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Thyroliberin stimulates rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phosphodiesterase in rat mammotropic pituitary cells

Evidence for an early Ca^{2+}-independent action

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Thyrotropin-releasing hormone (TRH; thyroliberin) stimulated rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by a phosphodiesterase (phospholipase C) in GH_3 cells, a prolactin-secreting rat pituitary tumour cell line. TRH caused a rapid decrease in the level of PtdIns(4,5)P_2 to 60% of control and stimulated a marked transient increase in inositol 1,4,5-trisphosphate, the unique product of phosphodiesteratic hydrolysis of PtdIns(4,5)P_2, to a peak of 410% of control at 15s. TRH also caused decreases in phosphatidylinositol 4-monophosphate (PtdIns4*P*) and phosphatidylinositol (PtdIns) to 65% and 93% of control at 15s respectively. Inositol 1,4-bisphosphate was increased to a peak of 450% at 30s; inositol 1-monophosphate and inositol were not elevated until 30s and 1min respectively after TRH addition. To study whether PtdIns(4,5)P_2 hydrolysis may be caused by an elevation in cytosolic Ca^{2+} concentration, the changes induced by TRH in the levels of inositol sugars were compared with the effects of membrane depolarization by high extracellular [K^+]. The elevation in cytosolic [Ca^{2+}] induced by K^+ depolarization did not change the level of inositol 1,4,5-trisphosphate. These data suggest that phosphodiesteratic hydrolysis of PtdIns(4,5)P_2 may be the initial event in TRH stimulation of inositol lipid metabolism in GH_3 cells and that PtdIns(4,5)P_2 hydrolysis is not stimulated by an elevation in cytosolic Ca^{2+} concentration. The decreases in PtdIns4*P* and PtdIns may be due to enhanced conversion of PtdIns into PtdIns4*P* into PtdIns(4,5)P_2 or to their direct hydrolysis by phosphomonoesterases and/or phosphodiesterases. These results are consistent with the hypothesis that TRH-stimulated PtdIns(4,5)P_2 breakdown causes Ca^{2+} mobilization leading to prolactin secretion

The mechanism(s) whereby ligands that interact with cell-surface receptors cause mobilization of cellular Ca^{2+} or stimulation of influx of extracellular Ca^{2+} or both, leading to elevation of the free cytosolic Ca^{2+} concentration, is not known (Rasmussen & Waisman, 1981). Several groups (Michell *et al.*, 1981; Michell, 1982*a*; Berridge, 1982) have recently suggested that these ligands may initiate their action by stimulating hydrolysis of PtdIns(4,5)P_2 by a specific phosphodiesterase (phospholipase C) which would serve to release membrane-bound Ca^{2+} and/or to activate channels within the plasma membrane and allow Ca^{2+} influx into cells. The decrease in the level of PtdIns commonly observed after stimulation by this class of ligand (Michell, 1975) is proposed to be due to conversion of PtdIns into PtdIns4*P* and then into PtdIns(4,5)P_2 by a kinase (or kinases). An important component of this hypothesis is that the initial event, phosphodiesteratic hydrolysis of PtdIns(4,5)P_2, should not be caused by the cytosolic [Ca^{2+}] elevation. Although a number of reports have documented decreases in PtdIns(4,5)P_2 and PtdIns4*P* during ligand stimulation (Downes & Michell, 1982), only in two cell types has the decrease in PtdIns(4,5)P_2 caused by ligand stimulation of intact cells been shown to be due to phosphodiesteratic hydrolysis of PtdIns(4,5)P_2 by

Abbreviations used: TRH, thyrotropin-releasing hormone (thyroliberin); PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

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the demonstration of an increase in the level of inositol 1,4,5-trisphosphate, its unique product (Akhtar & Abdel-Latif, 1980; Agranoff *et al.*, 1983). However, in rabbit iris smooth muscle (Akhtar & Abdel-Latif, 1980) PtdIns(4,5)P_2 hydrolysis appeared to be caused by an elevation in cytosolic Ca^{2+} concentration; the Ca^{2+}-dependency of PtdIns(4,5)P_2 hydrolysis in platelets was not studied (Agranoff *et al.*, 1983).

It has been observed (Rebecchi *et al.*, 1981; Drummond & MacPhee, 1981; Schlegel *et al.*, 1981; Sutton & Martin, 1982) that TRH stimulates ^{32}P-labelling of PtdIns and phosphatidic acid in GH_3 cells, a prolactin-secreting rat pituitary tumour cell line. More recently, we (Rebecchi *et al.*, 1983) provided evidence that TRH affected inositol lipid metabolism by stimulating a phosphodiesterase that resulted in a fall in the level of PtdIns and an increase in diacylglycerol, leading to formation of phosphatidic acid. On the basis of an increase in inositol 1-monophosphate, we suggested that the initial event in TRH-stimulated inositol lipid metabolism may be the hydrolysis of PtdIns by a specific phosphodiesterase; however, we considered the possibility that TRH stimulation may have been initiated by enhanced hydrolysis of PtdIns(4,5)P_2 and/or PtdIns4*P*.

