Dynamics of Platelet Glycoprotein IIb-IIIa Receptor Expression and Fibrinogen Binding. II. Quantal Activation Parallels Platelet Capture in Stir-Associated Microaggregation

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ABSTRACT There is broad agreement that platelet aggregation is generally dependent on fibrinogen (Fg) binding to the glycoprotein (GP) IIb-IIIa receptor expressed on the activated platelet surface. We therefore compared rates and extents of aggregation and of fibrinogen receptor expression and specific Fg binding to activated platelets, as a function of ADP concentration. Human citrated platelet-rich plasma (diluted 10-fold) was stirred with adenosine diphosphate (ADP) for 10 s or 2 min to measure rates and extent of aggregation, respectively, determined from the decrease in the total number of particles. The number of fibrinogen receptors and bound Fg were measured from mean fluorescence values obtained with FITC-labeled IgM monoclonal antibody PAC1 and the IgG monoclonal antibody, 9F9, respectively, using flow cytometry as presented in part I (Frojmovic et al., 1994). Because flow cytometric and aggregation measurements were routinely determined at room temperature and 37°C, respectively, we also compared and found temperature-independent initial rates of aggregation. The fraction of platelets with fluorescence values above one critical threshold value, corresponding to maximally "activated" platelets (P*), increased with increasing activator concentration and correlated linearly with the fraction of platelets recruited into aggregates for ADP ($r > 0.9$). Aggregation was not rate-limited by fibrinogen receptor expression or by Fg binding. It appears that each platelet expresses its maximal Fg receptors at a critical ADP concentration, i.e., occupancy of ADP receptors. This, in turn, leads to rapid Fg occupancy and capture of such "quantally activated" platelets into aggregates.

INTRODUCTION

It is widely recognized that platelet aggregation is centered on the activation of GPIIb-IIIa into a receptor specific for adhesive molecules like fibrinogen (Fg) and von Willebrand Factor (vWF), which can mediate cross-bridging of platelets (Plow and Ginsberg, 1989). In plasma, at the moderate shear rates associated with stirred platelet suspensions, it appears that Fg is the key adhesive protein allowing platelet-platelet capture (Farrell et al., 1992; Goldsmith et al., 1994; Ikeda et al., 1991; Marguerie et al., 1980; Peerschke, 1985; Plow and Ginsberg, 1989). Qualitative descriptions abound of the need for Fg in supporting turbidimetrically measured macroaggregation (Farrell et al., 1992; Marguerie et al., 1980; Peerschke, 1985; Plow and Ginsberg, 1989), but these studies are centered on bulk ^{125}I-Fg or antibodies measurements and on maximal activation. In addition, it is now widely recognized that turbidimetric measurements cannot describe early kinetics of microaggregation, which have been found to have distinct functional (Frojmovic et al., 1983) and pharmacologic (Pedvis et al., 1988) properties from macroaggregation measured from changes in light transmission. Recent reports have also shown that activated GPIIb-IIIa expressed in a nonplatelet environment, capable of binding Fg, can support Ca^{+2}/Fg-dependent aggregation (Frojmovic et al., 1991; Gawaz et al., 1991; Sung et al., 1993); this has also been most recently demonstrated for purified activated GPIIb-IIIa captured onto latex microspheres (2–6 μm diameter) (Frojmovic et al., 1991). Although theory predicts that the surface density of adhesive receptors and the rate of ligand binding such as Fg can determine kinetics of platelet aggregation (Bongrand, 1988), this simple analysis may be complicated by post-Fg events required for a functional expression of Fg in actual cross-bridging of platelets (see Fig. 1 in the companion paper): these may involve (i) further conformational changes in the Fg occupied GPIIb-IIIa receptor and the bound Fg (Plow and Ginsberg, 1989), (ii) cytoskeletal interactions associated with ADP activation (Newman et al., 1987), (iii) receptor clustering, including preferential concentration at surface projections (Hensler et al., 1992), and (iv) altered affinity of Fg binding with time (Peerschke, 1992).

