A Subpopulation of Platelets Responds to Thrombin- or SFLLRN-stimulation with Binding Sites for Factor IXa

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**Strong agonists cause platelets to expose a procoagulant surface supporting the assembly of two important coagulation enzyme complexes. Equilibrium binding has determined the density of high affinity saturable factor IXa binding sites to be 500–600 sites/platelet. We have now used flow cytometry to visualize the binding of factor IX and IXa to thrombin- or SFLLRN-activated platelets. Concentrations of these agonists that are half-maximal or maximal in kinetic studies resulted in only a small subpopulation (4–20%) of platelets binding factor IX or IXa with the density of binding sites for factor IX being about half of that for factor IXa, consistent with previous equilibrium binding studies. A small subpopulation (5 ± 1.5%) of platelets stimulated with either agonist also exposed annexin V binding sites, and this subpopulation of platelets also bound factor IXa. Annexin V decreased factor IXa binding in the presence or absence of factor VIIIa, and factor IXa could also decrease annexin V binding on some platelets indicating a common binding site in agreement with previous studies. All platelets binding factor IXa were positive for glycoprotein IX, at the same glycoprotein IX surface density as seen in platelets negative for factor IXa binding. These studies refine the results from equilibrium binding studies and suggest that, on average, only a small subpopulation (~10%) of PAR 1-stimulated platelets expose ~6000 factor IXa binding sites/platelet.**

Although normally circulating in a quiescent state, when activated with strong agonists, platelets provide an important surface for assembly of two major coagulation enzyme complexes, the intrinsic factor X-activating complex and the prothrombinase complex (1). In vitro kinetic assays have demonstrated that in the presence of activated platelets (or artificial phospholipid vesicles) factor Xa generation is enhanced several million-fold, and thrombin generation is enhanced 300,000-fold over solution phase activity (2–5). It is known that not all agonists are equivalent in their ability to stimulate all functional end points of platelet activation. Weak agonists (e.g. ADP and epinephrine) require feedback enhancement from secreted molecules or synthesized agonists such as thromboxane A2 to achieve stimulation of platelet aggregation and are unable to stimulate a procoagulant surface capable of supporting assembly of these enzyme complexes (6, 7). Strong agonists (e.g. thrombin and collagen) are capable of generating all platelet functional endpoints (8).

Are all platelets equivalent in their response to these agonists? Visualizations of in vitro clot formation show most platelets rolling, adhering, aggregating, and serving as the foundation for fibrin clot formation (9). However, circulating platelets may constitute a heterogeneous population. Their generation from megakaryocytes packages them in different sizes, with different granule loads, differing numbers of mitochondria, and other vestiges of the parent cells (10, 11). The circulation contains platelets of different ages as well as sizes. Flow cytometry analyses detect a wide range of forward and side scatter of blood platelets, necessitating the viewing of platelets on log scales (12). Flow cytometry provides an opportunity to view the homogeneity or heterogeneity of the incidence and density of platelet markers as well as the appearance of activation-dependent markers providing a clinical tool to probe for platelet activation in whole blood samples (13, 14).

We have formerly used both equilibrium binding studies and in vitro kinetic studies to investigate the nature of the factor IXa-factor VIIIa-catalyzed factor X-activating complex on thrombin- or SFLLRN-stimulated platelets. We determined that platelets upon activation expose 500–600 binding sites for the enzyme factor IXa (15), 1000–1200 binding sites for the cofactor factor VIIIa (6), and 1000–1200 specific binding sites for the substrate factor X (as well as 30,000 sites shared with prothrombin) (16). The affinity of each protein for its binding site as well as complete occupancy of sites are both optimal when all components of the enzyme complex are present. It has been assumed that the saturable binding sites determined from equilibrium binding studies are generated on all platelets as a result of agonist stimulation. We now present data from flow cytometry studies visualizing the binding of the enzyme and the zymogen that result in the surprising conclusion that although all platelets respond to the PAR 1 agonists with markers of activation, only a small subpopulation of platelets stimulated with thrombin or SFLLRN-amide responds with procoagulant surface changes allowing binding of factor IXa.

