Phospholipase C- β_1 is regulated by a pertussis toxin-insensitive G-protein

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Regulation of phospholipase C (PLC) by receptors is mediated either through protein tyrosine phosphorylation or by activation of GTP-binding proteins (G_p). For the latter, pertussis toxin (PT)-sensitive and -insensitive pathways have been described, indicating PLC regulation by at least two types of G-proteins. The identity of PLC isoenzymes which are regulated by either type of G_p remains to be determined. Thyrotropin-releasing hormone stimulates a PLC in GH_3 cells via a PT-insensitive G_p . Reconstitution methods for the assay of the GH_3 -cell G_p were developed. Previously, the membrane PLC was found to be reversibly extracted from membranes by high salt and to be activated by guanosine 5'-[γ -thio]triphosphate (GTP[S]) only when membrane-associated, suggesting that G_p was retained in salt-extracted membranes. In the present work, G_p was cholate-solubilized from PLC-deficient membranes and incorporated into phospholipid vesicles, which were found to confer GTP[S]- and AlF_4^- -stimulated activity on a solubilized membrane PLC. The reconstitution provided a direct assay for the GH_3 -cell G_p which was shown to be distinct from G_i , G_o and G_s proteins by immunodepletion studies. Incorporation of G-protein β - γ subunits into phospholipid vesicles with G_p inhibited GTP[S]-stimulated activity in the reconstitution. The results indicated that G_p is a heterotrimeric G-protein with the properties expected for the PT-insensitive GH_3 -cell G_p protein. PLC- β_1 was fully purified and shown to be regulated by G_p in the reconstitution. In contrast, PT-sensitive G-proteins failed to affect the activity of PLC- β_1 . The results indicate (1) that a PT-insensitive G_p regulates PLC- β_1 and (2) that PT-sensitive and -insensitive pathways of PLC regulation employ different PLC isoenzymes as well as different G-proteins.

INTRODUCTION

Two mechanisms have been identified by which hormone and growth-factor receptors regulate phosphoinositide hydrolysis. One of these involves growth-factor-receptor protein tyrosine kinase-mediated phosphorylation of phospholipase C (PLC)- γ [1,2]. The other mechanism is similar to that of receptor-regulated adenylate cyclase or photon-activated cyclic GMP phosphodiesterase and involves GTP-binding (G) proteins [2–5]. Hormone activation of phosphoinositide hydrolysis in membranes is potentiated by GTP and mimicked by hydrolysis-resistant GTP analogues [2–9]. Results of studies employing bacterial toxin ADP-ribosyltransferases to evaluate G-protein involvement suggest that several different G-proteins (G_p s) mediate receptor regulation of PLC. Agonist-specific and tissue-specific PLC regulation has been reported to be inhibited by either pertussis toxin (PT) [2,3] or cholera toxin (CT) [2,10], or to be unaffected by either toxin [2,4,7,9]. For PT-sensitive systems, PLC regulation appears to involve G_i and/or G_o [11–15]. PT-insensitive G-proteins that regulate PLC activity have recently been described [16,17].

The study of G_p regulation of PLC activity would be facilitated by the development of functional reconstitution methods analogous to those used for the study of the regulation of adenylate cyclase [18]. Several cytosolic and membrane PLCs have been purified and characterized [2,19,20]. Although studies have documented a role for PLC- γ in growth-factor signal transduction [1], the role of other PLC isoenzymes remains to be established. Attempts to regulate the activity of purified PLCs with purified heterotrimeric G proteins have met with limited success [17,19,20].

In the present paper, we describe a reconstitution assay for a membrane G_p present in GH_3 pituitary cells. Well-washed GH_3 -cell membranes exhibit PLC activity which is stimulated by thyrotropin-releasing hormone (plus GTP) or by non-hydrolysable GTP analogues [6,8]. The regulation of PLC activity by hormone (plus GTP) is PT-insensitive [7,21]. In a previous study [22], we found that the membrane PLC was solubilized by salt extraction and that the soluble enzyme lacked guanosine 5'-[γ -thio]triphosphate (GTP[S])-stimulated activity, suggesting that the PLC was separated from G_p . Incubation of the desalted membrane enzyme, or a similarly prepared brain membrane PLC, with extracted membranes resulted in the association of the PLC with the membrane and the reconstitution of GTP[S]-stimulated activity. It was concluded that G_p remained membrane-associated and was able to couple with the solubilized membrane PLC [22]. In the present study, G_p was cholate-solubilized from PLC-deficient GH_3 -cell membranes, incorporated into phospholipid vesicles and shown to confer GTP[S]-regulated activity on purified PLC- β_1 . In contrast, PT-sensitive G-proteins failed to affect the activity of PLC- β_1 . The results indicate that PT-sensitive and -insensitive pathways of PLC regulation employ distinct PLC isoenzymes as well as distinct heterotrimeric G-proteins.

