

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

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

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Thyrotropin-releasing hormone (TRH; thyroliberin) stimulated rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate [ $\text{PtdIns}(4,5)\text{P}_2$ ] by a phosphodiesterase (phospholipase C) in  $\text{GH}_3$  cells, a prolactin-secreting rat pituitary tumour cell line. TRH caused a rapid decrease in the level of  $\text{PtdIns}(4,5)\text{P}_2$  to 60% of control and stimulated a marked transient increase in inositol 1,4,5-trisphosphate, the unique product of phosphodiesteratic hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$ , to a peak of 410% of control at 15 s. TRH also caused decreases in phosphatidylinositol 4-monophosphate ( $\text{PtdIns}4\text{P}$ ) and phosphatidylinositol ( $\text{PtdIns}$ ) to 65% and 93% of control at 15 s respectively. Inositol 1,4-bisphosphate was increased to a peak of 450% at 30 s; inositol 1-monophosphate and inositol were not elevated until 30 s and 1 min respectively after TRH addition. To study whether  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis may be caused by an elevation in cytosolic  $\text{Ca}^{2+}$  concentration, the changes induced by TRH in the levels of inositol sugars were compared with the effects of membrane depolarization by high extracellular  $[\text{K}^+]$ . The elevation in cytosolic  $[\text{Ca}^{2+}]$  induced by  $\text{K}^+$  depolarization did not change the level of inositol 1,4,5-trisphosphate. These data suggest that phosphodiesteratic hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  may be the initial event in TRH stimulation of inositol lipid metabolism in  $\text{GH}_3$  cells and that  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis is not stimulated by an elevation in cytosolic  $\text{Ca}^{2+}$  concentration. The decreases in  $\text{PtdIns}4\text{P}$  and  $\text{PtdIns}$  may be due to enhanced conversion of  $\text{PtdIns}$  into  $\text{PtdIns}4\text{P}$  into  $\text{PtdIns}(4,5)\text{P}_2$  or to their direct hydrolysis by phosphomonoesterases and/or phosphodiesterases. These results are consistent with the hypothesis that TRH-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  breakdown causes  $\text{Ca}^{2+}$  mobilization leading to prolactin secretion

The mechanism(s) whereby ligands that interact with cell-surface receptors cause mobilization of cellular  $\text{Ca}^{2+}$  or stimulation of influx of extracellular  $\text{Ca}^{2+}$  or both, leading to elevation of the free cytosolic  $\text{Ca}^{2+}$  concentration, is not known (Rasmussen & Waisman, 1981). Several groups (Michell *et al.*, 1981; Michell, 1982a; Berridge, 1982) have recently suggested that these ligands may initiate their action by stimulating hydrolysis of

$\text{PtdIns}(4,5)\text{P}_2$  by a specific phosphodiesterase (phospholipase C) which would serve to release membrane-bound  $\text{Ca}^{2+}$  and/or to activate channels within the plasma membrane and allow  $\text{Ca}^{2+}$  influx into cells. The decrease in the level of  $\text{PtdIns}$  commonly observed after stimulation by this class of ligand (Michell, 1975) is proposed to be due to conversion of  $\text{PtdIns}$  into  $\text{PtdIns}4\text{P}$  and then into  $\text{PtdIns}(4,5)\text{P}_2$  by a kinase (or kinases). An important component of this hypothesis is that the initial event, phosphodiesteratic hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$ , should not be caused by the cytosolic  $[\text{Ca}^{2+}]$  elevation. Although a number of reports have documented decreases in  $\text{PtdIns}(4,5)\text{P}_2$  and  $\text{PtdIns}4\text{P}$  during ligand stimulation (Downes & Michell, 1982), only in two cell types has the decrease in  $\text{PtdIns}(4,5)\text{P}_2$  caused by ligand stimulation of intact cells been shown to be due to phosphodiesteratic hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by

