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# Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin.

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**Research Article**

Phosphoinositide hydrolysis in platelets stimulated by thrombin is thought to be regulated by a pertussis toxin-sensitive guanine nucleotide binding protein (G protein) referred to as Gp. The present studies examine the role of Gp in platelet responses to the thromboxane A2 analogue U46619 and in the pathway by which the phosphoinositide hydrolysis product inositol 1,4,5-triphosphate (IP3) causes secretion. In permeabilized platelets, U46619 caused phosphatidic acid formation and secretion, which were abolished by the G protein inhibitor, guanosine 5'-O-(2-thiophosphate) (GDP beta S). Unlike thrombin, however, U46619-induced phosphoinositide hydrolysis was unaffected by pertussis toxin, and U46619 was unable to inhibit the [32P]ADP ribosylation of the 42-kD pertussis toxin substrate in platelets. IP3-induced secretion, which is known to depend upon intracellular Ca release and subsequent arachidonic acid metabolism, was also inhibited by GDP beta S, as was Ca-induced secretion. These observations suggest that platelet thromboxane A2 (TxA2) receptors are coupled to a toxin-resistant form of Gp distinct from the one that is coupled to thrombin receptors, and that TxA2-stimulated phosphoinositide hydrolysis may serve as a feedback mechanism by which stimuli for arachidonic acid release, such as IP3 and Ca, amplify responses to agonists.



# Inositol 1,4,5-Triphosphate-induced Granule Secretion in Platelets

## Evidence That the Activation of Phospholipase C Mediated by Platelet Thromboxane Receptors Involves a Guanine Nucleotide Binding Protein-dependent Mechanism Distinct from That of Thrombin

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## Abstract

**Phosphoinositide hydrolysis in platelets stimulated by thrombin is thought to be regulated by a pertussis toxin-sensitive guanine nucleotide binding protein (G protein) referred to as $G_p$. The present studies examine the role of $G_p$ in platelet responses to the thromboxane $A_2$ analogue U46619 and in the pathway by which the phosphoinositide hydrolysis product inositol 1,4,5-triphosphate ($IP_3$) causes secretion. In permeabilized platelets, U46619 caused phosphatidic acid formation and secretion, which were abolished by the G protein inhibitor, guanosine 5′-*O*-(2-thiophosphate) (GDPβS). Unlike thrombin, however, U46619-induced phosphoinositide hydrolysis was unaffected by pertussis toxin, and U46619 was unable to inhibit the [$^{32}$P]ADP ribosylation of the 42-kD pertussis toxin substrate in platelets. $IP_3$-induced secretion, which is known to depend upon intracellular Ca release and subsequent arachidonic acid metabolism, was also inhibited by GDPβS, as was Ca-induced secretion. These observations suggest (*a*) that platelet thromboxane $A_2$ ($TxA_2$) receptors are coupled to a toxin-resistant form of $G_p$ distinct from the one that is coupled to thrombin receptors, and (*b*) that $TxA_2$-stimulated phosphoinositide hydrolysis may serve as a feedback mechanism by which stimuli for arachidonic acid release, such as $IP_3$ and Ca, amplify responses to agonists.**

## Introduction

The hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] by phospholipase C is one of the earliest events known to occur when platelets are activated by agonists such as thrombin (1–4). This process produced two key mediators of further platelet activation: diacylglycerol and inositol 1,4,5-triphosphate ($IP_3$). Diacylglycerol has been shown to cause protein phosphorylation and secretion and the exposure of fibrinogen receptors on the platelet surface, events thought to be mediated by protein kinase C (5–7). In platelets that have been permeabilized with saponin, exogenous $IP_3$ causes secretion, protein phosphorylation, fibrinogen receptor exposure, and aggregation (7–12). In this case, however, these events appear to be a consequence of the ability of $IP_3$ to trigger the release of Ca sequestered within the platelet-dense tubular system (7, 10, 13–15). The evidence that supports this conclusion is as follows. First, in platelets permeabilized with saponin, physiologically relevant concentrations of $IP_3$ cause both Ca release from the platelet dense tubular system and dense granule secretion. The dose-response curves for these two processes are essentially identical. Secondly, the addition of EGTA to the permeabilized platelets has no effect on $IP_3$-induced Ca release, but completely inhibits $IP_3$-induced secretion. Thirdly, in platelets permeabilized by voltage discharge, increases in the cytosolic free Ca concentration caused by manipulation of the extracellular Ca concentration also cause secretion (16–18).

