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Mechanisms of action of proteinase-activated receptor agonists on human platelets

[1]Ada W.Y Chung, [1]Paul Jurasz, [2]Morley D. Hollenberg & *,[1]Marek W. Radomski

[1]Department of Pharmacology, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada and [2]Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, T2N 4N1, Canada

1 We studied the activation of human platelets by thrombin and proteinase activated receptor (PAR)-activating peptides (PAR-APs) [SFLLRNPNDKYEPF-amide (TRAP), TFLLR-amide (PAR1AP) and AYPGKF-amide (PAR4AP)].

2 PAR agonist-induced platelet aggregation, glycoprotein (GP) Ib and GPIIb/IIIa surface expression and ADP release were measured by light aggregometry, flow cytometry and chemiluminescence.

3 Aggregation inhibitors, including prostacyclin (PGI_2), nitric oxide-releasing agent (S-nitroso-glutathione, GSNO), aspirin, apyrase, and phenanthroline were used to study the susceptibility of PAR agonist-induced aggregation to pharmacological inhibition.

4 Thrombin was the most potent platelet agonist, followed by PAR1AP, TRAP and PAR4AP.

5 The aggregatory potencies of PAR-APs were not modified by the aminopeptidase inhibitor, amastatin.

6 Subthreshold concentrations of PAR1AP potentiated the effects of PAR4AP to stimulate maximal aggregation.

7 Both PGI_2 and GSNO reduced PAR agonist-induced aggregation and diminished GPIIb/IIIa up-regulation.

8 PAR agonist-induced aggregation was aspirin-insensitive indicating a minor role for TXA_2.

9 In contrast, phenanthroline and apyrase significantly enhanced the anti-aggregatory effects of aspirin against thrombin-, PAR1AP- and TRAP-induced aggregation suggesting the involvement of ADP- and MMP-2-dependent pathways.

10 PAR4AP-induced aggregation (but not PAR1AP-induced aggregation) was entirely ADP-dependent (abolished by apyrase) and resistant to phenanthroline (MMP-2-independent).

11 Thus, the mechanisms of PAR1 and 4-induced platelet aggregation are distinct and depend differentially on their ability to interact with pathways of aggregation, along with the subsequent activation of GPIIb/IIIa receptors.

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Abbreviations: ASA, aspirin; GP, glycoprotein; GSNO, S-nitroso-glutathione; PAR, proteinase-activated receptor; PARAP, proteinase-activated receptor activating peptide; PGI_2, prostacyclin

Introduction

Thrombin is a serine proteinase generated during the stimulation of coagulation, in which the circulating clotting factors are activated by a proteolytic cascade. The main event in this cascade is the conversion of soluble fibrinogen into insoluble fibrin that is mediated by thrombin, thus reinforcing the hemostatic plug (Blomback, 1996). Moreover, thrombin plays an important role in platelet activation and aggregation being the most potent platelet agonist *in vitro* (McNicol & Gerrard, 1993). Thrombin initiates a wide range of platelet responses: shape change, the release from platelet granules of ADP, serotonin and thromboxane A_2 (TXA_2), mobilization of the adhesion molecule P-selectin to the platelet surface (Stenberg *et al*., 1985), and the activation of glycoprotein (GP) IIb/IIIa (integrin $\alpha IIb/\beta_3$) (Hughes & Pfaff, 1998). Therefore, thrombin can consolidate the platelet plug, rendering the aggregation irreversible (Blomback, 1996).

In human platelets, thrombin actions are mediated in large part by proteinase-activated receptors (PARs) 1 and 4 (Kahn *et al*., 1998; Vu *et al*., 1991; Xu *et al*., 1998). PARs 1 and 4 belong to Family 1 of the putative 7-transmembrane superfamily of G-protein-coupled receptors (Vu *et al*., 1991; Coughlin, 2000; O'Brien *et al*., 2001; Macfarlane *et al*., 2001). The activation of PARs is unique amongst G-protein-coupled receptors in that the process involves a proteolytic unmasking of a tethered ligand at the amino-terminal extracellular domain of the receptors (Vu *et al*., 1991; Coughlin, 2000). This proteolytic cleavage unmasks a new amino-terminal sequence (SFLLRNPNDKYEPF ... , in human PAR1) that serves as a tethered ligand, binding intramolecularly to the body of the receptor to stimulate the signal transduction pathway (Vu *et al*., 1991; Chen *et al*., 1994). Three of the four known PARs, PAR1, PAR3 and PAR4 are cleaved by

*Author for correspondence at: Department of Pharmacology, 9–50 Medical Sciences Building, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada; E-mail: Marek.Radomski@Ualberta.Ca

thrombin, but only PAR1 and PAR4, which have been identified in human platelets activate an intracellular signal (Nakanishi-Matsui *et al*., 2000). The activation of either PAR1 or PAR4 is sufficient to trigger platelet secretion and aggregation (Kahn *et al*., 1999). Specific agonist peptides have been designed for the thrombin-independent activation of PAR1 and PAR4. They mimic the tethered ligands, presumably by interacting with receptor extracellular domains and forcing the same conformational changes in the body of the receptor that are needed to activate the G-proteins located at the cytoplasmic face of the receptor (Bahou *et al*., 1993; Gerszten *et al*., 1994). Previous studies have shown that these synthetic peptides function as PAR agonists, and generate most of the biological actions of thrombin by a non-proteolytic mechanism (Vassallo *et al*., 1992; Vu *et al*., 1991).

In the present study, synthetic receptor-selective PAR-activating peptides (PAR-APs) were used as the pharmacological probes of PAR functions in human platelets. To activate PAR1, we used the originally described thrombin receptor-activating peptide (TRAP), SFLLRNPNDKYEPF-amide (Vu *et al*., 1991), as well as the more selective PAR1AP, TFLLR-amide (PAR1AP) (Kawabata *et al*., 1999). To stimulate PAR4 (Kahn *et al*., 1998; Xu *et al*., 1998), we used AYPGKF-amide (PAR4AP) that activates neither PAR1 (Faruqi *et al*., 2000) nor PAR2 (Hollenberg *et al*., 1999). We characterized the platelet aggregating effects and the interactions between these peptides and compared these actions with those of thrombin. We also investigated the relative contribution of the TXA_2-, ADP- and matrix metalloproteinase-2 (MMP-2)-dependent pathways to PAR agonist-induced aggregation (Needleman *et al*., 1976; Born, 1966; Sawicki *et al*., 1997). In addition, we studied the effects of these PAR agonists on the surface expression of platelet receptor glycoprotein (GP), GPIIb/IIIa and GPIb. Finally, we investigated the interactions between PAR-APs and the major inhibitors of platelet functions including prostacyclin (PGI_2) and nitric oxide (NO).

Methods

Preparation of human washed platelets

Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Washed platelets (2.5×10^{11} platelets l^{-1}) were isolated from blood as described before (Radomski & Moncada, 1983).

Platelet aggregation

Platelet aggregation was measured by light aggregometry as previously described (Sawicki *et al*., 1997; Jurasz *et al*., 2001) using a whole blood ionized calcium lumi-aggregometer (Chronolog Corp., Havertown, PA, U.S.A.). Washed platelets were preincubated at 37°C for 2 min. Aggregation was initiated by the addition of agonists, and monitored by Aggro-Link software for at least 9 min. For experiments using inhibitors, aggregation was initiated after 1–2 min preincubation with these compounds.