Abbreviations used: TRH, thyrotropin-releasing hormone (thyroliberin);  $\text{PtdIns}$ , phosphatidylinositol;  $\text{PtdIns}4\text{P}$ , phosphatidylinositol 4-monophosphate;  $\text{PtdIns}(4,5)\text{P}_2$ , 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<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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<sup>+</sup> 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. Cyclohexylenedinitrilotetraacetic acid was obtained from Eastman Kodak. AG 1X-2 resin was purchased from Bio-Rad. [ $^{32}P$ ]P<sub>i</sub> (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<sub>3</sub> 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<sub>i</sub> for less than 1 h, GH<sub>3</sub> 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<sub>2</sub>, 0.5 mM-MgCl<sub>2</sub>, 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<sub>i</sub>/ml at 26°C. After 40 min, TRH was added (1  $\mu$ M 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<sub>i</sub>, cells were incubated in growth medium supplemented with 10  $\mu$ Ci of [ $^{32}P$ ]P<sub>i</sub>/ml for 48 h. For experiments with [ $^3H$ ]inositol, GH<sub>3</sub> cells were incubated in growth medium containing 1  $\mu$ Ci 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  $\mu$ M- $Ca^{2+}$  as measured with a metallochromic dye (Rebecchi *et al.*, 1982). Medium containing 50 mM-K<sup>+</sup> 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<sub>2</sub> 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<sub>2</sub> 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<sub>3</sub> (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<sub>3</sub> (9:7:2, by vol.) containing 2 mM-cyclohexylenedinitrilotetraacetic 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 [ $^{32}\text{P}$ ]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<sub>3</sub> cells that had been labelled to isotopic steady-state with [ $^{32}\text{P}$ ]P<sub>i</sub> and [ $^3\text{H}$ ]inositol, and isolated using either system, the ratios of  $^{32}\text{P}/^3\text{H}$  radioactivity for the PtdIns(4,5) $P_2$  and PtdIns4P spots were  $3.00 \pm 0.22$  and  $2.04 \pm 0.04$  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<sub>2</sub> staining of lipid standards; the I<sub>2</sub> was always allowed to sublime before scraping. Spots were scraped into vials to which 0.2 ml of methanol/water/HCl (80:20:1, by vol.) and 6 ml of liquid-scintillation fluid were added and radioactivity was determined.

#### Inositol sugar analysis

$^3\text{H}$ -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<sub>2</sub> and resuspended in 0.12 ml of 0.1 M-formic acid; 0.1 ml was applied to a freshly prepared 1 ml column of resin (AG1 X-2; 200–400 mesh), which had been swollen in 0.1 M-formic acid containing unlabelled-5 mM-*myo*-inositol, and the column was eluted in a stepwise fashion. Four peaks of  $^3\text{H}$  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 [ $^{32}\text{P}$ ]P<sub>i</sub> and [ $^3\text{H}$ ]inositol, the lipids were extracted and [ $^{32}\text{P}$ ,  $^3\text{H}$ ]PtdIns(4,5) $P_2$ , [ $^{32}\text{P}$ ,  $^3\text{H}$ ]PtdIns4P and [ $^{32}\text{P}$ ,  $^3\text{H}$ ]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<sub>3</sub> cells.

Statistical analysis was performed by *t* test.

#### Results and discussion

Fig. 1 illustrates the time course of the effect of TRH on [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P in GH<sub>3</sub> cells labelled with [ $^{32}\text{P}$ ]P<sub>i</sub> for 40 min; under these conditions, phospholipids are not labelled to isotopic steady-state. TRH caused a rapid decrease by 15 s in the levels of [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P to 76% ( $P < 0.01$ ) and 71% of control ( $P < 0.025$ ) respectively. After 1 min, [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P increased again at a rate similar to that in control cells. In agreement with our

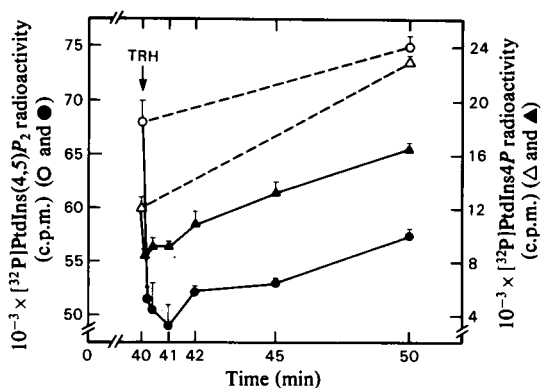


Fig. 1. Time course of the effect of TRH on [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P in GH<sub>3</sub> cells

