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Differential regulation by phosphatidylinositol 4,5-bisphosphate of pituitary plasma-membrane and cytosolic phosphoinositide kinases

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Regulation of phosphatidylinositol kinase (EC 2.7.1.67) and phosphatidylinositol 4-phosphate (PtdIns4*P*) kinase (EC 2.7.1.68) was investigated in highly enriched plasma-membrane and cytosolic fractions derived from cloned rat pituitary (GH_3) cells. In plasma membranes, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] added exogenously enhanced incorporation of [^{32}P]phosphate from [γ-^{32}P]MgATP^{2-} into PtdIns(4,5)P_2 and PtdIns4*P* to 150% of control; half-maximal effect occurred with 0.03 mM exogenous PtdIns(4,5)P_2. Exogenous PtdIns4*P* and phosphatidylinositol (PtdIns) had no effect. When plasma membranes prepared from cells prelabelled to isotopic steady state with [^{3}H]inositol were used, there was a MgATP^{2-}-dependent increase in the content of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* that was enhanced specifically by exogenous PtdIns(4,5)P_2 also. Degradation of ^{32}P- and ^{3}H-labelled PtdIns(4,5)P_2 and PtdIns4*P* within the plasma-membrane fraction was not affected by exogenous PtdIns(4,5)P_2. Phosphoinositide kinase activities in the cytosolic fraction were assayed by using exogenous substrates. Phosphoinositide kinase activities in cytosol were inhibited by exogenously added PtdIns(4,5)P_2. These findings demonstrate that exogenously added PtdIns(4,5)P_2 enhances phosphoinositide kinase activities (and formation of polyphosphoinositides) in plasma membranes, but decreases these kinase activities in cytosol derived from GH_3 cells. These data suggest that flux of PtdIns to PtdIns4*P* to PtdIns(4,5)P_2 in the plasma membrane cannot be increased simply by release of membrane-associated phosphoinositide kinases from product inhibition as PtdIns(4,5)P_2 is hydrolysed.

INTRODUCTION

In many cells the interaction of stimuli with cell-surface receptors enhances the turnover of membrane phosphoinositides. It is thought that this increased metabolism of phosphoinositides constitutes a signal-transducing mechanism that is initiated by stimulation of the hydrolysis of PtdIns(4,5)P_2, a minor but metabolically labile polyphosphoinositide, by a phospholipase C (or phosphodiesterase) to generate two putative intracellular messengers: 1,2-diacylglycerol and inositol trisphosphate (for a review, see [1]). This mechanism appears to be involved in the action of TRH in rat pituitary (GH_3) cells (for a review, see [2]). It has been proposed that, concomitant with the hydrolysis of PtdIns(4,5)P_2, there is enhanced flux of PtdIns to PtdIns4*P* to PtdIns(4,5)P_2 that provides additional precursor for the continued generation of these intracellular messengers [3]. Because PtdIns(4,5)P_2 is formed from PtdIns by stepwise phosphorylation catalysed by apparently distinct kinases first at the 4-position to form PtdIns4*P* and then at the 5-position (for a review, see ref. [4]), it was proposed that these kinases were activated during cell stimulation. A recent report, however, has presented evidence that these kinases are not activated during stimulation of human platelets by thrombin [5]. Similarly, we have been unable to measure any activation of PtdIns and PtdIns4*P* kinases in GH_3 cells during stimulation by TRH [5a].

Previous studies of phosphoinositide kinase activities from brain [6–8], kidney [9–11] and liver [12,13] suggested that they are present primarily, but not exclusively, in the plasma membrane. Although these kinases have been partially characterized, little information is available regarding regulation of these membrane-bound enzymes by factors that may be of physiological importance. Michell *et al.* [14] reported that PtdIns kinase activity within rat liver plasma membranes, measured by using exogenous PtdIns as substrate, was inhibited by several phospholipids, in particular by PtdIns4*P*. Inhibition by PtdIns4*P* of a PtdIns kinase assayed in a particulate fraction from transformed cells in the presence of detergents was also observed [15]. More recently, Van Rooijen *et al.* [16] observed that a soluble PtdIns4*P* kinase activity found in bovine retina, assayed with exogenous PtdIns4*P*, was inhibited by PtdIns(4,5)P_2. These observations suggested that these kinase activities were product-inhibited. This hypothesis is attractive, because it is consistent with the proposed enhancement of the flux of PtdIns to PtdIns4*P* and to PtdIns(4,5)P_2 in the intact cell as the levels of these lipids decrease. The present studies were undertaken to

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdA, phosphatidic acid; TRH, thyrotropin-releasing hormone (thyroliberin); $(Na^+ + K^+)$-ATPase, $(Na^+ + K^+)$-dependent ATPase.

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determine whether phosphoinositide kinase activities in the plasma membrane of pituitary cells may be regulated by the lipids of the polyphosphoinositide cycle.

