Action of guanosine 5'-[β -thio]diphosphate on thrombin-induced activation and Ca^{2+} mobilization in saponin-permeabilized and intact human platelets

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The non-hydrolysable guanine analogues guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[β -thio]diphosphate (GDP[S]) have been used extensively (as promoters and inhibitors respectively) to probe the importance of G-protein function. We report on the use of GDP[S] in permeabilized and intact platelets. The stimulatory analogue GTP[S] (9–60 μM) induces shape change, aggregation and 5-hydroxy[^{14}C]-tryptamine secretion when added to saponin (12–14 $\mu\text{g}/\text{ml}$)-permeabilized platelets, but not to intact platelets. In line with the activation responses in permeabilized cells, GTP[S] induces an increase in [^{32}P]-phosphatidic acid, which is indicative of phospholipase C activity. GDP[S] (> 400 μM) totally inhibits GTP[S] (90 μM)-stimulated phospholipase C activity and functional responses in saponinized platelets. GDP[S] (1 mM) was also effective at inhibiting low-dose thrombin (0.1 unit/ml)-induced aggregation and secretion responses (without affecting shape change) in permeabilized platelets with inhibition of [^{32}P]-phosphatidic acid formation. At higher doses of thrombin (> 0.5 unit/ml), both functional responses and [^{32}P]-phosphatidic acid formation are restored in the presence of GDP[S]. Studies on intact cells revealed that GDP[S] was as effective at inhibiting low-dose thrombin-induced functional responses as in the permeabilized cells, but there was no inhibition of [^{32}P]-phosphatidic acid formation, indicating that the agent is non-membrane-penetrating. This reflected the fact that GDP[S] has additional inhibitory sites on the surface of platelets. In Fura-2-loaded cells GDP[S] inhibited thrombin-induced Ca^{2+} mobilization, as measured by Fura-2 fluorescence, in a dose-dependent manner. In studies with and without Ca^{2+} present on the outside, the effect of GDP[S] was to block Ca^{2+} influx. These studies indicate that, although GDP[S] is a valuable tool in studying G-protein function in permeabilized cells, it also has inhibitory activities on the surface of platelets, and one of these has been identified as an effect on the Ca^{2+} -influx channel after agonist stimulation.

INTRODUCTION

One of the important events that follows an agonist-receptor interaction at the surface of many activatable cells is the phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate [1–3]. Both products of this reaction have important intracellular messenger functions. Inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] releases Ca^{2+} from intracellular stores, and diacylglycerol is involved in the activation of protein kinase C, and also may have a role in the membrane fusion events which occur during exocytosis in secreting cells. From recent studies using guanosine triphosphate and its non-hydrolysable analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]), there is increasing evidence for a key role for a guanine nucleotide-binding (G) protein as a link between occupancy of a surface receptor and polyphosphoinositide hydrolysis [4–6]. Studies on chick heart cells [7], rat liver membranes [8] and platelet plasma membranes [9] all support the involvement of a GTP-binding protein in the membrane signal-trans-

duction process (see also review by Cockcroft [10]), but exactly how this protein and its associated subunits regulate the phosphodiesterase activity has not yet been clearly defined.

In whole platelets in which the plasma membrane has been permeabilized by electrically-induced pore formation, GTP[S] has been shown to greatly enhance the Ca^{2+} -sensitivity for 5-hydroxytryptamine (5HT) secretion, and is also associated with an increased formation of diacylglycerol [11]. This GTP[S]-induced secretion phenomenon is essentially the same as that seen with certain agonists or with the addition of a synthetic analogue of diacylglycerol, oleoylacylglycerol [12,13].

Using a different permeabilization procedure, involving controlled exposure to saponin, we and others have reported that permeabilized platelets can be stimulated by $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from intracellular stores [14–17]. The subsequent activation of phospholipase A_2 , the release of arachidonic acid from phospholipids and the generation of thromboxanes results in a full activation response amplified by thromboxane leaving the cell and

Abbreviations used: G-protein, guanine nucleotide-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; 5HT, 5-hydroxytryptamine; [Ca^{2+}]_i, intracellular Ca^{2+} concn.; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate.

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binding to surface receptors [14,15,17]. This response is expressed as shape change, aggregation and the secretion of granule-stored 5-hydroxy[^{14}C]tryptamine ([^{14}C]5HT).

Recently we reported, in abstract form, that GTP[S] also induces similar aggregation and secretion responses in permeabilized platelets by activation of phospholipase C, probably through the effect of the nucleotide analogue on a GTP-binding protein at an intracellular site [18]. These GTP[S]-induced responses can be totally inhibited by pretreatment of the platelets with guanosine 5'-[β -thio]diphosphate (GDP[S]) before saponin treatment [18]. We also reported that GDP[S] was very effective at inhibiting platelet responses to low doses of thrombin and collagen in permeabilized cells and showed, for the first time to our knowledge, that GDP[S] was as effective on intact platelets as on the permeabilized platelets. We now report our detailed studies of these investigations, and include also further studies on the possible sites of action for the GDP[S] effects on intact platelets. Our studies suggest that some caution may be necessary when exploring the role of G-proteins in platelet function by use of GDP[S], although at present this is perhaps the best available inhibitor of G-protein functions.

