Activation of Na^+/H^+ exchange and Ca^{2+} mobilization start simultaneously in thrombin-stimulated platelets

Evidence that platelet shape change disturbs early rises of BCECF fluorescence which causes an underestimation of actual cytosolic alkalization

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Although an increase in cytosolic pH (pH_i) caused by Na^+/H^+ exchange enhances Ca^{2+} mobilization in platelets stimulated by low concentrations of thrombin [Siffert & Akkerman (1987) *Nature* (London) **325**, 456–458], studies using fluorescent indicators for pH_i (BCECF) and $[\text{Ca}^{2+}]_i$ (fura2) suggest that Ca^{2+} is mobilized while the cytosolic pH decreases. Several lines of evidence indicate that the initial fall in BCECF fluorescence is not due to cytosolic acidification but is caused by a platelet shape change. (1) Pulse stimulation of platelets by successive addition of hirudin (4 unit/ml) and thrombin (0.2 unit/ml) induced a shape change of $43 \pm 8\%$ and a fall in BCECF fluorescence, which both remained unchanged when Na^+/H^+ exchange was inhibited by ethylisopropylamiloride (EIPA, 100 μM). (2) Increasing the thrombin concentration to 0.4 unit/ml doubled the shape change and the fall in BCECF fluorescence, but again EIPA had no effect on these responses. (3) Treating platelets with 2 μM -ADP induced shape change and a decline in BCECF fluorescence that was unaffected by EIPA. (4) A second addition of thrombin to platelets that had already undergone shape change induced an immediate increase in BCECF fluorescence without a prior decrease. (5) Activation of protein kinase C by 1,2-dioctanoyl-*sn*-glycerol (DiC_8) neither induced shape change nor a decline in BCECF fluorescence; in contrast BCECF fluorescence rapidly increased indicating an immediate cytosolic alkalization. Concurrent analysis of $[\text{Ca}^{2+}]_i$ under conditions in which shape change did not interfere with BCECF fluorescence showed that cytosolic alkalization and Ca^{2+} mobilization started almost simultaneously. These observations suggest that cytosolic alkalization is not preceded by a fall in pH_i and can support Ca^{2+} mobilization induced by weak agonists.

INTRODUCTION

An early event in thrombin-stimulated platelets is a cytosolic alkalization mediated by Na^+/H^+ exchange (Horne *et al.*, 1981; Zavoico *et al.*, 1986; Siffert *et al.*, 1987a). Two lines of evidence indicate that this rise in cytosolic pH (pH_i) supports agonist-induced Ca^{2+} mobilization from intracellular storage sites. First, studies with permeabilized platelets have revealed that the inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$]-induced release of Ca^{2+} is pH-dependent. Elevating the pH of the suspending medium from 7.1 to 7.4 enhances Ca^{2+} release, especially at low concentrations of $\text{Ins}(1,4,5)\text{P}_3$ (Brass & Joseph, 1985). Secondly, studies on intact platelets loaded with the intracellular fluorescent Ca^{2+} dye quin2 have shown that inhibition of Na^+/H^+ exchange reduces thrombin-induced Ca^{2+} mobilization. Artificial cytosolic alkalization, on the other hand, amplifies the thrombin-induced rise in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Siffert & Akkerman, 1987; Siffert *et al.*, 1987b). This pH_i -dependence of thrombin-induced Ca^{2+} mobilization is especially seen at low agonist concentrations (< 0.1 unit/ml), but diminishes at higher thrombin concentrations (Sage & Rink, 1986; Zavoico

et al., 1986; Hunyady *et al.*, 1987; Siffert & Akkerman, 1987; Simpson & Rink, 1987; Sanchez *et al.*, 1988).

In contrast, studies with fluorescent indicators for both cytosolic pH (e.g. BCECF) and Ca^{2+} (fura2) suggest that Ca^{2+} is mobilized while pH_i decreases (Zavoico *et al.*, 1986; Rink, 1987; Simpson & Rink, 1987; Funder *et al.*, 1988; Sanchez *et al.*, 1988). In the present study we have attempted to resolve this apparent discrepancy.

