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COMMENTARY

ADP-Induced Platelet Aggregation and Inhibition of Adenylyl Cyclase Activity Stimulated by Prostaglandins

SIGNAL TRANSDUCTION MECHANISMS

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ABSTRACT. ADP is the oldest and one of the most important agonists of platelet activation. ADP induces platelet shape change, exposure of fibrinogen binding sites, aggregation, and influx and intracellular mobilization of Ca^{2+}. ADP-induced platelet aggregation is important for maintaining normal hemostasis, but aberrant platelet aggregation manifests itself pathophysiologically in myocardial ischemia, stroke, and atherosclerosis. Another important aspect of ADP-induced platelet activation is the ability of ADP to antagonize adenylyl cyclase activated by prostaglandins. ADP-induced inhibition of the stimulated adenylyl cyclase activity does not appear to play a role in ADP-induced platelet aggregation *in vitro* or *in vivo*. It is believed that a single ADP receptor mediates the above two ADP-induced platelet responses in platelets. The ADP receptor mediating ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity has not been purified. Therefore, the nature of molecular mechanisms underlying the two seemingly unrelated ADP-induced platelet responses remains either unclear or less well understood. The purpose of this commentary is to examine and make suggestions concerning the role of phospholipases and G-proteins in the molecular mechanisms of signal transduction underlying the two ADP-induced platelet responses. It is hoped that such discussion would stimulate thinking and invite future debates on this subject, and energize investigators in their efforts to advance our knowledge of the details of the molecular mechanisms of ADP-induced platelet activation. BIOCHEM PHARMACOL **57**;8:851–859, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ADP; platelet aggregation; adenylyl cyclase, activation and inhibition of; signal transduction; phospholipases; G-proteins

Platelets are derived from hematopoietic precursor cells, megakaryocytes, and circulate in blood in the nonadhesive state as discrete smooth discs. Following vascular injury, platelets in circulating blood come in contact with agonists, e.g. collagen fibrils underlying the subendothelium, ADP released from the platelet storage granules, and thrombin generated by the coagulation process, and undergo shape change. Agonist-induced platelet activation results in platelet–endothelium and platelet–platelet interactions that lead to platelet aggregation, a process responsible for thrombus or hemostatic plug formation and wound healing. On the other hand, aberrant platelet aggregation is pathophysiological for arterial thrombosis. ADP is the oldest and one of the most important agonists of platelet activation. ADP induces not only platelet shape change, exposure of fibrinogen binding sites, and aggregation, but also the influx and intracellular mobilization of Ca^{2+} [1–6]. Another important aspect of ADP-induced platelet activation is that ADP inhibits adenylyl cyclase activity stimulated by prostaglandins [7–9]. ADP-induced influx of Ca^{2+} is mediated by the $P2X_1$ purinergic receptor (for nomenclature of purinergic receptors, see Ref. 5) [10–12], which has been cloned recently [13]. However, the true physiological importance of the $P2X_1$ receptor in platelets remains unclear [6]. ADP-induced increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) [14, 15] and ADP-induced shape change are thought to be mediated by a platelet P2Y purinergic receptor similar to the human $P2Y_1$ receptor [15, 16]. However, more experimental work is needed to establish unequivocally the claims that the $P2Y_1$ receptor mediates ADP-induced platelet shape change [6]. It is believed that ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity are mediated by a P2T (T signifies thrombocytes) receptor or by a $P2Y_1$ receptor, which has not been purified [3, 6, 15–17]. However, no relationship has been established between the ability of ADP to aggregate platelets and to inhibit the stimulated adenylyl cyclase activity *in vivo* or *in vitro* [17, 18]. The signal transduction mechanism(s) underlying these two ADP-induced platelet responses remains either unclear or less well understood. The purpose of this commentary is to examine possibilities and make suggestions about the nature of the molecular mech-

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anism(s) underlying the two seemingly unrelated ADP-induced platelet responses.

ADP-INDUCED INHIBITION OF THE STIMULATED ADENYLYL CYCLASE ACTIVITY IN PLATELETS

ADP-β-S* is a partial agonist for platelet aggregation and inhibition of the stimulated adenylyl cyclase activity, but high concentrations of ADP-β-S inhibit the two ADP-induced platelet responses [19]. The (R_p) and (R_s) diastereomers of ADP-α-S competitively inhibit the two ADP-induced platelet responses [20]. A close agreement between the potencies of a number of ADP derivatives to competitively inhibit the two ADP-induced platelet responses strongly suggests that ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity are mediated by the same ADP receptor in platelets [3]. The fact that [β-^{32}P]-2-MeS-ADP [9] and [β-^{32}P]-2-AzPET-ADP [21], the two ADP-affinity analogs that aggregate platelets and inhibit the stimulated adenylyl cyclase activity, bind to a single class of ADP-binding sites in platelets and that 2-MeS-ADP inhibits the binding of [β-^{32}P]-2-AzPET-ADP to platelets, supports the view that the two ADP-induced platelet responses are mediated by the same ADP-receptor in platelets. Furthermore, the thienopyridines ticlopidine and clopidogrel are potent and specific inhibitors of ADP-induced platelet aggregation [22]; the two antithrombotic drugs also have been found to be inhibitors of the stimulated adenylyl cyclase activity in platelets [23, 24]. The potencies of the 2-substituted ADP analogs to inhibit the two ADP-induced platelet responses are very different. 2-MeS-ADP [9] and 2-N_3-ADP [25] are 10- and 5-fold more potent than ADP as platelet-aggregating agents, but 200- and 10- to 20-fold more potent as inhibitors of the stimulated adenylyl cyclase activity, respectively. Although 2-AzPET-ADP was somewhat less potent than ADP in inducing platelet aggregation, it was 120-fold more potent than ADP in inhibiting the stimulated adenylyl cyclase activity [21]. There is no satisfactory explanation or viable hypothesis that accommodates the experimental findings that (a) a single ADP receptor mediates ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity, and (b) the potencies of the 2-substituted ADP derivatives to elicit these two platelet responses are very different.

