Flow Cytometric Kinetic Assay of Calcium Mobilization in Whole Blood Platelets Using Fluo-3 and CD41

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Background: Platelet activation plays a major role in the physiology and pathology of hemostasis. Flow cytometry is a promising approach for the structural and functional analysis of platelets. However, the choice of adequate biological parameters and most technical issues are still under discussion. A rise in cytosolic free Ca2+ is a key early event that follows platelet stimulation and precedes several activation responses, including shape change, aggregation, secretion, and expression of procoagulant activity. Our objective was to set up a fast and sensitive flow cytometric method to determine the kinetics of intracellular Ca2+ mobilization in platelets, which could be performed with the least artificial perturbation of platelet function.

Methods: Anticoagulated blood was diluted in Tyrode’s buffer and incubated with Fluo-3-acetoxymethyl ester prior to staining with phycoerytrin-conjugated antiplatelet GPIIb/IIIa complex monoclonal antibody. Platelets were identified by a gate including only CD41 events. After the determination of baseline Fluo-3 green fluorescence on a flow cytometer (EPICS XL-MCL, Coulter Electronics, Hialeah, FL), adequate agonists were added and time-dependent changes in Fluo-3 fluorescence were recorded on-line for up to 3 min.

Results: In these conditions, a very fast and transient increase of cytosolic-free Ca2+ was observed following the addition of thrombin, a strong platelet agonist. Stimulation with adenosine diphosphate (ADP), a weak agonist, also resulted in evident increase of Ca2+ levels.

Conclusions: Our results show that this flow cytometric kinetic method provides a simple and sensitive tool to assess in vitro the time course and intensity of signal transduction responses to different platelet agonists under near physiological conditions. In this way, it may be useful to evaluate the degree of platelet reactivity and thus to monitor antiplatelet therapy. Cytometry 35:302–310, 1999.

Key terms: Ca2+; platelet activation; Fluo-3; CD41; GPIIb/IIIa; whole blood
tion in platelets, even though FCM has been successfully applied to such studies in a wide range of eukaryotic cells.

Platelet activation with most agonists results in a rise of free \( \text{Ca}^{2+} \), which appears to be due to both influx across plasma membrane and release from internal stores, mainly the dense tubular system (19–22). The increase of cytosolic free \( \text{Ca}^{2+} \) is a key early event that follows platelet stimulation and precedes several activation responses, including shape change, aggregation, secretion, and expression of procoagulant activity (21,23,24).

FCM provides a convenient method to evaluate kinetics of intracellular free \( \text{Ca}^{2+} \) when using fluorescent sensitive indicators that can be loaded into intact cells (25,26). Although whole blood techniques have obvious advantages to avoid artifactual platelet activation, the only FCM measurements of intracellular free \( \text{Ca}^{2+} \) in platelets have been reported in washed or gel-filtered platelets (20,23,25,27–32). In view of this, the purpose of the present study was to develop a sensitive FCM kinetic assay of intracellular platelet-free \( \text{Ca}^{2+} \) by using Fluo-3 and whole blood. In this method, platelets are identified on the
basis of their light-scatter profile and the specific constitutive antigen CD41 (GPIIb/IIIa complex). We have applied this technique to demonstrate fast and transient increases in cytosolic calcium, with stimulation with ADP and thrombin. Our results show that this method provides a simple and sensitive tool to assess, in vitro, the time course and intensity of signal transduction responses to different platelet agonists under near physiologic conditions.

**MATERIALS AND METHODS**

**Materials**

Fluo-3-acetoxymethyl ester (Fluo-3 AM) was obtained from Molecular Probes (Eugene, OR). Human \( \alpha \)-thrombin, ADP, glycyl-L-prolyl-L-arginyl-L-proline (GPRP), HEPES, bovine serum albumin (BSA), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibody specific for GPIIb/IIIa complex conjugated with phycoerytrin (CD41-PE) was from Immunotech (Marseille, France).

**Donors**

Whole blood was obtained from 10 healthy adult volunteers who had taken no medication for the preceding 2 weeks and had fasted overnight.

**Blood Collection**

To avoid artifactual platelet activation, whole blood was drawn without a tourniquet from an antecubital vein, and the phlebotomy was done with a 19-gauge needle. The first 2.5 ml were discarded, and 9 ml were collected directly into a tube containing 1 ml of 135-mM Na3 citrate.

**Preloading of Platelets With Fluo-3 AM**

Anticoagulated whole blood was diluted 1:10 in modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl2, 12 mM NaHCO3, 0.4 mM Na2HPO4, 0.35% BSA, 10 mM HEPES, 5.5 mM glucose, pH 7.4) and was incubated at 37°C for 15 min with 5 mM Fluo-3 AM from a 1-mM stock solution in DMSO.

