Thrombin-induced platelet activation and its inhibition by anticoagulants with different modes of action

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Thrombin-induced platelet activation involves cleavage of protease-activated receptors (PARs) 1 and 4, and interaction, via glycoprotein (Gp)Ibα, with the platelet GpIb/IX/V complex. This study investigated inhibition of platelet activation by thrombin inhibitors with different modes of action: two reversible direct thrombin inhibitors, melagatran and inogatran; hirudin, a tightly binding direct thrombin inhibitor; and two indirect thrombin inhibitors, heparin and dalteparin. Up-regulation of P-selectin (CD62P) and PAR-1 cleavage was measured in human whole blood, by flow cytometry. The thrombin concentration that induced 50% of maximum (EC_{50}) PAR-1 cleavage was 0.028 nmol/l, while that of platelet activation (CD62P) was over two-fold higher (0.64 nmol/l). The EC_{50} of a PAR-1-independent component, defined as a further activating effect of thrombin on top of the maximum PAR-1-activating peptide (AP) effect, was 3.2 nmol/l. All anticoagulants were concentration-dependent inhibitors of thrombin-induced platelet activation and PAR-1 cleavage, but none inhibited PAR-1-AP or PAR-4-AP induced activation. Melagatran and inogatran were more potent inhibitors of CD62P up-regulation than of PAR-1 cleavage; conversely, hirudin, heparin and dalteparin were more potent inhibitors of PAR-1 cleavage.Thus, reversible direct thrombin inhibitors, such as melagatran, are potent inhibitors of thrombin-induced platelet activation, acting mainly by inhibition of a PAR-1-independent component.
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Introduction

Thrombin not only catalyses the conversion of soluble fibrinogen into an insoluble fibrin clot, but it is also an extremely potent platelet activator. Thrombin induces a unique and specific proteolysis of cell surface receptors designated protease-activated receptors (PARs). Proteolysis of PARs by thrombin exposes a tethered ligand, known as the thrombin receptor-activating peptide, at the extracellular N-terminus of the receptor, resulting in intramolecular activation [1]. Three of the four known PARs, PAR-1, PAR-3 and PAR-4, are activated by thrombin [1–3]. Two of these, PAR-1 and PAR-4, are expressed by human platelets [4]. Apart from thrombin, synthetic peptides mimicking the exposed amino acid sequence of PAR-1 and PAR-4 can also activate the receptors, and have been used as tools to decipher the relative importance of these two pathways [3–5].

A study by Andersen *et al.* [5] suggested that thrombin mediates a strong platelet agonistic effect through the PAR-1 receptor, while activation of PAR-4 results in weak stimulation of platelets. PAR-1 has been described as having a higher affinity for thrombin than PAR-4 [4,6,7], and a sequential activation mechanism by thrombin was hypothesized by Covic *et al.* [6]. Thus, PAR-1 is cleaved first at low thrombin concentrations and then, as the thrombin concentration increases, the low-affinity PAR-4 is cleaved.

There is evidence that thrombin also binds to the platelet glycoprotein (GP)Ib/IX/V complex. The thrombin-binding site has been localized to the N-terminal region of the GPIbα chain [8,9], which also bears a binding site for von Willebrand factor [10]. Binding of thrombin to GPIbα occurs through an anion-binding exosite, which is also referred to as a heparin-binding site. It has been suggested that this interaction serves as a cofactor for PAR-1 cleavage [11] and that heparin, at least partly, inhibits thrombin-induced platelet activation via inhibition of this thrombin-receptor interaction [12]. Thrombin-mediated signalling via the GPIb/IX/V complex was described by Ramakrishnan *et al.* [13]. In this process, thrombin binds to GP1bα and cleaves GPV, which serves as an inhibitor of platelet activation, thereby resulting in platelet activation and aggregation. These results strongly support a role for GpIbα in thrombin-induced platelet activation. However, this is contradictory to the findings by Kahn *et al.* [4], who showed that aggregation induced by 30 nmol/l thrombin could be completely blocked by simultaneous inhibition of both PAR-1 and PAR-4.

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Theoretically, pharmacological intervention of thrombin-induced platelet activation can be achieved either via a specific blockade of one of PAR-1, PAR-4 or GPIbα or by inhibition of the enzymatic activity of thrombin. The latter approach would inhibit all pathways because they are all dependent on the enzymatic activity of thrombin. Receptor affinity for thrombin and thrombin concentration will both influence the relative importance of the different pathways. Therefore, thrombin inhibitors with different affinities for thrombin should have different potencies in inhibiting the different pathways. This study was undertaken to investigate whether anticoagulant drugs with different affinities for thrombin and different mechanisms of action, would have different effects with respect to inhibition of thrombin-induced platelet activation in human whole blood. The thrombin inhibitors studied were two reversible and competitive direct thrombin inhibitors, melagatran [14], the active form of ximelagatran [15], and inogatran [16]; hirudin, a specific and tightly binding direct thrombin inhibitor [17]; and two indirect thrombin inhibitors, heparin and dalteparin (a low-molecular-weight heparin) [18].

