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Stimulation of polyphosphoinositide hydrolysis by thrombin in membranes from human fibroblasts

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One of the earliest actions of thrombin in fibroblasts is stimulation of a phospholipase C (PLC) that hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. In membranes prepared from WI-38 human lung fibroblasts, thrombin activated an inositol-lipid-specific PLC that hydrolysed [^{32}P]PIP_2 and [^{32}P]phosphatidylinositol 4-monophosphate (PIP) to [^{32}P]IP_3 and [^{32}P]inositol 1,4-bisphosphate (IP_2) respectively. Degradation of [^{32}P]phosphatidylinositol was not detected. PLC activation by thrombin was dependent on GTP, and was completely inhibited by a 15-fold excess of the non-hydrolysable GDP analogue guanosine 5′-[β-thio]diphosphate (GDP[S]). Neither ATP nor cytosol was required. Guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) also stimulated polyphosphoinositide hydrolysis, and this activation was inhibited by GDP[S]. Stimulation of PLC by either thrombin or p[NH]ppG was dependent on Ca^{2+}. Activation by thrombin required Ca^{2+} concentrations between 1 and 100 nM, whereas stimulation of PLC activity by GTP required concentrations of Ca^{2+} above 100 nM. Thus the mitogen thrombin increased the sensitivity of PLC to concentrations of free Ca^{2+} similar to those found in quiescent fibroblasts. Under identical conditions, another mitogen, platelet-derived growth factor, did not stimulate polyphosphoinositide hydrolysis. It is concluded that an early post-receptor effect of thrombin is the activation of a Ca^{2+}- and GTP-dependent membrane-associated PLC that specifically cleaves PIP_2 and PIP. This result suggests that the cell-surface receptor for thrombin is coupled to a polyphosphoinositide-specific PLC by a GTP-binding protein that regulates PLC activity by increasing its sensitivity to Ca^{2+}.

INTRODUCTION

Thrombin, a potent mitogen and serine proteinase, initiates fibroblast proliferation by binding to specific high-affinity cell-surface receptors (Carney & Cunningham, 1978; Perez-Rodriguez *et al.*, 1981; Chen & Buchanan, 1985). Both receptor occupancy and proteolytic activity are essential for the progression from quiescence into the S phase of the active cell cycle (Glenn *et al.*, 1980; Perdue *et al.*, 1981). Although the precise nature of the post-receptor events that mediate mitogenesis are unknown, many growth factors, including thrombin, trigger the activation of a common set of biochemical pathways that are well correlated with the induction of DNA synthesis. One of the earliest events detected in thrombin-stimulated fibroblasts is the activation of a phospholipase C (PLC) that hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (Murayama & Ui, 1985; Carney *et al.*, 1986; Paris & Pouyssegur, 1986). IP_3 mobilizes Ca^{2+} from intracellular stores, elevating the cytoplasmic free Ca^{2+} concentration in a variety of cells (Berridge, 1984), including fibroblasts (Berridge *et al.*, 1984). DG activates protein kinase C (Nishizuka, 1986), which in turn stimulates the Na^+/H^+ antiporter and alkalinizes the cytoplasm (Pouyssegur, 1986).

Thrombin is best known for its role as a platelet activator in the process which leads to clot formation. As in fibroblasts, thrombin induces the rapid hydrolysis of PIP_2 to IP_3 and DG (Agranoff *et al.*, 1983; Watson *et al.*, 1984). In platelets permeabilized by electroporation, Haslam & Davidson (1984*b*) have observed that thrombin decreases the threshold for Ca^{2+}-stimulated DG accumulation. This findings suggested that Ca^{2+} could serve as an important regulatory ion in the process of PLC activation by thrombin.

Several studies have demonstrated that the Ca^{2+}-mobilizing hormones 5-hydroxytryptamine (Litosch & Fain, 1985; Litosch *et al.*, 1985), thyrotropin-releasing hormone (Straub & Gershengorn, 1986; Martin *et al.*, 1986), fMet-Leu-Phe (Smith *et al.*, 1985), vasopressin (Uhing *et al.*, 1986; Guillon *et al.*, 1986) and α-catecholamines (Uhing *et al.*, 1986) stimulate the GTP-dependent PLC hydrolysis of PIP_2 in membranes prepared from responsive tissues and cultured cells. A communication by Baldassare & Fisher (1986) indicates that thrombin and GTP stimulate polyphosphoinositide hydrolysis in platelet membranes. These observations have led to the suggestion that a GTP-binding protein couples the ligand–receptor complex to the PLC in a manner analogous to regulation of adenylate cyclase (Ross & Gilman, 1980) and cyclic GMP phosphodiesterase (Stryer *et al.*, 1981).

We now report that thrombin activates a GTP-dependent PLC in membranes prepared from WI-38

Abbreviations used: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; IP_3, inositol 1,4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; IP, inositol 1-phosphate; DG, diacylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; p[CH_2]ppA, adenosine 5′-[$\beta\gamma$-methylene] triphosphate; G-protein, guanine nucleotide binding protein.

fibroblasts by increasing its sensitivity to stimulation by Ca^{2+}. The concentration of Ca^{2+} required for maximal lipase activation is similar to that found in the cytoplasm of quiescent fibroblasts (Guillon *et al.*, 1986). In contrast, PDGF, a growth factor that also activates PLC in intact fibroblasts (Berridge *et al.*, 1984; Chu *et al.*, 1985), had no effect on PLC activity *in vitro*.

METHODS

Materials

Bovine thrombin (400 NIH units/mg of protein), human thrombin (4400 units/mg), hirudin, PIP_2, PIP, phytic acid and phytase were purchased from Sigma. Bovine serum albumin (fatty acid-free) was obtained from Miles Laboratories. GTP, GDP, GMP, p[NH]ppG and GDP[S] were supplied by Boehringer–Mannheim. Quin-2 (free acid) was obtained from Calbiochem–Behring. WI-38 normal (diploid) human lung fibroblasts were obtained from American Type Culture Collection. [γ-^{32}P]ATP (3000 Ci/mmol) and [*inositol*-2-^{3}H(n)]PIP_2 (2 Ci/mmol) were from New England Nuclear. [^{3}H]IP_3 (1 Ci/mmol), [^{3}H]PIP_2 (2 Ci/mmol) and [^{32}P]P_i (carrier-free) were purchased from Amersham. Highly purified human PDGF was kindly provided by Dr. Thomas Deuel, Washington University Medical School. Some of the PDGF was also purchased from PDGF Inc., Cambridge, MA, U.S.A. Centricon Microconcentrating Cells were purchased from Amicon. Silica H t.l.c. plates (LK5D) were from Whatman. Cyclohexylene-dinitrilotetra-acetic acid was from Kodak. Chloroform and methanol were h.p.l.c.-grade.

