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Thrombin-induced activation of calcium transport pathways and their role in platelet functions

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In human platelets thrombin-induced calcium release from intracellular stores, the consequent influx of extracellular calcium, as well as their role in the aggregation and ATP-secretion reactions were examined. In indo-1-loaded platelets intracellular calcium release was studied in the presence of excess EGTA in the incubation medium, while calcium influx was followed after a rapid repletion of external calcium. After thrombin-stimulation both calcium release and calcium influx produced about the same peak levels of cytoplasmic free calcium but in the first case it was only a transient response, while in the latter one a sustained calcium signal was observed. Increased calcium influx could be evoked for several minutes after the addition of thrombin, it was selectively inhibited by Mg^{2+} (20 mM) and Ni^{2+} (1 mM) ions, by neomycin and by PCMB, a non-penetrating SH-group reagent. This calcium influx was practically insensitive to organic calcium channel blockers. Thrombin-induced platelet aggregation was only partial in the absence of external calcium, even if excess magnesium was present in the media, while the aggregation response became complete if external calcium was repleted. A significantly reduced aggregation could be seen in calcium-containing media if calcium influx was selectively inhibited. Platelet ATP-secretion under the same conditions did not depend on external calcium or on calcium influx. These data indicate that in thrombin-stimulated platelets the opening of specific plasma membrane calcium channels can be selectively modulated and these channels play a major role in the development of a full-scale aggregation.

Introduction

In the regulation of platelet functions the changes in the cytoplasmic free calcium concentration play a fundamental role (see Refs. 1–3). These changes are governed by calcium influx and extrusion through the plasma membrane and by the sequestration and release of this ion by intracellular stores. It has been convincingly demonstrated that thrombin-activation of platelets induces a rapid increase in the cytoplasmic calcium and that this calcium signal is in direct relationship with the activation of platelet functions (see Refs. 1–4). Several data showed that after an initial rapid increase in cytoplasmic calcium, caused mostly by calcium liberation from intracellular stores, a sustained phase of this signal is observed if extracellular calcium is present [5–7]. Thus a role of calcium influx through receptor-operated plasma membrane calcium channels was postulated [7–10] and indeed such channels were demonstrated in patch-clamp experiments [11].

The fundamental role of inositol trisphosphate (IP_3) in intracellular calcium mobilization (see Refs. 12 and 13), the production of this compound in thrombin-stimulated platelets, as well as its direct calcium mobilizing effect in platelet membrane vesicles has been demonstrated [14–19]. However, the biochemical basis of the opening of plasma membrane calcium channels is still largely unknown: in various cell types the role of IP_3 [20,21], IP_4 [22], as well as PIP and PIP_2 [23,24] in this function were postulated.

In the present paper, by using indo-1 loaded intact platelets we report a relatively simple technique for the separation of thrombin-induced liberation of calcium from intracellular stores and of the activation of the

Abbreviations: BSA, bovine serum albumin; DAG, 1,2-diacylglycerol; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; fura-2/AM, acetoxymethyl ester of fura-2; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; indo-1/AM, acetoxymethyl ester of indo-1, NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; PGE_1, prostaglandin E_1; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride.

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calcium influx pathway. We also attempt to correlate the two phases of calcium movements to the platelet aggregation and ATP-secretion reactions.

Materials and Methods

Materials. ATP, BSA, EGTA, Hepes, luciferin-luciferase, neomycin sulfate, and PGE_1 were purchased from Sigma. Iodoacetamide, PCMB and NEM were obtained from Fluka AG. Cinnarizine and fendilinium were provided by Gedeon Richter Co. Ltd., fibrinogen was obtained from IMLO. Thrombin was purchased from Hoffmann LaRoche (Topostasin, 3000 NIH units/5 ml distilled water). Stock solution of indo-1/AM (Calbiochem) was prepared in DMSO (1 mg/ml) and stored at $-20°C$. All the basic chemicals used were of reagent grade.

Platelet preparation. Blood was freshly drawn into sodium citrate (0.4% final concentration) from healthy volunteers who had not taken any medication in the previous 10 days. Platelet-rich plasma was obtained by 15 min centrifugation at $160 \times g$. This suspension was supplemented with 50 nM final concentration of PGE_1, then centrifuged for 15 min at $750 \times g$. The pelleted platelets were resuspended in buffer A (containing 128 mM NaCl, 3 mM KCl, 0.5 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 10 mM Hepes, 3.5 g/l BSA, 1 g/l glucose and 50 nM PGE_1, pH 7.25) to obtain $3 \cdot 10^8$ platelets/ml medium.