MATERIALS AND METHODS

Materials

All experiments were carried out in solutions made up in AnalaR-grade water (BDH, Poole, Dorset, U.K.). Indomethacin, prostaglandin E_1 , saponin, human thrombin, GTP and GDP[S] were obtained from Sigma Chemical Co., Poole, Dorset, U.K., and GTP γS was obtained from Boehringer Corp. (London), Lewes, E. Sussex, U.K. $\text{Ins}(1,4,5)\text{P}_3$ was isolated from human red cells by the procedure of Downes *et al.* [19] as modified by Irvine *et al.* [20].

Preparation of washed human platelet suspensions

The collection of fresh human blood, preparation of platelet-rich plasma, prelabelling with 5-hydroxy[^{14}C]tryptamine ([^{14}C]5HT) and washing of the platelets were carried out exactly as described by Authi *et al.* [14,15]. The platelets were finally resuspended at a concentration of 2.5×10^8 cells/ml in a medium of the following composition: 140 mM-KCl, 1 mM-glucose, 1 mM- MgCl_2 , 0.42 mM- NaH_2PO_4 , 6 mM- NaHCO_3 and 10 mM-Hepes made up in AnalaR water and buffered to pH 7.4. The [Ca^{2+}] of this buffer varied between 3 and 6 μM . Platelet suspensions were kept at room temperature, gassed with O_2/CO_2 (19:1) at $\frac{1}{2}$ h intervals, and all experiments were completed within 4 h of blood donation.

Aggregation and [^{14}C]5HT secretion

Unless varied as indicated in the Results section, the following aggregometry procedure was carried out in a Payton 300B dual-channel aggregometer, with the cuvette maintained at 37 °C and the suspension stirred at 900 rev./min. For the permeabilization, the platelet suspensions were temperature-equilibrated for 2–3 min in the cuvette, and saponin (solubilized in 140 mM-KCl) was added to a final concentration of 12–14 μg of saponin/ml of suspension. After 2 min the inducer (e.g. GTP[S] or InsP_3) or agonist was added and the change in light transmission recorded for a further 4 min. In all experiments involving secretion of 5HT from [^{14}C]5HT-labelled cells, imipramine (2 μM) was added to the cell

suspension 2 min before addition of saponin, to inhibit amine re-uptake. After the 4 min recording, reactions were terminated by adding 500 μl of 3% (v/v) glutaraldehyde containing 6 mM-EDTA and 150 mM-KCl buffered to pH 7.4 with Hepes. The fixed cell suspensions were centrifuged at 12000 g for 3 min and the released [^{14}C]5HT in the supernatant was determined by liquid-scintillation counting. Released 5HT was expressed as a percentage of that released by total lysis of an equivalent volume of non-permeabilized unfixed cell suspension.

[^{32}P]Phosphate incorporation and assay of [^{32}P]phosphatidic acid production

Platelets were isolated from platelet-rich plasma by centrifugation (1200 g for 10 min) and resuspended in a medium containing 145 mM-NaCl, 5 mM-KCl, 1 mM- MgSO_4 , 10 mM-glucose, 1 mM-EGTA and 10 mM-Pipes, adjusted to pH 6.8. Apyrase (Grade V; Sigma) was added to a concentration of 0.1 mg/ml of suspension, and finally [^{32}P]P $_i$ (0.2 mCi/ml) was added. The mixture was incubated for 1 h at 37 °C, and after equilibration to room temperature the ^{32}P -labelled cell suspension was acidified to pH 6.5 with 0.3 M-citric acid and centrifuged to deposit the cells (500 g for 20 min). The cells were resuspended in high-KCl permeabilizing medium at a concentration of 2.5×10^8 cells/ml, and the saponin permeabilization and/or aggregation procedures were carried out exactly as described above. In these studies, however, reactions were terminated by the addition of 3 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.). Lipid extraction was performed by the procedure of Billah & Lapetina [21], and the extracts were separated on t.l.c. plates which had been previously impregnated with 1% potassium oxalate/2 mM-EDTA, pH 6.5. The solvent system was chloroform/methanol/4 M- NH_3 (9:7:2, by vol.). Areas corresponding to [^{32}P]phosphatidic acid were identified by autoradiography overnight, and comparison was made with a similarly chromatographed standard phospholipid mixture after scraping off the appropriate zones and liquid-scintillation counting.

Measurement of intracellular [Ca^{2+}] ($[\text{Ca}^{2+}]_i$) with Fura-2

Labelling of the platelets with the fluorescent dye Fura-2 acetoxymethyl ester was carried out exactly as described by Rao *et al.* [22]. Briefly, after the cells had been washed free of excess dye, they were resuspended in a buffer consisting of 145 mM-NaCl, 1 mM- MgSO_4 , 0.5 mM- NaH_2PO_4 , 5 mM-glucose, 1 mM- Ca^{2+} and 10 mM-Hepes adjusted to pH 7.55. Fluorescence measurements were made in a Perkin-Elmer LS 5B spectrofluorimeter, and the Ca^{2+} concentration was calculated by the procedure of Grynkiewicz *et al.* [23].

