Inhibition of agonist-induced platelet aggregation, Ca\(^{2+}\) mobilization and granule secretion by guanosine 5'-[\(\beta\)-thio]diphosphate and GDP in intact platelets

Evidence for an inhibitory mechanism unrelated to the inhibition of G-protein–GTP interaction

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The effect of guanosine 5'-[\(\beta\)-thio]diphosphate (GDP[\(\beta\)S]), reported to be an antagonist of GTP at the G-protein-binding site, on human platelet activation was examined. GDP[\(\beta\)S] (0.3–3 mM) had significant inhibitory effects on platelet aggregation and 5-hydroxytryptamine (5HT) secretion induced by thrombin, collagen, the thromboxane mimetic U46619 and 1,2-dioctanoylglycerol (diC\(_8\)) in intact platelets, as well as in saponin-permeabilized platelets. Similar inhibitory effects in intact platelets were also observed with ATP (over similar concentration ranges) and GDP and GTP (at 2- and 10-fold higher concentrations respectively). All four nucleotides also inhibited ADP-induced platelet aggregation in indomethacin-treated platelets under conditions where no 5HT secretion occurred. Inhibition of thrombin-induced aggregation and secretion by GDP[\(\beta\)S] and ATP in intact platelets was accompanied by a reduction in the thrombin-induced rise in intracellular Ca\(^{2+}\) levels and 45 kDa-protein phosphorylation. The results suggest that at least some of the effects of GDP[\(\beta\)S] may be unrelated to inhibition of G-protein–GTP interaction, but, instead, may be mediated via an extracellular site, common to all the nucleotides tested and perhaps via inhibition of the effects of endogenous/released ADP. The usefulness of GDP[\(\beta\)S] as a tool in studying G-protein–GTP interactions in platelets is thus questionable.

INTRODUCTION

Considerable evidence implicating GTP-binding proteins (G-proteins) as effectors of agonist-induced activation processes in blood platelets is now available. This has arisen principally from two sets of observations: (i) the stimulation of diacylglycerol formation and granule secretion by GTP and stable GTP analogues, and their inhibition by the GDP analogue GDP[\(\beta\)S], which prevents G-protein–GTP interaction in permeabilized platelets [1–5], and (ii) the inhibition of thrombin- and U46619-induced phosphoinositide breakdown and secretion by GDP[\(\beta\)S] in permeabilized platelets [6,7]. These findings have served to substantiate the generally accepted hypothesis on the role of a specific G-protein(s) (G\(_p\) or N\(_p\)) in the coupling of membrane receptors to the phosphoinositide phosphodiesterase [8,9] and in the regulation of phosphoinositide hydrolysis. In addition to the stimulation of platelet secretion, recent work [10] has implicated G-protein-dependent phosphoinositide hydrolysis in the exposure of fibrinogen receptors, achieved via the actions of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP\(_3\)) [11,12].

The aim of the present study was to examine the role of G-protein–GTP interaction in collagen-induced platelet-activation processes using GDP[\(\beta\)S], prompted by our recent observations on the different mechanisms involved in collagen- versus thrombin-induced arachidonate release [13], as well as the greater importance of arachidonate metabolites in the induction of collagen-induced secretion compared with secretion induced by thrombin [14]. Our results have demonstrated an inhibition of agonist (thrombin, collagen, U46619)-induced platelet aggregation and HT secretion by GDP[\(\beta\)S] in intact, as well as in saponin-permeabilized, platelets, over the concentration range used by earlier workers [6,7]. Similar inhibitory effects were also observed with GDP, GTP and ATP in intact platelets, suggesting a common extracellular site of action for these nucleotides, as these compounds are membrane-impermeable, and a need for re-interpretation of some of the earlier data obtained with GDP[\(\beta\)S] in permeabilized platelets. The full implications of the findings are discussed.

