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Thyrotropin-Releasing Hormone Stimulates Rapid Breakdown of Phosphatidylinositol 4,5-bisphosphate and Phosphatidylinositol 4-phosphate in GH_3 Pituitary Tumor Cells

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SUMMARY

Thyrotropin-releasing hormone (TRH) induced a rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5P_2) and phosphatidylinositol 4-phosphate (PtdIns 4P) in GH_3 cells labeled to isotopic equilibrium with [^{3}H]inositol. Within 10 sec of the addition of TRH (1 μM), there was a maximal 60% decrease in PtdIns 4,5P_2 and 40% decrease in PtdIns 4P. Breakdown of phosphatidylinositol (PtdIns) occurred only after a lag of 30 sec. While the reduced levels of the polyphosphoinositides had almost returned to control values by 5 min, the GH_3 cell PtdIns content remained at around 85% of controls for at least 2 hr. Both phosphatidic acid (PA) and 1,2-diacylglycerol levels increased in response to TRH in [^{32}P]PO_4- and [^{3}H]glycerol-labeled GH_3 cells. 1,2-Diacylglycerol accumulated in a biphasic manner with an early peak 10 sec after addition of the peptide. This early rise in 1,2-diacylglycerol levels coincided in time and was equivalent in lipid mass with the decrease in the polyphosphoinositide content, suggesting the involvement of a phospholipase C-type enzyme. 1,2-Diacylglycerol levels subsequently fell toward control values and, after 3 min of treatment with TRH, rose again to levels 50% above normal. PA levels reached a peak value approximately 2-fold above normal 1 min after the addition of TRH. At all times after TRH addition, the bulk of the inositol phospholipid lost was recovered as 1,2-diacylglycerol. These results suggest that TRH stimulates a cycle of events in which the breakdown of the polyphosphoinositides, PtdIns 4,5P_2 and, perhaps, PtdIns 4P by a phospholipase C enzyme could be the initiating event.

INTRODUCTION

All receptors which elicit Ca^{2+}-dependent responses share the common ability to enhance the breakdown of PtdIns,[1] an anionic phospholipid. This observation led Michell (1) in 1975 to postulate that the degradation of PtdIns is associated in some causative manner with the mobilization of cellular Ca^{2+}. More recently this view has been enlarged to include the possibility that the breakdown of the polyphosphoinositides, PtdIns 4,5P_2 and PtdIns 4P, may be the initial response to receptor activation (2). The polyphosphoinositides are synthesized by the sequential phosphorylation of PtdIns by the enzymes PtdIns kinase and PtdIns 4P kinase, and are thought to be largely confined to the plasma membrane (1). A specific phosphodiesterase has been reported to exist which acts on both PtdIns 4,5P_2 and PtdIns 4P with equal facility to form 1,2-diacylglycerol and to liberate inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate, respectively (3). Both 1,2-diacylglycerol and PA, formed from 1,2-diacylglycerol by diglyceride kinase, have been implicated as possible second messengers. A calcium-dependent protein kinase has been described whose affinity for Ca^{2+} is greatly enhanced by 1,2-diacylglycerol (4). Moreover, it has been proposed that PA, a putative calcium inophore (5), could gate the entry of calcium following receptor stimulation by Ca^{2+}-mobilizing agonists (6). Much of the cellular PtdIns contains the unsaturated fatty acid, arachidonate, at position 2. A further possible link between receptor occupation, PtdIns metabolism, and cellular response is the finding that 1,2-diacylglycerol and PA can supply arachidonate for prostaglandin biosynthesis (7, 8).

TRH has been shown to induce the release of prolactin from GH_3 pituitary tumor cells by a Ca^{2+}-dependent mechanism (9). Recent studies from a number of labo-

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[1] The abbreviations used are: PtdIns 4,5P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns 4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdC, phosphatidylcholine; PtdE, Phosphatidylethanolamine; PA, phosphatidic acid; TRH, Thyrotropin-releasing hormone; BSS, balanced salt solution; TLC, thin-layer chromatography; MEM, minimal essential medium (Earle's salts).

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ratories, including our own, indicate that this is associated with an increased turnover of PtdIns (10–13) and a net accumulation of PA (10, 14) and 1,2-diacylglycerol (14). The aim of this study was to compare the rates of breakdown of the three inositol-containing phospholipids with the rates of accumulation of PA and 1,2-diacylglycerol in GH_3 cells stimulated by TRH. These findings strongly suggest that one of the earliest cellular responses to TRH is the breakdown of PtdIns 4,5P_2 and PtdIns 4P by a phospholipase C-type enzyme.

EXPERIMENTAL PROCEDURES

Materials. TRH was obtained from Calbiochem-Behring, C.P. Laboratories (Bishops Stortford, England). Lipid standards were from Sigma (London, England). *myo*-[2-^{3}H]Inositol (16.4 Ci/mmole) and [2-^{3}H]glycerol (1 Ci/mmole) were from Amersham International (Amersham, England). [^{32}P]Orthophosphate, carrier-free, was from the Western Infirmary, Glasgow, Scotland. All cell culture reagents and materials were from Gibco-Europe (Paisley, Scotland) or Flow Laboratories (Irvine, Scotland). Silica gel 60 HP-TLC plates (Merck) were from MacFarlane Robson (Glasgow, Scotland). Silica gel G plates (Polygram) were purchased from Camlab (Cambridge, England). All organic solvents and other laboratory reagents were analytical-grade.