In the present paper the regulation of plasma-membrane-bound kinases derived from GH_3 pituitary cells acting on endogenous substrates is compared with that of cytosolic kinases acting on exogenous substrates. We show that PtdIns(4,5)P_2 stimulates the activities of membrane-bound kinases while inhibiting the activities of soluble kinases. These data suggest that flux of phosphatidylinositol to PtdIns4P to PtdIns(4,5)P_2 in the plasma membrane cannot be increased simply by release of membrane-associated phosphoinositide kinases from product inhibition as PtdIns(4,5)P_2 is hydrolysed.

EXPERIMENTAL PROCEDURES

Preparation of plasma membranes and cytosol from GH_3 cells

GH_3 cells were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal-bovine serum at 37 °C and harvested with 0.02% EDTA as previously described [17,18]. A volume of lysis buffer (0.5 mM-dithiothreitol/1 mM-EGTA/1 mM-$NaHCO_3$/10 mM-4-Hepes, pH 7.9) was added equal to 4–5 times the volume of the cell pellet. The cells ($\sim 4 \times 10^8$) were suspended and simultaneously lysed by incubating at 37 °C for 4.5 min, followed by addition of 15 vol. of ice-cold lysis buffer and rapid mixing on a vortex mixer for 0.5 min at 0–4 °C. The homogenate was centrifuged at 800 g for 5 min to remove nuclei and cell debris. The supernatant was centrifuged again at 31000 g for 10 min or at 100000 g for 60 min to yield the cytosol fraction. The resulting pellet was resuspended in 4 ml of lysis buffer that was then layered on top of a discontinuous sucrose density gradient consisting of 30, 36, 41, 45 and 50% (w/v) sucrose in lysis buffer and centrifuged at 100000 g for 1 h. Five narrow bands were obtained at the interfaces. The upper two bands were collected as the plasma-membrane fraction, diluted with lysis buffer and centrifuged at 31000 g for 10 min. The final pellet was resuspended in lysis buffer and used immediately or stored at −80 °C.

To examine the organellar contamination, marker enzyme analyses were performed by using $(Na^+ + K^+)$-dependent ATPase as a marker for plasma membranes [19], succinate dehydrogenase for mitochondria [20] and NADH dehydrogenase for endoplasmic reticulum [21]. Protein was determined by the method of Lowry *et al.* [22], with bovine serum albumin as standard. In the plasma-membrane fraction, the specific activity of $(Na^+ + K^+)$-ATPase was increased 18-fold from 20 to 350 nmol/min per mg of protein, succinate dehydrogenase was decreased from 650 to 68 pmol/min per mg of protein and NADH dehydrogenase was minimally increased from 13 to 21 pmol/min per mg of protein.

[^{32}P]Phosphate incorporation into phospholipids

The phosphorylation of endogenous substrates in plasma membranes was determined by measuring [^{32}P]phosphate incorporation from [γ-^{32}P]ATP. The reaction mixture consisted of 100 mM-Tris/HCl, pH 7.9, 1 mM-EGTA, 6.25 mM-$MgCl_2$ and 1.25 mM-[γ-^{32}P]ATP [$(1.0–2.0) \times 10^{14}$ c.p.m./mol] in a volume of 40 μl. In some experiments, an exogenous phosphoinositide or detergent was added or the concentration of ionized Ca^{2+} was varied with Ca^{2+}/EGTA buffers. After preincubation of assay mixtures for 2–5 min at 37 °C, the incubation was started by adding 10 μl of plasma-membrane suspension (30–50 μg of protein). The reaction was terminated by adding 1 ml of chloroform/methanol/conc. HCl (100:100:1, by vol.), followed by 250 μl of 10 mM-EDTA. After the two phases were separated, the upper phase was washed with 200 μl of pre-equilibrated lower phase. The lower phases were combined, washed twice with 1 ml of pre-equilibrated upper phase in order to remove [^{32}P]ATP and dried under N_2. The lipids were redissolved in small amounts of chloroform/methanol/conc. HCl (100:100:1, by vol.) and applied under N_2 to silica-gel type-H plates. Plates were developed in one dimension by sequential ascending chromatography in chloroform/methanol/4 M-NH_3 (9:7:2, by vol.) containing 2 mM-(1,2-cyclohexylenedinitrilo)tetra-acetic acid followed by chloroform/methanol/acetic acid/water (25:15:4:2, by vol. [23]. Dried plates were autoradiographed to localize PtdIns(4,5)P_2 and PtdIns4P (and PtdA) by comparison with standards. The areas corresponding to individual lipids were scraped into vials and the radioactivity was determined by liquid-scintillation counting. Under these conditions, less than 5% of the added ATP was consumed.

PtdIns4P and PtdIns kinase activities in the cytosol were assayed with exogenous substrate in the absence of detergents. The assay mixture contained 50 mM-Tris/HCl (pH 7.9)/10 mM-$MgCl_2$/0.5 mM-EGTA/0.5 mM-[γ-^{32}P]ATP [$(2.0–3.0 \times 10^{14}$ c.p.m./mol], cytosol sample (approx. 100 μg of protein) and 100 μM-PtdIns4P or -PtdIns. The incubation was performed at 37 °C for the times indicated and the reaction terminated and the lipids measured as described above.

