

Fluorescent Indicators Give Biased Estimates of Intracellular Free Calcium Change in Aggregating Platelets: Implication for Studies with Human von Willebrand Factor

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ABSTRACT: The ratiometric fluorescent indicators Fura-2 and Indo-1 are considered optimal probes for monitoring intracellular free calcium concentration ($[Ca^{2+}]_i$). Unique problems arise, however, in studying $[Ca^{2+}]_i$ changes induced in platelets by von Willebrand factor (vWF). Binding of native multimeric vWF causes extensive platelet aggregation, and is reported to evoke a gradual $[Ca^{2+}]_i$ increase. The present investigation examined the reliability of platelet $[Ca^{2+}]_i$ measurements in these circumstances. Ristocetin-mediated binding of vWF to human platelets promoted a slow rise in Fura-2 fluorescence ratio. Fura-2 extrusion contributed substantially to this rise, unless blocked by probenecid. Despite this precaution, the platelets were invariably contaminated slightly with extracellular indicator. As aggregation progressively reduced the number of platelets in the spectrofluorometer beam, through settling of the larger aggregates, such extracellular Fura-2 contributed proportionately more to the observed fluorescence. This extraneous signal accounted completely for the fluorescence ratio increase, and apparent $[Ca^{2+}]_i$ rise, in response to native multimeric vWF. The same problem arose with Indo-1, whereas the single wavelength indicator Fluo-3 showed the opposite pattern of apparent $[Ca^{2+}]_i$ changes. Thus, none of these indicators provides reliable data on $[Ca^{2+}]_i$ signals in aggregating platelets. Use of a dimeric form of vWF eliminated the problem of platelet aggregates settling out of suspension, but also virtually abolished the $[Ca^{2+}]_i$ increase. These observations may explain some of the inconsistencies among previous investigations of vWF-induced calcium signaling. Moreover, similar problems may arise in studies with other adhesive proteins.

Keywords: aggregation, fluorescence, Fura-2, glycoprotein Ib, Indo-1, intracellular calcium, platelet, von Willebrand factor

INTRODUCTION

Several technical problems may compromise the accuracy of measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) with a fluorescent indicator (1-3). Since the advent of the first such indicator, Quin-2 (4), attempts have been made to improve its properties through structural modification. This led to the development of indicators with a pronounced shift in peak excitation or emission wavelength upon binding of calcium (5); Fura-2 and Indo-1 are prominent

examples of such second generation calcium indicators. These allow $[Ca^{2+}]_i$ to be calculated from a ratio of fluorescence intensities at two excitation (Fura-2) or emission (Indo-1) wavelengths. This ratiometric approach offers several advantages; in particular, extrusion of indicator from the cell and changes in the number of cells monitored may have little influence on $[Ca^{2+}]_i$ estimation (1-3,5). The validation of these indicators is based solely on studies of the rapid calcium signals that are characteristic of many agonists. These indicators, however, are now being used

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increasingly to study the much slower, prolonged calcium signals that arise with other agents, despite a lack of appropriate validation for such usage.

One agent that is reported to cause a gradual, progressive $[Ca^{2+}]_i$ rise is the adhesive protein von Willebrand factor (vWF). This large, multimeric plasma glycoprotein plays a major role in hemostasis and thrombosis (6,7). It has the potential to interact with platelet membrane glycoprotein Ib (GpIb). Such interaction is not spontaneous, but can be instigated by immobilization of vWF on a surface (8,9), or by an exogenous modulator, such as the antibiotic ristocetin (10) or the snake venom protein botrocetin (originally denoted venom coagglutinin) (11,12). Adhesion of vWF to exposed subendothelial elements may play a similar role in initiating its binding to platelet GpIb *in vivo* (13–15). Recent studies indicate that vWF–GpIb binding also occurs when vWF and platelets are subjected to a high shear stress (16–18).

There is increasing evidence that vWF–GpIb binding activates platelets (8,16,17,19,20). Recent studies with the ratiometric fluorescent indicators Fura-2 (21–25) and Indo-1 (26–28) have examined the role of platelet $[Ca^{2+}]_i$ changes in such activation. These studies have employed two fundamentally different approaches to trigger vWF–GpIb interaction: use of an exogenous modulator or mutant vWF in a static system (21–25) and application of shear stress (26–28). Regardless of this fundamental difference in approach, each investigation revealed vWF to evoke a gradual and progressive $[Ca^{2+}]_i$ rise, which was concurrent with platelet aggregation. Another consistent finding was an absolute dependence on extracellular calcium. In other respects, however, these investigations have yielded conflicting data. For example, three studies reported a modest $[Ca^{2+}]_i$ rise to double or triple the resting concentration (25,26,28), whereas four others showed a 10-fold increase (22–24,27). Three studies reported that antibody or Arg-Gly-Asp-Ser blockade of vWF binding to its secondary platelet receptor, glycoprotein IIb/IIIa (GpIIb/IIIa), greatly diminished or

completely abrogated the $[Ca^{2+}]_i$ rise (22,24,27), whereas four others showed no inhibition with such blockade (21,25,26,28). The effects of cyclooxygenase inhibitors have also been contradictory: two reports indicated complete abolition of the vWF-induced $[Ca^{2+}]_i$ rise by indomethacin (21,22), two studies showed inhibition (at least partial) by aspirin (23,24), one study reported no inhibition with indomethacin (25), and two investigations found no reduction with aspirin (27,28).

The divergent findings amongst these previous studies with vWF show no clear pattern. Some apparent inconsistencies might be attributable to a difference in signaling mechanism with different means for initiating vWF binding. Nevertheless, other inconsistencies, such as the data on GpIIb/IIIa blockade, cannot be dismissed in this manner. A more likely explanation is that the discrepant findings reflect a technical problem in the measurement of gradual changes in $[Ca^{2+}]_i$ in aggregating platelets. The aim of the present investigation was to evaluate the reliability of ratiometric determination of $[Ca^{2+}]_i$ with Fura-2 or Indo-1 in platelets that are aggregating as a result of vWF–GpIb binding. Measurements with a single wavelength indicator, Fluo-3, were undertaken for the purpose of comparison.

MATERIALS AND METHODS

Purification of Multimeric vWF and Preparation of Reduced/Alkylated vWF Dimer

Human vWF was purified from cryoprecipitate by standard methods, involving differential polyethylene glycol precipitation and gel filtration (29). The final product had a ristocetin-cofactor activity of 100–150 plasma equivalent units/mg, showed the characteristic pattern of multimerization reported by other investigators (9,21), and exhibited a single band (purity > 95%) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions.

Molar vWF concentrations are expressed in terms of the vWF subunit (molecular weight \approx 225,000).

A dimeric form of vWF was prepared by limited disulfide reduction and alkylation, using the procedure of Loscalzo *et al.* (30). This vWF dimer retains the ability to bind to platelet GpIb, but aggregates platelets to a much lesser extent than native multimeric vWF (30).

Isolation of Platelets and Loading with Fluorescent Calcium Indicator

Venous blood was drawn from healthy adult volunteer donors who had not taken aspirin or ibuprofen within 10 days. The protocol was approved by the local Institutional Review Board. Blood was collected into 15% (V/V) acid–citrate–dextrose anticoagulant (65 mM citric acid, 85 mM sodium citrate, 110 mM D-glucose), and platelet-rich plasma obtained by centrifugation at 160 *g* (15 min, 23°C). The platelet-rich plasma was treated with 5 mM creatine phosphate and 25 U/ml creatine phosphokinase. Platelets were usually isolated by albumin density gradient centrifugation and gel filtration (31). In brief, platelet-rich plasma was centrifuged at 1,200 *g* (15 min, 23°C) on a discontinuous gradient (12%, 17%, 25% and 50% layers) of fatty-acid free bovine serum albumin (BSA). Platelets were collected from the 25%:50% BSA interface, and loaded with fluorescent calcium indicator. Gel filtration was effected on a 2 × 8 cm column of Sepharose 2B; it removed residual fluorescent indicator and remaining traces of plasma proteins. Platelets were eluted in calcium-free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM D- glucose), buffered with 10 mM HEPES, pH 7.35, and supplemented with 0.35% (W/V) BSA.