Loading of platelets with indo-1. Platelets resuspended in buffer A were incubated at 37°C for 30 min with 0.25 μM final concentration of indo-1/AM. After the loading period the platelets were kept at 20°C. Before each fluorescence measurement an aliquot of the platelet suspension was rapidly centrifuged (10 s at 12000 x g) in an Eppendorf microfuge and the pellet rinsed five times with buffer. B. The platelets were resuspended in 2 ml buffer B (10^8 cells/ml) for the fluorescence measurements. Buffer B had the same composition as buffer A, except that the concentration of Mg^{2+} was increased to 0.5 mM and that of BSA was reduced to 1 g/l.

Fluorescence measurements. Fluorescence was measured in a Hitachi F-4000 fluorescence spectrophotometer at 37°C with continuous gentle stirring. For indo-1 the excitation wavelength was 331 nm, emission was measured at 410 nm (bandwidth 5 nm). Cytoplasmic free calcium concentration was calculated by using the method of Tsien et al. [25], that is by lysing the cells in Triton X-100 in the presence of 0.5 mM free calcium in order to obtain maximum fluorescence, and then quenching the dye with manganese. In some experiments a similar calibration was carried out by the addition of digitonin instead of Triton X-100. By using the correction factor for manganese quenching of indo-1 (see Ref. 25), these calibrations gave similar intracellular free calcium values. The presence of extracellular fluorescent dye was checked by the addition of 0.5 mM EGTA and then 1 mM calcium to the control platelets. According to calibration of the loading procedure with free indo-1, and using the same amount of platelets and Triton X-100 in the medium, the intracellular concentration of indo-1 was between 30 and 50 μM in these experiments (the mean platelet volume was assumed to be $10 \cdot 10^{-15}$ l). Checking of the indo-1 response in the intact platelets by the addition of ionomycin (2 μM) indicated that the dye was fully responsive to the changes in cytoplasmic calcium concentrations (data not shown here).

The leakage of indo-1 proved to be very small during a 10–15 min experimental period, still, this leakage was taken into consideration in the calibration procedure. In the fluorescence measurements the thorough washing of platelets eliminated all fibrinogen and no aggregation occurred during the measurements. Platelet stimulation was evoked by 0.125 U/ml thrombin, which gave about 70–80% of the maximum response in the calcium signal development and a consistant, reproducible rate of aggregation (see later).

Platelet aggregation and ATP secretion. These were measured in a Chrono-Log luminescence - aggregometer (PICA) at 37°C with continuous stirring. Platelets were resuspended in buffer B with the same cell number as for the fluorescence measurements, except that the final washing and rinsing procedures were omitted. Aggregation was induced by the addition of 0.125 U/ml thrombin, in the presence of 1% platelet poor plasma, to assure the presence of fibrinogen. Aggregation was estimated by the continuously recorded changes in the transmission of the platelet suspension. The applied concentration of thrombin under the present conditions produced a reproducible rate and a full scale aggregation within 4 min (in the presence of calcium) and could be considered in the range of physiologically occurring thrombin concentrations.

For measuring ATP secretion the buffer was supplemented with 0.4 mg/ml luciferin-luciferase (Sigma) and the ATP-dependent luminescence was recorded in parallel with the light transmission changes. Calibration of the ATP-dependent luminescence was carried out in each experiment.

Results

Calcium-signal measurements in indo-1-loaded platelets

Fig. 1 demonstrates the thrombin-induced changes in the cytoplasmic free calcium concentration in indo-1-loaded human platelets. As seen in panel A, in the presence of 0.5 mM external free calcium the addition of thrombin evokes a rapid increase, followed by a slow and gradual decrease in cytoplasmic calcium level. This remains still above the non-stimulated levels after 5–8 min of incubation (the addition of 0.5 mM EGTA and

