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Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors

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Abstract

Human platelets express adenosine 5′-diphosphate (ADP)-specific purinoceptors of the P_{2X} and P_{2Y} receptor superfamily, but their structure, diversity, and precise pharmacological profile is not well understood. Here, functional assays with intact platelets and well-characterized nucleotide derivatives were performed in order to characterize the ligand specificity of these platelet-specific purinoceptors. For the signalling pathways investigated (aggregation, rapid Ca^{2+}-influx, desensitization of Ca^{2+}-influx, Ca^{2+}-mobilization, inhibition of adenylyl cyclase), significant differences in ligand specificity were demonstrated. ADP activated all purinoceptors of human platelets, while adenosine 5′-triphosphate (ATP) was a weak agonist for the P_{2X} receptor and an antagonist for the P_{2Y} receptors. The ADP-receptor pathway-antagonist ticlopidine inhibited ADP-evoked aggregation and adenylyl cyclase inhibition but did not affect platelet purinoceptors associated with Ca^{2+}-influx and Ca^{2+}-mobilization. These results indicate the presence of three distinct ADP-selective purinoceptors on human platelets.

Keywords: ADP derivative; Thienopyridine; Ca^{2+}; G-protein; Purinoceptor

1. Introduction

Human platelets are activated by numerous vasoactive agents, hormones and drugs. Platelet activation and aggregation by adenosine 5′-diphosphate (ADP) plays a key role in the development and pathogenesis of arterial thrombosis (Gaardner et al., 1961; Born, 1985). This concept is supported by the recent demonstration that thienopyridines, such as ticlopidine- and clopidogrel-specific inhibitors of the ADP activation pathways, are very effective in reducing the risk of atherosclerotic vascular disease (Schrör, 1993; Savi et al., 1996; CAPRIE Steering Committee, 1996). Therefore, the mechanism(s) of human platelet activation by ADP are of considerable biochemical, pharmacological and medical interest. Platelets are presently the only cells known which express ADP-specific, purinoceptors. Several purinoceptors of the P_2-subtype have been characterized and cloned within the last few years indicating the existence of the growing receptor superfamily of the P_{2X} and P_{2Y} subtypes (North and Barnard, 1997; Fredholm et al., 1997). However, very little is known about the structure, diversity and function of human platelet ADP (P_{2T}) receptors despite many attempts to determine the number and types of human platelet ADP receptors (Mills, 1996; Gachet et al., 1997). Major problems with respect to the analysis of platelet purinoceptors include the existence of several nonspecific nucleotide binding sites on platelet membranes, the rapid desensitization of platelet ADP receptors and the degradation and impurity of commercial nucleotide preparations (Gachet et al., 1997). Three main signalling pathways induced by ADP have been described for platelets: The activation of a putative receptor operated channel with low cation selectivity (Mahaut-Smith et al., 1992), the activation of intracellular Ca^{2+} mobilization probably via activation of phospholipase C and subsequent store dependent Ca^{2+} influx (Rink and Sage, 1990), and the inhibition of adenylyl cyclase by activation of G_i-protein (Cole et al., 1971). The activation of platelets by ADP ultimately results in shape change, adhesion, secretion and aggregation of the cells. In line with the recent classification of purinoceptors (Fredholm et al., 1997), the platelet purinoceptors mediating rapid Ca^{2+} influx have to be designated as P_{2X} receptors, the G-pro-

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tein coupled receptor/receptors mediating Ca^{2+} mobilization and adenylyl cyclase inhibition as P_{2Y} receptors. Here, we report the characterization of human platelet ADP receptors with respect to their ligand specificity and pharmacological profile of ADP evoked signalling in intact human platelets.

2. Materials and methods

2.1. Materials

6-Chloropurine 5′-diphosphate (6-Cl-PuDP), 7-deaazaadenosine 5′-diphosphate (tubercidine 5′-diphosphate, TuDP), 1-methyladenosine 5′-diphosphate (1-Me-ADP) and 2-chloroadenosine 5′-diphosphate (2-Cl-ADP) were obtained from Biolog (Bremen, Germany). Adenosine 5′-γ-thiotriphosphate (ATP-γ-S) was obtained from Boehringer (Mannheim, Germany), pyridoxal phosphate 6-azophenyl-2′,4′ disulfonate sodium salt (PPADS), reactive blue and suramin from RBI-Biotrend (Cologne, Germany). All other ADP derivatives and chemicals were obtained from Sigma (Deisenhofen, Germany). The purity of all derivatives was tested prior to use by high performance liquid chromatography (HPLC), and only substances with a purity better than 99% were used in the study. Ticlopidine (Tiklyd; Sanofi Winthrop) is commercially available.

2.2. Methods

2.2.1. Platelet preparation

Platelets were prepared from freshly drawn whole human blood obtained from healthy volunteers who had not received any medical treatment within two weeks before (Geiger et al., 1992). Briefly, the blood was collected in a citrate buffer (100-mM sodium citrate, 7-mM citric acid, 140-mM glucose, pH 6.5) containing 0.1-mM aspirine (except for aggregation experiments). Platelet-rich plasma was separated by centrifugation for 20 min at $300 \times g$ (Sigma 3K-1). The platelet-rich plasma (upper phase) was then removed with a plastic pipette and transferred into plastic tubes for further utilization. Washed platelets were prepared from platelet-rich plasma by centrifugation for 10 min at $500 \times g$ (Sigma 3K-1). The platelet pellet was resuspended in HEPES buffer (145-mM NaCl, 5-mM KCl, 1-mM $MgCl_2$, 10-mM HEPES, 10-mM glucose, pH 7.4). For Fura-2 measurements, the platelet-rich plasma was incubated with 4-μM Fura-2 acetoxymethylester (Fura-2/AM) (final concentration, 1% dimethylsulfoxide (DMSO) (v/v)) for 45 min at 37°C in presence of 0.1 u/ml apyrase and 0.1-mM aspirin. The platelets were then pelleted by centrifugation for 10 min at $500 \times g$ (Sigma 3K-1). The platelet pellet was resuspended in HEPES buffer.

2.2.2. Ticlopidine-treated platelets

The action of the platelet inhibitor ticlopidine was analysed using platelets obtained from healthy, ticlopidine-treated volunteers and patients undergoing an elective coronary stent implantation (Markert et al., 1996). Blood was taken with informed consent of both patients and volunteers. Patients received a 3-day treatment with ticlopidine (500 mg/day) and aspirin (100 mg/day) (Markert et al., 1996). Samples were taken before treatment and after 3 days of treatment. Volunteers received a 7-day ticlopidine medication with 500 mg/day. Samples were taken before treatment ((1) control), after 7 days of medication (during treatment) and 3 weeks after treatment with ticlopidine ((2) control). Determination of platelet aggregation, cAMP regulation and stimulated Ca^{2+} transients were performed analogous to the experiments with ADP derivatives. While the results of platelets from ticlopidine-treated patients (which also received other drugs) and ticlopidine-treated volunteers (which did not receive any other medication) were principally similar, only data from volunteer platelets are shown. All studies reported here were approved by the ethics committee of our university and performed according to the guidelines of the declaration of Helsinki.