Data are means  $\pm$  S.D. of [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P isolated from cells that had been pre-incubated in a balanced salt solution containing 0.5 mCi of [ $^{32}\text{P}$ ]P<sub>i</sub>/ml for 40 min and then incubated for various times in medium without (O and  $\Delta$ ) or with 1  $\mu\text{M}$ -TRH ( $\bullet$  and  $\blacktriangle$ ). The results shown are representative of three experiments.

previous observations (Rebecchi *et al.*, 1981), the levels of [ $^{32}\text{P}$ ]phosphatidic acid increased markedly by 15 s after TRH addition and that of [ $^{32}\text{P}$ ]PtdIns did not increase until after 2 min (results not shown). The rapid decreases in [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P in GH<sub>3</sub> 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 [ $^{32}\text{P}$ ]P<sub>i</sub>, TRH caused a decrease in PtdIns(4,5) $P_2$  from  $33 \pm 2.3$  pmol/ $10^6$  cells to  $22 \pm 1.1$  pmol at 30 s ( $P < 0.01$ ).

To determine whether the decreases in [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdIns4P induced by TRH were caused by losses of PtdIns(4,5) $P_2$  and PtdIns4P, cells were incubated with [ $^3\text{H}$ ]inositol for 48 h, 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  $^3\text{H}$ -labelled inositol lipids was determined (Fig. 2). The relative content (means  $\pm$  S.D.) of inositol lipids in cells prelabelled with [ $^3\text{H}$ ]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, [ $^3\text{H}$ ]PtdIns(4,5) $P_2$  radioactivity was  $92 \pm 2$  c.p.m./ $10^6$  cells, [ $^3\text{H}$ ]PtdIns4P was  $136 \pm 2$  c.p.m., [ $^3\text{H}$ ]lysophosphatidylinositol was  $337 \pm 54$  c.p.m. and [ $^3\text{H}$ ]PtdIns was  $4780 \pm 155$  c.p.m. (all means  $\pm$  S.E.M.). TRH stimulated rapid loss by 15 s of [ $^3\text{H}$ ]PtdIns(4,5) $P_2$  and [ $^3\text{H}$ ]PtdIns4P to 60% ( $P < 0.001$ ) and 65% of control ( $P < 0.001$ ) respectively; the nadirs in the levels of [ $^3\text{H}$ ]PtdIns(4,5) $P_2$  and [ $^3\text{H}$ ]PtdIns4P were

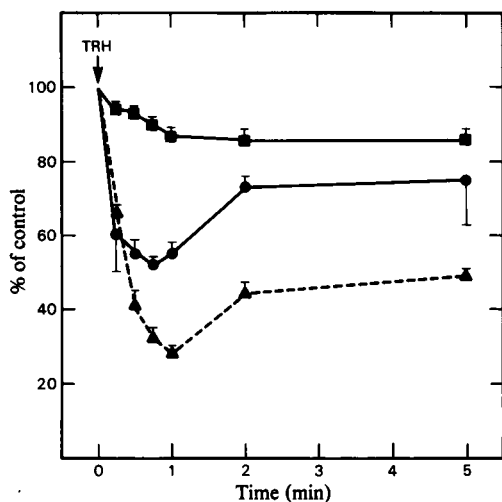


Fig. 2. Time course of the effect of TRH on the levels of [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> (●), [<sup>3</sup>H]PtdIns4P (▲) and [<sup>3</sup>H]-PtdIns (■) in GH<sub>3</sub> cells

The data are means  $\pm$  S.E.M. of triplicate determinations and are expressed as percentages of the levels of [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub>, [<sup>3</sup>H]PtdIns4P or [<sup>3</sup>H]-PtdIns isolated from unstimulated cells (control). Cells were incubated in growth medium with 1  $\mu$ Ci of [<sup>3</sup>H]inositol/ml for 48 h and then incubated in a balanced salt solution without [<sup>3</sup>H]inositol. After 20 min (zero time), TRH (final concentration 1  $\mu$ M) was added. In control cells, [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> radioactivity was  $92 \pm 2$  c.p.m./10<sup>6</sup> cells (means  $\pm$  S.E.M.), [<sup>3</sup>H]PtdIns4P was  $136 \pm 2$  c.p.m./10<sup>6</sup> cells and [<sup>3</sup>H]PtdIns was  $4780 \pm 155$  c.p.m./10<sup>6</sup> cells. The results shown are representative of three similar experiments.