Formation of [^{3}H]inositol-labelled polyphosphoinositides

Plasma membranes were prepared as described above from cells prelabelled with [^{3}H]inositol for 48 h, a time that is sufficient to attain isotopic steady state [23,24]. The [^{3}H]inositol-labelled membrane suspensions (70–100 μg of protein/10 μl) were added to 40 μl of assay mixture containing 100 mM-Tris/HCl, pH 7.9, 1 mM-EGTA, 6.25 mM-$MgCl_2$, 1.25 mM-ATP, with or without exogenous phosphoinositides and incubated at 37 °C. Lipid extraction and analysis were performed as described above. The specific radioactivity of the ^{3}H label in the phosphoinositides was determined for PtdIns as ^{3}H c.p.m./mol of lipid phosphorus after isolation by two-dimensional high-performance t.l.c. [25].

Degradation of plasma-membrane polyphosphoinositides

Plasma membranes from cells prelabelled with [^{3}H]inositol were also labelled with [^{32}P]phosphate by incubation with 1 mM-[γ-^{32}P]ATP for 5 min in the presence of 5 mM-Mg^{2+} as described above. The membranes were washed twice with lysis buffer and resuspended in the original volume of buffer. A 10 μl portion of plasma-membrane suspension was added to 40 μl of assay mixture containing 100 mM-Tris/HCl (pH 7.9)/1 mM-EGTA/6.25 mM-$MgCl_2$, with or without 1.25 mM-ATP and with or without exogenous PtdIns-(4,5)P_2 that had been preincubated at 37 °C for 2–5 min. The suspensions were rapidly mixed and incubated at 37 °C. Lipid extraction and analysis were performed as described above.

Materials

[γ-^{32}P]ATP and *myo*-[2-^{3}H]inositol (15 Ci/mmol) were obtained from New England Nuclear and American Radio-labelled Chemicals respectively. PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 were purchased from Sigma. Silica-gel type-H plates were products of Supelco. All other chemicals and solvents were of highest reagent grade.

Statistics

Statistical analysis was by Student's *t* test.

RESULTS

[^{32}P]Phosphate incorporation from [γ-^{32}P]ATP into PtdIns4*P* and PtdIns(4,5)P_2

When the plasma-membrane fraction isolated from GH_3 cells was incubated with [γ-^{32}P]ATP in the presence of Mg^{2+}, there was time-dependent incorporation of [^{32}P]-phosphate into PtdIns(4,5)P_2 and PtdIns4*P* (and also PtdA; results not shown). This indicated the presence of both the endogenous substrates, PtdIns4*P* and PtdIns (and also 1,2-diacylglycerol), and the kinases in the plasma-membrane fraction (Fig. 1). [^{32}P]Phosphate incorporation into each lipid increased over the first 1–2 min of incubation, attaining a steady-state level by 2–3 min that was maintained for at least 5 min. Approx. 95% of the radioactivity in the lipid fraction was accounted for by the two polyphosphoinositides PtdIns(4,5)P_2 and PtdIns4*P*; the remainder was present mainly as PtdA. The apparent activities of PtdIns4*P* and PtdIns kinases acting on endogenous substrates were increased from 2400 to 36000 c.p.m. of [^{32}P]phosphate incorporated/min per mg of protein (15-fold) and from 8400 to 80000 c.p.m. of [^{32}P]phosphate incorporated/min per mg of protein (10-fold) respectively, over that found in the cell homogenate. Half-maximal activation of PtdIns4*P* kinase activity occurred with 0.1 mM-Mg^{2+} and half-maximal activation of PtdIns kinase activity occurred with 0.5 mM-Mg^{2+}; both kinase activities were activated half-maximally by 0.5 mM-ATP. Both kinase activities were inhibited by Ca^{2+} (Table 1). As little as 0.45 μM free Ca^{2+} caused a decrease in incorporation of [^{32}P]phosphate into PtdIns(4,5)P_2 and PtdIns4*P*; maximal inhibition to 20% of control occurred with 1 mM-Ca^{2+}. Table 2 shows that both phosphoinositide kinase activities and 1,2-diacylglycerol kinase activity were inhibited in a concentration-dependent manner by the detergent alkyltrimethylammonium bromide; Triton X-100, digitonin and deoxycholate also inhibited these kinases. Hence it appears that there are kinase activities that phosphorylate endogenous PtdIns4*P* and PtdIns (and 1,2-diacylglycerol) that are associated with the plasma membrane of pituitary cells.

When the cytosol fraction isolated from GH_3 cells was incubated with 0.5 mM-[γ-^{32}P]ATP and 100 μM exogenous PtdIns4*P* or PtdIns in the presence of Mg^{2+}, there was a time-dependent incorporation of [^{32}P]phosphate into PtdIns(4,5)P_2 and PtdIns4*P* respectively (Fig. 1). [^{32}P]Phosphate incorporation into PtdIns(4,5)P_2 attained an apparent steady-state level after 5 min; incorporation into PtdIns4*P* increased for at least 10 min. Hence, there appears to be cytosolic (or soluble) kinase activities in pituitary cells also.

