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GTP AND CYTOSOL STIMULATE PHOSPHOINOSITIDE HYDROLYSIS IN ISOLATED PLATELET MEMBRANES

Joseph J. Baldassare* and Gary J. Fisher+

*American Red Cross Bi-State Chapter
4050 Lindell Blvd., St. Louis, MO 63108

+Department of Dermatology,
University of Michigan Medical School,
Ann Arbor, MI 48109

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Summary - Hydrolysis of polyphosphoinositides by phospholipase C was examined in isolated membranes prepared from [^{32}P]labelled platelets. In the presence of GTPγS, thrombin increased the release of inositol triphosphate and inositol biphosphate approximately 500%. GTPγS alone stimulated release 2 fold. Maximal activation of thrombin-induced phosphoinositide hydrolysis was observed at 10 uM GTP. Although addition of calcium had no effect, 2 mM EGTA completely inhibited inositolphosphate release. Addition of high speed supernatant to [^{32}P]labelled membranes stimulated the release of inositolphosphates. This hydrolysis was further enhanced by the addition of GTP. These data demonstrate that the breakdown of polyphosphoinositides in isolated platelet membranes is dependent on GTP and stimulated by platelet cytosol. © 1986 Academic Press, Inc.

In a variety of cell types agonist stimulation leads to the rapid hydrolysis of polyphosphoinositides by phospholipase C (1-5). The products of this reaction, inositol triphosphate and diacyglycerol have been identified as important intracellular messengers involved in the regulation of calcium homeostasis (6) and protein kinase activities (7).

Haslam and Davidson (8) recently reported that addition of nonhydrolyzable GTP analogues to permeabilized platelets enhanced thrombin-stimulated diacyglycerol formation. Recently several laboratories have demonstrated in a variety of cell types, that agonist-coupled polyphosphoinositide hydrolysis in isolated membranes is dependent on GTP (9-11). These data suggest that the phospholipase C responsible for agonist induced phosphoinositide breakdown is membrane associated and regulated by a guanine nucleotide binding protein.

Abbreviations: PIP_2, phosphotidylinositol-diphosphate; PIP, phosphotidylinositol-phosphate; PI, phosphotidylinositol; IP_3, inositol-triphosphate; IP_2, inositol-diphosphate; IP, inositol-phosphate, EGTA, ethylene glyco bis (β-aminoetylethen-N',N',N',N'-tetracetic acid; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; GTPγS, guanosine 5'-(3-0)-thio) triphosphate.

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In platelets, the majority of the phospholipase C activity is cytosolic. Whether this activity participates in agonist-induced phosphoinositide hydrolysis is currently unknown.

In order to investigate the mechanism of polyphosphoinositide breakdown in platelets, we have examined the effects of guanine nucleotides and cytosol on agonist (thrombin) stimulated polyphosphoinositide hydrolysis in isolated platelet membranes.

Materials and Methods - Blood was drawn from healthy volunteers into acid/citrate/dextrose (5 ml/60 ml blood) and then incubated with aspirin (0.5 mM) for 15 minutes at 37°C. Platelets were isolated by differential centrifugation (12). The platelets from one liter of blood were incubated in 30 ml of calcium free Hepes buffer pH 6.5 (12) with 2 mCi [^{32}P] PO_4 at room temperature for approximately 2 hours. The labelling was carried out in a platelet bag used for long term storage of platelets. The platelets were then gel filtered through a Sephorase 2B-CL column that had been equilibrated with calcium free Hepes buffer, pH 6.8. Membranes were then prepared as described by Wood and Steer (13). The final membrane pellet unless otherwise indicated was resuspended at between 2 to 4 mg/ml in cold buffer containing 20 mM Hepes with 100 mM NaCl, 25 mM LiCl, 2 mM $MgCl_2$, 0.3 mM EGTA pH 7.4 (Buffer A). TLC analysis of the [^{32}P]labelled phospholipids in our membrane preparations showed that each assay contained approximately 50 x 10^3 cpm, 41 x 10^3 cpm and 12 x 10^3 cpm in the PIP_2, PIP and PI , respectively. Membranes were incubated in a total volume of 500 ul at 37°C in Buffer A unless otherwise indicated. At the indicated times the incubations were terminated by the addition of 2 ml chloroform/methanol/12 N HCL (200:100:0.75 by volume). The [^{32}P]labelled inositolphosphates were extracted and analyzed on 0.5 ml columns of Dowex-1 (formate form) as described by Downes et al. (14). Since these conditions did not resolve inositol monophosphate from inorganic phosphate, data are presented for only inositol diphosphate and inositol triphosphate. Protein was determined according to Peterson (15). [^{32}P]orthophosphoric acid was obtained from ICN, Inc. GTP, GPP(NH)P and GTPγS were purchased from Boehringer Mannheim.

