Characterization of the Initial $\alpha$-Thrombin Interaction with Glycoprotein Ibα in Relation to Platelet Activation*

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We have evaluated the properties of $\alpha$-thrombin interaction with platelets within 1 min from exposure to the agonist, a time frame during which most induced activation responses are initiated and completed. Binding at 37 °C was rapidly reversible and completely blocked by a monoclonal antibody, LJ-Ib10, previously shown to be directed against the $\alpha$-thrombin interaction site on glycoprotein (GP) Ibα. By 2–5 min, however, binding was no longer fully reversible and was only partially inhibited by the anti-GP Ibα antibody. Results were similar at room temperature (22–25 °C), whereas the initial characteristics of $\alpha$-thrombin interaction with platelets were preserved for at least 20 min at 4 °C. Equilibrium binding isotherms obtained at the latter temperature were compatible with a two-site model, but the component ascribed to GP Ibα, completely inhibited by LJ-Ib10, had "moderate" affinity ($k_d$, on the order of $10^{-12}$ M) and relatively high capacity, rather than "high" affinity ($k_d$, on the order of $10^{-9}$ M) and low capacity as currently thought. The parameters of $\alpha$-thrombin binding to intact GP Ibα on platelets at 4 °C corresponded closely to those measured with isolated GP Ibα fragments regardless of temperature. Blocking the $\alpha$-thrombin-GP Ibα interaction caused partial inhibition of ATP release and prevented the association with platelets of measurable proteolytic activity. These results support the concept that GP Ibα contributes to the thrombogenic potential of $\alpha$-thrombin.

Platelet deposition at sites of vascular injury is thought to be enhanced by $\alpha$-thrombin during normal hemostasis as well as pathological arterial thrombosis (1–3), but the mechanisms responsible for this effect have yet to be fully understood. There is evidence that $\alpha$-thrombin-induced platelet activation is initiated by coupling of the agonist to specific receptors (4–6), whose nature is still a topic of debate. In this context, it is recognized that glycoprotein (GP) Ibα, a component of the GP Ib-IX-V complex (7, 8), binds $\alpha$-thrombin possibly with affinity higher than other sites on platelets (6, 9, 10). This interaction has been variably judged as functionally relevant (10–12) or irrelevant (13) or even as a negative regulator acting through sequestration of the enzyme (14). It is also known that $\alpha$-thrombin cleaves GP V (15, 16) but with no apparent relation to platelet activation (17, 18).

The agonist activity of $\alpha$-thrombin depends on proteolysis, a fact explained with the identification of a seven-transmembrane domain receptor stimulated by a tethered ligand sequence exposed as the new amino terminus of the molecule after cleavage of an internal Arg-Ser bond (19). This protease-activated receptor, PAR1, exemplifies an effector mechanism common to a family of related proteins exhibiting distinct specificities as substrates for different proteases (20). Because the function of PAR1 seemed to explain many of $\alpha$-thrombin effects on platelets, it was surprising that deletion of the homologous mouse gene, albeit lethal in many homozygous embryos, failed to result in decreased thrombogenic potential in the animals born alive (21). The subsequent demonstration on platelets of PAR3, another member of the family with specificity similar to PAR1 (22), provided a reasonable solution to the puzzle and reinforced the concept that a protease-activated receptor pathway is crucially involved in mediating responses to $\alpha$-thrombin. Yet the participation of GP Ibα in these processes remains a possibility that must be addressed conclusively.

There are apparent contradictions in the reported characteristics of $\alpha$-thrombin binding to platelets. Only few hundred high affinity sites have been ascribed to GP Ibα (6, 10), but the latter is present in greater number on the membrane (23). Moreover, a specific anti-GP Ibα antibody has been shown to block the interaction of approximately 5,000 $\alpha$-thrombin molecules with platelets, abolishing higher affinity sites but also decreasing markedly the moderate affinity ones (10). Finally, the apparent $k_d$ of the highest affinity sites on platelets, 0.25–1.3 nM (10), is substantially lower than that reported for $\alpha$-thrombin interaction with the isolated amino-terminal domain of GP Ibα, approximately 20 nM (24). Platelet binding parameters have been typically deduced from experiments with relatively long incubation times, in contrast with the rapidity of platelet responses to $\alpha$-thrombin stimulation (25–27), and may reflect events not relevant for activation. Indeed, the results presented here indicate that GP Ibα accounts for most of the initial $\alpha$-thrombin binding to platelets but apparently with the same "moderate" affinity assigned to the corresponding isolated functional domain (24). Different conclusions...
in this regard may be explained by time- and temperature-dependent deviations from equilibrium conditions. The fully reversible α-thrombin interaction with GP Ibα supports the association with platelets of a proteolytically active enzyme that may contribute to activation.