MATERIALS AND METHODS

PLC assay

Assays were conducted at 37 °C for 60 min (unless otherwise noted) in a total volume of 50 μ l containing 0.02 M-Tris, pH 7.0, 0.1 M-NaCl, 2.5 mM-MgCl₂, 2 mM-EGTA, CaCl₂ (added to provide 30 nM free Ca²⁺, [7]) and [³H]PtdInsP₂.

Abbreviations used: PLC, phospholipase C; NEtMal, *N*-ethylmaleimide; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; GTP[S], guanosine 5'-[γ -thio]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β -imidazole]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; G-protein, GTP-binding protein; AMF, 10 μ M-AlCl₃/6 mM-MgCl₂/10 mM-NaF; PT, pertussis toxin; CT, cholera toxin.

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[^3H]PtdIns P_2 substrate was prepared as previously described [22]; final concentrations of lipids in the assay were 100 μM -PtdIns P_2 (2×10^4 d.p.m./tube), 400 μM -phosphatidylethanolamine (PtdEtn), 400 μM -phosphatidylserine (PtdSer) and ~ 0.5 mM-sodium cholate. Ptd[2- ^3H]Ins(4,5) P_2 was purchased from DuPont-New England Nuclear, and other lipids were from Sigma Chemical Corp. A KCl extract prepared from rat brain membranes or purified PLC- β_1 (see below) was used as the source of PLC. Stripped GH $_3$ -cell membranes or phospholipid vesicles containing cholate-soluble membrane proteins (see below) were used as the source of G $_p$. Incubations also contained nucleotides (GTP[S] or adenosine 5'-[γ -thio]triphosphate (ATP[S]) from Boehringer Mannheim, or GTP or ATP from Sigma) at indicated concentrations, added with equimolar MgCl $_2$. Reactions at 37 $^{\circ}\text{C}$ were initiated after a 60 min preincubation of G $_p$ and PLC at 0 $^{\circ}\text{C}$ conducted in the absence of CaCl $_2$, MgCl $_2$, NaCl, nucleotides and [^3H]PtdIns P_2 . Incubations were terminated by addition of 100 μl of 10% (w/v) trichloroacetic acid plus 50 μl of BSA (10 mg/ml). After 10 min on ice, reaction tubes were centrifuged at 1000 g for 30 min and the supernatants were analysed by liquid-scintillation counting by using Ecolume (ICN-Biomedicals). Reaction products consisted exclusively of [^3H]Ins(1,4,5) P_3 and Ins(1,4) P_2 [22].

Preparation of stripped membranes and membrane cholate extracts

GH $_3$ -cell membranes were prepared as previously described [22] and were thoroughly washed by resuspension and centrifugation at 10000 g in 0.02 M-Tris/1 mM-MgCl $_2$ /2 mM-EGTA, pH 7.5, to remove cytosolic PLC. Membrane pellets were resuspended at 1–2 mg of protein/ml in 0.02 M-Tris, pH 7.5, and treated with 3 mM-*N*-ethylmaleimide (NEtMal) for 30 min at 0 $^{\circ}\text{C}$, followed by 3 mM-dithiothreitol to inactivate the guanine-nucleotide-insensitive portion of the membrane PLC [22]. The membranes were adjusted to 2 mM-EGTA and 1.0 M-KCl and placed on a platform rocker for 60 min at 4 $^{\circ}\text{C}$. Membranes were recovered by centrifugation at 20000 g for 45 min and resuspended to 1–2 mg of protein/ml in 0.02 M-Tris/3.3 mM-EGTA, pH 7.5. The NEtMal-treated KCl-extracted membranes are referred to as 'stripped', and were shown in our previous study [22] to lack PLC activity but to contain G $_p$ activity.

For preparation of cholate extracts, stripped membranes were resuspended to 1–2 mg of protein/ml in 0.02 M-Tris/1 mM-EDTA/1 mM-dithiothreitol, pH 8.0, containing 2.5 μg of aprotinin/ml. Solid sodium cholate was added to 1.0% (w/v) and the suspension was placed on a platform rocker for 60 min at 4 $^{\circ}\text{C}$. The membrane cholate extract was isolated as the supernatant of a centrifugation at 100000 g for 60 min. The cholate extract was concentrated to the desired volume with a Centriprep-10 device (Amicon Corp.).