Metabolic labelling of intact cells

Normal human lung fibroblasts (WI-38) were taken from passage 16–25 and grown to confluency in 150 mm culture dishes in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) fetal-bovine serum. Confluent cultures were rinsed once with the Dulbecco's medium containing 0.2 M-Na_2HPO_4, 0.1% bovine serum albumin, 5 mM-Hepes and 0.2% dialysed horse serum, and then incubated for 2 days in medium containing 12 mCi of [^{32}P]P_i/25 ml of medium. After 2 days the cultures were washed three times with balanced salt solution, containing 120 mM-NaCl, 5 mM-KCl and 10 mM-Hepes buffer, pH 7.4 at 37 °C, and then washed twice with ice-cold balanced salt solution. The cells were scraped into ice-cold Buffer A (80 mM-KCl, 25 mM-LiCl, 5 mM-$MgCl_2$, 0.5 mM-EDTA, 5 mM-EGTA, 2.5% sucrose, 0.5 mM-dithiothreitol, 50 μg of leupeptin/ml, 10 μg of aprotinin/ml, 10 μg of soya-bean trypsin inhibitor/ml and 10 mM-Tris buffer, pH 7.9) and disrupted with a Branson Cell Disrupter at 10% maximal tip power for 7 s. The homogenate was centrifuged at 400 *g* for 5 min. The supernatant was then centrifuged at 12000 *g* in a Microfuge at 4 °C for 30 min. The pellet was resuspended in Buffer A with 50 strokes of a Dounce homogenizer, and re-centrifuged at 12000 *g* for 30 min. The pellet was washed twice with Buffer A containing 1 mM-EGTA, and resuspended again in 400 μl of Buffer A with 50 strokes of a Dounce homogenizer. The membrane fraction (2.5–3.5 mg of protein/ml) was used immediately on preparation. Membrane and cytosolic fractions were obtained from unlabelled WI-38 cells incubated in parallel with labelled cell cultures. The cytosolic fraction was prepared from the first 12000 *g* supernatant by centrifugation at 100000 *g* for 60 min. The soluble material was then concentrated approx. 10-fold in a Centricon Concentrating Cell (10 kDa cutoff) to a protein concentration of 2.8 mg/ml.

Determination of PLC activity

Reactions were carried out in 12 mm × 75 mm silicone-treated glass tubes at 30 °C. To initiate the reaction, 10 μl of membrane in Buffer A (25–35 μg of protein) was added to 40 μl of Buffer B, which contained 80 mM-KCl, 1 mM-EGTA, 0.5 mM-$CaCl_2$, 1 mM-$MgCl_2$, 1 mM-2,3-bisphosphoglycerate, 0.5 mM-dithiothreitol and 50 mM-Tris buffer, pH 7.2. The final concentrations of the components of the reaction mixtures were 80 mM-KCl, 5 mM-LiCl, 1 mM-EGTA, 0.1 mM-EDTA, 0.5 mM-dithiothreitol, 0.4 mM-$CaCl_2$, 1.8 mM-$MgCl_2$, 0.8 mM-2,3-bisphosphoglycerate, 0.5% sucrose and 42 mM-Tris buffer, pH 7.3. Free Ca^{2+} concentration curves were constructed using Ca^{2+}/EGTA buffers that were calibrated to a known free Ca^{2+} concentration with Quin-2 (free acid) as described by Tsien *et al.* (1982). The free Ca^{2+} concentration in most experiments was 200 nM. Most reaction mixtures also contained 1 mM-ATP. In experiments designed to assess the effects of PDGF, 0.3% (w/v) bovine serum albumin was added to the reaction mixture. Reactions were terminated with 1 ml of ice-cold chloroform/methanol (2:1, v/v). In order to extract the water-soluble compounds from the lipids and precipitate most of the protein, 0.5 ml of water containing 0.2 mM-EDTA and 5 μM-phytic acid was added. A 0.5 ml portion of the upper phase was then used to determine the amounts of the inositol phosphates (see below). HCl (0.5 ml of 1 M) and 1 ml of chloroform/methanol (2:1, v/v) were added to the remainder of the sample, and the polyphosphoinositides were extracted into the lower phase.

Analysis of the inositol phosphates

The method of inositol phosphate analysis used was that described by Agranoff *et al.* (1983), but with slight modification. Samples were desalted batchwise with 0.2 ml of Bio-Rad AG50W-X8 cation-exchange resin (H^+ form) and re-extracted with 2 ml of chloroform equilibrated with water and methanol (10:5:3, by vol.). The concentration of H^+ resulting from the desalting process was less than 10 mM. The samples were then boiled at 100 °C for 8 min, dried in a Speed-vac at room temperature and finally resuspended in 20 μl of water. The black residue formed during the drying step was removed by centrifuging the samples at 12000 *g* for 5 min. A portion of the sample was applied to Whatman 3 MM paper and subjected to electrophoresis for 2.5 h at 2400 V in 50 mM-oxalate buffer, pH 1.5. The paper was then dried and subjected to radioautography. The spots corresponding to IP_3, IP_2 and InsP were cut out, minced and counted for radioactivity in a liquid-scintillation spectrometer. Recovery of [^{3}H]IP_3 standard was consistently about 85%. The identities of the spots eluted from the paper were confirmed by digestion with phytase, a highly active, though non-specific, phosphatase. Each compound yielded only the lower-order [^{32}P]inositol phosphate products and [^{32}P]P_i. [^{32}P]IP_3, [^{32}P]IP_2 and [^{32}P]IP standards were prepared from the corresponding lipids extracted from WI-38 cells by hydrolysing the lipids at 100 °C for 30 min in 1 M-HCl.

