The Thrombin High-Affinity Binding Site on Platelets Is a Negative Regulator of Thrombin-Induced Platelet Activation. Structure–Function Studies Using Two Mutant Thrombins, Quick I and Quick II†

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ABSTRACT: To elucidate the thrombin domains required for high-affinity binding and platelet activation, the platelet binding properties of thrombin and two mutant thrombins, thrombin Quick I and Quick II, were compared to their agonist effects in elevating intraplatelet [Ca^{2+}]. In Quick I, a mutation within the fibrinogen binding groove results in decreased clotting and platelet aggregating activities, whereas in Quick II, a mutation in the primary substrate binding pocket abolishes both activities. Dysthrombin binding was decreased compared to thrombin. The fibrinogen binding groove appeared more important than the primary substrate pocket for high-affinity binding since Quick I showed drastically reduced, and Quick II only slightly reduced, binding affinity (K_d ~200 and ~10 nM, respectively). The deduced interaction of thrombin with its high-affinity binding site indicated that the thrombin catalytic site is directed toward the platelet surface and therefore, when bound, is proteolytically inactive. Quick I (0.5–5 nM) elicited intraplatelet [Ca^{2+}] fluxes at concentrations where high-affinity binding was undetectable. Saturation of high-affinity binding sites with active-site-modified thrombin did not affect thrombin-induced (0.5 nM) or Quick I-induced (5 nM) responses. In contrast, addition of D-Phe-Pro-Arg chloromethyl ketone (FPRCK) subsequent to thrombin or Quick I stimulation of platelets abolished agonist-induced responses. Since Quick I was only 10–17% as effective as thrombin in increasing intraplatelet [Ca^{2+}], our data support a model in which thrombin acts enzymatically on a platelet membrane "substrate", through an interaction mediated in part by the fibrinogen binding groove of thrombin. This conclusion is consistent with the inhibition observed with high concentrations (>100 nM) of Quick II and FPRCK-modified thrombin (FPR-thrombin) in platelets stimulated with low concentrations of thrombin (<0.5 nM) or Quick I (<2 nM), consistent with inhibition by substrate depletion. In contrast, concentrations of FPR-thrombin or Quick II (<100 nM), which saturated predominantly the high-affinity binding sites, enhanced the platelet responses induced by thrombin (<0.5 nM). Thus, occupation of the high-affinity sites with inactive thrombin increased the concentration of active thrombin available for substrate interaction. Quick I-induced responses were not enhanced, consistent with its inability to interact with the high-affinity site. Since thrombin bound to the high-affinity site is proteolytically inactive, we hypothesize that the thrombin high-affinity binding site on platelets functions to alter thrombin activity and platelet activation.

Platelet interaction with thrombin is associated both with high-affinity binding and with cellular activation, which is characterized by a variety of biochemical and morphological changes, culminating in platelet aggregate formation (Siess, 1989). High-affinity, human thrombin binding to platelets has been demonstrated, and is characterized by K_d values ranging from 0.2 to 4.5 nM, with 50–1350 sites on the platelet surface (Shuman et al., 1976; Martin et al., 1976; Jamieson & Okumura, 1978; Harmon & Jamieson, 1985). Little is known about the thrombin domains required for high-affinity binding, although the data with γ-thrombin, which does not compete with thrombin for binding to the saturable, high-affinity site on platelets (Alexander et al., 1983), suggest that domains conferring specificity toward macromolecular substrates (Elion et al., 1986) may be involved. The active site of thrombin is not required for high-affinity binding, since active-site-blocked thrombins bind indistinguishably from the unmodified enzyme (Tollefsen et al., 1974; Shuman et al., 1976; Workman et al., 1977a; Alexander et al., 1983; Knupp & White, 1985).

In contrast to high-affinity, dissociable, equilibrium binding, thrombin activation of platelets requires the thrombin active site, since active-site-modified thrombin neither induces platelet activation nor, in combination with thrombin, inhibits thrombin-mediated activation (Tollefsen et al., 1974; Shuman et al., 1976; Workman et al., 1977a; Alexander et al., 1983; Knupp & White, 1985). These results indicate that thrombin proteolytic activity is essential for platelet activation. The activation of platelets by various proteases (Martin et al., 1975; Alexander et al., 1983) and the recent identification of a potential platelet "substrate" conferring thrombin responsiveness to *Xenopus* oocytes (Vu et al., 1991) are also consistent with a proteolytic mechanism of platelet activation.

The function of high-affinity binding in modulating platelet activation is less clear. The high-affinity binding of thrombin to platelets is characterized by a K_d in a concentration range

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that results in platelet activation. Studies with hirudin, which inactivates thrombin (Markwardt, 1970) and blocks binding to platelets (Tam et al., 1979), have been interpreted to indicate that specific activation events require continuous occupancy of the high-affinity dissociable sites while others do not (Holmsen et al., 1984; Huang & Detwiler, 1987). The loss of high-affinity binding in platelets treated with *Serratia marcescens* protease concomitant with a decrease in platelet responsiveness to thrombin (Cooper et al., 1982; Harmon & Jamieson, 1988; Yamamoto et al., 1991) likewise supports a facilitative role for high-affinity binding in platelet activation. However, a similar loss of platelet responsiveness to thrombin is observed in platelets treated with chymotrypsin or elastase (Tam et al., 1980; McGowan et al., 1983; McGowan & Detwiler, 1986; Brower et al., 1985), with minimal or undetectable alterations in thrombin binding characteristics or affinity (Tam et al., 1980; Brower et al., 1985). In this study, we have examined the thrombin domains required for high-affinity binding and platelet activation by using well-characterized mutant thrombins, and have assessed the functional significance of dissociable thrombin binding in platelet activation.

The two mutant thrombins, Quick I and Quick II, used in this study have been extensively characterized (Henriksen et al., 1980; Henriksen & Brotherton, 1983; Henriksen & Owen, 1987; Henriksen & Mann, 1988, 1989; Stone et al., 1991). Point mutations in different positions resulting in different functional defects have been identified in each of these mutant thrombins. In Quick I, an Arg to Cys substitution at position 382 [67][1] of prothrombin is within the secondary specificity site for fibrinogen in thrombin (Henriksen & Mann, 1988; Bode et al., 1989). Quick I has decreased clotting and platelet aggregating activities, but nearly normal activity toward chromogenic substrates (Henriksen & Brotherton, 1983; Henriksen & Owen, 1987). In Quick II, Gly-558 [226], within the arginine binding pocket of thrombin, is substituted by Val (Henriksen & Mann, 1989). Quick II lacks catalytic activity toward biologic and chromogenic substrates for thrombin (Henriksen & Owen, 1987; Henriksen & Mann, 1989). Our results demonstrated defects in platelet binding for both mutants which could be interpreted to indicate a requirement for the fibrinogen binding groove of thrombin in high-affinity, dissociable binding. Quick I activated platelets in the absence of high-affinity binding, indicating that high-affinity binding and cell activation are separate events. This conclusion is consistent with the observation that saturation of high-affinity sites with active-site-modified thrombin or Quick II had no effect on Quick I-induced activation, further indicating that high-affinity thrombin binding does not facilitate platelet activation. Further, a comparison of the binding interactions of the two mutant thrombins with hirudin and with the platelet high-affinity binding site indicated that thrombin bound to this site is proteolytically inactive. As a consequence of these combined observations, data are presented which indicate that the function of high-affinity thrombin binding to the platelet surface is to modulate the active thrombin concentration and platelet activation.