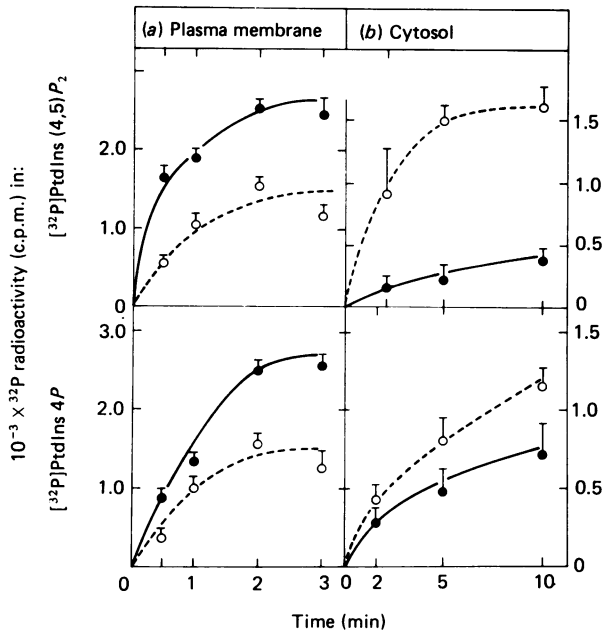

Fig. 1. Time courses of the effects of PtdIns(4,5)P_2 on incorporation of [^{32}P]phosphate into PtdIns(4,5)P_2 and PtdIns4*P* in plasma membranes and cytosol from GH_3 cells

(*a*) Plasma-membrane fractions from GH_3 cells (30 μg of protein) were incubated with 1 mM-[γ-^{32}P]$MgATP^{2-}$ in the presence (●) or absence (○, control) of 1 mM-PtdIns(4,5)P_2 at 37 °C for the times indicated. The reaction was terminated with acidic chloroform/methanol. The radioactivity in phospholipids was determined after t.l.c. Each point represents the mean ± S.D. of triplicate determinations from two experiments. Cytosol (50 μg of protein) was incubated with 0.5 mM-[γ-^{32}P]$MgATP^{2-}$ and 100 μM-PtdIns4*P* or PtdIns at 37 °C in the presence (●) or absence (○, control) of 100 μM-PtdIns(4,5)P_2 for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from two experiments.

Table 1. Effect of Ca^{2+} on [^{32}P]phosphate incorporation into PtdIns4*P* and PtdIns(4,5)P_2 in plasma membranes from GH_3 cells

Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into polyphosphoinositides in plasma membranes was assayed for 1 min as in Fig. 1 in buffers containing various concentrations of free (or unbound) Ca^{2+} ($[Ca^{2+}]_{free}$). These data are the means ± S.D. of triplicate determinations from two or three experiments and are expressed as a percentage of control in buffer containing $[Ca^{2+}]_{free} < 0.01$ μM.

$[Ca^{2+}]_{free}$ (μM)	Incorporation (% of control) into:	
	[^{32}P]PtdIns4*P*	[^{32}P]PtdIns(4,5)P_2
< 0.01	100 ± 5	100 ± 6
0.45	81 ± 8	76 ± 10
1.35	59 ± 11	47 ± 12
10	49 ± 4	40 ± 3
100	53 ± 12	49 + 13
1000	18 ± 3	25 ± 12

Table 2. Effect of alkyltrimethylammonium bromide on [^{32}P]phosphate incorporation into PtdIns4*P* and PtdIns(4,5)P_2 in plasma membranes from GH_3 cells

Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into polyphosphoinositides in plasma membranes was assayed for 1 min as in Fig. 1 in buffers containing various concentrations of alkyltrimethylammonium bromide without (control) or with 100 μM exogenous PtdIns(4,5)P_2. In incubations in buffer containing no detergent or PtdIns(4,5)P_2, [^{32}P]PtdIns4*P* and [^{32}P]PtdIns(4,5)P_2 had radioactivities of 1620 ± 97 and 873 ± 24 c.p.m./30 μg of protein respectively; these levels were set at 100%. [^{32}P]Phosphate incorporation into PtdA was also measured in these incubations. In control incubations, [^{32}P]PtdA had a radioactivity of 135 ± 26 c.p.m./30 μg of protein. Alkyltrimethylammonium bromide caused a concentration-dependent inhibition of ^{32}P-labelling of PtdA that paralleled the inhibition of the labelling of the polyphosphoinositides. There was no effect of exogenously added PtdIns (4,5)P_2 on the labelling of PtdA. The data are means $\pm$ S.D. of triplicate determinations from two experiments.