Materials and methods

Antibodies

The anti-CD62P-phycoerythrin (PE) (clone AK4) and anti-CD42b-fluorescein isothiocyanate (FITC) (clone HIP1) antibodies, and mouse immunoglobulin (Ig)G1κ-PE (clone MOPC-21), were purchased from Pharmingen (San Diego, California, USA). The SPAN12-PE antibody was obtained from Beckman Coulter (Fullerton, California, USA).

Reagents

Human thrombin and bovine serum albumin (BSA) were purchased from Sigma Chemical Company (St Louis, Missouri, USA). Thrombin receptor-activating peptide (TRAP)-14 (H-SFLLRNPNDKYEPF-OH), referred to in this study as PAR-1-AP, was purchased from Bachem (Bubendorf, Switzerland). AYPGKF-OH, referred to in this study as PAR-4-AP, was synthesized by Innovagen (Lund, Sweden). The fibrin polymerization inhibitor Pefablock FG (GPRP) was purchased from Pentapharm (Basel, Switzerland). The direct thrombin inhibitors, melagatran and inogatran, were produced by AstraZeneca (Mölndal, Sweden). Recombinant hirudin (recDNA [Tyr63]-Hirudin) was purchased from Sigma Chemical Company. Heparin and dalteparin were purchased from Lövens (Ballerup, Denmark), and Pharmacia-Upjohn (Uppsala, Sweden), respectively.

Blood collection

This study was carried out in blood from fasting, healthy adult males. All subjects gave informed consent to participate in the study, which was performed in accordance with local ethical regulations. Blood was collected by venipuncture through an 18-gauge butterfly needle, without a tourniquet to minimize platelet activation. The first 5 ml of blood was discarded before collecting 4.5 ml aliquots into tubes containing 0.5 ml of 3.8% sodium citrate. Within 2 min of collection, the blood was diluted 1:6 with modified Tyrodes buffer (137 mmol/l NaCl, 2.8 mmol/l KCl, 1 mmol/l $MgCl_2$, 12 mmol/l $NaHCO_3$, 0.4 mmol/l Na_2HPO_4, 0.35% BSA, 10 mmol/l HEPES, 5.5 mmol/l glucose, pH 7.4). Blood samples were used within 1 h of collection.

Platelet activation

Platelet activity was measured by means of a whole blood flow cytometry assay. CD42b, also known as GPIbα is a membrane glycoprotein found on both resting and activated platelets. It was therefore used as a general platelet marker. In contrast, P-selectin (CD62P) is normally stored in the α-granules, and is only expressed after platelet activation, leading to granule secretion. CD62P was therefore used as a marker of platelet activation [19]. GPRP, a fibrin polymerization inhibitor, was added in order to avoid clotting and platelet aggregation during activation in this study. GPRP does not inhibit either platelet activation or thrombin activity [20].

The following five thrombin inhibitors were investigated: melagatran ($K_d = 2 \times 10^{-9}$ nmol/l), a reversible direct thrombin inhibitor, and the active form of the oral direct thrombin inhibitor, ximelagatran [14,15]; inogatran ($K_d = 16 \times 10^{-9}$ nmol/l), a reversible, direct thrombin inhibitor [16]; hirudin (K_d approximately 10^{-14} nmol/l), a direct thrombin inhibitor (polypeptide with 65 amino acid residues) that forms an extremely tight complex via numerous interactions with thrombin [17]; and heparin and dalteparin, which are both irreversible indirect inhibitors of thrombin which act via potentiation of the anticoagulant effect of antithrombin [18].

All incubations were performed at room temperature, in the dark. The thrombin inhibitors were diluted in modified Tyrodes buffer, and 10 µl was added to each tube. The following reagents were then added to each tube: 47 µl diluted human whole blood, 5 µl mouse anti-human CD42b-FITC, 5 µl mouse anti-human CD62P-PE, and 3 µl of 66 mmol/l Pefabloc FG. The mixture was pre-incubated for 5 min before 10 µl of either thrombin or PAR-AP was added. The samples were then incubated with thrombin or PAR-AP for 15 min. The reaction was stopped by fixing the cells for 30 min with 80 µl of 1% paraformaldehyde in modified Tyrodes buffer. The samples were diluted with 2 ml modified Tyrodes buffer prior to analysis on a flow cytometer. In order to avoid non-thrombin/PAR-AP-

induced platelet activation, the procedure did not involve any washing, centrifugation or vortex-mixing.

