Reconstitution of thromboxane A₂ receptor-stimulated phosphoinositide hydrolysis in isolated platelet membranes: involvement of phosphoinositide-specific phospholipase C- β and GTP-binding protein G_q

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Activation of human platelets by the arachidonic acid metabolite thromboxane A₂ and the thromboxane A₂ mimic U46619 is mediated through phosphoinositide-specific phospholipase C-catalysed hydrolysis of phosphoinositides. We have established conditions to reconstitute U46619-stimulated phosphoinositide breakdown by addition of guanine nucleotides and soluble platelet phospholipase C activities to isolated ³²P-labelled membranes. Receptor-activated phosphoinositide hydrolysis was observed in the presence of guanosine 5'-[γ -thio]triphosphate (GTP[S]) or GTP plus U46619. Phosphoinositide hydrolysis was dependent on both GTP and U46619, with half-maximal stimulation observed at 5 μ M and 500 nM respectively. Phospholipase C isoenzymes β , γ_1 , γ_2 and δ were purified from platelet cytosol and their ability to reconstitute GTP[S]-dependent and GTP/U46619-dependent phosphoinositide hydrolysis determined. Phospholipase C- β and - δ , but not phospholipase C- γ_1 or - γ_2 ,

catalysed phosphoinositide breakdown in the presence of GTP[S]. In contrast, only phospholipase C- β was able to reconstitute GTP-dependent U46619-induced hydrolysis. The participation of GTP-regulatory proteins in the reconstitution of GTP[S]- and GTP/U46619-induced phosphoinositide hydrolysis was examined using antibodies to the C-terminals of the α -subunits of three of the heterotrimeric GTP-binding proteins expressed in human platelets G_q, G_{i2} and G_{i3}. Anti-G_q antibody, but not anti-G_{i2} or G_{i3} antibody, inhibited both GTP[S]- and GTP/U46619-dependent reconstitution of phosphoinositide hydrolysis with phospholipase C- β . In contrast GTP[S]-stimulated hydrolysis by phospholipase C- δ was not inhibited by any of the G-protein antibodies. These results show the functional specificity of GTP-binding proteins and phospholipase C isoenzymes in mediating agonist-induced phosphoinositide hydrolysis in human platelets.

INTRODUCTION

Thromboxane A₂ (TXA₂) and its prostaglandin precursor prostaglandin H₂ (PGH₂) elicit a variety of responses in human platelets including shape change, secretion and aggregation [1–3]. These responses are mediated, at least in part, through phosphoinositide metabolism. In platelets, TXA₂, PGH₂ and stable analogues of PGH₂, U46619 and I-BOP, induce the rapid hydrolysis of phosphoinositides by phosphoinositide-specific phospholipase C (PtdIns-PLC) with the formation of diacylglycerol, inositol phosphates and increased intracellular calcium [4,5].

In human platelets, TXA₂-mediated breakdown of phosphoinositides is G-protein dependent, and insensitive to inhibition by pertussis toxin [6]. Until recently, G_z was the leading candidate for the G-protein involved in phosphoinositide metabolism in human platelets since this G-protein is pertussis-toxin-insensitive [7–9]. Recent results, however, demonstrate that platelets express another pertussis-toxin-insensitive G-protein, G_q [10]. Using antibodies specific for the C-terminals of the α subunits of the major G-proteins expressed in human platelets, Shenker et al. [10] found that only antibodies to G_q α inhibited TXA₂-stimulated GTPase activity. These results suggest that in human platelets G_q is coupled to the TXA₂ receptor.

Thomas et al. [11] have demonstrated that addition of a

mixture of PtdIns-PLC isoenzymes from rat brain to permeabilized HL-60 cells enhanced GTP-dependent release of inositol phosphates. It was further shown that PtdIns-PLC- β and a novel 86 kDa PtdIns-PLC were capable of stimulating GTP-dependent phosphoinositide hydrolysis, while PtdIns-PLC- γ was not. These results indicate that the functional specificities of individual PtdIns-PLC isoenzymes can be investigated using a reconstituted, cell-free system.