To study the aggregatory potency of PAR agonists, the concentration-response curves for thrombin, TRAP, PAR1 and PAR4APs were generated. The submaximal concentrations of agonists, i.e. the concentrations that gave approximately 95% of the maximal aggregation were used to study the effects of inhibitors of aggregation. The subthreshold concentrations (i.e. non-aggregatory concentrations only promoting shape change) of PAR1 and PAR4 APs were used to study the interactions between PAR1 and PAR4. Results were expressed in per cent changes in maximal light transmission, with 100% representing light transmission of platelet medium alone.

Flow cytometry analysis of platelet receptor glycoproteins Ib and IIb/IIIa

In order to analyse receptor expression on the surface of individual platelets and to minimize platelet activation caused by sample preparation procedures, no stirring or vortexing steps were used. Platelet samples were placed in 12×75 mm polypropylene, round-bottom culture tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The samples were first activated with PAR agonists for 10 min, and then diluted 10 times with physiological saline. In some experiments, platelets were preincubated with inhibitors for 1–2 min prior to the addition of agonists. Platelet samples were then incubated in the dark without stirring for 15 min at room temperature in the presence of saturating concentrations (10 μg ml^{-1}) of one of the three fluorescence-conjugated antibodies, as indicated below. Following incubation, platelets were diluted in FACS Flow fluid and analysed immediately by flow cytometer (Becton Dickinson, CA, U.S.A.) equipped with a 488 nm wavelength argon laser. The 525 nm and 575 nm band pass filters were used for detecting FITC- and PE-fluorescence. The instrument was set up to measure the size (forward scatter), granularity (side scatter) and platelet fluorescence. A two-dimensional analysis gate of forward and side scatter was drawn in order to include single platelets and exclude platelet aggregates and microparticles. Antibody binding was measured by analysing individual platelets for fluorescence. Mean fluorescence intensity (MFI) was determined after correction for cell autofluorescence. For each sample, the fluorescence was analysed using a logarithmic scale, and a fluorescence histogram was obtained for 10,000 individual events. Data were analysed using CELLQUEST software and expressed as arbitrary units of fluorescence or normalized to control.

Measurement of ATP release by chemiluminescence

ADP secreted from dense granules in stimulated platelets was measured by a whole blood ionized calcium lumi-aggregometer as previously described (Radomski *et al*., 1992; Sawicki *et al*., 1997). Briefly, platelets were incubated with luciferin-luciferase reagent (440 luciferase units ml^{-1} and 4 μg ml^{-1} of luciferin) for 2 min at 37°C in order to convert ADP to ATP and generate chemiluminescence. Following incubation, the agonist was added and luminescence was monitored. To quantify the generation of platelet ATP, standard curves were constructed with standard ATP. The data were expressed as nM ATP.

Statistical analysis

All results were given as mean ± s.e.mean of at least three independent experiments. They were analysed by one-way analysis of variance using Prism software (GraphPad, San Diego, CA, U.S.A.). The EC_{50} and IC_{50} values were also calculated using Prism software. P value less than 0.05 was considered to be statistically significant.

Reagents, antibodies and peptides

Collagen, thrombin, ATP standard and luciferin-luciferase reagent were obtained from Chronolog., apyrase, aspirin, prostacyclin, S-nitroso-glutathione, phenanthroline and amastatin, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fluorescein-isothiocyanate (FIT)-conjugated monoclonal mouse antibodies (MoAbs) directed against GPIIb (CD41-FITC) and R phycoerythrin R (PE)-conjugated MoAbs against human platelet GPIb (CD42-PE) were from DAKO Diagnostics Canada Inc. (Ontario, Canada).

Monoclonal antibody directed against activated GPIIb/IIIa (PAC-1-FITC) was purchased from Becton Dickinson Biosciences (Ontario, Canada). TRAP (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-amide) was purchased from Sigma. PAR1AP (Thr-Phe-Leu-Leu-Arg-amide) and PAR4AP (Ala-Tyr-Pro-Gly-Lys-Phe-amide) were synthesized by the University of Calgary Peptide synthesis Facility (Director, Dr Denis McMaster). The purity (>95% by HPLC) and composition of all peptides were verified by mass spectrometry and the concentrations of stock solutions dissolved in 25 mM HEPES buffer were measured by quantitative amino acid analysis. All other reagents were analytical grade.

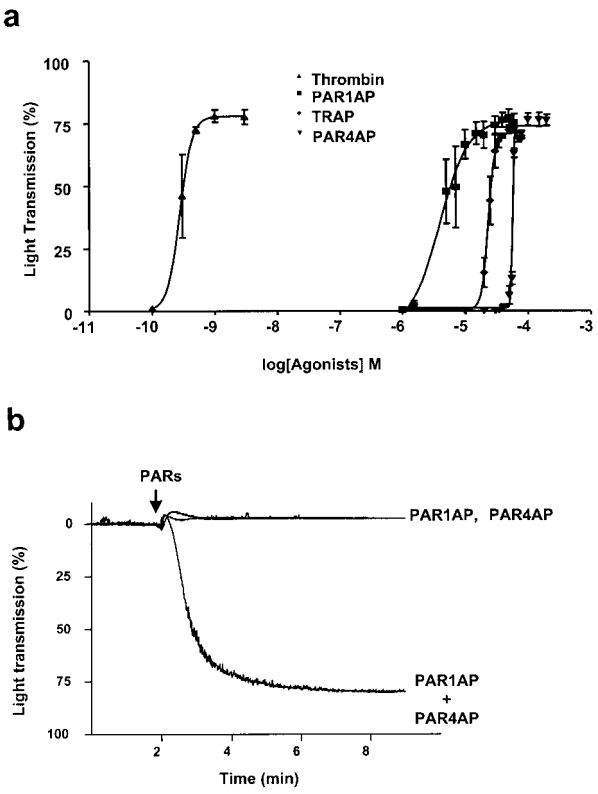

Figure 1 PAR agonist-induced platelet aggregation. Panel a shows concentration-response curves to PAR agonists. Data points are mean ± s.e.mean, $n=5$. Superimposed aggregatory tracings (panel b) demonstrate amplification of platelet aggregation by PAR1AP (3 μM) and PAR4AP (30 μM). Tracings are representative of six similar experiments.

Results

Effects of PAR agonists on platelet aggregation

Figure 1a shows concentration-response curves for thrombin, TRAP, PAR1AP and PAR4 AP. The order of aggregatory potency was: thrombin > PAR1AP > TRAP > PAR4AP, as determined by EC_{50} values of 0.29 nM ± 0.00, 3.9 μM ± 1.1, 24 μM ± 1.7 and 60 μM ± 1.9, respectively, $P<0.05$, $n=5$. Subthreshold concentrations of PAR1AP (3 μM) potentiated the effects of PAR4AP (30 μM) to promote maximal and irreversible platelet aggregation (Figure 1b).

The addition of aminopeptidase inhibitor, amastatin (10 μM), did not significantly affect ($P>0.05$; $n=4$) the aggregatory activity of PAR agonists (EC_{50}s for PAR agonists in the presence of amastatin: TRAP = 24 μM ± 2.1, PAR1AP = 3.7 μM ± 1.6, PAR4AP = 58 μM ± 2.5).

