Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP_2

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Background: Phosphatidylinositol transfer protein (PI-TP), which has the ability to transfer phosphatidylinositol (PI) from one membrane compartment to another, is required in the inositol lipid signalling pathway through phospholipase C–β (PLC–β) that is regulated by GTP-binding protein(s) in response to extracellular signals. Here, we test the hypothesis that the principal role of PI-TP is to couple sites of lipid hydrolysis to sites of synthesis, and so to replenish depleted substrate for PLC–β.
Results: We have designed an experimental protocol that takes advantage of the different rates of release of endogenous PI-TP and PLC-β from HL60 cells permeabilized with streptolysin O. We have examined the kinetics of stimulated inositol lipid hydrolysis in cells depleted of PI-TP, but not of endogenous PLC-β, in the presence and absence of exogenous PI-TP. Linear time-courses were observed in the absence of any added protein, and the rate was accelerated by PI-TP using either guanosine 5′[γ-thio]-triphosphate (GTPγS) or the receptor-directed agonist fMetLeuPhe as activators. In addition, depletion from the cells of both PI-TP and PLC-β isoforms by extended permeabilization (40 minutes) allowed us to control the levels of PLC–β present in the cells. Once again, PI-TP increased the rates of reactions. To identify whether the role of PI-TP was to make available the substrate phosphatidylinositol bisphosphate (PIP_2) for the PLC, we examined the synthesis of PIP_2 in cells depleted of PI-TP. We found that PI-TP was essential for the synthesis of PIP_2.
Conclusions: The predicted function of PI-TP in inositol lipid signalling is the provision of substrate for PLC–β from intracellular sites where PI is synthesized. We propose that PI-TP is in fact a co-factor in inositol lipid signalling and acts by interacting with the inositol lipid kinases. We hypothesize that the preferred substrate for PLC–β is not the lipid that is resident in the membrane but that provided through PI-TP.

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Background

Purification of mammalian phospholipases (PLCs) and the molecular cloning of their genes have led to the identification of at least three PLC families — β, γ and δ [1,2]. Recent studies indicate that members of the PLC–β family are regulated either by the α subunits of heterotrimeric GTP-binding (G) proteins belonging to the G_q family, or by G-protein βγ subunits [3,4]. Specifically, G_q α subunits have been shown to activate PLC–β isozymes in the order PLC–β1 ≥ PLC–β3 ≫ PLC–β2, which differs from the order for activation by βγ subunits, PLC–β3 > PLC–β2 > PLC–β1 [3,5].

We have identified a requirement for cytosolic component(s) for the efficient activation of the G-protein-regulated PLC in HL60 promyelotic cells [6,7]. Depletion of cytosol from HL60 cells leads to an attenuation of guanosine 5′[γ-thio]-triphosphate (GTPγS)-dependent activation of PLC, and this activation can be reconstituted with exogenously added cytosol. Thomas *et al.* [6] first reported that rat brain cytosol contains two factors that can reconstitute GTPγS-mediated inositol lipid signalling in cytosol-depleted HL60 cells. The minor reconstituting factor was tentatively identified as PLC–β1 [6], and the major factor, a 35 kD protein, has been identified as the phosphatidylinositol-transfer protein (PI-TP) [8].

PI-TP was originally identified because of its ability to transfer phosphatidylinositol (PI) or phosphatidylcholine (PC) from one membrane compartment to another, a function that depends on the ratio of PI:PC in the two compartments [9,10]. This dependence suggested that the ability of PI-TP to restore inositol lipid signalling is related to its ability to deliver PI to the site of hydrolysis by PLC [11]. The requirement for PI-TP therefore suggests that the plasma membrane has a limited supply of substrate for PLC, and that replenishment from internal membranes is necessary for continued inositol phosphate production. So, we expected that PI-TP would not affect the initial rate of inositol lipid hydrolysis, but would function in maintaining that rate. We have examined the kinetics of inositol phosphate production in response to both the receptor-directed agonist, fMetLeuPhe, and GTPγS, a direct activator of G proteins. The effect of both PI-TP and PLC–β on the rate of inositol phosphate production was examined. Contrary to expectations, PI-TP was found strongly to influence the initial rate of

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inositol phosphate production, either with GTPγS or with fMetLeuPhe as the activator. We conclude that PI-TP acts as a cofactor by interacting with the lipid kinases, and hypothesize that the preferred substrate for PLC is the lipid transported by PI-TP.

Results and discussion

PI-TP increases the rate of inositol phosphate production

Upon permeabilization of HL60 cells with streptolysin O, PI-TP leaks out of the cells. Near-maximal release of PI-TP occurs at 2 minutes, as observed by western immunoblotting (Fig. 1a) and the measurement of PI transfer activity *in vitro* (Fig. 1d). Release of PI-TP protein into the extracellular medium is concurrent with the decline in PI-TP protein levels within the cells; by 2 minutes there is no detectable PI-TP left in the permeabilized cells (Fig. 1b). It has been reported previously that, in Swiss 3T3 cells, part of the PI-TP remains in the permeabilized cell, associated with the perinuclear Golgi apparatus [12]. In HL60 cells, permeabilization appears to lead to the near-total removal of the cellular PI-TP.

In contrast to PI-TP, the elution of PLC-β2/β3 from the same cells is much slower ([8] and see Fig. 1c). Release of PLC-β2 is insignificant at 10 minutes, but maximal release is observed by 30 minutes (Fig. 1c). The elution pattern for PLC-β3 was identical (data not shown); in further contrast to PI-TP, a significant proportion of this enzyme remains cell-associated [8]. By choosing the time of permeabilization, therefore, cells can be prepared that contain no PI-TP, and either the full complement of PLC-β2/β3 (10 minutes permeabilization) or reduced amounts of PLC-β2/β3 (40 minutes permeabilization).

GTPγS can gain rapid entry (within 10 seconds) to the cell interior when added in the presence of streptolysin O, and it stimulates inositol lipid hydrolysis [13]. This response to GTPγS diminishes with a time-course that exactly matches the loss of PI-TP from the cells (compare Figs 1a and 1d).

