Calcium Mobilization in Human Platelets Using Indo-1 and Flow Cytometry

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Regulation of cytoplasmic free calcium concentration is believed to be important in the response of platelets to external stimuli. A relatively new fluorescent calcium indicator, indo-1, has properties by which alterations of cytoplasmic calcium can be evaluated in single platelets by flow cytometry. Activation of platelets at a temperature lower than 37°C allows examination of the heterogeneity of intracellular free calcium levels and can distinguish variations among platelets in the initiation, duration, and magnitude of calcium fluxes. The clear advantage of flow cytometric analysis of platelet cytosolic calcium is that stimulus–response coupling can now be studied on a single cell basis. Platelets were activated by addition of human α-thrombin or ADP at 37°C or at room temperature (22°C). Activation at 37°C approaches more closely an in vivo response and, as expected, increases in cytosolic calcium occurred within seconds of agonist addition. Transient increases in cytoplasmic calcium levels occurred when platelets were challenged with a low concentration of agonist. Heterogeneity in cytoplasmic calcium levels was also observed at 10^{-6} mol/L ADP and 0.1 U/mL α-thrombin. Some of this heterogeneity was no longer observed at higher concentrations of agonist (10^{-4} mol/L ADP and 0.5 U/mL thrombin), suggesting that a sufficient magnitude of signal is required to induce changes in platelet cytosolic calcium. Light-scatter properties of the activated platelets were also monitored simultaneously and showed changes in response to both agonists. The ability to measure changes in cytoplasmic free calcium by ratio flow cytofluorimetry provides a new approach to study of the role of alterations in intracellular calcium in response to agonists acting through different membrane receptors as well as providing a sensitive technique to detect functional subpopulations of platelets.

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MATERIALS AND METHODS

Preparation of indo-1–labeled platelets. Blood drawn from medication-free healthy volunteer donors by venipuncture was anticoagulated with either 3.8% sodium citrate (one part sodium citrate
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to nine parts blood) or with ACD (1.4 mL ACD to 8.6 mL blood). The platelet-rich plasma (PRP) was isolated by centrifugation of the whole blood at 800 rpm for 20 minutes at room temperature in an IEC 8R centrifuge (Needham Heights, MS). One milliliter PRP (approximately 3.0 x 10^6 platelets) was routinely used for the labeling procedure and transferred to a 1.5-mL conical microcentrifuge tube. Platelets were incubated with 20 μmol/L indo-1 at 37°C. After 30-minute incubation, the labeled PRP sample was used in our flow cytometric studies. Depending on the platelet count, 2 to 3 μL PRP was added to 990 μL buffer for analysis.

Uptake of indo-1 by platelets in plasma was measured by spectrofluorimetry. Aliquots of indo-1-loaded platelets were removed at the incubation times indicated (0 to 90 minutes), washed, and resuspended in an EDTA-Tris-saline buffer (0.008 mol/L EDTA, 0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.4) containing 0.5% Triton X-100 (Sigma Chemical Co, St Louis, MO). After a centrifugation step, the fluorescence of the supernatant was measured and the fraction of indo-1 taken up by the platelets was determined on a Perkin Elmer MPF-4a fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT) using an excitation wavelength of 356 nm and an emission wavelength of 485 nm.