EXPERIMENTAL

Materials

GDP[\(\beta\)S] and ATP (special quality) were obtained from Boehringer, and GTP and GDP were from Sigma. Thrombin and collagen were obtained respectively from Parke–Davis and Hormon Chemie, München, Germany. U46619 was purchased from Cayman Chemicals, Ann Arbor, MI, U.S.A., and the membrane-permeable DAG analogue sn-1,2-dioctanoylglycerol (diC\(_8\)) was from Novabiochem, Laufelfingen, Switzerland. All radiochemicals were from Amersham International, and materials for gel electrophoresis were from Bio-Rad.

Abbreviations used: GDP[\(\beta\)S], guanosine 5'-[\(\beta\)-thio]diphosphate; DAG, diacylglycerol; InsP\(_3\), inositol 1,4,5-trisphosphate; 5HT, 5-hydroxytryptamine; diC\(_8\), sn-1,2-dioctanoylglycerol; [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; GTP[\(\gamma\)S], guanosine 5'-[\(\gamma\)-thio]triphosphate.
Preparation of washed platelets

Blood (9 vol.) collection into 3.2% trisodium citrate (1 vol.) was via forearm venepuncture from apparently healthy human (male) volunteers, who had denied taking any medication for at least 2 weeks. Preparation of washed platelets resuspended in a pH 7.4 buffer composed of 10 mM-Hepes, 103 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 1 mM-CaCl₂, 0.09% glucose and hirudin (0.1 unit/ml) [platelet count of (2-4)×10⁹/ml for all the experiments] and pre-labelled with [¹⁴C]5HT or [³²P]Pₐ was carried out as described previously [13]. For experiments where saponin was used to permeabilize platelets, Ca²⁺ was omitted from this resuspension medium.

Platelet aggregation, [¹⁴C]5HT secretion and 45 kDa-protein phosphorylation

Platelet aggregation and the release of [¹⁴C]5HT, or aggregation and 45 kDa-protein phosphorylation, were studied simultaneously in a sample of washed platelets pre-labelled with [¹⁴C]5HT or [³²P]Pₐ respectively [13]. In all the experiments where U46619, collagen or diC₄ were the agonists studied, human fibrinogen (Kabi), at a concentration of 0.5 mg/ml, was added 90 s before the agonist. GDP[βS], GDP, ATP or GTP were added 60 s before the agonist or, in experiments where saponin was employed, the nucleotides were added 10 s before saponin, followed 60 s later by addition of the appropriate agonist. All incubations were terminated 3 min after agonist addition. The cuvette containing the platelets was placed in a Payton Aggregometer Module and stirred at 800 rev./min. Aggregation was measured as the percentage increase in light transmission by using a chart recorder linked to the aggregometer. The release of [¹⁴C]5HT was quantified by stopping the reaction 3 min after addition of the agonist(s) with ice-cold EDTA (16 mM) plus 1% formaldehyde. Protein phosphorylation was studied by removing portions of ³²P-labelled platelets from the aggregometer cuvette 3 min after agonist addition and adding them to 0.5 vol. of an SDS stopping solution [9.5% (w/v) SDS/6% (w/v) 2-mercaptoethanol/15% trisodium phosphate] and boiling for 2 min. Samples were then electrophoresed in a SDS-10% polyacrylamide gel and visualized by autoradiography.

Fig. 1. (a) Effect of GDP[βS] (3 mM) on thrombin (Thro) (0.2 unit/ml)- and collagen (Coll) (20 µg/ml)-induced aggregation and [¹⁴C]5HT secretion in saponin (Sap)-permeabilized platelets, and (b) effect of GDP[βS], ATP and GTP on ADP (10 µM)-induced aggregation and [¹⁴C]5HT secretion in intact platelets

(a) GDP[βS] was added 10 s before the saponin, as indicated by the first vertical line. The values in parentheses beside each aggregation trace (which is a representative one from six determinations) represent the percentage of [¹⁴C]5HT released (mean ± S.E.M.) for six determinations from three experiments 3 min after agonist addition into the platelet supernatants.