Materials and Methods

Materials. Taipan snake venom, potato apyrase (grade VIII), Sephadex G-25-150, sulfopropyl-Sephadex, and digitonin (50% purity) were purchased from Sigma. Digitonin was purified further by recrystallization with 100% ethanol. Benzamidine–Sepharose was obtained from Pharmacia. $Na^{125}I$ was purchased from Amersham and IODO-GEN from Pierce. D-Phe-Pro-Arg chloromethyl ketone (FPRCK)[2] and platelet activating factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), were obtained from Calbiochem; ChromozymTH was from Boehringer Mannheim, S2238 was from Kabi Pharmaceuticals, SpectrozymTH was from American Diagnostica, and Centricon-10 microconcentrators were from Amicon. Crystallized bovine serum albumin was purchased from ICN ImmunoBiologicals, *n*-butyl phthalate was from Fisher, and Apiezon A was from James G. Biddle. Fura-2 AM was from Molecular Probes, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619) was from Cayman Chemicals, and thapsigargin was from GIBCO BRL.

Proteins. Thrombin was prepared by activating human prothrombin with Taipan snake venom according to described methods (Owen & Jackson, 1973). Thrombin Quick was prepared as described (Henriksen et al., 1980) from prothrombin Quick which was isolated from plasma obtained by pheresis from the donor, V.A. (Quick et al., 1955; Quick, 1974). Approval of the University of Iowa Human Subjects Committee (Iowa City, IA) and informed consent of the donor were obtained. The thrombin Quick preparation was applied to benzamidine–Sepharose, equilibrated in 20 mM Tris–60 mM NaCl, pH 7.5. The column was washed with the same buffer to remove Quick II, following which the buffer was brought to 1 M arginine to elute Quick I. Arginine was removed from the Quick I preparation by gel filtration on Sephadex G-25 in 10 mM Tris–200 mM NaCl, pH 7.5. Quick II was rechromatographed on sulfopropyl-Sephadex (Henriksen & Brotherton, 1983). Aliquots of Quick I and Quick II were stored at −70 °C. The activity of Quick I was determined by ChromozymTH hydrolysis and by fibrinogen clotting. Quick I had 80% amidolytic activity and 1% clotting activity of thrombin. Quick II showed <1% contamination with Quick I. Inactive thrombin and Quick I were prepared by active-site modification with FPRCK. To eliminate the possibility of contaminating Quick I, Quick II preparations were inactivated using the same protocol. A 210–240-fold molar excess of FPRCK was required to completely inactivate thrombin, whereas a 90-fold molar excess was sufficient to inactivate Quick I. FPRCK, neutralized to pH 7 immediately before addition, was added at 30 times the protein concentration, and incubated at room temperature for 15 min. Excess FPRCK was removed by gel filtration, or by repeated concentration and dilution in Centricon-10 microconcentrators. The process of FPRCK addition and removal was repeated 7–8 times for thrombin and 2 times for Quick I to eliminate residual enzyme activity. Residual FPRCK was finally removed by extensive dialysis against 20 mM Tris–300 mM NaCl, pH 7.4. Possible FPRCK contamination of the FPR-thrombin preparations was assayed by monitoring hydrolysis of chromogenic substrate by 0.2 nM thrombin in the presence of 1 μM FPR-thrombin. Complete inactivation of the various thrombins was assayed by two methods. High concentrations of modified thrombin (1 μM) neither induced intraplatelet $[Ca^{2+}]$ fluxes (see below) over the course of 5 min nor cata-

[1] "Human thrombin residues are numbered according to the human prothrombin sequence" (Degen et al., 1983). The corresponding chymotrypsin-based numbering (Bode et al., 1989) is given in brackets.

[2] Abbreviations: FPRCK, D-Phe-Pro-Arg chloromethyl ketone; FPR-thrombin and FPR-Quick I, FPRCK-modified thrombin and Quick I, respectively; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PGE_1, prostaglandin E_1; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP_3, inositol 1,4,5-trisphosphate; PAF, platelet activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); U46619, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$.

lyzed chromogenic substrate hydrolysis over the course of 30 min. The modified and unmodified thrombins migrated identically on 10% gels in SDS–PAGE (Laemmli, 1970), indicating that the lengthy inactivation procedure did not result in detectable protein degradation.

Thrombin, FPR-thrombin, Quick I, and Quick II were iodinated according to a modified (Monkovic & Tracy, 1990) IODO-GEN transfer technique (Dewanjee et al., 1986). Briefly, $Na^{125}I$ (1.9–6.5 μCi/μg of protein) was added to a test tube coated with 125 μg of IODO-GEN and containing 100 μL of 20 mM Tris–150 mM NaCl, pH 7.4. After 5 min, the oxidized iodonium ion was transferred to the protein (0.185–10 mg/mL in 20 mM Tris–150 mM NaCl, pH 7.4) solution. After 15 min, labeled protein was isolated by gel filtration with a Sephadex G-25-150 column, equilibrated and developed with 150 mM sodium phosphate buffer, pH 7.6, or 20 mM Tris–300 mM NaCl, pH 7.4. Labeled protein fractions (>98% precipitable with 10% trichloroacetic acid) were pooled and dialyzed overnight against 20 mM Tris–300 mM NaCl, pH 7.4. Protein concentration was determined using an extinction coefficient $E^{1\%}_{280nm,1cm} = 17.4$ and $M_r = 37\,000$ (Fenton et al., 1977). Specific radioactivity of labeled proteins varied from 800–3000 cpm/ng for thrombin, 3100 cpm/ng for FPR-thrombin, 450–3300 cpm/ng for Quick I, and 380–6000 cpm/ng for Quick II. Radiolabeled proteins were stored at −20 °C.

Platelet Preparations. Blood was obtained, after informed consent, from healthy adults denying aspirin and ibuprofen ingestion 10–14 days prior to phlebotomy, using acid–citrate–dextrose as an anticoagulant (1:6 v/v, anticoagulant to blood). Platelet-rich plasma was isolated from whole blood, and platelets were washed as described (Mustard et al., 1972), with minor modifications. Apyrase was omitted, and the wash solutions were acidified to ~pH 6.8 with 2.9% (v/v) acid–citrate–dextrose. In some cases, platelets were treated with 1 mM aspirin and 0.5 μM PGE_1 in platelet-rich plasma before being washed (Rittenhouse, 1983). Washed platelets were resuspended in 5 mM HEPES-buffered Tyrode's solution, pH 7.35, with 0.35% bovine serum albumin. For fluorescence studies measuring intraplatelet $[Ca^{2+}]$, $CaCl_2$ was omitted from the wash and resuspension solutions.

Platelet Binding. Platelets [(2–4) × 10^8/mL] were incubated with radiolabeled thrombin, FPR-thrombin, Quick I, or Quick II for 2 min at room temperature. In order to examine high-affinity interactions of the various thrombins with platelets, the concentrations of ligands to be studied in the binding assays were restricted to ≤100 nM. The concentration range for thrombin and FPR-thrombin was from 0.1 to 90 nM, while for Quick I and Quick II, the range varied from 0.2 to 100 nM. Bound ligand was separated from free by centrifugation through an Apiezon A–*n*-butyl phthalate mixture (1:9 v/v) at 13000*g* for 2 min (Miletich et al., 1977; Tracy et al., 1979). Radioactivity associated with pellets, supernatants, and aliquots of the reaction mixture was determined. Total and nonspecific binding was determined by measuring radioligand binding in the absence and presence, respectively, of a 50-fold molar excess of unlabeled FPRCK-modified ligand for thrombin or Quick I, and unmodified ligand for Quick II. Specific binding was calculated by subtracting nonspecific binding from total binding. For competition studies, various concentrations of unlabeled FPR-thrombin, FPR-Quick I, or Quick II (0.25–500 nM) were added to a fixed concentration (0.2 nM) of ^{125}I-thrombin, and the reactions were initiated by the addition of platelets (2 × 10^8/mL). FPRCK-modified thrombin and Quick I were used to prevent nonspecific proteolysis that would otherwise occur with high concentrations of enzyme. FPRCK modification of Quick II was unnecessary since Quick II lacked proteolytic activity toward thrombin substrates (Henriksen & Owen, 1987; Henriksen & Mann, 1989).