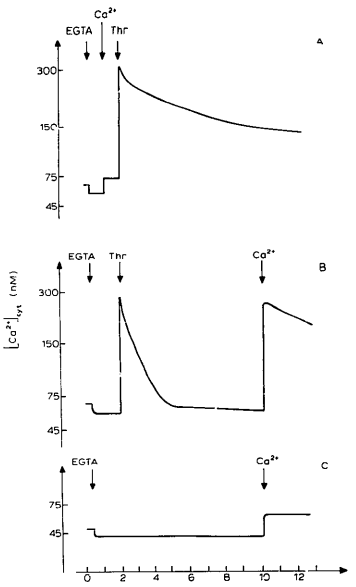

Fig. 1. Changes in platelet cytoplasmic calcium concentration during thrombin activation. Effects of external calcium. Fluorescence was measured in indo-1-loaded platelets as described in Methods. The cells were stimulated by thrombin in the presence (Panel A) or in the absence (Panel B) of extracellular calcium. In Panel B external calcium was repleted after 8 min of thrombin stimulation. Panel C shows similar platelets without the addition of thrombin. Additions: thrombin = 0.125 U/ml; EGTA = 0.5 mM, $CaCl_2$ = 1.0 mM (yielding about 0.5 mM free Ca^{2+}).

then 1.0 mM calcium at the beginning of the experiment demonstrates the absence of a significant indo-1 fluorescence outside the platelets). In panels B and C the platelets are incubated in the presence of 0.5 mM EGTA (that is in the virtual absence of free calcium in the incubation media) and in panel B thrombin is added under these conditions. The thrombin-induced increase in cytoplasmic calcium, which in this case reflects the calcium liberation from internal stores, reaches about the same peak level as seen in the presence of extracellular calcium. However, under these conditions the spike is followed by a rapid decline. After reduction of the cytoplasmic calcium to the original baseline level a rapid increase of the calcium concentration in the incubation medium (yielding about 0.5 mM free calcium) induces a second peak in the cytoplasmic calcium, which is indistinguishable from the calcium signal seen in panel A; that is a sustained increase in cellular calcium level occurs. The peak level of the cytoplasmic calcium concentration, produced in this case by a calcium influx, is about the same as that seen in the absence of external calcium and is not modified significantly if external calcium is varied between 0.5 and 2 mM (data not shown). This calcium influx reaction occurs with a similar magnitude if external calcium is added within 2–8 min after thrombin stimulation. Increasing thrombin concentrations (up to 1 U/ml) slightly elevate the maximum levels of the calcium signals but do not cause a major change in the overall picture described. In the control platelets (panel C) an increase in extracellular calcium concentration within 10–15 min of the incubation period produces only a small rise in the fluorescence levels (note the logarithmic scale of the calculated cytoplasmic calcium concentrations), which may be due to some leakage of free indo-1 into the medium, or to a slight increase in the number of non-viable cells under these conditions.

The above data demonstrate that by using this simple technique of avoiding or allowing calcium influx during thrombin stimulation, it is possible to separate the phenomena of calcium release from internal stores and of thrombin-induced calcium influx from the extracellular fluids, respectively. The opening of calcium influx pathways is reflected by the plateau-phase of the calcium signal in the presence of external calcium and by the rapid rise of cytoplasmic calcium after calcium repletion in the incubation medium.

Earlier data indicated [23,24], that neomycin and higher concentrations of magnesium preferentially inhibit a PIP_2-induced calcium leakage pathway in isolated platelet membrane vesicles. In the experiments shown in Fig. 2, we analyzed the effects of these agents on thrombin-activation of intracellular calcium release and of the calcium influx pathways in intact platelets loaded with indo-1.

As demonstrated in panel A of Fig. 2, in the presence of extracellular calcium, 1 mM neomycin only slightly reduces the peak level of the calcium signal (in various experiments this reduction was found to be between 15 and 20%) but the recovery of the low internal calcium concentration is significantly faster and the plateau is significantly lower in the presence of this drug. It has to be mentioned that higher neomycin concentrations (2–5 mM) significantly decrease the calcium signal peak as well. In the experiment shown in panel B, neomycin was added after the transient calcium signal, induced by thrombin in the absence of extracellular calcium, and

