The human platelet ADP receptor activates G_{i2} proteins

Philippe OHLMANN,* Karl-Ludwig LAUGWITZ,† Bernd NÜRNBERG,† Karsten SPICHER,† Günter SCHULTZ,† Jean-Pierre CAZENAVE* and Christian GACHET*‡

*INSERM U.311, Biologie et Pharmacologie des Interactions du Sang avec les Vaisseaux et les Biomatériaux, Etablissement de Transfusion Sanguine de Strasbourg, 10, rue Spielmann, B.P. no. 36, F-67065 Strasbourg Cédex, France, and †Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69-73, D-14195 Berlin, Germany.

We have previously shown that platelet ADP receptors are coupled to G-proteins by measuring the binding of [^{35}S]guanosine-5′-[γ-thio]triphosphate ([^{35}S]GTPγS) to human platelet membranes stimulated with ADP. In order to identify the activated G-proteins, we used an approach which combines photolabelling of receptor-activated G-proteins with 4-azidoanilido-[α-^{32}P]GTP and immunoprecipitation of the G-protein α-subunits with subtype-specific antibodies. Stimulation of human platelet membranes with ADP resulted in an increase in 4-azidoanilido-[α-^{32}P]GTP incorporation into the immunoprecipitates of $G\alpha_i$ but not of $G\alpha_q$ proteins, whereas stimulation with the thromboxane analogue U46619 resulted in an increase in 4-azidoanilido-[α-^{32}P]GTP incorporation into the immunoprecipitates of $G\alpha_q$ but not of $G\alpha_i$ proteins, and thrombin activated both G-proteins. This effect of ADP was concentration dependent and inhibited by the class P_2 purinoceptor (P_{2T}) antagonist ATP. Using specific antisera against subtypes of G_i proteins, we found that ADP stimulated labelling of the $G\alpha_{i2}$ immunoprecipitate, but not of the $G\alpha_{i3}$ precipitate. $G\alpha_{i1}$ was not detectable by immunoblotting of platelet membrane proteins. These data suggest that ADP inhibits cAMP formation by activation of $G\alpha_{i2}$ proteins and add evidence in support of the hypothesis that human platelet ADP receptors do not activate PLC through G_q activation.

INTRODUCTION

Platelet aggregation by ADP plays a key role in the development and extension of arterial thrombosis. Stored at very high concentrations in platelet dense granules, ADP is released when platelets are stimulated by other aggregating agents, such as thrombin or collagen, and so contributes to and reinforces platelet aggregation. In addition, ADP at low concentrations potentiates or amplifies the effects of all other stimuli, even weak agonists such as adrenaline or serotonin [1–3]. Addition of ADP to washed human platelet suspensions results in shape change, exposure of the fibrinogen-binding site on the $\alpha_{IIb}\cdot\beta_3$ integrin and reversible aggregation in the presence of fibrinogen and physiological concentrations of Ca^{2+}. At the intracellular level, platelet activation following ADP binding to its receptor leads to a transient rise in free cytoplasmic Ca^{2+}, resulting from both Ca^{2+} influx and mobilization of internal stores. ADP also inhibits stimulated adenylyl cyclase [1], but this is not causally involved in platelet aggregation.