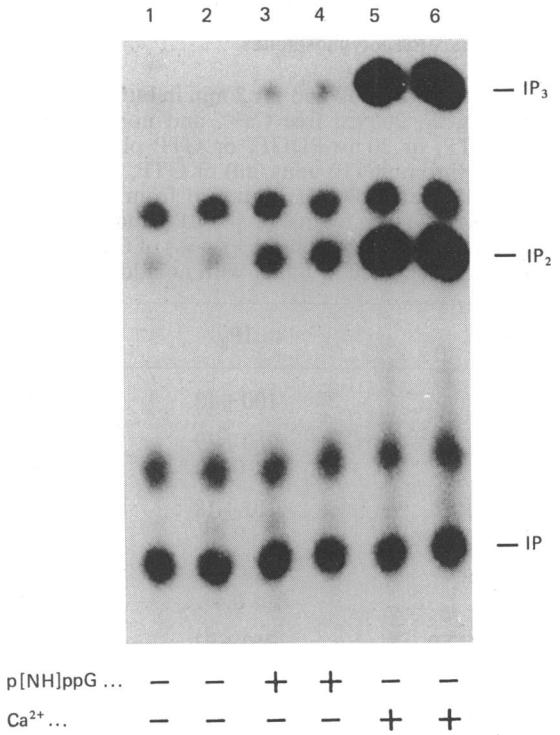

Fig. 1. Stimulation of inositol polyphosphate generation by p[NH]ppG and Ca^{2+}

Autoradiogram of samples analysed by high-voltage paper electrophoresis. Membranes were prepared from WI-38 fibroblasts and incubated for 2 min in buffer containing 200 nM free Ca^{2+} (lanes 1 and 2), 300 μM-p[NH]ppG and 200 nM-Ca^{2+} (lanes 3 and 4), or 1 mM-Ca^{2+} (lanes 5 and 6).

The hydrolysate was then extracted with chloroform/methanol (2:1, v/v). HCl was removed by drying the standards in a Speed-vac at room temperature.

Analysis of the polyphosphoinositides

The polyphosphoinositides were separated by t.l.c. on silica H plates developed in chloroform/methanol/ammonia/water (40:35:4:5, by vol.) which contained 10 mM-cyclohexylenedinitrilotetraacetic acid. PIP_2 and PIP were identified by co-migration with authentic standards.

Measurement of protein tyrosine kinase activity

The ability of PDGF to stimulate protein kinase activity was assessed in unlabelled membranes prepared in parallel with the labelled membranes used to assay PLC activation. The same reaction mixture was used for both experiments, except for the addition of 200 μM-[γ-^{32}P]ATP (10 d.p.m./mol) in the protein kinase assay. The reactions were carried out at 4 °C for 10 min and terminated by addition of 200 μl of 10% (v/v) trichloroacetic acid containing 10 mM-sodium pyrophosphate. Samples were incubated on ice for 20 min and then centrifuged at 12000 *g* for 15 min. The precipitate was washed once with 10% trichloroacetic acid, twice with diethyl ether and redissolved in SDS. The samples were analysed by SDS/polyacrylamide-gel electrophoresis on 7.5% or 10% polyacrylamide gels (Laemmli, 1970), The gels were stained, destained, soaked in 1 M-NaOH for 1 h at 55 °C (Cooper & Hunter, 1981), dried and subjected to radioautography. The radioactivity in the phosphoprotein bands was quantified by densitometry with an LKB soft-laser scanning densitomer.

Table 1. Effects of thrombin, GTP and p[NH]ppG on the generation of inositol polyphosphates

Membranes were incubated for 2 min in buffer with 1.8 mM-$MgCl_2$, 200 nM free Ca^{2+}, and no additions, or 300 μM GTP, or thrombin (30 units/ml), or GTP plus thrombin, or GTP, thrombin and 5 mM-GDP[S]. The results are the averages of six separate experiments and are expressed as percentages (mean ± S.E.M.) of the amounts of [^{32}P]IP_3 or [^{32}P]IP_2 in membranes incubated in buffer with no additions.

Additions	IP_3	IP_2
None	100 ± 11	100 ± 11
GTP	242 ± 21 $P < 0.001$*	262 ± 27 $P < 0.001$*
Thrombin	150 ± 24 $P > 0.05$*	122 ± 15 $P > 0.05$*
GTP, thrombin	583 ± 41 $P < 0.001$†	889 ± 134 $P < 0.001$†
GTP, thrombin, GDP[S]	174 ± 51	50 ± 18
p[NH]ppG	345 ± 47 $P < 0.001$	556 ± 98 $P < 0.001$*

* Compared with control membranes incubated in buffer with no additions.
† Compared with membranes incubated in buffer containing GTP.

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951).

Statistical analysis

The significance of the differences between means was assessed by Student's *t* test.

RESULTS

Hydrolysis of PIP_2 and PIP

Membranes prepared from ^{32}P-labelled WI-38 fibroblasts were used to study the PLC that hydrolyses PIP_2 and PIP. Enzyme activity was assessed by measuring the generation of the reaction products, [^{32}P]IP_3 and [^{32}P]IP_2. Initial experiments demonstrated that Ca^{2+} could stimulate PLC activity in fibroblast membranes. The amounts of [^{32}P]IP_3 and [^{32}P]IP_2 were dramatically increased by 1 mM-Ca^{2+} (Fig. 1, lanes 5 and 6) as compared with 200 nM-Ca^{2+} (lanes 1 and 2). This increase was accompanied by a fall in the amounts of [^{32}P]PIP_2 and [^{32}P]PIP, to 44% and 74% of their respective control values, whereas the amount of [^{32}P]PI remained constant. The amounts of [^{32}P]IP_3 and [^{32}P]IP_2 in membranes incubated in buffer with 200 nM-Ca^{2+} were not significantly different from those found in membranes incubated with excess EGTA (pCa < 1 nM) (see below). Stimulation by Ca^{2+} was unaffected by washing the

membranes with 0.2% (w/v) of the detergent octyl glucoside in buffer A, or with a solution containing 500 mM-KCl, 2 mM-EDTA, 1 mM-dithiothreitol and 20 mM-Tris, pH 7.8, or with a solution containing 0.5 mM-$MgCl_2$, 1 mM-dithiothreitol and 0.5 mM-Tris buffer, pH 7.4 (results not shown). Thus the PLC activity in the membrane fraction appeared to be tightly bound.