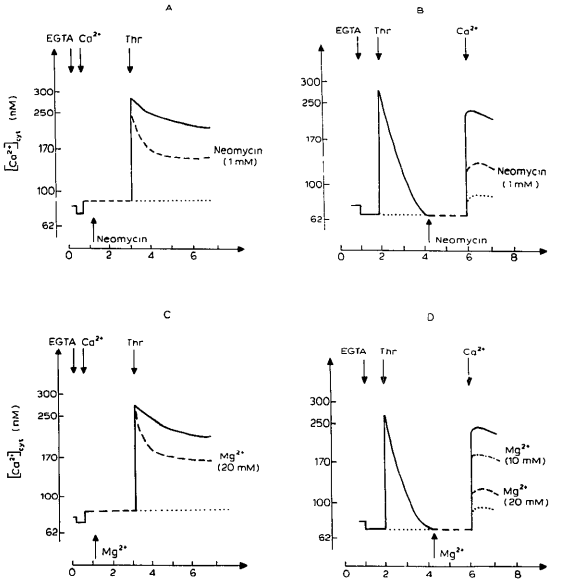

Fig. 2. Effects of neomycin and magnesium on the thrombin-induced changes in platelet cytoplasmic calcium concentration. Fluorescence was measured in indo-1-loaded platelets as described in Methods. Solid lines represent the experiments with thrombin but without added drugs. Dotted lines show the controls without thrombin. Neomycin (1 mM) or Mg^{2+} (10 mM or 20 mM) were added as indicated on the figure (dashed lines). Thrombin-stimulation was carried out in the presence of 0.5 mM external calcium (Panels A and C) or in the presence of EGTA (Panels B and D). In these latter experiments external free calcium was repleted to about 0.5 mM after 4 min of thrombin stimulation. Additions: EGTA = 0.5 mM, $CaCl_2$ = 1.0 mM, thrombin = 0.125 U/ml.

before calcium influx was initiated by adding calcium to the medium. This calcium influx was found to be substantially (by 60–70%) reduced by 1 mM neomycin, although the calcium signal seen in the absence of external calcium is hardly influenced by the drug (maximum peak reduction is between 8 and 10% at 1 mM neomycin, data not shown).

Panels C and D show similar experiments by altering the magnesium concentration in the media between 0.5 to 20 mM. As demonstrated, high magnesium concentrations reduce the plateau level of the calcium signal in the presence of external calcium and almost eliminate the thrombin-induced calcium influx, while they do not significantly modulate the peak level of the first calcium response either in the presence or absence of external calcium. According to these data both 1 mM neomycin and high extracellular magnesium preferentially inhibit the opening of thrombin-induced calcium influx pathways.

In the experiments summarized in Table I, we have examined the effects of several organic 'calcium antagonists' on the thrombin-induced calcium liberation and calcium influx in platelets. The first calcium signal, reflecting calcium release from internal stores, was measured in the presence of 0.5 mM EGTA (virtually 0 calcium) in the media, while a second calcium signal (reflecting calcium influx, see Fig. 1, panel B) was induced by an increase of the extracellular free calcium

concentration to 0.5 mM. The drugs were added 2 min before thrombin stimulation. The data presented in the table depict the percent changes in the peak levels of the calcium signals as reported by the fluorescence of indo-1. We found that nifedipine or verapamil, even in relatively high concentrations, did not reduce thrombin-induced calcium influx, while at these high concentrations a small reduction in the calcium liberation response occurred (when nifedipine was used, the photo-sensitive compound was stored in the dark and preincubation of the platelets for 2 min before applying thrombin or excess calcium was also performed by removing the cuvettes from the light path). In the case of fendilinium (sensit) and cinnarizine these changes were even more pronounced: calcium liberation from internal stores was significantly reduced, while calcium influx was unchanged or (at higher drug concentrations), significantly increased. This latter phenomenon probably reflects a calcium leakage induced by these drugs at the plasma membrane.

Table II demonstrates experimental data obtained with a similar technique for several compounds, reported to influence the calcium transport pathways. As shown, lanthanum produced a similar decrease both in the calcium signals reflecting calcium liberation and calcium influx, while nickel ions almost exclusively inhibited calcium influx. Indomethacin had no significant effect on the thrombin-induced calcium movements under these conditions, while several SH-group reagents strongly reduced the magnitude of the calcium signals. The penetrating SH blockers, NEM and iodoacetamide, produced a non-selective inhibition of both calcium liberation and influx, while the non-penetrating PCMB selectively inhibited the calcium influx pathway.

TABLE I

Effects of organic calcium antagonists on thrombin-induced calcium liberation and calcium influx in platelets

Indo-1-loaded platelets were prepared as described in Materials and Methods. Calcium release from intracellular stores was determined by indo-1 fluorescence measurements in the absence of external Ca^{2+} (0.5 mM EGTA) after thrombin stimulation (0.125 U/ml). Calcium influx from the medium was induced by calcium repletion to 0.5 mM, 5 min after thrombin stimulation. The calcium antagonists were added 2 min before applying thrombin or excess calcium, respectively. For details see text. Results are given as means (±S.D.) of three independent experiments.

