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Variability of the thrombin- and ADP-induced Ca^{2+} response among human platelets measured using fluo-3 and fluorescent videomicroscopy

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Abstract

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) of individual human platelets localized between siliconized glass cover slips was determined at rest and after stimulation with thrombin and ADP using the Ca^{2+} indicator fluo-3 (0.97 ± 0.30 mmol/l cell volume) with fluorescence video microscopy. Resting $[Ca^{2+}]_{cyt}$ in the presence of 2 mM external Ca^{2+} showed only small inter-platelet variability ($[Ca^{2+}]_{cyt}$ = 86 ± 30 (S.D.) nM). Resting $[Ca^{2+}]_{cyt}$ of individual fluo-3-loaded platelets measured as a function of time had a S.D. of 10 nM or 12% (S.D./mean). Individual platelets showed no affinity for the siliconized support and their $[Ca^{2+}]_{cyt}$ showed no tendency to oscillate in either the resting or in the activated state. When 0.2 U/ml thrombin or 20 μM ADP were added, all platelets showed a characteristic Ca^{2+} transient whereby $[Ca^{2+}]_{cyt}$ increased to peak values within 8–12 sec and then declined. The Ca^{2+} transients measured with fluo-3 were in approximate synchrony but peak $[Ca^{2+}]_{cyt}$ values showed large inter-platelet variability. The ensemble average peak $[Ca^{2+}]_{cyt}$ for thrombin and ADP were 672 ± 619 (S.D.) nM and 640 ± 642 (S.D.) nM, respectively. Thus inter-platelet variations (S.D./mean) were 92% or 100% as large as the average measured values. Mathematically-constructed averages of the single platelet experiments agreed reasonably well with platelet-averaged values obtained in parallel experiments with stirred platelet suspensions in a plastic cuvette, measured with a conventional spectrofluorometer. Peak $[Ca^{2+}]_{cyt}$ values reflecting dense tubular Ca^{2+} release alone (external Ca^{2+} removed) also showed large interplatelet variation (171 ± 105 (S.D.) nM with thrombin and 183 ± 134 (S.D.) nM with ADP). Dense tubular Ca^{2+} release induced by cyclopiazonic acid (a dense tubular Ca^{2+}-ATPase inhibitor) gave peak $[Ca^{2+}]_{cyt}$ of 289 ± 170 nM. Thus the size of the dense tubular Ca^{2+} pool has an inter-platelet variation of 59% (S.D./mean). Variability of the dense tubular pool size accounts for some, but not all, of the large interplatelet variation in peak $[Ca^{2+}]_{cyt}$ seen with thrombin and ADP activation.

Keywords: Platelet; Thrombin; ADP; Fluo-3; Calcium imaging; Calcium; Cytoplasmic; (Human)

1. Introduction

Agonist-activated Ca^{2+} influx and dense tubular release play an important role in platelet activation. These processes elevate the free concentration of Ca^{2+} in the cytoplasm ($[Ca^{2+}]_{cyt}$), leading to a shape change and exocytotic release processes which, in turn, cause platelets to aggregate [1,2]. Both $[Ca^{2+}]_{cyt}$ and Ca^{2+} concentration in the dense tubular store are tightly controlled by a system of pumps, leaks and receptor-operated channels. Spectrofluorometer studies of platelets in suspension using entrapped indicators of $[Ca^{2+}]_{cyt}$ have characterized the Ca^{2+} activation process in response to activators such as thrombin and ADP in terms of Ca^{2+} influx across plasma membrane and Ca^{2+} release from dense tubules [3]. The time-dependent $[Ca^{2+}]_{cyt}$ values obtained in cuvette spectrofluorometer studies are weighted averages, calculated from the average degree of indicator dye concentration averaged over all of the platelets in the sample. This technique does not reveal whether all platelets are behaving identically at each instant in time.

The activation process has also been studied with flow

Abbreviations: DT, Dense tubules; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $[Ca^{2+}]_{cyt}$, the free ('ionized') Ca^{2+} concentration in the cytoplasm; $[Ca^{2+}]_o$, the external Ca^{2+} concentration; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid; ADP, adenosine 5'-diphosphate; CPA, cyclopiazonic acid; thromboxane A_2, TXA_2.

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cytometry using Indo-1 in experiments in which suspensions of platelets were activated with ADP and thrombin and $[Ca^{2+}]_{cyt}$ values were determined for large numbers of individual platelets at the instant they flowed through the capillary [4,5]. These studies showed that the *average* $[Ca^{2+}]_{cyt}$ exhibits the same biphasic response as in a stirred cuvette measured by spectrofluorometers. Flow cytometric measurement showed large variation in the $[Ca^{2+}]_{cyt}$ values of individual platelets measured at specific times after activation [4,5]. However, the experiments did not reveal whether the variation is due to differences in peak $[Ca^{2+}]_{cyt}$ (in individual platelets) or is due to asynchrony between individual platelets in attaining the peak $[Ca^{2+}]_{cyt}$.

The present study addresses the question of inter-platelet variability and asynchrony using fluorescence video microscopy. The method is capable of reporting the activation behavior of individual platelets as a function of time after activation. Conditions were chosen to eliminate contact activation with the slide (siliconization) and to reduce interaction between platelets (dilution).

The present study was designed to elicit behavior of an ADP or thrombin-stimulated human platelet in absence of surface attachment. We make use of fluo-3, a high quantum yield fluorescent Ca^{2+} indicator [6]. These data are directly compared with the behavior of the platelets in suspension measured by fluorescence spectrophotometer. The present study will show that thrombin- or ADP-activated, unattached platelets undergo a simple $[Ca^{2+}]_{cyt}$ increase and decline in synchrony, but that the peak $[Ca^{2+}]_{cyt}$ values show large variation.

2. Materials and methods

2.1. Materials

Glucose, EGTA, Hepes, Human Thrombin (Cat. No. T3010), ADP and Cyclopiazonic acid were purchased from Sigma Chemical Co., St. Louis, MO. Ionomycin from Calbiochem, La Jolla, CA. Fluo-3/AM was obtained from Molecular Probes (Eugene, OR, USA). Fluoresbrite™ Plain Microspheres were from Polysciences, (Warrington, PA). The reagents used in the preparation of Tyrode's solution were supplied by Mallinkrodt, Paris, KY.

2.2. Platelet preparation

Blood was drawn from normal donors who had not taken aspirin, non-steroidal anti-inflammatory drugs or other medications for at least 1 wk prior to the donation. After the first 2 ml of blood was discarded, the blood was collected into anti-coagulant citrate dextrose. Washed platelets were prepared as described previously [7]. Fluo-3 loading was achieved by incubating the washed-platelets ($4 \cdot 10^8$/ml) in 20 μM Fluo-3/AM for 45 min at 37°C, giving a final intracellular fluo-3 concentration of 0.97 ± 0.30 m mol/l cell volume (see below). Its buffer capacity is lower than the intrinsic Ca^{2+} buffer capacity of the cytoplasm (ca. 3.0 mM for $0 < [Ca^{2+}]_{cyt} < 1.5$ μM [8,9]). The cells were washed twice and resuspended in a nominally Ca^{2+}-free Tyrode's solution of the following composition: 138 mM NaCl/3 mM KCl/10 mM glucose/2 mM $NaHCO_3$/25 mM Hepes with the pH adjusted to 7.35. After isolation, platelets will lose Ca^{2+} as they approach a new steady-state with this low external Ca^{2+} concentration ($[Ca^{2+}]_o$). The cells are therefore referred to as being Ca^{2+}-depleted platelets at the end of the isolation procedure. A platelet concentration of 1.6×10^7 per ml was used for all fluorometric experimentation. This was routinely measured turbidimetrically as an $OD_{600\ nm} = 0.20$ using a Beckman DB-G grating Spectrophotometer.