RESULTS

Stimulation of saponin-permeabilized platelets with GTP[S]

Washed human platelets permeabilized by carefully controlled exposure to saponin are responsive, showing full aggregation and secretion, to the intracellular Ca^{2+} -mobilizing agent $\text{Ins}(1,4,5)\text{P}_3$ [14,15]. The permeabilization of the plasma membrane is usually maximum within 45 s of adding the detergent, and full activation responses

Fig. 1. (a) Activation of saponin-permeabilized platelets by GTP[S] and (b) its inhibition by GDP[S]

(a) Samples of platelet suspensions were equilibrated at 37 °C in aggregation cuvettes. Various concentrations of GTP[S] were added 2 min after addition of saponin (14 $\mu\text{g}/\text{ml}$), and aggregation was monitored by light-transmission measurements. 5HT-release measurements (values in parentheses) were made in the presence of 2 μM -imipramine by stopping the reactions, 4 min after GTP[S] addition, with a fixative (see the Materials and methods section), and the released [^{14}C]5HT was measured in the supernatants (values represent means $\pm$ S.E.M.; $n = 4$). Traces are representative of those obtained from at least ten preparations. (b) Reaction conditions were similar to those described in Fig. 1(a). All reactions contained 14 μg of saponin/ml and 90 μM -GTP[S] added 2 min after saponin. GDP[S] (various concentrations) was added 2 min before saponin. Traces are representative of at least three preparations, and [^{14}C]5HT release was determined 4 min after GTP[S] addition as described in the Materials and methods section. Values represent means $\pm$ S.E.M.

occur if $\text{Ins}(1,4,5)\text{P}_3$ is added 1–2 min after saponin permeabilization. Fig. 1(a) shows the effects of adding various amounts of GTP[S] 2 min after addition of saponin. In the control trace (no GTP[S]) there is a slow increase in light transmission, but this does not lead to an

aggregation response for at least 8 min, and secretion of [^{14}C]5HT into the medium is also minimal over this period (e.g. approx. 18 % lost after 6 min). Addition of GTP[S] (9–60 μM) to saponized platelets induces a dose-related shape change, aggregation and 5HT release, which at maximal doses $> 60 \mu\text{M}$ is essentially the same response as that observed with 0.1 unit of thrombin/ml. In six different platelet preparations 90 μM -GTP[S] effected the secretion of $50 \pm 4\%$ of the [^{14}C]5HT into the supernatant. Onset of shape change occurred 25–30 s after addition of GTP[S], and full aggregation was complete within 2–3 min and was irreversible. No activation responses were observed with GTP[S] concentrations even as high as 540 μM added to non-saponized (intact) cells.

Responses to GTP[S] were also observed with higher concentrations of saponin (16–19 $\mu\text{g}/\text{ml}$) under conditions where 70–80 % of previously incorporated [^3H]-adenine metabolites (primarily ATP) are released into the medium (Authi *et al.* [14]). However, here the high-saponin controls gave a greater increase in light-transmission than when saponin was added in the 14 $\mu\text{g}/\text{ml}$ range. Another non-hydrolysable GTP analogue, guanosine 5'-[$\beta\gamma$ -imido]triphosphate, gave (only occasionally) very weak aggregation responses and secretion, usually 10 % above those of control platelets treated only with saponin. These weak effects, however, were not reproducible with different platelet preparations.

GTP tested up to a concentration of 135 μM on saponized platelets (14 μg of saponin/ml) produced no functional responses (aggregation or secretion), and the traces were essentially the same as those seen with the saponin-only controls.

Inhibition by GDP[S] of GTP[S]-induced activation of saponin-permeabilized platelets

GDP[S] has been reported to be the best structurally related inhibitor of the action of GTP[S] in permeabilized neutrophils [24]. Fig. 1(b) shows the results of adding to the platelet suspensions various concentrations of GDP[S] 2 min before permeabilization by saponin. Inhibition of responses induced by 90 μM -GTP[S] (i.e. shape change, aggregation and secretion) was dose-related in the range 0–480 μM -GDP[S], with the high concentration totally inhibiting all responses. Addition of GDP[S] alone (up to 1.4 mM) to the saponin-treated cells did not cause any activation responses, and in fact further suppressed the secretion of [^{14}C]5HT, from control values of 18 % down to 13 %.

Effect of GDP[S] on GTP[S]-induced formation of [^{32}P]-phosphatidic acid in saponin-permeabilized platelets

The incorporation of ^{32}P into the phosphatidic acid formed after an activation stimulus to cells has been extensively used as an index of phospholipase C activity (see [25]). One of the phospholipid hydrolysis products, diacylglycerol, appears to be rapidly converted into phosphatidic acid by diacylglycerol kinase. Prelabelling of the cell nucleotide pool with [^{32}P]P_i allows a quantitative expression of the formation of phosphatidic acid.

