Dynamics of Platelet Glycoprotein IIb-IIIa Receptor Expression and Fibrinogen Binding. I. Quantal Activation of Platelet Subpopulations Varies with Adenosine Diphosphate Concentration

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ABSTRACT We have previously reported that maximal platelet activation with adenosine diphosphate (100 μM ADP) causes rapid expression of all GPIIb-IIIa receptors for fibrinogen (FgR) (<1–3 s), measured with FITC-labeled PAC1 by flow cytometry. We have extended these studies to examine the effects of ADP concentration on the graded expression and Fg occupancy of GPIIb-IIIa receptors. Human citrated platelet-rich plasma, diluted 10-fold with Walsh-albumin-Mg^{+2} (2 mM), was treated with ADP (0.1–100 μM). The rates of GPIIb-IIIa receptor expression or Fg binding were measured in unstirred samples by flow cytometry, using FITC-labeled monoclonal antibodies (mAb) PAC1 and 9F9, respectively, from on-rates, using increasing times between mAb and ADP additions. Fibrinogen receptors were all expressed rapidly at low (1 μM) or high (100 μM) ADP (few seconds), whereas Fg occupancy was 50% of maximal by about 2 min. The maximal extent of GPIIb-IIIa receptor expression and Fg occupancy was determined from maximal binding (Fl_{max}) at 30 min incubation with PAC1 or 9F9. On-rates and maximal extents of binding for either PAC1 or 9F9 probes showed identical [ADP]-response profiles ("K_D" ~ 1.4 ± 0.1 μM). However, Fl_{max} studies showed bimodal histograms consisting of "resting" (P^o) and maximally "activated" (P^*) platelets for both PAC1 and 9F9 binding, with the fraction of "activated" platelets increasing with ADP concentration. The data best fit a model where platelet subpopulations are "quantally" transformed from P^o to P^*, expressing all GPIIb-IIIa receptors, rapidly filled by Fg, but "triggered" at critical ADP concentrations. Larger, but not the largest, platelets appear to be the most sensitive subpopulation. The implications for clinical studies are discussed, and the relationship to dynamics of aggregation are described in a companion paper.

INTRODUCTION

Mammalian blood platelets circulate as inert, discoid cells until activated by a variety of physiological agonists, including adenosine diphosphate (ADP), thrombin, or collagen, which all cause rapid shape changes (Frojmovic and Milton, 1982; Gear, 1984; Pedvis et al., 1988) and activation of GPIIb-IIIa by altered conformation and/or microenvironment in the membrane (Plow and Ginsberg, 1989; Shattil et al., 1987; Shattil et al., 1989). Subsequent platelet aggregation is generally considered to be mediated by fibrinogen (Fg) binding to the activated GPIIb-IIIa complex (Marguerie et al., 1980; Niewiarowski et al., 1983; Peerschke, 1985) with ensuing cross-bridging of platelets, as shown schematically in Fig. 1. It is expected that any mechanisms that increase the number of fibrinogen receptors per unit area of membrane at the site of bond formation will enhance rates and extent of aggregation (Bell, 1988). Many studies have been reported for ADP-induced macroaggregation using turbidimetrically determined light transmission changes, suggesting that the quantity of fibrinogen bound is related to the degree of platelet aggregation (Marguerie et al., 1980; Niewiarowski et al., 1983; Peerschke, 1985): however, maximal ADP activation and bulk, average Fg measurements using I^{125}-Fg were generally made, with any subpopulation behavior undetected by this approach.

It is widely known that kinetics of platelet activation and aggregation are markedly dependent on the concentration of ADP as activator, as determined by direct particle counting techniques, and on size and density-dependent subpopulations (Carty and Gear, 1986; Frojmovic and Milton, 1982; Frojmovic, 1994; Wong et al., 1989). We therefore opted to evaluate the dynamics of GPIIb-IIIa receptor expression and fibrinogen occupancy as a function of ADP concentration by flow cytometry, which allows both rapid dynamical measurements and detection of platelet subpopulation behavior (Frojmovic et al., 1991; Frojmovic and Wong, 1991). We recently reported a novel theoretical and experimental approach for measuring the rapid expression of activated GPIIb-IIIa receptors for Fg on platelets maximally activated with ADP (100 μM); we used the FITC-PAC1 monoclonal IgM antibody, which binds specifically to the activated form of the GPIIb-IIIa complex (Fig. 1). By measuring initial on-rates of PAC1 binding, as a function of varying time interval between ADP and reporting PAC1 addition, we discovered that platelets appear to express maximally all of their GPIIb-IIIa receptors within 1–3 s of ADP addition (Frojmovic et al., 1991), essentially poised for optimal rates of fibrinogen binding and rapid recruitment into aggregates. In addition, we found that for maximal activation with ADP (100 μM), large platelets expressed higher surface densities of GPIIb-IIIa receptors than did smaller platelets (Frojmovic and Wong, 1991), consistent with reported size-dependent subpopulation responses of platelets to microaggregation (Wong et al., 1989).

We have extended the above flow cytometric studies to an analysis of the kinetics of expression of GPIIb-IIIa receptor and its occupancy by fibrinogen as a function of varying ADP

Received for publication 12 November 1993 and in final form 2 May 1994.
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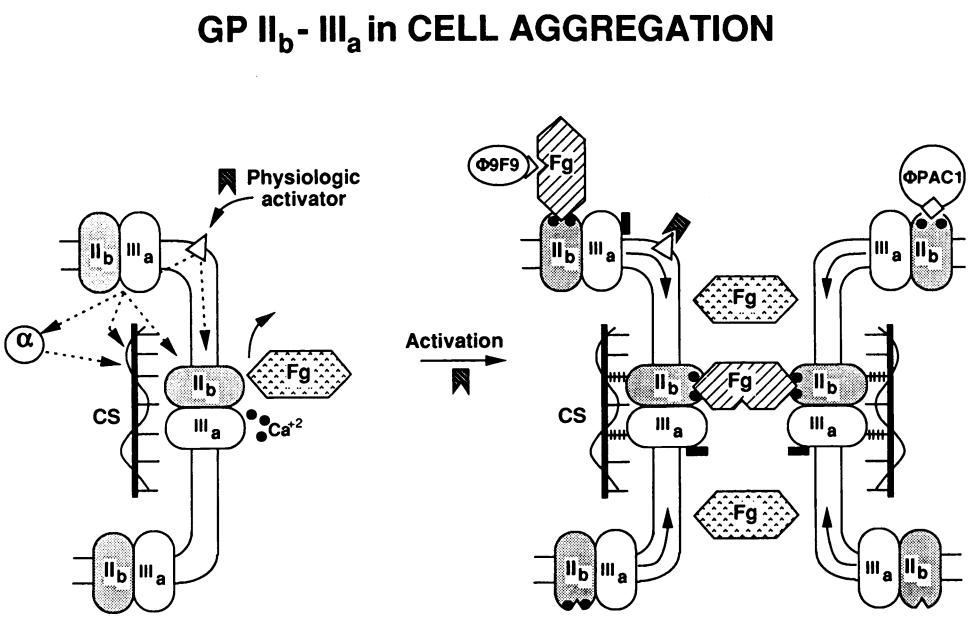


