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Antagonists of platelet fibrinogen receptor are less effective in carriers of Pl^{A2} polymorphism of β_3 integrin

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Abstract

The polymorphism $Leu^{33} \rightarrow Pro$ (platelet-specific antigen; $Pl^{A1/A2}$) of platelet GPIIIa is a potential risk factor for arterial thrombosis. However, its influence on platelet function remains controversial and little is known about its impact on platelet sensitivity to GPIIb-IIIa antagonists. Our objective was to compare the effectiveness of various GPIIb-IIIa antagonists in $Pl^{A2}(+)$ and $Pl^{A2}(-)$ carriers. Platelet aggregation was monitored in healthy donors including $Pl^{A2}(-)$ ($N=31$) and $Pl^{A2}(+)$ subjects ($N=27$; 23 $Pl^{A1/A2}$, 4 $Pl^{A2/A2}$) using the impedance and turbidimetric aggregation techniques. We evaluated the inhibition of ADP- and collagen-induced aggregation by disintegrins, kistrin and echistatin, and the low-molecular-weight blockers, GR144053F ((4-[4-[4-(aminoiminomethyl]-1-piperazinyl]-1-piperidineacetic acid, hydrochloride trihydrate) and eptifibatide (N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-propyl-L-cysteinamide, cyclic (1 → 6)-disulfide). Kistrin (10–30 nmol/l) inhibited ADP- and collagen-induced aggregation stronger in $Pl^{A2}(-)$ donors than in $Pl^{A2}(+)$ donors; there was a significant difference between 50% inhibitory concentrations (IC_{50}). The same tendency occurred with moderate concentrations of eptifibatide (40–100 nmol/l) and also at low concentrations of GR144053F (5–10 nmol/l) and high concentrations of echistatin (80–150 nmol/l), although in the case of the two latter inhibitors, the estimated IC_{50} values were not significantly different. In conclusion, GPIIb-IIIa blockers representing various classes are less effective inhibitors of platelet aggregation in $Pl^{A2}(+)$ carriers; however, the effect of the genotype is both agonist- and antagonist-dependent.

Keywords: GPIIb-IIIa antagonist; Disintegrin; Integrilin; Eptifibatide; GR144053F; Polymorphism $Pl^{A1/A2}$ (platelet-specific antigen)

1. Introduction

The heterodimer glycoprotein Iib-IIIa (GPIIb-IIIa; $\alpha_{IIb}\beta_3$) is the major blood platelet membrane receptor for fibrinogen and is involved in platelet aggregation and adhesion. Since blood platelets are main factors contributing to arterial thrombosis, GPIIb-IIIa has some time ago become an attractive target for blockade with various antagonists. The general action of GPIIb-IIIa antagonists is to block the receptor, which leads to the inability of activated platelets to bind fibrinogen and thus prevents platelet aggregation and thrombus formation (Nurden, 1996; Coller, 1997). The subunit β_3 (GPIIIa) of this platelet membrane integrin appears to be dimorphic at the position of $Leu^{33} \rightarrow Pro$, which results from a single nucleotide $C^{1565} \rightarrow T$ transition in the GPIIIa gene and is commonly known as platelet-specific antigen ($Pl^{A1/A2}$) polymorphism (Newman et al., 1989).

Weiss et al. (1996), based on the observation of the higher frequency of the Pl^{A2} allele in patients with unstable angina and myocardial infarction in comparison with healthy donors, suggested that the carriers of the Pl^{A2} allele may be at potentially increased risk of arterial thrombosis. Since then, however, conflicting results on the role of this polymorphism have been reported in a number of case-control studies. Many investigators found no differences in $Pl^{A1/A2}$ variant frequency between healthy donors and patients with myocardial infarction (Samani and Lodwick, 1997; Ridker et al., 1997; Hato et al., 1997; Herrmann et al., 1997; Zhu et al., 2000; Durante-Mangoni et al., 1998; Gardemann et al., 1998), ischaemic heart disease (IHD) (Corral et al., 1997; Ridker et al., 1997; Durante-Mangoni et al., 1998; Mamotte et al., 1998) or stroke (Corral et al., 1997; Ridker et al., 1997; Carlsson et al., 1997). On the other hand, in other reports, a higher

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prevalence of the Pl^{A2} allele was demonstrated in stroke (Carter et al., 1998; Wagner et al., 1998), ischaemic heart disease and myocardial infarction (Carter et al., 1997; Durante-Mangoni et al., 1998; Goldschmidt-Clermont et al., 1999).

