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Activation of phospholipase C associated with isolated rabbit platelet membranes by guanosine 5′-[γ-thio]triphosphate and by thrombin in the presence of GTP

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Rabbit platelets were labelled with [^{3}H]inositol and a membrane fraction was isolated in the presence of ATP, $MgCl_2$ and EGTA. Incubation of samples for 10 min with 0.1 μM-Ca^{2+}_{free} released [^{3}H]inositol phosphates equivalent to about 2.0% of the membrane [^{3}H]phosphoinositides. Addition of 10 μM-guanosine 5′-[γ-thio]triphosphate (GTP[S]) caused an additional formation of [^{3}H]inositol phosphates equivalent to 6.6% of the [^{3}H]phosphoinositides. A half-maximal effect was observed with 0.4 μM-GTP[S]. The [^{3}H]inositol phosphates that accumulated consisted of 10% [^{3}H]inositol monophosphate, 88% [^{3}H]inositol bisphosphate ([^{3}H]IP_2) and 2% [^{3}H]inositol trisphosphate ([^{3}H]IP_3). Omission of ATP and $MgCl_2$ led to depletion of membrane [^{3}H]polyphosphoinositides and marked decreases in the formation of [^{3}H]inositol phosphates. Thrombin (2 units/ml) or GTP (4–100 μM) alone weakly stimulated [^{3}H]IP_2 formation, but together they acted synergistically to exert an effect comparable with that of 10 μM-GTP[S]. The action of thrombin was also potentiated by 0.1 μM-GTP[S]. Guanosine 5′-[β-thio]diphosphate not only inhibited the effects of GTP[S], GTP and GTP with thrombin, but also blocked the action of thrombin alone, suggesting that this depended on residual GTP. Incubation with either GTP[S] or thrombin and GTP decreased membrane [^{3}H]phosphatidylinositol 4-phosphate ([^{3}H]PIP) and prevented an increase in [^{3}H]phosphatidylinositol 4,5-bisphosphate ([^{3}H]PIP_2) observed in controls. Addition of unlabelled IP_3 to trap [^{3}H]IP_3 before it was degraded to [^{3}H]IP_2 showed that only about 20% of the additional [^{3}H]inositol phosphates that accumulated with GTP[S] or thrombin and GTP were derived from the action of phospholipase C on [^{3}H]PIP_2. The results provide further evidence that a guanine-nucleotide-binding protein mediates signal transduction between the thrombin receptor and phospholipase C, and suggest that PIP may be a major substrate of this enzyme in the platelet.

INTRODUCTION

Activation of a phosphoinositide-specific phospholipase C plays a key role in mediating the effects of many physiological stimuli on cells [1,2]. Hydrolysis of any phosphoinositide (PI, PIP or PIP_2) can, in principle, generate diacylglycerol and so activate protein kinase C, whereas hydrolysis of PIP_2 alone can generate the IP_3 that releases Ca^{2+} ions from the endoplasmic reticulum into the cytosol. However, in many cells, PI may act as a reservoir for regeneration of PIP_2, rather than as an immediate substrate of phospholipase C [2–4]. In human platelets, thrombin is known to cause a transient decrease in PIP_2 [5–7] and the rapid formation of both diacylglycerol [8] and inositol phosphates, particularly IP_2 and IP_3 [6,9–11]. Similar changes in PIP_2 and inositol phosphates have been observed after addition of thrombin to rabbit platelets [12–14]. Thrombin also causes a marked loss of platelet PI that has been attributed largely to its hydrolysis by phospholipase C rather than to replenishment of PIP_2 [8,15,16]. However, as little IP accumulates immediately after addition of thrombin, even when Li^+ is added to inhibit IP breakdown [9–11,13], and part of the PI broken down is converted into lyso-PI [11,17], the relative importance of different phosphoinositides as substrates of phospholipase C in platelets remains uncertain. In any case, there is abundant evidence from the above studies that it is the hydrolysis of polyphosphoinositides, rather than that of PI, that is most closely related to receptor action in platelets, as in other cells [2,18].

Signal transduction between receptors and phospholipase C appears to be mediated by guanine-nucleotide-binding proteins [19]. The first indication of this came from the finding that guanine nucleotides stimulate the secretion of serotonin from permeabilized platelets at physiological pCa values and enhance the ability of agonists that activate phospholipase C to exert the same effect [20]. Addition of the non-hydrolysable GTP analogue GTP[S], or of thrombin with GTP, was then found to increase [^{3}H]diacylglycerol formation in permeabilized platelets that had been labelled with [^{3}H]arachidonate [21]. Experiments with permeabilized GH_3 cells [22] or pancreatic acinar cells [23] containing [^{3}H]inositol-labelled phosphoinositides have also demonstrated the guanine-nucleotide-dependent activation of

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; pCa, $-\log [Ca^{2+}_{free}]$; G_i, guanine-nucleotide-binding protein mediating inhibition of adenylate cyclase.

phospholipase C, and we have found that GTP[S] or thrombin and GTP stimulate [^{3}H]inositol phosphate formation from [^{3}H]phosphoinositides in permeabilized human platelets [24]. GTP[S] has been shown to enhance the hydrolysis of labelled polyphosphoinositides in isolated membranes from several cells and tissues [25–29], and a GTP-dependent hormonal activation of membrane-bound phospholipase C has also been demonstrated in a few instances, most notably by 5-hydroxytryptamine in blowfly salivary-gland membranes [26], by *N*-formyl-methionyl-leucyl-phenylalanine in neutrophil membranes [30], by vasopressin in hepatocyte membranes [31] and by thyrotropin-releasing hormone in GH_3-cell membranes [29,32,33]. In the present study, we sought to obtain a platelet membrane preparation in which the guanine-nucleotide-dependent activation of phospholipase C by physiological agonists was preserved and could be studied easily. This was achieved by using membranes from rabbit platelets labelled with [^{3}H]inositol, provided that ATP was added to maintain [^{3}H]polyphosphoinositide concentrations. However, [^{3}H]PIP proved to be a more important substrate of the membrane-bound phospholipase C than was [^{3}H]PIP_2. Some of our results have been published in a preliminary form [34].