Results and Discussion - In the presence of 2 mM $MgCl_2$, addition of GTPγS (100 uM) to membranes from platelets prelabelled with [^{32}P] resulted in a small enhancement of release of inositolphosphates compared to control incubations (Fig. 1). Incubation of membranes with thrombin (1 U/ml) plus GTPγS (100 uM) lead to a much larger increase of both inositol triphosphate and inositol diphosphate (Figure 1). No lag was observed in the formation of either IP_2 or IP_3. Analysis of the individual inositol phosphates indicated that 30 seconds after the addition of thrombin, the release of IP3 and IP2 were enhanced 800% compared to GTPγS alone.

It cannot be concluded, however, that both PIP and PIP_2 are hydrolyzed at the same rate since the appearance of IP_2 may result from either phospholipase C hydrolysis of PIP or dephosphorylation of IP_3 by monoesterase activity. It has been shown that platelets contain a very active, LiCl insensitive, soluble phosphomonoesterase that specifically hydrolyzes the 5-phosphate of inositol 1,4,5 triphosphate (16). In addition, lithium insensitive monophosphatases have been identified in erythrocyte (14) and the liver membranes (17).

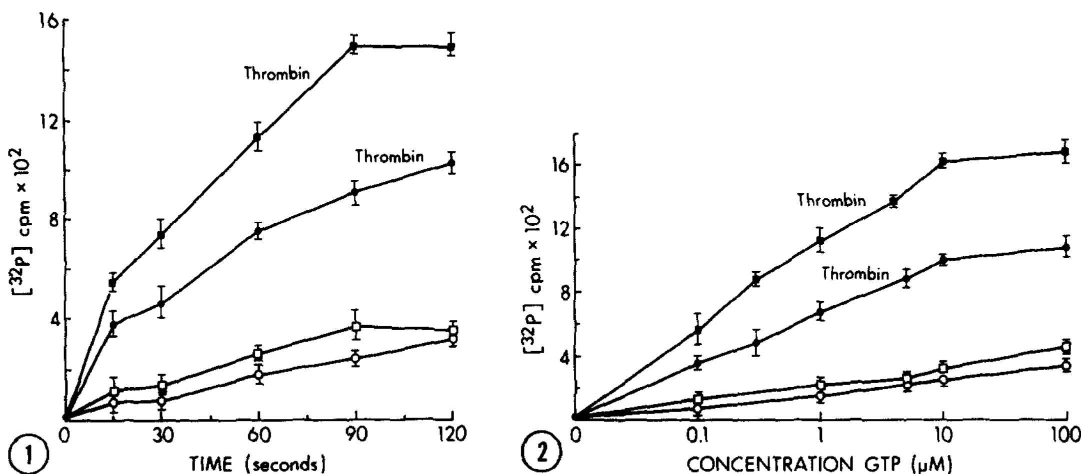

Figure 1. Time course of thrombin-stimulated inosititolphosphate release from $[^{32}P]$labelled platelet membranes. Membranes (approximately 2-4 mg protein/ml) isolated from $[^{32}P]$labelled intact platelet were incubated in the absence or presence of thrombin (1 Unit/ml) for the indicated time at 37°C in Buffer A (see Materials and Methods) and 100 uM GTPγS. $[^{32}P]IP_3$ (□-□, ■-■) and $[^{32}P]IP_2$ (○-○,●-●) were isolated as described under Materials and Methods. The value at each time point is expressed as the difference of the sample value minus the control value (absence of GTP and Thrombin). Results shown are the mean ± SE of three seperate experiments.

Figure 2. GTP-dependence of the release of inositolphophates from $[^{32}P]$labelled platelet membranes. $[^{32}P]$labelled platelet membranes were incubated in the absence and presence of thrombin (1 Unit/ml). at 37°C in buffer A and the indicated concentrations of GTP. $[^{32}P]IP_3$ (□-□, ■-■) and $[^{32}P]IP_2$ (○-○,●-●) were isolated as described under Materials and Methods Results shown are the mean ± SE of three seperate experiments.

