Thyrotropin-releasing hormone receptor occupancy determines the fraction of the responsive pool of inositol lipids hydrolysed in rat pituitary tumour cells

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We report that there are distinct thyrotropin-releasing hormone (TRH)-responsive and -unresponsive pools of inositol (Ins) lipids in rat pituitary tumour (GH₃) cells, and present evidence that the size of the responsive pool is determined by the number of activated TRH-receptor complexes. By use of an experimental protocol in which cycling of [³H]Ins is inhibited and resynthesis occurs with unlabelled Ins only, we were able to measure specifically the effects of TRH on the hydrolysis of the Ins lipids present before stimulation. A maximally effective dose of TRH (1 μM) caused a time-dependent decrease in ³H-labelled Ins lipids that attained a steady-state value of 42 ± 1 % of the initial level between 1.5 and 2 h. After 2 h, even though there was no further decrease in ³H-labelled Ins lipids, and no increase in [³H]Ins or [³H]Ins phosphates, turnover of Ins lipids, as assessed as incorporation of [³²P]P_i into PtdIns, continued at a rate similar to that in cells incubated without LiCl or unlabelled Ins. These data indicate that Ins lipid turnover was not desensitized during prolonged TRH stimulation. Depletion of lipid ³H radioactivity by TRH occurred at higher TRH doses on addition of the competitive antagonist chlordiazepoxide. Addition of 1 μM-TRH after 3 h of stimulation by a sub-maximal (0.3 nM) TRH dose caused a further decrease in ³H radioactivity to the minimum level (40 % of initial value). We propose that the TRH-responsive pool of Ins lipids in GH₃ cells is composed of the complement of Ins lipids that are within functional proximity of activated TRH-receptor complexes.

INTRODUCTION

Inositol lipid-derived intracellular messenger molecules mediate the actions of a diverse group of hormones, neurotransmitters and growth factors in many cell types [1–3]. It has been shown that a proximal step in this pathway is activation of a phospholipase C which hydrolyses PtdIns(4,5)P₂, and perhaps PtdIns [4–7]. However, it is not clear whether there is a distinct pool of Ins lipids which undergoes rapid hydrolysis and resynthesis during stimulation, or whether the entire cell content of Ins lipids can be used to generate mediator molecules for signal transduction [8–14], but see [15]. It is important to note that the definition of a stimulus-responsive pool is a functional one which may or may not have a readily detectable anatomical counterpart.

We have presented evidence that a major enzyme of Ins lipid synthesis, CDP-diacylglycerol:Ins phosphatidyltransferase (PtdIns synthase; EC 2.7.8.11), is found in the plasma membrane and endoplasmic reticulum of GH₃ cells, and have suggested that functionally distinct Ins lipid pools may be formed at these two sites [16,17]. Herein we study the effects of thyrotropin-releasing hormone (TRH) in GH₃ cells prelabelled with [³H]Ins and use an experimental protocol in which recycling of [³H]Ins is inhibited and resynthesis of Ins lipids during stimulation occurs only with unlabelled Ins [18,19]. We were able to measure specifically the effects of TRH on the hydrolysis of Ins lipids present before stimulation, without the interpretive complications introduced by recycling of [³H]Ins. We show that a distinct fraction (approx. 60 %) of Ins lipids may be hydrolysed during stimulation by TRH. We show, moreover, that the quantity of Ins lipids hydrolysed within this pool is proportional to the number of receptors occupied by TRH, and not to the duration of TRH

stimulation, and we present an hypothesis to explain the genesis of the TRH-responsive pool.