These studies used three different calcium indicators, Fura-2, Indo-1 and Fluo-3. Platelets were loaded by a 30 min (Fura-2 and Indo-1) or 1 h (Fluo-3) incubation at 37°C with a 2 μ M solution of acetoxymethyl (AM) ester of the calcium

indicator (Molecular Probes Inc, Eugene, OR). Unless otherwise indicated, the loading buffer and all subsequent buffers included 2.5 mM probenecid to minimize extrusion of the indicator after hydrolysis of its AM ester groups (1,32,33).

Fluorescence Measurement of Platelet [Ca²⁺]_i

Platelet [Ca²⁺]_i was measured on either a Photon Technology International (South Brunswick, NJ) RF-M2004 or a Perkin-Elmer (Norwalk, CT) LS-5 spectrofluorometer; studies were conducted in either a standard quartz (10 mm square) or a cylindrical glass (7 mm diameter) cuvette. The choice of instrument and cuvette did not affect the pattern of results; the excitation and emission beams have a perpendicular orientation (L-format) in both instruments. Platelets loaded with calcium indicator were adjusted to 200,000 cells/ μ l, and stirred continuously at 37°C. The platelets were equilibrated briefly (2-5 min) with extracellular calcium (1 mM CaCl₂), then stimulated by sequential addition of purified multimeric vWF (usually 50 nM) and 1 mg/ml ristocetin (Bio/Data Corp, Hatboro, PA). Studies with Fura-2 involved measurements of fluorescence intensity at 510 nm emission with alternating excitation wavelengths of 340 and 380 nm (achieved by slewing the monochromator grating); data with this indicator are generally expressed as a fluorescence ratio (340/380). Some measurements were also made with excitation at the isosbestic wavelength (360 nm). Simultaneous dual emission data (400 and 480 nm) were collected for Indo-1, with excitation at 340 nm. Fluorescence was monitored at fixed excitation (505 nm) and emission (530 nm) wavelengths in studies with Fluo-3. Auto-fluorescence was determined in platelets that had not been loaded with indicator, and was subtracted from each fluorescence measurement (before ratio calculation in studies with Fura-2 and Indo-1).

Calibration data were obtained at the end of each study, using the procedure of Merritt *et al.* (32). Maximum fluorescence ratio (R_{max})

with Fura-2 or Indo-1, or fluorescence intensity (F_{max}) with Fluo-3, was defined by adding 30 μ M digitonin (Calbiochem, San Diego, CA) in the presence of 1 mM CaCl_2 . Minimum fluorescence ratio (R_{min}) or intensity (F_{min}) was assessed after addition of excess EGTA (20 mM), buffered to pH 8.5. For the studies with Fura-2, experimental measurements of fluorescence ratio (R) were converted to $[\text{Ca}^{2+}]_i$ values by the equation (5):

$$[\text{Ca}^{2+}]_i = K_d(F_f/F_b)(R - R_{min})/(R_{max} - R),$$

where F_f and F_b are fluorescence intensities with 380 nm excitation for free and calcium-bound Fura-2, respectively. An analogous conversion was applied to the Indo-1 data. K_d values of 224 and 250 nM, respectively, were assumed for the binding of calcium to Fura-2 and Indo-1 at 37°C (5). Fluorescence intensity (F) data obtained with Fluo-3 were converted to $[\text{Ca}^{2+}]_i$ values by the equation (5):

$$[\text{Ca}^{2+}]_i = K_d(F - F_{min})/(F_{max} - F),$$

assuming a K_d value of 407 nM for the binding of calcium to Fluo-3 at 37°C (34).

Assessment of Indicator Extrusion from Platelets

Two methods were used to quantify extrusion of Fura-2 from the platelet. [Expulsion of fluorescent indicator from the cytosol is referred to by the term “extrusion”, rather than “leakage”, because it is largely an active process mediated by an organic anion transporter (33). Extrusion neither requires nor is affected by stimulation of the platelet by an agonist.] The first method involved assessment of changes in fluorescence intensity with 340 nm excitation for a platelet suspension in 0.1 mM extracellular calcium. (Studies with 1 mM extracellular calcium yielded equivalent data.) Indicator extrusion was defined from

the rate of increase in fluorescence, expressed as a percentage of the increase on release of cytosolic indicator by digitonin. An analogous strategy was used with Indo-1 and Fluo-3. The second approach comprised measurement of the quenching of fluorescence intensity at the isosbestic wavelength (360 nm excitation for Fura-2) that occurred when 5 mM NiCl_2 was added to the platelet suspension (in 1 mM extracellular calcium) after different incubation periods. Nickel quenches only the extracellular fluorescent indicator, as it does not permeate ion channels in the platelet plasma membrane (35). With the latter approach, indicator extrusion was quantified from the rate of increase in quenching as the incubation period was lengthened, expressed as a percentage of the quenching seen with digitonin. The first method allows continuous monitoring of extrusion, but assumes that platelet $[\text{Ca}^{2+}]_i$ remains constant; the second strategy is independent of platelet $[\text{Ca}^{2+}]_i$, but unsuited to continuous monitoring.

Platelet Aggregometry

Aggregation of human platelets in response to ADP was assessed in a Sienco (Morrison, CO) dual-channel aggregometer. The platelet-rich plasma was adjusted to a platelet count of 200,000 cells/ μ l and samples were stirred continuously at 900 rpm throughout these studies.

Simultaneous measurements of platelet aggregation and $[\text{Ca}^{2+}]_i$ were undertaken in a few studies with multimeric vWF. The Photon Technology RF-M2004 spectrofluorometer was used in an unorthodox configuration for the latter studies; one emission monochromator (380 nm wavelength) was repositioned to monitor transmitted light and thereby assess aggregation, while platelet $[\text{Ca}^{2+}]_i$ was evaluated from Fura-2 emission measurements (510 nm) perpendicular to the excitation beam. The excitation wavelength alternated between 340 and 380 nm; transmitted light was only collected during the 380 nm excitation phase.