One mechanism by which the Ca discharged by $IP_3$ might stimulate secretion is by stimulating arachidonic acid release from phospholipids such as phosphatidylcholine. It has recently been shown using platelets permeabilized with saponin that $IP_3$-induced secretion, protein phosphorylation, and platelet aggregation are inhibited by aspirin, suggesting that these processes are partially dependent upon the products of arachidonic acid metabolism such as thromboxane $A_2$ ($TxA_2$) (10–12, 19). It has also been shown that $TxA_2$ analogues bind to specific receptors on the platelet surface (20) and stimulate phosphoinositide hydrolysis (21–23). Collectively, these observations suggest a model in which the Ca released from the platelet-dense tubular system by $IP_3$ stimulates phospholipase $A_2$ (24) leading to arachidonic acid release, thromboxane formation, and amplification of the original stimulus for phosphoinositide hydrolysis. If this hypothesis is correct, then the mechanism that couples phospholipase C activation to thromboxane receptor occupation plays an important role and is a potential site for the regulation of platelet activation. In the case of thrombin, recent evidence suggests that phosphoinositide hydrolysis is regulated by a guanine nucleotide binding protein (G protein) that is commonly referred to as $G_p$. Specifically, it has been shown that thrombin stimulates GTPase activity in platelets (e.g., 25), that the addition of GTP or nonhydrolyzable GTP analogues to permeabilized platelets causes phosphoinositide hydrolysis and Ca release and secretion (16–18, 26), and that inhibitors of G protein function, such as guanosine 5′-*O*-(2-thiophosphate) (GDPβS) (27, 28) and pertussis toxin, will inhibit platelet responses to thrombin (26). In a previous study from this laboratory (26), inhibition of thrombin by pertussis toxin was associated with the ADP ribosylation of a protein with an apparent molecular mass of 42 kD that comigrates with the α subunit of $G_i$, the G protein that mediates inhibition of adenylate cyclase. The extent of inhibition by per-

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1. *Abbreviations used in this paper:* G protein, guanine nucleotide binding protein; GDPβS, guanosine 5′-*O*-(2-thiophosphate); $G_i$, guanine nucleotide binding protein that mediates inhibition of adenylate cyclase; $G_p$, guanine nucleotide binding protein that regulates phospholipase C activation: Gpp(NH)p, guanyl-5′-yl imidodiphosphate; $IP_3$, inositol 1,4,5-triphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $TxA_2$, thromboxane $A_2$.

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tussis toxin of thrombin-stimulated phosphoinositide hydrolysis paralleled the inhibition of thrombin's ability to suppress prostacyclin ($PGI_2$)-stimulated cyclic AMP (cAMP) formation and correlated with the extent of labeling of the 42-kD protein.

With this background, the present studies were designed to address two aspects of the regulation of platelet activation: (*a*) to determine whether $TxA_2$-stimulated phosphoinositide hydrolysis in platelets is, like thrombin-stimulated phosphoinositide hydrolysis, mediated by a G protein and, if so, to examine the properties of that G protein, and (*b*) to determine whether $IP_3$-stimulated arachidonic acid metabolism is able to amplify platelet responses to agonists by "feedback" activation of phosphoinositide hydrolysis.

## Methods

*Platelet preparation.* Washed platelets were prepared from fresh human blood as previously described (8). In brief, the platelets were sedimented from platelet-rich plasma, washed once in buffer containing 140 mM NaCl, 20 mM Hepes, and 1 mM EDTA, pH 7.1, and then resuspended in buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM $NaH_2PO_4$, and 20 mM Hepes, pH 7.4. The final cell count was adjusted to $1.4 \times 10^9$/ml. For the studies of secretion, the platelets were loaded with [$^{14}$C]serotonin (New England Nuclear, Boston, MA) by addition of the labeled compound to platelet-rich plasma (8). When noted, the platelet-rich plasma was also incubated with 1 mM aspirin for 30 min to inhibit cyclooxygenase. In some experiments, successful inhibition of eicosanoid synthesis was confirmed by measuring arachidonic acid-induced platelet aggregation (8).

*Serotonin release from saponin-treated platelets.* The washed platelet suspension was diluted to one fourth of the original concentration using buffer containing 160 mM KCl, 5.3 mM $MgCl_2$, 3.3 mM ATP, and 13.3 mM Hepes, pH 7.1. The free Ca concentration was adjusted by the addition of EGTA and/or $CaCl_2$ and was either measured with a Ca electrode or calculated as previously described (29). Afterwards, the platelets were preincubated with saponin (13–15 μg/ml) for 1 min before the addition of an agonist. When indicated, GDPβS was added at the same time as the saponin. The reaction was terminated after 3–10 min by sedimenting the platelets at 14,000 *g* for 3 min in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY). Serotonin release was quantitated by measuring $^{14}$C in aliquots of the supernate.