EXPERIMENTAL

GH₃ cells were grown at 37 °C in a 5 % CO₂ atmosphere in Ham's F10 medium containing 15 % horse serum and 2.5 % fetal calf serum. Cells were prelabelled by incubation in growth medium supplemented with 1 μCi of [³H]Ins/ml for 40–72 h to label Ins lipids and Ins phosphates to isotopic steady-state [20]. Cells were detached from the flasks by addition of 0.02 % EDTA in phosphate-buffered saline (100 mM-NaCl/50 mM-Sodium phosphate, pH 7.4) and were washed twice with, and then incubated [(2–3) × 10⁶ cells/0.2 ml] in, a buffer containing 5.6 mM-glucose, 4 mM-NaHCO₃ and 10 mM-Hepes, pH 7.4, without (control) or with 100 mM unlabelled Ins at 37 °C for 60 min before the start of the experiment. Cells incubated in this buffer without or with LiCl for up to 6 h remain viable, i.e. more than 95 % exclude Trypan Blue, and retain an Ins lipid response to TRH that is indistinguishable from that of control cells incubated in buffer without 100 mM-Ins or LiCl (see the Results section). LiCl (10 mM) and/or TRH, at the concentrations noted, were added to portions of the cell suspensions at the times indicated. Incubations were terminated by addition of ice-cold solvent (chloroform/methanol/conc. HCl; 100:100:1, by vol). EDTA (10 mM, 0.2 ml/ml of solvent) was added, and the phases were separated and washed with pre-equilibrated upper and lower phase solvents as described [20]. Ins and Ins phosphates from the washed upper phases were concentrated under N₂ and separated by anion-exchange column chromatography by the

Abbreviations used: TRH, thyrotropin-releasing hormone; PA, phosphatidic acid; G-protein, GTP-binding protein.

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method of Downes & Michell [21] or by h.p.l.c. by the method of Morgan and coworkers [22], with minor modification. ^3H -labelled Ins lipids were estimated as ^3H radioactivity in the lower phases, or were measured individually by t.l.c. [20]. In some experiments, $[^{32}\text{P}]\text{P}_i$ (0.2 mCi/ml) was added to the cell suspensions for the times indicated to assess phospholipid turnover by ^{32}P labelling.

Materials

TRH was obtained from Calbiochem. Tissue culture supplies were from Gibco. $[^3\text{H}]\text{Ins}$ (19 Ci/mmol) was from Amersham and $[^{32}\text{P}]\text{P}_i$ (carrier-free) was from New England Nuclear. Reagent-grade chemicals were from Sigma.

RESULTS

In these studies we incubated GH_3 cells in buffer containing 10 mM-LiCl and 100 mM unlabelled Ins so as to inhibit recycling of released ^3H -labelled Ins phosphates to Ins lipids during stimulation by TRH. This strategy is based on our previous findings [19] that GH_3 cells incubated in Ins-free buffer containing 10 mM-LiCl show diminished resynthesis of Ins lipids when stimulated by TRH, but that cells incubated in buffer containing 100 mM-Ins and LiCl display normal rates of PtdIns resynthesis during TRH stimulation. Thus extracellular Ins can serve as substrate for PtdIns resynthesis, and at high concentrations it dilutes the residual intracellular pool of ^3H -labelled Ins, thereby redirecting resynthesis of PtdIns with unlabelled Ins. Fig. 1 illustrates the results of an experiment in which prelabelled cells were incubated in buffer without or with 10 mM-LiCl or 100 mM unlabelled Ins, or both, and were stimulated with a maximally effective dose of TRH (1 μM) for 120 min. As found previously [23], TRH caused a small transient decrease in ^3H -labelled Ins lipids and increases in $[^3\text{H}]\text{Ins}$ and $[^3\text{H}]\text{Ins}$ phosphates in cells incubated in Ins-free buffer without LiCl (Fig. 1*a*). The small magnitude and transient nature of these changes were due to enhanced resynthesis during stimulation [24–26]. In contrast,

TRH stimulated a time-dependent decrease and then a persistent decrease, to $42 \pm 1\%$ of the initial value, in ^3H -labelled Ins lipids in the presence of 10 mM-LiCl, 100 mM-Ins, or both. In the presence of 10 mM-LiCl the decrease in lipid radioactivity was accounted for by an increase in ^3H -labelled Ins phosphates (Fig. 1*b*). In the presence of 100 mM-Ins the decrease in lipid radioactivity was accounted for by an increase in $[^3\text{H}]\text{Ins}$; $[^3\text{H}]\text{Ins}$ phosphates were not elevated (Fig. 1*c*). In the presence of both LiCl and unlabelled Ins the decrease in lipid radioactivity was accounted for by increases in both $[^3\text{H}]\text{Ins}$ and $[^3\text{H}]\text{Ins}$ phosphates (Fig. 1*d*). It is of note that, in cells incubated in buffer with unlabelled Ins, the decrease in ^3H -labelled Ins lipids did not represent depletion of a TRH-responsive pool but rather replacement by unlabelled lipids, as stimulated Ins lipid turnover persisted (see below). Hence, under three conditions in which resynthesis of Ins lipids using released ^3H -labelled Ins was inhibited there was a decrease in ^3H -labelled Ins lipids to a similar level between 90 and 120 min after TRH addition. After this time, there was no further decrease in ^3H -labelled Ins lipids or increases in ^3H -labelled Ins or Ins phosphates.