*P < 0.001 compared with control agonist-induced secretion. (b) All experiments with ADP as the agonist were carried out in indomethacin (19 µM)-treated platelets. Nucleotides were added 60 s before the agonist, as indicated by the first vertical line. a, b and d represent responses to ADP in the presence of 0.15 mM-GDP[βS], 0.075 mM-ATP, 0.3 mM-GDP[βS] and 0.15 mM-ATP respectively, whereas e and f represent responses to U46619 in the presence of 3 mM- and 1.5 mM-GTP respectively. The percentage [¹⁴C]5HT release in each case is shown in parentheses beside each aggregation trace. *P < 0.001 compared with control agonist-induced secretion (Student's t test for unpaired data).
(v/v)glycerol/0.186 M-Tris (pH 6.8)/0.03 % (w/v) Bromophenol Blue. Immediately after this, the samples were boiled at 100 °C for 3 min and the 32P-labelled proteins were separated by SDS/polyacrylamide-gel electrophoresis [4 % (w/v) stacking and 12 % (w/v) separating gels]. The increase in 32P labelling in the 45 kDa protein, the major substrate of protein kinase C [12], was quantified by autoradiography of the dried gels, and counting the radioactivity in appropriate areas cut out from the gels.

Measurement of [Ca2+]i

Fluorescence measurements on quin 2-loaded washed platelets, which were incubated at 37 °C and not stirred, were carried out by using a Perkin–Elmer LS5 luminescence spectrometer, with excitation at 339 nm and emission at 500 nm. Calibration of the signal and calculation of the [Ca2+]i in nM were carried out as described previously [15]. The intracellular concentration of quin 2 ranged between 0.5 and 1 mM.

RESULTS

Saponin concentrations

Two methods were used to determine the appropriate saponin concentrations to use: (i) the extent of ATP released 30–60 s after saponin addition was measured by using the luciferase–luciferin reagent in a Lumi Aggregometer (Payton) [16] and (ii) release of [3H]adenine (and metabolites) from pre-labelleed platelets was measured 30–60 s after saponin addition [17]. The latter method was preferred to the former, as it was more sensitive. A 70–80 % release of [3H]adenine metabolites was obtained with 10–15 µg of saponin/ml, whereas detectable (20–30 %) ATP release was only observed with concentrations of saponin in excess of 18 µg/ml. In all subsequent experiments, the concentration of saponin used was 10–15 µg/ml, and this was a concentration which caused less than 20 % [14C]SHT release and less than 20 % increase in light transmission over a 4 min period.

Effect of GDP[βS] on thrombin- and collagen-induced aggregation and [14C]SHT secretion in permeabilized platelets

Initial experiments on saponization of platelets were carried out with platelets resuspended in buffer containing 3.3 mM-ATP, as used by Brass et al. [6,7]. However, as these platelets failed to respond to thrombin (0.2 unit/ml), collagen (20 µg/ml) or U46619 (1 µM), and did so only when ATP was omitted from the resuspension medium, the use of ATP in the medium was discontinued (see below for details on the effects of ATP). Aggregation and secretory responses to maximally effective concentrations of thrombin (0.2 unit/ml) and collagen (20 µg/ml) were unchanged in platelets pre-treated with saponin for 1 min (Fig. 1a) compared with that in untreated, intact platelets (Fig. 2). Significant inhibition (50–60 %) of both responses induced by both the agonists in saponin-treated platelets was observed by pre-treatment of the platelets with GDP[βS] (1.5 or 3 mM) before saponin addition (Fig. 1a).