*Phosphatidic acid formation.* Washed platelets were preincubated for 1 min with saponin (15 μg/ml), ATP (0.5 mM), and ~ 4 μCi/ml of γ-$^{32}$P-labeled ATP (sp act 10–40 Ci/mmol, New England Nuclear) before adding an agonist. The reaction was terminated after 3 min by the addition of chloroform/methanol and [$^{32}$P]phosphatidic acid formation was detected by thin layer chromatography as previously described (6). Preliminary studies showed that phosphatidic acid formation in the saponin-treated platelets was maximal within 60 s of the addition of thrombin and the amount of [$^{32}$P]phosphatidic acid remained constant for at least 5 min. In the studies that examined the effects of pertussis toxin on phosphatidic acid formation, the toxin and 0.2 mM NADH were added to the platelet suspension at the same time as the saponin and the preincubation period increased to 5 min.

*Detection of pertussis toxin substrates.* [$^{32}$P]ADP ribosylation was performed as previously described (26). Washed platelets ($3.5 \times 10^8$/ml) were incubated with saponin (15 μg/ml), NAD (0.2 mM), [$^{32}$P]NAD (sp act 25 Ci/mmol, final concentration 70 μCi/ml, New England Nuclear), pertussis toxin (15 μg/ml, List Biologicals, Campbell, CA), and EGTA (10 μM). When indicated, thrombin or U46619 was added before the saponin. At the end of the incubation period, the platelets were sedimented and then analyzed by one-dimensional polyacrylamide electrophoresis in the presence of sodium dodecylsulfate (26). Protein bands were identified using Coomasie blue stain, and $^{32}$P-labeled proteins were detected by autoradiography.

*cAMP formation.* cAMP formation was measured using a radioimmunoassay kit (New England Nuclear) after extracting aliquots of the platelet suspension with 6% trichloroacetic acid.

*Other materials.* $IP_3$, U46619, and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO). Gpp(NH)p and GDPβS were obtained from Boehringer Mannheim (Indianapolis, IN). Pertussis toxin was obtained from List Biologicals. Purified human α-thrombin was the gift of Dr. John Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY.

## Results

*G-protein interactions with platelet thromboxane receptors.* Previous studies have demonstrated that $TxA_2$/$PGH_2$ analogues stimulate phosphoinositide hydrolysis (21–23, 30) and GTPase activity (31) in platelets. In order to determine whether these responses reflect the coupling of one or more G proteins to platelet $TxA_2$ receptors, we first examined the effects of the G protein inhibitor GDPβS on phosphoinositide hydrolysis and secretion in permeabilized platelets stimulated with the $TxA_2$ analogue, U46619. In the studies shown in Fig. 1, saponin-treated platelets were stimulated with 10 μM U46619 in the presence of various concentrations of GDPβS. Phosphoinositide hydrolysis was detected by measuring phosphatidic acid formation. In the absence of GDPβS, U46619 caused a 2.5-fold increase in phosphatidic acid formation. GDPβS inhibited this increase in a concentration-dependent manner. Half-maximal inhibition required ~ 40 μM GDPβS, which is similar to the GDPβS concentration required to inhibit thrombin-induced diacylglycerol formation and $PGI_2$-stimulated cAMP formation in saponin-treated platelets (26). GDPβS also inhibited U46619-induced secretion (Fig. 2). At 1 μM U46619, inhibition of secretion was also half-maximal at ~ 40 μM GDPβS (not shown).

Pertussis toxin has no apparent effect on intact platelets. However, we have recently demonstrated that the addition of the toxin to platelets permeabilized with saponin results in the inhibition of thrombin-stimulated phosphoinositide hydrolysis and of thrombin's ability to suppress $PGI_2$-stimulated cAMP formation (26). This suggests that both $G_p$ and $G_i$ are affected by the toxin. In order better to characterize the G proteins coupled to platelet $TxA_2$ receptors, we measured U46619-induced phosphatidic acid formation in saponin-treated platelets preincubated with pertussis toxin. Under conditions in which thrombin-induced phosphatidic acid formation was inhibited by

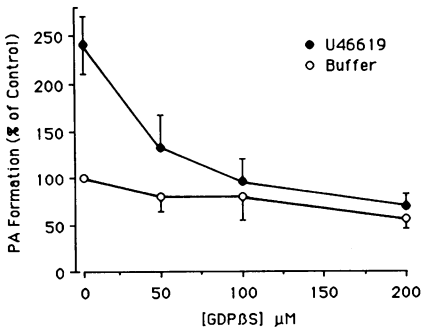


*Figure 1.* Phosphatidic acid formation in response to U46619. Washed platelets were incubated for 1 min with saponin, [$^{32}$P]ATP, and GDPβS (final concentration shown) before adding 10 μM U46619 (*closed circles*) or solvent (*open circles*). The reaction was stopped 3 min later by the addition of chloroform/methanol and the [$^{32}$P]phosphatidic acid isolated by thin-layer chromatography. The results shown are mean±standard error of the mean for four studies.