Effect of guanine nucleotides

The possibility that fibroblast membranes contained a GTP-binding protein that can stimulate PLC to degrade polyphosphoinositides was initially tested by using p[NH]ppG, the non-hydrolysable GTP derivative, which is known to activate G-proteins even in the absence of an appropriate hormone–receptor complex (Ross & Gilman, 1980). p[NH]ppG stimulated polyphosphoinositide hydrolysis, but was less effective than 1 mM-Ca^{2+} (Fig. 1, lanes 3 and 4). Stimulation by p[NH]ppG was dependent on Ca^{2+}; PLC activation could only be detected when the free Ca^{2+} concentration was between 10 and 1000 nM (see below). Activation of PLC by p[NH]ppG also required Mg^{2+}, whereas stimulation by 1 mM-Ca^{2+} alone was unaffected by Mg^{2+} ions (results not shown). The stimulation of PLC by p[NH]ppG, like that by Ca^{2+}, was unaffected by concentrations of octyl glucoside up to 0.2% or by buffer conditions known to solubilize transducin, the G-protein of retinal rod-outer-segment membranes (Kuhn, 1980). Thus the components required for p[NH]ppG activation were also tightly associated with the particulate fraction. The addition of cytosol (560 μg of protein/ml) to the reaction mixture had no effect on either basal or p[NH]ppG-stimulated polyphosphoinositide hydrolysis.

PLC activation by thrombin

Thrombin, in the presence of GTP, markedly stimulated the formation of [^{32}P]IP_3 and [^{32}P]IP_2. A summary of experiments in which the effects of thrombin, GTP and p[NH]ppG were compared is presented in Table 1. The effect of thrombin and GTP was greater than that of either thrombin or GTP alone. The effect of thrombin was inhibited by the GDP analogue GDP[S], which blocks the binding of GTP to G-proteins (Eckstein *et al.*, 1979). Thrombin stimulation was also blocked by hirudin, a specific thrombin antagonist (Table 2). In contrast, hirudrin did not affect p[NH]ppG-stimulated polyphosphoinositide hydrolysis, nor did GDP[S] affect PLC activation by Ca^{2+} (results not shown).

The potentiation of thrombin stimulation by GTP was not mimicked by ATP, GMP, GDP or 3′,5′-cyclic GMP. However, dGTP was approx. 75% as effective as GTP. p[NH]ppG was also effective in stimulating polyphosphoinositide hydrolysis (Table 1). Although GTP alone increased polyphosphoinositide hydrolysis, it was less effective than its non-hydrolysable analogue. Stimulation by thrombin was not mimicked by another serine proteinase, trypsin, at 10 or 100 μg/ml. No significant effects of thrombin, GTP or p[NH]ppG on amounts of inositol 1-[^{32}P]monophosphate or inositol 1,3,4,5-[^{32}P]-tetrakisphosphate were observed. There were also no detectable effects of thrombin, GTP or p[NH]ppG on the amounts of polyphosphoinositides. This is consistent with the limited effects of thrombin or p[NH]ppG on inositol polyphosphate production compared with the effect induced by 1 mM-Ca^{2+} (see above). The difficulty in detecting a fall in the amounts of these lipids during thrombin stimulation of intact fibroblasts has been noted (Murayama & Ui, 1985).

Table 2. Effect of PDGF, thrombin and GTP on the production of inositol polyphosphates

Membranes were incubated for 2 min in buffer containing 1.8 mM-$MgCl_2$, 200 nM free Ca^{2+}, and no additions, or 300 μM-GTP, or 20 nM-PDGF, or GTP plus PDGF, or GTP plus thrombin (10 units/ml) or GTP, thrombin and hirudin (30 units/ml). The data are from two separate experiments. The amounts of [^{32}P]IP_3 and [^{32}P]IP_2 are expressed as percentages (mean ± S.E.M.) of the amounts in membranes incubated in buffer with no additions.

Additions	IP_3	IP_2
None	100 ± 13	100 ± 11
GTP	260 ± 39 $P < 0.005$*	225 ± 40 $P < 0.025$*
PDGF	101 ± 18 $P > 0.4$*	100 ± 16 $P > 0.4$*
PDGF, GTP	244 ± 25 $P > 0.4$†	297 ± 30 $P > 0.05$†
Thrombin, GTP	752 ± 93 $P > 0.005$†	876 ± 109 $P < 0.005$†
Thrombin, GTP, hirudin	153 ± 25 $P > 0.1$*	178 ± 40 $P > 0.1$*

* Compared with membranes incubated in buffer with no additions.

† Compared with membranes incubated in buffer containing GTP.

The polyphosphoinositides are formed through successive phosphorylations of the inositol ring by PI and PIP kinases (Irvine, 1982). The possibility that one or both of the inositol lipid kinases is activated by thrombin or p[NH]ppG appears unlikely, since (1) the addition of ATP was not required for PLC activation, and (2) the non-hydrolysable ATP analogue p[CH_2]ppA (5 mM) did not affect stimulation by thrombin. In the presence of p[CH_2]ppA, the amounts of [^{32}P]IP_3 and [^{32}P]IP_2 were 112 ± 3% and 92 ± 8% (mean ± S.D.) respectively of those stimulated by thrombin in the absence of this analogue. Thus it seems more plausible that a polyphosphoinositide-specific PLC is the metabolic enzyme activated by thrombin and p[NH]ppG.

