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Thyrotropin-Releasing Hormone-Stimulated [^{3}H]Inositol Metabolism in GH_3 Pituitary Tumor Cells

Studies with Lithium

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SUMMARY

GH_3 pituitary tumor cells were labeled to isotopic equilibrium with [^{3}H]inositol. Thyrotropin-releasing hormone (TRH), which has been shown to stimulate inositol phospholipid metabolism in these cells, enhanced the accumulation of [^{3}H]inositol-derived radioactivity in the cell's acid-soluble fraction. Separation of the [^{3}H]inositol metabolites by ion-exchange chromatography revealed that TRH induced a rapid rise in the cellular content of [^{3}H]inositol mono-, bis-, and trisphosphate. The latter two metabolites accumulated in a multiphasic manner with an initial peak 5–10 sec after TRH addition. This was followed by a short-lived decline and a secondary rise which left the metabolite levels elevated for at least 50 min. The GH_3 cell [^{3}H]inositol monophosphate and [^{3}H]inositol content also rose in response to TRH, but the latter accumulated with a considerably slower time course than the phosphorylated derivatives. None of these responses could be mimicked by the calcium ionophore A23187. Incubation of GH_3 cells with TRH in the presence of lithium led to an enhanced accumulation of [^{3}H]inositol monophosphate and, to a lesser extent, of [^{3}H]inositol bis- and trisphosphate. This accumulation rose in a linear fashion with time for at least 20 min, by which point 50% of the [^{3}H]inositol-containing phospholipids had been depleted. When lithium was added 30 min after TRH, [^{3}H]inositol monophosphate accumulated at the same rate as was found when TRH and lithium were added together, indicating that the TRH-induced phospholipid response in GH_3 cells does not desensitize. Under normal conditions, approximately equal amounts of the three [^{3}H]inositol phosphates were formed within 5 sec of TRH addition. However, when TRH was added to cells grown chronically in lithium-containing medium, or to cells incubating at a subphysiological temperature (25°), greater than 90% of the metabolites formed were the bis- or trisphosphates. This indicates that the primary event stimulated by TRH is the breakdown by phospholipase C of phosphatidylinositol 4,5-bisphosphate and, perhaps also, of phosphatidylinositol 4-phosphate.

INTRODUCTION

In 1975, Michell (1) proposed that the breakdown of PtdIns[1] is related in a causative manner to the agonist-induced mobilization of calcium. More recently, this view has been modified to include the possibility that the initial receptor-mediated response may be a phospholipase C-catalyzed hydrolysis of the polyphosphoinositides, PtdIns 4,5P_2 and PtdIns 4P (2).

GH_3 pituitary tumor cells, a clonal cell line that releases prolactin in a Ca^{2+}-dependent manner (3), have been shown to be a useful model system for the elucidation of the role of PtdIns turnover in Ca^{2+} gating (4–8). In the preceding paper (9), it has been demonstrated that TRH induces a rapid (<10 sec) reduction in the GH_3 cell content of PtdIns 4,5P_2 and PtdIns 4P. This decrease is simultaneous with a rise in the cellular content of 1,2-diacylglycerol, indicating that a phospholipase C enzyme might be involved in the initial receptor-mediated process.

In this study we have investigated the fate of the inositol moiety of these phospholipids when GH_3 cells

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[1] The abbreviations used are: PtdIns, phosphatidylinositol; PtdIns 4,5P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns 4P, phosphatidylinositol 4-phosphate; TRH, thyrotropin-releasing hormone; InMP, inositol monophosphate; InDP, inositol bisphosphate; InTP, inositol trisphosphate.

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are stimulated with TRH. Berridge and colleagues (10) have recently demonstrated that lithium inhibits the enzyme *myo*-inositol-1-phosphatase. This represents the first example of selective pharmacological interference with the "PtdIns cycle," and lithium has been used in this work to demonstrate that the hydrolysis of PtdIns 4,5P_2 and, perhaps, of PtdIns 4P by a phospholipase C enzyme is the initial response to TRH receptor occupancy. Some of these data have been presented in a preliminary form (11).

EXPERIMENTAL PROCEDURES

Materials. *myo*-[2-^{3}H]Inositol (16.4 Ci/mmole) was obtained from Amersham International (Amersham, England). TRH was Calbiochem-Behring, C. P. Laboratories (Bishops Stortford, England). Dowex 1-X8 (Cl^- form, 100–200 mesh) anion-exchange resin was purchased from Sigma Chemical Company (Kingston-upon-Thames, England). All other chemicals were of the highest purity available. The source of cell culture materials is fully described in the preceding paper (9).

Cell culture. The methods used for the production of GH_3 cells have been previously outlined (9). On the day of the second medium change, usually 7 days after plating, [^{3}H]inositol (5 μCi) was added to each tissue culture dish (10 ml). The cells were allowed to grow for at least another 3 days before use. In the experiments in which the effect of chronic lithium treatment of GH_3 cells was investigated, the cells which had previously been labeled to isotopic equilibirum with [^{3}H]inositol were grown for an additional 24 hr in Ham's F10 medium (supplemented with antibiotics and serum) plus 10 mM lithium chloride. Control cells received an additional 10 mM sodium chloride (to control for any effect of the increased osmotic pressure), which did not appreciably affect the cells' subsequent responses to TRH (data not shown). Cell suspensions were prepared as described (9).

