Direct labelling of hormone-sensitive phosphoinositides by a plasma-membrane-associated PtdIns synthase in turkey erythrocytes

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We have previously characterized phosphatidylinositol (PtdIns) synthase and PtdIns/*myo*-inositol-exchange enzyme activities in ghost membranes prepared by hypotonic lysis of turkey erythrocytes [McPhee, Lowe, Vaziri and Downes (1991) *Biochem. J.* **275**, 187–192]. Here we show that PtdIns synthase activity is relatively enriched in plasma-membrane preparations of turkey erythrocytes and that inositol phospholipids labelled by both PtdIns synthase and PtdIns *myo*-inositol exchange enzymes are susceptible to hydrolysis by the receptor- and G-protein-regulated phospholipase C (PLC), which is present also in ghost preparations. Specific-radioactivity measurements of [³H]PtdIns from ghosts labelled to equilibrium under conditions favouring [³H]inositol incorporation by PtdIns synthase activity indicate that PtdIns synthase can directly access approx. 14% of the total erythrocyte ghost PtdIns. Approx. 16% of the [³H]PtdIns labelled by the PtdIns synthase reaction can be phosphorylated to polyphosphoinositides, which are then

hydrolysed by the receptor- and G-protein-stimulated PLC. Since the mass of PtdIns declines to a similar extent as [³H]PtdIns during stimulation in the presence of guanine nucleotides and ATP, it is evident that both the labelled and unlabelled phosphoinositides are susceptible to hydrolysis by the relevant PLC. Phosphoinositides present in nuclei-free plasma membranes were also labelled by [³H]inositol under conditions favouring PtdIns synthase and PtdIns/*myo*-inositol-exchange enzyme activities respectively. These membranes lack PLC activity [Vaziri and Downes (1992) *J. Biol. Chem.* **267**, 22973–22981], but the labelled lipids were sensitive to purinergic-receptor-stimulated hydrolysis in reconstitution assays using partially purified turkey erythrocyte PLC. The results strongly suggest that at least a portion of the PtdIns synthase in turkey erythrocytes is located in the plasma membrane and has direct access to an agonist-sensitive pool of inositol phospholipids.

INTRODUCTION

Receptor-regulated PtdIns₂ hydrolysis, in response to a variety of agonists, generates the ubiquitous second messengers, InsP₃ (Berridge and Irvine, 1984, 1989) and diacylglycerol (DAG) (Nishizuka, 1988). Replenishment of the finite pool of hormone-sensitive polyphosphoinositides following receptor-regulated PtdIns₂ hydrolysis is likely to require the efficient recycling of InsP₃ and DAG (Berridge et al., 1989). InsP₃ is metabolized via a complex series of reactions, eventually resulting in free intracellular inositol (Berridge and Irvine, 1989). DAG is phosphorylated by DAG kinase to generate phosphatidic acid, which is further metabolized to CMP-phosphatidate (CMP-PA), a reaction catalysed by phosphatidate:CTP cytidylyltransferase (reviewed by Hokin, 1985). PtdIns is synthesized from CMP-PA and inositol in a reaction catalysed by PtdIns synthase. Agonists which stimulate PtdIns₂ hydrolysis cause increased synthesis of PtdIns, as evidenced by increased incorporation of [³H]inositol or of ³²P into PtdIns (reviewed by Downes and Michell, 1985). The mechanism of the receptor-stimulated increase in PtdIns synthesis is not understood, although Imai and Gershengorn (1987b) have suggested that it may be due to relief of product inhibition of PtdIns synthase by PtdIns.

There is still some uncertainty whether the entire phosphoinositide metabolic cycle can occur in a single membrane compartment. The synthesis of PtdIns₂ from PtdIns and receptor-regulated hydrolysis of PtdIns₂ have generally been

thought to occur at the plasma membrane. The enzymes which catalyse the stepwise phosphorylation of PtdIns to PtdIns₄P and PtdIns_(4,5)P₂ are thought to be present in plasma membranes (reviewed by Carpenter and Cantley, 1990). However, recent studies also indicate that there may be polyphosphoinositide synthesis and PtdIns₂ hydrolysis in the nucleus of some cells (Smith and Wells, 1983; Cocco et al., 1987; Divecha et al., 1991). PtdIns is widely distributed among the membranes of eukaryotic cells (Bishop and Bell, 1988). In contrast, PtdIns synthase appears to be present exclusively in endoplasmic-reticulum (E.R.)-derived membranes, in some studies employing subcellular-fractionation techniques (Morris et al., 1990; Helms et al., 1991). One possible mechanism by which the widespread distribution of PtdIns may be achieved is by the transfer of lipid molecules through the cytoplasm to the plasma membrane and other intracellular compartments. A family of cytosolic proteins has been characterized which will catalyse the transfer of PtdIns *in vitro* between donor lipid vesicles and biological membranes (Van Paridon et al., 1987). An alternative possible mechanism to explain the subcellular distribution of PtdIns was suggested by Imai and Gershengorn (1987a), who showed that membrane fractions from GH₃ cells, which were enriched in plasma-membrane markers, contained PtdIns synthase activity. It was therefore proposed that a plasma-membrane-associated PtdIns synthase may be responsible for the resynthesis of PtdIns that occurs following hormonal stimulation of cells. Studies in mutants of *Saccharomyces cerevisiae* have shown PtdIns synthase

Abbreviations used: DAG, diacylglycerol; PLC, phospholipase C; CMP-PA, CMP-phosphatidate; GTP[S], guanosine 5'-[γ-thio]triphosphate; E.R., endoplasmic reticulum.