In the presence of GTP, thrombin significantly increased the rates of [^{32}P]IP_3 and [^{32}P]IP_2 generation over those stimulated by GTP or thrombin alone (Figs. 2*a* and 2*b*). The effect of thrombin was transient. After 2 min the rate of inositol polyphosphate production was similar to that in membranes incubated with GTP alone. In the experiment depicted in Fig. 2, thrombin increased polyphosphoinositide hydrolysis in the absence of added GTP; however, this effect was not consistently observed. In all experiments, GTP significantly potentiated the stimulation by thrombin. Moreover, the effect of thrombin was completely blocked by GDP[S] (see above). The variability in the requirement for added GTP may reflect the presence of contaminating endogenous GTP in some membrane preparations.

The quantity of [^{32}P]IP_2 produced by PLC activation

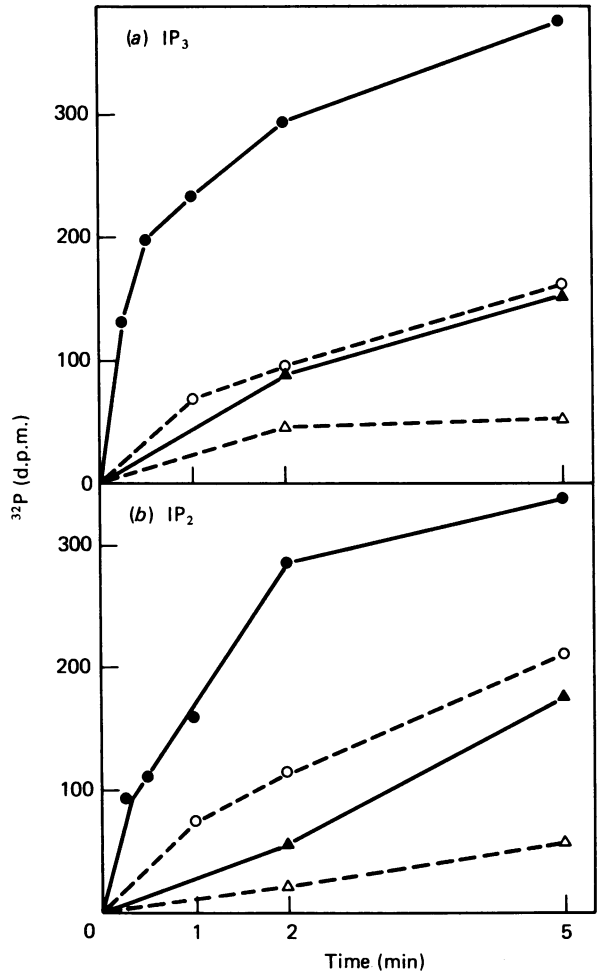

Fig. 2. Time course of the effects of thrombin and GTP on the generation of inositol polyphosphates

Membranes were incubated in buffer containing 1.8 mM-$MgCl_2$ and 200 nM free Ca^{2+} and no additions (△), thrombin (30 units/ml) (▲), GTP (300 μM) (○), or thrombin and GTP (●). The results shown are the means of duplicate determinations which did not differ from each other by more than 15%.

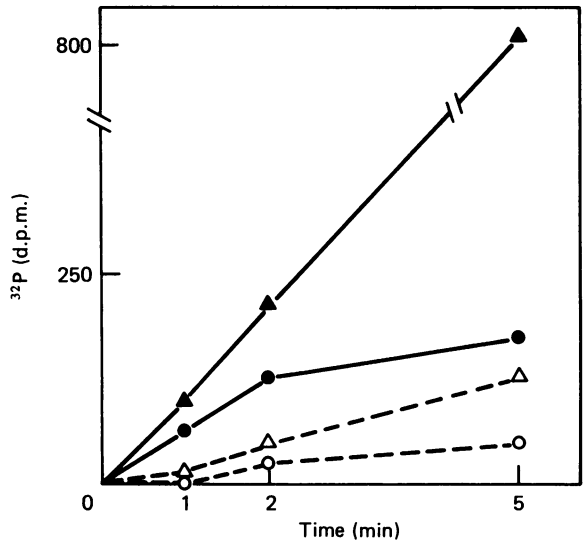

Fig. 3. Time course of the effect of p[NH]ppG on the amounts of [^{32}P]IP_3 (○, ●) and [^{32}P]IP_2 (△, ▲)

Membranes were incubated in buffer containing 1.8 mM-$MgCl_2$ and 200 nM Ca^{2+} alone (○, △) or 300 μM-p[NH]ppG plus 200 nM-Ca^{2+} (●, ▲). The results are the means of duplicate determinations which did not differ from each other by more than 12%.

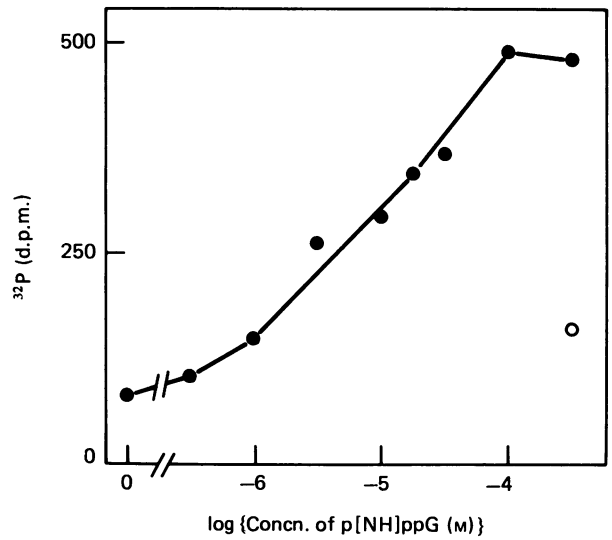

Fig. 4. Effect of p[NH]ppG concentration on the generation of inositol polyphosphates

Membranes were incubated for 2 min in buffers containing 1.8 mM-$MgCl_2$ and 200 nM free Ca^{2+} and various concentrations of p[NH]ppG (●). Membranes were also incubated with 300 μM-p[NH]ppG and 3 mM-GDP[S] (○) and the same concentrations of $MgCl_2$ and Ca^{2+}. Each point is the mean of the sum of the radioactivity in [^{32}P]IP_3 and [^{32}P]IP_2 determined in duplicates which did not vary by more than 15% from the mean. The amounts of inositol polyphosphates stimulated by p[NH]ppG concentrations greater than 1 μM were significantly greater than in controls without p[NH]ppG (least significance $P < 0.05$).

could not be accurately determined, because a portion of this IP_2 may be generated by dephosphorylation of [^{32}P]IP_3 to IP_2 by a specific phosphatase, originally described by Downes *et al.* (1982). In fact, under these experimental conditions added [^{3}H]IP_3 was rapidly degraded to [^{3}H]IP_2 (results not shown). Hence it is possible that the primary substrate hydrolysed by the thrombin- and GTP-activated PLC is PIP_2 and that IP_3 is the source of IP_2.