Interactions of PAR agonists with GPIb and GPIIb/IIIa

Stimulation of platelets with thrombin, TRAP, PAR1AP, PAR4AP, as well as with the potentiating combination of PAR1AP and PAR4AP resulted in a significant down-regulation of expression of GPIb (Figure 2a). This reduction was approximately 40% of GPIb levels in unstimulated platelets and was similar for all tested agonists.

In contrast, thrombin and PARAP-induced platelet activation were associated with up-regulation of GPIIb/IIIa expression. Using antibody directed against GPIIb that does not differentiate between the activated and non-activated conformation of GPIIb/IIIa receptors, it was found that PAR agonists resulted in approximately a 50% increase in the surface expression of this receptor (Figure 2b). This up-regulation was similar for all agonist treatments. However, the expression of activated GPIIb/IIIa, as measured with selective (differentiating) PAC-1 antibody, was increased 20–80 times compared to control (Figure 2b).

Effects of PGI_2 and GSNO on PAR agonist-stimulated platelet aggregation and GPIIb/IIIa

Incubation of platelets with PGI_2 or GSNO resulted in a concentration-dependent inhibition of platelet aggregation induced by PAR agonists. Prostacyclin was more potent than GSNO as an inhibitor of PAR agonist-induced aggregation (Table 1 and Figure 3).

Platelet aggregation induced by synthetic PAR agonists was more sensitive to inhibition with PGI_2 than those induced by thrombin and collagen (Table 1). These differences were not detected for inhibition of PAR agonist-induced aggregation by GSNO. Prostacyclin and GSNO inhibited increases in activated and non-activated

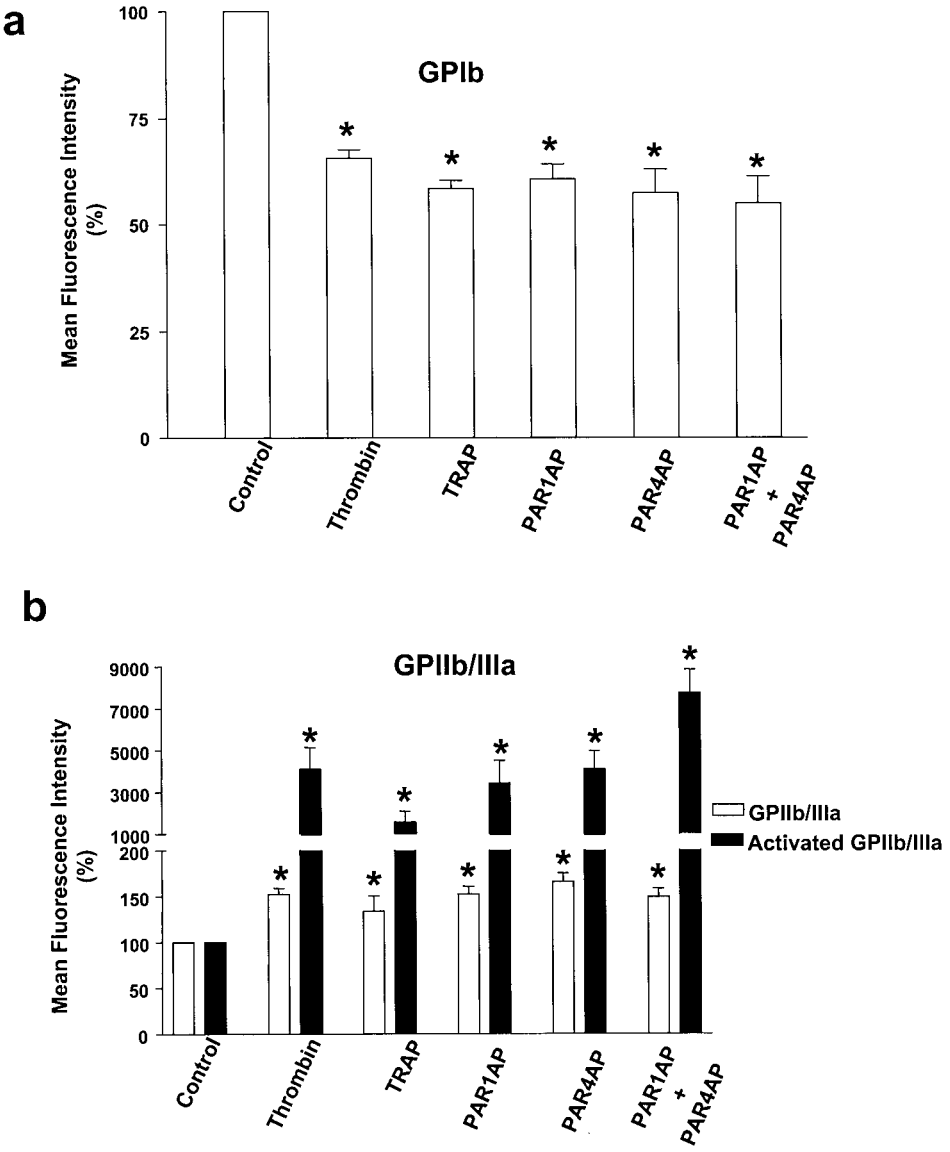

Figure 2 Flow cytometry analysis of GPIb, GPIIb/IIIa and activated GPIIb/IIIa on platelets stimulated with PAR agonists. PAR agonists result in a significant reduction of GPIb (panel a) and up-regulation of GPIIb/IIIa and activated GPIIb/IIIa (panel b). PAR agonists were used at concentrations that resulted in submaximal (95%) aggregatory response (shown in Table 1). Control: unstimulated platelets. Bars are mean ± s.e.mean, $n = 3-9$. $^*P < 0.05$ treatments versus control.

Table 1 IC_{50} for inhibition of PAR agonist- and collagen-induced aggregation by PGI_2 and GSNO

Aggregatory agonists	*PGI_2* (nM)	*GSNO* (μM)
Thrombin (0.1 U ml^{-1})	0.93 ± 0.02	0.34 ± 0.16
TRAP (35 μM)	0.08 ± 0.01*	0.11 ± 0.11
PAR1AP (10 μM)	0.19 ± 0.14*	0.21 ± 0.15
PAR4AP (70 μM)	0.09 ± 0.15*	0.18 ± 0.08
PAR1AP (3 μM) + PAR4AP (30 μM)	0.12 ± 0.10*	0.11 ± 0.09
Collagen (4 μg ml^{-1})	0.73 ± 0.02	0.33 ± 0.09

Data are IC_{50} representing mean ± s.e.mean, $n = 3-7$. $^*P < 0.05$ PARAPs v/s thrombin or collagen.

GPIIb/IIIa in some, but not all PAR agonists (Figure 4). This inhibition was more pronounced for the activated GPIIb/IIIa ($P < 0.05$, $n = 3-9$).

The effects of aspirin, phenanthroline and apyrase on PAR agonist-induced aggregation

To study the dependence of PAR agonist-induced aggregation on the activation of the TXA_2, MMP-2 and ADP-mediated pathways of aggregation, respective inhibitors of these pathways such as aspirin, phenanthroline and apyrase (Sawicki *et al*., 1997; Jurasz *et al*., 2001; Radomski *et al*., 2001) were used. ASA exerted no significant effects on thrombin or PAR-AP-induced aggregation (Figure 5). Phenanthroline partially inhibited thrombin-, PAR1AP-, PAR1AP + PAR4AP- and TRAP-, but not PAR4AP-induced aggregation. The effects of apyrase were most pronounced against PAR4AP-induced aggregation. Moreover, PAR4AP-induced aggregation was abolished by the inhibitor. A combined administration of these inhibitors enhanced the effects of single compounds (Figure 5).