We have examined the kinetics of inositol lipid hydrolysis by endogenous PLC-β2/β3 in the complete absence of PI-TP by using cells permeabilized for 10 minutes (Fig. 2a,b). Inositol phosphate production stimulated by GTPγS was linear for at least 20 minutes (Fig. 2b). This linear time-course is possible as the levels of the primary substrate, phosphatidylinositol bisphosphate (PIP_2; or phosphatidylinositol phosphate, PIP), do not decline markedly during the 20 minutes of incubation (Fig. 3, and see also Table 1). As Mg^{2+}–ATP is present, the primary substrate PIP_2 can be constantly replenished, ultimately from PI. When PI-TP is added with GTPγS, after a short delay, an eight-fold increase in the rate of production of inositol phosphate is observed (Fig. 2a). The rate of hydrolysis is linear for 20 minutes, after which it begins to slow down.

Reconstitution of receptor-mediated signalling with PI-TP

When GTPγS is used as the stimulus for PLC, it will activate, non-selectively, all the available G proteins present in HL60 cells. This will include members of the G_q family and the more abundant G_i family. In addition, GTPγS is a poorly hydrolyzable analogue: activation cannot be terminated and the resultant stimulation is supramaximal. On average, GTPγS can cause the hydrolysis of 24 ± 5 % (mean ± SEM, n = 7) of cellular inositol lipids when Ca^{2+} is buffered at 1 μM. In contrast, the maximal amount of inositol lipid hydrolyzed by fMetLeuPhe, a receptor-directed agonist, is limited to 3.4 ± 0.8% (mean ± SEM, n = 20) in intact cells. In

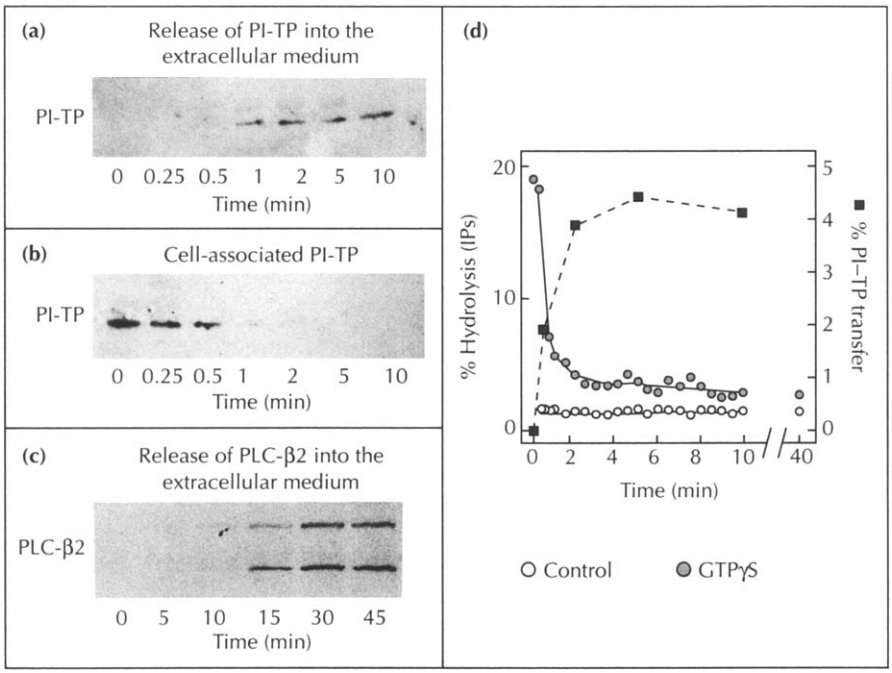

Fig. 1. Release of PI-TP, but not of PLC-β2, is associated with the loss of GTPγS-stimulated PLC activity from HL60 cells upon permeabilization with streptolysin O. HL60 cells were permeabilized with 0.6 IU ml^{-1} streptolysin O, and aliquots were removed at the indicated time points. Cells were separated from the supernatant by centrifugation. The supernatant and the cells were analyzed by western immunoblotting (see Materials and methods), using anti-PI-TP and anti-PLC-β2 antibodies. **(a)** PI-TP in the extracellular medium and **(b)** cell-associated PI-TP. **(c)** PLC-β2 in the extracellular medium. **(d)** Release of PI–TP into the medium as assayed by transfer activity monitored simultaneously with loss of GTPγS-stimulated PLC activity. The *x*-axis indicates the duration of permeabilization before assay with GTPγS.

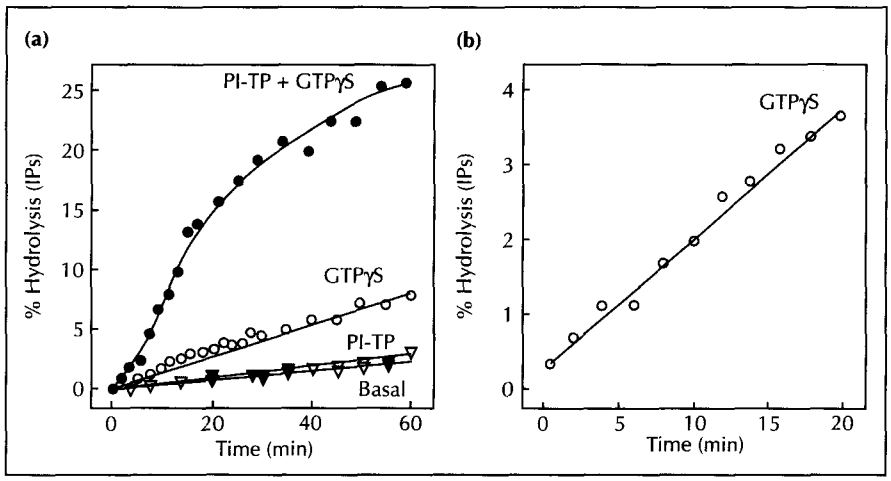

Fig. 2. **(a)** Time-course of inositol phosphate (IP) production in the presence and absence of PI-TP. HL60 cells were permeabilized for 10 min, washed and subsequently incubated at 37 °C, with or without 220 μg ml^{-1} PI-TP ± 10 μM GTPγS. **(b)** Detail of the time-course of inositol phosphate production stimulated by GTPγS in the absence of PI-TP.

principle, it could be argued that PI-TP is required only when the system is pushed to extremes with GTPγS, and that we are creating an artificial condition by making the system dependent on PI-TP [11]. It was important, therefore, to examine the requirement for PI-TP in a receptor-driven system.

