawa-Sasaki, H. (1985) *Biochim. Biophys. Acta* **833**, 316–322
- Schacht, J. (1981) *Methods Enzymol.* **72**, 626–631
- Sugiura, T. & Waku, K. (1984) *Biochim. Biophys. Acta* **796**, 190–198
- Thomas, J. M. F., Hullin, F., Chap, H. & Douste-Blasy, L. (1984) *Thrombosis Res.* **34**, 117–123
- Turk, J., Colca, J. R., Kotagal, N. & McDaniel, M. L. (1984) *Biochim. Biophys. Acta* **794**, 125–136
- Turk, J., Colca, J. R. & McDaniel, M. L. (1985) *Biochim. Biophys. Acta* **834**, 23–36
- Waku, K., Shibata, T., Kato, H., Tsutsui, K. & Nakazawa, Y. (1982) *Biochim. Biophys. Acta* **710**, 39–44

Received 21 October 1985/17 December 1985; accepted 10 January 1986