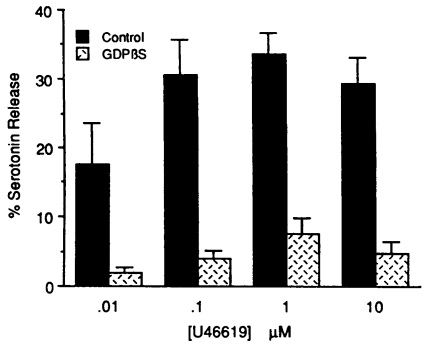


*Figure 2.* Secretion in response to U46619. U46619 at the final concentrations shown was added to washed platelets 2 min after the addition of (*left*) 3 mM GDPβS or (*right*) 10 μM pinane $TxA_2$. The results obtained in the presence of the inhibitors are shown in the hatched bars. Those obtained in the absence of the inhibitors are shown in the solid bars. The data shown are the mean of three studies of each type.

~ 60%, the toxin had no effect on U46619-induced phosphatidic acid formation (Fig. 3, *top*). We also compared the ability of U46619 and thrombin to cause $G_i$-dependent inhibition of $PGI_2$-stimulated cAMP formation. Intact platelets were preincubated with thrombin or U46619 before adding 1 μM $PGI_2$. The formation of cAMP was followed for the next 2 min. Thrombin suppressed cAMP formation by > 80%. Under the same conditions, U46619 had no effect (Fig. 3, *bottom*).

These observations suggest that platelet $TxA_2$ receptors are coupled to a toxin-insensitive form of $G_p$ and not coupled to $G_i$ at all. Incubation of permeabilized platelets with pertussin toxin and [$^{32}$P]NAD results in the ADP ribosylation of a protein with an approximate molecular mass of 42 kD (26). Preincubation of the platelets with thrombin abolishes the labeling of this protein, presumably because occupation of the thrombin receptor results in the dissociation of any coupled G proteins into toxin-insensitive subunits (see Discussion). Similar studies were performed with U46619. Under conditions in which thrombin abolished [$^{32}$P]ADP ribosylation of the 42-kD protein, a concentration of U46619 which maximally stimulates phosphoinositide hydrolysis and secretion had no effect (Fig. 4). In agreement with the functional studies, this suggests that $TxA_2$ receptors are not coupled to either toxin-sensitive G protein.

*Role of $TxA_2$-stimulated phosphoinositide hydrolysis in $IP_3$-induced secretion.* Several investigators have demonstrated that $IP_3$-induced secretion in permeabilized platelets is blocked by inhibitors of cyclooxygenase such as aspirin or indomethacin (10–12), observations that we have confirmed (19). Aspirin has no effect on Ca release from the platelet-dense tubular system in response to $IP_3$ (8). Therefore, in order to assess the contribution of G protein-dependent phosphoinositide hydrolysis in $IP_3$-induced secretion, we examined the effect of GDPβS on $IP_3$-induced secretion and the effect of GDPβS and aspirin on Ca-induced secretion. In the studies shown in Fig. 5, $IP_3$-induced secretion was measured at various GDPβS concentrations. GDPβS completely inhibited $IP_3$-induced secretion. Inhibition was half-maximal at approximately the same GDPβS concentration as that which inhibited U46619-induced phosphatidic acid formation and secretion. GDPβS also inhibited Ca-induced secretion. The inhibition was not complete, but closely resembled the inhibition of Ca-induced secretion seen with aspirin (Fig. 6). In contrast to these results, we have previously shown that