EXPERIMENTAL

Materials

Fura2-acetoxymethylester (fura2-am) was purchased from Calbiochem (Frankfurt, Germany). 2,7-Bis-(2-carboxethyl)-5(6)-carboxyfluorescein tetraacetoxymethylester (BCECF-am) was obtained from HSC Research Development Corporation (Toronto, Canada). Both indicators were stored frozen as 5 mM-stock solutions in dimethyl sulphoxide. Ethylisopropylamiloride (EIPA) was provided by Dr. T. Friedrich (Max-Planck-Institut für Biophysik, Frankfurt, Germany). A 50 mM-stock solution was prepared in dimethyl sulphoxide. Thrombin (bovine), hirudin, 1,2-

Abbreviations used: pH_i , cytosolic pH; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; BCECF, 2,7-bis-(2-carboxethyl)-5(6)-carboxyfluorescein; EIPA, ethylisopropylamiloride; DiC_8 , 1,2-dioctanoyl-*sn*-glycerol; PRP, platelet-rich plasma; *F*, fluorescence; Na_o^+ , extracellular Na^+ .

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dioctanoyl-*sn*-glycerol (DiC₈), ionomycin, monensin, nigericin and prostaglandin I₂ were obtained from Sigma. All other chemicals were of analytical grade and obtained from Sigma or Merck (Darmstadt, Germany).

Preparation of platelet-rich plasma

Venous blood was obtained from healthy human volunteers who denied having taken any medication for the previous 2 weeks. The blood was anticoagulated by addition of 0.2 vol. of acid citrate/dextrose (2.5 g of sodium citrate, 1.5 g of citric acid and 2.0 g of glucose in 100 ml of distilled water). Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200 *g* for 15 min at room temperature. The upper two-thirds of the supernatant was used for the preparation of either fura2- or BCECF-loaded platelets.

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was determined after loading platelets with the fluorescent dye fura2 essentially as described by Pollock *et al.* (1986). PRP was incubated with 2 μ M-fura2-am for 45 min at room temperature. Thereafter, 1 μ M-prostaglandin I₂ was added and the PRP was centrifuged at 700 *g* for 20 min. The supernatant plasma was discarded and the platelet pellet was resuspended in Hepes buffer, pH 7.4, at 37 °C, consisting of 140 mM-NaCl, 5 mM-KCl, 5 mM-KH₂PO₄, 1 mM-MgSO₄, 10 mM-Hepes (free acid) and 5 mM-glucose. The platelet concentration in these stock suspensions was 5 $\times$ 10⁹/ml. Samples of these suspensions (approx. 40–50 μ l) were then transferred to a cuvette containing 2 ml of Hepes buffer and prewarmed to 37 °C for 5 min. Measurements of the fura2 fluorescence were performed in a SFM 25 spectrofluorimeter (Kontron, Düsseldorf, Germany) equipped with a thermostated cell holder and a stirring device. Immediately before addition of agonist, 1 mM-EGTA was added to chelate extracellular Ca²⁺, making all fura2 signals in the present study solely dependent on the mobilization of Ca²⁺ from internal storage sites. Agonist was added directly to the cuvette through a hole in the cover of the apparatus while continuously stirring the sample. The monochromator settings were 339 nm for excitation and 500 nm for emission, respectively. After each single experiment the fura2 responses were calibrated to obtain [Ca²⁺]_i by the following procedure. The cells were lysed with 50 μ M-digtonin followed by addition of 1 mM-CaCl₂ to obtain the maximal fluorescence, $F_{\max}$. Thereafter, the pH of the lysed cells was adjusted to 8.5 with 20 mM-Tris base followed by the addition of 10 mM-EGTA to yield the Ca²⁺-independent fluorescence of fura2, $F_{\min}$. [Ca²⁺]_i was then calculated according to the formula:

$$[\text{Ca}^{2+}]_i = K_d \times (F - F_{\min}) / (F_{\max} - F)$$

where F reflects the fura2 fluorescence of resting or stimulated platelets. K_d was taken to be 224 nM (Gryniewicz *et al.*, 1985; Pollock *et al.*, 1986). No platelet aggregation occurred in these experiments.