2.2.3. Platelet aggregation

Platelet aggregation was measured with platelet aggregation profiler (PAP-4, Biodata). For the experiments, 0.3-ml platelet-rich plasma were transferred into siliconized glass cuvettes with a plastic pipette and a magnetic stir bar added. Data were captured via the analog output port of the PAP-4, digitised with an A/D converter, and stored on a microcomputer. For the inhibition experiments, platelets were pretreated 10 s with the appropriate derivative and then stimulated with ADP.

2.2.4. Platelet cAMP content

Both platelet-rich plasma and washed platelets were used for the determination of G_i-protein stimulation. For the experiments, 0.3-ml aliquots of platelet suspension in siliconized Eppendorf caps were either incubated with ethanol (1% ethanol (v/v)) alone for 1 min (base control), 10-μM prostaglandine E_1 (ethanol solved, final concentration 1% ethanol (v/v)) for 1 min or 10-μM prostaglandine E_1 for 1 min (maximum control) and an ADP derivative, added 15 s after addition of prostaglandine E_1, for 45 s. Prostaglandine E_1 was added prior to ADP to prevent secondary changes of platelet responses due to platelet activation by ADP. In inhibition experiments, the derivative was added immediately after the addition of prostaglandine E_1 and ADP was added 15 s after addition of prostaglandine E_1. The cells were stopped with 0.5-ml 70% (v/v) ice-cold ethanol and kept on ice for 30 min. The precipitate was pelleted by centrifugation for 10 min at $5000 \times g$ at 4°C. The supernatant was transferred to an

Eppendorf cap and the precipitate was extracted twice with 0.5-ml 70% (v/v) ethanol. The extracts were combined and evaporated in membrane pump vacuum. The resulting extract was solved in 0.5 ml of assay buffer (50-mM sodium acetate, pH 5.8). The cAMP determination was performed using the Amersham Biotrak cAMP RIA kit. Dose–response curves were determined by weighted Marquardt–Levenberg approximation of the raw data.

2.2.5. Ca^{2+} regulation

Ca^{2+} concentration transients were observed either with a Perkin Elmer LS-50 fluorescence spectrometer or a Schoeffl fluorescence spectrometer (Geiger et al., 1992). For the experiments with the LS-50 fluorimeter, 2 ml of a suspension of Fura-2-loaded platelets were transferred in 10-mm pathlength quartz cuvettes (Hellma, Germany), with a magnetic stir bar added, and placed in a thermostated (37°C) and stirring cuvette holder.

The total Ca^{2+} response of human platelets was determined with Fura-2-loaded human platelets, stimulated in presence of 1-mM $CaCl_2$ with ADP or ADP derivatives. The fluorescence emission was measured at an excitation wavelength of 340 nm (slit width 5 nm) and an emission wavelength of 510 nm (slit width 5 nm). If the substances applied showed spectral interference with Fura-2, the excitation wavelength was set to 380 nm (slit width 7 nm).

For the Ca^{2+}-mobilization experiments, extracellular Ca^{2+} was removed by addition of 4-mM sodium EGTA. Fluorescence measurement was performed as with extracellular Ca^{2+}.

For the separation of Ca^{2+}-influx from Ca^{2+}-mobilization, the manganese quench method (Geiger et al., 1992) was applied. Prior to stimulation, 0.5-mM $MnCl_2$ and 0.5-mM $CaCl_2$ were added to the platelet suspension and the fluorescence signal was observed at the isosbestic point of the Fura-2 spectrum (excitation: 360 nm, slit width: 5 nm; emission: 510 nm, slit width: 5 nm).

In order to distinguish the Ca^{2+}-influx through the receptor-operated Ca^{2+}-channel from store dependent Ca^{2+}-influx, two methods were applied. Platelet Ca^{2+}-mobilization and store-dependent Ca^{2+}-influx were inhibited by a 3-min preincubation with 10-μM prostaglandine E_1 at 37°C (Geiger et al., 1994), and then the cells were stimulated by ADP and/or the appropriate derivative. The different phases of stimulated increase of the intracellular Ca^{2+}-concentration could also be separated on the basis of their time constants by stopped-flow fluorometry (Sage et al., 1990). Stopped-flow fluorometry was performed with a Schoeffl fluorescence spectrometer fitted with a stopped-flow setup constructed at our institute. The setup consists of two gastight 200-μl Hamilton syringes (providing the reaction components) and one 500-μl Hamilton syringe serving as stop syringe, the piston being connected to a microswitch. The microswitch provides the necessary trigger signal. The syringes are connected by teflon tubes to the Y-shaped mixing chamber and a 75-μl Hellma flow through quartz cuvette. The syringes are manually driven. In previous tests, the dead time of the stopped-flow setup was determined to be 10–20 ms.

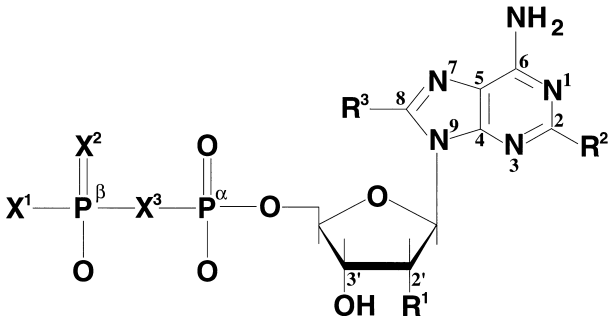

Fig. 1. Structure of ADP and the ADP derivatives used in the study. Substituents: X^1 = -O$^-$, $-PO_3^{2-}$ (ATP), $-P_2O_6^{3-}$ (AP_4), $-NH_2$ (β-NH_2-ADP); X^2 = -O, -S (ADP-β-S); X^3 = -O-, $-CH_2$-(AMPCP); R^1 = -OH, -H (dADP); R^2 = -H, -Cl (2-Cl-ADP), $-SCH_3$ (2-MeS-ADP); R^3 = -H, -Br (8-Br-ADP), $-N_3$ (8-N_3-ADP).

One of the syringes was filled with a suspension of Indo-1-loaded human platelets in HEPES buffer, the other one with the agonist solved in HEPES buffer. Loading with Indo-1 was performed identical to Fura-2 loading of platelets. Fluorescence emission was determined at an excitation wavelength of 350 nm (slit width 5 nm) and an emission wavelength of 460 nm (slit width 5 nm) for the free dye and 410 nm for the Ca^{2+} bound complex. The ratio was evaluated in real time with an analogue computer. Data were collected with a Linseis L8200 data acquisition system at an acquisition rate of 1 kHz and transferred to a microcomputer.