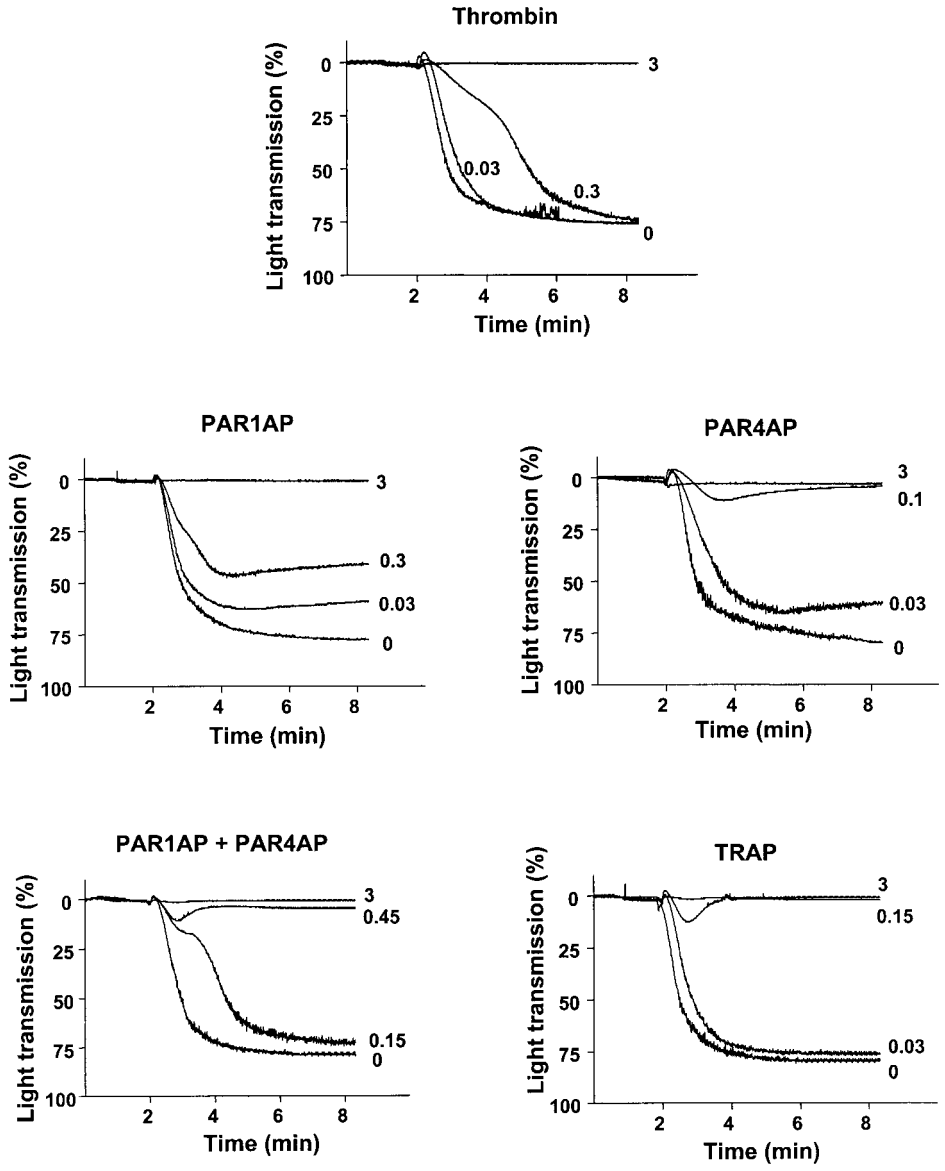

Figure 3 Inhibition of PAR agonist-induced aggregation by PGI_2. Aggregation was induced by submaximal concentrations of PAR agonists. Numbers beside the tracings show the concentrations of PGI_2 in nM. Traces are representative of 3–7 independent experiments.

The release of ADP in PAR4AP-stimulated platelets

Since PAR4AP-induced aggregation was entirely apyrase-sensitive, we studied further, the contribution of ADP pathway to this reaction. PAR4AP-induced aggregation was associated with the generation of ATP and there was a significant correlation ($r^2 = 0.95$, $P<0.05$, $n=3$) between inhibition of aggregation and reduction of ATP release caused by apyrase during PAR4AP-mediated aggregation (Figure 6a–c). Figure 6d shows that apyrase abolished PAR4AP-mediated activation of GPIIb/IIIa.

Discussion

We investigated the mechanisms of actions of PAR agonists including thrombin, TRAP, PAR1AP and PAR4AP on human platelets. These agonists concentration-dependently induced platelet aggregation. To our knowledge, this is the first report showing a comparison of the concentration-response curve for a PAR4AP relative to PAR1 agonists in human platelets. The challenges associated with this classical pharmacological approach are due to the complex nature of interactions between thrombin, PAR peptides, their receptors and the down-stream cellular signalling pathways. In addition, PAR agonists such as SFLLRN can be readily cleaved and inactivated by plasma- and serum- associated aminopeptidase leading to decreased aggregating activity. Indeed, the treatment of platelets with amastatin, an aminopeptidase inhibitor, has been shown to enhance SFLLRN-induced aggregation in platelet-rich plasma (Coller *et al*., 1993). However, in washed human platelets aminopeptidase did not affect the pharmacological activity of the PAR-APs, as shown by the lack of amastatin effect on PAR-