The hydrolysis of each Ins lipid and the generation of the individual Ins phosphates were also measured under these conditions. Table 1 shows the amounts and distribution of ^3H radioactivity in PtdIns, lyso-PtdIns, PtdIns(4)*P* and PtdIns(4,5)*P*₂ in cells incubated in buffer containing 10 mM-LiCl and 100 mM unlabelled Ins and in cells incubated in this buffer after stimulation with 1 μM -TRH for 3 h. The distribution of ^3H radioactivity in the four lipids was similar in control and TRH-stimulated cells, even though there was a mean decrease in ^3H radioactivity of 59% in each lipid upon TRH stimulation. This distribution of ^3H labelling of Ins lipids was indistinguishable from that in cells before incubation in buffer with LiCl and unlabelled Ins: PtdIns, $0.94 \pm 0.6\%$; lyso-PtdIns, $2.6 \pm 0.3\%$; PtdIns(4)*P*, $2.4 \pm 0.4\%$; PtdIns(4,5)*P*₂, $1.3 \pm 0.01\%$. Fig. 2 shows the effects of TRH on $[^3\text{H}]\text{Ins}(1)\text{P}$ and $[^3\text{H}]\text{Ins}(4)\text{P}$ in cells incubated in buffer without or with 10 mM-LiCl. In cells incubated in buffer without LiCl there was a much greater relative increase

Fig. 1. Effects of TRH on levels of ^3H -labelled Ins lipids (■, □), Ins (●, ○) and Ins phosphates (▲, △) in GH_3 cells

Cells were prelabelled with $[^3\text{H}]\text{Ins}$ and incubated in buffer without LiCl or unlabelled Ins (*a*), in buffer with 10 mM-LiCl and without unlabelled Ins (*b*), in buffer without LiCl and with 100 mM-Ins (*c*), or in buffer with 10 mM-LiCl plus 100 mM unlabelled Ins (*d*). Prelabelled cells were incubated in buffer without or with 100 mM-Ins for 1 h. TRH (1 μM) without or with 10 mM-LiCl was then added (time zero). Incubations were terminated at the times indicated and ^3H radioactivity in lipids, Ins and Ins phosphate was measured. □, ○, △, Without TRH (controls); ■, ●, ▲, with TRH. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times; where not shown, S.D. is within symbols.

Table 1. Comparison of the ^3H -labelled Ins lipids in control GH_3 cells incubated in buffer containing 10 mM-LiCl and 100 mM unlabelled Ins and in cells stimulated by TRH for 3 h

Values in parentheses indicate ^3H labelling as a percentage of the total

	^3H -labelled Ins lipids (d.p.m.)			
	PtdIns	Lyso-PtdIns	PtdIns(4)P	PtdIns(4,5)P ₂
Control	111 040 $\pm$ 2695 (93.0 $\pm$ 2.2)	3855 $\pm$ 430 (3.2 $\pm$ 0.4)	1950 $\pm$ 70 (1.6 $\pm$ 0.1)	2410 $\pm$ 35 (2.0 $\pm$ 0.03)
TRH	45 575 $\pm$ 3620 (93.0 $\pm$ 7.4)	2120 $\pm$ 40 (4.3 $\pm$ 0.1)	720 $\pm$ 65 (1.5 $\pm$ 0.1)	745 $\pm$ 30 (1.5 $\pm$ 0.1)

Fig. 2. Effects of TRH on $[^3\text{H}]\text{Ins}(4)\text{P}$ and $[^3\text{H}]\text{Ins}(1)\text{P}$ levels in GH_3 cells