2.2.6. Selection of ADP derivatives

A wide variety of ADP derivatives was used in this study in order to characterize the interaction of the nucleotide with the receptor protein (Fig. 1). The influence of the phosphate group was tested with elongated or shortened phosphate chains (e.g., ATP, AMP) and substitutions of oxygen in the phosphate chain by sulfur, amino groups or methylene groups. The ribose ring is expected to interact mainly via the 2′- and 3′-hydroxo groups. Therefore the reduced form (2′-deoxy) and the oxidized and acyclic forms (dialdehyde and dialcohol) of ADP were applied. The interaction of the purine base with the receptor protein was tested by exchange or blocking of potential hydrogen bond donors and acceptors, i.e., nitrogen in the heterocycle (positions 1 and 7) or amino substituents (position 6). The 1, 2, 6 and 8 positions of the purine ring were tested for their sterical susceptibility by use of bulky substituents.

3. Results

3.1. Aggregation

Compared to the well-known effect of ADP, only 2-substituted ADP derivatives stimulated a similar extent of

Table 1
Stimulation of human platelet aggregation by ADP derivatives

Derivative	Aggr. (rel.%)	±S.E.
ADP-β-S	6.6	±3.1
2-Cl-ADP	125.0	±14
dADP	−6.5[a]	±1.2
1-Me-ADP	8.9	±2.7
N-etheno-ADP	11.3	±4.0
TuDP	12.5	±2.8

Human platelets were stimulated with 20 μM of the appropriate ADP derivative. Aggregation responses given are the maxima reached within 3 min after stimulation relative to the aggregation response evoked by 20-μM ADP. Data are means of five individual platelet preparations ± S.E.
[a]With dADP, only shape change was observed.

platelet aggregation (Table 1). 2-Cl-ADP had a similar, 2-methylthioadenosine 5′-diphosphate (2-MeS-ADP) an even higher potency when compared to ADP (Fig. 2). 1-Me ADP and the $1,N^6$-ethenoadenosine 5′-diphosphate (*N*-etheno ADP) caused very weak and reversible aggregation responses (Fig. 2C and D; Table 1). The 6-substituted 6-Cl-PuDP did not show any effects on platelet aggregation (data not shown). TuDP at low concentration (10 μM) produced only weak, reversible or no platelet aggregation at all while a biphasic aggregation response of 40% was observed at higher (100 μM) concentration (Fig. 2C and D). The inhibitory effects of 8-substituted ADP derivatives already observed previously (Hourani and Hall, 1994) could be confirmed although this inhibition was less effective in our hands (Table 2). An acyclic ADP derivative, adenosine 5′-diphosphate acyclic 2′3′-dialcohol (ADP-dialcohol), was neither stimulatory nor inhibitory (data not shown). Adenosine 5′-diphosphate acyclic 2′3′-dialdehyde (ADP-dialdehyde) was a weak inhibitor of ADP induced platelet aggregation (Table 2). 2′-Deoxyadenosine 5′-diphosphate (dADP) stimulated a weak, reversible aggregation response at high concentration (100 μM) and only

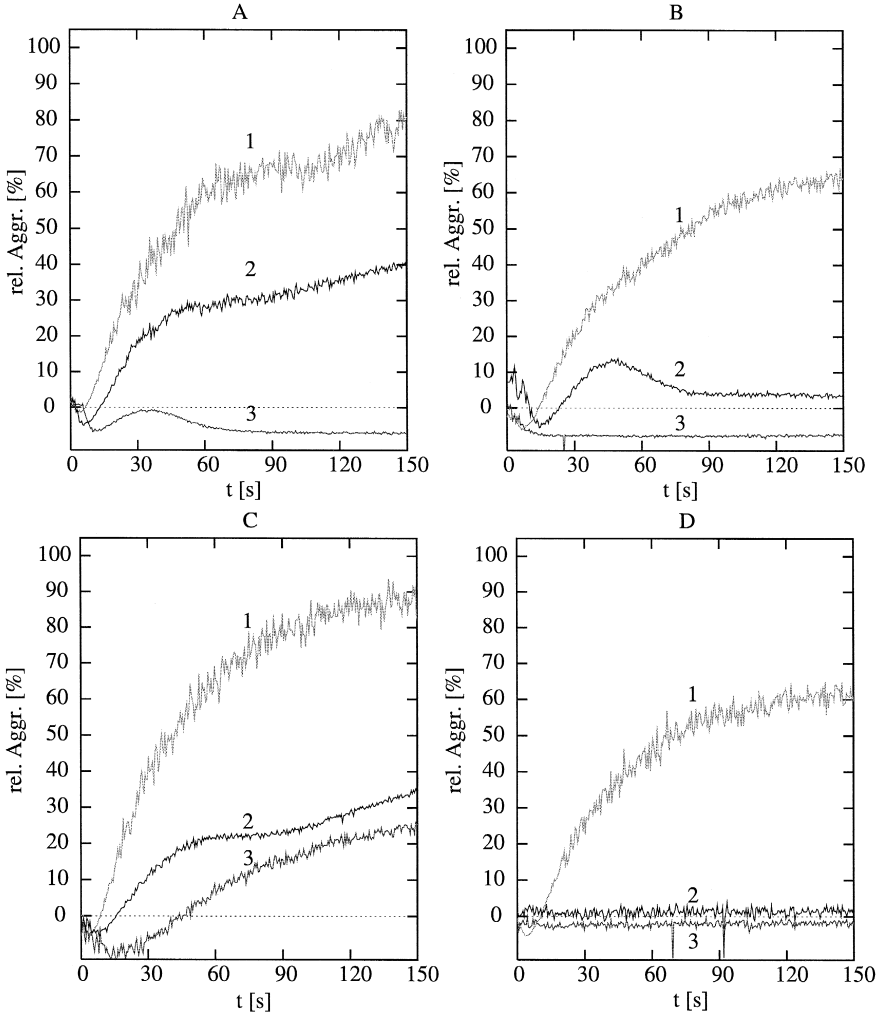

Fig. 2. Platelet aggregation caused by ADP and ADP derivatives. Platelets of freshly prepared platelet rich plasma were stimulated with ADP (trace 1), ADP-β-S (trace 2) or dADP (trace 3) (panel (A): 100 μM, panel (B): 10 μM) or 2-Cl-ADP (trace 1), TuDP (trace 2) or 1-Me-ADP (trace 3) (panel (C): 100 μM, panel (D): 10 μM). The stimulant was added at time point 0. The results are representative for several independent experiments also summarized in Table 5.

Table 2
Inhibition of ADP-induced human platelet aggregation by ADP derivatives

Derivative	Aggr. (rel.%)	±S.E.
AP_4	38.3	±4.2
AMPPNP	55.1	±1.4
ATP	57.9	±2.5
8-Br-ADP	79.0	±9.2
ADP-dialdehyde	79.8	±6.2

Human platelets were treated 10 s with 20 μM of the appropriate ADP derivative prior to stimulation with 20-μM ADP. Aggregation responses are given relative to the aggregation response evoked by 20-μM ADP alone (100%). Data are means of five individual platelet preparations ± S.E.