Abbreviations: ADP-β-S, adenosine 5′-*O*-(2-thiodiphosphate); ADP-α-S, adenosine 5′-*S*-(2-*O*-diphosphate); PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; PLC, phospholipase C; PKC, protein kinase C; CO, cyclooxygenase; TS, thromboxane synthetase; MAPK, mitogen activated protein kinase; $\alpha_{IIb}\beta_3$ or GPIIb-IIIa, glycoprotein IIb-IIIa complex; GPIb-IX-V complex, a complex of glycoproteins Ib, IX, and V; PC, phosphatidylcholine; AA, arachidonic acid; TXA_2, thromboxane A_2; DAG, diacylglycerol; IP_3, inositol (1,4,5)-triphosphate; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; CTX, cholera toxin; PTX, pertussis toxin; 2-MeS-ADP, 2-methylthioadenosine 5′-diphosphate; 2-N_3-ADP, 2-azidoadenosine 5′-diphosphate; and 2-AzPET-ADP, 2-(*p*-azidophenyl) ethylthioadenosine 5′-diphosphate.

ADP-INDUCED PLATELET AGGREGATION

ADP-induced platelet aggregation requires influx of Ca^{2+} as well as mobilization of Ca^{2+} from the intracellular stores. ADP-induced platelet aggregation also requires (a) adhesion of platelets to the endothelium mediated by the binding of GPIb-IX-V complex in platelets to von Willebrand factor (vWf) on the endothelium [3, 5], and (b) exposure of GPIIb-IIIa complex (or $\alpha_{IIb}\beta_3$ integrin), the fibrinogen receptor on the platelet surface [26]. The platelet–platelet interactions are mediated by the binding of fibrinogen (a *polydentate* ligand), which is released by ADP-induced secretion from the platelet dense granules, to the conformationally competent integrin $\alpha_{IIb}\beta_3$ expressed on the surface of adjacent platelets, causing them to aggregate. Ca^{2+} is required for the binding of fibrinogen to its receptors on the platelet surface. ADP-induced influx of Ca^{2+} is mediated by the $P2X_1$ purinoreceptors [10, 13]. ADP-induced mobilization of Ca^{2+} from the intracellular stores is a complex process that involves a number of steps leading to the activation of PLC (Fig. 1). Hydrolysis products of the PLC reaction, IP_3 and DAG, mobilize Ca^{2+} from the intracellular stores and cause secretion from the dense granules, respectively, in platelets [29] (Fig. 1). ADP-induced influx of Ca^{2+} and activation of the Na^+/H^+ exchanger, the latter mediated by the ADP-induced influx of Na^+, lead to the activation of $cPLA_2$, which causes the hydrolytic cleavage of PC to AA. AA is converted into prostaglandin endoperoxides G_2 and H_2 (PGG_2 and PGH_2) by CO; TS converts PGG_2 and PGH_2 to TXA_2 (Fig. 1). In a feedback mechanism, PGG_2, PGH_2, and TXA_2 diffuse out of the platelet membrane and bind to their receptors, leading to the receptor-mediated activation of PLC [29] (Fig. 1). An important feature of this mechanism is that the ADP-induced activation of $cPLA_2$ precedes PLC activation [29]. This sequence of events probably involves binding of ADP to the P2T receptor in platelets [29]. There are several lines of evidence that support this mechanism. First, ADP-induced platelet aggregation is accompanied by an increase in $^{22}Na^+$ but not $^{36}Cl^-$ [30]. Second, inhibition of the Na^+/H^+ exchanger has been shown to be accompanied by loss of ADP-induced platelet aggregation and secretion [31–34]. Third, ethylospropylamiloride, a perturbant of the Na^+/H^+ exchanger, has been shown to lower the intracellular pH and block ADP-induced activation, which can be restored by artificial alkalinization of the platelet interior by methylamine [32]. Fourth, ADP and a low concentration of thrombin have been shown to induce platelet aggregation, which requires transient alkalinization of the platelet interior [30–35]. Fifth, an increase in the intracellular concentration of Ca^{2+} and pH, mediated by the ADP-induced influx of Ca^{2+} and Na^+, in the microenvironment of $cPLA_2$ is essential for its activation [36, 37]. Sixth, indomethacin and SQ29548, inhibitor of CO and antagonist of the endoperoxide/TXA_2 receptor, respectively, block ADP-induced mobilization of AA as well as ADP-induced formation of the PLC hydrolysis products [33]. Seventh,