On the basis of agonist selectivity and signalling properties [4,5], the platelet receptor for ADP has been classified as a P_{2T} receptor of the P_2 purinoceptor family. Its main characteristic is that ADP is its natural agonist while ATP is a competitive antagonist. However the biochemical structure of this receptor remains unknown. Most of the platelet membrane receptors such as platelet-activating factor, thromboxane A_2 (TXA_2), thrombin, adrenaline and 5′-hydroxytryptamine belong to the seven transmembrane domain G-protein-coupled receptor family, and many have been cloned and sequenced [6]. Several ADP-binding proteins have been proposed as putative ADP receptors, for instance the 100 kDa protein called aggregin [7] or more recently a 40 kDa protein which incorporates a photoaffinity analogue of ADP [8]. Nevertheless, the P_{2T} receptor has not yet been precisely identified by biochemical or molecular biological techniques. Three receptor subtypes of the P_2 family have been cloned, sequenced and expressed: G-protein-coupled receptors in the form of mouse and human P_{2U} receptors and a chicken P_{2Y} receptor, and recently, channel-linked P_{2X} receptors from rat vas deferens and pheochrocytoma PC12 cells [9]. Whether the P_{2T} receptor is a G-protein-coupled or a channel-linked receptor is not known. It has been proposed that there could exist more than one type of ADP receptor in blood platelets, since the ADP-binding protein seems to possess characteristics of both known types [3]. In contrast to the other P_2 purinoceptors, which are broadly expressed in many tissues, the P_{2T} receptor(s) appears to be specific for platelets and has never been found in other cells, apart from certain studies where functional properties suggested that this receptor could be expressed by K562 [10] and Dami leukaemia cells [11], or where functional and binding characteristics indicated its presence in the megakaryoblastic cell line Meg-01 [12]. Obviously, a better knowledge of the ADP platelet-activation pathway is of major importance for the identification of this receptor and the understanding of the physiology of primary haemostasis.

We have previously shown that ADP stimulates the binding of [^{35}S]guanosine-5′-[α-thio]triphosphate ([^{35}S]GTPγS) to platelet membranes in humans and in rats [13,14], suggesting that the ADP receptor is coupled to G-proteins, the nature of which remain to be identified. The aim of the present study was the identification of these activated G-proteins. For this purpose, we used an approach which combines photolabelling of receptor-activated G-proteins with 4-azidoanilido-[α-^{32}P]GTP and immunoprecipitation of the G-protein α-subunits with subtype-specific antibodies [15].

MATERIALS AND METHODS

Chemicals

[α-^{32}P]GTP was obtained from Du Pont-New England Nuclear

Abbreviations used: GTP[γ]S, guanosine-5′-*O*-(3-thiotriphosphate); $G\alpha_i$, α-subunit of the G_i class of G-proteins; P_{2T}, platelet purinoceptor of class P_2; cAMP, cyclic AMP; PLC, phospholipase C; IP_3, D-*myo*-inositol 1,4,5-triphosphate; TXA_2, thromboxane A_2; ECL, enhanced chemiluminescence; KLH, keyhole limpet haemocyanin; *N*-DEC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; NP 40, Nonidet P.40.

‡ To whom correspondence should be addressed.

(Boston, MA, U.S.A.) and 4-azidoaniline HCl and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (*N*-DEC) from Fluka (Buchs, Switzerland). All nucleotides were from Boehringer Mannheim (Mannheim, Germany). U46619 (TXA_2 receptor agonist) was from Sigma (St. Louis, MO, U.S.A.), while the thrombin receptor-activating peptide (TRAP-SF7) was synthesized by Neosystem (Strasbourg, France).

Antibodies

Rabbit polyclonal antibodies were raised against peptides corresponding to specific regions of G-protein α-subunits and coupled to keyhole limpet haemocyanin (KLH) with an additional cysteine at the N-terminus as previously described [16]. The antisera were designated as follows: AS 8 (anti-α common); AS 190 (anti-α_{i1}); AS 269 (anti-α_{i2}); AS 105 (anti-α_{i3}); AS 266 (anti-α_{i3}); AS 266 (anti-α_i common); AS 227 (anti-α_z); AS 369 (anti-$\alpha_{9/11}$); AS 232 (anti-α_{12}); and AS 343 (anti-α_{13}). All were of known characteristics [15,17,18] and were employed as crude sera for immunoprecipitation and immunoblotting.