Cell culture. GH_3 cells, a clonal strain of rat pituitary tumor cells secreting growth hormone and prolactin (15), were obtained from Flow Laboratories (Irvine, Scotland). Cells were grown in monolayer culture at 37° under 95% air/5% CO_2 in either Ham's F10 medium or MEM supplemented with donor horse serum (15%), fetal calf serum (2.5%), penicillin (100 units/ml), and streptomycin (100 μg/ml). GH_3 cells were seeded at 2–5 × 10^5 cells per 90-mm Petri dish in 10 ml of culture medium and allowed to grow for 8–12 days. The medium was changed every 3 days. Two general types of experiment were performed: equilibrium studies and incorporation studies. In the equilibrium studies, the desired radioactive precursor, e.g., [^{32}P]orthophosphate, was added to the growth medium (10 ml) on the day of the last medium change. After a further 2 days of growth, the [^{32}P]orthophosphate (10 μCi/dish) had reached isotopic equilibrium with the phospholipids and cellular ATP (see Results). As a consequence, changes in phospholipid radioactivity were taken as reflecting changes in the absolute cellular content of the phospholipid. [^{3}H]Inositol (5 μCi/dish) and [^{3}H]Glycerol (50–100 μCi/dish) were added to the growth medium in a similar manner. With these latter two precursors, however, a prelabeling period of 3 days was found necessary to allow equilibration with their endogenous counterparts (data not shown). For the incorporation studies, 2–3 days after the final medium change, [^{32}P]orthophosphate (10 μCi/5 ml) was added 30 min prior to TRH addition. Long-term incubations (≤120 min) were performed on monolayer cells in Petri dishes still containing normal growth medium from the final medium change. Short-term (≤5 min) incubations were performed on cells suspended in BSS [135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose; 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.1% (w/v) bovine serum albumin (pH 7.4, 37°)] (11). Cells in suspension were prepared on the day of the experiment by first removing the cells from the substrate, using a rubber policeman, and then washing them three times by centrifugation (200 × g for 3 min at 25°)/resuspension in BSS at 37°. The cells were finally resuspended in BSS at a protein concentration of 0.4–0.8 mg/0.5-ml aliquot. In one instance, incorporation studies were carried out on cells suspended in BSS. On this occasion the [^{32}P]orthophosphate (15 μCi/ml) was added during the final resuspension, and 15 min later, TRH was added.

Phospholipid analysis. Cells in suspension (at 37°) were split into two portions, one of which received TRH (1 μM). Aliquots (0.5 ml) were then removed from control or TRH-treated cells at the appropriate time and immediately added to glass test tubes containing 1.5 ml of chloroform/methanol (1:2). Lipids were extracted using acid chloroform/methanol by the method of Schacht (16). For monolayer cells, the available Petri dishes were divided into two groups, control or TRH-treated, and replaced in the incubator at 37°. At the appropriate times thereafter the incubations were terminated by placing the dishes on ice and adding 1 volume of ice-cold Hanks' solution. The monolayers were scraped, the cells were centrifuged (500 × g for 5 min at 4°), and the resulting pellets were washed once more with ice-cold Hanks' solution. Finally, 0.5 ml of (10% w/v) ice-cold trichloroacetic acid was added to the cell pellet, and phospholipids were extracted by the method of Griffin and Hawthorne (17). A portion of the trichloroacetic acid-soluble fraction was also counted for radioactivity.

Extracted GH_3 lipids were dried at 40° under N_2, redissolved in 0.1 ml of chloroform/methanol (9:1, v/v), and stored at −20° until separation. [^{3}H]Inositol-containing phospholipids were separated on oxalate-impregnated HP-TLC plates (20 cm × 10 cm) following the procedure of Jolles *et al.* (18) but running the plates in the longer dimension. An unknown, non-inositol-containing, phospholipid comigrated with PtdIns 4P in this system. Thus, TRH-mediated changes in the metabolism of PtdIns 4P using this procedure were reliable only with [^{3}H]inositol as the radioactive label. 1,2-Diacylglycerol, 1,3-diacylglycerol, and triacylglycerols were separated on activated silica gel G thin-layer plates (0.25 mm thick, 20 cm × 20 cm) by the method of Calderon *et al.* (19). All other phospholipids (including PtdIns) were separated by a rapid 2-dimensional TLC method on silica gel G plates (0.25 mm, 10 × 10 cm) as described by Yavin and Zutra (20). In all cases, lipids were identified by comparison with reference standards using I_2 vapor and (for ^{3}H) by using a radiochromatogram scanner (Packard Model 7230, Reading, England) or (for ^{32}P) autoradiography on Ilford 25 EP X-ray film (24 hr). Spots/bands were scraped, and incorporated radioactivity was quantified by liquid scintillation spectrometry.

Protein and phosphorus determination. Proteins were determined by the method of Lowry *et al.* (21), using bovine serum albumin (Miles) as standard. In experiments using suspension cells, separate 0.5-ml aliquots were taken to determine the cellular protein content. These aliquots were washed twice by centrifugation (200 × g for 3 min)/resuspension in 0.9% (w/v) saline to remove the 0.1% (w/v) bovine serum albumin present in the BSS. Phospholipid spots were assayed for phosphorus content by the method of Bowyer and King (22).

