Simultaneous Flow Cytometric Measurements of Thrombin-induced Cytosolic pH and Ca\(^{2+}\) Fluxes in Human Platelets*

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Human platelets exhibit an extremely rapid increase in cytoplasmic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{i}\)]\(_{o}\)) and a dose-dependent cytoplasmic pH change ([pH]\(_{i}\)) upon thrombin stimulation. A cytoplasmic alkalization, maximal by 60 s, is preceded by a very rapid acidification, which is masked by the alkalization when saturating thrombin doses are used. Using the pH probe 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein we report here the kinetics of simultaneous cytoplasmic pH and Ca\(^{2+}\) changes in thrombin-stimulated platelets, measured in single cells by flow cytometry. This permits analysis of the responding subpopulation. Maximal thrombin stimulation ([pH]\(_{i}\)) induces a dose-dependent increase in pH\(_{i}\) from approximately 7.0 to 7.30 and a maximal [Ca\(^{2+}\)\(_{i}\), transient of up to 800 nM. The Ca\(^{2+}\) transient coincides temporally with the rapid initial acidification, while the alkalization is maximal considerably later. The Ca\(^{2+}\) transients occur max- imally in each responding cell, but occur only in a subpopulation of the platelets at subsaturating (<4.5 nM) thrombin doses; in contrast, the dose-dependent cytoplasmic acidification appears to occur uniformly in all platelets. The rapid increase in [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) is not dependent on the alkalization, and the former occurs maximally in amiloride treated, Na\(^+\)/H\(^+\) exchange inhibited human platelets. These results indicate that the acidification and the rise in [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) may be interre- lated, whereas the cytoplasmic alkalinization (maxi- mal considerably later than either the acidification or the [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) rise) may be independent of these earlier, temporally correlated increases in H\(^{+}\) and Ca\(^{2+}\) concentra- tions.

We have previously shown that stimulation of human platelets by \(\alpha\)-thrombin is accompanied by a rapid dose-dependent membrane depolarization (1–3) and a cytoplasmic alkalinization (4–7). These changes start immediately, but attain their maxima later than the temporally correlated rapid Ca\(^{2+}\) transient (8 11) and the formation of products of phosphoi- nositide metabolism (11–20); however, they precede platelet lysosomal granule release (6) and aggregation (20). While the role of the cytoplasmic alkalinization in the overall mechanism of platelet activation is unclear, it appears to be coupled to a concomitant sodium influx since both are blocked with dimethyl amiloride (3, 5, 21, 23). The acidification is not abolished by blockage of Na\(^+\)/H\(^+\) countertransport nor by chelation of cytoplasmic Ca\(^{2+}\) after amiloride (21) and BAPTA (5,5'-dimethyl-bis-(o-aminophenoxy)ethane-N,N',N\(^\prime\),N\(^\prime\)-te- racteacetic acid) (22) pretreatment, respectively; the acidification thus appears to be independent of the sequential H\(^{+}\) efflux-Na\(^{+}\) influx (23) as well as the rapid [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) transient (22).

There is considerable controversy regarding the role and interdependence of the cytoplasmic alkalinization and [Ca\(^{2+}\)\(_{i}\)\(_{o}\)]\(_{i}\) fluxes. Siffert and colleagues (7, 24–26) have suggested that Na\(^+\)/H\(^+\) exchange is a necessary prerequisite for Ca\(^{2+}\) mobilization since the Ca\(^{2+}\) rise in addition to the alkalization is inhibited after pretreatment by amiloride. Additionally, they demonstrated that artificial alkalinization alone is not suffi- cient (27) to induce Ca\(^{2+}\) mobilization. In contradiction to Siffert, Sage and Rink (28) have shown that the thrombin-induced Ca\(^{2+}\) rise is present after substitution of Na\(^{+}\) with choline in normal buffers and, using stopped flow techniques, have demonstrated the presence of a Ca\(^{2+}\) transient within 200–300 ms. Recently, Zavoico and Cragoe (29) presented simultaneous [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) and [pH]\(_{i}\) measurements in human platelets in suspension: they concluded that Ca\(^{2+}\) mobilization does not require activation of the Na\(^{+}\)/H\(^{+}\) exchanger. The data presented here corroborate the work by Zavoico and Cragoe (29). In addition, our single cell studies provide evidence for the presence of platelet subpopulations, in contrast to studies of whole cell suspensions measured by other inves- tigators. We demonstrate here that the thrombin-induced platelet cytoplasmic acidification is not dependent on the presence of a [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) rise.