Flow cytometric analysis. Flow cytometric analysis was performed on a Coulter corporation EPICS 753 flow cytometer (Coulter, Hialeah, FL). The front 5-W (all lines) argon ion laser of this instrument was used in the ultraviolet mode (351 to 364 nm) at a power of 50 mW. Three signals were measured on each platelet passing through the instrument. Forward low-angle light scatter was measured as a logarithmic signal without use of a neutral density filter in front of the detector. The fluorescence signals produced by the internalized indo-1 were measured as two separate detectors after the signal was split with a 453-nm dichroic mirror. The shorter wavelengths were measured as linear integrated signals after passing through a 400-nm bandpass filter. This signal is predominantly the fluorescence of the Ca^{2+}-indomethacin complex. The longer wavelengths were measured as a linear integrated signal after passing through a 486-nm bandpass filter. This fluorescence is that produced predominantly from the Ca^{2+} free form of the internalized indo-1. Two additional parameters were generated during each set of measurements. The first of these was time. The second was the ratio measurement, data were also analyzed by the instrument's analog ratio circuitry and was gated on the 486-nm signal to exclude instrument noise and debris observed in the lower fluorescence channels and platelet clumps at the highest fluorescence channels. This ratio measurement is related to the intracellular calcium concentration, with higher ratios indicating higher calcium concentrations. Calcium fluorescence is measured as a logarithmic signal after passing through a 486-nm bandpass filter. This fluorescence is that produced predominantly from the Ca^{2+} free form of the internalized indo-1. Two additional parameters were generated during each set of measurements. The first of these was time. The second was the ratio measurement, data were also analyzed by the instrument's analog ratio circuitry and was gated on the 486-nm signal to exclude instrument noise and debris observed in the lower fluorescence channels and platelet clumps at the highest fluorescence channels. This ratio measurement is related to the intracellular calcium concentration, with higher ratios indicating higher calcium concentrations.

Voltagess and gains of the two fluorescence detectors were adjusted from run to run to obtain similar low ratio values for resting platelets. Cells were run at an approximate rate of 600/s with a transit time through the sample tube of 25 seconds. The total time of data collection for each sample was 600 seconds. For measurements at 37°C, the temperature of the chamber jacket was regulated with a circulating water bath.

A standard of Hoechst 33342 stained microbead alignment standards (Flow Cytometry Standards, Research Triangle Park, NC) was run for each set of experiments to calibrate fluorescence intensity and obtain coefficient of variation (CV) on the ratio measurement. The CV of the ratio for these beads ran between 2.6 and 3.6 with a mean of 3.2. Control unstimulated platelets in the indicated buffer were run before each set of experiments to detect any platelet activation in buffer alone. For each experiment, baseline data of unstimulated platelets were collected for either 60 or 120 seconds. Sample flow was then halted while the stimulus was added, and flow was resumed within 30 seconds. The experiment was then continued, collecting data on the stimulated cells for the remainder of the 600 seconds. For measurements at 37°C, the agonist was injected by syringe to avoid interruption of cell flow. In addition to the ratio (FL400/FL486) measurement, data were also analyzed by obtaining the percent of platelets responding over continuous time.

Activation of platelets. Platelets in PRP were activated by addition of the following agonists: human α-thrombin (0.01 to 1.0 U/mL), ADP (2 x 10^{-4} to 10^{-3} mol/L), and 4-bromo-A23187 (75 mmol/L) in either calcium-containing or EGTA-containing buffers. Free Ca^{2+} concentrations less than micromolar were buffered with EGTA. Platelets loaded with indo-1 were tested for aggregation function as compared with untreated platelets. Indo-1–loaded platelets were first washed free of indo-1 and resuspended in 0.002 mol/L calcium chloride, 0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.4 (CaTS buffer) for aggregation studies. Aggregation experiments were performed with a Payton aggregometer (Payton, Buffalo, NY).

RESULTS

Platelets were loaded with graded amounts of indo-1 and then examined for indo-1 fluorescence. Little difference was observed at loading concentrations between 20 and 30 μmol/L indo-1. At concentrations ≤10 μmol/L, the fluorescent signal was too low for accurate determination. On the basis of these findings, 20 μmol/L indo-1 was added to 3 x 10^6/mL platelets as standard procedure. A maximum uptake (ie, loading efficiency) of 12% was obtained by 30 minutes at 37°C with no further significant increase after 2 hours. This was used as the standard incubation time. Preparations of platelets in PRP could be used for activation studies for as long as 8 hours. Platelets loaded with indo-1 aggregated in response to physiologic stimuli in an identical manner to unlabeled platelet (data not shown).

Indo-1 fluorescence was assessed by determining the coefficient of variation on the ratio of the unstimulated platelet control. The CV of the ratio varied between 9.3 and 15.7 (mean 13.5). Controls were also analyzed to detect platelet activation before addition of agonist. A two-parameter histogram was collected to measure time elapsed on the x axis and ratio fluorescence on the y axis. Platelet size was measured on a one-parameter histogram by log forward-angle light scatter. In addition, change in platelet light scatter in response to stimuli was monitored by a histogram measuring elapsed time on the x axis and log forward-angle light scatter on the y axis.