Measurement of pH_i

Cytosolic pH was determined using the intracellularly trappable fluorescent pH indicator BCECF (Rink *et al.*, 1982). Pelleted platelets prepared from PRP as described above were resuspended in Hepes buffer (see above), pH 6.5. BCECF-am (final concn. 10 μ M) was added and the cells were incubated for 30 min at 37 °C. Thereafter,

1 μ M-PGI₂ was added and the platelets were then washed twice in Hepes buffer, pH 6.5, by repeated centrifugation at 650 *g* for 20 min and resuspension at room temperature. After the last washing step, the final pellet was taken up into Hepes buffer, pH 7.4, at a concentration of 5 $\times$ 10⁹ cells/ml. Samples of these suspensions (30–40 μ l) were then transferred to 2 ml of Hepes buffer in a cuvette and prewarmed at 37 °C. Other details of the experimental conditions were identical to those for the Ca²⁺ measurements. The fluorescence of BCECF was measured using wavelengths for excitation and emission of 495 nm and 530 nm respectively. The calibration of the fluorescence signals was carried out by (1) suspending platelets in high K⁺/nigericin medium at defined pH values, and by (2) lysis of platelets with 50 μ M-digtonin followed by adjustment of the pH to defined values exactly as described in the literature (e.g. Rink *et al.*, 1982; Zavoico *et al.*, 1986; Siffert *et al.*, 1987a).

Measurement of shape change

Platelet shape change was measured in a Chronolog P.I.C.A. aggregometer and quantified as described in the literature (Latimer *et al.*, 1977; Holme & Murphy, 1978; Patscheke *et al.*, 1984; Verhoeven *et al.*, 1985). Briefly, PGI₂-treated pelleted platelets were resuspended in Hepes buffer, pH 7.4, at a concentration of 1 $\times$ 10⁸ cells/ml. After prewarming the suspensions to 37 °C, the platelets were transferred to the aggregometer and intermittently stirred at 100 and 800 rev./min. Lowering the stirring speed to 100 rev./min increased the absorbance of the suspensions, whereas at 800 rev./min the absorbance decreased. The difference in absorbance measured at both stirring speeds, which reflects the sphere-disc transformation, was used to quantify the shape change observed after addition of agonists (Verhoeven *et al.*, 1985). All measurements were performed in the presence of 2 mM-EDTA added immediately before addition of agonist to prevent platelet aggregation.

Manipulation of Na⁺/H⁺ exchange

Two methods were used to inhibit the platelet Na⁺/H⁺ exchanger. First, the Na⁺ of the final Hepes buffer was iso-osmotically replaced by choline chloride. In these experiments the platelets were kept as a concentrated suspension (approx. 5 $\times$ 10⁹ cells/ml) in 140 mM-Na⁺ medium in order to prevent spontaneous cytosolic acidification. Samples (approx. 50 μ l) were then added to 2 ml of Na⁺-free Hepes buffer, pH 7.4. Thus, the final Na⁺ concentration was approx. 3 mM, which inhibits Na⁺/H⁺ exchange by about 90% (Siffert *et al.*, 1987a). Secondly, the exchanger was inhibited by addition of 100 μ M-EIPA which is thought to be a rather specific blocker of this ion transport (Vigne *et al.*, 1983; Besterman *et al.*, 1985), although recent reports point at a few significant side effects (Simchowitz *et al.*, 1987; Yoshida *et al.*, 1988).

Presentation of data

Data are expressed as means $\pm$ s.d. unless otherwise stated. Statistical significances were determined by Student's *t* test, and were considered not significant at $P > 0.05$. Original traces are from one experiment that was typical for at least five similar determinations in two or three preparations.