To study the PAR-1- or PAR-4-independent component, human whole blood was pre-incubated for 5 min with a saturating concentration of PAR-1-AP (100 μmol/l) or PAR-4-AP (1200 μmol/l) prior to activation with thrombin. Platelet activation by the PAR-APs in the absence of thrombin was set as background (Fig. 1). Thereafter, these experiments were conducted as described above. This made it possible to construct dose–response curves for the PAR-1- and PAR-4 independent components (Fig. 2).

The possibility of direct platelet activation by the thrombin inhibitors was evaluated by incubating each thrombin inhibitor with human whole blood for 15 min in the absence of agonist, before analysis for expression of CD62P. In these experiments, the final plasma concentration of heparin used was 3 IU/ml, which approximates to a high concentration used in clinical practice. The corresponding concentrations of melagatran, inogatran, hirudin and dalteparin were 1 μmol/l, 2 μmol/l, 1 μmol/l, and 3 IU/ml, respectively. Possible inhibitory effects of the anti-CD42b antibody were also tested.

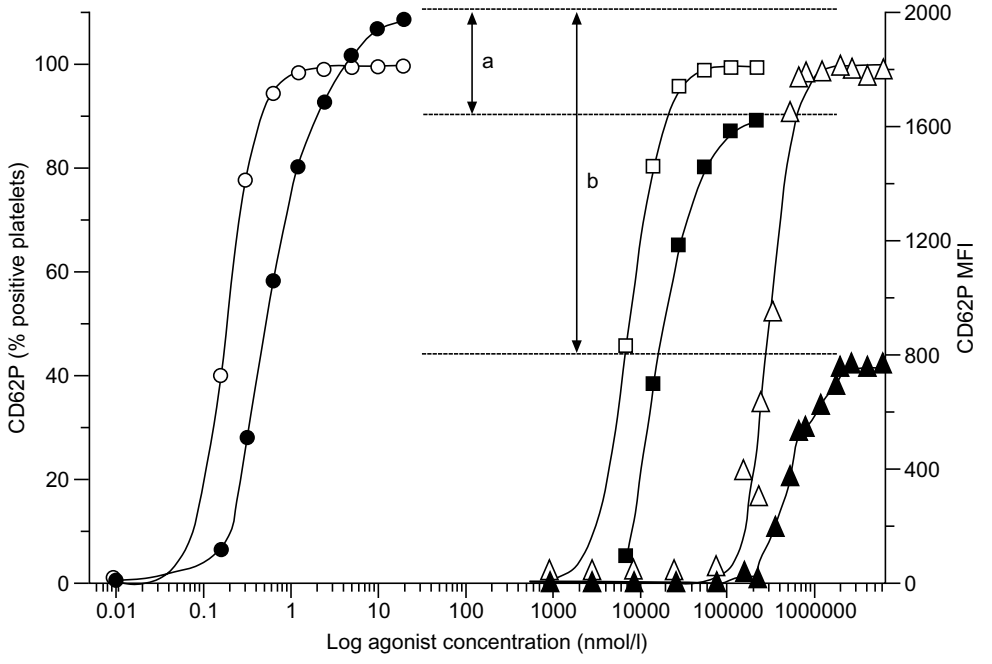
Protease-activated receptor-1 cleavage

PAR-1 cleavage was assayed using SPAN12, an antibody that recognizes an N-terminal peptide of PAR-1, which spans the cleavage site for thrombin. Thus, SPAN12 can only bind to uncleaved PAR-1 [21–23] . The following reagents were added to each tube: 57 μl of diluted human blood and 3 μl of 66 mmol/l Pefabloc FG. Possible inhibitory effects of the SPAN12 antibody were also tested. To further avoid possible inhibition, the SPAN12-PE antibody (5 μl) was added after 5 min of thrombin incubation. Thereafter, these experiments were conducted as above.