Drug	Concn. (μM)	Percent	
		calcium release from internal stores	calcium influx from the medium
No addition	–	100	100
Nifedipine	5	100	100
	25	80±4	95± 3
Verapamil	20	100	100
	100	85±3	100
Fendilinium	25	50±5	100
	50	9±8	600±41
Cinnarizine	2	58±5	100
	5	11±8	410±40

TABLE II

Effects of calcium-transport modifying agents on the thrombin-induced calcium liberation and calcium influx in platelets

Indo-1-loaded platelets were prepared as described in Materials and Methods. Thrombin-induced (0.125 U/ml) calcium release from intracellular stores was measured in Ca^{2+} free medium (0.5 mM EGTA). 5 min after the thrombin stimulation the medium was repleted by calcium (0.5 mM free Ca^{2+}) and the calcium influx from the medium was measured. The calcium-transport modifying agents were added 2 min before thrombin stimulation or applying excess calcium, respectively. Results are given as means (±S.D.) of three independent experiments.

Agent	Concn. (μM)	Percent	
		calcium release from internal stores	calcium influx from the medium
No addition	–	100	100
$LaCl_3$	25	100	70±6
	50	50±8	50±8
$NiSO_4$	1000	100	12±7
Indomethacin	50	100	100
NEM	50	40±6	30±7
	100	30±8	20±7
Iodoacetamide	500	86±5	81±5
	1000	56±6	49±6
PCMB	50	95±3	25±8
	100	92±4	18±8

Fig. 3 shows in detail the experiments analyzing the effects of PCMB on the thrombin-induced calcium signal in indo-1-loaded platelets. The addition of 100 μM PCMB does not effect the calcium signal in the absence of extracellular calcium but eliminates external calcium influx. This PCMB effect is entirely reversed by the addition of dithiothreitol, either before or after thrombin stimulation (see panels C and D).

Platelet aggregation and ATP-secretion measurements

In the following experiments we examined the aggregation and ATP secretion of indo-1-loaded platelets, with the intention to separate the roles of calcium liberation and of calcium influx in these phenomena. According to several control experiments, there was no significant difference in the response of untreated and indo-1-loaded platelets, respectively (data not shown).

Fig. 4 shows the thrombin-induced aggregation (Panel A), and ATP-secretion (Panel B) of indo-1-loaded platelets under similar conditions as used for measuring the fluorescent calcium signals – except that in this case the media are supplemented with fibrinogen (a small amount of plasma) to allow aggregation. In the presence of external calcium both aggregation and ATP secretion are complete within 2 min. In the absence of external calcium (with EGTA in the medium), aggregation is much slower and only partial, while ATP secretion is

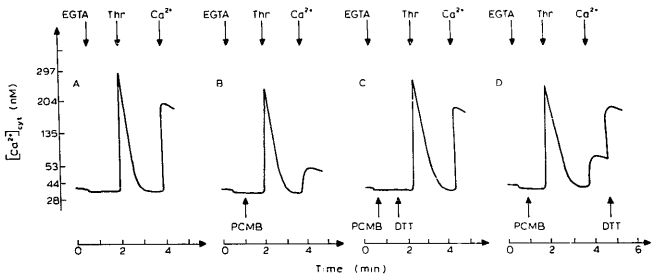

Fig. 3. Inhibition of thrombin-induced calcium influx by PCMB and its reversal by dithiothreitol (DTT). Fluorescence in indo-1-loaded platelets was measured as described in Methods. Additions: EGTA = 0.5 mM, Ca^{2+} = 1.0 mM, thrombin = 0.125 U/ml, PCMB = 100 μM, DTT = 1 mM.

practically unaffected. When external calcium is absent, the presence of Mg^{2+} is essential to obtain any aggregation. According to several experiments, 0.5 mM Mg^{2+} is sufficient to allow aggregation and changes in the free magnesium concentration between 0.5 and 2 mM have no significant effect on the speed or magnitude of this reaction. In EGTA-containing media the addition of excess calcium after the partial aggregation response induces a rapid and full-scale aggregation - corresponding to the calcium influx seen in the fluorescence measurements.