We have previously reported our preliminary findings that the formation of [^{32}P]phosphatidic acid is enhanced in saponin-treated platelets exposed to GTP[S] (Authi *et al.* [18]). By using platelets prelabelled with [^{32}P]P_i

Table 1. [32 P]Phosphatidic acid formation in saponin-permeabilized platelets

Values are for triplicate preparations (means $\pm$ S.E.M.). Values in parentheses represent the control radioactivity present in the extracted and separated phosphatidic acid (see the Materials and methods section). GTP[S] and GDP[S] were added at 90 μ M and 1.0 mM respectively.

Experimental condition	[32 P]Phosphatidic acid (% of saponin control)
Saponin only (control)	100 (3867 $\pm$ 402 c.p.m.)
Saponin + GTP[S]	293 $\pm$ 35
Saponin + GTP[S] + GDP[S]	105 $\pm$ 15

and permeabilized with saponin, the effect of the two nucleotide analogues on [32 P]phosphatidic acid was investigated. Table 1 shows that when GTP[S] is introduced into the previously permeabilized platelets there is a 3-fold increase in the formation of [32 P]-phosphatidic acid. Pretreatment of the saponized cells with GDP[S] before the addition of GTP[S] results in values for [32 P]phosphatidic acid formation closely approximating to those of the 'saponin only' control platelets. It is therefore clear that the GTP[S] stimulation of phosphatidic acid production can be totally inhibited by GDP[S] pretreatment.

Inhibition of thrombin-induced aggregation and [14 C]5HT secretion by GDP[S]

The effect of GDP[S] on the platelet aggregation and secretion responses to a conventional surface-membrane agonist, thrombin, was also investigated by using the same saponin-permeabilization protocol as described above.

Fig. 2(a) shows the effect of pretreating the washed platelet suspensions with GDP[S] on the thrombin-induced activation of saponized and intact platelets. The dose of GDP[S] (960 μ M) was in excess of that required to inhibit totally GTP[S]-induced activation of permeabilized cells. In Fig. 2(a) the control aggregometer traces (ii and iv) were essentially the same for both the intact and permeabilized platelet preparations. Each showed good shape change and aggregation responses to 0.1 unit of thrombin/ml, although the amount of [14 C]-5HT released was slightly higher in the intact cells (58%) than the saponized cells (52%). Pretreatment with GDP[S] totally inhibited the aggregation response to 0.1 unit of thrombin/ml in both the intact and permeabilized platelets (traces i and iii in Fig. 2a). Secretion was also inhibited, and in fact the amount secreted by the GDP[S]-treated intact cells was substantially lower (6%) than observed with the GDP[S]-treated permeabilized cells (16%). Shape change, as indicated by the small change in light transmission immediately after adding thrombin, appeared to be unaffected by GDP[S] in both the intact and the permeabilized cell suspensions.

When the secretion phenomenon was investigated in more detail at a range of thrombin concentrations (0.01–2.0 unit/ml), whereas pretreatment with GDP[S] totally inhibited the induced secretion of [14 C]5HT at all concentrations of the agonist up to 0.2 unit/ml,

Fig. 2. (a) Effects of GDP[S] on thrombin-induced activation of saponin-permeabilized and intact cells and (b) effect of GDP[S] (0.96 mM) on [14 C]5HT release induced by various concentrations of thrombin on intact and saponin-permeabilized cells

(a) Curves (i) and (ii) are for saponized cells. GDP[S] (0.96 mM; i) or KCl control (ii) was added 3 min before saponin (14 μ g/ml). Thrombin (0.1 unit/ml) was added 2 min after saponin and reactions were stopped by a fixative after a further 4 min. Curves (iii) and (iv) are for intact cells. GDP[S] was added 3 min before thrombin (0.1 unit/ml) addition. Reactions were followed for a further 4 min. [14 C]5HT release (values in parentheses) was determined as above. Traces are representative of at least five different platelet preparations. (b) Reactions were carried out in aggregation cuvettes as above with ($\square$, $\blacksquare$) or without ($\triangle$, $\blacktriangle$) GDP[S], with various concentrations of thrombin for 4 min incubation on intact ($\blacksquare$, $\blacktriangle$) or saponized ($\square$, $\triangle$) cells. GDP[S] or KCl control was added 3 min before thrombin. Points represent means of duplicates or means $\pm$ S.E.M. ($n = 3$). Similar results were obtained in three other preparations.

this inhibitory effect diminished with increasing concentrations of thrombin such that at 2 units/ml the percentage of [14 C]5HT secreted came within a few per cent of the values recorded for platelets exposed to the same concentration of thrombin but not pretreated with

Table 2. Effect of GDP[S] on the formation of [^{32}P]phosphatidic acid in (a) saponin-permeabilized and (b) intact platelets exposed to low (0.2 unit/ml) and high (2.0 units/ml) thrombin concentrations

Values are from triplicate experiments (means $\pm$ S.E.M.). Values in parentheses are the control radioactivity for the extracted and separated phosphatidic acid (see the Materials and methods section).