To optimize Fluo-3 AM concentration and incubation time, samples with different concentrations of the fluorochrome (0.5, 1, 2, 5, and 10 mM) were prepared and analyzed in the flow cytometer every 5 min.

**Immunostaining of Platelets With Monoclonal Antibody CD41-PE**

Twenty-five-microliter samples of whole blood preloaded with Fluo-3 were directly labeled with 5 µl of CD41-PE to identify the platelet population. In samples to be stimulated with \( \alpha \)-thrombin, the synthetic tetrapeptide GPRP was added at 2.5 mM final concentration to inhibit fibrin polymerization (33). After 15 min of incubation at room temperature in the dark, modified Tyrode's buffer (1 ml) was added, and samples of 500 µl were used to determine kinetic variations of intracellular Ca\(^{2+}\).

**Flow Cytometric Kinetic Measurement of Intracellular Ca\(^{2+}\) in Platelets Activated With ADP and Thrombin**

All the analyses were performed with an EPICS XL-MCL Flow Cytometer (Coulter Electronics, Hialeah, FL) with a 15-mW argon laser tuned at 488 nm. The instrument was set up to measure forward angle scattered light (FS), side angle scattered light (SS), Fluo-3 (FL1), and PE (FL2).
fluorescence intensities. Fluorescence was collected through a 488-nm blocking filter via a 550-nm long-pass dichroic through a 525-nm bandpass (Fluo-3) and via a 600-nm long-pass dichroic through a 575-nm bandpass (PE).

Correlated histograms and list mode data were collected for logarithmically amplified signals (FS, SS, Fluo-3, and PE). Time was acquired as a cytometric parameter. Platelets were identified by using analytical gates based on FS log and SS log signals and on CD41⁺ events.

Kinetics of intracellular Ca²⁺ mobilization were evaluated in whole blood samples, double stained with Fluo-3 and CD41, and activated with ADP and thrombin. The following agonists were tested: α-thrombin (0.005, 0.05, 0.5, and 1 U/ml) and ADP (5, 12.5, 25, 50, and 100 µM).

After the determination for about 20 s of baseline Fluo-3 fluorescence (log FL1) from the platelet population, cell aspiration into the flow cytometer was briefly paused, and the appropriate concentration of agonist (25 µl) was added.

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**Fig. 3.** Dual staining of whole blood samples with Fluo-3 and the monoclonal antibody specific for GPIIb/IIIa complex conjugated with phycoerytrin (CD41-PE). Histograms A and B show that only the CD41⁺ population changes green fluorescence when stimulated with thrombin. A: Unstimulated sample (time: 0 s). B: After stimulation with thrombin 1 U/ml (time: 20 sec). Histograms C and D show the light scatter properties of the CD41⁺ subpopulation gated in region A before (C) and after (D) thrombin stimulation.
to the whole blood sample. The acquisition was then resumed, and changes in log FL1 versus time were recorded up to 3 min.

For each plot, rectangular analysis regions were defined over the time axis, each covering the whole length of the fluorescence axis. The AUTOANALYSIS option in the EPICS XL-MCL software printed the statistics of each analysis region. When necessary, data analysis and reprocssing was performed off-line with System II® software (Coulter Electronics).

RESULTS
Platelet Identification in Whole Blood With CD41-PE
Platelet population was identified in whole blood (Fig. 1A) by using a PE-conjugated monoclonal antibody against the platelet-specific constitutive antigen CD41 (GPIIb/IIIa complex) (Fig. 1B). According to what has been previously described (3,12,24), CD41 specifically identifies the platelet population, which appears in Figure 1B as a low side scatter and high PE-fluorescence population in unstimulated whole blood. Thus, CD41-PE fluorescence was used to gate platelets for further analysis (Fig. 1D). A small percentage of CD41+ events appears with a high forward and side scatter similar to those displayed by red blood cells and leukocytes in the logarithmic representation shown in Figure 1A. This discrete population could represent either platelets coincident in flow with other blood cells or activated platelets specifically bound to leukocytes, as has been reported (34,35).

Optimization of Platelet Loading With Fluo-3
To determine the adequate incubation time and dye-to-cell concentration ratio for Fluo-3 in the kinetic assay, whole blood samples from normal donors (platelet count range: 150,000–450,000/µL) were diluted 1:10 in Tyrode’s buffer and incubated up to 35 min with different concentrations of Fluo-3 AM (0.5–10 µM). Figure 2 shows the time and concentration dependence of Fluo-3 loading in whole blood platelets. In this series of experiments, platelets were selected by their light scatter properties previously identified by CD41-PE backgating (Fig. 1D). As can be seen, 5 µM Fluo-3 AM was the lower concentration, producing both a significant and stable fluorescence at this incubation time. With lower dye concentrations, fluorescence was too low for accurate determination, but at 10 µM Fluo-3 fluorescence was still steadily increasing.