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to be present in secretory vesicles destined for plasma membranes (Kinney and Carman, 1990), indicating that the plasma membranes from these cells may be capable also of synthesizing PtdIns. A plasma-membrane-associated PtdIns synthase could circumvent the need for lipid transfer proteins, and might provide a basis for metabolically segregated pools of phosphoinositides, which have been proposed by some workers (Monaco, 1982, 1987).

We have sought direct evidence for the existence of a PtdIns synthase that can access hormone-sensitive pools of phosphoinositides. Turkey erythrocyte ghosts have provided a uniquely responsive cell free model system for the study of receptor- and G-protein-regulated phospholipase C (PLC). We have previously characterized the PtdIns synthase activity present in turkey erythrocyte ghost membranes (McPhee et al., 1991). In this report we show that PtdIns synthase activity can be exploited to label directly hormone-responsive pools of inositol lipids, in cytosol-free turkey erythrocyte ghost and plasma-membrane preparations. These results suggest the existence of a plasma-membrane-associated PtdIns synthase that is responsible for the synthesis of hormone-labile phosphoinositides in turkey erythrocytes.

MATERIALS AND METHODS

Materials

myo-[2-³H]Inositol (sp. radioactivity 4.19×10^{16} d.p.m./mol of inositol) was purchased from Amersham International, Amersham, Bucks., U.K., or from NEN DuPont, Boston, MA, U.S.A. Silica-gel 60 t.l.c. plates (20 cm $\times$ 20 cm) were purchased from Merck, U.K. Dipalmitoyl phosphatidic acid was purchased from Calbiochem-Novabiochem, Nottingham, U.K. All other reagents were obtained from previously described sources (Harden et al., 1988; Vaziri and Downes, 1992a,b).

Preparation of subcellular fractions from turkey erythrocytes

Ghosts and plasma membranes were prepared from washed turkey erythrocytes as described previously (Vaziri and Downes, 1992b).

Labelling of PtdIns under exchange and synthase conditions

PtdIns synthase and PtdIns/inositol exchange reactions were assayed essentially as described by MCPhee et al. (1991). Typically, turkey erythrocyte ghosts (0.5–1.5 mg of protein) were labelled with [³H]inositol (0.5–1 μ Ci) in a basic incubation buffer consisting of 50 mM Tris/HCl (pH 8.0) and 10 mM MgCl₂ in the absence or presence of 1 mM CMP for labelling via the PtdIns synthase and PtdIns/Ins exchange reactions respectively, in a final assay volume of 0.2 ml. In some experiments the incubation buffer was also supplemented with unlabelled inositol, as indicated in the legends to Figures and Tables. PtdIns synthase was assayed in the forward direction by the synthesis of radiolabelled PtdIns from [³H]inositol and CMP-PA under similar conditions to those described above. However, the basic incubation buffer replaced CMP with 0.1 mM CMP-PA in 0.2 % Triton. CMP-PA was synthesized from dipalmitoyl phosphatidic acid by the method of Carman and Fischl (1980). The reactions were terminated and phases split as described previously (McPhee et al., 1991). Radioactivity incorporated into the lower chloroform phase was quantified by scintillation counting (McPhee et

al., 1991). Further details of labelling conditions are indicated in the legends to Figures and Tables.

Assay of adenylate cyclase

Turkey erythrocyte subcellular fractions were assayed for fluoride-stimulated adenylate cyclase activity as described previously (Boyer et al., 1989).

Assay of agonist- and guanine-nucleotide-regulated PLC

Turkey erythrocyte ghosts were labelled with [³H]inositol *in vitro* by methods similar to those previously described (Vaziri and Downes, 1992a). For labelling via PtdIns synthase, the incubation was supplemented with 1 mM unlabelled inositol and 1 mM CMP. These additions were omitted for labelling via the PtdIns/Ins exchange reaction. The labelled ghosts were washed extensively in 10 mM Hepes (pH 7.0)/5 mM MgCl₂ and diluted in this buffer. Ghosts (typically 100 μ l) were assayed for agonist- and guanine-nucleotide-stimulated PLC activity in an intracellular-type buffer (Harden et al., 1988), containing ATP and guanosine 5'-[γ -thio]triphosphate (GTP[S]) at concentrations indicated in the legends to Figures, in a final incubation volume of 0.2 ml.