FIGURE 1 Schematic for glycoprotein IIb-IIIa activation, fibrinogen binding, and fibrinogen-mediated platelet aggregation. The resting platelet has GP IIb-IIIa randomly distributed in the plasma membrane, unattached to cytoskeleton (CS), with negligible specific affinity for soluble fibrinogen (Fg). Activation with ADP generates signals transforming GPIIb-IIIa into an activated receptor that now expresses an accessible binding pocket for adhesive ligands including Fg; FITC-labeled monoclonal antibodies PAC1 and 9F9 bind respectively to the activated, conformationally transformed GPIIb-IIIa and Fg. Note that ligand-induced binding sites (LIBS) are shown for GPIIb-IIIa occupied by Fg (*filled rectangles*) (4). The activated GPIIb-IIIa is largely "anchored" to cytoskeleton (CS), whereas one or more Fg may cross-link two or more sets of receptors on adjacent platelets.

concentration. We have used both FITC-PAC1, as well as FITC-9F9, which reports specifically on Fg bound to its activated GPIIb-IIIa receptor (Fig. 1) (Shattil et al., 1989). Initial on-rates for both probes were used to evaluate the rapidity of receptor expression, whereas equilibrium binding measurements were used to evaluate the maximal numbers per platelet of free and Fg-occupied receptors for PAC1 and 9F9, respectively. Our flow cytometric approach revealed that (i) rapid expression of GPIIb-IIIa receptors occurs at varying ADP concentrations, but (ii) subpopulation responses occurred rather than a graded response of the entire platelet population. Such a graded response to increasing ADP concentration might be expected from a model presenting a pseudo-linkage between ADP binding to its receptor and transformation of GPIIb-IIIa into an active receptor for specific binding of Fg (De Cristofaro et al., 1988). Thus, it appears that platelets are "quantally" transformed from "resting" cells (P°) to "maximally activated" cells (P*) with each platelet "triggered" at a critical ADP concentration. In part II, we demonstrate that the fraction of P* present at any given ADP concentration corresponds to the actual platelet number participating in rates and extent of platelet microaggregation (PA) for stirred platelet suspensions (Frojmovic et al., 1994).

MATERIALS AND METHODS

Materials and chemicals

All chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). The platelet activators ADP was diluted in Walsh buffer from a stock of 0.02 M (in Tyrode's pH 7.4) stored at −20° celsius. The vehicle of additions of activator to 10-fold diluted platelet-rich plasma was <10% (v/v). Fluorescein isothiocyanate (FITC; Isomer I, on celite 10%) used for monoclonal antibody labeling was purchased from Behring Diagnostics (La Jolla, CA). Aldehyde solutions used for platelet fixation were freshly prepared by diluting 8% glutaraldehyde solution 10-fold with double distilled de-ionized water. The anticoagulant trisodium citrate was prepared from Sigma, whereas the fatty acid free bovine serum albumin was prepared from ICN Biochemicals (Cleveland, OH). ADP, or trisodium citrate (all from Sigma) or glutaraldehyde (8% aqueous stock in sealed ampoules, Polysciences Inc., Warrington, PA) were separately diluted in Ca^{2+}/Mg^{2+}-free Tyrode's solution (NaCl 140 mM, KCl 2.7 mM, $NaHCO_3$ 0.4 mM; pH 7.4). Walsh-albumin solution was freshly made up as needed from Tyrode's containing Mg^{2+} (2 mM), albumin (1 mg/ml) [bovine albumin, Fraction V (Sigma)], and glucose (1 mg/ml).

Monoclonal antibodies

PAC1, a murine monoclonal IgM antibody specific for GP IIb-IIIa in its activated state (Shattil et al., 1987, 1989), and 9F9, a murine monoclonal IgG antibody specific for the D-domain of fibrinogen bound to its GPIIb-IIIa receptor (Shattil et al., 1989), were purchased from Dr. Sanford Shattil (University of Pennsylvania, Philadelphia, PA) and Dr. Andrei Budzynski (Temple University, Philadelphia, PA), respectively. The antibodies were conjugated with FITC (Shattil et al., 1985), and aliquoted stocks of this material were stored at −70° celsius. On the day of use, these stocks were freshly thawed and spun for 5 min at 8700 × *g* to remove antibody aggregates. The fluorescein/protein (F:P) molar ratio varied between 7.0 and 7.7 for the three different preparations of FITC-PAC1 used, and from 2.9 to 3.6 for FITC-9F9.

Preparation of platelet-rich plasma

Healthy males and females, between the ages of 19 and 45 and not on any medication, were used as donors with their informed consent. Blood was collected from the antecubital vein by venipuncture. It was then added directly into 3.8% sodium citrate (1 vol to 9 vol blood), and platelet-rich plasma (PRP) was prepared and maintained at 37°C for 30 min before use, as previously reported (Frojmovic et al., 1991); the PRP was then diluted 10-fold (PRP (1:10)) with Walsh-albumin at room temperature. All studies with PRP were made within 2–4 h of blood collection.