Little is known about an influence of the $Pl^{A1/A2}$ polymorphism on functional platelet parameters, such as ligand binding, reactivity and sensitivity to antiplatelet agents. Articles concerning the functional differences between platelets from the carriers of various allelic forms of the $Pl^{A1/A2}$ polymorphism, most of which focused on differences in the binding of naturally occurring ligands (mainly fibrinogen), are scarce and the findings are inconsistent. Some authors found moderately but significantly enhanced binding of fibrinogen to Pl^{A2}-positive cells (Goodall et al., 1999; Vijayan et al., 2000), while others failed to observe any association (Meiklejohn et al., 1999; Corral et al., 1997; Michelson et al., 2000). Despite a number of published reports pointing to either hyperreacivity (Feng et al., 1999; Michelson et al., 2000) or hyporeactivity (Andrioli et al., 2000; Lasne et al., 1997) of Pl^{A2}-positive platelets, there exist only incidental studies focusing on the association between Pl^{A} genotype and platelet sensitivity to antiplatelet drugs and GPIIb-IIIa blockers (Michelson et al., 2000).

The aim of our study was to investigate whether the occurrence of various allelic forms of the $Pl^{A1/A2}$ GPIIIa polymorphism affects platelet sensitivity to various GPIIb-IIIa antagonists, including disintegrins (kistrin and echistatin) and synthetic arginine-glutamate-aspartate (RGD) peptidomimetics:GR144053F((4-[4-[4-(aminoiminomethyl]-1-piperazinyl]-1-piperidineacetic acid, hydrochloride trihydrate) and eptifibatide ([N^6- (aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-propyl-L-cysteinamide, cyclic (1 → 6)-disulfide]; Integrilin).

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma (St. Louis, MO, USA) unless stated otherwise. Collagen fibrils (type I) from equine tendons were from Chrono-Log (USA). GR144053F (4-[4-[4-(aminoiminomethyl]-1-piperazinyl]-1-piperidineacetic acid, hydrochloride trihydrate) was a kind gift from Glaxo Wellcome (UK). Eptifibatide ([N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-propyl-L-cysteinamide,cyclic (1 → 6)-disulfide]; Integrilin) was from COR Therapeutics (CA, USA).

2.2. Blood donors

We genotyped a group of healthy volunteers ($N=286$; 139 women and 147 men; mean age ± S.D. = 30 ± 12 years) of Polish origin. The distribution of genotypes was $Pl^{A1/A1}$ 76.5%, $Pl^{A1/A2}$ 21.7%, $Pl^{A2/A2}$ 1.8%, and allele frequencies were Pl^{A1} 0.873, Pl^{A2} 0.127. Studies of platelet aggregation were performed in two subgroups of the whole population, referred to as Pl^{A2}-negative ($Pl^{A1/A1}$) ($N=31$) and Pl^{A2}-positive individuals ($N=27$; 23 $Pl^{A1/A2}$, 4 $Pl^{A2/A2}$). The subgroups were matched according to sex and age and their characteristics were $Pl^{A1/A1}$ subjects ($N=31$, 14 women and 17 men; mean age ± S.D. = 26.4 ± 4.0 years) and Pl^{A2}-positive donors ($N=27$; 23 $Pl^{A1/A2}$, 4 $Pl^{A2/A2}$; 12 women and 15 men; mean age ± S.D. = 25.2 ± 4.0 years). $Pl^{A1/A2}$ polymorphism was genotyped using the polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) method, as described previously (Ridker et al., 1997). Experiments were performed in a pair-wise mode. None of the selected individuals had a history suggestive of an underlying haemostatic disorder, and within 10 days prior to the study, none of them was known to have taken aspirin or any other drug that affects platelet function. All donors were nonsmokers. Blood was collected on 0.105 mol/l sodium citrate under the guidelines of the Helsinki Declaration for human research, and the studies were approved by the committee on the Ethics of Research in Human Experimentation at Medical University of Łódź.

2.3. Whole blood aggregometry

In whole blood, platelet aggregation was studied by using an impedance method with Whole Blood Aggregometer Chrono-Log 592 and two concentrations of collagen (2 and 5 μg/ml) as a platelet agonist. Measurements were taken according to the Chrono-Log protocol (Ray et al., 1994). Shortly, whole blood diluted 1:1 with 0.85% saline was incubated with the respective amount of a given antagonist for 6 min at 37 °C, then supplemented with an agonist, and the impedance, reflecting the extent of platelet aggregation, was read after an additional 6 min. The extent of platelet aggregation inhibition was calculated for various concentrations of the used antagonists.