Preparation of phospholipid vesicles reconstituted with GH $_3$ -cell membrane cholate extract

Cholate extracts from stripped membranes were mixed with a suspension of PtdSer and PtdEtn to form a mixed detergent-lipid-protein micelle. A cholate extract prepared from membranes of 5×10^7 cells was generally concentrated to 0.5–1.0 ml and mixed with 0.15 ml of a sonicated PtdSer/PtdEtn preparation (prepared by vortex-mixing and bath-sonicating 2.5 mg of each lipid in 1 ml of 0.02 M-Tris/1 mM-EDTA/1 mM-dithiothreitol, pH 8.0, plus 2.5 μg of aprotinin/ml). Phospholipid vesicles (also termed 'liposomes') were formed by dialysis for 18 h against 1000 vol. of 0.02 M-Tris/1 mM-EDTA/1 mM-dithiothreitol, pH 8.0. Vesicles were isolated by centrifugation at 100000 g for 120 min and resuspended in 0.02 M Tris/3.3 mM-EGTA, pH 7.5, to a lipid concentration of 10 mg/ml. When unpurified cholate

membrane extracts were used as the source of protein, vesicles incorporated approx. 0.1 mg of protein/mg of lipid. Purified bovine brain G $_o$ (generously provided by R. Iyengar, Mount Sinai School of Medicine, New York, NY, U.S.A.), recombinant G $_{\alpha}$ subunit (generously provided by P. Casey, Duke University, Durham, NC, U.S.A.) and purified brain β - γ subunits (also provided by P. Casey) were incorporated into phospholipid vesicles by similar dialysis techniques.

Preparation of crude and purified PLC- β_1

The rat brain membrane PLC was prepared by homogenizing fresh rat brains in 7 vol. of 0.02 M-Hepes/0.25 M-sucrose/2 mM-EGTA/2 mM-EDTA/0.1 mM-dithiothreitol/0.4 mM-phenylmethanesulphonyl fluoride plus 0.5 μg of leupeptin/ml and the homogenate was centrifuged at 10000 g for 30 min. Pellets were washed twice by centrifugation in a large volume of 0.02 M Tris/2 mM-EGTA/1 mM-MgCl $_2$, pH 7.5, to remove cytosolic PLC thoroughly. The final membrane pellet was resuspended in 0.02 M-Tris/2 mM-EGTA/0.5 M-KCl, pH 7.5, placed on a platform rocker for 60 min at 4 $^{\circ}\text{C}$ and centrifuged at 100000 g for 60 min. The supernatant was removed, diluted 5-fold to decrease the KCl to 0.1 M, concentrated to 1–2 mg of protein/ml with a Centricon-10 apparatus and stored frozen at -20 $^{\circ}\text{C}$. This PLC preparation is referred to as 'KCl extract' and is known to contain principally PLC- β_1 (see the Results section). PLC- β_1 was purified from a fresh KCl extract by the procedure of Lee *et al.* [23] with modifications. Sequential chromatography on DEAE-cellulose, heparin-agarose, phenyl-Sepharose and Mono Q was employed. The chromatography on phenyl-Sepharose (Pharmacia LKB Biotechnology) replaced the TSK-Phenyl-5PW step of Lee *et al.* [23]. Material from the heparin-agarose step was adjusted to 3 M-KCl, and the pool was

Fig. 1. The solubilized membrane PLC regains GTP[S]-stimulated activity upon co-incubation with stripped membranes

The solubilized membrane PLC was prepared from rat brain membranes by KCl extraction (KCl extract) as described in the Materials and methods section. Stripped membranes from GH $_3$ cells were prepared by treatment with NEtMal and extraction with KCl as described. Hydrolysis of [^3H]PtdIns P_2 was determined in standard incubations conducted in the absence (○) or presence (●) of 10 μM -GTP[S]. Panels (a) and (b) show incubations with indicated amounts of KCl extract incubated alone or with 30 μg of stripped membranes, respectively. A zero-time incubation value representing less than 2% of the total [^3H]PtdIns P_2 substrate has been subtracted from all values. Ordinate values represent $100 \times$ d.p.m. of acid-soluble ^3H /total d.p.m. of [^3H]PtdIns P_2 (%). Note that membranes alone (ordinate value in b) lacked PLC activity.

Fig. 2. Reconstitution of GTP[S] stimulation of PLC activity with phospholipid vesicles containing G_p

$[^3H]PtdInsP_2$ hydrolysis was determined for incubations which included 2 μg of rat brain KCl extract plus the indicated amounts of phospholipid vesicles (liposomes) containing cholera-soluble membrane proteins: 100 μM -GTP[S] was included where indicated by ●. Phospholipid vesicles were prepared as described in the Materials and methods section by using a membrane cholera extract.

applied at 1 ml/min to a 15 ml phenyl-Sepharose column equilibrated in 0.02 M-Hepes/3.3 mM-EGTA/0.1 mM-dithiothreitol/3.0 M-KCl/0.5 μg of leupeptin/ml, pH 7.0. The column was washed in 50 ml of this buffer, followed by 150 ml of a similar buffer adjusted to 2.0 M-KCl. The column was eluted with

a 200 ml linear gradient of a similar buffer containing from 2.0 M- to 0 KCl, followed by a 200 ml wash in the buffer at 0 KCl. Fractions eluted at 0.3–0 M-KCl containing PLC- β_1 were pooled, dialysed to 0 KCl, concentrated and loaded on to the Mono Q column as described by Lee *et al.* [23].