Platelet-rich plasma processing for activation and flow cytometric measurements

Quench-dilution measurements for PAC1

As previously reported, samples typically consisted of 22.5 μl of PRP (1:10), plus 2.5 μl of activator (ADP) added at room temperature to 12x75 mm

polystyrene tubes for varying incubation times (τ) before the addition of FITC-antibody (4 μl). Saturating concentrations of antibody were used to yield >90% of maximal fluorescence after 30 min incubation for maximally activated platelets: typically 0.1 μM for PAC1. Samples were hand-swirled rapidly for <1 s to ensure complete reagent mixing but negligible aggregation, verified by direct microscopic counting. After incubating the above PRP (1:10) with ADP and antibody for incubation times, τ, varying from 5 s to 60 min, each sample was quench-diluted 10-fold with Walsh-albumin and measured by flow cytometry within 30 s to minimize any post-dilution, time-dependent changes as previously reported (Frojmovic et al., 1991). These studies were performed for varying τ at selected concentrations of ADP (1, 10, or 100 μM). We confirmed our previous observations that 10-fold dilution of PRP (1:10) containing 100 μM ADP and FITC-PAC1 alters neither Fl_{max} (Frojmovic et al., 1991) nor the fraction of activated platelets (P*) caused by dilution, whether activating with 100 or 1 μM ADP. All of the above procedures were conducted at room temperature.

Direct measurements without dilution for 9F9

Samples were processed as in Quench-Dilution Measurements for PAC1 above, except that six volumes (typically 135 μl) of PRP 1:10 was used for direct measurements with the flow cytometer without any further sample dilution to avoid underestimates caused by significant off-rates of 9F9 with sample dilution. Typically, 0.6 μM 9F9 monoclonal antibody was used for saturation conditions.

Flow cytometry measurements

Platelet samples were analyzed for 2500–5000 cells in a FACSCAN flow cytometer (Becton-Dickinson Canada, Mississauga, Ontario). Light scatter (forward scatter (FSC), side scatter (SSC)), and one color fluorescence (FL) were obtained with flow cytometer gain settings in the logarithmic mode and analyzed with Becton-Dickinson C30 software on a Hewlett-Packard 300 computer to yield cross-plots, histograms, or mean values, as previously reported (Frojmovic et al., 1991). In the case of both unactivated and maximally activated platelets, the mean and mode values for *Fl* and FSC (forward scatter) were within <5% of each other because of the symmetrical shapes of the histograms. *Fl* values associated with FITC-antibody binding to activated platelets were corrected for nonspecific or background binding by subtracting *Fl* "control" values obtained in the absence of activator or by the addition of 5 mM EDTA 10 s before addition of the activator. Thus, parallel samples were incubated with PAC 1 or 9F9 for identical times without activator, as for the activator studies, and background *Fl* values were obtained.

Time courses of FITC-PAC1/9F9 binding were obtained for samples pre-activated with ADP for different times, τ, from the mean platelet-bound fluorescence (Fl), determined for ≥15 s of antibody addition, as previously reported (Frojmovic et al., 1991). Both rapid, initial rate of increase in fluorescence (Fl) (related to antibody on-rates) and maximal extent of increase (Fl_{max}) were thus determined for different τ values. These measurements yield the rate of formation of fibrinogen receptors (k_1) and the maximal number of free and fibrinogen-occupied receptors, respectively, for PAC1 and 9F9, as a function of activator concentration and time of action (Frojmovic et al., 1991).

For the ADP dose response studies, platelets were pre-activated for 3 or 10 s with varying ADP concentrations (specified in legends to figures), and on-rates and maximal equilibrium binding (maximal extent; Fl_{max}) of binding of PAC1 or 9F9 were respectively measured from *Fl* values determined for 30 s and 30 min incubations with the reporting antibody.

For analyses of maximal, equilibrium binding of antibodies, Fl_{max} values were analyzed by two different methods. One method, as used previously for platelets maximally activated with 100 μM ADP (Frojmovic et al., 1991), uses the mean value for the entire platelet population. A second method is based on the observation that at high ADP concentrations most of the platelets have *Fl* values greater than a critical threshhold value, Fl_c, but decreasing fractions of these platelets remained above this threshhold with decreasing ADP, i.e., there was a bimodal distribution of "resting" (P^o) and "maximally activated" (P*) platelets. For this type of subpopulation analysis, we report the fraction of platelets having $Fl > Fl_c$, i.e., the fraction of "maximally activated" platelets as a function of varying ADP concentration. Fl_c was experimentally selected such that >95% of all platelets had *Fl* values $<Fl_c$ in PRP 1:10 in the absence of added ADP.

RESULTS

Kinetics of binding of PAC1 and 9F9 to platelets maximally activated with ADP

For platelets maximally pre-activated with 100 μM ADP (τ = 3 s), a time course of FITC-PAC1 or 9F9 binding to platelets was obtained by dilution-quenching or "live" measurements, respectively, at t-values ranging from 0 to 30 min, as shown for the fluorescence histograms in Fig. 2. There was a net shift in fluorescence profiles to equilibrium, maximal values within 30 min incubation (profiles were unchanged for 45 and 60 min points, not shown) for both PAC1 and 9F9 binding, as previously reported for PAC1 (Frojmovic et al., 1991).

We confirmed that *Fl* increased linearly with PAC1 incubation times for varying τ values after 100 μM ADP additions as previously reported (Frojmovic et al., 1991) and showed linear changes for 9F9 reporting on bound Fg for 100 μM ADP (Fig. 3), allowing measures of on-rates and Fl_{max} (≥30 min) for PAC1 or 9F9 for varying ADP concentrations. The time needed for maximal expression of receptors at decreasing activating ADP concentrations needed first to be determined.

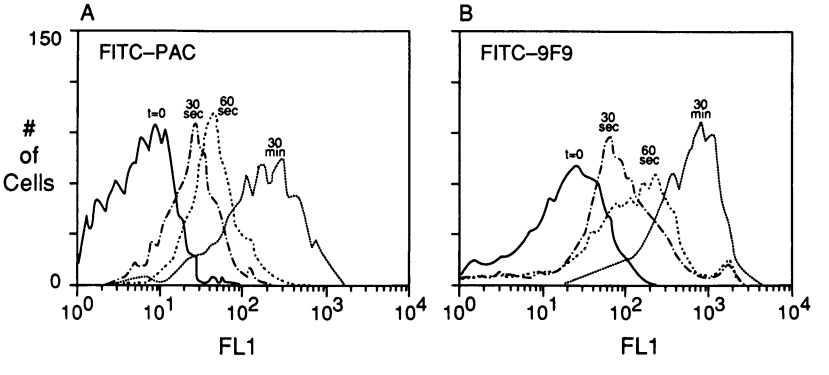


FIGURE 2 Dynamics of fluorescence histograms of platelets maximally pre-activated with 100 μM ADP (τ = 3 s) with time-dependent binding of (*A*) FITC-PAC1 and (*B*) FITC-9F9. From left to right, incubation times with antibodies were as follows: (*A*) control, 30 s, 60 s, and 30 min and (*B*) control, 15 s, 30 s, and 30 min.