Statistics. Statistical analysis was performed by Student's t-test.

RESULTS

GH_3 Cell Phospholipid Content

The data in Table 1 compare the phospholipid composition of GH_3 cells as measured by inorganic phosphorus content or as ^{32}P radioactivity following a 48-hr prelabeling with [^{32}P]PO_4. The percentage distribution of each phospholipid was independent of the measuring technique employed. This suggests that, 48 hr after adding the isotope to GH_3 cells, the diester phosphate groups of the phospholipids have come to isotopic equilibrium with the γ-phosphate of ATP. Changes in the radioactive labeling of phospholipids following a 48-hr prelabeling period with [^{32}P]phosphate reflect, therefore, proportional changes in the cellular content of the phospholipids.

PtdC and PtdE together constitute approximately 66% of the total phospholipid content of GH_3 cells. PtdIns comprises around 10.5% of the phospholipid distribution and PA only 1% (Table 1).

Long-Term Studies

Effect of TRH on GH_3 cell PtdIns and PA metabolism. Within 10 min of TRH (1 μM) addition, incorporation of [^{32}P]PO_4 into PtdIns and PA was markedly enhanced (5- and 3-fold, respectively, above controls) (Fig. 1A). Incor-

TABLE 1

Phospholipid composition of GH_3 cells

GH_3 cells were grown for 8 days in MEM and fed twice in the interim. Monolayer cells were used; phospholipid [^{32}P]PO_4-derived radioactivity, following a 48-hr prelabeling, and phospholipid phosphorus content were measured as described under Experimental Procedures. Each value represents the mean from three experiments. Values for GH_3 cell phosphatidylcholine content (± standard error of the mean) were 148.1 ± 6.2 nmoles/10^7 cells and 244,000 ± 39,000 cpm/10^7 cells.

Phospholipid	% Distribution	
	Measured as inorganic phosphorus	Measured by 48-hr prelabeling with [^{32}P]PO_4
Phosphatidylcholine	47.7	46.1
Phosphatidylethanolamine	18.6	19.9
Lysophosphatidylethanolamine and sphingomyelin	10.3	11.5
Phosphatidylinositol	10.5	10.8
Phosphatidylserine	4.0	3.8
Lysophosphatidylcholine	2.1	2.4
Cardiolipin	2.2	1.5
Phosphatidylglycerol	1.2	0.9
Phosphatic acid	1.2	0.7
Others	2.2	2.4

poration into all other identifiable phospholipids was unaffected by TRH at this time (data not shown). At 30 min, the effect appeared to be maximal (10.5- and 4-fold increased incorporation into PtdIns and PA, respectively, versus controls) and by 2 hr the TRH-treated cells showed a rate of [^{32}P]PO_4 incorporation into PtdIns that was slowing, reflecting the fact, perhaps, that a substantial proportion of the cellular PtdIns was now labeled (see below). At incubation times beyond 10 min, there was a small (1.5- to 2.5-fold) TRH-induced increase in incorporation of [^{32}P]PO_4 into many other phospholipids. This occurred later than the effects of PtdIns and PA and coincided with a concentration-dependent enhancement by TRH of [^{32}P]PO_4 uptake into the trichloroacetic acid-soluble compartments of the cell (ref. 11, and data not shown).

The data in Fig. 1B show the effect of TRH (1 μM) on PtdIns and PA levels in GH_3 cells prelabeled to isotopic equilibrium with [^{32}P]PO_4. TRH caused a fall in the level of PtdIns (10–20%) which was apparent by 10 min but only significant after a 2-hr incubation. An approximate 2-fold accumulation of PA occurred concomitantly with the fall in PtdIns. This increase was obtained within 10 min and was maintained for at least 120 min. All other phospholipids which could be identified using the 2-dimensional TLC system (this does not include PtdIns 4,5P_2 and PtdIns 4P) were unaffected by TRH addition (data not shown).

The relative specific radioactivities for both PtdIns and PA could be calculated for the time course studies (Fig. 1C) by combining the data shown in Fig. 1A and B. Basal PtdIns specific radioactivity slowly rose over the 2-hr time course, presumably reflecting both the increase in specific radioactivity of [γ-^{32}P]ATP and basal turnover of PtdIns. When TRH was added, the specific radioactivity rapidly rose to a value indicating that at least 19% of cellular PtdIns had turned over after 30 min. After 2 hr, the specific radioactivity of the [^{32}P] PtdIns in the TRH-treated cells demonstrated that at least half of the cellular PtdIns had become involved in the PtdIns response. Unstimulated PA specific radioactivity tended to rise faster during the 30-min prelabeling period than during the 2-hr incubation period (Fig. 1C). This is likely to result from the presence of at least two different cellular pools of PA which label with [^{32}P]PO_4