Experimental conditions	Formation of [^{32}P]phosphatidic acid (%)
(a) Control saponized platelets	100 (4006 $\pm$ 328 c.p.m.)
Saponized + 0.2 unit of thrombin/ml	399 $\pm$ 33
Saponized + 0.2 unit of thrombin/ml + GDP[S] (1 mM)	86 $\pm$ 5
Saponized + 2.0 units of thrombin/ml	829 $\pm$ 73
Saponized + 2.0 units of thrombin/ml + GDP[S] (1 mM)	820 $\pm$ 75
(b) Control intact platelets	100 (3373 $\pm$ 350 c.p.m.)
Intact platelets + 0.2 unit of thrombin/ml	501 $\pm$ 34
Intact platelets + 0.2 unit of thrombin/ml + GDP[S] (1 mM)	419 $\pm$ 29
Intact platelets + 2.0 units of thrombin/ml	669 $\pm$ 59
Intact platelets + 2.0 units of thrombin/ml + GDP[S] (1 mM)	682 $\pm$ 63

GDP[S] (Fig. 2b). This apparent neutralization of the GDP[S] inhibitory effect was observed for both the intact and permeabilized platelet preparations.

Effect of GDP[S] on thrombin-induced formation of [^{32}P]phosphatidic acid

To complement the secretion-inhibition studies with GDP[S] at low and high doses of thrombin, the formation of [^{32}P]phosphatidic acid was carried out in platelets prelabelled with [^{32}P]P_i and washed before saponization. The amount of [^{32}P]phosphatidic acid formed after stimulation with low (0.2 unit/ml) and high (2.0 units/ml) concentrations of thrombin was measured in control platelets and platelets pretreated with GDP[S] before addition of the agonist. At low doses of thrombin, the production of [^{32}P]phosphatidic acid was increased 4-fold above control platelets (saponin only), and at the high dose of thrombin the increase was $\sim$ 8-fold (Table 2). Pretreatment with GDP[S] resulted in total inhibition of the low-dose thrombin-induced production of [^{32}P]phosphatidic acid, whereas at the higher dose of thrombin the GDP[S] had no effect and an 8-fold enhancement of [^{32}P]phosphatidic acid formation was recorded. These results were to some extent analogous to the secretion data, where the GDP[S] inhibitory effect was neutralized at high thrombin concentrations. However, when the formation of [^{32}P]phosphatidic acid was measured in intact platelets stimulated with low and high thrombin concentrations, no significant difference was observed in the presence or the absence of GDP[S]. The ranges of enhancement of [^{32}P]phosphatidic acid formation were 5-fold at 0.2 unit of thrombin/ml and 7-fold with 2.0 units of the agonist/ml.

Effect of GDP[S] on [Ca^{2+}]_i measured with Fura-2

Since some of the effects of GDP[S] so far observed could be demonstrated on intact platelets, a series of experiments were carried out using the fluorescence Ca^{2+} -binding dye Fura-2 to monitor the [Ca^{2+}]_i of intact platelets on exposure to thrombin in the presence and the absence of GDP[S] and with the medium containing 1 mM- Ca^{2+} . Increases in [Ca^{2+}]_i within intact cells preloaded with Fura-2 are revealed by a shift in the fluorescence-excitation spectrum. This results in an increase in

the emission signal when the sample is excited at 340 nm and a decrease in emission at an excitation wavelength of 380 nm with increased [Ca^{2+}]_i. Fig. 3(a) shows these spectral changes in Fura-2-loaded platelets exposed to thrombin when the fluorescence emission is scanned through the excitation-wavelength range 300–400 nm. It can be seen, by comparison with the control trace, that thrombin added to the intact platelets markedly increased the fluorescence signal emitted at an excitation wavelength of 340 nm. The addition of GDP[S] 3 min before addition of the agonist inhibited the effect of thrombin and gave a trace closely approximating to that of the control platelets. This inhibition by GDP[S] of a thrombin-induced rise in [Ca^{2+}]_i as reflected in the fluorescence emission changes was dose-dependent. Fig. 3(b) shows the values for cytosol [Ca^{2+}] calculated by the previous procedures [23,26]. In the control experiments 0.2 unit of thrombin/ml induced a rise in cytosol [Ca^{2+}] from a basal value of around 60 nM to 900 nM. Pretreatment with GDP[S] over the concentration range 0.1–1.5 mM showed a progressive inhibition of the thrombin-induced rise in [Ca^{2+}]_i. At the highest GDP[S] concentration (1.5 mM) the inhibition was $\sim$ 80% with respect to the [Ca^{2+}]_i in the thrombin-induced control platelets. With a similar experimental protocol but with the GDP[S] concentration fixed at 1.5 mM and with different concentrations of thrombin in the range 0–2 units/ml, Fig. 3(c) shows that in the control experiments without pretreatment with GDP[S] the rise in [Ca^{2+}]_i varies with concentration of agonist up to a maximum of $> 1 \mu\text{M}$ -[Ca^{2+}]_i at around 0.5 unit of thrombin/ml. However, when the platelets have been pre-exposed to GDP[S] before adding the agonist, it is clear not only that this nucleotide analogue is particularly effective at inhibiting the Ca^{2+} mobilization at low doses of thrombin, but also that its effect diminishes as the agonist concentration is increased. To exclude the possibility that the inhibition of Ca^{2+} mobilization by GDP[S] was a chelation effect, the Ca^{2+} concentration in the external medium was increased to 3 mM. No effect was observed on the inhibition of Ca^{2+} mobilization by GDP[S] (results not shown). Since GDP[S] does not shift the fluorescence spectrum of Fura-2-labelled platelets and does not absorb between 310 and 600 nm, inter-