Isolation of [^{3}H]inositol metabolites. Cell suspensions (0.5 ml containing 0.4–0.8 mg of protein) were incubated at 37° (unless otherwise stated) with TRH or vehicle. The reactions were stopped by adding 0.5 ml of 20% (w/v) trichloracetic acid (ice-cold). After 15 min at 0°, precipitated proteins were removed by centrifugation (650 × g at 4° for 15 min). The acid-soluble fraction was collected and frozen until [^{3}H] inositol metabolites were to be assayed. In some experiments the protein pellet was also extracted with 3 ml of chloroform/methanol (2:1) to yield an estimate of total [^{3}H]inositol in the phospholipid fraction of GH_3 cells. For the separation of acid-soluble [^{3}H]inositol metabolites, frozen aliquots were thawed and extracted three times with 4 volumes of water-saturated diethyl ether. After driving off the residual ether in a boiling water bath, the extracts were placed onto columns (1 × 2 cm) of Dowex 1-X8 anion-exchange resin. The method used thereafter was identical with that described by Griffin and Hawthorn (12): [^{3}H]inositol was eluted with 6 ml of distilled water, [^{3}H] InMP with 7 ml of 30 mM HCl, [^{3}H]InDP with 7 ml of 90 mM HCl, and [^{3}H]InTP with 7 ml of 500 mM HCl. Preliminary experiments using the method of Downes and Michell (13) to separate glycerophosphoinositol and inositol 1,2-cyclic phosphate from the other inositol metabolites revealed that the cellular contents of the former metabolites were unaffected by TRH (data not shown).

RESULTS

Experiments conducted in the absence of lithium. The data in Fig. 1 show the effects of TRH (0.1 μM) on the accumulation of the GH_3 cell [^{3}H]inositol metabolites separated by Dowex ion-exchange chromatography. It is clear that the four metabolites accumulate at different rates in response to TRH. Note that control values do not change over the course of a 5-min incubation at 37°. [^{3}H]InMP (B), [^{3}H]InDP (C), and [^{3}H]InTP (D) accumulate very rapidly in the acid-soluble fraction of GH_3 cells after the addition of TRH (Fig. 1B, C, and D). The accumulation of the latter two metabolites is multiphasic, with an early peak at 10 sec. This is followed by a decline in levels by 1–2 min, and then a secondary rise to a new peak by around 3–4 min. These elevated levels are maintained for at least 50 min (see Fig. 5 below). [^{3}H]InMP (B) and [^{3}H]inositol (A) also rose after TRH addition with significant changes in [^{3}H]InMP, in particular, occurring within 10 sec. Both of these metabolites accumulate in a monophasic manner in response to TRH, and their levels reach a plateau within 60–90 sec (InMP, B) and 3 min (inositol, A).

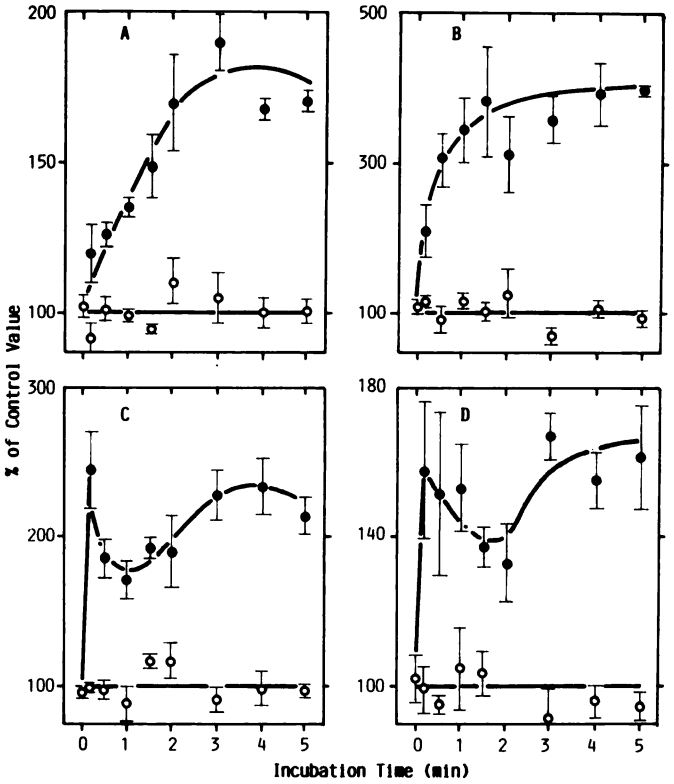

FIG. 1. *Effect of TRH on GH_3 cell acid-soluble [^{3}H]inositol metabolites*

GH_3 cells, suspended in balanced salt solution, were exposed to TRH (0.1 μM) at 37° for the times indicated. Reactions were terminated and acid-soluble [^{3}H]inositol metabolites separated as described under Experimental Procedures. A, [^{3}H]Inositol (control value, 15,400 ± 2,000 dpm/mg of protein); B, [^{3}H]InMP (2,600 ± 700 dpm/mg of protein); C, [^{3}H]InDP (2,000 ± 500 dpm/mg of protein); D, [^{3}H]InTP (3,100 ± 1,000 dpm/mg of protein). Values shown are means ± standard error of the mean for five experiments. O——O, control; ●——●, TRH.

The pattern of metabolite accumulation is concentration-dependent (Fig. 2). Low concentrations of TRH (3 nM, the half-maximal concentration for PtdIns turnover; see refs. 4 and 7) do not elicit the characteristic early peaks in InDP and InTP to nearly the same extent as higher concentrations [0.1 μM (Fig. 1) and 1 μM (Fig. 2)]. This presumably indicates that the enzymes that catabolize InTP to InDP and InDP to InMP are relatively easily saturated with substrate during the early stages after stimulation with TRH at high concentrations.