Depletion of cytosol by permeabilization of the cell leads to a rapid decline in inositol phosphate production stimulated by fMetLeuPhe (Fig. 4a). In cells permeabilized for 10 minutes, fMetLeuPhe stimulates a constant rate of inositol phosphate production for the first 20 minutes (Fig. 4b), and this is enhanced greatly in the presence of PI-TP (Fig. 4c). The absolute amount of inositol lipid hydrolyzed by fMetLeuPhe in the presence of PI-TP is 2.5 %, as compared to 0.5 % in its absence. The response to fMetLeuPhe in the presence of PI-TP is linear for 20 minutes, after which a plateau is observed (Fig. 4c). These results indicate that PI-TP is a critical component, not only when the system is activated supramaximally with GTPγS, but also with receptor-mediated activation.

Interplay between exogenously added PLC-β1 and PI-TP

From our previous studies using rat brain cytosol, we had established the presence of two separate reconstituting activities, one of which was PLC–β1 [6]. We examined, therefore, the nature of the interaction between PI-TP and PLC-β1 by extending the period of permeabilization to 40 minutes in order to deplete the endogenous PLC-β2/β3. Both PI-TP (presumably by interacting with the residual PLC-β2/β3) and PLC-β1 alone are capable of restoring GTPγS-stimulated inositol phosphate production (Fig. 5a). The percentage of lipid hydrolysed by PLC-β1 was always smaller than that caused by PI-TP when maximal concentrations of each component were used. The concentration of PI-TP used was deliberately kept low in this experiment (Fig. 5a). We have shown previously that reconstitution with PI-TP increases linearly as more PI-TP is added [8]. Reconstitution with PLC-β1 reaches a plateau as the concentration of enzyme is increased (Fig. 5b). Reconstitution with PLC-β1 or with PI-TP is entirely dependent on the presence of a G-protein activator, GTPγS (Fig. 5a).

Table 1. Production of inositol phosphates and the level of inositol lipids in response to PI-TP and GTPγS.

	Control	GTPγS	PI-TP	PI-TP + GTPγS
IP_3	405 ±15	3 639 ± 129	1 191 ± 23	16 090 ± 830
IP_2	250 ± 20	2 588 ± 32	633 ± 16	7 840 ± 110
IP_1	510 ±10	3 515 ± 193	730 ± 22	6 170 ± 390
$IP_3 + IP_2 + IP_1$	1 240	9 742	2 554	30 100
PIP_2	5 870 ± 65	5 436 ± 68	5 949 ± 264	4 440 ± 160
PIP	5 435 ± 340	5 877 ± 125	6 900 ± 333	5 445 ± 120
PI	91 780 ± 2 410	92 055 ± 388	82 051 ± 1 861	64 920 ± 1 560
$PIP_2 + PIP + PI$	103 085	103 268	94 900	74 805

HL60 cells were labelled with [^{3}H]inositol for 48 h. The cells were permeabilized for 10 min to deplete the cytosol and washed. The cytosol-depleted cells were incubated with either buffer, 10 μM GTPγS, 370 μg ml^{-1} PI-TP or PI-TP plus GTPγS at pCa 6 for 20 min. Production of the inositol phosphates and the level of the inositol lipids were analyzed as described in the results section. The assay was carried in triplicate and the mean ± SEM is shown.

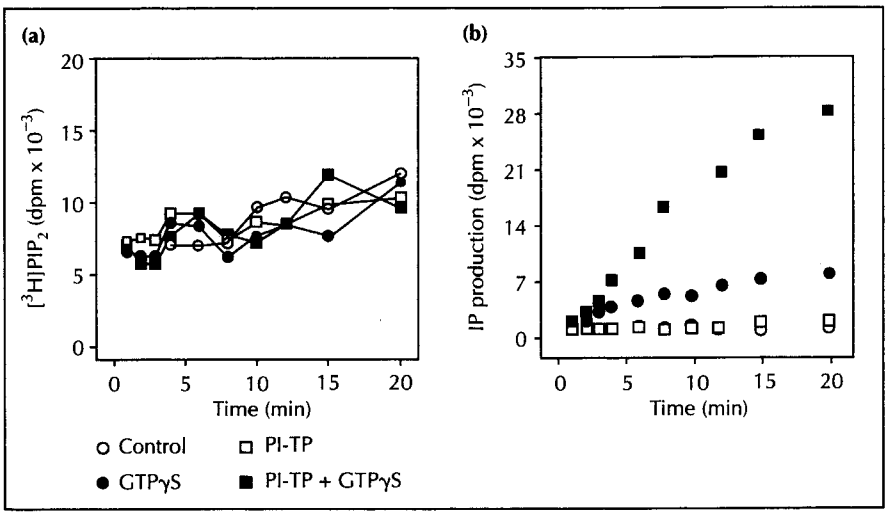

Fig. 3. (a) The levels of $[^3H]PIP_2$ remain unchanged over time during stimulation with GTPγS and PI–TP. HL60 cells, labelled with $[^3H]$inositol for 48 h, were washed and permeabilized for 10 min and subsequently stimulated with 10 μM GTPγS, in the presence or absence of 75 μg ml^{-1} PI-TP. **(b)** The production of inositol phosphates (IP) upon stimulation in this experiment.

Figure 5a illustrates that when PI-TP and PLC-β1 are introduced into the cytosol-depleted cells together, the response is not additive but synergistic, giving nearly 20 % hydrolysis. This synergy is observed only if the concentration of PI-TP is kept low. Analysis of 11 such experiments shows that when the response to GTPγS is set to 100 %, the additional increase due to the presence of PI-TP was 167 ± 25 % (that is, 2.67 times that in the presence of GTPγS alone). The additional increase in the presence of PLC-β1 was 128 ± 27 %. When PI-TP and PLC-β1 were added together, the additional increase amounted to 543 ± 150 %, which is greater than the sum of the individual responses. We next examined the time-course of inositol lipid hydrolysis in the presence of PI-TP and PLC-β1, individually and in combination (Fig. 5c). In the absence of PI-TP, cytosol-depleted HL60 cells incubated with GTPγS show a linear increase in inositol lipid hydrolysis (Fig. 5c). The rate of this hydrolysis can be increased further by adding PLC-β1 but, significantly, the time-course remains linear for 30 minutes (Fig. 5c). The effect of PI-TP is to accelerate the reaction both in the presence and in the absence of PLC-β1.