**Experimental Procedures**

Reagents—Bovine serum albumin, buffer reagents, disodium EDTA, dimethyl sulfoxide (Me2SO), calcium chloride, and Sepharose 2B-CL, were obtained from Sigma. Electrophoresis reagents were from Bio-Rad Laboratories, Inc. Chromogenic substrates Nα-benzylxoycarbonyl-D-arginyl-t-gluclyl-t-arginine-p-nitroanilene-dihydrochloride (S-2765) and H-D-phenylalanyl-t-piperyl-t-arginine-p-nitroaniline dihydrochloride (S-2238) were purchased from DiaPharma (West Chester, OH). The thrombin receptor agonist peptide, SFLLRN-amide, was synthesized using Fmoc (9-fluorenymethylxoycarbonyl) chemistry on an Applied Biosystems 430A synthesizer and reverse-phase high pressure liquid chromatography purified to greater than 99% homogeneity. A fluores-
Flow Cytometry Detection of Factor IXa Binding to Platelets

Platelets activated with low levels of thrombin or PAR 1-activating peptide (SFLRN) support intrinsic factor Xa generation (5) and expose high affinity saturable binding sites for factor X-activating complex components (6, 7, 15, 20). We have now used flow cytometry studies to visualize the effect of PAR 1 stimulation on both factor IXa binding and on exposure of procoagulant lipids and compared this with the ability of PAR 1 agonists to stimulate platelet support for factor Xa generation.

Platelets stimulated with increasing amounts of thrombin or SFLRN-amide for 10 min at 37°C supported increasing factor Xa generation saturating at 14.4 ± 2 nM/min in response to thrombin at 1 units/ml and at 9.7 ± 0.9 nM/min in response to 150 μM PAR 1 agonist peptide (Fig. 1). The EC50 for thrombin was 0.02 ± 0.02 units/ml (1.4 nM) and for SFLRN-amide was 2 ± 0.5 μM. When incubated with an FITC-labeled antibody to P-selectin (CD62P), platelets stimulated with 35 μM SFLRN-

FIG. 1. Agonist stimulation of platelet-supported factor IXa-catalyzed factor X activation. Platelets prepared as described under “Experimental Procedures” were combined with calcium (5 mM) and factor IXa (1 nM) and activated with varying amounts of either thrombin (a) or SFLRN-amide (b) before the addition of the cofactor and substrate. Factor VIII (200 units/ml) was incubated 1 min at 37°C with thrombin (0.1 units/ml) and an additional minute with hirudin (5 units/ml) immediately before addition to reaction mixtures. Reaction mixtures were stopped after 3 min and were analyzed for factor Xα generated by chromogenic substrate S-2765 cleavage as described (19).

1 The abbreviations used are: FITC, fluorescein isothiocyanate; GPIX, glycoprotein IX; GP Ibα, glycoprotein Ibα; PE, phycoerythrin; FL, fluorescence channel.

Data Analysis—Data from experiments were converted to factor Xα formed/min by comparison to a standard curve of factor Xα cleavage of S-2765 and plotted using KaleidaGraph software (Synergy, Reading, PA) to derive kinetic parameters. Results from multiple experiments were pooled and analyzed for means and standard errors.

Flow Cytometry—Washed, aspirin-treated, gel-filtered platelets were prepared from human whole blood as described previously (18) with the following modifications. Platelet-rich plasma was incubated with 1 mM acetylsalicylic acid for 30 min to inhibit feedback activation through prostaglandin synthesis, with prostaglandin E1 (2 μM) added 5 min before layering over the bovine serum albumin gradient for the wash step. Platelet Preparation—Washed, aspirin-treated, gel-filtered platelets were prepared from human whole blood as described previously (18) with the following modifications. Platelet-rich plasma was incubated with 1 mM acetylsalicylic acid for 30 min to inhibit feedback activation through prostaglandin synthesis, with prostaglandin E1 (2 μM) added 5 min before layering over the bovine serum albumin gradient for the wash step.