EXPERIMENTAL PROCEDURES

 Purification and Iodination of α-Thrombin—Purified human α-thrombin with specific clotting activity between 2,180 and 2,800 NIH units/mg (28) (a gift of Dr. John W. Fenton II, Wadsworth Center for Laboratories and Research, New York Department of Health, Albany) was radiolabeled with 125I (Amersham Corp.) using IODOGEN (Pierce) (29). The radiolabeled ligand, with specific radioactivity between 5 and 7 mCi/mg, was characterized and stored as described (10) and was used within 2 weeks of iodination.

 Preparation of Washed Platelets—Blood was drawn from normal volunteers, who had denied ingestion of drugs known to interfere with platelet function for at least 1 week and given their informed consent to these experimental studies according to the declaration of Helsinki, and was collected into one-sixth final volume of citric acid/citrate/dextrose, pH 4.5, containing 25 mM prostaglandin E1 (Sigma). Platelets were washed free of plasma constituents using the albumin density gradient method (30), with modifications previously described (31).

 Binding of 125I-Thrombin to Immobilized Glycocalicin—The extracytoplasmic domain of GP Ibα was about 30% lower at 4 °C (Fig. 1). Concurrent addition of nonlabeled ligand 20 min after 125I-thrombin was removed, the sucrose layer was discarded, and the platelet pellet was resuspended with 600 μl of binding buffer. The chromogenic substrate 5-ASA was added to the supernatant as well as the resuspended platelets at the concentration of 0.4 mM, and the incubation was continued for 5 min at 37 °C. The hydrolysis reaction was then stopped with 4% acetic acid, and the release of p-nitroaniline was measured at 405 nm in a spectrophotometer (Beckman DU-65) after removing the platelets by centrifugation at 12,000 × g for 2 min.

 RESULTS

Time Course and Reversibility of α-Thrombin Binding to Platelets—The binding of 125I-α-thrombin to washed platelets reached a maximum in 5 min at 37 °C but required 10 min and was about 30% lower at 4 °C (Fig. 1). Concurrent addition of 1000-fold excess of nonlabeled α-thrombin inhibited the binding of labeled ligand by greater than 90% at either temperature (not shown). In contrast, addition of nonlabeled ligand 20 min after 125I-α-thrombin, when binding of the latter was maximal, resulted in 80–95% dissociation of bound ligand at 4 °C but only about 50–60% at room temperature (22–25 °C) and 25–30% at 37 °C (Fig. 2). At the latter temperature, dissociation was approximately 70% when nonlabeled ligand was added 1 min after 125I-α-thrombin, 60% when it was added after 2 min, and 45% when it was added after 10 min (Fig. 2). The dissociation of bound ligand at 4 °C was not only greater in extent but occurred more rapidly than at higher temperatures, being almost maximal in 1 min as opposed to 5 min (Fig. 2).

Inhibitory Effect of Antibody LJ-Ib10 on α-Thrombin Binding to Platelets and Purified Glycocalicin as a Function of Incubation Time and Temperature—The anti-GP Ibα monoclonal antibody, LJ-Ib10, mimicked the maximum binding of 125I-α-thrombin to platelets, measured after incubation of 20 min, by approximately 75% at 4 °C but only 60% at 22–25 °C and less than 50% at 37 °C (Fig. 3). The same antibody inhibited the maximum binding to glycocalicin, the isolated extracytoplasmic domain of GP Ibα, by at least 90% at all temperatures tested (Fig. 3). In the latter case, the degree of inhibition

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terminated by centrifuging the platelets through 20% sucrose in modified Tyrode buffer (31) for 4 min at 12,000 × g, and the radioactivity in the pellet was measured in a γ counter. Non-specific binding, determined by mixing a 1000-fold excess of nonlabeled α-thrombin with the labeled ligand, was less than 0.02% of the total counts added and 10% or less of total bound ligand both at 37 and 4 °C and was subtracted from total binding to give the values shown in the figure. The number of 125I-α-thrombin molecules bound per platelet was calculated assuming a molecular mass of 37 kDa. Each point represents the mean ± S.E. of three experiments.