Identification of PIP_2 as the major substrate for PLC

In the experiments described so far, we have used the production of total inositol phosphates as a measure of PLC activity. As PLC is capable of hydrolysing all inositol lipids *in vitro*, we investigated the substrate requirement for the PLC in the presence or absence of PI-TP. Table 1 illustrates that the addition of PI-TP alone marginally increases the production of IP_2 and IP_3. In the presence of GTPγS alone, there is an increase not only in IP_2 and IP_3, but also in IP_1. In the presence of GTPγS and PI-TP, there is a substantial increase in the production of all three inositol phosphates, with the largest increase being in IP_3.

Table 1 illustrates the relationship between the amount of IP_3 formed and the level of PIP_2 present before and

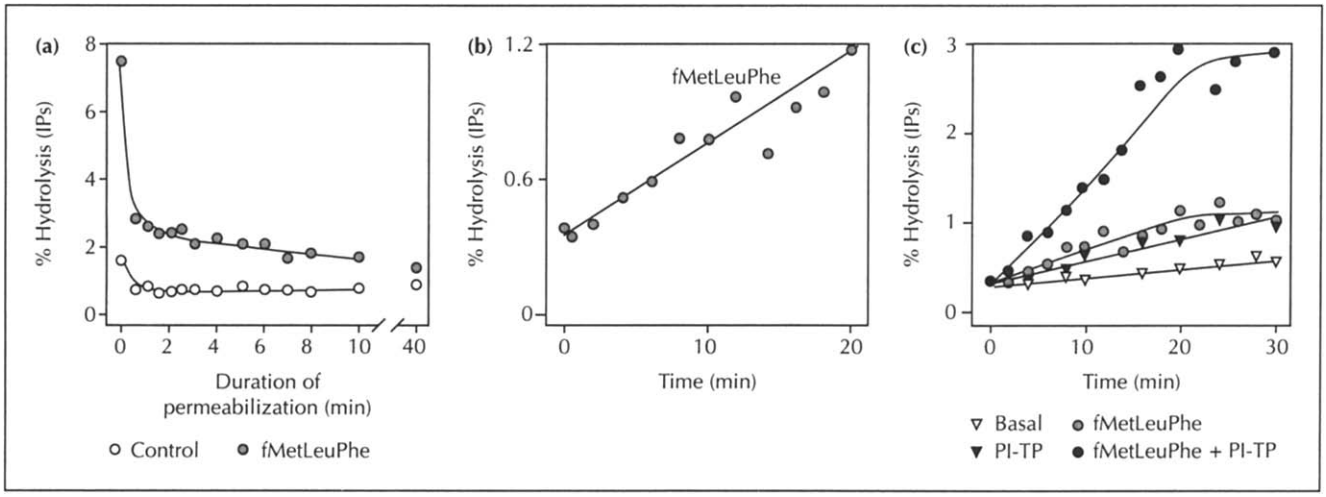

Fig. 4. (a) PLC activity is not stimulated by fMetLeuPhe when HL60 cells are permeabilized with streptolysin O to deplete the cytosol. HL60 cells were permeabilized with streptolysin O and, at the indicated time intervals, aliquots were removed and assayed for fMetLeuPhe-stimulated PLC activity. Samples were incubated for 20 min at 37 °C in the presence or absence of 1 μM fMetLeuPhe at pCa 6. **(b)** Detail of the time-course of inositol phosphate production stimulated by fMetLeuPhe. HL60 cells were permeabilized for 10 min at pCa 7, washed, and subsequently incubated ± 1 μM fMetLeuPhe. **(c)** Time-course of PLC activation by fMetLeuPhe in the presence or absence of 250 μg ml^{-1} PI-TP ± 1 μM fMetLeuPhe, as indicated.

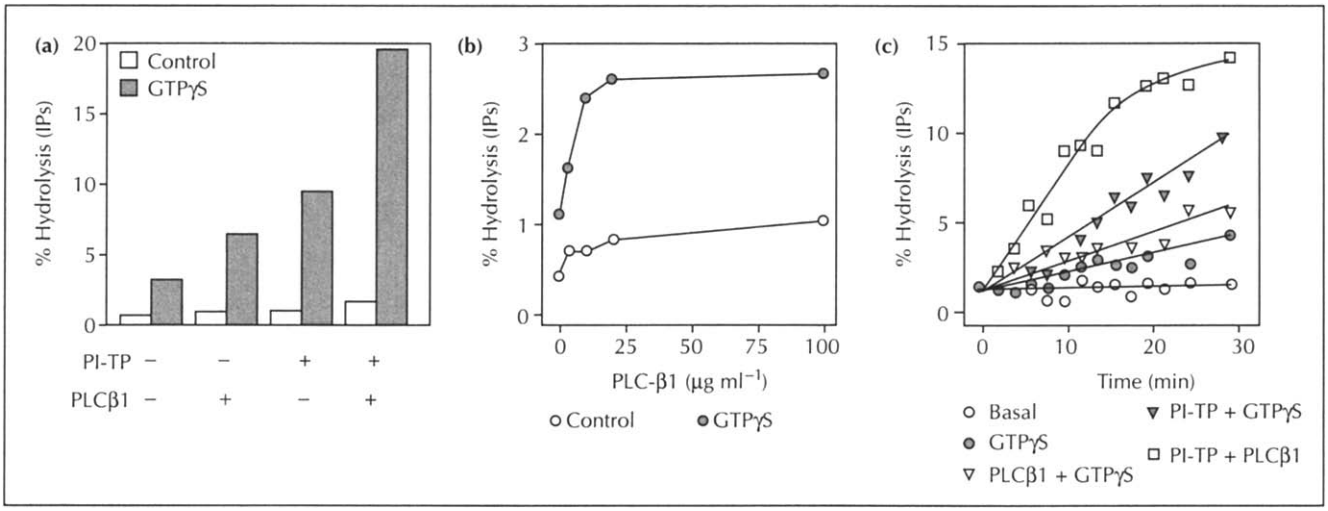

Fig. 5. **(a)** Reconstitution of G-protein-regulated inositol phosphate production with PI-TP and PLC-β1 in HL60 cells permeabilized for 40 min. Cytosol-depleted cells were incubated with 20 μg ml^{-1} PI-TP, 34 μg ml^{-1} PLC-β1 (specific activity 2.2 μmol min^{-1} mg^{-1}) and 10 μM GTPγS, as indicated. After incubation at 37 °C for 20 min, the reaction was terminated with 1 ml ice-cold 0.9 % saline and analyzed for released inositol phosphates. **(b)** Reconstitution of GTPγS-regulated inositol phosphate production with increasing concentrations of PLC-β1. **(c)** Time-course of GTPγS-stimulated inositol phosphate production in the presence of PI-TP or PLC-β1, or both. HL60 cells were permeabilized for 40 min, washed and subsequently incubated with 10 μM GTPγS, 40 μg ml^{-1} PI-TP and/or 14 μg ml^{-1} PLC-β1, as indicated.

after stimulation. In control cells, PIP_2 represents 5.8 % of the total inositol lipids. The amount of IP_3 made upon stimulation with GTPγS in the presence of PI-TP far exceeds the amount of PIP_2 present in the cells. In addition, despite the extensive production of IP_3, the level of PIP_2 changes little, whilst that of PI decreases significantly. This result indicates that the lipid kinases must be able to synthesize PIP_2 from PI on demand.