2.3. Mixing and containment of platelets

Containment of the platelets was achieved by either of two techniques. In the siliconized coverslip 'sandwich' technique, 10 μl of the fluo-3-loaded platelet suspension was placed between two glass cover slips which were previously siliconized. The sandwich was rapidly mounted, such that the cells could be visualized within 3 s of addition. All platelets were free-floating for the first 6–10 s, and settled, remaining well-separated during the time of measurement (6–60 s). The fluorescent measurements were made on those cells for which movement was slight, commencing 6 s after mixing and generally extending to 60 sec. During this period, movement of all cells ceased and fluo-3 leakage from platelets is negligible. Manipulation of the cover slips was sufficient to cause all platelets to move, indicating that none of them was firmly attached. (For this reason it was not possible to do experiments observing a particular cell before and after a solution change.) Control experiments observing the cells for 60 sec or more gave no indication of contact activation or spontaneous oscillation of fluorescent signal or cell shape. This indicates that siliconization was effective in preventing contact activation and firm attachment to the glass. To study the thrombin- or ADP-activated Ca^{2+} transient with this technique, the agents were quickly mixed with the platelets on the cover slip and then images were taken.

A second method, the siliconized Millipore® filter/glass cover slip 'sandwich' technique, had the advantage that particular cells could be monitored before, during and after activation with thrombin or ADP. The fluo-3-loaded platelet suspension was placed on a siliconized glass cover slip and a siliconized Millipore® filter (0.5 μm) was placed on the top. Control experiments showed that the filter did not activate the cells. Addition of thrombin or ADP on the *top* of the Millipore® filter resulted in its rapid spreading and diffusion to the cells, which were monitored before and at 6-s intervals.

2.4. Digital fluorescence imaging with fluo-3

The instrumentation and techniques have been previously described [10]. The experiments used a Nikon Diaphot Fluorescence Microscope in the inverted, epi-fluorescence mode with 100 × oil immersion objective. Stage temperature was 25 ± 2°C. Platelets were excited at 490 nm and the emitted light was collected at 530 nm by an intensified television camera (DAGE 66SIT, at fixed gain and voltage). ImagePro software was used to define a field containing as many as 5–10 cells and to track the individual cell fluorescence intensity at measured intervals. Images were captured and digitized by an Image Processor (Model 151; Imaging Technology, Woburn, MA) and stored on an AT microcomputer. For analysis of data, the experimental images were retrieved and the average pixel intensity of each cell was quantified by bracketing the cell with a cursor box and determining mean pixel intensity of the cell (after substraction of background level).

2.5. Quantitation of fluorescence

The pixel intensity (PI; camera output) increases with increasing impinging fluorescent intensity (F) from the cells and examined materials, but saturates at a limiting value for high values of F. The characteristic of PI vs. F was determined at fixed camera gain and voltage by systematically varying the fluorescent intensity (F) of emitting fluorescein-labelled microspheres (Fluoresbrite™ Plain Microspheres, cf. Materials) by insertion of neutral density filters of known transmittance in the excitation beam. The relationship between the pixel intensity minus background (PI) and fluorescence intensity (F) is hyperbolic and can be described by

$$PI = \frac{PI_{max} * F}{F_{1/2} + F} \quad (1)$$

where PI_{max} is the maximal value of pixel intensity and $F_{1/2}$ value is fluorescence intensity for half maximal pixel intensity (PI_{max}). The best fit values were $PI_{max} = 178$ and $F_{1/2} = 0.68$. Rearrangement of Eq. (1) gives,

$$F = \frac{0.68 * PI}{178 - PI} \quad (2)$$

allowing the fluorescence intensity (F) of individual platelets was calculated from PI.

Photobleaching of fluo-3 in the platelets was assessed and corrected by determining F vs time of exposure, for fluo-3 loaded platelets exposed to 3 μM ionomycin in the presence of 2 mM external Ca^{2+}. This put 100% of the cytoplasmically-entrapped indicator in the Ca^{2+}-complexed state. Under these conditions and continuous illumination, pixel intensity was lost with a $t_{1/2}$ of 30 s. Time-dependent platelet changes in PI and F were corrected for this effect.

Pixel intensity analysis gave no evidence of 'hot spots' or sequestration of fluo-3 by organelles. The kinetics of response of the dye to changes in external Ca^{2+} concentration were also approprite for cytoplasmic localization [8].

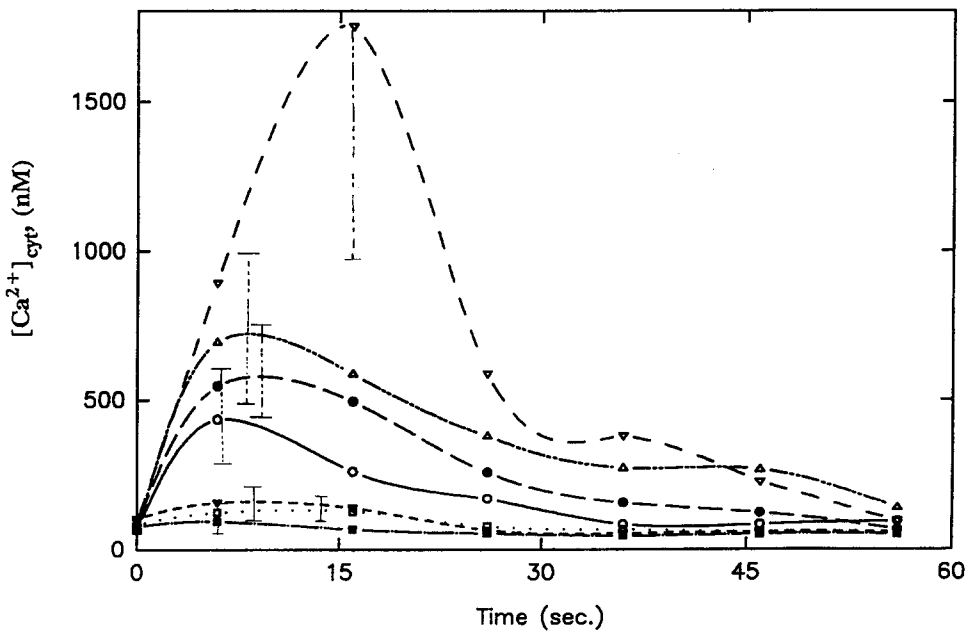

Fig. 1. Typical experiment showing resting $[Ca^{2+}]_{cyt}$ and transient changes in $[Ca^{2+}]_{cyt}$ in 7 platelets in response to 0.2 U/ml thrombin in the presence of 2 mM external Ca^{2+}. The experiment was performed by the Millipore® filter/glass coverslip 'sandwich' configuration described in Section 2. The thrombin addition was made to the top of the Millipore® filter after initial visualization of the 7 platelets. The error bars (± SD) show the domain of 68% certainty in the *absolute* values of $[Ca^{2+}]_{cyt}$ for each platelet. The possibility of any two platelets with non-overlapping error bars having identical $[Ca^{2+}]_{cyt}$ is ≤ 2.5%. No rank correlation was observed between the resting and the peak values of $[Ca^{2+}]_{cyt}$ among individual platelets.