In an effort to determine whether the rapid accumu-

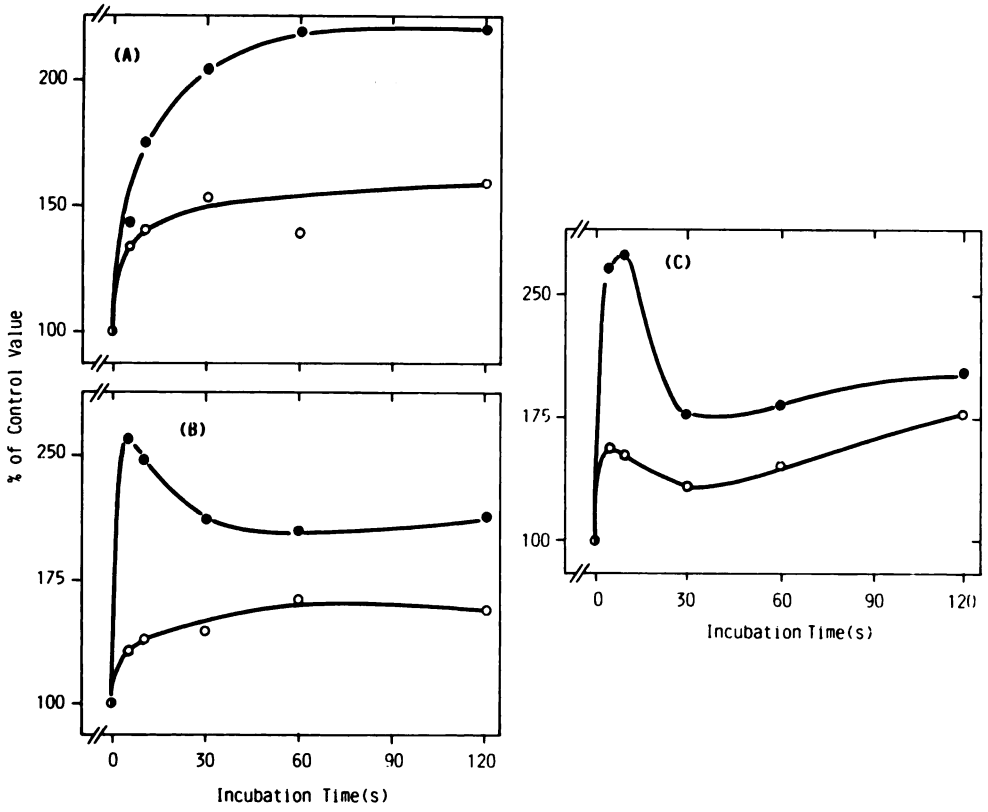

FIG. 2. *Concentration dependence of TRH-induced accumulation of different [^{3}H]inositol metabolites in GH$_3$ cells*

GH$_3$ cells, suspended in balanced salt solution, were exposed to TRH at two different concentrations for the times indicated. See Experimental Procedures for experimental details. Values shown are means for a representative experiment, carried out in duplicate, which has been replicated twice. O——O, TRH (3 nM); ●——●, TRH (1 μM). Note that control values for the different [^{3}H]inositol metabolites do not change over the incubation period used (see legend to Fig. 1). A, [^{3}H]InMP (control value, 5,000 dpm/mg of protein); B, [^{3}H]InDP (1,450 dpm/mg of protein); C, [^{3}H]InTP (1,500 dpm/mg of protein).

lation of [^{3}H]inositol metabolites depends on a previous rise in intracellular free calcium, the effect of the Ca^{2+}-ionophore A23187 was investigated. A23187, at a concentration (4 μM) which induces prolactin release in GH$_3$ cells (14) (see legend to Table 1), did not affect the level of any [^{3}H]inositol metabolite under conditions in which TRH was effective (Table 1).

Experiments conducted in the presence of lithium. Berridge and collaborators (10) have previously demonstrated that 10 mM lithium is maximal with respect to InMP accumulation. The data presented in Fig. 3 demonstrate that TRH-induced InMP accumulation in GH$_3$ cells is enhanced by lithium concentrations as low as 0.3 mM. Concentrations of Li^+ in excess of 10 mM would appear to be necessary to block *myo*-inositol 1-phosphomonoesterase completely.

One of the attractions of lithium as a research tool in PtdIns metabolism is its ability to transform a complex metabolic cycle into a situation in which the cycle has one accumulating end-product, namely, InMP. Thus the rate of InMP accumulation in lithium-treated cells should reflect the rate at which the PtdIns cycle is functioning. Lithium can therefore be used to investigate whether long-term stimulation of TRH receptors on GH$_3$ cells leads to desensitization of the cycle. These data are presented in Figs. 4 and 5. In the absence of lithium, TRH induces a long-lasting 10%–20% decrease in GH$_3$ cell PtdIns (9); this is confirmed in Fig. 4, which also shows a corresponding increase in acid-soluble [^{3}H]inositol metabolites. When lithium and TRH are added simultaneously, there is a gradual decrease in total lipid [^{3}H]inositol, and 50% of its content is depleted by 20 min of treatment (Fig. 4). The lost radioactivity is quantitatively recovered in the acid-soluble fraction. When lithium is added to GH$_3$ cells 30 min after TRH, the rate of depletion of lipid [^{3}H]inositol and increase in acid-

TABLE 1

Effect of TRH and A23187 on GH$_3$ cell [^{3}H]inositol metabolites

Incubations were conducted for 2 min at 37°. The results shown are means ± standard error of the mean for triplicate estimations. In parallel experiments, A23187 (4 μM) increased prolactin release from GH$_3$ cells from a control value of 59 ± 6 ng/mg of protein to 86 ± 7 ng/mg of protein, using a 10-min incubation period.

Addition	[^{3}H]Inositol monophosphate	[^{3}H]Inositol bisphosphate	[^{3}H]Inositol trisphosphate
	dpm/mg protein		
Control	2400 ± 165	922 ± 107	1380 ± 77
TRH (1 μM)	6640 ± 127[a]	1970 ± 107[a]	2000 ± 48[a]
EtOH (0.2%, v/v)	2110 ± 42	879 ± 97	1470 ± 73
A23187 (4 μM)	2130 ± 236	943 ± 193	1470 ± 61

[a] $p < 0.001$.