Table 3
Concentration of ADP-derivatives (EC_{50} values) causing half-maximal inhibition of prostaglandine E_1-stimulated adenylyl cyclase in intact human platelets

Derivative	EC_{50} (μM)	±S.E.
2-Cl-ADP	0.2748	±0.0021
ADP	0.8804	±0.019
ADP-β-S	2.1543	±0.054
TUDP	2.778	±0.045
N-etheno-ADP	5.003	±0.063
1-Me-ADP	50.82	±0.62

Prostaglandine E_1 (10 μM) was added to human platelets (0 min of the incubation time), followed by the addition of ADP or the appropriate ADP derivative at 15 s. After an additional 45 s (60 s total incubation time) cells were stopped with ethanol, and their cAMP content was determined. Data represent means of 5–7 experiments ± 66% confidence interval.

shape change or no response at all at lower (20 μM, 10 μM) concentrations (Table 1; Fig. 2A and B). Adenosine 5′-triphosphate (ATP) and its derivatives acted, similar to other adenosine polyphosphates like adenosine 5′-tetraphosphate (AP_4) and diadenosine polyphosphates like P^1,P^4-diadenosine 5′-tetraphosphate (A_2P_4), as inhibitors of platelet aggregation (Table 2). Adenosine 5′-monophosphate (AMP) exhibited neither a stimulatory nor an inhibitory effect on platelet aggregation (data not shown). Substitution at the terminal ADP phosphate with hydrogen bond donors other than oxygen reduced the potency to cause aggregation. Adenosine 5′-β-thiodiphosphate (ADP-β-S) and adenylyl 5′-phosphoramidate (ADP-β-NH_2) (at 100 μM each) stimulated a biphasic aggregation response of about 40–50% and, at 10 μM, only a weak and reversible aggregation (Fig. 2A and B). Lipophilic substitution at the bridging oxygen of the diphosphate (adenosine 5′-αβ-methylenediphosphonate (AMPCP)) produced a platelet aggregation inhibitor of medium strength (Table 2). Other nucleotide 5′-diphosphates and triphosphates like guanosine 5-diphosphate (GDP), inosine 5-diphosphate (IDP) and uridine 5″-triphosphate (UTP) had no effect on platelet aggregation (data not shown). Pretreatment of platelets with the prominent purinoceptor antagonists PPADS, suramine and reactive blue only slightly affected ADP-evoked platelet aggregation even when used at high concentrations up to 1 mM (data not shown). Platelets of ticlopidine-treated individuals did show only weak, reversible aggregation or no aggregation at all in response to ADP (Fig. 3A), while ADP-caused shape change (Fig. 3A) and the thrombin-induced response was almost intact (Fig. 3B).

3.2. Inhibition of adenylyl cyclase

The inhibition of prostaglandine-E_1-stimulated cAMP elevation (due to G_i-protein activation) by ADP and ADP derivatives exhibited a similar selectivity for ADP derivatives as the platelet aggregation in response to purinergic agents (Tables 3 and 5). Also, most substances which

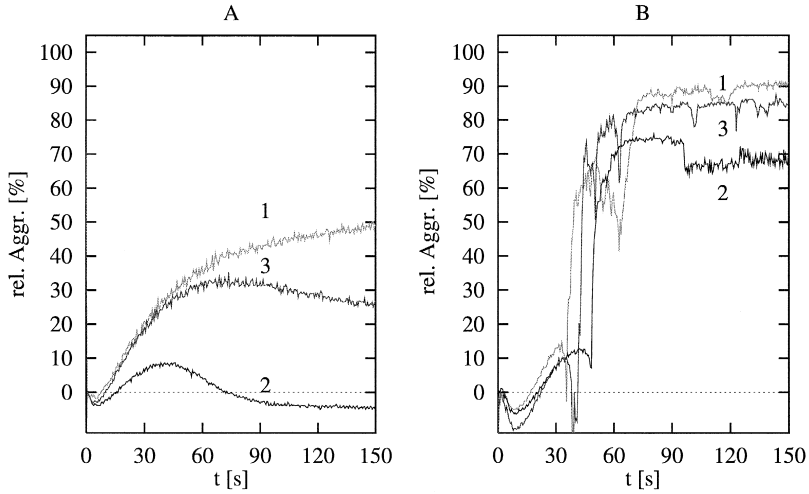

Fig. 3. Effect of ticlopidine treatment on human platelet aggregation. Platelets were prepared from ticlopidine-treated volunteers as described in Section 2.2.2 and stimulated with 10 μM ADP (panel (A)) or 1 u/ml thrombin (panel (B)) before (trace 1), after 1 week treatment (trace 2) and 2 weeks after treatment had been discontinued (trace 3). The results are representative for several independent experiments.

Table 4
Concentration of purinergic derivatives (IC_{50} values) resulting in half-maximal inhibition of the ADP-caused downregulation of adenylyl cyclase in intact human platelets

Derivative	IC_{50} (μM)	±S.E.
ATP	61.31	±1.1
6-Cl-PuDP	64.12	±1.1
AP_4	80.70	±0.6
ADP-dialdehyde	138.7	±4.5
A_2P_4	152.9	±0.7
AMPPNP	167.3	±4.8
AMPPCP	183.9	±5.3
FSBA	210.9	±4.7
dADP	333.8	±6.0
AMPCPP	465.2	±1.7
AMPCP	> 0.5 mM	
8-Br-ADP	> 0.5 mM	
Suramin	> 1 mM	
PPADS	> 2 mM	

Prostaglandine E_1 (10 μM) was added to human platelets (0 min of the incubation time) followed the addition of the tested ADP derivative at 10 s and 20-μM ADP at 15 s. After an additional 45 s (60 s total incubation time), platelets were stopped with ethanol, and their cAMP content was determined. Data represent means of 5–7 experiments ± 66% confidence interval.

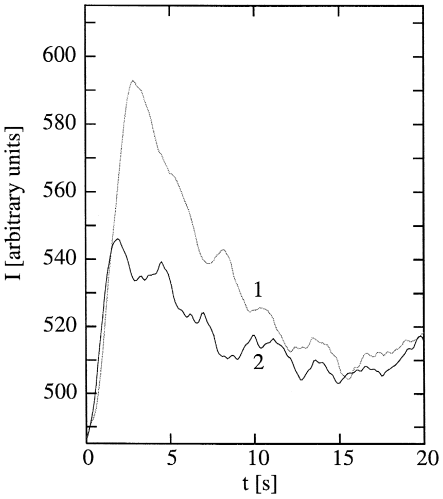

Fig. 4. Inhibition of ADP stimulated Ca^{2+}-influx by dADP in prostaglandine E_1 pretreated platelets. Fura-2 loaded human platelets were preincubated with 10 μM prostaglandine E_1 for 3 min and then stimulated with ADP (20 μM) alone (trace 1) or ADP (20 μM) + dADP (20 μM) (trace 2) in the presence of 1-mM $CaCl_2$. The stimulants were added at time point 0. The results are representative for several independent experiments.