at 45 s and 60 s respectively. After 1 min in the continued presence of TRH, the levels of [<sup>3</sup>H]-PtdIns(4,5)P<sub>2</sub> and [<sup>3</sup>H]PtdIns4P increased but remained below control for at least 5 min. Concomitant with the fall in [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> and [<sup>3</sup>H]-PtdIns4P and in agreement with our previous observations (Rebecchi *et al.*, 1983), TRH caused a decrease in [<sup>3</sup>H]PtdIns to 86% of control by 2 min ( $P < 0.001$ ); there was no measurable change in the level of [<sup>3</sup>H]lysophosphatidylinositol (results not shown). The effect of TRH to decrease [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> was concentration-dependent; half-maximal effect occurred with approx. 5 nM-TRH (results not shown), a concentration similar to that needed for half-maximal loss of [<sup>3</sup>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<sub>3</sub> 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<sub>2</sub> or PtdIns4P because diacylglycerol can be formed by hydrolysis of PtdIns(4,5)P<sub>2</sub>, PtdIns4P 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 PtdIns4P, or sequentially from inositol 1,4,5-trisphosphate, a product of phosphodiesterase hydrolysis of PtdIns(4,5)P<sub>2</sub>. Hence it was important to monitor the levels of all the inositol sugars in GH<sub>3</sub> 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<sub>2</sub> by a phosphodiesterase in mammalian cells.

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

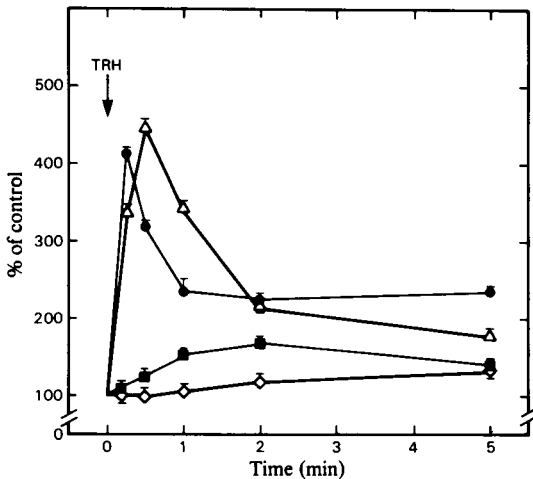


Fig. 3. Time course of the effect of TRH on the levels of  $[^3\text{H}]$ inositol phosphate sugars in  $\text{GH}_3$  cells

Data are means  $\pm$  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  $1\ \mu\text{Ci}$  of  $[^3\text{H}]$ inositol/ml for 48 h and then incubated in a balanced salt solution without  $[^3\text{H}]$ inositol. After 20 min (zero time), TRH (final concentration  $1\ \mu\text{M}$ ) was added. The levels of inositol phosphate sugars in unstimulated cells expressed as  $^3\text{H}$  c.p.m./ $5 \times 10^6$  cells (means  $\pm$  S.E.M.) were:  $[^3\text{H}]$ inositol,  $9660 \pm 117$ ;  $[^3\text{H}]$ inositol 1-monophosphate,  $2020 \pm 22$ ;  $[^3\text{H}]$ inositol 1,4-bisphosphate,  $481 \pm 7$ ;  $[^3\text{H}]$ inositol 1,4,5-trisphosphate,  $154 \pm 2$ . The results shown are representative of two similar experiments. Symbols:  $\bullet$ ,  $[^3\text{H}]$ inositol 1,4,5-trisphosphate;  $\Delta$ ,  $[^3\text{H}]$ inositol 1,4-bisphosphate;  $\blacksquare$ ,  $[^3\text{H}]$ inositol 1-monophosphate;  $\circ$ ,  $[^3\text{H}]$ inositol.

