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Identification of a novel cytosolic poly-phosphoinositide-specific phospholipase C (PLC-86) as the major G-protein-regulated enzyme

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Activation of phosphoinositide-specific phospholipase C (PLC) generates two intracellular signals which play major roles in many cellular processes including secretion, proliferation and contraction. PLC activation by many receptors occurs via a guanine nucleotide regulatory protein, G_p. PLCs are found predominantly in the cytosolic fraction though some activity is membrane-associated. At least four families of isoenzymes of PLC (α, β, γ and δ) have been identified, but there is only scant evidence to indicate that any of the mammalian cytosolic activities are involved in G-protein-regulated signalling. In this study we demonstrate that the PLC activity from rat brain cytosol can be regulated in a G-protein-dependent manner in a reconstituted system using pre-permeabilized HL60 cells. We identify two enzymes, PLC-β and a novel 86 kDa enzyme (designated PLC-ϵ) as the G-protein-regulated enzymes. PLC-ϵ was found to be the major G-protein-regulated enzyme.

Key words: isoforms of brain phospholipases C/permeabilized HL60 cells/reconstitution of phospholipase C

Introduction

Phosphoinositide-specific phospholipase Cs (PLCs) are known to be present in the cytosol of many cells but their role in receptor/G-protein-stimulated transmembrane signalling is not yet clear. Purification and molecular cloning has revealed that PLCs constitute several families of isoenzymes designated α, β, γ and δ (Rhee *et al.*, 1989; Kritz *et al.*, 1990; Meldrum *et al.*, 1991). The β, γ and δ isoforms of PLC each contain two regions of high homology, which are considered to be important for the catalytic activity of these proteins. Of these isoforms only the regulation of PLC-γ is understood. This isoform is thought to be activated by growth factor receptors (EGF and PDGF) by tyrosine phosphorylation (Wahl *et al.*, 1989).

Activation of many cell surface receptors stimulates PLC activity via G-proteins (G_ps) (Cockcroft, 1987). Studies on the identification of the G-protein-regulated PLC activity have been impaired because membrane preparations do not appear to contain all the components of the inositide signalling system. This has been demonstrated by comparing the fMetLeuPhe- and the GTPγS-regulated PLC activity in HL60 membrane preparations and in streptolysin O permeabilized HL60 cells (Cockcroft and Stutchfield, 1988; Stutchfield and Cockcroft, 1991). The main difference between these two systems is the extent of the response to stimulation. The response in membranes is only a small fraction of that observed in permeabilized cells. This strongly argues in favour of component(s) that are present in the cell that are lost during the preparation of the membranes.

Evidence is now provided that this component is the cytosolic PLC. The cytosolic PLC (prepared from rat brain) could be used to restore the lost GTPγS responses in a reconstitution system. One of the PLC isoforms that is linked to G_p is identified to belong to the β family. This concurs with similar observations in rabbit brain (Carter *et al.*, 1990), turkey erythrocytes (Morris *et al.*, 1990) and *Drosophila* retina (Bloomquist *et al.*, 1988). PLC-γ isoform was unable to be activated by G-proteins. Additionally, another PLC possibly not belonging to either the α, β, γ or δ family was also found to be linked to G-proteins. This novel PLC (PLC-86) is the most efficient activity that is able to restore the responsiveness to GTPγS in a reconstitution system consisting of HL60 cells depleted of endogenous PLCs.

Results and discussion

Incubation of HL60 cells with streptolysin O causes lesions in the plasma membrane, large enough for cytosolic proteins to leak out of the cells within 5 min (Stutchfield and Cockcroft, 1988). Introduction of GTPγS at the time of permeabilization leads to the activation of PLC (Stutchfield and Cockcroft, 1988). Figure 1A illustrates that, if addition of GTPγS is delayed after permeabilization, then the ability of GTPγS to activate PLC diminishes as the permeabilization interval increases. Ten minutes after permeabilization, there is no further loss of GTPγS-stimulated PLC activity. A residual component of GTPγS-stimulated PLC activity remains even when assayed 60 min later. This residual component is similar in magnitude to the GTPγS-stimulated PLC activity observed previously in membrane preparations (Cockcroft and Stutchfield, 1988).