EXPERIMENTAL

Materials

myo-[2-^{3}H]Inositol (15 Ci/mmol) was from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.), D-*myo*-[2-^{3}H]inositol 1,4-bisphosphate (1 Ci/mmol) and ACS scintillant were from Amersham Canada Ltd. (Oakville, Ont., Canada) and [^{32}P]P_i (carrier-free) was from Du Pont Canada (Dorval, Que., Canada). [^{32}P]IP_3 and [^{32}P]IP_2 were prepared from ^{32}P-labelled red cells [35]. Ready-Solv HP/b scintillant was from Beckman Instruments Ltd. (Toronto, Ont., Canada). GTP[S] (tetralithium salt) and GDP[S] (trilithium salt) were obtained from Boehringer Mannheim Canada Ltd. (Dorval, Que., Canada), and ATP (disodium salt, prepared by phosphorylation of adenosine), GTP, EGTA, Pipes, unlabelled IP_3, brain phosphoinositides and protein standard solution were from Sigma (St. Louis, MO, U.S.A.). Dowex-1 anion-exchange resin (AG 1-X8; 100–200 mesh, formate form) was obtained from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ont., Canada), and silica-gel t.l.c. plates (Si250) were from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Apyrase was prepared as described by Molnar & Lorand [36]. Human α-thrombin (2700 units/mg) and α-thrombin inactivated by treatment with di-isopropyl fluorophosphate were kindly provided by Dr. J. W. Fenton II of New York State Department of Health (Albany, NY, U.S.A.).

Preparation of platelet membranes containing [^{3}H]phosphoinositides

Rabbit platelets were isolated and washed in Ca^{2+}-free Tyrode's solution containing 0.35% bovine serum albumin as described in [37], apart from the following modifications. Thus, all washing solutions were supplemented with 30 μg of apyrase/ml and 10 mM-Pipes (buffered to pH 6.5 with NaOH). After the first wash, the platelets (usually 4×10^{10}–6×10^{10}) were resuspended at 5×10^9/ml in fresh medium and incubated for 2 h at 37 °C with 25 μCi of [^{3}H]inositol/ml. The platelets were then washed once more to remove [^{3}H]inositol and finally resuspended at 2×10^9/ml in a solution containing 100 mM-KCl, 25 mM-Hepes and 2.5 mM-EGTA (adjusted to pH 7.4 with KOH to give Buffer A), which was nomally supplemented with 1.23 mM-ATP and 1.56 mM-$MgCl_2$ (Buffer B). The suspension was frozen and thawed twice (with solid CO_2/acetone), and the platelet particulate fraction was then isolated by centrifugation at 37500 $g_{av.}$ for 40 min at 4 °C. The pellet was resuspended in the same volume of Buffer B without KCl by using a Dounce homogenizer with an A pestle and re-centrifuged. This procedure was repeated and the membrane pellet was finally suspended in complete Buffer B at a protein concentration of 0.5–0.75 mg/ml (equivalent to 2×10^9–3×10^9 platelets/ml). Protein in the suspension was determined as in [38], by using a standard containing 62.5% human albumin and 37.5% human globulin.

Incubations

Incubation mixtures contained 400 μl of membrane suspension in Buffer B and a total of 100 μl of additions dissolved in Buffer A. These included a concentrated solution of ATP and $MgCl_2$ to give the same final concentrations of each as in Buffer B, a freshly prepared solution containing $CaCl_2$ and KOH to give the required pCa value at pH 7.4 and solutions containing guanine nucleotides, thrombin etc. Incubations were started by simultaneous addition of membranes and $CaCl_2$/KOH to the other components, followed by rapid mixing. In most experiments, a pCa of 7 was used; this required a final $CaCl_2$ concentration of 1.48 mM, calculated according to [39]. The final concentrations of MgATP and $Mg^{2+}{}_{free}$ were 1.0 mM and 0.5 mM respectively. After incubation of samples for 0–10 min at 25 °C, reactions were stopped by addition of 0.25 ml of 30% (w/v) trichloroacetic acid.

Measurement of labelled inositol phosphates and phosphoinositides

After centrifugation of the acidified samples, the supernatants were neutralized with NaOH by using Bromothymol Blue as an indicator and diluted to 5 ml with water. Labelled IP, IP_2 and IP_3 in these extracts were then separated by chromatography on columns containing 1.25 ml of Dowex-1 resin, as described by Berridge *et al.* [40], with minor modifications. Thus, after elution of inositol and glycerophosphoinositol, IP was eluted with 3×5 ml of 0.15 M-ammonium formate in 0.1 M-formic acid, as significant amounts of IP_2 appeared in the final IP fraction when 0.2 M-ammonium formate was used. IP_2 was eluted with 4×5 ml of 0.4 M-ammonium formate in 0.1 M-formic acid, and IP_3 by 3×5 ml of 1.0 M-ammonium formate in 0.1 M-formic acid. Individual 5 ml fractions were adjusted to contain 1 M-ammonium formate, mixed with 15 ml of ACS scintillant and counted for ^{3}H (efficiency 14%) and, when present, ^{32}P. ^{3}H found in inositol phosphates was corrected for quenching and cross-over of ^{32}P (if any) and expressed as d.p.m./mg of membrane protein. This stepwise-elution method was validated by gradient elution of the [^{3}H]inositol phosphates from Dowex-1 resin (0.1–1.2 M-ammonium formate in 0.1 M-formic acid) and by h.p.l.c. of the fractions obtained on a Waters Radial-PAK cartridge containing Partisil SAX, which was eluted as in [41]. [^{32}P]IP_2 and [^{32}P]IP_3 prepared from