2.6. Measurements in spectrofluorometer

For all reported experimental conditions, parallel experiments were conducted with platelet suspensions in a cuvette at 37°C in a Perkin Elmer (Model MPF-3L) spectrofluorometer at 490 nm excitation and 530 nm emission, allowing comparison with the video fluorescent microscopy results. The total cell-associated fluorescence was determined by digitonin lysis in 2 mM Ca^{2+}. An average intracellular fluo-3 concentration is 0.93 ± 0.30 (S.D.) mmol/l cell volume (averaged over five preparations) was determined from total fluorescence together with the cell number and volume, determined by Coulter counting.

2.7. Determination of cytoplasmic Ca^{2+} concentration with fluo-3

In vitro Ca^{2+} titrations of fluo-3 free acid were done in the spectrofluorometer to determine the K_d of the indicator in 155 mM KCl, 15 mM NaCl, 0.1 mM Mg^{2+}, 50 mM Hepes, pH 6.9. The fluorescence of the Ca^{2+}-free form, F_{min}, was determined by adding 2.0 mM EGTA. Measured

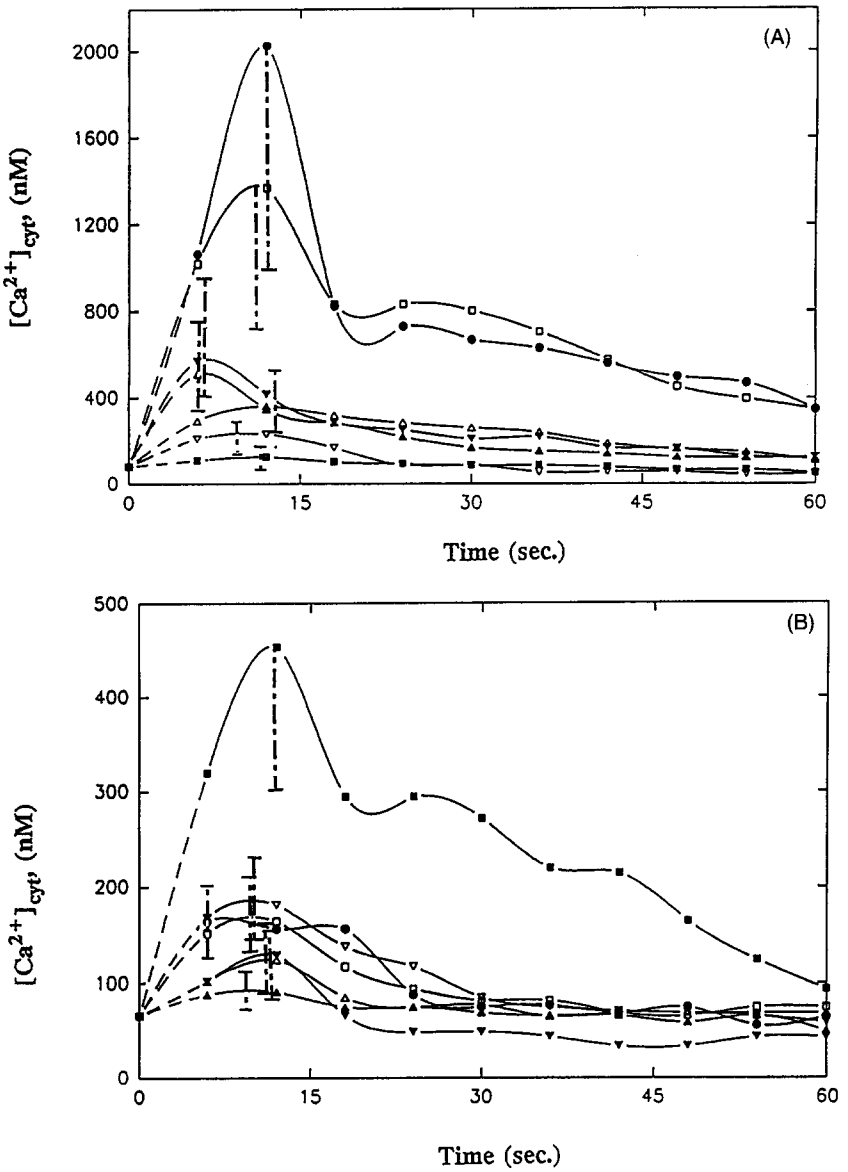

Fig. 2. Typical experiment in the siliconized cover slip configuration, showing $[Ca^{2+}]_{cyt}$ transient in individual platelets in response to 0.2 U/ml thrombin in the presence (A) and absence (B) of 2 mM external Ca^{2+}. In Panel A, platelets were preincubated with 2 mM external Ca^{2+} for 15 min and then thrombin was added. In Panel B (expanded $[Ca^{2+}]_{cyt}$ scale), the platelets were also preincubated with 2 mM external Ca^{2+} for 15 min, then 2.5 mM EGTA was added, and thrombin was added 0.5 min later. In both experiments, the specimens were mounted immediately after thrombin addition ($t = 0$) and the first measurement was made at $t = 6$ s. The plotted values for $t = 0$ are the means determined from platelets to which thrombin was not added. The error bars ($\pm$SD) show the domain of 68% certainty in the *absolute* values of $[Ca^{2+}]_{cyt}$ for each platelet. The possibility of any two platelets with non-overlapping error bars having identical $[Ca^{2+}]_{cyt}$ is $\leq 2.5\%$. Occasional traces seem to reveal secondary peaks, but it was not possible to study the same systematically, owing to their low amplitude.

Table 1
Distribution of peak $[Ca^{2+}]_{cyt}$ in response to activators in the presence of 2 mM external Ca^{2+}

	Total cells	Number of cells showing peak $[Ca^{2+}]_{cyt}$			Ave. $[Ca^{2+}]_{cyt}$ ± S.D. (nM)
		80–200 nM	200–600 nM	> 600 nM	
thrombin	19	6	6	7	672 ± 619
ADP	14	3	7	4	640 ± 642
CPA	13	3	5	5	735 ± 640

Peak $[Ca^{2+}]_{cyt}$ values were determined for each cell in the field after addition of thrombin (0.2 U/ml), ADP (20 μM) or cyclopizonic acid (CPA, 50 μM) in the presence of external Ca^{2+}. Data are cumulative of 3–4 experiments with 4 preparations.