The progressive loss of GTPγS-stimulated PLC activity from the pre-permeabilized cells correlates with the loss of the PLC enzyme(s) from the cell (Figure 1B). The release of enzyme(s) into the external medium (determined *in vitro* using PIP_2 as substrate) is essentially complete within 20 min. Although the exit of the enzyme is slower than the loss of GTPγS-stimulated PLC (compare Figure 1A and B), these results do suggest that the exit of the enzyme(s) could explain the loss of responsiveness to GTPγS. Alternatively, loss of GTP-binding protein(s) could also account for the results. However, this is unlikely since the heterotrimeric G-proteins are generally membrane-anchored by myristoylation (Jones *et al.*, 1990; Mumby *et al.*, 1990). Other possibilities such as depletion of substrate for PLC were also considered; this was not found to be a major contributory factor. The ratio of the three inositol lipids, PI:PIP:PIP_2, before and after permeabilization for 30 min was 93.8:3.9:2.3 and 92:6.1:1.8 respectively.

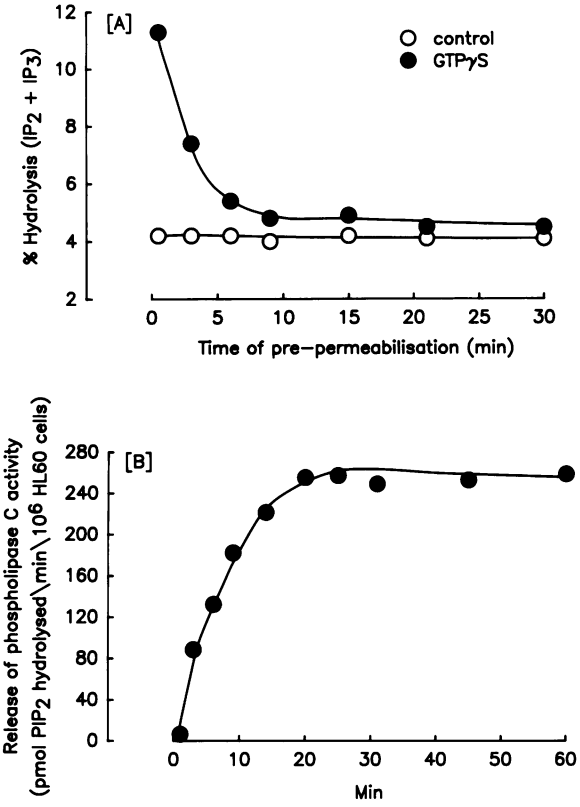

Fig. 1. [A] HL60 cells were permeabilized with streptolysin O for different lengths of time and then transferred to tubes containing GTPγS (60 μM) and Ca^{2+} (100 nM) for determination of GTPγS-stimulated PLC activity. [B] HL60 cells were permeabilized for different lengths of time and the cells sedimented by centrifugation. The supernatant was analysed for PLC activity measured against phosphatidylinositol bisphosphate. Total PLC activity was also determined after sonication and was found to be 300 pmol/min/10^6 HL60 cells.

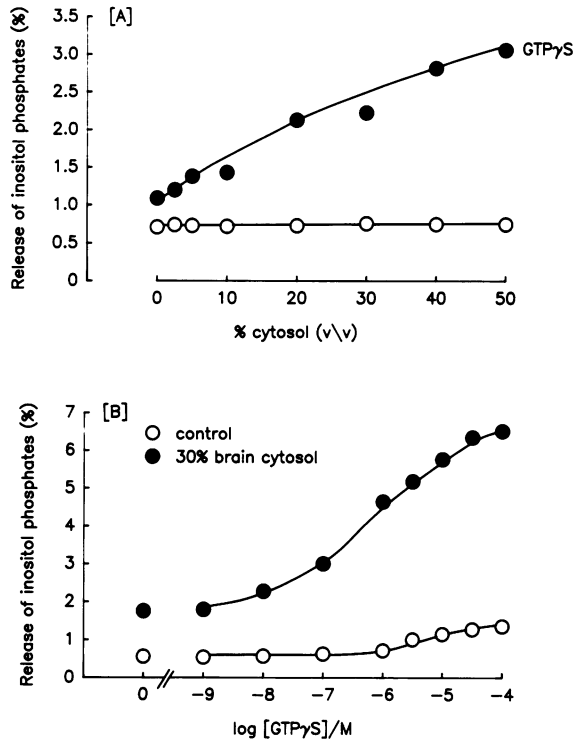

Fig. 2. Restoration of PLC responsiveness to GTPγS in pre-permeabilized HL60 cells with rat brain cytosol. [A] Dependence on cytosol concentration, [B] dependence on GTPγS concentration. In [A] 90 μl of pre-permeabilized HL60 cells plus 10 μl of GTPγS (final concentration 10 μM) were incubated with cytosol diluted (v/v) as indicated in a total reaction volume of 200 μl. The activity of the PLC in undiluted cytosol was 300 ± 6 nmol PIP_2 hydrolysed/min/ml (specific activity 50 nmol/min/mg). In [B] cytosol was diluted to 30% (v/v) and the concentration of GTPγS was varied as indicated. After incubating the samples for 10 min at 37°C, the samples were analysed for inositol phosphates.