Table 5
Effects of nucleotide derivatives and purinoceptor inhibitors on human platelet purinergic responses

Derivative	Ca^{2+}-regulation		Adenylyl cyclase inhibition (G_i)	Aggregation
	ROC	Mobilization		
Stimulators				
ADP	+++	+++	+++	+++
2-Cl-ADP	+++	+++	+++	+++
2-MeS-ADP	+++	+++	+++	++++
ADP-β-S	++	+++	++	++[b]
TuDP	+	+	+	+[b]
1-Me-ADP	0	++	+	+/0[b]
Inhibitors				
8-Br-ADP	0	−	0	−
AP4	−	−−	−−−	−−
A_2P_4	0	−−	−−	−−
ADP-dialdehyde	0	−	−−	−−
AMPPCP	0	−	−−	−
AMPCP	0	0	0	−
PPADS	N.D.	0	0	−−
6-Cl-PuDP	0	0	−−−	0
Stimulators and inhibitors				
AMPCPP	++	−−	−	−−
ATP	+	−−	−−−	−−
AMPPNP	+[c]	−−	−−	−−−
dADP	−	++	−	0[d]
Inactive				
ADP-dialcohol	0	0	0	0
GDP	0	0	0	0
UDP	0	0	0	0
IDP	0	0	0	0

Qualitative representation of nucleotide derivative effects on human platelets are presented. (+)-signs indicate stimulatory effects, (−)-signs inhibitory effects and (0) no effect on platelet purinoceptor mediated responses. The number of (+) and (−)-signs are a qualitative representation of strong (+++; −−−) or relatively weak (+; −) effects observed.
[b]Reversible aggregation; [c]unclear effect; [d]only shape change observed.

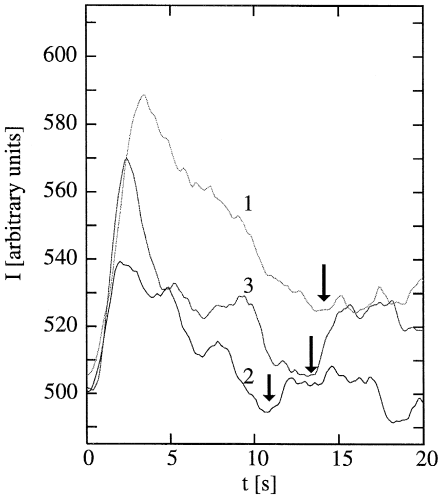

Fig. 5. Stimulation of Ca^{2+}-influx by adenosine triphosphates in prostaglandine E_1-pretreated platelets. Fura-2-loaded human platelets were preincubated with 10-μM prostaglandine E_1 for 3 min and, then, stimulated with ADP (20 μM) (trace 1), ATP (100 μM) (trace 2) or AMPCPP (100 μM) (trace 3) in the presence of 1-mM $CaCl_2$. The stimulant was added at time point 0. Further 20-μM ADP was added at the point indicated with arrows. The results are representative for several independent experiments.

inhibited ADP-induced platelet aggregation were also effective inhibitors of the ADP-caused G_i-protein activation (e.g., adenosine 5′-triphosphate and adenosine 5′-polyphosphates) (Table 4). The 8-substituted ADP derivatives did not show any significant inhibition of the ADP evoked down regulation of adenylyl cyclase up to 0.5 mM concentration (Table 4). However some ADP derivatives (e.g., dADP) which had only minor effects on platelet aggregation inhibited G_i-protein activation by ADP. Other substances (e.g., ADP-β-S) which induced only weak aggregation responses could stimulate G_i-protein quite effectively (Tables 3 and 5). Treatment with AMP lead to an increase of cAMP, most likely due to AMP breakdown and subsequent G_s-stimulation by the adenosine formed (not shown). Suramin and other nonnucleotide purinoceptor antagonists PPADS and reactive blue were without effect (Table 4). In agreement with the effects reported for the thienopyridine clopidogrel (Savi et al., 1996), ticlopidine treatment inhibited the ADP-caused downregulation of prostaglandine E_1-stimulated adenylyl cyclase although the effects were somewhat variable when relatively high concentrations (i.e., 10 μM) of prostaglandine E_1 were used (data not shown). Also, prostaglandine E_1-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Geiger et al., 1994) was significantly inhibited by ADP, which was not observed in ticlopidine-treated platelets (Geiger, unpublished data).

3.3. Ca^{2+} regulation

3.3.1. Ca^{2+}-influx

Only a small number of ADP derivatives clearly stimulated ligand-gated Ca^{2+}-influx detected by both the stopped-flow approach and by the analysis of Ca^{2+}-influx during prostaglandine E_1-inhibited Ca^{2+}-mobilization/store-dependent influx. The 2-substituted ADP derivatives were as efficient as ADP itself, while derivatives substituted in the phosphate moiety (e.g., ADP-β-S) and TuDP were less effective than ADP in stimulating Ca^{2+}-influx through the receptor-operated cation channel (Table 5). Most other derivatives did not influence the ligand-gated Ca^{2+}-influx at all or showed only a weak inhibitory action similar to that of dADP, which caused a 30% inhibition of the ADP-induced Fura-2-fluorescence-

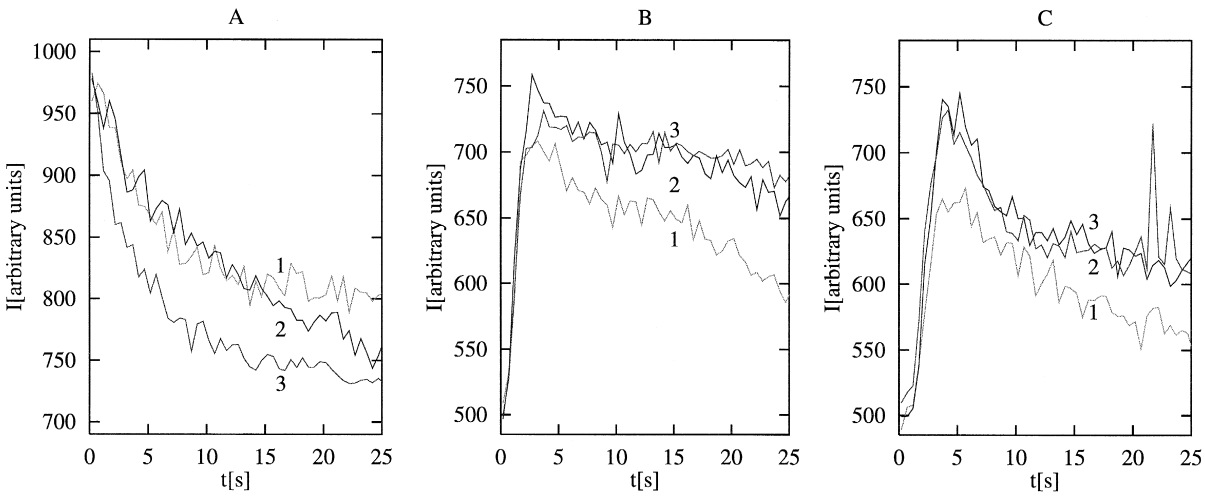

Fig. 6. Lack of ticlopidine effects on stimulated Ca^{2+}-influx and Ca^{2+}-mobilization in human platelets. Platelets from ticlopidine treated volunteers were loaded with Fura-2 AM and stimulated with 10-μM ADP in the presence of 0.5-mM $MnCl_2$ and $CaCl_2$ (manganese influx, fluorescence measurement at 360 nm; panel (A)), 1-mM $CaCl_2$ (total Ca^{2+} signal; panel (B)) or 4-mM EGTA (Ca^{2+}-mobilization; panel (C)). Platelets were obtained before medication (trace 1), after 1 week of medication (trace 2) and 2 weeks after the treatment had been discontinued (trace 3). The results are representative for several independent experiments.