Immunoprecipitation of PLC and G-proteins

Immunodepletion studies were conducted with rabbit polyclonal antisera purified by chromatography on Protein A-Sepharose [24]. For each experiment, an equivalent protein amount of purified IgG prepared in parallel from non-immune rabbit serum was used as a control. Purified IgG fractions were concentrated to the original serum volume. IgG purification was essential, since proteins in crude serum were incorporated into phospholipid vesicles and strongly inhibited GTP[S]-stimulated PLC activity in the reconstituted system. Immunoprecipitations of G-proteins from cholera extracts or of PLC from KCl extracts were conducted under similar antibody-excess conditions. For PLC- β_1 immunoprecipitations, 60 μg of purified rabbit anti-PLC IgG was incubated with 40 μg of KCl extract. For G-protein immunoprecipitation, 120 μg of purified rabbit anti-G-protein peptide IgG was incubated with 160 μg of cholera-extracted membrane protein. Fractions were incubated with primary antibodies for 1 h on ice, followed by a second 1 h incubation with Protein A-Sepharose (obtained from Sigma). Immunodepleted supernatants were obtained by centrifugation at 12000 g for 20 min. The extent of immunodepletion was evaluated by analysis of supernatants by SDS/PAGE on 10%-polyacrylamide gels, followed by immunoblotting analysis by described methods [25], except that ^{125}I -Protein A (DuPont-New England Nuclear; 0.1 $\mu Ci/ml$) was used as the secondary reagent for antibody detection. Antisera used in this study were; anti-PLC- β_1 antibody, generously provided by S. G. Rhee, N.I.H., Bethesda, MD, U.S.A.; antisera GO/1 (against $G_{\alpha C}$ C-terminal peptide), EC/1

Fig. 3. Properties of the reconstituted G_p -regulated PLC

(a) $[^3H]PtdInsP_2$ hydrolysis was determined for complete incubations containing rat brain PLC and membrane-protein-containing liposomes as described in the legend to Fig. 2. Incubations were conducted for the indicated times either without (○) or with (●) 100 μM -GTP[S]. (b) The requirements for GTP[S]-stimulated hydrolysis in the reconstituted system were determined in 60 min incubations which included the components indicated. (c) The requirement of PLC- β_1 for GTP[S]-stimulated hydrolysis in the reconstituted system was determined in 60 min incubations which included 100 μM -GTP[S], 2 μg of phospholipid-vesicle-incorporated membrane protein and the indicated amounts of rat brain PLC preparation. PLC preparations were treated before assay with either non-immune (●) or PLC β_1 -specific immune sera (○) to immunoprecipitate PLC- β_1 as described in the Materials and methods section. The extent of immunodepletion was assessed in Western-blotting studies, which indicated 90% removal of PLC- β_1 (insets show 150 kDa region of immunoblot).

(against $G_{13\alpha}$ C-terminal peptide), AS/7 (against $G_{12\alpha}$ C-terminal peptide) and SW/1 (against β -subunit C-terminal peptide) generously provided by A. Spiegel, N.I.H., Bethesda, MD, U.S.A.; and antiserum RM/1 (against $G_{13\alpha}$ C-terminal peptide) purchased from DuPont-New England Nuclear.

RESULTS

Reconstitution of solubilized membrane PLC with membrane-associated G_p

Most of the PLC activity detected in GH_3 -cell membranes was shown to be solubilized by salt extraction [22]. The soluble PLC

Fig. 4. G_p regulates the activity of purified PLC- β_1

PLC- β_1 was purified as described in the Materials and methods section. (a) Fractions from the final Mono Q column step of purification were tested for PLC activity in the presence of G_p -containing liposomes as described in the legend to Fig. 2. Incubations were conducted either without (○) or with (●) $100 \mu\text{M}$ -GTP[S]. A_{280} values of column fractions are indicated (---). PLC elution in fractions 49–51 occurred at 0.29 M-KCl. (b) Column fractions representing about $10 \mu\text{g}$ of protein were analysed by SDS/PAGE on 8% polyacrylamide gels. Protein was stained with Coomassie Blue. (c) Western-blotting studies were conducted after gel electrophoresis as in (b). Immunoblotting with PLC- β_1 -specific rabbit antisera was conducted as described in the Materials and methods section.

lacked GTP[S]-regulated activity, suggesting that it was extracted free of G_p . Restoration of the ionic strength of the GH_3 -cell enzyme preparation, or of a similarly prepared rat brain membrane enzyme, allowed reassociation of the soluble PLC with stripped membranes, and the PLC regained GTP[S]-stimulated activity. That G_p was present in the PLC-deficient membrane was indicated by the fact that stripped membranes could be preactivated by GTP[S] [22]. In more recent studies, G_p -PLC coupling was found to be reconstituted by simply co-incubating the PLC with stripped GH_3 -cell membranes, as shown in Fig. 1. The soluble membrane PLC was unresponsive to GTP[S] (Fig. 1a) and stripped GH_3 -cell membranes lacked PLC activity (Fig. 1b, point on ordinate). However, incubation of the PLC (KCl extract) with stripped membranes restored GTP[S]-stimulated PLC activity (Fig. 1b). The results indicated that the GH_3 -cell membrane G_p could functionally couple with a solubilized brain membrane PLC.