In the present study, we have investigated whether TRH affects PtdIns(4,5)P_2 and PtdIns4*P* metabolism in GH_3 cells, whether this may have resulted from stimulation of a phosphodiesterase that hydrolyses PtdIns(4,5)P_2 and whether this action was dependent on Ca^{2+}. We find that TRH initially affects inositol lipid metabolism in GH_3 cells, at least in part, by stimulating a phosphodiesterase that hydrolyses PtdIns(4,5)P_2 to yield inositol 1,4,5-trisphosphate and that this reaction is not stimulated by an elevation of cytosolic [Ca^{2+}] induced by K^+ depolarization.

Experimental procedures

Materials

TRH was purchased from Beckman. Lipid standards were obtained from Sigma. T.l.c. plates (silica gel type G and silica gel type H; 0.25 mm thick) were from Analabs. Cyclohexylenedinitrilotetra-acetic acid was obtained from Eastman Kodak. AG 1X-2 resin was purchased from Bio-Rad. [^{32}P]P_i (carrier-free), *myo*-[2-^3H]inositol (sp. radioactivity 15 Ci/mmol), ^3H_2O and [^3H]inulin (270 mCi/g) were from New England Nuclear.

Cell studies

GH_3 cells were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% horse serum and 2.5% foetal-bovine serum as described by Tashjian *et al.* (1968) and Gershengorn *et al.* (1979). To determine the effect of TRH on lipids labelled with [^{32}P]P_i for less than 1 h, GH_3 cells were harvested with 0.02% EDTA, washed and incubated in a balanced salt solution (BSS), consisting of 135 mM-NaCl, 4.5 mM-KCl, 1.5 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 5.6 mM-glucose and 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, at $(1–3) \times 10^6$ cells/0.1 ml, also containing 0.5 mCi of [^{32}P]P_i/ml at 26°C. After 40 min, TRH was added (1 μM final concentration) and portions of the cell suspension were removed at the indicated times as described by Rebecchi *et al.* (1981). For experiments in which lipids were labelled to isotopic steady-state with [^{32}P]P_i, cells were incubated in growth medium supplemented with 10 μCi of [^{32}P]-P_i/ml for 48 h. For experiments with [^3H]inositol, GH_3 cells were incubated in growth medium containing 1 μCi of [^3H]inositol/ml for 48 h, a time that is sufficient to attain isotopic steady-state (Rebecchi *et al.*, 1983). Under these conditions, greater than 98% of lipid-soluble ^3H radioactivity is present in PtdIns, PtdIns4*P*, PtdIns(4,5)P_2 and lysophosphatidylinositol. After harvesting, cells were incubated in BSS [$(3–5) \times 10^6$ cells/0.1 ml] at 26°C. After 20 min, TRH was added and portions of the cell suspension were removed in triplicate at the indicated times. Medium 'without Ca^{2+}' was BSS carefully prepared to exclude Ca^{2+}; BSS without Ca^{2+} contained approx. 3 μM-Ca^{2+} as measured with a metallochromic dye (Rebecchi *et al.*, 1982). Medium containing 50 mM-K^+ was prepared by substituting KCl for NaCl in BSS.

Lipid analysis

Lipids were extracted from cells with 1 ml of chloroform/methanol/conc. HCl (10:10:1; by vol.) and 0.25 ml of 10 mM-EDTA solution. The upper and lower phases were separated and each phase was washed once in 1 ml of pre-equilibrated lower and upper phase respectively. Portions of the lower phase were dried on ice under N_2 to inhibit acid hydrolysis of lipids. Extractions were also performed using chloroform/methanol/conc. HCl (100:100:1, by vol.), which yielded similar results. The lipids were redissolved in ice-cold chloroform/methanol (1:1, v/v) and applied under N_2 to silica gel type G plates or to silica gel type H plates. Plates were developed in one dimension by sequential ascending chromatography in either chloroform/methanol/28% NH_3 (13:5:1, by vol.) followed by chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) for silica gel G or chloroform/methanol/4 M-NH_3 (9:7:2, by vol.) containing 2 mM-cyclohexylenedinitrilotetra-acetic acid followed by chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) for silica gel H. The R_F values for PtdIns(4,5)P_2, PtdIns4*P*, lysophosphatidylinositol and PtdIns were 0, 0.20, 0.47 and 0.81 respectively, on

