Evidence for a model of integrated inositol phospholipid pools implies an essential role for lipid transport in the maintenance of receptor-mediated phospholipase C activity in 1321N1 cells

Ian H. BATTY¹, Richard A. CURRIE and C. Peter DOWNES

Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

The compartmentation of inositol phospholipids was examined by using a combination of radiolabelling approaches in intact and permeabilized 1321N1 astrocytoma cells. A 'chase' protocol was developed with whole cells in which phosphoinositide (PI) pools were labelled to steady state with [³H]inositol and the cellular [³H]inositol pool was then diluted selectively with non-radioactive inositol. In these cells muscarinic-receptor-stimulated phospholipase C (PLC) hydrolysed [³H]PI at approx. 1–2 %/min. However, after the chase procedure the relative specific radioactivity of [³H]Ins(1,3,4)P₃, a rapidly metabolized and sensitive marker of PLC activity, decreased only after more than 5 min and over a time course similar to that during which the labelling of each [³H]PtdIns, [³H]PtdInsP and [³H]PtdInsP₂ declined by at least 50 %. These results demonstrate a large receptor-responsive [³H]PI pool that is accessed by stimulated PLC without apparent metabolic compartmentation, despite its probable distribution between different membrane fractions. Support for this was obtained in intact cells by using an acute [³H]inositol labelling

method in which increases in the specific radioactivity of [³H]-inositol phosphates stimulated by carbachol occurred only in parallel with similar increases in the labelling of the bulk of cellular [³H]PI. In [³H]inositol-prelabelled cells permeabilized to deplete cytosolic proteins, carbachol and guanosine 5'-[γ-thio]triphosphate stimulated the endogenous PLC to degrade only approx. 5 % of [³H]PI. This was increased to approx. 30 % in the presence of exogenous PtdIns transfer protein, which, at a concentration approx. 5–10 % of that in 1321N1 cell cytosol, was sufficient to support PLC activity comparable with that observed in response to carbachol in whole cells. These and earlier results in 1321N1 cells suggest a model of integrated PI pools involving an obligatory role for lipid transport. Given the multifunctional capacity of PI in cellular signalling mechanisms, this model has important implications, particularly for the hypothesis that the ability of Li⁺ ions to influence these selectively might account for its therapeutic actions.

INTRODUCTION

Interest in inositol phospholipids has focused on their role as substrates for phospholipases C (PLC) and phosphoinositide (PI) 3-kinases, two important classes of enzyme involved in cellular signalling [1–4]. Additional functions for inositol phospholipids have been proposed, including the regulation of phospholipase D [5,6], the cytoskeletal matrix [7] and exocytosis [8]. This multiplicity of functions suggests that mechanisms exist that allow these to be co-ordinated and/or segregated, perhaps involving functionally shared and/or discrete lipid pools. The identification of a wortmannin-sensitive PtdIns 4-kinase that selectively provides substrate to receptor-activated PLC supports the latter possibility [9,10].

The metabolic compartmentation of inositol phospholipids within cells, however, is still far from being clearly understood. Indeed the subcellular organization of the 'PI cycle' initiated by stimulation of receptor-mediated PLC has long been debated [11–14] and is still not established. Thus, although it is clear that PLC hydrolyses PtdIns(4,5)P₂ at the plasma membrane and that replenishment of this substrate by phosphorylation of PtdIns and PtdIns4P occurs at the same location, it remains uncertain whether the ultimate resynthesis of PtdIns is also co-localized or occurs within a separate, intracellular membrane (reviewed in [14]. Recent studies [15–17] have cast fresh light on this issue by

providing support for the long-standing proposal [11] that PtdIns transfer proteins (PtdIns-TP) might have an important role. These proteins facilitate the exchange between membranes of phosphatidylcholine or sphingomyelin and PtdIns, but not polyphosphoinositides [18,19]. In permeabilized [15,16] or broken cells [17], exogenous PtdIns-TP is able to enhance or sustain receptor-mediated PLC activity. The mechanisms invoked to account for these effects differ (compare [16] with [17]), but the simpler interpretation [17] is that by delivering PtdIns from intracellular membranes to locally depleted sites reflecting PLC activity at the plasma membrane, PtdIns-TP fuels the resynthesis of requisite PtdIns(4,5)P₂.

However, although these studies illustrate clearly the potential of PtdIns-TP, it is important to emphasize that the cellular preparations and/or assay conditions that they employed are likely to compromise or preclude endogenous PtdIns synthesis. Consequently, it remains unclear whether the effects of PtdIns-TP observed reflect an important role of this protein in intact cells or simply the ability of exogenous PtdIns-TP to compensate for preparational deficiencies in PtdIns synthase activity.

We have attempted to resolve this matter and gain further insight into the compartmentation of inositol lipids in intact 1321N1 astrocytoma cells, a system in which we have previously characterized both receptor-mediated PI 3-kinase and PLC responses [20,21] and the mechanisms underlying the stimulated

Abbreviations used: PI, phosphoinositide(s); DG, diacylglycerol; PA, phosphatidic acid; PLC, phospholipase C; PtdIns-TP, phosphatidylinositol transfer protein; GTP[S] guanosine 5'-[γ-thio]triphosphate.

¹ To whom correspondence should be addressed.

re-synthesis of PtdIns [22]. The results presented here show that muscarinic-receptor-stimulated PLC gains wide, rapid access to cellular PI and, together with other kinetic and metabolic data from intact and permeabilized 1321N1 cells, support a detailed model of PI pooling. This model is based on that proposed originally by Michell [11] and defines an obligatory role for PtdIns-TP in the maintenance of stimulated PLC activity, but also describes novel features likely to have important implications for the multifunctional role of inositol phospholipids in cellular signalling.