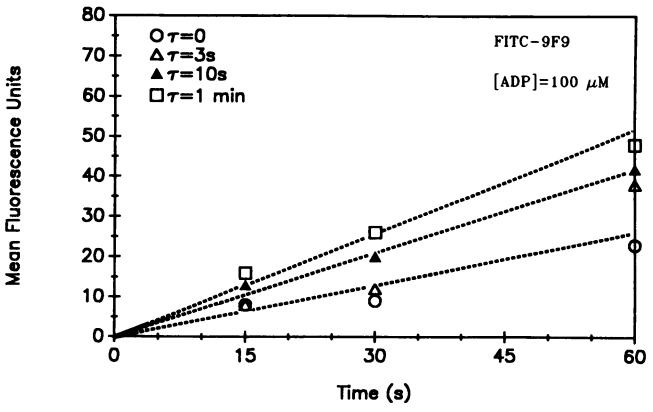
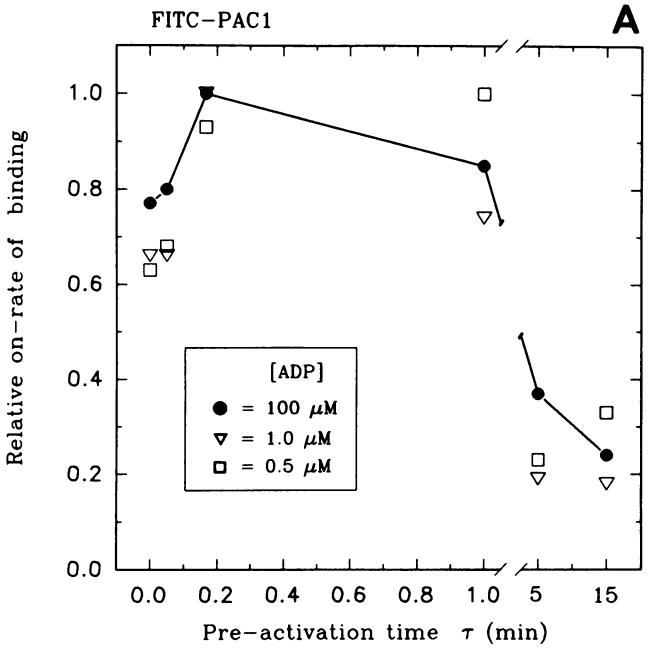


FIGURE 3 Kinetics of the increase in mean fluorescence (Fl) of FITC-9F9 to platelets maximally activated with 100 μM ADP as a function of increasing preactivation time τ. The initial rate of increase in Fl represents the 9F9 on-rate (v_τ) for binding of the probe for time τ. Fl values corrected for nonspecific binding are shown for one of six donors evaluated. Linear regression lines have been drawn for each set of data points, with r values of 0.996, 0.997, and 0.935 for decreasing τ.



Rapidity of expression of Fg receptors and of Fg binding with varying ADP

We previously reported that activation of platelets with 100 μM ADP (maximal activation) causes rapid expression of all fibrinogen receptors within 3 s of activator addition (Frojmovic et al., 1991). This was concluded from the observation that the same initial on-rate (v) for PAC1 binding was seen for pre-activation times of 1, 3, or 10 s. We have confirmed that rapid expression of fibrinogen receptors occurs at decreasing ADP concentrations to 1 μM (Fig. 4 *A*) with <10% differences between 0.5 and 1 μM ADP and 100 μM ADP. The donor's platelets used for this study showed a small delay for 100% activation of all GPIIb-IIIa compared with the three donors previously reported (Frojmovic et al., 1991), but >60–80% of all receptors were expressed within 3 s for 0.5–100 μM ADP evaluated, with ≥95% by 10 s. In addition, as previously reported (Frojmovic et al., 1991), Fl_{max} values were independent of varying τ values, even though on-rates (v) did decrease with τ beyond 1 min for PAC1 binding (Fig. 4 *A*). This decay in accessibility of the activated GPIIb-IIIa beyond 1 min of ADP activation occurred for the range of 0.5–100 μM evaluated, with 2 μM ADP showing the same results as that reported for 100 μM ADP.

Fig. 3 shows linear increases for fluorescence for 9F9, with incubation time of platelets after maximal activation with 100 μM ADP, out to 1 min, because more Fg will bind during increasing τ times between addition of ADP and final addition of the reporting 9F9 mAb. A plot of initial on-rate (v_τ) as a function of τ normalized to maximal v_τ values found for τ = 60 s, was previously shown to be a relative measure of the number of fibrinogen receptors (FgR) using PAC1 as the reporting mAb (Frojmovic et al., 1991) and receptor accessibility. A similar plot for receptors occupied by Fg and accessible to the reporting mAb at time τ of addition of 9F9 has

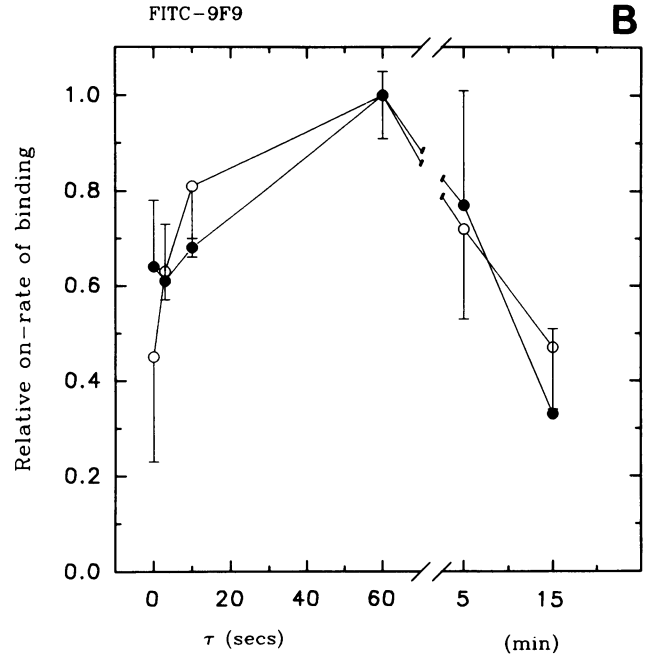


FIGURE 4 Relative on-rates (v_τ) of (*A*) FITC-PAC1 or (*B*) FITC-9F9 for different periods of pre-activation (τ) with varying concentrations of ADP. The data have been normalized to v_τ-max values obtained for $\tau \leq 60$ s. One representative donor is shown for PAC1, and mean ± 1 SD for 4 ± 2 donors are shown for 9F9.