Recent studies [12–14] have shown that platelets express PtdIns-PLC isoenzymes β , γ_1 , γ_2 , δ and two novel forms. In the present study, we have employed a reconstituted assay system consisting of isolated platelet membranes and purified platelet PtdIns-PLC isoenzymes to investigate the mechanism of TXA₂-stimulated phosphoinositide hydrolysis. Our results demonstrate that: (1) TXA₂-stimulated phosphoinositide hydrolysis is GTP dependent; (2) PtdIns-PLC- β , but not PtdIns-PLC- γ_1 , - γ_2 or - δ , isolated from human platelets markedly enhances GTP-dependent TXA₂-induced phosphoinositide hydrolysis; and (3) antibodies directed against G_q α , but not G_{i2} α or G_{i3} α , block U46619-dependent formation of inositol phosphates.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP (500 Ci/mmol) and an enhanced chemiluminescence

Abbreviations used: TXA₂, thromboxane A₂; PGH₂, prostaglandin H₂; PtdIns-PLC, phosphoinositide-specific phospholipase C; PMSF, phenylmethanesulphonyl fluoride; HA-cytosol, heparin-agarose-chromatographed platelet cytosol; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate.

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Table 1 PtdIns-PLC sequences used to generate anti-peptide antibodies

Sequence	PtdIns-PLC	Location	Designation
CLRIYPCQRTDSSNY	β , γ , δ	Y-region	1109
KDGTFLVRES	γ_2	557–566*	1114
DSRERRAPRRTRVNGD	γ_1	1272–1287*	1215
VKSSSWRRERFYKLQED	δ_1	31–47*	1179
LTRVYPKGQRVDSSNY	β , γ , δ	Y-region	1237

* Residue number

detection kit (ECL) for immunoblotting were obtained from Amersham. Thrombin (4580 units/mg of protein) was purchased from Sigma. Anti-(PtdIns-PLC- β) and anti-(PtdIns-PLC- γ_1) antibodies were generated against PtdIns-PLC- β and - γ_1 isolated from rat brain. Antibodies to the C-terminals of $G_{1\alpha}$ were generously provided by Dr. David Manning, Pharmacology Department, University of Pennsylvania, Philadelphia, PA, U.S.A. Organic solvents were g.c. grade from Fisher Scientific. Immobilon-P was purchased from Millipore Corporation. All other compounds were of at least reagent grade.

Platelet preparation

Whole blood was collected from healthy volunteers and platelet-rich plasma was prepared by centrifugation at 150 *g* for 15 min at room temperature. Platelets were prepared as described by Nakashima et al. [15]. Briefly, prostaglandin I_2 and apyrase (1.0 unit/ml) were added to platelet-rich plasma, the mixture was layered on to a 40% (w/v) BSA cushion and centrifuged at room temperature for 10 min at 1000 *g*. The platelet pellet was resuspended in Hepes/Tyrod buffer containing 134 mM NaCl, 12 mM NaHCO_3 , 3.0 mM KCl, 0.35 mM NaH_2PO_4 , 1.0 mM MgCl_2 , 5.6 mM dextrose, and 10 mM Hepes, pH 7.4. Platelets were then further purified on a Sepharose 4B-CL column (2.5 cm $\times$ 15 cm) equilibrated with Hepes/Tyrod buffer.

^{32}P -labelling of platelets

Platelets were incubated in phosphate-free Higuchi media with carrier-free $^{32}\text{P}_i$ (1 $\mu\text{Ci/ml}$) overnight at 37 °C with gently rocking. The labelling was carried out in plastic bags used for long-term platelet storage.

Preparation of platelet cytosol and isolation of platelet membranes

Platelets were lysed by sonication (Sonic 300 Dismembrator, Artek Systems Corp., NY) at 90 W on ice for four 5 s intervals (5 s on, 60 s off) in 20 mM Tris/HCl buffer, pH 7.5, with 1 mM EGTA, 0.1 mM dithiothreitol, 0.9 mM phenylmethanesulphonyl fluoride (PMSF), 2 $\mu\text{g/ml}$ leupeptin and 60 $\mu\text{g/ml}$ aprotinin (Buffer A). The platelet homogenate was centrifuged at 100 000 *g* for 2 h at 4 °C to obtain the cytosolic fraction. The 100 000 *g* pellet was resuspended in Buffer A containing 0.25 M sucrose, layered on to 25% (w/v) sucrose and spun at 100 000 *g* at 4 °C for approx. 18 h. Membranes were collected from the 25% (w/v) sucrose/buffer interface, diluted approx. 3-fold with Buffer A

and centrifuged at 100 000 *g* at 4 °C for 1 h. The membrane pellet was then resuspended in Buffer A with 2 M KCl, incubated for 30 min at 4 °C and centrifuged for 1 h at 100 000 *g* at 4 °C. The final platelet membrane pellet was resuspended at a concentration of 10–15 mg of protein/ml in Buffer A and used immediately.