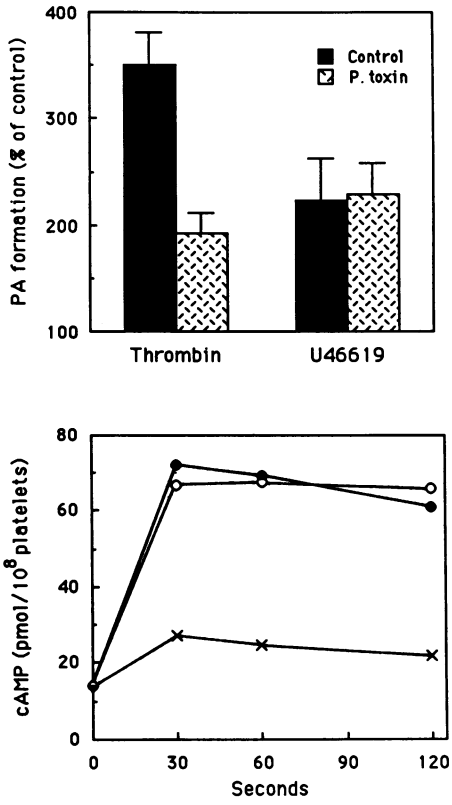


*Figure 3.* Comparisons between the effects of thrombin and U46619 on phosphatidic acid and cAMP formation. In the studies shown in the upper half of the figure, washed platelets were incubated for 5 min with saponin, 0.5 mM [$^{32}$P]ATP and 0.2 mM NAD with (*hatched bars*) or without (*solid bars*) 15 μg/ml of pertussis toxin before adding thrombin (0.2 μM) or U46619 (10 μM). [$^{32}$P]phosphatidic acid formation was measured 3 min later. The results shown are the mean±standard error of the mean of four studies. In the lower half of the figure the time course of cAMP formation is shown in platelets stimulated with 1 μM $PGI_2$. Intact platelets were preincubated for 15 s with buffer (*closed circles*), 0.2 μM thrombin (×), or 10 μM U46619 (*open circles*) before the addition of the $PGI_2$. The amount of cAMP present in the cells was measured 30, 60, and 120 s after the $PGI_2$ was added. The results shown are the mean of three studies.

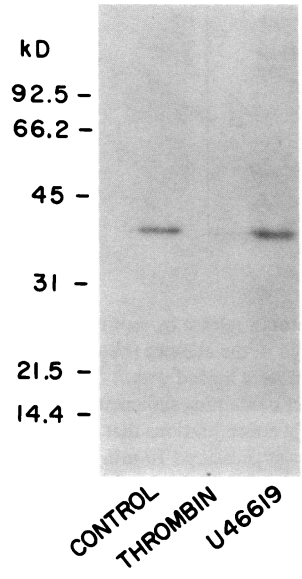


*Figure 4.* Inhibition of ADP ribosylation by thrombin and U46619. Washed platelets were preincubated with or without thrombin (1 U/ml) for 5 s or U46619 (10 μM) for 15 s before adding saponin (15 μg/ml), NAD (50 μM), [$^{32}$P]NAD and EGTA (10 μM). After an additional 30 min, the platelets were sedimented and then analyzed by polyacrylamide electrophoresis. The figure shows an autoradiogram of the $^{32}$P-labeled proteins. Variations in the intensity of labeling were confirmed by densitometry.

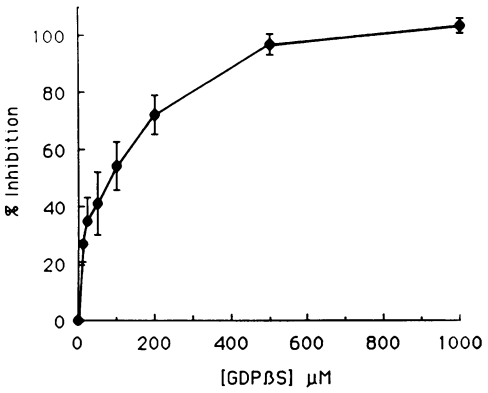


*Figure 5.* Effect of GDPβS on $IP_3$-induced serotonin release. [$^{14}$C]serotonin-loaded platelets were preincubated with saponin and GDPβS (final concentration shown) for 1 min before adding $IP_3$ (40 μM). Secretion was measured 5 min later. The results shown are expressed as the % inhibition of the results obtained with $IP_3$ alone and are the mean±standard error of the mean of four studies.

GDPβS has no effect on secretion caused by the diacylglycerol analogue 1-oleoyl-2-acetyl-glycerol (OAG) or on Ca release in response to $IP_3$ (26). Taken together, these observations suggest that effects of GDPβS are on phosphoinositide hydrolysis and not on the secretory mechanism initiated by newly-formed diacylglycerol.