Total binding data were analyzed by means of the program LIGAND (Munson & Rodbard, 1980), using a model of one (or two) specific site(s) together with a nonsaturable binding component. The nonsaturable component was regarded as a parameter whose value was adjusted, together with the values for binding affinity and capacity, to obtain the optimal fit to the total binding data; this approach avoids the problems that are intrinsic to any experimental measurement of nonspecific binding (Mendel & Mendel, 1985; Kermode, 1989). All the data presented were obtained using this computer-fitting approach, but comparable results were obtained by fitting specific data calculated by using the traditional method of correcting for nonspecific binding, i.e., measuring radioligand binding in the presence of excess unlabeled ligand, which were FPRCK-modified, as appropriate.

Platelet Intracellular $[Ca^{2+}]$ Measurements. Preparation of washed platelets for intraplatelet $[Ca^{2+}]$ measurements was performed as described (Pollock et al., 1986) in 5 mM HEPES-buffered Tyrode's solution, pH 7.35, containing 0.35% bovine serum albumin. Fluorescence was measured at 500 nm on a Perkin-Elmer LS-3B fluorometer (Perkin-Elmer). Excitation was at 339 nm. Maximum fluorescence was determined by adding 0.5 mM digitonin (3 times recrystallized) and 1 mM $CaCl_2$ to the platelet suspension. Minimum fluorescence was determined by adding 10 mM Tris and 5 mM EGTA to the cell suspension containing digitonin and $CaCl_2$. Maximum and minimum fluorescence was determined for each sample. Fluorescence data were analyzed as described for single-wavelength measurements (Grynkiewicz et al., 1985). In experiments to determine the role of external Ca^{2+} on agonist-induced $[Ca^{2+}]$ elevations, 0.1 mM EGTA was added to the platelet suspension 3 min prior to agonist addition. To determine the role of inositol 1,4,5-trisphosphate (IP_3) in agonist-induced Ca^{2+} mobilization, IP_3-sensitive stores were depleted with 0.1 μM thapsigargin preincubation (Thastrup, 1987; Thastrup et al., 1990) for 3 min, followed by 0.5 nM thrombin or 5 nM Quick I addition. Thapsigargin preincubation was performed in the presence of 0.1 mM EGTA to minimize the contribution of Ca^{2+} influx to intracellular $[Ca^{2+}]$ increases. In experiments examining the effect of agonist inactivation, platelets were stimulated with either thrombin (0.2 nM) or Quick I (2 nM), followed by 200 nM FPRCK addition after various intervals. The effect of FPRCK addition on Ca^{2+} flux mediated by PAF and U46619 was also assessed to determine the specificity of the FPRCK effect. The concentrations of PAF and U46619 used in these assays have been demonstrated by other investigators to elicit Ca^{2+} flux in human platelets (Hallam et al., 1984; Powling & Hardisty, 1986). To assess the function of high-affinity thrombin binding, FPR-thrombin either was added 5 min before or was added simultaneously with either thrombin or Quick I. In some cases, Quick II pretreated with FPRCK to remove contaminating Quick I was added simultaneously with agonist in similar protocols.

Results

Dissociable Thrombin, Quick I, and Quick II Platelet Interactions. Equilibrium binding of thrombin, FPR-thrombin, and the two dysthrombins, Quick I and Quick II, to platelets was measured as a function of ligand concentration, from 0.1 to 100 nM. Thrombin binding data, analyzed using LIGAND,

Table I: High-Affinity Thrombin and Dysthrombin Interactions with Human Platelets: Relative Affinity and Number of Sites

ligand	K_a (M^{-1})[a]	K_d ($\times 10^9$ M)	no. of sites[b]
IIa	$(8.6 \times 10^8) \pm (1.6 \times 10^8)$	1.0–1.5 (0.2–4.5)[c]	180–1200 (50–1350)[c]
QI	$(4.8 \times 10^6) \pm (1.1 \times 10^6)$	170–270	170–270
QII	$(2 \times 10^8) \pm (9.5 \times 10^7)$	3.3–9.5	160–500

[a] Binding parameters were derived from analyzing accumulated direct binding or competition data, unless otherwise indicated. Parameter values determined from direct binding data are consistent with those derived from displacement studies. For thrombin, the results were determined by analyzing the cumulative data from seven experiments with different donors. For Quick I, the results were derived from analysis of cumulative data from two competition experiments with different donors, since direct Quick I binding data did not allow accurate determination of either an affinity constant or the number of binding sites. The results for Quick II were derived from five direct binding or two competition experiments with different donors. Mean and standard errors of K_a are shown, and corresponding K_d values are expressed as ranges. [b] The number of sites is expressed as a range and indicates the number present per platelet. [c] The binding affinity of the high-affinity thrombin site is expressed as a range of K_d values which are derived from studies using human thrombin (Shuman et al., 1976; Martin et al., 1976; Jamieson & Okumura, 1978; Harmon & Jamieson, 1985).

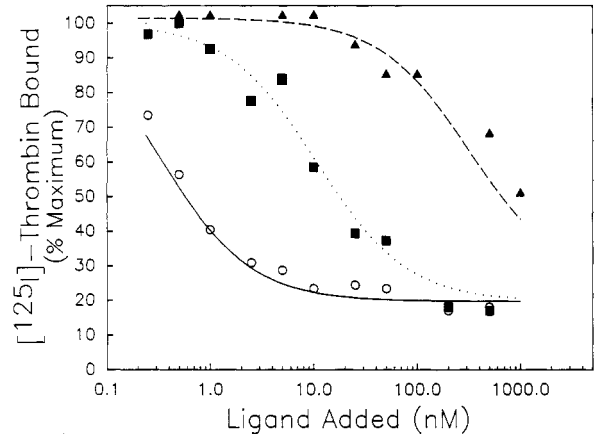

FIGURE 1: Displacement of ^{125}I-thrombin bound to the high-affinity site by FPR-thrombin, FPR-Quick I, or Quick II under equilibrium conditions. The ability of FPR-Quick I and Quick II, relative to FPR-thrombin, to compete with ^{125}I-thrombin (0.2 nM) for binding to platelets was assessed as outlined under Materials and Methods. Binding is expressed as a percentage of maximum ^{125}I-thrombin bound, with 100% being ^{125}I-thrombin binding in the absence of unlabeled ligand. FPR-Thrombin binding is represented by (○), FPR-Quick I by (▲), and Quick II by (■). The binding curves represent optimal fit of binding parameters generated using LIGAND. The figure is a composite of two different competition experiments, performed with FPR-thrombin and FPR-Quick I, or with FPR-thrombin and Quick II. The second FPR-thrombin displacement curve has been omitted for clarity of presentation, and is described by similar binding parameters as the FPR-thrombin curve shown. Thrombin binding in the presence of FPR-thrombin has the following parameters: binding affinity, $K_a = 6.69 \times 10^8$ M^{-1}; binding capacity, $R = 1.77 \times 10^{-10}$ M; nonsaturable fraction, $N = 0.031$. For thrombin binding in the presence of FPR-Quick I, the displacement curve is described by $K_a = 4.3 \times 10^6$ M^{-1}, $R = 1.77 \times 10^{-10}$ M, and $N = 0.031$. The curve describing Quick II competition for the thrombin site used $K_a = 1.1 \times 10^8$ M^{-1}, $R = 2.8 \times 10^{-11}$ M, and $N = 0.02$.