Factor Xα Generation—Factor Xα was generated as described previously (18) with the following modifications. Washed, aspirin-treated, gel-filtered platelets (10^6/ml) were added to wells of a microtitration plate. Factor IXα and varying concentrations of agonists (thrombin or SFLLRN-amide) were added, and activation proceeded for 10 min before the addition of the cofactor and substrate. Factor VIII (200 units/ml) was incubated 1 min at 37°C with thrombin (0.1 units/ml) and an additional minute with hirudin (5 units/ml) immediately before addition to reaction mixtures. Reaction mixtures were stopped after 3 min and were analyzed for factor Xα generated by chromogenic substrate S-2765 cleavage as described (19).

Data Analysis—Results from enzyme assays were converted to factor Xα formed/min by comparison to a standard curve of factor Xα cleavage of S-2765 and plotted using KaleidaGraph software (Synergy, Reading, PA) to derive kinetic parameters. Results from multiple experiments were pooled and analyzed for means and standard errors.

Flow Cytometry—Washed, aspirin-treated, gel-filtered platelets (10^6) were incubated with FITC-labeled CD42b against platelet GP Ibα to define the platelet population. Platelets were incubated with FITC- or PE-labeled annexin V at specified concentrations or with various concentrations of factor IXα or IX, and half of each sample was activated with thrombin (0.2 or 2 units/ml) or with SFLLRN-amide (30–150 μM) for 10 min. Some samples contained thrombin-activated factor IXα at 10 units/ml. Samples containing factor IXα or IXa were incubated with either FITC-labeled C10D or biotinylated C10D for 15 min, and the latter was detected with streptavidin-PE-cy5. Some samples were dual-labeled to simultaneously detect PE-annexin V binding and factor IXα binding (through FITC-C10D). Other samples were dual-labeled to simultaneously detect platelet GP IX and factor IXα binding. All samples were diluted 5-fold and analyzed on a Facsscan flow cytometer (Pharmingen) equipped with a 488-nm-emitting laser. Data were collected using either E00 or E01 voltage settings for forward scatter statistics. Events measured with buffer alone were subtracted from events measured with platelet samples. Data from different experiments collected under the same conditions were averaged and statistically analyzed.

RESULTS

Platelets activated with low levels of thrombin or PAR 1-activating peptide (SFLRN) support intrinsic factor X generation (5) and expose high affinity saturable binding sites for factor X-activating complex components (6, 7, 15, 20). We have now used flow cytometry studies to visualize the effect of PAR 1 stimulation on both factor IXa binding and on exposure of procoagulant lipids and compared this with the ability of PAR 1 agonists to stimulate platelet support for factor Xa generation.

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amidine showed a 5-fold fluorescence shift of the entire population of platelets indicating that all platelets had responded to the agonist peptide with secretion from α-granules and incorporation of membrane-bound P-selectin into the plasma membrane. The same effect on P-selectin exposure was found in response to thrombin at 0.2 units/ml, and these platelets aggregated fully without exogenous fibrinogen when stimulated with as little as 10 nM SFLLRN or thrombin at 0.2 units/ml (data not shown) indicating that all platelets are capable of aggregation as well as secretion in response to PAR 1 stimulation.

The assembly of the factor X-activating complex on platelets was probed by flow cytometry for factor IXa binding. The monoclonal antibody C10D to the heavy chain of factor IX was either fluoresceinated with FITC for detection in the FL-1 window or was biotinylated and detected with streptavidin conjugated to PE-cy5, a tandem dye absorbing at 488 nm and fluorescing by resonance energy transfer from PE to cy5 in the FL-3 window.