[Alkyltrimethyl-ammonium bromide] (%)	Incorporation (% of control) into:			
	[^{32}P]PtdIns4*P*		[^{32}P]PtdIns(4,5)P_2	
	Control	+PtdIns(4,5)P_2	Control	+PtdIns(4,5)P_2
0	100 ± 6	135 ± 7	100 ± 3	164 ± 21
0.0003	86 ± 16	134 ± 17	111 ± 16	177 ± 17
0.001	106 ± 9	134 ± 8	101 ± 6	156 ± 14
0.003	108 ± 12	132 ± 7	$84 + 5$	165 ± 9
0.01	70 ± 8	87 ± 12	$51 + 8$	87 ± 7
0.03	50 ± 8	41 ± 4	38 ± 3	58 ± 5
0.1	3 ± 1	2 ± 1	24 ± 6	12 ± 6

Effects of exogenous PtdIns(4,5)P_2 on [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into PtdIns4*P* and PtdIns(4,5)P_2

To begin to investigate the mechanism of regulation of phosphoinoisitide kinases, the effects of exogenous phosphoinositides, their substrates and products on ^{32}P-labelling of PtdIns4*P* and PtdIns(4,5)P_2 were examined. In plasma membranes, exogenous PtdIns(4,5)P_2 increased incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into PtdIns(4,5)P_2 and PtdIns4*P* (Fig. 1); there was no effect on the labelling of PtdA (Table 2). The effect to increase the level of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4*P* was present at the earliest time point measured and persisted throughout the incubation. In a separate experiment, PtdIns(4,5)P_2 increased the level of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4*P* at 10 s by 1.4- and 1.6-fold respectively. In contrast, PtdIns(4,5)P_2 inhibited incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into PtdIns(4,5)P_2 and PtdIns4*P* when the cytosolic kinase activities were assayed. Hence, there are opposite effects of PtdIns(4,5)P_2 on the plasma-membrane and cytosolic phosphoinositide kinases when assayed under these conditions.

Fig. 2 illustrates the concentration-dependency of these effects. Half-maximal enhancement of ^{32}P-labelling of both polyphosphoinositides in the plasma membranes occurred at approx. 0.03 mM exogenous PtdIns(4,5)P_2. Half-maximal inhibition of cytosolic kinase activities occurred at approx. 0.01 mM-PtdIns(4,5)P_2. Similar effects of exogenously added PtdIns(4,5)P_2 to enhance [^{32}P]phosphate incorporation into PtdIns(4,5)P_2 and PtdIns4*P* were obtained when membranes were preincubated with PtdIns(4,5)P_2 for 1 min at 37 °C, washed, and then incubated in buffer containing [^{32}P]ATP but without PtdIns(4,5)P_2 (results not shown). This finding suggests that the effect of PtdIns(4,5)P_2 occurs after it intercalates into the membrane.

The effect of PtdIns(4,5)P_2 to enhance [^{32}P]phosphate incorporation into the polyphosphoinositides in the plasma-membrane fraction appeared to be specific, because exogenously added PtdIns and PtdIns4*P*, the usual substrates for these kinases, failed to stimulate ^{32}P-labelling of PtdIns4*P* or PtdIns(4,5)P_2 (Table 3); exogenous PtdIns and PtdIns4*P* did not appear to serve as substrates for the kinase activities in the plasma membrane under these conditions. The effect of PtdIns(4,5)P_2 was abolished by high concentrations of alkyltrimethylammonium bromide as the kinase activities were inhibited (Table 2).

Effects of exogenous PtdIns(4,5)P_2 on the content of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* in plasma membranes

To demonstrate that the effect of exogenous PtdIns(4,5)P_2 was to increase the plasma-membrane content of the polyphosphoinositides, the levels of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* were measured in membranes from cells prelabelled to isotopic steady state with [^{3}H]inositol. In these experiments, the phosphoinositides were labelled to constant specific radioactivity [11,12] and, therefore, the changes in the levels of the [^{3}H]phosphoinositides accurately reflect changes in lipid mass. In control incubations, [^{3}H]PtdIns(4,5)P_2 increased 0.6 nmol/mg of protein and [^{3}H]PtdIns4*P* increased by 1.7 nmol/mg of protein. Exogenous PtdIns(4,5)P_2 caused a further increase in the content of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4*P* to 142% and 146% of control levels respectively (Table 3). The effect of exogenous PtdIns(4,5)P_2 was specific, because PtdIns and PtdIns4*P* had no effect on the level of [^{3}H]polyphosphoinositides. These data complement the findings of the ^{32}P-labelling experiments and demonstrate that PtdIns(4,5)P_2 increases the content of the polyphosphoinositides in pituitary plasma membranes.

Degradation of polyphosphoinositides

To demonstrate that the effect of exogenous PtdIns(4,5)P_2 to increase the levels of polyphosphoino-

