Polyphosphoinositide hydrolysis by phospholipase C is accelerated by thyrotropin releasing hormone (TRH) in clonal rat pituitary cells (GH_3 cells)

Werner Schlegel, Claude Roduit and Gaston R. Zahnd

Fondation pour Recherches Médicales, Department of Medicine, University of Geneva, 64, av. de la Roseraie, CH-1211 Geneva 4, Switzerland

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Thyrotropin releasing hormone (TRH) accelerates the turnover of phosphatidylinositol in GH_3 cells ('phospholipid response') From the analysis of inositol phosphates in the presence of Li^+ which inhibits their dephosphorylation, it can be concluded that the hydrolysis of phosphatidylinositol 4,5-biphosphate, and possibly of phosphatidylinositol 4-phosphate by phospholipase C is markedly accelerated by TRH. It appears that this reaction initiates the acceleration of phosphatidylinositol turnover. The specificity of hormonally regulated phospholipase C reaction for polyphosphoinositides has important implications for the potential role of the phospholipid response as a mechanism of membrane signal transduction.

Lithium *Phospholipid response* *Ca^{2+} mobilization* *Second messenger*

1. INTRODUCTION

GH_3 cells are clonal rat pituitary tumor cells that have been widely used as a model system to study the mode of action of TRH [1]. It was shown that TRH increases the secretion of prolactin (PRL) in a Ca^{2+}-dependent manner [2] and there is now direct evidence that TRH raises $[Ca^{2+}]_i$ by mobilization of intracellular calcium [3]. TRH also accelerates the turnover of PtdIns [4–8]; this phospholipid response has been observed in a wide variety of cells and tissues in parallel to changes in Ca^{2+} distribution brought about by the same stimuli [9]. The phospholipid response in GH_3 cells is not a consequence of increased $[Ca^{2+}]_i$ since depolarization of the cells leading to an increase in $[Ca^{2+}]_i$ comparable to or greater than that after TRH stimulation [3] does not change PtdIns turnover [4,7]. In many systems hormones or neurotransmitters eliciting a phospholipid response also stimulate the hydrolysis of polyphosphoinositides, PtdIns 4P and PtdIns(4,5)P_2 [10–15]. Polyphosphoinositide hydrolysis can occur through the action of phosphomonoesterases or by a phospholipase C reaction [16], and the latter pathways give rise to Ins(1,4)P_2 and Ins(1,4,5)P_3 from PtdIns 4P and PtdIns(4,5)P_2, respectively.

It appears from the analysis of the phospholipids that TRH stimulates hydrolysis of PtdIns by phospholipase C: PtdIns is decreased concomitantly with an increase of PA and a transient rise of DG [8]. However, we present here evidence from the analysis of inositol phosphates that PtdIns(4,5)P_2 and possibly PtdIns 4P are the major substrates in the hormonally accelerated reaction. As will be discussed, this observation has important implications for the potential role of the phospholipid response for the generation of an in-

Abbreviations: TRH, thyrotropin releasing hormone, $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PtdIns, phosphatidylinositol; PtdIns 4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-biphosphate; Ins 1P, *myo*-inositol 1-phosphate; Ins(1,4)P_2, *myo*-inositol 1,4-biphosphate; Ins(1,4,5)P_3, *myo*-inositol 1,4,5-triphosphate; PA, phosphatidic acid; DG, diacylglycerol

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tracellular signal (second messenger), i.e., as a mechanism of membrane signal transduction.

2. MATERIALS AND METHODS

GH_3 cells were grown attached in cell culture flasks (25 cm^2) in Ham F-10 medium supplemented with 15% horse and 2.5% fetal calf serum as in [1].

^{32}P-labelling of phospholipids was obtained by exposure of GH_3 cells to carrier-free [^{32}P]orthophosphoric acid (Amersham) (5 μCi/ml) in Hepes (30 mM) buffered Ham F-10 culture medium (pH 7.4). Phosphoinositides and inositol phosphates were labelled by incubating GH_3 cells for 16–18 h in serum-free Hepes buffered Ham F-10 culture medium with *myo*-[2-^{3}H]inositol (Amersham, 17 Ci/mmol) (0.5 μCi/ml). Cells were incubated in Hepes buffered Ham F-10 medium at 37°C, and TRH or KCl were added in a small volume at 50-fold the final concentration. Incubations were stopped by withdrawal of the medium and addition of 2 ml of 0.25% 12 N HCl in methanol. After transferring the denatured cells in the methanol to stoppered glass tubes, the phospholipids were extracted with acidified chloroform–methanol and analysed by one-dimensional thin-layer chromatography according to [17] as in [4].