been determined (Figs. 3 and 4 *B*). Analysis of such plots obtained for both 100 μM and 1 μM ADP as shown in Fig.4 *B*, showed (i) similar initial rates of Fg-occupancy for 1 and 100 μM ADP, (ii) a half-time ($t_{1/2}$) for maximal on-rates for 9F9 binding to bound Fg of about 10 s, peaking at about 60 s, with on-rates decaying by 5 and 15 min, as previously observed with PAC1 binding (Frojmovic et al., 1991), whether activating with 1 or 100 μM ADP. The half-

time ($t_{1/2}$) for maximal occupancy by Fg is 2 ± 0.5 min, measured from Fl_{max} values as shown in Fig. 2 for 100 μM ADP, with data pooled and analyzed for three donors, yielding a plot from which the time for 50% of Fl_{max} to be reached could be graphically determined as $t_{1/2}$. Note that the same Fl_{max} values were obtained for varying τ (as previously reported for PAC1) (Frojmovic et al., 1991).

Dependence of PAC1 and 9F9 binding (rates and extent) on ADP concentration used for activating platelets

A series of fluorescence histograms were next obtained as shown in Fig. 2 for 100 μM but now determined for concentrations of ADP varying from 0.1 to 100 μM, with pre-activation times of 3 s: such a series of histograms is shown for *Fl* measured for 30 s incubation of platelets with PAC1 and 9F9 chosen to reflect initial on-rates (v_r) (Fig. 5). A plot of mean *Fl* values, determined from profiles as shown in Fig. 5, but determined for pre-activation times of 10 s to ensure maximal receptor expression, was then obtained to show the time-dependence for PAC1 or 9F9 binding to platelets for different ADP activator concentrations. It is seen that *Fl* varies linearly with time over the first 30 s of PAC incubation (Fig. 6), such that initial on-rates (v) can faithfully be obtained fom 30 s measurements of *Fl*. Similar results were obtained for 9F9 (data not shown). A plot of the initial rate of increase in specific *Fl* due to PAC1 or 9F9 binding determined from these curves (v) as a function of ADP concentration (Fig. 6) yields similar log-dose-response curves (Fig. 7).

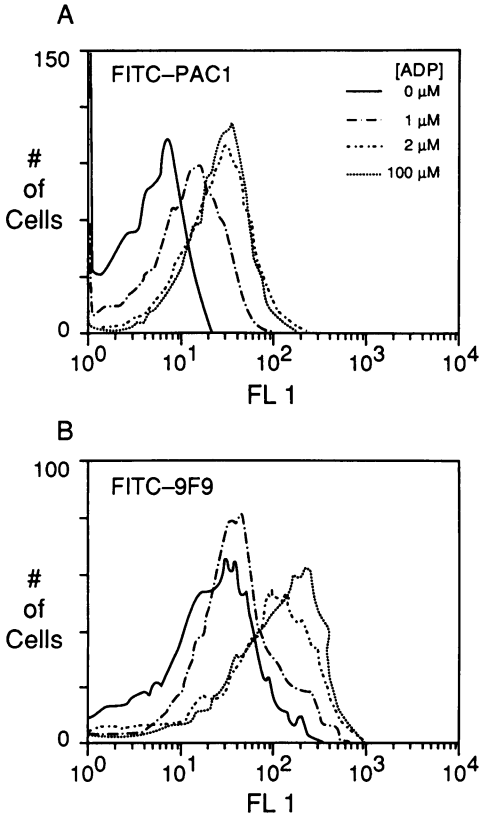


FIGURE 5 Fluorescence profiles for the effect of varying the ADP concentration on the binding of (*A*) FITC-PAC1 (reflecting fibrinogen receptor expression) and (*B*) of FITC-9F9 (fibrinogen binding), measured during the initial linear phase of binding (for τ = 3 s, t = 30 s). These mean fluorescence values are used to represent initial on-rates (v).

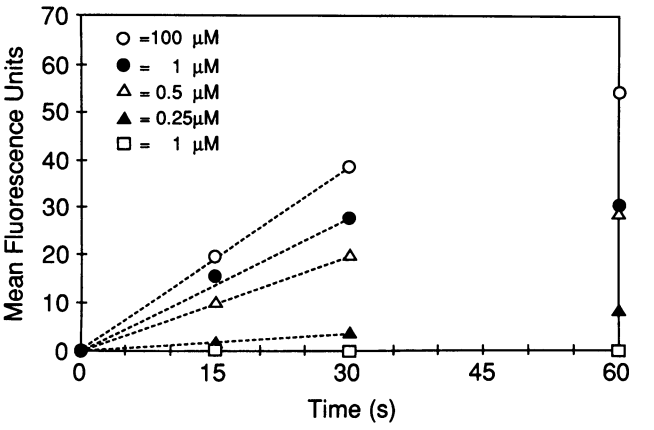


FIGURE 6 Dependence of the initial rate of binding of PAC1 to platelets activated with varying ADP concentration. Corrected F1 values versus incubation time with FITC-PAC1 are shown for one typical donor from a pool of 10 donors. Similar curves were obtained with 9F9 for five donors. The initial slopes represent on-rates (v), determined from Fl values at 30 s, as reported in Fig. 7.