The difference in time dependence of the loss of responsiveness to GTPγS and exit of PLC into the medium is probably accounted for by the presence of multiple species of PLC of which only some are regulated by G-proteins. From cDNA cloning, HL60 cells are known to contain at least two PLC genes, most closely related to PLC-γ (PLC-γ2) and PLC-β (PLC-β3) (Kritz *et al.*, 1990). These isoenzymes possess only a limited overall sequence homology (Katan *et al.*, 1988; Suh *et al.*, 1988b) and it is also clear that they are subject to different forms of control, e.g. tyrosine phosphorylation for PLC-γ (Nishibe *et al.*, 1990).

If the exit of PLCs accounts for the loss of responsiveness to GTPγS, then it should be possible to restore responsiveness to GTPγS by adding back exogenous PLC. PLCs from brain are best characterized (Ryu *et al.*, 1987b; Suh *et al.*, 1988b) and therefore rat brain cytosol was selected as a source of these enzymes. For reconstitution, we used HL60 cells which were pre-permeabilized with streptolysin O for 30 min, conditions under which the majority of the GTPγS-stimulated PLC activity is lost. Figure 2A illustrates that the brain cytosol is able to stimulate release of inositol phosphates in the presence of GTPγS. Cytosol alone had a marginal effect in the absence of GTPγS and this varied from preparation to preparation. The restoration of the response is dependent on the concentration of GTPγS (Figure 2B), is specific for GTPγS (GTP, GDP, ATPγS, AppNHp, ITP or XTP were inactive; tested at 100 μM) and is inhibited by GDPβS (data not shown). All these characteristics are similar to the previously described G-protein regulation of PLC in streptolysin O permeabilized HL60 cells and in HL60 membranes (Cockcroft and Stutchfield, 1988; Stutchfield and Cockcroft, 1988).

Release of all three inositol phosphates (IP_1, IP_2 and IP_3) is stimulated by GTPγS plus cytosol (Figure 3A and B). After a delay of 2 min, an increase in all three inositol phosphates is apparent. The increase in IP_2 plus IP_3 declines after 10 min (Figure 3A) but the increase in IP_1 remains linear for 20 min (Figure 3B). The presence of phosphatases that can degrade the inositol polyphosphates is most likely to account for the production of inositol monophosphates. HPLC analysis of the monophosphates shows the presence of both I-1-P/I-3-P and I-4-P isomers (data not shown) indicating that the monophosphates were probably derived from inositol polyphosphates. This would suggest that the polyphosphoinositides are the major substrates for the PLC. It should be noted that GTPγS (in the absence of cytosol) stimulates the release of IP_2 and IP_3 only, supporting the notion that polyphosphoinositides are the main substrates for PLC when presented in a biological membrane (Figure 3B).

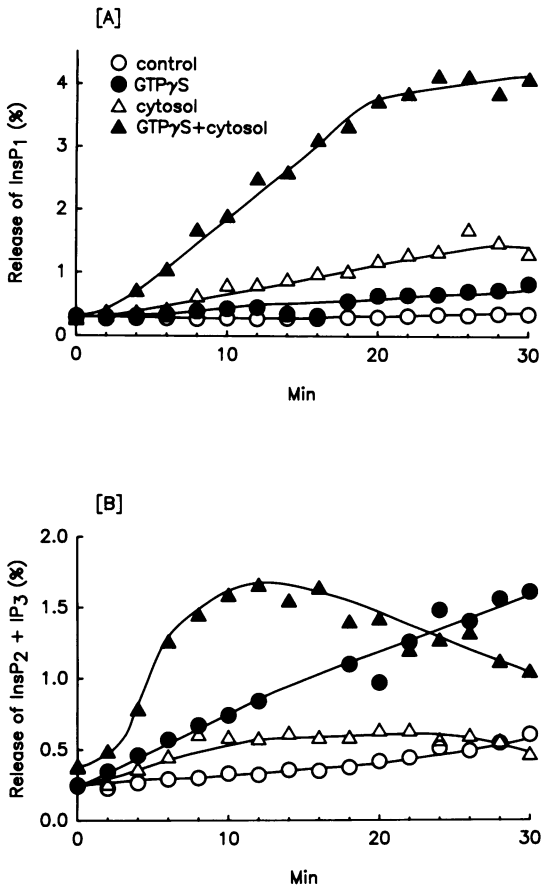

Fig. 3. Time-dependence of [**A**] IP_1 and [**B**] IP_2 plus IP_3 production. Pre-permeabilized HL60 cells were stimulated with GTPγS (10 μM) in the presence and absence of rat brain cytosol (30% v/v). 90 μl of pre-permeabilized HL60 cells plus 10 μl of GTPγS (final concentration 10 μM) were incubated with 60 μl cytosol to give 30% (v/v) in a total reaction volume of 200 μl. The activity of the PLC in undiluted cytosol was 300 ± 6 nmol PIP_2 hydrolysed/min/ml (specific activity 50 nmol PIP_2/min/mg). After incubating the samples for the indicated period at 37°C, the samples were analysed for inositol phosphates.