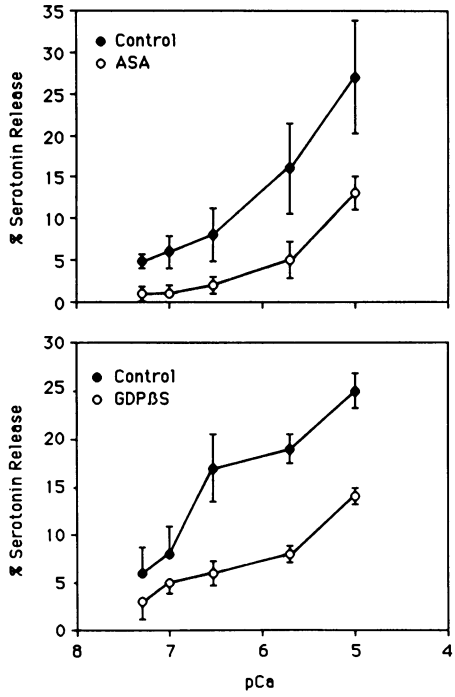


*Figure 6.* Inhibition of Ca-induced serotonin release by aspirin and GDPβS. (*Top*) Washed platelets prepared in the absence (*closed circles*) or presence (*open circles*) of aspirin were loaded with [$^{14}$C]serotonin and resuspended in buffer containing sufficient EGTA or $CaCl_2$ to give the approximate free Ca concentrations that are shown. The extent of serotonin release was measured 10 min after the addition of saponin. The results shown are the mean±standard error of the mean of three studies. (*Bottom*) GDPβS (3 mM) was added to the platelets at the same time as the saponin. Mean±standard error of the mean of four studies.

If, as is suggested by these data, agonist-mediated phosphoinositide hydrolysis can cause secretion either directly via diacylglycerol or indirectly via $IP_3$/Ca-stimulated arachidonic acid metabolism, then the effects of aspirin on agonist-induced secretion might be expected to vary with the strength of the initial stimulus for phosphoinositide hydrolysis. This is known to be the case for thrombin. However, in the case of thrombin, there is also evidence that secretion can occur in the absence of phosphoinositide hydrolysis (26). Therefore, in order to have as specific a stimulus for G protein-mediated phosphoinositide hydrolysis as possible, we examined the effects of aspirin on secretion caused by the nonhydrolyzable GTP analogue, Gpp(NH)p. As is shown in Fig. 7, at low Gpp(NH)p concentrations serotonin release was markedly inhibited by aspirin. At higher Gpp(NH)p concentrations, however, maximal secretion occurred despite the aspirin.

## Discussion

There is now good evidence in platelets that phospholipase C is coupled to thrombin receptors by a G protein. Studies from this laboratory suggest that the α subunit of this G protein has a molecular mass of ~ 42 kD and is a substrate for pertussis toxin (26). Based upon similar types of evidence, it appears that a G protein, commonly referred to as $G_p$, is also responsible for phospholipase C activation in an ever-growing list of tissues, including insect salivary glands, mast cells, neutrophils, adipocytes, and hepatocytes (31–35). To date, $G_p$ has been characterized functionally, but has not been isolated. Adding to the uncertainties about the properties of $G_p$ is the observation that in some tissues, including platelets, neutrophils, HL-60 cells, and mast cells, agonist or hormone-induced phosphoinositide hydrolysis is inhibited by pertussis toxin, whereas in other tissues it is not. This difference does not appear to be due to differences in the ability of the pertussis toxin to enter cells. In several of the cases in which it was not possible to demonstrate inhibition of phosphoinositide hydrolysis, it was possible to demonstrate other effects of the toxin, such as ADP ribosylation of $G_i$.

It has recently been shown that platelets have specific, high-affinity receptors for $TxA_2/PGH_2$ analogues (20). Although the precise location of these receptors has yet to be determined, the available data suggest that they are located on the platelet surface. Two pieces of evidence suggest that these receptors are also coupled to phospholipase C by a G protein. First, Houslay and co-

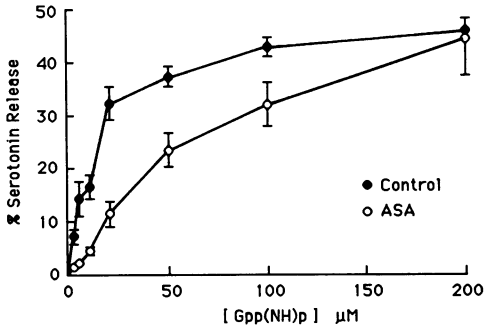


*Figure 7.* Effect of aspirin on Gpp(NH)p-induced secretion. Washed platelets prepared in the absence (*solid symbols*) or presence (*open symbols*) of aspirin were loaded with [$^{14}$C]serotonin and preincubated with saponin for 2 min before the addition of Gpp(NH)p. The results shown are the mean±standard error of the mean of five studies.