[^{3}H]Inositol phosphates were analysed in the combined aqueous phases of the phospholipid extraction, adjusted to pH 9.0 with NaOH, diluted to 3 ml, by ion exchange chromatography on Dowex 1X8 columns, 1 ml bed volume; elution was carried out stepwise, 5 ml per step, with the following solutions: (I) H_2O; (II) 5 mM $Na_2B_4O_7$, 60 mM sodium formate; (III) 5 mM $Na_2B_4O_7$, 150 mM sodium formate; (IV) 0.1 M formic acid, 0.3 M ammonium formate; (V) 0.1 M formic acid, 0.55 M ammonium formate. Inositol, Ins 1P, Ins(1,4)P_2, and Ins(1,4,5)P_3 are eluted in steps I, III, IV, and V, respectively. A nearly identical procedure has been described in detail [18]. Separation of [^{3}H]inositol phosphates was verified with high-voltage paper electrophoresis [11]. More than 80% of the radioactivity eluted in steps III, IV, and V was recovered after electrophoresis in spots corresponding to the position of the respective standards [Ins 1P (Sigma), inositol di- and triphosphates in impure phytic acid (Fluka)].

3. RESULTS

Incorporation of [^{32}P]phosphate into phosphoinositides and PA is shown in fig.1. Basal turnover of these phospholipids is reflected by the labelling during the first 60 min. Upon addition of TRH, ^{32}P-labelling of PA and PtdIns increases rapidly due to the accelerated turnover of PtdIns – the phospholipid response – described previously [4–8]. In contrast, TRH leads to a transient decrease of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns 4P. Since the labelling of the precursor of polyphosphoinositides, PtdIns, is increasing, the transient loss of ^{32}P-labelling indicates a decrease in the concentration of PtdIns(4,5)P_2 and PtdIns 4P.

To investigate whether this decrease is due to increased hydrolysis of polyphosphoinositides by phospholipase C, inositol phosphate levels in GH_3 cells were analysed. Inositol phosphates are normally dephosphorylated rapidly. Li^+, an inhibitor of Ins 1P phosphatase [19], can block the breakdown of Ins 1P in intact cells and tissues, and it has been reported that Li^+ amplified the increased Ins

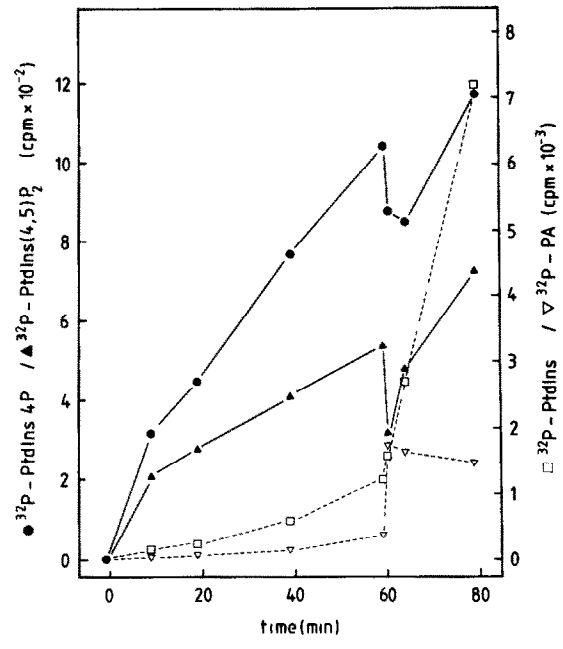


Fig.1. Effect of TRH on phosphoinositides and PA [^{32}P]Phosphate incorporation into PtdIns 4P (●) PtdIns(4,5)P_2 (▲), PtdIns (□), and PA (▽) of GH_3 cells cpm per flask (2.5 × 10^6 cells); TRH, 10^{-7} M fina concentration, was added after 60 min.