Fig. 3. Effect of GDP[S] on thrombin-induced Ca^{2+} mobilization in Fura-2-labelled platelets

(a) Excitation spectra of Fura-2-loaded platelets. Thrombin (0.2 unit/ml) induces the characteristic spectral shift (see also Rao *et al.* [22]). GDP[S] concentration is 1.5 mM, added 3 min before thrombin. (b) Dose-dependent inhibition of thrombin (0.2 unit/ml)-induced Ca^{2+} mobilization by GDP[S]. Various concentrations of GDP[S] were added 3 min before thrombin. Resuspension medium contains 1 mM- Ca^{2+} . Points represent peak values of Ca^{2+} obtained, and are means $\pm$ S.E.M. (c) Effects of 1.5 mM-GDP[S] (○) on Ca^{2+} mobilization induced by various concentrations of thrombin (▽). Reaction conditions are as in (b).

Table 3. Effect of GDP[S] on thrombin-induced Ca^{2+} mobilization in the presence and the absence of phosphocreatine/creatine kinase (CP/CPK)

Concentrations used were thrombin (0.2 unit/ml), GDP[S] (1.5 mM), CP (4 mM)/CPK (2 units/ml).

Conditions	Inhibition of thrombin-induced rise in fluorescence above basal* (%)	
	Preparation A	Preparation B
Thrombin + CP/CPK	13	—
Thrombin + GDP[S]	73	83
Thrombin + CP/CPK + GDP[S]	87	87

* Basal values were unaffected by GDP[S], CP/CPK or both.

ference in the Fura-2 assay was similarly excluded. Of other nucleoside phosphates tested (at 1.5 mM concentrations), GDP showed a minor inhibitory effect (16%) and GTP had no effect on thrombin-induced Ca^{2+} mobilization (results not shown).

Since Ca^{2+} -influx channels are known to be activated by ADP [27], experiments were performed in the presence of an enzyme scavenger system for released ADP. The results presented in Table 3 for two platelet preparations show that in the presence of phosphocreatine (4 mM) and creatine kinase (2 units/ml) the thrombin-induced rise in $[\text{Ca}^{2+}]_i$ was only 13% lower than in the absence of phosphocreatine/creatine kinase. In contrast, GDP[S] (1.5 mM) inhibited Ca^{2+} mobilization induced

by thrombin (0.2 unit/ml) by 73% in the absence of phosphocreatine/creatine kinase and 87% in the presence of the ADP scavenger system (Preparation A). Similar results were observed with a second platelet preparation (B, in Table 3). This would suggest that released ADP plays only a minor role in responses induced by thrombin.

Effect of GDP[S] on the influx of Ca^{2+} through the plasma membrane

In an attempt to define the site of action on the intact platelet of GDP[S] for its effect in inhibiting the thrombin-induced rise in cytosolic $[\text{Ca}^{2+}]$, the change in fluorescent signal (wavelength 340 nm) was studied with either 1 mM- Ca^{2+} in the surrounding medium or 1 mM-EGTA. In the presence of 1 mM external Ca^{2+} , 0.2 unit of thrombin/ml induced a rapid rise in the fluorescence emission signal, which then decreased gradually over the succeeding 4 min (Fig. 4a). Pretreatment with GDP[S] (1 mM) before exposure to the agonist substantially decreased the fluorescence signal. However, with 1 mM-EGTA in the external medium, where the height of the initial rise in fluorescent emission was significantly lower than that in the presence of external 1 mM Ca^{2+} (Fig. 4b), the pretreatment with GDP[S] had little effect either on the initial fluorescence response or on the rate of decrease of the signal over the course of the following 1 min. In a further experiment with 1 mM- Ca^{2+} in the medium, GDP[S] was added to cells in the fluorimeter cuvettes just after the addition of thrombin and as close as possible to the peak of the rise in the Ca^{2+} -fluorescence signal. GDP[S] substantially accelerated the rate at which the emission decreased over the following 4 min (Fig. 4c).

In a further series of experiments, bivalent-cation influx was investigated by the Mn^{2+} -quenching procedure of Hallam & Rink [28], by using quin-2-labelled cells. Fig. 5 shows a typical set of fluorescence traces. GDP[S] inhibits the thrombin-induced rise in the quin-2 fluorescence signal in a manner similar to the effects seen with

Fig. 4. Time course of thrombin-induced changes in Fura-2 fluorescence in the presence and absence of GDP[S] and with and without Ca^{2+} in the external medium

Traces in (a) contained 1 mM- Ca^{2+} . Thrombin concentration used was 0.2 unit/ml. A representative trace is shown by the continuous line. Incubations containing GDP[S] (1 mM) are shown by the broken line. Both traces are representative of three experiments. For traces in (b), reaction conditions were identical with those for (a), except that 1 mM-EGTA was present in the external media. For traces in (c), 1 mM- Ca^{2+} was present in the medium. In these experiments GDP[S] or vehicle (KCl) was added as close as possible to the peak of Ca^{2+} rise induced by thrombin. Traces are representative of three experiments.