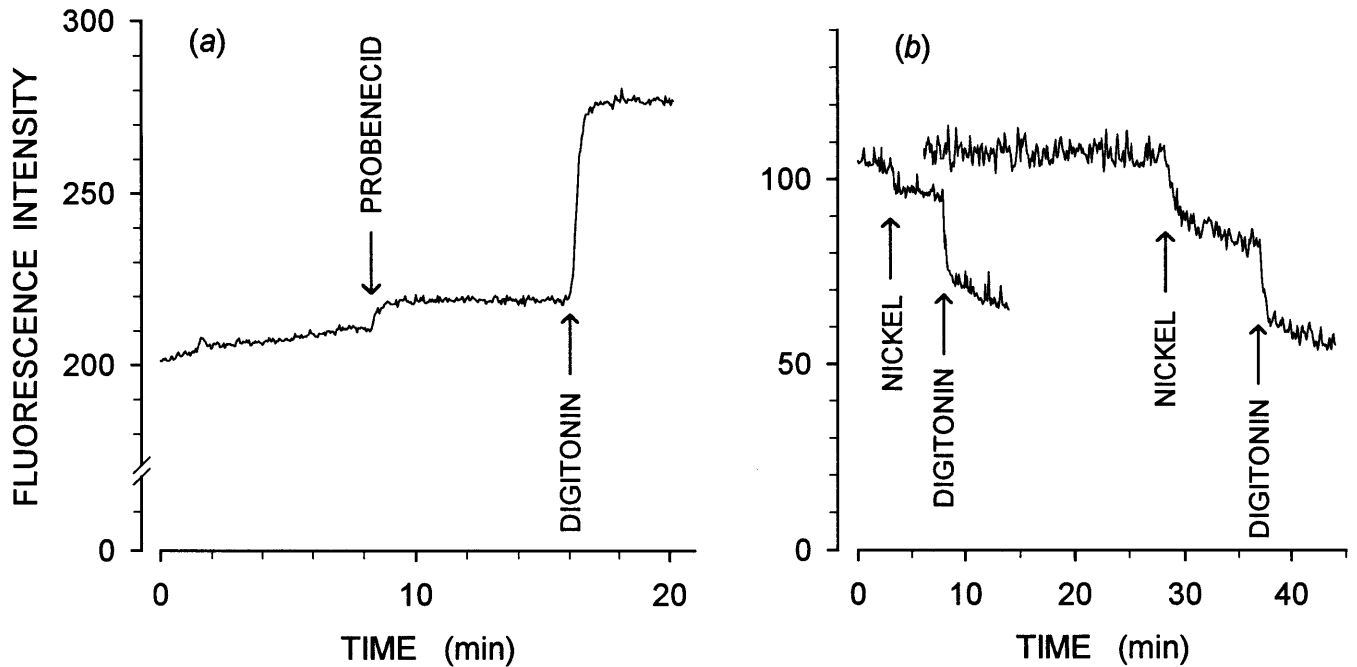


Figure 1. Effect of probenecid on Fura-2 extrusion from platelets. Human platelets were loaded with Fura-2 in the absence of probenecid. (a) Extrusion was assessed from changes in fluorescence intensity at 340 nm excitation and 510 nm emission in the presence of extracellular calcium (0.1 mM). Subsequent addition of probenecid (2.5 mM) substantially slowed Fura-2 extrusion. (The abrupt fluorescence increase on addition of probenecid results from its intrinsic fluorescence.) Data were calibrated in relation to the fluorescence signal attained when all the Fura-2 was released by treatment with digitonin (30 μ M). These data yielded an extrusion rate of 1.58 % per min without probenecid, and 0.11 % per min with probenecid. (b) Extrusion was evaluated by the nickel quench method in the absence of probenecid. Fluorescence intensity was measured at the isosbestic excitation wavelength (360 nm). Extracellular Fura-2 was quantified after different incubation periods (3 and 28 min) through its sensitivity to quenching by nickel (5 mM). (Data for the first 6 min are omitted from the trace for the longer incubation for clarity.) Digitonin (30 μ M) was again used for calibration. An extrusion rate of 1.33 % per min was calculated from these nickel quench data. Results from typical studies conducted on the Photon Technology International RF-M2004 spectrofluorometer are shown.

RESULTS

Inhibition of Fura-2 Extrusion from Platelets by Probenecid

The fluorescence intensity at 340 nm excitation rose gradually and progressively when platelets loaded with Fura-2 were incubated at 37°C in the presence of 0.1 mM extracellular calcium, but without probenecid or a platelet agonist (Fig. 1a). This rise was attributed to extrusion of indicator from the platelets into the extracellular milieu. The rate of Fura-2 extrusion, estimated from the change in fluorescence intensity, averaged 1.20 ± 0.36 % per min (mean \pm SD, $n = 8$). Similar estimates of Fura-2 extrusion (1.13 ± 0.29 % per

min) were obtained (in the presence of 1 mM extracellular calcium) by the nickel quench method (Fig. 1b).

Extrusion of Fura-2 at this rate is a major obstacle to the assessment of platelet $[Ca^{2+}]_i$ changes induced by vWF-GpIb binding: it would typically result in an artificial 70-110% rise in platelet $[Ca^{2+}]_i$ (assayed in the presence of extracellular calcium) during the 15 min incubation required for such studies. Other investigators, however, have found that the organic anion transport inhibitor probenecid suppresses Fura-2 extrusion from various cells (33,36). Its effectiveness at preventing extrusion from the platelet was thus assessed; probenecid immediately slowed Fura-2 extrusion (Fig. 1a). Measurements at 340 nm

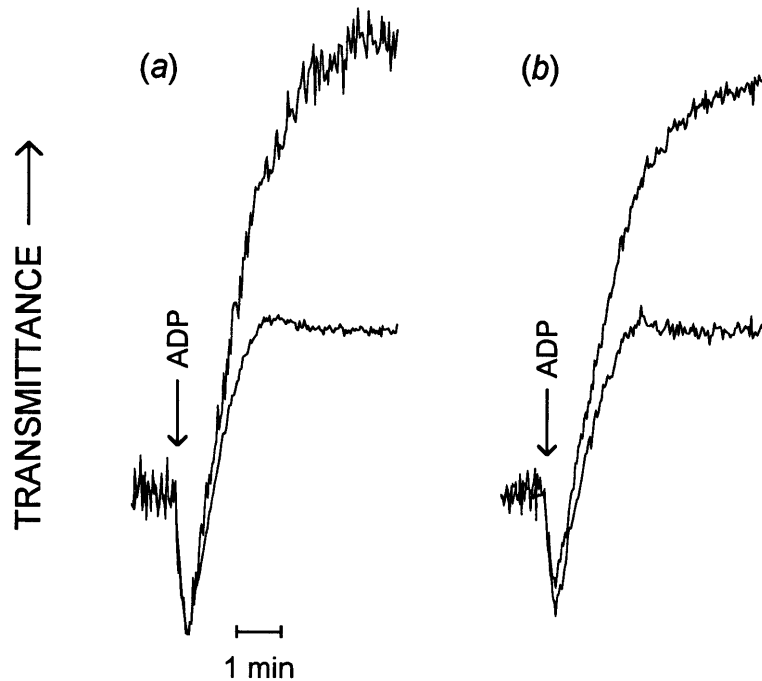


Figure 2. Effect of probenecid on platelet aggregation. Platelets (200,000 cells/ μ l) were pre-incubated (10 min) either (a) without or (b) with probenecid (2.5 mM). Platelet aggregation in response to ADP stimulation was then assessed; the lower trace in each panel shows the response to 2.5 μ M ADP, and the upper trace that to 5 μ M ADP. Responses to other ADP concentrations were also unaffected by probenecid. Results from a typical study are shown.

excitation gave an average extrusion rate of 0.27 ± 0.18 % per min ($n = 5$) for Fura-2 in the presence of 2.5 mM probenecid ($p < 0.005$, paired t -test, compared with control extrusion in the same platelets). This inhibition by probenecid confirms that an organic anion transporter is largely responsible for removal of Fura-2 from the platelet cytosol; passive diffusion plays little, if any, role. Use of probenecid should limit the artificial $[Ca^{2+}]_i$ rise to 15–25% during a 15 min incubation.

Lack of Influence of Probenecid on Platelet Activation

Use of probenecid in studies of calcium signaling is only appropriate if it does not interfere with the signaling process. Previous studies have shown $[Ca^{2+}]_i$ signals and aggregation responses induced by several platelet agonists to be unaffected by probenecid (32,37), though one earlier investigation reported inhibition of the responses to thrombin (38). Confirmation that probenecid does not interfere with platelet activation was sought in the

present study. The aggregatory response of platelets (in platelet-rich plasma) to ADP was found to be unaffected by the addition of 2.5 mM probenecid (Fig. 2).