MATERIALS AND METHODS

Materials

1321N1 human astrocytoma cells were obtained from the European Collection of Animal Cell Cultures. Other materials for cell culture were from either Gibco or Sigma. AG1 × 8 resin (200–400 mesh, formate form) was from Bio-Rad, and Partisphere SAX HPLC columns were from LaserChrom. Streptolysin O was purchased from Research Biochemicals International, and *myo*-[2-³H]inositol, *myo*-[2-³H]inositol 1,3,4-trisphosphate and *myo*-[2-³H]inositol 1,4,5-trisphosphate were from NEN-Dupont. Frozen bovine brains were obtained from Advanced Protein Products Ltd. Creatine phosphate, creatine kinase and guanosine 5'-[γ-thio]trisphosphate (GTP[S]) were from Boehringer-Mannheim. Kits for assay of lactate dehydrogenase were from Sigma. Other reagents were of analytical grade and were obtained from Sigma or BDH.

Cell culture

Inositol-replete and inositol-depleted cells respectively were grown on 24-well plates in medium containing or lacking *myo*-inositol as described previously [21], with the modifications indicated where appropriate to the [³H]inositol labelling procedures described below. In inositol-depleted cells the cellular inositol, but not phosphoinositide, pools are markedly decreased [21]. Experiments involving comparison between cells labelled by different approaches were conducted in parallel with age-matched cultures.

Radiolabelling procedures

Confluent cell cultures (approx. 0.25–0.35 mg of protein) were labelled with [³H]inositol by three separate approaches.

(1) Steady-state protocol: inositol-replete or inositol-depleted cells were labelled with [³H]inositol (2–10 μCi/ml) for approx. 2–3 days as described previously [21] to allow [³H]PI pools to achieve steady state.

(2) Chase protocol: inositol-depleted cells were first labelled with [³H]inositol for 2 days as above; the labelling medium was then removed and the cells were washed four times with 2 ml of inositol-free medium before incubation for approx. 16–20 h in 1 ml of the same medium with or without 1 mM unlabelled *myo*-inositol.

(3) Acute labelling protocol: this approach has been described previously [22]. Briefly, inositol-replete cells were first transferred to incubation buffer, then maintained at 37 °C for 30 min in 0.5 ml of the same buffer supplemented with [³H]inositol (50 μCi). The labelling buffer was then aspirated, the cells were washed rapidly five times with 2 ml of incubation buffer to remove extracellular [³H]inositol, and incubations were continued as described below.

Measurement of [³H]PI turnover

After labelling by protocols (1) and (2) or immediately before protocol (3), the culture medium was aspirated and the cells were washed four times with 2 ml of incubation buffer (118 mM NaCl/4.69 mM KCl/1.18 mM MgSO₄/1.29 mM CaCl₂/1.18 mM KH₂PO₄/11.67 mM glucose/25 mM Hepes, adjusted to pH 7.4 with NaOH) and equilibrated at 37 °C for 30 min in 0.4–0.5 ml of this buffer. When cells were labelled acutely, this equilibration period was omitted to allow comparative parallel incubations with steady-state-labelled cells. Drugs or buffer vehicle were then added to give a final assay volume of 0.5 ml; incubations were continued at 37 °C as indicated. Reactions were stopped by aspiration of the incubation buffer and the addition of 0.6 ml of ice-cold 0.5 M trichloroacetic acid; samples were transferred immediately to ice. The [³H]PI and water-soluble [³H]inositol metabolites were then extracted and analysed by Dowex or HPLC anion-exchange chromatography as described previously [21,23].

Experiments with permeabilized cells

Inositol-depleted cells were labelled with [³H]inositol to steady state as described above. The labelling medium was then aspirated and the cells were washed four times with 2 ml of incubation buffer and once with 0.25 ml of assay buffer (10 mM NaCl/115 mM KCl/5.9 mM MgSO₄/0.424 mM CaCl₂/2 mM EGTA/5 mM KH₂PO₄/25 mM Hepes, adjusted to pH 7.0 with KOH) supplemented with ATP (5 mM), creatine phosphate (5 mM) creatine kinase (10 μg/ml) and BSA (1 mg/ml) and prewarmed to 37 °C. The free Ca²⁺ and Mg²⁺ ion concentrations of this buffer were calculated to be approx. 100 nM and 1 mM respectively [24]. The washed cells were then transferred to 0.2 ml of the same supplemented buffer containing 3 units/ml streptolysin O (units as defined by the supplier) and maintained at 37 °C for 45 min. Permeabilization under these conditions typically resulted in a quantitative release of cytosolic lactate dehydrogenase from 1321N1 cells. After permeabilization, the buffer and released cytosolic constituents were carefully removed and replaced with 0.2 ml of the same buffer lacking streptolysin O but supplemented additionally with stimuli as shown. Incubations were continued for the times indicated; the [³H]PI and [³H]metabolites were then extracted and analysed as described above.

Other methods

PtdIns-TP was partly purified from bovine brain and assayed as described previously [17]. Protein was assayed by the method of either Lowry et al. [25] or Bradford [26]. Where indicated, data from paired experiments were compared by Student's *t* test and values were taken as significantly different at *P* ≤ 0.05.

RESULTS

1321N1 cells are a useful model system in which muscarinic receptor stimulation elicits sustained PLC responses [21,27]. We have used a combination of labelling approaches in intact 1321N1 cells together with complementary studies after permeabilization of these to examine three key features of inositol phospholipid compartmentation: (1) the fraction of cellular PI accessible to receptor-activated PLC; (2) the metabolic/kinetic characteristics of such pool(s); and (3) its physical integrity or sub-division and accessibility to PtdIns-TP.

Figure 1 Muscarinic-receptor-responsive fraction of [^3H]PI

Cells labelled with [^3H]inositol (10 $\mu\text{Ci}/\text{ml}$) for 2 days and loaded with non-radioactive inositol overnight, as described for the chase protocol, were then further incubated in the absence ($\circ$) or presence ($\bullet$) of 1 mM carbachol for the times indicated before the extraction and measurement of [^3H]PtdIns, [^3H]PtdInsP or [^3H]PtdInsP₂. Results are the means from duplicate determinations made in a single experiment, which was representative of several (see the text). The labelling of each [^3H]lipid is expressed as a percentage of the mean value of that in control samples at 0 and 120 min. Values representing 100% are: [^3H]PtdIns, 4442849 d.p.m.; [^3H]PtdInsP, 131576 d.p.m.; [^3H]PtdInsP₂, 187313 d.p.m.

