Molecular Mechanism of Thromboxane A$_2$-induced Platelet Aggregation

ESSENTIAL ROLE FOR P2T$_{AC}$ and $\alpha$$_2$A RECEPTORS

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Thromboxane A$_2$ is a positive feedback lipid mediator produced following platelet activation. The $G_q$-coupled thromboxane A$_2$ receptor subtype, TP$_{a}$, and $G_i$-coupled TP$_b$ subtype have been shown in human platelets. ADP-induced platelet aggregation requires concomitant signaling from two P2 receptor subtypes, P2Y$_1$ and P2T$_{AC}$, coupled to $G_q$ and $G_i$, respectively. We investigated whether the stable thromboxane A$_2$ mimetic, (15S)-hydroxy-9,11-epoxymethanoprost-5Z,13E-dienoic acid (U46619), also causes platelet aggregation by concomitant signaling through $G_q$ and $G_i$, through co-activation of TP$_a$ and TP$_b$ receptor subtypes. Here we report that secretion blockade with Ro 31-8220, a protein kinase C inhibitor, completely inhibited U46619-induced, but not ADP- or thrombin-induced, platelet aggregation. Ro 31-8220 had no effect on U46619-induced intracellular calcium mobilization or platelet shape change. Furthermore, U46619-induced intracellular calcium mobilization and shape change were unaffected by A3P5P, a P2Y$_1$ receptor-selective antagonist, and/or cyproheptadine, a 5-hydroxytryptamine subtype 2A receptor antagonist. Either Ro 31-8220 or AR-C66096, a P2T$_{AC}$ receptor selective antagonist, abolished U46619-induced inhibition of adenylyl cyclase. In addition, AR-C66096 drastically inhibited U46619-mediated platelet aggregation, which was further inhibited by yohimbine, an $\alpha$$_2$A-adrenergic receptor antagonist. Furthermore, inhibition of U46619-induced platelet aggregation by Ro 31-8220 was relieved by activation of the $G_i$ pathway by selective activation of either the P2T$_{AC}$ receptor or the $\alpha$$_2$A-adrenergic receptor. We conclude that whereas thromboxane A$_2$ causes intracellular calcium mobilization and shape change independently, thromboxane A$_2$-induced inhibition of adenylyl cyclase and platelet aggregation depends exclusively upon secretion of other agonists that stimulate $G_i$-coupled receptors.

Upon exposure to activating agonists (e.g. thrombin, ADP, and collagen), platelets liberate arachidonic acid stored as phospholipid in the platelet plasma membrane that is converted into thromboxane A$_2$ by sequential oxygenation of arachidonic acid by cyclooxygenase and thromboxane A$_2$ synthase (1). The released thromboxane A$_2$ acts as a positive feedback mediator in the activation and recruitment of more platelets to the primary hemostatic plug (2). Thromboxane A$_2$ exerts its actions via specific $G$ protein-coupled receptors and has been described as either a potent platelet agonist (2, 3) or as a weak agonist with an important role in amplifying the response of platelets to more potent agonists (4).

Pharmacological studies indicate the presence of two potential thromboxane A$_2$ receptor (TP receptor) subtypes on human platelets (5, 6). The TP receptor gene has been cloned and encodes two subtypes of the TP receptor that result from alternative splicing of the primary transcript (7). The subtypes share the identical first 239 amino acids but possess different carboxyl-terminal domains. A complete cDNA of the 343 amino acid TP$_{a}$ isoform was isolated from both a placental cDNA library and human megakaryocytic leukemia cells (8, 9) and a chronic myelogenous leukemia cell line (10). A cDNA for the 407-amino acid TP$_b$ subtype was cloned from a vascular endothelial library (11, 12). Both the TP$_a$ and TP$_b$ subtypes mediate the stimulation of phospholipase C and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate induces an increase in the cytosolic concentration of Ca$^{2+}$, whereas the release of diacylglycerol activates PKC (13–16). In transfected cell lines the two subtypes were shown to oppositely regulate levels of cAMP. The TP$_a$ receptor stimulated cAMP formation in contrast to the TP$_b$ receptor that inhibited the level of intracellular cAMP (15). Pertussis toxin was shown to block TP$_{b}$ receptor-mediated inhibition of adenylyl cyclase; however, its effect on phospholipase C activation was not determined (15). By using isoform-specific antibodies Habib et al. (17) only detected the presence of the TP$_{a}$ receptor in human platelets. Hirata et al. (15) have shown the presence of mRNA encoding both TP$_a$ and TP$_b$ subtypes in platelets using reverse transcriptase-polymerase chain reaction.