Fig. 1. Time course of α-thrombin binding to platelets. Washed platelets, 2.8 × 10^9/ml final count, were incubated at 37 °C (filled circles) or 4 °C (open circles) with a constant concentration (1 nM) of 125I-labeled α-thrombin. At each indicated time point, the reaction was terminated by centrifuging the platelets through 20% sucrose in modified Tyrode buffer (31) for 4 min at 12,000 × g, and the radioactivity in the pellet was measured in a γ counter. Non-specific binding, determined by mixing a 1000-fold excess of nonlabeled α-thrombin with the labeled ligand, was less than 0.02% of the total counts added and 10% or less of total bound ligand both at 37 and 4 °C and was subtracted from total binding to give the values shown in the figure. The number of 125I-α-thrombin molecules bound per platelet was calculated assuming a molecular mass of 37 kDa. Each point represents the mean ± S.E. of three experiments.

Fig. 2. Dissociation of bound α-thrombin from platelets. α–c, washed platelets were mixed with 1 nM 125I-labeled α-thrombin, as indicated in the legend to Fig. 1. After 20 min at either 4 °C (a), 22–25 °C (room temperature; (b) or 37 °C (c), 1 μM nonlabeled α-thrombin (filled circles) or the same volume of binding buffer (open circles) was added, and the incubation was continued. All indicated concentrations were in the final mixture. Bound 125I-α-thrombin was calculated (see legend to Fig. 1) immediately before and at selected time intervals after addition of the nonlabeled ligand. Each point represents the mean ± S.E. of three experiments at 4 °C or two each at 37 °C and room temperature. d, washed platelets were incubated at 37 °C with 125I-α-thrombin; nonlabeled ligand was added after 1, 2, or 10 min (arrows). Binding was measured immediately before and at selected time points after addition of the nonlabeled ligand. Similar results were obtained in two experiments.

was equivalent to that produced by a 1000-fold excess of nonlabeled ligand added concurrently with 125I-α-thrombin (not shown). At 37 °C, the time of incubation between 125I-α-thrombin and platelets influenced the inhibitory effect of LJ-Ib10 on the interaction. Inhibition was essentially complete, i.e. equivalent to that caused by a 1000-fold excess of unlabeled ligand, during the first 2 min of incubation, when binding reached approximately 50% of maximum (Fig. 4). With continuing incubation, however, the inhibitory effect of the antibody progressively decreased as compared with that seen with excess unlabeled ligand (Fig. 4). Altogether, the results shown in Figs. 1–4 are compatible with the hypothesis that α-thrombin binding to GP Ibα on platelets becomes progressively less reversible as a consequence of changes related to activation, as they occur better at 37 °C than at lower temperatures. The fact that...


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**Fig. 5.** Platelet membrane expression of P-selectin. Upper panel, washed platelets (final count 2.8 x 10⁹/ml) were incubated for 20 min at 37 °C (filled circles) or 4 °C (open circles) with increasing concentrations of α-thrombin (0.01–100 nM, corresponding approximately to 0.001–10 NIH units/ml); at the end of the incubation, hirudin was added at the final concentration of 200 units/ml. After 2 min, platelets were fixed with 1% paraformaldehyde, washed twice with Tris-buffered saline, added at the initial count of 2.8 x 10⁹/ml, and then mixed with 5 µl of PE-labeled anti-CD62P (P-selectin) monoclonal antibody (corresponding to a final concentration of 10 µg/ml purified IgG). After 30 min at 22–25 °C, positive (fluorescent) platelets that had bound the anti-P-selectin antibody were detected by flow cytometric analysis. Background fluorescence was measured by adding the same concentration of PE-labeled nonspecific mouse IgG and corresponding to that measured with the anti-CD62P antibody in the absence of α-thrombin stimulation. At either temperature less than 10% of untreated washed platelets reacted with the anti-P-selectin antibody. Each point represents the mean ± S.E. of the percent of fluorescence-positive platelets (see “Experimental Procedures”) measured in two experiments at 4 °C and six at 37 °C. Lower panel, these experiments were performed at 37 °C as described above, except that a constant concentration of 1 nM α-thrombin was incubated with platelets for the indicated periods before adding hirudin. The results shown represent the mean ± S.E. of three separate experiments.