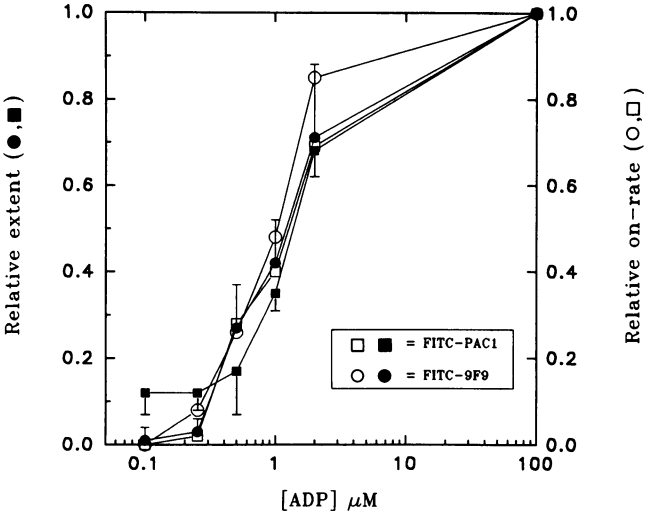


FIGURE 7 Log dose-response curves for the relative on-rates and maximal extent of PAC1 or 9F9 binding for platelets activated with increasing ADP concentrations. Fluorescence parameters have been normalized to maximal values obtained at 100 μM ADP. Platelets were pre-activated with varying ADP for 10 s (τ value). On-rates were determined for 30 s antibody incubation (linear portion as in Fig. 6) with relative mean ± SD shown for each antibody for three donors. Maximum fluorescence (Fl) values were obtained for 30 min incubations with antibody, with the mean Fl values recorded for the entire platelet population, irrespective of apparent subpopulations shown in Fig. 8 (see Materials and Methods); relative mean values are shown for 10 and 5 donors, respectively, for PAC1 and 9F9, with plus and minus standard SDs, respectively, shown for 9F9 and PAC1 for filled symbols (very similar SDs were obtained for the open symbols).

A series of fluorescence profiles were also obtained to compare the maximal extent of fluorescence obtained with various concentrations of ADP using both FITC-9F9 and FITC-PAC1 (Fig. 8) reporting on the extent of fibrinogen receptor expression and of fibrinogen binding, respectively. For both probes, the maximal extent of specific binding was observed for 10–100 μM ADP. However, it must be noted that unlike the *Fl* profiles shown for early binding times (Fig. 5), corresponding to initial on-rates (v), the *Fl* histograms at equilibrium, maximal binding show evidence of at least two distinct subpopulations of cells. We therefore conducted the following two types of analyses of the data: (i) measurement of the mean fluorescence values (*Fl*) for the entire population, neglecting the possibility of subpopulation behavior, and (ii) measurement of the percent of cells with fluorescence values above a critical threshold (Fl_c), determined from the apparent shift of most cells to one uniform histogram at maximal ADP activation. Log-dose-response curves are shown for Fl_{max} vs. [ADP] for both PAC1 and 9F9 binding, determined from overall mean *Fl* values (Fig. 7).

Log-dose-response curves for both v and Fl_{max} show that similar sensitivity to ADP was observed for the expression of fibrinogen receptors whether determined from on-rates (v) or Fl_{max} for FITC-PAC1 binding, or whether using 9F9 or PAC1 as the reporting probes (Fig. 7). The percent of cell with $Fl > Fl_c$ was also found to yield identical log dose-response curves for PAC1 and 9F9 for *Fl* or for relative percent of activated cells, shown for data using FITC-9F9 as the reporting probe (Fig. 9). Similar threshold values were observed whether examining fibrinogen receptor expression or Fg binding as determined from both on-rates and maximal extent, typically 0.2–0.3 μM ADP. The ADP concentrations causing 50% of maximal increases in the above parameters, $[ADP]_{1/2}$ values, in all cases were 1.3 ± 0.3 μM (range 0.9–1.6 μM). In addition, it appears tht mean Fl_{max} values are exact predictors of the relative percent of activated platelets (percent of cells above a critical threshold seen in Fig. 8). We therefore next examined the quantal nature of resting P° to activated P* transformation.

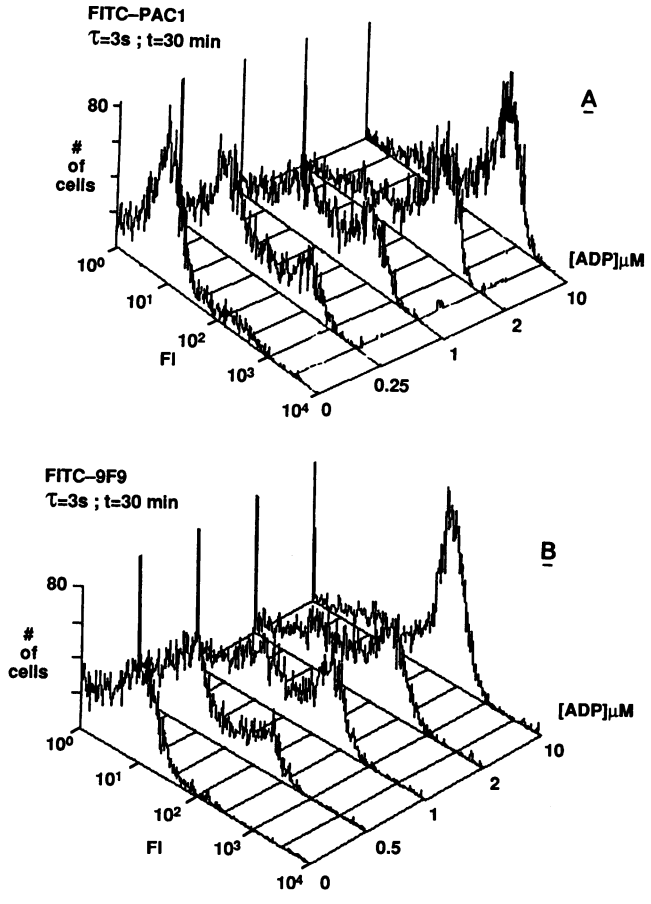


FIGURE 8 Fluorescence profiles for maximal equilibrium binding of (*A*) FITC-PAC1 and (*B*) FITC-9F9, to platelets pre-activated for 3 s with varying ADP concentrations and then incubated with the antibody for 30 min. Data are for one typical donor. Note subpopulation responses. No differences were observed whether preactivating for 0 or 10 s.