Partial purification of turkey erythrocyte cytosolic PLC

Cytosolic PLC was partially purified from turkey erythrocytes by (NH₄)₂SO₄ precipitation and DEAE-cellulose chromatography as previously described (Morris et al., 1990). Active fractions were dialysed extensively against 10 mM Hepes (pH 7.0)/0.1 mM EDTA and concentrated by using a Centricon 30 filtration device.

Reconstitution of PLC

Plasma membranes were labelled with [³H]inositol under conditions favouring PtdIns synthase or PtdIns/Ins exchange as described above for agonist stimulation of ghosts. Reconstitution of PLC was carried out by a similar approach to that described previously (Vaziri and Downes, 1992b). Plasma membranes (0.5 μ g of protein) were reconstituted with PLC (12 nmol of PtdInsP hydrolysed/min under standard conditions as described previously; Vaziri and Downes, 1992b) or with Hepes/EDTA for controls. Incubations were carried out under basal conditions or with 5 mM Mg-ATP and 10 μ M GTP[S] for times indicated on the Figures.

Measurement of PtdIns

The mass of PtdIns was determined after labelling of turkey ghosts with [³H]inositol either via PtdIns synthase or the PtdIns/Ins exchange reaction, as described above, and also after agonist stimulation. The reactions were terminated and phases split as described previously (McPhee et al., 1991). The lower phase was dried *in vacuo*, taken up in chloroform and spotted on pre-activated (100 °C, 10 min) silica-gel t.l.c. plates. Typically each lane on the t.l.c. plate was derived from approx. 0.2–0.3 ml of packed ghosts. A portion of this sample was subjected to scintillation counting to determine the total initial radioactivity applied to the plate. PtdIns was purified by using a double solvent system composed of (solvent 1) chloroform/methanol/acetone/trifluoroacetic acid/water (40:13:15:12:4, by vol.) and (solvent 2) chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8, by vol.) (Jolles et al., 1981).

After development in the first solvent system, the PtdIns band was identified by using a Raytest t.l.c. scanner, scraped out and extracted from the silica by vortex-mixing with 0.75 ml of chloroform/methanol/water (40:80:1, by vol.), followed by a phase split with 0.25 ml each of 0.1 M HCl and chloroform. After a brief centrifugation, the lower phase was dried down as above and re-run on a second t.l.c. using solvent 2. The PtdIns was re-extracted from the silica, and the chloroform phase was dried down under nitrogen into HClO₄-cleaned Pyrex test tubes. The PtdIns was hydrolysed by refluxing with 70% (v/v) HClO₄ (144 μ l, 250 °C, 2 h). P_i was determined by the method of Bartlett (1959). The samples were mixed with 10 ml of Triton X-100/xylene (1:1, v/v) containing 2,5-diphenyloxazole (3.75 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.093 g/l) and the radioactivity was quantified by scintillation counting. The recovery through the mass assay was determined by comparing the initial radioactivity applied to the t.l.c. with the radioactivity finally recovered after the P_i assay. The specific radioactivity of PtdIns is expressed as the recovered radioactivity divided by the mass of recovered PtdIns.

RESULTS

Incorporation of [³H]inositol into PtdIns in turkey erythrocyte ghost membranes involves a nucleotide-independent PtdIns/*myo*-inositol base-exchange activity, and a distinct CMP-dependent base-exchange reaction (McPhee et al., 1991). Net synthesis of PtdIns from CMP-PA and inositol is the physiologically important reaction catalysed by PtdIns synthase. However, the CMP-dependent incorporation of [³H]inositol into PtdIns is thought to represent a base-exchange activity catalysed by PtdIns synthase (Berry et al., 1983; Fischl et al., 1986; MCPhee et al., 1991). The CMP-dependent reaction in turkey erythrocytes can be distinguished from PtdIns/*myo*-inositol base-exchange activity by its low affinity for inositol (K_m of 0.3 mM for CMP-dependent versus K_m of 0.012 mM for CMP-independent exchange) and distinctive bivalent-cation sensitivity. Although CMP-dependent exchange of free for lipid-associated inositol does not prove the presence of PtdIns synthase activity in turkey erythrocytes, the latter was demonstrated by the inositol-reversible incorporation of [¹⁴C]CMP into a lipid which was identified as CMP-PA (McPhee et al., 1991) and by direct assay of PtdIns synthase in the forward reaction using CMP-PA as substrate (Vaziri and Downes, 1992b).