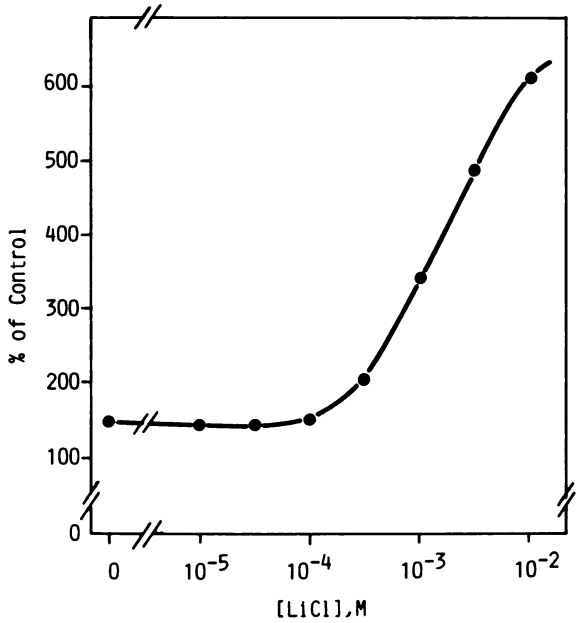

FIG. 3. *Effect of lithium on the TRH-induced accumulation of GH_3 cell [^{3}H]InMP*

GH_3 cells, suspended in balanced salt solution, were exposed to TRH (0.1 μM) for 30 min at 37° prior to the addition of lithium chloride at the concentrations indicated. The incubations were terminated 15 min later, and [^{3}H]InMP was isolated as described under Experimental Procedures. All samples were made hypertonic by 10 mM, and the proportion of this which is lithium chloride and *not* sodium chloride is shown on the abscissa. Sodium chloride (10 mM) had no effect on the GH_3 cell content of [^{3}H]InMP. One hundred per cent control represents the content of [^{3}H]InMP in the absence of TRH (4810 dpm/mg of protein). The data shown are the mean of an experiment carried out in duplicate, which has been replicated once.

soluble [^{3}H]inositol are similar to that found when the two agents are added together. The PtdIns cycle, therefore, is functioning as strongly at 30 min as it is during the first few minutes after TRH addition. It should be noted that, in the interest of clarity, the untreated and lithium-treated controls have been omitted from Fig. 4. The changes that occur are relatively small and are documented in the legends to Figs. 4 and 5. When similar experiments were performed in which the individual [^{3}H] inositol phosphates were measured (Fig. 5), the data were qualitatively similar to those presented in Fig. 4. However, GH_3 cells which received lithium 30 min after TRH showed an even greater accumulation of [^{3}H]InTP and, in particular, [^{3}H]InDP than when lithium was added together with TRH (Fig. 5; see legend for changes with lithium alone).

One major difference between the two situations prevailing at the time when lithium is added to the cells in the experiment shown in Fig. 5, i.e., along with TRH or 30 min after TRH, is that in the latter situation [^{3}H] inositol phosphates are already elevated above resting levels. It seemed possible that the elevated level of [^{3}H] InMP, in particular, might be able to alter the activity of the *myo*-inositol 1,4-phosphomonoesterase by product inhibition. This was investigated in another way by growing GH_3 cells for 48 hr in the *absence* of TRH but in the *presence* of 10 mM lithium. Under those circumstances the cellular content of [^{3}H]InMP was elevated over 4-fold (Fig. 6). When these cells were exposed to TRH, the former cells accumulated more [^{3}H]InDP and less [^{3}H]InMP 5 sec after the addition of TRH. This finding is consistent with the idea of product inhibition of *myo*-inositol 1,4-phosphomonoesterase.

Since these data tend to suggest that PtdIns breakdown by a phospholipase C enzyme is *not* an initial response to TRH action, other conditions were sought which mimicked this situation. Treatment of GH_3 cells with TRH at 25° rather than 37° markedly altered the pattern of [^{3}H]inositol metabolites formed in response to the agonist: in particular, 5 sec after its addition at 25°, TRH induced a normal elevation of [^{3}H]InTP levels with little elevation of the other metabolites (see Fig. 7D, InTP, versus Fig. 7B, InMP). Longer incubations with TRH at 25° enhanced its ability to elevate [^{3}H] InTP while slowing or reducing its ability to elevate the other derivatives (Fig. 7).

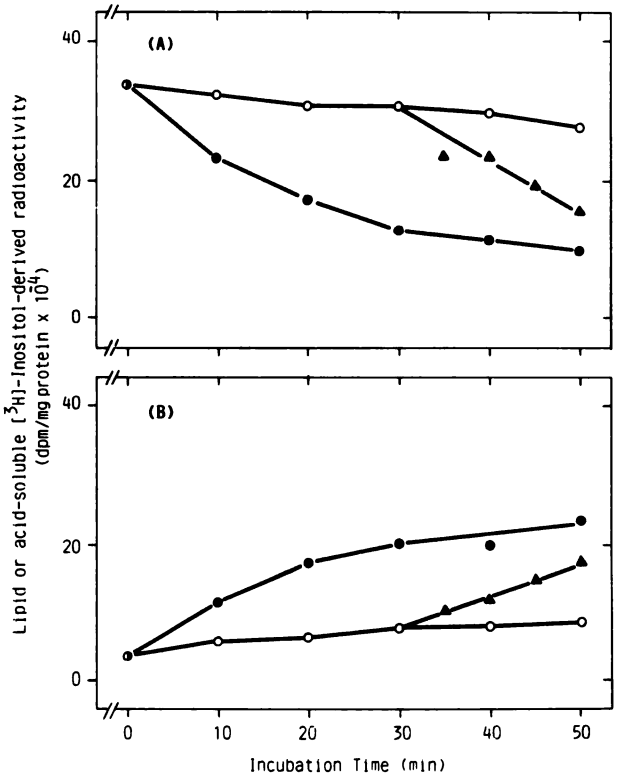

FIG. 4. *Effect of lithium added at the same time and 30 min after TRH on GH_3 cell lipid and acid-soluble [^{3}H]inositol-derived radioactivity*

