Subsecond Calcium Dynamics in ADP- and Thrombin-Stimulated Platelets: A Continuous-Flow Approach Using Indo-1

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The regulation and kinetics (<5 seconds) of cytosolic calcium changes ([Ca^{2+}]_{i}) in stimulated blood platelets have been investigated under physiological blood-flow conditions. Using a newly-developed continuous-flow approach with indo-1-loaded human platelets, adenosine diphosphate (ADP, 10 µmol/L) and thrombin (5 U/mL) were equally effective in significantly increasing [Ca^{2+}]_{i} by 0.5 seconds. ADP induced a transient [Ca^{2+}]_{i} peak of 1 to 2 µmol/L near 2 seconds, whereas thrombin caused a sustained and larger response. The first phase (<2 seconds) was not influenced by a lack of extracellular Ca^{2+}, in contrast to the subsequent [Ca^{2+}]_{i} increase that only reached about 0.7 µmol/L for either ADP or thrombin. The shear rates used in our continuous-flow apparatus were physiological (<1,258 sec^{-1}) and only slightly increased the basal [Ca^{2+}]_{i}, of 0.1 µmol/L. Platelet aggregation (<5 seconds), assessed by single-particle counting, was not altered in platelets loaded with indo-1/AM (2.5 µmol/L).

C RITICAL EVALUATION of the role of calcium as an intracellular messenger requires kinetic analysis of changes in the cytosolic free calcium concentration ([Ca^{2+}]_{i}) in response to various stimuli.1,2 A variety of agonists including adenosine diphosphate (ADP) and thrombin are known to increase platelet [Ca^{2+}]_{i}.3,9 These calcium fluxes are usually accompanied by phosphorylation of specific proteins, increased turnover of phosphatidylserine (PI), and other intracellular responses.10 However, the specific sources, precise functions, and kinetics of calcium fluxes in platelet physiology are not well defined, partly because of the rapidity with which these changes occur. Morphological changes and aggregation are complete within seconds of an inducing stimulus.1,12

The important question of whether extracellular calcium and opening of plasma membrane channels serve as a major way for raising cytoplasmic calcium levels is supported by earlier studies using quin2.13 Thrombin raises the [Ca^{2+}]_{i}, to only 0.3 µmol/L in the absence of extracellular calcium but to near 3 µmol/L in its presence. Other studies with quin2 have shown that ADP can induce similar increases in [Ca^{2+}]_{i}, whereas epinephrine or lower doses of ADP fail to raise [Ca^{2+}]_{i}, even though aggregation may occur.14 However, second-generation fluorescent dyes such as fura-2 and indo-1 provide several advantages over quin2.14 Much lower dye concentrations are needed, thus reducing both internal buffering artifacts and functional inhibition.13

The quenched-flow approach15 has previously been used to study platelet “shape change,”12 aggregation,11 secretion,15 and protein phosphorylation16,17 within the time scale required for efficient hemostasis, which is estimated to be about 0.1 second.18 Because substantial differences appear to exist between the mechanisms of thrombin- and ADP-induced platelet activation,14,19 we examined the rapid kinetics (<5 seconds) of [Ca^{2+}]_{i} changes in human platelets stimulated under physiological blood-flow conditions.11,20

MATERIALS AND METHODS
Platelet preparation and dye loading. Human platelet-rich plasma (PRP) was isolated as previously described.11 The platelets were loaded by incubating PRP containing 8 U/mL apyrase (Sigma Chemical Co, St Louis, grade VII), prostacyclin (0.3 µg/mL), indomethacin (Sigma, 1 µg/mL), and extra acid-citrate-dextrose (ACD) (1:10 final volume), with 2.5 µmol/L indo-1/AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Uptake studies suggested that typically 35% of the dye became associated with the platelets. After centrifugation for 20 minutes at 350 g, the platelets were suspended in ACD containing 8 U/mL apyrase and essentially fatty acid–free albumin (3 mg/mL, Sigma), centrifuged again, and finally suspended in 0.5 vol Eagle’s balanced salt solution (pH 7.2, GIBCO, Grand Island, NY) containing sodium bicarbonate (21 mmol/L), HEPES (5 mmol/L), albumin (3 mg/mL), and hirudin (0.1 U/mL, Sigma, grade IV). The [Ca^{2+}]_{i} in the Eagle’s solution was 1.8 mmol/L. Human fibrinogen (0.5 mg/mL) was added to portions of the platelet-rich Eagle’s (PRE) to check platelet aggregation kinetics11; otherwise it was omitted for the [Ca^{2+}]_{i} measurements. Platelet aggregation. The kinetics of platelet aggregation were evaluated as previously described.11,16