Influence of PI-TP on PIP_2 synthesis

GTPγS-stimulated synthesis of PIP_2 was analyzed in detail in human neutrophils by Stephens *et al.* [14]; they concluded that GTPγS can cause a large activation of PIP-5-kinase. We also monitored the turnover of PIP_2 in HL60 cells permeabilized in the presence of 100 μM Mg^{2+}–ATP and 10 μCi [γ-^{32}P]ATP. In control cells, the radioactivity associated with PIP_2 increased with time; this reflects turnover by phosphatases and kinases, as the actual concentration of PIP_2 does not change under these conditions. In the presence of GTPγS, the radioactivity associated with PIP_2 increased more rapidly compared to control cells (Fig. 6a). Measurements of radioactivity in PIP_2 from [^{3}H]inositol-labelled cells showed little change (Fig. 3 and also Table 1). As the HL60 cells were grown in [^{3}H]inositol for 48 hours, the label in PIP_2 reflects closely any changes in the actual mass of PIP_2. Thus the increase in the labelling of PIP_2 with [γ-^{32}P]ATP in the presence of GTPγS reflects the synthesis of PIP_2.

When HL60 cells were depleted of PI-TP by permeabilization for 10 minutes, the GTPγS-stimulated synthesis of PIP_2 was impaired. This phenomenon could be due to a number of factors, including the loss of the inositol lipid kinases during permeabilization. PI-4-kinase(s) have been generally found to be membrane-associated [15], whereas PIP-5-kinase(s) have been found both in membranes [16,17] and in the cytosol [18]. In order to examine the location of the inositol lipid kinases in HL60 cells, we monitored the activity of PI-4-kinase(s) and PIP-5-kinase(s) using an *in vitro* assay with exogenous substrate. HL60 cells were disrupted and separated into particulate membrane and supernatant fractions. Both lipid kinase activities were found to be associated with membranes. This result is in agreement with previous work on human neutrophils [19]. In addition, the permeabilization protocol used did not decrease the level of PIP_2. The radioactive counts (disintegrations per minute) in the inositol lipids PIP_2, PIP and PI in intact HL60 cells were 13 943 ± 287, 26 410 ± 1198 and 1 063 360 ± 40 686, respectively; the values for cells which were permeabilized for 10 minutes

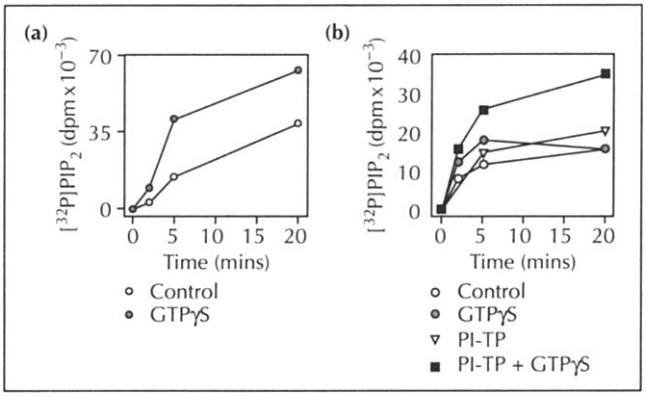

Fig. 6. Restoration of PIP_2 synthesis by PI-TP in cytosol-depleted HL60 cells. **(a)** HL60 cells were permeabilized in the presence of 100 μM Mg^{2+}–ATP plus [γ-^{32}P]ATP and 10 mM $MgCl_2$ at pCa 7, in the presence or absence of 10 μM GTPγS. **(b)** Cytosol-depleted cells were washed and subsequently incubated in the presence or absence of 10 μM GTPγS ± 250 μg ml^{-1} PI-TP, as indicated, and 100 μM Mg^{2+}–ATP plus [γ-^{32}P]ATP and 10 mM $MgCl_2$ at pCa 7. In each case, aliquots (100 μl) were removed at the indicated time points and quenched with chloroform–methanol.

and washed were 15 397 ± 425, 34 143 ± 927 and 953 926 ± 10 902, respectively (means ± SEM, $n = 4$).

The loss of a cytosolic factor could also account for the impairment of the GTPγS-stimulated PIP_2 synthesis. PI-TP is an obvious candidate. PI-TP was added back to cytosol-depleted cells and tested for its ability to restore the GTPγS-stimulated synthesis of PIP_2. Figure 6b shows that it does have this ability.

In the experimental system used here, the cytosol-depleted HL60 cells do not have the ability to resynthesize PI, because CTP and inositol, the soluble components required for PI synthesis, are absent. We have been unable to monitor the resynthesis of PI after stimulation with GTPγS in permeabilized HL60 cells when the system was supplemented with CTP and inositol (data not shown). It is most likely that this is due to impaired transport of phosphatidic acid (PA) from the plasma membrane to the endoplasmic reticulum where the enzymes for PI biosynthesis are located [20,21]. The membrane compartment in which PI synthesis occurs has not been described for HL60 cells but it is expected to occur at the endoplasmic reticulum, the predominant site of PI synthesis in other cell types [20,21]. There has been one report identifying PI-synthase activity at the plasma membrane of GH_3 rat pituitary cells [22], but these results have not been substantiated in later studies [20].