6-Carboxyfluorescein has previously been used to measure cytoplasmic pH changes (5, 7, 30) in suspension, yielding results similar to the improved 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)\(^1\) technique (7, 30). We have previously described the necessary correction for the extensive probe leakage encountered in human platelets (30). Here we use flow cytometric analysis which offers a distinct advantage over our previous suspension studies since one looks only at intracellular fluorescences (and thus no leakage corrections are needed).

Simultaneous measurements of Ca\(^{2+}\) and pH in human platelets using a dual laser flow cytometer permit single cell analysis of two activation parameters simultaneously. Our previous studies with Indo-1 (11, 22) have demonstrated the advantages of flow cytometric subpopulation analysis of the [Ca\(^{2+}\)\(_{i}\)]\(_{o}\) changes observed upon platelet stimulation with sub- saturating concentrations of \(\alpha\)-thrombin. We show here that simultaneous pH and Ca\(^{2+}\) measurements on the FACS permit subpopulation analysis without extracellular probe interfer- ence and allow the temporal relationship between these activ- ity parameters to be investigated.

\(^{1}\) The abbreviations used are: BCECF, 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein; FACCS, fluorescence activated cell sorter; HEPES, N-2-hydroxyethylpiperazine-N\(^\prime\)-2-ethanesulfonic acid; AM, acetoxymethyl ester; MCF, mean channel fluorescence.
EXPERIMENTAL PROCEDURES

Materials

Sepharose, apyrase, HEPES, nigericin, and dimethyl sulfoxide were purchased from Sigma. The fluorescent probes 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM), and Indo-1 acetoxymethyl ester were obtained from Molecular Probes Inc. (Eugene, OR). Dimethylmethyloleat was the gift of Dr. W. Hendler, Merck, Sharp and Dohme. All other chemicals were reagent grade.

Methods

Platelet Preparation—Blood was drawn from normal human volunteers and anticoagulated with 0.38% sodium citrate. Platelets were isolated by gel filtration using a Sepharose 2B column preequilibrated with HEPES buffer (137 mM NaCl, 3.3 mM NaH2PO4, 5.5 mM D-glucose, 2.7 mM KCl, 3.8 mM HEPES, 0.98 mM MgCl2, and 0.15 unit/ml apyrase), pH 7.4, as described previously (2, 9).

Thrombin Preparation—Parte-Davis bovine topical thrombin was further purified according to Lundblad et al. (31). Thrombin activity was expressed in fibrinogen clotting units (31).

BCECF-AM Loading—Platelets were washed at pH 7.0 were incubated at $6 \times 10^{11}$/ml with 1 $\mu$m BCECF-AM (1 mm stock in dimethyl sulfoxide) for 12 min at 37°C. After the incubation, 50 $\mu$m EDTA was added, and the platelets were centrifuged for 10 min at 25°C, 128 X g. The supernatant was discarded and the pellet gently resuspended in 0.2 ml of HEPES buffer, pH 7.4, with 3 mM EDTA. The resuspended pellet was then diluted to a final concentration of $3 \times 10^{10}$/ml for suspension studies or $1 \times 10^{10}$/ml for individual cell studies. Platelets/ml with HEPES buffer, pH 7.4, for thrombin response measurements, or K+HEPES (140 mM KCI, 3.8 mM HEPES, 5.5 mM D-glucose, 3.3 mM KH2PO4, 1 mM MgCl2, and 0.15 unit/ml apyrase) at varying pH values, immediately before use, for pH calibration curves (32).

Measurement of Cytosolic pH Changes in Suspension and in Individual Cells—Platelets loaded with BCECF as described previously (30) were diluted to $3 \times 10^{10}$/ml for suspension studies or $1 \times 10^{10}$/ml for FACs studies. Suspension studies were performed as described (30); the rate and the extent of the pH change were evaluated by measuring either the slope normalized to the baseline fluorescence at 450 nm (slope/450) or the relative change in emission at 530 nm (excitation = 488 nm) is expressed in fibrinogen clotting units (31).