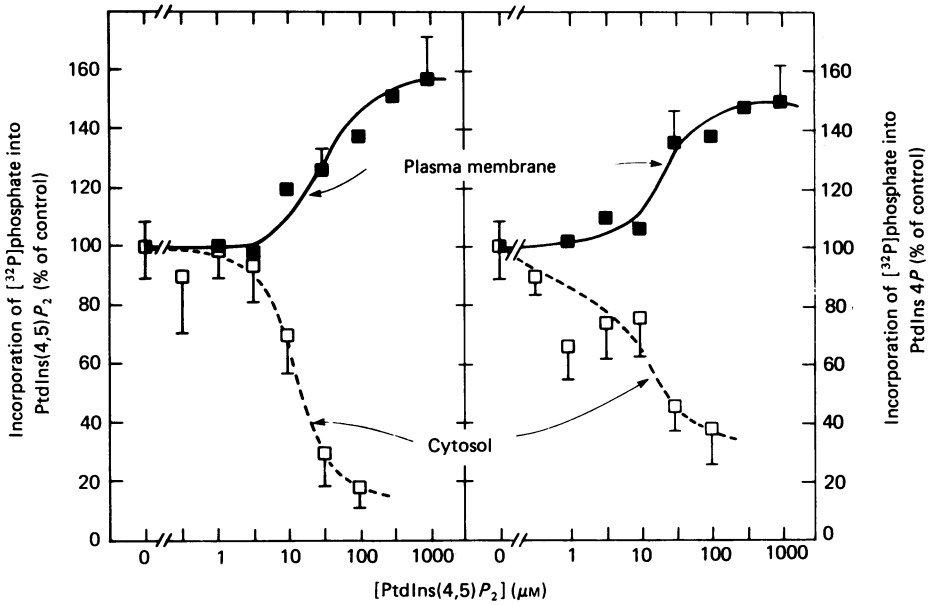

Fig. 2. Concentration-dependencies of the effects of exogenous PtdIns(4,5)P_2 on incorporation of [^{32}P]phosphate into PtdIns(4,5)P_2 and PtdIns4P in plasma membranes and cytosol from GH_3 cells

The experiments were performed as in Fig. 1, except that the buffers contained various concentrations of exogenous PtdIns(4,5)P_2 as indicated. The incubation was for 1 min with plasma membranes and for 5 min with cytosol. The points represents the means ± S.D. of triplicate determinations from two experiments and are expressed as percentages of control [no exogenous PtdIns(4,5)P_2].

Table 3. Effects of exogenous phosphoinositides on [^{32}P]phosphate incorporation into PtdIns4P and PtdIns(4,5)P_2 in unlabelled plasma membranes and on the content of [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 in plasma membranes from GH_3 cells prelabelled with [^{3}H]inositol

Plasma membranes (70–100 μg of protein) from cells prelabelled to isotopic steady state with [^{3}H]inositol were incubated for 1 min with or without 1 mM exogenous phosphoinositides in the presence of 1 mM-ATP at 37 °C. The content of [^{3}H]polyphosphoinositides was calculated from the specific radioactivity of PtdIns [(4.55 ± 0.38) × 10^3 c.p.m./nmol]. In control incubations, the content of [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 increased from 2.1 ± 0.07 and 2.3 ± 0.08 to 3.8 ± 0.41 and 2.9 ± 0.28 nmol/mg of protein respectively. The data represent the means ± S.D. of triplicate determinations from two experiments and are expressed as a percentage of the control increase above the initial levels. Incorporation of [^{32}P]phosphate into polyphosphoinositides in plasma membranes were assayed for 1 min as in Fig. 1 in the presence or absence of exogenous phosphoinositides. In control incubations, [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 had specific radioactivities of 1330 ± 97 and 760 ± 53 c.p.m./30 μg of protein respectively. The data represent the means ± S.D. of triplicate determinations from two experiments and are expressed as percentages of control (no exogenous phosphoinositides).

Exogenous phosphoinositide	Incorporation (% of control) into: PtdIns4P ^{3}H	PtdIns4P ^{32}P	PtdIns(4,5)P_2 ^{3}H	PtdIns(4,5)P_2 ^{32}P
None	100 ± 9	100 ± 7	100 ± 11	100 ± 7
PtdIns	98 ± 13	102 ± 12	98 ± 6	96 ± 6
PtdIns4P	113 ± 7	97 ± 18	109 ± 8	108 ± 5
PtdIns(4,5)P_2	146 ± 12*	132 ± 20*	142 ± 11*	152 ± 10*

*$P < 0.025$ versus control.

sitides in the plasma-membrane fractions was not due to inhibition of their degradation, the losses of ^{3}H- and ^{32}P-prelabelled PtdIns(4,5)P_2 and PtdIns4P were measured under conditions identical with those used for assay of the kinase activities. Plasma membranes from cells prelabelled with [^{3}H]inositol were labelled with [^{32}P]ATP, washed, and then incubated in buffer with or without unlabelled ATP and with or without exogenous PtdIns(4,5)P_2. There were slow decreases of ^{3}H- and ^{32}P-labelled PtdIns(4,5)P_2 and PtdIns4P during the first 2 min in the presence (results not shown) or absence of ATP and no further decreases after 5 min of incubation; only approx. 30% of the labeled PtdIns(4,5)P_2 and PtdIns4P was degraded during this time (Fig. 3). Under