As the reconstituted system used here has lost the ability to resynthesize PI, it could be argued that the system is artificially dependent on PI-TP. Studies in *Drosophila* indicate that flies with mutations in the enzyme CDP-diacylglycerol synthase, responsible for PI synthesis, cannot sustain a light-activated current in their photoreceptor cells [23]. However, PI-TP accelerates the rate of lipid hydrolysis in HL60 cells, not only with GTPγS but also with fMetLeuPhe, in which case the amount of lipid hydrolyzed is limited to a few percent. The inability to resynthesize PI, therefore, should not be a limiting factor, particularly when fMetLeuPhe is used as the stimulus; regardless, PI-TP has very significant effects on IP_3 production.

When we first observed the dramatic effects of PI-TP on stimulated inositol lipid hydrolysis, it was clear that our observations could be explained in the light of the known properties of this protein *in vitro* [8]. Principally, PI-TP could couple PI synthesis at the endoplasmic reticulum to the sites of hydrolysis at remote membranes. While PLC consumed inositol lipids, the PI concentration at the site of hydrolysis would be buffered by the transfer of PI from PI-TP into the membrane. One observable consequence of this would be a decrease in the rate of stimulated inositol lipid hydrolysis, in cells depleted of PI-TP, as the concentration of substrate decreased in the membrane compartment(s) where PLC was active. We were unable to demonstrate this decline in activity, either under conditions of supramaximal stimulation with GTPγS, or with submaximal and compartment-specific hydrolysis induced by fMetLeuPhe. In addition, hydrolysis induced by either stimulus was linear with time, and this was the case even if the rate of GTPγS-stimulated hydrolysis was enhanced by the addition of exogenous PLC-β. However, PI-TP was able to accelerate greatly all of these reactions to new maximal rates. These observations are inconsistent with a simple 'substrate re-supply' model for the role of PI-TP [11]. Our data support the conclusion that PI-TP dictates the rate of IP_3 production by promoting the synthesis of PIP_2, and that the newly synthesized PIP_2 is preferentially hydrolyzed by PLC.

A model for PI-TP action

If PI-TP is not acting simply to replenish depleted substrate, how might it function? One hypothesis is that PI-TP is an important cofactor in inositol lipid signalling. We define the polyphosphoinositides and the more abundant PI as the potential substrates for PLC, although the enzyme only hydrolyses the polyphosphoinositides (see Table 1). As reconstitution with PI-TP is absolutely dependent on the availability of Mg^{2+}–ATP [8], we assume that the lipid kinases required for phosphorylation of PI are not limiting factors for substrate provision. Examination of PI-4-kinase activity and PIP-5-kinase activity using an *in vitro* assay confirmed that the majority of the lipid kinase(s) in HL60 cells are membrane-associated.

It is also reasonable to regard PI-4-kinase, PIP-5-kinase and PLC-β as a single coupled-enzyme system at the plasma membrane compartment. It is hypothesized that PI-TP serves to accelerate inositol lipid hydrolysis by acting as a cofactor for one of the reactions in this pathway. As PI-TP is required not only for inositol lipid hydrolysis but also for synthesis of PIP_2, we can exclude PLC-β as a possible target for PI-TP. This conclusion is consistent with our recent observations concerning the requirement for PI-TP in order for the EGF receptor to activate lipid hydrolysis by PLC-γ [24]. In addition, an interaction between PI-TP and lipid kinases, as suggested here, is in line with the conclusions of Hay *et al.* [25]: that the requirement for PI-TP in the priming of exocytosis is due to a requirement for the synthesis of PIP_2.

We can narrow down the possible target(s) for PI-TP, therefore, to PI-4-kinase and PIP-5-kinase. Synthesis of PIP_2 is dependent on the sequential phosphorylation of PI by PI-4-kinase, and of the resulting PIP by PIP-5-kinase. Multiple forms of PI-4-kinase(s) have been purified and characterized, and have been classified into two categories, types II and III, on the basis of differences in sensitivity to adenosine and nonionic detergents [26]. In A431 cells, the EGF receptor associates with both type II PI-4-kinase and PI-TP [24,27]. Multiple isoforms of PIP-5-kinase are also present [16,18], of which one form appears to be regulated by GTPγS, at least in human neutrophils [14] and HL60 cells (this paper). Although the GTP-binding protein responsible for this regulation has not been identified in neutrophils or HL60 cells, a possible candidate is the small GTP-binding protein, Rho, which has been recently found to regulate a PIP-5-kinase [28].

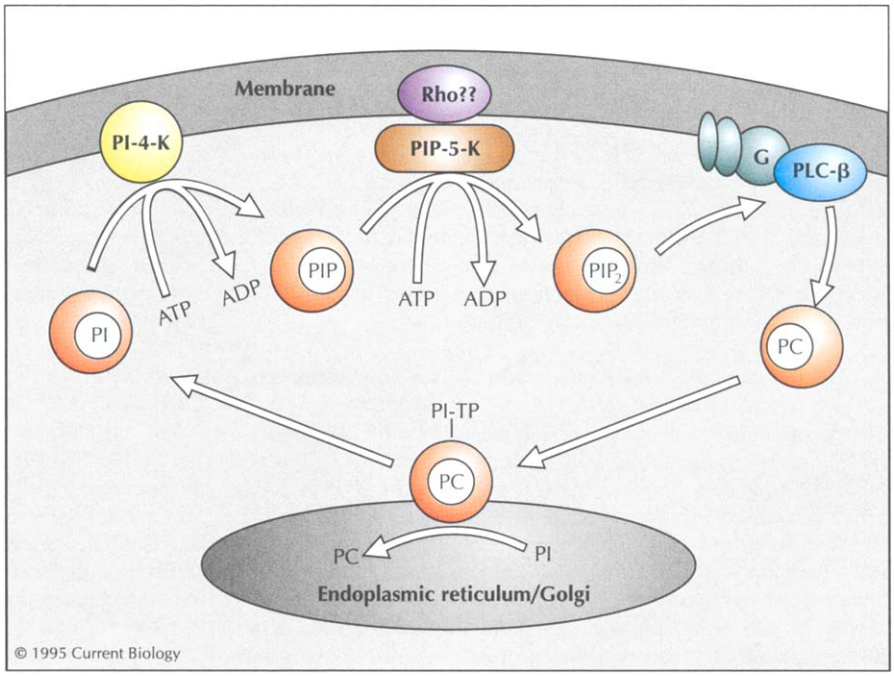

Fig. 7. Working model for PI-TP involvement in inositol lipid signalling. This model combines elements of lipid transfer and cofactor activity. The model proposes that the lipid carried by PI-TP, rather than the PIP_2 resident in the membrane bilayer, is the preferred substrate for PI-4-kinase (PI-4-K) and PIP-5-kinase (PIP-5-K). PI-TP could be seen as a soluble vector for PI, therefore, that allows the provision of inositol lipids at remote sites (PI-transfer activity), and that possibly channels PI directly into the inositol lipid kinase pathway (cofactor activity).