Fig. 1. Effect of thrombin on the fluorescence of BCECF- or fura2-loaded human platelets

Representative fluorescence traces obtained from BCECF-loaded (upper panels, F_{BCECF}) or fura2-loaded (lower panels, F_{fura2}) platelets. The scales to the right indicate estimations of pH_i and $[\text{Ca}^{2+}]_i$ which were obtained after directly interpreting the changes in BCECF or fura2 fluorescence without correction for shape change. All experiments were performed in the presence of 1 mM-EGTA. The following experimental conditions are displayed: (a) 140 mM- Na^+ ; (b) 3 mM- Na^+ ; (c) 140 mM- Na^+ with 100 μM -EIPA. Thrombin, 0.05 unit/ml (T), was added to continuously stirred suspensions. Platelet concentration was $10^8/\text{ml}$, and temperature was 37 °C. Each trace is representative of at least seven determinations performed in three different preparations.

RESULTS

Effect of thrombin on the fluorescence of BCECF- and fura2-loaded platelets

In a first set of experiments we monitored the effect of thrombin (0.05 unit/ml) on the fluorescence of BCECF- and fura2-loaded platelets in the absence of extracellular Ca^{2+} (Fig. 1). Unstimulated platelets had a pH_i of 7.15 ± 0.08 ($n = 48$), and $[\text{Ca}^{2+}]_i$ was 55 ± 17 nM ($n = 32$). In the presence of 140 mM-external Na^+ (Na^+_{o}) and intact Na^+/H^+ exchange (Fig. 1a), addition of thrombin resulted in an immediate drop in the BCECF fluorescence which was followed by an increase, suggesting an acidification phase which was followed by alkalization above the pH_i of resting platelets. The same treatment evoked a rapid rise in the fura2 fluorescence indicating a rise in $[\text{Ca}^{2+}]_i$ to 210 ± 25 nM ($n = 8$). After blocking Na^+/H^+ exchange by lowering Na^+_{o} to 3 mM (Fig. 1b), the initial thrombin-induced fall in BCECF fluorescence was more pronounced, but returned to its original value within 30 s. This would suggest that thrombin initially induced a decrease in pH_i followed by recovery to its starting value. Under identical conditions, the thrombin-induced rise in $[\text{Ca}^{2+}]_i$ was lowered by $70 \pm 12\%$ ($n = 4$). Complete blockade of Na^+/H^+ exchange by pretreatment of platelets with 100 μM -EIPA for 1 min (Fig. 1c) resulted in a sharp and immediate fall in BCECF fluorescence, suggesting a rapid acidification of the cytosol. This apparent cytosolic acidification correlated with a suppression of Ca^{2+} mobilization by $84 \pm 12\%$ ($n = 10$).

Interference of shape change with BCECF measurements

In order to determine whether platelet shape change interfered with the measurement of pH_i by the BCECF

technique, we next tried to separate shape change from the subsequent biochemical and functional responses occurring upon platelet stimulation. Previous studies have shown that different degrees of platelet activation can be induced by adding increasing amounts of thrombin to platelets suspended in medium containing excess hirudin (Verhoeven *et al.*, 1985). Under these conditions thrombin is rapidly bound by hirudin, leaving only a short period in which it can activate the platelets. Fig. 2 illustrates a comparison between BCECF and fura2 signals and changes in the absorbance of platelet suspensions preincubated with 4 units of hirudin/ml and subsequently stimulated by 0.2–0.4 unit of thrombin/ml. In the absence of hirudin, the platelets are disc-shaped as illustrated by an increase in absorbance of the suspension when the stirring speed is decreased from 800 to 100 revs./min. (Fig. 2a, lower panel). Activation of platelets by 0.2 unit of thrombin/ml again resulted in a sharp fall in BCECF fluorescence that was followed by an increase. The same treatment raised $[\text{Ca}^{2+}]_i$ to 560 ± 44 nM. Under similar conditions, 0.2 unit of thrombin/ml induced complete shape change as indicated by an increase in the absorbance which thereafter was independent of the stirring speed. When the platelets were incubated with 4 units of hirudin/ml before stimulation with 0.2 unit of thrombin/ml (Fig. 2b), the BCECF fluorescence declined and remained low after 30 s. This process was not accompanied by Ca^{2+} mobilization. However, the absorbance of the suspension increased, and within 30 s a platelet shape change of $43 \pm 8\%$ ($n = 6$) had occurred.