Incubation of platelet membranes

^{32}P -labelled platelet membranes (50 μg of protein) containing between 1×10^5 and 1×10^6 c.p.m. were equilibrated for approx. 5 min at 37 °C with the indicated nucleotides before addition of the TXA_2 mimic U46619 (500 nM). In experimental mixtures that contained either cytosol or purified PtdIns-PLC isoenzymes the membranes were incubated for approx. 1 min with GTP plus U46619 before the addition of PtdIns-PLC activity.

Analysis of [^{32}P]inositol phosphates

At the times indicated in the text, incubations were terminated by addition of 200 μl of 10% (v/v) perchloric acid containing 3 mM EGTA and 20 μg of phytic acid, and samples were centrifuged at 15000 *g*. The perchloric acid extracts were neutralized and inositol polyphosphates analysed by chromatography on a Dowex AG 1-X8 column as described previously [16,17].

Purification of PtdIns-PLC isoenzymes β , γ_1 , γ_2 and δ

Supernatant fractions were prepared from approx. 1000 outdated platelet concentrates as described previously [18]. The supernatant was applied to a Fast-Q column (2.5 cm $\times$ 30 cm) that had been equilibrated with 20 mM Tris/HCl containing 1 mM EGTA, 0.1 mM dithiothreitol, 5% (v/v) glycerol, pH 7.6 (Buffer B) plus 100 mM NaCl. The column was eluted with a 6 litre linear gradient from 100 to 400 mM NaCl in Buffer A. This procedure yielded four peaks of PtdIns-PLC activity [14]. The first peak, which eluted between 100 and 150 mM NaCl, contained PtdIns-PLC isoenzymes γ_2 and δ . PtdIns-PLC- γ_2 was further purified as described previously by Banno et al. [12]. PtdIns-PLC- δ was isolated as described by Nozawa and Banno [13]. We [18] had previously reported the isolation of a 57 kDa PtdIns-PLC isoenzyme from human platelet cytosol. Immunoblot analysis with the anti(PtdIns-PLC) antibodies shows that the 55 kDa PtdIns-PLC cross-reacts with anti-(PtdIns-PLC- δ) antibodies and suggests that the 55 kDa PtdIns-PLC is a proteolytic cleavage product of PtdIns-PLC- δ . PtdIns-PLC- γ_1 eluted in the second fraction between 150 and 250 mM NaCl, and was further purified as described by Nozawa and Banno [13]. The third fraction from the Fast-Q column contained several unidentified PtdIns-PLC activities [14]. These were not subjected to further purification. The PtdIns-PLC- β eluted between 400 and 500 mM NaCl on the Fast-Q column, and was further purified as described previously [13].

Preparation of PtdIns-PLC antisera

Synthetic peptides containing amino acid sequences specific for the rat cDNA sequences of the PtdIns-PLC- β_1 , - γ_1 , and - δ isoenzymes and from the human cDNA sequence of PtdIns-PLC- γ_2 were used to generate PtdIns-PLC isoenzyme-specific antibodies (Table 1). In addition, two additional peptides with amino acid sequences corresponding to highly conserved portions of the Y domains found in PtdIns-PLC- β , - γ , and - δ (Table 1) were also used as immunogens. Each peptide was conjugated to keyhole limpet haemocyanin and rabbit antisera were prepared by Hazelton Research Products Inc. as described for the prep-

aration of 1109 antiserum [19]. Antisera 1109, 1114, 1215 and 1237 (Table 1) were purified by affinity chromatography on a column of the corresponding antigenic peptide (10 mg) coupled to activated CH-Sepharose (Pharmacia).

Immunoblot analysis of PtdIns-PLC isoenzymes

Proteins were resolved by SDS/PAGE [20] and transferred to Immobilon-P membranes according to the method of Towbin et al. [21]. The membranes were blocked in 10 mM Tris/HCl, pH 8.0, containing 2.5% (w/v) BSA, 150 mM NaCl, and 0.1% Tween 20 overnight at 4 °C, followed by incubation with antisera, diluted as indicated in the text, in blocking buffer for 1 h. The blots were then washed twice for 15 min in blocking buffer and twice for 15 min in blocking buffer without BSA, and then incubated for 40 min with horseradish peroxidase-conjugated goat anti(rabbit IgG) antibody. The anti-IgG-treated blots were washed with blocking buffer for 15 min, then with blocking buffer without BSA for 2 × 15 min. Blots were developed using chemiluminescence detection (Amersham Corp.).