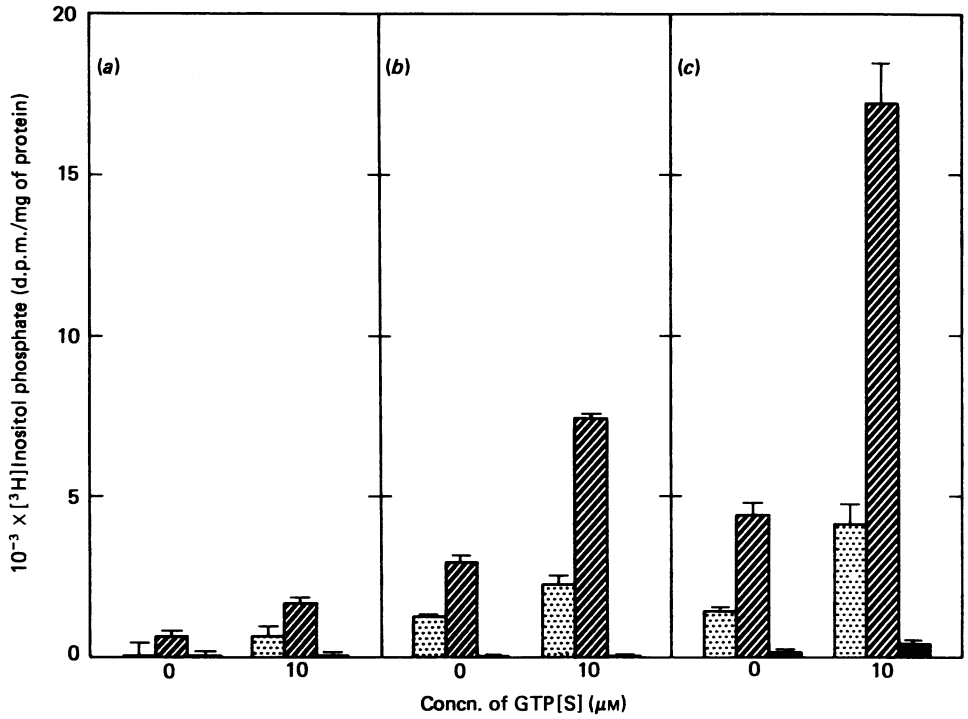

Fig. 1. Effects of addition of ATP during the preparation and incubation of platelet membranes on the formation of [^{3}H]inositol phosphates from endogenous [^{3}H]phosphoinositides

Membranes were prepared from [^{3}H]inositol-labelled platelets, as follows: (*a*) omitting ATP and $MgCl_2$ from the lysis, washing and incubation media; (*b*) omitting ATP and $MgCl_2$ from only the final resuspension and incubation medium; (*c*) with ATP and $MgCl_2$ present in all media, as described in the Experimental section. Samples (0.25 mg of membrane protein) were incubated for 10 min at 25 °C with $CaCl_2$ (pCa 7) in the absence or presence of 10 μM-GTP[S]. [^{3}H]Inositol phosphates were isolated and counted for radioactivity; small amounts present before incubation were subtracted. Values are means ± S.E. of the difference from three identical incubation mixtures. ▨, IP; ▩, IP_2; ■, IP_3.

red cells and commercial [^{3}H]IP_2 were used as standards. Their recovery on stepwise elution from Dowex-1 was 95–100%.

Phosphoinositides were extracted by a modification of the method of Rittenhouse [42]. The trichloroacetic acid pellets were washed with 1 ml of water, resuspended in a further 1 ml of water and mixed with 3.75 ml of chloroform/methanol/conc. HCl (100:200:3, by vol.). After 15 min at room temperature, 1 ml of chloroform and 1 ml of 1.8 M-KCl were mixed in, and the lower chloroform phase was removed for analysis. In some experiments, a further extraction was carried out to optimize the recovery of phosphoinositides. The aqueous phase was then mixed first with 1.1 ml of chloroform, 1.4 ml of methanol and 0.02 ml of conc. HCl and, after 15 min, with a further 1.0 ml of chloroform, 0.5 ml of water and 0.5 ml of 1.8 M-KCl. The lower phase, containing about 5% of the total phosphoinositides, was again removed for analysis. The phospholipid extracts were dried in a stream of N_2, dissolved in 80 μl of chloroform/methanol (2:1, v/v), mixed with 25 μg of carrier phosphoinositides and analysed by t.l.c. on silica-gel plates [5]. Silica gel from the areas containing PI, PIP and PIP_2 was scraped into scintillation vials, mixed with 0.5 ml of methanol/conc. HCl (25:1, v/v) and 8 ml of HP/b scintillant, and counted for ^{3}H (efficiency 25%). The results were expressed as d.p.m./mg of membrane protein for comparison with [^{3}H]inositol phosphates formed in the same experiments.

RESULTS

ATP requirement for the formation of [^{3}H]inositol phosphates from isolated membranes containing [^{3}H]phosphoinositides

In rabbit platelets labelled with [^{3}H]inositol as described in the Experimental section, ^{3}H was distributed between the phosphoinositides with 79.2±1.4% in PI, 13.4±1.0% in PIP and 7.4±0.9% in PIP_2 (mean values ± S.E.M., six expts.). Preparation of a platelet mixed-membrane fraction by freezing and thawing the platelets in Buffer A, which contained no ATP or $MgCl_2$, followed by two washes of the membranes in the same medium without KCl, diminished [^{3}H]PIP and [^{3}H]PIP_2 to 9.6±1.0% and 3.3±0.6% of the total [^{3}H]phosphoinositides respectively (mean values ± S.E.M., four expts.). Incubation of these membranes at a pCa of 7 led to the formation of only trace amounts of [^{3}H]inositol phosphates in the absence of GTP[S] and of only slightly more in the presence of 10 μM-GTP[S] (Fig. 1*a*). To test the possibility that this weak response to GTP[S] was due to depletion of membrane [^{3}H]polyphosphoinositides, both ATP and $MgCl_2$ were included in the membrane isolation and incubation media (see the Experimental section). This procedure yielded membrane preparations in which [^{3}H]PIP and [^{3}H]PIP_2 amounted to 19.6±0.6% and 6.3±0.4% of total [^{3}H]phosphoinositides respectively (mean values ± S.E.M., 13 expts.). Thus membrane [^{3}H]PIP_2 was largely