1P accumulating resulting from a phospholipid response [20].

The effects of Li^+ (15 mM) on [^{3}H]inositol phosphates in GH_3 cells prelabelled with *myo*-[^{3}H]inositol are presented in table 1. Incubation of GH_3 cells with Li^+ leads to an increase of all [^{3}H]inositol phosphates, presumably by inhibition of their dephosphorylation. Note that so far only inhibition of Ins 1P phosphatase by Li^+ has been directly demonstrated [19,20]. The effect of Li^+ is most pronounced after TRH stimulation. While in the absence of Li^+ TRH increases all [^{3}H]inositol phosphates only slightly (1.4–2-fold) the levels of [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 are markedly higher (4–10-fold) after TRH action in the presence of Li^+ when compared to non-stimulated cells; in contrast the TRH effect on [^{3}H]Ins 1P is not enhanced by Li^+. Hence the 'amplification' of TRH-induced changes in inositol phosphates by Li^+ allows one to demonstrate that the major effect of TRH is the increase in Ins(1,4,5)P_3 and Ins(1,4)P_2, consistent with a stimulation of the breakdown of PtdIns(4,5)P_2 and possibly PtdIns 4P by phospholipase C.

The time course of [^{3}H]inositol phosphate accumulation in the presence of Li^+ is shown in fig.2. As can be seen in panel A, TRH (10^{-7} M) added after 20 min of preincubation with LiCl (15 mM) leads rapidly to a marked increase in [^{3}H]Ins (1,4,5)P_3. Depolarization with KCl (50 mM), which has been shown to increase $[Ca^{2+}]_i$ [3], fails to affect [^{3}H]Ins(1,4,5)P_3 levels. Hence the accumulation of [^{3}H]Ins(1,4,5)P_3 is not a consequence of the rise in $[Ca^{2+}]_i$. The onset of this response is as rapid as the effects of TRH on ^{32}P-labelling of phosphoinositides and PA (fig.1, [6–8]). This suggests that the changes in [^{3}H]inositol phosphates and the changes in [^{32}P]phospholipids both arise directly from TRH acceleration of PtdIns(4,5)P_2 hydrolysis by phospholipase C.

Table 1

Effects of TRH and Li^+ on [^{3}H]inositol phosphates in GH_3 cells prelabelled with [^{3}H]inositol [dpm × 10^{-3}, ± SE (n = 3) per flask (1 5 × 10^6 cells)]

Condition	Ins 1P	Ins(1,4)P_2	Ins(1,4,5)P_3
Control	3.9 ± 0.2	7.7 ± 0.2	2.8 ± 0 1
LiCl	6 0 ± 2.5	12.9 ± 2.5	4.2 ± 0.6
LiCl, TRH	16.0 ± 4.0	57.4 ± 5.7	47.2 ± 2.4
TRH	10.5 ± 2.2	12.3 ± 2.3	6 8 ± 0.3

GH_3 cells prelabelled with [^{3}H]inositol were incubated for 30 min in the presence or absence of LiCl (15 mM); TRH (10^{-7} M) was added after 20 min

In the same experiment [^{3}H]PtdIns(4,5)P_2 was determined, and a transient decrease, similar to that shown in fig.1 for [^{32}P]PtdIns(4,5)P_2, was observed. Combining these data with those in fig.2A, approximative hydrolysis rates for PtdIns(4,5)P_2 in different time intervals following TRH addition were calculated: the increment of [^{3}H]Ins(1,4,5)P_3 in each time interval is expressed as percent of [^{3}H]PtdIns(4,5)P_2 determined at the beginning of the interval and related to the length of the interval (min). This approximation, presented in fig.2B, is based on the assumption that the dephosphorylation of Ins(1,4,5)P_3 is completely inhibited, and hence probably underestimates the exact hydrolysis rates. Such a calculation shows that PtdIns(4,5)P_2 hydrolysis is very rapid immediately following TRH action, such that without resynthesis all PtdIns(4,5)P_2 would be hydrolysed within less than 30 s. It is therefore possible that, despite the fact that polyphosphoinositides represent only a minor fraction of the phosphoinositides [9], acceleration of their hydrolysis would be sufficient to increase the turnover of PtdIns markedly. The high rates of hydrolysis of PtdIns(4,5)P_2 are not maintained and an apparent desensitization of the phospholipid response is observed. The transient nature of both the increase in $[Ca^{2+}]_i$ [3] and the loss of [^{32}P]polyphosphoinositides (fig.1) may reflect this.