Protein determination

Protein was determined as described by Bradford et al. [22] using BSA as the standard.

RESULTS

Reconstitution of GTP-dependent phosphoinositide hydrolysis with platelet cytosolic PtdIns-PLC

We first investigated whether GTP-dependent hydrolysis of phosphoinositides in isolated platelet membranes could be reconstituted upon addition of an unfractionated mixture of human platelet cytosolic PtdIns-PLC activities. For these experiments, platelet cytosol was chromatographed on heparin-agarose (HA-cytosol), as described previously [23,24], before addition to isolated platelet membranes. This procedure has been shown to effectively separate soluble PtdIns-PLC activity from soluble GTP-binding activity [23]. Incubation of platelet membranes with HA-cytosol did not significantly enhance inositol polyphosphate formation (Figure 1). Addition of the stable GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]) to isolated platelet membranes stimulated a small, time-dependent, increase in the release of [^{32} P]inositol phosphates (Figure 1). This release was substantially increased when platelet membranes were incubated in the presence of both GTP[S] and HA-cytosol. Under these conditions phosphoinositide hydrolysis was 2-fold greater (at 5 min) than that observed with GTP[S] alone (Figure 1). The increase in inositol polyphosphates observed with addition of HA-cytosol and GTP[S] was dependent on the amount of HA-cytosol added, and was maximal upon addition of 50 μ g of protein (results not shown).

Reconstitution of U46619-stimulated phosphoinositide hydrolysis with platelet cytosolic PtdIns-PLC

We next examined whether agonist-stimulated phosphoinositide hydrolysis could be reconstituted with addition of HA-cytosol to isolated platelet membranes. For these experiments, ^{32} P-labelled platelet membranes were incubated alone or in the presence of GTP, HA-cytosol, and the TXA₂ mimic U46619. U46619 (1 μ M) was ineffective in increasing inositol polyphosphate release when added alone to platelet membranes (Figure 2). Addition of GTP,

at a concentration of 10 μ M, resulted in a small, time-dependent, enhancement of [^{32} P]inositol polyphosphate release, which was not affected by addition of U46619 (Figure 2). Addition of HA-cytosol plus GTP stimulated a small increase in phosphoinositide hydrolysis above that seen with GTP alone (Figure 2). Inositol phosphate release was increased an additional 3–4-fold when U46619 was added together with GTP and HA-cytosol (Figure 2).

The increase in phosphoinositide hydrolysis observed in the presence of HA-cytosol, GTP, and U46619 was dependent on the concentrations of both GTP and U46619. At a fixed concentration of U46619 (1 μ M), half-maximal stimulation of

Figure 1 Stimulation of the release of inositol phosphates from platelet membranes with platelet cytosol

Isolated ^{32} P-labelled platelet membranes were incubated for the indicated times at 37 °C with no additions (○), 500 μ M GTP[S] (●), HA-cytosol (50 μ g) (▼), or HA-cytosol (50 μ g) + 500 μ M GTP[S] (▽). Membranes were pre-incubated for 5 min at 37 °C with HA-cytosol before the addition of GTP[S]. Free Ca²⁺ was maintained at approx. 100 nM in EGTA-containing buffer. Inositol phosphates were determined as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

Figure 2 Reconstitution of TXA₂ receptor-coupled inositol phosphate formation with platelet cytosol

^{32} P-labelled platelet membranes were incubated for the indicated times at 37 °C with no additions (○), 1 μ M U46619 (▼), 10 μ M GTP (●), U46619 + GTP (□), or HA-cytosol (90 μ g) + GTP + U46619 (▽). Results are means $\pm$ S.E.M. of three experiments.