Cells were prelabelled with $[^3\text{H}]\text{Ins}$ and incubated in buffer with 100 mM unlabelled Ins (without LiCl) (a) or in buffer with 10 mM-LiCl plus 100 mM unlabelled Ins (b). The experiment was performed as described in the legend to Fig. 1. $\text{Ins}(1)\text{P}$ (■) and $\text{Ins}(4)\text{P}$ (●) were measured by h.p.l.c.

in $\text{Ins}(4)\text{P}$ than in $\text{Ins}(1)\text{P}$, whereas in cells incubated in buffer with LiCl there was a more rapid increase in $\text{Ins}(4)\text{P}$ but a delayed increase in $\text{Ins}(1)\text{P}$. However, the increase in $\text{Ins}(1)\text{P}$ exceeded that in $\text{Ins}(4)\text{P}$ at the later time points. In the absence of LiCl, treatment with 100 mM-Ins for 180 min decreased $[^3\text{H}]\text{Ins}(4)\text{P}$ levels to control values, showing that the ^3H -labelled TRH-responsive lipid pool has been completely replaced by unlabelled Ins lipids (results not shown).

It was important to determine whether termination of the effect of TRH in decreasing ^3H -labelled Ins lipids and increasing ^3H -labelled Ins phosphates and/or Ins was caused by depletion of ^3H radioactivity from a responsive Ins lipid pool or by

Fig. 3. Effects of prolonged stimulation by TRH on formation of $[^3\text{H}]\text{Ins}$ phosphates in GH_3 cells prelabelled with $[^3\text{H}]\text{Ins}$

Prelabelled cells were incubated in buffer without (a) or with (b) 100 mM-Ins for 1 h. TRH ($1 \mu\text{M}$) was then added (time zero) and 10 mM-LiCl (●, ▲) was added after 5 or 120 min (○, △, no LiCl addition). Incubations were terminated at the times indicated, and ^3H radioactivity in Ins phosphates and lipids was measured. ^3H -labelled Ins lipids were 50400 ± 560 d.p.m. at zero time, and decreased to 40500 ± 2600 d.p.m. after stimulation by TRH for 120 min in buffer without unlabelled Ins, and to 18500 ± 420 d.p.m. after stimulation by TRH for 120 min in buffer with 100 mM unlabelled Ins. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment which was repeated three times; where not shown, S.D. is within symbols.

desensitization. We approached this important point in two series of experiments. In the first, we found that when $1 \mu\text{M}$ -TRH was added to prelabelled cells incubated in buffer without Ins, and then 10 mM-LiCl was added after 5 or 120 min, there were only small differences in the rates of accumulation of ^3H -labelled Ins phosphates, of 1650 and 1480 d.p.m./min respectively, over the next 10 min (Fig. 3). A similar small decrease of between 10 and 15% in the rate of Ins phosphate accumulation after exposure to TRH for 120 min compared with the rate after 5 min was observed in three out of five experiments. These data showed that stimulation by TRH of Ins phosphate formation is not markedly diminished after a 2 h exposure to TRH in control cells under these conditions. By contrast, when 10 mM-LiCl was added 5 min after TRH to prelabelled cells incubated in buffer with 100 mM unlabelled Ins, ^3H -labelled Ins phosphates accumulated at a rate of 1300 d.p.m./min, whereas when LiCl was added after 120 min there was no significant accumulation of ^3H -labelled Ins phosphates. These findings confirm the results of the experiments shown in Fig. 1, i.e. when re-utilization of $[^3\text{H}]\text{Ins}$ is inhibited by dilution with unlabelled Ins, formation of ^3H -labelled Ins phosphates stimulated by TRH is abolished after 2 h.