The dependence of rates of microaggregation on both ADP activator concentration (Frojmovic et al., 1989; Pedvis et al., 1988) and on size-dependent platelet subpopulations (Wong et al., 1989) have previously been reported for studies with undiluted citrated platelet-rich (PRP). It has been reported that the equilibrium dissociation constant, K_D, for specific Fg binding to its platelet receptor (Marguerie et al., 1980; Niewiarowski et al., 1983; Peerschke, 1985; Plow and Ginsberg, 1989), is in the same range as apparent "K_M" values driving platelet micro- and macroaggregation, 0.1–0.5 and 1–2 μM range, respectively (Frojmovic et al., 1989; Pedvis et al., 1988; Wong et al., 1989). However, microaggregation in normal, stirred PRP occurs within seconds of ADP addition (Frojmovic et al., 1989; Milton and Frojmovic, 1984), i.e., far removed from equilibrium conditions, whereas Fg binding measurements using I^{125} Fg represent

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bulk, average measurements that do not detect platelet subpopulation behavior (Frojmovic, 1995).

We therefore sought to obtain, for the first time, data that would allow a direct correlation between the dynamics of GPIIb-IIIa receptor expression, Fg occupancy, platelet subpopulation responses, and actual recruitment of stirred platelets into microaggregates. We therefore compared rates and extent of aggregation with the percent of "quantally activated" platelets, P*, present in PRP (1:10) according to the concentration of ADP used for activation as shown in the companion paper (Frojmovic et al., 1994). We found that the data best fit a model where increasing ADP concentrations generates increasing numbers of P* that require only a small fraction of their activated GPIIb-IIIa receptors to be filled with Fg to yield an optimal aggregation response for the stirred suspension. The aggregation dynamics are predicted by simple second-order kinetics for the reactive P* platelets, in the presence of unreactive P° platelets.

MATERIALS AND METHODS

Platelet isolation and analysis by flow cytometry

The procedures and materials used are exactly as described in the companion paper for the flow cytometric studies (Frojmovic et al., 1994), with parallel studies usually made on the same donor's PRP (1:10) for flow cytometry and aggregation. Glutaraldehyde was freshly diluted in Ca^{2+}/Mg^{2+}-free Tyrode's solution from an 8% aqueous stock in sealed ampoules (Polysciences Itk;2nc., Warrington, PA). Although samples for flow cytometry were kept at room temperature, studies of aggregation were generally made at 37°C, with one set of comparative studies done at room temperature versus 37°C (see Fig. 3).

Platelet aggregation by particle counting

Both the kinetics and sensitivity of platelet aggregation were determined by measuring the change in platelet particle number per unit volume with the Elzone resistive particle counter (Model 80XY, Particle Data Inc., Elmhurst, IL) at various times after the addition of varying concentrations of ADP to suspensions stirred at 1000 rpm, as previously reported (Frojmovic et al., 1989; Wong, 1989). Counts are corrected for background (<5% of total). The percent of platelets aggregated after a time t after addition of ADP to the stirred platelet suspensions, %PA, was calculated using the relation

$$\%PA = [1 - (N_t/N_0)] \times 100\%, \quad (1)$$

where N_0 and N_t are platelet particle counts, respectively, at times 0 and t after ADP addition.

ADP (1–5 μl) was added by rapid injection with a microsyringe to stirred PRP (1:10) (0.1 ml) in an aggregometry cuvette, normally at 37°C or, when so indicated, at room temperature as used for flow cytometry studies. Subsequent aggregation was arrested with the addition of 0.4 ml of 0.8% glutaraldehyde to the PRP (1:10) at times, t, ranging from 0 s to ≥2 min after the addition of ADP. These fixed samples were further diluted with isotonic saline to final counts of <70 particles/μl and counted within 2 h of fixation. Each sample was counted twice, with <5% deviations from mean values. The initial rate and maximal extent of aggregation were typically measured at 10 s (PA_i) and 2 min (PA_{max}), respectively. Log-dose response curves were obtained for these two parameters for ADP varying from 0 to 100 μM; ADP concentrations corresponding to half-maximal rate or extent of PA, designated as $[ADP]_{1/2}$, were thus obtained.

RESULTS

Platelet aggregation

To compare fluorescence measurements to platelet aggregation, studies were conducted for PRP samples diluted 1:10 with Tyrodes-albumin exactly as for the flow cytometric studies in the companion paper (Frojmovic et al., 1994), except that the suspensions were stirred at 1000 rpm, normally at 37°C, after ADP addition ($\tau = 0$). These studies allowed the determination of the initial rate (PA_i) and maximal extent (PA_{max}) of aggregation for varying ADP concentrations (Fig. 1). A log-dose-response curve is shown for a typical donor in Fig. 2 for PA_i and PA_{max} measured at 10 s and 2 min of stir, respectively. We found that $[ADP]_{1/2}$ for PA_i (1.6 ± 0.4 μM), and for PA_{max} (1.4 ± 0.6 μM), evaluated at 37°C for nine donors, were not significantly different (unpaired t test, $p = 0.25$). We initially chose 37°C as a more physiologic temperature for aggregation studies. However, fibrinogen binding and flow cytometric measurements have routinely been conducted at room temperature (RT) (Frojmovic, 1994; Marguerie et al., 1979, 1980; Shattil et al., 1989), even for comparisons with turbidimetric aggregation generally done at 37°C (Marguerie et al., 1979; Shattil et al., 1989); moreover, it has been reported that there is about one-half of the on-rate and maximal extent of I^{125}-Fg binding to activated platelets at 37°C compared with RT (Marguerie and Plow, 1981; Marguerie et al., 1982; Peerschke, 1992). Therefore, we next compared aggregation dynamics for 37°C and RT.