preserved and [^{3}H]PIP was increased above that found in intact platelets. With these membrane preparations, the formation of [^{3}H]inositol phosphates at a pCa of 7 was appreciable in control incubations, and the stimulatory effect of GTP[S] was enhanced in percentage and particularly in absolute terms (Fig. 1*c*). The control and additional GTP[S]-stimulated formation of [^{3}H]inositol phosphates was equivalent to $2.0 \pm 0.5\%$ and $6.6 \pm 0.6\%$ of the total membrane [^{3}H]phosphoinositides respectively (mean values $\pm$ S.E.M., 11 expts.). The effects of addition of thrombin (2 units/ml) with 10 μM-GTP were also studied. No significant increase in [^{3}H]inositol phosphates was observed in the absence of ATP and $MgCl_2$, but an increase equivalent to $6.3 \pm 1.0\%$ of the [^{3}H]phosphoinositides (mean $\pm$ S.E.M., five expts.) was seen when ATP and $MgCl_2$ were present. Omission of both $MgCl_2$ and ATP from the final resuspension and incubation medium alone gave intermediate results with respect to the effect of GTP[S] (Fig. 1*b*), but permitted only a very weak stimulatory effect of thrombin and GTP. In subsequent experiments, ATP and $MgCl_2$ were added during both the isolation and the incubation of platelet membranes.

Effects of GTP[S] on the formation of [^{3}H]inositol phosphates

Although GTP[S] caused reproducible increases in the formation of [^{3}H]inositol phosphates, when these were expressed as percentages of the membrane [^{3}H]phosphoinositides (see above), the stimulation of phospholipase C activity was more variable when expressed as multiples of the control values, which appeared to vary independently. On this basis, the stimulatory effect of GTP[S] ranged from 2- to 18-fold (mean 4-fold) in 11 experiments. In both the absence and the presence of GTP[S], most of the [^{3}H]inositol phosphates that accumulated were [^{3}H]IP_2, with some [^{3}H]IP (Fig. 1*c*). After 10 min with 10 μM-GTP[S], the additional [^{3}H]inositol phosphates comprised $10 \pm 1\%$ [^{3}H]IP, $88 \pm 1\%$ [^{3}H]IP_2 and $2 \pm 0\%$ [^{3}H]IP_3 (mean values $\pm$ S.E.M., 11 expts.). The [^{3}H]IP_2 accumulating in the presence of GTP[S] was analysed by h.p.l.c. and was shown to co-chromatograph with commercial [^{3}H](1,4)IP_2. In the absence of added $CaCl_2$ (pCa > 8), the total [^{3}H]inositol phosphates formed in 10 min in the presence of GTP[S] amounted to less than 15% of those formed at a pCa of 7. The accumulation of [^{3}H]IP and [^{3}H]IP_2 was greater at a pCa of 6 than 7 in the absence, but not in the presence, of 10 μM-GTP[S]. The time course of [^{3}H]inositol phosphate accumulation at a pCa of 7 was similar in the presence and absence of 10 μM-GTP[S] (Fig. 2). [^{3}H]IP_2 formation was linear for about 5 min and then slowed, whereas [^{3}H]IP increased mainly between 5 and 10 min, suggesting a precursor–product relationship between the two compounds. [^{3}H]IP_3 accumulation was significant in 10 min, but not in 2 min, incubations. At a pCa of 7, increases in the accumulation of [^{3}H]IP_2 were detectable with 0.04 μM-GTP[S] and nearly maximal with 10 μM-GTP[S] (Fig. 3). A half-maximal effect required 0.38 μM-GTP[S] (mean from two expts.).

Synergistic effects of thrombin and GTP

Addition of thrombin concentrations above 0.2 unit/ml increased the formation of [^{3}H]inositol phosphates in the absence of GTP; in 10 min incubations with 2

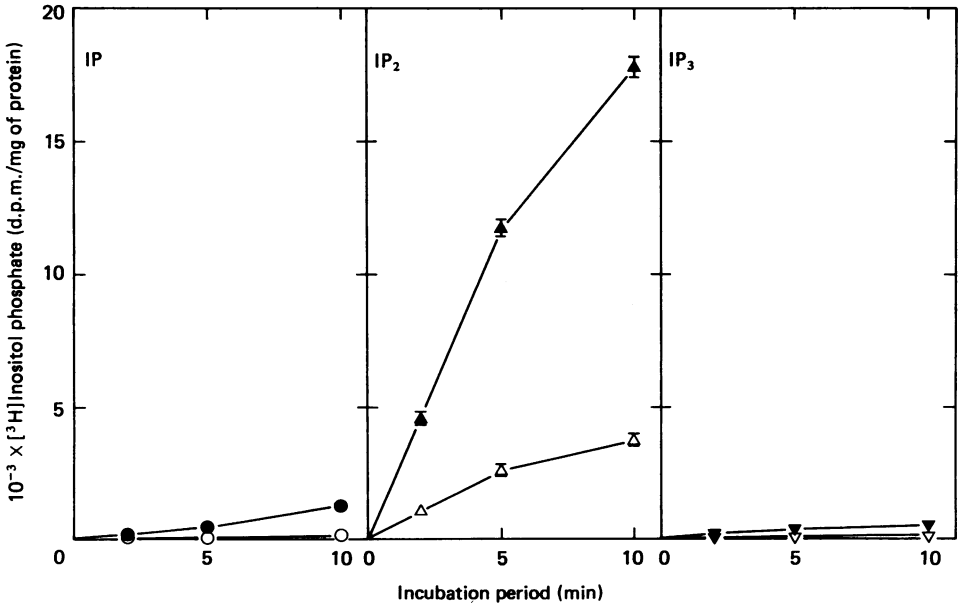

Fig. 2. Time course of the formation of [^{3}H]inositol phosphates from membrane [^{3}H]phosphoinositides in the presence and absence of GTP[S]

Membranes from platelets labelled with [^{3}H]inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.23 mg of membrane protein were incubated at 25 °C with ATP, $MgCl_2$ and $CaCl_2$ (pCa 7) in the presence (black symbols) or absence (white symbols) of 10 μM-GTP[S]. Incubations were terminated after the periods indicated and [^{3}H]inositol phosphates determined. Amounts present before incubation were subtracted. Values are means $\pm$ S.E. of the difference from three identical incubation mixtures.