Table 2
Distribution of peak $[Ca^{2+}]_{cyt}$ in response to activators in the absence of external Ca^{2+}

	Total cells	Number of cells showing peak $[Ca^{2+}]_{cyt}$			Average $[Ca^{2+}]_{cyt}$ (nM)
		80–100 nM	100–300 nM	> 300 nM	
thrombin	15	4	9	2	171 ± 105
ADP	16	3	10	3	183 ± 134
CPA	11	0	8	3	289 ± 170

Peak $[Ca^{2+}]_{cyt}$ values were determined for each cell in the field after addition of thrombin (0.2 U/ml), ADP (20 μM) and cyclopizonic acid (CPA, 50 μM) in the absence of external Ca^{2+}. Data are cumulative of 3–4 experiments with 4 preparations.

quantities of Ca^{2+} were added (with NaOH additions to maintain constant pH) to obtain the systematically increasing $[Ca^{2+}]$ values, which were calculated using stability constants obtained from Alexandre Fabiato-Computer Programs [28,29]. The titration curve conformed to Eq. (3) where F is the fluorescent intensity and ΔF_{max} is maximal change of fluorescence from F_{min} after addition of Ca^{2+}.

$$[Ca^{2+}]_{cyt} = K_d \frac{(F - F_{min})}{(F_{max} - F)} \quad (3)$$

The F_{min} was 1.6%–3% of F_{max}. Five repetitions of this experiment gave $K_d = 525 \pm 51$ nM. Repetitions of the experiments in digitonin lysate of fluo-3/AM-loaded platelets gave identical results, indicating that the dye was fully hydrolyzed.

The free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was determined in fluo-3 loaded cells using Eq. (3) using measured F values, F_{max} values obtained in the presence of 2 mM Ca^{2+} and 3 μM ionomycin. In the cells F_{min} was taken as 2% of F_{max} and was not measurably larger than the background seen with unloaded cells, which, in turn, was not larger than the background of the field.

Values of F were determined for individual platelets by video fluorescent microscopy. In each experiment (repeated many times), separate fields of 5–10 platelets were imaged and the average pixel intensity (PI) of each platelet was quantified by bracketing the cell with a cursor box and determining mean pixel intensity of the cell (after substraction of background level). This was converted to F, Eq. (2), which was converted to $[Ca^{2+}]_{cyt}$ Eq. (3). Separate slides prepared from the same sample of platelets treated with 3 μM ionomycin in the presence of 2 mM external Ca^{2+} were used to determine F_{max}. This quantity showed a small inter-platelet variance (SD/mean) of 22% (n = 30). The fluorescence of resting platelets in the presence of 2 mM Ca^{2+} showed still smaller variation (16%). Thus the resting values of $[Ca^{2+}]_{cyt}$ for individual platelets was calculated from Eq. (3), using the ±1.0 SD variation in F_{max} to define the limits of confidence.

The same procedure was applied to experiments reporting $[Ca^{2+}]_{cyt}$ and its ±1.0 SD band of uncertainty as a

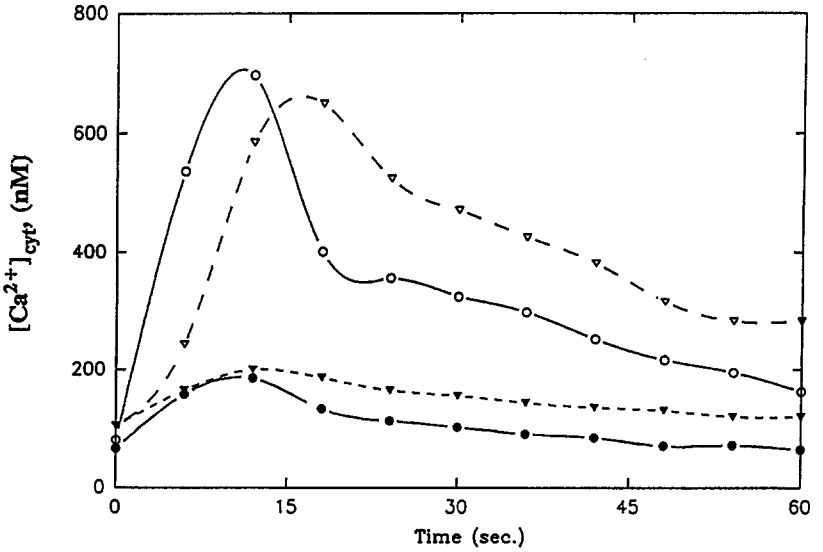

Fig. 3. Comparison of average thrombin-activated $[Ca^{2+}]_{cyt}$ transients measured by fluorescence video microscopy (solid lines) vs spectrofluorimetry (dashed lines). Open symbols are for 2 mM external Ca^{2+}; Solid symbols are for the absence of external Ca^{2+}. The fluorescent video data were taken from Fig. 2. The spectrofluorometer experiments were for stirred platelet suspensions (1.6×10^7/ml) which were otherwise manipulated in a manner identical to that of Fig. 2. The presented experiments were repeated at least three times, yielding similar results.

function of time after activation. Since the uncertainty of $[Ca^{2+}]_{cyt}$ becomes very large for $[Ca^{2+}]_{cyt} \gg K_d$, we have taken 2.1 μM as the highest value which could be accurately reported. When the error bars surrounding $[Ca^{2+}]_{cyt}$ values of two different cells do not overlap, it is 97.5% certain that the $[Ca^{2+}]_{cyt}$ values of the two cells are different.

Further experimentation supports the above-stated limits of confidence. In the experiment of Fig. 1, individual platelets were observed before and during activation with thrombin. Repetition of that experiment, stimulating with ionomycin instead of thrombin causes the intracellular fluo-3 of all platelets to be Ca^{2+}-saturated. That experiment gave no evidence that interplatelet variation in F_{max} contributed systematically to variation in calculated resting $[Ca^{2+}]_{cyt}$ values beyond the predictions of Eq. (3). Furthermore, the experiment of Fig. 1 with thromin gave no evidence for correlation between calculated resting and peak $[Ca^{2+}]_{cyt}$ values. This constitutes further support of the SD-defined confidence limits for $[Ca^{2+}]_{cyt}$ in the presented figures.