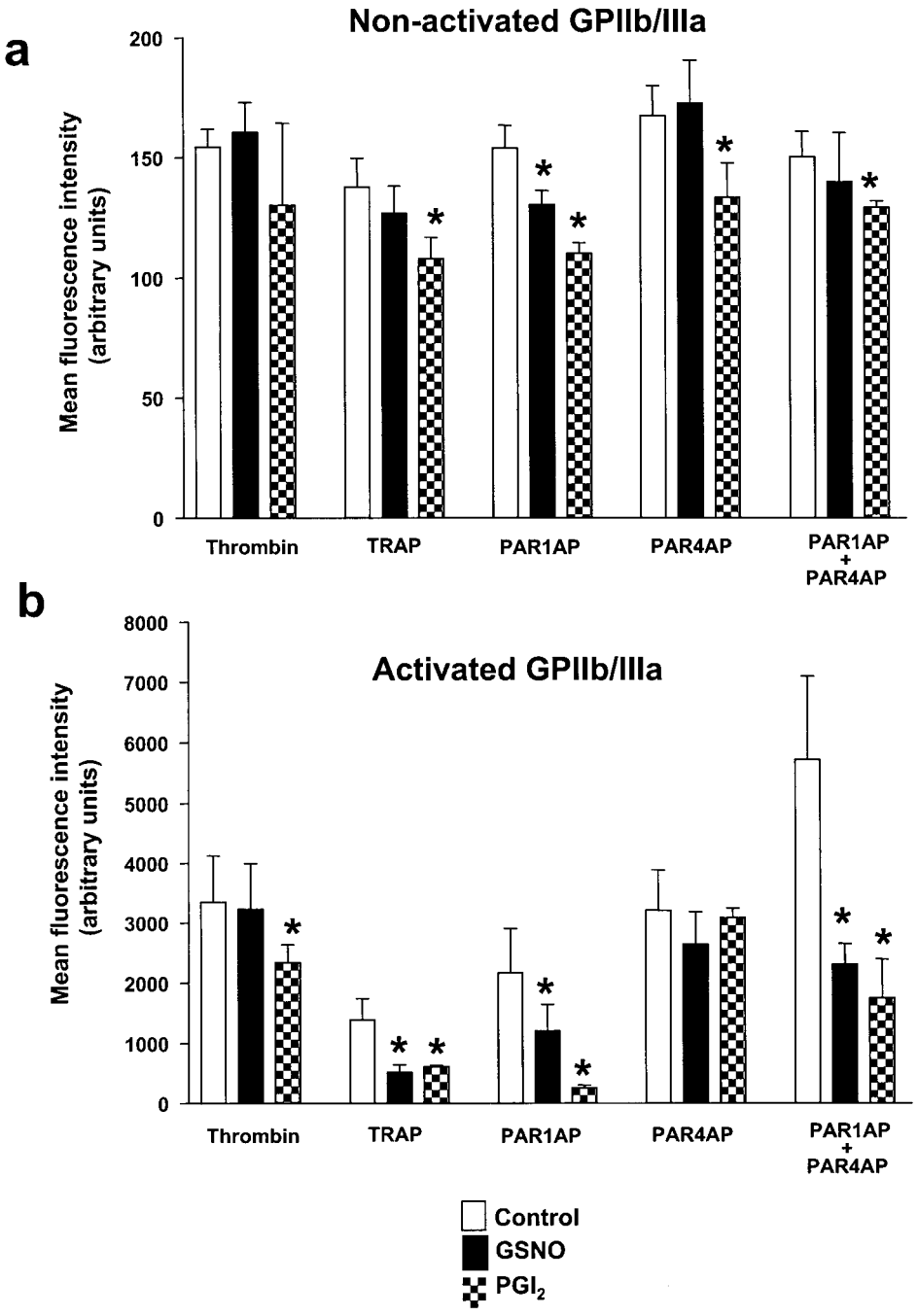

Figure 4 Flow cytometry analysis of GPIIb/IIIa (panel a) and activated GPIIb/IIIa (panel b) in platelets stimulated with PAR agonists in the presence of PGI_2 (3 nM) or GSNO (5 μM). Data are mean$\pm$s.e.mean, $n=3-9$. *$P<0.05$ treatments versus control (PAR agonists in the absence of inhibitors).

AP-induced aggregation. These results allowed us to construct concentration-response curves to PAR peptides over a broad range of agonist concentrations and analyse the corresponding EC_{50} values. Although all PAR agonists were equally efficacious, they differed in their aggregating potency. The comparison of EC_{50}s showed that thrombin was the most potent agonist followed by PAR1AP, TRAP and PAR4AP. The high potency of thrombin can be explained largely by its unusually high affinity for the hirudin-like domain in PAR1 and its non-proteolytic interaction with GPIb (McNicol *et al*., 1989). Added to this high binding affinity, the potency of thrombin is enhanced by its ability to act *via* a 'dual' PAR1/PAR4 receptor system in human platelets (Kahn *et al*., 1998; 1999). Thrombin-mediated proteolysis of PAR1 and PAR4 generates tethered ligands autostimulating both receptors; and it has been discovered that in addition, the PAR1 amino-terminal peptide released by thrombin action can also activate platelets (Furman *et al*., 1998; 2000). Thus, thrombin-induced proteolysis of PARs 1 and 4 can generate both the tethered ligands and the cleaved

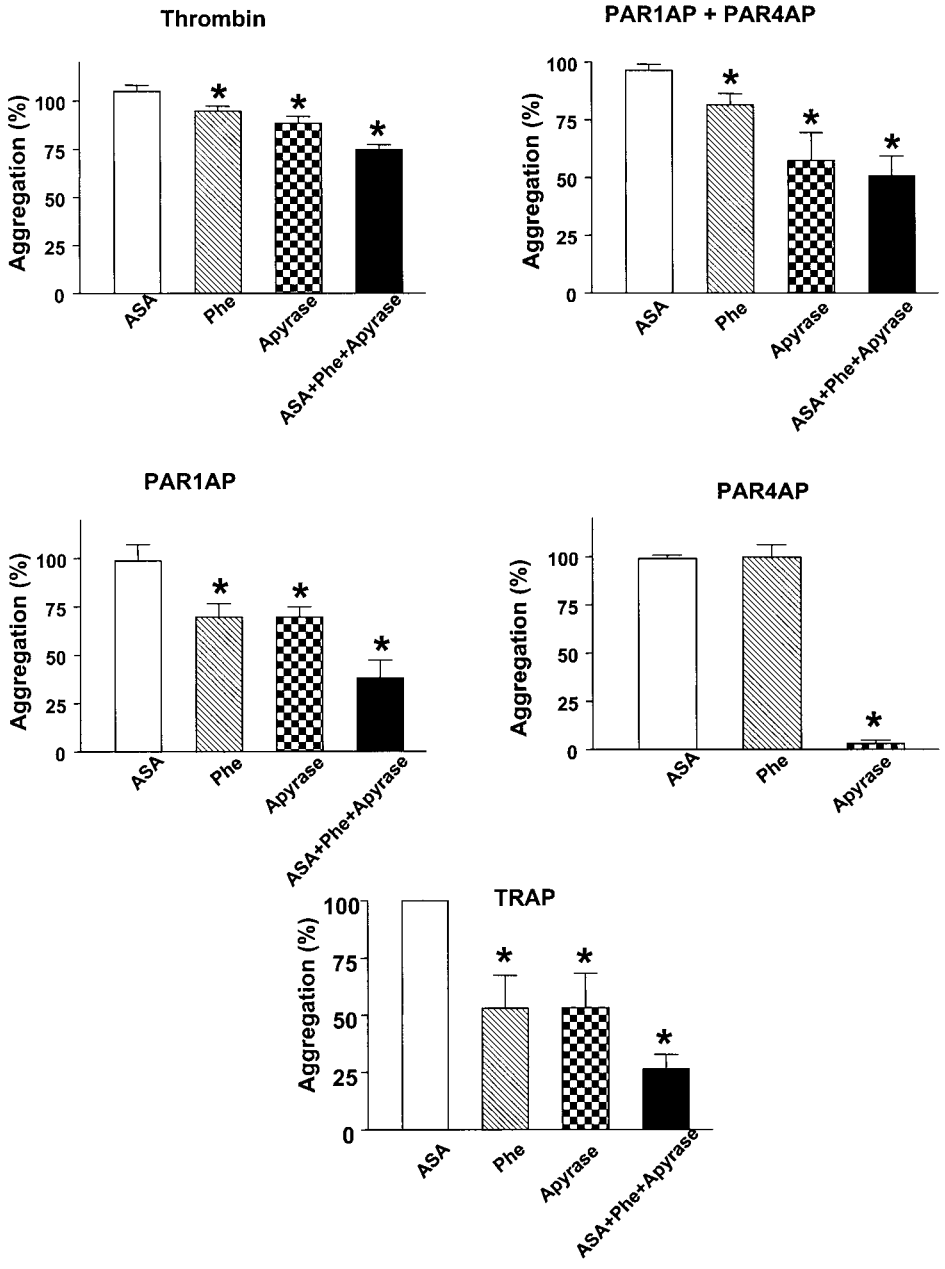

Figure 5 Effects of aspirin (ASA, 300 μM), phenanthroline (Phe, 100 μM) and apyrase (300 μg ml^{-1}) on PAR agonist-induced aggregation. Results are expressed as a per cent of aggregation induced by the corresponding agonist without inhibitors. Data are mean $\pm$ s.e.mean, $n = 3-9$.