RESULTS

BCECF-loaded platelets were stimulated with various doses of $\alpha$-thrombin and monitored at 4-s intervals (4000 cells/interval) for 1 min on the FACs. Loading of the platelet with probe did not alter the platelets' ability to exhibit normal depolarization or to degranulate (30). The platelets did not exhibit any significant autofluorescence in the BCECF-sensitive wavelengths. Quantitation of actual pH changes was achieved from a calibration curve prepared by adding the ionophore nigericin (2 $\mu$m) to cells in K+HEPES buffer at varying pH values as described previously (30). The calibration curve utilizing the ratio technique (Fig. 1a) indicated that the resting pH of the platelet (the pH at which nigericin yields no change in fluorescence) was 6.97 ± 0.04 (n = 26, mean ± S.D.); a comparable curve using only the relative change in fluorescence at 530 nm (excitation = 488 nm) is shown in Fig. 1b and resulted in a resting pH of 7.01 ± 0.02 (n = 26, mean ± S.D.). Both values correlate well with our previously published value of 6.99 ± 0.07, found in suspension studies (30). The calibration curve in Fig. 1b was used to quantitate pH change in all experiments when simultaneous [Ca2+]i and [pH]i were measured, since the ratio technique could not be used due to the limitations of our present flow cytometric set-up.

A typical histogram representative of our flow cytometric
Cytoplasmic pH and Ca\(^{2+}\) in Human Platelets

Data is shown in Fig. 2. A shift in the entire population of platelets was observed upon addition of thrombin. A change in pH was observed at our first time point (15 s), the time to maximal change being dose-dependent; the time varied from 40 to 60 s. The broad population distribution in platelet resting internal pH is similar to that of resting [Ca\(^{2+}\)]\(_i\); both are probably attributable to the broad platelet size distribution and to the variability in individual cell organelar contents. The thrombin dose dependence, as calculated by both quantitation techniques described above, is shown in Fig. 3; the curves are nearly superimposable and demonstrate a maximal pH change of 0.27 ± 0.06 (n = 8, mean ± S.D., relative emission 530 nm) and 0.24 ± 0.02 (n = 12, mean ± S.D., 488/454 excitation ratio) when a saturating dose of thrombin (9.0 nM) was used as the stimulus. The large error bars reflect significant donor variability as well as the broad resting pH\(_m\) (Fig. 2). For any one donor, the standard error of the mean was much lower (8.3 to 11.1%) than that observed for four donors (n = 8, maximal S.E. = 23%). Thus, flow cytometric analysis enables us to conclude that the thrombin-induced alkalinization is maximal within 60 s and that the time to maximal alkalinization and the final extent are both dose-dependent.

We further investigated the rapid platelet acidification by amiloride pretreatment prior to thrombin stimulation. Since the acidification is extremely rapid and therefore is masked at saturating thrombin doses by the alkalinization, blockage of Na\(^+\)/H\(^+\) countertransport with amiloride permitted accurate quantitation of its dose dependence.

Flow cytometry further permitted the determination of the presence or absence of subpopulations exhibiting acidification. Single cell analysis of the thrombin dose dependence of the acidification by flow cytometry and suspension analysis by fluorometry are shown in Fig. 4. Simultaneous experiments were performed on the cytometer and in suspension. The dose dependence was comparable by both techniques (Table I). The acidification was observed in the entire platelet population even at submaximally activating thrombin doses (0.45 nM) indicating that, unlike the Ca\(^{2+}\) response, the thrombin dose-dependent cytoplasmic acidification was exhibited in every cell in response to low thrombin doses. The addition of 10\(^{-4}\) M dimethylamiloride reduced the platelet resting pH by approximately 0.05 pH units. Subsequent thrombin stimulation elicited a further 0.25-0.4-unit pH decrease. In summary, amiloride pretreatment resulted in a thrombin-induced acidifica-
Table I
Suspension analysis of platelet pH dose response to thrombin in presence of dimethylamiloride

| Thrombin unit/ml | Relative rate of acidification | ΔpH | pH change |
|------------------|------------------------------|-----|-----------|
| 0.0025           | -0.207 ± 0.049               | -0.066 ± 0.009 | -0.25 |
| 0.005            | -0.233 ± 0.042               | -0.061 ± 0.014 | -0.28 |
| 0.01             | -0.251 ± 0.054               | -0.080 ± 0.021 | -0.30 |
| 0.025            | -0.334 ± 0.059               | -0.103 ± 0.023 | -0.39 |
| 0.05             | -0.342 ± 0.059               | -0.113 ± 0.024 | -0.41 |