Studies in intact cells

Determination of the receptor-responsive fraction of PI

This issue was addressed by using a 'chase' protocol in which cellular PI was first labelled with [^3H]inositol and cells were then loaded with non-radioactive inositol under conditions designed to achieve a gradient of specific radioactivity from low to high between the cellular inositol and lipid pools respectively. Parallel experiments using methods described previously [28] showed that 'chased' cells accumulated approx. 50–100 nmol of unlabelled inositol per well. In prelabelled cells, where the cellular [^3H]PI mass was approx. ~ 2 –3 nmol per well [21], this loading with unlabelled inositol typically resulted in a redistribution of label such that prior labelling of [^3H]PtdIns, [^3H]PtdInsP and [^3H]PtdInsP₂ decreased to $49.9 \pm 2.8\%$, $47.5 \pm 3.0\%$ and $42.6 \pm 3.9\%$ respectively (mean $\pm$ S.E.M. for 17 experiments). The label lost from [^3H]lipids accumulated predominantly in the cellular inositol pool, contributing most ($79.1 \pm 3.0\%$) of the label then present in the latter. Thus after incubation with unlabelled inositol the majority of cellular radiolabel was distributed approximately evenly between the [^3H]inositol and [^3H]PI pools, despite the disparity in the masses of these components, suggesting that subsequent stimulated turnover of [^3H]lipid should result in an effective chase of radiolabel from [^3H]PI.

Figure 1 illustrates this, showing that activation of PLC by the addition of the muscarinic-receptor agonist carbachol caused a decrease in the labelling of each [^3H]PI over a time course compatible with responses reported previously [21,27]. In inositol-replete 1321N1 cells the mass of PtdIns and of PtdInsP₂ decreased by only 5–10% in response to maximally effective concentrations of carbachol [21,22] (see Figure 3c). Therefore the decreased labelling of these lipids shown in Figure 1 must reflect the decreased specific radioactivity that occurred as lipid consumed initially through PLC activity was replenished by the

synthesis of PtdIns from the less highly labelled inositol pool. The changes in [^3H]PtdInsP labelling are a result of both a decrease in specific radioactivity and a modest decrease (approx. 25%) in mass. The maximum extent of the agonist-dependent changes in the labelling of [^3H]PtdIns thus provides a measure of the cellular total of this lipid that is available to the stimulated PLC. Changes in [^3H]PtdInsP and [^3H]PtdInsP₂ also indicate the proportion of these that is accessible to PLC either directly or indirectly through equilibration with the receptor-sensitive PtdIns pool. The accessible fractions of [^3H]PtdIns, [^3H]PtdInsP and [^3H]PtdInsP₂ were $51.1 \pm 4.9\%$, $52.0 \pm 6.7\%$ and $50.0 \pm 5.4\%$ of their totals respectively (means $\pm$ S.E.M. for six experiments). These values are of a similar order to those determined previously by independent methods in these cells [21,22] and are intermediate within the wide range (approx. 5–90%) suggested by observations in other systems (compare [29] with [30]). The precision of the current values is dependent largely on the effectiveness of the chase, which might be less than expected (see below); a consensus of our present and previous results [20–22] suggests that up to 70–80% of cellular PtdIns and a similar fraction of PtdInsP and PtdInsP₂ is accessible to receptor-stimulated PLC in 1321N1 cells. As most PtdIns and its synthesis *de novo* occur at intracellular membranes [12,31], these observations, like others [14], imply that PLC is able to recruit PtdIns in addition to that present in the plasma membrane. This suggests either that a means of lipid transport between the plasma membrane and interior membranes is essential to the integrity of the 'PI cycle' or that stimulated PLC activity at the plasma membrane is coupled to local resynthesis of PtdIns, which is then able to equilibrate with other pools of this lipid by appropriate transport mechanisms. To distinguish between these, and so evaluate the potential role of PtdIns-TP, we examined the kinetic characteristics of the agonist-sensitive fraction of the PI by using two

Figure 2 Effects of an unlabelled inositol chase on the accumulation of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ stimulated by carbachol

(a) Cells were labelled with $[^3\text{H}]\text{inositol}$ ($10 \mu\text{Ci}/\text{ml}$) for 2 days and then incubated overnight in the absence ($\circ$, $\bullet$) or presence ($\triangle$, $\blacktriangle$) of non-radioactive inositol. Incubations were then continued in the absence ($\circ$, $\triangle$) or presence ($\bullet$, $\blacktriangle$) of 1 mM carbachol and the $[^3\text{H}]$ -metabolites were then extracted as described in the Materials and methods section. Extracts from duplicate incubations were pooled and the $[^3\text{H}]\text{inositol}$ triphosphates were then analysed by HPLC. Results are from the same experiment as those illustrated in Figure 1. Similar results were obtained on several occasions. Additional results are shown in Table 1. (b) The results from (a) are represented as a ratio of values from cells incubated overnight in the presence/absence of non-radioactive inositol to give a measure of changes in relative specific radioactivity.

complementary approaches designed to resolve a rapidly cycling pool consistent with a plasma-membrane location.

The kinetic characteristics of the receptor-responsive PI pool

The first of these approaches involved the use of the chase procedure. To achieve high kinetic resolution we examined the effects of this on the labelling of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$. This is a specific marker of $[^3\text{H}]\text{PtdInsP}_2$ hydrolysis whose turnover is very rapid ($t_{1/2} \approx 30 \text{ s}$ [32]) and whose concentration is highly responsive to the degree of muscarinic-receptor stimulation in 1321N1 cells [21]. Figure 2(a) illustrates the time course of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ accumulation in response to carbachol in cells previously labelled with $[^3\text{H}]\text{inositol}$ and then incubated overnight with or without non-radioactive inositol. In cells not loaded with unlabelled inositol, in which changes in radiolabelling reflect equivalent changes in the mass of labelled components, carbachol evoked a marked initial increase in $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ concentrations that peaked between 1 and 5 min and then declined but remained many-fold above basal values even after 2 h.