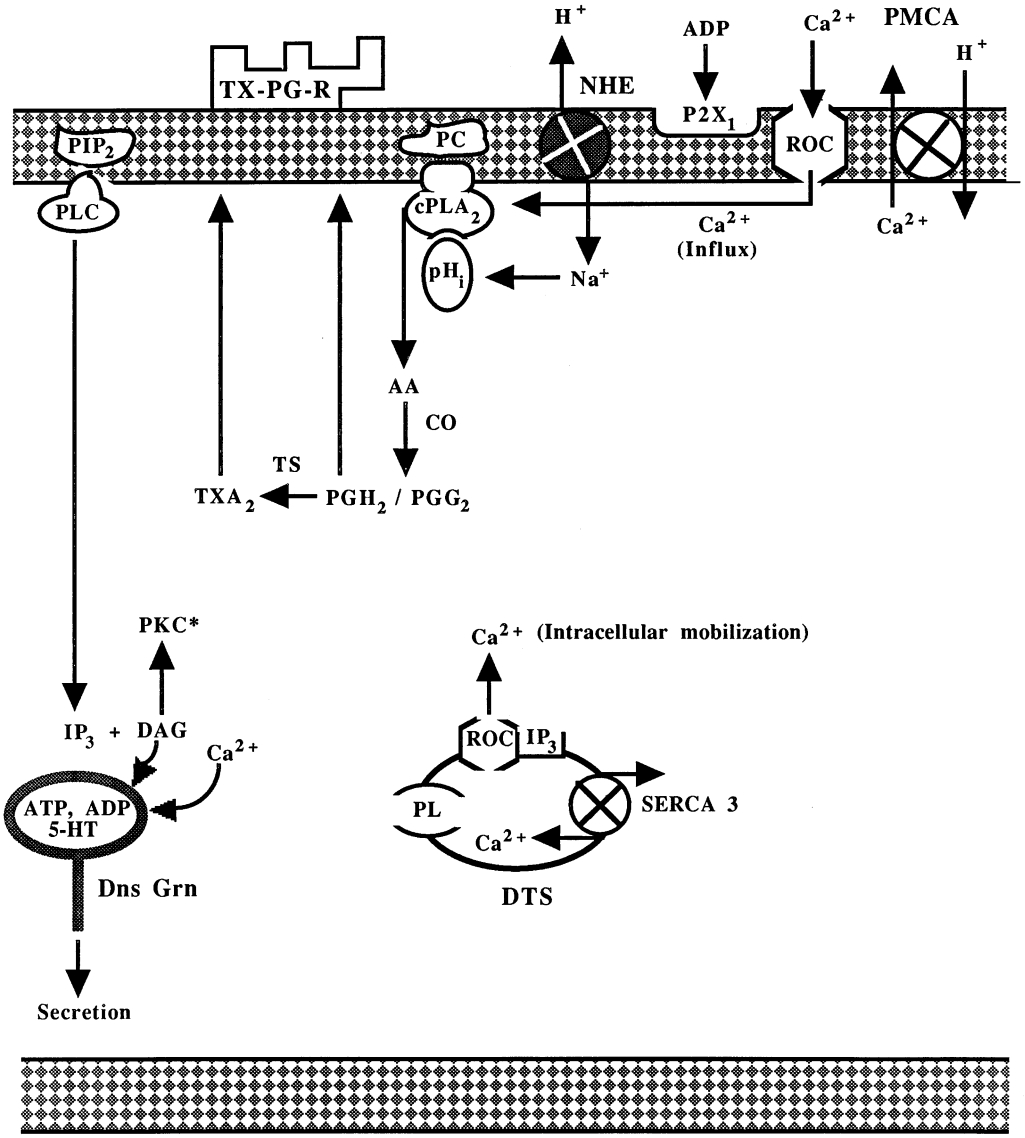

FIG. 1. Mechanism of activation of $cPLA_2$ and PLC in ADP-induced platelet aggregation. Binding of ADP to its receptor on the platelet surface activates the Na^+/H^+ exchanger, leading to a cytosolic pH gradient (pH_i) favorable for the activation of $cPLA_2$. ADP-induced activation of the Na^+/H^+ exchanger (NHE) in platelets is probably mediated by the P2T receptors (not shown). Activation of the $cPLA_2$ is also aided by the ADP-induced influx of Ca^{2+} by a receptor-operated-channel (ROC) mechanism involving the $P2X_1$ purinoreceptors. The activated $cPLA_2$ catalyzes the hydrolysis of phospholipids such as phosphatidylcholine (PC) to arachidonic acid (AA), which is cyclized to prostaglandin endoperoxides PGG_2/PGH_2 by cyclooxygenase (CO) followed by their conversion to thromboxane A_2 (TXA_2) catalyzed by thromboxane synthetase (TS). In a feedback mechanism, the thromboxane-prostaglandin-receptor (TX-PG-R) mediates activation of PLC by prostaglandin endoperoxides PGG_2/PGH_2 and TXA_2 (which diffuse out of the membrane), leading to the formation of PLC hydrolysis products IP_3 and DAG from phosphatidylinositol 4,5-bisphosphate (PIP_2). PKC is activated to PKC* by DAG, its physiological activator. DAG, Ca^{2+}, and PKC* mediate secretion of small molecules, e.g. ADP, ATP, and 5-HT (5-hydroxytryptamine, serotonin), from the dense granules (Dns Grn). IP_3 also releases Ca^{2+} by binding to its receptors through ROC in the IP_3-sensitive storage granules [dense tubular system (DTS)]. Depleted stores in DTS can be replenished with Ca^{2+} by a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 3 isoform (SERCA 3 isoform) [27]. A passive leak (PL) mechanism and the SERCA 3 work together to maintain steady-state levels of Ca^{2+} in the DTS in platelets in the resting state [28]. The plasma membrane Ca^{2+}-ATPase (PMCA) along with a passive leak (not shown) mechanism in the membrane are involved in maintaining steady-state levels of Ca^{2+} in the resting platelets [28]. ADP-induced activation of the Na^+/H^+ exchanger also leads to the exposure of fibrinogen-binding sites, the $\alpha_{IIb}\beta_3$ integrin (glycoprotein IIb-IIIa complex) (not shown). In the presence of Ca^{2+}, binding of fibrinogen to its receptor (the $\alpha_{IIb}\beta_3$ integrin) leads to platelet aggregation. The sequence of events shows that the activation of $cPLA_2$ precedes the activation of PLC in platelets stimulated by ADP.

ADP-induced exposure of the $\alpha_{IIb}\beta_3$ integrin is mediated by the ADP-induced activation of the Na^+/H^+ exchanger, and inhibition of the exchanger blocks this ADP-induced platelet response [38]. This mechanism of ADP-induced platelet activation does not explain the nature of the molecular mechanism by which ADP inhibits the stimulated adenylyl cyclase activity while aggregating platelets at the same time. The mechanism *does* lead to the suggestion