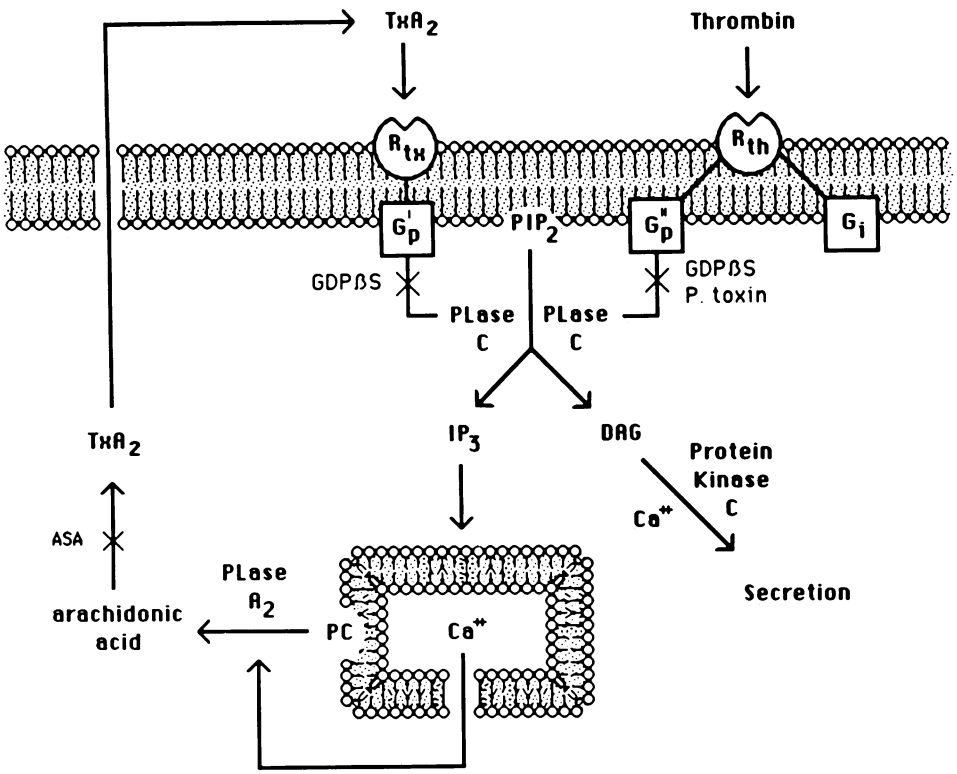


*Figure 8.* A scheme for the interaction between the phosphoinositide and arachidonic acid pathways during platelet activation. The shaded band at the top represents the platelet plasma membrane. The dense tubular system is shown at the bottom. The receptor abbreviation ($R_{th}$ and $R_{tx}$) represent the receptors for thrombin and $TxA_2$ linked to phospholipase C (*PLase C*) by guanine nucleotide-dependent regulatory proteins ($G_p'$ *and* $G_p''$). $G_i$, protein that mediates inhibition of adenylate cyclase; $IP_3$, inositol 1,4,5-triphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; AA, arachidonic acid formed from phosphatidylcholine (*PC*) among other sources. The proposed sites of inhibition by aspirin (*ASA*), pertussis toxin (*P. Toxin*) and GDPßS are shown.

workers (31) have shown that endoperoxide analogues stimulate GTPase activity in platelet membranes. Second, the present studies show that GDPßS completely inhibits U46619-induced diacylglycerol formation and secretion. The concentration of GDPßS required is similar to that which inhibits thrombin-induced phosphoinositide hydrolysis and $PGI_2$-stimulated cAMP formation in permeabilized platelets (26). Up to this point these results parallel those obtained with thrombin. In contrast to thrombin, however, the ability of U46619 to stimulate phosphatidic acid formation was unaffected by pertussis toxin, an observation in agreement with Houslay's (31) finding that endoperoxide-stimulated GTPase activity in platelet membranes is unaffected by pertussis toxin.