- Rate of pH change measured as the rate of change of slope (cm/min) normalized to the baseline emission (excitation 450, emission 530 nm, i.e., slope/450 nm).
- Relative change in emission at excitation 590 nm before and after thrombin stimulation, normalized to the baseline emission at excitation 450 nm.

FIG. 5. Simultaneous pH and Ca++ time course tracings of thrombin-induced, amiloride-pretreated platelets. Platelets loaded simultaneously with 2 μM Indo-1 and 1 μM BCECF were stimulated with 0.9 mM and 9 mM thrombin and their fluorescence emissions monitored continuously for 90 s. The Indo-1 tracings (a) and the BCECF tracings (b) represent the time course after thrombin stimulation of the Ca++ responding cells. Also included are tracings after pretreatment with 10^-4 M amiloride for 2 min for 0.9 mM thrombin + amiloride (O) and 9.0 mM thrombin + amiloride (□). Data are representative tracings from one of three separate experiments.

DISCUSSION
Flow cytometry permits responses of subpopulations of cells to be measured continuously. BCECF has been used previously to measure pH change on the FACS (33) as has Indo-1 to measure Ca++ (11, 30, 34), but this is the first report of simultaneous observations, permitting analysis of the interrelationships between [Ca++]i transients and cytoplasmic pH changes. Flow cytometry offers several advantages over suspension fluorometric techniques including 1) a smaller number of cells required per run; 2) continuous monitoring of subpopulation responses without the intrinsic errors induced by probe leakage; and 3) simultaneous observation of several activation parameters. Limitations include the time required to stimulate, mix, and collect the first data point, usually 10–15 s, and the availability of only three photomultipliers and two lasers, requiring pH to be evaluated from the reading of the pH-sensitive wavelength rather than a ratio. Our results demonstrate a rapid (within 15 s) cytoplasmic acidification coincident with a [Ca++]i transient increase upon thrombin stimulation, and a dose-dependent alkalinization becoming dependent, the proportion of cells responding being dose-dependent. In addition, platelets pretreated with amiloride prior to addition of thrombin (0.9, 9.0 nM) also exhibited a near normal, maximal Ca++ rise, indicating that amiloride pretreatment did not affect the Ca++ fluxes. In all experiments, the maximal Ca++ level was observed at the first time point, i.e. 15 s (due to instrumental limitations, earlier time points could not be attained).

Fig. 5b demonstrates the relative pH change in these same, simultaneously analyzed thrombin-stimulated and amiloride pretreated, Ca++ responding platelets. When 0.9 nM thrombin (a subsaturating dose) was used, a rapid acidification (0.10 pH units) was detected prior to the alkalinization. The alkalinization was dose-dependent (0.143 ± 0.086 versus 0.098 ± 0.098 pH units for 0.9 and 9.0 nM α-thrombin, respectively) and was maximal by 40–60 s. The acidification was not detectable in the 9.0 nM tracing, possibly due to masking of the much smaller acidification by the large rapid alkalinization. The thrombin-induced alkalinization was totally inhibited after preincubation with 10^-4 M amiloride for 2 min (Fig. 5b) and the acidification was significantly prolonged. These data represent only the cells exhibiting a Ca++ rise. We also analyzed the pH change in those cells not exhibiting a Ca++ rise and found the pH curves to be identical. The acidification was therefore thrombin dose-dependent and exhibited in the entire population of platelets. Our results prevented us from drawing any definitive conclusions regarding the presence or absence of subpopulations exhibiting the alkalinization, since subsaturating thrombin concentrations induce a alkalinization too small to be analyzed by our present instrumentation (≤-0.26 versus ≤0.15 pH units, for 0.9 nM + amiloride and 0.9 nM, respectively).