Effect of GDP[βS] on agonist-induced aggregation and [14C]SHT secretion in intact platelets: comparison with the effects of GDP, GTP and ATP

The possibility that GDP[βS] might have a direct inhibitory effect on platelet aggregation, unrelated to any effect on G-protein–GTP interaction was considered by examining the effect of this compound on agonist-induced aggregation and secretion in intact platelets, in the absence of saponin. Fig. 2 demonstrates that GDP[βS] (0.3–3 mM) has strong inhibitory effects on aggregation and [14C]SHT secretion induced by thrombin, collagen, diC₄ and the thromboxane mimetic U46619. The extent of inhibition by GDP[βS] in intact platelets was similar to that seen in saponinized platelets (Fig. 1a). GDP[βS] was considerably more potent against U46619 of all the agonists examined, and less so against diC₄. Thus, with GDP[βS] concentrations of 0.6–1.5 mM, complete inhibition of U46619-induced responses was achieved, whereas with 3 mM-GDP[βS], only 40–50 % inhibition of diC₄-induced responses was observed. Interestingly, the
which induced more of concentration platelets in suspensions of \([\text{Ca}^{2+}]_0\), suspensions of \(\text{GTP}\) GDP

...other nucleotides. Measurement of aggregation by induced conditions under concentrations also induced though aggregation with (3 mM) inhibition of thrombin (0.2 unit/ml)-induced secretion by GDP[γS] (3 mM) was partially overcome by an increase in the thrombin concentration to 1 unit/ml, which agrees with earlier findings in permeabilized platelets [6].

Inhibitory effects on agonist-induced aggregation and secretion in intact platelets was also observed with ATP (0.3-3 mM), which was approximately equipotent with GDP[γS], and GDP (1.5–6 mM, which was approximately half as potent as GDP[γS] or ATP) (Fig. 2). Secretion induced by thrombin at 0.2 unit/ml was unaffected by addition of EGTA (5 mM) or EDTA (5 mM) (even though aggregation was completely inhibited); however, both GDP[γS] and ATP strongly inhibited thrombin-induced secretion in the presence of EGTA or EDTA (results not shown). All three nucleotides at somewhat lower concentrations also inhibited ADP (10 µM)-induced aggregation in indomethacin-treated platelets (Fig. 1b) under conditions where no \(^{[3]P}\)SHT secretion was observed or significant phosphoinositide hydrolysis is known to occur [18,19]. GTP (1.5–6 mM) also had an inhibitory effect, although this was weaker than that of the other nucleotides. The inhibitory effect of GTP was more pronounced at lower agonist concentrations, and Fig. 1(b) demonstrates complete inhibition by 1.5–3 mM GTP of aggregation and secretion induced by a threshold concentration of U46619 (i.e. 0.1 µM). None of the nucleotides tested altered significantly the pH of the platelet suspensions upon addition. The intactness of the platelets in our preparations was confirmed by the lack of secretion with the stable GTP analogue (10–100 µM), which induced 30–40% \(^{[3]P}\)SHT secretion when the platelets were saponized.

**Effect of GDP[γS] and ATP on thrombin-induced rises in [Ca\(^{2+}\)]\(_0\).**

In intact quin 2-loaded platelets, which allowed measurement of thrombin-induced rises in [Ca\(^{2+}\)]\(_0\), GDP[γS] (1.5 or 3 mM) and ATP (0.6–3 mM) greatly reduced the extent to which thrombin (0.2 or 1 unit/ml) elevated [Ca\(^{2+}\)]\(_0\) (Fig. 3).

![Fig. 3](image)

**Fig. 3. Effect of GDP[γS] and ATP on thrombin (Thro) (0.2 unit/ml and 1 unit/ml)-induced rise in [Ca\(^{2+}\)]\(_0\), in quin 2-loaded platelets**

a and g represent control responses to thrombin at 0.2 and 1 unit/ml respectively. b, c, d, e and f represent responses to thrombin (0.2 unit/ml) in the presence of 0.75 mM-ATP, 1.5 mM-ATP, 3 mM-ATP, 1.5 mM-GDP[γS] and 3 mM-GDP[γS] respectively, whereas h, i and j represent responses to thrombin (1 unit/ml) in the presence of 3 mM-ATP, 1.5 mM-GDP[γS] and 3 mM-GDP[γS] respectively. The mean peak [Ca\(^{2+}\)]\(_0\) value (±SEM) is shown beside each trace.