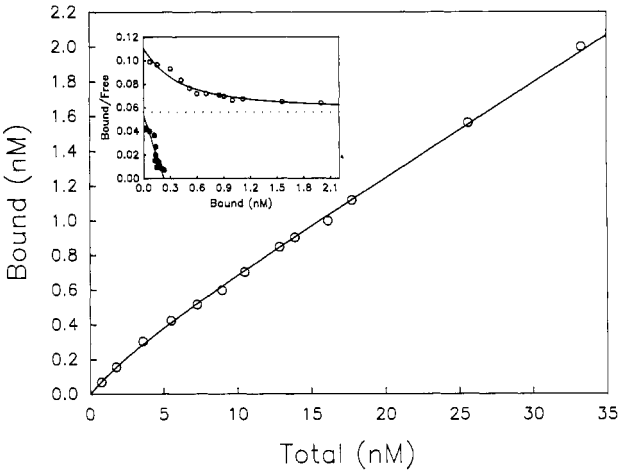

FIGURE 2: Concentration-dependent binding of Quick II to human platelets. Quick II binding to platelets (4×10^8/mL) was performed as outlined under Materials and Methods. Total binding data are shown (○) in the form of a saturation isotherm. The inset shows the data in the form of a Scatchard plot (Scatchard, 1949), illustrating the breakdown of the total binding data (○) into its specific (●) and nonsaturable (···) components. The curves represent optimal fits using the binding parameters generated by LIGAND: $K_a = 2.4 \times 10^8$ M^{-1}; $R = 2.3 \times 10^{-10}$ M; $N = 0.057$. The data shown are obtained from a single experiment and are representative of five studies with different donors.

fit a one-site model with a nonsaturable component. Thrombin interaction with the high-affinity site was governed by a K_d of ~1 nM (range = 0.3–5 nM for different platelet preparations), with ~500 sites (range = 200–1200 sites). These values are consistent with reported values for the thrombin high-affinity site (Table I). There was no improvement in the fit to these data when a two-site model was employed. The one-site model with a nonsaturable component and a two-site model were indistinguishable on the basis of residual sum of squares, whether data derived from different donors were analyzed separately or together. Therefore, the two-site model was rejected (Munson & Rodbard, 1980). Our inability to detect a second class of thrombin binding sites reported by other investigators is likely due to the limited concentration range (≤100 nM) employed in our studies. The nonsaturable component detected by our binding data is likely to represent the low-affinity thrombin binding site (K_d in the micromolar range) reported by other investigators. Likewise, FPR-thrombin binding to platelets also fit a one-site model with a nonsaturable component, characterized by a K_d of ~0.2 nM, a binding affinity comparable to that of thrombin (Table I).

The binding affinity of Quick I and Quick II for platelets was substantially less than that observed for thrombin (Figure 1). FPR-Thrombin, FPR-Quick I, and Quick II displaced ^{125}I-thrombin (0.2 nM) binding to platelets, indicating that these ligands competed for binding to the same site as thrombin. Analysis of competition data indicated that the platelet–Quick I interaction is governed by a K_d of 170–270 nM (Table I) whereas a K_d of 8–13 nM was obtained for the Quick II interaction with platelets. Competition studies with FPR-thrombin gave similar binding parameter values as those obtained from direct binding studies (K_d of 0.6–1 nM, with 150–240 sites). Data obtained using Quick II in direct binding studies gave similar results as those obtained from competition studies (Figure 2, Table I). Quick II bound saturably to a single class of sites on platelets, with a K_d of 3–10 nM and 160–460 sites. Direct Quick I binding to platelets was barely detectable, and specific Quick I binding was undetectable at concentrations ≤5 nM (data not shown).

A previous study has indicated that Quick I induces platelet aggregation, however, at concentrations considerably lower than those required to effect dissociable binding to platelets (Henriksen & Brotherton, 1983). In contrast, Quick II fails to activate platelets at concentrations where significant dis-

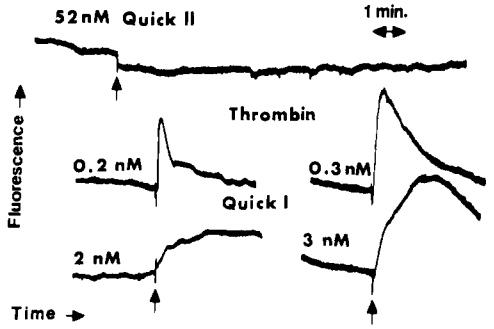

FIGURE 3: Elevation of platelet intracellular [Ca^{2+}] by thrombin, Quick I, or Quick II. Platelets (2×10^8/mL) loaded with 1 μM Fura-2 AM were stimulated with varying thrombin, Quick I, or Quick II concentrations as described under Materials and Methods. The typical pattern of Ca^{2+} response with the indicated concentrations of Quick II, thrombin, or Quick I is shown. Agonist addition is indicated by arrows beneath the traces.

sociable platelet binding is observed (Henriksen & Brotherton, 1983), as determined by these studies. Since platelet aggregation is a relatively late activation response and is the culmination of a variety of metabolic changes, including IP_3 generation (Siess, 1989), the ability of Quick I and Quick II to elicit an earlier activation response, IP_3-mediated Ca^{2+} mobilization, was examined.

Effect of Thrombin, Quick I, and Quick II on Platelet Ca^{2+} Mobilization. Typically, basal intraplatelet Ca^{2+} concentrations were ~100 nM. Representative fluorescence tracings show the effects of thrombin, Quick I, and Quick II on intraplatelet Ca^{2+} levels (Figure 3). Thrombin, at 0.05–0.5 nM, induced a rapid spike in intracellular [Ca^{2+}], followed by a gradual decline toward basal levels. Unlike thrombin, Quick I at concentrations of 1–5 nM induced a biphasic [Ca^{2+}] rise. The initial [Ca^{2+}] rise was rapid, although slower than that observed with thrombin, and this rapid [Ca^{2+}] rise was followed by a slower rise to peak levels. Once peak levels were achieved in Quick I-stimulated platelets, the Ca^{2+} levels either remained elevated or declined to basal levels, depending on the concentration of Quick I used. High concentrations of Quick I ($\geq$10 nM) induced a Ca^{2+} spike qualitatively similar to thrombin, although the rate of rise was still slower (data not shown). Treatment of platelets with aspirin and PGE_1 prior to isolation and washing did not affect the shape of the Ca^{2+} curve when platelets were stimulated with either thrombin or Quick I, suggesting that thromboxane A_2 release had no effect on agonist-induced Ca^{2+} elevation in our studies. Neither Quick II (52 nM, Figure 3) nor FPR-thrombin (1 μM, data not shown) elevated intracellular [Ca^{2+}].