The data comparing platelet aggregation at room temperature (RT) versus 37°C, at two different ADP concentrations (Fig. 3), indicate that the initial rates of platelet aggregation are the same for both temperatures, whereas maximal extent (PA_{max} at 2 min) is ~33% lower at 1.5 μM ADP at 37°C compared with RT averaged for five donors, although similar at 100 μM ADP. There was negligible reversal in aggregation between 2 and 5 min stir at 100 μM ADP for 37°C or RT. However, at 1.5 μM ADP (Fig. 3 *B*), PA_{max} reached at 2 min at RT remained unchanged out to 5 min, but variable

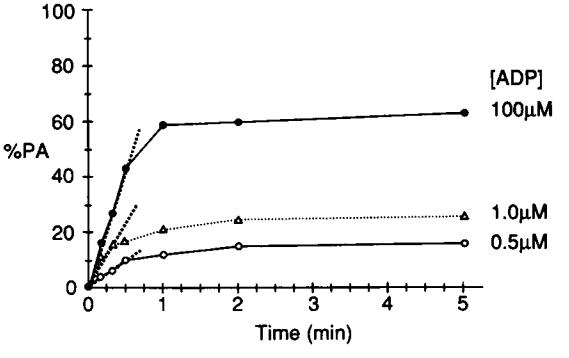

FIGURE 1 Dependence of the initial rate (PA_i) and maximal extent (PA_{max}) of platelet aggregation on ADP concentration. Platelet aggregation (PA) was determined from the change in particle count for PRP (1:10) stirred at 1000 rpm at 37°C for increasing times after addition of varying concentrations of ADP. Initial slopes (·····) typically obtained from PA at 10 s, and maximum extent, typically at 2 min, yield PA_i and PA_{max}, respectively (see Materials and Methods).

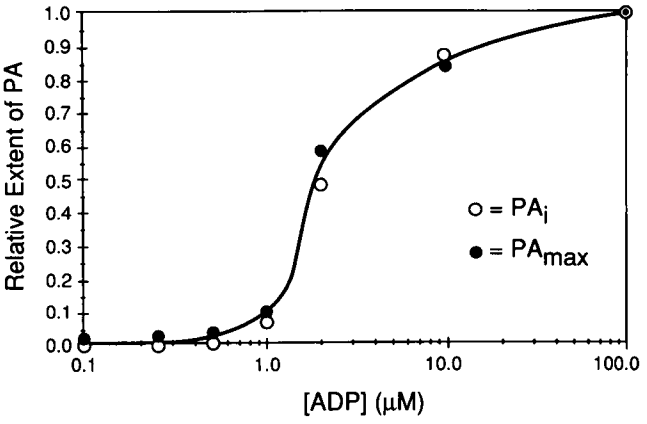

FIGURE 2 Log-dose-response analysis for the initial rates (PA_i) as well as the maximal extent (PA_{max}) of platelet aggregation measured at 37°C (shown for one typical donor). Measurements were taken after 10 s (PA_i) and 2 min (PA_{max}) of stirring at 1000 rpm; data were normalized to maximal values obtained at 100 μM ADP.

reversal was observed at 37°C: in one of five donors there was no reversal, but on average there was about 20% decrease in PA_{max}.

Comparison of aggregation and fibrinogen-receptor dynamics

Comparison of on-rates (v) for PAC1/9F9 with rates and extent of aggregation

We found comparable log dose response profiles for rates of platelet aggregation (PA_i) and for initial on-rates (v) determined for both 9F9 and PAC1 as a function of varying ADP activator concentration, with $[ADP]_{1/2}$ = 1.4 ± 0.1 μM (Fig. 4 *A*).