that there might be two different signal transduction mechanisms for ADP-induced platelet aggregation and ADP-induced inhibition of the stimulated adenylyl cyclase activity.

ADP-INDUCED PLATELET AGGREGATION AND INHIBITION OF STIMULATED ADENYLYL CYCLASE ACTIVITY: SIGNAL TRANSDUCTION MECHANISMS

The GTP-binding proteins (G-proteins) are heterotrimeric proteins that consist of a complex of large α- and β-subunits, and a much smaller γ-subunit [39]. G-proteins act as signal transducers in receptor-mediated stimulus–response coupling. The role of G-proteins in stimulus–response coupling in general [39–42] and in platelet activation by various agonists specifically [43, 44] has been reviewed previously. Briefly, in hormone-induced cellular responses, reversible binding of a hormone to its receptor on the cell surface results in orienting the receptor in a conformational state that is favorable for its interaction with a G-protein [39]. GTP displaces GDP bound to an α-subunit of a G-protein, and this results in lowering the affinity of a hormone for its receptor as well as the affinity of an α-subunit for a βγ-subunit complex. An α-subunit bound to GTP dissociates from the βγ-subunit and is ready to modulate the activity of an effector. The change in effector activity lasts until GTP is hydrolyzed to GDP. An α-subunit bound to GDP then reassociates with a βγ-subunit complex of the G-protein. The same is true for the G-protein-mediated stimulus–response coupling by platelet agonists like thrombin, platelet activation factor, and TXA_2. However, in several cellular systems investigated during the last decade, it is the βγ-subunit complex that mediates stimulus–response coupling [41, 45–47]. Using antisense probes [48], it has been possible to demonstrate that in some other cellular systems it is the β- [49] or the γ-subunit [50] that determines the specificity of a G-protein-mediated stimulus–response coupling. Furthermore, some G-proteins need not reside in association with cell membranes, but they or their subunits can initiate a cellular response by translocating from cytosol to membrane. Indeed, previous studies have shown the presence of G-proteins in association with cytoplasmic structures [51, 52]. G-proteins stimulating cAMP formation by the activated adenylate cyclase are referred to as G_s, and are regulated by CTX. G-proteins mediating inhibition of cAMP formation by inhibiting adenylyl cyclase are termed G_i, and are regulated by PTX (PTX-sensitive). $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ are three platelet proteins that show considerable amino acid sequence homology among themselves [43]. G_p proteins correspond to those G-proteins that mediate phospholipid hydrolysis by the stimulated PLC. G_p proteins may or may not be ADP-ribosylated by PTX; it varies with the cell type. G_q, another closely related platelet membrane protein that also mediates phospholipid hydrolysis by the stimulated PLC, is not regulated by ADP ribosylation catalyzed by PTX (PTX-insensitive).