Fig. 5 shows the effects of PCMB, neomycin, and high external Mg^{2+} on the aggregation of indo-1-loaded platelets, measured either in the absence or presence of

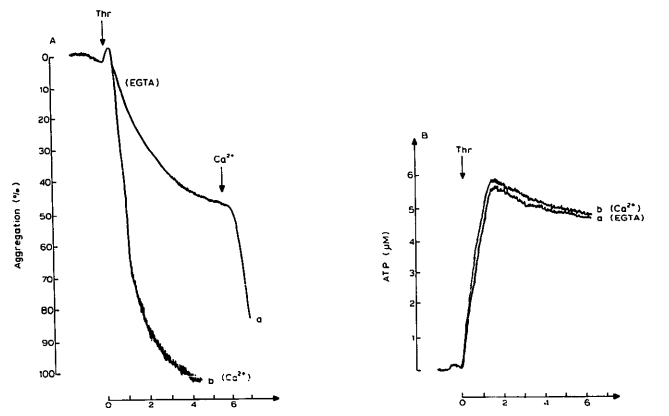

Fig. 4. Thrombin-induced aggregation (A) and ATP secretion (B) in indo-1-loaded platelets. Effects of extracellular calcium. Aggregation and ATP secretion were measured as described in Methods. Line a shows aggregation in the presence of 0.5 mM EGTA. In this experiment after 6 min of thrombin stimulation 1 mM $CaCl_2$ is added to obtain about 0.5 mM free calcium. Line b shows a similar experiment in the presence of 0.5 mM EGTA and 1 mM $CaCl_2$ (about 0.5 mM free calcium).

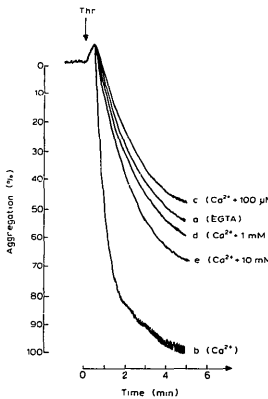

Fig. 5. Effects of drugs modifying calcium influx on thrombin-induced platelet aggregation. Platelet aggregation was measured as described in the Methods. The media contained 0.5 mM EGTA (a), or 0.5 mM $CaCl_2$ (b–e). Additions: line c = 100 μM PCMBS; line d = 1 mM neomycin, line e = 10 mM $MgCl_2$.

external calcium. The data indicate that these agents, which inhibit calcium influx, reduce platelet aggregation in calcium-containing media to about the level seen in the absence of external calcium. A similar inhibitory effect of 1 mM nickel ion has also been observed (data not shown). The penetrating SH-group inhibitors, NEM or iodoacetamide, in the concentrations which eliminate both intracellular calcium release and calcium influx, completely abolish the aggregation response (data not shown).

Discussion

The data presented above indicate that by using a relatively simple technique, in indo-1-loaded platelets it is possible to separate the thrombin-induced opening of calcium transport pathways in the membranes of internal calcium storages (mostly the dense tubular system – see Refs. 26 and 27) and in the plasma membrane, respectively. Under physiological conditions probably both types of calcium movements, that is the release from intracellular stores and influx from the extracellular medium, contribute to the normal 'suicidal' response of platelets after thrombin stimulation. Earlier experiments, by using quin2 as an intracellular calcium indicator, showed that a thrombin-induced calcium signal was much greater if extracellular calcium was present [5,6,8–10]. Our results, in accordance with those obtained by using fura-2 [7] demonstrate that, when indicators with high fluorescence yields, such as fura-2 or indo-1 are used (their low intracellular concentration does not produce a significant calcium buffering), the calcium release from intracellular stores produces about the same peak level of cytoplasmic calcium as that seen when calcium influx from the medium is also allowed. In our studies we preferred to use indo-1, because, in contrast to that experienced with fura-2, there was no significant leakage of the free dye from the platelets. In the present experiments the peak values of the observed calcium signals and the resting cytoplasmic calcium levels are somewhat lower than those reported in the literature, especially for quin2-loaded cells [6,8,10]. This may be partially due to the better condition of our cells and a faster response of the calcium-restoration mechanisms after the careful loading procedure which yielded a relatively low cellular dye concentration (30–50 μM, see Methods). The thrombin concentration applied also did not give a maximum response, but allowed the examination of thrombin effects under near physiological conditions.