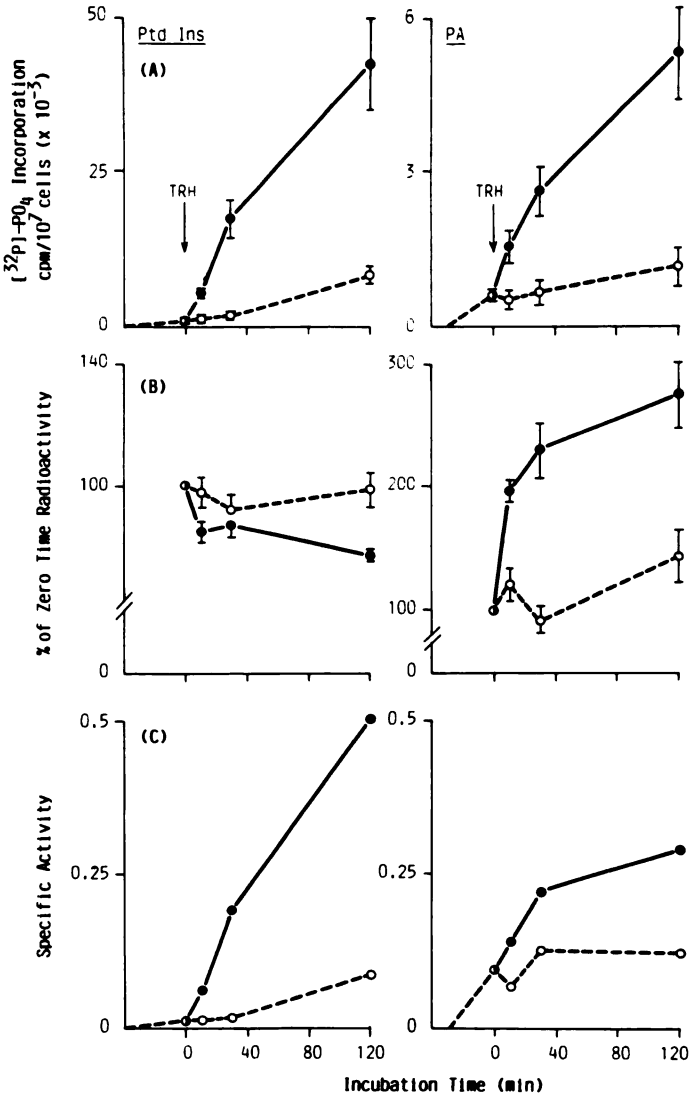

FIG. 1. *Effect of TRH on GH_3 cell PtdIns and PA metabolism*

A, Incorporation study; B, equilibrium study; C, specific activity. GH_3 cells were grown for 8 days in MEM and fed twice in the interim. Monolayer cells were used; [^{32}P]PO_4 incorporation and equilibrium studies were carried out as described under Experimental Procedures. Note that the specific activity of the [^{32}P]PO_4 added to the Petri dishes in the incorporation studies was twice that used in the equilibrium studies. This difference has been taken into account in calculating the data shown in C. A and B values are expressed as means ± standard error of the mean from three experiments, each performed in duplicate. Each point in C represents the mean values shown in A divided by those shown in B. For the equilibrium study, zero-time radioactivities for PtdIns and PA were 50,800 ± 7,800 and 3,300 ± 1,030 cpm/10^7 cells, respectively. O– – –O, Control; ●——●, 1 μM TRH.

at different rates. The addition of TRH caused an initial rapid increase in PA specific radioactivity during the first 30 min which appeared to plateau thereafter. Unlike PtdIns, only around 25% of total cellular PA was involved in the TRH response after 2 hr. The calculated specific radioactivities (Fig. 1C) represent minimal estimates of the proportion of cellular phospholipid involved in the "PtdIns cycle," since at the early time points in the turnover experiments (Fig. 1A) the γ-phosphate of ATP would not be in equilibrium with the added [^{32}P] PO_4.

Monolayer cells versus suspension cells. In order to facilitate the measurement of small changes in phospholipid content and to improve the speed with which the reaction was terminated, especially at early time points, cells suspended in BSS were used for subsequent experiments. This methodological change did not markedly affect cellular responsiveness. In monolayers, after 30 min, TRH increased [^{32}P]PO_4 incorporation into PtdIns 11-fold above controls, and in suspension cells the increase was 16-fold. In both cases, maximal stimulation by TRH was observed at 30 min and by 2 hr the cells showed a rate of incorporation approaching control values (data not shown).

Short-Term Studies

Effect of TRH on inositol phospholipid composition. TRH induced the breakdown of all three inositol-containing phospholipids in GH_3 cells (Fig. 2). The levels of PtdIns 4,5P_2 and PtdIns 4P declined rapidly, reaching a minimum at 10 sec, the earliest time measured. PtdIns 4,5P_2 decreased to 40% of control levels, then gradually recovered to near-normal around 5 min. Only 60% of the unstimulated level of PtdIns 4P remained at 10 sec, and the minimum was maintained over a 4-min period before returning toward normal by 5 min (Fig. 2). In contrast, breakdown of PtdIns was not observed in the first 30 sec after TRH stimulation. Thereafter, however, its rate of degradation increased over the following 3 min to give a PtdIns content which was only 85% of normal. This diminution of PtdIns content is similar to that observed on TRH addition in the experiment shown in Fig. 1B. However, the increased accuracy obtained with suspension cells permitted the detection of statistically significant differences at earlier times (2–3 min).