Comparison of maximal binding (Fl_{max}) of PAC1 and 9F9 with rates and extent of aggregation

a) Whole population studies

Similar ADP concentration-dependent profiles were obtained for the extent of platelet aggregation (PA_{max}) and the extent of both PAC1 and 9F9 maximal binding, which are used to measure fibrinogen receptor expression and fibrinogen binding, respectively (Fig. 4 *B*). The $[ADP]_{1/2}$ values for the extents of platelet aggregation (PA_{max}), fibrinogen binding, and fibrinogen receptor expression were all approximately 1.5 μM (Fig. 4 *B*).

b) Subpopulation analysis

An analysis of mean fluorescence was done in terms of the percentage of cells with fluorescence (Fl) above a critical threshold (Fl_c). Similar log-dose-response curves were obtained for parallel studies of the percentage of cells with $Fl > Fl_c$ (Fig. 5) and the percent of maximal extent of platelet aggregation (PA_{max}) for varying concentrations of ADP as predicted in the companion paper for comparisons of Fl_{max} and %P* ($Fl > Fl_c$). Cross-plots comparing the extent of platelet aggregation (PA_{max}) against the fraction of cells with

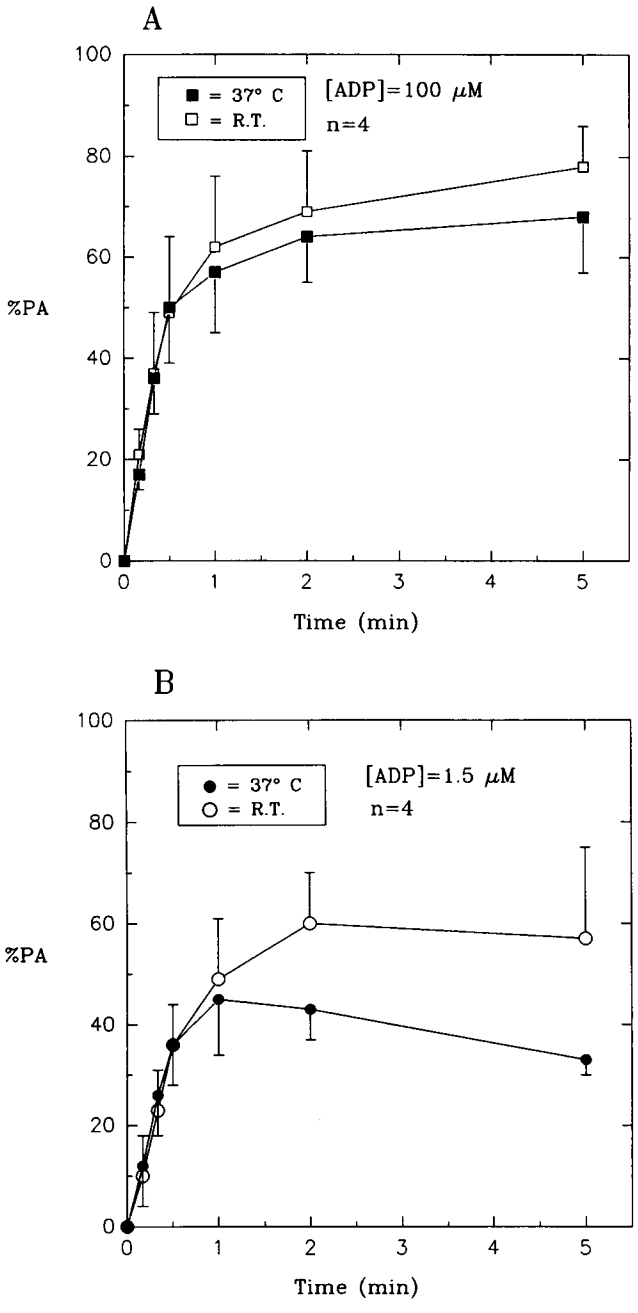

FIGURE 3 Kinetics of platelet aggregation at 37°C versus room temperature for (*A*) maximal (100 μM) and (*B*) intermediate (1.5 μM) ADP concentrations. The same PRP (1:10) was used for the two temperatures and ADP conditions. The data have been pooled for four donors studied in parallel, with mean and SD bars.

fluorescence above a critical threshold demonstrate a linear relationship between the two variables for either PAC1 (Fig. 5 *A*) or 9F9 binding (Fig. 5 *B*) with %PA generally ~0.80 of %P* (percent of cells with $Fl > Fl_c$).