2.4. Turbidimetric aggregation assay

Platelet aggregation was monitored according to the microplate method (Walkowiak et al., 1997) in platelet-rich plasma obtained by centrifugation (190 × g, 15 min at 37 °C). Briefly, wells of microtiter plates were loaded with 150-μl platelet-rich plasma and supplemented with the appropriate amount of a given blocker; the total volume was adjusted to 300 μl with 0.85% saline. The plate was incubated for 10 min at 37 °C, and then the content of each well was transferred to a fresh well containing a given platelet agonist to give the final agonist concentration of either 20 μmol/l ADP or 10 μg/ml collagen. Subsequently, platelet aggregation was monitored for 22 min at 12-s intervals (including 4-s agitation) as a decrease in extinc-

tion read at 655 nm. The rates of platelet aggregation inhibition at a given concentration of either antagonist were calculated from the maximal decrease in $A_{655\ nm}$ recorded for the control samples (no antagonist present), which was considered as 100% aggregation (and 0% inhibition). All the measurements were performed in duplicate for each blood donor, and data gathered for each platelet-rich plasma sample were used for further analysis as described below.

2.5. Statistical analysis

Means ± S.E.M. are given for all parameters. Data which showed a right-skewed distribution and met the remaining criteria of normal distribution were transformed logarithmically and analysed with the relevant parametric tests. Variables for which the transformed data showed departures from normality or homoscedasticity (according to Shapiro–Wilk's and Levene's tests, respectively) were analysed using Mann–Whitney *U*-test, otherwise Student's *t*-test was applied. Raw data gathered for each platelet-rich plasma sample incubated with increasing concentrations of antagonists were used to generate inhibition curves and to estimate the individual 50% inhibitory concentration (IC_{50}) values, using a nonlinear estimation (quasi-Newton algorithm) (Zar, 1984). Bioequivalence tests were applied to assess differences between inhibition curves for $PI^{A2}(-)$ and $PI^{A2}(+)$ donors. According to the accepted statistical consensus, the compared curves were considered different if at least one of the coefficients of the bioequivalence analysis indicated statistical dissimilarity, i.e. $f_{differences} < 15\%$ or/and $f_{similarities} > 50\%$ (Simpfendorfer et al., 1997).

3. Results

3.1. Whole blood aggregometry

In whole blood, platelets from PI^{A2}-positive individuals gave a slightly stronger response to collagen than platelets from PI^{A2}-negative individuals (15.4 ± 1.9 Ω vs. 11.1 ± 1.5 Ω, respectively, for 2 μg/ml collagen and 17.7 ± 2.1 Ω vs. 14.3 ± 1.8 Ω, respectively, for 5 μg/ml collagen); however, the differences were not statistically significant.

The lowest concentration of echistatin (10 nmol/l) was shown to be ineffective in blocking whole blood platelet aggregation, whereas kistrin at its highest concentration (100 nmol/l) nearly completely blocked the platelet response. Therefore, in further experiments, we applied only the concentrations of 50 and 100 nmol/l echistatin or 10 and 50 nmol/l kistrin. At 10 nmol/l, kistrin was able to block platelet aggregation significantly stronger in PI^{A2}-negative donors than in PI^{A2}-positive individuals, whereas no statistically significant differences between the two polymorphic groups were found for 50 nmol/l kistrin and either 50 or 100 nmol/l echistatin (Fig. 1).

3.2. Platelet-rich plasma aggregometry

3.2.1. $PI^{A1/A2}$ genotype and effectiveness of kistrin

Using the microplate turbidimetric method of platelet aggregation, we demonstrated that the ability of kistrin to inhibit platelet aggregation discriminated well between the polymorphic groups. The significant differences in the efficacy to block the platelet response to ADP, observed between PI^{A2}-positive and PI^{A2}-negative donors over a wide