It was decided to determine the size of the pool of PtdIns accessible to PtdIns synthase in turkey erythrocyte ghosts. Ghosts were labelled with [³H]inositol in the presence of 1 mM CMP and 1 mM unlabelled inositol, thus favouring the CMP-dependent PtdIns synthase-catalysed reaction. Incorporation via the CMP-independent PtdIns/inositol base-exchange reaction was assessed in the absence of CMP. The PtdIns labelled by these base-exchange mechanisms does not result in significant net synthesis of PtdIns even after a 1 h incubation (paired *t* test, *P* < 0.005). That incorporation occurs via an exchange process was confirmed by the ability of unlabelled inositol to chase out the label from PtdIns in a CMP-dependent manner (McPhee et al., 1991; Figure 1b). The rate of incorporation of [³H]inositol into PtdIns by the PtdIns synthase declines to zero after 60 min of incubation (Figure 1a). This is not due to the inactivation of the PtdIns synthase, or resealing of the ghost preparation, as approx. 80% of the radiolabel can be chased from the prelabelled PtdIns under PtdIns synthase conditions in the presence of excess unlabelled inositol (Figure 1b). Therefore, after 60 min the pool of PtdIns accessed by PtdIns synthase must have achieved isotopic equi-

librium with the exogenously added [³H]inositol in the labelling medium. Since the concentration of inositol in the medium is sufficiently large (1 mM) relative to that of the PtdIns in the ghosts (approx. 0.39 mM), changes in the specific radioactivity of the inositol due to exchange of unlabelled PtdIns after a 60 min incubation is calculated to be less than 10%. As shown in Table 1, the specific radioactivity of the [³H]PtdIns extracted from ghosts labelled to equilibrium reaches almost 14% of that of the [³H]inositol in the incubation medium, thus suggesting that 14% of the PtdIns present in the turkey ghosts is accessible to PtdIns synthase. In contrast, the specific radioactivity of PtdIns labelled via the CMP-independent exchange reaction reached only approx. 4% of that in the incubation medium. However, under exchange conditions, radioactivity in PtdIns was still increasing at 60 min, so this experiment gives no information about the size of the PtdIns pool accessed by the exchange enzyme. These data demonstrate that a significant proportion of the total cellular PtdIns can be labelled by an exchange process catalysed by PtdIns synthase.

Turkey erythrocyte ghosts contain predominantly a nucleus and a plasma membrane (Beam et al., 1979). It is therefore likely

Figure 1 (a) Time course of [³H]inositol incorporation into turkey erythrocyte ghost PtdIns and (b) time-dependent chase of radioactivity from PtdIns labelled via PtdIns synthase

(a) Ghosts (approx. 1 mg) were labelled with [³H]inositol (1 μ Ci) via PtdIns synthase (+ CMP) (●) or the PtdIns/inositol exchange enzyme (—CMP) (○) in the presence of 1 mM unlabelled inositol as described in the Materials and methods section. Each data point is the mean of duplicate incubations which differed by no more than 5%. Similar results were obtained in two experiments. (b) Turkey erythrocyte ghosts (approx. 60 mg of protein) were labelled with [³H]inositol (100 μ Ci) via PtdIns synthase for 1 h, then washed extensively in 50 mM Tris/HCl (pH 8.0) and 5 mM MgCl₂ and diluted in this buffer. The ghosts (approx. 1 mg) were resuspended in basic incubation buffer (see the Materials and methods section) and the radiolabel was chased from PtdIns via PtdIns synthase (+1 mM CMP +10 mM unlabelled inositol) (●). Controls (○) contained no unlabelled inositol or CMP. Each data point is the mean of duplicate incubations which differed by no more than 5%. The results are from a single experiment which was reproduced several times with similar results.

Table 1 Proportion of PtdIns labelled via PtdIns synthase and PtdIns/inositol exchange reactions

The mass of PtdIns was measured after incubation of turkey erythrocyte ghosts (approx. 6 mg of ghost protein) with [3 H]inositol (4 μ Ci) in the presence of 1 mM unlabelled inositol under PtdIns synthase (+CMP) and PtdIns/Ins headgroup exchange conditions (—CMP) for 10 and 60 min in a final incubation volume of 0.8 ml, as described in the Materials and methods section. The data represent means $\pm$ S.D. from n experiments, which were conducted in triplicate. The proportion of PtdIns labelled is expressed as a percentage of the specific radioactivity of PtdIns (see the Materials and methods section) divided by the specific radioactivity of inositol in the incubation medium.

Labelling conditions	Labelling time (min)	Proportion of PtdIns labelled (%)
—CMP	10	0.61 $\pm$ 0.17 (n = 3)
	60	3.99 $\pm$ 1.57 (n = 2)
+CMP	10	9.98 $\pm$ 2.07 (n = 4)
	60	13.52 $\pm$ 0.99 (n = 2)

Table 2 Subcellular distribution of PtdIns synthase in turkey erythrocytes

Subcellular fractions of turkey erythrocytes were assayed for PtdIns synthase (via the forward reaction) and adenylate cyclase activity as described in the Materials and methods section. The results represent means $\pm$ S.E.M. from four separate experiments which were carried out in triplicate. The overall fold enrichment of PtdIns synthase and adenylate cyclase activities in plasma membranes and nuclear pellet is expressed relative to ghosts. The DNA content of the plasma-membrane preparations was typically 40-fold less than that in ghosts (Vaziri and Downes, 1992b).