2-MeS-ADP, ADP, and GDP were shown to stimulate the binding of [^{32}S]-GTP-γ-S to platelet membranes, a process that is antagonized by ATP-α-S [53]. ATP-α-S, a potent inhibitor of ADP-induced platelet activation [3, 5], did not antagonize the binding of [^{32}S]-GTP-γ-S to platelets stimulated by agonists other than ADP. Photoaffinity labeling of platelets with 4-azidoanilido-[α-^{32}P]-GTP followed by immunoprecipitation with a subtype-specific antibody [54] revealed that the G-protein activated in platelets stimulated by ADP is $G_{i\alpha2}$ [55]. Ticlopidine [56] has been shown to inhibit ADP-induced platelet aggregation and stimulated adenylyl cyclase activity [23]. Clopidogrel [55], a thienopyridine similar to ticlopidine [57], was shown to block the binding of 2-MeS-[^{32}P]ADP to platelets [58] and to inhibit ADP-induced stimulation of a G-protein [53]. 2-MeS-ADP induces platelet aggregation as well as inhibition of the stimulated adenylyl cyclase activity [9]. Together these results have been taken to imply that $G_{i\alpha2}$ represents the G-protein that mediates ADP-induced inhibition of adenylyl cyclase activity [55].

Aggregin (a 100-kDa surface-membrane protein) [5, 6], a 61 kDa protein [59], a 43 kDa protein [3], and a 28 kDa protein [60] have been proposed as candidates for the platelet ADP receptor. In light of the fact that the true molecular identity of the ADP receptor (P2T) mediating ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity remains controversial [3–6], and that the receptor has not been purified, it is desirable to examine possibilities and make suggestions that might shed light on the nature of the molecular mechanism(s) underlying the two seemingly unrelated ADP-induced platelet responses. Although Ohlmann *et al.* [55] have identified the activation of $G_{i\alpha2}$ protein in human platelet membranes exposed to ADP, there is no direct experimental evidence that shows that $G_{i\alpha2}$ participates in ADP-induced inhibition of the stimulated adenylyl cyclase activity in human platelets. G_{i2} is a major platelet protein [43, 44], and in platelets stimulated by epinephrine, a greater proportion of the $G_{i\alpha2}$ was found in Triton X-100 insoluble fraction than in Triton X-100 soluble fraction [61]. PTX was shown to catalyze ADP-ribosylation of $G_{i\alpha2}$ in intact platelets [61]. A critical observation was that epinephrine (a weak platelet agonist like ADP), but not thrombin (a strong platelet agonist) stimulated the translocation of $G_{i\alpha2}$ from Triton-soluble to Triton-insoluble fraction in human platelets [61]. G_{i2} has been shown to be activated, in part, by a post-receptor signal that can be mimicked by PKC in growth factor-stimulated fibroblasts, and has been shown to migrate in undissociated form to the nucleus in fibroblasts stimulated by either thrombin or epidermal growth factor [62]. G_{i2} has been shown to mediate adrenergic inhibition of adenylyl cyclase in platelet membranes [63], and also has been shown to play a functional role in transmembrane signal transduction in human platelets stimulated by TXA_2 [64]. Collectively, these investigations suggest that (a) G_{i2} can mediate agonist-induced cellular responses in cytosol as well as in

platelet membranes, and (b) G_{i2} or its subunits can be translocated from cytosol to membranes or to the nucleus in response to cell activation by various stimuli.

Epinephrine acting through α_2-adrenergic receptors has been shown to activate PLA_2 in platelets [65]. A link between G-proteins and PLA_2 activation has been provided by the GTP-induced arachidonate release in saponin-permeabilized platelets [66]. Experiments with norepinephrine-stimulated thyroid cells have provided evidence for a direct G-protein coupled regulation of PLA_2 independent of PLC [67]. In a nicely executed set of experiments, Gupta *et al.* [68] demonstrated that $G_{i\alpha2}$ inhibited PLA_2 activity in Chinese hamster ovary (CHO) cells transfected with this protein and stimulated by thrombin and ATP. Furthermore, $G_{i\alpha2}$ involved in the receptor-mediated regulation of PLA_2 activity in CHO cells was shown to be PTX-sensitive [68]. These findings suggest that the G-proteins, including $G_{i\alpha2}$, can modulate PLA_2 activity in platelets and other cell types.

In the absence of ADP, prostaglandins stimulate binding of an α-subunit of a G_s protein to adenylyl cyclase leading to its activation and, hence, an increase in intracellular cAMP levels (Fig. 2; top panel). The α-subunit of a G_s protein has been shown previously to be involved in the adrenergic stimulation of adenylyl cyclase in platelets [69].