the silica gel G system and 0.11, 0.29, 0.68 and 0.92 respectively, on the silica gel H system. In order to isolate [^{32}P]PtdIns from the other lipids, two-dimensional t.l.c. was used as previously described (Rebecchi *et al.*, 1981). As predicted, when lipids were extracted from GH_3 cells that had been labelled to isotopic steady-state with [^{32}P]P_i and [^{3}H]inositol, and isolated using either system, the ratios of $^{32}P/^3H$ radioactivity for the PtdIns(4,5)P_2 and PtdIns4P spots were 3.00 ± 0.22 and 2.04 ± 0.04 times the ratio in PtdIns respectively, thus confirming separation of the lipids and the identities of these spots. Lipid spots were visually detected by radioautography or I_2 staining of lipid standards; the I_2 was always allowed to sublime before scraping. Spots were scraped into vials to which 0.2ml of methanol/water/HCl (80:20:1, by vol.) and 6ml of liquid-scintillation fluid were added and radioactivity was determined.

Inositol sugar analysis

^{3}H-labelled inositol sugars were measured by anion-exchange chromatography by the method of Downes & Michell (1981). A portion of the upper phase of each sample was dried on ice under N_2 and resuspended in 0.12ml of 0.1M-formic acid; 0.1ml was applied to a freshly prepared 1ml column of resin (AG1 X-2; 200–400mesh), which had been swollen in 0.1M-formic acid containing unlabelled-5mM-*myo*-inositol, and the column was eluted in a stepwise fashion. Four peaks of ^{3}H radioactivity were identified corresponding to free inositol, inositol 1-monophosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate. The identities of these peaks were confirmed in the following manner. Cells were labelled with [^{32}P]P_i and [^{3}H]inositol, the lipids were extracted and [^{32}P,^{3}H]PtdIns(4,5)P_2, [^{32}P,^{3}H]-PtdIns4P and [^{32}P,^{3}H]PtdIns were isolated by t.l.c. as described above. Each inositol lipid was hydrolysed with acid by the method of Dawson & Dittmer (1961) and the corresponding water-soluble inositol sugar products were shown to elute from the anion-exchange column at the same positions as the inositol sugars isolated directly from GH_3 cells.

Statistical analysis was performed by *t* test.

Results and discussion

Fig. 1 illustrates the time course of the effect of TRH on [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P in GH_3 cells labelled with [^{32}P]P_i for 40min; under these conditions, phospholipids are not labelled to isotopic steady-state. TRH caused a rapid decrease by 15s in the levels of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P to 76% ($P < 0.01$) and 71% of control ($P < 0.025$) respectively. After 1min, [^{32}P]PtdIns-(4,5)P_2 and [^{32}P]PtdIns4P increased again at a rate similar to that in control cells. In agreement with our

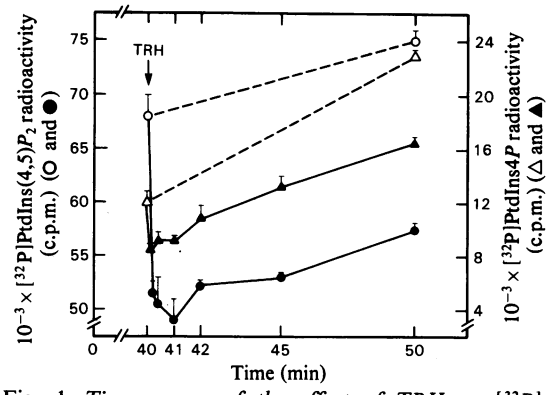

Fig. 1. *Time course of the effect of TRH on* [^{32}P]-*PtdIns(4,5)P_2 and* [^{32}P]*PtdIns4P in GH_3 cells*

Data are means ± S.D. of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P isolated from cells that had been pre-incubated in a balanced salt solution containing 0.5mCi of [^{32}P]P_i/ml for 40min and then incubated for various times in medium without (○ and △) or with 1μM-TRH (● and ▲). The results shown are representative of three experiments.

previous observations (Rebecchi *et al.*, 1981), the levels of [^{32}P]phosphatidic acid increased markedly by 15s after TRH addition and that of [^{32}P]PtdIns did not increase until after 2min (results not shown). The rapid decreases in [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P in GH_3 cells after TRH stimulation are similar to recent observations in rat hepatocytes stimulated by vasopressin (Kirk *et al.*, 1981; Thomas *et al.*, 1983) and in horse platelets stimulated by thrombin (Billah & Lapetina, 1982; Vickers *et al.*, 1982). In cells labelled to isotopic steady-state with [^{32}P]P_i, TRH caused a decrease in PtdIns(4,5)P_2 from 33 ± 2.3pmol/10^6 cells to 22 ± 1.1pmol at 30s ($P < 0.01$).