ADP-induced platelet aggregation results from concomitant signaling through the P2Y$_1$ and P2T$_{AC}$ receptors that couple to $G_q$ and $G_i$, respectively (18–21). Thrombin has been shown to activate both $G_q$ and $G_i$-signaling cascades (22, 23). Contrary to this, the $\alpha$$_2$A-receptor mediated aggregation is not inhibited by ADP. Therefore, if a $G_i$-mediated pathway were to exist in platelets, it would not be activated by ADP.
to previous studies, we have demonstrated that epinephrine and serotonin activating only $G_i$ or $G_s$ pathways, respectively, are not true platelet-aggregating agents (18). Offermanns et al. (24) have provided evidence showing that U46619 couples to $G_t$. Since thromboxane $A_2$ couples to two TP receptor subtypes and TP$\beta$ has been shown to inhibit adenyl cyclase, we investigated whether U46619 (a stable thromboxane $A_2$ analog) also causes platelet aggregation by co-activation of TP$\alpha$ and TP$\beta$ receptor subtypes coupled to $G_t$ and $G_s$, respectively.

We report here that U46619 causes intracellular calcium mobilization and shape change in human platelets independently of secretion. However, Tx$A_2$-induced platelet aggregation depends upon secretion of other platelet agonists capable of coupling to $G_s$ pathways. In the absence of $G_t$ signaling by other agonists, U46619 cannot cause inhibition of adenyl cyclase or platelet aggregation. We provide evidence for the involvement of the P2T$_{AC}$ and $\delta_3_A$-adrenergic receptors as well as other $G_t$-coupled receptors in U46619-induced platelet aggregation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adenosine-5'-phosphate-5'-phosphate (A3P5P), epinephrine, apyrase (type V), ADP, fibrinogen (type I), and bovine serum albumin (fraction V) were from Sigma. The acetoxyethyl ester of Fura PE-3 was from Tedla (Austin, TX). The stable thromboxane/prostaglandin endoperoxide analogue 9,11-dieoxy-9,11-epoxymethanoprostaglandin $F_2_{\alpha}$ (U46619) and Ro 31-8220 (bisindolylmaleimide IX) were from Biomol (Plymouth Meeting, PA). Imipramine was purchased from ICN (Costa Mesa, CA). Bovine thrombin was from Parke-Davis. SC-57101 was a gift from Searle and Co. AR-C66096 (previously known as ARL 66096) was a gift from Astra Research Laboratories—Charnwood, Loughborough, UK (formerly Fisons). Yohimbine and cyproheptadine were purchased from Research Biologicals International (Natick, MA). All other chemicals were reagent grade, and deionized water was used throughout.

**Isolation of Platelets**—Human blood was collected from a pool of informed healthy volunteers all of whom are students or staff at Temple University School of Medicine. The donated blood was collected into a one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of deionized H$_2$O). Platelet-rich plasma (PRP) was isolated by centrifugation of citrated blood at 180 g for 15 min at room temperature. PRP was incubated with 1 mM acetylsalicylic acid (aspirin treated) for 1 h at 37 °C followed by centrifugation at 1000 g for 10 min at room temperature. The platelet pellet was resuspended in HEPES-buffered Tyrode's solution (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 3.0 mM NaH$_2$PO$_4$, 5 mM glucose, 10 mM HEPES, adjusted to pH 7.4) supplemented with 0.2% bovine serum albumin, and 0.05 units/ml apyrase. The platelet count was adjusted to 2 x 10$^9$ cells/ml. All experiments were repeated at least three times using platelets from different donors.