ADP, like epinephrine, is a weak platelet agonist; ADP may also translocate $G_{i\alpha2}$ to the membrane, where its binding to adenylyl cyclase results in the inhibition of this effector. Alternatively, binding of ADP to its receptor may activate G_{i2} directly in membranes. The $\beta\gamma$-subunit complex of G_{i2} may bind to PLA_2, leading to a conformational change in this effector (Fig. 2; middle panel). However, the conformational change induced in PLA_2 by binding of the $\beta\gamma$-subunit complex may not be sufficient to render the enzyme catalytically functional, but is essential for the α-subunit to remain dissociated from the $\beta\gamma$-subunit complex of G_{i2}. By using an antibody to PLA_2, it was demonstrated that the $\beta\gamma$-subunit complex of a G-protein activates the cardiac muscarinic K^+-channel by directly activating PLA_2 [72]. The $\beta\gamma$-subunit complex of a G_i protein has been shown to stimulate PLA_2 activity in G_i-depleted dark-adapted retinal rod outer segment [73]. The $\beta\gamma$-subunit complex of G-proteins has been shown to activate voltage-sensitive muscarinic K^+ channels [74], calmodulin [75], $p21^{ras}$ [76], and MAPK [77]. ADP-induced activation of the Na^+/H^+ exchanger (which leads to alkalinization of the platelet interior) and the influx of Ca^{2+} provide additional mechanisms that make it possible for PLA_2 to catalyze hydrolysis of PC to AA. There are several forms of PLA_2 in different cell types, and they differ in their requirements for catalytic function [36, 37]. It is known that the platelet $cPLA_2$ has strict requirements for Ca^{2+} and alkaline pH [36, 37]. ADP-induced activation of $cPLA_2$ is central to ADP-induced platelet aggregation [29]. Since G-proteins are membrane associated or can translocate from cytosol to the cell membrane in response to an external stimulus, it is expected that they would more readily interact with a membrane-bound effector. Although $cPLA_2$ is the predominant form of PLA_2 that catalyzes the hydrolytic cleavage of PC to AA in platelets [29], $cPLA_2$ has been shown to translocate to platelet membrane in response to an increase in the $[Ca^{2+}]_i$ [37, 78]. Furthermore, PLA_2 activity has been shown to exist in isolated platelet membranes [79], and isolated platelet membranes have been used as a source of PLA_2 activity, the substrate, and the G-protein in an investigation of the effect of GTP-binding protein that regulates PLA_2 in human platelets [80]. An important feature of the hypothetical model described to explain the two ADP-induced platelet responses is that a single ADP-receptor in platelets activates a single G-protein (G_{i2}): the α- and $\beta\gamma$-subunits of G_{i2} mediate ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity, respectively. The activation of two isozymes, PLCβ1 and PLCβ2, by the α- and $\beta\gamma$-subunit complex, respectively, derived from the same G-protein has been described previously [81]. The hypothesis that each receptor system couples to a unique G-protein, which then interacts with multiple effector systems, was proposed earlier by Cotecchia *et al.* [71] and reviewed in detail in Refs. 40 and 41. Regulation of $cPLA_2$ by MAPKs also has been reported in several cell types, but depends on the nature of the cell type and the stimulus [29]. There is no experimental evidence that shows or suggests that in platelets $cPLA_2$ is regulated by MAPKs [29]. The possibility that the ADP-receptor couples to two different G-proteins [40, 41], and that the $\beta\gamma$-subunit complex stimulating PLA_2 is derived from a G-protein other than G_{i2}, should not be ruled out.

The disparity between the potencies of 2-substituted ADP derivatives for the induction of platelet aggregation and for inhibition of the stimulated adenylyl cyclase activity may be explained on the basis of a hypothetical model that suggests that $G_{i\alpha2}$ and $G_{i\beta\gamma2}$ may have differential potency in inhibiting the stimulated adenylyl cyclase and activating PLA_2, respectively (Fig. 2; middle panel). Some reports have indicated that the activity of the $\beta\gamma$-subunit complex of G-proteins is more pronounced than that of the α-subunit [82, 83], and this may be attributed to the ability of an α-subunit to recombine with the $\beta\gamma$-subunit complex [84]. Thus, the potency of an α-subunit and a $\beta\gamma$-subunit complex to modulate the activity of effectors could be very different. It is also possible that during the recombination process between the $\beta\gamma$- and α-subunits of G_{i2}, the α-subunit may, in some way, modulate the activity of PLA_2 (Fig. 2; bottom panel) [63, 68], and this may shift the balance of ADP-induced inhibition of the stimulated adenylyl cyclase in its favor compared with ADP-induced platelet aggregation. Furthermore, in the absence of an α-subunit, $\beta\gamma$-subunit complex may be degraded at a faster rate, and this may tend to lower its concentration relative to an α-subunit.

Recently, Offermanns *et al.* [85] demonstrated that ADP failed to elicit aggregation, increase in the $[Ca^{2+}]_i$, and formation of IP_3 in platelets obtained from mice deficient in $G_{\alpha q}$. Shape change induced by most platelet agonists was unaffected in platelets obtained from $G_{\alpha q}$ (−/−) mice [85]. These findings suggest that the ADP-induced platelet