To determine whether the decreases in [^{32}P]-PtdIns(4,5)P_2 and [^{32}P]PtdIns4P induced by TRH were caused by losses of PtdIns(4,5)P_2 and PtdIns4P, cells were incubated with [^{3}H]inositol for 48h, a time that we (Rebecchi *et al.*, 1983) have shown previously is sufficient to label PtdIns to isotopic steady-state. The effect of TRH on ^{3}H-labelled inositol lipids was determined (Fig. 2). The relative content (means ± S.D.) of inositol lipids in cells prelabelled with [^{3}H]inositol was: PtdIns(4,5)-P_2, $2.5 \pm 1\%$; PtdIns4P, $2.8 \pm 1\%$; lysophosphatidylinositol, $6.2 \pm 1\%$; PtdIns, $88 \pm 1\%$. In a representative experiment, [^{3}H]PtdIns(4,5)P_2 radioactivity was 92 ± 2 c.p.m./10^6 cells, [^{3}H]PtdIns4P was 136 ± 2c.p.m., [^{3}H]lysophosphatidylinositol was 337 ± 54c.p.m. and [^{3}H]PtdIns was 4780 ± 155c.p.m. (all means ± S.E.M.). TRH stimulated rapid loss by 15s of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]-PtdIns4P to 60% ($P < 0.001$) and 65% of control ($P < 0.001$) respectively; the nadirs in the levels of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4P were

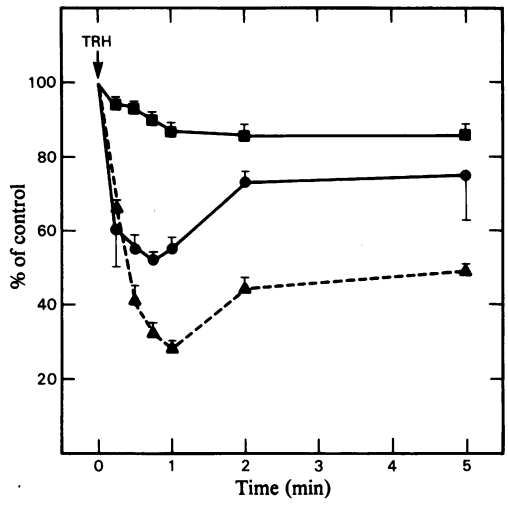

Fig. 2. *Time course of the effect of TRH on the levels of* [^{3}H]*PtdIns(4,5)*P_2 (●), [^{3}H]*PtdIns4P* (▲) *and* [^{3}H]*PtdIns* (■) *in* GH_3 *cells*

The data are means ± S.E.M. of triplicate determinations and are expressed as percentages of the levels of [^{3}H]PtdIns(4,5)P_2, [^{3}H]PtdIns4*P* or [^{3}H]PtdIns isolated from unstimulated cells (control). Cells were incubated in growth medium with 1 μCi of [^{3}H]inositol/ml for 48h and then incubated in a balanced salt solution without [^{3}H]inositol. After 20min (zero time), TRH (final concentration 1 μM) was added. In control cells, [^{3}H]PtdIns(4,5)P_2 radioactivity was 92 ± 2c.p.m./10^6 cells (means ± S.E.M.), [^{3}H]PtdIns4*P* was 136 ± 2c.p.m./10^6 cells and [^{3}H]PtdIns was 4780 ± 155c.p.m./10^6 cells. The results shown are representative of three similar experiments.

at 45s and 60s respectively. After 1min in the continued presence of TRH, the levels of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* increased but remained below control for at least 5min. Concomitant with the fall in [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* and in agreement with our previous observations (Rebecchi *et al.*, 1983), TRH caused a decrease in [^{3}H]PtdIns to 86% of control by 2min ($P<0.001$); there was no measurable change in the level of [^{3}H]lysophosphatidylinositol (results not shown). The effect of TRH to decrease [^{3}H]PtdIns(4,5)P_2 was concentration-dependent; half-maximal effect occurred with approx. 5nM-TRH (results not shown), a concentration similar to that needed for half-maximal loss of [^{3}H]PtdIns (Rebecchi *et al.*, 1983).