From our data it is not possible to decide whether TRH accelerates exclusively the hydrolysis of PtdIns(4,5)P_2, since the inhibition of dephosphorylation of Ins(1,4,5)P_3 by Li^+ cannot be verified and may be incomplete. As shown in fig.2C,D, TRH also increases [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins 1P, which could stem from either dephosphorylation of [^{3}H]Ins(1,4,5)P_3 or from hydrolysis of [^{3}H]PtdIns 4P and [^{3}H]PtdIns by phospholipase C. Note that the time course of [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins 1P accumulation after TRH action differs slightly from that observed for [^{3}H]PtdIns(1,4,5)P_3 and that early after TRH addition the rise in [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns 1P is insignificant. In addition, given the rapid equilibrium between all phosphoinositides [9],

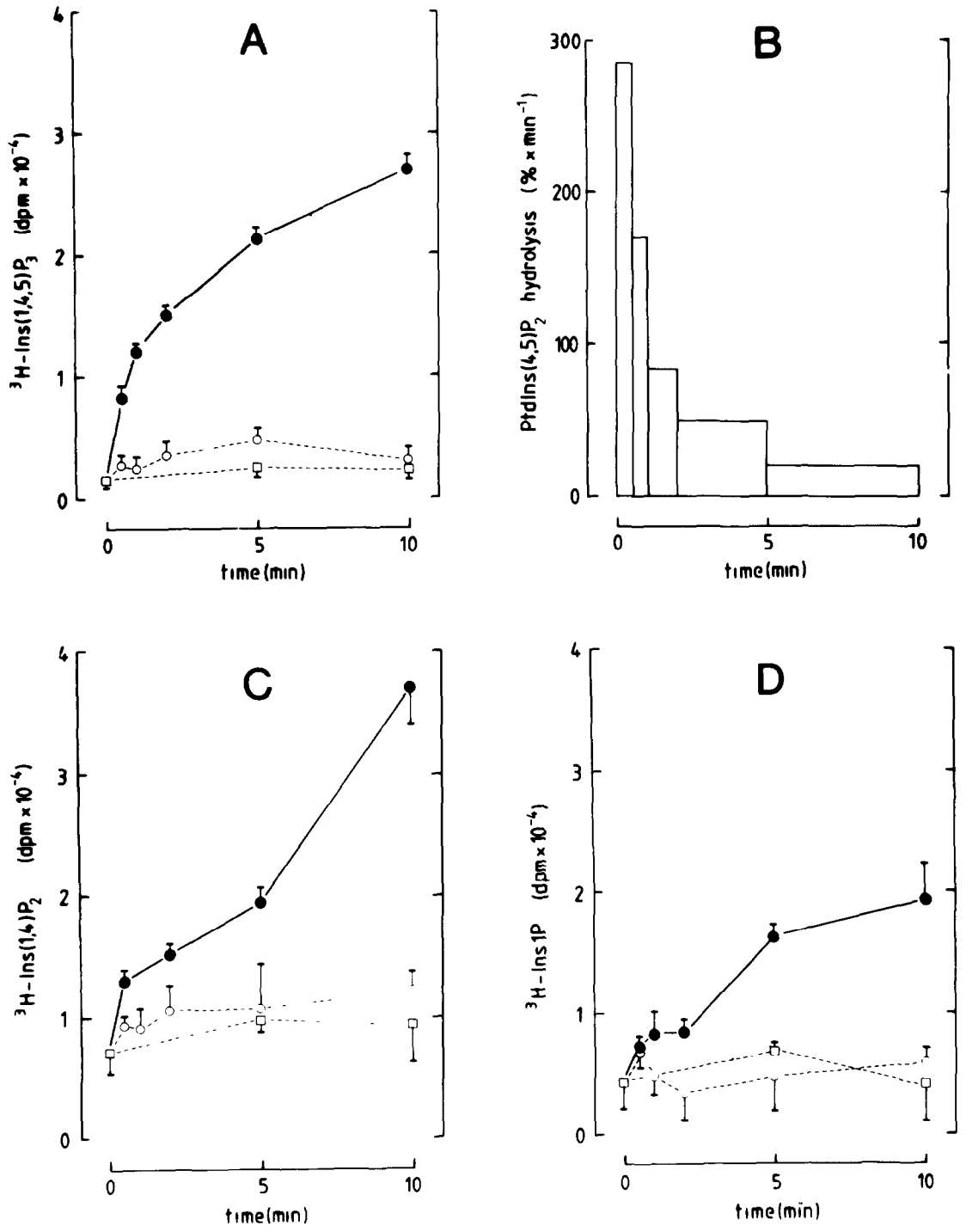


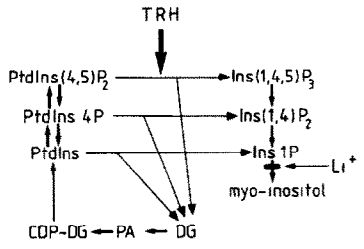
Fig.2 (A,C,D) [^{3}H]Inositol phosphates in GH_3 cells labelled with [^{3}H]inositol; dpm ± SE (n = 3) per flask (1.5 × 10^6 cells). Stimuli added after 20 min preincubation with LiCl (15 mM): (●) TRH, 10^{-7} M; (○) KCl, 50 mM: (□) no addition. (B) Approximative hydrolysis rates of PtdIns(4,5)P_2 in different time intervals following TRH addition (for details see text)

breakdown of either of these phospholipids could lead to a decrease in the concentrations of the others, and explain the loss of PtdIns 1P (fig.1) and PtdIns [8] without a stimulation of phospholipase C hydrolysis of these phosphoinositides.

4. DISCUSSION

Acceleration of the turnover of phosphoinositides (scheme 1) by hormones and neurotransmitters has been proposed as a mechanism of membrane signal transduction [9]. To substantiate this proposal, we analysed the biochemistry of the phospholipid response to TRH in GH_3 cells and determined the critical step in the pathways involved that is probably under direct hormonal control.