GH_3 cells, suspended in balanced salt solution, were exposed to TRH (0.1 μM) plus or minus lithium chloride (10 mM) for the times indicated. The reaction was terminated, and *total* lipid and acid-soluble [^{3}H] inositol-derived radioactivity was measured as described under Experimental Procedures. A, Total lipid [^{3}H]inositol-derived radioactivity; B, total acid-soluble [^{3}H]inositol-derived radioactivity. ●——●, TRH (0.1 μM) and lithium chloride (10 mM) added simultaneously; ○——○, TRH (0.1 μM) alone; ▲——▲, TRH (0.1 μM) added at time 0, lithium chloride (10 mM) added 30 min later. The values shown are means in a representative experiment, carried out in duplicate, which has been replicated three times.

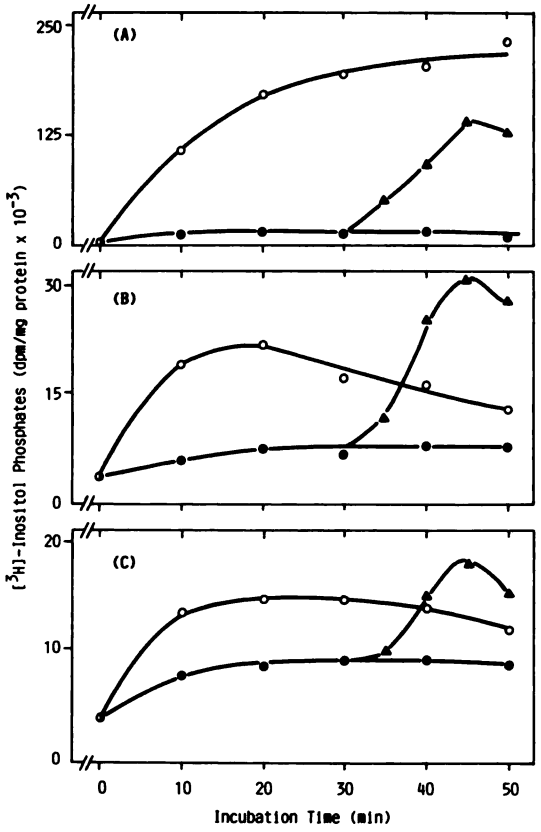

FIG. 5. *Effect of lithium added at the same time and 30 min after TRH on GH₃ cell [³H]inositol metabolites*

The data shown are derived from the same experiment as those presented in Fig. 4. A, [³H]InMP; B, [³H]InDP; C, [³H]InTP. O——O, TRH (0.1 μM) and lithium chloride (10 mM) added simultaneously; ●——●, TRH (0.1 μM) alone; ▲——▲, TRH (0.1 μM) added at time 0, lithium chloride (10 mM) added 30 min later. The data shown are means in a representative experiment, carried out in duplicate, which has been replicated three times. Treatment of GH_3 cells with 10 mM lithium chloride itself for 30 or 50 min caused 2.5-fold and 3.2-fold increases in [³H]InMP levels, respectively (data not shown).

DISCUSSION

There is still considerable debate regarding the initial, receptor-mediated, event in stimulated PtdIns metabolism. The original proposition (1) that receptor occupancy leads to the breakdown of PtdIns by phospholipase C has been called into question by findings that show a more rapid agonist-induced breakdown of PtdIns 4,5P_2 and PtdIns 4P in a number of tissues (see ref. 15 for a recent review). In certain tissues, such as iris smooth muscle and synaptosomes, these responses appear to be dependent upon a previous rise in cytoplasmic free calcium concentration (12, 16)—a finding that would tend to obviate a role for stimulated PtdIns metabolism in Ca^{2+} mobilization. In GH_3 cells, as in a number of tissues, the breakdown of the higher PtdIns derivatives does not appear to be the result of a change in cytoplasmic free calcium. Thus, the calcium ionophore, A23187, at a concentration which is adequate for the stimulation of prolactin release from GH_3 cells, does not increase the rate of appearance of any [³H]inositol metabolite in these cells. One is led to conclude that, despite the Ca^{2+} dependence of phospholipase C (13), overt changes in cytoplasmic free calcium levels are insufficient to activate the enzyme. Sutton and Martin (7) have reported a small stimulation of PtdIns turnover by A23187 in GH_3 cells. We have confirmed these data, but have been unable to detect changes in the levels of any acid-soluble [³H] inositol metabolite using incubation periods up to 10 min duration (Table 1 and data not shown). In our experience, the PtdIns turnover assay is a more sensitive method of detecting changes in PtdIns metabolism than following the mass of the various metabolites, and it remains possible that A23187 can, by whatever means, elicit a small breakdown of the inositol phospholipids.