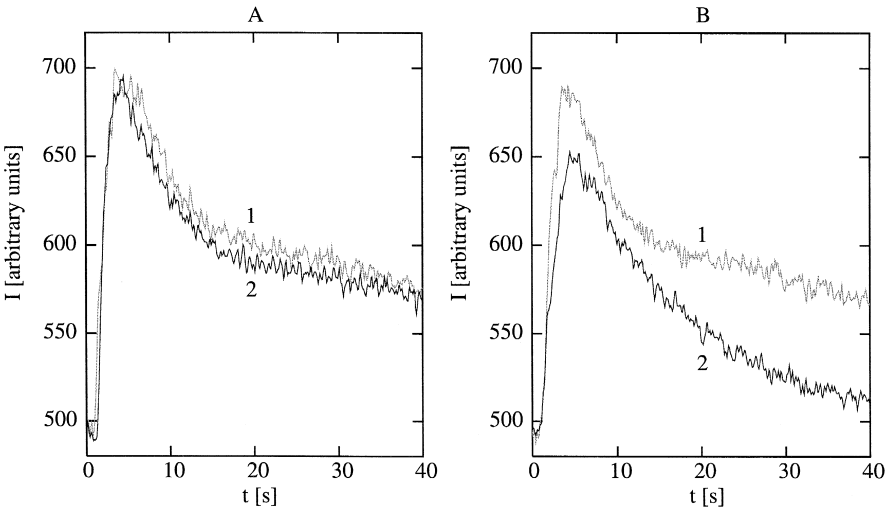

Fig. 7. Stimulation of platelet Ca^{2+}-mobilization from intracellular stores by ADP derivatives. Fura-2-loaded human platelets were stimulated with 20-μM ADP (trace 1), 2-Cl-ADP (trace 2) (panel (A)), 1-Me-ADP (trace 1) or dADP (trace 2) (panel (B)) in the presence of 4-mM EGTA. The stimulants were added at time point 0. The results are representative for several independent experiments.

increase at a concentration of 20 μM (Fig. 4). ATP and adenosine 5′-$\alpha\beta$-methylenetriphosphate (AMPCPP) acted as weak activators of human platelet Ca^{2+}-influx (Fig. 5). 100 μM of AMPCPP, an established activator of P_{2X1}-receptors (Evans et al., 1995), and ATP stimulated Ca^{2+}-influx of about 70% or 40%, respectively, when compared to the maximal response evoked by 20-μM ADP (Fig. 5; Table 5). Suramin (1 μM), adenosine 5′-$\beta\gamma$-methylenetriphosphate (AMPPCP) and adenosine polyphosphates (e.g., AP_4) inhibited ADP-stimulated Ca^{2+}-influx, whereas reactive blue at concentrations up to 0.5 mM was without effect (not shown). Ticlopidine treatment had no significant effect on ADP induced Ca^{2+}-influx of human platelets (Fig. 6A).

3.3.2. Desensitization of Ca^{2+}-influx

The fast desensitization of the ligand-gated Ca^{2+}-influx is one of the main characteristics of the associated ADP receptor in human platelets (Mahaut-Smith et al., 1992). All agonists of the rapid Ca^{2+}-influx in human platelets caused a comparable rate of desensitization. Whereas platelets stimulated with ADP no longer respond to a second stimulation with ADP or ADP derivatives, the application of 20-μM ADP 10 s after stimulation with some ADP derivatives lead to weak but significant Ca^{2+}-influx in human platelets as shown for ATP and AMPCPP (Fig. 5) and with 20-μM ADP-β-S (not shown).

3.3.3. Ca^{2+}-mobilization

Platelet Ca^{2+}-mobilization from intracellular stores could be observed in response to most ADP derivatives, thus demonstrating the least nucleotide specificity (Table 5). Derivatives which activated G_i-proteins (as measured by the inhibition of prostaglandine E_1-stimulated adenylyl cyclase) were also activators of Ca^{2+}-mobilization in platelets (Fig. 7) but their relative potency was higher for Ca^{2+}-mobilization (e.g., 1-Me-ADP, Fig. 7B). Also, most derivatives which inhibited G_i-protein activation were also inhibitors of Ca^{2+}-mobilization (Table 5). However, dADP was an exception, as it was a relatively good activator of platelet Ca^{2+}-mobilization (Fig. 7B). The 8-Bromoadenosine 5′-diphosphate (8-Br-ADP) turned out being a weak inhibitor of ADP induced Ca^{2+}-mobilization. The P_2-receptor antagonists PPADS, suramin and reactive blue did not remarkably influence platelet Ca^{2+}-mobilization (not shown). Ticlopidine treatment did not significantly affect ADP induced Ca^{2+}-mobilization (Fig. 6C).

4. Discussion

Platelet receptors responsive to the small-molecule ADP have been collectively termed P_{2T} receptors (Fredholm et al., 1997). However, the P_{2T} receptor was very recently considered more as a pharmacological concept rather that a molecular entity (Gachet et al., 1997) despite multiple attempts to identify platelet ADP receptors by pharmacological, biochemical and molecular biology methods. Considerable evidence supports the concept of more than one ADP receptor including a P_{2X} ionotropic receptor (Mahaut-Smith et al., 1992; MacKenzie et al., 1996; Gachet et al., 1997) and P_{2Y} receptor(s) linked to G-proteins which stimulate phospholipase C/Ca^{2+}-mobilization from intracellular stores and/or inhibit adenylyl cyclase (Cole et al., 1971; Geiger and Walter, 1993; Geiger et al., 1994; Hourani and Hall, 1994; Savi et al., 1996; Gachet et al., 1997; Savi et al., 1998). However, whether these effects are mediated by two or more receptors which can be distinguished by biochemical or pharmacological methods is not established. One major problem in defining ADP-

selective receptors has been the impurity and contamination of commercial nucleotides (Gachet et al., 1997). Here, we attempted to define the human platelet ADP receptors by a variety of biochemical and pharmacological functional assays with intact human platelets using a series of pure (HPLC criteria: $>99\%$) nucleotides in combination with short incubation times and other inhibitors of ADP stimulated pathways such as thienopyridines (Savi et al., 1996) and prostaglandine E_1 (Geiger and Walter, 1993; Geiger et al., 1994).