In contrast with the rapid and transient effect of thrombin, p[NH]ppG stimulated IP_2 production for at least 5 min (Fig. 3). This difference may be due to the nearly irreversible nature of G-protein activation by p[NH]ppG (Schramm & Rodbell, 1975). The increased [^{32}P]IP_2/[^{32}P]IP_3 ratio induced by p[NH]ppG at later time points (2 and 5 min) compared with that observed with thrombin stimulation was consistent. PLC activation by p[NH]ppG was concentration-dependent; it was maximal at 100 μM-p[NH]ppG and inhibited by 3 mM-GDP[S] (Fig. 4).

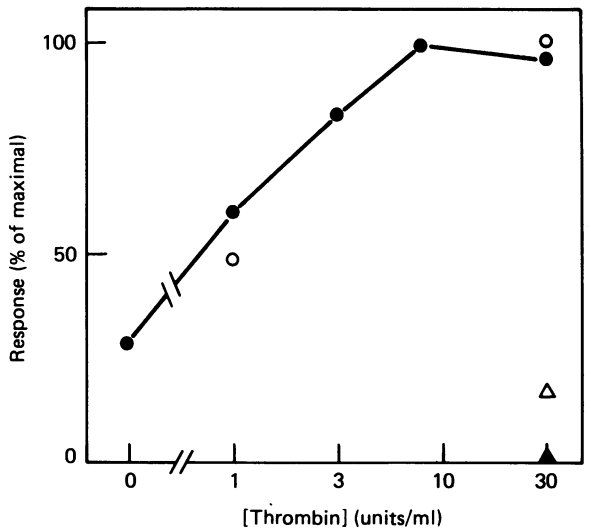

Fig. 5. Effect of thrombin concentration on the generation of inositol polyphosphates

Membranes were incubated for 2 min in buffer with 1.8 mM-$MgCl_2$, 200 nM free Ca^{2+}, 300 μM-GTP and increasing concentrations of bovine thrombin (●) or human thrombin (○). Membranes were also incubated with 30 units of bovine thrombin/ml without GTP (△), or with 30 units of bovine thrombin/ml, 300 μM-GTP and 5 mM-GDP[S] (▲). Each point is calculated as the sum of the radioactivities in $[^{32}P]IP_3$ and $[^{32}P]IP_2$ determined in duplicate and expressed as a percentage of maximal stimulation. Thrombin from 1 to 30 units/ml significantly stimulated inositol phosphate production when compared with controls without thrombin (least significance $P < 0.005$). Bovine thrombin at 30 units/ml, in the presence of 300 μM-GTP, increased the inositol polyphosphates to 953 ± 24 d.p.m. (mean ± S.E.M.).

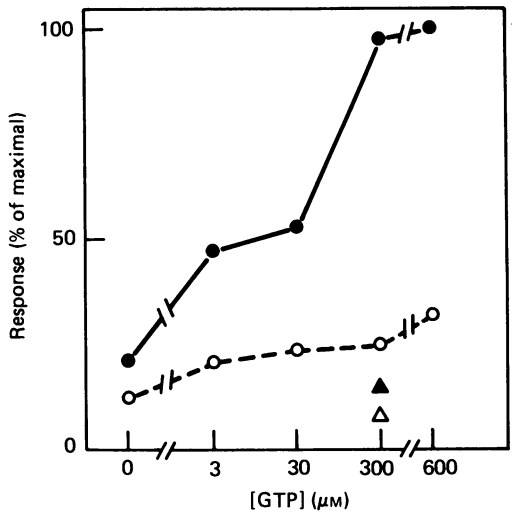

Fig. 6. Effect of GTP concentration on generation of inositol polyphosphates in the presence or absence of thrombin

Membranes were incubated for 2 min in buffer containing 1.8 mM-$MgCl_2$, 200 nM-Ca^{2+} and increasing concentrations of GTP without (○) or with (●) thrombin (30 units/ml). Membranes were also incubated with 300 μM-GTP and 5 mM-GDP[S] in either the presence (▲) or the absence (△) of thrombin. Each point is the mean of the sum of the radioactivities in $[^{32}P]IP_3$ and $[^{32}P]IP_2$ determined in duplicate from three separate experiments and is expressed as a percentage of the maximal stimulation. Thrombin in the presence of 3–600 μM-GTP significantly increased the amounts of inositol polyphosphates compared with controls with GTP alone (least significance $P < 0.025$). In a typical experiment thrombin, in the presence of 300 μM-GTP, increased $[^{32}P]IP_3$ and $[^{32}P]IP_2$ to 620 ± 37 and 993 ± 33 d.p.m. (mean ± S.D.) respectively.

The ability of thrombin to activate the polyphosphoinositide PLC in the presence of GTP was dependent on thrombin concentrations between 1 and 10 units/ml (Fig. 5). This is similar to the concentration of thrombin needed to stimulate maximally phosphoinositide hydrolysis and DNA synthesis in intact fibroblasts (Glenn *et al.*, 1980; Carney *et al.*, 1985). Although bovine thrombin (400 NIH units/mg of protein) was used in most of the experiments shown, highly purified human thrombin (4400 units/mg of protein) stimulated PLC activity equivalently (Fig. 5).

Activation of PLC by thrombin was dependent on the concentration of GTP (Fig. 6). The response appeared to be biphasic, with a maximally effective GTP concentration of 300 μM. GTP alone (30–600 μM) also stimulated the PLC. The ability of thrombin to stimulate polyphosphoinositide hydrolysis in the presence of 300 μM-GTP was completely inhibited by 5 mM-GDP[S] (Fig. 6). These results suggest that stimulation by the thrombin–receptor complex or p[NH]ppG requires a G-protein.