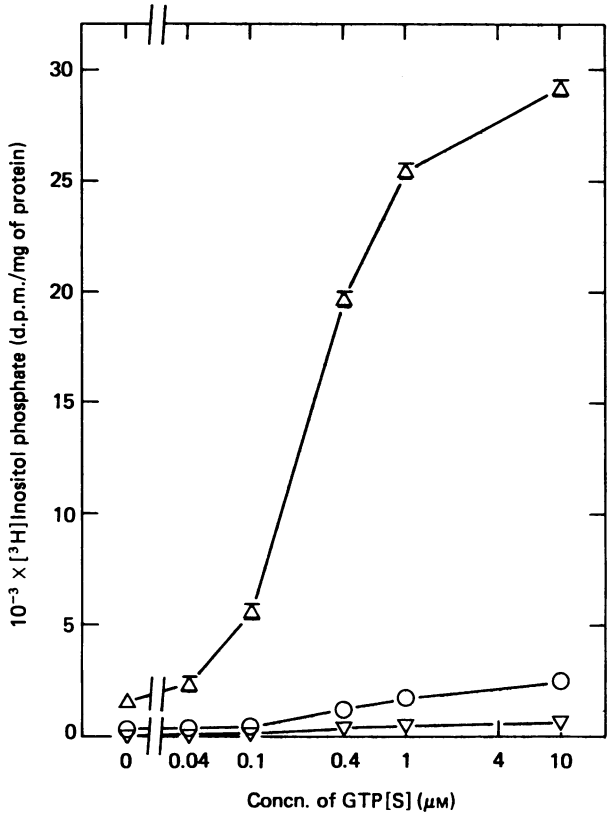

Fig. 3. Effects of different GTP[S] concentrations on the formation of [^{3}H]inositol phosphates from membrane phosphoinositides

Membranes from platelets labelled with [^{3}H]inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.27 mg of membrane protein were incubated for 10 min at 25 °C with ATP, $MgCl_2$, $CaCl_2$ (pCa 7) and the indicated concentrations of GTP[S]. [^{3}H]Inositol phosphates were determined and the amounts present before incubation subtracted. Values are means ± S.E. of the difference from three identical incubation mixtures. ○, IP; △, IP_2; ▽, IP_3.

units/ml, the increase was equivalent to 27 ± 3% of that observed with 10 μM-GTP[S] (mean ± S.E.M., five expts.). Similarly, GTP alone increased the release of [^{3}H]inositol phosphates, causing a half-maximal effect at 5.1 μM (mean from two expts.). The maximal effect of GTP was equivalent to 20% of that caused by GTP[S]. Additions of both thrombin (2 units/ml) and 4 μM-GTP increased the additional formation of [^{3}H]inositol phosphates over 10 min to 85 ± 3% of the value obtained with 10 μM-GTP[S] (mean ± S.E.M., five expts.), whereas with thrombin (2 units/ml) and 10 μM-GTP a value equivalent to 101 ± 2% of the GTP[S] effect was obtained (mean ± S.E.M., six expts.). Higher GTP concentrations had no further effect (Tables 1–3). Thus thrombin and GTP acted synergistically to promote [^{3}H]phosphoinositide breakdown and exerted a maximum effect over 10 min similar to that obtained with GTP[S]. In shorter incubations, thrombin and GTP stimulated the formation of [^{3}H]inositol phosphates slightly more than did 10 μM-GTP[S] (e.g. Table 4). The proportions in which [^{3}H]inositol phosphates accumulated after addition of thrombin and GTP were identical with those observed

Table 1. Potentiation by GTP or GTP[S] of the stimulatory effect of thrombin on [^{3}H]IP_2 formation from membrane [^{3}H]phosphoinositides

Membranes from platelets labelled with [^{3}H]inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.27 mg of membrane protein were incubated for 10 min at 25 °C with ATP, $MgCl_2$ and sufficient $CaCl_2$ to give a pCa of 7 (see the Experimental section). Other additions to incubation mixtures were guanine nucleotides and thrombin, as indicated. [^{3}H]IP_2 was isolated and counted for radioactivity; that present before incubation was subtracted. Values are means ± S.E. of the difference from three identical incubation mixtures.

Guanine nucleotide	10^{-3} × [^{3}H]IP_2 formed (d.p.m./mg of protein)		
	Without thrombin	With thrombin (2 units/ml)	Increase
None	1.5 ± 0.1	7.7 ± 0.5	6.2 ± 0.5
GTP (4 μM)	4.0 ± 0.1	23.7 ± 0.5	19.7 ± 0.5
GTP (40 μM)	6.7 ± 0.1	27.4 ± 1.2	20.7 ± 1.2
GTP[S] (0.1 μM)	5.5 ± 0.2	23.4 ± 0.8	17.9 ± 0.8
GTP[S] (10 μM)	29.2 ± 0.3	34.5 ± 0.6	5.3 ± 0.7

with GTP[S], with [^{3}H]IP_2 accounting for about 90% of the total after 10 min. A low concentration of GTP[S] (0.1 μM) also potentiated the action of thrombin, but no synergism was seen between concentrations of thrombin and GTP[S] that had optimal effects alone (Table 1). Addition of thrombin that had been inactivated with di-isopropyl fluorophosphate (the equivalent of 2 units of the active enzyme/ml) had no effects on the formation of [^{3}H]inositol phosphates, in either the presence or the absence of 4 μM-GTP.