**Analysis of Platelet Aggregation and Shape Change**—Agonist-induced platelet aggregation was determined by measuring the transmission of light through a 0.5-ml sample of aspirin-treated washed platelets (2 x 10$^9$ cells/ml) with stirring (900 rpm) in a lumi-aggregometer at 37 °C (Chrono-Log, Havertown, PA). The recorder output speed was set to 0.2 mm/s. The base line was set using 0.5 ml of HEPES-buffered Tyrode's solution as a blank. Aggregation of washed platelets required the addition of fibrinogen (1 mg/ml) prior to the addition of an agonist at 1000 µm. The recorder output speed was set to 0.2 mm/s. The base line was set using 0.5 ml of HEPES-buffered Tyrode's solution as a blank. Aggregation of washed platelets required the addition of fibrinogen (1 mg/ml) prior to the addition of an agonist. Platelet shape change was observed by the addition of 1 mM acetylsalicylic acid (aspirin treated) for 1 h at 37 °C followed by centrifugation at 1000 g for 10 min at room temperature. The platelet pellet was resuspended in HEPES-buffered Tyrode's solution (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 3.0 mM NaH$_2$PO$_4$, 5 mM glucose, 10 mM HEPES, adjusted to pH 7.4) supplemented with 0.2% bovine serum albumin, and 0.05 units/ml apyrase. The platelet count was adjusted to 2 x 10$^9$ cells/ml. All experiments were repeated at least three times using platelets from different donors.

**Measurement of Platelet Secretion**—Platelet secretion was determined by measuring the release of [$^{14}$C]5-HT and expressed as the percentage of the total [$^{14}$C]5-HT content. The activation of labeled [$^{14}$C]5-HT platelets was performed in the lumi-aggregometer at 37 °C with stirring (900 rpm) and was stopped after 2 min with the addition of formylmethionyl-leucyl-phenylalanine (FMLP) according to the method of Costa and Murphy (26). Imipramine was added to the HEPES-buffered Tyrode's solution at a final concentration of 1 µM in order to prevent re-uptake of secreted [$^{14}$C]5-HT. Samples were collected and centrifuged at 5000 x g for 1 min, and the radioactivity of the supernatant was measured using an LKB (Amersham Pharmacia Biotech) liquid scintillation counter.

**Measurement of Cytoplasmic Concentrations of Ionized Ca$^{2+}$**—Platelet-rich plasma was incubated at 37 °C with 3 µM Fura PE-3 acetoxyethyl ester and 1 mM acetylsalicylic acid for 45 min followed by 15 min at room temperature. The platelet-rich plasma was centrifuged at 1000 x g for 10 min at room temperature. The platelet pellet was resuspended in HEPES-buffered Tyrode's solution supplemented with 0.2% bovine serum albumin, and 0.05 units/ml apyrase. The platelet count was adjusted to 2 x 10$^8$ cells/ml. Aliquots (1 ml) of the platelet suspension were stirred (900 rpm) in a water-jacketed cuvette maintained at 37 °C during activation. Fluorescence was constantly measured using a Perkin-Elmer LS-5 spectrofluorimeter with settings of 340
(excitation) and 510 nm (emission). Fura PE-3 fluorescence signals were calibrated as described previously (27). $F_{\text{min}}$ was determined by the addition of 2 mM EGTA, 20 mM Tris base, and 40 µM digitonin. $F_{\text{max}}$ was determined by addition of a saturating concentration of CaCl$_2$ to the lysed cells. All experiments were performed in the presence of 2 mM CaCl$_2$ and repeated at least three times using platelets from different donors. Calibration curves for experiments that included Ro 31-8220 were performed in the presence of Ro 31-8220 due to its slight quenching of the fluorescent signal.