Flow cytometry

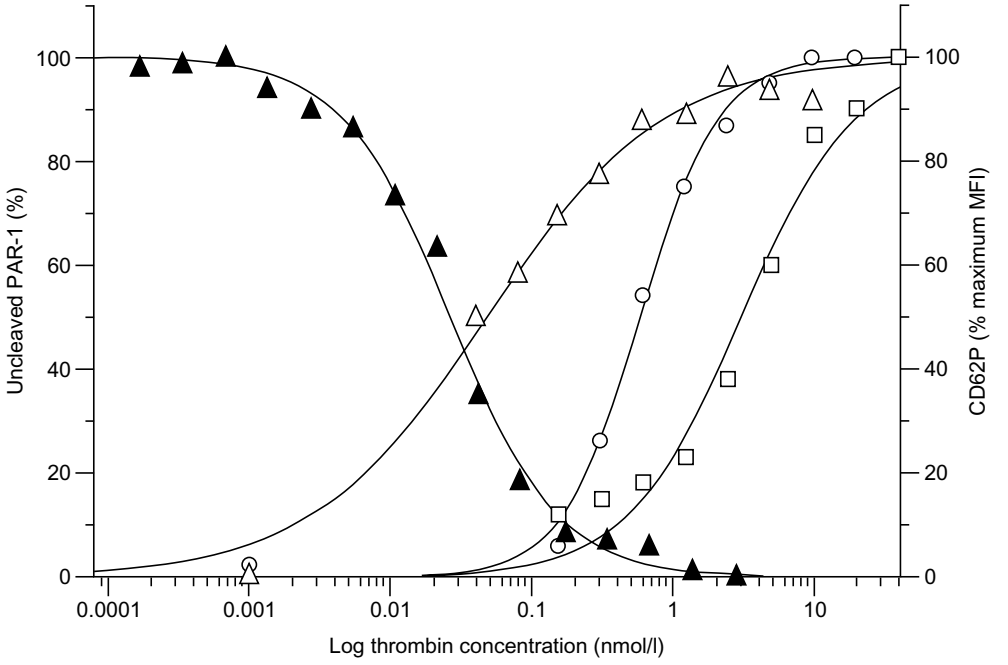
Samples from experiments were analysed within 1 h of fixing with a FACSCalibur using CellQuest software (Becton Dickinson, Palo Alto, California, USA). The threshold was set at fluorescence 1 (CD42b-FITC), and platelets were defined by fluorescence 1 and forward and side-scatter gating. Data on 5000 platelets were acquired for each sample. The data were analysed using WinList 4.0 software (Verity Software House, Topsham, Maine, USA) and platelets were defined as CD42b-positive and within the platelet cluster in a forward versus side-scatter dot plot. The platelet population was analysed with respect to CD62P mean

Fig. 1



Thrombin (○/●), protease-activated receptor (PAR)-1-activating peptideAP (□/■) and PAR-4-AP (△/▲)-induced activation of human platelets, expressed as CD62P mean fluorescence intensity (MFI; filled symbols) and percentage of CD62P positive platelets (open symbols). (a) The PAR-1-independent component. (b) The PAR-4-independent component.

Fig. 2



Dose–response of the different thrombin components: total thrombin (○), protease-activated receptor (PAR)-1-independent (□) and PAR-4-independent (△), expressed as percentages of maximum mean fluorescence intensity (MFI). Cleavage of PAR-1 (▲), expressed as a percentage of uncleaved PAR-1. SPAN12 MFI was set to 100% for platelets in the absence of thrombin.

fluorescence intensity (MFI) and the percentage CD62P positive.

The threshold was set on forward scatter for analysis of samples from the SPAN12 experiments, and platelets were defined as within the platelet cluster in a forward versus side-scatter dot plot. The platelet population was analysed with respect to SPAN12 MFI.

Data analysis

Inhibition of thrombin and PAR-AP-induced platelet activation were assessed as the down-regulation of CD62P MFI in the platelet population, and expressed as a percentage of the CD62P MFI in the absence of inhibitor. All data were corrected for background, which was defined as the MFI in the absence of agonist. In the experiments investigating the PAR-1- or PAR-4-independent components, all data were corrected for background, which was defined as the CD62P expression generated in the presence of saturating concentrations of PAR-1-AP or PAR-4-AP alone. Thus, only the interval between maximum PAR-1- or PAR-4-mediated and maximum thrombin-mediated activation was investigated (Figs 1, 2).

Inhibition of thrombin-induced PAR-1 cleavage was assessed as the up-regulation of SPAN12 MFI in the platelet population, and expressed as a percentage of the up-regulation of SPAN12 MFI in the absence of thrombin inhibitor. The latter was assigned an arbitrary activity of 100%. The percentage of inhibition was calculated for platelet activation as (CD62P $MFI_{thrombin+inhibitor}$/CD62P $MFI_{thrombin}$) × 100, and for PAR-1 cleavage as (SPAN12 MFI_{unstim} − SPAN12 $MFI_{thrombin+inhibitor}$)/(SPAN12 MFI_{unstim} − SPAN12 $MFI_{thrombin}$) × 100. The percentage of inhibition was plotted against the thrombin inhibitor concentration (log_{10} transformed) and fitted to sigmoidal concentration–response curves using Grafit Version 4.10 (Erytacus Software, London, UK). The thrombin inhibitor concentrations giving half-maximum inhibition effect (IC_{50}) were calculated according to the equation,

$$y = a/[1 + (x/IC_{50})^s],$$

where y = percentage inhibition (range 100–0); a = maximum range, i.e., 100 in the absence of inhibitor; s = the slope of the concentration–response curve; x = inhibitor concentration; and IC_{50} = inhibitor concentration that inhibits up-regulation of CD62P, or down-regulation of SPAN12 by 50%.