Regulation of solubilized membrane PLC by detergent-solubilized G_p incorporated in phospholipid vesicles

In additional experiments (results not shown), cholate extraction was found to remove the membrane component, G_p , essential for restoring the GTP[S] regulation of PLC activity. This result suggested the possibility of reconstituting this component into phospholipid vesicles. Membrane proteins were cholate-solubilized from stripped membranes and incorporated by dialysis into PtdEtn/PtdSer vesicles. As shown in Fig. 2, the activity of the solubilized PLC was not altered by GTP[S] when tested alone (points on ordinate), but addition of membrane protein in phospholipid vesicles conferred GTP[S] stimulation. Phospholipid vesicles lacking membrane protein or containing heat-denatured (65°C for 15 min) protein did not restore GTP[S] regulation. GTP[S]-stimulated PtdIns P_2 hydrolysis was linear for at least 60 min in the reconstitution (Fig. 3a). GTP[S]-stimulated PLC activity required both the soluble membrane PLC and vesicle-incorporated membrane protein (Fig. 3b). Vesicles tested in the absence of the soluble PLC exhibited only low basal or GTP[S]-stimulated activity, resulting from residual PLC present in the stripped GH_3 -cell membranes from which cholate extracts were prepared.

Reconstitution of GTP[S]-regulated activity with purified PLC- β_1

Most of the stimulated PLC activity detected in the reconstitution assay was due to the rat brain PLC- β_1 isoenzyme. PLC preparations immunodepleted of PLC- β_1 exhibited only background activity in the reconstitution (Fig. 3c). Additional evidence that G_p regulated the activity of the PLC- β_1 isoenzyme was obtained by testing the purified enzyme. Fig. 4 shows the final step of PLC- β_1 purification on a Mono Q f.p.l.c. column. PLC activity in fractions 49–53 was GTP[S]-stimulated when G_p -containing vesicles were included in the reaction mixtures. Analysis of these column fractions by SDS/PAGE revealed one major 145 kDa protein band, a protein doublet at about 90 kDa and several minor lower-molecular-mass proteins. Immunoreactivity detected with PLC- β_1 -specific antibody corresponded to the 145 kDa, 90 kDa and several lower-molecular-mass proteins, indicating that PLC fragments were present in the purified fraction, as previously reported [23]. The results indicated that the activity of highly purified PLC- β_1 can be regulated by G_p .

Reconstituted G_p -PLC coupling is similar to that observed in membranes

As shown in Fig. 5, PLC- β_1 activity was Ca^{2+} -activated but not GTP[S]-regulated when assayed alone (Fig. 5a). With G_p -con-

Fig. 5. Ca^{2+} stimulation of PLC activity in the absence or presence of G_p

$[^3\text{H}]\text{PtdInsP}_2$ hydrolysis was determined in parallel incubations which included PLC- β_1 without (a) or with (b) phospholipid vesicles containing membrane proteins. Reactions containing the indicated concentrations of free Ca^{2+} and $100\ \mu\text{M}$ -GTP[S] where indicated (●). Other conditions were as specified in the legend to Fig. 2.

taining vesicles, GTP[S] stimulation was observed at low Ca^{2+} concentrations (Fig. 5b). Preferential stimulation by GTP[S] at low Ca^{2+} concentrations indicated that the reconstitution exhibited characteristics similar to those reported for studies of G-protein-regulated PLC in intact membranes [6,7,21].

Nucleotide stimulation of PLC activity in the reconstitution exhibited characteristics expected for G-proteins [18]. GTP[S] stimulation of hydrolysis was observed at 0.1 – $100\ \mu\text{M}$ (Fig. 6a). Guanosine 5'-[β -imido]triphosphate (p[NH]ppG) was a much less effective activator (Table 1). Stimulation of PLC activity was observed with $\text{AlCl}_3/\text{Mg}^{2+}/\text{NaF}$ (AMF; Table 1), presumably acting as the AlF_4^- complex [18]. Activation by nucleotides and

Table 1. Nucleotide stimulation of PLC activity reconstituted with phospholipid vesicles containing cholate-soluble proteins

$[^3\text{H}]\text{PtdInsP}_2$ hydrolysis was determined in standard reconstitution assays with phospholipid vesicles containing membrane proteins and rat brain PLC. Incubations were for 60 min at 37°C and contained the indicated concentrations of nucleotides along with equimolar MgCl_2 . The results from three independent experiments are shown. Means and ranges of duplicate determinations are indicated.