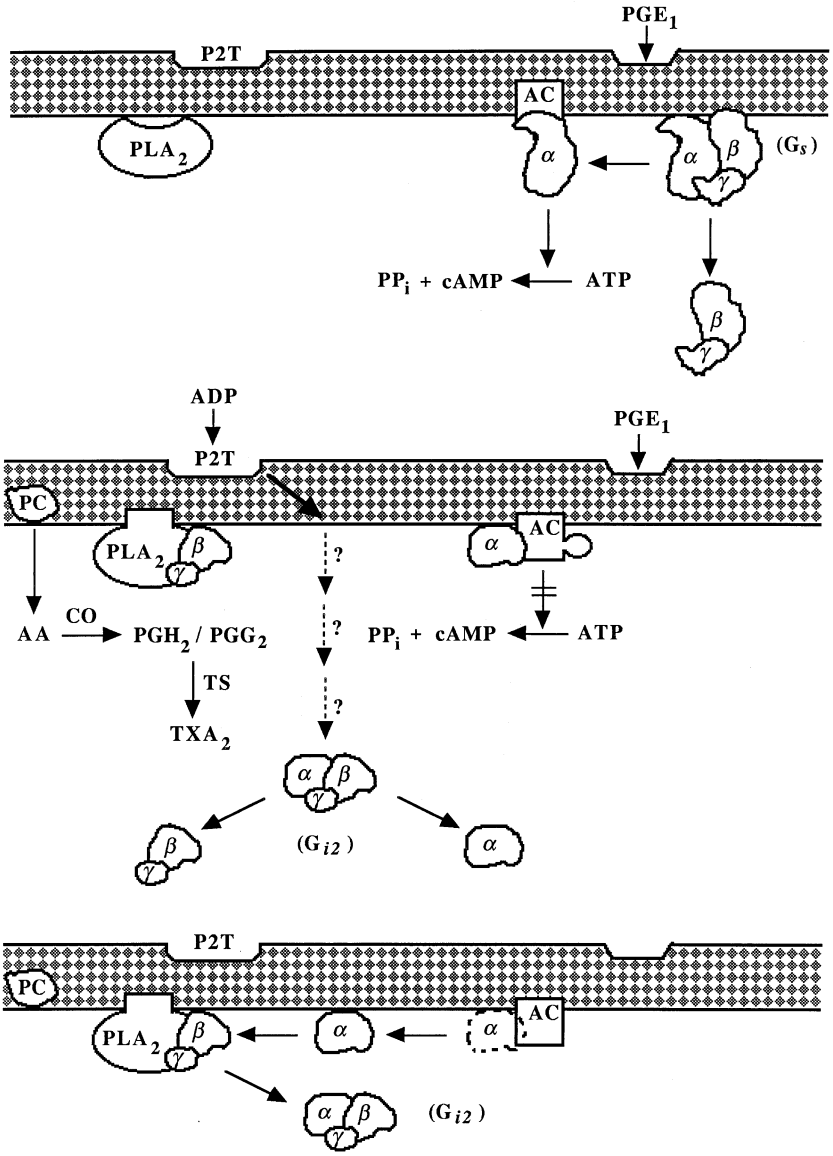

FIG. 2. Possible mechanism of signal transduction in ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity. (Top panel): In an analogy to adrenergic stimulation of adenylyl (adenylate) cyclase (AC) [69], binding of PGE_1 to its receptor leads to binding of an α-subunit of a G_s protein to AC, resulting in its activation and formation of cAMP. Caution should be exercised in drawing such analogies. For example, PGE_1 has been shown to increase the potency and the maximum extent of stimulation of AC activity by the βγ-subunit complex isolated from illuminated bovine rod outer segment in the presence of GTPγS, while under identical experimental conditions the βγ-subunit complex in the presence of GTPγS inhibits forskolin-stimulated AC activity [70]. Furthermore, Cotecchia *et al.* [71] have described and emphasized the need to appreciate multiple effector second messenger pathways associated with adrenergic receptor subtypes in eukaryotic cells. (Middle panel): ADP-induced influx of $[Ca^{2+}]_i$ translocates PLA_2 from cytosol to the platelet membrane. ADP-induced stimulation of PLA_2 may involve translocation of the βγ-subunit complex of G_{i2} to the membrane where it binds to PLA_2. Then the primed PLA_2 is activated by the alkaline pH gradient generated by activation of the Na^+/H^+ exchanger and ROC-mediated increase in $[Ca^{2+}]_i$ (see Fig. 1 and discussion in Signal Transduction Mechanisms). Activated PLA_2 catalyzes the formation of AA from PC. This reaction is followed by the formation of TXA_2 by the sequential action of CO and TS. In a feedback mechanism, TXA_2 mediates activation of PLC by binding to the surface receptor. The hydrolysis products of the PLC reaction cause secretion from the dense granules and release of Ca^{2+} from the DTS (Fig. 1). ADP-induced influx of Ca^{2+} and an increase in the $[Ca^{2+}]_i$, as well as exposure of the fibrinogen binding sites, lead to platelet aggregation, as described in Fig. 1. ADP-induced inhibition of adenylyl cyclase stimulated by prostaglandins may involve binding of the α-subunit of G_{i2} to AC, thus, blocking the stimulatory effect of prostaglandins. It is also possible that the βγ-subunit complex and α-subunit are derived from different G-proteins, which are well known for their promiscuous character. In the absence of the true molecular identity of an ADP-receptor inducing platelet aggregation, the events leading to the receptor-mediated activation of G_{i2} are not known, but the mechanisms of action of all other components shown in this figure have precedents. (Bottom panel): Dissociation of the α-subunit from AC and its approach toward the βγ-subunit complex in order to reconstitute the heterotrimeric G_{i2} may result in the inhibition of PLA_2 ([68]; see also discussion in Signal Transduction Mechanisms). This could explain the differential potencies of ADP in inducing platelet aggregation and inhibition of stimulated adenylate cyclase activity. Differences in the potency of ADP to aggregate platelets and to inhibit stimulated adenylyl cyclase activity may also be due to (a) the differences in the potency of the α-subunit and βγ-subunit complex of G_{i2} to interact with effectors, and (b) the faster rate of degradation of a βγ-subunit complex in the absence of an α-subunit.