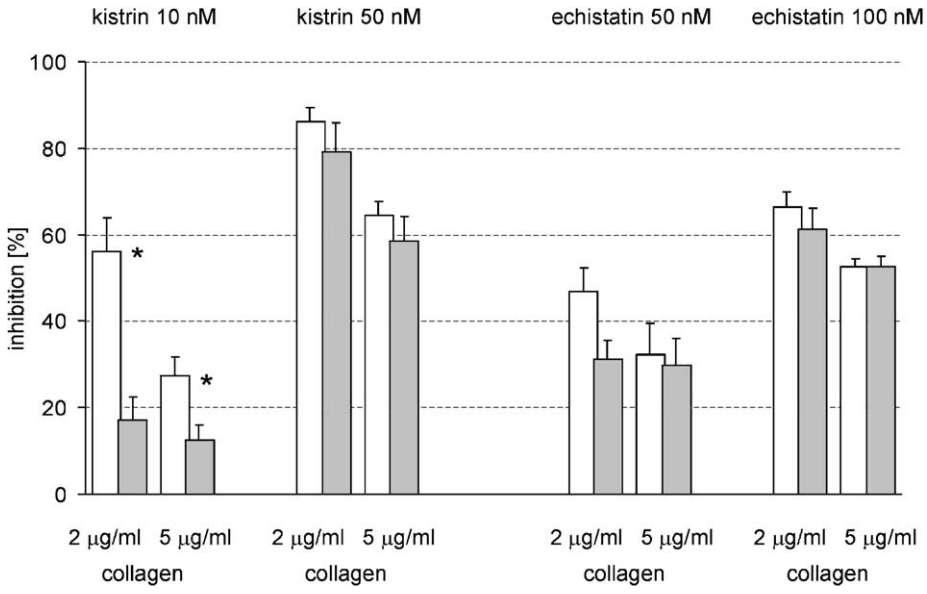

Fig. 1. Inhibition of collagen-induced (2 and 5 μg/ml) platelet aggregation by kistrin and echistatin monitored by whole blood aggregometry in blood from PI^{A2}-negative (white bars, $N = 11$) and PI^{A2}-positive individuals (grey bars, $N = 10$). Error bars indicate S.E.M. (*) $P < 0.01$ as estimated by Mann–Whitney *U*-test.

range of kistrin concentrations (1–100 nmol/l), resulted in significantly higher IC_{50} values in the PI^{A2}-positive donors (Fig. 2A). Likewise, when platelet aggregation was evoked by collagen, the overall efficacy of kistrin was significantly higher in PI^{A2}-negative donors (significantly lower IC_{50}), although the particular differences remained significant only at kistrin concentrations of 10 and 30 nmol/l (Fig. 2B). The significantly higher IC_{50} values revealed in PI^{A2}-positive donors for both platelet agonists corresponded well with the outcomes of bioequivalence tests, which enabled us to compare the characteristics of the inhibition curves obtained for PI^{A2}-positive and PI^{A2}-negative donors for both platelet agonists. The coefficient of differences ($f_{differences}$) exceeded 15% (24.7% and 23.9% for ADP and collagen, respectively), whereas the coefficient of similarities ($f_{similarities}$)

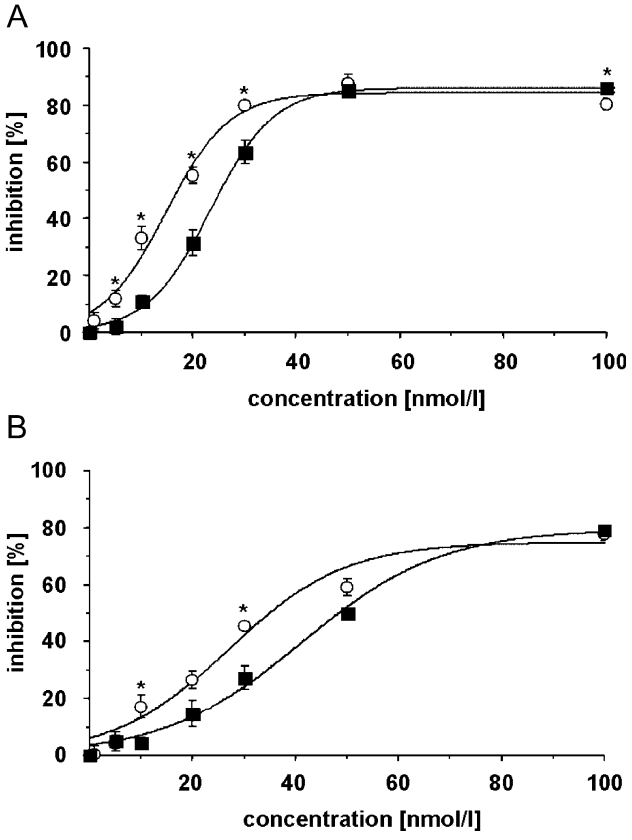

Fig. 2. Inhibition of ADP-induced (A) and collagen-induced (B) platelet aggregation by kistrin monitored by turbidimetric method in platelets from PI^{A2}-negative ($N=8$) (open circles) and PI^{A2}-positive donors ($N=8$) (solid squares). Each point represents the group-averaged extent of inhibition at a given antagonist concentration. Sigmoidal inhibition curves were plotted based on the calculation of nonlinear regression coefficients, obtained for each group of donors using a quasi-Newton algorithm for nonlinear estimation. Error bars indicate S.E.M. Significance of differences estimated by Student's *t*-test (*) or Mann–Whitney *U*-test were as follows:

Kistrin [nmol/l]	1	5	10	20	30	50	100
ADP	0.06	0.02*	0.0006	0.0003	0.0001	n.s.	0.03
Collagen	n.s.	n.s.	0.04	n.s.	0.01	n.s.	n.s.