The distribution of resting cytoplasmic calcium of intact platelets in a Ca^{2+}-containing buffer (CaTS) is shown in Fig 1A. A positive control was the addition of ionophore, 4-bromo-A23187, to platelets in CaTS buffer (Fig 1B). Ionophore was used as a positive control because A23187 causes platelet activation by directly increasing the calcium ion concentration in the cytoplasm, thus bypassing ligand–receptor interactions.

In an attempt to correlate the fluorescent ratio measurements to an absolute calcium concentration, graded Ca^{2+} buffers were added to a series of tubes and the distribution of the ratio FL400/FL486 was evaluated before and after addition of ionophore. This evaluation could not be made since a simple equilibrium of calcium (ie, constant FL400/
FL486) was not established after addition of ionophore over the 10-minute analysis period. Calibration of the level of [Ca$^{2+}$]i in lymphocytes using the mean ratio channel number obtained after ionophore addition was also attempted, but as other investigators have reported, this approach did not yield accurate standardization technique for similar reasons. Calibration of [Ca$^{2+}$]i in intact cells does not appear to be accurate in that certain assumptions which cannot be tested must be made: First, it must be assumed that all of the indo-1 loaded into cells is fully hydrolyzed to the free acid form; second, it is not known how the microviscosity of the cytoplasm or its refractive index affects the kd of the indo-1; and third, it must be assumed that the ionophore used in calibrating [Ca$^{2+}$]i causes total equilibrium across all calcium storage areas. In view of these problems, we determined the mean ratio channel number for resting platelets and for platelets activated by varying concentrations of ADP or thrombin in calcium- or EGTA-containing buffers. A ratio of these values, a calcium mobilization index (CMI), was used for standardization of this cytofluorimetric method. This ratio provides a method of comparison of platelet response with agonists (Table 1). The mean channel ratio was determined for the entire analysis period for this study, although a CMI at peak platelet response or other time points may be determined by this method as well. These data suggested that thrombin at 0.1 U/mL and ADP at $10^{-4}$ mol/L invoked a similar overall response in calcium-containing buffers whereas thrombin at 0.05 U/mL caused increases in platelet cytosolic calcium comparable with that observed with $10^{-6}$ mol/L ADP. Lower CMI values were observed at high agonist concentration when platelets were activated in EGTA-containing buffers.

Platelets are heterologous in response to stimuli. Mobilization of calcium was examined in response to the addition of a variety of agonists and agonist concentrations. The flow cytometric technique demonstrated that agonists evoked different distributions of platelet cytoplasmic calcium, varied in the percentage of responsive platelets, and differed in their time course of response. Increases of platelet cytosolic calcium on addition of 0.2 U/mL thrombin and $10^{-3}$ mol/L were measured at room temperature (22°C) and at 37°C (Fig 2). At 37°C, a subpopulation of platelets had already begun to mobilize calcium during cell transit and before cell interrogation on the flow cytometer had been resumed (approximately 30 seconds). To capture some of these early events, we added agonist with a syringe directly into the pressurized sample compartment during analysis instead of interrupting cell flow.

Changes in platelet cytosolic calcium were routinely analyzed at room temperature to evaluate early events in platelet activation. Maintaining platelets at room temperature sufficiently slowed down the reaction time of the platelet so that events taking place normally in the framework of seconds at physiologic temperature could be evaluated and compared for a longer time. Zavoico and Cragoe previously showed that studies of changes in cytoplasmic calcium levels and internal pH in platelets at lower temperature (10 to 15°C) only delayed response and that the intensity of response was unaffected as compared with response at 37°C. Therefore, one of the significant features of this technique is that it provides a more detailed analysis of platelet events that normally occur within seconds after ligand binding. At concentrations of 0.1 U/mL thrombin and $10^{-5}$ mol/L ADP in calcium-containing buffers, heterogeneity of platelet response was observed (Fig 2). Transient increases in cytoplasmic calcium were measured in only a subpopulation of platelets when these agonists were added to platelets in
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Fig 2. Heterogeneity of [Ca2+]i of indo-1-labeled platelets in CaTS buffer in response to 10^{-8} mol/L ADP and 0.2 U/mL α-thrombin at room temperature (22°C) (A) and 37°C (B). Differences were observed among individual platelets in duration, magnitude, and intensity of response. At 37°C, most platelets had responded by the time cell interrogation had resumed: x-axis, ratio (FL400/FL486); y-axis, cell number; z-axis, time in minutes.