Measurement of cAMP—PRP was incubated with 2 µCi/ml [3H]adenine and aspirin (1 mM) for 1 h at 37 °C. Platelets were isolated from PRP by centrifugation as described above and resuspended in HEPES-buffered Tyrode’s solution. Reactions were stopped with 1 M HCl, and 4,000 dpm of [14C]cAMP was added as the recovery standard. The level of cAMP was determined as described previously (28) and measured as a fraction of total [3H]adenine nucleotides. Results are normalized to the level of forskolin (20 µM)-stimulated cAMP and expressed as a percentage.

RESULTS

Effect of Ro 31-8220, a Protein Kinase C Inhibitor, on U46619-induced Platelet Responses—Platelets respond to increasing concentrations of ADP by first undergoing shape change and then, at a higher concentrations, aggregation (29). This is because ADP-induced platelet shape change results from activation of the high affinity P2Y1 receptor (19), and higher concentrations of ADP are needed for co-stimulation of both the high affinity P2Y1 receptor and a low affinity P2Y$_{AC}$ receptor to induce aggregation (19). In order to determine if concomitant higher affinity G$_i$-coupled signaling and lower affinity G$_i$-coupled signaling also occurs in response to U46619 and to determine whether aggregation requires lower concentration of U46619 than secretion, we exposed platelets to different concentrations of the agonist. Similar to the response observed for ADP, the platelets first responded to lower concentrations of U46619 (100 nM) by changing shape. Aggregation occurred at significantly higher concentrations (300 nM) (Fig. 1A). Secretion did not occur at concentrations of U46619 below 300 nM (Fig. 1B); furthermore, the onset of aggregation appears to correlate with the initiation of secretion. PKC has been shown to play an important role in the induction of platelet secretion, and secretion can be blocked using the cell-permeable inhibitor of PKC, Ro 31-8220 (30–32). We investigated the role of secretion in platelet aggregation in response to ADP, thrombin, and U46619. Secretion in response to U46619 is totally abolished by 10 µM Ro 31-8220 (Fig. 1B). In the presence of Ro 31-8220, U46619 caused shape change but did not induce aggregation (Fig. 2). Platelet aggregation induced by thrombin...
ADP and serotonin at the site of injury in order to activate and recruit more platelets into the forming primary hemostatic plug (2). By using receptor-selective antagonists, we investigated the contribution of these agonists to U46619-induced G_q-coupled responses. The compound A3P5P is an antagonist of the G_i-coupled P2Y1 receptor (33). Cyproheptadine is an antagonist at the 5-HT_2A receptor (34–37). Aggregation was not affected by the presence of either compound (data not shown). U46619-induced platelet shape change was not affected by the presence of A3P5P (Fig. 4A) or cyproheptadine (not shown) indicating the lack of any contribution by the P2Y1 or serotonin receptors to this event. The possible contribution of both the P2Y1 and 5-HT_2A receptors in the mobilization of intracellular Ca^{2+} was investigated. Intracellular Ca^{2+} mobilization in response to U46619 was not affected by A3P5P and/or cyproheptadine (Fig. 4B).