3. Results

3.1. Invariance of $[Ca^{2+}]_{cyt}$ in resting platelets

The resting value of $[Ca^{2+}]_{cyt}$ was determined by video fluorescent microscopy in multiple preparations and fields

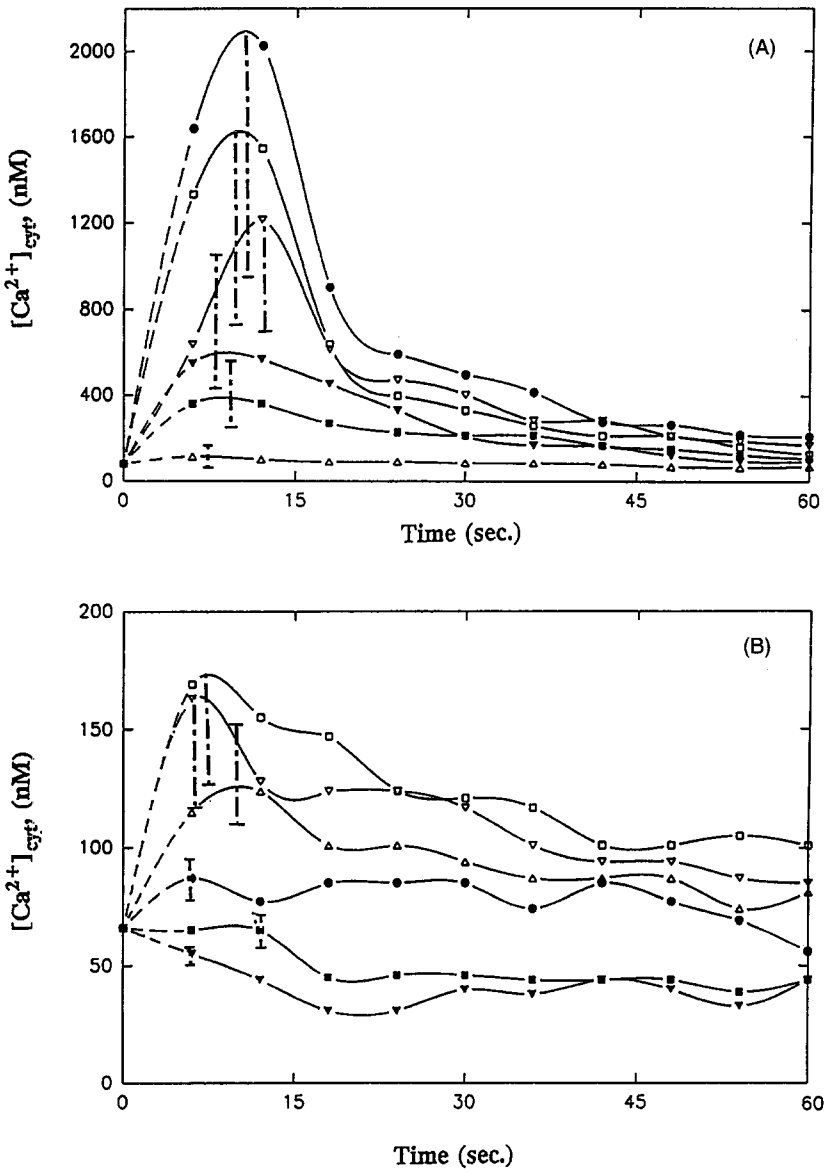

Fig. 4. Typical experiment showing changes in Ca^{2+} concentration in individual platelets stimulated with 20 μM ADP. The protocol of Fig. 2 was repeated except that 6 platelets were viewed and the activator was ADP. Panel A, in the presence of 2 mM external Ca^{2+}; Panel B (expanded scale), 2.5 mM EGTA was added 0.5 min. before addition of ADP, $[Ca^{2+}]_{cyt}$ is plotted scale. Similar results were seen in at least three experiments. The error bars ($\pm$SD) show the domain of 68% certainty in the *absolute* values of $[Ca^{2+}]_{cyt}$ for each platelet. The possibility of any two platelets with non-overlapping error bars having identical $[Ca^{2+}]_{cyt}$ is $\leq 2.5\%$.

of platelets, as described in Section 2. In the presence of 2 mM external Ca^{2+} the average value of $[Ca^{2+}]_{cyt}$ was 86 ± 30 nM ($n = 26$). The S.D. of the calculated $[Ca^{2+}]_{cyt}$ values is 35% of the mean. Of this, 17% is attributable to inter-platelet variation in F_{max}. Thus, the inter-platelet variation of resting $[Ca^{2+}]_{cyt}$ in normal platelets is ≤ 35%, possibly ≤ 18%. In the absence of external Ca^{2+} we obtained $[Ca^{2+}]_{cyt}$ 44 ± 15 nM ($n = 16$).

In separate experiments, continuous monitoring of $[Ca^{2+}]_{cyt}$ in individual platelets showed that $[Ca^{2+}]_{cyt}$ is held constant as a function of time: 30 repeated measurements of fields of platelets ($n = 6$) were made at 3-s intervals. Corrected fluorescent intensities of the cells remained constant and in constant ratio to each other during this time. Time-averaged $[Ca^{2+}]_{cyt}$ values were calculated for each individual platelet together with the S.D. for the repeated measurements. The latter reflects the time-dependent variation of the $[Ca^{2+}]_{cyt}$ of each platelet. The average S.D. value for an individual platelet was 4 nM in the absence of external Ca^{2+} and 10 nM in the presence of 2 mM Ca^{2+}. (The S.D. values of the standard deviations between cells were 1 nM and 3 nM, respectively.) Thus, the time-dependent variation in $[Ca^{2+}]_{cyt}$ was always less than 15% of the platelet's average value, indicating that it is under tight control. We found no evidence for oscillations in the resting state.

3.2. Substantial platelet-to-platelet variation in the $[Ca^{2+}]_{cyt}$ transients upon stimulation with thrombin and ADP

As described earlier, cuvette spectrofluorometer and flow cytometer experiments have given evidence that after thrombin and ADP addition, $[Ca^{2+}]_{cyt}$ increases, reaches a peak value and then undergoes a gradual decline. Our video fluorescence microscope experiments have shown that the platelets do this in approximate synchrony, but with large platelet-to-platelet variation in peak $[Ca^{2+}]_{cyt}$. Fig. 1 presents a typical experiment showing thrombin activation in the siliconized Millipore^{R}/glass cover slip 'sandwich' configuration (Methods). The platelets were incubated in the presence of 2 mM external Ca^{2+} for sufficient time (15 min) to achieve normal resting $[Ca^{2+}]_{cyt}$ and dense tubular Ca^{2+} uptake, and were imaged and at rest, yielding $[Ca^{2+}]_{cyt}$ = 86 ± 13 nM (mean ± S.D.) for 7 platelets. Then 0.2 U/ml thrombin was applied and $[Ca^{2+}]_{cyt}$ was determined for the same 7 platelets at timed intervals. The platelets showed essentially synchronous Ca^{2+} transients, with peak $[Ca^{2+}]_{cyt}$ values reached within 18 sec after thrombin addition. The peak $[Ca^{2+}]_{cyt}$ values in this typical experiment ranged from 110 nM to 1,781 nM, with $[Ca^{2+}]_{cyt}$ = 539 ± 578 nM (mean ± S.D.). The differences in peak $[Ca^{2+}]_{cyt}$ are much larger than the uncertainty of the individual measurements, indicated by the error bars. The above experiment was repeated three times, in three separate preparations, yielding similar results.

When the platelets were activated in the siliconized coverslip 'sandwich' configuration (Methods), large variation in peak $[Ca^{2+}]_{cyt}$ was also seen. Fig. 2A presents a typical experiment showing thrombin-induced changes in $[Ca^{2+}]_{cyt}$ of 7 individual platelets within the same viewing field. A biphasic response was also observed, with peak $[Ca^{2+}]_{cyt}$ values reached between 8 and 12 sec after mixing. The peak $[Ca^{2+}]_{cyt}$ in this experiment ranged from 124 nM to 2050 nM (mean = 740 ± 696 nM, S.D.).