The question of substrate specificity of agonist-induced phosphoinositide hydrolysis might better be addressed by analysis of the changes in the individual phosphoinositides. In our experiments, however, the changes in amounts of phosphoinositides were too small for accurate determination. In addition, it is difficult to ascribe significance to measurements of net loss of $[^{32}P]$labelled phosphoinositides, since there are appreciable PI and PIP phosphatase and kinase activities associated with isolated membranes (9).

The effect of GTP concentration on the basal and thrombin-stimulated release of inositolphosphates is shown in Figure 2. GTP, in the presence or absence of thrombin, caused a significant, dose dependent, increase in release of inositolphosphates. The accumulation of IP_2 and IP_3 showed similar GTP dependencies. In the presence of thrombin half maximal and maximal stimulation were observed at 0.2 and 10 uM GTP, respectively. These values are similar to those reported by Litosch et al. (9) for 5-methyltryptamine-stimulated release of inositolphosphates from blow fly salivary gland membranes. Thrombin-stimulated phosphoinositide hydrolysis specifically required GTP or its nonhydrolyzable analogues, since no stimulation of hydrolysis was observed in the presence of GDP (data not

Table I. Effect of platelet high speed supernatant on inositolphosphate release from [^{32}P]labelled platelet membranes. [^{32}P]labelled platelet membranes (4 mg membrane protein/ml) were incubated as described under the Materials and Methods. The reaction was stopped after 120 seconds and [^{32}P]labelled IP_2 and IP_3 were quantitated as described under Material and Methods. Results shown are mean ± SE of three seperate experiments.

Conditions	IP_2 cpm	IP_3 cpm
none	95±53	122±78
GTPγS (10 uM) + Thrombin (1 Unit/ml)	1018±156	1393±139
Cytosol (final conc. 200 ug protein/ml)	1837±182	2377±301
Cytosol + GTPγS	4973±210	6531±199
Cytosol + GTPγS + Thrombin	4318±258	6299±311

shown). We consistently observed that the nonhydrolyzable GTP analogue GTPγS was a more potent stimulator of phosphoinositide hydrolysis, both in the absence and presence of thrombin. Nonhydrolyzable GTP analogues have also been demonstrated to be more potent effectors of adenylate cyclase activity (18). This is believed to be due to their resistance to hydrolysis by the GTPase activity associated with the guanine nucleotide regulatory proteins. The above data demonstrate that polyphosphoinositide hydrolysis in isolated membranes requires GTP and is stimulated by thrombin.

In platelets the vast majority of phospholipse C activity is found in the cytosol (19). Since this activity has traditionally been assayed in the presence of calcium and without added magnesium, the relationship between this activity and the thrombin-stimulated GTP-dependent phospholipase C activity we observed in isolated platelet membranes is not clear. Therefore, we were interested in investigating the effect of platelet cytosol on phosphoinositide hydrolysis of platelet membranes. Incubation of membranes with platelet cytosol resulted in even greater accumulation of inositolphosphates than membranes exposed to thrombin and GTPγS (Table I). Unexpectedly addition of GTPγS and cytosol further stimulated phosphoinositide hydrolysis 300%. No further stimulation, however, was observed with the addition of thrombin.

The finding that platelet cytosol stimulated inositolphosphate release in the absence of added calcium is consistent with the characteristics of a soluble phospholipase C from seminal vesicles described by Wilson et al. (20). These investigators found that PI hydrolysis, but not hydrolysis of PIP or PIP_2 required calcium. What is novel, however, is that the addition of GTPγS further enhanced the effect of cytosol on accumulation of inositol-phosphates.

At the present time there are several possible explanations for this intriguing observation. The data presented above (Fig. 2) suggest that GTP

can act directly on isolated membranes to stimulate thrombin-induced inositol-phosphate release. This is consistent with data from other laboratories suggesting that phospholipase C-catalyzed hydrolysis of polyphosphoinositides is regulated by a membrane bound guanine nucleotide regulatory protein (9-11). Our data suggest that some additional component(s) in the cytosol is involved in GTP-regulated polyphosphoinositide breakdown. It is reasonable to suggest that this component is the soluble phospholipase C, however, our data do not preclude other cytosolic components. The membrane associated phospholipase C appears to be stimulated by GTP (9-11). One possible interpretation of our data is that the soluble phospholipase C might also be regulated by GTP. This hypothesis can be tested by examination of the effect of GTP on the hydrolysis of polyphosphoinositides in phospholipid vesicles by the cytosolic phospholipase C. It should also be possible to identify the soluble stimulatory factor(s) by fractionation of the cytosol and assaying for GTP-dependent phosphoinositide hydrolysis. We are presently pursuing both these courses of action in our laboratories.

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