Calcium-containing buffer. This transient release has been suggested to correspond in part to release from the dense tubular system and evacuation by the Ca^{2+}-Mg^{2+}-ATPase. As concentrations of agonist increased, most platelets demonstrated a more homogenous response (Fig 3). At high-dose thrombin (1 U/mL), platelets in CaTS buffer at room temperature often formed aggregates that prevented analysis of single cells for the entire 600-second period. However, at 0.5 U/mL, platelets were analyzed for the entire period, with 93% of platelets responding. Thrombin addition (0.5 U/mL) resulted in the greatest number of platelets responding over continuous time, with few platelets showing decreased levels of cytosolic calcium at the end of the analysis period. In contrast, lower concentrations of thrombin (0.1 U/mL) had a slightly lower response rate: Increases in cytosolic calcium levels were detected in only 70% of the platelets. At this concentration, transient increases in calcium levels were evident: At the end of the analysis period, approximately 40% of the platelets were analyzed as responders. Similar results were observed with ADP. At 10^{-4} mol/L ADP, more than 90% of the platelets were responders, with more platelets having transient increases over time. At 10^{-5} mol/L ADP, the time to response and the percentage of responders reflected lower agonist concentration in that ≥30 seconds was required to detect responders and the maximum percentage of responders was 53%. Approximately 20% of the platelets were positive at the end of the analysis period. The percentage of platelets responding to ADP and thrombin over continuous time was determined by analyzing the percentage of platelets responding above as established baseline. Analysis gates were positioned where approximately 3% of the untreated (resting) platelet population were analyzed as positive. Percentage of responders measured over continuous time on addition of thrombin (0.5 and 0.1 U/mL) and ADP (10^{-4} and 10^{-5} mol/L) are shown in Fig 4.

We were also able to distinguish between mobilization of calcium from internal stores (+ EGTA) and mobilization resulting from influx from the media and release from stores combined (Fig 5). When low concentrations of ADP (2 × 10^{-9} mol/L) and thrombin (0.05 U/mL) were added to platelets in EGTS media, we observed no measurable changes in cytoplasmic calcium levels. However, when high concentrations of agonists were added, calcium mobilization was observed. The overall response of platelets to ADP (10^{-4} mol/L) and α-thrombin (1 U/mL) in EGTA-containing buffer was 14% and 29%, respectively, of the total platelets analyzed.

In addition to the ability of this technique to assess platelet activation either before or subsequent to agonist addition, we were also able to monitor other parameters of platelet function such as change in light-scatter properties. Holme et al12 recently reported that platelets that had undergone shape change in response to ADP showed as an increase in forward-angle light scatter. We observed an increase in platelet light scatter at both low and high concentrations of agonist. The extent of change in light scatter was dependent...
was observed even in the absence of detectable mobilization of calcium. Therefore, both agonists evoked response when increases in [Ca^{2+}]_i may be small or localized.

**DISCUSSION**

Using ratio cytofluorimetry and the internalized calcium chelating dye indo-1, we demonstrated that individual platelets differ in distribution of [Ca^{2+}]_i when exposed to agonists. Depending on the agonist used and its concentration, the percentage that responded and the time course of response was heterogeneous. With this technique, we observed subpopulations of platelets that were unresponsive to high concentrations of agonists. More typically, we observed subpopulations of platelets that did not respond to a particular agonist but did respond when a higher concentration was used. Transient release of calcium from internal stores probably corresponds to calcium released from the dense tubular system and then evacuated by the Ca^{2+}-Mg^{2+}-ATPase.