The data presented above indicate that PtdIns(4,5)P_2 and possibly PtdIns 4P are the major substrates for the phospholipase C reaction stimulated by TRH. The same conclusion was reached in a study [21] published during the revision of this paper, which was carried out without the use of Li^+ to block inositol phosphate hydrolysis. Under these experimental conditions, a transient rise in Ins(1,4,5)P_3 and Ins(1,4)P_2 was observed; early changes in inositol phosphates thus reflect the specificity of the hormonally accelerated phospholipase C reaction despite the rapid dephosphorylation of inositol phosphates in the absence of Li^+ [20], whereas after 10 min the substrate specificity of the phospholipid response is no longer apparent ([21], table 1). Blockage of inositol phosphate hydrolysis with Li^+ is thus not a prerequisite, but may facilitate the analysis of phosphoinositide metabolism, especially in systems which react less homogeneously to stimulation than a clonal cell line.



Scheme 1. Turnover cycle of phosphoinositides: Site of action of TRH.

The specificity of the hormonally accelerated phospholipase C reaction for polyphosphoinositides implies that only a minor fraction of the phospholipids in the plasma membrane is involved in membrane signal transduction. From the rapid apparent desensitization of the response (fig.2B), it follows that this pool of polyphosphoinositides in the plasma membrane of GH_3 cells can be depleted upon maximal stimulation with TRH; this is similar to the phospholipid response in blowfly salivary gland, which has been shown to be limited to a specific pool of PtdIns [22]. The transient nature of the rise in Ins(1,4,5)P_3 and Ins(1,4)P_2 observed in the absence of Li^+ [21] may also reflect this limitation.