Changes in Intracellular Ca2+ in CD41+ and CD41− Cells in Whole Blood Following Thrombin Stimulation
Incubation of diluted whole blood samples with 5 µM Fluo-3 AM resulted in the loading of platelets (Fig. 2) and the rest of the blood cells. To improve the detection of Fluo-3-fluorescence changes expected following activation with Ca2+-mobilizing agonists such as thrombin, the FL1 detector was set up such that cellular green fluorescence was brought to the first logarithmic decade (Fig. 3A). Under these conditions, CD41+ platelets exhibited a slightly higher green fluorescence due to the cross-talk of bound CD41-PE and were clearly distinguished from the remaining blood cells, which were CD41−. As seen in Figure 3B, stimulation of whole blood with 1 U/ml thrombin induced a significant increase in Fluo-3 green fluorescence only in the CD41+ population; the CD41− cells remained unchanged. This result shows that under these conditions the mobilization of intracellular Ca2+ can
be detected in single platelets and that these are the only cells that respond to the agonist.

A small percentage of CD41+ events appears with a high Fluo-3 fluorescence previous to thrombin activation, which may indicate that some platelets are activated, possibly due to sample manipulation. Because the light scatter properties shown in Figure 1D suggested the presence of leukocyte-bound, activated platelets, we identified the light scatter properties of these CD41+/Fluo-3 bright cells. As shown in Figure 3C,D, this subpopulation of platelets is associated with light scatter characteristics similar to those displayed by erythrocytes and leukocytes (Fig. 1A).

**Kinetic Assay of Intracellular Ca2+ Mobilization in Platelets**

After determining the sensitivity of the method to detect changes in intracellular Ca2+ concentration in whole blood platelets, we tested its suitability to the kinetic assay...
of intracellular Ca\textsuperscript{2+} mobilization. Testing was performed by adding platelet agonists to whole blood stained with Fluo-3 and CD41-PE monoclonal antibody and recording the time variations in green fluorescence of the CD41\textsuperscript{+} platelet population.

Figure 4 describes the procedure and time course of a typical experiment when using the kinetic assay. To determine the baseline, green fluorescence of unstimulated platelets was recorded for about 20 s. The sample aspiration was then briefly paused while agonist was added, and the acquisition resumed immediately up to 3 min. By means of rectangular analysis regions defined over the time axis and covering the whole length of the fluorescence axis, the cytometer software automatically displays the statistical data for each time slice as it is crossed by the plot. These regions can be displaced after the run, if necessary, for more accurate numerical analysis. Furthermore, these time regions can be used as gating windows to display and analyze kinetic changes in relevant features. In addition, such data representation may permit the comparison and quantification of subpopulations of platelets that are different in terms related to the activation process, such as maximal increase of cytosolic free Ca\textsuperscript{2+}, or reversibility of the process.

**Intracellular Ca\textsuperscript{2+} Mobilization Induced by Thrombin- and ADP Stimulation of Platelets in Whole Blood**

The applicability of this method to the study of signal transduction in platelets was assessed on activation induced by agonists that were different both in the mechanisms of signal transduction and the intensity and duration of Ca\textsuperscript{2+} mobilization. Thus, ADP and thrombin were used as weak and strong agonists, respectively (1).

Although previous experiments (Fig. 2) indicated that 5 \(\mu\)M Fluo-3 AM was the adequate loading concentration, this concentration may not have been ideal for a sensitive...
and reversibility of free Ca\(^{2+}\) showed the best resolution for the evaluation of intensity of Fluo-3 (2–10 µM). Figure 5 shows that 5 µM Fluo-3 (50 µM) in samples incubated with different concentrations of Thrombin (1 U/ml) and ADP caused fast and dose-dependent elevation in free Ca\(^{2+}\) and ADP. The normalized data shown in Table 1 demonstrate that the immediate, dose-dependent increase in Ca\(^{2+}\) induced by thrombin can be directly monitored in whole blood by using the tetrapeptide GPRP, which inhibits fibrin polymerization and platelet aggregation. In addition, we tested another physiologic but weak agonist, ADP. The normalized data shown in Table 1 demonstrate that the immediate, dose-dependent increase in Ca\(^{2+}\) is of similar intensity across normal donors and across different experiments.