Points with significant differences between groups marked with asterisks. The IC_{50} calculated for platelets from PI^{A2}(+) and PI^{A2}(−) individuals were (mean ± S.E.M.) 23.5 ± 1.9 nmol/l vs. 14.8 ± 1.9 nmol/l; $P<0.006$ (ADP) and 41.5 ± 6.0 nmol/l vs. 27.2 ± 3.8 nmol/l; $P<0.01$ (collagen), respectively; significance of differences estimated by Student's *t*-test (ADP) or Mann–Whitney *U*-test (collagen). The coefficients of bioequivalence analysis were:

$f_{differences}$: 24.7% (ADP); 23.9% (collagen)

$f_{similarities}$: 41.6% (ADP); 49.6% (collagen).

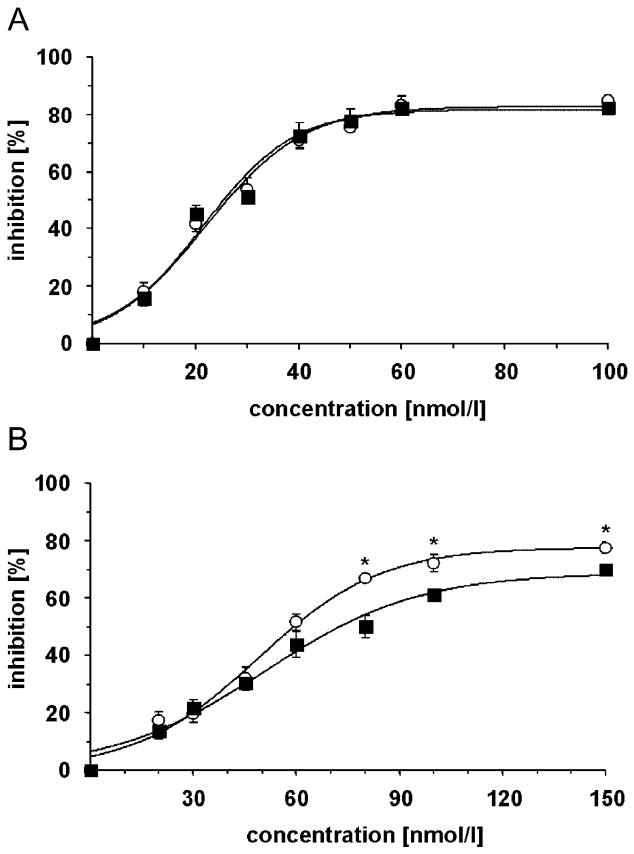

Fig. 3. Inhibition of ADP-induced (A) and collagen-induced (B) platelet aggregation by echistatin monitored by turbidimetric method in platelets from PI^{A2}-negative ($N=8$) (open circles) and PI^{A2}-positive donors ($N=6$) (solid squares). Each point represents the group-averaged extent of the inhibition at a given antagonist concentration. Error bars indicate S.E.M. Sigmoidal inhibition curves estimated as described in Fig. 2. Differences for ADP-induced platelet aggregation were insignificant. Significance of differences for platelets stimulated with collagen, estimated by Student's *t*-test, was as follows:

Echistatin [nmol/l]	1	20	30	40	60	80	100	150
	n.s.	n.s.	n.s.	n.s.	n.s.	0.02	0.04	0.02

Points with significant differences between groups are marked with asterisks. The IC_{50} values for platelets from PI^{A2}(+) and PI^{A2}(−) individuals were (mean ± S.E.M.) 21.5 ± 2.8 nmol/l vs. 22.2 ± 3.0 nmol/l; n.s. (ADP) and 50.8 ± 5.8 nmol/l vs. 48.8 ± 3.9 nmol/l; n.s. (collagen), respectively; significance of differences estimated by Student's *t*-test. The coefficients of bioequivalence analysis were:

$f_{differences}$: 3.6% (ADP); 14.9% (collagen)

$f_{similarities}$: 79.7% (ADP); 52.8% (collagen).

remained lower than 50% (41.6% and 49.6% for ADP and collagen, respectively), which allowed us to reason that the kistrin-mediated inhibition of platelet aggregation stimulated by either ADP or collagen was different in PI^{A2}-negative and PI^{A2}-positive individuals.