Continuous-flow fluorescence methods. The modifications to our general quenched-flow approach are illustrated in Fig 1. One syringe was filled with PRE and the other with either saline (control) or agonist. These solutions combined at a “T” junction and were pumped through a reaction tube (0.3-mm internal-diameter teflon tubing) that passed through a specially designed flow-through, fluorescence microcuvette. The entire apparatus was maintained at 37°C for five minutes before each experiment. The cuvette was mounted in a T-format SLM 4800 spectrophluorimeter (SLM Instruments, Urbana, IL). Excitation light (335 nm) was focused on the reaction tube and fluorescence emission at both 400 and 480 nm simultaneously monitored at right angles. These signals were continuously averaged and the 400/480 ratio electronically computed after subtraction of the emission signals from platelet-poor plasma blanks. After resuspension of the platelets into Eagle’s solution, approximately 6% of the total indo-1 in the PRE leaked from the platelets each hour. Therefore, new blanks were prepared before each set of measurements to compensate for this leakage of dye; control values were constant during this time. The observed cuvette volume was 0.1 μL, which corresponded to a time resolution of 0.03 seconds.

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**RESULTS**

We used indo-1–loaded platelets and a continuous-flow approach (Fig 1) to investigate the early kinetics of \([Ca^{2+}]_i\), mobilization. This modification of our general quenched-flow approach\(^\text{11}\) enables monitoring of rapid “real-time” changes in the \([Ca^{2+}]_i\) of platelets under flow conditions similar to those in arterial blood vessels.\(^\text{11,20}\) Our shear rates varied from 1,258 to 236 sec\(^{-1}\) at the slowest pumping speed. Because both the emission intensity and wavelength of indo-1 fluorescence change after binding calcium, we used the “ratio mode” to determine \([Ca^{2+}]_i\), independently of the dye concentration.\(^\text{14}\) The observed reaction time was selected by varying either the syringe pump speed or the length of the reaction tube that precedes the optical aperture of the cuvette. Reaction times beginning at about 0.3 seconds can be studied.

Platelet functionality was assessed by monitoring the ADP-induced aggregation of washed, indo-1–loaded platelets in the presence of fibrinogen.\(^\text{11}\) Five seconds after the addition of 10 \(\mu\text{mol/L}\) ADP, only 38.3\% (± 11.7\% SEM) of the singlet platelets remained. This efficiency is similar to the 33\% reported for control platelets not loaded with indo-1/AM.\(^\text{17}\) In addition, the secretion efficiency of platelets isolated by our washing procedure in ACD, and assessed by thrombin-stimulated serotonin release, is nearly the same\(^\text{16}\) as for control platelets in plasma.\(^\text{15}\) Control experiments revealed that the physiological shear stresses of continuously pumping platelets through the reaction tube slightly increased the \([Ca^{2+}]_i\), to 120 \(\text{nmol/L}\) on average, from a static level of 100 \(\text{nmol/L}\). Either ADP (10 \(\mu\text{mol/L}\)) or thrombin (5 U/mL) rapidly increased the \([Ca^{2+}]_i\), above control levels (\(P < .001\)) within 0.5 seconds (Fig 2). This maximal dose of thrombin was sufficient to prevent any effect of the 0.1 U/mL hirudin previously added to the PRE. Both agonists were equally effective for increasing \([Ca^{2+}]_i\), during the first 2 to 3 seconds of platelet activation. The ADP-induced \([Ca^{2+}]_i\) rise was transient, peaked between 1 and 2 \(\mu\text{mol/L}\) at 2 to 3 seconds, and then dropped below 0.5 \(\mu\text{mol/L}\) by eight seconds (Fig 3). Thrombin, on the other hand, elicited a sustained \([Ca^{2+}]_i\) rise that was much larger than that for ADP (Fig 3). The \([Ca^{2+}]_i\) trends with either ADP or thrombin both extrapolated very close to zero reaction time at basal \([Ca^{2+}]_i\), 0.06 seconds ± 0.09 SEM and 0.19 seconds ± 0.14 SEM, respectively. The slight drop in the control values at 2 seconds corresponded to a change in the length of the reaction tube that precedes the cuvette aperture and may be due to regional differences in the flow dynamics.