All values were expressed as mean ± standard deviation

(SD). Values for thrombin and PAR-AP concentrations giving half-maximum effect (EC_{50}), were calculated using the same equation as described for determination of IC_{50} values.

Results

Platelet activation by thrombin and PAR-AP

Thrombin, PAR-1-AP and PAR-4-AP all activated platelets, as shown by concentration-dependent increases in CD62P expression on the cell surface (Fig. 1). Up-regulation of CD62P can be expressed either as the number of platelets with exposed CD62P (as a percentage of total), or as the MFI of the entire platelet population. The percentage of positive platelets data provide information on whether platelets are activated or not, while MFI gives a value proportional to the mean density of CD62P expression per platelet. Thus there can be 100% CD62P-positive platelets at agonist concentrations that give only sub-maximum CD62P MFI (Fig. 1). CD62P MFI thus enables more precise resolution of the platelet activation response. The platelet activation results from this study are therefore presented as CD62P MFI. PAR-1-AP and PAR-4-AP could induce 80 and 40% of maximum thrombin stimulation, respectively (Fig. 1). Half-maximum CD62P MFI (EC_{50}) for thrombin, PAR-1-AP, and PAR-4-AP were obtained at concentrations of 0.64 ± 0.15 nmol/l ($n = 5$), 19 ± 9 μmol/l ($n = 5$) and 530 ± 110 μmol/l ($n = 4$), respectively. The concentrations of thrombin, PAR-1-AP and PAR-4-AP that induced 80–90% of maximum platelet activation were used in the subsequent thrombin inhibitor experiments. These were 2.7 nmol/l, 25 μmol/l, and 1000 μmol/l for thrombin, PAR-1-AP, and PAR-4-AP, respectively (Fig. 1).

A PAR-1-independent component was defined as the residual activating effect when increasing concentrations of thrombin were added to platelets after maximum PAR-1 activation (Fig. 1a). This effect was obtained by pre-incubation with 100 μmol/l PAR-1-AP. The dose–response curve for thrombin-induced activation of this component is shown in Figure 2. An EC_{50} of 3.2 ± 0.8 nmol/l ($n = 6$) for this component was calculated from this curve (Table 1) [24].

A PAR-4-independent component was similarly defined as the residual activating effect when increasing concentrations of thrombin were added to platelets after maximum PAR-4 activation (Fig. 1b). This effect was obtained by pre-incubation with 1200 μmol/l PAR-4-AP. This component had an EC_{50} of 0.048 nmol/l (Fig. 2; Table 1).

Thrombin-induced cleavage of PAR-1 was studied using SPAN12, an antibody that only binds to intact PAR-1. Thus, a concentration-dependent decrease in binding of SPAN12 to platelets reflects a concentration-dependent increase in cleavage of PAR-1. The EC_{50} for thrombin-induced cleavage of PAR-1 was 0.028 ± 0.007 nmol/l ($n = 4$) (Fig. 2; Table 1).

Figure 2 was used to select the thrombin concentrations that induced 80–90% of the maximum thrombin effect, for use in the subsequent experiments with thrombin inhibitors. These were 0.05 nmol/l and 10 nmol/l, for cleavage of PAR-1 and activation of the PAR-1-independent component, respectively. The effects of thrombin inhibitors on the PAR-4-independent component were not investigated.

Differential responses of the platelet activation components to thrombin

The dose–response curves for the various thrombin components (Fig. 2) were used to calculate to what

Table 1. Thrombin half-maximum effect (EC_{50}) values for the different assays, and the corresponding half-maximum inhibition (IC_{50}) values for the various thrombin inhibitors

	Assay			
	PAR-1 cleavage (0.05 nmol/l thrombin) ($n = 4$)	Platelet activation (2.7 nmol/l thrombin) ($n = 5$)	PAR-1-independent component (10 nmol/l thrombin) ($n = 6$)	PAR-4-independent component
EC_{50} thrombin	0.028 ± 0.007 nmol/l	0.64 ± 0.15 nmol/l	3.2 ± 0.8 nmol/l	0.048 nmol/l
Thrombin inhibitor	IC_{50} values			
Melagatran	30 ± 7 nmol/l	11 ± 3 nmol/l	13 ± 4 nmol/l	ND
Inogatran	143 ± 24 nmol/l	32 ± 9 nmol/l	ND	ND
Hirudin	0.35 ± 0.23 nmol/l	4 ± 1 nmol/l	6 ± 3 nmol/l	ND
Heparin	0.006 ± 0.003 IU/ml (3 ± 2 nmol/l*)	0.016 ± 0.003 IU/ml (9 ± 2 nmol/l*)	ND	ND
Dalteparin	0.022 ± 0.005 IU/ml (31 ± 7 nmol/l*)	0.048 ± 0.030 IU/ml (67 ± 42 nmol/l*)	ND	ND