inositol lipid metabolism. at least in part, by causing hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by a phosphodiesterase (phospholipase C) and that the time course of  $[^3\text{H}]$ inositol 1,4,5-trisphosphate accumulation is consistent with the notion that  $\text{PtdIns}(4,5)\text{P}_2$  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  $[^3\text{H}]$ inositol 1,4-bisphosphate and  $[^3\text{H}]$ inositol 1-monophosphate during TRH stimulation of  $\text{GH}_3$  cells may have been due to either hydrolysis by a phosphodiesterase of  $\text{PtdIns}4\text{P}$  and  $\text{PtdIns}$  respectively, or dephosphorylation by a phosphatase of  $[^3\text{H}]$ inositol 1,4,5-trisphosphate and  $[^3\text{H}]$ 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  $\text{GH}_3$  cells, is whether the initial enzyme reaction that is induced by ligand is dependent on a rise in the free (or unbound) concentration of  $\text{Ca}^{2+}$  in the cytosol (Michell, 1982a; Berridge, 1982). This is important because if stimulated inositol lipid metabolism were induced by an elevation in cytosolic  $[\text{Ca}^{2+}]$ , it could not be the initial event after ligand receptor interaction that causes or triggers  $\text{Ca}^{2+}$  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  $\text{GH}_3$  cells was to stimulate phosphodiesteratic hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  (Fig. 3), it was important to attempt to determine whether TRH-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis was secondary to an elevation in cytosolic  $\text{Ca}^{2+}$  concentration that

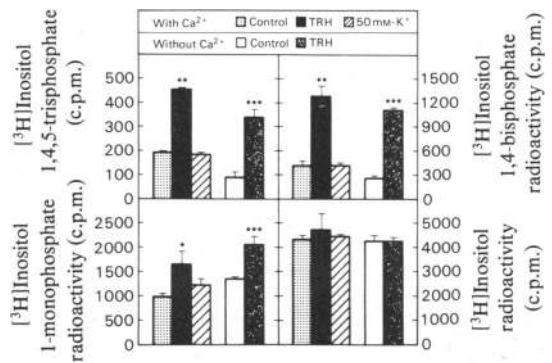


Fig. 4. Effects of TRH, in medium without or with added  $\text{Ca}^{2+}$ , and  $50\ \text{mM-K}^+$ , in medium with  $\text{Ca}^{2+}$ , on the levels of  $[^3\text{H}]$ inositol phosphate sugars after 30 s in  $\text{GH}_3$  cells

Cells were incubated in growth medium with  $1.4\ \mu\text{Ci}$  of  $[^3\text{H}]$ inositol/ml for 48 h and then incubated in a balanced salt solution (BSS) without  $[^3\text{H}]$ inositol containing  $1.5\ \text{mM-Ca}^{2+}$  or without added  $\text{Ca}^{2+}$ ; BSS without added  $\text{Ca}^{2+}$  contained approx.  $3\ \mu\text{M-Ca}^{2+}$ . After 20 min, TRH (final concentration  $1\ \mu\text{M}$ ),  $\text{KCl}$  (final concentration  $50\ \text{mM-K}^+$ ) or BSS without or with  $\text{Ca}^{2+}$  (controls) was added. Results are means  $\pm$  S.D. (represented by the half-bar) of triplicate determinations of  $^3\text{H}$ -labelled sugars isolated from cells after 30 s. The levels of significance, when data from cells incubated with TRH or  $50\ \text{mM-K}^+$  were compared with controls with or without  $\text{Ca}^{2+}$ , 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  $\text{PtdIns}(4,5)\text{P}_2$  induced by TRH may have been caused by an elevation in cytosolic free  $[\text{Ca}^{2+}]$ , TRH-induced changes in inositol lipids were compared with the effects of membrane depolarization by high extracellular  $[\text{K}^+]$ .  $\text{K}^+$  depolarization of  $\text{GH}_3$  cells causes influx of extracellular  $\text{Ca}^{2+}$ , which increases cytosolic  $[\text{Ca}^{2+}]$  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  $50\text{mM-K}^+$  after 30s in cells prelabelled with  $^3\text{H}$ inositol (Fig. 4). TRH markedly increased the levels of  $^3\text{H}$ inositol 1,4,5-trisphosphate and  $^3\text{H}$ inositol 1,4-bisphosphate, minimally increased  $^3\text{H}$ inositol 1-monophosphate and had no effect on  $^3\text{H}$ inositol after 30s as was shown in separate experiments in Fig. 3. In contrast,  $50\text{mM-K}^+$  had no effect on the levels of  $^3\text{H}$ inositol 1,4,5-trisphosphate,  $^3\text{H}$ inositol 1,4-bisphosphate or  $^3\text{H}$ inositol; a small increase in  $^3\text{H}$ 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  $50\text{mM-K}^+$  in medium without added  $\text{Ca}^{2+}$  (results not shown). This increase in inositol 1-monophosphate caused by  $50\text{mM-K}^+$  may have been due to hydrolysis of a small amount of  $\text{PtdIns}$  by a phosphodiesterase stimulated by  $\text{Ca}^{2+}$  or to  $\text{Ca}^{2+}$ -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  $^3\text{H}$ inositol 1,4,5-trisphosphate and  $^3\text{H}$ inositol 1,4-bisphosphate were caused by accelerated dephosphorylation of these sugars to  $^3\text{H}$ inositol 1-monophosphate induced by  $50\text{mM-K}^+$ , cells were exposed to TRH and  $50\text{mM-K}^+$  simultaneously. There was no effect of  $50\text{mM-K}^+$  on TRH-stimulated increases in  $^3\text{H}$ inositol 1,4,5-trisphosphate or  $^3\text{H}$ inositol 1,4-bisphosphate after 30s (results not shown). Also, in contrast with TRH,  $50\text{mM-K}^+$  did not measurably change the levels of any of the  $^3\text{H}$ -labelled inositol lipids (results not shown). Hence, the elevation in cytosolic  $[\text{Ca}^{2+}]$  induced by  $\text{K}^+$  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  $\text{PtdIns}(4,5)\text{P}_2$  by a phosphodiesterase.