In 'chased' cells the early rise in $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ accumulation followed a similar pattern, although the absolute labelling was decreased as expected. After 5 min, however, these cells showed

Table 1 Non-radioactive inositol chase does not reduce the relative specific radioactivity of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ accumulated rapidly in response to carbachol

Cells were labelled with $[^3\text{H}]\text{inositol}$ and subsequently incubated overnight with or without non-radioactive inositol, as described in the legend to Figure 2, before exposure to 1 mM carbachol for the times indicated. The ^3H -labelled metabolites were then extracted and analysed and the relative specific radioactivities of $[^3\text{H}]$ products derived from cells loaded with non-radioactive inositol were calculated as described in the legend to Figure 2. Data are means $\pm$ S.E.M. for four experiments. Comparison by paired Student's *t* test revealed no significant decrease in the specific radioactivities of any of the $[^3\text{H}]$ metabolites shown over the period indicated. Basal concentrations of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ were too low to be measured accurately. Abbreviation: n.d., not determined.

Time (min)	Relative specific radioactivity			
	PtdIns	PtdInsP	PtdInsP ₂	Ins(1,3,4)P ₃
0.00	0.52 $\pm$ 0.03	0.44 $\pm$ 0.05	0.41 $\pm$ 0.06	n.d.
0.25	0.54 $\pm$ 0.02	0.51 $\pm$ 0.08	0.50 $\pm$ 0.07	0.57 $\pm$ 0.06
1.00	0.54 $\pm$ 0.04	0.48 $\pm$ 0.06	0.47 $\pm$ 0.07	0.45 $\pm$ 0.07
5.00	0.52 $\pm$ 0.03	0.53 $\pm$ 0.04	0.47 $\pm$ 0.06	0.48 $\pm$ 0.06

both an accelerated rate and extent of decline in $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ levels, reflecting a decrease in specific radioactivity. Figure 2(b) presents the ratio of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ labelling in chased to non-chased cells to reveal this effect in chased cells in terms of the decrease in relative specific radioactivity. This demonstrates three important points: (1) no decrease in relative specific radioactivity was apparent over the first 5 min; (2) a subsequent decrease was then evident that paralleled that shown for labelling of $[^3\text{H}]\text{PI}$; and (3) the maximum decrease in relative specific radioactivity was approx. five-fold. The last of these points shows that the chase was less effective than might have been predicted, perhaps implying a preferential loss of label from the receptor-responsive $[^3\text{H}]\text{PI}$ pool when the cells were loaded with unlabelled inositol; it nevertheless demonstrates the utility of this approach. Most importantly, however, the first of these points argues against the view [13] that stimulated PLC activity might be associated with a small, metabolically isolated pool of PI recycled rapidly at the plasma membrane. As the muscarinic-receptor-stimulated PLC in 1321N1 cells has the capacity to hydrolyse at least 1.5–2.0 % of the total cellular $[^3\text{H}]\text{PI}/\text{min}$ [21], it would be expected that a pool constituting approx. 10–20 % of this total would be revealed by a rapid decline in the relative radioactivity of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ by a factor of approx. 2–4-fold within 5 min. Table 1 extends these observations, showing that there was no measurable decrease in the relative specific radioactivity of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$, $[^3\text{H}]\text{PtdInsP}$ or $[^3\text{H}]\text{PtdInsP}_2$ over the first 5 min after carbachol addition. As the last two $[^3\text{H}]$ lipids account collectively for only approx. 5–10 % of the total $[^3\text{H}]\text{PI}$, this implies that the later effects of the chase must be buffered initially by a substantial pool of $[^3\text{H}]\text{PtdIns}$, which must be more than 10–15 % of the cellular total of this lipid, even assuming that all the cellular $[^3\text{H}]\text{polyphosphoinositide}$ participates in this response. Additionally, the similarity in the time courses over which the labelling of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and of $[^3\text{H}]\text{PI}$ decreases in cells loaded with non-radioactive inositol suggests that the pool of $[^3\text{H}]\text{PtdIns}$ that is intimately associated with the receptor mechanism is either a large fraction of the cellular total or equilibrates rapidly with such a fraction.

We sought to confirm the latter conclusion using an alternative, acute labelling approach that avoids the prior manipulations of the cellular inositol and PI pools entailed by the chase procedure. Figure 3 illustrates the results obtained when cells were incubated

Figure 3 Time course of carbachol-stimulated $[^3\text{H}]\text{InsP}$ accumulation is similar to that for $[^3\text{H}]\text{PI}$ labelling in acutely labelled cells

Cells were labelled with $[^3\text{H}]\text{inositol}$ (5 or 50 $\mu\text{Ci}/\text{ml}$) for either 3 days (**a, c**) or 30 min (**b, d**), then incubated in the absence (○) or presence (●) of 1 mM carbachol before the extraction and analysis of $[^3\text{H}]\text{InsP}_{1-4}$ (**a, b**) or $[^3\text{H}]\text{PI}$ (**c, d**). Values (**a-d**) are expressed relative to the labelling of cellular $[^3\text{H}]\text{inositol}$ within the appropriate sample and are means $\pm$ S.E.M. for five experiments, each performed in duplicate. (**e**) Increases in the relative specific radioactivity of $[^3\text{H}]\text{PI}$ (Δ , $\blacktriangle$) and $[^3\text{H}]\text{InsP}_{1-4}$ (○, ●) that occur in acutely labelled cells after agonist addition. These were calculated for $[^3\text{H}]\text{PI}$ from the ratio of $[^3\text{H}]\text{lipid}$ labelling in acute/chronically labelled cells, and for $[^3\text{H}]\text{InsP}_{1-4}$ from a similar ratio of the stimulated increment in labelling.

briefly under conditions that allowed the almost exclusive labelling of the cellular $[^3\text{H}]\text{inositol}$ pool [22]. Under these conditions, activation of PLC causes markedly enhanced labelling of $[^3\text{H}]\text{PI}$ and $[^3\text{H}]\text{inositol}$ phosphate metabolites, permitting sensitive measurements. This is possible owing to the large cellular inositol pool (10–20 $\times$ PI) present in these cells. In addition, the expression of $[^3\text{H}]\text{metabolite}$ concentrations relative to the labelling of this pool facilitates comparisons with corresponding changes in their mass determined separately in cells labelled with $[^3\text{H}]\text{inositol}$ to steady state.