We have previously observed that TRH caused increases in the levels of inositol 1-monophosphate, diacylglycerol and phosphatidic acid in GH_3 cells and suggested that TRH may stimulate inositol lipid hydrolysis specifically by stimulating the activity of a phosphodiesterase (phospholipase C) which hydrolysed PtdIns (Rebecchi *et al.*, 1983). We also presented evidence that strongly suggested that the diacylglycerol and phosphatidic acid formed during TRH stimulation were derived from inositol lipids. However, we considered the possibility that TRH could have stimulated a phosphodiesterase specific for either PtdIns(4,5)P_2 or PtdIns4*P* because diacylglycerol can be formed by hydrolysis of PtdIns(4,5)P_2, PtdIns4*P* or PtdIns and inositol 1-monophosphate can be formed directly from the hydrolysis of PtdIns or by dephosphorylation from inositol 1,4-bisphosphate, a product of phosphodiesterase hydrolysis of PtdIns4*P*, or sequentially from inositol 1,4,5-trisphosphate, a product of phosphodiesterase hydrolysis of PtdIns(4,5)P_2. Hence it was important to monitor the levels of all the inositol sugars in GH_3 cells during TRH stimulation. In particular, inositol 1,4,5-trisphosphate was measured since it is the only sugar that is a specific reaction product in the metabolism of the inositol lipids; that is, inositol 1,4,5-trisphosphate appears only to be formed by hydrolysis of PtdIns(4,5)P_2 by a phosphodiesterase in mammalian cells.

The inositol sugars were measured in cells prelabelled to isotopic steady-state with [^{3}H]inositol. In unstimulated cells the relative content (means ± S.D.) of the inositol sugars was: [^{3}H]inositol 1,4,5-trisphosphate, 1.3 ± 0.1%; [^{3}H]inositol 1,4-bisphosphate, 3.9 ± 0.1; [^{3}H]inositol 1-monophosphate, 16 ± 0.3; [^{3}H]inositol, 78 ± 1.6%. In a representative experiment, [^{3}H]inositol 1,4,5-trisphosphate radioactivity was 31 ± 0.4c.p.m./10^6 cells, [^{3}H]inositol 1,4-bisphosphate was 96 ± 2c.p.m. [^{3}H]inositol 1-monophosphate was 404 ± 4c.p.m. and [^{3}H]inositol was 1930 ± 29c.p.m. The effect of TRH on the levels of ^{3}H-labelled inositol sugars is illustrated in Fig. 3. TRH stimulated a rapid, transient increase in [^{3}H]inositol 1,4,5-trisphosphate to 410% of control ($P<0.001$) at 15s; the level declined after 15s but was still above control after 5min. The level of [^{3}H]inositol 1,4-bisphosphate was also increased by 15s ($P<0.001$) and attained its highest level of 450% of control at 30s after TRH addition ($P<0.001$); its level declined after 30s but was above control at 5min. In contrast, [^{3}H]inositol 1-monophosphate was not elevated until 30s after TRH and [^{3}H]inositol until 1min after TRH addition; these findings are similar to our previous observations in which [^{3}H]inositol 1-monophosphate and [^{3}H]inositol were measured by a different method (Rebecchi *et al.*, 1983). In four separate experiments, the effects of TRH on the levels of [^{3}H]inositol sugars after 30s, expressed as percentages of unstimulated levels (means ± S.E.M.), were: [^{3}H]inositol 1,4,5,-trisphosphate, 246 ± 19 ($P<0.001$); [^{3}H]inositol 1,4-bisphosphate, 316 ± 21 ($P<0.001$); [^{3}H]inositol 1-monophosphate, 138 ± 7 ($P<0.001$); and [^{3}H]inositol, 100 ± 3 ($P>0.2$). These results demonstrate that TRH stimulates

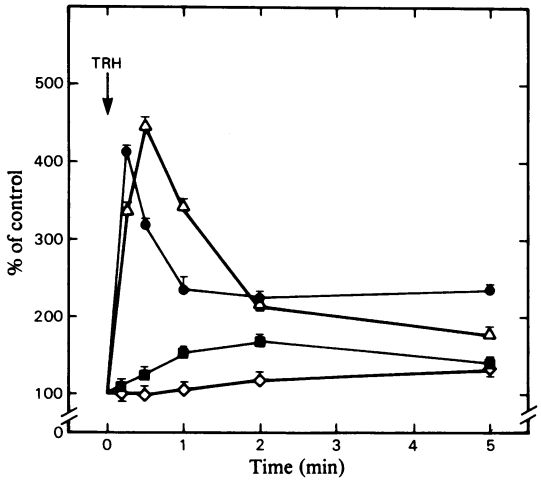

Fig. 3. *Time course of the effect of TRH on the levels of [^{3}H]inositol phosphate sugars in GH_3 cells*