PAR1 peptide, which may potentiate each other and amplify thrombin-mediated aggregation (Furman *et al*., 1998).

Although PAR1, PAR4 and TRAP can fully activate human platelets, their absolute potencies are relatively low, with EC_{50}s in the micromolar range. It is generally accepted that these comparatively low potencies are due to differences between a built-in tethered ligand and a ligand free in solution, which would gain an extra thermodynamic degree of freedom and be able to diffuse away from the receptor. Since human platelets do not express PAR2, the ability of TRAP to activate PAR2 (Blackhart *et al*., 1996) was not an issue in our study. We also found that PAR4 was less potent than PAR1 on platelet aggregation. This most likely relates to the differential coupling of the two receptors to their target G-proteins. In this regard, PAR1 can couple to either G_q or G_i (Hung *et al*., 1992), whereas PAR4 in the same cellular context as PAR1 cannot couple to G_i (Faruqi *et al*., 2000). Further, quite different desensitization properties have been documented for PAR1 and PAR4, in that the signal generated by PAR4 is more persistent than that of PAR1 and that activation-dependent internalization of PAR4 is slower than that for PAR1 (Shapiro *et al*., 2000). These differences in desensitization/internalization kinetics suggest that PARs 1 and 4 may subserve distinct functions in platelets or their megakaryocyte precursors.

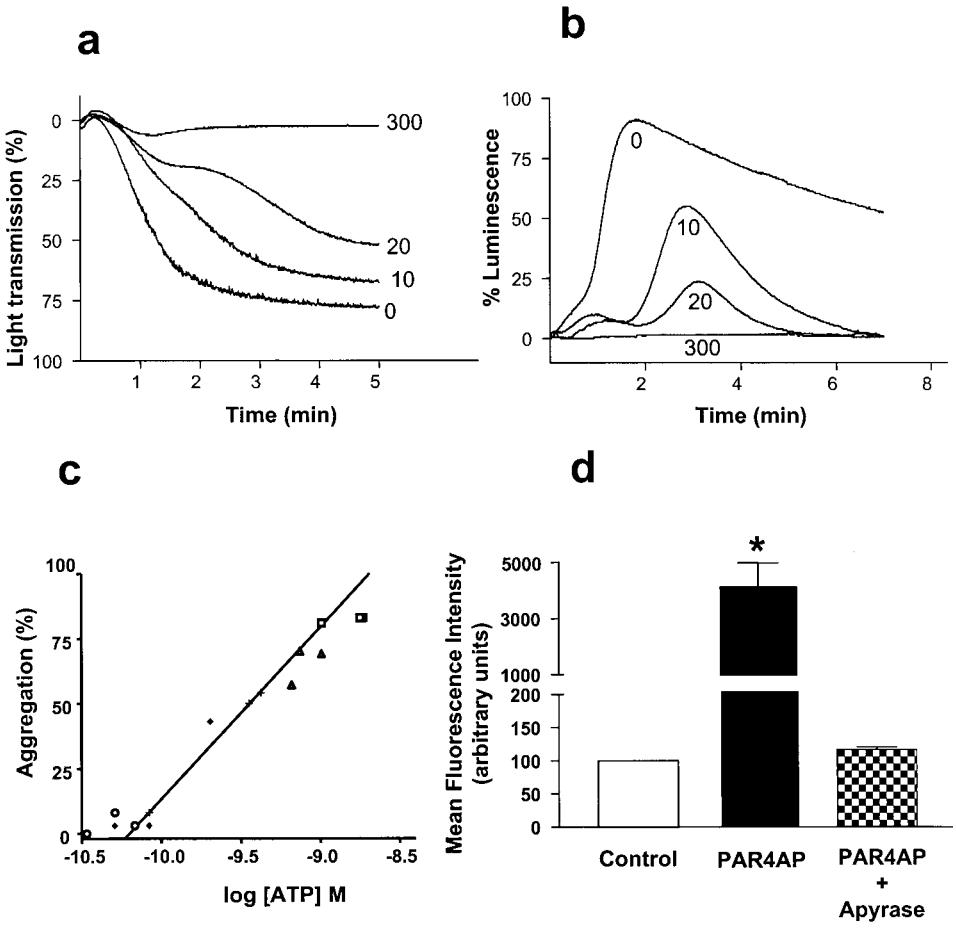

Figure 6 Role of ADP in PAR4AP-induced aggregation. Superimposed aggregatory and the corresponding chemiluminescence tracings showing inhibition of PAR4AP-stimulated aggregation with apyrase (panel a and b). Panel c shows correlation between the concentrations of ATP and the aggregatory responses during inhibition of aggregation induced by apyrase (square: 0, triangle: 10, cross: 20, diamond: 35 and circle: 300 μg ml^{-1}). Panel d demonstrates inhibition of PAR4AP-induced GPIIb/IIIa activation by apyrase (50 μg ml^{-1}). Data are mean $\pm$ s.e.mean, $n=3$. *$P<0.05$ treatments versus control.

We also demonstrated that concomitant administration of subthreshold concentrations of PAR1AP and PAR4AP caused maximal aggregation. It has been shown that thrombin has a differential affinity for PAR1 and PAR4, such that activation of the latter receptor takes place only at comparatively high concentrations of thrombin (Kahn *et al*., 1999). However, the potentiating interactions between PAR1AP and PAR4AP observed in our study further underscore the importance of PAR4 in aggregation of human platelets by thrombin, even at concentrations that only minimally activate PAR4. Interestingly, mouse platelets that do not express PAR1 rely on collaborative interactions between PAR3 and PAR4 during thrombin-induced aggregation (Nakanishi-Matsui *et al*., 2000). Moreover, the PAR4-deficient mouse shows a prolonged bleeding time, which is consistent with a key role of PAR4 in mouse haemostasis and thrombosis (Sambrano *et al*., 2001). Thus, activation of platelets by thrombin involves an interaction between PAR4 and either PAR1 (human) or PAR3 (mouse) to amplify aggregation.

Thrombin-induced aggregation is dependent upon glycoprotein receptors such as GPIb and GPIIb/IIIa. Therefore, we measured the expression of GP receptors on platelets stimulated with PAR agonists by flow cytometry. GPIb is a positive effector of PAR1-driven platelet activation by localizing thrombin to sites that facilitate cleavage of the nearby PARs (Gralnick *et al*., 1994). Thrombin down-regulates the surface expression of GPIb (De Marco *et al*., 1994; Michelson & Barnard, 1987). Our results showed a similar down-regulation of GPIb receptors after stimulation with thrombin and all PAR-APs. All of the synthetic peptides induced a 40–50% reduction in GPIb surface expression, in association with platelet aggregation. The mechanism(s) of this down-regulation induced by PAR-APs remains to be studied, but this may be similar to that seen with thrombin, which is known to cause a rapid redistribution of GPIb complex into the surface-connected canalicular system (Hourdille *et al*., 1990).