Measurement of vWF-Induced Platelet $[Ca^{2+}]_i$ Change with Fura-2

The consequences of ristocetin-mediated vWF–GpIb interaction on platelet $[Ca^{2+}]_i$ were examined by ratiometric measurements with Fura-2. This strategy was chosen because it is claimed to minimize distortion of the measured $[Ca^{2+}]_i$ as a result of a change in effective cell number (1,5). The principal studies were done in the presence of 2.5 mM probenecid, as otherwise the rapid extrusion of Fura-2 compromised quantitative assessment of $[Ca^{2+}]_i$.

Addition of native multimeric vWF (10 μ g/ml; approx. 50 nM subunit concentration) and ristocetin (1 mg/ml) to a stirred platelet suspension promoted a gradual and progressive rise in the Fura-2 fluorescence ratio. Platelet $[Ca^{2+}]_i$ thus

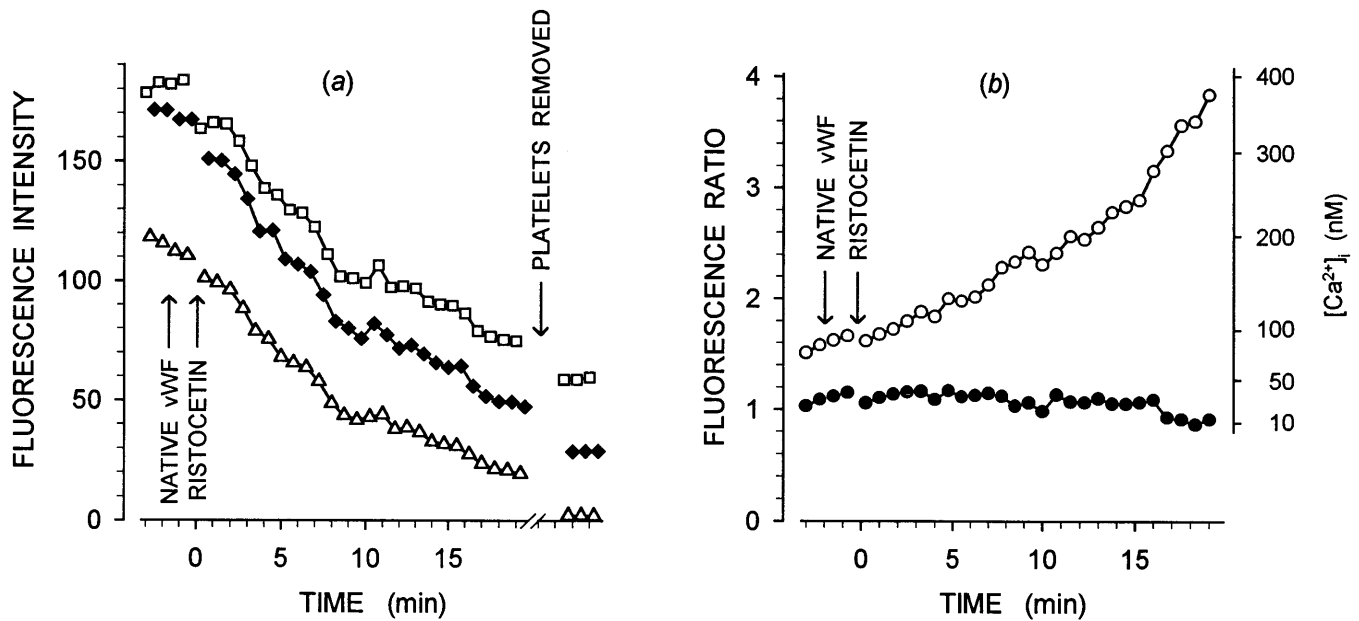


Figure 3. Contribution of extracellular Fura-2 to ratiometric estimates of changes in platelet $[Ca^{2+}]_i$ induced by ristocetin-mediated vWF–GpIb binding. Platelets were loaded with Fura-2, and measurements conducted in the presence of extracellular calcium (1 mM $CaCl_2$) and probenecid (2.5 mM). Interaction of native multimeric vWF (50 nM) with platelet GpIb was instigated by the subsequent addition of ristocetin (1 mg/ml). Results from a typical study conducted on the Perkin-Elmer LS-5 spectrofluorometer are shown. (a) Data are displayed in terms of Fura-2 fluorescence intensities (at 510 nm emission) for individual excitation wavelengths (\square , 340 nm; \blacklozenge , 360 nm; \triangle , 380 nm). Measurements at the isosbestic excitation wavelength (360 nm) essentially reflect the effective platelet number (those in the spectrofluorometer excitation beam). The platelets were removed by centrifugation at the end of the study; fluorescence measurements on the supernatant fraction provided an estimate of extracellular Fura-2. (Fluorescence was not significantly perturbed if unlabeled platelets were added back.) (b) Data are presented in terms of the Fura-2 fluorescence ratio (ratio of fluorescence at 510 nm emission wavelength with excitation at 340 and 380 nm). This ratio was calculated with the standard correction for autofluorescence at each excitation wavelength (\circ). The ratio was also recalculated (\bullet) after correction for the contribution from extracellular Fura-2, assuming a constant contribution throughout the measurements (because probenecid limited the extrusion of Fura-2). Platelet $[Ca^{2+}]_i$ was estimated by standard methods, using calibration data acquired on digitonin (30 μ M) treatment of a separate portion of the platelet suspension (as the extensive aggregation that arose with vWF precluded accurate calibration on the actual sample). Fluorescence ratios that have been corrected for the signal from extracellular Fura-2 do not show the progressive $[Ca^{2+}]_i$ rise that is apparent without this correction.

appeared to rise gradually throughout the measurements (Fig. 3b), when it was calculated from the fluorescence ratio by the standard formula (5).

Close inspection of the Fura-2 data raised questions about the validity of the deduced rise in platelet $[Ca^{2+}]_i$. The fluorescence intensity for each excitation wavelength (340 and 380 nm) declined progressively with time throughout the measurement period (Fig. 3a). The patterns of decline at these two wavelengths were parallel; the increased fluorescence ratio (340/380) arose from the closer approach of the fluorescence at 380 nm

excitation to the autofluorescence background by the end of the observation period. A similar decline in fluorescence intensity was seen at the isosbestic excitation wavelength of 360 nm (Fig. 3a), which essentially monitors the effective platelet number (those in the spectrofluorometer beam). These patterns *might* reflect a platelet $[Ca^{2+}]_i$ rise in combination with an aggregation-dependent decline in effective platelet number. Alternatively, however, they could reflect a declining fluorescence signal from the platelets together with a constant signal of high 340/380 ratio from extracel-

ular Fura-2. The latter possibility was evaluated through measuring the extracellular indicator at the end of the observation period; this was accomplished after removing the platelets by centrifugation (500 g, 15 min, 23°C). Although the amount of extracellular Fura-2 after a 15–20 min incubation was modest in relation to the initial platelet Fura-2 content (typically 10–15%, based on fluorescence at the isosbestic wavelength), it made a substantial contribution to the fluorescence signal with excitation at 340 nm (Fig. 3a). The high 340/380 ratio for this extracellular Fura-2, and its reversal by EGTA (data not shown), confirmed that the extruded indicator was functional in terms of calcium binding.