Not only was the maximal effect of GTP on [^{3}H]IP_2 formation considerably less than that of GTP[S], but GTP inhibited the action of GTP[S] when both were added together (Table 2), suggesting that these compounds act through the same guanine-nucleotide-binding protein, but with differing efficacies. Moreover, addition of 400 μM-GDP[S], which had no effect on control [^{3}H]IP_2 formation, inhibited the stimulatory effects of GTP, GTP[S] or thrombin, with or without GTP (Table 2). The last finding indicates that stimulation of [^{3}H]IP_2 accumulation by thrombin alone depends on the presence of traces of GTP in the membrane preparations.

Pathway of [^{3}H]phosphoinositide breakdown

To identify the metabolic precursor of the [^{3}H]IP_2 accumulating in the above experiments, parallel measurements of the changes in membrane [^{3}H]phosphoinositides were made (Table 3). Incubation of membranes with ATP and $MgCl_2$ at a pCa of 7, but without GTP[S] or thrombin and GTP, led to a significant increase in their content of [^{3}H]PIP_2 (27 ± 4%; mean ± S.E.M., four expts.), but no corresponding decrease in the amount of [^{3}H]PIP. The amount of [^{3}H]PI present was too large for an equivalent decrease to be measurable, but it is likely that this [^{3}H]PIP_2 was formed from [^{3}H]PI via [^{3}H]PIP. On addition of either 10 μM-GTP[S] or thrombin (2 units/ml) with

Table 2. Effects of GDP[S] on the stimulation of [^{3}H]IP_2 formation from membrane [^{3}H]phosphoinositides by thrombin, GTP and GTP[S]

Membranes from platelets labelled with [^{3}H]inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.29 mg of membrane protein were incubated for 10 min at 25 °C with ATP, $MgCl_2$ and sufficient $CaCl_2$ to give a pCa of 7 (see the Experimental section). Other additions to incubation mixtures were guanine nucleotides and thrombin, as indicated. [^{3}H]IP_2 was isolated and counted for radioactivity; that present before incubation was subtracted. Values are means ± S.E. of the difference from three identical incubation mixtures.

Other additions	10^{-3} × [^{3}H]IP_2 formed (d.p.m./mg of protein)	
	Without GDP[S]	With 400 μM-GDP[S]
None	5.0±0.3	5.2±0.2
Thrombin (2 units/ml)	8.2±0.1	5.6±0.3
GTP (100 μM)	9.4±0.6	6.7±0.2
GTP (100 μM)+thrombin (2 units/ml)	23.3±0.8	14.1±0.3
GTP[S] (10 μM)	22.9±0.9	9.1±0.2
GTP[S] (10 μM)+GTP (100 μM)	12.6±0.2	9.1±0.5

100 μM-GTP, this increase in [^{3}H]PIP_2 was decreased or abolished, and a substantial decrease in the amount of [^{3}H]PIP present was observed (Table 3). In 10 min incubations, this decrease in [^{3}H]PIP amounted to 17±3% with 10 μM-GTP[S] (mean ± S.E.M., four expts.), relative to incubated controls. Table 2 shows that this loss of [^{3}H]PIP was equivalent to about 60% of the additional [^{3}H]inositol phosphates that accumulated and that a further 15–20% of the latter can be accounted for by the breakdown (or decreased formation) of [^{3}H]PIP_2. We conclude that the source of most of the [^{3}H]IP_2 is [^{3}H]PIP and that the remainder probably originates from [^{3}H]PI.

It is possible that all or most of the [^{3}H]PIP or [^{3}H]PI that is converted into [^{3}H]IP_2 is metabolized via [^{3}H]PIP_2, which would then be the sole substrate of phospholipase C in these membranes. To investigate this question, we added a high concentration of unlabelled IP_3 to our incubation mixtures to trap [^{3}H]IP_3 and, at the same time, measured the breakdown of tracer [^{32}P]IP_3 to monitor possible losses of [^{3}H]IP_3 (Table 4). Degradation of tracer [^{32}P]IP_3 over 2 min was decreased from about 90% to 30% by addition of 0.3 mM-IP_3, indicating the presence of a membrane-bound IP_3ase. At the same time, the unlabelled IP_3 trapped small amounts of [^{3}H]IP_3 when GTP[S] or thrombin and GTP were present (but not in their absence) and caused corresponding decreases in the accumulation of [^{3}H]IP_2 (Table 4). Assuming, as a first approximation, that the rates of [^{3}H]IP_3 formation and of total IP_3 breakdown are linear and that mixing of the two is instantaneous, the total [^{3}H]IP_3 formed is given by the expression:

$$y = \frac{x(P-1)}{P \ln P}$$

where x is the [^{3}H]IP_3 accumulating in the presence of unlabelled IP_3 and P is the fraction of the [^{32}P]IP_3 remaining at the end of the incubation. We calculate from this equation that, although none of the [^{3}H]IP_2 formed in the control incubations was derived from [^{3}H]IP_3, about 23% of the additional [^{3}H]inositol phosphates that accumulated in the presence of GTP[S] were initially [^{3}H]IP_3. For thrombin and GTP, the corresponding value was 18%. In two further experiments, the effects of unlabelled IP_3 were studied in 5 min incubations, during which 74% of the added [^{32}P]IP_3 was broken down in the presence of unlabelled IP_3. Average values for [^{3}H]IP_3 formation in the latter experiments were 23% of the additional [^{3}H]inositol phosphates with GTP[S] and 19% with thrombin and GTP. In contrast with unlabelled IP_3, 10 mM-LiCl had no effect on the amounts of [^{3}H]inositol phosphates that accumulated over 5 min. We conclude that in these membrane preparations the guanine-nucleotide-mediated activation of phospholipase C led to the hydrolysis of [^{3}H]PIP and [^{3}H]PIP_2 in a ratio of about 4:1.

Table 3. Effects of GTP[S] and of thrombin with GTP on the metabolism of endogenous [^{3}H]phosphoinositides in isolated platelet membranes

Membranes from platelets labelled with [^{3}H]inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.27 mg of membrane protein were incubated at 25 °C with ATP, $MgCl_2$ and sufficient $CaCl_2$ to give a pCa of 7 (see the Experimental section). Other additions to incubation mixtures were 10 μM-GTP[S] or 2 units of thrombin/ml with 100 μM-GTP, as indicated. The ^{3}H present in phosphoinositides (extracted twice) and inositol phosphates was then determined. Values are means ± S.E.M. from four identical incubation mixtures. Significant changes are indicated (*$P < 0.01$; unpaired t test).