In a parallel experiment, the effect on TRH-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis of incubating cells in medium with no added  $\text{Ca}^{2+}$  was determined.

Incubation of  $\text{GH}_3$  cells in medium without added  $\text{Ca}^{2+}$  decreased the content of  $^3\text{H}$ inositol 1,4,5-trisphosphate and  $^3\text{H}$ inositol 1,4-bisphosphate and increased  $^3\text{H}$ inositol 1-monophosphate in unstimulated cells; there was no measurable effect on  $^3\text{H}$ inositol (Fig. 4). TRH stimulated an increase in the amounts of  $^3\text{H}$ inositol 1,4,5-trisphosphate,  $^3\text{H}$ inositol 1,4-bisphosphate and  $^3\text{H}$ inositol 1-monophosphate in cells incubated in medium with no added  $\text{Ca}^{2+}$  that was almost identical with that in cells stimulated by TRH in medium with  $1.5\text{mM-Ca}^{2+}$  (Fig. 4). Incubation of cells in medium with no added  $\text{Ca}^{2+}$  did not interfere with the TRH-stimulated decrease in  $^3\text{H}$ inositol 1,4,5-trisphosphate,  $^3\text{H}$ inositol 1,4-bisphosphate and  $^3\text{H}$ inositol 1-monophosphate (results not shown). Hence, TRH-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis was not affected by very low  $[\text{Ca}^{2+}]$  in the medium, which is known to lead to depletion of cellular  $\text{Ca}^{2+}$  and to prevent influx of extracellular  $\text{Ca}^{2+}$  into  $\text{GH}_3$  cells (Gershengorn, 1982) and provides further indirect evidence that TRH-induced  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis may not be dependent on  $\text{Ca}^{2+}$ . 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  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis in rabbit iris smooth muscle was secondary to enhanced  $\text{Ca}^{2+}$  influx.