	Ghosts	Plasma membranes	Nuclear pellet
PtdIns synthase			
Specific activity (nmol of PtdIns/min per mg)	0.99 $\pm$ 0.08	2.32 $\pm$ 0.09	0.34 $\pm$ 0.03
Fold enrichment	1	2.34	0.34
Adenylate cyclase			
Specific activity (pmol of cyclic AMP/min per mg)	5.26 $\pm$ 0.38	11.09 $\pm$ 0.54	1.06 $\pm$ 0.07
Fold enrichment	1	2.11	0.20

that the PtdIns synthase responsible for labelling the PtdIns is associated with either the plasma membrane or nucleus, as this proportion (14%) of labelled PtdIns is unlikely to be derived solely from the relatively small amount of E.R. or other intracellular organelles present in avian erythrocytes. Subcellular-localization experiments revealed that anucleate plasma-membrane preparations, generated during vigorous agitation of turkey erythrocytes with glass beads, are enriched relative to ghosts approx. 2-fold in PtdIns synthase activity (see Table 2 and also Vaziri and Downes, 1992b). This fold enrichment parallels almost exactly that of fluoride-stimulated adenylate cyclase activity, a marker enzyme for plasma membranes. The plasma-membrane preparations typically contain 40 times less DNA/mg of protein than is present in ghosts, indicating that they lack nuclei (Vaziri and Downes, 1992b). Indeed, nuclei prepared by differential centrifugation of turkey erythrocyte lysates have substantially decreased PtdIns synthase specific activity compared with ghosts and especially compared with the plasma membrane preparation

Figure 2 PLC activity in turkey erythrocyte ghosts

Ghosts were labelled for 1 h with [3 H]inositol at 30 °C and under PtdIns synthase (+1 mM CMP, +1 mM inositol) (●, ■) or PtdIns/inositol exchange (no CMP, no unlabelled inositol) conditions (□, ○). After extensive washing, the labelled ghosts were assayed for ATP (5 mM)- and GTP[S] (10 μ M)-stimulated PLC activity at 30 °C in an intracellular-type buffer in a final incubation volume of 0.2 ml (Harden et al., 1988). At the indicated times, [3 H]inositol phosphate production was measured. In some incubations (□, ■), ATP (5 mM) and GTP[S] (10 μ M) were added to the incubations after 40 min, as indicated by the arrow, or omitted from the incubations (▲).

(Table 2). Any PtdIns synthase activity present in the nuclear pellet can be at least partially accounted for by contaminating plasma-membrane activity, as indicated by the retention of contaminating adenylate cyclase activity in this fraction. PtdIns synthase activity can be solubilized from ghosts using non-ionic detergents such as Triton X-100, but is not detectable in the detergent-insoluble residue containing cytoskeletal proteins (results not shown).

To provide further evidence for the existence of a plasma-membrane-associated PtdIns synthase, we sought to demonstrate receptor- and G-protein-regulated hydrolysis of [3 H]phosphoinositides which had been labelled directly by the PtdIns synthase activity present in washed membrane preparations. In order to label hormone-sensitive pools of phosphoinositides accessed by PtdIns synthase, it was necessary to exploit the CMP-dependent incorporation of [3 H]inositol into PtdIns. It was not appropriate to label PtdIns by using the CMP-PA-dependent incorporation of [3 H]inositol, as this reaction requires the CMP-PA to be present as detergent-solubilized micelles, and exposure of ghosts to detergents uncouples PLC from regulation by the receptor-linked G-protein.

Ghosts were labelled under conditions favouring incorporation of [3 H]inositol into PtdIns by PtdIns synthase (incubations containing 1 mM CMP and 1 mM inositol) or by the base-exchange enzyme (incubations containing no added unlabelled inositol and no CMP). Receptor- and G-protein-regulated PLC activity was subsequently determined by measuring the production of [3 H]InsPs from the labelled ghosts in response to various stimuli.

The results in Figure 2 clearly confirm previous observations that the G-protein-regulated PLC activity in turkey erythrocytes does not recognize PtdIns itself. Radiolabelled inositol phosphates are only produced in response to guanine nucleotides if the ghosts are also incubated with ATP to convert some of the PtdIns into polyphosphoinositides. However, both PtdIns4P and PtdIns(4,5)P₂ appear to be effective substrates for this enzyme (Harden et al., 1987, 1988; Morris et al., 1990). Therefore, the appearance of radiolabelled inositol phosphates in response to guanine nucleotides and ATP reflects the co-localization of both

Table 3 Mass of PtdIns and PLC activity after agonist and guanine nucleotide stimulation

Turkey erythrocyte ghosts were labelled with [^3H]inositol via PtdIns synthase (1 mM CMP, 1 mM inositol) as described in the Materials and methods section. Labelled ghosts were assayed for ATP (5 mM)- and GTP[S] (10 μM)-stimulated PLC activity, and the mass of PtdIns was determined as described in the Materials and methods section. The data for the mass measurements are from a single experiment and are expressed as means $\pm$ S.E.M. for the numbers of replicates in parentheses. The measurements of PtdIns and inositol phosphates are means of four incubations in a single experiment which is representative of four experiments (see the text). *Significantly different at $P < 0.05$ by the one-tail unpaired t test.