Dependence of rate of platelet aggregation on initial platelet count and fibrinogen concentration

Because we observed subpopulation responses for platelet aggregation, we needed to identify the kinetic order of

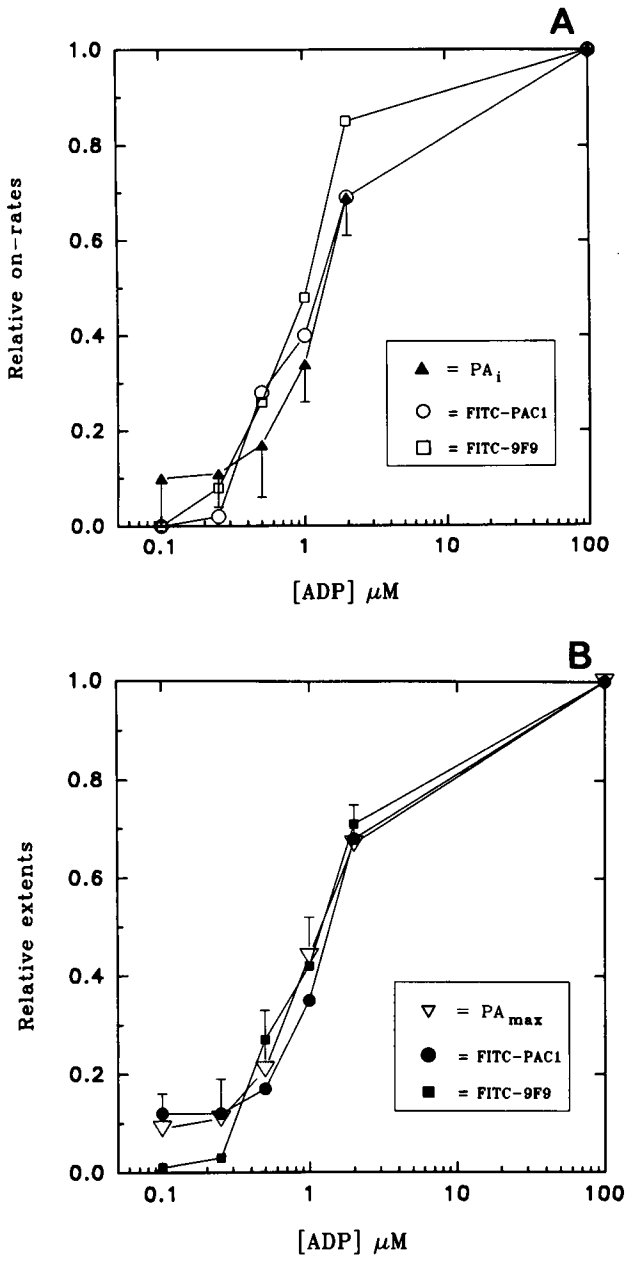

FIGURE 4 A comparison of the (*A*) rates and (*B*) extent of platelet aggregation and PAC1/9F9 binding to platelets as a function of varying ADP concentrations. On rates (v) and maximal fluorescence were determined as for Fig. 7 in the companion paper, ignoring any subpopulation behavior (1), and comparisons were made for parallel PRP samples from the same donor. Data have been normalized to maximal values obtained for each parameter at 100 μM ADP; mean values are shown at each ADP concentration, pooled for 3 and 10 donors, respectively for *A* and *B*; SD are indicated only for aggregation measurements (PA), because these were previously shown in the companion paper (1) for antibody binding.