Additions	PtdInsP ₂ hydrolysis (% of control)
None	100 ± 16
GTP[S] (100 μM)	368 ± 40
GTP (100 μM)	116 ± 12
ATP[S] (100 μM)	100 ± 12
None	100 ± 13
GTP[S] (100 μM)	370 ± 27
AMF	538 ± 22
None	100 ± 7
GTP[S] (100 μM)	354 ± 34
p[NH]ppG (1 mM)	178 ± 29
GTP (1 mM)	44 ± 7
GTP[S] + p[NH]ppG	206 ± 15
GTP[S] + GTP	100 ± 8

AMF required phospholipid vesicles containing G_p , ATP[S], GTP (Table 1), GDP (results not shown) and guanosine 5'-[β -thio]diphosphate (GDP[S]) (Fig. 6a) did not activate the reconstituted PLC.

GTP and GDP[S] antagonized the stimulatory effects of GTP[S] (Table 1 and Fig. 6a). The antagonistic properties of GTP were used to characterize GTP[S] activation. If added at zero

Fig. 6. Nucleotide stimulation of reconstituted PLC- β_1

(a) $[^3\text{H}]\text{PtdInsP}_2$ hydrolysis was determined in incubations similar to those described in the legend of Fig. 2, except that the concentrations of GTP[S] were varied as indicated. Incubations also included $1\ \text{mM}$ -GDP[S] as indicated (●). (b) GTP[S] stimulation of the reconstituted PLC is quasi-irreversible. Incubations containing PLC and phospholipid vesicles were conducted for the indicated times in the absence (○) or in the presence (●, ■, ▲) of $100\ \mu\text{M}$ -MgGTP[S]. At the time indicated by the arrow, parallel incubations were supplemented with $1\ \text{mM}$ -Mg Cl_2 (▲) or $1\ \text{mM}$ -MgGTP (■). Data shown are means of duplicate determinations which differed from each other by less than 15% of the absolute value. (c) GTP[S] stimulation of the reconstituted PLC exhibits a latency. Reactions containing PLC and G_p -containing liposomes were incubated for the indicated times in the absence (○) or presence (●) of $100\ \mu\text{M}$ -GTP[S]. A parallel incubation was conducted (▲) in which phospholipid vesicles were preincubated with GTP[S] for 7.5 min at 37°C before addition of PLC. Additional control experiments (results not shown) indicated that removal of the lag for activation required inclusion of GTP[S] in the preincubation with vesicles. Data shown are means of duplicate determinations which differed from each other by less than 15% of the absolute value.

Fig. 7. G_p activity is present in cholate extracts immunodepleted of G_0 and G_i

(a) and (b) Cholate extracts of stripped GH_3 -cell membranes were treated with purified IgG fractions of non-immune (a) or G_{13} -peptide-specific immune (b) sera. After Protein A-Sepharose adsorption, depleted supernatants were incorporated into phospholipid vesicles by dialysis. The recovered liposomes were assayed with 2 μ g of rat brain PLC in the absence (○) or presence (●) of 100 μ M-GTP[S] for ability to hydrolyse [3 H]PtdInsP₂. Data shown are means of duplicate determinations with range indicated. (c) Treated cholate extracts were analysed by Western-blotting procedures for their G-protein content. Anti-peptide antisera GO/1, AS/7 and EC/1 were used at 1:100 dilution for detection of G_0 , G_{12} and G_{13} respectively.

time, GTP completely blocked stimulation by GTP[S] (Table 1), whereas delayed addition of GTP had no effect on the rate of GTP[S]-stimulated PtdInsP₂ hydrolysis (Fig. 6b). This result indicated that GTP[S] stimulation was quasi-irreversible, as had been observed for other G-protein-regulated processes [18]. Activation by GTP[S] exhibited a distinct lag of 2–3 min (Fig. 6c, ○). Preincubation of phospholipid vesicles with GTP[S] before PLC addition eliminated the latency to activation (Fig. 6c, ▲).

Characterization of GH_3 -cell membrane G_p

The reconstitution of GTP[S]-activated PLC activity provided an assay to monitor the purification of the GH_3 -cell G_p and has been used to establish the chromatographic properties of G_p on DEAE-Sepharose, Ultrogel AcA 34, heptylamine-Sepharose and hydroxyapatite columns (results not shown). In each case, G_p was co-eluted with the major peak of GTP[35 S]-binding, which corresponded to known heterotrimeric G-proteins (G_0 , G_i and G_s) [26,27]. Previous studies had demonstrated that regulation by thyrotropin-releasing hormone (plus GTP) of PLC activity in GH_3 -cell membranes was CT- and PT-insensitive [6,7,21]. The apparent co-elution of G_p with known G-proteins on several columns prompted studies to examine the similarity of G_p to these G-proteins. In addition, it was important to verify that the G_p detected by reconstitution with PLC- β_1 corresponded to the PT- and CT-insensitive G_p inferred from intact GH_3 -cell and membrane studies [7,21]. GH_4C_1 cells, closely related to GH_3 cells, are known to contain G_{13} , G_{12} , G_0 and G_s ([28]; S. E. Senogoles, unpublished work). Peptide-specific antisera to known G-proteins were used to immunodeplete G-proteins from GH_3 -cell membrane cholate extracts and the extracts were incorporated into phospholipid vesicles for testing with PLC- β_1 .