Figure 3 shows changes in $[^3\text{H}]\text{PI}$ and $[^3\text{H}]\text{InsP}_{1-4}$ concentrations in steady-state (Figures 3a and 3c) and acutely (Figures 3b and 3d) labelled cells. Figures 3(a) and 3(c) respectively show that carbachol evoked an approx. 4-fold increase in $[^3\text{H}]\text{InsP}_{1-4}$ concentrations, which achieved a new steady state within 10 min and that this was accompanied by an approx. 5–10% fall in $[^3\text{H}]\text{PI}$ concentrations. In parallel 60 min incubations, the inclusion of 10 mM LiCl to inhibit inositol monophosphatase resulted in a stimulated accumulation of $[^3\text{H}]\text{InsP}_{1-4}$ above controls, equivalent to $8.73 \pm 0.72\%$ of $[^3\text{H}]\text{inositol}$ (mean $\pm$ S.E.M. for five experiments). This shows that the amount of $[^3\text{H}]\text{PI}$ turned over by PLC in this time is equal to at least the total cellular mass of this lipid. In contrast, in cells labelled acutely, stimulation evoked progressive increases in the labelling of both $[^3\text{H}]\text{InsP}_{1-4}$ and $[^3\text{H}]\text{PI}$ (Figures 3b and 3d). The enhanced labelling of the latter reflects predominantly the increase in specific radioactivity that occurs when $[^3\text{H}]\text{PtdIns}$ consumed through PLC activity is resynthesized from the heavily labelled $[^3\text{H}]\text{inositol}$ pool. Earlier studies with this approach [22] have shown that carbachol-stimulated incorporation of $[^3\text{H}]\text{inositol}$ into $[^3\text{H}]\text{PtdIns}$ is maximal by 90–120 min, when approx. 80% of the cellular total of this lipid is labelled, implying that this fraction of PtdIns is accessible to PLC. However, as the current results show that within the same period the stimulated PLC

turns over the equivalent of approx. 1.5–2.0 times this lipid mass, it is probable that a subfraction of the receptor-responsive lipid pool is metabolized more rapidly (see the Discussion section). Nevertheless the results in Figure 3(e) support the view that any such subpool must represent a substantial fraction of the total cellular $[^3\text{H}]\text{PI}$.

Figure 3(e) presents the stimulated changes in $[^3\text{H}]\text{InsP}_{1-4}$ and in $[^3\text{H}]\text{PI}$ that occur in acutely labelled cells in terms of relative specific radioactivity. This shows that after an initial delay of approx. 5 min after agonist addition, the relative specific radioactivities of both $[^3\text{H}]\text{InsP}_{1-4}$ and of $[^3\text{H}]\text{PI}$ increased in tandem at a linear rate for approx. 30–40 min. The initial lag phase is consistent with the results obtained with the chase procedure, whereas the sustained increase in the relative specific radioactivity of $[^3\text{H}]\text{InsP}_{1-4}$ demonstrates that the $[^3\text{H}]\text{lipid}$ hydrolysed by PLC does not equilibrate fully with the cellular $[^3\text{H}]\text{inositol}$ pool even within 30–40 min. As the stimulated activity of PLC is sufficient to metabolize approx. 60% of the total cellular $[^3\text{H}]\text{PI}$ over this period, a fraction of about this size must be readily accessible to the receptor mechanism. This is also consistent with our earlier conclusions, indicating that if a restricted pool of PI exists within the plasma membrane its rate of agonist-stimulated turnover is not measurably different from its rate of equilibration with a much larger pool whose size implies its predominant location in a distinct membrane compartment(s). Earlier studies also failed to identify a metabolically isolated and receptor-responsive PI pool compatible with a plasma-membrane location [33]. On the contrary, such studies have indicated that PtdIns initially present in several intracellular membrane fractions might participate in receptor-mediated PLC responses [34].

Studies in permeabilized cells

Figure 4 summarizes the results of experiments in which the

Figure 4 PtdIns-TP augments carbachol- and GTP[S]-stimulated PLC activity in permeabilized cells

Cells labelled for 2–3 days with [3 H]inositol (4 μ Ci/ml) were permeabilized, then equilibrated without (○) or with (●) PtdIns-TP, then incubated for the times shown in the presence of 1 mM carbachol and 100 μ M GTP[S] before the extraction and analysis of [3 H]Ins P_{1-4} (top panel) or [3 H]PI (bottom panel). Results are means $\pm$ S.E.M. for three experiments.

physical organization of the receptor-responsive PI pool and the ability of PtdIns-TP to access this were examined. For these studies 1321N1 cells were prelabelled to steady state with [3 H]inositol and the plasma membrane was then permeabilized with streptolysin O under conditions that allowed the quantitative release of cytosolic lactate dehydrogenase. These permeable preparations were then equilibrated in the absence or presence of partially purified PtdIns-TP at a concentration approx. 5–10 % of that in the cytosol of intact 1321N1 cells, calculated on the basis of comparative activity assays [17]. Permeabilized cells showed similar total [3 H]PI levels to those of intact cells. Permeabilization and preincubation with PtdIns-TP caused separate, modest increases in the proportion of [3 H]polyphosphoinositides (results not shown), but the sum of [3 H]PtdIns P and [3 H]PtdIns P_2 did not exceed approx. 10–15 % of the total [3 H]lipid. As the purified PtdIns-TP did not contain PtdIns kinase activity, these changes probably reflect the loss of cytosolic PtdIns P and/or PtdIns P_2 phosphatases on permeabilization.