Fura-2. When Mn^{2+} is present with Ca^{2+} in the external medium, after the initial rise in fluorescence signal there is a rapid quenching as Mn^{2+} displaces Ca^{2+} from the fluorophor. When GDP[S] is also present, the initial fluorescence peak is higher and the rate of Mn^{2+} quenching of the signal is substantially decreased.

DISCUSSION

Platelets are highly responsive cells and have been used in a number of studies involving guanine nucleotides and their stable or non-hydrolysable analogues [9,11–13,

Fig. 5. Effects of GDP[S] and Mn^{2+} on quin-2 fluorescence changes induced by thrombin

Reaction conditions were similar to those in Fig. 3, except that platelets were loaded with quin-2 instead of Fura-2. All incubations contained 1 mM- Ca^{2+} , and on the indicated traces GDP[S] and Mn^{2+} were added 3 min before thrombin. Traces are typical of at least three determinations.

29–31]. Strong evidence is emerging for G-protein involvement in at least some of the agonist-induced responses. Although caution may be required in interpreting data from permeabilized cell preparations, such model systems are now widely used for studies involving the control of the concentration of cytosol constituents and for investigations on the intracellular action of non-membrane-penetrating molecules. We, and others, have previously shown that $\text{Ins}(1,4,5)\text{P}_3$ can be introduced into saponin-permeabilized platelets and, through its direct action on intracellular Ca^{2+} stores, initiate release of the cation and trigger intracellular processes. These then lead to a full expression of functional responses, such as aggregation and secretion [14–17].

In the present investigations we report the use of GTP[S] and GDP[S] in attempts to probe their sites of action as promoters or inhibitors of activation responses in intact and saponin-permeabilized platelets. Addition of GTP[S] induces aggregation and secretion responses in saponin-permeabilized platelets, and these responses are essentially of the same magnitude as those seen with thrombin activation. This nucleotide, being non-penetrating, has no effect on intact platelets. That the action of GTP[S] is mediated through a G-protein stimulation of phospholipase C activity is indicated by the experiments in which we measured the formation of [^{32}P]-

phosphatidic acid. These findings support the observations by others, using human and rabbit platelet systems [31–35], that GTP[S] causes the formation of inositol phosphates, including $\text{Ins}(1,4,5)\text{P}_3$. At a dose of GTP[S] that elicits maximum aggregation and secretion responses in permeabilized cells, GDP[S] is totally inhibitory to these expressions of activation. The inhibitory action is associated with a suppression of phospholipase C, as monitored by [^{32}P]phosphatidic acid formation. Thus the use of GDP[S] as an inhibitor of phospholipase C activity [24,29] appears to be justified in permeabilized platelet preparations.

The use of GDP[S] has allowed investigations to explore the functions of G-proteins in agonist-induced responses of permeabilized platelet preparations. The earlier observations by Haslam & Davidson [11] showed that at high doses of thrombin (2 units/ml) the secretion of 5HT by electro-permeabilized platelets was unaffected by GDP[S] (0.4 mM). This led them to suggest that both G-protein-dependent and -independent mechanisms may operate in platelet stimulus-response coupling. Later studies by Brass *et al.* [29] on saponin-permeabilized platelets showed that GDP[S] was effective at inhibiting the secretion of [^{14}C]5HT at low doses of thrombin, in keeping with its inhibition of phosphoinositide metabolism, and that at higher thrombin concentrations (> 0.5 unit/ml) secretion and fibrinogen-receptor expression [36] were restored, but phosphoinositide metabolism was still inhibited. This suggests that secretion and fibrinogen binding can occur through a phosphoinositide-independent mechanism. Our data would support this concept only in part, since at low thrombin concentrations (< 0.2 units/ml) secretion, aggregation and phospholipase C activation are all inhibited totally by GDP[S], whereas at high thrombin concentrations both the phosphoinositide metabolism and secretion are restored. We therefore suggest that at high thrombin concentrations (e.g. 2 units/ml) phospholipase C activation may also occur through a G-protein-independent mechanism, explaining the lack of an effect by GDP[S] under these conditions. Several recent reports provide some support for this view. In studies by Oberdisse & Lapetina [31] using saponin-permeabilized platelets GDP[S] failed to inhibit thrombin (1 unit/ml)-induced release of [^3H]inositol phosphates, and in fact enhanced it to some degree, even though GDP[S] was effective in inhibiting GTP[S]-stimulated inositol phosphate release. They suggested that perhaps two types of phospholipase C enzyme may exist: one which is stimulated by G-protein and one which is stimulated by other mechanisms. What these other mechanisms may be is at present unknown, but one possibility is that high concentrations of thrombin are able to induce phospholipase C activation and 5HT secretion via a proteinase action. Indeed, in one recent study [37] the serine proteinase trypsin (at $1\ \mu\text{M}$) induced a 30-fold increase in [^{32}P]phosphatidic acid, compared with thrombin (1 unit/ml), which resulted in only a 15-fold increase. Other receptor-operating agonists, such as 5HT, platelet-activating factor and the thromboxane analogue EP171, induced only a 2–6-fold increase in [^{32}P]phosphatidic acid production. It is possible therefore that thrombin at low doses (≤ 0.2 unit/ml) activates phospholipase C via a receptor-mediated G-protein-dependent mechanism, whereas at higher doses (0.5–2.0 units/ml) it is able in some way to stimulate phospholipase C and generate the subsequent responses via a

proteinase action, as typified by trypsin. Also, in our studies with saponin-permeabilized platelets, GDP[S] was unable to inhibit the shape change induced by thrombin at all doses of agonist studied.