Influx of extracellular Ca^{2+} produces a slower rate of [Ca^{2+}] increase than that observed with release from internal, dense tubule stores (Davies et al., 1988). Since the pattern of [Ca^{2+}] rise with Quick I had a slow component, the effect of EGTA on Quick I-induced [Ca^{2+}] elevation was examined. In these experiments, extracellular EGTA decreased basal Ca^{2+} levels to ~60 nM, and did not affect the qualitative pattern of [Ca^{2+}] rise, except that the decline from peak levels was more rapid (data not shown). Extracellular EGTA decreased the magnitude of the [Ca^{2+}] rise induced by thrombin and Quick I to the same extent (69 $\pm$ 4% of 0.5 nM IIa alone; 62 $\pm$ 3% of 5 nM Quick I alone, n = 8). The similarity of effects observed with EGTA indicated that the qualitative difference in Ca^{2+} response with Quick I as an agonist, when compared to thrombin, was not due to an increase in extracellular Ca^{2+} influx.

To demonstrate that the increase in intracellular [Ca^{2+}] is a result of IP_3-induced release of Ca^{2+} from dense tubules

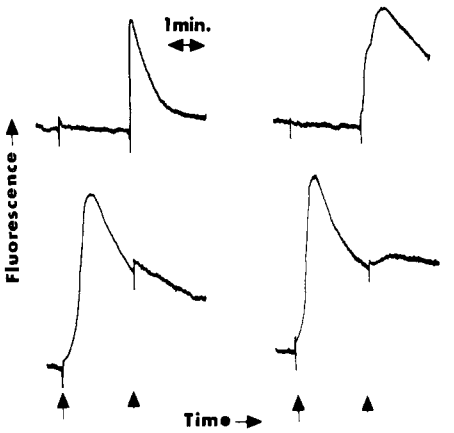

FIGURE 4: Effect of thapsigargin depletion of IP_3-sensitive Ca^{2+} pools on thrombin-induced or Quick I-induced Ca^{2+} mobilization. The effect of thapsigargin on agonist-induced Ca^{2+} responses was examined in platelets as indicated under Materials and Methods. The top traces show the effect of the vehicle on 0.5 nM thrombin-induced and 5 nM Quick I-induced Ca^{2+} responses (left and right traces, respectively). The bottom traces show the effect of thapsigargin, followed by 0.5 nM thrombin (left) or 5 nM Quick I (right) stimulation. Additions are indicated by arrows and arrowheads, respectively.

during Quick I stimulation, dense tubule Ca^{2+} was depleted by prior addition of a high concentration of thapsigargin, in the presence of extracellular EGTA. Thapsigargin is a microsomal Ca^{2+}-ATPase inhibitor, which releases Ca^{2+} from IP_3-sensitive intracellular stores by an, as yet, unknown mechanism that is IP_3-independent (Thastrup et al., 1987, 1990). Preincubation with the vehicle for 3 min had no effect on either the thrombin-induced or Quick I-induced Ca^{2+} responses (Figure 4, top traces). In contrast, 0.1 μM thapsigargin preincubation for the same duration totally abolished both thrombin-induced and Quick I-induced Ca^{2+} responses (Figure 4, bottom traces), indicating that both thrombin and Quick I elevate intraplatelet [Ca^{2+}] through IP_3 generation. All these data indicate that Quick I, in the absence of dissociable binding, induced intracellular [Ca^{2+}] increases by a pathway indistinguishable from that utilized by thrombin.

To examine the relative effectiveness of Quick I in mobilizing intracellular Ca^{2+}, increases in intraplatelet [Ca^{2+}] were determined from the difference between basal and peak Ca^{2+} levels. The concentration-dependent effect of thrombin or Quick I on intraplatelet Ca^{2+} mobilization is shown in Figure 5. The dose–response curves for thrombin-induced and Quick I-induced Ca^{2+} elevation appeared sigmoidal. The threshold for the thrombin-induced response was ~0.05–0.1 nM, since at these concentrations, only slight increases ($\leq$50 nM) in intracellular [Ca^{2+}] were observed (data not shown). At concentrations as low as 0.5 nM, thrombin induced substantial increases (~200 nM) in intraplatelet [Ca^{2+}]. By 10 nM, a maximal ~800 nM increase in platelet [Ca^{2+}] was observed. Quick I was a less effective agonist than thrombin, and the dose–response curve for Quick I was shifted to the right relative to thrombin. Below 0.5 nM Quick I, no Ca^{2+} response could be observed. Approximately 2 nM Quick I was required to increase platelet [Ca^{2+}] by 200 nM, and 60–100 nM was required to mobilize a maximal increase in intracellular [Ca^{2+}]. Therefore, 6–10 times more Quick I was required to induce a comparable rise in [Ca^{2+}] when compared to thrombin. The quantitative increase in platelet intracellular [Ca^{2+}] induced by thrombin or Quick I was unaffected by aspirin and PGE_1 pretreatment prior to washing. The ability of Quick I and the inability of FPR-thrombin and Quick II to elicit a Ca^{2+} response are consistent with the well-recognized importance of

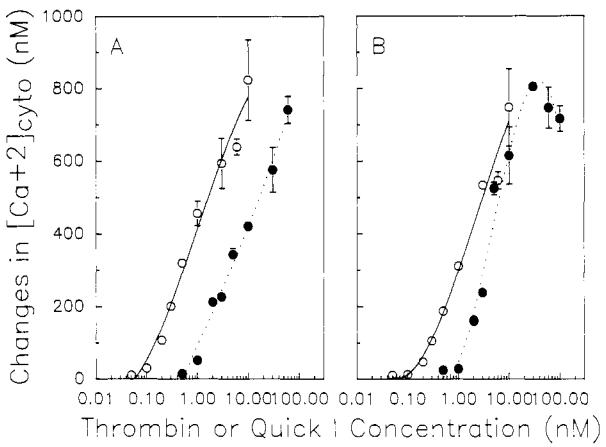

FIGURE 5: Dose-dependent increases in intraplatelet [Ca^{2+}] mediated by thrombin or Quick I. Platelets (2×10^8/mL) were stimulated with thrombin (0.05–10 nM) or Quick I (0.5–100 nM) as indicated under Materials and Methods. Results of representative experiments with two different donors are shown in panels A and B. Increases in intracellular [Ca^{2+}] induced by thrombin (○) and Quick I (●) are the means of duplicate determinations, with the error bars indicating the upper and lower limits of the range of values obtained.

the thrombin active site in platelet activation (Shuman et al., 1976).

To determine if thrombin or Quick I proteolytic activity alone was inducing activation-dependent [Ca^{2+}] elevation, 200 nM FPRCK was added to platelets activated for varying durations with 0.2 nM thrombin, or 2 nM Quick I. Regardless of the magnitude of the [Ca^{2+}] increase induced by Quick I, FPRCK addition, up to 1 min after Quick I stimulation, totally prevented additional increases in intraplatelet [Ca^{2+}], and returned intracellular Ca^{2+} levels rapidly to basal values (Figure 6). When the agonist was thrombin, FPRCK addition within 10–20 s of thrombin stimulation likewise inhibited further increases in intracellular [Ca^{2+}] (data not shown). FPRCK inhibition of either thrombin-induced or Quick I-induced effects was the result of thrombin or Quick I inactivation and not a result of nonspecific platelet inhibition, since FPRCK at the same concentration failed to inhibit the Ca^{2+} flux induced by 76.4 nM PAF, or 0.5 μM U46619 (data not shown). These data indicate that a proteolytic mechanism was mediating thrombin-induced and Quick I-induced activation and are consistent with the inhibition of thrombin-induced phosphatidate and 3-phosphorylated phosphoinositide accumulation with the addition of the thrombin inhibitor dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (Huang et al., 1991).