In the preceding paper (9), it was demonstrated that TRH induces a rapid breakdown of PtdIns 4,5P_2 and PtdIns 4P and a simultaneous rise in the cellular content of 1,2-diacylglycerol. These data, which suggest that a phosphodiesterase enzyme is responsible, are corrobo-

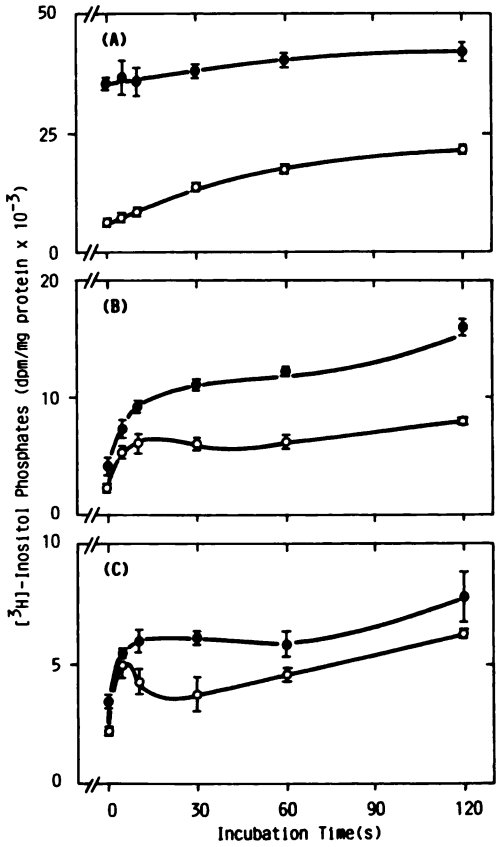

FIG. 6. *Effect of acute or chronic treatment with lithium on the GH_3 cell content of [³H]inositol metabolites in TRH-treated cells*

GH_3 cells were grown for their final 48 hr, prior to the preparation of cells, in medium supplemented with either 10 mM sodium chloride or 10 mM lithium chloride. Both types of cells were finally resuspended in balanced salt solution containing 10 mM lithium chloride. The sodium chloride content of the balanced salt solution was correspondingly reduced. The cells were then exposed to TRH (1 μM) for the times indicated, the reaction was terminated, and [³H]inositol metabolites were measured as described under Experimental Procedures. A, [³H]InMP; B, [³H]InDP; C, [³H]InTP. O——O, Cells grown in the absence of lithium; ●——● cells grown in the presence of lithium. The values shown are means ± standard error of the mean in a representative experiment, carried out in triplicate, which was replicated twice.

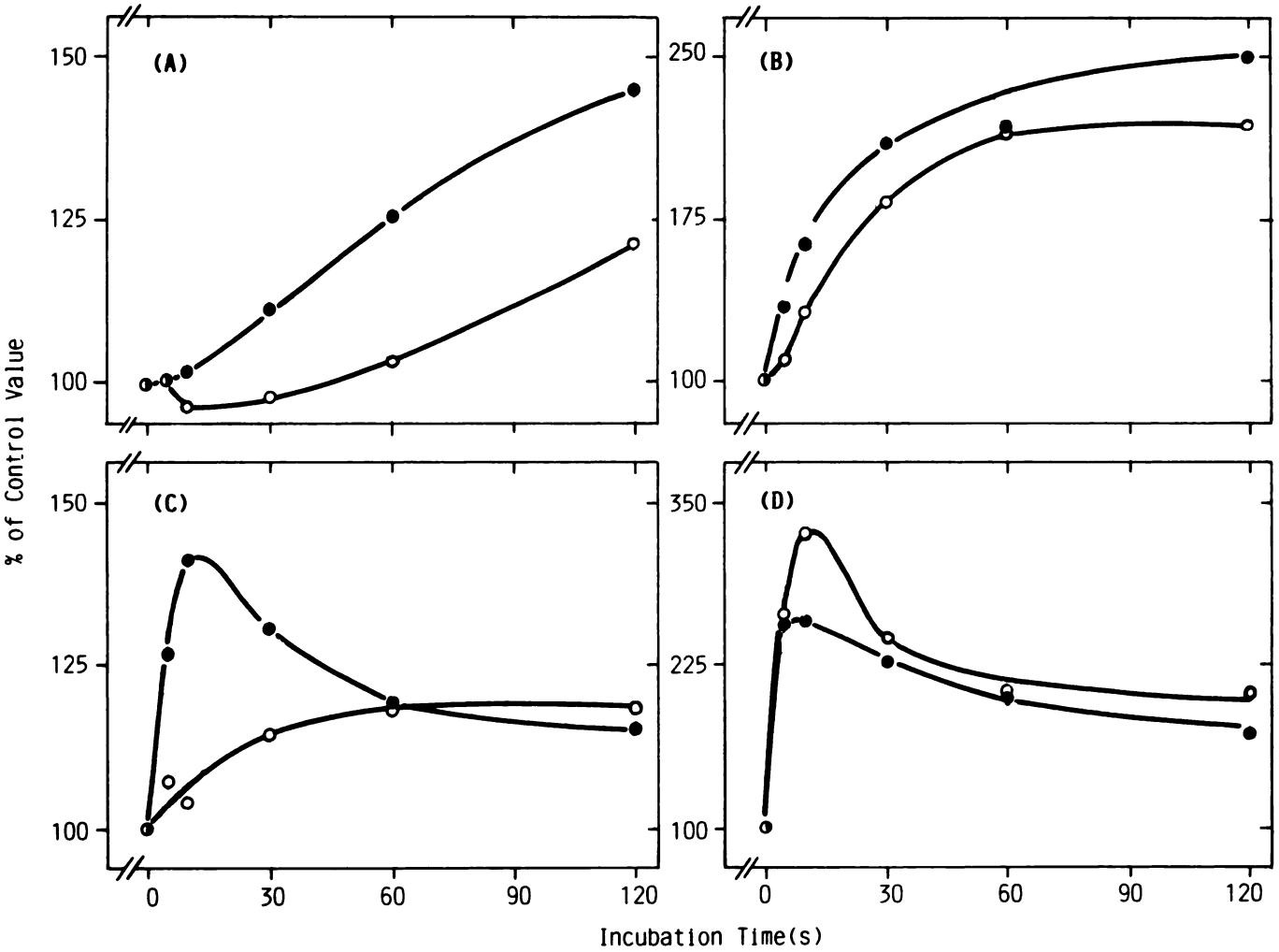

FIG. 7. *Effect of TRH on GH_3 cell acid-soluble [^{3}H]inositol metabolites: temperature dependence*