To identify which of the isoforms of PLC present in brain cytosol is activated by the G-protein, the cytosol was fractionated on a heparin–Sepharose column (Figure 4). PLC activity was assessed in the column fractions with phosphatidylinositol (PI) and phosphatidylinositol bisphosphate (PIP_2) as substrates. Three major peaks (I, II and III) of activity could be identified. The fractions containing the activity were pooled, concentrated and tested for their ability to restore GTPγS-stimulated PLC activation in pre-permeabilized HL60 cells.

Peaks I and III were effective at reconstitution (Figure 5) but not peak II. The hydrolysis of total inositol lipids was 1.9% and 2.01% in the absence and presence of 20 μl of peak II respectively when assayed in the presence of GTPγS. Peak II was identified as PLC-γ by immunoblotting with specific antibodies (Figure 6B). Results from five separate preparations showed that the fraction identified as PLC-γ was totally ineffective in restoring the responsiveness to GTPγS. The highest activity of the PLC-γ preparation tested was comparable with the total activity present in the cytosol preparations previously described. The PLC-γ isoform has been demonstrated to be regulated by tyrosine phosphorylation by EGF and PDGF receptor (Wahl *et al.*, 1989) and therefore it is not surprising that it is not under G-protein regulation.

Peak III contains both PLC-β1 and PLC-δ1 as determined by immunoblotting (Figure 6A and C). Maximal reconstitution with this peak is observed when the final PLC activity present in a 45 μl reaction volume was 0.75 nmol PIP_2 hydrolysed/min (Figure 5B and D). The extent of reconstitution was much smaller than that observed with the crude cytosol. PLC-β1 purified from bovine brain was also effective at reconstitution. The reconstitution observed with bovine brain PLC-β1 (final activity present in a 45 μl incubation was 1 nmol/min) was similar in magnitude to that observed for peak III. PLC-β1 is susceptible to proteolytic cleavage to yield a smaller fragment of 100 kDa (Ryu *et al.*, 1987a). In our early experiments we found that reconstitution with this fraction was poor, and this coincided with proteo-

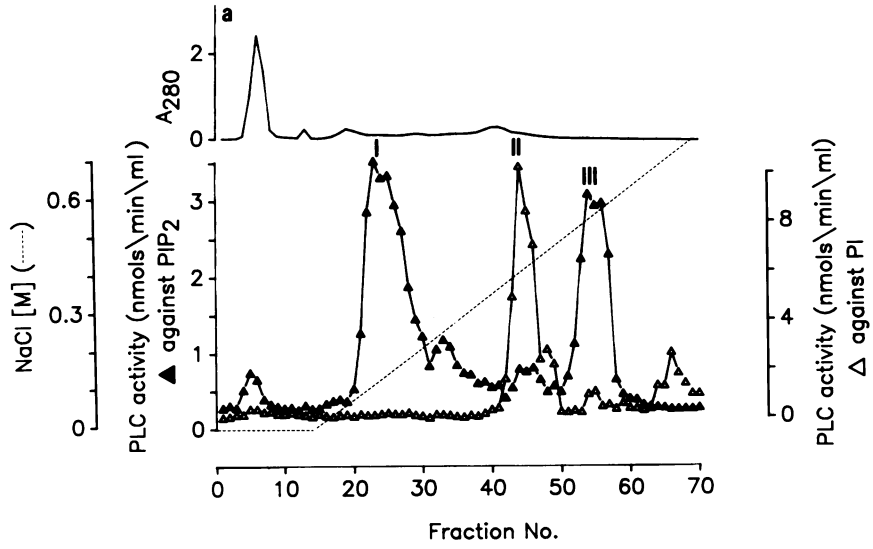

Fig. 4. Fractionation of rat brain cytosol on heparin–Sepharose. PLC activity was assayed against phosphatidylinositol bisphosphate or against phosphatidylinositol. Three peaks of activity were identified, labelled peaks I, II and III.