Effect of FPR-Thrombin or Quick II on Agonist-Induced Ca^{2+} Responses. The contribution of high-affinity, dissociable binding to agonist-induced platelet activation was assessed by using FPR-thrombin to saturate high-affinity sites prior to agonist addition. FPR-thrombin, in 50-fold molar excess, did not significantly affect thrombin-induced or Quick I-induced Ca^{2+} mobilization. The magnitude of the Ca^{2+} response observed in thrombin-stimulated or Quick I-stimulated platelets following FPR-thrombin preincubation was 115 ± 6% (n = 13) or 100 ± 4% (n = 13) of the response observed with thrombin or Quick I alone, respectively. The lack of effect with FPR-thrombin preincubation indicates that dissociable binding did not affect the thrombin-induced or Quick I-induced Ca^{2+} response. To verify this observation, FPR-thrombin concentration was varied from 100 to 500 nM. Again, FPR-thrombin, at concentrations resulting in ≥95% receptor occupancy, did not affect the qualitative or quantitative Ca^{2+} response to 0.5 nM thrombin when added prior to thrombin (Table II).

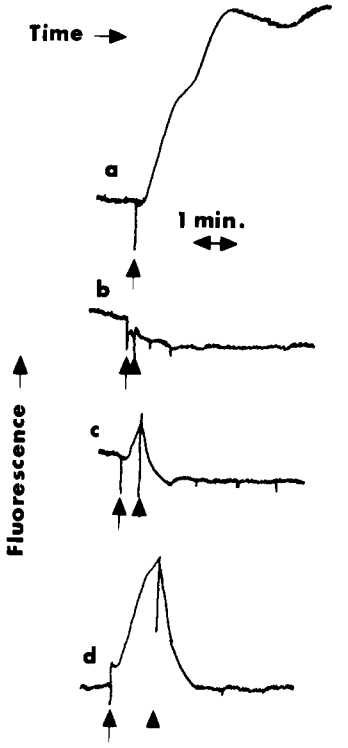

FIGURE 6: Effect of Quick I inactivation on agonist-induced Ca^{2+} mobilization. The Ca^{2+} responses of platelets (2×10^8/mL) stimulated for various durations with 2 nM Quick I before FPRCK (200 nM) addition are shown. FPRCK was omitted in "a", while in "b"–"d", FPRCK was added at the following intervals after agonist: "b", 10 s; "c", 30 s; and "d", 60 s. Agonist additions and subsequent FPRCK additions are indicated by arrows and arrowheads, respectively.

Table II: Time- and Dose-Dependent Effect of FPR-Thrombin on Thrombin-Induced Elevation in Intraplatelet [Ca^{2+}]

[FPR-thrombin] (nM)[a]	preincubation time (min)	% control[b]
100	5	117 ● 11
200	5	110 ● 11
500	5	96 ± 13

[a] Platelets (2×10^8/mL) were preincubated with varying FPR-thrombin concentrations for the designated time, followed by stimulation with 0.5 nM thrombin. [b] These values are expressed as a percentage of the response with thrombin alone, and were calculated from duplicate sample determinations using two different donors, with mean control values determined for each donor; results represent mean ± standard error of the mean.

However, since the agonist concentrations used in these studies elicited substantial increases in intraplatelet [Ca^{2+}], the effect of high-affinity binding, if slight, may not be evident at these concentrations. Therefore, agonist concentration was reduced to threshold levels, such that intraplatelet [Ca^{2+}] increased by ~50 nM. The effect of various FPR-thrombin or Quick II concentrations on agonist-induced increases in intraplatelet [Ca^{2+}] was again assessed. Various concentrations of FPR-thrombin or Quick II (≤1 μM) were added simultaneously with fixed concentrations of thrombin (0.05–0.2 nM, Figure 7A). FPR-Thrombin, at concentrations below 100 nM, enhanced the thrombin-induced Ca^{2+} response. As the FPR-thrombin concentration approached 100 nM, the enhancement observed was progressively abolished. Inhibition of both the rate and the amplitude of the Ca^{2+} response was observed at FPR-thrombin concentrations ≥250 nM. Maximal inhibition was observed at 1 μM, when the agonist-induced response was decreased to 15–35% of the control. When Quick II replaced FPR-thrombin, similar results were obtained,

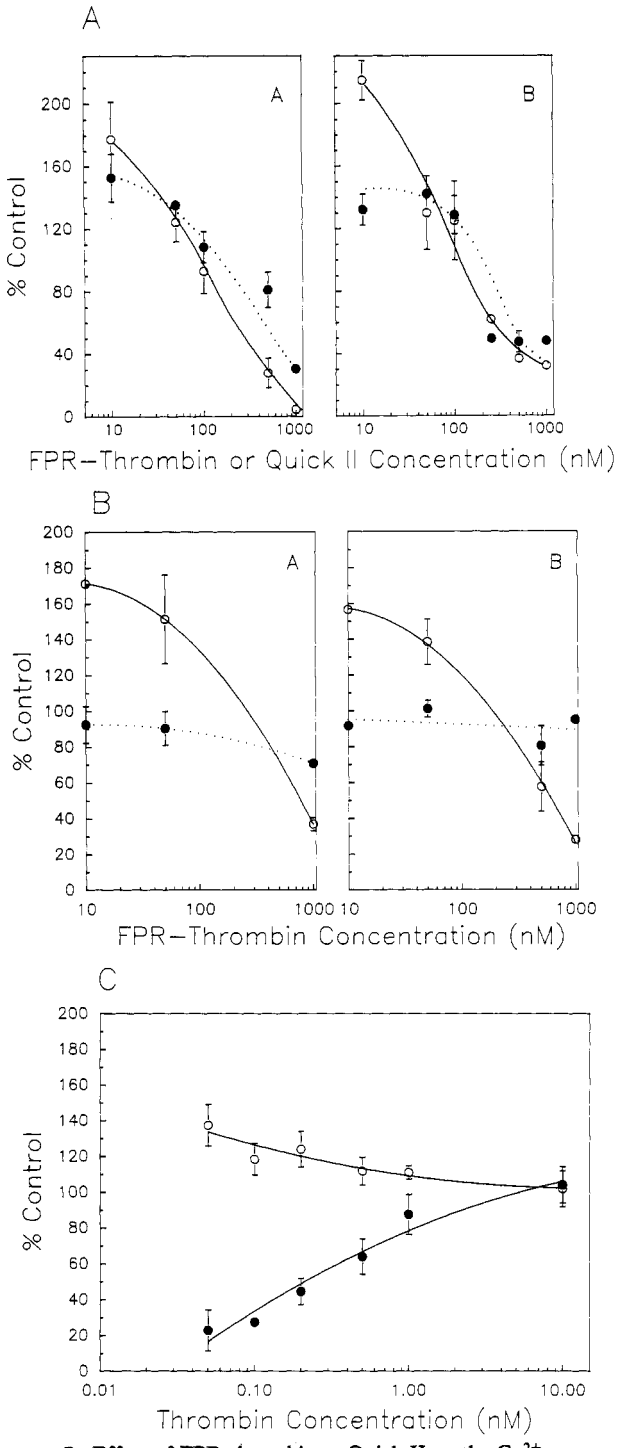

FIGURE 7: Effect of FPR-thrombin or Quick II on the Ca^{2+} response elicited by thrombin or Quick I. (A) Varying concentrations of FPR-thrombin (○) or Quick II (●) and thrombin (0.05 nM, panel A; 0.2 nM, panel B) were added simultaneously to platelets (2×10^8/mL), washed in the presence of 1 mM aspirin and 0.5 μM PGE_1. The results obtained from representative experiments with two different donors are shown. (B) Varying concentrations of FPR-thrombin were added simultaneously with thrombin (○) (0.1 nM, panel A; 0.15 nM, panel B) or Quick I (●) (1 nM, panels A and B) to platelets (2×10^8/mL). The concentrations chosen gave comparable [Ca^{2+}] increases. Results from two representative experiments are shown. All data in Figure 7A,B are expressed as a percent of the response observed with agonist alone and indicate the mean of duplicate determinations, with the error bars indicating the upper and lower limits of the range of values obtained. (C) FPR-thrombin [10 nM (○) or 1 μM (●)] was added simultaneously with varying concentrations of thrombin to platelets (2×10^8/mL). Results from experiments with three different donors are shown. Data are expressed as the percent of the response observed with agonist alone and are the means ± standard errors of three to five determinations.