To examine the sources of \(Ca^{2+}\) that contributed to the platelet \([Ca^{2+}]_i\), fluxes, we investigated the effect of chelating the extracellular \(Ca^{2+}\) by preincubating the platelets with EGTA for five minutes at 37°C (Fig 3). Incubation with
EGTA did not significantly affect control [Ca$^{2+}$] values (data not shown). The values for EGTA-incubated platelets induced by either ADP or thrombin, however, were significantly lower after 2.0 seconds ($P<.05$) relative to platelets in Ca$^{2+}$-containing medium. Two distinct phases were observed during the first eight seconds of platelet activation. The earliest phase (<2 seconds) induced by either ADP or thrombin was not inhibited by EGTA. In the absence of external Ca$^{2+}$, ADP released sufficient internal pool(s) of Ca$^{2+}$ to raise the [Ca$^{2+}$], to 0.6 μmol/L within 2 seconds, whereas thrombin only raised the [Ca$^{2+}$] to 0.8 μmol/L by eight seconds.

**DISCUSSION**

We have reported rapid [Ca$^{2+}$]$_{1}$ fluxes in platelets under physiological blood-flow conditions. Our basal [Ca$^{2+}$]$_{1}$ levels are equivalent to those indicated by quin2. The peak [Ca$^{2+}$]$_{1}$, however, was much higher than that indicated by quin2. The possible influences of cytoplasmic Ca$^{2+}$ heterogeneity and changes in platelet volume or microviscosity have not been determined. Our data with thrombin generally agree with Pollock and Rink who reported that in fura-2–loaded platelets, internal Ca$^{2+}$ stores may provide an initial spike in the [Ca$^{2+}$]$_{1}$ to about 1 μmol/L that is prolonged and somewhat higher in the presence of external Ca$^{2+}$. Sage and Rink have recently reported data on subsecond calcium dynamics in platelets when using a stopped-flow technique that involves high shear rates for ramming the solutions into a mixing chamber that is subsequently monitored in the absence of any shear force. This procedure may have caused some platelet lysis, activation, and rises in the [Ca$^{2+}$]$_{1}$. Also, their responses were uncalibrated, and control data were not reported for the absence of agonist. They consider that an initial Ca$^{2+}$ influx across the plasma membrane precedes Ca$^{2+}$ release from internal stores. Our observations suggested the opposite for both ADP- and thrombin-stimulated platelets under physiological flow conditions (Fig 3). Only after 2 seconds was there evidence for opening of plasma-membrane calcium channels.

Our results reveal that Ca$^{2+}$ fluxes in platelets, under rheological conditions close to those in vivo, can occur very rapidly, beginning within 0.5 seconds after activation with ADP or thrombin. The first phase (<2 seconds) appeared to originate from intracellular stores, which is supported by the fact that the Ca$^{2+}$-dependent phosphorylation (<2 seconds) of myosin light chain (20 Kd) does not require extracellular Ca$^{2+}$. The data also suggested that this early phase might involve similar mechanisms for ADP and thrombin. The interrelationships between the PI cycle and [Ca$^{2+}$]$_{1}$ fluxes in ADP- and thrombin-activated platelets have not been clearly established. Relevant to the functions of the PI cycle are the sources of calcium that contribute to [Ca$^{2+}$] dynamics, ie, extracellular, intracellu-
lar, or plasma membrane-bound pools.\textsuperscript{27-35} Our results demonstrate that both ADP and thrombin caused a rapid initial increase in [Ca\textsuperscript{2+}], (Fig 2) that requires neither extracellular calcium (Fig 3) nor the formation of inositol-triphosphate (IP\textsubscript{3}).\textsuperscript{4} Both external and internal sources of Ca\textsuperscript{2+} apparently contributed to the second phase of the [Ca\textsuperscript{2+}], rise beginning near 2 seconds. These studies provide evidence for early biochemical events in the stimulus-response sequence in blood platelets. We have previously found that major morphological platelet changes are nearly complete within 2 seconds after activation by ADP or thrombin\textsuperscript{13} and that aggregation, as monitored by resistive-particle counting, is about 80\% complete within 5 seconds.\textsuperscript{12,15} Phosphorylation of platelet proteins begins within less than 1 second.\textsuperscript{16,17} Our quenched-flow and continuous-flow approaches are complementary methods that enable kinetic analysis of the earliest events in platelet function.

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