In order to address this issue further, we also compared the ability of thrombin and U46619 to cause inhibition of $PGI_2$-stimulated cAMP formation, a process known to involve the pertussis toxin-sensitive G protein, $G_i$. Thrombin inhibited cAMP formation. U46619 did not.[2] Further evidence that the G protein(s) coupled to platelet thromboxane receptors are not sensitive to pertussis toxin was obtained by examining the effect of U46619 on the [$^{32}$P]ADP ribosylation of the single 42-kD pertussis toxin substrate found in platelets. Thrombin, which has toxin-sensitive effects on both phosphoinositide hydrolysis and cAMP formation, completely inhibits labeling of this protein (26). Based upon studies performed with the well-characterized adenylate cyclase regulatory system, this appears to be due to the ability of thrombin to dissociate the heterotrimeric G proteins coupled to thrombin receptors into $\beta/\gamma$ dimers and free $\alpha$ subunits which are no longer substrates for the toxin (38). We found that U46619 had no effect on $PGI_2$-stimulated cAMP formation and caused toxin-insensitive phosphoinositide hydrolysis and, in contrast to thrombin, U46619 had no effect on the [$^{32}$P]ADP ribosylation of the 42-kD protein.

It is not immediately obvious why within a single tissue the G protein responsible for the same set of responses to different agonists should be affected by pertussis toxin in some cases, but not in others—yet this appears to be the case in platelets. It may also be the case in hepatocytes where Johnson and co-workers (39) have recently reported that pertussis toxin inhibits phospholipase C activation by epidermal growth factor but not by angiotensin II. It is also not clear why the function associated with $G_p$ should be sensitive to pertussis toxin in some tissues, but not others. $G_p$ has yet to be isolated from any tissue in which there is functional evidence for its existence. The sole identifiable pertussis toxin substrate in platelets co-migrates on SDS gels with the $\alpha$ subunit of $G_i$. Two recent studies have shown that hormone-stimulated phosphoinositide hydrolysis can be restored in pertussis toxin-treated membranes with HL-60 cells and neutrophils by adding back purified $G_i$ (40, 41). In one of the studies, phosphoinositide hydrolysis was also restored by $G_o$, a well-characterized G protein from brain whose function has yet to be determined. One possible explanation for these observations is that the role and the toxin-sensitivity of G proteins may depend in part upon the characteristics of the receptor to which the G protein is coupled. This remains to be determined.

*Feedback amplification of phosphoinositide hydrolysis.* The second issue that was addressed in the present studies is the

2. The literature contains conflicting reports on the ability of arachidonic acid metabolites to inhibit $PGE_1$-stimulated cAMP formation. An earlier report by Miller et al. (36) suggested that platelet endoperoxides can in fact inhibit cAMP formation. However, those studies, in contrast to the present studies and those of Best et al. (37), were performed using stirred platelets in plasma at 37°C, conditions in which platelet aggregates were formed. Under these circumstances, released ADP, not $TxA_2$, may have mediated the suppression of adenylate cyclase.

mechanism by which $IP_3$ causes secretion in platelets. The data obtained are consistent with a model in which $IP_3$ causes secretion by an "indirect" pathway in which the Ca released from the dense tubular system activates phospholipase $A_2$, initiating the arachidonic acid pathway which, in turn, stimulates receptor-linked, guanine nucleotide-regulated phosphoinositide hydrolysis leading to diacylglycerol formation and, finally, granule secretion. Specifically, our data, in combination with those of previous investigators show: (*a*) that $IP_3$-induced serotonin release, but not $IP_3$-induced Ca release, is inhibited by aspirin, $TxA_2$ antagonists, and GDP$\beta$S and (*b*) that Ca-induced secretion is also inhibited by aspirin and GDP$\beta$S. The effect of GDP$\beta$S in this case appears to be confined to inhibition of phosphoinositide hydrolysis because GDP$\beta$S was found to inhibit neither $IP_3$-induced Ca release nor secretion in response to the synthetic diacylglycerol, OAG. These observations are incorporated into the model shown in Fig. 8. In this model positive feedback from the phosphoinositide hydrolysis pathway through the arachidonic acid pathway causes further phosphoinositide hydrolysis by stimulating platelet thromboxane receptors. In order to distinguish the toxin-insensitive G protein coupled to $TxA_2$ receptors from the toxin-sensitive G protein coupled to thrombin receptors, the former is identified as $G'_p$ and the latter as $G''_p$ in Fig. 8.

In a platelet maximally stimulated with high concentrations of an agonist such as thrombin, such feedback is probably superfluous. However, with low concentrations of thrombin or with agonists that are weaker stimuli for phosphoinositide hydrolysis or with agonists, such as epinephrine, which do not appear to have a primary effect on phosphoinositide hydrolysis (42, 43), this pathway may serve to reinforce platelet activation. In fact, in epinephrine-stimulated platelets phosphoinositide hydrolysis appears to be entirely attributable to the activation of phospholipase $A_2$ and thromboxane formation (43). In addition, because $TxA_2$ is released from activated platelets, this process, along with ADP released from platelet-dense granules, would provide a mechanism for the recruitment of additional platelets into a growing platelet plug.

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