Data are means ± S.E.M. of triplicate determinations and are expressed as percentages of the level of the corresponding inositol sugar isolated from unstimulated cells (control). Cells were incubated in a growth medium with 1 μCi of [^{3}H]inositol/ml for 48 h and then incubated in a balanced salt solution without [^{3}H]inositol. After 20 min (zero time), TRH (final concentration 1 μM) was added. The levels of inositol phosphate sugars in unstimulated cells expressed as ^{3}H c.p.m./5 × 10^6 cells (means ± S.E.M.) were: [^{3}H]inositol, 9660 ± 117; [^{3}H]inositol 1-monophosphate, 2020 ± 22; [^{3}H]inositol 1,4-bisphosphate, 481 ± 7; [^{3}H]inositol 1,4,5-trisphosphate, 154 ± 2. The results shown are representative of two similar experiments. Symbols: ●, [^{3}H]inositol 1,4,5-trisphosphate; △, [^{3}H]inositol 1,4-bisphosphate; ■, [^{3}H]inositol 1-monophosphate; ◇, [^{3}H]inositol.

inositol lipid metabolism, at least in part, by causing hydrolysis of PtdIns(4,5)P_2 by a phosphodiesterase (phospholipase C) and that the time course of [^{3}H]inositol 1,4,5-trisphosphate accumulation is consistent with the notion that PtdIns(4,5)P_2 hydrolysis is the initial event in TRH stimulation of inositol lipid metabolism. Because there is no evidence in mammalian cells that the levels of inositol 1,4-bisphosphate or inositol 1-monophosphate can be increased by ligand stimulation of synthesis *de novo*, accumulation of [^{3}H]inositol 1,4-bisphosphate and [^{3}H]inositol 1-monophosphate during TRH stimulation of GH_3 cells may have been due to either hydrolysis by a phosphodiesterase of PtdIns4*P* and PtdIns respectively, or dephosphorylation by a phosphatase of [^{3}H]-inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,4-bisphosphate respectively. In support of the second possibility, a phosphomonoesterase specific for the conversion of inositol 1,4,5-trisphosphate into inositol 1,4-bisphosphate has been recently described in human erythrocyte membranes by Downes *et al.* (1982).

A major question regarding stimulated inositol lipid metabolism in many cell types, and TRH-stimulated phosphoinositide metabolism in GH_3 cells, is whether the initial enzyme reaction that is induced by ligand is dependent on a rise in the free (or unbound) concentration of Ca^{2+} in the cytosol (Michell, 1982*a*; Berridge, 1982). This is important because if stimulated inositol lipid metabolism were induced by an elevation in cytosolic [Ca^{2+}], it could not be the initial event after ligand receptor interaction that causes or triggers Ca^{2+} mobilization. Although many previous workers have attempted to address this question it remains unresolved (Irvine, 1982). Because it appeared likely that the primary effect of TRH on inositol lipids in GH_3 cells was to stimulate phosphodiesteratic hydrolysis of PtdIns(4,5)P_2 (Fig. 3), it was important to attempt to determine whether TRH-stimulated PtdIns(4,5)P_2 hydrolysis was secondary to an elevation in cytosolic Ca^{2+} concentration that

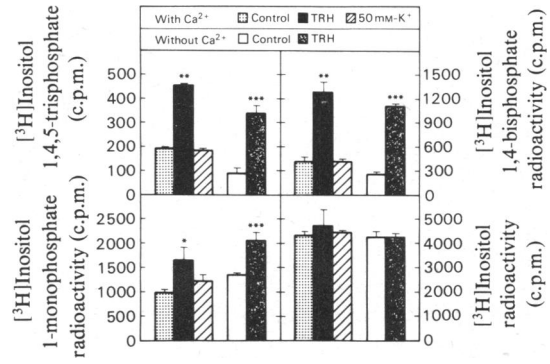

Fig. 4. *Effects of TRH, in medium without or with added Ca^{2+}, and 50 mM-K^+, in medium with Ca^{2+}, on the levels of [^{3}H]inositol phosphate sugars after 30 s in GH_3 cells*

Cells were incubated in growth medium with 1.4 μCi of [^{3}H]inositol/ml for 48 h and then incubated in a balanced salt solution (BSS) without [^{3}H]inositol containing 1.5 mM-Ca^{2+} or without added Ca^{2+}; BSS without added Ca^{2+} contained approx. 3 μM-Ca^{2+}. After 20 min, TRH (final concentration 1 μM), KCl (final concentration 50 mM-K^+) or BSS without or with Ca^{2+} (controls) was added. Results are means ± S.D (represented by the half-bar) of triplicate determinations of ^{3}H-labelled sugars isolated from cells after 30 s. The levels of significance, when data from cells incubated with TRH or 50 mM-K^+ were compared with controls with or without Ca^{2+}, are represented by: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.02$. These results are representative of two similar experiments.

is sufficient to stimulate the biological response, prolactin secretion.