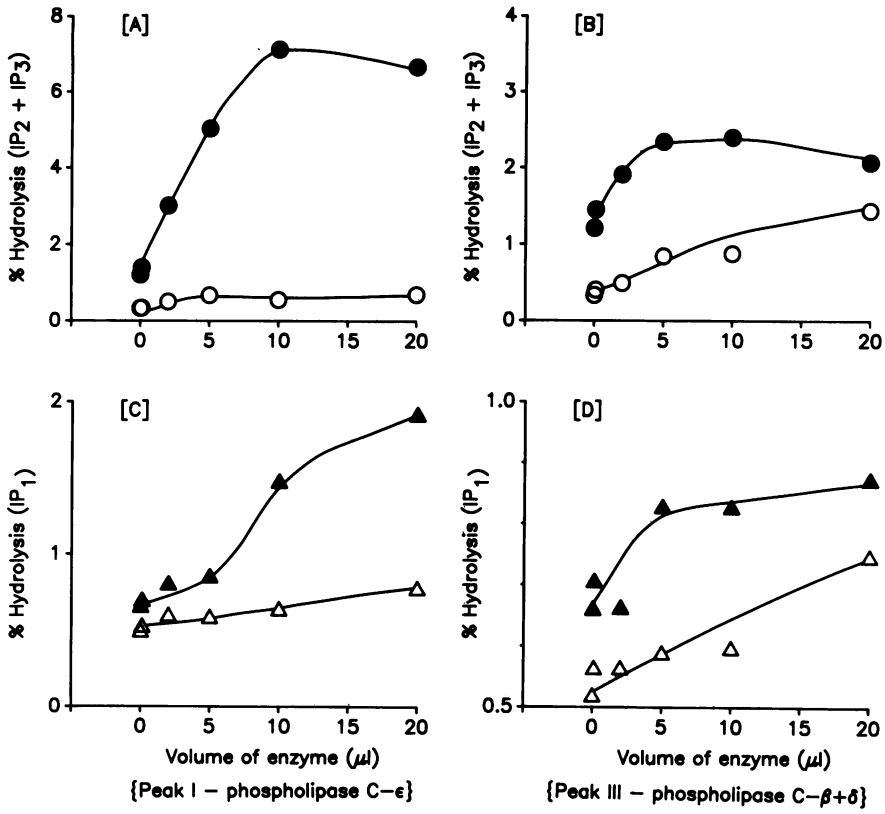

Fig. 5. Assay for reconstitution of GTPγS-stimulated PLC activation with peaks of activity separated on heparin–Sepharose. Peaks I, II and III were assayed for their ability to restore GTPγS responses after concentration and dialysis. 20 μl of pre-permeabilized cells were incubated with the indicated volume of enzyme in a final incubation volume of 45 μl in the absence (open circles) or presence (closed circles) of 10 μM GTPγS. After 20 min incubation at 37°C, the samples were assayed for released inositol phosphates. [**A**] and [**B**], IP_2 plus IP_3; [**C**] and [**D**], IP_1. [A] and [C], PLC activity from peak I (PLC-ε) and [B] and [D], PLC activity from peak III (PLC-β plus PLC-δ). The activities (assayed against PIP_2) in peaks I and III were 160 nmol/min/ml (specific activity 37 nmol/min/mg) and 150 nmol/min/ml (specific activity 50 nmol/min/mg) respectively. Peak II (identified as PLC-γ) with an activity of 228 nmol PIP_2 hydrolysed/min/ml (specific activity 31 nmol/min/mg) was also tested and was not effective. The hydrolysis of total inositol lipids was 1.9% and 2.01% in the absence and presence of 20 μl of peak II respectively, when assayed in the presence of GTPγS.

lytic cleavage of PLC-β to a truncated form of molecular weight 100 kDa. Proteolytic cleavage of PLC-δ1 was not observed in the same preparation. This would indicate that PLC-δ1 cannot be regulated in a G-protein-dependent manner.

Peak I was most effective at restoring responsiveness to GTPγS (Figure 5A). It predominantly released IP_2 plus IP_3 in contrast to cytosol (compare Figures 3A and B and 5A and C) presumably due to the absence of inositol phosphatases. Although maximal reconstitution was observed at activity levels similar to those for PLC-β1, the response obtained was much larger in magnitude. The net hydrolysis of inositol lipids observed with peak I was 7% compared with 1.7% with peak III. The magnitude of the reconstitution observed with this fraction is similar to the response observed in the HL60 cells measured at the time of permeabilization (compare Figure 1A and Figures 5A and C) as well as the reconstitution observed with cytosol.