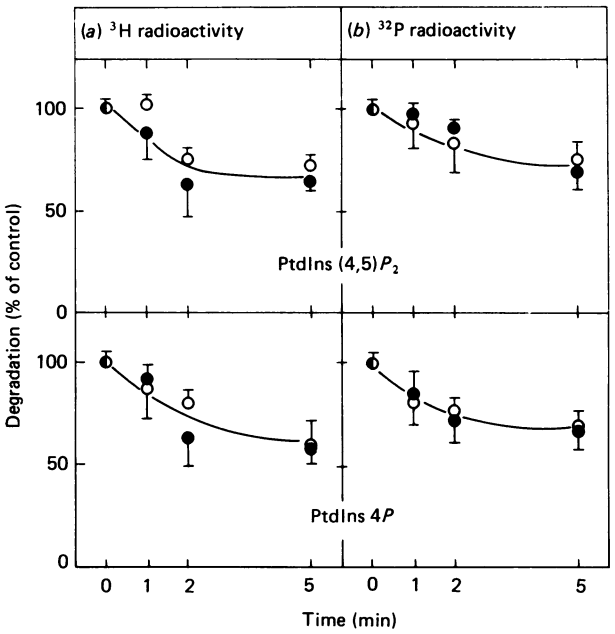

Fig. 3. Lack of effect of exogenous PtdIns(4,5)P_2 on degradation of endogenous PtdIns(4,5)P_2 and PtdIns4P, doubly labelled with [^{3}H]inositol *in vivo* and with [^{32}P]phosphate *in vitro*, in plasma membranes from GH_3 cells

Plasma membranes from GH_3 cells prelabelled to isotopic steady state with [^{3}H]inositol *in vivo* were also labelled with [^{32}P]phosphate by incubation for 5 min with [γ-^{32}P]-$MgATP^{2-}$ *in vitro* as described in the legend to Fig. 1. The membranes were washed and resuspended in fresh buffer without [^{32}P]$MgATP^{2-}$ and without (○, control) or with (●) 1 mM-PtdIns(4,5)P_2 and incubated at 37 °C for the times indicated. Each point represents the mean ± S.D. of triplicate determinations expressed as a percentage of the inital levels, which were: (*a*) [^{3}H]PtdIns4P, 3.23 ± 0.08 nmol/mg of protein; [^{3}H]PtdIns(4,5)P_2, 3.03 ± 0.06 nmol/mg of protein; (*b*) [^{32}P]PtdIns4P, 661 ± 7 c.p.m.; [^{32}P]PtdIns(4,5)P_2, 341 ± 16 c.p.m.

these conditions of extremely low free Ca^{2+} concentration (the buffer contained 1 mM-EGTA and no added Ca^{2+}), degradation of the polyphosphoinositides is catalysed almost exclusively by phosphomonoesterases; there is very little phospholipase C activity detectable [26]. Exogenously added PtdIns(4,5)P_2 had no effect on the degradation of either polyphosphoinositide.

DISCUSSION

Previous studies of PtdIns and PtdIns4P kinase activities in several tissues concentrated to a large extent on determining the intracellular localization of these enzymes. From studies in liver, brain and kidney, it appears that PtdIns kinase is located primarily within the plasma membrane [7–9] on the cytoplasmic leaflet [27], but there is evidence to suggest that it may be present in other sites, for example, within lysosomes in liver [28]. The subcellular distribution of PtdIns4P kinase appears less certain. In kidney, it is found in both the particulate and soluble fractions [10], whereas in brain most of the activity appears to be soluble [29,30], but there is a significant activity associated with myelin [31,32]. More recent data obtained by using membranes derived from liver cells suggest that both PtdIns and PtdIns4P kinase activities may be present in many membrane fractions within cells, but that the most active are present within the plasma membrane [12,13]. In fact, one group has suggested that these kinase activities may be used as markers for plasma membrane in Friend erythroleukaemia cells [33]. In the present case we studied the regulation of PtdIns4P and PtdIns kinase activities in the plasma-membrane fraction derived from cloned rat pituitary (GH_3) cells because of the accumulating evidence that it is this subcellular site that contains polyphosphoinositides with important roles in cell regulation in general [1], and in regulation of GH_3-pituitary-cell function in particular [2]. We studied regulation of plasma-membrane-bound and cytosolic kinases from pituitary cells so that we could compare our findings with those previously published [14–16].

The plasma-membrane and cytosolic fractions isolated from GH_3 cells contained PtdIns4P and PtdIns kinase activities. (The plasma membrane contained 1,2-diacylglycerol kinase activitiy also.) PtdIns4P kinase activity, and to a lesser extent PtdIns kinase activity, appeared to co-purify with the plasma-membrane enzyme marker ($Na^+ + K^+$)-ATPase. In our experiments using this highly enriched plasma-membrane fraction, PtdIns(4,5)P_2 and PtdIns4P accounted for more than 95% of the phosphorylated lipid products when kinase activities were measured as incorporation of [^{32}P]phosphate from the γ-position of ATP into endogenous substrates. We also monitored phosphoinositide kinase activities as increases in the content of [^{3}H]polyphosphoinositides in membranes prelabelled to isotopic steady state *in vivo* with [^{3}H]inositol. Exogenous PtdIns4P and PtdIns did not serve as substrates under the conditions of the assay used with the plasma-membrane fraction. The apparent activities of the kinases in the plasma membranes, based on the specific radioactivities of [^{3}H]inositol and [^{32}P]ATP, are among the highest reported for PtdIns4P and PtdIns kinase activities [33–35]. The phosphoinositide kinase activities in the cytosolic fraction appear similar to those reported previously [4,15,16].