The relative contribution from extracellular Fura-2 became exacerbated as the incubation with vWF and ristocetin progressed. Large platelet aggregates formed and, despite the stirring, tended to settle to the bottom of the cuvette below the spectrofluorometer excitation beam. The decline in fluorescence at the isosbestic wavelength (360 nm) is a direct measure of such aggregation and settling of the platelets (Fig. 3a). ('Aggregation' is used here in an inclusive sense to encompass both true aggregation that involves platelet activation and also physical agglutination through binding of vWF to GpIb moieties on different platelets.) Fluorescence from the extracellular Fura-2 contributed proportionately more to the overall signal as these large aggregates settled out of suspension. A correction was thus made for the fluorescence signal from extracellular Fura-2, which was assumed to be constant throughout the measurement period (as probenecid was used to minimize Fura-2 extrusion). This correction eliminated the apparent vWF-induced rise in platelet $[Ca^{2+}]_i$ (Fig. 3b), implying that the observed rise was an artifact. The corrected data provide no evidence that vWF–GpIb binding triggers a transient $[Ca^{2+}]_i$ signal; nor do they suggest a gradual elevation of platelet $[Ca^{2+}]_i$.

Additional studies were conducted with human vWF obtained from a commercial supplier (Calbiochem), and using platelets isolated by repeated centrifugation and resuspension (39).

Analogous patterns of aggregate settling and apparent $[Ca^{2+}]_i$ rise were invariably observed. Limited studies were also conducted without probenecid; the apparent platelet $[Ca^{2+}]_i$ rise tended to be faster or greater, owing to the increased amount of Fura-2 in the extracellular milieu when extrusion was not restricted. Furthermore, the time-course for formation and settling of the large platelet aggregates varied when the vWF concentration, the stirrer used, or the stirring speed were altered. Regardless of such changes, the time-course of the apparent platelet $[Ca^{2+}]_i$ rise evoked by vWF invariably paralleled the aggregation of platelets and their loss from the spectrofluorometer beam (Fig. 4).

Measurement of vWF-Induced Platelet $[Ca^{2+}]_i$ Change with Indo-1

Fluorescence intensity measurements at 400 nm emission wavelength in the presence of 1 mM extracellular calcium indicated that Indo-1 is extruded from the platelets as fast as Fura-2. Indo-1 was extruded at an average rate of 1.14 ± 0.23 % per min ($n = 4$) at 37°C in the absence of probenecid.

The $[Ca^{2+}]_i$ signal evoked by vWF–GpIb binding was examined in platelets loaded with Indo-1 without using probenecid. Addition of multimeric vWF and ristocetin to a stirred platelet suspension resulted in a progressive decline in fluorescence intensity at both 400 and 480 nm emission (Fig. 5a). Platelet $[Ca^{2+}]_i$ showed an *apparent* progressive increase, after a lag phase, when it was calculated from the Indo-1 fluorescence ratio in the normal manner (Fig. 5b). Just as in the case of the studies with Fura-2, however, extracellular indicator made a substantial contribution to the fluorescence signal at the end of the observation period when most of the platelets had aggregated and settled out of suspension (Fig. 5a). Moreover, the signal from this extracellular Indo-1 virtually accounted for the fluorescence ratio increase as the platelets aggregated. Correction for this signal essentially abolished the apparent $[Ca^{2+}]_i$ rise (Fig. 5b).

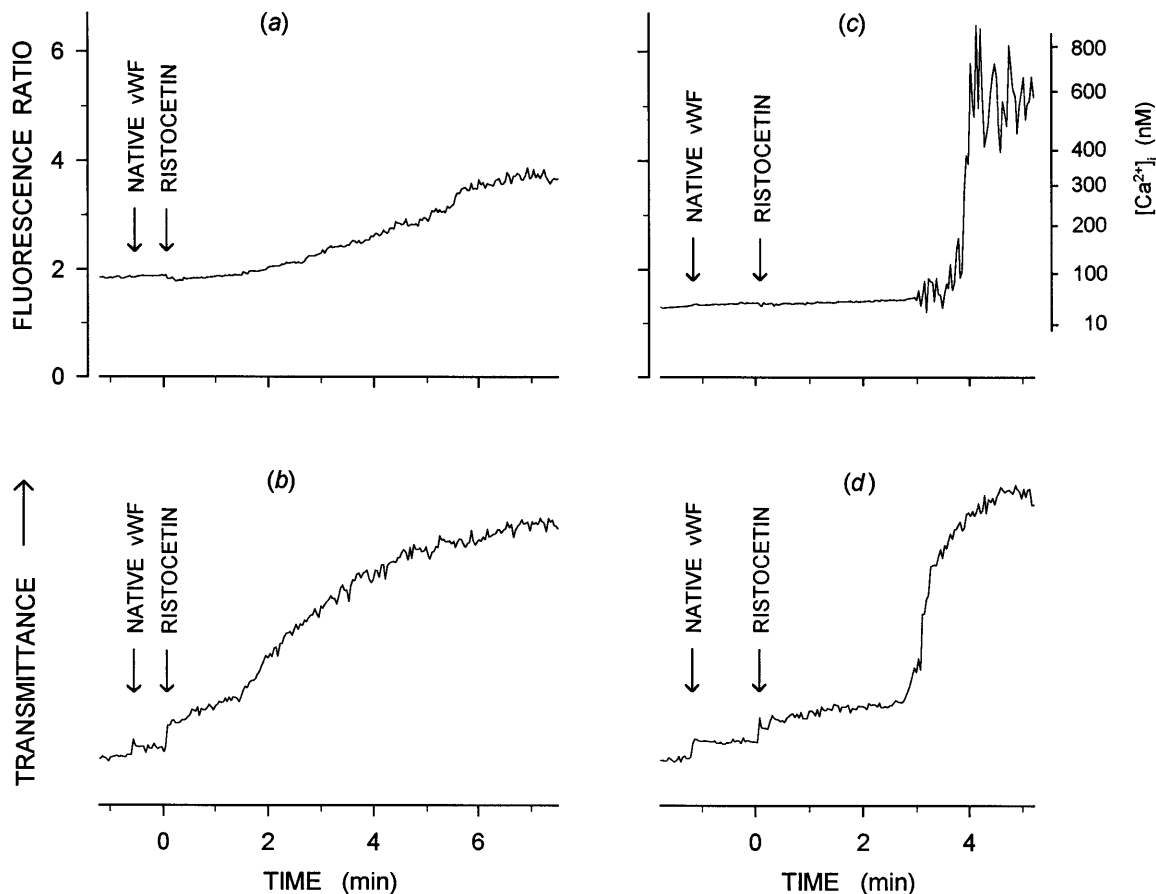


Figure 4. Relationship between apparent changes in $[Ca^{2+}]_i$ and platelet aggregation with ristocetin-mediated vWF-GpIb binding. Platelets were loaded with Fura-2, and aggregation was monitored simultaneously with $[Ca^{2+}]_i$ on a re-configured RF-M2004 spectrofluorometer. The excitation wavelength alternated between 340 and 380 nm, and fluorescence emission was measured in a perpendicular orientation. The instrument's second emission monochromator was repositioned for direct assessment of transmitted light (at 380 nm); this provided data on platelet aggregation. All measurements were conducted in the presence of extracellular calcium (1 mM), but without probenecid. Binding of native multimeric vWF (Calbiochem; 25 nM) with GpIb was instigated by the addition of ristocetin (1 mg/ml). Results from a typical study are shown. The platelet suspension was either stirred slowly with a 6×1 mm siliconized metal aggregometer stir bar (a,b) or stirred rapidly with a 7×2 mm Teflon-coated magnetic stir bar (c,d). Simultaneous measurements were made of the apparent changes in platelet $[Ca^{2+}]_i$ (a,c) and platelet aggregation (b,d). Platelet $[Ca^{2+}]_i$ was calculated from the Fura-2 fluorescence ratio (after correction for autofluorescence), and aggregation from the transmitted light measurements. (Fluorescence ratios in this figure have not been corrected for the extracellular Fura-2 signal.) In this study, slow stirring promoted gradual aggregation and apparent $[Ca^{2+}]_i$ change, whereas rapid stirring triggered more abrupt aggregation and apparent $[Ca^{2+}]_i$ change after a lag phase.