As shown in Fig. 7(c), antibodies to a G_{13} peptide completely depleted G_{13} and G_0 from cholate extracts; G_{12} was depleted by over 80%. The results were consistent with the reported cross-reactivity of this antibody [29]. Phospholipid vesicles prepared with the depleted extracts (Fig. 7b) exhibited G_p activity that was indistinguishable from that of vesicles incorporated with control extracts (Fig. 7a). Since G_{12} was not completely removed, an anti- G_i -peptide antibody was also used and was found to deplete 96% of the G_{12} , with no resulting change in the apparent abundance of G_p in the reconstitution assay (results not shown). Similar experiments were conducted with G_s -peptide-specific antisera, which depleted extracts of G_s by 99% without loss of G_p activity (results not shown). The results indicated that the GH_3 -cell G_p is immunologically distinct from known G-proteins present in these cells [28].

The reconstitution assay was also used to test the ability of several known G-proteins to regulate PLC- β_1 activity. As shown in Fig. 8(a), purified brain G_0 failed to confer GTP[S] regulation on PLC- β_1 . However, inclusion of G_0 in phospholipid vesicles along with G_p resulted in inhibition of G_p activity. G_z , a G-protein lacking sites for PT-catalysed ADP ribosylation, has been proposed as a possible PT-insensitive G_p [30,31], and appears to be expressed in GH_3 cells [31]. However, G_z failed to support GTP[S] stimulation of PLC activity, but also inhibited G_p activity when incorporated into phospholipid vesicles along with GH_3 -cell G_p (Fig. 8b). A likely explanation for the inhibitory effects of these G-proteins was the inclusion of additional β - γ subunits provided by these heterotrimers. This suggestion was confirmed by the experiment shown in Fig. 8(c), where inclusion of β - γ subunits was found to inhibit the stimulation of PLC- β_1 by G_p .

Fig. 8. β - γ subunits inhibit G_p activity in liposomes

Phospholipid vesicles of different compositions were prepared by detergent-dialysis methods. In each case, proteins were incorporated with 0.5–0.75 mg of total lipid (PtdEtn plus PtdSer). After recovery by centrifugation, the liposomes were resuspended to 10 mg of lipid/ml and tested for activity in the absence (○, △, □) or in the presence of (●, ▲, ■) of 100 μ M-GTP[S], by using 2 μ g of PLC. (a) Liposomes were prepared with ~150 μ g of cholate-soluble GH₃-cell membrane protein (○, ●; G_p), with 1.25 μ g purified G_0 (△, ▲; G_0) or with both (□, ■; $G_p + G_0$). The amount of purified G_0 incorporated was equivalent to about 6 times that incorporated into the liposomes prepared from cholate extracts, as estimated from immunoblotting analysis (results not shown). (b) Liposomes were prepared with ~150 μ g of cholate-soluble membrane protein from GH₃-cell membranes (○, ●; G_p), with 0.5 μ g of recombinant $G_2\alpha$ plus 1.5 μ g of purified β - γ subunits (△, ▲; G_2) or with both (□, ■; $G_p + G_2$). Incorporation of $G_2\alpha$ alone with G_p produced liposomes which were not detectably different from those prepared with G_p alone (results not shown). (c) Liposomes were prepared with ~150 μ g of cholate-soluble membrane protein from GH₃-cell membranes (○, ●; G_p) or with cholate extract plus purified β - γ subunits (□, ■, 0.5 μ g; or △, ▲, 1.5 μ g). Liposomes prepared with purified β - γ subunits contained about 3 times (■) and 6 times (▲) the amount present in the liposomes prepared with cholate extract alone, as estimated from immunoblotting analysis (results not shown).

DISCUSSION

Although there is substantial indirect experimental evidence implicating GTP-binding proteins in receptor-mediated activation of PLC [2–5], the G_p s and PLCs involved in transduction have not been completely identified. The mechanisms of PLC activation by G_p s have also not been fully elucidated. Identification of the components, as well as studies of mechanism, require the development of functional reconstitution assays into which G_p s and PLCs can be separately introduced. Such assays have been difficult to develop for the phosphoinositide signalling system, since all of the required components, including substrate, are membrane-associated.