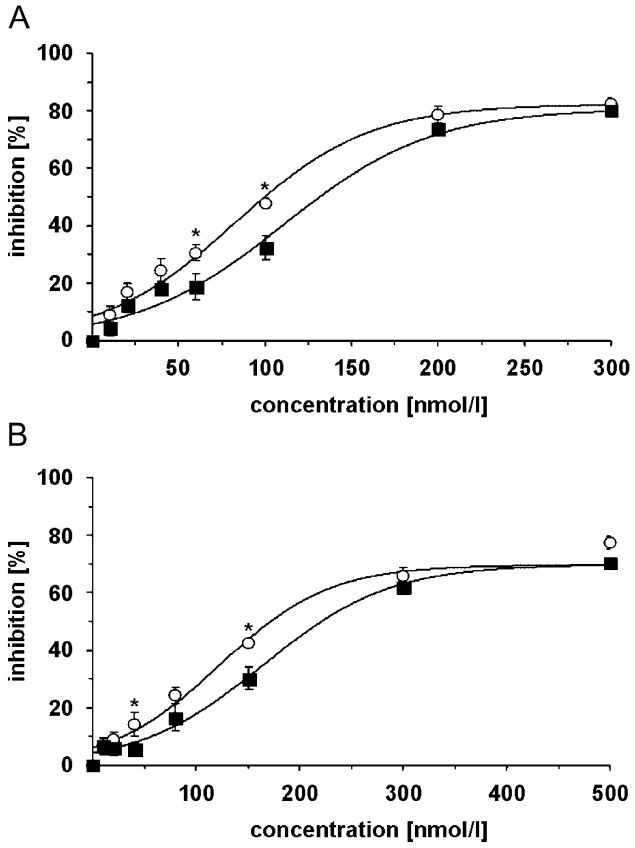

Fig. 4. Inhibition of ADP-induced (A) and collagen-induced (B) platelet aggregation by eptifibatide monitored by turbidimetric method in platelets from PI^{A2}-negative ($N=8$) (open circles) and PI^{A2}-positive donors ($N=8$) (solid squares). Each point represents the group-averaged extent of the inhibition at a given antagonist concentration. Error bars indicate S.E.M. Sigmoidal inhibition curves estimated as described in Fig. 2. Significance of differences, estimated by Student's *t*-test (*) or Mann–Whitney *U*-test, was as follows:

Eptifibatide [nmol/l]	10	20	40	60	100	200	300
ADP	n.s.	n.s.	n.s.	0.009 *	0.02 *	n.s.	n.s.
Eptifibatide [nmol/l]	10	20	40	80	150	300	500
Collagen	n.s.	n.s.	0.02 *	n.s.	0.06 *	n.s.	n.s.

Points with significant differences between groups marked with asterisks. The IC_{50} values for platelets from PI^{A2}(+) and PI^{A2}(−) individuals were (mean ± S.E.M.) 110.9 ± 11.3 nmol/l vs. 82.9 ± 9.5 nmol/l; $P<0.02$ (ADP) and 165.3 ± 21.4 nmol/l vs. 123.0 ± 9.0 nmol/l; $P<0.05$ (collagen), respectively; significance of differences estimated by Student's *t*-test. The coefficients of bioequivalence analysis were:

$f_{differences}$: 17.6% (ADP); 15.4% (collagen)

$f_{similarities}$: 53.9% (ADP); 59.1% (collagen).

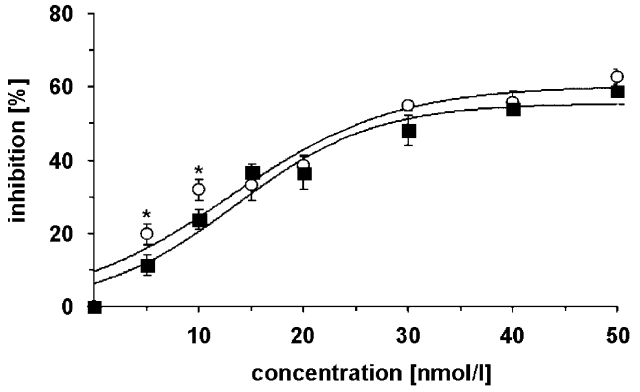

Fig. 5. Inhibition of ADP-induced platelet aggregation by GR144053F monitored by turbidimetric method in platelets from PI^{A2}-negative ($N=7$) (open circles) and PI^{A2}-positive donors ($N=7$) (solid squares). Each point represents the group-averaged extent of the inhibition at a given antagonist concentration. Error bars indicate S.E.M. Sigmoidal inhibition curves estimated as described in Fig. 2. Significance of differences, estimated by Mann–Whitney *U*-test, was as follows:

GR144053F [nmol/l]	5	10	15	20	30	40	50
	0.02	0.04	n.s.	n.s.	n.s.	n.s.	n.s.

Points with significant differences between groups marked with asterisks. The IC_{50} values for platelets from PI^{A2}(+) and PI^{A2}(−) individuals were (mean ± S.E.M.) 13.6 ± 3.3 nmol/l vs. 13.1 ± 3.0 nmol/l, n.s., respectively; significance of differences estimated by Student's *t*-test. The coefficients of bioequivalence analysis were:

$f_{differences}$: 11.8%

$f_{similarities}$: 62.3%.