Platelet membrane preparation

Human platelets were isolated and washed as previously described [19], and resuspended in Tyrode's buffer, without Ca^{2+}, containing 2 mM EDTA, apyrase (2 μg/ml) and protease inhibitors [leupeptin (10 μM), aprotinin (1 μM), PMSF (20 μM)]. The platelet suspension was frozen and thawed before being homogenized in a glass–Teflon homogenizer on ice. Membranes were pelleted by centrifugation (30000 *g* for 15 min) at +4 °C and the final pellet was resuspended in 50 mM Tris/HCl (pH 7.5) containing 2 mM EDTA and stored at −80 °C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL, USA).

Synthesis and purification of [α-^{32}P]GTP-azidoanilide

[α-^{32}P]GTP-azidoanilide was synthesized using a modification of a standard method [20]. Briefly, [α-^{32}P]GTP (5 mCi/111 TBq/mmol) was evaporated to dryness under vacuum and dissolved in 120 μl of 100 mM Mes buffer (pH 5.5) containing 1.2 mg of *N*-DEC. After 15 min incubation under rotation at room temperature in the dark, 1.3 mg of azidoaniline was added and the reaction was allowed to proceed for 8 h. Under these conditions, the synthesis yield was 70 %. Purification was performed by HPLC [20], [α-^{32}P]GTP-azidoanilide was adjusted to 74 kBq/μl and aliquots were stored at −80 °C.

Photolabelling of membrane proteins

Platelet membranes (400 μg of protein per sample) were suspended in ice-cold incubation buffer containing 50 mM Hepes (pH 7.4), 0.1 mM EDTA, 10 mM $MgCl_2$, 30 mM NaCl, 1 mM benzamidine and 0.2 % (w/v) BSA. In some cases, GDP (1–10 μM) was added to the incubation buffer [15]. After a 10 min preincubation in the absence or presence of agonists, the samples were incubated for a further 10–20 min, depending on the G_α-subunit, with [α-^{32}P]GTP-azidoanilide (5 μCi/tube). The reaction was stopped by cooling on ice and all subsequent procedures were performed at +4 °C. Samples were centrifuged (12000 *g* for 5 min), the membrane pellets were resuspended in the same buffer supplemented with 2 mM glutathione and these samples were then irradiated for 30 s at +4 °C with a UV lamp (254 nm, 100 W) at a distance of 3 cm. After irradiation, the samples were centrifuged again (12000 *g* for 5 min) and solubilized in SDS buffer as described below.

Immunoprecipitation

Immunoprecipitation was performed by standard techniques [15,17]. Pellets of photolabelled membranes were solubilized in 40 μl of 2 % (w/v) SDS at room temperature. Following addition of 120 μl of precipitating buffer containing 1 % (v/v) Nonidet NP.40 (NP40) or 1 % (w/v) Chaps, 1 % (w/v) deoxycholate, 0.5 % (w/v) SDS, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 20 μM PMSF, 1 μM aprotinin and 10 mM Tris/HCl (pH 7.4), samples were incubated with 15–20 μl of antiserum for at least 90 min at +4 °C under constant rotation. Washed Protein A-Sepharose beads [60 μl of a 10 % (w/v) suspension in precipitating buffer] were added to the supernatants and incubated overnight at +4 °C, still under rotation. The Sepharose beads were then pelleted (12000 *g* for 5 min) and washed, first with 2 × 1 ml of high-salt buffer containing 1 % (v/v) NP40, 0.5 % (w/v) SDS, 600 mM NaCl and 50 mM Tris/HCl (pH 7.4), and then with 1 ml of low-salt buffer containing 300 mM NaCl, 10 mM EDTA and 100 mM Tris/HCl (pH 7.4). Finally, the samples were solubilized in Laemmli buffer and subjected to SDS/PAGE [21]. Electrophoresis was performed on 10 % (w/v) acrylamide gels and run until the 30 kDa standard protein reached the bottom of the gel. The photolabelled proteins were visualized by autoradiography of the dried gels, densitometric analyses being carried out by means of an image analyser (Biocom, Paris, France).