In order to investigate whether the initial decrease in BCECF fluorescence represented a true fall in pH_i caused by an increased intracellular H^+ generation, the same experiment was performed after inhibition of Na^+/H^+ exchange by 100 μM -EIPA. Fig. 2(c) illustrates that EIPA had no effect on the thrombin-induced BCECF response. Furthermore, Ca^{2+} mobilization was not detectable and the shape change averaged $34 \pm 17\%$ ($n = 7$) which is not significantly different from that observed in the absence of EIPA ($P > 0.3$).

Increasing the thrombin concentration to 0.4 unit/ml (Fig. 2d) enhanced the fall in BCECF fluorescence by a factor of 2.3 ± 0.3 ($n = 6$). Again, $[\text{Ca}^{2+}]_i$ did not change, but shape change amounted to $92 \pm 18\%$ ($n = 7$). These observations were not affected by addition of 100 μM -EIPA (Fig. 2e), except for a slightly smaller shape change, to $69 \pm 18\%$ ($n = 7$; $P < 0.05$). These findings suggest that the initial fall in BCECF fluorescence is not caused by cytosolic acidification since it is not potentiated by EIPA. In contrast, there is a close correlation between the changes in BCECF fluorescence and platelet shape change.

Next, we investigated the effect of a second stimulation of platelets that had already undergone shape change after the sequential treatment with hirudin and a low concentration of thrombin. Fig. 3(a) shows that addition of 0.4 unit of thrombin/ml to platelets pretreated with 4 units of hirudin/ml again decreased the BCECF fluorescence without raising $[\text{Ca}^{2+}]_i$. The second addition of 2.0 units of thrombin/ml to these platelets caused an immediate rise in BCECF fluorescence suggesting an alkalization by 0.23 ± 0.03 pH unit ($n = 5$) within less than 8 s. Simultaneously, $[\text{Ca}^{2+}]_i$ rose to 263 ± 48 nM ($n = 5$). Measurements of absorbance at the two stirring speeds showed that most of the platelets remained disc-shaped after hirudin addition. Following stimulation

Fig. 2. Changes in the fluorescence of BCECF and fura2, and in the absorbance of thrombin-activated platelets

Shown are representative fluorescence traces obtained from BCECF-loaded platelets (upper panel, F_{BCECF}), fura2-loaded platelets (middle panel, F_{fura2}) together with changes in the suspensions' absorbance (A , lower panel). Separate experiments were carried out for BCECF fluorescence, fura2 fluorescence and absorbance. The scales on the right are as in Fig. 1. The following additions were made at the corresponding arrows: (a) addition of 0.2 unit of thrombin/ml ($T_{0.2}$); (b), (c), addition of 4 units of hirudin/ml (H), followed by addition of 0.2 unit of thrombin/ml ($T_{0.2}$); (d), (e), addition of 4 units of hirudin/ml, followed by 0.4 unit of thrombin/ml ($T_{0.4}$). 100 μM -EIPA was administered in (c) and (e). All fluorescence experiments were performed in the presence of 1 mM-EGTA. 2 mM-EDTA was added for the measurements of shape change. The platelet concentration was $10^8/\text{ml}$; temperature was 37°C . Changes in the absorbance were recorded with alternate stirring at 800 and 100 rev./min (closed and open bars respectively). Data originate from platelet suspensions obtained from three different donors and are representative of eight similar experiments. The results show that the rapid fall in BCECF fluorescence which is not accompanied by changes in $[\text{Ca}^{2+}]_i$ results from platelet shape change and does not reflect true cytosolic acidification.