GH_3 cells, suspended in balanced salt, were exposed to TRH (1 μM) at 37° (●——●) or at 25° (○——○) for the times indicated. Reactions were terminated and acid-soluble [^{3}H]inositol metabolites separated as described under Experimental Procedures. A, [^{3}H]inositol (control value, 22,100 dpm/mg of protein); B, [^{3}H]InMP (6,050 dpm/mg of protein); C, [^{3}H]InDP (1,500 dpm/mg of protein); D, [^{3}H]InTP (3,800 dpm/mg of protein). Values shown are means for a representative experiment, carried out in duplicate, which has been replicated twice.

rated by the findings reported here which demonstrate a rapid accumulation of [^{3}H]InMP, [^{3}H]InDP, and [^{3}H]InTP in the acid-soluble fraction of GH_3 cells. During the first 10 sec after the addition of TRH, approximately equal amounts of all three [^{3}H]inositol metabolites are formed. These data might suggest that all three inositol-containing phospholipids are broken down by phosphodiesterase action under the influence of TRH. The lack of a significant breakdown of PtdIns over this period (9) could simply reflect the fact that only a small pool of plasma membrane-located PtdIns is susceptible to this attack. A second explanation for the finding that equal amounts of the three [^{3}H]inositol phosphates are formed within 10 sec of TRH addition is that the enzymes of inositol polyphosphate catabolism are very active and quickly convert the [^{3}H]InTP derived from the breakdown of PtdIns 4,5P_2 to [^{3}H]InDP and [^{3}H]InMP. In favor of this view is the fact that [^{3}H]InTP levels, in particular, are already as high 5 sec after TRH addition as they are 5 sec later (see Figs. 2 and 7, for example). This is not the case with [^{3}H]InMP levels, which rise in a linear fashion over this time. Further evidence in favor of the latter view, that the breakdown of a polyphosphoinositide is the initial response to receptor occupancy, comes from studies conducted at 25° and from the work using lithium. When the incubation with TRH was carried out at 25°, the cellular content of [^{3}H]inositol, [^{3}H]InMP, and [^{3}H]InDP increased much more slowly after TRH addition, as might be expected for any enzyme-linked event. On the contrary, however, cellular levels of [^{3}H]InTP increased to a greater extent at 25° than at 37°. The implication here is that the enzymes of inositol polyphosphate catabolism are more dependent on the reaction temperature than is phospholipase C. An alternative explanation for this result, that the reduction in temperature affects the physicochemical properties of the membrane in such a way as to render PtdIns and PtdIns 4*P* less sensitive and PtdIns 4,5P_2 more sensitive to attack by phospholipase C, seems less likely because the fatty acid composition of the three inositol lipids

should be identical given the synthetic route. In the experiment shown in Fig. 7, 90% of the inositol metabolites formed 5 sec after the addition of TRH at 25° were recovered as inositol trisphosphate.

A surprising aspect of the experiments which were designed to determine whether the "PtdIns cycle" desensitizes upon prolonged agonist stimulation (see below) was that larger rises in [^{3}H]InTP and, in particular, [^{3}H]InDP occurred when lithium was added 30 min after TRH rather than at the same time. We considered it possible that the elevated levels of [^{3}H]InMP, which are present in the former situation, might decrease the activity of *myo*-inositol 1,4-phosphomonoesterase by product inhibition. This remains a speculative explanation, although similar results were obtained when the [^{3}H]InMP was elevated by chronic lithium treatment in the absence of TRH. The subsequent addition of the peptide produced much less [^{3}H]InMP and more of the [^{3}H]inositol polyphosphates than normal; as before, the prime beneficiary from the reduction of [^{3}H]InMP production was [^{3}H]InDP. It is also possible that Li^+ can inhibit *myo*-inositol 1,4-phosphomonoesterase directly. However, this cannot resolve the dilemma of why more [^{3}H]InDP is formed when lithium is added after, rather than along with, TRH. The relative insensitivity of [^{3}H]InTP production to these manipulations is consistent with the results of studies using erythrocyte *myo*-inositol 1,4,5-phosphomonoesterase which indicate that the enzyme is insensitive to product inhibition (17). The evidence accumulated in this and in the accompanying paper (9) thus provide good evidence that the initiating event of the "PtdIns cycle" is the breakdown of the polyphosphoinositides—in particular, PtdIns 4,5P_2. A similar conclusion has recently been reached by Berridge (18), who investigated the stimulation of the cycle by 5HT in *Calliphora* salivary gland.

Our studies with lithium confirm much of the data originally presented by Berridge and his collaborators (10). Thus, the concentration dependence of the lithium effect and the selective, but not specific, action on [^{3}H]InMP accumulation appear similar in the two studies. The work, using lithium-induced [^{3}H]InMP accumulation to measure the activity of the cycle, shows that the TRH-stimulated "PtdIns cycle" does not densensitize in the first 30 min after its initiation. This is of interest, since the changes in the cellular mass of the cycle's intermediates such as PtdIns 4,5P_2, PtdIns 4P, PtdIns, and 1,2-diacylglycerol are complete within 5 min (9). Details of the mechanisms that control the "PtdIns cycle" once it is initiated remain obscure. However, it is evident that some feature of the cycle, perhaps the activity of PtdIns 4P kinase, must increase over the early minutes after TRH addition so that the intermediates of the cycle attain a new equilibrium.