Effect of Ro 31-8220 or Receptor-selective Antagonists on U46619-induced Inhibition of Platelet Adenylyl Cyclase—Previous studies have shown that U46619 causes a decrease in the intracellular concentration of cAMP in platelets (38, 39). In order to determine whether TP receptors can couple to G_i-signaling pathways, we utilized two approaches. The first was to block secretion using Ro 31-8220. In the absence of Ro 31-8220, U46619 inhibited forskolin-stimulated adenylyl cyclase (Fig. 5). In the presence of Ro 31-8220, U46619 failed to inhibit adenylyl cyclase. The second approach utilized receptor-selective antagonists to the P2T_A, and α_2A-adrenergic receptors. AR-C66096 is an antagonist at the G_i-coupled P2T_A receptor (28), and yohimbine is an antagonist at the G_i-coupled α_2-adrenergic receptor (40, 41). Platelet dense granules contain both ADP and epinephrine which cause the inhibition of cAMP following activation at their respective receptors (2). The level of cAMP was measured following stimulation of platelets in the absence and presence of the antagonists AR-C66096 and yohimbine. These antagonists effectively prevented the contribution of G_i-coupled signaling by either the P2T_A or the α_2-adrenergic receptor, respectively. As shown in Fig. 5, U46619-induced adenylyl cyclase inhibition was also blocked by these receptor antagonists, suggesting that U46619-induced G_i-stimulation depends on secreted ADP and epinephrine.
Effect of Receptor-selective Antagonists on U46619-induced Platelet Aggregation—We (18) and others (20, 21) have provided evidence that concomitant signaling through both G\(_i\)-coupled and G\(_q\)-coupled receptors is required for platelet aggregation. Since the TP receptor does not couple to G\(_i\), these Gi-coupled receptors in U46619-induced platelet aggregation were studied. Four explanations for the stimulatory action caused by U46619 or other thromboxane A\(_2\) mimetics are possible. First, U46619 may activate G\(_q\) and G\(_i\) through the TP\(_\alpha\) and TP\(_\beta\) receptors, respectively. Second, it is conceivable that U46619 only activates the G\(_q\) pathway and that secreted ADP activates the G\(_i\) pathway. Although unlikely, a third explanation is that U46619 activates G\(_q\) or G\(_i\) through TP\(_\beta\) leading to the activation of phospholipase C and the inhibition of cyclase. Following secretion, released ADP would activate the G\(_i\) pathway. Finally, U46619 may activate an unidentified G protein-coupled pathway that results in secretion of ADP which activates both G\(_q\) and G\(_i\) through the P2Y1 and the P2T\(_{AC}\) receptors, respectively. We used three complementary approaches to identify the molecular mechanisms of U46619-induced platelet aggregation as follows: 1) determination of the minimum concentration required for platelet aggregation and secretion by U46619, 2) blockade of secretion, and 3) receptor subtype-selective antagonists in order to determine if the positive feedback from granule contents. Here we report that although thromboxane A\(_2\) causes intracellular calcium mobilization and shape change independently, thromboxane A\(_2\)-induced inhibition of adenylyl cyclase and platelet aggregation depend exclusively on ADP and other released granule contents.

Evidence exists for a dissociation of platelet activation responses following stimulation of the TP receptor. First, the EC\(_{50}\) values of the TP receptor agonists, U46619 (42) and STA\(_2\) (43), for an increase in cytosolic Ca\(^{2+}\) and platelet shape change are lower than the EC\(_{50}\) values for secretion and aggregation. Our data indicate that platelet aggregation correlates with the occurrence of secretion. We observed that platelet shape change occurs at lower concentrations of U46619 and that aggregation occurs at higher concentrations (Fig. 1A). Furthermore, the same concentration of U46619 that leads to the initiation of aggregation also initiates secretion (Fig. 1B). However, from this evidence it is not clear if platelet aggregation results in part from P2 receptor stimulation.

Substantial evidence exists that PKC activation is required for platelet secretion (31). In platelets activated by U46619 in the presence of Ro 31-8220, it was reported that P47 phosphorylation, fibrinogen binding, and serotonin release were all inhibited (32). In agreement with previous studies, our results show that Ro 31-8220 prevented U46619-induced platelet aggregation (Fig. 2). We observed that Ro 31-8220 inhibited U46619-induced secretion in platelets loaded with \(^{14}\)C-serotonin in the presence of 2 mM Ca\(^{2+}\) (Fig. 1B) and that Ro 31-8220 did not inhibit the increase in cytosolic Ca\(^{2+}\) induced by U46619 (Fig. 3B).

Ro 31-8220 failed to inhibit ADP- or thrombin-induced platelet aggregation (Fig. 3) suggesting that the Ro 31-8220 inhibitable PKC isoforms do not directly contribute to fibrinogen receptor activation. Ro 31-8220 has been shown to block PKC isoforms \(\alpha, \beta, \gamma, \text{ and } \epsilon\) (44). Hence these PKC isoforms do not contribute to the inside-out signaling leading to fibrinogen receptor activation by either ADP or thrombin.