**Fig. 6.** Effect of α-thrombin on anti-GP Ibα antibody binding to platelets. a and b, these experiments were performed as described in the legend to Fig. 5, upper panel, except that 100 µg/ml of fluorescein isothiocyanate-labeled anti-GP Ibα antibody, LJ-Ib1, was used instead of the anti-CD62P antibody. a, platelets stimulated at 37 °C; b, platelets stimulated at 4 °C. Curve 1, platelets stimulated with 1 nM α-thrombin; curve 2, platelets stimulated with 100 nM α-thrombin. The curves obtained at other thrombin concentrations have been omitted for graphical clarity. Curve NS, fluorescence distribution of nonstimulated platelets. The curve at the extreme left corresponds to background fluorescence. Similar results were obtained in three separate experiments. c, platelets were incubated with 1 nM α-thrombin at 37 °C for the indicated periods before measuring anti-GP Ibα antibody binding (see above). Each point is the mean ± S.E. of three experiments in which the median fluorescence intensity of stimulated platelets was expressed as percentage of that of nonstimulated ones.

α-thrombin binding to isolated glycopnicin was inhibited by the antibody LJ-Ib10 in identical manner at all the temperatures tested is in agreement with such a concept.

**Markers of α-Thrombin-induced Platelet Activation**—The following experiments were performed to evaluate the time course of platelet stimulation by α-thrombin and correlate the membrane expression of an activation marker, P-selectin, with changes in the accessibility of GP Ibα to antibody probes. Greater than 50% of platelets incubated with α-thrombin concentrations as low as 0.1 nM for 20 min at 37 °C exhibited increased P-selectin membrane expression, and greater than 80% was positive when the agonist concentration was in the range of 1–10 nM; in contrast, at 4 °C there was no significant change relative to nonstimulated platelets even with concentrations as high as 100 nM (Fig. 5). The number of platelets displaying surface expression of P-selectin increased rapidly after stimulation with α-thrombin, reaching a maximum in 20–40 s at 37 °C (Fig. 5).

Exposure of platelets to α-thrombin at 4 °C had no significant effect on the binding of an anti-GP Ibα antibody, whereas progressively lower binding as a function of agonist concentration was seen at 37 °C (Fig. 6). Identical results were observed whether or not 2 mM Ca²⁺ and/or 1 mM Mg²⁺ was present in the incubation mixtures (data not shown). The observed changes in anti-GP Ibα antibody binding started after a time interval approximately 10-fold longer (Fig. 6) than required for increase in P-selectin surface expression (Fig. 5) following α-thrombin stimulation.

**Effects of Temperature on α-Thrombin Binding to Platelets and Immobilized Glycocalicin**—The concentration-dependent binding of α-thrombin to platelets was different at 4 °C as compared with 37 °C (Fig. 7). Scatchard-type analysis of data generated at 37 °C, performed for comparative purposes even
Control indicates normal platelets; LJ-Ib10 and anti-TR\textsuperscript{1-160} indicate normal platelets treated with saturating amounts (150 \(\mu\)g/ml) of F(\(\text{ab}^\prime\))\textsubscript{2} fragment of the anti-GP Ib or anti-PAR1 antibody, respectively. All parameters and the corresponding standard error of estimated values were calculated with LIGAND (37, 38).