Effect of TRH on triacylglycerol, 1,2-diacylglycerol, and PA content. TRH caused no significant change in the levels of triacylglycerol in GH_3 cells (Fig. 3). However, it

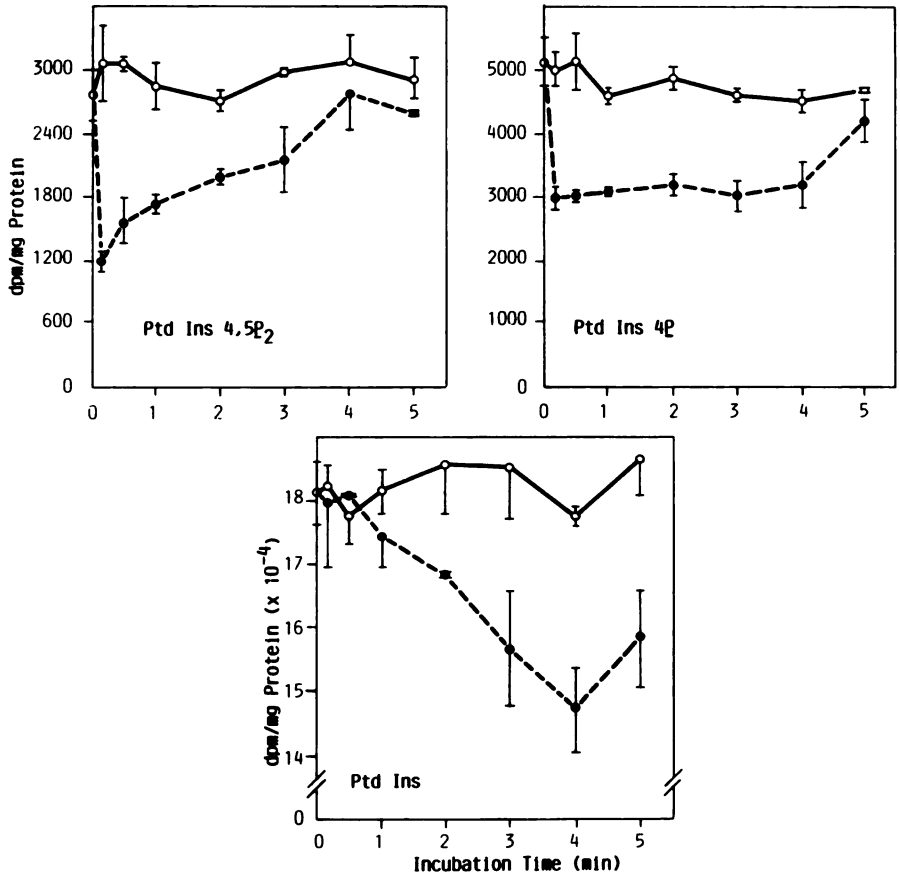

FIG. 2. *Effect of TRH on inositol phospholipid composition of GH_3 cells*

GH_3 cells were grown for 12 days in Ham's F10 medium and fed three times in the interim. Cells suspended in BSS, which had been prelabeled to equilibrium with [^{3}H]inositol (5 μCi/dish), were used. For experimental details see Experimental Procedures. The data represent means ± standard error of the mean for a representative experiment carried out in triplicate and replicated once. O——O, Control; ●– – –●, 1 μM TRH.

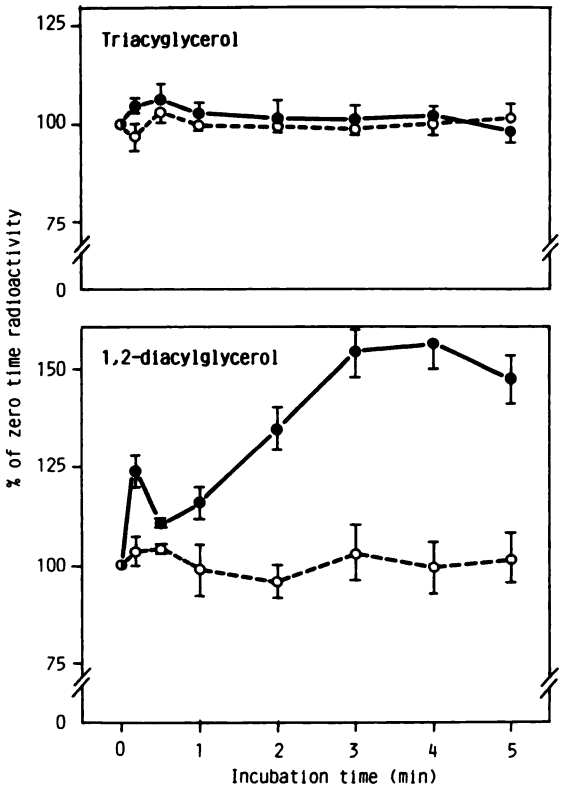

FIG. 3. *Effect of TRH on GH_3 cell triacylglycerol and 1,2-diacylglycerol content*

GH_3 cells were grown for 12 days in Ham's F10 medium and fed three times in the interim. Cells suspended in BSS, which had been prelabeled to equilibrium with [^{3}H]glycerol (50 μCi/dish) were used. For experimental details see Experiment Procedures. The data represent means ± standard error of the mean for two experiments, each performed in duplicate. The zero-time radioactivities for triacylglycerol and 1,2-diacylglycerol, in disintegrations per minute per milligram of protein, were 70,400 ± 10,700 and 3,450 ± 730, respectively. O– – –O, Control; ●——●, 1 μM TRH.

did provoke a biphasic change in the 1,2-diacylglycerol content which was reproducible. Within 10 sec of TRH addition, 1,2-diacylglycerol levels rose to an initial peak value 24% above control levels. This level then fell to a value of 11% above control levels at 30 sec before steadily rising again over the next 2.5 min to a new peak of 50% above controls (Fig. 3). This level was maintained for a further 2 min.