Because of this transient release, there was a measurable heterogeneity in duration of calcium response in single cells. Calcium mobilization was observed at a thrombin concentration of 0.01 U/mL with an overall response of 11%. Approximately 35% of platelets were responding at ADP concentrations of 10^{-4} mol/L. With EGTA added, increases in cytoplasmic calcium were not observed at 2 \times 10^{-4} mol/L ADP or 0.05 U/mL thrombin. Ware et al. reported a similar threshold concentration of 5 \times 10^{-4} mol/L ADP in EGTA-containing buffers for both quin 2- and aequorin-loaded platelets but observed a lower threshold for thrombin. However, we observed changes in light scatter properties with both agonists in calcium-containing buffers. Both ADP and thrombin at low concentrations evoked a platelet response under conditions in which increases in cytoplasmic calcium were not detected by our methods and therefore may reflect localized changes in platelet cytosolic calcium. Rink et al. also reported that thrombin (0.4 U/mL) could induce shape change in quin 2-loaded platelets even when [Ca^{2+}]_i remained below the resting level. Although their results lend supporting evidence for thrombin activation by calcium-independent pathways or an increase in calcium sensitivity of rate-limiting steps, other investigators have shown that
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Fig 5. Effect of external calcium on changes in [Ca^{2+}]_i on exposure to agonists: 2 x 10^{-4} mol/L ADP (A), 0.5 U/mL α-thrombin (B). 10^{-4} mol/L ADP (C) or 1 U/mL α-thrombin (D) was added to indo-1-labeled platelets in EGTA buffer in which the free Ca^{2+} concentration was less than 0.1 μmol/L. Increasing intensity of shading reflects five levels of increasing cell density: less than 2 cells, 2 to 50 cells, 50 to 100 cells, 100 to 200 cells, and more than 200 cells. Line plot represents the change in the mean channel ratio versus time.

Fig 6. Changes in the forward-angle light-scatter properties of indo-1-labeled platelets activated by thrombin or ADP: 2 x 10^{-4} mol/L ADP (A), 0.05 U/mL α-thrombin (B). Control in which buffer instead of agonist was added (C). Data are expressed as the mean (O) and peak (A) light scatter channel versus time.

Increases in cytoplasmic calcium in aequorin-loaded platelets occurred at agonist concentrations that were the same or lower than that which induces shape change, aggregation, or secretion. Thus, thrombin at a concentration of 0.05 U/mL may have induced localized increases in cytosolic calcium that cannot be detected by single cell measurements using indo-1 and ratio flow cytofluorimetry but can be detected by use of aequorin.

High concentrations of both ADP (10^{-4} mol/L) and thrombin (1 U/mL) initiated calcium mobilization in the absence of external calcium. While extracellular EGTA can reduce the Ca^{2+} pool within the dense tubular system and therefore lower the amount of Ca^{2+} available for release, an increase in [Ca^{2+}]_i was observed in EGTA-containing buffers. Therefore, these results argue for the contribution of both the dense tubular system and the influx of extracellular Ca^{2+} in mediating platelet response upon exposure to ADP or thrombin. Our results also suggest that an increase in cytoplasmic calcium on exposure to low levels of agonist is mainly due to the presence of external calcium in the media.

With indo-1 and ratio cytofluorimetry, calcium mobilization can now be correlated with other physiologic parameters and with expression of surface antigens. Such techniques should enhance the basic understanding of transmembrane signalling since regulation of intracellular free calcium concentration has been considered an important mechanism for transmission of cell-surface receptor signals. Objective measurements of changes in platelet cytosolic calcium can now be correlated with other physiologic parameters and with expression of surface antigens. The observed heterogeneity of [Ca^{2+}]_i on platelet activation requires careful examination of activation pathways that may or may not be calcium dependent. Correlative studies will aid in distinguishing diverse processes that culminate in platelet aggregation. Another application of this technique is the in vitro detection of in vivo activated platelets. Preliminary studies indicate that this
technique can detect platelets from individuals with alterations in cytoplasmic calcium levels and therefore can potentially identify individuals who have abnormal platelet function.

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