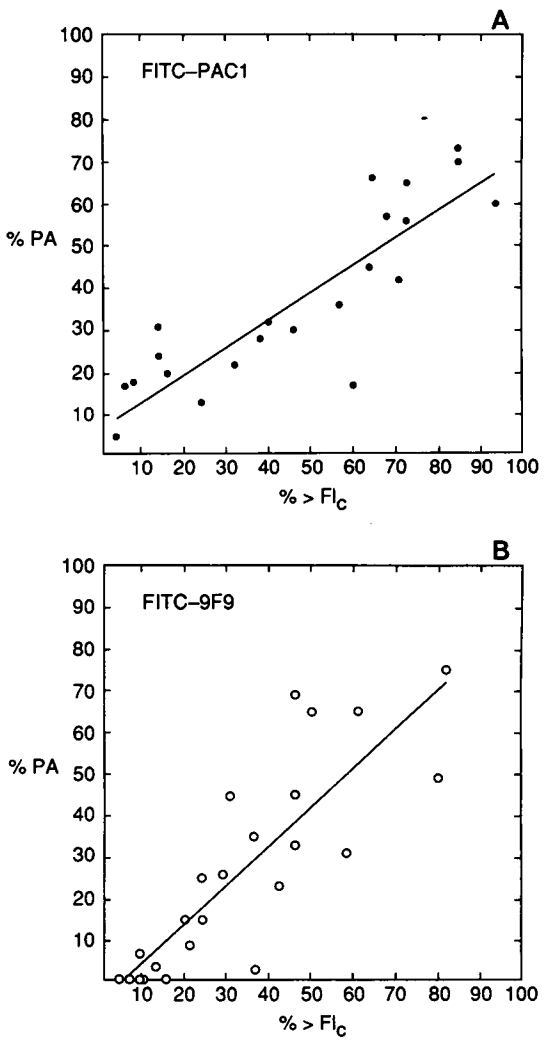

FIGURE 5 The maximal extent of platelet aggregation (*%PA*) versus the percent of cells "quantally" transformed into the activated state (P*) as reported by (*A*) FITC-PAC1 and (*B*) FITC-9F9. PA was measured at 2 min stir for varying concentrations of ADP in parallel with measures of the percent of cells with fluorescence (Fl) above a critical threshold (Fl_c) corresponding to the activated platelet subpopulation (P*) as described in the companion paper (1). The same donors were compared for PA and Fl measures; actual parameter values (not normalized) are shown for seven and four donors, respectively, for PAC1 and 9F9 studies. Lines drawn are the best fits for a linear regression, with r values of 0.7 and slopes of 0.8 for both *A* and *B*.

platelet aggregation with respect to initial platelet count (N_o) over the range of initial platelet counts studied (~25,000–50,000 μl^{-1} for PRP (1:10)). We found that aggregation was second order, because $\%PA_i$ varied linearly with N_o for the range from PRP to PRP (1:10) with an essentially zero intercept (r = 0.99): (Fig. 6); $\%PA_i = cN_o$, but expressing the rate of aggregation as actual number of cells/unit volume/s requires $\%PA_i \times N_o = (cN_o)^2$, actually second order kinetics. It follows that $\%PA_i$ will decrease linearly as the fraction of P* available to participate in aggregation decreases, assuming that "resting" P° platelets cannot coaggregate with P* with any significant capture efficiency.

Because our studies were all conducted for a 10-fold dilution of PRP with buffer, the Fg concentration was ~0.7 μM in all cases, given that normal donors have plasma Fg concentrations of 7.1 μM (± 0.3 μM for 2 SD) (Bakker et al., 1992). We evaluated the possible rate-determining effect of

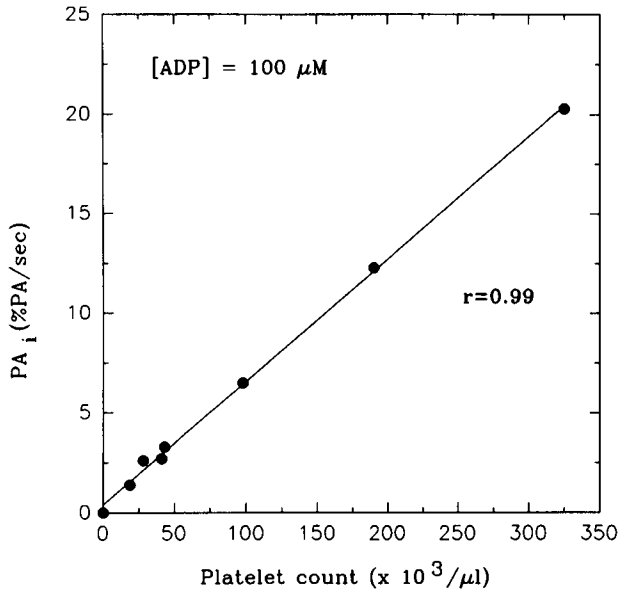

FIGURE 6 Dependence of the rate of platelet aggregation on initial platelet count (No). PRP was increasingly diluted with walsh-albumin (containing 2 mM Mg^{2+}) at 37°C and the initial rate of aggregation (PA_i) measured for stir times between 3 and 20 s, depending on No. Data shown are typical for one of two experiments.

more physiologic Fg by adding pure Fg to PRP (1:10) to give a final Fg concentration of 3.5 μM (5× more). This is expected to increase early linear on-rates for Fg by fivefold and affect the initial rate of aggregation (PA_i) if Fg is rate-determining. No significant effect was seen (Fig. 7).