Thrombin up-regulates and activates the GPIIb/IIIa complex (Suzuki *et al*., 1992). We demonstrated that the synthetic peptides also increased surface expression of non-activated GPIIb/IIIa by 1.5 fold, and caused a dramatic up-regulation of activated GPIIb/IIIa by 20–80 fold. These results emphasize the importance of activation of GPIIb/IIIa receptors in mediating aggregation induced by PAR agonists. Similar to other ligands such as collagen and von Willebrand

factor (Radomski *et al*., 2001), the changes in the ratio of GPIb to GPIIb/IIIa could be important in shifting platelet reactivity away from GPIb-mediated adhesion to GPIIb/IIIa-mediated aggregation.

Prostacyclin and NO are the two major platelet inhibitors generated from endothelium (Jurasz *et al*., 2000). We used PGI_2 and NO-releasing agent, GSNO (Radomski *et al*., 1992), to study their effects on PAR-induced aggregation. We found that although these inhibitors could effectively suppress aggregation, their effects on GPIIb/IIIa activation were variable. Indeed, inhibition of aggregation by PGI_2 correlated well with the reduction of IIb/IIIa activation in thrombin-, TRAP-, PAR1AP- and PAR1AP+PAR4AP-induced aggregation, but not with PAR4AP-induced aggregation. Moreover, GSNO caused only down-regulation of GPIIb/IIIa activation in platelets stimulated with TRAP, PAR-1AP and PAR1AP+PAR4AP. These results indicate that PGI_2 and NO, in addition to inhibition of GPIIb/IIIa activation, affect other stages of platelet activation (Jurasz *et al*., 2000).

We also found that aggregation induced by synthetic PAR-APs is significantly more sensitive to the inhibitory effects of PGI_2, as compared with those induced by thrombin and collagen. These differences were not detectable for inhibition of platelet aggregation by GSNO. The reasons for this differential reactivity are not clear.

We used ASA, phenanthroline and apyrase to explore a relative contribution of TXA_2, MMP-2 and ADP release to PAR agonist-induced aggregation. We observed that platelet aggregation stimulated by thrombin, TRAP, PAR1AP and PAR4AP was ASA-insensitive, indicating that these reactions were TXA_2-independent. In contrast to ASA, apyrase and phenanthroline partially inhibited aggregation induced by thrombin, TRAP and PAR1AP indicating that MMP-2 and ADP are mediators of these aggregatory reactions. However, even the three inhibitors were not capable to inhibit completely thrombin-, TRAP- or PAR1-induced aggregatory responses, suggesting the involvement of TXA_2-, ADP- and MMP-2-independent mechanism(s). Interestingly, as outlined above, PAR4AP-induced aggregation was (1) abolished in the presence of apyrase, an agent that degrades ADP and (2) was insensitive to phenanthroline. Moreover, there was a strong correlation between aggregation and ATP release during PAR4AP-induced aggregation. These data suggest that PAR4AP-mediated platelet aggregation is entirely ADP-dependent and metalloproteinase-independent.

Our results demonstrated that PAR agonist-induced aggregation was ASA-insensitive. Despite its effectiveness in treating and preventing the thrombotic complications of atherosclerotic disease, a minority of patients appears to be relatively ASA- resistant, even when it is administered in large doses (Helgason *et al*., 1993). Platelet aggregation studies have demonstrated the incomplete inhibition of aggregation in 25% of patients with prior ischaemic stroke who were receiving long-term ASA therapy (Helgason *et al*., 1994). The mechanism(s) of the aspirin-resistance is not clear, but probably is due to the TXA_2-independent activators of platelet aggregation (i.e. thrombin) bypassing the ASA-inhibitory effect and resulting in thrombosis (Radomski *et al*., 2000).

We also showed that, although PAR agonist-induced aggregation is ASA-insensitive, a combination of this compound with ADP and MMP-2 inhibitors reduced aggregation. Thus, MMP-2 inhibitors may have a potential to be used in the anti-platelet therapy.

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References

BAHOU, W.F., COLLER, B.S., POTTER, C.L., NORTON, K.J., KUTOK, J.L. & GOLIGORSKY, M.S. (1993). The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. *J. Clin. Invest.,* **91,** 1405 – 1413.

BLACKHART, B.D., EMILSSON, K., NGUYEN, D., TENG, W., MARTELLI, A.J., NYSTEDT, S., SUNDELIN, J. & SCARBOROUGH, R.M. (1996). Ligand cross-reactivity within the proteinase-activated receptor family. *J. Biol. Chem.,* **271,** 16466 – 16471.

BLOMBACK, B. (1996). Fibrinogen and fibrin-proteins with complex roles in hemostasis and thrombosis. *Thromb. Res.,* **83,** 1 – 75.

BORN, G.V. (1966). Effects of adenosine diphosphate (ADP) and related substances on the adhesiveness of platelets in vitro and in vivo. *Br. J. Haematol.,* **12,** 37 – 38.

CHEN, J., ISHII, M., WANG, L., ISHII, K. & COUGHLIN, S.R. (1994). Thrombin receptor activation: confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J. Biol.Chem.,* **268,** 16041 – 16045.

COLLER, B.S., SPRINGER, K.T., SCUDDER, L.E., KUFOK, J.L., CERUSO, M. & PRESTWICH, G.D. (1993). Substituting isoserine for serine in the thrombin receptor activation peptide SFLLRN confers resistance to aminopeptidase M-induced cleavage and inactivation. *J. Biol. Chem.,* **268,** 20741 – 20743.

COUGHLIN, S.R. (2000). Thrombin signalling and proteinase-activated receptors. [Review] *Nature,* **407,** 258 – 264.

DE MARCO, L., MAZZUCATO, M., MASOTTI, A. & RUGGERI, Z.M. (1994). Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. *J. Biol. Chem.,* **269,** 6478 – 6484.

FARUQI, T.R., WEISS, E.J., SHAPIRO, M.J., HUANG, W. & COUGHLIN, S.R. (2000). Structure-function analysis of proteinase-activated receptor 4 tethered ligand peptides. Determinants of specificity and utility in assays of receptor function. *J. Biol. Chem.,* **275,** 19728 – 19734.

FURMAN, M.I., LIU, L., BENOIT, S.E., BECKER, R.C., BARNARD, M.R. & MICHELSON, A.D. (1998). The cleaved peptide of the thrombin receptor is a strong platelet agonist. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 3082 – 3087.

FURMAN, M.I., NURDEN, P., BERNDT, M.C., NURDEN, A.T., BENOIT, S.E., BARNARD, M.R., OFOSU, F.A. & MICHELSON, A.D. (2000). The cleaved peptide of PAR1 results in a redistribution of the platelet surface GPIb-IX-V complex to the surface-connected canalicular system. *Thromb Haemost.,* **84,** 897 – 903.

GERSZTEN, R.E., CHEN, J., ISHII, M., ISHII, K., WANG, L., NANEVICZ, T., TURCK, C.W., VU, T.K. & COUGHLIN, S.R. (1994). Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface. *Nature,* **368,** 648 – 651.

GRALNICK, H.R., WILLIAMS, S., MCKEOWN, L.P., HANSMANN, K., FENTON, II J.W. & KRUTZSCH, H. (1994). High-affinity alpha-thrombin binding to platelet glycoprotein Ib alpha: identification of two binding domains. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 6334–6338.