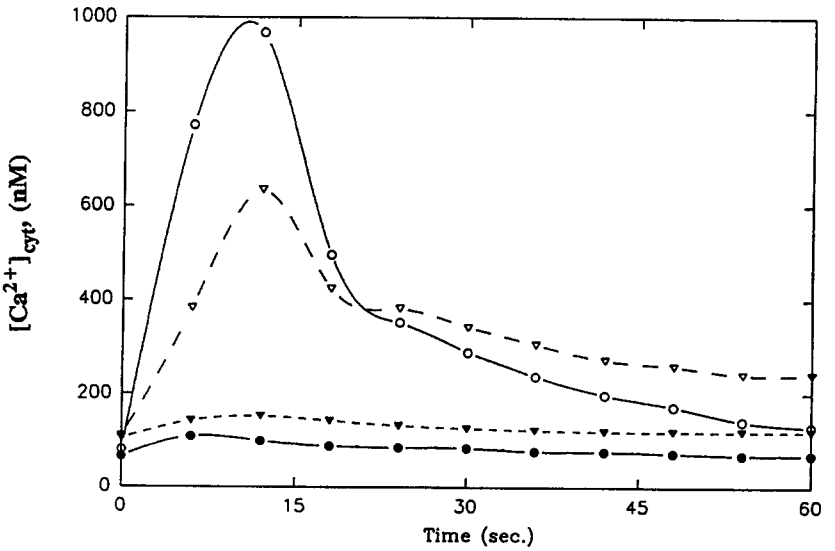

Fig. 5. Comparison of average $[Ca^{2+}]_{cyt}$ transients measured by fluorescence video microscopy (solid lines) vs spectrofluorimetry (dashed lines). Open symbols are for 2 mM external Ca^{2+}; Solid symbols are for the absence of external Ca^{2+}. The fluorescent video data were taken from Fig. 4. The same protocol as in Fig. 3 was repeated except that ADP was added instead of thrombin. The presented experiments were repeated three times, yielding similar results.

The experiment of Fig. 2A was repeated four times with four different donors, and similar time courses were observed. The peak $[Ca^{2+}]_{cyt}$ values were determined and their average values and distributions are presented in Table 1. The average peak $[Ca^{2+}]_{cyt}$ was 672 ± 619. Thus, the variation (S.D./mean) is 92%. The Table also gives information on the distribution of values, showing that approximately equal numbers of platelets have peak $[Ca^{2+}]_{cyt}$ values in the 80–200 nM, 200–600 nM and > 600 nM ranges. Thus the thrombin-stimulated elevation of $[Ca^{2+}]_{cyt}$ shows much greater heterogeneity than does the resting $[Ca^{2+}]_{cyt}$ value.

In the above experiments with activation in the presence of external Ca^{2+}, the Ca^{2+} transient reflects both dense tubular Ca^{2+} release and Ca^{2+} influx. Fig. 2, Panel B presents a typical experiment designed to measure thrombin-stimulated dense tubular Ca^{2+} release alone. The platelets were first exposed to 2 mM external Ca^{2+} for sufficient time (15 min.) to achieve normal resting $[Ca^{2+}]_{cyt}$ and dense tubular Ca^{2+} uptake [7,8] and then external Ca^{2+} was removed with EGTA (to disable influx) before thrombin was added. The figure shows rapid increases in $[Ca^{2+}]_{cyt}$ resulting from Ca^{2+} release from the dense tubule, followed by a decline as Ca^{2+} is actively extruded across the plasma membrane [7]. The peak values also showed a high degree of variation (S.D./mean = 65%).

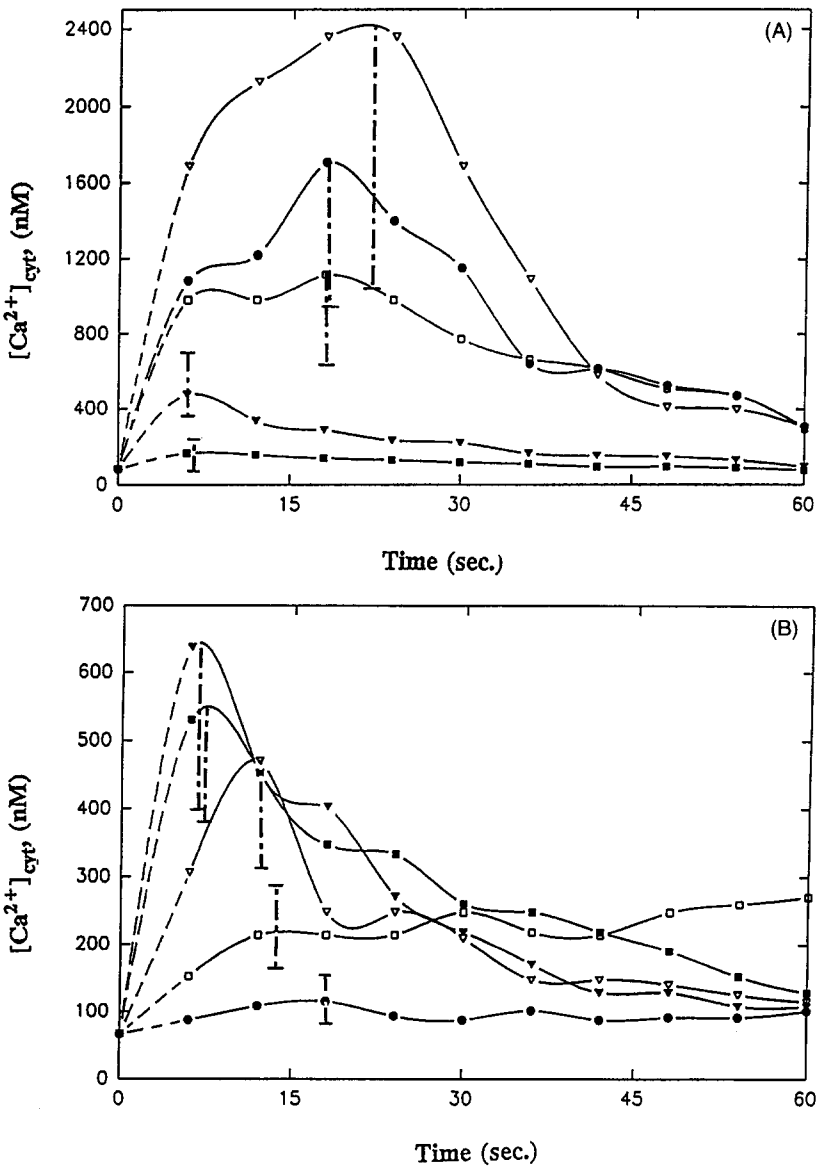

Fig. 6. Typical experiment showing changes in Ca^{2+} concentration in individual platelets treated with 50 μM cyclopiazonic acid (CPA). The protocol of Fig. 2 was repeated except that five platelets were viewed and CPA was added instead of thrombin. Panel A: in the presence of 2 mM external Ca^{2+}. Panel B (expanded scale): 2.5 mM EGTA was added 0.5 min. before addition of CPA. Similar results were seen in at least three experiments. The error bars (± SD) show the domain of 68% certainty in the *absolute* values of $[Ca^{2+}]_{cyt}$ for each platelet. The possibility of any two platelets with non-overlapping error bars having identical $[Ca^{2+}]_{cyt}$ is ≤ 2.5%.