In the second series of experiments, we showed that the inhibition of $[^3\text{H}]\text{Ins}$ phosphate formation in cells incubated in buffer containing 100 mM unlabelled Ins and 10 mM-LiCl was due to loss of ^3H radioactivity from Ins lipids and not to desensitization. $[^{32}\text{P}]\text{P}_i$ and $1 \mu\text{M}$ -TRH were added simultaneously (at zero time) to $[^3\text{H}]\text{Ins}$ -prelabelled cells incubated in buffer with 10 mM-LiCl, without or with 100 mM unlabelled Ins, or in buffer alone (control). In cells incubated in buffer alone (Fig. 4a), TRH stimulated ^{32}P labelling of PtdIns at a nearly constant rate for the

Fig. 4. Effects of LiCl and unlabelled Ins on ^{32}P labelling of PtdIns during prolonged stimulation of GH₃ cells by TRH

Cells prelabelled with [^3H]Ins were incubated in buffer without or with 100 mM-Ins for 1 h. Cells were then incubated in buffer without ($\circ$, Δ) or with ($\bullet$, $\blacktriangle$) 1 μM -TRH, and with [^{32}P] P_i (time zero) for an additional 3 h. (a) No further additions; (b) plus 10 mM-LiCl; (c) plus 10 mM-LiCl and 100 mM unlabelled Ins. At the times indicated, the incubations were terminated and ^{32}P and ^3H radioactivities in PtdIns ($\circ$, $\bullet$) and ^{32}P radioactivity in PA (Δ , $\blacktriangle$) were measured (^3H radioactivity shown in insets). Points represent means $\pm$ S.D. of triplicate determinations in one of two experiments.

entire duration of the experiment. TRH caused only a small increase in the rate of ^{32}P labelling of phosphatidic acid (PA), which accumulates in TRH-stimulated GH₃ cells when the content of Ins is insufficient to sustain PtdIns resynthesis [19]. There was only a small decrease in ^3H -labelled PtdIns under these conditions (Fig. 4a, inset). By contrast, in [^3H]Ins-prelabelled cells incubated in buffer containing 10 mM-LiCl, TRH caused a smaller stimulation of the ^{32}P labelling of PtdIns, and the ^{32}P labelling of PA was increased whereas the level of [^3H]PtdIns was decreased (Fig. 4b). In [^3H]Ins-prelabelled cells incubated in buffer containing 10 mM-LiCl and 100 mM unlabelled Ins, the rates of ^{32}P labelling of PtdIns and PA stimulated by TRH were similar to those in control cells, even though the level of [^3H]PtdIns was decreased (Fig. 4c). Hence prolonged stimulation by TRH does not markedly desensitize Ins lipid hydrolysis and resynthesis. These findings show, therefore, that termination of [^3H]Ins phosphate formation represents depletion of ^3H radioactivity, not Ins lipid mass, from a distinct TRH-responsive Ins lipid pool.

To determine whether the apparent size of the TRH-responsive pool under conditions of prolonged stimulation was related to the number of activated TRH-receptor complexes, we performed extended time course experiments in the presence of unlabelled Ins and different doses of TRH. Fig. 5 illustrates the time course of the effect of two doses of TRH on ^3H -labelled Ins lipids in

Fig. 5. Time course of the effect of two doses of TRH on ^3H -labelled Ins lipids in GH₃ cells prelabelled with [^3H]Ins and incubated in buffer with 10 mM-LiCl + 100 mM unlabelled Ins.

Prelabelled cells were incubated in buffer with 10 mM-LiCl and 100 mM-Ins for 1 h. TRH [0.3 nM ($\blacksquare$) or 1 μM ($\blacktriangle$)] was then added (time zero) to separate portions of the cell suspension ($\bullet$, control). TRH (1 μM) was added to a separate portion of cells 3 h after additions of 0.3 nM-TRH ($\square$). Incubations were terminated at the times indicated and ^3H radioactivity in PtdIns was determined. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times.

Fig. 6. Effect of receptor occupancy on TRH-stimulated decreases in ^3H -labelled Ins lipids in GH_3 cells prelabelled with ^3H Ins and incubated in buffer with 10 mM-LiCl plus 100 mM unlabelled Ins

Prelabelled cells were incubated in buffer with 10 mM-LiCl and 100 mM-Ins for 1 h. Cells were then incubated for an additional 2 h with TRH (0–1 μM) without (●) or with (□) 50 μM -chlordiazepoxide. Points represent means $\pm$ S.D. of triplicate determinations in one of two similar experiments.