Table II shows a summary of the simultaneous pH and Ca++ data. The Ca++ transient was maximal in responding control and amiloride-treated platelets, and its magnitude was dose-independent. The percent of cells exhibiting a Ca++ rise in control cells was dose-dependent, and, after amiloride pretreatment, there was a significant reduction in this percentage. This reduction is due to amiloride pretreatment and not due to a lower resting pH since addition of ammonium chloride (6 mM) to readjust the resting pH (after amiloride pretreatment) did not increase the percentage of the cells responding (data not shown). The alkalinization was thrombin dose-dependent in control platelets, as was the acidification in amiloride-treated platelets.
maximal considerably later than this acidification and Ca\(^{2+}\) transient. In many cases, especially at saturating thrombin doses when the rate of alkalinization was near maximal, the elapsed time prior to collection of the first time point was too long to permit observation of the initial rapid acidification. Since the acidification was obscured by the alkalinization, its long duration 

\[ \text{alkalinization} \]

was also a significant reduction in the percentage of cells responding with a Ca\(^{2+}\) rise. All these results also explain why other investigators, not able to examine Ca\(^{2+}\) subpopulations by flow cytometry and therefore analyzing the average of cells in suspension (responding and not responding), have reported a decreased magnitude of Ca\(^{2+}\) transient in the presence of amiloride. Our findings imply that the thrombin-induced acidification and Ca\(^{2+}\) transient are independent of the alkalinization and that the two H\(^{+}\) fluxes observed upon thrombin stimulation are controlled differently.

Finally, previous studies (22) totally chelating intracellular Ca\(^{2+}\) demonstrated the presence of the acidification (and absence of subsequent alkalinization) after thrombin stimulation. We show here that this acidification was dose-dependent in a uniform platelet population (in contrast to the Ca\(^{2+}\) rise which was dose-independent and which occurred in a dose-dependent percentage of the population). It should be noted that cytoplasmic acidification is the only activation parameter, of those we have measured so far, that occurs in the absence of a membrane depolarization, a cytosolic alkalinization, and a rapid cytosolic [Ca\(^{2+}\)]\(_{in}\) rise. We conclude that not only is the alkalinization independent of the Ca\(^{2+}\) and H\(^{+}\) concentration increases but that the accumulation of cytoplasmic H\(^{+}\) may be one of the early thrombin-induced activation signals.

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**Table II**

Simultaneous single cell analysis, pH and Ca\(^{2+}\) dose response to thrombin in the presence of amiloride

Platelets simultaneously loaded with Indo-1-AM (2 μM) and BCECF-AM (1 μM) were subsequently stimulated with varying doses of thrombin. Their relative intracellular pH and Ca\(^{2+}\) changes were monitored on the fluorescence activated cell sorter. The Ca\(^{2+}\) data presented are the maximal Ca\(^{2+}\) levels in the subpopulation exhibiting a change. The pH data are representative of the entire population. All values are represented as mean ± S.D. (n ≥ 5).

| Thrombin Maximal Ca\(^{2+}\) | Maximal Ca\(^{2+}\) | Relative pH change | Relative pH change | % responding | % responding |
|-----------------------------|---------------------|--------------------|--------------------|--------------|--------------|
| units/ml | nm | nm | | | |
| 0.0025 | 808.14 ± 31.66 | 815.27 ± 85.08 | 0.119 ± 0.086 | -0.323 ± 0.088 | 77.14 ± 5.9 | 47.02 ± 14.2 |
| 0.05 | 777.79 ± 66.35 | 815.28 ± 121.8 | 0.143 ± 0.086 | -0.357 ± 0.137 | 80.73 ± 3.7 | 44.76 ± 10.9 |
| 0.01 | 793.60 ± 23.91 | 810.06 ± 118.6 | 0.243 ± 0.084 | -0.379 ± 0.124 | 81.69 ± 5.9 | 66.73 ± 21.0 |
| 0.05 | 812.56 ± 115.7 | 815.20 ± 112.8 | 0.299 ± 0.098 | -0.407 ± 0.086 | 86.31 ± 4.3 | 71.68 ± 9.84 |

* Without amiloride.
* With 10^-4 M amiloride.
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