Factor IXa binding results were equivalent using either detection system. Table I shows binding results determined in the presence of 10 nM factor IXa. Factor IXa was probed by flow cytometry for factor IXa binding. The monoclonal antibody C10D to the heavy chain of factor IX was either fluoresceinated with FITC for detection in the FL-1 window or was biotinylated and detected with streptavidin conjugated to PE-cy5, a tandem dye absorbing at 488 nm and fluorescing by resonance energy transfer from PE to cy5 in the FL-3 window.

Table I: Detection of factor IXa binding to activated platelets

| Cofactor factor VIIIa | Ligand factor IXa | Positive platelets | Fluorescence peak intensity |
|----------------------|------------------|--------------------|--------------------------|
| 6 units/ml           | nm               | %                  | nm                       |
| Absent               | 0.5              | 2                  | 66                       |
|                      | 3                | 3.4                | 300                      |
| (n = 12)             | 10               | 9 (±2.4)           | 1000 (±147)              |
| Present              | 0.5              | 2.5                | 200                      |
|                      | 3                | 5.5                | 650                      |
|                      | 10               | 9                  | 1400                     |
| Absent Factor IX     | 10               | 6.5 (±2.3)         | 304 (±147)               |

When saturating (10 nm) factor IXa was incubated with platelets in the presence of half-maximal or saturating levels of thrombin (0.2 and 2 units/ml) or SFLLRN-amide (30 and 150 μM), there was little effect on mean fluorescence density/platelet (900 ± 25–1000 ± 50 in both cases). The population of platelets responding, however, increased from 4.2 ± 1.3 to 7.0 ± 1.5 with thrombin and from 4.4 ± 1.3 to 8.4 ± 2.5 with SFLLRN-amide.

When probed for exposure of annexin V binding sites after stimulation with thrombin or SFLLRN at either their EC50 or at saturation levels, only a small subpopulation of platelets was positive with a high density of annexin V binding sites/platelet (Fig. 2). Annexin V binding occurred in few (1.8%) unstimulated platelets (Fig. 2b) and increased as platelets were activated with half-maximal to maximal levels of both PAR 1 agonists, thrombin (4–7%) and SFLLRN-amide (3.8–5.7%). In five experiments with PE-annexin V and seven experiments with FITC-annexin V performed with the EC50 concentrations of the agonists, the mean (±S.E.) percentage of platelets positive with thrombin stimulation of annexin V binding was 5% ± 1.5 and with SFLLRN-amide stimulation was 4.2% ± 0.5, whereas maximal levels of SFLLRN-amide (150 μM) resulted in 7% ± 1.8. Therefore, activation with PAR 1 agonists was accompanied by a 3-fold increase in the population positive for annexin V binding with a 2-fold increased density of binding sites.

Dual-labeling studies were performed to investigate the relationship between annexin V binding and factor IXa binding to SFLLRN-activated platelets. Platelets were stimulated with saturating SFLLRN-amide (150 μM) and exposed to 10 nm factor IXa (∼ thrombin-activated factor VIII, 6 units/ml) in the presence of varying concentrations of annexin V. Increasing annexin V concentrations from 1 to 5.7 nM resulted in increased density of bound annexin V/platelet (data not shown). Fig. 3a shows that the density of bound factor IXa decreased with increasing annexin V concentration in the absence (gray bars) or presence (black bars) of factor VIIIa. The simultaneous presence of both ligands had no effect on the percentage of platelets positive for either annexin V (6–7%) or for factor IXa (6–8%). It was noted, however, that of eight experiments all showing a decrease in factor IXa binding in the presence of annexin V, only five showed no change in annexin V binding in the presence of 10 nm factor IXa. Fig. 3b presents the data for samples incubated with 3.8 nm annexin V in the absence (open bars) or presence (black bars) of factors IXa/VIIIa. The bars on the left represent all data analyzed together for annexin V binding. Five experiments (Fig. 3b, middle) showed no change in annexin V bound in the presence of factors IXa/VIIIa. Three experiments showed that annexin V binding could be substantially decreased by the presence of factors IXa/VIIIa (Fig. 3b, right). These results were found using PE-annexin V with FITC detection of factor IXa and using FITC-annexin V with PE-cy5 detection of factor IXa. Factor IXa in the absence of factor VIIIa showed the same effect. The ability of factor IXa to reduce binding sites for annexin V was more noted with annexin V concentrations at or below the previously determined kinetic IC50 (19).