The first few steps of the signal-transduction pathway in the thrombin-stimulation of platelets are relatively well established: the ligand induced breakdown of PIP_2 results in IP_3 and DAG liberation and most probably IP_3 is the major second messenger in inducing calcium liberation from intracellular stores (for reviews see Refs. 12 and 13). However, the opening of the plasma membrane calcium influx pathway, which seems to play a major role in the biological response, is relatively poorly characterized. In the present report we looked for some basic features of this transport pathway.

It has been indicated in the literature that 'classical' voltage-sensitive calcium channels are not involved in platelet activation as organic calcium channel inhibitors do not affect this phenomenon [28–30]. The experiments shown above reinforce this conclusion by demonstrating that the thrombin-induced calcium influx, studied separately from the intracellular calcium release in indo-1-loaded platelets, is practically insensitive to several organic calcium channel blockers. The reported inhibitory effect of verapamil on calcium signal development in quin2-loaded platelets may be due to the different experimental conditions (greater thrombin, external calcium and verapamil concentrations, longer preincubations, etc.) used in this previous study. In accordance with the data in Ref. 30, several 'calcium antagonists', such as cinnarizine, preferentially block the internal calcium release phenomenon (in high concentrations they also induce a non-specific calcium influx, see Table I). Indomethacin does not significantly modulate either calcium release or calcium influx during thrombin stimulation, thus, in accordance with the data

in literature [1,4,10] the development of the calcium signal is not critically dependent on the formation of thromboxanes (a measurable effect of indomethacin was found on the calcium signal in quin-2-loaded platelets in Ref. 10.

The slowly penetrating nickel ions effectively blocked thrombin-induced plasma membrane calcium channels and reduced external calcium dependent aggregation, in good agreement with the data presented in Refs. 8 and 28. Neomycin, although reported to affect several biochemical mechanisms in platelets [31], in the relatively low concentrations used here, may also be applied for studying the calcium influx phenomenon.

As shown in the Results, penetrating SH-group reagents inhibited both thrombin-induced internal calcium release and calcium influx, as expected from their inhibitory effects on calcium release in platelet membrane vesicles induced by IP_3 [32] or PIP_2 [24], and the reported NEM-inhibition of platelet calcium uptake under certain conditions [33]. In contrast, PCMB was found to be a selective and reversible inhibitor of the thrombin-induced calcium influx pathway, most probably because of the slow permeation of this agent into cells (in membrane vesicles PCMB is also effective in inhibiting IP_3-induced calcium release [32]). Thus PCMB in intact cells may be used as an effective tool for the separation of various calcium transport pathways.

As to the mediators of the opening of the plasma membrane calcium channels, the possible role of IP_3 in this process (as suggested in Refs. 20 and 21), is questioned by the observation that such channels are open for several minutes after thrombin-stimulation (see Fig. 1). Data in the literature indicate that the calcium-releasing isomer of IP_3 has a relatively short rise in platelets and within about 1–2 min it is eliminated by phosphatase and kinase actions [34,35]. In fact, Sage and Rink [36] suggested that during platelet activation the development of a calcium influx may precede the calcium release from internal stores.

The data presented in this paper suggest a possible role of polyphosphoinositides in the induction of plasma membrane calcium channels. Earlier experiments showed that polyphosphoinositides, especially PIP_2, induce a rapid calcium leakage in mixed platelet membrane vesicles [23,24] and this pathway is blocked by neomycin, high concentrations of Mg^{2+} and by SH-group reagents. The inhibition of the calcium influx by the same drugs in intact platelets (see Results), as well as the data in the literature showing an overshoot in the polyphosphoinositide concentration in thrombin-stimulated platelets [37,38] further support this idea.

In this report we find that thrombin-induced secretion of platelet granule contents (measured here as ATP secretion), in accordance with the data in the literature (see Refs. 1 and 4) is practically independent of the presence and influx of external calcium. This means either that calcium release from internal stores is sufficient to induce such a release, or that calcium is not a key mediator in this response. In contrast, during thrombin stimulation, full aggregation response requires the presence of extracellular calcium and a secondary calcium influx. In the aggregation response external calcium may also be required for the appearance of the high-affinity fibrinogen receptors, while for the formation of fibrinogen bridges between platelets calcium can be replaced by magnesium ions (see Ref. 39). Our present studies, by using the inhibitors of calcium influx, e.g. PCMB, neomycin, Ni, and high concentration of Mg^{2+}, strongly suggest that it is the influx of calcium which is a prerequisite of a full scale aggregation.

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