![Fig. 4](image)

**Fig. 4. Effect of GDP[γS] (3 mM) and ATP (3 mM) on thrombin (0.2 and 1 unit/ml)-induced 45 kDa protein phosphorylation**

An autoradiograph of a dried gel demonstrating changes in \(^{32}P\) labelling of the 45 kDa protein is shown. The lanes represent: a, resting platelets; b, thrombin (0.2 unit/ml); c, 3 mM-GDP[γS]+thrombin (0.2 unit/ml); d, 3 mM-ATP+thrombin (0.2 unit/ml); e, 3 mM-ATP+thrombin (1 unit/ml); f, thrombin (1 unit/ml); g, 3 mM-GDP[γS]+thrombin (1 unit/ml); h, 3 mM-ATP alone; i, 60 µM-diC\(_4\); j, 3 mM-GDP[γS]+60 µM-diC\(_4\); k, 3 mM-ATP+60 µM-diC\(_4\).

**Effect of GDP[γS] and ATP on thrombin-induced 45 kDa-protein phosphorylation**

In intact \(^{32}P\)-labelled platelets, thrombin (0.2 unit/ml) induced a 2–3-fold increase in \(^{32}P\) labelling of the 45 kDa protein kinase C substrate, indicating phosphorylation of this protein (Fig. 4). Paralleling the inhibition of thrombin (0.2 unit/ml)-induced aggregation by GDP[γS] (3 mM) and ATP (3 mM) in these platelets, almost total
inhibition (90–100%) of 45 kDa phosphorylation was observed (Fig. 4). The inhibitory effect of these compounds was much less marked against thrombin (at 1 unit/ml) (Fig. 4). Partial inhibition (20–30%) of diC4-induced 45 kDa-protein phosphorylation by GDP[βS] and ATP was also observed (Fig. 4).