Considering that secretion and aggregation both occur at the same concentration of U46619 (Fig. 1) and that blocking secretion prevents aggregation (Fig. 2), it is reasonable to suggest that thromboxane A\(_2\)-induced aggregation is dependent upon secretion. The role of ADP in thromboxane A\(_2\)-induced platelet aggregation has been investigated using enzymes that deplete released ADP. This work suggested that the aggregation response is mediated by the secretion of platelet ADP (45–49). It was concluded that U46619-induced platelet aggregation depends on the release of stored ADP. The use of apyrase could have enhanced the generation of adenosine from AMP. Adenosine binds to the G\(_i\)-coupled A\(_2\) receptor resulting in an in-

**DISCUSSION**

The molecular mechanisms leading to aggregation following platelet exposure to thromboxane A\(_2\) have yet to be clearly elucidated. Four explanations for the stimulatory action caused by U46619 or other thromboxane A\(_2\) mimetics are possible. First, U46619 may activate G\(_q\) and G\(_i\) through the TP\(_\alpha\) and TP\(_\beta\) receptors, respectively. Second, it is conceivable that U46619 only activates the G\(_q\) pathway and that secreted ADP activates the G\(_i\) pathway. Although unlikely, a third explanation is that U46619 activates G\(_q\) or G\(_i\) through TP\(_\beta\) leading to the activation of phospholipase C and the inhibition of cyclase. Following secretion, released ADP would activate the G\(_i\) pathway. Finally, U46619 may activate an unidentified G protein-coupled pathway that results in secretion of ADP which activates both G\(_q\) and G\(_i\) through the P2Y1 and the P2T\(_{AC}\) receptors, respectively. We used three complementary approaches to identify the molecular mechanisms of U46619-induced platelet aggregation as follows: 1) determination of the minimum concentration required for platelet aggregation and secretion by U46619, 2) blockade of secretion, and 3) receptor subtype-selective antagonists in order to determine if the positive feedback from granule contents.
may be affected by levels of heterologous receptor expression; furthermore, high levels of receptor expression can lead to promiscuous coupling to multiple G proteins.

U46619-induced aggregation requires concomitant stimulation of both a Gq-coupled receptor and a Gi-coupled receptor. Granule contents appear to mediate the stimulation of Gq-coupled signaling as is evident by the lack of cyclase inhibition when U46619-induced platelet secretion is prevented (Fig. 5). The fact that signaling through the Gi-coupled TP receptor is unaffected under such conditions is apparent by both the robust shape change response (Fig. 3A) and the normal level of cytosolic Ca^{2+} mobilization (Fig. 3B).

An alternative explanation for the effect of Ro 31-8220 on U46619-induced platelet aggregation is that U46619 causes platelet aggregation involving activation of a PKC isoform through a mechanism different from that of ADP. Hence, Ro 31-8220 would inhibit U46619-induced aggregation by inhibiting this PKC isoform in addition to blocking secretion. This possibility was ruled out using receptor-selective antagonists.

Through the use of receptor-selective antagonists, we were able to identify clearly the contribution of receptors mediating aggregation following U46619-induced secretion. Antagonists at Gq-coupled receptors such as cypromeptadine and A3PSP had no effect on aggregation, shape change, or the increase in cytosolic Ca^{2+} concentration. In contrast, both of the Gi-coupled P2TAC and Gq-adrenergic receptors were found to mediate aggregation and inhibition of adenyly cyclase, following U46619-induced secretion (Fig. 6). The compound AR-C66096 had the greatest inhibitory effect indicating the large contribution to Gi-coupled signaling by the P2TAC receptor. In the absence of AR-C66096, yohimbine failed to affect U46619-induced aggregation, indicating that Gi stimulation could be compensated by P2TAC receptor stimulation. The amount of epinephrine found in platelets is extremely small (1.1–3.8 pmol/10^8 platelets) (56); however, the initial concentration of this secreted amount in the microenvironment of the platelet could be much greater. As observed, the secreted epinephrine makes a significant contribution as revealed by the inhibition of aggregation by yohimbine only in the absence of P2TAC receptor stimulation (Fig. 6). This suggests that secretion of the Gi-coupled receptor stimulating agonists (ADP and epinephrine) are required for full aggregation following activation of Gi-coupled signaling by thromboxane A2. When U46619-induced secretion was blocked by Ro 31-8220 aggregation was pre-