PAR, protease-activated receptor; ND, no data.*IC_{50} values for heparin and dalteparin expressed in nmol/l, assuming specific activities of 130 and 120 IU/mg and mean molecular weights of 14 000 and 6000 Da, respectively [24].

extent the different components were activated at various thrombin concentrations (Table 2). The thrombin EC_{50} for platelet activation was 0.64 nmol/l. At this concentration approximately 95% of PAR-1 was cleaved and the PAR-1- and PAR-4-independent components were activated to 16 and 86% of their maximum values, respectively. In contrast, the thrombin EC_{50} for cleavage of PAR-1 (0.028 nmol/l) induced approximately 1% of maximum CD62P expression and less than 1 and 41% activation of the PAR-1- and PAR-4-independent components, respectively. Complete (around 99%) cleavage of PAR-1 was obtained with a thrombin concentration (1.5 nmol/l) that induced 80% of maximum thrombin-induced expression of CD62P, and which was equal to the maximum platelet activation induced by PAR-1-AP (Fig. 1). At this thrombin concentration (1.5 nmol/l), the PAR-1-independent component was activated to 32% of maximum, while the PAR-4-independent component was almost completely activated (92%). Maximum activation of the PAR-1-independent component was achieved at a thrombin concentration of 45 nmol/l. Maximum thrombin-induced expression of CD62P was also achieved at this thrombin concentration.

Inhibition of thrombin and PAR-AP-induced platelet activation

Dose–response curves were generated for the inhibition of thrombin-induced CD62P expression (with 2.7 nmol/l thrombin) by direct and indirect thrombin inhibitors. All five thrombin inhibitors were potent inhibitors of thrombin-induced CD62P expression (Table 1). None of the anticoagulants evaluated (melagatran, inogatran and hirudin) inhibited PAR-1AP- (25 μmol/l) or PAR-4-AP-induced (1000 μmol/l) platelet activation at concentrations up to 100 mmol/l (data not shown).

The addition of thrombin inhibitors resulted in increased binding of SPAN12 to PAR-1 with increasing inhibitor concentrations. Reversible and tightly binding or irreversible inhibitors gave different results in this assay. The IC_{50} values for inhibition of thrombin-induced PAR-1 cleavage by the two reversible inhibitors, melagatran and inogatran, were three to five times higher than the corresponding IC_{50} values for inhibition of platelet activation, even though the thrombin concentration was over fifty-fold higher in the latter assay (Table 1). In contrast, hirudin, heparin and dalteparin were more potent inhibitors of PAR-1 cleavage than of platelet activation (Table 1). The mean IC_{50} values ($n = 6$) for inhibition of the PAR-1-independent up-regulation of CD62P by melagatran and hirudin are shown in Table 1.

Table 2. The responses of the different components at relevant thrombin concentrations

	Effect on the different components (EC_{50} values)			
Thrombin concentration (nmol/l)	PAR-1 cleavage	Platelet activation	PAR-1-independent component	PAR-4-independent component
0.028*	50	<1	<1	41
0.048**	66	2	1	50
0.64***	95	50	16	86
3.2†	>99	94	50	95
1.5‡	99	80	32	92

PAR, protease-activated receptor. *Half-maximum efective value (EC_{50}) for PAR-1 cleavage; **EC_{50} for the PAR-4-independent component. ***EC_{50} for total thrombin-induced platelet activation. †EC_{50} for the PAR-1-independent component. ‡The thrombin concentration equal to the maximum response (80%) of PAR-1-activating peptide.

Potential thrombin inhibitor-induced platelet activation

Melagatran, inogatran and hirudin did not induce platelet activation *per se*, whereas both heparin and dalteparin induced six-fold ($P = 0.001$) and two-fold ($P = 0.007$) increases in CD62P MFI, respectively, compared with control platelets (Fig. 3).

Potential antibody-mediated inhibition of platelet activation

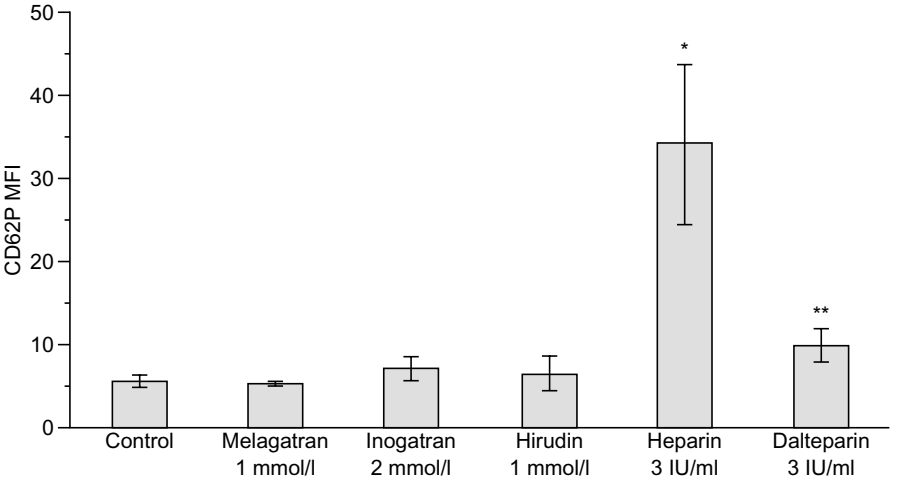
The anti-CD42b and SPAN12 antibodies did not inhibit platelet activation at the thrombin concentrations used in these experiments.