In all ADP responses analysed (rapid Ca^{2+}-influx, Ca^{2+}-mobilization from intracellular stores, inhibition of adenlyl cyclase, shape change, aggregation), the purine ring is obviously essential for receptor interaction. Non-purine nucleotides are completely inactive at human platelet purinoceptors (Table 5). Substitution of the 2-position of the purine ring produced a stronger agonist while substitution at the 8-position resulted in inactive derivatives or even inhibitors (Tables 1–5). This can be explained by a conformational change of the ADP molecule. Substitution at the 8-position leads to a rotation of the purine ring out of the anti conformation towards syn conformation, while large 2-substituents stabilize the anti conformation. Therefore, the platelet ADP receptor(s) appear to prefer the anti conformation of ADP. In addition, there may also be lipophilic interaction of the 2-substituent with the receptor protein. The 6-amino group appears to be essential for receptor interaction due to hydrogen bridges, while the 1- and the 7-positions are less sensitive. Obviously, no essential interaction of the agonists with the ADP receptor take place via the ring nitrogen. Binding of the agonist via the ribose 2′-and 3′-hydroxo groups is necessary for stimulation of rapid Ca^{2+}-influx (ROC), while the activation of Ca^{2+}-mobilization from intracellular stores does not depend on the formation of hydrogen bridges with the ribose ring. However, the rigid structure of the ribose ring is essential in terms of receptor interaction. The acyclic ADP derivative ADP-dialcohol has neither stimulatory nor inhibitory effects on human platelet ADP receptors. The inhibition of human platelet ADP responses by ADP-dialdehyde may be due to the reactivity of the aldehyde groups with proteins. Also, the length of the phosphate chain appears to be essential for receptor interaction. Only diphosphates are able to stimulate aggregation, Ca^{2+}-mobilization and G_i-protein, while polyphosphates do inhibit these responses and monophosphates are inactive. The substitution of the phosphate oxygen by polar substituents only slightly affects the activity of the agonist whereas substitution of the P_1,P_2 bridging oxygen with the unpolar methylene group gives a weak inhibitor of platelet aggregation. There is presently no clear explanation for this effect since the methylene group does not significantly influence both bond angle and polarity of the neighbouring phosphates. Though most adenosine 5′-triphosphate derivatives and adenosine 5′-polyphosphates act as antagonists at the platelet ADP receptors, ATP and AMPCPP stimulated a small extent of rapid Ca^{2+}-influx in agreement with the results of a recent study (MacKenzie et al., 1996). However, the Ca^{2+} responses evoked by ATP and AMPCPP were weaker than the ADP responses at the same concentration (Fig. 5). Nevertheless, it is difficult to rule out in these types of experiments that the effects of ATP and its derivatives on the ligand-gated Ca^{2+} channel of platelets are due to minor impurities present in the derivatives applied and/or produced during the incubation period (e.g., by ectonucleotidases). As mentioned before, here we used only HPLC checked nucleotides (purity $>99\%$) and short incubation times. Commercial nucleotides such as ATP and ATP-γ-S are known to contain biologically active impurities (Geiger and Walter, 1993; Gachet et al., 1997). In our present HPLC analysis, most commercially available ATP derivatives contained ATP and ADP up to 5% (data not shown) requiring additional purification procedures.

The results obtained with dADP clearly indicate that the purinoceptors mediating G_i-protein activation and Ca^{2+}-mobilization differ in their binding characteristics while G_i-protein activation and aggregation responses appear to be similar with all ADP derivatives applied (Figs. 2 and 7; Tables 1, 4 and 5). Therefore, G_i-protein stimulation and platelet aggregation are closely connected.

Our present data with ADP, ATP, and their derivatives already suggest the existence of three different ADP-binding sites on human platelets. All three sites do show high selectivity for ADP, since ATP and ATP derivatives (which are activators for most purinoceptors known) are only relatively weak inhibitors of platelet purinergic responses except for Ca^{2+}-influx. Here, ATP appears to be a weak activator (Fig. 5). Clearly, binding properties of platelet purinergic binding sites responsible for rapid Ca^{2+}-influx, Ca^{2+}-mobilization and adenylyl cyclase inhibition differ in their affinity and specificity for nucleotides. Conferring to the rules for the nomenclature of purinoceptors (Kennedy and Leff, 1995; Fredholm et al., 1997), the ligand-gated Ca^{2+} channel belongs to the P_{2X} receptor family while the receptors involved in Ca^{2+}-mobilization and adenylyl cyclase inhibition (G-protein mediated responses) belong to the P_{2Y} receptor family. However, the pharmacology of human platelet ADP receptors does not indicate any clear similarity to any of the known P_2 receptors. Although our Ca^{2+}-influx data do, in part, resemble the pharmacological profile of P_{2X1} receptors, they do not entirely support the hypothesis put forward by MacKenzie et al. (1996) who suggested that the human platelet P_{2X} receptor is identical to the P_{2X1} receptor already described by Valera et al. (1994). Our experiments show that ATP and AMPCPP are much weaker agonists than ADP at the platelet receptor (Fig. 5), whereas ATP is clearly a stronger agonist than ADP at the authentic P_{2X1} receptor (Valera et al., 1994; Evans et al., 1995). Our observation of an AMPCPP-insensitive component of platelet Ca^{2+}-influx which is stimulated only by ADP perhaps indicates the presence of a

Table 6
Characteristics of human platelet purinoceptors

Sensitivity for:	Ca^{2+}-regulation		G_i-stimulation	Aggregation
	ROC	Mobilization		
ADP	+	+	+	+
ATP	+	−	−	−
AMPCPP	+	−	−	−
Suramin	−	0	0	−
PPADS	N.D.	0	0	−
Ticlopidine	0	0	−	−
Desensitization	+	+/0	0	0

Summary of the effects of ADP, ATP, AMPCPP, suramin PPADS and ticlopidine treatment and of desensitization with respect to human platelet purinergic responses. (+)-signs indicate stimulatory, (−)-signs indicate inhibitory, (0) no effects on human platelet purinergic responses.

heteromultimeric assembly of purinoceptors in human platelets which has been described recently for other systems (Radford et al., 1997).

With respect to P_{2Y} responses, the only P_{2Y} purinoceptors known to be activated by ADP are the P_{2Y1} and P_{2Y3} subtypes (Schachter et al., 1996; Webb et al., 1996). But even at these purinoceptors, ATP acts more as an agonist rather than antagonist. Recently, Gachet et al. (1997) proposed the P_{2Y1} receptor as the major, if not only, P_{2Y} receptor present on human platelets. Our data suggest the existence of two different binding sites mediating either Ca^{2+}-mobilization or adenylyl cyclase down regulation. However, pharmacological data alone cannot distinguish between the possibilities that these G-protein coupled responses are mediated by distinct receptors or by only one receptor whose binding characteristics changes upon binding to different G-proteins. Evidence was presented for mice that a common G-protein (G_q) mediates both Ca^{2+}-mobilization and aggregation (Offermanns et al., 1997), supporting our present conclusion that two different P_{2Y} receptors are present on human platelets, although species differences are possible. However, the possibility of two distinct P_{2Y} receptors is also supported by the observed effects of the thienopyridine ticlopidine. Ticlopidine treatment inhibited ADP-induced platelet aggregation (Fig. 3) and ADP-evoked adenylyl cyclase inhibition (Savi et al., 1996; data not shown) but did not affect ADP-induced rapid Ca^{2+}-influx and Ca^{2+}-mobilization from intracellular stores (Fig. 6). Whereas a pretreatment with a cAMP-elevating agent such as prostaglandine E_1 can distinguish between ROC Ca^{2+}-influx and Ca^{2+}-mobilization (Geiger and Walter, 1993; Geiger et al., 1994), a pretreatment with a thienopyridine (ticlopidine or clopidogrel) can distinguish ADP responses coupled to Ca^{2+}-mobilization and adenylyl cyclase inhibition. The extremely high concentrations of other purinoceptor antagonists required to inhibit platelet ADP responses (Tables 4–6) indicate a low specificity of these drugs. It is possible that these antagonists do not interact directly with the ADP receptors at the agonist-binding site but affect the receptor conformation or a later step of the signal-transduction cascade. For suramin and PPADS, it has already been shown by binding assays that the competition with ATP is not competitive but allosteric (Michel et al., 1997).