Effect of PDGF

PDGF (20 nM) did not induce the release of inositol polyphosphate products from the membrane fraction (Table 2). In parallel experiments carried out at 4 °C for 10 min, PDGF stimulated the alkali-stable phosphorylation of a 185 kDa membrane protein, probably the PDGF receptor (Heldin *et al.*, 1983), 8.0 ± 1.9-fold (means ± S.E.M.). These results suggest that, although both thrombin and PDGF stimulate polyphosphoinositide hydrolysis in intact fibroblasts, these two mitogens may activate PLC by somewhat different mechanisms.

Regulation by Ca^{2+}

The free Ca^{2+} concentration was a critical factor in PLC regulation. Thrombin, in the presence of GTP, stimulated polyphosphoinositide hydrolysis at Ca^{2+} concentrations between 1 and 100 nM (Fig. 7). In contrast, GTP alone significantly activated PLC only at Ca^{2+} concentrations above 100 nM. Similar results were obtained with p[NH]ppG (Fig. 8), although the enhancement of Ca^{2+}-sensitivity was less dramatic with this analogue. Stimulation by either thrombin or p[NH]ppG diminished at Ca^{2+} concentrations above 300 nM. Thus it appears that both thrombin and p[NH]ppG act to increase the affinity of the PLC for Ca^{2+}, a bivalent cation required for enzyme activity.

DISCUSSION

We have demonstrated that thrombin stimulates a PLC activity that hydrolyses PIP_2 and PIP to IP_3 and IP_2 in isolated fibroblast membranes. This effect was inhibited by hirudin, a specific thrombin antagonist that

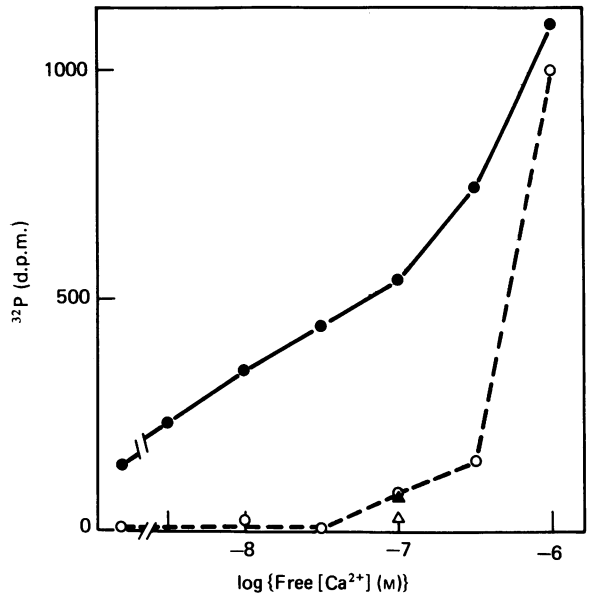

Fig. 7. Effect of thrombin on the concentration of Ca^{2+} needed to stimulate inositol polyphosphate production

Membranes were incubated for 2 min in buffer containing 1.8 mM-$MgCl_2$, various free Ca^{2+} concentrations and 300 μM-GTP in either the presence (●) or the absence (○) of thrombin (30 units/ml). Membranes were also incubated in buffer with 100 nM free Ca^{2+} without GTP in the presence (▲) or absence (△) of thrombin. Each point is the mean of the sums of the radioactivities in [^{32}P]IP_3 and [^{32}P]IP_2, which were determined in duplicate and did not vary by more than 10% from the mean. The amounts of inositol polyphosphates in the presence of thrombin and GTP were significantly greater than those in the presence of GTP alone (least significance $P < 0.025$ at all Ca^{2+} concentrations except 1 μM, for which $P > 0.05$).

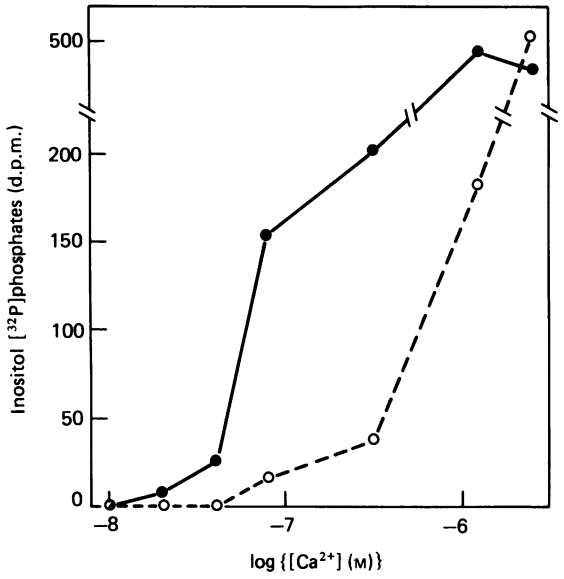

Fig. 8. Effect of p[NH]ppG on the Ca^{2+} concentration required to stimulate inositol polyphosphate production

Membranes were incubated in buffers containing 1.8 mM-$MgCl_2$ and various free Ca^{2+} concentrations in either the presence (●) or the absence (○) of 300 μM-p[NH]ppG. Each point is the mean of the sum of the radioactivities in [^{32}P]IP_3 and [^{32}P]IP_2 determined in duplicates which did not vary by more than 20% from the mean. p[NH]ppG stimulated significantly greater release of inositol polyphosphates than in controls without it, at Ca^{2+} concentrations of 40–1000 nM (least significance $P < 0.05$).

dissociates thrombin from its receptor (Van Obberghen-Schilling *et al.*, 1985). In intact fibroblasts (Carney *et al.*, 1985; Murayama & Ui, 1985; Paris & Pouyssegur, 1986) and platelets (Agranoff *et al.*, 1983; Watson *et al.*, 1984), thrombin stimulates the rapid hydrolysis of PIP_2 and PIP to IP_3, IP_2 and DG. We have shown that these reactions take place in a membrane compartment, where all of the necessary components reside.