### Table I

Parameters of \(\alpha\)-thrombin binding to platelets

|                | Higher affinity |       | Lower affinity |       | Nonsaturable |       |
|----------------|-----------------|-------|----------------|-------|--------------|-------|
|                | \(k_d\) \(10^{-6}\) M | \(B_{max}\) sites/platelet | \(k_d\) \(10^{-6}\) M | \(B_{max}\) sites/platelet | \(N_1\) \(10^{-2}\) |
| Control (range of 3 assays) | 1.10 \(\pm\) 0.63–2.70 \(\pm\) 1.61 | 80 \(\pm\) 13–211 \(\pm\) 103 | 4.08 \(\pm\) 0.23–9.22 \(\pm\) 0.13 | 6055 \(\pm\) 2151–9436 \(\pm\) 5270 | 1.24 \(\pm\) 0.41–2.03 \(\pm\) 0.22 |
| LJ-Ib10 (range of 2 assays) | 2.73 \(\pm\) 1.21–6.70 \(\pm\) 2.51 | 82 \(\pm\) 20–237 \(\pm\) 107 | 0 | 0 | 1.50 \(\pm\) 0.19–2.15 \(\pm\) 0.10 |
| Anti-TR\textsuperscript{1-160} (1 assay) | 7.44 \(\pm\) 3.01 | 86 \(\pm\) 6 | 6.80 \(\pm\) 2.29 | 4195 \(\pm\) 1363 | 0.97 \(\pm\) 0.13 |
| anti-TR\textsuperscript{1-160} + LJ-Ib10 (1 assay) | 5.89 \(\pm\) 1.58 | 87 \(\pm\) 14 | 0 | 0 | 1.35 \(\pm\) 0.04 |

This is the Scatchard-type plot of the data. Similar results were obtained in four experiments.

### Discussion

The effects of \(\alpha\)-thrombin on platelets is rapidly manifest (27), and most of the induced responses may reach completion in a time frame of seconds (25, 26). Here we show that within 1 min from exposure to the agonist at 37 °C, a time sufficient for maximal activation as judged by surface expression of P-selectin, the initial \(\alpha\)-thrombin interaction with platelets is...
rapidly reversible and completely blocked by a specific anti-GP Iba antibody. By 5–10 min, however, α-thrombin binding to platelets is less promptly reversible and only partially blocked by the anti-GP Iba antibody. These changes occur in temporal relationship with decreased accessibility of GP Iba to antibody probes (43, 47–49), an event that is also known to correlate with decreased von Willebrand factor binding (50), and both are prevented at 4 °C. The latter temperature, therefore, appears to preserve in time the characteristics of α-thrombin binding to GP Iba as during the initial interaction with platelets, thus providing appropriate conditions to obtain equilibrium binding isotherms. Accordingly, in the time frame relevant for agonist-induced activation, GP Iba may bind α-thrombin with “intermediate” or moderate affinity ($k_d$ on the order of $10^{-8}$ M), rather than being the “highest” affinity site ($k_d$ on the order of $10^{-10}$ M) as currently thought (6, 10, 12). Our findings also indicate that selective blockade of α-thrombin interaction with GP Iba dampens responses to the agonist and prevents the association with platelets of a proteolytically active, thus potentially procoagulant, enzyme. Such conclusions are in agreement with recent evidence showing that kininogens inhibit α-thrombin-induced platelet aggregation because they interfere with agonist binding to GP Iba (51). Indeed, owing to its relatively high membrane density, the function of GP Iba may be relevant in localizing α-thrombin at sites of vascular injury, thus facilitating its action on specific substrates.

Application of Scatchard-type analysis to α-thrombin-platelet binding isotherms has usually resulted in curvilinear, upward concave plots indicative of a deviation from the simplest model of reversible ligand interaction with a homogeneous...
class of noninteracting receptors (52). This lack of uniformity has been interpreted as evidence for the presence of more than one type of receptor (6, 10, 12), leading to the proposed existence of three α-thrombin binding sites with high, intermediate, and low affinity ($k_d$ of approximately 0.3, 30, and 3000 nM, respectively) without nonspecific binding (6). Even considering the more likely possibility that the low affinity site represents nonspecific binding with respect to physiologic significance (32), accepting the existence of the other two sites with the reported characteristics requires prior exclusion of alternative explanations for the observed curvilinear Scatchard plots and more direct experimental evidence. Regardless of the method used for analysis of experimental results, the validity of estimated binding parameters, such as dissociation constant and receptor density, depends on the assumption that ideal thermodynamic conditions, including reversibility of ligand-receptor coupling (37, 38), are satisfied in the assay. We show here that, using intact platelets and active α-thrombin, this condition is met at 4 °C but not at 22–25 or 37 °C, owing to partly irreversible ligand binding at 37 °C as well as room temperature (usually 22–25 °C). Notably, previous studies proposing the concept that GP Ibα is the high affinity α-thrombin binding site were performed at room temperature (6, 10, 12) and, thus, may have resulted in incorrect estimates for the parameters of interaction.