Discussion

In this investigation, thrombin-induced human platelet activation was measured in whole blood as up-regulation of CD62P and cleavage of PAR-1, using flow cytometry. Blood samples were processed without the use of centrifugation and washing procedures, in order to minimize artefactual platelet activation. As expected, a dose-dependent increase in CD62P expression was observed with both thrombin and PAR-APs. Thrombin was the most potent agonist, with PAR-1-AP and PAR-4-AP inducing approximately 80 and 40% CD62P expression, respectively, compared with thrombin (Fig. 1).

The differential responses of the platelet activation components at various thrombin concentrations (Table 2), shows that components other than PAR-1 are required to induce maximum platelet activation. These experiments show that thrombin cleaves PAR-1 at very low concentrations ($EC_{50} = 0.028$ nmol/l) and that up-regulation of CD62P is not seen until 80% of PAR-1 is cleaved. When PAR-1 was completely cleaved (at 1.5 nmol/l thrombin), the expression of CD62P was only 80% of maximum. The similar effects of thrombin on PAR-1 cleavage and on the PAR-4-independent component indicate that the PAR-4-independent component is likely to depend primarily on PAR-1. Complete expression of CD62P was obtained at the same thrombin concentration (45 nmol/l) as was required to maximally activate the PAR-1-independent component.

Fig. 3



Platelet activation by the different thrombin inhibitors tested expressed as CD62P mean fluorescence intensity (MFI). $^{*}P = 0.001$; $^{**}P = 0.007$.

This suggests that maximum platelet activation is dependent on a PAR-1-independent pathway. These results are in good agreement with the findings of Covic *et al.* [6], who described platelet activation as a sequential process. Initially, a low concentration of thrombin preferentially cleaves the high-affinity PAR-1, resulting in a rapid, but transient, Ca^{2+} flux that mediates full adenosine diphosphate (ADP) release [25]. The presence of higher concentrations of thrombin, which are generated at the site of injury, would activate the lower-affinity PAR-4, to produce a sustained high intracellular Ca^{2+} concentration.

When the IC_{50} values for the various inhibitors with respect to inhibition of PAR-1 cleavage and platelet activation are compared (Table 1), it is evident that the reversible direct thrombin inhibitors, melagatran and inogatran behave differently to the tightly binding thrombin inhibitor hirudin, and the indirect irreversible inhibitors heparin and dalteparin. The IC_{50} value for PAR-1 cleavage for the reversible direct thrombin inhibitor melagatran was approximately three-fold higher ($IC_{50} = 30$ nmol/l) than the corresponding value for inhibition of platelet activation ($IC_{50} = 11$ nmol/l), although the thrombin concentration in the PAR-1 assay was over fifty-fold lower (0.05 versus 2.7 nmol/l) than in the CD62P assay. In contrast, the tightly binding direct thrombin inhibitor hirudin, as well as the indirect irreversible thrombin inhibitors heparin and dalteparin, were more effective at inhibiting thrombin-catalysed cleavage of PAR-1 than inhibiting platelet activation. The observation that both heparin and dalteparin in the absence of agonist can induce increases in platelet activation is in agreement with previous findings [26].

A thrombin concentration of 2.7 nmol/l was used to study the inhibition of platelet activation. In the absence of a thrombin inhibitor, this concentration produced an up-regulation of CD62P to approximately 85% of maximum (Fig. 2). It was calculated from the dose–response curve for thrombin (Fig. 2), that for an inhibitor to reduce the expression of CD62P to 50% of this sub-maximal value (85%), the thrombin concentration would have to be reduced to 0.60 nmol/l. This concentration is very similar to the EC_{50} for thrombin (0.64 nmol/l). PAR-1 is almost completely cleaved at this thrombin concentration. The inhibitor-induced down-regulation of platelet activation is therefore most likely to be due to a potent inhibition of the PAR-1 independent pathway (i.e. of PAR-4 or GPIbα which is only activated to 16% of its capacity at this same thrombin concentration (0.60 nmol/l). It can, therefore, be concluded that the IC_{50} values obtained for the inhibition of platelet activation result mainly from inhibition of the PAR-1-independent pathway.