	Control	Stimulated
Mass of PtdIns (nmol)	233 $\pm$ 10 (6)	199 $\pm$ 9* (7)
Radiolabelled PtdIns (d.p.m.)	281 682 $\pm$ 18 994	239 893 $\pm$ 11 355
InsP production (d.p.m.)	720 $\pm$ 25	37 223 $\pm$ 4100
PLC activity (% of lipid hydrolysed)	0.44	15.5

Figure 3 Reconstitution of cytosolic PLC with plasma membranes

Plasma membranes were labelled with [^3H]inositol under conditions favouring PtdIns synthase (+1 mM CMP, +1 mM unlabelled inositol) (a) or PtdIns/inositol exchange (no CMP, no unlabelled inositol) (b) as described in the Materials and methods section. Plasma membranes were reconstituted with PLC ($\blacktriangle$, $\bullet$) or HEPES/EDTA for controls ($\triangle$, $\circ$) as described in the Materials and methods section. Incubations were carried out under basal conditions ($\bullet$, $\circ$) or with 5 mM Mg-ATP and 10 μM GTP[S] ($\blacktriangle$, $\triangle$) for the times indicated in the Figure.

PtdIns synthase and PtdIns and PtdIns4P kinases. After 40 min of stimulation with ATP and GTP[S], approx. 16% of the PtdIns labelled by the PtdIns synthase was indirectly converted into [^3H]InsPs in this way (Figure 2; see also Table 3). Re-addition of ATP and GTP[S] at this time (indicated by the arrow in Figure 2) did not result in further inositol phosphate accumulation, indicating that depletion of ATP or GTP[S] was not responsible

for the decline in PLC activity, and that approx. 84% of the lipid pool labelled by the PtdIns synthase was not susceptible to hydrolysis, either because it was not available for PtdIns kinase and/or because the polyphosphoinositides derived from it were not accessible to PLC. It was consistently found that slightly more of the [^3H]PtdIns was converted into InsPs on stimulation with ATP + GTP[S] when the ghosts had been labelled under PtdIns synthase conditions, implying that less of the pool labelled by the PtdIns/inositol exchange enzyme is accessible to PtdIns kinase and/or PLC.

Unlike turkey erythrocyte ghosts, plasma-membrane preparations do not contain detectable PLC activity, yet are enriched in P_{2y} -receptor-coupled G-protein (Vaziri and Downes, 1992a). Therefore, in order to demonstrate that the PtdIns synthase activity present in plasma membranes can directly label hormone-sensitive pools of phosphoinositides, a sensitive reconstitution assay for the detection of receptor- and G-protein-regulated PLC activity was devised. Plasma membranes were labelled with [^3H]inositol under conditions favouring PtdIns synthase (Figure 3a) or PtdIns/inositol exchange (Figure 3b) activity. As previously shown (Vaziri and Downes, 1992b), [^3H]InsP production was barely detectable after stimulation of the labelled membranes with ATP + GTP[S], in the absence of exogenously added PLC (Figures 3a and 3b). However, in incubations containing partially purified turkey erythrocyte cytosolic PLC, membranes labelled under PtdIns synthase or exchange conditions generated [^3H]InsPs in response to ATP and GTP[S].

After 10 min of stimulation with ATP and GTP[S] in the presence of exogenously added PLC, 4.8% and 5.2% of the [^3H]PtdIns was converted into InsPs in membranes labelled by PtdIns synthase and PtdIns/Ins exchange conditions respectively (Figures 3a and 3b). The rate of ATP + GTP[S]-stimulated hydrolysis of labelled lipid, on reconstitution of a maximally effective amount of PLC with the plasma membranes, is less than that hydrolysed by the endogenous PLC in fully responsive ghost preparations (e.g. Figure 2). It was routinely observed that, in the presence of ATP, less of the [^3H]PtdIns was converted into polyphosphoinositides, by the endogenous PtdIns and PtdInsP kinases, in plasma membranes than in ghost preparations. Typically, the proportions of [^3H]PtdIns:[^3H]PtdInsP:[^3H]Ptd-Ins P_2 , at steady state, are 12:3:1 in ghosts and 120:14:1 in plasma-membrane preparations. This may be because the plasma membranes are deficient in PtdIns 4-kinase and PtdIns4P 5-kinase activities relative to ghosts, or because less of the pool of labelled PtdIns is accessible to these enzymes in plasma-membrane preparations. Regardless of why the labelled membranes contain less [^3H]polyphosphoinositide, this provides a possible explanation for the decreased rate of reconstituted PLC activity in plasma membranes, as compared with the rate of agonist- and guanine-nucleotide-stimulated phosphoinositide hydrolysis in intact ghosts.