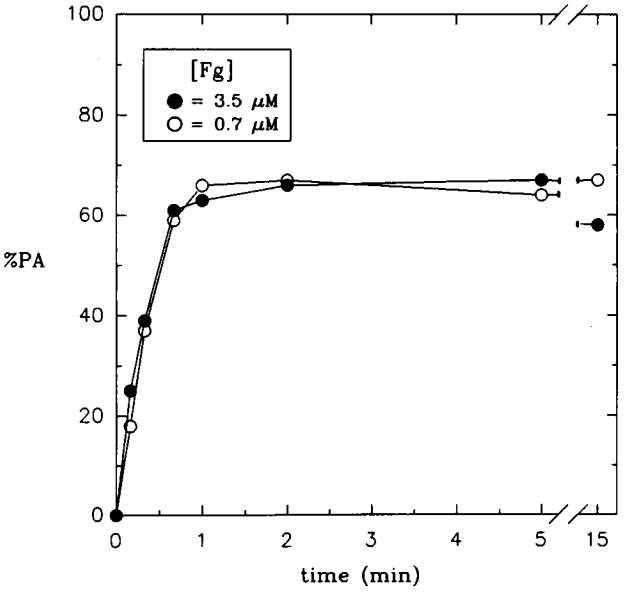

FIGURE 7 Dependence of platelet aggregation on plasma fibrinogen concentration. The aggregation time course is compared for free fibrinogen concentration in PRP (1:10) of 0.7 μM (○) with that of a fivefold increase in concentration (3.5 μM; ●) obtained by addition of pure human fibrinogen to the PRP (1:10). Data were obtained for the same donor's PRP at 37°C.

DISCUSSION

We have found that the "quantal" transformation of "resting" platelets (P^o) to "activated" platelets (P*) by critical concentrations of ADP present in platelet suspensions described in the companion paper (Frojmovic et al., 1994) determines the dynamics of platelet microaggregation in stirred suspensions. Identical log-dose-responses were obtained for PA_i and PA_{max} as a function of activating ADP concentrations. Thus, we found that both rates (PA_i) and maximal extents of aggregation (PA_{max}) varied directly and linearly with the %P* present in PRP (1:10), determined by the [ADP] added. This observation is most simply consistent with a model where only those platelets that are activated, P*, (i) can participate in platelet aggregation, (ii) do not lose their aggregability with time, (iii) do not co-aggregate significantly with resting platelets, P^o, and (iv) follow essentially second order kinetics with a simple dependence of PA_i, expressed as percent aggregation, on the number of P* present per unit volume. We have indeed confirmed that maximally activated platelets, P*, do follow second-order kinetics for N_o values down to PRP (1:10) and below (Fig. 6); and that the rates and extent of aggregation, previously shown not to exhibit time-dependent refractoriness out to >10 min of incubation of PRP with 10 μM ADP (Frojmovic et al., 1983), are equally unaffected for 10-fold diluted PRP as used in our flow cytometry/aggregation studies (data not shown). If platelets are not refractory to aggregation, and if the entire population were only partially activated at intermediate ADP concentrations, or even if P* could coaggregate more slowly with P^o than with other P*, we would expect lowered rates of aggregation (PA_i), but the same eventual maximal extents of aggregation (PA_{max}) at lower ADP concentrations. In fact, we observed only partial PA_{max} at intermediate ADP concentrations, even when PA was monitored out to 15–30 min of stir after ADP addition.

Analysis of our fluorescence data in the companion paper as a function of platelet size (from forward scatter profiles) indicated that the 20% most sensitive platelets (20% P*) recruited into aggregates at the lowest ADP concentrations contain the intermediate to largest sized platelets, with the heterogeneity in platelet responses associated with additional platelet parameters likely related to platelet production and platelet age (Penington et al., 1976; Corash et al., 1977). These findings are consistent with previous reports that "larger" platelets isolated by elutriation (Wong et al., 1989), by differential centrifugation (Carter and Gear, 1986) or by aggregation (Haver and Gear, 1982), are recruited into aggregates at lower ADP concentrations than are "smaller" platelets; the considerable overlap observed in size histograms for the "larger" and "smaller" populations in the latter two methods have also suggested major roles for size-independent heterogeneity in platelet biochemistry and aggregability. On the basis of platelet functional heterogeneity, we propose the following model for ADP concentration-dependent activation/aggregation of platelet subpopulations.