Conclusions

We have provided evidence that PI-TP is essential for the synthesis of PIP_2 under certain conditions, and that this is how PI-TP dictates the rate of IP_3 production. By combining elements of lipid transfer and cofactor activity, we propose the model outlined in Figure 7. The model proposes that the lipid carried by PI-TP, rather than the PIP_2 resident in the membrane bilayer, is the preferred substrate for PI-4-kinase and PIP-5-kinase. In this way, PI-TP could be seen as a soluble vector for PI that allows provision of inositol lipids at remote sites (PI-transfer activity) and that possibly channels PI directly into the inositol lipid kinase pathway (cofactor activity). Implicit in the model is the ability of PI-TP to bind PIP and PIP_2. Wirtz and colleagues [29] have demonstrated that PI-TP has a very high affinity for PIP_2. Significantly, PI-TP does not have the ability to transfer this lipid between membrane compartments [9]. We would also predict that other reactions that consume PIP_2 as a substrate, such as PI-3-kinase [30], or that require PIP_2 [25], as in exocytosis and actin assembly [31], may be dependent on PI-TP, and we are currently investigating these possibilities.

Materials and methods

Materials

Materials were from previously defined sources [6,8].

Loss of receptor- and GTPγS-activated PLC activity upon permeabilization

HL60 cells were maintained in suspension culture and labelled with 1 μCi ml^{-1} [^{3}H]inositol for 48 h [7]. For differentiation, 300 μM dibutyryl cyclic AMP was also present for 48 h. The [^{3}H]inositol-labelled cells (10^7 cells per ml) were permeabilized with streptolysin O (0.6 IU ml^{-1}) in PIPES buffer (20 mM PIPES, 137 mM NaCl, 3 mM KCl, 1 mg ml^{-1} glucose, 1 mg ml^{-1} albumin, pH 6.8) supplemented with 1 mM Mg^{2+}–ATP at pCa 7, in a volume of 4–5 ml. At the times indicated in the figures (Figs 1d and 4a), 50 μl aliquots were transferred to tubes for assay of PLC activity as described previously [6]. The assay conditions were: 10 μM GTPγS or 1 μM fMetLeuPhe; 2 mM Mg^{2+}–ATP; 2 mM $MgCl_2$; 10 mM LiCl; pCa 6 (buffered with 3 mM EGTA as previously described [32]). After 20 min incubation at 37 °C, samples were quenched with chloroform:methanol and the inositol phosphates analysed as described previously [33].

Purification of PI-TP

PI-TP was purified from bovine brain cytosol as described previously [8], except for the experiment in Fig. 6a, in which PI-TP was purified from rat brain cytosol [34]. Briefly, a high-speed supernatant was prepared from one homogenized bovine brain. A 40–60 % saturated ammonium precipitate was prepared from the supernatant and dialyzed extensively. The resultant protein preparation was chromatographed on DE52 weak anion-exchange resin. All fractions were assayed for PI-transfer activity [8]. Those fractions with the PI-TP activity were pooled, concentrated and chromatographed isocratically on HiTrap Heparin Sepharose. In a modification to the previously described method, the PI-TP activity was then passed through a Superdex 75 gel filtration column. Finally, the relevant gel-filtration fractions were pooled and chromatogaphed on a hydrophobic interaction column, Phenyl Superose HR 5/5. Purity was established by analyzing the protein by SDS polyacrylamide gel electrophoresis followed by silver staining. The concentration of PI-TP was obtained by assay with a Pierce Kit using BSA as a standard. This assay over-estimates the amount of PI-TP by a factor of 20 when compared with that determined by amino-acid analysis [8]. In all the experiments reported here, PI-TP was freshly purified and stored at 4 °C for a maximum of 3 days.

Production of antibody to recombinant PI-TP

Rat PI-TP was expressed as a GST-fusion protein. The coding region for rat PI-TP was amplified by reverse transcription and the polymerase chain reaction (RT–PCR), and cloned into pGEX-2T (Pharmacia) to create a GST–PI-TP fusion protein expression construct. Briefly, 0.5 μg rat brain poly(A) RNA

was reverse transcribed using the cDNA Cycle system (Invitrogen). PCR was performed using 2.5 μl first-strand cDNA as template and the following primers: 5′-AAAAAGGATC-CATGGTGCTGCTCAAGGAA and 3′-AAAAAGAATTC-TAGCGCTAGTCATCTGCTG (PCR conditions: 92 °C, 1 min; 56 °C, 30 sec; 72 °C, 2 min; 35 cycles). The 844 base-pair PCR product was gel-purified and cloned into pGEX-2T using *Bam*HI and *Eco*RI restriction sites added to the ends of the 5′ and 3′ PCR primers, respectively. The pGEX-2T.PI-TP expression construct was transformed into *Escherichia coli* XLI-Blue (Stratagene), and expression of the fusion protein was carried out as described previously [35].

In order to raise anti-PI-TP antiserum, the fusion protein was purified from 2 l bacterial culture. Cells were harvested by centrifugation and sonicated in RIPA buffer (150 mM NaCl, 1 % (v/v) Nonidet p-40, 0.05 % (w/v) deoxycholate, 0.1 % (w/v) SDS, 50 mM Tris pH 8.0). After centrifugation (20 000 x *g*, 4 °C, 20 min) the pellets were washed once in RIPA buffer and then sonicated in SDS electrophoresis buffer (7 ml), boiled and stored at −20 °C. Aliquots (300 μl) were thawed and purified by electrophoresis on 1 mm thick 12 % cross-linking polyacrylamid gels. The gel was stained in 300 mM $CuCl_2$, the band corresponding to the fusion protein excised, destained in 50 mM EDTA and 50 mM EGTA, and finally equilibriated in water. The gel slice was homogenized in Freunds complete adjuvant and injected subcutaneously into three female New Zealand white rabbits. Boosts were administered at four-weekly intervals with test bleeds following after 12 days. Antisera were affinity-purified on polyvinyldifluoride membranes containing rPI-TP–GST fusion protein, eluted in 0.2 M glycerine pH 3.0, neutralized with 1 M Tris pH 9.0 and stored at −20 °C.