Our results suggest that thrombin activates a PLC which is associated with the plasma membrane, since the thrombin receptor is localized to the cell surface (Carney & Cunningham, 1978). Although we have been unable to prepare highly purified plasma membrane from WI-38 fibroblasts, several studies demonstrate the presence of a PLC activity associated with the plasma membrane. Uhing *et al.* (1986) have reported that PIP_2 hydrolysis to IP_3 and DG is stimulated by guanosine 5′-[γ-thio]-triphosphate and Ca^{2+} in highly purified plasma membranes prepared from rat livers. Similar results have been reported by Kikuchi *et al.* (1986), using plasma membranes derived from HL-60 cells, a differentiated human leukaemia cell line.

Numerous studies have described both soluble and membrane-bound PLC activities that hydrolyse the polyphosphoinositides (Keough & Thompson, 1972; Akhtar & Abdel-Latif, 1978; Low & Weglicki, 1983; Wilson *et al.*, 1984; Banno *et al.*, 1986). The relationship between the soluble and membrane-bound lipases is not clear. Our results in fibroblast membranes demonstrate the presence of an associated PLC capable of rapidly hydrolysing up to 50% of the endogenous labelled polyphosphoinositides. Addition of cytosol neither increased the basal activity of PLC nor affected the stimulation of this enzyme activity by thrombin or p[NH]ppG. Several reports have described a membrane-associated PLC that is regulated, *in vitro*, by Ca^{2+}-mobilizing hormones. In contrast, Baldassare & Fisher (1986) have reported that cytosol added to platelet membranes stimulates basal PLC activity and acts synergistically with GTP to increase inositol polyphosphate production. However, the active cytosolic component(s) was not identified. Clearly the activity that we have measured in these membranes is not simply the result of contamination by a soluble PLC. Resolution of this issue will require purification of the membrane-associated lipase and comparison of this protein with the purified soluble enzymes.

Several growth-factor receptors possess intrinsic tyrosine kinase activity. These include receptors for insulin, PDGF and EGF (see Hunter & Cooper, 1985). Of these, only PDGF has clearly been shown to stimulate polyphosphoinositide hydrolysis (Habenicht *et al.*, 1981; Berridge *et al.* 1984; Chu *et al.*, 1985). The thrombin receptor has been identified as a 185 kDa glycoprotein in platelet membranes (Tollefsen & Majerus, 1976; Takamatsu *et al.*, 1986). It is not known whether it possesses protein tyrosine kinase activity, and thus far thrombin action in platelets or fibroblasts has not been correlated with an increase in protein phosphorylation

on tyrosine residues. In this study we compared PLC activation by PDGF and thrombin in fibroblasts membranes. Under conditions in which thrombin stimulated polyphosphoinositide hydrolysis, PDGF had no effect, despite evidence that PDGF could stimulate PDGF-receptor kinase activity in the membranes. The explanation for the failure of PDGF to stimulate polyphosphoinositide hydrolysis in fibroblast membranes is unclear. It is possible that the PDGF receptor requires tyrosine kinase substrates not present in the membrane preparation, or that there are separate pools of PIP_2 and PIP for thrombin- and PDGF-stimulated lipases. The results suggest a difference in at least some requirement for thrombin- and PDGF-stimulated polyphosphoinositide hydrolysis *in vitro*. However, these negative results do not allow us to draw any conclusions about the mechanism of PLC activation by PDGF.

Stimulation of polyphosphoinositide hydrolysis by thrombin was dependent on GTP. The non-hydrolysable GTP analogue p[NH]ppG, known to activate G-proteins in the absence of the hormone–receptor complex, also stimulated the hydrolysis of these lipids. Activation by thrombin or p[NH]ppG was blocked by GDP[S], a non-hydrolysable GDP analogue which is known to inhibit the activation of G-proteins (Eckstein *et al.*, 1979). These results suggest that in fibroblasts a GTP-binding protein is required to couple the thrombin receptor to the PLC in a manner analogous to regulation of adenylate cyclase (Ross & Gilman, 1980) and cyclic GMP phosphodiesterase (Stryer *et al.*, 1981). Several reports provide evidence to support this hypothesis. Houslay *et al.* (1986) have demonstrated that thrombin activates a GTPase in membranes prepared from human platelets. Moreover, Baldassare & Fisher (1986) have observed in platelet membranes that thrombin-stimulated hydrolysis of PIP_2 is potentiated by GTP and mimicked by a non-hydrolysable GTP analogue.

Thrombin, in the presence of GTP, maximally activated PLC at 100 nM-Ca^{2+} whereas GTP alone stimulated only at concentrations above 100 nM. The concentration of Ca^{2+} required for the thrombin-coupled reaction is similar to the estimated free Ca^{2+} concentration in quiescent fibroblasts (Moolenaar *et al.*, 1984). Ca^{2+} itself was stimulatory only at concentrations far beyond those required for thrombin stimulation, similar to that required to activate PIP_2 hydrolysis in erythrocyte membranes (Downes & Michell, 1982). The results suggest that thrombin activates PLC by lowering the threshold for Ca^{2+} stimulation. Previous studies in electrically permeabilized platelets by Knight *et al.* (1982) and Haslam & Davidson (1984*a*) have shown that thrombin increases the sensitivity of 5-hydroxytryptamine secretion to stimulation by Ca^{2+}. A portion of this effect required GTP, was blocked by GDP[S] and could be simulated by non-hydrolysable GTP analogues. Stimulation of secretion by thrombin was attributed to activation of protein kinase C (Knight & Scrutton, 1986). Haslam & Davidson (1984*b*) also demonstrated that thrombin decreased the threshold concentration for Ca^{2+}-stimulated DG generation. This effect was simulated by GTP and guanosine 5′-[γ-thio]triphosphate. The latter has also been shown to increase the sensitivity of polyphosphoinositide hydrolysis to Ca^{2+} activation in liver membranes (Uhing *et al.*, 1986). Our findings extend these observations by demonstrating that thrombin, a fibroblast mitogen, activates a polyphosphoinositide-specific PLC at physiological concentrations of Ca^{2+} in fibroblast membranes. We conclude that the thrombin–receptor complex is coupled to a GTP-binding protein that activates PLC by increasing the affinity of this enzyme for Ca^{2+}.

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