As shown for monolayer cells (Fig. 1B), TRH stimulated an approximate 2-fold accumulation of PA in suspension cells (Fig. 4). The improved precision obtained with the suspension cells revealed that the increase in PA is rapid, being statistically significant with 30 sec.

Quantitative aspects of TRH-induced changes in cell lipid metabolism. TRH causes net changes in the levels of five GH_3 cell lipids: PtdIns 4,5P_2, PtdIns 4P, PtdIns, PA, and 1,2-diacylglycerol. The data presented above indicate that the time-course for these TRH-induced changes varies from lipid to lipid. In an effort to determine net changes in lipid levels, [^{3}H]glycerol was used to label all five lipids to isotopic equilibrium prior to TRH stimulation. Two time points, 10 sec and 5 min after TRH addition, were investigated (Table 2).

At 10 sec, the GH_3 cell contents of two of the five lipids were altered significantly in response to TRH. Levels of cell PtdIns, 4,5P_2 had declined to 56% of control levels, and the content of 1,2-diacylglycerol had risen 13% over controls (Table 2). The TRH-stimulated level of PtdIns 4P (84% of controls) undoubtedly would have been lower but for the presence of an unidentified phospholipid (see Experimental Procedures) which co-migrates with PtdIns 4P on the TLC system. PA levels also appeared to increase at 10 sec in response to TRH; however, this 13% rise above controls was not statistically significant. In terms of net lipid movement, >90% of the lipid lost from the inositol phospholipids at 10 sec was recovered as 1,2-diacylglycerol rather than as PA. No significant breakdown of PtdIns was observed with TRH at 10 sec, which is in agreement with the data shown in Fig. 2.

GH_3 cell PtdIns 4,5P_2, PtdIns, PA, and 1,2-diacylglycerol contents had significantly changed 5 min following TRH addition (Table 2). The decline in PtdIns 4,5P_2 content observed at 10 sec was far less marked 5 min after TRH treatment (only 24% reduced relative to control values). In contrast, both PA and 1,2-diacylglycerol levels rose significantly to 190% and 130% of control levels, respectively (Table 2). Again, although the net percentage rise above controls was greater for PA than for 1,2-diacylglycerol, >75% of the lipid mass recovered after inositol phospholipid breakdown was 1,2-diacylglycerol. Almost all of the augmentation in PA and 1,2-diacylglycerol levels at 5 min could be accounted for by the significant TRH-induced decline in PtdIns levels to 88% of control values (Table 2).

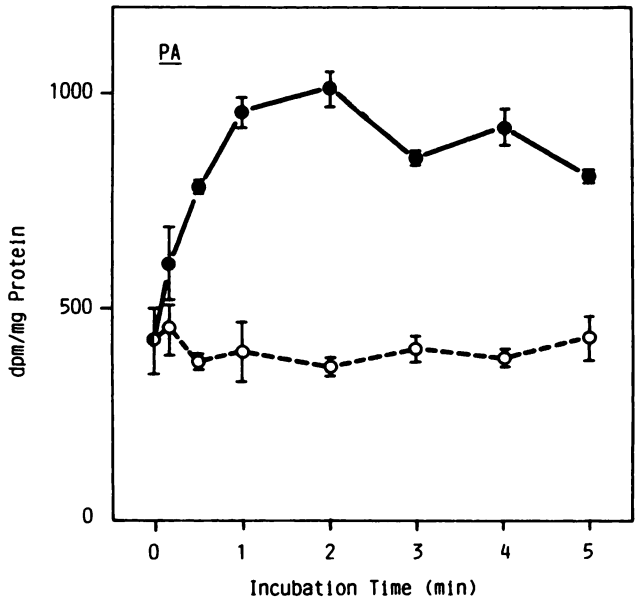

FIG. 4. *Effect of TRH on PA levels in GH_3 cells*

GH_3 cells were grown for 12 days in Ham's F10 medium and fed three times in the interim. Cells suspended in BSS, which had been prelabled to equilibrium with [^{32}P]PO_4 (10 μCi/dish), were used. For experimental details see Experimental Procedures. The data represent means ± standard error of the mean for a representative experiment carried out in triplicate, which was replicated twice. O– – –O, Control; ●——●, 1 μM TRH.

TABLE 2

Changes in lipid composition of GH_3 cells following incubation with TRH (1 μM)

GH_3 cells were grown for 12 days in Ham's F10 medium and fed three times in the interim, with [^{3}H]glycerol [100 μCi/dish (0.1% ethanol)] included in the last medium change. Cells suspended in BSS were used, and lipid radioactivity was quantified, as outlined under Experimental Procedures. Values are means ± standard error of the mean for a representative experiment carried out in quadruplicate. This experiment has been repeated with similar results.