Additions	Incubation period (min)	10^{-3} × ^{3}H present (d.p.m./mg of protein)					
		PI	PIP	PIP_2	IP	IP_2	IP_3
None	0	102.3±2.4	31.1±1.0	9.9±0.0	0.5±0.1	0.3±0.0	0.0±0.0
None	10	109.8±5.5	31.6±0.5	13.1±0.2*	0.5±0.1	1.0±0.0*	0.0±0.0
GTP[S]	10	110.9±3.8	23.9±0.6*	10.6±0.1*	1.6±0.1*	11.8±0.1*	0.3±0.0*
Thrombin+GTP	10	104.9±1.1	24.6±0.5*	11.3±0.6	1.5±0.0*	12.0±0.3*	0.2±0.0*

Table 4. Effects of a high concentration of unlabelled IP_3 on the breakdown of $[^{32}P]IP_3$ by platelet membranes and on the accumulation of $[^3H]$inositol phosphates released from membrane $[^3H]$phosphoinositides

Membranes from platelets labelled with $[^3H]$inositol were isolated in the presence of ATP and $MgCl_2$. Samples containing 0.22 mg of membrane protein were incubated for 2 min at 25 °C with ATP, $MgCl_2$ and sufficient $CaCl_2$ to give a pCa of 7 (see the Experimental section). Incubation mixtures also contained 0.5 μM-$[^{32}P]IP_3$ and one or more of the following additions, as indicated; unlabelled IP_3 (0.3 mM), GTP[S] (10 μM), thrombin (2 units/ml) and GTP (100 μM). Inositol phosphates were isolated and counted for ^3H and ^{32}P. Values are means ± S.E.M. for three identical incubation mixtures.

Additions	Incubation period (min)	Radioactivity (d.p.m./sample)		
		$[^{32}P]IP_3$	$[^3H]IP_3$	$[^3H]IP_2$
None	0	852 ± 15	186 ± 6	362 ± 29
None	2	76 ± 2	182 ± 28	1358 ± 52
IP_3	2	601 ± 9	148 ± 25	1222 ± 48
GTP[S]	2	60 ± 16	144 ± 38	2363 ± 24
GTP[S] + IP_3	2	599 ± 1	348 ± 36	2058 ± 40
Thrombin + GTP	2	87 ± 3	152 ± 31	3223 ± 60
Thrombin + GTP + IP_3	2	605 ± 5	417 ± 53	2766 ± 55

DISCUSSION

Demonstration of an appreciable guanine-nucleotide-dependent release of $[^3H]$inositol phosphates from platelet membranes containing $[^3H]$phosphoinositides required inclusion of ATP and $MgCl_2$ in the media used for isolation and incubation of the membranes. This procedure enhanced the membrane contents of $[^3H]$PIP and $[^3H]PIP_2$. However, the resulting increase in $[^3H]$inositol phosphate formation was proportionately much greater than the increases in membrane $[^3H]$phosphoinositides. This implies that only specific pools of the latter, that are readily depleted and resynthesized, are utilized for signal transduction. This is not surprising, in view of the heterogeneity of the membranes we have used, and is consistent with a study demonstrating compartmentation of phosphoinositides in rabbit platelets [43]. Although some groups studying phosphoinositide breakdown in isolated membranes have included ATP in the membrane isolation or incubation media [26,27,29,30,32], others have not done so [25,28,31]. In only one system, the GH_3-cell membrane [32], was the requirement for ATP studied in detail. In intact human platelets, depletion of metabolic ATP has been shown to prevent the loss of PI caused by thrombin, but not that of PIP_2 [44]. The second important factor required for demonstration of a guanine-nucleotide-dependent activation of phospholipase C was an appropriate pCa. As observed by others [26,29–32,45], the formation of inositol phosphates was greatly decreased in the absence of added Ca^{2+} ions, whereas stimulation by agonists with GTP was diminished at high Ca^{2+} concentrations as a result of increases in control enzyme activity. A pCa of about 7 proved optimal in most studies.

The results show that in platelet membranes the major $[^3H]$phosphoinositide substrate of phospholipase C was $[^3H]$PIP. Hydrolysis of $[^3H]PIP_2$ accounted for only about 20% of the total $[^3H]$inositol phosphates formed, and no detectable breakdown of $[^3H]$PI occurred. This pattern must reflect both the substrate specificity of this phospholipase C and the substrate availability in the membrane compartment containing the enzyme. Although the large amounts of $[^3H]$PIP in our membranes may favour hydrolysis of this substrate, the pattern of $[^3H]$inositol phosphates formed was very similar to that reported in several studies with $[^3H]$inositol-labelled platelets. In human platelets, $[^3H]IP_2$ formation immediately after addition of thrombin exceeded that of $[^3H]IP_3$ by a factor of 2 or more [9–11]. In experiments with $[^3H]$inositol-labelled rabbit platelets, it has been shown [14] that 10 s after the addition of thrombin the $[^3H]IP_2$ formed was 4–5-fold greater than $[^3H]IP_3$, though in a study on the effects of platelet-activating factor [46] $[^3H]IP_3$ formation initially exceeded that of $[^3H]IP_2$. It has been widely assumed that increases in platelet $[^3H]IP_2$ largely reflect the hydrolysis of $[^3H]IP_3$, though there is no direct evidence for this. It appears equally likely that PIP is a major substrate of phospholipase C in the platelet. Our results differ from those obtained in intact platelets in one significant respect. Thus, in neither human nor rabbit platelets was activation of phospholipase C by thrombin associated with an appreciable decrease in PIP, and increases have often been observed after a short interval [6,11,12,47]. It follows that, if PIP is a major substrate of phospholipase C in the intact platelet, it must be rapidly resynthesized from PI. Moreover, to accommodate results indicating that little newly synthesized PIP accumulates after addition of thrombin [16], the pool susceptible to phospholipase C must be a small fraction of the total. This pool may have expanded in our isolated membrane preparations, which contained more $[^3H]$PIP than do intact platelets. PIP hydrolysis could thus be a major source of diacylglycerol for activation of protein kinase C in the intact platelet. The formation of diacylglycerol by a mechanism that does not simultaneously generate IP_3 could help to explain why platelet responses to physiological stimuli can occur without increases in cytosolic Ca^{2+} [48].