Under these experimental conditions, the time and concentration dependence of platelet response to the agonists could be determined quantitatively (Fig. 6). Thrombin induced a fast and dose-dependent elevation in free intracellular Ca\(^{2+}\), from a resting level corresponding to less than 2 fluorescence arbitrary units (FAU) to a maximal value of more than 8 FAU (Table 1). Thereafter, Ca\(^{2+}\) decreased to reach almost the resting level. ADP also induced fast and transient increases in cytosolic free Ca\(^{2+}\) level, although of lesser intensity than those induced by thrombin (Table 1).

Because the elevations in Ca\(^{2+}\) caused by both thrombin and ADP were observed in a Ca\(^{2+}\)-free buffer, they must be the consequence of Ca\(^{2+}\) mobilization from internal stores. The subsequent decrease in Fluo-3 fluorescence indicates removal of Ca\(^{2+}\) from the cytosol into internal stores or extrusion across the plasma membrane, as previously reported (20,21).

**DISCUSSION**

The study of early biochemical markers of platelet activation may be relevant to assess in vivo platelet activation and the changes in platelet reactivity that are associated with hemostasis disorders and their therapy. Among the earliest events, a rise of cytosolic Ca\(^{2+}\) is a key phenomenon, which follows platelet activation with most, if not all, agonists. Conversely, many platelet functions require a minimal threshold level of intracellular Ca\(^{2+}\) for their occurrence.

In the present study, we sought to develop a fast FCM kinetic method to evaluate platelet intracellular Ca\(^{2+}\) mobilization in whole blood. Several studies using fluorescent Ca\(^{2+}\)-sensitive indicators and FCM in samples of washed or gel-filtered platelets have been reported (27–30), although the approach in whole blood presents obvious advantages. In this method, sample manipulation was minimal in contrast to other methods involving separation procedures that cause artifactual platelet activation and eventual loss of platelet subpopulations.

Whole blood platelets are analyzed in a more physiologic milieu, where erythrocytes and white blood cells are included that can affect platelet responses to activation (35,36). In this experimental situation, it is possible to differentiate clearly the platelet population from other blood cells on the basis of their light scatter profile and by using a monoclonal antibody against a platelet-specific antigen conjugated with PE. Simultaneous analysis of whole blood light scatter properties and fluorescence emission of PE (CD41+ cells) allows one to follow the interaction of platelets with other blood cells at platelet activation.

Thrombin is the most important physiologic and potent agonist of platelets, and interaction with its receptor triggers different mechanisms of signal transduction, including activation of phospholipase C. The rise in cytosolic free Ca\(^{2+}\) induced by thrombin can be directly monitored in whole blood by using the tetrapeptide GPRP, which inhibits fibrin polymerization and platelet aggregation. In addition, we tested another physiologic but weak agonist, ADP. The normalized data shown in Table 1 demonstrate that the immediate, dose-dependent increase in Ca\(^{2+}\) is of similar intensity across normal donors and across different experiments.

Our findings are in agreement with those of some previous studies by other investigators (20,21,28,30), indicating a very fast and transient rise in intracellular free Ca\(^{2+}\) in platelets stimulated with thrombin or ADP; but, depending on the agonist used and its concentration, the time course and intensity of response were heterogeneous.

The heterogeneous pattern of platelet response to agonists is an important aspect to be considered. In the kinetic assay presented in this study, because intracellular Ca\(^{2+}\) mobilization is quantitated in real time in single platelets, it is possible to detect and to compare platelet subpopulations differing in their response behavior, including transient changes.

Our results show that this method provides a simple and sensitive tool to assess in vitro the time course and intensity of signal transduction responses to different platelet agonists under near physiologic conditions. Also, the present method should be broadly applicable to studies of platelet reactivity as a mean to identify individuals with abnormal platelet function and to monitor platelet-directed therapies.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ca(^{2+}) Maximal Responses in Whole Blood Platelets After Stimulation With Different Concentrations of ADP and Thrombin*</th>
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<tbody>
<tr>
<td>Agonists</td>
<td>Ca(^{2+}) maximal response (fluorescence ratio)</td>
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<td>---------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>ADP (µM)</td>
<td></td>
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<tr>
<td>5</td>
<td>2.16 ± 0.64</td>
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<tr>
<td>12.5</td>
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<tr>
<td>25</td>
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</tr>
<tr>
<td>50</td>
<td>2.49 ± 0.61</td>
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<td>Thrombin (U/ml)</td>
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<tr>
<td>0.005</td>
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<tr>
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<td>4.07 ± 1.12</td>
</tr>
<tr>
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</table>

*Data are presented as mean ± SD for at least three experiments with different donors. **Fluorescence ratio = maximal value of mean FL1 in agonist-stimulated platelets/mean FL1 in unstimulated platelets.
ACKNOWLEDGMENTS

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LITERATURE CITED