The PLC activity present in peak I was not recognized by antibodies against PLC isoforms β1, γ1 or δ1 (Figure 6A–C). This PLC activity could not hydrolyse PI (Figure 4) or be precipitated at pH 5 (data not shown). Most of the previously characterized PLCs from brain (β1, γ or δ1/δ2) have been purified after acid precipitation of proteins (Ryu *et al.*, 1987b; Meldrum *et al.*, 1989). These two characteristics distinguish this enzyme from the previously characterized PLCs. The molecular weight of the PLC

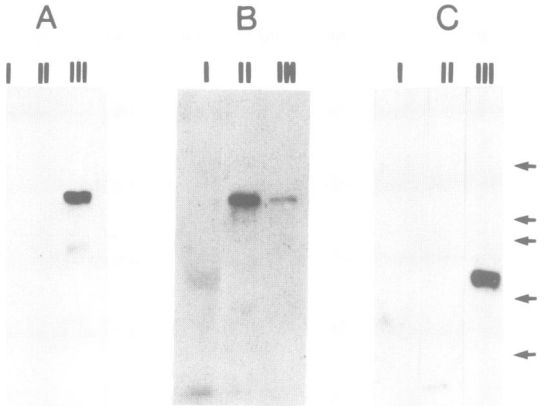

Fig. 6. Western blotting of peaks I, II and III. The fractions containing PLC activity from the heparin–Sepharose column were pooled and concentrated 40- to 50-fold (peaks I, II and III). Peaks I, II and III were subjected to electrophoretic separation on SDS–gel, blotting and analysis with a mixture of monoclonal antibodies against PLC-β_1 [**A**], PLC-γ_1 [**B**] and PLC-δ_1 [**C**]. To detect proteins recognized by the antibodies, the blot was exposed to ^{125}I-labelled goat anti-mouse IgG. The molecular weight markers used were 200, 116, 97, 66 and 42 kDa.

activity determined by gel permeation chromatography was estimated to be 86 kDa (Figure 7). These characteristics suggest that it may be one of the two immunologically and

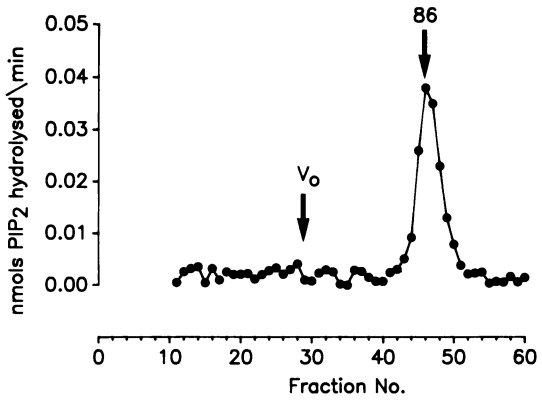

Fig. 7. Gel permeation chromatography of peak I (designated PLC-ϵ). Peak I (100 μl) was loaded onto a Superose-12 column (Pharmacia) and eluted with 20 mM PIPES, 2.7 mM KCl and 10% glycerol, pH 6.8. Molecular size was determined by reference to a calibration curve constructed using proteins of known molecular size. Fractions were assayed for PIP_2-hydrolysing activity.

Table I. Effect of GTPγS on cytosolic PLC activity

Assay conditions	GTPγS (10 μM)	Cytosolic PLC activity (nmol/min/ml)	
		Peak I (PLC-ϵ)	Peak III (PLC-β+δ)
Pure PIP_2 + 100 nM Ca^{2+}	−	5.82	1.2
	+	5.74	1.0
Pure PIP_2 + 200 μM Ca^{2+}	−	103	94
	+	112	91
Liposomes made from HL60 cell lipids + PIP_2 + 100 nM Ca^{2+}	−	n.d.	n.d.
	+	n.d.	n.d.

The two peaks of activity that could reconstitute with pre-permeabilized HL60 cells in the presence of GTPγS were tested for their ability to be activated by GTPγS using pure PIP_2 as substrate as well as liposomes containing PIP_2 plus extracted HL60 lipids. The assays were done under similar conditions to the reconstitution experiments with permeabilized cells, i.e. in the presence of 1 mM MgATP and 100 nM Ca^{2+} (pH 6.8) except that sodium cholate was also present when pure PIP_2 was used as substrate. Enzyme activity was also tested under the usual assay conditions, i.e. 200 μM Ca^{2+} with pure PIP_2 as substrate.
n.d., not detected.

structurally distinct 85 kDa enzymes previously purified from rat brain by Homma *et al.* (1988). We tentatively designate this enzyme to belong to a different family (PLC-ϵ) rather than a member of the δ family because of the substantial biochemical (e.g. substrate specificity) differences as well as different peptide patterns after proteolytic cleavage (Homma *et al.*, 1988).