Although at present, GDP[S] seems to be the best inhibitor available for probing G-protein functions at the intracellular level, it is by no means specific, as our studies show. The inhibitory effects of this agent on low-dose thrombin-induced [^{14}C]5HT secretion could be demonstrated equally well with either intact or permeabilized platelets. We have also reported that GDP[S] inhibits aggregation and secretion induced by other agonists, such as low doses of collagen [18], and GDP[S] (1 mM) totally inhibits shape change, aggregation and 5HT secretion when washed intact platelets are exposed to $20\ \mu\text{M}$ -ADP (results not shown). The nucleotide analogue is non-penetrating, and in intact cells phospholipase C activity is unaltered, as shown by the data for the thrombin induction of [^{32}P]phosphatidic acid formation (Table 2). Thus GDP[S] may have additional binding sites at the surface of platelets, where the results of the inhibition can be as profound as when the compound interferes with phospholipase C activity in the permeabilized platelet preparations.

Our studies with Fura-2-labelled intact platelets suggest an identity for one of these surface sites, since GDP[S] was found to be most effective in inhibiting the low-dose thrombin-induced elevation of cytosolic [Ca^{2+}]. As with the [^{14}C]5HT-secretion experiments, described above, this inhibitory action is decreased as the thrombin concentrations are increased. The experiments using thrombin with and without Ca^{2+} in the external medium suggest that GDP[S] can block Ca^{2+} influx across the plasma membrane rather than affect its release from intracellular stores. If GDP[S] is added as close as possible to the peak of the rise in [Ca^{2+}], with 1 mM- Ca^{2+} present on the outside of the cell, then the result is further to suppress Ca^{2+} entry, and there is an apparent enhancement of the removal of Ca^{2+} from the cytosol. The effect cannot be attributed to chelation of Ca^{2+} by the nucleotide analogue, since higher concentrations of extracellular Ca^{2+} (3 mM) have no effect on the inhibitory action of GDP[S]. Moreover, neither GDP nor GTP has any significant inhibitory effects. Thus GDP[S] may modulate the status of a thrombin-regulated Ca^{2+} channel in the plasma membrane. Further support for this concept is provided by the Mn^{2+} -quenching experiment. The ability of Mn^{2+} to quench quin-2 fluorescence has been used to monitor agonist-induced bivalent-cation entry into cells [28]. In our experiments the decrease by GDP[S] of the rate of quenching suggests an action of the nucleotide to inhibit agonist-induced cation entry.

An additional site of action for GDP[S] may be the ADP receptor and its triggered functions, since 1 mM-GDP[S] totally inhibits all responses even at $20\ \mu\text{M}$ -ADP. Since ADP is the only platelet agonist that we have studied in which GDP[S] inhibits the shape change, a direct action here at the surface receptor may be indicated. However, the experiments performed in the presence and the absence of the ADP scavenger system phosphocreatine/creatine kinase suggest that secreted ADP plays only a marginal role, if any, in the action of maximally effective thrombin concentrations.

In conclusion, these studies have substantiated the view that thrombin-induced platelet activation can be directly attributed to a receptor-linked G-protein-

dependent stimulation (or relief of inhibition) of phospholipase C activity. This sequence of events can be explored experimentally in permeabilized platelets by using the inhibitory non-hydrolysable nucleotide analogue GDP[S]. However, the findings that this agent is equally effective in inhibiting low-dose thrombin-induced responses in intact platelets as in permeabilized platelets suggests there may be accessible sites for its action at the surface membrane. One of these sites, for which we have provided evidence for a GDP[S]-inhibitory effect, may be the thrombin-regulated influx channel for Ca^{2+} . Although GDP[S] may well be one of the best inhibitors of G-protein-linked phospholipase C-mediated intracellular processes. [i.e. production of diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$, and their consequences], clearly its action at other cellular sites cannot be discounted. Until more specific inhibitory agents become available, some caution should perhaps be exercised in interpreting the data derived from experiments using this nucleotide analogue if we are to progress towards a better molecular understanding of the platelet's complex G-protein-associated activity profile.

Note added in proof (received 8 August 1988)

Recently two other reports have been published concerning the use of GDP[S] with platelet function in agreement with our findings. The first highlights non-specific inhibitory properties of GDP[S] on intact platelets [38], and the second reports that inhibition of phospholipase C activity by GDP[S] is only seen in permeabilized platelets [39].

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