When maximally effective concentrations of carbachol plus GTP[S] were added to these permeabilized cells to activate endogenous PLC, there was a rapid accumulation of [3 H]Ins P (s) and a corresponding fall in [3 H]PI concentrations, as indicated in Figure 4. The [3 H]Ins P (s) were a mixture of [3 H]Ins P_{2-4} , showing that the stimulated PLC hydrolysed only [3 H]polyphosphoinositides and that cytosolic inositol mono- and bis-phosphatases were lost during prior permeabilization. Most pertinently, Figure 4 shows that the size and time course of these responses were dependent on PtdIns-TP. Thus, when this was absent, the PLC response was complete by approx. 5 min, when approx. 10 % of [3 H]PI had been degraded. In contrast, in the presence of PtdIns-TP the response was larger and better sustained, resulting in the hydrolysis of approx. 30 % of [3 H]lipid within 30 min. Approxi-

mately 90 % of the additional [3 H]lipid consumed in the latter instance was [3 H]PtdIns, whereas the final concentrations of [3 H]PtdIns P and [3 H]PtdIns P_2 were at least 50 % of their initial values with or without PtdIns-TP (results not shown). The changes in [3 H]polyphosphoinositide concentrations probably reflect their elevation on prior permeabilization to levels not representative of intact cells.

These results are reminiscent of those presented in recent, similar studies [15–17]. They indicate that in the absence of exogenous PtdIns-TP, the receptor-activated PLC has access to only a limited fraction of cellular [3 H]PI, which presumably reflects some or all of that present in the plasma membrane. This is consistent with our assumption that the large proportion of receptor-responsive PI observed in intact cells is distributed between two or more membrane compartments. The ability of exogenous PtdIns-TP to augment stimulated PLC responses lends further support to this view. In addition, it is clear from Figure 4 that the initial PLC response was greater in the presence than in the absence of PtdIns-TP but subsequently declined to a slower rate, which was maintained even when 30 % of [3 H]PI had been degraded. The faster initial rate in the presence of PtdIns-TP is accounted for, at least in part, by the elevated concentrations of [3 H]polyphosphoinositides before stimulation, whereas the secondary rate probably reflects that of inter-membrane [3 H]-PtdIns transport. As the latter rate of approx. 1 % of [3 H]PI/min is similar to that of maximal, muscarinic-receptor-stimulated PLC activity in intact cells (approx. 1–2 % of PI/min), these results support our conclusion from studies in whole cells that stimulated PLC gains efficient access to PtdIns located predominantly other than in the plasma membrane.

DISCUSSION

Other studies [15,16] have presented novel evidence regarding the function of PtdIns-TP in the provision of substrate to receptor-mediated PLC and have implied an essential role for this protein. However, although these and other studies [35–37] indicate the potential of PtdIns-TP in support of this and perhaps further PI-dependent processes, it is premature to infer an absolute requirement for PtdIns-TP from observations arising from systems lacking the means to support endogenous PtdIns synthase activity. We have used a combination of approaches to evaluate the likely capacity of PtdIns-TP to support muscarinic-receptor-activated PLC in intact 1321N1 cells in which stimulated PtdIns P_2 hydrolysis and PtdIns resynthesis are efficiently coupled by established mechanisms [22].

Two complementary labelling approaches have been used in intact cells to determine whether stimulated PLC activity is associated with the selective, rapid hydrolysis and resynthesis of a restricted fraction of PI compatible with a local plasma-membrane pool. The chase protocol provides good kinetic resolution but limited sensitivity, whereas the acute labelling approach has complementary advantages and limitations. The results from neither approach support the possibility of a limited plasma-membrane substrate pool, instead suggesting strongly that stimulated PLC can gain far wider access to cellular PI. This process must therefore involve inter-membrane lipid transport, at a rate indistinguishable from that of any plasma-membrane-localized resynthesis. Separate experiments with permeabilized cells further strengthen this view, showing that in the absence of cytosolic components, stimulated PLC hydrolysed a very limited proportion of PI and that the addition of exogenous PtdIns-TP not only restored wider access to PtdIns but also allowed lipid to be supplied at a rate similar to that of PtdIns P_2 hydrolysis in intact, stimulated cells. Thus the high capacity for lipid transport

inferred from our studies with whole cells might be accounted for by PtdIns-TP, which is likely to contribute significantly towards the provision of substrate to stimulated PLC.

The central question arising from this conclusion is whether PtdIns-TP is the primary means of supplying the PtdIns required to sustain stimulated PLC activity or acts in an auxiliary capacity. Thus if resynthesis of PtdIns can occur in the plasma membrane, where PtdIns P_2 is hydrolysed, PtdIns-TP might be better considered as fulfilling an auxiliary role, allowing the efficient equilibration of locally resynthesized lipid with PtdIns in other membranes. Alternatively, in the absence of PtdIns synthase at the plasma membrane, PtdIns-TP would be the only means of supplying PtdIns to PLC and would represent an essential component of the PI cycle. A necessary corollary of the latter is the requirement for a means to transport lipid metabolites of PtdIns P_2 [diacylglycerol (DG), phosphatidic acid (PA) or CMP-PA] to the site(s) of PtdIns synthesis [11,12], a need that might be met either by specific transfer proteins or through constitutive vesicle flow [38].

Several lines of evidence suggest an essential rather than a secondary role for PtdIns-TP. First, neither the present nor earlier metabolic studies [33] have been able to reveal the preferential turnover of a subfraction of PI expected for a rapidly metabolized plasma-membrane pool, and direct evidence for PtdIns synthase in this membrane is also scarce [14,39]. Secondly, in 1321N1 cells muscarinic-receptor-stimulated PLC and PtdIns synthase activity are closely co-ordinated via changes in the concentrations of inositol and/or CMP-PA [22], and receptor stimulation results in the selective accumulation of CMP-PA in a light vesicle fraction [40], perhaps indicating material in transit to one or more sites of PtdIns synthesis distinct from the plasma membrane. Thirdly, the use of a chase protocol similar to that described here has established that the extent of the receptor-responsive fraction of PI in GH $_3$ cells responding to stimulation of PLC by thyrotropin-releasing hormone is proportionate to agonist/receptor occupancy [41]. We have also obtained preliminary results similar to this in carbachol-stimulated 1321N1 cells (I. H. Batty and C. P. Downes, unpublished work). This observation is particularly pertinent, because it would not be predicted if PtdIns-TP served simply to equilibrate PtdIns rapidly between a locally recycled pool and the larger cellular fraction, because under these conditions maximal and half-maximal activation of PLC would be expected to access the same fraction of PI but at different rates. A complex explanation of the latter phenomenon has been presented [14], but we believe that this and both the present and most previous metabolic results on PI pooling can be accommodated by a simpler model (see Figure 5) based on that discussed originally by Michell [11].