Immunoblotting

Platelet membrane proteins were separated on 9 % polyacrylamide gels containing 6 M urea for the $G\alpha_i$ subunits and on 13 % polyacrylamide gels without urea for members of the $G\alpha_{q/11}$ and $G\alpha_{12}$ families. Immunodetection of $G\alpha$ subunits was performed by the enhanced chemiluminescence (ECL) procedure (Amersham, Little Chalfont, U.K.) as described in [17].

Reproducibility

The experiments shown are representative of at least three independent assays using different membrane preparations.

RESULTS

Immunoblotting

Immunoblotting of platelet membranes with antisera raised against peptides corresponding to specific regions of G-protein α-subunits (see Table 1) confirmed that platelets express G_i-,

Table 1 Peptide antisera used for the detection of G-protein α-subunits

(C) indicates that an N-terminal cysteine was added to the original peptide sequence in order to facilitate the coupling to KLH

Antiserum	Peptide sequence	G-protein α-subunit recognized
AS 8	(C) GAGESGKSTIVKQM	$\alpha_{i1}/\alpha_{i2}/\alpha_{i3}$
AS 266	(C) NLREDGEKAAREV	$\alpha_{i1}/\alpha_{i2}/\alpha_{i3}$
AS 190	(C) LDRIAQPNYI	α_{i1}
AS 269	(C) TGANKYDEAAS	α_{i2}
AS 105	(C) LDRISQSNYI	α_{i3}
AS 369	(C) LQLNLKEYNLV	α_{q11}
AS 232	(C) QENLKDIMLQ	α_{12}
AS 343	(C) LHDNLKQLMLQ	α_{13}
AS 227	(C) HLRSESQRQRREI	α_z

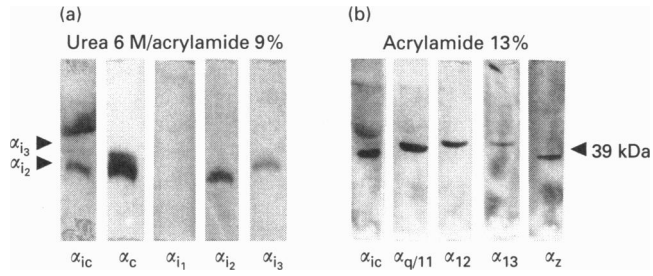

Figure 1 Immunoblot analysis G-protein α-subunits in platelet membranes

Platelet membrane proteins (200 μg) were separated on SDS/9% acrylamide gels containing 6 M urea for the $G\alpha_i$ subtype (**a**) and on SDS/13% acrylamide gels for the other subtypes (**b**) and blotted onto nitrocellulose filters. Filter strips were incubated with the following antisera (specificity, dilution): AS 266 (anti-α_{ic}, 1:25); AS 8 (anti-α_c, 1:50); AS 190 (anti)α_{i1}, 1:25); AS 269 (anti-α_{i2}, 1:50); AS 105 (anti-α_{i3}, 1:50); AS 369 (anti-$\alpha_{q/11}$, 1:150); AS 232 (anti-α_{12}, 1:50); AS 343 (anti-α_{13}, 1:50); and AS 227 (anti-α_z, 1:100). Bound antibodies were visualized by the ECO technique as described in the Materials and methods section.

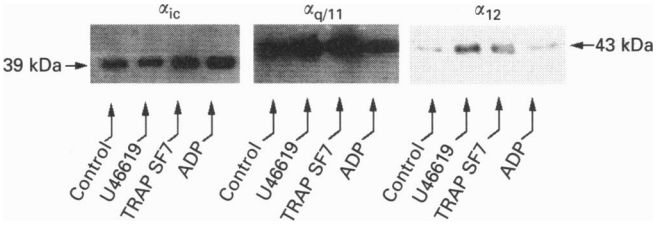

Figure 2 Photolabelling and immunoprecipitation of $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$

Platelet membranes (400 μg) were photolabelled with 5 mCi of [α-^{32}P]GTP-azidoanilide, in the absence of a stimulator (control) or in the presence of 5 μM U46619, 10 μM TRAP-SF7 or 10 μM ADP. Proteins were immunoprecipitated with antisera AS 266 (anti-α_{ic}, 20 μl), AS 369 (anti-$\alpha_{q/11}$, 15 μl) and AS 232 (anti-α_{12}, 15 μl), separated on SDS/10% acrylamide gels and revealed by autoradiography.