Figure 3 GTP- and U46619-dependence of inositol phosphate release from platelet membranes catalysed by platelet cytosol

(a) Platelet membranes were pre-incubated at 37 °C with HA-cytosol (90 μ g), U46619 (1 μ M), and the indicated concentrations of GTP. (b) Platelet membranes were incubated at 37 °C with HA-cytosol (90 μ g), GTP (10 μ M), and the indicated concentrations of U46619. Reactions were terminated after 5 min and [^{32} P]inositol phosphates quantitated as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

Table 2 Nucleotide specificity of U46619-dependent inositol phosphate release from ^{32} P-labelled platelet membranes

[^{32} P]Phosphate-labelled platelet membranes were incubated with the thromboxane mimic U46619 (1 μ M) plus HA-cytosol for 5 min at 37 °C. Reactions were initiated by the addition of nucleotide and terminated after 5 min. Inositol phosphates were analysed as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

Additions	Inositol phosphates (c.p.m.)
None	1784 $\pm$ 377
GTP (10 μ M)	6203 $\pm$ 402
GDP (10 μ M)	1654 $\pm$ 288
CTP (10 μ M)	1794 $\pm$ 385
ITP (10 μ M)	1692 $\pm$ 433
GTP (10 μ M) + GDP[S] (1 mM)	1547 $\pm$ 419

phosphoinositide hydrolysis occurred at 5 μ M GTP (Figure 3a), and at a fixed concentration of GTP (10 μ M), half-maximal stimulation was observed at 500 nM U46619 (Figure 3b). Stimulation of phosphoinositide hydrolysis was specific for GTP, since ITP, GDP and XTP did not substitute for GTP, and guanosine 5'-[β -thio]diphosphate (GDP[S]) blocked the effect of GTP (Table 2). These results indicate that HA-cytosol from human platelets stimulates U46619-induced GTP-dependent phosphoinositide hydrolysis.

Purification and characterization of cytosolic PtdIns-PLC isoenzymes from human platelets

Human platelets are known to express several distinct forms of PtdIns-PLC [14]. The HA-cytosol used in the above experiments therefore contains a mixture of PtdIns-PLC isoenzymes. To determine the identity of the PtdIns-PLC isoenzyme(s) that catalyse U46619-dependent phosphoinositide hydrolysis, PtdIns-PLC isoenzymes were purified from platelet cytosol and used to reconstitute U46619-stimulated phosphoinositide hydrolysis. Employing previously described procedures [12–14,16], PtdIns-PLC isoenzymes β , γ_1 , γ_2 , and δ were purified to apparent homogeneity (results not shown), and characterized by immunoblot analysis. Each of the four purified PtdIns-PLC isoenzymes cross-reacted with antibody 1109 (Table 1), which

Figure 4 Western-blot analysis of PtdIns-PLC isoenzymes in human platelet cytosol with anti-(Y-region) antibody 1109

Four PtdIns-PLC activities (lanes 1–4) were chromatographically resolved and purified from human platelets as described in the Materials and methods section. Each PtdIns-PLC isoenzyme was subjected to SDS/PAGE, and immunoblotted with anti-peptide antibody 1109, which recognizes conserved sequences in the Y region found in PtdIns-PLC- β , γ and δ .

was generated against conserved amino acid sequences found in rat brain PtdIns-PLC- β , γ_1 and δ (Figure 4). The apparent molecular mass of each isoenzyme detected by immunoblot (PtdIns-PLC- β , 150 kDa; PtdIns-PLC- γ_1 , 145 kDa; PtdIns-PLC- γ_2 , 145 kDa; and PtdIns-PLC- δ , 85 kDa) is in good agreement with the reported molecular masses of these PtdIns-PLC isoenzymes [25–29]. Similar results were obtained with antibody 1237 (Table 1), which also recognizes highly conserved amino acid sequences found in the Y region of PtdIns-PLC- β , γ_1 , γ_2 and δ (results not shown). The 150 kDa and 85 kDa PtdIns-PLC isoenzymes were recognized by anti-PtdIns-(PLC) antibodies directed against rat brain PtdIns-PLC- β and δ (antibody 1179) respectively [Figures 5(a) and 5(d)]. Antibody 1215 (Table 1) specifically recognized PtdIns-PLC- γ_1 (Figure 5b), while antibody 1114 (Table 1) cross-reacted specifically with PtdIns-PLC- γ_2 (Figure 5c). These results confirm that human platelets expressed cytosolic PtdIns-PLC isoforms β , γ_1 , γ_2 and δ .