Studies on the GTP-dependent activation of phospholipase C in membranes from GH_3 cells also suggest that $[^3H]$PIP may be a more important substrate of the enzyme than is $[^3H]PIP_2$ [32,33]. However, with membranes from blowfly salivary glands [26] and rat hepatocytes

[31], the formation of [^{3}H]IP_3 exceeded that of [^{3}H]IP_2. In the first system, as in the platelet, the membranes contained much more [^{3}H]PIP than [^{3}H]PIP_2, whereas in the latter instances the reverse was the case. These correlations strongly suggest that the relative amounts of the two [^{3}H]polyphosphoinositides in membranes may determine the ratio in which they are hydrolysed.

The phospholipase C of human platelets, assayed with exogenous PI in the presence of 1 mM-Ca^{2+}, appears to be entirely soluble [49]. This activity has been resolved into multiple forms, at least one of which is a proteolytic fragment [50]. Both crude enzyme [51,52] and the purified forms [50] can hydrolyse PI, PIP and PIP_2 in a Ca^{2+}-dependent manner. Under physiological ionic conditions at a pCa of 7–6, pure PIP appears to be the preferred substrate [50], though it is difficult to extrapolate from this result to the mixed phospholipid environment of the intact membrane. However, evidence has been presented that PIP_2 can suppress the action of the soluble platelet phospholipase C on PI, with the result that PIP may be selectively degraded in mixtures of phosphoinositides similar to those present in platelets [52]. Thus the pattern of guanine-nucleotide-activated phosphoinositide breakdown that we have observed may be consistent with the properties of soluble platelet phospholipase C, and it is possible that our membranes contain firmly adsorbed soluble enzyme. However, in addition to earlier observations in erythrocytes and other cells of a membrane-bound phospholipase C that is specific for polyphosphoinositides and activated by high Ca^{2+} concentrations [53–55], it is now clear that membranes from many cells possess related phospholipase C activities that can be activated by agonists and guanine nucleotides at physiological pCa values [25–33]. It is therefore also possible that a specific membrane-bound phospholipase C mediates signal transduction. This question will only be resolved by careful comparison of the properties of the soluble and membrane-bound enzymes under identical assay conditions.

The present results extend those obtained with permeabilized platelets [20,21,24], whch first demonstrated a role for a guanine-nucleotide-binding protein in the activation of phospholipase C by thrombin. The observation that thrombin induces a GTP-dependent hydrolysis of polyphosphoinositides in isolated platelet membranes emphasizes the parallel with the hormonal activation or inhibition of adenylate cyclase [56], and suggests that protein–protein interactions between a thrombin receptor, a G-protein and phospholipase C may occur in the membrane. Features common to the two systems included the dependence of agonist effects on GTP, the more potent action of the stable GTP analogue GTP[S], and the inhibitory effects of GDP[S]. In these respects, our results are in good agreement with those of other workers who have demonstrated a GTP-dependent hormonal activation of phospholipase C in isolated membranes [26,29–33]. In the present study, GDP[S] prevented the weak activation of phospholipase C by thrombin, suggesting that this depended on residual GTP in the membranes. In contrast, the stimulation of 5-hydroxytryptamine secretion from permeabilized platelets by thrombin was not affected by GDP[S], though the action of GTP in potentiating secretion was blocked [20,21].

Baldassare & Fisher [57] have reported that thrombin causes a GTP- or GTP[S]-dependent release of [^{32}P]IP_3 and [^{32}P]IP_2 from membranes isolated from ^{32}P-labelled human platelets. Although clearly attributable to the same phenomenon, their results differ significantly from ours. Thus ATP was not present during the isolation or incubation of the membranes and, perhaps as a result, any changes in [^{32}P]phosphoinositides were too small to be measured. More [^{32}P]PIP_2 than [^{32}P]PIP remained in the membranes, and [^{32}P]IP_3 formation exceeded that of [^{32}P]IP_2. Release of these inositol [^{32}P]phosphates occurred in the absence of added Ca^{2+}. Finally, addition of cytosol released [^{32}P]IP_3 and [^{32}P]IP_2 from the membranes, and this was potentiated by GTP[S]. The mechanism responsible for the latter effect was not determined, but may not be related to signal transduction, as a variety of nucleoside triphosphates seem to activate soluble phospholipase C in a relatively non-specific manner [58].

The identity of the G-protein mediating activation of phospholipase C in platelets remains uncertain. In mast cells [59] and neutrophils [30] the agonist-induced activation of phospholipase C is blocked by ADP-ribosylation of G_i-related proteins by pertussis toxin, but, in many other systems, ADP-ribosylation of G_i is without effect (e.g. [23,31,33]). We have argued that G_i is unlikely to activate phospholipase C in platelets, since adrenaline powerfully inhibits adenylate cyclase without inducing phosphoinositide breakdown [21]. This conclusion is supported by observations that only one component of the increase in platelet membrane GTPase activity caused by thrombin, that associated with inhibition of adenylate cyclase, is blocked by prior treatment of the membranes with pertussis toxin [60,61]. Unfortunately, the prolonged incubations required for complete ADP-ribosylation of pertussis-toxin substrates in our platelet membrane preparations alone affected the GTP-dependent activation of phospholipase C by thrombin, so that direct evidence for or against a role for a pertussis-toxin substrate is not yet forthcoming. However, if a new G-protein is involved, it is relevant that a number of guanine-nucleotide-binding proteins with lower M_r values than the classical G-proteins have now been identified in platelets and other cells [62,63].

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