3.2.2. $PI^{A1/A2}$ genotype and effectiveness of echistatin

Another tested disintegrin, echistatin (10–150 nmol/l), inhibited platelet aggregation induced by ADP to approximately the same extent in both groups of donors, and the estimated IC_{50} values were not statistically different (Fig. 3A). However, the similarities between the groups of PI^{A2}-negative and PI^{A2}-positive donors appeared much less distinct when platelets were activated with collagen, especially at high concentrations of the antagonist (80–100–150 nmol/l), when platelet aggregation remained significantly more inhibited in PI^{A2}-negative individuals (Fig. 3B). Nevertheless, the overall efficacy of echistatin remained not statistically different in both genotype groups, as evidenced by the comparison of the IC_{50} values and given by the estimates of bioequivalence analysis of the inhibition curves for PI^{A2}-positive and PI^{A2}-negative donors: $f_{differences}<15\%$ (3.6% and 14.9% for ADP and collagen, respectively) and $f_{similarities}>50\%$ (79.7% and 52.8% for ADP and collagen, respectively) (Fig. 3B).

3.2.3. $PI^{A1/A2}$ genotype and effectiveness of eptifibatide

The effect of eptifibatide was tested over the range of antagonist concentrations of either 10–300 or 10–500 nmol/l for ADP- and collagen-induced aggregation, respec-

tively. The antagonist significantly inhibited both ADP- and collagen-induced platelet aggregation, and its efficacy was significantly higher in the noncarriers of the Pl^{A2} allele (Fig. 4A and B). For both platelet agonists, the IC_{50} values of the eptifibatide-mediated inhibition of platelet aggregation were significantly greater in Pl^{A2}-positive individuals. Also, the bioequivalence tests indicated that the inhibition curves obtained for Pl^{A2}-positive and Pl^{A2}-negative donors differed for both ADP- and collagen-induced platelet aggregation (Fig. 4).

3.2.4. $Pl^{A1/A2}$ genotype and effectiveness of GR144053F

In addition, we studied the influence of GR144053F on platelet aggregation stimulated with ADP. As demonstrated in the bioequivalence tests, there were no significant differences between inhibition curves obtained for Pl^{A2}-positive and Pl^{A2}-negative donors; $f_{differences} < 15\%$ (11.8%) and $f_{similarities} > 50\%$ (62.3%). Likewise, the mean values of IC_{50} for GR144053F did not differ significantly between Pl^{A2}-positive and Pl^{A2}-negative individuals. However, at the lowest GR144053F concentrations (5 and 10 nmol/l), the antagonist was significantly less effective in blocking the ADP-induced aggregation of platelets from the carriers of the Pl^{A2} allele (Fig. 5).

4. Discussion

In this study, we investigated the association between Pl^{A} genotype and susceptibility of platelets to aggregate in response to various GPIIb-IIIa antagonists, including the naturally occurring snake venom disintegrins, kistrin and echistatin, as well as synthetic, low-molecular weight compounds, such as eptifibatide and GR144053F. Using whole blood and platelet-rich plasma aggregometry, we revealed that kistrin was the most effective inhibitor of platelet aggregation and it was much more effective in Pl^{A2}-negative individuals. Another disintegrin—echistatin—appeared effective at much higher concentrations and discriminated between the polymorphic groups only at high concentrations, and only in the case when platelets were activated with collagen. Such a discrepancy could be reasonably attributed to differences in the structure and properties of both antagonists. Kistrin belongs to the group of long disintegrins (68 amino acids) and contains six disulfide bonds (Adler et al., 1991), whereas echistatin is the shortest of all known disintegrins (49 amino acids), with a distinct stereochemistry and containing only four disulfide bonds (Gray, 1993; Cooke et al., 1992). Since it was demonstrated that the amino acids flanking the common RGD motif of disintegrins can modulate their specific binding to integrins (Rahman et al., 2000), we cannot exclude that the $Leu^{33} \rightarrow$ Pro substitution in the GPIIIa chain divergently modulates the inhibitory action of kistrin and echistatin. Further, we found that eptifibatide, a cyclic heptapeptide, which was designed by mimicking the structure of barbourin—a member of disintegrins (Scarborough, 1999), was much more effective in Pl^{A2}-negative individuals, whereas the carriers of the Pl^{A2} allele were more resistant to the blocker. Noteworthily, the efficacy of eptifibatide, one of a few antiplatelet drugs approved for clinical use in the treatment of cardiovascular disorders (Goa and Noble, 1999), discriminated between Pl^{A2}-positive and Pl^{A2}-negative subjects at concentrations comparable to those occurring in the circulation after intravenous administration of eptifibatide in vivo (Alton et al., 1998). GR144053F, although not accepted for therapy in clinical practice, has been shown to be a potent inhibitor of the GPIIb-IIIa receptor and remains a useful tool for in vitro studies (Matsuno et al., 1998; Rozalski et al., 2001). In our present study, we demonstrated that the inhibitory effects of GR144053 were much stronger in Pl^{A2}-negative subjects but only at low antagonist concentrations, which might reasonably explain the lack of statistically significant differences between the IC_{50} values estimated for $Pl^{A2}(-)$ and $Pl^{A2}(+)$ individuals. The lower molecular weight of GR144053F and a stereochemistry different from that of eptifibatide might also explain the differences in the efficacy of both antagonists.