Reconstitution of U46619-stimulated phosphoinositide hydrolysis with purified PtdIns-PLC isoenzymes

^{32} P-labelled platelet membranes were incubated with each of the four purified platelet PtdIns-PLC isoenzymes in the presence of either a high concentration (100 μ M) of GTP[S] (Figure 6a), or of GTP (10 μ M) plus U46619 (1 μ M) (Figure 6b). In the presence of GTP[S], PtdIns-PLC- β and δ , but not PtdIns-PLC- γ_1 or γ_2 , stimulated phosphoinositide hydrolysis (Figure 6a). In contrast, reconstitution of U46619-mediated phosphoinositide hydrolysis was observed only in the presence of PtdIns-PLC- β ; additions of PtdIns-PLC- γ_1 , γ_2 , or δ were without effect (Figure 6b). These

Figure 5 Immunoblot analysis of PtdIns-PLC isoenzymes in human platelet cytosol

Four platelet PtdIns-PLC activities were isolated from human platelets as described in the Materials and methods section. Each PtdIns-PLC isoenzyme (lanes 1–4) was subjected to SDS/PAGE, and immunoblotted with isoenzyme specific antibodies. (a) Anti-PtdIns-PLC- β , (b) anti-PtdIns-PLC- γ_1 , (c) anti-PtdIns-PLC- γ_2 and (d) anti-PtdIns-PLC- δ .

Figure 6 Reconstitution of phosphoinositide hydrolysis in platelet membranes with purified platelet PtdIns-PLC isoenzymes

Platelet membranes were pre-incubated with no addition (1), or purified PtdIns-PLC- β (2), - γ_1 (3), - γ_2 (4) and - δ (5) at 37 °C for 5 min. (a) Reactions were initiated by the addition of 100 μ M GTP[S], or (b) 10 μ M GTP + 1 μ M U46619. After 5 min, reactions were terminated and [^{32} P]inositol phosphates quantitated as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

results indicate that PtdIns-PLC- β specifically catalyses GTP-dependent, U46619-stimulated phosphoinositide hydrolysis in isolated platelet membranes.

Table 3 Effect of C-terminal G_q antibodies on U46619-dependent activation of PtdIns-PLC- β

[^{32} P]Phosphate-labelled platelet membranes were pre-incubated with antibody or normal rabbit serum (NRS) (20 μ g/ml) for 1 h at 4 °C. Treated membranes were then incubated with U46619 (1 μ M) plus PtdIns-PLC- β for 5 min at 37 °C. Inositol phosphates were analysed as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

Additions	Inositol phosphates (c.p.m.)
None	1689 $\pm$ 209
PtdIns-PLC- β	5371 $\pm$ 396
PtdIns-PLC- β + NRS	5347 $\pm$ 417
PtdIns-PLC- β + anti- $G_q2\alpha$	5528 $\pm$ 374
PtdIns-PLC- β + anti- $G_q3\alpha$	5173 $\pm$ 415
PtdIns-PLC- β + anti- $G_q\alpha$	2538 $\pm$ 386

Table 4 Effect of C-terminal G_q antibodies on GTP[S]-dependent activation of PtdIns-PLC- δ

[^{32} P]Phosphate-labelled platelet membranes were pre-incubated for 1 h at 4 °C with or without anti- $G_q\alpha$ antibody. Treated membranes were then incubated with GTP[S] plus PtdIns-PLC- β or - δ for 5 min at 37 °C. Inositol phosphates were analysed as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three experiments.

Additions	Inositol phosphates (c.p.m.)
None	1289 $\pm$ 422
PtdIns-PLC- δ	4517 $\pm$ 449
PtdIns-PLC- δ + anti- $G_q\alpha$	4508 $\pm$ 397
PtdIns-PLC- β	5112 $\pm$ 383
PtdIns-PLC- β + anti- $G_q\alpha$	2651 $\pm$ 441

Involvement of G_q in GTP-dependent phosphoinositide hydrolysis in platelet membranes

The above results demonstrating that GTP is required for reconstitution of U46619-stimulated phosphoinositide hydrolysis suggest the involvement of a G-protein(s) in coupling the TXA₂ receptor to PtdIns-PLC- β . To determine the identity of the G-protein involved, we utilized specific antibodies to G_q , G_{i2} and G_{i3} , three heterotrimeric G-proteins expressed in human platelets [7,30]. The antibodies recognize the C-terminal of the α -subunit of each G-protein. The effect of each G-protein antibody on reconstitution of GTP-dependent, U46619-stimulated phosphoinositide hydrolysis with isolated platelet membranes and purified PtdIns-PLC- β was determined. Pre-incubation of membranes with either normal rabbit serum or antibodies made against the C-terminals of $G_{i2\alpha}$ or $G_{i3\alpha}$ had no effect on basal or U46619/GTP-stimulated release of inositol polyphosphates (Table 3). In contrast, pretreatment of platelet membranes with antibody directed against the C-terminal of G_q resulted in a 72 % inhibition of a U46619/GTP-dependent increase in polyinositol phosphates (Table 3). These results indicate that G_q , or an immunologically related G-protein, functions in the coupling of the TXA₂ receptor to PtdIns-PLC- β in human platelets.