In support of this evidence for competition, it was clear that the platelet populations responding to PAR 1 stimulation with binding of either annexin V or factor IXa were almost totally overlapping. Fig. 4 shows that almost all positive events in the dual-labeled sample (Fig. 4d) appeared in the upper right quadrant that was set to depict dual-labeled platelets. This overlap in binding determinants was consistent with previously reported kinetic studies (19) of annexin V inhibition of factor IXa-catalyzed factor X activation, which predicted an overlap in platelet binding sites for both ligands. Thus, platelets re-
sponding to thrombin with the appearance of aminophospholipids that bind annexin V are the same platelets capable of binding the enzyme factor IXa.

Activation of platelets can lead to microvesiculation (21), resulting in procoagulant vesicles displaying exposed aminophospholipids that can bind coagulation proteins. To determine whether activation of platelets with the EC\textsubscript{50} concentration of agonists leads to extra platelet procoagulant surfaces, data from single and dual-label platelet samples from factor IXa/annexin V experiments were analyzed for positive events inside and outside of the defined platelet population. It was found that 95% of all events positive for factor IXa binding, 94% of all events positive for annexin V binding, and 97% of all events positive for both labels were within the platelet population. It was previously published data (21) reporting barely detectable microvesicle formation in unstimulated platelets stimulated with low thrombin agonist concentration support our observation that factor IXa binding stimulated by PAR 1 activation occurs on the activated platelet surface.

To confirm that all events positive for factor IXa binding were platelets, dual staining was performed with PE-cy5-labeled anti-factor IXa and FITC-labeled anti-GPIX. A representative experiment is shown in Fig. 5. The quadrants were drawn to define the negative population of platelets incubated with both FITC-anti-GPIX in the presence of excess unlabeled anti-GPIX and with biotinylated C10D and streptavidin-PE-cy5 in the absence of factor IXa (Fig. 5a). 6% of activated platelets were positive for factor IXa (Fig. 5b), whereas all platelets were positive for GPIX (Fig. 5c). Fig. 5d shows that all platelets positive for factor IXa contained the same density of GPIX (upper right quadrant) as the activated platelet population that remained negative for factor IXa binding (upper left quadrant).

In summary, these experiments produced evidence that platelet activation with doses of PAR 1 agonists mid to maximally effective at eliciting platelet procoagulant activity resulted in only a subpopulation of platelets exposing binding sites for factor IXa and for annexin V. This population displayed the same surface density of GPIb-IX-V complex as seen in the population of activated platelets negative for factor IXa binding.

**DISCUSSION**

Biochemical techniques for determining specific ligand binding rely on statistical averaging over the entire population of...
cells studied. Using these techniques, it was determined that factor IXa binds to thrombin- or SFLLRN-amide-activated platelets with 500–600 sites/platelet and that the inactivated zymogen binds to one-half that number of sites (15). Flow cytometry provides the opportunity to analyze the total cell population as individual events and thus can help determine whether all cells provide binding sites or whether activation events are specific to portions of the cell population. It can also determine how uniformly distributed are the binding events. Although it has been demonstrated that thrombin, a strong agonist for many platelet activation events, is a weak agonist for activation-dependent exposure of procoagulant phospholipids (22), we have previously used thrombin or SFLLRN-amide to stimulate robust platelet support of factor Xa generation at one-fifth to one-tenth the concentrations that had shown little ability to stimulate exposure of procoagulant phospholipids (23). Here we correlate dose-response curves for the stimulation of factor Xa generation with the development of factor IXa binding sites and exposure of procoagulant phospholipids detected by flow cytometry.