To study whether hydrolysis of PtdIns(4,5)P_2 induced by TRH may have been caused by an elevation in cytosolic free [Ca^{2+}], TRH-induced changes in inositol lipids were compared with the effects of membrane depolarization by high extracellular [K^+]. K^+ depolarization of GH_3 cells causes influx of extracellular Ca^{2+}, which increases cytosolic [Ca^{2+}] and leads to marked stimulation of phosphorylation of several proteins (Drust & Martin, 1982) and of prolactin secretion (Gershengorn, 1982). The effects of TRH were compared with those of depolarization by 50mM-K^+ after 30s in cells prelabelled with [^{3}H]inositol (Fig. 4). TRH markedly increased the levels of [^{3}H]inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,4-bisphosphate, minimally increased [^{3}H]inositol 1-monophosphate and had no effect on [^{3}H]inositol after 30s as was shown in separate experiments in Fig. 3. In contrast, 50mM-K^+ had no effect on the levels of [^{3}H]inositol 1,4,5-trisphosphate, [^{3}H]inositol 1,4-bisphosphate or [^{3}H]inositol; a small increase in [^{3}H]inositol 1-monophosphate, was observed, to $126 \pm 6\%$ of control (mean $\pm$ S.E.M.), which attained significance ($P < 0.01$) when two experiments were analysed. This effect was abolished when cells were stimulated by 50mM-K^+ in medium without added Ca^{2+} (results not shown). This increase in inositol 1-monophosphate caused by 50mM-K^+ may have been due to hydrolysis of a small amount of PtdIns by a phosphodiesterase stimulated by Ca^{2+} or to Ca^{2+}-dependent inhibition of conversion of inositol 1-monophosphate into free inositol by a phosphatase, or both. It is not possible, at present, to distinguish between these possibilities. To determine whether the lack of measurable increases in [^{3}H]-inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,4-bisphosphate were caused by accelerated dephosphorylation of these sugars to [^{3}H]inositol 1-monophosphate induced by 50mM-K^+, cells were exposed to TRH and 50mM-K^+ simultaneously. There was no effect of 50mM-K^+ on TRH-stimulated increases in [^{3}H]inositol 1,4,5-trisphosphate or [^{3}H]inositol 1,4-bisphosphate after 30s (results not shown). Also, in contrast with TRH, 50mM-K^+ did not measurably change the levels of any of the ^{3}H-labelled inositol lipids (results not shown). Hence, the elevation in cytosolic [Ca^{2+}] induced by K^+ depolarization that is sufficient to increase the level of inositol 1-monophosphate, to cause phosphorylation of several proteins (Drust & Martin, 1982) and to stimulate prolactin secretion (Gershengorn, 1982) does not cause hydrolysis of PtdIns(4,5)P_2 by a phosphodiesterase.

In a parallel experiment, the effect on TRH-stimulated PtdIns(4,5)P_2 hydrolysis of incubating cells in medium with no added Ca^{2+} was determined. Incubation of GH_3 cells in medium without added Ca^{2+} decreased the content of [^{3}H]inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,4-bisphosphate and increased [^{3}H]inositol 1-monophosphate in unstimulated cells; there was no measurable effect on [^{3}H]inositol (Fig. 4). TRH stimulated an increase in the amounts of [^{3}H]inositol 1,4,5-trisphosphate, [^{3}H]inositol 1,4-bisphosphate and [^{3}H]inositol 1-monophosphate in cells incubated in medium with no added Ca^{2+} that was almost identical with that in cells stimulated by TRH in medium with 1.5mM-Ca^{2+} (Fig. 4). Incubation of cells in medium with no added Ca^{2+} did not interfere with the TRH-stimulated decrease in [^{3}H]PtdIns(4,5)P_2, [^{3}H]-PtdIns4P and [^{3}H]PtdIns (results not shown). Hence, TRH-stimulated PtdIns(4,5)P_2 hydrolysis was not affected by very low [Ca^{2+}] in the medium, which is known to lead to depletion of cellular Ca^{2+} and to prevent influx of extracellular Ca^{2+} into GH_3 cells (Gershengorn, 1982) and provides further indirect evidence that TRH-induced PtdIns(4,5)P_2 hydrolysis may not be dependent on Ca^{2+}. By contrast, in another study in which inositol 1,4,5-trisphosphate was measured in intact cells, Akhtar & Abdel-Latif (1980) concluded that acetylcholine-stimulated PtdIns(4,5)P_2 hydrolysis in rabbit iris smooth muscle was secondary to enhanced Ca^{2+} influx.