A simple model of occupancy of any one platelet by a critical threshold number of ADP molecules is proposed as a pre-requisite for "firing" an "all-or-none" expression of GPIIb-IIIa receptors. We propose that mid-size to larger platelets contain ADP receptors more sensitive for coupling to activation of GPIIb-IIIa, as proposed for a pseudo-linkage between ADP binding and GPIIb-IIIa activation (De Cristofaro et al., 1988), rather than any heterogeneity in ADP receptor density or affinity (K_D) for ADP binding, which have been proposed to be 0.4 ± 0.1 μM with ~60,000 sites per platelet averaged for the entire platelet population (Cattaneo et al., 1991; Jefferson et al., 1988). This would be consistent with a report of receptor homogeneity for thrombin binding to platelets but, in the case of thrombin, responses only occurred for distinct platelet subpopulations in intracellular calcium increases (Davies et al., 1990). Thus, rapid equilibrium binding of ADP to the most sensitive platelets responding to 0.4 μM ADP will yield 50% occupancy or 30,000 ADP-occupied sites per platelet, predicted to "fire" all of the ≥30,000 GPIIb-IIIa (Plow and Ginsberg, 1989) into high affinity receptors for Fg. Less sensitive platelets would require greater numbers of bound ADP to "fire" the GPIIb-IIIa complexes into competent receptors for Fg. Direct studies of ADP occupancy and platelet functional heterogeneity will yield the correct details for this model.

We have previously reported that the rate of transformation of resting platelets (discocytes) to cells with one or more pseudopods (echinocytes) generally parallels initial rates of microaggregation for 1–10 μM ADP with an onset time for aggregation of 1 s (Milton and Frojmovic, 1984). We have also recently demonstrated that these pseudopods can concentrate GPIIb-IIIa receptors and fibrinogen on their tips within 3 s of ADP activation, which would facilitate pseudopod-mediated platelet aggregation (Hensler et al., 1992). The fact that adrenaline is associated with less than one-third of all platelets as echinocytes, with corresponding partial aggregation, and that low ADP (0.1–0.5 μM) showed ADP-dependent partial conversion of discocytes to echinocytes (Milton et al., 1980), is consistent with the idea that activator concentration may yield only subpopulations of shape-changed platelets and corresponding partial aggregation. Pseudopod formation and expression of fibrinogen receptors will occur maximally within seconds of ADP activation for increasing fractions of platelets with increasing ADP addition.

Our comparison of microaggregation dynamics for RT versus 37°C show temperature-independent initial rates of aggregation for ~50% or >80–90% conversion of resting to activated platelets (1.5 vs. 100 μM ADP in Fig. 3). It appears that 50% decreases in on-rates or numbers of Fg-accessible receptors, reported for studies at 37°C versus RT (Gawaz et al., 1991; Marguerie and Plow, 1981; Marguerie et al., 1982; Peerschke, 1992), do not provide any limitation on kinetics of aggregation at 37°C versus RT. This is also consistent with the observations that i) a fivefold increase in free Fg concentration in PRP (1:10) did not alter aggregation dynamics (Fig. 7), and ii) the apparent decrease, but not total loss, in accessibility of the GPIIb-IIIa receptor for PAC1 or Fg, with a latent time of about 60 s (Fig. 4 in the companion paper) is not reflected in any loss in platelet aggregability under conditions where PA_{max} is achieved within 1–2 min.

Our results using 9F9 to probe the on-rates of Fg to rapidly expressed GPIIb-IIIa receptors on platelets demonstrate that for PRP (1:10), associated with 0.7 μM free Fg in the 10-fold diluted plasma (Bakker et al., 1992), the half-time ($t_{1/2}$) for full occupancy is on the order of 2–3 min, which we have confirmed using FITC-labeled fibrinogen (Frojmovic, 1995), with <20% of full Fg receptor occupancy within the 30 s of ADP addition used for measuring rates of aggregation. This data, along with other studies using FITC-Fg (Xia and Frojmovic, 1994), suggest that <1–5% occupancy of Fg receptors is actually needed to provide >50% of capture efficiencies for microaggregate formation. It is therefore expected that at physiologic Fg concentrations of 7 μM, ADP concentrations will determine the fraction of platelets rapidly expressing essentially all of their Fg receptors, with rates of aggregation limited by the fraction of such P expressed, and not limited by on-rates of Fg binding that can yield >20% occupancy of receptors within 3 s of ADP activation (10 times faster at 7 μM Fg). Direct measurements of Fg receptor occupancy and capture efficiencies have been reported as a function of shear stresses in well defined, homogeneous laminar flow (Xia and Frojmovic, 1994).

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