We have found that although thrombin or SFLLRN stimulates all platelets to aggregate and to expose P-selectin through externalization of α-granules, only 4–20% of the platelets respond to thrombin or SFLLRN with exposure of factor IXa binding sites. These platelets responding with factor IXa binding sites overlap almost entirely with the entire population exposing aminophospholipids detected with annexin V binding. The subpopulation varies widely in single-donor platelets, the reasons for which are unknown. Because Feng and Tracy (24) found that factor Xa binding was confined to a subpopulation of stimulated platelets, it would be interesting to determine whether there is a correlation between a tendency toward thrombotic events and the percentage of platelets responding to
thrombin stimulation with exposure of factor IXa or Xa binding sites. Previous flow cytometric detection of factor IXa binding to thrombin-stimulated platelets provided dose-response data supporting the existence of two sites, a high affinity site comparable with what was found here and a low affinity site found with higher factor IXa concentrations (25).

Factor IXa binding results were similar for two different probes. Both fluorescein modification of a monoclonal antibody to the factor IX catalytic domain and biotinylation of that antibody detected with streptavidin-PE-cy5 allowed robust detection of 0.5 nM factor IXa in the presence of the cofactor and a weak detection of that concentration in the absence of the cofactor. Predictable from equilibrium binding results, 10 nM factor IXa represented a saturating level where binding-site density was not changed by the presence of the cofactor. The K_d was not measured in these flow cytometry studies, but it was noted that although the presence of both cofactor and substrate in equilibrium binding studies lowered the K_d from 2.5–3 nM to 0.5 nM (20), an inclusion of the cofactor in the absence of substrate here did not result in half-maximal binding density at 0.5 nM factor IXa. This may reflect either a lack of sensitivity at low binding-site density or the requirement for the substrate to stabilize the complex. However, the presence of the cofactor increased the density of binding sites/platelet most at a ligand concentration around the equilibrium K_d. As predicted from the titrations of agonists in kinetic assays, maximal concentrations of both SFLLRN-amide and thrombin elicited twice the factor IXa binding as seen with half-maximal concentrations. The additional binding resulted from recruitment of twice the number of platelets to bind factor IXa rather than from increases in the number of factor IXa binding sites/platelet. In these studies, factor IX bound to almost the same percentage of platelets to bind factor IXa rather than from increases in the number of factor IXa binding sites/platelet.

Also in agreement with previous studies (19) is the overlap in binding determinants for annexin V and factor IXa. In kinetic studies, annexin V was found to inhibit factor Xa generation with a K_I of 3.5 nM. In these binding studies, although the density of factor IXa was reduced by the presence of annexin V, especially with annexin V present at concentrations above its K_I for kinetic studies, annexin V density could also be reduced by the presence of factor IXa. These results suggest that there is true competition for overlapping binding determinants, with factor IXa as a less effective competitor for annexin V binding sites than annexin V is for factor IXa binding sites.

Binding sites for factor IXa and for annexin V were located primarily within the defined platelet population, which is in agreement with the previous observation that <5% of total factor IXa binding was attributed to platelet microparticles (25). This was predictable from the weak ability of thrombin to generate microparticles from activated platelets (21). The dual-labeling studies detecting both GPIb/IX and factor IXa binding revealed that all platelets binding factor IXa contain an equivalent surface density of GPIb/IX/V complexes as the bulk of the platelet population that remains negative for factor IXa binding.

These flow cytometry studies visualizing factor IXa binding to thrombin- or SFLLRN-stimulated platelets suggest a modification of the previous conclusions about intrinsic factor X-activating complex binding sites. It appears from the present results that instead of all platelets presenting ~600 factor IXa binding sites/platelet, an average of 10% of activated platelets presents an average ~6000 factor IXa binding sites/platelet. Flow cytometry detection of platelet binding of other factor Xa complex components should reveal interesting data to augment and refine information obtained from equilibrium binding studies.

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