^3H Ins-prelabelled cells incubated in buffer containing 10 mM-LiCl and 100 mM unlabelled Ins. Under these conditions, there was a slow but continuous decrease in ^3H PtdIns in control cells incubated without TRH, whereas 1 μM -TRH caused a marked decline in ^3H PtdIns, which attained an apparent steady-state level after 2 h. By contrast, 0.3 nM-TRH caused a less marked decrease in ^3H PtdIns which attained its apparent steady-state level after 4 h. After about 5 h (up to 8 h), the small continuing decrease in ^3H radioactivity in PtdIns in TRH-stimulated cells paralleled that in control cells (results not shown). In separate experiments, doses of 1 and 3 nM-TRH yielded similar results (results not shown). When 1 μM -TRH was added to cells which had been incubated with 0.3 nM-TRH for 3 h there was a rapid decrease in ^3H PtdIns to a level similar to that obtained by addition of 1 μM -TRH at zero time (Fig. 5).

Fig. 6 shows that the decrease in ^3H PtdIns caused by TRH was dose-dependent. The half-maximally effective dose was between 0.3 and 1 nM-TRH. When ^3H Ins-prelabelled cells were incubated in buffer containing 10 mM-LiCl, 100 mM unlabelled Ins and 50 μM -chlordiazepoxide (a competitive antagonist of TRH action [27,28]), the TRH effect still occurred, but at higher doses. In addition, the effect of 1 nM-TRH in decreasing ^3H PtdIns was antagonized progressively by increasing doses of chlordiazepoxide (results not shown).

DISCUSSION

We have shown that, during prolonged (5 h) stimulation by a maximally effective dose of TRH, approx. 60% of the total content of Ins lipids in a population of GH_3 cells can be hydrolysed and resynthesized. The remaining 40% of Ins lipids were not hydrolysed, even though the pool of Ins lipids that was responsive was depleted of its initial complement of Ins lipids within 2 h and was apparently resynthesized and rehydrolysed during the subsequent 3 h. The continued action of TRH in GH_3 cells is, therefore, dependent on the resynthesis of Ins lipids, a finding that has been observed previously [24–26]. Our findings extend previous observations to show that resynthesis occurs in a distinct TRH-responsive pool in GH_3 cells. Although it is theoretically possible that the two functionally distinct pools reflect two populations of cells, one which can be stimulated by TRH and a second which cannot, this is highly unlikely because

GH_3 cells are a cloned cell line [29] in which the vast majority of cells respond to TRH [30]. Furthermore, newly isolated GH_3 cell clones also possess these two pools (A. B. Cubitt, E. Geras-Raaka & M. C. Gershengorn, unpublished work). Moreover, the observation that the fraction of total cell Ins lipids that can be hydrolysed and resynthesized in response to TRH even after prolonged stimulation is dependent on the number of receptors occupied is strong evidence against the existence of two cell populations, because only cells possessing functional TRH receptors would respond. Hence we conclude that there are distinct functional pools of Ins lipids in individual GH_3 cells and that only a part of total cell Ins lipids can be used for signal transduction on TRH stimulation.

The rate of PtdIns resynthesis was decreased by only 10–15% in the presence of 100 mM-Ins after 2 h of stimulation by TRH compared with 5 min after TRH addition. Also, TRH stimulation of Ins phosphate formation was only minimally decreased after 2 h of incubation as compared with 5 min after TRH addition. These small decreases may have been secondary to a decrease in TRH receptor number [31,32]. Therefore these data show that prolonged stimulation by TRH does not markedly desensitize Ins lipid hydrolysis and resynthesis in GH_3 cells under these conditions. This finding appears to agree with some previous observations [33,34] and conflict with others [35]. The reason(s) for these apparent discrepancies is not clear, but they may be caused by the differences in incubation conditions of the cells in the different reports.

In cells incubated in buffer without LiCl we found that the major InsP was Ins(4)P, even after a prolonged duration of stimulation. Moreover, the time course of accumulation of Ins(1)P was similar to that of Ins(4)P, and the maximum increase in Ins(1)P was only 16% of that in Ins(4)P. Although this type of analysis cannot exclude the possibility that PtdIns is hydrolysed directly during stimulation by TRH, the data are more consistent with the idea that PtdIns(4,5) P_2 is the major, if not the sole, lipid hydrolysed under these conditions [36]. We had concluded previously [6] that PtdIns was hydrolysed predominantly at later times during TRH action, based on experiments in which we found that ^{32}P -labelling of PtdIns was persistent whereas that of PtdIns(4,5) P_2 was transient. However, we discussed the possibility that this interpretation may not have been correct if there were distinct TRH-responsive and -unresponsive Ins lipid pools in GH_3 cells. Hence our present results are important in allowing re-interpretation of our previous findings.