As summarized in Fig. 8, our data provide evidence for the existence of at least three different functional nu-

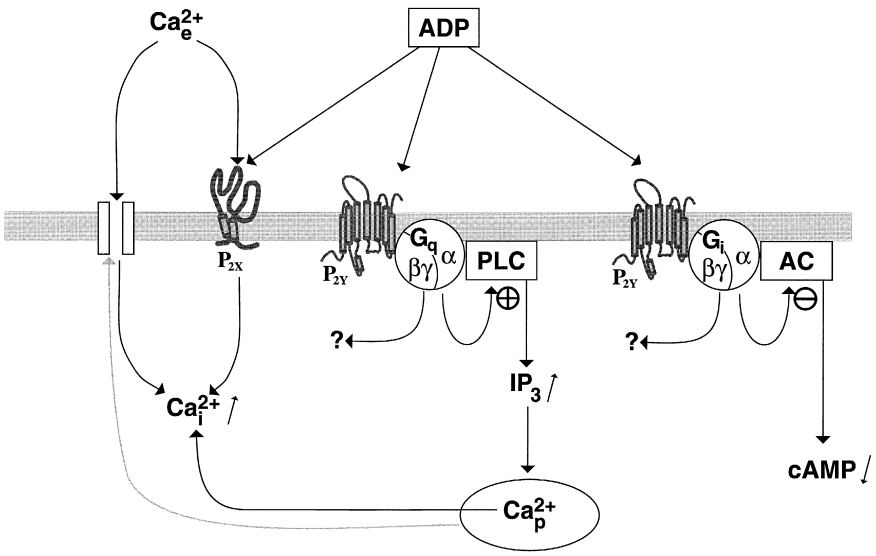

Fig. 8. Pathways affected by ADP receptors in human platelets. ADP activates a P_{2X} receptor-operated Ca^{2+} channel on the platelet membrane (ROC) leading to a fast Ca^{2+}-influx (Ca_e^{2+} (Ca_i^{2+})). Additionally, phospholipase C (PLC) is activated via G-protein stimulation by a P_{2Y} receptor on the plasma membrane. PLC activation leads to the formation of inositolphosphates (IP_3) and liberation of intracellular bound (Ca^{2+}-pools) Ca^{2+}-ions (Ca_p^{2+}). Further Ca^{2+} influx through a store-dependent Ca^{2+} channel by a yet unknown mechanism is then induced by the loss of Ca^{2+}-ions from the intracellular stores. Activation of G_i-proteins by stimulation of another P_{2Y} receptor results in an inhibition of adenylyl cyclase (AC), leading to a reduction of intracellular cAMP levels. Functional and pharmacological evidence indicates that these ADP effects are mediated by three distinct purinoceptors.

cleotide binding sites on human platelets. One important unresolved issue is the coupling of these platelet purinoceptors to G-proteins and G-protein effector systems. Human platelets are known to express heterotrimeric G-proteins of all four major subfamilies which, upon activation, release the different classes of G_α subunits (G_s, G_i, G_q and G_{12}) and concomitantly the $\beta\gamma$-complexes (Koesling and Nürnberg, 1997). Receptors which directly inhibit platelets (receptors for adenosine, β-adrenergic agents, prostacyclin, prostaglandine E_1) stimulate adenylyl cyclase via G_s-proteins, while platelet activating receptors are thought to couple to more than one G-protein, i.e., G_q, G_{12}, G_{13}, G_{i2}, G_{i3} (in the case of thrombin), G_q, G_{12}, G_{13} (in the case of thromboxane A_2) and G_{i2} (in the case of ADP) although it should be emphasized that many of these conclusions are based on in vitro experiments with platelet membranes (Ohlmann et al., 1995; Koesling and Nürnberg, 1997). This distinct, platelet-specific pattern of G-protein coupling may be the basis why a ticlopidine/clopidogrel treatment (which impairs the ADP inhibition of adenylyl cyclase) selectively impairs the ADP-induced aggregation with no or minor effect on thrombin or thromboxane A_2-induced aggregation of human platelets, respectively (Fig. 4; see also Savi et al. (1996)). The observation that ticlopidine treatment neither affects ADP-stimulated rapid Ca^{2+}-influx (ROC) and Ca^{2+}-mobilization from intracellular stores (Fig. 6) nor ADP-induced shape change (Fig. 3) but impairs ADP-evoked aggregation and adenylyl cyclase inhibition strongly suggests a prominent role for an ADP-activated, G_i-protein coupled purinoceptor with respect to the activation of human platelet aggregation. Whether this ADP receptor which remains to be identified at the molecular level achieves its effect on aggregation by the G-protein alpha subunits (α_{i2}, α_{i3}) or by $\beta\gamma$-complexes remains to be elucidated. The activation and release of the α_{i2}/α_{i3}-subunits would inhibit the adenylyl cyclase, decrease the intracellular level of cAMP which is associated with a decreased extent of vasodilator stimulated phosphoprotein (VASP) phosphorylation (Geiger, unpublished experiments). Interestingly, the extent of vasodilator stimulated phosphoprotein phosphorylation closely correlated with the inhibition of platelet aggregation (Horstrup et al., 1994). Alternatively, the release of β-complexes are known to separately stimulate phospholipase C, in particular PLC β_2 and PLC β_3, (Koesling and Nürnberg, 1997) but also interact with other targets such as phosphoinositide 3-kinases (Thomason et al., 1994). Recently, further evidence was provided that phosphoinositide 3-kinase activation is an important component for integrin $\alpha_{IIb}\beta_3$ activation and associated platelet aggregation (Zhang et al., 1996). Although our data presented here strongly suggest the presence of 3 distinct human platelet ADP receptors as summarized in Fig. 8, it remains a challenging task to identify the molecular details of these three receptors and their signal transduction pathways coupled to the various aspects of platelet activation.

5. Note added in proof

During the review/revision process of this paper, three publications appeared dealing with related topics. Jin and Kunapuli (1998) and Daniel et al. (1998) also presented pharmacological evidence for the presence of three distinct platelet ADP receptors and actually identified a new P_{2Y1} receptor which mediates ADP-induced calcium mobilization. Savi et al. (1998) also demonstrated the presence of a P_{2Y1} receptor on human platelets and showed that activation of this receptor is not sufficient for platelet aggregation. All three papers and our present paper, although based on different experimental approaches and results, support the concept of three distinct ADP receptors on human platelets.

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