aggregation, the increase in the $[Ca^{2+}]_i$, and the formation of the PLC hydrolysis products are coupled to the activation of $G_{\alpha q}$ [85]. These results and those described earlier suggest that ADP-induced platelet aggregation and ADP-induced inhibition of the stimulated adenylyl cyclase activity may be mediated by $G_{\alpha q}$ [85] and $G_{i\alpha 2}$ [55], respectively. This is in accord with the hypothesis that (a) there are two different signaling mechanisms for ADP-induced platelet aggregation and ADP-induced inhibition of the stimulated adenylyl cyclase activity [86], and (b) the two different ADP-induced platelet responses may be mediated by two different G-proteins that may be faithfully coupled to two different effector systems [40]. However, ADP-induced activation of the Na^+/H^+ exchanger and formation of AA (which are essential for ADP-induced platelet aggregation), and inhibition of the stimulated adenylyl cyclase activity were not investigated in platelets obtained from $G_{\alpha q}$ (−/−) mice [85]. An answer to these questions would be helpful to confirm or refute whether or not ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity are mediated by the same receptor and by direct activation of the two different G-proteins. Furthermore, identification of $G_{\alpha q}$, by photoaffinity labeling and immunoprecipitation with a specific subclass antibody [54], in ADP-stimulated platelets would be even more desirable. Nevertheless, it is possible that ADP-induced primary activation of G_{i2} leads to secondary activation of $G_{\alpha q}$ by its occupancy by PGG_2/PGH_2-TXA_2 by a feedback mechanism described earlier. G_q has been shown to be associated with the receptor-mediated activation of PLC in platelets stimulated by various agonists [85]. The results obtained by Offermanns *et al.* [85] fit into the model of ADP-induced platelet aggregation based on the experimental findings that suggest that the activation of PLA_2 precedes the activation of PLC in ADP-stimulated platelets [29] and that PLC does not play a primary role in ADP-induced platelet aggregation and secretion [30, 35, 87, 88]. More recently, Jin *et al.* [15] demonstrated that ADP-induced platelet shape change, but not platelet aggregation and inhibition of the stimulated adenylyl cyclase activity, is mediated by the platelet $P2Y_1$ receptor, which also mediates an ADP-induced increase in $[Ca^{2+}]_i$ and IP_3 formation. These results are in marked contrast to those obtained by Offermanns *et al.* [85], which show that the receptor-mediated activation of PLC contributes to ADP-induced platelet aggregation but not to platelet shape change.

CONCLUSIONS

Available experimental evidence favors the view that ADP-induced activation of $cPLA_2$ precedes PLC activation. ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity are mediated by a single ADP receptor in platelets but may proceed by two signaling mechanisms. Whether or not the two signaling mechanisms are mediated directly by the receptor activation of two different G-proteins remains to be ascertained. The two G-proteins $G_{i\alpha 2}$ and $G_{\alpha q}$ have been shown to be involved in ADP-induced platelet activation, but their precise role in ADP-induced platelet aggregation and inhibition of the adenylyl cyclase activity is unclear. Successful cloning of the $P2X_1$ and $P2Y_1$ purinergic receptors in platelets has contributed to a better understanding of the nature of molecular mechanisms involved in ADP-induced influx of Ca^{2+} and platelet shape change. However, additional experimental work is needed to define more clearly the physiological importance of $P2X_1$ and $P2Y_1$ receptors in platelets. Purification of the platelet receptor(s) mediating ADP-induced platelet aggregation and/or inhibition of the stimulated adenylyl cyclase activity would not only provide the molecular identity of the receptor but also will help advance our knowledge of the molecular mechanisms underlying the two seemingly unrelated ADP-induced platelet responses.

NOTE ADDED AT PROOF

After this manuscript was submitted for publication, two reports describing the possible role of G_i and G_q proteins in ADP-induced platelet responses have appeared in the literature. One of the reports (Geiger *et al.*) shows that ADP-induced platelet aggregation and inhibition of the stimulated adenylyl cyclase activity are mediated by the same ADP receptor that may be coupled to a G_i protein.

Jin J and Kunapuli SP, Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* **95:** 8070–8074, 1998.

Geiger J, Hönig-Liedi P, Schanzenbächer P and Walter U, Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* **251:** 235–245, 1998.

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