The mechanism by which the effect of one amino acid substitution in β_3 integrin can modulate the sensitivity of GPIIb-IIIa receptor to some antagonists still remains obscure. It has been established that the $Leu^{33} \rightarrow$ Pro substitution is not located within the RGD binding regions in GPIIIa (Calvete, 1995), and hence any attempts to relate $Pl^{A1/A2}$ polymorphism to the altered function of GPIIb-IIIa might seem unjustified. However, as speculated by Feng et al. (1999), the tertiary structure of GPIIIa, which is maintained by cysteine pairing, causes the Leu/Pro^{33} residue to come into close proximity with GPIIIa binding regions. Thus, it is likely that the substitution of Leu^{33} by Pro, due to the unique chemical properties of the latter, might affect the conformation of this GPIIIa microregion and thus influence the binding of RGD-containing ligands.

Some of the examined GPIIb-IIIa antagonists had a reduced inhibitory efficacy in platelets from the Pl^{A2}-positive donors. The second important finding is that this reduced sensitivity of platelets from Pl^{A2}-positive carriers was antagonist-specific and depended on the type and concentration of the agonist. The latter observation is consistent with some other reports. It was reported (Michelson et al., 2000) that $Pl^{A1/A2}$ heterozygotes were more sensitive to abciximab and aspirin. The increased sensitivity to the anti-aggregatory effect of aspirin has been incidentally reported in other studies (Cooke et al., 1998; Andrioli et al., 2000), whereas quite opposite results, pointing to a higher aspirin resistance in the Pl^{A2}-positive carriers, have been reported by other authors (Szczeklik et al., 2000). It should be noticed, however, that these observations neither question nor oppose our present findings. Abciximab is a large molecule with a structure and chemical characteristics completely distinct from those of the blockers tested in our study. Aspirin, in turn, although it has recognised antiplatelet

activity, is not a GPIIb-IIIa antagonist and its altered efficacy in Pl^{A2} carriers is due to unexplained mechanism(s), which are likely to be divergent from those of GPIIb-IIIa blockers.

Although it was not a major aim of this work, we also made an attempt to analyse the influence of the $Leu^{33} \rightarrow Pro$ substitution in GPIIIa on the rate of collagen-stimulated whole blood platelet aggregation. We did not detect significant alterations between the aggregation rates of platelets from Pl^{A2}-positive and Pl^{A2}-negative individuals. However, a tendency to hyperaggregability associated with the Pl^{A2}-allele was found, particularly at low collagen concentrations. To date, opposing results have been published, showing either hypo- (Lasne et al., 1997) or hyperaggregability (Feng et al., 1999) of Pl^{A2}-positive platelets. Our present results support the reasoning that the impact of the $Leu^{33} \rightarrow Pro$ substitution is subtle and concerns subthreshold concentrations of weak agonists, and thus seems to be detectable only in large cohorts of subjects. Similar conclusions were recently drawn by Vijayan et al. (2000), who studied the adhesive properties of GPIIb-IIIa using CHO and human kidney embryonal 293 cells overexpressing the Pl^{A1} or Pl^{A2} forms of the GPIIb-IIIa. In this model, they demonstrated that the $\alpha_{IIb}\beta_3$-mediated binding of soluble fibrinogen was not different between Pl^{A1} and Pl^{A2} forms. However, the Pl^{A2}-positive cells bound more easily to immobilized fibrinogen, which was related to increased actin polymerization, greater cell spreading, enhanced tyrosine phosphorylation of $pp125^{FAK}$ and greater fibrin clot retraction. Our present work supports the opinion presented by Vijayan et al. that, taking into account the relatively high prevalence of Pl^{A2} in the population, it is not surprising that the functional differences between the polymorphic forms of GPIIIa are modest, simply because more profound alterations would have resulted in the elimination of Pl^{A2}-positive phenotypes by natural selection.

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