HELGASON, C.M., BOLIN, K.M., HOFF, J.A., WINKLER, S.R., MANGAT, A., TOTORICE, K.L. & BRACE, L.D. (1994). Development of aspirin resistance in persons with previous ischemic stroke. *Stroke,* **25,** 2331–2336.

HELGASON. C.M., TORTORICE, K.L., WINKLER, S.R., PENNEY, D.W., SCHULER, J.J., MCCLELLAND, T.J. & BRACE, L.D. (1993). Aspirin response and failure in cerebral infarction. *Stroke,* **24,** 345–350.

HOLLENBERG, M.D., SAIFEDDINE, M., AL-ANI, B. & GUI, Y. (1999). Proteinase-activated receptor 4 (PAR4): action of PAR4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR1 and PAR2. *Can. J. Physiol. Pharmacol.,* **77,** 458–464.

HOURDILLE, P., HEILMANN, E., COMBRIE, R., WINCKLER, J., CLEMETSON, K.J. & NURDEN, A.T. (1990). Thrombin induces a rapid redistribution of glycoprotein Ib-IX complexes within the membrane systems of activated human platelets. *Blood,* **76,** 1503–1513.

HUGHES, P.E. & PFAFF, M. (1998). Integrin affinity modulation. *Trends Cell Biol.,* **8,** 359–364.

HUNG, D.T, WONG, Y.H., VU, T.K. & COUGHLIN, S.R. (1992). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J. Biol. Chem.,* **267,** 20831–20834.

JURASZ, P., RADOMSKI, A., SAWICKI, G., MAYERS, I. & RADOMSKI, M.W. (2000). Nitric oxide and platelet function. In *Nitric Oxide Biology and Pathology*. ed. Ignarro, L.J. pp. 823–840. San Diego: Academic Press.

JURASZ, P., SAWICKI, G., DUSZYK, M., SAWICKA, J., MIRANDA, C., MAYERS, I. & RADOMSKI, M.W. (2001). Matrix metalloproteinase 2 in tumor cell-induced platelet aggregation: regulation by nitric oxide. *Cancer Res.,* **61,** 376–382.

KAHN, M.L., NAKANISHI-MATSUI, M., SHAPIRO, M.J., ISHIHARA, H. & COUGHLIN, S.R. (1999). Proteinase-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J. Clin. Invest.,* **103,** 879–887.

KAHN, M.L., ZHENG, Y.W., HUANG, W., BIGORNIA, V., ZENG, D., MOFF, S., FARESE JR., R.V., TAM, C. & COUGHLIN, S.R. (1998). A dual thrombin receptor system for platelet activation. *Nature,* **394,** 690–694.

KAWABATA, A., KURODA, R., NISHIKAWA, H. & KAWAI, K. (1999). Modulation by proteinase-activated receptors of the rat duodenal motility in vitro: possible mechanisms underlying the evoked contraction and relaxation. *Br. J. Pharmacol.,* **128,** 865–872.

MACFARLANE, S.R., SEATTER, M.J., KANKE, T., HUNTER, G.D. & PLEVIN, R. (2001). Proteinase-activated receptors. *Pharmacol. Rev.,* **53,** 245–282.

MCNICOL, A. & GERRARD, J.M. (1993). Post-receptor events associated with thrombin-induced platelet activation. *Blood Coag. Fibrinol.,* **4,** 975–991.

MCNICOL, A., GERRARD, J.M. & MACINTYRE, D.E. (1989). Evidence for two mechanisms of thrombin-induced platelet activation: one proteolytic, one receptor mediated. *Biochem. Cell Biol.,* **67,** 332–336.

MICHELSON, A.D. & BARNARD, M.R. (1987). Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. *Blood,* **70,** 1673–1678.

NAKANISHI-MATSUI, M., ZHENG, Y.-W., SCLCINER, D.J., WEISS, E.J., LUDEMAN, M.J. & COUGHLIN, S.R. (2000). PAR3 is a cofactor for PAR4 activation by thrombin. *Nature,* **404,** 609–613.

NEEDLEMAN, P., MONCADA, S., BUNTING, S., VANE, J.R., HAMBERG, M. & SAMUELSSON, B. (1976). Identification of an enzyme in platelet microsomes which generates thromboxane A2 from prostaglandin endoperoxides. *Nature,* **261,** 558–560.

O'BRIEN, P.J., MOLINO, M., KAHN, M. & BRASS, L.F. (2001). Proteinase activated receptors: theme and variations. *Oncogene,* **20,** 1570–1581.

RADOMSKI, A., STEWART, M.W., JURASZ, P. & RADOMSKI, M.W. (2001). Pharmacological characteristics of solid-phase von Willebrand factor in human platelets. *Br. J. Pharmacol.,* **134,** 1013–1020.

RADOMSKI, M.W., GORDON, P.A., RADOMSKI, A. & ARMSTRONG, P.W. (2000). Modulation by aspirin of platelet aggregatory pathways. *Can. J. Cardiol.,* **16** (Suppl. F), 221.

RADOMSKI, M.W. & MONCADA, S. (1983). An improved method for washing of human platelets with prostacyclin. *Thromb. Res.,* **30,** 383–389.

RADOMSKI, M.W., REES, D.D., DUTRA, A. & MONCADA, S. (1992). S-nitroso-glutathione inhibits platelet activation in vitro and in vivo. *Br. J. Pharmacol.,* **107,** 745–749.

SAMBRANO, G.R., WEISS, E.J., ZHENG, Y.W., HUANG, W. & COUGHLIN, S.R. (2001). Role of thrombin signaling in platelets in haemostasis and thrombosis. *Nature,* **413,** 74–78.

SAWICKI, G., SALAS, E., MURAT, J., MISZTA-LANE, H. & RADOMSKI, M.W. (1997). Release of gelatinase A during platelet activation mediates aggregation. *Nature,* **386,** 616–618.

SHAPIRO, M.J., WEISS, E.J., FARUQI, T.R & COUGHLIN, S.R. (2000). Proteinase-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J. Biol. Chem.,* **275,** 25216–25221.

STENBERG, P.E., MCEVER, R.P., SHUMAN, M.A., JACQUES, Y.V. & BAINTON, D.F. (1985). A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J. Cell Biol.,* **101,** 880–886.

SUZUKI, H., NAKAMURA, S., ITOH, Y., TANAKA, T., YAMAZAKI, H. & TANOUE, K. (1992). Immunocytochemical evidence for the translocation of alpha-granule membrane glycoprotein IIb/IIIa (integrin alpha IIb beta 3) of human platelets to the surface membrane during the release reaction. *Histochemistry,* **97,** 381–388.

VASSALLO, R.R., KIEBER-EMMONS, T., CICHOWSKI, K. & BRASS, L.F. (1992). Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.,* **267,** 6081–6085.

VU, T.K., HUNG, D.T., WHEATON, V.I. & COUGHLIN, S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell,* **64,** 1057–1068.

XU, W.F., ANDERSEN, H., WHITMORE, T.E., PRESNELL, S.R., YEE, D.P., CHING, A., GILBERT, T., DAVIE, E.W. & FOSTER, D.C. (1998). Cloning and characterization of human proteinase-activated receptor 4. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 6642–6646.

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