The experiment was repeated three times with three different blood donors and the cumulative data are entered in Table 2. The average peak value was 171 ± 105 nM and showed large variation between platelets (S.D./mean = 61%). Thus there are large differences between individual platelets in the thrombin-stimulated dense tubular Ca^{2+} release.

As noted earlier, cuvette spectrofluorometer experiments average over tens of thousands of platelets and can not reveal such differences. This leads to the question of how accurately they report the time course of the average $[Ca^{2+}]_{cyt}$ during activation. For this purpose, we conducted spectrofluorometer experiments in parallel under essentially the same conditions. Fig. 3 compares the mathematically averaged $[Ca^{2+}]_{cyt}$ from video microscopy with the $[Ca^{2+}]_{cyt}$ from the spectrofluorometer experiments. The comparison shows that the two methods deliver *average* $[Ca^{2+}]_{cyt}$ transients of similar shape and magnitude. The figure also illustrates the fact that thrombin-induced Ca^{2+} increase in the presence of external Ca^{2+} (representing Ca^{2+} influx and intracellular Ca^{2+} release) is larger than the increase in the absence of external Ca^{2+} (representing release only), as has been observed previously using quin-2 in platelet suspensions [7].

The above experimentation was repeated using 20 μM ADP as agonist. Fig. 4 presents a typical experiment showing the time courses of ADP-induced changes. The procedures and format are otherwise identical to Fig. 2 (thrombin). The peak value of the ADP-induced $[Ca^{2+}]_{cyt}$ transient also shows considerable platelet-to-platelet variation both in the presence and absence of extracellular Ca^{2+}. Tables 1 and 2 present the results of three repetitions of the experiment. Peak values showed large variation both in the presence of Ca^{2+} (S.D./mean = 100%) and in the absence of Ca^{2+} (S.D./mean = 73%).

Fig. 5 compares the above platelet-averaged ADP-stimulated $[Ca^{2+}]_{cyt}$ transient with a cuvette spectrofluorometer experiment. As was the case for thrombin, the two methods deliver *average* $[Ca^{2+}]_{cyt}$ transients of similar shape and magnitude.

3.3. Inter-platelet variation in the size of the CPA-releasable internal Ca^{2+} pool

A remaining question was whether the large inter-platelet variability in thrombin- or ADP-stimulated dense tubular Ca^{2+} release (Fig. 2B and 4B) was due differences in dense tubular pool size, or resulted from differences in its releasability. We probed the dense tubular pool size using cyclopiazonic acid (CPA). This agent causes intracellularly stored Ca^{2+} to be lost to the cytoplasm where it is registered as an increased $[Ca^{2+}]_{cyt}$. The compound has been shown to be an effective inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase [11,12] and is seeing widespread use as a means of releasing ER-stored Ca^{2+}. Fig. 6B is a typical experiment showing the time course of the increase in $[Ca^{2+}]_{cyt}$ resulting from CPA-induced loss of Ca^{2+} from the internal store to the cytoplasm when platelets are challenged with 50 μM CAP in the *absence* of external Ca^{2+}. The inhibitor causes $[Ca^{2+}]_{cyt}$ to increase, reaching peak values between 6 and 24 s. This is followed by a decline in $[Ca^{2+}]_{cyt}$ corresponding to active Ca^{2+} extrusion across the plasma membrane. Fig. 6B shows that the peak values exhibit large differences be-

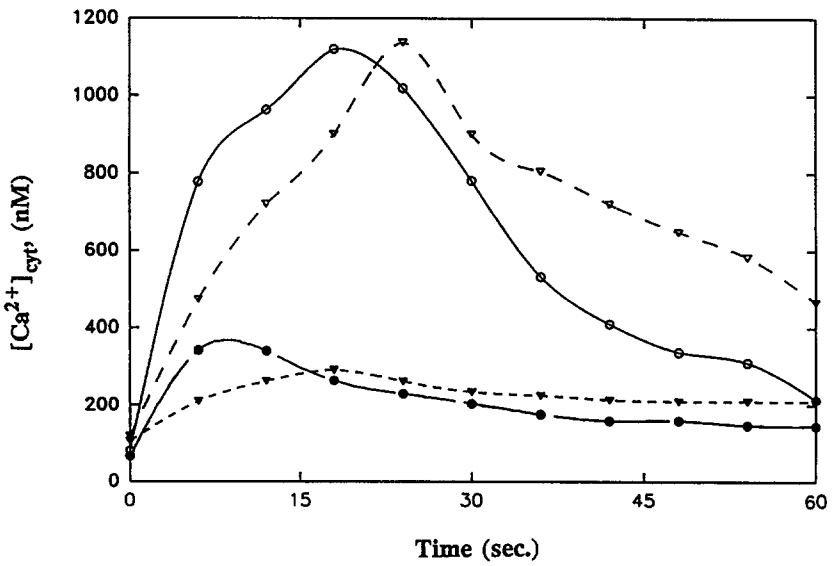

Fig. 7. Comparison of average CPA-induced $[Ca^{2+}]_{cyt}$ transients measured by fluorescence video microscopy (solid lines) vs spectrofluorimetry (dashed lines). Open symbols are for 2 mM external Ca^{2+}. Solid symbols are for the absence of external Ca^{2+}. The fluorescent video data were taken from Fig. 6 and the spectrofluorometer experiment was performed in parallel as in Fig. 3. The presented experiments were repeated three times, yielding similar results.

tween individual platelets, suggesting that the size of the CPA-inhibitable intracellular store is quite variable between platelets. Table 1 gives data for three repetitions of the experiment. The average elevation in $[Ca^{2+}]_{cyt}$ is 289 ± 170 nM, corresponding to a 59% variation in this property (S.D./mean).

Fig. 6A shows the time-course of the above experiment repeated in the *presence* of 2 mM external Ca^{2+}. Similar but larger $[Ca^{2+}]_{cyt}$ transients are observed. Table 1 gives data for 3 repetitions of this experiment. The average elevation in $[Ca^{2+}]_{cyt}$ is 735 ± 640 nM, corresponding to an 87% variation in the amplitude of this phenomenon (S.D./mean). The larger amplitude may reflect Ca^{2+}-induced Ca^{2+} influx, although the experiment does not rule out CPA effects on pumps or channels in the plasma membrane.

The platelet-averaged $[Ca^{2+}]_{cyt}$ data were also compared with data obtained from a spectrofluorometer experiment. Fig. 7 presents this comparison in the same format as Fig. 3. Once again, the two methods are found to deliver *average* $[Ca^{2+}]_{cyt}$ transients of similar shape and magnitude.