It is interesting that the Ins lipids in the unresponsive pool in GH_3 cells, i.e. those lipids not hydrolysed after several hours of stimulation, contained the same proportions of PtdIns, lyso-PtdIns, PtdIns(4)P and PtdIns(4,5) P_2 as those in the TRH-responsive lipid pool (Table 1). It was previously thought possible that this pool was not hydrolysed because it was composed of different lipids. In particular, the unresponsive pool may not have contained PtdIns(4,5) P_2 , and may have been present in an environment in which PtdIns(4)P kinase, the enzyme that synthesizes PtdIns(4,5) P_2 , was deficient. However, our data appear to exclude this possibility as an explanation for the genesis of the unresponsive pool.

The most important novel finding in our report is that the fraction of Ins lipids in the responsive pool available for hydrolysis is dependent on receptor occupancy by TRH, even under conditions of persistent stimulation. This observation seems inconsistent with accepted concepts that ligand–receptor and receptor–GTP-binding protein (G-protein) interactions are rapidly and completely reversible. It would have been predicted that, at a submaximally effective concentration of TRH, only a fraction of receptors would be occupied at any given time, but

this would be a different population of receptors at different times during the incubation, as TRH dissociated from and re-associated with receptors. Thus, given sufficient time, all receptors would be transiently occupied and lipids in the entire responsive pool would be hydrolysed. Our observations are not consistent with this hypothesis, at least for the duration of our experiments. Indeed, it has been shown that the interaction of TRH with its receptors in GH₃ cells with time leads to an increase in receptor affinity, as indicated by a slower rate of dissociation of bound TRH that can be inhibited by incubation at low temperature [37,38]; A. B. Cubitt, E. Geras-Raaka & M. C. Gershengorn, unpublished work). This is followed by receptor sequestration, as shown by resistance to acid washing [37,38], which does not appear to be receptor internalization by endocytosis [39]. Similar observations of a higher-affinity receptor form after agonist occupation and of functional sequestration have been made with gonadotropin-releasing hormone receptors in pituitary cells [40]. The generation of a higher-affinity form of the TRH receptor shortly after TRH binding may be secondary to formation of a receptor-G-protein complex or a receptor-G-protein-phospholipase C complex. Indeed, TRH receptors have been solubilized in complexes with G-proteins [41,42], and vasopressin receptors, which mediate Ins lipid hydrolysis in the liver, have been solubilized in ternary complexes with G-proteins and phospholipases C [43]. These observations are consistent with the idea that receptors, G-proteins and phospholipases C interact with high affinity and that these complexes are stable. It is possible, therefore, that the population of receptors occupied by TRH does not change during the course of incubation. Even upon dissociation, TRH would preferentially bind to receptors that are of higher affinity in a complex with G-protein and phospholipase C (the previously occupied receptors) rather than to receptors that were unoccupied and not in a complex with G-protein and phospholipase C. These data are consistent with the idea that the responsive pool of Ins lipids contains those lipids within cellular domains that are accessible to persistently (or preferentially) occupied receptors and need not be present in a compartment within cells that is anatomically distinct from that of the unresponsive pool. We propose that the TRH-responsive pool of Ins lipids in GH₃ cells is composed of the complement of Ins lipids that are within functional proximity to the activated TRH-receptor complex. A corollary to this is that Ins lipids within the microenvironment of unactivated (unoccupied) receptors are not fully accessible to other activated TRH receptors, at least under the conditions of these experiments. We think there may be some overlap of these Ins lipid domains, because half of the responsive pool can be hydrolysed when fewer than half of the receptors are occupied. Lastly, because of the continued hydrolysis and resynthesis of a fraction of the Ins lipids without affecting the other Ins lipids, we suggest that PtdIns resynthesis is likely to occur within the same microenvironment as hydrolysis, i.e. within close proximity of the activated TRH-receptor complex.

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