It has been proposed that ligands that interact with cell-surface receptors and cause mobilization of cellular Ca^{2+} or influx of extracellular Ca^{2+}, or both, do so by stimulating hydrolysis of PtdIns(4,5)P_2 by a specific phosphodiesterase (Michell *et al.*, 1981; Michell, 1982*a*; Berridge, 1982). According to this hypothesis, mobilization of Ca^{2+} caused by PtdIns-(4,5)P_2 breakdown leads to an elevation of the free cytoplasmic Ca^{2+} concentration and to stimulation of the biological response. A major question regarding the association between PtdIns(4,5)P_2 breakdown and Ca^{2+} mobilization is whether the activity of the phosphodiesterase that cleaves PtdIns(4,5)P_2 is stimulated by an elevation of the free cytoplasmic Ca^{2+} concentration or whether its stimulation may occur before or in the absence of an elevation of free cytoplasmic [Ca^{2+}]. In the present study, we demonstrated that the elevation of cytoplasmic Ca^{2+} concentration induced by K^+ depolarization that is comparable in magnitude and longer in duration to that induced by TRH stimulation (M. C. Gershengorn & C. Thaw, unpublished work) and sufficient to stimulate prolactin secretion did not activate hydrolysis of PtdIns(4,5)P_2 by a phosphodiesterase. We also found that the time course of TRH stimulation of PtdIns(4,5)P_2 hydrolysis was almost identical with that of cellular Ca^{2+} mobilization (Moriarty & Leuschen, 1981; Gershengorn *et al.*, 1981; Tan & Tashjian, 1981; Rebecchi *et al.*, 1982; Thaw *et al.*, 1982) and

elevation of free cytoplasmic Ca^{2+} concentration (M. C. Gershengorn & C. Thaw, unpublished work). Hence, these data are consistent with the hypothesis that ligand-stimulated PtdIns(4,5)P_2 hydrolysis by a phosphodiesterase causes Ca^{2+} mobilization. However, the mechanism whereby PtdIns(4,5)P_2 hydrolysis causes Ca^{2+} mobilization is unclear. Because it appears that most cellular PtdIns(4,5)P_2 is in the plasma membrane (Griffin & Hawthorne, 1978; Rawyler *et al.*, 1982), it has been suggested that the ligand-stimulated decrease in PtdIns(4,5)P_2 concentration may release a sequestered pool of plasma-membrane-bound Ca^{2+} or increase the plasma-membrane permeability to Ca^{2+}, or both (Michell, 1982*b*). In this regard, it has been shown that TRH mobilizes membrane-associated Ca^{2+} in GH_3 cells, perhaps from the plasma membrane (Tan & Tashjian, 1981; Thaw *et al.*, 1982). However, it has also been observed that TRH stimulates mobilization of Ca^{2+} from the endoplasmic reticulum and mitochondria of GH_3 cells (Ronning *et al.*, 1982). In order to explain fully TRH-induced mobilization of cellular Ca^{2+}, we further propose that inositol 1,4,5-trisphosphate may act as an intracellular messenger to mobilize Ca^{2+} from the endoplasmic reticulum and mitochondria. Hence, we propose that TRH-stimulated hydrolysis of PtdIns-(4,5)P_2 by a phosphodiesterase may cause an elevation in free cytoplasmic Ca^{2+} concentration by either releasing plasma-membrane-bound Ca^{2+} or allowing influx of extracellular Ca^{2+}, or both, and by mobilizing Ca^{2+} from intracellular pools.

In conclusion the data herein demonstrate that TRH stimulates the rapid loss of PtdIns(4,5)P_2 and PtdIns4P, as well as PtdIns, and the accumulation of inositol 1,4,5-trisphosphate and inositol 1,4-bis-phosphate, as well as inositol-1-monophosphate and free inositol, in GH_3 cells. Because inositol 1,4,5-trisphosphate is a unique product of hydrolysis of PtdIns(4,5)P_2 by a phosphodiesterase (phospholipase C), it can be concluded that TRH stimulates the activity of a phosphodiesterase that hydrolyses PtdIns(4,5)P_2. It is not possible to draw similar conclusions regarding the effects of TRH on PtdIns4P or PtdIns because the sugar products of their hydrolysis may be formed also by sequential dephosphorylation of inositol 1,4,5-trisphosphate; the decreases observed in PtdIns4P and PtdIns may have been secondary to their conversion into Ptdins(4,5)P_2 by kinases. Moreover, TRH-stimulated PtdIns(4,5)P_2 hydrolysis does not appear to be secondary to an elevation of free cytosolic [Ca^{2+}] and, therefore, as suggested by Michell *et al.* (1981), may be involved in causing cellular Ca^{2+} mobilization.

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