Although the factors that regulate PtdIns4P and PtdIns kinases *in vivo* are unknown, there are several reports that address the question of regulation of phosphoinositide kinases *in vitro*. Jolles, Gispen and their colleagues [34, 36] observed stimulation by adrenocorticotropin of a PtdIns4P kinase activity in a membrane fraction derived from rat brain; however, the concentration of adrenocorticotropin required for this effect was very high (greater than 10 μM). Torda [37] reported stimulation of a soluble PtdIns4P kinase isolated from rat brain by cyclic AMP. In an early study, Michell *et al.* [14] reported that PtdIns kinase activity in a plasma-membrane fraction from liver was inhibited by several phospholipids, in particular by PtdIns4P. However, Michell *et al.* [14] measured PtdIns kinase activity by using exogenous PtdIns as substrate, and the correlation with regulation in the milieu of the plasma membrane is uncertain. MacDonald *et al.* [15] observed that, in the presence of detergents, PtdIns4P inhibited PtdIns kinase activity acting on exogenous and endogenous PtdIns. More recently, Van Rooijen *et al.* [16] observed that PtdIns(4,5)P_2 inhibited the activity of a soluble PtdIns4P kinase derived from bovine retina. We also found that PtdIns(4,5)P_2 inhibited the activity of a PtdIns4P kinase

that was present in a soluble form in the cytosolic fraction derived from GH_3 cells. In contrast, we found that PtdIns(4,5)P_2 added exogenously to pituitary plasma membranes had the following effects: (1) it increased incorporation of [^{32}P]phosphate from the γ-position of [^{32}P]ATP into endogenous PtdIns(4,5)P_2 and PtdIns4P without affecting its incorporation into PtdA; (2) it increased the content of [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4P in membranes from cells prelabelled to isotopic steady state with [^{3}H]inositol; and (3) it had no effect on the degradation of ^{3}H- and ^{32}P-labelled polyphosphoinositides. Because these findings show that PtdIns(4,5)P_2 increased the content of polyphosphoinositides without affecting their degradation and increased [^{32}P]phosphate incorporation into PtdIns(4,5)P_2 and PtdIns4P, it may be concluded that the effect of PtdIns(4,5)P_2 was to increase the formation of the polyphosphoinositides by their respective kinases. The effect was specific for PtdIns(4,5)P_2, because other phosphoinositides, PtdIns4P and PtdIns, did not affect the kinases. Also, it was not caused by non-specific perturbation of the structure of the plasma membrane, because neutral, anionic and cationic detergents at broad ranges of concentrations caused inhibition, not stimulation of these activities. Hence, the most likely explanation for our findings is that PtdIns(4,5)P_2 specifically stimulated phosphoinositide kinase activities in plasma membranes derived from pituitary cells. It is important to note that our data do not definitively exclude the possibility that dephosphorylation of exogenous PtdIns(4,5)P_2 served simply to provide added substrate for the kinase rather than enhancing the activity of the kinases. However, this explanation seems very unlikely because: (1) exogenously added PtdIns4P and PtdIns did not serve as substrates themselves; and (2) the effect was maximal at the earliest time measured (10 s) rather than increasing with time as more exogenous PtdIns(4,5)P_2 could have become dephosphorylated.

The physiological significance of the finding that PtdIns(4,5)P_2 stimulates phosphoinositide kinase activities within the plasma membrane is uncertain. Previous observations that PtdIns(4,5)P_2 inhibits a soluble PtdIns4P kinase [16] and that PtdIns4P inhibits PtdIns kinase activities [14,15] had suggested that the phosphoinositide kinases were enzymes that were product-inhibited. Those findings supported the hypothesis that increased flux from PtdIns to PtdIns4P to PtdIns(4,5)P_2 may occur during cell stimulation by Ca^{2+}-mobilizing hormones and neurotransmitters [1], including TRH stimulation of GH_3 cells [2], as the enzymes were released from product inhibition when PtdIns(4,5)P_2 was hydrolysed. However, a recent report in which thrombin stimulation of human platelets was monitored [5] and our findings during TRH stimulation of GH_3 cells [5a] have provided evidence that there is no measurable increase in the rate of formation of PtdIns4P and PtdIns(4,5)P_2. In fact, the findings reported herein suggest that, as the level of PtdIns(4,5)P_2 decreases, the activities of the phosphoinositide kinases *in vivo* may decrease rather than increase. This effect, of inhibiting the resynthesis of PtdIns(4,5)P_2, may be a mechanism to limit the generation of inositol trisphosphate during prolonged exposure of GH_3 cells to TRH. It is possible that these findings in GH_3 cells may have general applicability to the mechanism of action of all Ca^{2+}-mobilizing hormones and neurotransmitters.

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