crease in the intracellular concentration of cAMP and inhibits platelet activation (50, 51). Moreover, these studies did not clearly determine how ADP and the other components of the dense and a-granules contribute to TxA2-induced platelet aggregation. The use of creatine phosphate/creatine phosphokinase converts ADP to ATP, an agonist at the platelet ADP receptors (2). ATP can also potentially stimulate adenylyl cyclase activity resulting in inhibition of platelet activation (52, 53). Our experiments make use of the receptor subtype-selective antagonists AR-C66096 and yohimbine, which block stimulation of Gi signaling.

Evidence exists to support the presence of the TPα and TPβ receptor subtypes in platelets (8, 17); these isoforms, when expressed in Chinese hamster ovary cells, have been shown to couple to Gi, and Gq pathways, respectively. However, in the presence of Ro 31-8220, high concentrations of U46619 did not alter the level of cAMP, indicating that TP receptor subtypes do not couple to adenylyl cyclase in platelets. Our observation also is supported by two studies. By using platelet membranes, U46619 was found to have no effect upon levels of cAMP (54). Furthermore, Klages et al. (55) have shown that U46619 does not stimulate Gi proteins in mouse platelets. G protein coupling
vented. Under these conditions the selective activation of either the Gβγ-coupled P2Y1 receptor or the α2A-adrenergic receptor restored aggregation (Fig. 7).

Further evidence for the important role of the P2Y1 receptor in mediating the platelet response to Txα2 is provided by reports of patients with congenital ADP receptor defects (57–59). In these cases the shape change and cytosolic Ca2+ mobilization responses to ADP are present indicating function of the P2Y1 receptor, whereas ADP-induced aggregation and inhibition of adenyl cyclase are absent. Such findings suggest that the defect involves the P2Y1 receptor. The lack of signaling due to a defective P2Y1 receptor affects the response of these platelets to thromboxane A2 mimetics. In both cases, U46619-induced activation of the integrin αIIbβ3 was inhibited (58, 59). Inhibition of adenyl cyclase by epinephrine in platelets from both patients was normal, suggesting that the residual fibrinogen receptor activation could be due to activation of α2A-adrenergic receptors by secreted epinephrine. On the other hand, we predict that in the case of a hypothetical P2Y1 receptor defect, platelet aggregation in response to U46619 would appear normal as Gα stimulation, although the P2Y1 receptor and the α2A-adrenergic receptor would be intact.

Even in the presence of both AR-C66906 and yohimbine we still observed some residual aggregation (Fig. 6). We propose that this residual aggregation results from Gβγ signaling by other components of the granules. This prediction is supported by the fact that secretion blockade completely eliminates U46619-induced platelet aggregation. Based on previous and recent reports describing the mechanism of action by thrombospondin, a major constituent of the α granules, in platelet activation and aggregation (60–62), we suggest that it too may be mediating Txα2 mimetic-induced aggregation. A recent study has shown that thrombospondin can stimulate the Gβγ-signalizing pathways (60).

In conclusion, as outlined in Fig. 8, our results show that U46619 causes platelet shape change and intracellular Ca2+ mobilization independently of secreted granule contents. However, U46619-induced platelet aggregation depends exclusively on Gα stimulation by ADP and other released granule contents. The P2Y2 receptor appears to be the predominant stimulator of the Gα pathway. These results further support the hypothesis that platelet fibrinogen receptor activation requires concomitant signaling from the Gβγ and Gα-signalizing pathways.

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