Release of PI-TP and PLC-β2 into the medium upon permeabilization

Antisera selective for PLC-β2 were raised against a synthetic oligopeptide based on carboxy-terminal sequences of these proteins and were obtained from P. Parker [36]. Western immunoblotting was performed by established procedures. For detection, the ECL system (Amersham) was used.

To assay for released PLC-β2 and PI-TP in the supernatant, HL60 cells were permeabilized for varying lengths of time and the medium tested for the presence of PLC-β2 and PI-TP by western blotting. Cells (5 x 10^7) were washed in PIPES buffer and permeabilized with streptolysin O (O.6 IU ml^{-1}) in 5 ml total volume. Samples (500 μl) were removed at the indicated times, and the cells sedimented by centrifugation. The proteins in the supernatant were precipitated with TCA and redissolved in 100 μl sample buffer. The proteins were then run on a 12 % polyacrylamide gel, blotted, and probed with the appropriate antibodies. The antibodies against PI-TP and PLC-β2 were used at dilutions of 1:2 500 and 1:1 000, respectively. Anti-PLC-β2 antibody always recognized two proteins of approximately 150 kD and 100 kD; the 100 kD protein represents a proteolytic fragment of the 150 kD protein.

Cell-associated PI-TP was solubilized with 100 μl RIPA buffer on ice for 30 min, centrifuged for 15 min to remove insoluble material and analysed as above. Release of PI-TP was also measured by determining the lipid-transfer activity of PI-TP, as described previously [8].

Purification of PLC-β1

PLC-β1 was purified from bovine brain membranes by a modification of a published procedure [37]. The membranes (from three bovine brains) were extracted with 2 M KCl and the proteins precipitated with an ammonium sulphate 60 % saturation. The following modifications were made to the published procedure. Hydrophobic interaction chromatography was performed on a phenylsuperose HR 10/10 column (Pharmacia) and this was followed by anion-exchange chromatography at pH 7.6 with a 0–500 mM NaCl gradient on a Mono Q HR 5/5 column (Pharmacia) after dialysis. The identity of the purified protein was confirmed by western blotting using monoclonal antibodies specific to PLC-β1 [38]. Upon electrophoresis, a doublet band at approximately 140 kD and 150 kD was observed.

Reconstitution with PI-TP and PLC-β1

Labelled HL60 cells (5 x 10^7) were permeabilized with 0.6 IU ml^{-1} streptolysin O in PIPES buffer, supplemented with 1 mM Mg^{2+}–ATP at pCa 7, for 10 min or 40 min as indicated in the figure legends. The permeabilized cells were washed and finally resuspended in PIPES buffer supplemented with 4 mM Mg^{2+}–ATP, 20 mM LiCl and 4 mM $MgCl_2$ at pCa 6. Permeabilized cells (20 μl) were incubated with 20–30 μl protein(s) in the presence of activators where indicated. All additions were done on ice. The tubes were transferred from 4 °C to 37 °C and incubated for 20 min. At the end of the incubation, the reaction was quenched with 1 ml ice-cold 0.9 % NaCl, and the cells sedimented at 2 000 x *g* for 5 min at 4 °C. Supernatant (0.9 ml) was removed and used for the assay of released inositol phosphates [33].

For the time-course experiments, equal volumes of cytosol-depleted cells and protein(s) were added to tubes at 4 °C. The reaction tubes were transferred to 37 °C and the assay initiated by addition of 3 μl fMetLeuPhe or GTPγS, as indicated. Samples (20 μl) were removed and quenched directly into chloroform–methanol and processed as above.

Expression of data

The increase in total [^{3}H]inositol phosphates (IP_1 + IP_2 + IP_3) is expressed as a function of the total radioactivity (disintegrations per minute) incorporated in the inositol lipids. In some experiments the inositol phosphates were separated individually (see Table 1) and the majority of the increase in inositol phosphates was due to IP_3. All experiments were repeated on 3–4 occasions (except that shown in Fig. 3) and representative experiments are illustrated here. The experiment in Fig. 3 was carried out on two separate occasions with identical results. Determinations were carried out in duplicate or triplicate, except for the time-course experiments.

Assay for PIP_2 production in permeabilized HL60 cells

HL60 cells (5 x 10^7) were permeabilized with 0.6 IU ml^{-1} streptolysin O in PIPES buffer, supplemented with 100 μM Mg^{2+}–ATP, 10 μCi [γ-^{32}P]ATP) and 10 mM $MgCl_2$ at pCa 7, in the presence or absence of 10 μM GTPγS. At the indicated times, 100 μl samples were withdrawn and quenched into 750 μl acidified chloroform:methanol (1:2) to provide a single phase; 250 μl chloroform and 250 μl aqueous Solution A (0.1 M HCl, 5 mM EDTA, 0.5 M NaCl) were added to obtain a two-phase system. The upper aqueous phase was carefully removed and replaced with a synthetic upper phase of chloroform:methanol:Solution A (3:48:47). This was repeated once more to reduce the amount of radiolabelled ATP in the chloroform phase. The chloroform phase was dried down and resuspended in a small volume of chloroform and samples spotted on a thin-layer chromatography (TLC) plate impregnated with oxalate, as described previously. The polyphosphoinositides

were separated using chloroform:methanol:acetic acid:acetone: water (40:13:12:15:8). The TLC plate was autoradiographed and PIP_2 identified using authentic lipid standards. The spot containing PIP_2 was excised and counted by liquid scintillation counting.

When cytosol-depleted cells were used, the cells were initially permeabilized with streptolysin O in the presence of 1 mM Mg^{2+}–ATP at pCa 7. The permeabilized cells were washed twice and resuspended in PIPES buffer supplemented with 100 μM Mg^{2+}–ATP (10 μCi ATP), 10 mM $MgCl_2$ at pCa 7, in the presence or absence of 150 μg ml^{-1} PI-TP and 30 μM GTPγS. At the indicated times, duplicate samples were quenched and phosphoinositides separated by TLC as described above.

For the experiments shown in Table 1 and Fig. 3, HL60 cells were labelled with [^{3}H]inositol for 48 h and the inositol lipid levels were analyzed for radioactivity after TLC as described above.

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