Lipid	Incubation time				
	0 Min	10 Sec		5 Min	
	Control	Control	+1 μM TRH	Control	+1 μM TRH
			dpm/mg protein		
PtdIns	30300 ± 2200	30300 ± 1000	30000 ± 1900	30600 ± 1200	26900 ± 600[a]
PtdIns 4*P*	4790 ± 240	4780 ± 340	4030 ± 110	4670 ± 320	4570 ± 280
PtdIns 4,5P_2	880 ± 13	845 ± 48	472 ± 74[b]	876 ± 64	665 ± 24[a]
1,2-Diacylglycerol	13000 ± 1300	13000 ± 200	14700 ± 400[a]	13300 ± 500	17300 ± 400[b]
Phosphatidic acid	1200 ± 220	1390 ± 410	1570 ± 190	1360 ± 240	2580 ± 220[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

CDP-diacylglycerol, formed from PA, could not be detected on any autoradiograms of separated GH_3 cell lipid extracts. It must, therefore, be a quantitatively minor lipid with a very high turnover rate (data not shown).

DISCUSSION

In an effort to understand the molecular basis of receptor-mediated calcium mobilization, we and others have investigated the potential of GH_3 cells as a convenient model system. These findings demonstrate that TRH stimulates rapid changes in the metabolism of five GH_3 lipids: PtdIns, PtdIns 4*P*, PtdIns 4,5P_2, PA, and 1,2-diacylglycerol. All of these lipids are constituents of the same cycle of events (2) that is activated when TRH interacts with its plasma membrane receptor.

We confirm that the TRH-enhanced rates of [^{32}P]PO_4 incorporation into PtdIns and PA is a response to the net loss of PtdIns and net accumulation of PA (10, 14). These changes in phospholipid metabolism are the same for either GH_3 cell monolayers in normal growth medium or for GH_3 cell suspensions in BSS. By combining the long-term and short-term experiments we find that the TRH-induced breakdown of PtdIns occurs only after a lag of 30 sec. PtdIns levels then decline over the next 2–3 min to give eventually a PtdIns content which is 80–90% of normal. This lower level is maintained for at least 2 hr in the presence of TRH. The specific activity data demonstrate that by 2 hr at least 50% of the cellular PtdIns content has become involved in the TRH response. In contrast to the data of Rebecchi *et al.* (14), we reproducibly observe a lag period of approximately 30 sec before PtdIns levels begin to fall in TRH-treated GH_3 cells. This observation is discussed more fully in the subsequent paper. PA levels, on the other hand, rise rapidly to give a level 2-fold above controls. This level is attained within 0.5–1 min and is maintained for at least 2 hr, by which time at least 25% of GH_3 cell PA is involved. The specific radioactivity data indicate that a smaller proportion of the cellular PA than PtdIns is involved in the TRH-induced phospholipid response. This most probably indicates that cellular PA pools are heterogeneous in nature. This PA pool is likely to be located in the endoplasmic reticulum and plasma membrane. At these sites, PA acts as an intermediate in the resynthesis of the inositol-containing phospholipids which are degraded following the occupation of certain receptor types (1, 2), e.g., TRH receptors. Since PA possesses Ca^{2+} ionophoric properties (5), its accumulation could conceivably play an important role in mediating the TRH-induced Ca^{2+} mobilization in GH_3 cells. PA could gate the entry of Ca^{2+} from the extracellular medium down its concentration gradient through the plasma membrane and/or from cytoplasmic Ca^{2+}-sequestering organelles such as the endoplasmic reticulum. Some workers have suggested that, in GH_3 cells, TRH promotes influx of extracellular Ca^{2+} (23, 24), whereas others have proposed that the hormone mobilizes intracellular Ca^{2+} (25–28). Ronning *et al.* (28) have recently shown that TRH mobilizes Ca^{2+} from endoplasmic reticulum and mitochondria of GH_3 cells. These workers noted a rapid onset (<10 sec) and early maximum (<1 min) of the TRH effect, which was followed immediately by a recovery phase. Other researchers (23, 27) are in close agreement with the rapid and transient nature of the TRH effect on Ca^2 mobilization. The speed with which Ca^{2+} is mobilized in GH_3 cells tends to negate a role for PA as the Ca^{2+} ionophore mediating such Ca^{2+} movements. Certainly, the bulk of PA is formed too slowly to account for the early onset of the Ca^{2+} changes initiated by TRH stimulation. Moreover, PA levels remain elevated during and after the recovery phase of the Ca^{2+} signal. Thus, if these elevated levels of PA are still gating Ca^{2+}, compensatory, energy-dependent mechanisms for lowering the elevated intracellular Ca^{2+} levels would be required—a wasteful and unnecessary process for the cell.