Thus we propose that the receptor-mediated hydrolysis of PtdIns P_2 in the plasma membrane is accompanied by compensatory resynthesis of PtdIns in one or more distinct, intracellular membrane compartments and that communication between these sites is mediated inwardly by an essential means for efficient transport of DG, PA or CMP-PA and outwardly by requisite PtdIns-TP. The extent of PtdIns resynthesis is co-ordinated with that of PtdIns P_2 hydrolysis by changes in inositol and/or CMP-PA, the concentrations of which reflect a descending gradient away from the site(s) of PLC activity. The relationship between receptor occupancy and the apparent size of the agonist-sensitive PI pool [41] could be explained if PtdIns synthase that was located at intracellular membrane sites closer to the stimulated PLC experienced saturating substrate concentrations more readily than those at greater distance. Thus resynthesis of PI would proceed simultaneously at multiple intracellular locations distributed across a concentration gradient

of CMP-PA and/or inositol such that segregated pools of PtdIns turn over concomitantly at graduated rates and are therefore not readily resolved kinetically. Newly synthesized PtdIns is then transferred rapidly by PtdIns-TP to sites of local plasma membrane deficit created by activation of PLC, as suggested previously [17]. As efficient transfer will occur along acute PtdIns concentration gradients, lipid will be most readily transported back to the plasma membrane from those sites at which PtdIns synthase activity is most enhanced. This would account for the semi-closed cycle discussed previously [14], provided that the exchange of PtdIns between distinct intracellular membrane pools was slow, a view that is consistent with the present labelling data.

We would also propose that multiple, separate pools of phosphoinositide, each comprising PtdIns, PtdIns P and PtdIns P_2 , exist within the plasma membrane and that the PtdIns component of each is rapidly equilibrated by PtdIns-TP with PtdIns at intracellular sites but without net flux between membranes except at points of lipid consumption. This is because 70–80% of PtdIns P and PtdIns P_2 is accessible to thrombin-receptor-stimulated PLC within 30 s in 1321N1 cell [20], and so must reside within the plasma membrane. In contrast, muscarinic-receptor-stimulated PLC turns over a similar fraction of these lipids (see above and [22]) only with a time course consistent with a mechanism involving equilibration via the bulk of cellular PtdIns. Thus it is probable that even maximal muscarinic-receptor-activated PLC acts directly on only a subfraction of PtdIns P and PtdIns P_2 , which then equilibrates with the majority of cellular polyphosphoinositides through the means described for PtdIns resynthesis. It follows that even plasma-membrane pools of PI not turned over directly by PLC must equilibrate efficiently with the bulk PtdIns pool. As this movement of PtdIns between the plasma membrane and intracellular membranes must occur in the absence of a concentration gradient and without concomitant intermixing of PtdIns within intracellular pools, we propose that this might be achieved by the targeting of PtdIns-TP-dependent transport, perhaps directed by specific recognition of receptors of PtdIns kinase(s). This would ensure the supply of PtdIns to essential, dependent mechanisms in the plasma membrane and perhaps elsewhere. This possibility is consistent with a number of recent observations indicating that PtdIns-TP might associate with activated epidermal-growth-factor receptors [36] and with PI 3-kinase [37], whereas its ability to reconstitute stimulated PLC activity in cytosol-depleted cells is dependent on sequence-specific features that are non-essential for its lipid transfer capacity [42]. Finally, as a smaller proportion of cellular PI (approx. 20–30%) is not readily accessible to the receptor-stimulated PLC, it is probable that this is associated with more strictly compartmentalized functions such as the nuclear PI cycle described recently [43].

Although this model is based primarily on our kinetic and metabolic studies in 1321N1 cells, it also draws on numerous observations made in other systems [14]. It is therefore likely to be widely applicable. Taken in conjunction with the established diversity of cellular structure and the probable variation in the expression or efficiency of the appropriate lipid transport mechanisms, we expect that it can accommodate most of the observed aspects of inositol lipid pooling. It should be emphasized, however, the mechanisms such as those proposed might serve to integrate wider aspects of lipid signalling, because PtdIns-TP(s) counter-transport phosphatidylcholine or sphingomyelin [18,19], two other lipids believed to have important signalling functions [44,45]. Our model should therefore provide a useful framework for future studies and provide valuable insight to issues of broader general interest. For example, it

Figure 5 Schematic model of integrated PI pools

The activated receptor R_1 is coupled to PLC-mediated hydrolysis of PtdIns P_2 (PIP $_2$) at the plasma membrane. The Ins P_3 and DG released are recycled to inositol and CMP-PA, the co-substrates for PtdIns synthase. PtdIns resynthesis occurs on intracellular membranes at numerous sites distributed across a descending concentration gradient of these substrates, as indicated by the large shaded arrow. PtdIns resynthesized most locally to the site of PLC activity is then most efficiently transported by PtdIns-TP to the plasma membrane such that the complete 'PI cycle' involves the lipid transfer of DG, PA or CMP-PA from here to the sites of PtdIns synthesis and the net transfer of PtdIns in the reverse direction. In addition, although exchange of PtdIns between distinct intracellular membrane pools is slow, there is a rapid equilibration of most of this PtdIns with that in the plasma membrane. Thus inositol lipid in the plasma membrane but not turned over directly in response to stimulation of R_1 is nevertheless integrated with PI metabolized immediately by the activated PLC through the mechanism for the graduated resynthesis of PtdIns. This leads to the graded rate of equilibration indicated by the shading.

predicts the presence of physically discrete pools of PtdIns P_2 , which, although differently accessible to key signalling enzymes activated acutely through distinct cell-surface receptors, are nevertheless closely integrated metabolically through the major cellular fraction of PtdIns. It follows that when PtdIns synthesis is impaired, as can occur when inositol monophosphatase is inhibited by Li^+ ions [46,47], the chronic consequences are likely to reflect the multifunctional capacity of inositol phospholipids. Equally, the restricted, immediate access of particular receptors to a limited fraction of PtdIns P_2 might account for the minimal acute secondary effects of Li^+ on the cellular concentration of this lipid, despite apparent effects on downstream signals [46,47]. These observations suggest that the model of integrated PI pools inferred from the present studies is likely to have important implications for the future understanding of PI-dependent sig-

nalling mechanisms and for the potential that the ability of Li^+ ions to disrupt these underlies its therapeutic usefulness.