Measurement of vWF-Induced Platelet $[Ca^{2+}]_i$ Change with Fluo-3

As ratiometric assessment of platelet $[Ca^{2+}]_i$ with Fura-2 or Indo-1 did not yield reliable data on any changes induced by multimeric vWF, further studies were conducted with Fluo-3. The advantages of Fluo-3 include its 40-fold increase in

fluorescence intensity on binding calcium, and its relatively long excitation wavelength (32,40). Its major disadvantage is that it does not exhibit a spectral shift on binding calcium.

Other investigators have reported an absolute requirement for probenecid when Fluo-3 is used to monitor platelet $[Ca^{2+}]_i$ (32). Our observations confirmed this. Fluo-3 was extruded too rapidly

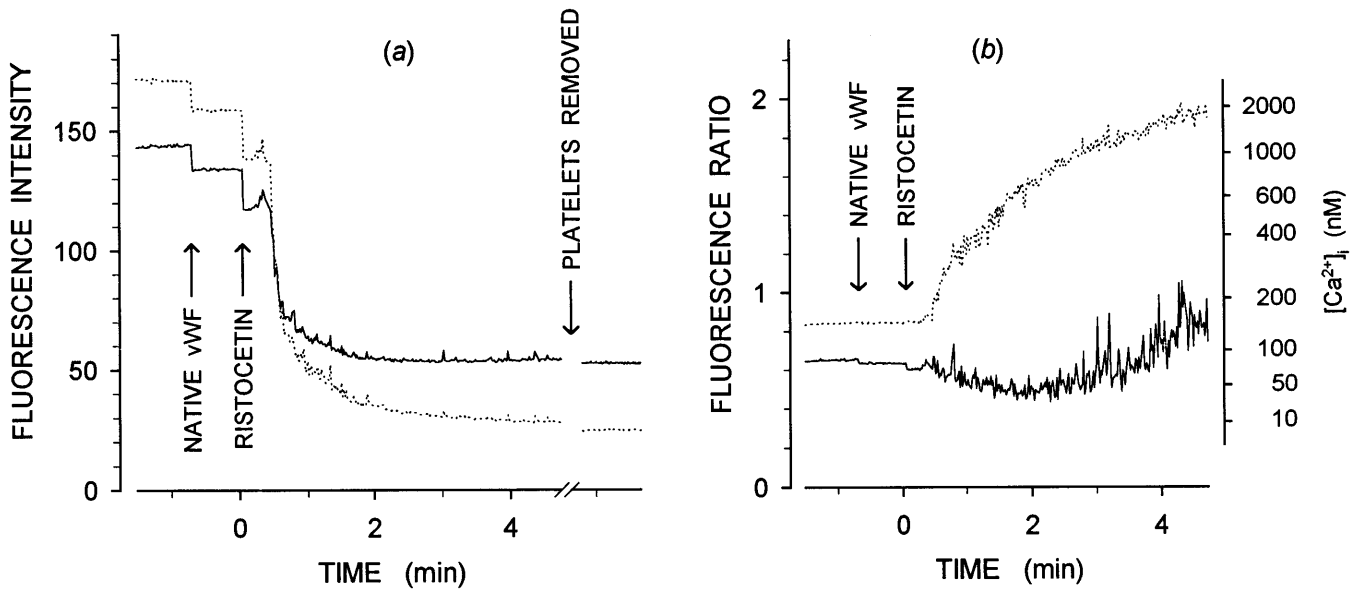


Figure 5. Ratiometric estimation with Indo-1 of platelet $[Ca^{2+}]_i$ changes induced by vWF-GpIb binding. Platelets were loaded with Indo-1, and measurements conducted in the presence of extracellular calcium (1 mM), but without probenecid. Binding of native multimeric vWF (25 nM) to GpIb was instigated by the subsequent addition of ristocetin (1 mg/ml). Results from a typical study conducted on the RF-M2004 spectrofluorometer are shown. (a) Data are displayed in terms of Indo-1 fluorescence intensities at individual emission wavelengths (solid line, 400 nm; broken line, 480 nm); excitation was at 340 nm. The platelets were removed by centrifugation at the end of the study; fluorescence measurements on the supernatant fraction provided an estimate of extracellular Indo-1. (b) Data are presented in terms of the fluorescence emission ratio (ratio of fluorescence intensities at 400 and 480 nm). This ratio was calculated with the standard correction for autofluorescence at each wavelength (broken line). It was also recalculated (solid line) after correction for the estimated contribution from extracellular Indo-1, assuming extrusion of Indo-1 at a constant rate throughout the measurements. Calibration data were acquired with a separate portion of the platelet suspension. Fluorescence ratios that have been corrected for the extracellular Indo-1 signal do not show the dramatic $[Ca^{2+}]_i$ rise that is apparent without this correction. (The slight $[Ca^{2+}]_i$ changes apparent in the corrected data can probably be attributed to the inaccuracy of this correction in the absence of probenecid.)

(~10 % per min) to allow measurement of platelet $[Ca^{2+}]_i$ in the absence of probenecid. Significant extrusion (at a rate of 2.0 ± 1.1 % per min; $n = 4$) occurred at 37°C even in the presence of 2.5 mM probenecid.

Addition of multimeric vWF and ristocetin to a stirred suspension of Fluo-3-loaded platelets caused a progressive decrease in fluorescence intensity, and thus an apparent decline in platelet $[Ca^{2+}]_i$ (Fig. 6a). The reduction in fluorescence was attributed to a gradual loss of platelets from the spectrofluorometer beam as large aggregates formed (just as occurred in the studies with Fura-2 and Indo-1). It was not possible with Fluo-3, however, to correct for the loss of platelets and estimate the true platelet $[Ca^{2+}]_i$. Despite the

greater fluorescence response of Fluo-3 (40-fold) than Fura-2 or Indo-1 to calcium, there was no evidence of a transient fluorescence increase prior to the aggregation-dependent decline. Thrombin-induced platelet $[Ca^{2+}]_i$ transients monitored with Fluo-3 in the presence of probenecid (Fig. 6b) were similar to those detected using other calcium indicators without probenecid (28,41). This similarity validates the measurement of platelet $[Ca^{2+}]_i$ with Fluo-3 in the absence of aggregation.