G_q- and G_{12}-proteins [17], with the exception of $G\alpha_{i1}$ which was not recognized by our antibody (Figure 1). This absence of $G\alpha_{i1}$ was supported by the observation that purification of pertussis toxin-sensitive G-proteins from human platelets yielded only $G\alpha_{i2}$ and $G\alpha_{i3}$ (results not shown). Using a 6 M urea/9% acrylamide separating gel, we were able to distinguish between $G\alpha_{i2}$ and $G\alpha_{i3}$ with an anti-$G\alpha_i$ common antibody (AS 266) and this result was confirmed with the subtype-specific antibodies raised against $G\alpha_{i2}$ (AS 269) and $G\alpha_{i3}$ (AS 105). Once again, $G\alpha_{i1}$ was not detectable. In addition, the antibody (AS 369) specific for the G-protein α-subunits α_q and α_{11} showed the presence of a 42 kDa protein corresponding to α_q, as reported earlier for α_{12}, α_{13} and α_z [22].

Photolabelling of membrane proteins and immunoprecipitation

In order to identify the G-protein(s) activated by ADP, platelet membranes were photolabelled with [α-^{32}P]GTP-azidoanilide in the absence and presence of 10 μM ADP and immunoprecipitation was performed using different subtype-specific antibodies. As controls, platelet membranes were also stimulated with the TXA_2 receptor agonist U46619 (5 μM) and the thrombin receptor agonist TRAP-SF7 (10 μM). As expected from previous studies [17], U46619 stimulated the photolabelling of $G\alpha_q$, $G\alpha_{i2}$ (Figure 2) and $G\alpha_{13}$ proteins (results not shown) without any effect on $G\alpha_i$, where TRAP-SF7 stimulated $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$

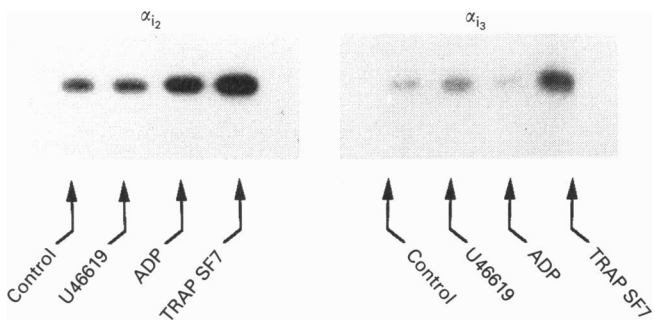

Figure 3 Photolabelling of $G\alpha_i$ subtypes under ADP and thrombin receptor stimulation

Membranes (400 μg) were incubated in a medium containing 10 μM GDP and photolabelled as described in Figure 2 in the absence of a stimulator (control) or in the presence of 10 μM ADP, 10 μM TRAP-SF7 or 5 μM U46619 (negative control). The samples were then solubilized in a medium containing 1% (w/v) Chaps instead of NP40. Proteins were immunoprecipitated with antisera As 269 (anti-α_{i2}, 20 μl) and AS 105 (anti-α_{i3}, 20 μl), separated on SDS/10% acrylamide gels and revealed by autoradiography of the dried gels.