The reconstitution experiments utilized the HL60 pre-permeabilized cells as a source of both the substrate and the G-protein(s). Whilst there is no ambiguity regarding the HL60 cell preparation as being the source of the substrate for the PLC, additional experiments were done to eliminate the possibility that the partially pure enzymes could also be the source of G-proteins. It has been reported that the soluble PLCs can be activated by GTPγS in the absence of membranes (Banno *et al.*, 1986; Dekmyn *et al.*, 1986; Baldassare *et al.*, 1988). We have therefore tested for the effect of GTPγS on the fractions containing peaks I and III using the pure PIP_2 or liposomes containing PIP_2 combined with HL60 lipids. The assays were conducted at 100 nM Ca^{2+} in the presence of 1 mM MgATP, the conditions used for the reconstitution experiments. Table I illustrates that GTPγS did not have any effect on the cytosolic PLC activity in either peak I (PLC-ϵ) or peak III (PLC-β+δ). The activity of the preparations used for the assays was kept at a similar concentration to that which gave maximal reconstitution in pre-permeabilized cell preparation (Table I).

The results described here demonstrate that at least two cytosolic PLC activities can be regulated by G-protein(s). Support for this conclusion comes from the observation that exit of endogenous PLC from the HL60 cells correlates with the loss of GTPγS-stimulated PLC activation. Direct evidence that the cytosolic PLCs can hydrolyse polyphosphoinositides is provided by the studies utilizing cytosol prepared from rat brain. Of the different PLCs present in the cytosol, PLC-β1 and PLC-ϵ (designated PLC-ϵ to distinguish it from PLC-δ1/δ2) are identified as the G-protein-regulated PLCs. Evidence that a membrane-bound form of PLC-β is linked to G-proteins has been demonstrated recently in studies in rabbit brain (Carter *et al.*, 1990). However, the PLC-ϵ identified here appears to be the major cytosolic PLC that can be regulated by the G-proteins present in HL60 cells.

Materials and methods

Materials

Streptolysin O was obtained from Wellcome Diagnostics, Dartford, Kent, UK. Antibodies against PLC isoenzymes (Suh *et al.*, 1988a) were a gift from Dr S.G.Rhee.

Measurement of GTPγS-stimulated PLC activity in permeabilized HL60 cells

HL60 cells were maintained in suspension culture and labelled with 1 μCi/ml [^{3}H]inositol for 48 h. Determination of GTPγS-stimulated PLC activity from HL60 cells was assessed as described previously (Cockcroft and Stutchfield, 1988; Stutchfield and Cockcroft, 1988). In brief, the cells were permeabilized with streptolysin O (0.4 i.u./ml) in the presence of 100 nM Ca^{2+} (buffered with 100 μM EGTA) and 2 mM MgATP. At times indicated the permeabilized cells were transferred to tubes containing 100 nM Ca^{2+} (buffered with 3 mM EGTA) alone or 100 nM Ca^{2+} plus GTPγS (60 μM) for determination of GTPγS-stimulated PLC activation. The permeabilized cells were incubated for 10 min at 37°C, quenched with 1 ml of ice-cold 0.9% NaCl and sedimented at 1000 *g* for 5 min at 4°C. One ml of the supernatant was analysed for the inositol phosphates as described previously (Cockcroft and Stutchfield, 1988). The radioactivity in the inositol phosphates is expressed as the percentage of the total radioactivity present in the inositol lipids.

In vitro assay of PLC

Release of PLC enzyme was measured from HL60 cells after permeabilization with streptolysin O (0.4 i.u./ml) in the presence of 100 nM Ca^{2+} (buffered with 100 μM EGTA) at 37°C. At the times indicated, duplicate samples were withdrawn and centrifuged. The supernatant was assayed for PLC activity measured against PIP_2 as described previously (Katan and Parker, 1987). After sonication the total PLC activity was found to be 300 pmol/min/10^6 HL60 cells.

Preparation of rat brain cytosol

Rat brain cytosol was prepared from two brains. After homogenization in 8 ml buffer (20 mM PIPES, pH 6.8, containing 50 μg/ml PMSF) (20 strokes) at 4°C, the homogenate was centrifuged (1 h, 100 000 g_{av}) to pellet the membrane. The supernatant was dialysed overnight in 500 ml of buffer containing 20 mM PIPES, 137 mM NaCl and 2.7 mM KCl.

Reconstitution of GTPγS-stimulated PLC activation in pre-permeabilized HL60 cells with rat brain cytosol

[^{3}H]Inositol-labelled HL60 cells were permeabilized with streptolysin O for 30 min and washed at 4°C. Pre-permeabilized cells (100 μl) were

incubated in the presence of cytosol in the presence or absence of GTPγS as indicated in the individual figures in a total incubation volume of 200 μl. Activity of the PLC in the cytosol was 300 ± 6 nmol PIP_2 hydrolysed/min/ml (specific activity 50 nmol/min/mg). After incubating the samples for 10 min (or as indicated in the figure) at 37°C, the samples were quenched with 0.9% saline and analysed for inositol phosphates.