Our previous studies [22] demonstrated that G_p -PLC coupling in GH₃-cell membranes could be reversibly dissociated by high salt, which solubilized the PLC. The reconstitution of GTP[S]-regulated PLC activity in the membrane required both the solubilized PLC and a membrane component, G_p . The present studies extended these findings, demonstrating that the G_p required for reconstituting GTP[S]-stimulated PLC activity could be extracted with cholate and incorporated into phospholipid vesicles which conferred GTP[S] regulation on a solubilized membrane PLC. Reconstitution of regulated PLC activity convincingly displayed the nucleotide-specificity and fluoride-activation characteristics of the intact membrane system [6,7] and of other G-protein-regulated processes [18]. This was important to establish, since numerous reports have documented non-specific effects of nucleotides or fluoride on phosphoinositide hydrolysis which do not involve G-proteins [32–35].

The previous studies showed that an extrinsic GH₃-cell membrane PLC could undergo reversible coupling with G_p [22]. In the present studies, a heterologous reconstitution was achieved in which the GH₃-cell G_p regulated the activity of a brain extrinsic membrane PLC. Immunodepletion studies demonstrated that the isoenzyme responsible for activity in the reconstitution was PLC- β_1 . In addition, it was shown that the membrane form of PLC- β_1 could be purified essentially to homogeneity without loss of its ability to couple with G_p . Since PLC- β_1 has not been detected immunologically in GH₃ cells (T. F. J. Martin & J. A. Kowalchuk, unpublished work), it is probable that the pituitary cell G_p can regulate the activity of at least two distinct extrinsic membrane PLC isoenzymes, although the identity of the GH₃-pituitary-cell enzyme remains to be established.

There may be several tissue-specific modes of G_p -PLC coupling with employ distinct PLCs. At least five PLC isoenzymes have been fully purified and characterized [2,19]. Although guanine nucleotide stimulation of cytosolic PLC activity [36–38] has been reported, the role of cytosolic PLCs in signal transduction by G-protein-linked receptors remains uncertain. Since GTP-dependent agonist regulation of PLC activity occurs in purified membrane preparations [2–5], it is important to elucidate the regulation of membrane PLC isoenzymes. Guanine nucleotide stimulation of activity has been reported for partially characterized platelet intrinsic membrane PLCs [12,20,38]. A soluble, possibly extrinsic membrane, 150 kDa PLC distinct from PLC- β was reported to be regulated by an erythrocyte membrane G_p [39]. Guanine-nucleotide-regulated PLC activity has been described for brain membranes [40,41], where the

extrinsic membrane form of PLC- β_1 predominates [23]; anti-PLC- β antibody was shown to inhibit GTP[S] stimulation [42]. Recently, Smrcka *et al.* [17] demonstrated the regulation of a partially purified PLC- β_1 by purified G_q . These and the results provided here for the highly purified enzyme indicate that PLC- β_1 is one of the enzymes that is regulated by a PT-insensitive G_p .

Several distinct G_p proteins regulate PLC activity in different systems. For systems in which agonist-regulated phosphoinositide hydrolysis is PT-sensitive, roles for G_o and G_i in coupling have received strong experimental support [11–15]. G_o and G_i substitute for the endogenous G_p in PT-treated HL-60 cell membranes [11], and both were reported to activate a partially purified platelet membrane PLC [12]. In *Xenopus* oocytes, G_o , but not G_i , was found to enhance PT-sensitive receptor-mediated activation of a presumed PLC [15]. The identity of G_p s in PT-insensitive systems has also recently been clarified. Evidence that β - γ subunits affected PT-insensitive receptor-mediated activation suggested that a heterotrimeric G protein was involved [43,44]. Recently, Taylor *et al.* [16] and Smrcka *et al.* [17] reported the purification of G-proteins with 42 kDa α -subunits that were capable of activating PLCs. Smrcka *et al.* [17] reported that the purified G-proteins corresponded to G_q/G_{11} , proteins inferred from cDNA sequencing [45], which lacked a site for ADP-ribosylation by PT. The GH₃-cell G_p identified by reconstitution in the present study is likely to be a related PT-insensitive G_p , since quantitative immunodepletion of G_{12} , G_{13} , G_o and G_s from cholera extracts failed to affect G_p activity.

Our results suggest that PT-sensitive and -insensitive modes of G_p -PLC coupling may differ with respect to both the G-proteins and the PLC isoenzymes employed. PLC- β_1 was found not to be regulated by purified brain G_o . In addition, depletion of GH₃-cell G_o and G_i proteins did not affect activity in the reconstitution. Lastly, during purification efforts, column fractions which contained G_o or G_i , but not G_p , failed to confer guanine nucleotide regulation on PLC- β_1 . These results stand in contrast with the evidence reviewed above for the role of G_i and/or G_o proteins in PT-sensitive PLC regulation in other tissues. These observations indicate that PT-sensitive PLC regulation must employ an isoenzyme other than PLC- β_1 . Hence, the available evidence indicates that at least two distinct PLCs are regulated by distinct PT-sensitive and -insensitive G_p proteins.

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