DISCUSSION

Recent work demonstrating stimulation of phosphoinositide hydrolysis and platelet secretion by GTP and its stable analogues [1–5], and its inhibition by GDP[βS] [1–7], has served to establish firmly a key role for GTP-binding proteins in stimulus–response coupling in platelets. In the present study, we sought to examine the role of these proteins in collagen-induced platelet activation using GDP[βS] and saponin-permeabilized platelets. Our results demonstrate that, far from being a useful tool in the elucidation of the role of G-proteins, GDP[βS] can inhibit all the stages of platelet activation in intact platelets, and independently of any effect on G-protein–GTP interaction. The evidence for the latter comes mainly from two of our findings: (i) inhibition of agonist-induced platelet activation in intact platelets in the absence of permeabilization makes it unlikely that inhibition is at the site of the G-protein, as these proteins are believed to be located at the intracellular surface of the plasma membrane, or even in the cytoplasm [20,21]; (ii) inhibitory effects of GDP[βS] in intact platelets are mimicked by other nucleotides, such as ATP, GDP and, most notably, GTP itself, which is suggestive of inhibitory effects directed at ADP, a phenomenon reported several years ago in rabbit platelets by Packham et al. [22]. These workers showed that nucleoside di- and tri-phosphates such as GDP, CDP and UDP, and ATP, GTP, CTP and UTP, inhibit the conversion of ADP into ATP by the platelet enzyme nucleoside diphosphokinase and proposed that this reaction could be central to the pro-aggregatory effects of ADP on platelets. Moreover, Shattil & Brass [10] have suggested that inhibitory effects of GDP[βS] at the fibrinogen receptor are mediated via inhibition of phosphoinositide hydrolysis and DAG/InsP₃ formation at the Gₛ or Nₛ site; our results, which demonstrate inhibition of ADP-induced aggregation in indomethacin-treated platelets, however, suggest otherwise, and imply a direct inhibitory effect on ADP-induced aggregation/fibrinogen binding, as ADP induces very little or no phosphoinositide hydrolysis in indomethacin-treated platelets [18,19]. The possibility that the platelets in our preparation were not intact and allowed permeation of the nucleotides can be discounted, as the stable GTP analogue, GTP[γS], which is known to induce secretion [1–3], only did so when the platelets were permeabilized. Thus our data strongly suggest a need for re-interpretation of earlier data from Brass et al. [6,7,10], particularly on U46619 [7] and fibrinogen binding [10], and the recent data of Authi et al. [23] on collagen, where, solely on the basis of inhibitory effects of GDP[βS], a role for G-proteins in fibrinogen binding and collagen- and U46619-induced activation has been proposed. The almost total inhibition of collagen- and U46619-induced aggregation and secretion by GDP[βS] that we observe in intact platelets would make it impossible to detect any additional effects on G-protein–GTP interactions and phosphoinositide hydrolysis in saponized platelets. It is most likely that inhibition of collagen- and U46619-induced secretion by GDP[βS] and the other nucleotides examined is mediated via inhibition of aggregation and/or inhibition of the effects of endogenous/released ADP. This would agree with earlier views [14,24,25] on the role of aggregation as well as ADP in these processes. The partial inhibition of diC₄’s effects (aggregation, 45 kDa-protein phosphorylation and secretion) by GDP[βS] and ATP would also agree with the findings of Ashby et al. [26], who demonstrated a clear role for ADP in mediating the effects of 1-oleoyl-2-acetylgllycerol. However, it would be surprising if the inhibitory effects of these nucleotides are mediated entirely via inhibition of the effects of ADP, as previous work [14,24] demonstrated a role for ADP only at low thrombin concentrations, whereas, in our study, GDP[βS] and ATP were able to abolish all the effects of thrombin (0.2 unit/ml), which was a maximally effective concentration for inducing 5HT secretion. In this context it is unlikely that chelation of extracellular bivalent cations, such as Mg²⁺ or Ca²⁺, would be the cause of the inhibitory effects of the nucleotides tested, as the extent to which the extracellular Mg²⁺ and Ca²⁺ concentrations would be reduced by 3 mM-ATP in our resuspension medium would still be high enough to support aggregation as well as secretion induced by all the agonists tested, particularly thrombin. Moreover, although addition of EDTA or EGTA did not affect thrombin (0.2 unit/ml)-induced secretion, ATP and GDP[βS] had strong inhibitory effects on secretion in the presence of EDTA or EGTA.

It should be mentioned that an entirely surprising finding to us is that U46619 (0.01–3 μM), in the study by Brass et al. [7], was able to induce any 5HT secretion at all with 3.3 mM-ATP present in the resuspension medium, as the duration of saponization in the secretion studies was similar to that in our study, and U46619, in our study, was the most sensitive of all the agonists examined to inhibition by ATP and other nucleotides, in intact as well as in saponized platelets. In other studies with permeabilized platelets, where a different permeabilization period was used, (e.g. other assays in [6]) and in electropermeabilization studies [1–4], the inhibitory effects of ATP may not be so pronounced.

In conclusion, our results suggest that, even though GDP[βS] may exert inhibitory effects mediated via G-protein–GTP interaction inhibition in permeabilized platelets, these results should be interpreted with great caution, as this compound, as well as other related nucleotides, can exert inhibitory effects on agonist-induced platelet functions mediated via an extracellular site of action. This point, which makes GDP[βS] not such a useful tool in studying G-protein–GTP interactions, at least in human platelets, as well as the role of endogenous/released ADP in platelet activation induced by other agonists, are issues of great importance which have to be resolved.

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