It has been proposed that ligands that interact with cell-surface receptors and cause mobilization of cellular  $\text{Ca}^{2+}$  or influx of extracellular  $\text{Ca}^{2+}$ , or both, do so by stimulating hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by a specific phosphodiesterase (Michell *et al.*, 1981; Michell, 1982a; Berridge, 1982). According to this hypothesis, mobilization of  $\text{Ca}^{2+}$  caused by  $\text{PtdIns}(4,5)\text{P}_2$  breakdown leads to an elevation of the free cytoplasmic  $\text{Ca}^{2+}$  concentration and to stimulation of the biological response. A major question regarding the association between  $\text{PtdIns}(4,5)\text{P}_2$  breakdown and  $\text{Ca}^{2+}$  mobilization is whether the activity of the phosphodiesterase that cleaves  $\text{PtdIns}(4,5)\text{P}_2$  is stimulated by an elevation of the free cytoplasmic  $\text{Ca}^{2+}$  concentration or whether its stimulation may occur before or in the absence of an elevation of free cytoplasmic  $[\text{Ca}^{2+}]$ . In the present study, we demonstrated that the elevation of cytoplasmic  $\text{Ca}^{2+}$  concentration induced by  $\text{K}^+$  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  $\text{PtdIns}(4,5)\text{P}_2$  by a phosphodiesterase. We also found that the time course of TRH stimulation of  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis was almost identical with that of cellular  $\text{Ca}^{2+}$  mobilization (Moriarty & Leuschen, 1981; Gershengorn *et al.*, 1981; Tan & Tashjian, 1981; Rebecchi *et al.*, 1982; Thaw *et al.*, 1982) and

elevation of free cytoplasmic  $\text{Ca}^{2+}$  concentration (M. C. Gershengorn & C. Thaw, unpublished work). Hence, these data are consistent with the hypothesis that ligand-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis by a phosphodiesterase causes  $\text{Ca}^{2+}$  mobilization. However, the mechanism whereby  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis causes  $\text{Ca}^{2+}$  mobilization is unclear. Because it appears that most cellular  $\text{PtdIns}(4,5)\text{P}_2$  is in the plasma membrane (Griffin & Hawthorne, 1978; Rawlyer *et al.*, 1982), it has been suggested that the ligand-stimulated decrease in  $\text{PtdIns}(4,5)\text{P}_2$  concentration may release a sequestered pool of plasma-membrane-bound  $\text{Ca}^{2+}$  or increase the plasma-membrane permeability to  $\text{Ca}^{2+}$ , or both (Michell, 1982*b*). In this regard, it has been shown that TRH mobilizes membrane-associated  $\text{Ca}^{2+}$  in  $\text{GH}_3$  cells, perhaps from the plasma membrane (Tan & Tashjian, 1981; Thaw *et al.*, 1982). However, it has also been observed that TRH stimulates mobilization of  $\text{Ca}^{2+}$  from the endoplasmic reticulum and mitochondria of  $\text{GH}_3$  cells (Ronning *et al.*, 1982). In order to explain fully TRH-induced mobilization of cellular  $\text{Ca}^{2+}$ , we further propose that inositol 1,4,5-trisphosphate may act as an intracellular messenger to mobilize  $\text{Ca}^{2+}$  from the endoplasmic reticulum and mitochondria. Hence, we propose that TRH-stimulated hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by a phosphodiesterase may cause an elevation in free cytoplasmic  $\text{Ca}^{2+}$  concentration by either releasing plasma-membrane-bound  $\text{Ca}^{2+}$  or allowing influx of extracellular  $\text{Ca}^{2+}$ , or both, and by mobilizing  $\text{Ca}^{2+}$  from intracellular pools.

In conclusion the data herein demonstrate that TRH stimulates the rapid loss of  $\text{PtdIns}(4,5)\text{P}_2$  and  $\text{PtdIns}4\text{P}$ , as well as  $\text{PtdIns}$ , and the accumulation of inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate, as well as inositol-1-monophosphate and free inositol, in  $\text{GH}_3$  cells. Because inositol 1,4,5-trisphosphate is a unique product of hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by a phosphodiesterase (phospholipase C), it can be concluded that TRH stimulates the activity of a phosphodiesterase that hydrolyses  $\text{PtdIns}(4,5)\text{P}_2$ . It is not possible to draw similar conclusions regarding the effects of TRH on  $\text{PtdIns}4\text{P}$  or  $\text{PtdIns}$  because the sugar products of their hydrolysis may be formed also by sequential dephosphorylation of inositol 1,4,5-trisphosphate; the decreases observed in  $\text{PtdIns}4\text{P}$  and  $\text{PtdIns}$  may have been secondary to their conversion into  $\text{PtdIns}(4,5)\text{P}_2$  by kinases. Moreover, TRH-stimulated  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis does not appear to be secondary to an elevation of free cytosolic  $[\text{Ca}^{2+}]$  and, therefore, as suggested by Michell *et al.* (1981), may be involved in causing cellular  $\text{Ca}^{2+}$  mobilization.

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