Fig. 3. Effect of thrombin on pH_i , $[\text{Ca}^{2+}]_i$ and absorbance in platelets pretreated with hirudin and thrombin

Shown are representative fluorescence traces obtained from BCECF-loaded platelets (upper panel, F_{BCECF}), fura2-loaded platelets (middle panel, F_{fura2}) along with changes in the suspensions' absorbance (A , lower panel). The scales on the right are as in Fig. 1. Platelets were suspended in 140 mM- Na^+ medium in the presence of 1 mM-EGTA or 2 mM-EDTA, and pretreated with 4 unit of hirudin/ml (H), followed by addition of 0.4 unit of thrombin ($T_{0.4}$), as shown in Figs. 2(d) and 2(e). After establishment of a stable BCECF fluorescence, a second dose of thrombin (2 unit/ml; $T_{2.0}$) was added. (a) Na^+/H^+ exchange intact; (b) presence of 100 μM -EIPA. Changes in absorbance were recorded with alternate stirring as described in the legend to Fig. 2. All traces are representative of at least five similar experiments performed on two or three different platelet preparations. The results show that activation by thrombin of platelets that have already undergone shape change raises both pH_i and $[\text{Ca}^{2+}]_i$ with a similar time course. Blocking Na^+/H^+ exchange by EIPA results in cytosolic acidification and suppression of Ca^{2+} mobilization.

Fig. 4. Effect of a sequential addition of ADP and thrombin on the fluorescence of BCECF- and fura2-loaded platelets

Shown are representative fluorescence traces obtained from BCECF-loaded platelets (upper panel, F_{BCECF}) and fura2-loaded platelets (middle panel, F_{fura2}), together with changes in the suspensions' absorbance (A , lower panel). The scales on the right are as in Fig. 1. Addition of ADP ($2 \mu\text{M}$) was followed by 0.1 unit of thrombin/ml (T) 1 – 5 min later. (a) intact Na^+/H^+ exchange; (b) presence of $100 \mu\text{M}$ -EIPA. Stirring and incubation conditions were identical to those described for Figs. 2 and 3.

with 0.4 unit of thrombin/ml, shape change was almost complete. No further change in absorbance occurred at the time when BCECF and fura2 fluorescence changed.

When the same procedure was repeated after blockade of Na^+/H^+ exchange with $100 \mu\text{M}$ -EIPA (Fig. 3b), the second addition of thrombin caused an abrupt fall in BCECF fluorescence suggesting a decrease in pH_i by 0.40 ± 0.05 unit ($n = 6$). In these cells, Ca^{2+} mobilization was almost negligible ($9 \pm 3\%$ of control; $n = 7$). A similar series of experiments was carried out with platelets that had completed shape change after stimulation with a low concentration of ADP. The results are shown in Fig. 4. Upon addition of $2 \mu\text{M}$ -ADP to BCECF-loaded platelets, the fluorescence decreased within 20 – 30 s, and thereafter remained stable for at least 5 min (Fig. 4a). This response was accompanied by a small increase in $[\text{Ca}^{2+}]_i$ amounting to 26 ± 14 nM ($n = 6$) above the resting value, and by a $94 \pm 12\%$ ($n = 6$) shape change. Addition of 0.1 unit of thrombin/ml 2 – 5 min after ADP evoked an immediate rise in BCECF fluorescence indicative of a rise of pH_i by 0.26 ± 0.08 unit, as well as an increase in $[\text{Ca}^{2+}]_i$ to 282 ± 36 nM ($n = 4$). In platelets pretreated with $100 \mu\text{M}$ -EIPA (Fig. 4b) the ADP-induced fall in BCECF fluorescence and shape change remained unaffected, whereas Ca^{2+} mobilization was completely suppressed. Addition of 0.1 unit of thrombin/ml to these platelets caused a further drop in BCECF fluorescence equivalent to a decrease in pH_i of 0.18 ± 0.03 unit ($n = 7$). The thrombin-induced rise in $[\text{Ca}^{2+}]_i$ was strongly reduced and amounted to 79 ± 22 nM ($n = 3$).