To determine whether G_q was coupled to PtdIns-PLC- δ , we examined the effect of anti- G_q antibody on GTP[S]-dependent hydrolysis with purified PtdIns-PLC- β and - δ (Table 4). Pretreatment of membranes with anti- G_q antibody significantly inhibited phosphoinositide hydrolysis stimulated by PtdIns-PLC- β . In contrast, pre-incubation of membranes with anti- G_q anti-

body did not affect inositol polyphosphate release due to PtdIns-PLC- δ -stimulated phosphoinositide hydrolysis (Table 4). These results indicate that PtdIns-PLC- δ is not coupled to G_q .

DISCUSSION

We have demonstrated that GTP[S]-dependent and GTP/U46619-dependent phosphoinositide hydrolysis can be reconstituted using isolated [^{32} P]phosphate-labelled platelet membranes and soluble platelet PtdIns-PLC activities. Phosphoinositide hydrolysis was dependent on the concentration of both GTP and U46619, with half-maximal stimulation observed at concentrations consistent with the binding of these ligands to their cognate receptors, G-proteins and the TXA_2 receptor respectively.

Human platelets were found to contain at least four soluble PtdIns-PLC isoenzymes, which by immunological criteria were identified as PtdIns-PLC- β , γ_1 , γ_2 , and δ . At least one additional PtdIns-PLC activity was also observed, which did not cross-react with antibodies to any of the known PtdIns-PLC isoenzymes (results not shown). This unidentified PtdIns-PLC activity was also not recognized by antibodies to the conserved Y regions of PtdIns-PLC- β , γ and δ , which have been demonstrated to be required for catalytic activity of these PtdIns-PLC isoenzymes. These results suggest that the unidentified PtdIns-PLC activity is not a degradation product of one of the other known PtdIns-PLC isoenzymes, and therefore may be a novel PtdIns-PLC isoenzyme. Banno et al. [14] have also recently reported expression of PtdIns-PLC- β , γ_1 , γ_2 , δ and two unidentified PtdIns-PLC activities in human platelets.

Among the four PtdIns-PLC isoenzymes examined, only PtdIns-PLC- β was able to reconstitute GTP-dependent, U46619-stimulated phosphoinositide hydrolysis. These results are consistent with evidence from other laboratories demonstrating that PtdIns-PLC- β can function to catalyse phosphoinositide hydrolysis in a G-protein- and agonist-dependent manner [31–33]. In contrast with GTP/U46619, GTP[S] stimulated both PtdIns-PLC- β and δ . This suggests that although PtdIns-PLC- δ may be activated through a G-protein-dependent mechanism, it is probably not coupled to the TXA_2 receptor. GTP[S]-dependent activation of PtdIns-PLC- δ was not inhibited by anti- $G_q\alpha$ antibody, which suggests PtdIns-PLC- δ is not coupled to G_q . Presumably PtdIns-PLC- δ functions to catalyse phosphoinositide hydrolysis in response to activation of one or more distinct receptors.

Recently, Shenker et al. [8] have shown that TXA_2 -stimulated GTPase activity was inhibited by pre-incubation of platelet membranes with anti- G_q antibodies, suggesting that a member of the G_q family of heterotrimeric G-proteins is coupled to the TXA_2 receptor. Our results with C-terminal antibody to G_q are in agreement with this conclusion, and support the notion that TXA_2 -stimulated phosphoinositide hydrolysis is mediated through G_q in human platelets. Since G_q is not ADP-ribosylated by pertussis toxin, our finding that U46619-induced phosphoinositide hydrolysis in isolated platelet membranes is insensitive to pertussis toxin (results not shown) provides additional evidence for this conclusion.

In conclusion, the above results indicate that platelets contain several GTP-dependent PtdIns-PLC isoenzymes. The TXA_2 receptor activates PtdIns-PLC- β . Which PtdIns-PLC isoenzyme is activated by other agonists that are known to activate phosphoinositide hydrolysis awaits further study.

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