The initial proposition that the breakdown of PtdIns is intimately involved in Ca^{2+} mobilization (1) has recently been modified in the light of findings that, in a number of tissues, the breakdown of the polyphosphoinositides, PtdIns 4,5P_2 and PtdIns 4*P*, precedes PtdIns breakdown (see ref. 29 for recent review). This study confirms the above findings inasmuch as the TRH-induced breakdown of PtdIns 4,5P_2 and PtdIns 4*P* in GH_3 cells is clearly faster than the decline in PtdIns. As far as can be determined from this experimental protocol, both polyphosphoinositides break down equally fast,

their contents reaching a minimum within 10 sec of TRH addition. The simultaneous appearance of inositol-1,4,5-trisphosphate, inositol-1,4-bisphosphate (30), and 1,2-diacylglycerol at 10 sec may be taken as evidence that the breakdown of PtdIns 4,5P_2 and PtdIns 4P occurs by the action of a phospholipase C-type enzyme. This contrasts with the recent proposal for the platelet (31) that the levels decrease either because of decreased synthesis of the polyphosphoinositides or by phosphomonoesterase action. One point which cannot be unequivocally answered by these data is the mechanism underlying the breakdown of PtdIns. It is, in theory, possible that the PtdIns does break down immediately after TRH addition but that this is masked by compensatory resynthesis. This is unlikely because (a) resynthesis is a relatively slow event (11, 13) and (b) chronic treatment with lithium ions, which block *myo*-inositol-l-phosphatase actually decreases the early (<30-sec) accumulation of inositol monophosphate resulting from TRH treatment (30). In fact, studies using lithium or reduced temperature (30) lead us to conclude that PtdIns is not a substrate for the phospholipase C enzyme(s) that cleaves PtdIns 4,5P_2 or PtdIns 4P. An interesting observation is that the decline in PtdIns occurs at a time when there is active resynthesis of PtdIns 4,5P_2 and PtdIns 4P, presumably from PtdIns. If initial rates of TRH-induced PtdIns 4,5P_2 and PtdIns 4P breakdown were to be maintained for 4 min, then the measured decrease in PtdIns could be entirely explained by conversion to PtdIns 4,5P_2 and PtdIns 4P. These findings suggest that the role of PtdIns may be, at least in part, as a precursor for the higher polyphosphoinositides, as suggested recently by Michell and co-workers (2). Further support for this idea comes from work cited in the following paper (30) on the effects of TRH on the [^{3}H]inositol metabolites in GH_3 cells.

Based on the fact that the polyphosphoinositides have a high affinity for Ca^{2+}, Kretsinger (32) has suggested that the breakdown of PtdIns 4,5P_2 is of sufficient magnitude to mobilize Ca^{2+} and initiate cellular responses. Quantitative analysis of the data in GH_3 cells indicates that this could, indeed be a possibility (see discussion in ref. 30 concerning the relevant calculation). It is certainly true that the maximal breakdown of PtdIns 4,5P_2 and PtdIns 4P precedes the maximal release of Ca^{2+} from superficially located sites in GH_3 cells (23).

The early accumulation of 1,2-diacylglycerol, 10 sec after the addition of TRH, is not maintained, and the level declines toward control at 30 sec before rising again to a new level. The fall in the GH_3 cell content of 1,2-diacylglycerol after 10 sec most likely reflects its conversion to PA by diglyceride kinase. The secondary rise in 1,2-diacylglycerol (>30 sec) coincides with a plateau in the cellular PA levels. The reason why PA does not continue to accumulate after 1 mn is not known, although an obvious explanation would be that the activity of diglyceride kinase declines. The coincidental fall in cellular PtdIns (>30 sec) can be explained by its utilization as a precursor for the resynthesis of the polyphosphoinositides, and the PtdIns lost at 5 min can be largely accounted for by the gain in 1,2-diaclyglycerol content. When the net lipid movement is expressed as 1,2-diacylglycerol gained/PA gained, values of 9.5 and 3.3 for 10-sec and 5-min TRH incubations are obtained, respectively. On quantitative grounds alone, then, a role for 1,2-diacylglycerol as a second messenger seems more likely than for PA. A mechanism by which 1,2-diacylglycerol could act has been recently suggested: it may serve as a signal for the transmembrane control of protein phosphorylation by activating a novel protein kinase (4, 33). This protein kinase has an absolute requirement for Ca^{2+} and phospholipid for its activation, and is referred to as protein kinase C. Drust *et al.* (34, 35) have reported that TRH exerts Ca^{2+}-dependent protein phosphorylation in GH_3 cells which is distinctively different from cyclic AMP-dependent phosphorylation induced by vasoactive intestinal polypeptide. Further work is necessary to elucidate whether such a protein kinase C exists and functions in the TRH-stimulated protein phosphorylation in GH_3 cells.

The results emphasize the utility of GH_3 cells as a model system for investigating the mechanisms governing receptor-mediated changes in inositol lipid metabolism and their relevance to calcium mobilization. A further advantage of the GH_3 cell system is that, unlike the situation found in a variety of tissues and cells, the intermediates of the "PtdIns cycle" do not appear to be subject to attack by enzymes such as phospholipase A_2, which, by releasing arachidonic acid, would remove the intermediate from the cycle (14).[2]

In conclusion, TRH stimulates the rapid breakdown of the polyphosphoinositides, PtdIns 4,5P_2 and PtdIns 4P, in GH_3 cells. The role of PtdIns in the response may simply be as a reservoir to fuel the resynthesis of the polyphosphoinositides.

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