These findings illustrate that elevation of pH_i in thrombin-stimulated platelets starts at the same time as the rise in $[\text{Ca}^{2+}]_i$, provided that simultaneous shape change and the concomitant disturbance of BCECF fluorescence is prevented.

Finally, we investigated how activation of protein kinase C by DiC_8 , which is known not to induce immediate shape change (Friesen & Gerrard, 1985), affected the BCECF fluorescence. Activation of protein kinase C by $25 \mu\text{M}$ - DiC_8 (Fig. 5) increased the BCECF fluorescence without a preceding fall, and the final increase in pH_i amounted to 0.17 ± 0.05 unit ($n = 6$) above the resting value. Neither Ca^{2+} mobilization nor any changes in the suspensions' absorbance occurred upon this treatment.

DISCUSSION

Shape change decreases BCECF fluorescence without a change in pH_i

The present study shows that the initial decrease in BCECF fluorescence is not a reflection of cytosolic acidification but is caused by platelet shape change which changes the light-transmitting properties of the cell suspension. This conclusion is based on the following observations:

1. The time course of the decrease in BCECF fluorescence paralleled that of shape change. Thereafter, the BCECF signals remained stable and did not return to original values although the Na^+/H^+ exchanger was not inhibited. This is in strict contrast to what one observes if the cytosol is artificially acidified (e.g. by nigericin), since the increase in intracellular H^+ is rapidly counteracted by an increased activity of the Na^+/H^+ antiport (Zavoico *et al.*, 1986). Furthermore, complete blockade of the exchanger by $100 \mu\text{M}$ -EIPA did not increase the initial fall in BCECF fluorescence evoked by thrombin or ADP. This again is in contrast to what is observed after an acid load, where EIPA both potentiates and prolongs the decrease in BCECF fluorescence due to inhibition of the H^+ -removing system (Zavoico *et al.*, 1986).

Fig. 5. Effect of DiC_8 on BCECF and fura2 fluorescence, and absorbance

Shown are representative fluorescence traces obtained from BCECF-loaded platelets (upper panel, F_{BCECF}) and fura2-loaded platelets (middle panel, F_{fura2}), together with changes in the suspensions' absorbance (A , lower panel). The scales on the right are as in Fig. 1. The arrow indicates addition of $25 \mu\text{M}$ - DiC_8 . For incubation and stirring conditions see Fig. 2. Each trace is representative for at least six determinations from two or three different preparations.

2. Addition of a second dose of thrombin to platelets that had already undergone shape change after either pulse activation (by pretreatment with thrombin/hirudin) or addition of ADP did not induce a second decrease in BCECF fluorescence in the absence of EIPA (Figs. 3a and 4a). In contrast, under these conditions, the BCECF fluorescence increased instantly after thrombin addition, except when the Na^+/H^+ exchanger was inhibited.

3. Activation of protein kinase C by DiC_8 neither induced immediate shape change, in agreement with the literature (Rink *et al.*, 1983; Friesen & Gerrard, 1985; Lapetina *et al.*, 1985), nor led to a decrease in BCECF fluorescence. In contrast, the BCECF fluorescence increased promptly upon this treatment (Fig. 5b).

BCECF and measurement of pH_i

The effect of shape change on BCECF fluorescence leads to a significant underestimation of the agonist-induced alkalization in human platelets. Similar problems are likely to arise in ADP-stimulated platelets (Sweatt *et al.*, 1986). This may explain why Funder *et al.* (1988) recently reported that stimulation of platelets with ADP induces cytosolic acidification with almost no elevation of pH_i above the resting value.