4. Discussion

The present study has demonstrated that $[Ca^{2+}]_{cyt}$ is tightly controlled in the resting state. The variation in $[Ca^{2+}]_{cyt}$ measured by fluo-3 between individual unstimulated platelets is 35%; the variation in $[Ca^{2+}]_{cyt}$ for an individual platelet as a function of time is 12%. For proper hemostatic function it is important that platelet $[Ca^{2+}]_{cyt}$ be reliably maintained below the activation threshold, since platelet activation is an essentially irreversible phenomenon. Also, the resting $[Ca^{2+}]_{cyt}$ influences the level of releasable Ca^{2+} in the dense tubular store and thus determines the strength of stimulus necessary to trigger platelet aggregation [13].

The present study has shown that the $[Ca^{2+}]_{cyt}$ vs time profile measured with fluo-3 after thrombin- or ADP-stimulated activation is similar for all platelets, but that peak $[Ca^{2+}]_{cyt}$ values show wide variation. This is true when the $[Ca^{2+}]_{cyt}$ increase is driven by Ca^{2+} influx plus release ($[Ca^{2+}]_o = 2$ mM) or by release alone ($[Ca^{2+}]_o = 0$ mM). Thrombin activation was at 0.2 U/ml, which is close to its EC_{50} value [7]; ADP was applied at a maximally-effective concentration.

As noted earlier, experiments using cuvette spectrofluorometers are not capable of showing these differences between platelets. However, the *average* $[Ca^{2+}]_{cyt}$ calculated by the two methods is in surprisingly good agreement. There is also good agreement with time-dependent changes in $[Ca^{2+}]_{cyt}$ distribution observed in flow cytometric studies under similar conditions [4,5]. These studies showed that the distribution of $[Ca^{2+}]_{cyt}$ values is broadened and shifted to higher levels after activation [4,5]. The present study indicates the broadening must be the result of differences in peak amplitude, and not differences in time course. The present study shows that the platelets are essentially synchronized in their increase and subsequent decline in $[Ca^{2+}]_{cyt}$.

A significant finding in the present study was the absence of oscillations in $[Ca^{2+}]_{cyt}$. Our cell containment system used silicone-coated cover slips in order to minimize platelet interaction with the glass and subsequent activation. A video microscope study by other groups [14–16] reported oscillations of platelets on fibringen-coated glass cover slips. Placement on the fibrinogen-coated cover slips was itself sufficient to produce 'spiking' of $[Ca^{2+}]_{cyt}$ and oscillations in its level [14]. In the above-cited study, the agonists ADP and thrombin increased the frequency of the oscillation. A study of rabbit platelets immobilized on a poly-ethyleneimine-coated surface showed that addition of serotonin can initiate oscillatory behavior of $[Ca^{2+}]_{cyt}$ [17]. Two possible explanations for the difference can be considered: (a) that oscillatory behavior of $[Ca^{2+}]_{cyt}$ is more likely to be associated with activation in the attached mode whereas the biphasic $[Ca^{2+}]_{cyt}$ transient (observed here) may be more characteristic of platelets activated in suspension or (b) that the Ca^{2+} buffering contribution of fluo-3 eliminated oscillations which would otherwise have occurred. Disfavoring interpretation (b) are calculations which show that the fluo-3 buffer capacity is small compared to that of the cytoplasm under our conditions. Use of the equilibrium equation and constant for Ca^{2+}-fluo-3 binding together with 0.97 ± 0.30 mmol fluo-3 per liter cell volume ('mM') predicts that 0.2 mM, 0.5 mM and 0.9 mM Ca^{2+} will be bound to fluo-3 for $[Ca^{2+}]_{cyt} = 200$, 600 and > 1500 nM, respectively. The corresponding amounts of Ca^{2+} bound to the platelet's intrinsic binding sites is estimated as 0.5 mM, 2.0 mM and ≥ 2.9 mM, respectively (cf. Fig. 5, ref. [9]).

It is interesting that inter-platelet variation in agonist-stimulated peak $[Ca^{2+}]_{cyt}$ was found under all conditions: For both thrombin and for ADP activation, and under experimental conditions allowing both influx plus dense tubular release or dense tubular release only. The size of the dense tubular store is itself subject to wide inter-platelet variation, as revealed in the CPA experiments. The biochemical events giving rise to the $[Ca^{2+}]_{cyt}$ transient (including agonist binding, G-protein coupled to phospholipase C, production of inositol 1,4,5-trisphosphate and diacylglycerol, etc.) have been recently reviewed [1,2,18]. Differences in any of these components might show platelet to platelet variation if these biochemical reactions were experimentally accessible on the single cell level. However, variation in the dense tubular Ca^{2+} pool may be a sufficient explanation since the elevation of $[Ca^{2+}]_{cyt}$ is subject to positive feedback through the Ca^{2+}-dependent activation of phospholipase A_2 [18] and Ca^{2+}/Ca^{2+} positive feedback on the level of the thrombin-activated recep-

tor [19]. Further manipulation of the dense tubular pool size by combinations of agents [20] may be informative.

The origins of the decline in $[Ca^{2+}]_{cyt}$ subsequent to achieving peak value have not received much attention. In fact, its physiological significance remains essentially unexplained. On the Ca^{2+} handling level, we have suggested that the decline in $[Ca^{2+}]_{cyt}$ is due to the combined effects of Ca^{2+} sequestration, Ca^{2+} extrusion, plus the inactivation of the Ca^{2+} influx and Ca^{2+} release processes [7,18]. Resequestration by the dense tubules was seen with thrombin [7]. We have recently presented evidence that elevated $[Ca^{2+}]_{cyt}$ increases the rate of its own extrusion across the plasma membrane by the mechanism of calcium-calmodulin activation of the Ca^{2+} pump located in the plasma membrane [9]. Murray et al [21], have shown that the thromboxane A_2 (TXA_2) receptor rapidly desensitizes after its activation, providing a precedent for channel desensitization. We believe that desensitization of the ADP-operated channel [22] and GTP-binding protein-operated channels [23] are also possibilities which should be investigated [18].

Heterogeneity in the thrombin- and ADP-activated Ca^{2+} transients has direct relevance to platelet physiology and pathology. Spectrofluorometer studies [24] of platelet suspensions have shown that the *average* $[Ca^{2+}]_{cyt}$ of the population must increase to certain critical levels for the population to exhibit the shape change ($[Ca^{2+}]_{cyt} = 0.3–0.5$ μM), the exocytotic release reaction (ca. 1 μM) and aggregation (ca. 1 μM). If these thresholds are the same for all platelets, then our data suggest that the platelets which show higher peak $[Ca^{2+}]_{cyt}$ values will be preferentially activated and will then activate the other platelets (via ADP, 5-HT and TXA_2 release). The average resting $[Ca^{2+}]_{cyt}$ for patients with hypertension [25], and arterial or venous thrombosis [26,27] are elevated. This leads to the question of whether the higher than average resting $[Ca^{2+}]_{cyt}$ in these disorders is due a specific subpopulation with high resting $[Ca^{2+}]_{cyt}$ and increased reactivity to activating stimuli.

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