It appears from the data in fig.1 that the transient decrease in [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns 4P is rather small and since the specific radioactivities of the polyphosphoinositides are probably higher than that of PtdIns one would estimate that the quantity of DG produced by this reaction is too small to explain the massive increase in ^{32}P-labelling of PtdIns. However, as shown in fig.2B, the hydrolysis rate of PtdIns(4,5)P_2 reaches 300% per min when estimated on the assumption of a complete block of Ins(1,4,5)P_3 hydrolysis by Li^+; i.e., the real hydrolysis rate could be even higher. Hence, PtdIns(4,5)P_2 must be replenished rapidly by phosphorylation of PtdIns 4P and PtdIns. Since this replenishment reaction also implies accelerated resynthesis of PtdIns to maintain the level of this phospholipid, it can explain the massive increase in ^{32}P-labelling presented in fig.1 and shown previously [4–8]. The constant resynthesis of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns 4P from [^{32}P]PtdIns of increasing radioactivity leads to a transient lowering of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns 4P which does not reflect the amount of DG generated during the process. Thus despite the discrepancy in the changes of ^{32}P-labelling between polyphosphoinositides and PtdIns (fig.1), the data presented above are consistent with the notion that accelerated hydrolysis of PtdIns(4,5)P_2 and possibly PtdIns 4P leads to the increased turnover of PtdIns and PA reported in [4–8]; however, the possibility that the latter event is triggered independently, i.e., by the lowering of PtdIns concentration [8,21], has to be kept in mind.

The rapidity, selectivity, and independence of $[Ca^{2+}]_i$ of the phospholipid response in GH_3 cells

(see above and [4–8,21]) suggest a direct action of TRH receptors on PtdIns(4,5)P_2 hydrolysis; yet the mechanism of stimulation of the phospholipase C remains unknown. Since it is very difficult to demonstrate a phospholipid response in broken cells, where hormone sensitivity and maximal effects are greatly reduced [23], the cellular localization of either PtdIns(4,5)P_2 and/or the regulated phospholipase C appear to be important for the 'coupling' of the hormone–receptor interaction to the hydrolysis of PtdIns(4,5)P_2.

It was proposed that the phospholipid response mediates the changes in $[Ca^{2+}]_i$, either via interference with Ca^{2+} gating mechanisms [9] or by the liberation of Ca^{2+} bound to polyphosphoinositides [24] upon their hydrolysis. Polyphosphoinositides are good chelators for divalent cations; however they show little specificity of ion binding [25]. Exposed to the cytoplasm they would be complexed mainly with Mg^{2+} and their hydrolysis would not liberate substantial amounts of Ca^{2+} as has been proposed [10], unless complex mechanisms of transfer of polyphosphoinositides from the extracellular side to the cytoplasmatic side of the plasma membrane are invoked. The liberation of Ca^{2+} bound to polyphosphoinositides upon their hydrolysis is thus an unlikely explanation for the mobilization of Ca^{2+}.

From the data presented above, the working hypothesis can be derived that Ins(1,4,5)P_3 is a second messenger; it is produced uniquely through breakdown of PtdIns(4,5)P_2 and reflects specifically TRH action (fig.2A). It has been shown very recently in pancreatic acinar cells that Ins(1,4,5)P_3 can release Ca^{2+} from a non-mitochondrial intracellular pool [26]; the rise in Ins(1,4,5)P_3 following TRH stimulation of PtdIns(4,5)P_2 hydrolysis could thus provide the link between the phospholipid response and the mobilization of intracellular Ca^{2+} [2,3].

Enhanced hydrolysis of polyphosphoinositides raises the concentration of two other potential intracellular mediators of hormone action, namely DG and PA. DG, increasing transiently after TRH action [8], is an activator of protein kinase C [27,28]. PA, increased in steady state in the presence of TRH [8], is a Ca^{2+} ionophore [29] and a main source for the precursor arachidonic acid utilized in prostaglandin and leukotriene synthesis [30,31]. Both protein kinase C and arachidonic acid metabolism are also regulated by Ca^{2+} [25,26,29] such that $[Ca^{2+}]_i$ could modulate these processes in the stimulated cells. It seems therefore that the correlation of Ca^{2+} mobilization with the phospholipid response [9,12] also reflects coordinate control of cellular responses.

In conclusion, our data demonstrate that TRH accelerates selectively the breakdown of polyphosphoinositides in GH_3 cells. This observation points to a specific role of PtdIns(4,5)P_2 and possibly PtdIns 4P in membrane signal transduction; however more information, in particular on the cellular localization of these phospholipids, is required to understand the mechanisms involved.

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