although the magnitude of the effect was slightly diminished. When Quick I (1–2 nM) replaced thrombin as agonist, FPR-thrombin did not enhance the Quick I-induced response (Figure 7B). Only slight inhibition of the Quick I response was observed at 1 μM FPR-thrombin, since the concentration of Quick I used was ~10-fold greater than that of thrombin.

To determine whether the effects of FPR-thrombin or Quick II are consistently observed over a wide range of agonist concentration, fixed concentrations of FPR-thrombin (10 nM or 1 μM) were added simultaneously with varying concentrations of thrombin (0.05–10 nM). With increasing agonist concentrations, the enhancement observed with 10 nM FPR-thrombin and the inhibition observed with 1 μM FPR-thrombin progressively diminished (Figure 7C).

Discussion

In this study, the platelet binding properties of two mutant thrombins, Quick I and Quick II, have been determined and the results compared to those obtained for thrombin. Dys-thrombin binding to platelets was examined in relation to an early activation event, agonist-induced elevations in intraplatelet [Ca^{2+}]. As discussed below, our results identify specific structural features of thrombin required for high-affinity platelet binding and platelet activation. The results a'so indicate that thrombin-mediated platelet activation does not involve the high-affinity binding site even in a facilitative manner, but rather support the hypothesis that the high-affinity binding site serves to modulate thrombin activity and thus platelet activation.

Thrombin Domains Required for High-Affinity Platelet Binding. Consistent with earlier reports, thrombin bound to a single class of high-affinity sites (Table I). The substitution of Val for Gly-558 [226] in the primary substrate binding pocket (Henriksen & Mann, 1989) reduced the binding affinity of Quick II by approximately an order of magnitude. This result was somewhat unexpected since thrombin and FPR-thrombin, where the primary substrate binding pocket is occupied, bind equivalently to the high-affinity site on platelets. However, the results are consistent with the decreased binding affinity of Quick II for hirudin and the predicted distortion of the Quick II surface near the primary substrate binding pocket, as predicted by modeling studies in which Gly-558 [226] of FPR-thrombin was substituted by Val (Stone et al., 1991).

For Quick I, the substitution of Cys for Arg-382 [67] within the postulated fibrinogen binding groove (Henriksen & Mann, 1988; Bode et al., 1989) has reduced its binding affinity for platelets to 1/500 of that for thrombin and indicates a critical role for the fibrinogen binding groove in the binding of thrombin at the platelet surface. This conclusion is based on the functional properties of Quick I in its interaction with fibrinogen, where the K_m of fibrinopeptides A and B release by Quick I is decreased relative to thrombin (Henriksen & Owen, 1987; Henriksen, 1990), and on the location of Arg-382 [67] within a major groove on the thrombin surface (Bode et al., 1989). Although a significant role for the fibrinogen binding groove in high-affinity binding of thrombin to platelets has been indicated by previous studies with both chemically and enzymatically modified thrombins (Workman et al., 1977b; White et al., 1981; Alexander et al., 1983; McGowan & Detwiler, 1986), modifications in the former case are either less well characterized, less specific, or both, and in the latter case, more extensive than for Quick I (Lundblad et al., 1988; Elion et al., 1986).

Taken together, the data for the two mutant thrombins are consistent with a model in which thrombin binding to platelets

is mediated through an extensive portion of the thrombin surface, involving residues on both sides of the catalytic site and including the fibrinogen binding groove. This interaction would be similar to thrombin–hirudin binding, where the C-terminal region of hirudin binds within the fibrinogen binding groove and the N-terminal domain binds a portion of the thrombin surface near the catalytic site (Rydel et al., 1990), thus blocking access to the catalytic site. This interpretation is also consistent with the earlier report that hirudin displaces thrombin from the platelet surface (Tam et al., 1979). This would indicate that thrombin is bound to platelets with the catalytic site directed toward the platelet surface. Therefore, the catalytic site is inaccessible to both thrombin substrates and plasma inhibitors.

Thrombin High-Affinity Binding Does Not Contribute to Platelet Activation. In view of the conclusion that thrombin bound to the high-affinity site is proteolytically inactive and the previous demonstration that active thrombin is required for activation, dysthrombin binding to platelets was compared to the efficacy of these molecules in mediating increases in intraplatelet [Ca^{2+}]. As anticipated, Quick II, which lacks proteolytic activity but exhibits high-affinity binding, did not induce platelet activation. The important result of these studies is that 10 nM Quick I, where no dissociable platelet binding was observed ($K_d > 0.2$ μM), maximally increased intraplatelet [Ca^{2+}]. Saturation of high-affinity binding sites with FPR-thrombin prior to Quick I addition had no effect on the agonist response, either the rate or the magnitude, further indicating a lack of interaction between occupation of the high-affinity binding site and the activation event. A similar dissociation of saturable, high-affinity binding from platelet activation has been reported with more extensively modified thrombins such as nitro-thrombin and γ-thrombin (Workman et al., 1977; Alexander et al., 1983; Lundblad et al., 1988).

Our results are consistent with a model in which thrombin acts enzymatically on a platelet membrane "substrate" and not via a classical ligand–receptor interaction. An enzyme–substrate (Michaelis) complex would not have sufficient lifetime to be characterized by classical methods for studying ligand–receptor interaction. Data shown in Figure 7A,B also suggest that the affinity of this interaction may be 2 orders of magnitude weaker than the high-affinity binding. The abolition of Quick I-induced or thrombin-induced Ca^{2+} responses by subsequent FPRCK addition provides the strongest evidence for a proteolytic mechanism of thrombin activation, and further discounts the hypothesis that high-affinity, dissociable binding facilitates activation, since FPR-thrombin generation in situ was without effect on platelets stimulated previously with thrombin. Thus, Quick I and thrombin may both hydrolyze a "substrate", such as that recently described by Vu et al. to effect platelet activation (Vu et al., 1991).

The Fibrinogen Binding Groove and Platelet Activation. Our data indicate that Quick I induced increases in intraplatelet [Ca^{2+}] less effectively than thrombin at equivalent concentrations, such that Quick I was 10–17% as effective as thrombin in producing maximal Ca^{2+} flux, which is achieved at a slower rate than with thrombin. The decreased rate and extent of the activation reaction is most probably due to the fibrinogen binding groove defect of Quick I (Henriksen & Mann, 1988; Bode et al., 1989), which results both in decreased affinity for and rate of hydrolysis of fibrinogen (Henriksen & Owen, 1987) and in a reduced affinity for the C-terminal fragment of hirudin (Stone et al., 1991). Consequently, the fibrinogen binding groove of thrombin appears to be an important part of the specificity site mediating the interaction of thrombin with its platelet membrane "substrate". A similar conclusion was derived from the studies with hirugen, a hirudin-derived peptide which inhibited thrombin-induced aggregation in platelets and Ca^{2+} flux in oocytes expressing the putative platelet "substrate" (Jakubowski & Maraganore, 1990; Vu et al., 1991).