(Figure 2) or $G\alpha_{13}$ photolabelling (results not shown). Platelet membrane stimulation with ADP resulted in an increase in [α-^{32}P]GTP-azidoanilide incorporation in the immunoprecipitates of $G\alpha_i$ but not of $G\alpha_q$ proteins (Figure 2). Using $G\alpha_{i2}$ and $G\alpha_{i3}$ subtype-specific antisera, we observed that ADP stimulated the photolabelling of $G\alpha_{i2}$but not of $G\alpha_{i3}$, while TRAP-SF7 stimulated the labelling of both $G\alpha_{i2}$ and $G\alpha_{i3}$, (Figure 3). In $G\alpha_i$ subtype-specific immunoprecipitation experiments, the platelet membranes were solubilized with Chaps instead of NP40, which improved the immunoprecipitation of $G\alpha_{i2}$ and $G\alpha_{i3}$ with our antibodies. The stimulatory effect of ADP was concentration dependent from 0.1–10 μM and was inhibited by simultaneous incubation with the P_{2T} receptor antagonist ATP (10 μM). This is illustrated in Figure 4, where Figure 4(a) shows an autoradiogram of a dried gel and Figure 4(b) the corresponding densitogram obtained by image analysis.

DISCUSSION

We have previously shown that ADP stimulates the binding of [^{35}S]GTP[γS] to platelet membranes from humans and rats [13,14], suggesting that the ADP receptor is coupled to G-proteins, the nature of which remain unknown. In order to identify these proteins, we combined photolabelling of receptor-activated G-proteins with immunoprecipitation of the labelled G-protein α-subunits. This method allows exact identification of the receptor-activated G-proteins, provided the necessary subtype-specific antisera are available [15]. All the antibodies used recognized the corresponding G-protein α-subunit in platelets except the anti-$G\alpha_{i1}$ AS 190 antibody. This antibody recognizes $G\alpha_{i1}$ in the human neuroblastoma cell line SH-SY5Y [18] and purified $G\alpha_{i1}$ from bovine brain [23]. The reason why it did not detect any platelet protein is unclear, since small amounts of $G\alpha_{i1}$ have been shown to be present in platelets [24], even though the functional role of $G\alpha_{i1}$ in these cells is not known. However, purification of pertussis toxin sensitive G-proteins from 7 g of platelet membranes yielded $G\alpha_{i2}$ and $G\alpha_{i3}$ but no detectable $G\alpha_{i1}$ (results not shown).

Using the antibodies described, we found that stimulation of the platelet ADP receptor led to an increase in the incorporation of [α-^{32}P]GTP-azidoanilide into the $G\alpha_{i2}$ subunit, while, as expected from previous reports, U46619 activated $G\alpha_q$ and

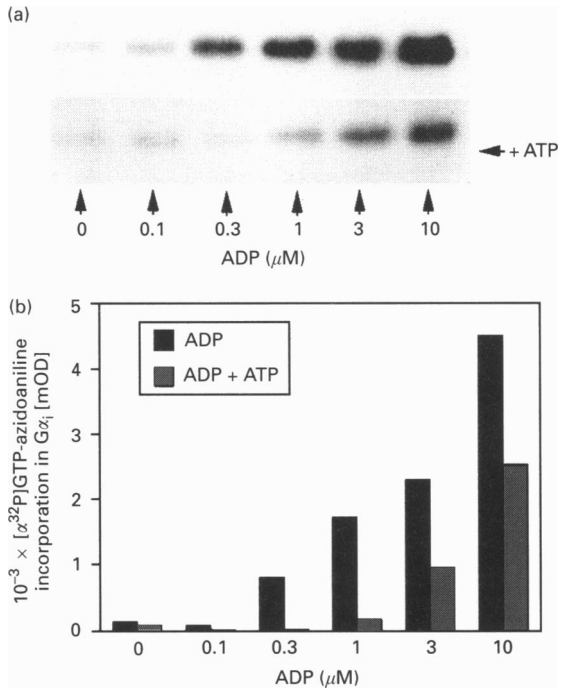

Figure 4 Concentration dependence of the ADP-stimulated photolabelling of $G\alpha_i$