Fractionation of rat brain cytosol on heparin – Sepharose

Three rat brains were homogenized in 25 ml buffer (20 mM PIPES, 2.7 mM KCl, pH 6.8, containing 5 mM EGTA, 5 mM EDTA, 10 mM benzamidine, 1 mM dithiothreitol, 1 μg/ml soyabean trypsin inhibitor, 5 μg/ml aprotinin, 2 μM pepstatin A, 100 μM TLCK, 0.1 mM leupeptin and 1 mM PMSF), (20 strokes) at 4°C. The homogenate was centrifuged (1 h, 150 000 g_{av}) to pellet the membranes. The supernatant (14 ml) was filtered (0.45 μm) and loaded on a heparin–Sepharose column. After washing the column with buffer (20 mM PIPES, 2.7 mM KCl, pH 6.8), the adsorbed proteins were eluted with a gradient of NaCl (0–700 mM). Six-ml fractions were collected and 25 μl of each column fraction was analysed for PLC activity against PI and PIP_2. In brief, PI was assayed at pH 5.5 in 50 mM Tris-maleate buffer containing 129 nmol of a mixture of ^{3}H-labelled and non-radioactive PI, 1 mM $CaCl_2$ and enzyme fraction (25 μl) in a final incubation volume of 50 μl (Hirasawa *et al.*, 1982). PIP_2 was assayed at pH 6.8 in 20 mM PIPES buffer containing 100 μM of a mixture of ^{3}H-labelled and non-radioactive PIP_2, 0.6% sodium cholate (w/v), 200 μM $CaCl_2$, 5 mM mercaptoethanol, 100 mM NaCl and enzyme fraction (20 μl) in a final incubation volume of 50 μl (Katan and Parker, 1987).

Reconstitution of GTPγS-stimulated PLC activation in pre-permeabilized HL60 cells with peaks of activity separated on heparin – Sepharose

The fractions containing PLC activity were pooled and concentrated 40- to 50-fold (peaks I, II and III). The concentrated samples were dialysed into 20 mM PIPES, 137 mM NaCl and 2.7 mM KCl prior to use. The enzyme preparation was added to pre-permeabilized cells as described for the cytosol except that 20 μl of pre-permeabilized cells were incubated with the indicated volume of enzyme in a final incubation volume of 45 μl. After 20 min of incubation at 37°C, the samples were quenched and assayed for released inositol phosphates. The activities (assayed against PIP_2) in peaks I and III were 160 nmol/min/ml (specific activity 37 nmol/min/mg) and 150 nmol/min/ml (specific activity 50 nmol/min/mg) respectively. Peak II (identified as PLC-γ) with an activity of 228 nmol/min/ml (specific activity 31 nmol/min/mg) was also tested and was not effective (data not shown).

Western blotting of peaks I, II and III

Antibodies to phospholipase β_1, γ_1 and δ_1 (Suh *et al.*, 1988) were used to identify the isoforms present in the pooled fractions, peaks I, II and III. Peaks I, II and III were subjected to SDS–PAGE followed by immunoblotting with a mixture of monoclonal antibodies against PLC-β_1 [A], PLC-γ_1 [B] and PLC-δ_1 [C]. To detect proteins recognized by the antibodies, the blot was exposed to ^{125}I-labelled goat anti-mouse IgG. The molecular weight markers used were 200, 116, 97, 66 and 42 kDa.

Gel permeation chromatography of peak I (designated PLC-ε)

Peak I (100 μl) was chromatographed on a Superose-12 column (Pharmacia) in a buffer consisting of 20 mM PIPES, 2.7 mM KCl and 10% glycerol, pH 6.8. Molecular size was determined by reference to a calibration curve constructed using proteins of known molecular size. Fractions were assayed for PIP_2-hydrolysing activity.

Effect of GTPγS on PLC activity in the absence of membranes

Peaks I and III (after heparin–Sepharose chromatography) were assayed for PLC activity against PIP_2 as substrate in the presence and absence of 10 μM GTPγS at two Ca^{2+} concentrations, 100 nM (conditions used for the reconstitution assay) and 200 μM (conditions used for the usual determination of PLC activity) (Katan and Parker, 1987) (pH 6.8). In addition 1 mM MgATP was also included. The substrate, PIP_2, was presented either as a pure substrate as described (Katan and Parker, 1987) or was presented as liposomes prepared as described by Deckmyn *et al.* (1986) except that lipids were extracted from 10^8 HL60 cells.

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