Mass assays of PtdIns were performed to determine whether the 16% of PtdIns that was converted to InsPs, after ATP and GTP[S] stimulation of ghosts, consisted solely of the PtdIns labelled by the PtdIns synthase or of the total cellular PtdIns (i.e. both labelled and unlabelled PtdIns). Ghosts labelled under PtdIns synthase conditions were stimulated with supraphysiological concentrations of ATP and GTP[S] for 60 min as described above, and the mass of PtdIns was determined. A decrease in mass of PtdIns of 14% was measured after prolonged stimulation of PtdIns synthase-labelled ghosts (Table 3), which is of a similar magnitude to the decrease in [^3H]PtdIns (14.8%) under the same labelling and stimulation conditions (Table 3). The results in Table 3 are an example of one experiment which

was repeated four times. A significant decrease in the mass of PtdIns, after ATP and GTP[S] stimulation of ghosts, was also found when applying a paired *t* test to four separate experiments ($P < 0.05$). This result indicates that PtdIns synthase does not label all of the hormone-sensitive pool of PtdIns, since, if it did, only 2.2 % loss of PtdIns mass would have been detected (16 % of the 14 % of total PtdIns that was labelled by PtdIns synthase).

DISCUSSION

Previous workers have investigated the location of PtdIns synthase only by direct assay of enzyme activity in subcellular fractions. Imai and Gershengorn (1987a,b) characterized a PtdIns synthase activity present in plasma-membrane preparations from GH₃ cells. The plasma-membrane-associated PtdIns synthase was found to be distinct from an enzyme present in E.R./microsomal fractions, on the basis of its susceptibility to extraction with high salt, its K_m values for CMP-PA and inositol, as well as its responsiveness to stimulation by Mg²⁺. It was suggested that a plasma-membrane-associated PtdIns synthase would allow localized synthesis of hormone-sensitive inositol lipids, thereby circumventing the proposed requirement for reciprocal transport of phosphatidic acid and PtdIns between E.R. and plasma membrane (Michell, 1975).

We have exploited the relatively simple structure of turkey erythrocytes to establish that at least a portion of the PtdIns synthase activity which these cells possess must be located in the plasma membrane. Conventional subcellular-fractionation techniques showed that anucleate plasma-membrane preparations are enriched in PtdIns synthase to a similar extent as is fluoride-stimulated adenylate cyclase. It is possible that this fraction was contaminated with fragments of nuclear membrane which might not be associated with DNA, the marker that we used to detect intact nuclei. However, since the specific activity of PtdIns synthase in the nuclear pellet was 7-fold lower than that in the plasma-membrane preparation, this would seem to be unlikely. Nevertheless, our results do not rule out the possibility that some PtdIns synthase activity might be also present in the nuclei, although this fraction clearly contains contaminating plasma membrane, as indicated by the presence of fluoride-stimulated adenylate cyclase.

Measurements of specific radioactivity of [³H]PtdIns from equilibrium-labelled ghosts indicate that PtdIns synthase can access only 14 % of the total PtdIns pool in ghost preparations. It therefore appears that this pool of lipid is in a subcellular compartment distinct from the bulk of the cellular PtdIns. It is unlikely that the 14 % of the PtdIns in turkey erythrocytes accessible to PtdIns synthase might be contributed by an intracellular non-nuclear-membrane PtdIns synthase, since turkey erythrocytes contain relatively little E.R. and the nucleus is the only large membrane compartment, other than the plasma membrane, in these cells (Beam et al., 1979).

To obtain further evidence that at least some of the PtdIns synthase activity is present in plasma membranes, we assessed whether any of the PtdIns labelled by PtdIns synthase could be converted into polyphosphoinositides that were hydrolysed by purinergic-receptor- and G-protein-regulated PLC. This approach clearly only identifies a pool of PtdIns synthase that is co-localized with PtdIns kinase and/or PtdIns4P kinase, since PtdIns itself is not a substrate for the G-protein-regulated PLC, but has the advantage that the location of the relevant receptor and G-protein is clearly defined. Since this system is sensitive to sub-millimolar levels of ATP/ADP and is activated when intact cells are exposed to these levels of purinergic ligands, the agonist-binding site must be located on the cell surface. Moreover, G₁₁,

the G-protein which couples purinergic receptors to PLC in turkey erythrocytes (Waldo et al., 1991), has previously been shown to be present in plasma membranes of these cells (Vaziri and Downes, 1992b). The results clearly show that radiolabelled polyphosphoinositides, which must be derived from PtdIns labelled by a CMP-dependent process previously shown to be due to PtdIns synthase, are hydrolysed in an agonist- and guanine-nucleotide-dependent manner by the endogenous PLC in ghosts, or when plasma membranes are reconstituted with purified PLC.