Platelet $[Ca^{2+}]_i$ Measurements with Reduced/Alkylated vWF Dimer

Visual inspection indicated that the problem of platelet settling could be eliminated by substituting

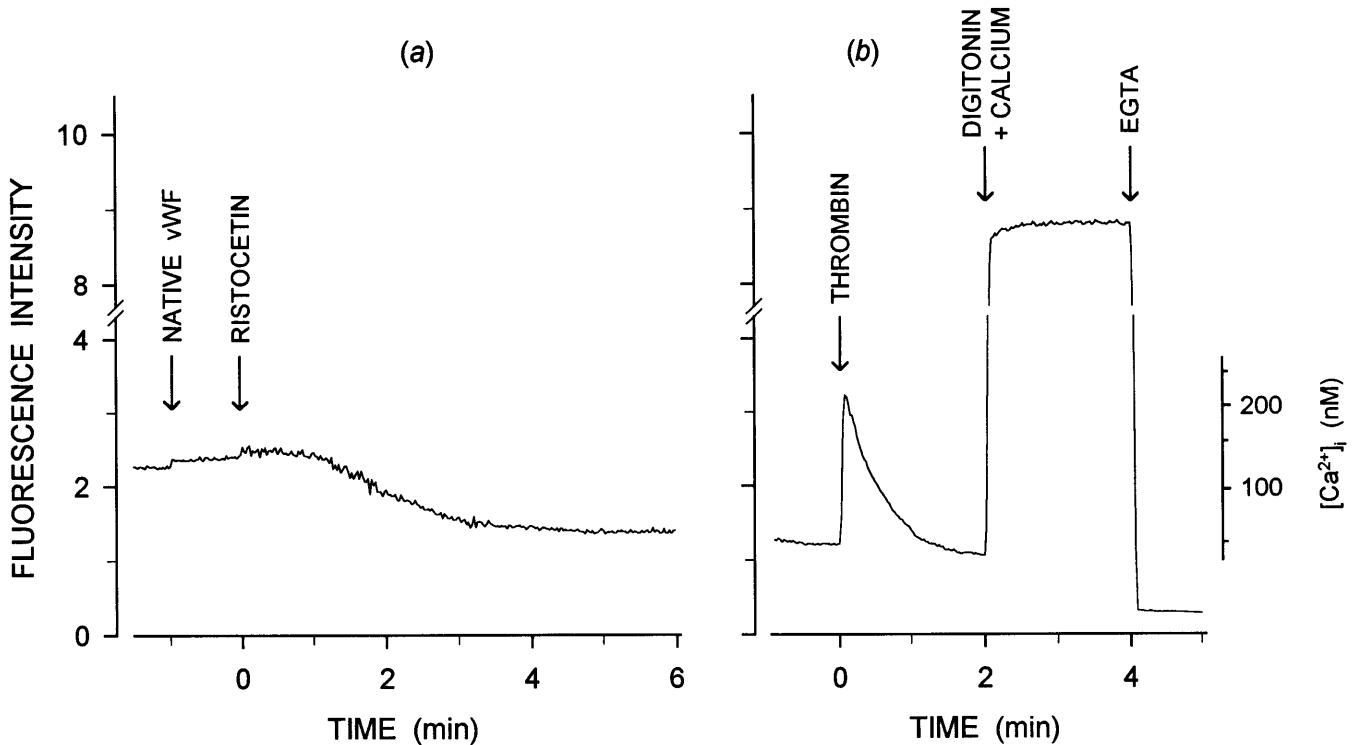


Figure 6. Estimation of platelet $[Ca^{2+}]_i$ signals with the single wavelength indicator Fluo-3. Platelets were loaded with Fluo-3 and measurements performed in the presence of probenecid (2.5 mM). Traces of fluorescence intensity (505 nm excitation, 530 nm emission) from typical studies conducted on the LS-5 spectrofluorometer are shown. (a) Binding of native multimeric vWF (50 nM) to platelet GpIb was instigated in the presence of extracellular calcium (1 mM) by the subsequent addition of ristocetin (1 mg/ml). (Fluorescence data have been corrected for the dilutions on addition of vWF and ristocetin.) As extensive platelet aggregation occurred and the larger aggregates tended to settle to the bottom of the cuvette below the spectrofluorometer's excitation beam, the data do not reliably reflect changes in platelet $[Ca^{2+}]_i$. (b) Platelets were stimulated with thrombin (1U/ml) in the absence of extracellular calcium (1 mM EGTA in the extracellular milieu). Fluorescence data for this agonist validate the assay of platelet $[Ca^{2+}]_i$ with Fluo-3 in the absence of aggregation. Calibration data are also illustrated.

tion of a reduced and alkylated dimeric form of vWF for the native multimeric ligand. Fluorescence measurements at the isosbestic excitation wavelength for Fura-2 (360 nm) confirmed that ristocetin-mediated binding of this vWF dimer to platelet GpIb did not diminish the number of platelets in the spectrofluorometer excitation beam over an observation period of 20 min (Fig. 7a). Platelet $[Ca^{2+}]_i$, however, changed little during this period (Fig. 7b). Correction for the extracellular Fura-2 reduced the absolute estimates of platelet $[Ca^{2+}]_i$, but did not alter the pattern over time. Similar patterns were observed with 5 nM and 50 nM reduced/alkylated vWF.

DISCUSSION

Several studies have reported that binding of native vWF to platelet GpIb induces a rise in $[Ca^{2+}]_i$ (21–28). This rise is believed to play a role in activation of the platelet by vWF (7,28), as it does with other agonists (42). Assay of $[Ca^{2+}]_i$ in platelets that are aggregating as a consequence of vWF–GpIb binding, however, presents unique technical problems. Though the ratiometric indicators Fura-2 and Indo-1 have proven invaluable for detecting agonist-induced $[Ca^{2+}]_i$ change (1,2,5), this does *not* mean that they can be applied unquestioningly to a situation as novel as vWF-induced signal transduction. The platelet $[Ca^{2+}]_i$