Support for this model is provided by Figure 7. High concentrations of FPR-thrombin and Quick II inhibited the magnitude and the rate of thrombin-induced increases in intraplatelet [Ca^{2+}]. Since an intact fibrinogen binding groove is present in each molecule, FPR-thrombin or Quick II would be expected to bind the "substrate" to form in inactive "substrate"–inhibitor complex, thereby decreasing the effective "substrate" concentration. The inhibition observed was not due to inactivation of thrombin by residual FPRCK or to receptor desensitization induced by residual, contaminating thrombin or Quick I, respectively, in the FPR-thrombin or Quick II preparations (see Materials and Methods). The FPR-thrombin inhibition was overcome with increasing thrombin concentrations (Figure 7C), and is consistent with inhibition due to substrate depletion (Segel, 1975).

The magnitude of the Quick I-induced response was not inhibited to the same extent with FPR-thrombin, since ~10-fold greater concentrations of Quick I were used to elicit comparable responses as thrombin. However, a decrease in the rate of the Quick I-stimulated response (data not shown) was observed, indicating that high FPR-thrombin concentrations are inhibitory toward this agonist as well. This observation is consistent with a model in which a threshold number of hydrolytic events must occur to stimulate an intracellular Ca^{2+} flux.

Function of the Thrombin High-Affinity Binding Site on Platelets. Our results indicate that thrombin bound to the high-affinity site does not contribute to platelet activation and, perhaps more importantly. is proteolytically inactive, as indicated by the thrombin domains required for dissociable binding (see above). Consequently, thrombin binding to the high-affinity site would decrease the concentration of free, active thrombin available for interaction with its membrane "substrate". Data shown in Figure 7 support this conclusion. With low agonist concentrations (<0.5 nM), enhancement of thrombin activation of platelets was observed when FPR-thrombin or Quick II concentrations <100 nM were used to occupy the high-affinity binding sites. Since the platelet response is proportional to the agonist concentration, such an enhancement indicates an increase in the effective concentration of active thrombin capable of interacting with the platelet "substrate" (Figure 7A). As FPR-thrombin or Quick II concentrations were increased, the enhancement due to displacement of thrombin from the high-affinity site was abrogated by the ability of FPR-thrombin or Quick II to compete with thrombin for interaction with its membrane "substrate". This interpretation is consistent with the apparent lack of an effect observed when FPR-thrombin was added before 0.5 nM thrombin, a concentration eliciting substantial increases in intraplatelet [Ca^{2+}]. However, the loss of the enhancing effect of 10 nM FPR-thrombin at a mere 2.5-fold increase in thrombin concentration (Figure 7C) indicates that the modulatory effect of high-affinity thrombin binding is most significant at very low agonist concentrations and at these concentrations high-affinity binding determines whether local concentrations of thrombin achieve threshold levels to effect platelet activation.

Observations made by other investigators support this concept. Phillips demonstrated that preincubation of platelets

with a 5–130-fold molar excess of active-site-blocked thrombin facilitates thrombin-induced platelet aggregation and secretion at low thrombin concentrations (Phillips, 1974). On the other hand, a high concentration of FPR-thrombin (at ~1200-fold molar excess over thrombin) inhibits platelet aggregation and secretion, whether the FPR-thrombin is preincubated or added simultaneously with thrombin (Greco et al., 1990). In contrast, preincubation of FPR-thrombin at the same molar excess over thrombin is required to inhibit thrombin-induced Ca^{2+} flux. The enhancing effect of active-site-blocked thrombin reported by Phillips is in agreement with our results with FPR-thrombin (<100 nM). Also, although both we and Greco et al. observed inhibition of thrombin-induced responses at high FPR-thrombin concentrations, there are, nevertheless, significant discrepancies between our results and theirs, which cannot be explained at present, but may be partially accounted for by differences in FPR-thrombin preparation.

In summary, thrombin generation at the platelet surface by the prothrombinase complex (Tracy & Mann, 1986) would be distributed between an inactive bound form at the platelet high-affinity binding site and its platelet membrane "substrate". Interaction with the high-affinity binding site results in temporary "sequestration" of the enzyme. The immediate consequence of this "sequestration" is decreased local concentration of active enzyme, thus increasing the nominal concentration of thrombin required to achieve platelet activation, therefore either preventing or dampening the magnitude of the activation response. Consequently, thrombin bound to the high-affinity site acts to down-regulate thrombin-induced platelet activation. In addition, this "sequestration" may also dampen the amplitude of the coagulation response since additional thrombin functions include cofactor activation. However, subsequent to platelet activation, thrombin bound dissociably to this site may prolong the duration of thrombin action by acting as a "reservoir". Thrombin bound at the platelet surface would be protected from clearance and from physiologic inhibitors, particularly antithrombin III, which interacts with thrombin through the catalytic site (Harpel, 1987). The platelet membrane could continue to release active thrombin to enhance a hemostatic or prothrombotic condition at the site of platelet activation. Thus, the platelet high-affinity binding site of thrombin may have a significant role in the regulation of hemostasis and thrombosis.

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Registry No. Ca, 7440-70-2; thrombin, 9002-04-4.

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Peptide Secondary Structure Induced by a Micellar Phospholipidic Interface: Proton NMR Conformational Study of a Lipopeptide

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ABSTRACT: The conformational change of the model peptide Ac-K-G-R-G-D-G-amide induced by a phospholipidic interface was investigated by proton nuclear magnetic resonance (^{1}H NMR). In aqueous solution, the free peptide is highly flexible and disordered, even in the presence of deuterated dodecylphosphocholine (DPC-d_{38}) micelles which mimic a membrane interface. The lipopeptide, obtained by grafting a lipid anchor [2,3-dipalmitoyl-D-(+)-glyceric acid] to the lysine side chain of the peptide, was studied by standard 2D ^{1}H NMR spectroscopy combined with distance geometry and simulated annealing calculations. When anchored to a micelle interface, the peptide acquires a definite turn (II/I′) conformation. We were also able to describe precisely the conformation of the diacylglyceric fragment of the lipopeptide in a lipid environment and to establish the average orientation of the peptide segment with respect to the micelle surface.

The ability of membranes to alter the conformation of peptides is currently being illustrated by an increasing number of examples: biologically active peptides such as hormones or neuropeptides, etc. (Wider et al., 1982; Zetta et al., 1983; Kaiser et al., 1984; Wakamatsu et al., 1987; Wooley & Deber, 1987; Olejniczak et al., 1988; Naider et al., 1989; Beschiaschvili & Seelig, 1991), toxins or antibiotics (Brown et al., 1982; Arseniev et al., 1985; Lee et al., 1986; Cavatorta et al., 1989; Inagaki et al., 1989; Bairaktari et al., 1990; Lakey et al. 1991), peptide signals (Giersasch, 1989; von Heijne, 1990; Karslake et al., 1990), and coat proteins (Schiksnis et al., 1987; O'Neil & Sykes, 1988; Shon et al., 1991). The interactions of a peptide ligand with a membrane interface and the re-

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