The most important question that remains unanswered is, how, if at all, alterations in PtdIns metabolism are linked to Ca^{2+} mobilization. A variety of proposals have been made in this respect. PA, which has Ca^{2+}-ionophoric properties (19), might accumulate in the membrane and allow Ca^{2+} to enter the cell down its electrochemical gradient (20, 21). This proposal seems unlikely in view of the transient nature of the Ca^{2+} signal, which is unlikely to be produced by an ionophore, and the temporal delay in the appearance of PA (9). In fact, the key question is to explain how the breakdown of inositol phospholipids can generate a phasic Ca^{2+} signal: all of the TRH-induced changes in the mass of the various inositol-related metabolites present in GH_3 cells are complete within 3–5 min, and these persist, in most cases, for more than 1 hr (9). Recent data on receptor mobilization of Ca^{2+} pools are illuminating in this respect. A number of reports describe a limited "trigger pool" of nonmitochondrial Ca^{2+} which is rapidly depleted upon receptor activation and refilled, from the extracellular space, upon dissociation of the agonist from its receptor (22, 23). Similar data have been provided for GH_3 cells (24). The major attraction of these data is the finite nature of this pool. If it is readily depleted by agonist-receptor occupation, then the changes in PtdIns metabolism may only be coupled to Ca^{2+} mobilization during the first seconds after receptor stimulation, i.e., until the Ca^{2+}-pool is depleted. The persistent changes in the intermediates of the PtdIns cycle may, thus, be redundant as far as Ca^{2+} signaling is concerned unless the "trigger pool" can be repleted in some way. Alternatively, since the cycle's intermediates accumulate at varying rates, it is possible that the increased mass of one metabolite constitutes a signal for Ca^{2+} mobilization while another is a signal for termination of this process.

One biochemical event which occurs fast enough to be responsible for Ca^{2+} mobilization is the hydrolysis of PtdIns 4,5P_2 and the consequent accumulation of 1,2-diacylglycerol and InTP as a result of phospholipase C action. Both products have already been implicated in this process. Thomas *et al.* (25) have suggested that 1,2-diacylglycerol, acting on protein kinase C (26), could influence Ca^{2+} availability. One problem with this proposal is that phorbol esters, which also activate protein kinase C (27), do not increase cytoplasmic free Ca^{2+} concentrations (28). Indeed, if anything, the published data suggest that these esters decrease cytoplasmic Ca^{2+} levels (28), which might favor the suggestion that the PtdIns cycle contains both an on- and off-signal for Ca^{2+} mobilization. Berridge (18) has suggested that InTP might act as a second messenger molecule in a manner analogous to cyclic AMP, and, more recently, has provided data indicating that InTP can release Ca^{2+} from intracellular pools in pancreatic acinar cells (29).

Another proposal was made by Kretsinger (30), that Ca^{2+} ions bound to PtdIns 4,5P_2 are released inside the cell as the phospholipid is degraded. Such a process should be fast enough to be responsible for Ca^{2+} mobilization, but suffers the disadvantage that the specificity of PtdIns 4,5P_2 for Ca^{2+} over Mg^{2+} is not of sufficient magnitude to yield much Ca^{2+}, given the overwhelming excess of Mg^{2+} present in the cytoplasm (31). A modification of this hypothesis, which incorporates the fact that the Ca^{2+} "trigger" pool fills up from the extracellular space (22), is to assume that PtdIns 4,5P_2 also fills with Ca^{2+} from the extracellular space and cannot exchange it with intracellular ions. The concentrations of Ca^{2+} and Mg^{2+} in the medium are roughly equivalent, ensuring that the bulk of the PtdIns 4,5P_2 will load with Ca^{2+}. Calculations of the amount of Ca^{2+} which can be released

by the hydrolysis of such Ca^{2+}-loaded PtdIns 4,5P_2 indicate that the cytoplasmic free Ca^{2+} might rise to around 30 μM.[2] There are, however, also problems with this modified hypothesis in that the rank order of potency for the ability of a variety of cations to bind to PtdIns 4,5P_2 does not parallel completely their ability to inhibit Ca^{2+}-dependent responses (33). Moreover, the work outlined in this paper suggests that PtdIns 4,5P_2 is being degraded and resynthesized for at least 50 min after the addition of TRH, a fact incompatible with the phasic nature of calcium gating unless it is assumed that resynthesized PtdIns 4,5P_2 cannot load with extracellular Ca^{2+} ions.

It remains to be seen which (if any) of these proposals underlies receptor-mediated Ca^{2+}-mobilization. This and the accompanying paper (9) indicate that such proposals must take account of the fact that receptor-stimulated hydrolysis of PtdIns 4,5P_2 does not desensitize and that the elevated levels of a number of metabolites of the PtdIns cycle are maintained for considerable periods of time.

[2] Using the data presented in table 1 of ref. 9, GH_3 cells contain 32 nmoles of PtdIns/10^7 cells. Since the cellular PtdIns 4,5P_2 content is around 3% of PtdIns (table 2 of ref. 9), 0.92 nmole of PtdIns 4,5P_2 is contained in 10^7 GH_3 cells. The intracellular volume of GH_3 cells is 17 μl/10^7 cells (32). If one molecule of PtdIns 4,5P_2 binds one ion of Ca^{2+}, the amount released will be equivalent to 0.55 nanoequivalent of Ca^{2+}/10^7 cells [60% of the cellular content of PtdIns 4,5P_2 breaks down within 10 sec of TRH addition (9)]. Thus the maximal increase in intracellular free calcium concentration due to PtdIns 4,5P_2 hydrolysis will be around 30 μM.

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