Platelet membranes (400 μg) were incubated in a medium containing 10 μM GDP and photolabelled as described for Figure 2 in the presence of increasing concentrations of ADP (0.1–10 μM), in the absence or presence of 10 μM ATP. Proteins were immunoprecipitated with antiserum AS 266 (anti-α_{ic}, 20 μl) and separated on SDS/10% acrylamide gels. The photolabelled proteins were then visualized by autoradiography (**a**) and their relative abundance determined by densitometric image analysis (**b**), results shown here being representative of three identical independent experiments.

thrombin activated both $G\alpha_q$ and $G\alpha_i$ [17]. U46619 activates phospholipase C (PLC) in a pertussis toxin independent manner through G_q. Thrombin or the thrombin receptor agonist peptide (TRAP-SF7) simultaneously inhibits adenylyl cyclase through G_i proteins and activates phospholipase C (PLC) through G_q and presumably by means of $\beta\gamma$ dimers of pertussis toxin sensitive G-proteins [6]. Whereas ADP is known to inhibit platelet adenylyl cyclase, it is a matter of debate whether or not this agonist causes activation of PLC [3]. Our results support the idea that ADP does not activate PLC through the G_q pathway. On the other hand, adrenaline, which is not itself an aggregating agent [25], has been shown to inhibit adenylyl cyclase in human platelets through α_2 adrenergic receptors which are coupled to $G\alpha_{i2}$ [26], without causing any change in cytoplasmic Ca^{2+} levels. Thus, it could be suggested that ADP acts on adenylyl cyclase through $G\alpha_{i2}$ and that this particular G-protein does not provide sufficient concentrations of free $\beta\gamma$ complexes for PLC activation in response to adrenaline or ADP stimulation. Alternatively, PLC-β_2 and -β_3 may not be expressed in platelets. Further studies are needed to answer these points.

Since the activation of $G\alpha_{i2}$ by ADP cannot account alone for its aggregatory effect, how then can the stimulation of ADP receptor(s) result in mobilization of intracellular Ca^{2+} stores? In contrast to most aggregating agents which mobilize Ca^{2+} through D-*myo*-inositol 1,4,5-triphosphate, the signal transduction pathways of ADP are poorly understood [3]. ADP induces an increase in intracellular Ca^{2+} in a unique manner, involving both activation of non-selective cation channels [27] and mobilization of internal stores by a still unidentified pathway. Studies of the mechanism of action of the anti-thrombotic drugs ticlopidine and clophidogrel, which are specific inhibitors of ADP-induced platelet aggregation [28], have shown that in the case of rat platelets, the inhibition of the increase in intracellular Ca^{2+} is due to blockade of the mobilization of internal stores [29]. Although we did not find the same effect in human volunteers [30], this discrepancy could arise from the fact that the doses of drugs given to the animals were very much higher than those permissible in humans. These drugs do inhibit the ADP receptor-induced activation of G-proteins [14]. However, whether the Ca^{2+}-mobilizing effect of ADP receptor stimulation is a G-protein-dependent effect remains to be established. Studies of patients with selective deficiency of ADP-induced platelet aggregation [31–32], who have reduced numbers of binding sites for 2MeSADP, a specific P_{2T} agonist, should be of interest in this respect, and we are currently investigating such a patient with our system [33].

CONCLUSIONS

Our results confirm that ADP-induced platelet activation is partly due to a G-protein-coupled receptor, which interacts with the $G\alpha_{i2}$ heterotrimeric G-protein. This may explain how ADP inhibits adenylyl cyclase. On the other hand, the absence of activation of the G_q pathway raises the question of the mechanism of Ca^{2+} mobilization and the subsequent aggregation process for which, as in case of adrenaline, inhibition of adenylyl cyclase is not sufficient.

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