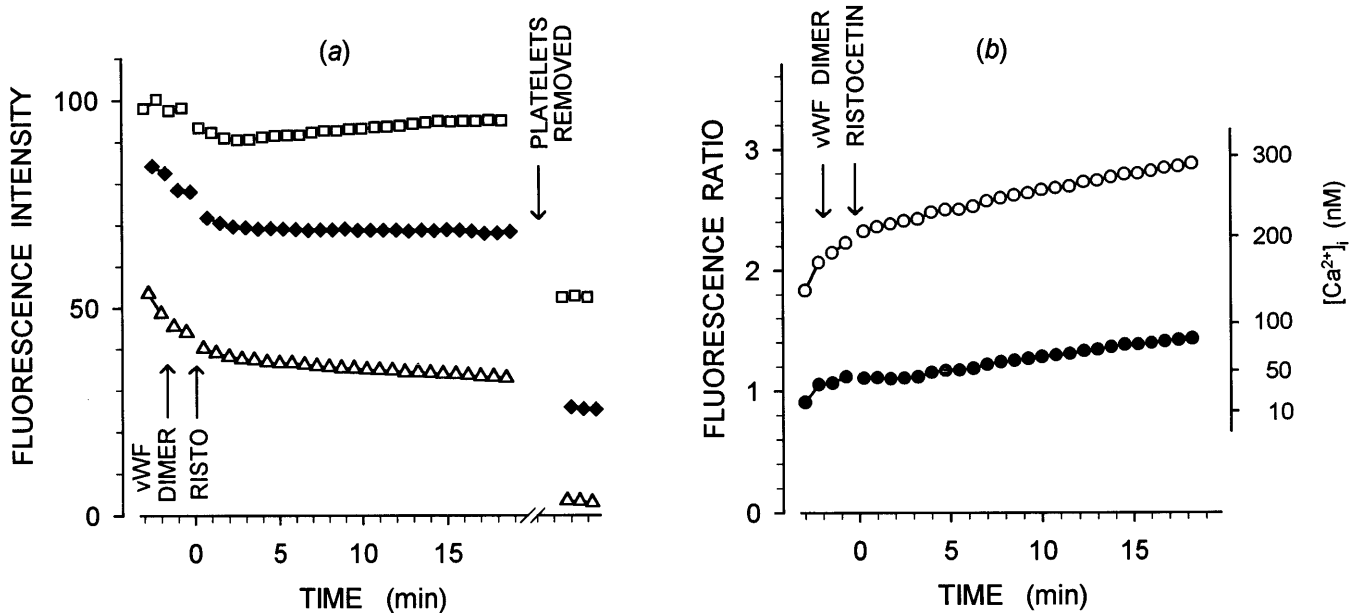


Figure 7. Ratiometric assessment of changes in platelet $[Ca^{2+}]_i$ on ristocetin-mediated binding of reduced and alkylated vWF dimer. (a) The experimental protocol paralleled that of Fig. 3, except that reduced/alkylated vWF dimer (5 nM) was used instead of multimeric vWF. Measurements with Fura-2 were conducted in the presence of extracellular calcium (1 mM) and probenecid (2.5 mM). Data from a typical study conducted on the LS-5 spectrofluorometer are displayed in terms of fluorescence intensities for individual excitation wavelengths (\square , 340 nm; \blacklozenge , 360 nm; \triangle , 380 nm). Measurements at 360 nm excitation wavelength monitor the effective platelet number. The platelets were removed by centrifugation at the end of the study to estimate extracellular Fura-2. (b) Fluorescence ratios (340/380) were calculated after correction for the signal from extracellular Fura-2 (\bullet), and without such correction (\circ). This correction diminished the estimate of resting platelet $[Ca^{2+}]_i$, but had little influence on the very modest rise evoked by the reduced and alkylated vWF dimer.

rise attributed to vWF differs markedly from that induced by most other agonists; it is much slower (reaching a plateau after 1-5 min) and occurs concurrently with platelet aggregation (21-23,25-28). Such differences pose substantial obstacles to quantifying vWF-induced platelet $[Ca^{2+}]_i$ changes. Moreover, ratiometric fluorescence measurements do not automatically overcome these obstacles; they eliminate some problems, but exacerbate others. Extrusion of fluorescent indicator from the cell seriously compromises $[Ca^{2+}]_i$ assessment, particularly in the presence of extracellular calcium (1,2,32,33). Use of a ratiometric indicator, such as Fura-2 or Indo-1, does *not* alleviate this difficulty, unless the cells are perfused continuously to remove extruded indicator. The problem of indicator extrusion is accentuated in studies with vWF, as a prolonged incubation is needed to follow the gradual $[Ca^{2+}]_i$ rise. Platelet aggregation further complicates studies with vWF. The

present study shows that vWF and ristocetin promote the formation of platelet aggregates so massive that, despite continuous stirring, they tend to settle to the bottom of the cuvette below the spectrofluorometer excitation beam. These aggregates also tend to adhere to the magnetic stir bar, despite its Teflon coating. Use of a ratiometric indicator would appear to resolve this problem, as the fluorescence ratio should be unaffected by a decline in cell number. This ideal is not realized, however, when the extracellular milieu contains a significant amount of fluorescent indicator. Under such circumstances, the fluorescence signal from extracellular indicator contributes proportionately more to the overall fluorescence as the signal from the platelets declines; this *inevitably* results in a rise in the observed fluorescence ratio whenever the extracellular milieu contains calcium.

In the present study, 10-20% of the Fura-2 or

Indo-1 was typically recovered in the extracellular milieu at the end of a 15-20 min incubation at 37°C. Other investigators have reported similar findings (33,43,44). Despite this modest percentage, the signal from this extracellular indicator can explain the apparent rise of up to 10-fold in platelet $[Ca^{2+}]_i$ induced by vWF and ristocetin (see Figs. 3-5). Thus, measures to limit the extracellular indicator are an essential precaution for any assessment of $[Ca^{2+}]_i$ in aggregating platelets. Probenecid is an invaluable aid towards achieving this goal. Not only does it minimize indicator extrusion (~4% over 15 min) during the observations *per se*, it also limits the calcium-sensitive (esterase-cleaved) form available for carry-over from the loading solution. Probenecid, moreover, appears not to interfere with platelet activation or $[Ca^{2+}]_i$ signaling evoked by ADP (see Fig. 2) or other agonists (32,37). Its use may thus be both justifiable and advisable in any $[Ca^{2+}]_i$ signaling study in aggregating platelets.

The present observations may explain many of the discrepancies in the characteristics of the vWF-induced platelet $[Ca^{2+}]_i$ rise among previous studies (21-28). Different methods for instigating vWF-GpIb binding (with ristocetin, botrocetin, shear stress, a mutant form of vWF, or porcine vWF) might evoke a platelet $[Ca^{2+}]_i$ signal with distinct characteristics. The present data, however, imply that at least part of the signal reported in earlier ratiometric studies (21,23,25-28) is an artifact resulting from extracellular fluorescent indicator. These findings apply most directly to the previous cuvette-based studies conducted under static (minimal shear) conditions (21,23,25). However, in view of the extent of platelet aggregation, and despite the differences in methodology, it would be naïve to deny the possibility that the platelet $[Ca^{2+}]_i$ changes were also overestimated in the investigations where shear stress triggered vWF binding (26-28). The degree of overestimation of the $[Ca^{2+}]_i$ rise in a particular study depends on many factors, including the extent of indicator extrusion and the geometry of

the spectrofluorometer beam in relation to the measuring chamber. Variation in this overestimation may explain the variation among studies in the observed platelet $[Ca^{2+}]_i$ rise with vWF, suggesting that reports of a more modest $[Ca^{2+}]_i$ rise (25,26,28) might be more reliable.

These findings pose a further question: is the *entire* vWF-induced platelet $[Ca^{2+}]_i$ increase seen in previous studies an artifact? Does it merely reflect the proportionately greater signal from extracellular indicator as platelets aggregate? The failure of previous investigators to inhibit indicator extrusion implies that extracellular indicator made a major contribution in those studies. That extracellular calcium was mandatory for a $[Ca^{2+}]_i$ rise to be observed (22-28) fits the artifactual explanation. Also consistent is the present finding that the apparent $[Ca^{2+}]_i$ rise invariably parallels the aggregation-dependent loss of platelets from the spectrofluorometer beam (see Fig. 4). The lack of a significant $[Ca^{2+}]_i$ signal on spontaneous binding of asialo-vWF (45) or ristocetin-mediated binding of reduced/alkylated vWF dimer (see Fig. 7 in this study) further supports this explanation. It should, nonetheless, be emphasized that the present data do *not* necessarily preclude an effect of native vWF on platelet $[Ca^{2+}]_i$; it is only appropriate to conclude that there is no $[Ca^{2+}]_i$ change in the few platelets remaining in suspension. Moreover, other studies have provided some evidence of a real $[Ca^{2+}]_i$ increase. For example, a vWF-induced $[Ca^{2+}]_i$ rise in the presence of ristocetin has been recorded using the photoprotein aequorin (46). In addition, two previous Fura-2 studies that showed a vWF-induced $[Ca^{2+}]_i$ rise utilized single-wavelength fluorescence measurements (22,24), whereby aggregation leads to *underestimation* of the rise (as in Fig. 6a). The evidence in favor of a true platelet $[Ca^{2+}]_i$ increase with vWF currently balances the data arguing that the whole increase is an artifact. A definitive answer to this question requires a method to quantify $[Ca^{2+}]_i$ reliably in aggregating platelets. Moreover, although the present findings question the ability of vWF to increase platelet $[Ca^{2+}]_i$, they do not, in

any way, question its ability to activate the platelet.

The difficulties encountered in measuring vWF-induced platelet $[Ca^{2+}]_i$ changes may not be limited to this particular combination of agonist and cell. Similar problems may arise with ratiometric assessment of $[Ca^{2+}]_i$ signals induced by other adhesive proteins. This is most likely when the agent used induces a gradual $[Ca^{2+}]_i$ rise and causes extensive aggregation of the cells.

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