Mediation of the PAR-1-independent activation pathway, primarily via PAR-4, would also account for the dramatic difference between melagatran and hirudin with respect to inhibition of cleavage of PAR-1 and the inhibition of platelet activation. PAR-1 with its 'hirudin-like' thrombin-binding site has a very high affinity for thrombin [27]. A tightly binding direct thrombin inhibitor, such as hirudin, will therefore be able to compete effectively with PAR-1 for thrombin, thus hirudin could 'irreversibly' inhibit thrombin, and prevent cleavage of PAR-1. In the presence of a reversible direct thrombin inhibitor such as melagatran, there will be a limited amount of free thrombin available to bind to, cleave and thus activate PAR-1. However, although

hirudin was more potent than melagatran with respect to inhibiting cleavage of PAR-1 (IC_{50} ratio melagatran : hirudin = 86), the difference in IC_{50} with respect to inhibition of platelet activation was much lower (IC_{50} ratio melagatran : hirudin = 2.75). The corresponding IC_{50} ratio for the PAR-1-independent component was 2.17. This provides further support for the hypothesis that inhibition of thrombin-induced platelet activation is mediated primarily via inhibition of the PAR-1-independent pathway, as PAR-4 has a much lower affinity for thrombin than PAR-1 [4,6,7]. A potent and reversible direct thrombin inhibitor with a rapid onset of action, such as melagatran, can compete more effectively with this low-affinity receptor. The difference in affinity between hirudin and melagatran for thrombin has, therefore, limited importance with respect to PAR-4 inhibition.

The combination of PAR-1-AP and PAR-4-AP at suboptimal concentrations produced an effect on platelet activation that was as potent as the maximum stimulatory effect of thrombin (data not shown). This observation is in agreement with that of Chung *et al.* [28]. However, although this finding does not preclude a role for GPIbα in platelet activation, the interaction between thrombin and GPIbα does not appear to be a prerequisite for obtaining a complete thrombin response with respect to platelet activation. GPIbα may still have an important role in potentiation of the other pathways, either by acting as a cofactor for PAR-1 cleavage [11], or via a thrombin-mediated signalling mechanism [13].

In a recent study, Klement *et al.* [29] compared the antithrombotic and bleeding effects, of melagatran and hirudin in a rabbit model. They found that melagatran caused two- to three-fold less bleeding than hirudin when the compounds were compared at doses that resulted in 80–100% antithrombotic potency, and complete thrombus resolution. Klement *et al.* suggested that the difference in safety profile between the two drugs may reflect the fact that melagatran inhibits both fibrin-bound and fluid-phase thrombin equally well, whereas hirudin is less effective at inhibiting fibrin-bound thrombin. The observations in the present study, that melagatran mainly inhibits platelet activation via inhibition of a PAR-1-independent component (PAR-4), whereas hirudin is an effective inhibitor of both PAR-1 cleavage and the PAR-1-independent component, may also explain why there is less bleeding associated with melagatran than with hirudin. It may be that activation of PAR-1 is sufficient to reduce the bleeding from a cut in a rabbit's ear, without altering the antithrombotic effect. Similarly, Sambrano *et al.* [30] found that mice lacking functional PAR-4 had markedly prolonged bleeding times. Lack of PAR-4 function in mice can be considered analogous to inhibiting both PAR-1 and PAR-4 function in human platelets [31].

In conclusion, all the thrombin inhibitors tested in this study inhibited both thrombin-induced PAR-1 cleavage and platelet activation in a dose-dependent manner, but none had a direct effect on PAR-1 or PAR-4. PAR-1 cleavage alone is not sufficient to achieve a maximum thrombin response, and approximately 80% of this receptor must be cleaved before up-regulation of expression of CD62P is observed. Higher thrombin concentrations than those required to cleave 100% of PAR-1 are necessary for complete thrombin-induced platelet activation. The extra effect of this high thrombin concentration is mediated via a PAR-1-independent component, comprising PAR-4 and possibly also GPIbα. The low affinity of thrombin for PAR-4 results in reversible direct thrombin inhibitors, such as melagatran and inogatran, being efficient inhibitors of this PAR-1-independent component. This, and the apparent need for only a partial inhibition of PAR-1 cleavage in order to achieve a strong inhibition of platelet activation, explains why reversible direct thrombin inhibitors are potent inhibitors of thrombin-induced platelet activation, even though they are relatively poor inhibitors of PAR-1 cleavage. Furthermore, the finding that reversible direct thrombin inhibitors, such as melagatran, are only weak inhibitors of PAR-1 cleavage might be beneficial in a clinical situation, as complete inhibition is likely to increase bleeding.

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