The results obtained with platelets at 4 °C are still best fitted with a two-site model represented by an upwardly concave Scatchard plot but are compatible with the conclusion that the binding of α-thrombin to GP Ibα occurs with a $k_d$ of between 4 and $9 \times 10^{-8}$ M, as shown by obliteration of this class of sites by the monoclonal antibody LJ-Ib10 without any influence on the putative higher affinity sites. The latter observation is relevant, since previous evaluation of the effects of this antibody at room temperature had shown apparent inhibition of both high and intermediate affinity receptors, as well as appearance of a new class of binding sites, not present on control platelets, with affinity halfway between high and intermediate (10). This finding, later confirmed independently (12), could be taken to reflect the existence of negative cooperativity between two distinct receptors but, in view of the above considerations on equilibrium binding, is more likely to indicate that the parameters estimated at 22–25 °C were erroneous. It is also relevant to note that the sum of presumed high and intermediate affinity sites inhibited by LJ-Ib10 at room temperature (10, 32) is of the same order of magnitude as the number of homogeneously intermediate affinity sites inhibited at 4 °C, in agreement with the notion that the sites may be the same and the estimated affinities at room temperature may be misleading.

The conclusion that α-thrombin interaction with GP Ibα on platelets has a $k_d$ on the order of $10^{-8}$ M is substantiated both by results obtained with isolated glycoprotein, as shown here and in agreement with independent data reported elsewhere (24, 32), and by previous findings with the recombinant aminoterminal domain of GP Ibα (24). In the case of isolated receptor fragments containing the α-thrombin binding site, interactions with the ligand are fully reversible at 4 °C as well as 37 °C and occur with $k_d$ between 1 and $5 \times 10^{-8}$ M, reflecting the initial attributes of α-thrombin pairing with GP Ibα on platelets. These results cannot support the proposed alternative possibility that high and intermediate affinity sites are both expressed on GP Ibα (9). They also indicate that other components of the GP Ib-IX-V complex have no direct influence on the function of the α-thrombin binding site on GP Ibα, suggesting that data to the contrary obtained in heterologous expression systems (53) may depend on specific experimental conditions. The nature and physiologic significance of the α-thrombin binding sites not inhibited by LJ-Ib10, of higher affinity than GP Ibα sites on the basis of the results obtained at 4 °C, remain undetermined at present. Candidates for their identification may include PAR1 (13, 19), PAR3 (22), and protease nexin 1 (54).

Despite evidence to the contrary from independently performed experiments (10, 12), others have reached the conclusion that antibodies against the proposed α-thrombin binding site on GP Ibα, such as LJ-Ib10, have no inhibitory effect on platelet interaction with the agonist nor on activation (13). As in the case of studies aimed at determining binding characteristics, the methodology used may have influenced the conclusions reached. Lack of inhibitory effect by LJ-Ib10 was reported in experiments in which platelets were fixed after incubation with α-thrombin, then processed for indirect detection of bound ligand after repeated washing steps. It may be that, after such a procedure, the α-thrombin remaining associated with platelets, not defined in terms of quantity or binding characteristics, is interacting with PAR1 rather than GP Ibα, as suggested (13); such a conclusion is compatible with the two-site model discussed here. On the other hand, the present studies provide direct evidence that the reversible α-thrombin binding to platelets at 37 °C can be blocked by LJ-Ib10 within the first 60 s of incubation. The same antibody reduces dense granule ATP release, acting with an anti-PAR1 antibody to yield more efficient inhibition. Moreover, it is apparent that the α-thrombin associated with platelets through a GP Ibα-dependent mechanism remains available as an active enzyme. One function of this relatively high capacity site, therefore, may be that of increasing the concentration of α-thrombin onto or in proximity of the platelet membrane for subsequent proteolytic cleavage of appropriate substrates. This latter event may take place not while the enzyme is bound to GP Ibα, assuming that the association prevents catalytic function (14), but after dissociation from the receptor that may occur rapidly during the time frame of interaction relevant for platelet activation and clotting. Therefore, even without considering the possibility of coupling to a distinct signaling pathway that remains to be proven directly (12), our present findings add evidence to the previously proposed concept (10, 12) that α-thrombin interaction with GP Ibα has a net prothrombotic effect.

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