It is unlikely that cytosolic lipid-transfer proteins are responsible for shuttling [³H]phosphoinositides from intracellular membrane compartments to the plasma membrane in our experiments, since the ghosts used for these experiments are routinely washed extensively in hypotonic and hypertonic buffers. Any cytosolic lipid-transfer proteins remaining in the ghosts would be subject to very large dilutions. Moreover, it was also possible to label an agonist- and guanine-nucleotide-sensitive inositol lipid pool in anucleate plasma membranes. Although a soluble G-protein-regulated PLC was used for these experiments, activation of this enzyme requires it to associate with the GTP-liganded G-protein α -subunit (Waldo et al., 1991), and previous studies showed this α -subunit to be present in the plasma membrane (Vaziri and Downes, 1992b). In addition, the proportion of inositol lipid hydrolysed on stimulation of ghosts labelled *in vitro* (approx. 16 % of total labelled lipid) is similar to that hydrolysed on stimulation of ghosts prepared from [³H]inositol-labelled intact erythrocytes (C. Vaziri and C. P. Downes, results not shown), suggesting that labelling of the agonist-sensitive pool does not require cytosolic components or a restricted intracellular space.

In ghosts, a proportion (16 %) of the labelled PtdIns is converted into InsPs on stimulation of PLC with ATP and GTP[S]. If the 14 % of total PtdIns labelled by PtdIns synthase was the only hormone-sensitive pool, then there would be an overall estimated 2.2 % decrease in mass of total cellular PtdIns. However, mass measurements of PtdIns indicate that both the PtdIns labelled by the PtdIns synthase and a proportion of unlabelled PtdIns are accessible to agonist- and guanine-nucleotide-stimulated PLC, since a significant decrease in cellular PtdIns mass was observed on stimulation. It appears therefore that PtdIns synthase can only label directly a proportion of the hormone-sensitive PtdIns in turkey erythrocyte ghosts.

Metabolic heterogeneity of phosphoinositides has previously been reported in human erythrocytes (King et al., 1987). Phosphoinositide kinases access only a fraction (25–30 %) of inositol lipids in such cells, and only 50 % of PtdInsP₂ was accessible to the Ca²⁺-activated PLC in erythrocyte ghosts. Since the anatomically simple human erythrocytes lack nuclear and other intracellular membranes, it was suggested that stable associations between the lipids and sub-membranous or cytoskeletal proteins might be responsible for the segregation of lipid pools in these cells. It is possible that such a mechanism might account for the distinct pools of PtdIns in turkey erythrocytes. Indeed, we have presented data indicating that the receptor- and G-protein-regulated PLC is tightly and specifically associated with a component of the detergent-insoluble cytoskeleton in turkey erythrocytes, and that the cytoskeleton plays a critical role in localizing PLC to its coupling site at the inner leaflet of the plasma membrane (Vaziri and Downes, 1992b). The restricted lateral mobility of PLC is likely to limit the amount of phosphoinositide substrate available to the activated PLC, thereby preventing uncontrolled hydrolysis of inositol lipid constituents of the plasma membrane. It is tempting to speculate that the cytoskeleton is responsible for maintaining a discrete

signalling compartment within the cell, allowing localized synthesis of second-messenger molecules. However, it remains possible that the metabolically separate compartments might simply reflect variance in the competence of PLC and/or PtdIns synthase within populations of erythrocytes, for example, of differing age.

Nevertheless, the results clearly show that turkey erythrocytes contain a plasma-membrane-associated PtdIns synthase that can directly label hormone-sensitive pools of inositol phospholipids. Unlike many animal cells, turkey erythrocytes contain little E.R. (Beam et al., 1979). It is therefore possible that these specialized cells possess a plasma-membrane-associated PtdIns synthase in order to fulfil their requirements for PtdIns. However, since mature erythrocytes no longer synthesize new protein, it is evident that the PtdIns synthase present in plasma membranes must have been synthesized at an earlier stage in haemopoiesis when the E.R. was present. This suggests that distinct targeting mechanisms may exist for the plasma-membrane and E.R. enzymes. It would also be of interest to determine whether a PtdIns synthase reaction can be exploited to label hormone-sensitive phosphoinositides in plasma-membrane preparations from cells which contain extensively developed E.R. A potential problem with this approach is the lability of receptor- and G-protein-regulated PLC in membrane preparations from most cell types. Nevertheless, from preliminary studies it is clear that well-washed membranes from rat hepatocytes also incorporate [³H]inositol into an agonist- and GTP[S]-sensitive lipid pool via a CMP-dependent reaction with a high capacity for inositol (D. Hollyman, C. Vaziri and C. P. Downes, unpublished work). Generation of antisera to purified PtdIns synthase isoenzymes should allow immunolocalization of the enzyme in sections from whole cells, and may provide an approach to resolve the controversy concerning the subcellular site(s) of PtdIns synthesis.

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