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REFERENCES

- Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- Stephens, L. R., Jackson, T. R. and Hawkins, P. T. (1993) *Biochim. Biophys. Acta* **1179**, 27–75
- Fry, M. J. (1994) *Biochim. Biophys. Acta* **1226**, 237–268
- Toker, A. and Cantley, L. C. (1997) *Nature (London)* **387**, 673–676
- Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) *Cell* **75**, 1137–1144
- Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-C. and Cantley, L. C. (1994) *J. Biol. Chem.* **269**, 21403–21406

- 7 Janmey, P. A. (1994) *Annu. Rev. Physiol.* **56**, 169–191
- 8 Hay, J. C., Fiset, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A. and Martin, T. F. J. (1995) *Nature (London)* **374**, 173–177
- 9 Nakanishi, S., Catt, J. K. and Balla, T. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5317–5321
- 10 Meyers, R. and Cantley, L. C. (1997) *J. Biol. Chem.* **272**, 4384–4390
- 11 Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- 12 Hokin, L. E. (1985) *Annu. Rev. Biochem.* **54**, 205–235
- 13 Downes, C. P. and Michell, R. H. (1985) in *Molecular Aspects of Cellular Regulation*, vol. 4 (Cohen, P. and Houslay, M. D., eds.), pp. 3–56, Elsevier, Amsterdam
- 14 Monaco, M. E. and Gershengorn, M. C. (1992) *Endocr. Rev.* **13**, 707–718
- 15 Thomas, G. M. H., Cunningham, E., Fensome, A., Ball, A., Totty, N. F., Truong, O., Hsuan, J. J. and Cockcroft, S. (1993) *Cell* **74**, 919–928
- 16 Cunningham, E., Thomas, G. M. H., Ball, A., Hiles, I. and Cockcroft, S. (1995) *Curr. Biol.* **5**, 775–783
- 17 Currie, R. A., MacLeod, B. M. G. and Downes, C. P. (1997) *Curr. Biol.* **7**, 184–190
- 18 Wirtz, K. W. A. (1991) *Annu. Rev. Biochem.* **60**, 73–99
- 19 Wirtz, K. W. A. (1997) *Biochem. J.* **324**, 353–360
- 20 Batty, I. H. and Downes, C. P. (1996) *Biochem. J.* **317**, 529–537
- 21 Batty, I. H. and Downes, C. P. (1994) *Biochem. J.* **297**, 529–537
- 22 Batty, I. H. and Downes, C. P. (1995) *J. Neurochem.* **65**, 2279–2289
- 23 Batty, I. H., Carter, A. N., Challiss, R. A. J. and Hawthorne, J. N. (1997) in *Neurochemistry: A Practical Approach*, (Turner, A. J. and Bachelard, H. S., eds.), pp. 229–268, IRL Press, Oxford
- 24 Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L. and Downes, C. P. (1988) *Biochem. J.* **252**, 583–593
- 25 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 26 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 27 Masters, S. B., Harden, T. K. and Brown, J. H. (1984) *Mol. Pharmacol.* **26**, 149–155
- 28 Batty, I. H., Michie, A., Fennel, M. and Downes, C. P. (1993) *Biochem. J.* **294**, 49–55
- 29 Fain, J. N. and Berridge, M. J. (1979) *Biochem. J.* **180**, 655–661
- 30 Monaco, M. E., Attinasi, M. and Koreh, K. (1990) *Biochem. J.* **269**, 633–637
- 31 Bell, R. M. and Coleman, R. A. (1980) *Annu. Rev. Biochem.* **49**, 459–487
- 32 Nakahata, N. and Harden, T. K. (1987) *Biochem. J.* **241**, 337–344
- 33 MacCallum, S. H., Barker, C. J., Hunt, P. A., Wong, N. S., Kirk, C. J. and Michell, R. H. (1989) *J. Endocrinol.* **122**, 379–389
- 34 Kirk, C. J., Michell, R. H. and Hems, D. A. (1981) *Biochem. J.* **194**, 155–165
- 35 Hay, J. C. and Martin, T. F. J. (1993) *Nature (London)* **366**, 572–575
- 36 Kauffmann-Zeh, A., Thomas, G. M. H., Ball, A., Prosser, S., Cunningham, E., Cockcroft, S. and Hsuan, J. J. (1995) *Science* **268**, 1188–1190
- 37 Panaretou, C., Domin, J., Cockcroft, S. and Waterfield, M. D. (1997) *J. Biol. Chem.* **272**, 2477–2485
- 38 Downes, C. P. and Batty, I. H. (1993) *Curr. Biol.* **3**, 794–796
- 39 Santiago, O. M., Rosenberg, L. I. and Monaco, M. E. (1993) *Biochem. J.* **290**, 179–183
- 40 Sillence, D. J. and Downes, C. P. (1993) *Biochem. J.* **290**, 381–387
- 41 Cubitt, A. B., Geras-Raaka, E. and Gershengorn, M. C. (1990) *Biochem. J.* **271**, 331–336
- 42 Hara, S., Swigart, P., Jones, D. and Cockcroft, S. (1997) *J. Biol. Chem.* **272**, 14908–14915
- 43 Divecha, N. and Irvine, R. F. (1995) *Cell* **80**, 269–278
- 44 Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212**, 26–42
- 45 Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 3125–3128
- 46 Nahorski, S. R., Ragan, C. I. and Challiss, R. A. J. (1991) *Trends Pharmacol. Sci.* **12**, 297–303
- 47 Gani, D., Downes, C. P., Batty, I. H. and Bramham, J. (1993) *Biochim. Biophys. Acta* **1177**, 253–269