"Quantal" activation of platelets

We found that "activated" (P*) and "resting" (P°) platelets displayed distinct and constant values of Fl_{max} (Fig. 10). Thus, the mean maximal fluorescence for "resting" platelets ($Fl°$) incubated with PAC1 or 9F9 changed very little over the entire range of cell activation as the percent of such cells decreased from >95% to <10–20% (Fig. 10). There appears to be an effective "quantal" activation from P° to P* cells expressing maximal PAC1 or 9F9, with the percent of such cells driven by the ADP concentration. In contrast to the minor changes in Fl° values with varying percent of activated cells, it is seen that *Fl** increased by 1.7-fold for *Fl** values

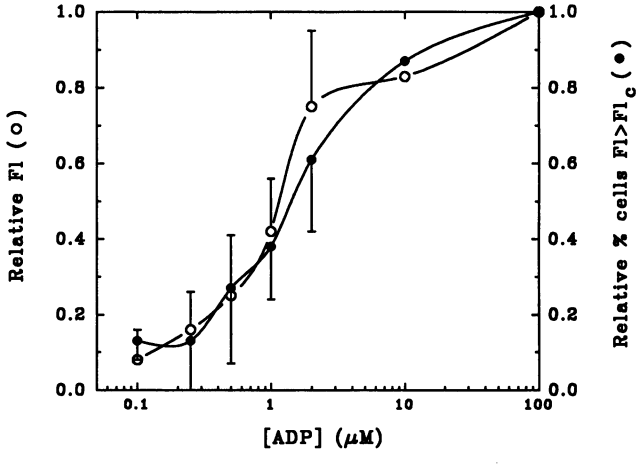


FIGURE 9 Comparison of the ADP concentration dependence of the overall mean fluorescence (Fl) for maximal binding of FITC-9F9 (disregarding subpopulation behavior) to the percent of cells "quantally" activated (P* with $Fl > Fl_c$ (see Fig. 8 and Materials and Methods)). *Fl* and % cells $Fl > Fl_c$ have been normalized to 1 for 100 μM ADP, with the mean shown for the same four donors studied in parallel for each of the two parameters; plus and minus SD bars are respectively shown for the open and filled symbols, except for the data at 10 μM ADP (one donor). Spline curves were drawn by running interpolation of cubic polynomials. Similar results were obtained with FITC-PAC1 as the reporting probe.

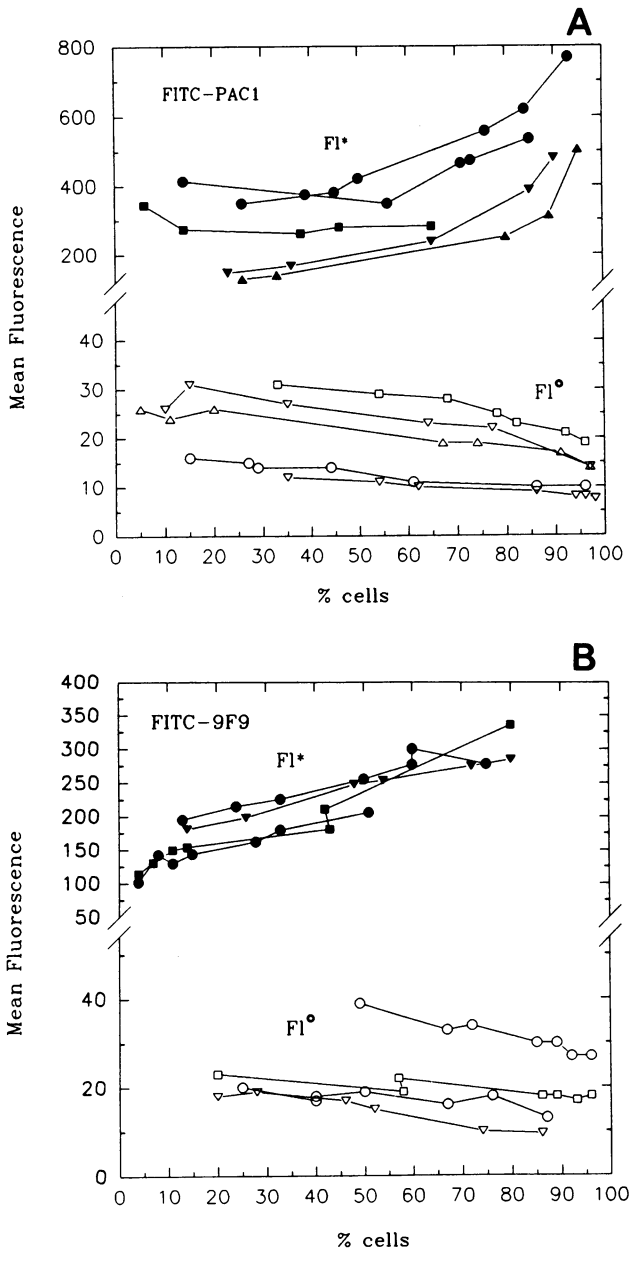


FIGURE 10 Dependence of the mean fluorescence for the "resting" (Fl^o) and "activated" (Fl^*) platelet subpopulations on the % of cells, respectively, in the "resting" or "activated" state, shown for both (*A*) PAC1 and (*B*) 9F9 reporting probes. Fl° and Fl* were obtained by gating on the two subpopulations shown in Fig. 8 (see Materials and Methods), as a function of varying ADP concentrations that determine the actual % of all cells present in the second Fl profile corresponding to quantally activated platelets. Each curve represents a distinct donor's PRP evaluated for both Fl^o and Fl^* in (*A*) four of five donors and (*B*) four of four donors.

for ≥80% activated cells vs. ≤20% of activated cells (SD ± 0.3 and 0.6 for 9F9 and PAC1, respectively, for the data shown in Fig. 10).

DISCUSSION

We previously reported that the resting GPIIb-IIIa complexes on platelets maximally activated with ADP (100 μM) are all transformed into high affinity receptors for fibrinogen, as probed by the PAC1 IgM monoclonal antibody and rapid flow cytometric measurements, within seconds of ADP addition to the platelets in platelet-rich plasma (PRP) (Frojmovic et al., 1991). We have now confirmed this rapid expression of receptors for varying ADP concentrations using PAC1 as reporting probe. Thus, 67 to >80% of maximal on-rates for either probe were obtained within seconds of ADP addition for ADP concentrations varying from <1–100 μM ADP (Fig. 4). The somewhat "apparently" slower expression at 0.5–1 μM ADP (≤10% slower over the first 10 s) compared with 100 μM ADP may arise from the heterogeneous response of platelets, yielding mean fluorescence values (*Fl*) consisting of both "resting" (P^o) and "activated" (P^*) platelets, discussed below in greater detail.

We have also confirmed, for both PAC1 and 9F9 probes, that the accessibility of the activated GPIIb-IIIa receptor to the IgM PACl, or to 9F9 IgG binding to Fg, measured from on-rates of these probes, decreases between 1 and 15 min after ADP binding for varying ADP concentrations; this was previously reported for PACl binding to platelets activated with 100 μM ADP (Frojmovic et al., 1991). In all cases, as previously reported, maximal, equilibrium fluorescence values (Fl_{max}) were unaffected with varying incubation times, τ, between ADP and reporting probe additions. Finally, the measured $t_{1/2}$ of about 2 min for Fg occupancy in PRP (1:10) is comparable with that reported elsewhere using I^{125}-Fg (Plow and Marguerie, 1980) or FITC-labeled Fg (Xia and Frojmovic, 1994).