An estimation of the actual increase in pH_i can be made by comparing the changes in BCECF fluorescence in untreated platelets and in platelets that have completed shape change (Figs. 3a and 4a). These experiments demonstrate that activation of platelets by thrombin actually raises pH_i from 7.15 to approx. 7.4, thereby approaching the value of the extracellular pH. Hence, the actual increase in pH_i is approx. 2-fold higher than

previously reported (Zavoico *et al.*, 1986; Siffert *et al.*, 1987a). It should be stressed that shape change increases the fall in BCECF fluorescence after blockade of Na^+/H^+ exchange by EIPA and thus leads to an overestimation of the actual fall in pH_i (Fig. 3b).

An alternative way to correct BCECF fluorescence for interference by shape change has been proposed by Davies *et al.* (1987), who recorded the fluorescence with excitation at 500 nm for maximal pH-sensitive emission and at 450 nm, the isosbestic wavelength at which there are no pH-induced changes. Unfortunately, the intensity of emitted light at an excitation wavelength of 450 nm is much lower than at 500 nm which complicates estimation of the contribution of shape change to total fluorescence changes. We found no effect of shape change on the fluorescence of fura2. The signal-to-noise ratio is rather low in BCECF-loaded platelets as compared to fura2-loaded platelets. Under our experimental conditions the fluorescence of BCECF-loaded platelets is about ten-fold higher than the autofluorescence of unlabelled cells, whereas for fura2-loaded platelets this ratio is at least 30.

It is also possible to alter the light-transmitting properties of the platelet suspension by inducing hypo-osmotic swelling. In preliminary experiments we found that decreasing the osmotic strength of the incubation buffer by 30% reduced the decrease in BCECF fluorescence by more than 80% but left rapid alkalization intact. However, this procedure also induced platelet lysis. Furthermore, artificial manipulation of the cell volume may itself alter the activity of the Na^+/H^+ exchanger (for review see Grinstein & Rothstein, 1986), so this method was not routinely employed.

It is noteworthy that similar decreases in BCECF fluorescence have been observed in suspensions of various other cells upon activation. The stimulation of vascular smooth muscle cells with thrombin, Ca^{2+} ionophore and angiotensin II has been reported to induce an initial acidification followed by alkalization due to Na^+/H^+ exchange (Berk *et al.*, 1987a,b; Hatori *et al.*, 1987; Huang *et al.*, 1987). Interestingly, when these cells were activated with phorbol ester or 1-oleoyl-2-acetyl-glycerol, no initial decrease in BCECF fluorescence was detectable, which resembles our observations in platelets. Also, activation of BCECF-loaded neutrophils with the chemotactic peptide fMet-Leu-Phe resulted in an initial fall in fluorescence, followed by a later increase (Grinstein & Furuya, 1984; Molski & Shaafi, 1987; Weisman *et al.*, 1987). It would be interesting to re-investigate whether activation of these cells by different agonists actually acidifies the cytosol or whether early occurring changes in cell morphology, such as swelling, interfere with the measurement of pH_i .

Interrelationship between Na^+/H^+ exchange and Ca^{2+} mobilization

In conditions where the influence of shape change on changes of BCECF fluorescence was largely eliminated, we found that cytosolic alkalization and Ca^{2+} mobilization started almost simultaneously (Figs. 3a and 4a). When under these conditions Na^+/H^+ exchange was inhibited by EIPA, activation of platelets by thrombin caused cytosolic acidification and a concomitant reduction of Ca^{2+} mobilization (Figs. 3b and 4b). Hence, the present data strengthen our earlier findings that activation of Na^+/H^+ exchange enhances the rise in

[Ca²⁺]_i in thrombin-stimulated platelets (Siffert & Akkerman, 1987, 1988*a,b*). This effect would be abolished by EIPA or, more specifically, by removal of extracellular Na⁺ (Siffert & Akkerman, 1987; Ghigo *et al.*, 1988). It should be noted, however, that the increase in fura2 fluorescence is still somewhat faster than the increase in BCECF fluorescence. This agrees with recent observations by Zavoico & Cragoe (1988), who included both fura2 and BCECF in the same platelet preparation. A more precise analysis of both events awaits more accurate analysis, e.g. by stopped-flow spectrofluorimetry.

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