Fluorescence profiles for early binding times of PACl or 9F9 to platelets activated with 1–100 μM ADP show broad distributions with suggestions of "shouldering" and subpopulation responses (Fig. 5). Equilibrium binding studies showed unequivocally for either probe that platelets appear to be transformed "quantally" from "resting" (P^o) to "activated" (P^*) platelets, increasing with more elevated concentrations of ADP. Both on rates (v) and Fl_{max} values for mean *Fl* used without accounting for subpopulation responses, varied identically with [ADP], as well as with the actual percent of P* measured from equilibrium binding as a function of ADP. This is most simply explained by the fact that *Fl* values are likely

$$Fl = f^oFl^o + f^*Fl^*,$$

where f^o and f^* are the fraction of resting and activated platelets with mean fluorescence Fl^o and Fl^*, respectively. Therefore,

$$Fl = Fl^o + f^*(Fl^* - Fl^o) = a + bf^*$$

where Fl^o and Fl^* are considered constants for "resting" and quantally activated platelets, i.e., *Fl* values directly predict f^*, the fraction of activated cells, as shown experimentally (see Fig. 9). This relationship is expected to apply equally to early on-rate measurements, consistent with results showing identity between the ADP concentration-dependence for on-rates and Fl_{max}.

Analysis of the data shown in Fig. 10 for "resting" (P^o) versus "maximally activated" (P*) platelets shows that Fl^o are less than 5% of Fl^* values, but Fl^* increases with the fraction of P*: Fl^* for 80% P* is 1.7 times greater than Fl^* for only 20% P*. This increase in Fl^* values occurring with increasing ADP could be largely associated with larger platelet size and mean surface area (SA) and/or higher surface density of receptors. Thus, if the first 20% of activated platelets were the largest platelets, predicted from the reported greater sensitivity of larger platelets to aggregation (Wong et al., 1989), then $Fl^*(0.8)/Fl^*(0.2)$ would be expected to be <0.7 rather than the observed 1.7. However, analyses of the forward scatter (FSC) profile for the 20% P* formed at the lowest ADP show these platelets to have only intermediate to higher FSC values compared with the resting population (data not shown), consistent with intermediate-larger sized platelets (Frojmovic and Wong, 1991), i.e., the largest platelets do not appear to be exclusively recruited at the low ADP concentrations associated with formation of 20% P*. In addition, it was found that the width of the histograms for Fl^* actually decreased by 22 ± 13% for PAC1 and 9F9 as the percent of P* increased from 20 to ≥80%. Therefore, the increase in Fl^* with increasing percent of P* largely reflects parameters other than size, such as heterogeneity in platelet production and platelet age (Carty and Gear, 1986; Corash and Gralnick, 1977; Frojmovic and Milton, 1982; Penington et al., 1976; Wong and Frojmovic, 1989).

The subpopulation response (P^o and P*) associated with expression of the GPIIb-IIIa receptor and its occupancy by Fg has never been observed for α-granule secretion of GMP-140 or GPIIb-IIIa, i.e., there is an ADP dose-dependent but unimodal increase in fluorescence for antibody probes reporting on α-granule secretion (S12 mAb for GMP 140) or GPIIb-IIIa secretion (AP2), with all of the platelets responding in a graded fashion (data not shown). The ~20% of platelets remaining as "resting" P^o, with respect to PAC1 and 9F9 binding, up to 100 μM ADP, are expected to undergo the partial secretion observed homogeneously for the entire platelet population.

The support for ADP-driven "quantal" activation of platelet subpopulations can be found upon careful analysis of histograms published by Warkentin et al. (1990) for FITC-labeled polyclonal antifibrinogen antibody binding at equilibrium conditions (30 min) to platelets in anticoagulated blood activated with varying ADP concentrations. Jackson and Jennings (1989), using a similar approach with plasma diluted PRP, found that the proportion of platelets "showing positive antifibrinogen antibody binding" increased with ADP concentration, but they did not quantitate the "cutoff" for positive cells, and additionally observed a more than four-fold increase in Fl for the "positive" platelets with increasing ADP concentration. The capacity for platelets to respond in an "all or none" fashion has been reported for calcium transients occurring maximally in a subpopulation of platelets: 77–86% of all cells responding over a 20-fold increase in thrombin concentrations (Davies et al., 1990). Changes in calcium are expected to be related to fibrinogen receptor expression in the responding platelet subpopulations (Kroll and Schafer, 1989).

It has been proposed that GPIIb-IIIa is conformationally transformed from a low affinity state (T) to a high affinity state (R) capable of specific binding of adhesive proteins such as Fg, via pseudo-linkage between ADP receptor binding and Fg binding via a third component yet to be characterized (De Cristofaro et al., 1988). Such a model could allow for (i) a continuous, graded expression of receptor R on each platelet with increasing ADP receptor occupancy or (ii) and "all-or-none" response occurring above a critical ADP receptor occupancy varying for platelet subpopulations. Our data favor the second possibility, although De Cristofaro et al. (1988) could only interpret their data in terms of bulk, average measurement, because they used ^{125}I-Fg for measuring Fg binding to platelets.

Increasing numbers of investigators are using fluorescent probes in the clinical setting for detection of platelet activation in vivo (Abrams and Shattil, 1991; Ault et al., 1989; Frojmovic, 1995; Ginsberg et al., 1990; Plow and Ginsberg, 1989; Scharf and Clemetson, 1995; Shattil et al., 1987; Warkentin et al., 1990). Because "irreversibly" bound Fg can inhibit the binding of PAC1 to activated platelets (Shattil et al., 1987), both PAC1 and a monoclonal antibody to receptor-bound Fg, such as 9F9, should be used to detect free and occupied activated GPIIb-IIIa. Our studies underline the power and sensitivity of flow cytometry for the detection of only a few percent of circulating, activated platelets, as first suggested by Shattil et al. (Shattil et al., 1987), given that low activator concentrations do not yield "partially" activated platelets, but rather a small fraction of readily detected, fully activated P*.

We appreciate the useful discussions with Professor Theo Van de Ven, Chemistry Department, McGill University.

This work was supported by a grant from the Medical Research Council of Canada.

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