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ON THE SIGNIFICANCE OF THE INFLUX OF CALCIUM IONS INTO STIMULATED HUMAN BLOOD PLATELETS

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SUMMARY

Blood platelets, upon stimulation with various substances, take up calcium ions from the suspending medium. This influx occurs simultaneously with the release reaction, i.e. the specific secretion of a variety of substances from storage organelles and the second wave of aggregation. Various inhibitors of the release reaction inhibit this Ca^{2+} influx. Platelets previously loaded with ^{45}Ca show an increased efflux of the cation upon stimulation by thrombin. These results suggest that the plasma membrane acquires an increased permeability to Ca^{2+} only in a later phase of platelet activation, in most cases after the earlier release of Ca^{2+} into the cytoplasm from Ca-storing organelles. Rapid shape change and release proceed independently of external calcium, whereas clot retraction depends upon a prolonged increased permeability of the plasma membrane to this cation.

INTRODUCTION

Thrombin induces, besides the release reaction [1], an influx of Ca^{2+} into blood platelets [2, 3]. The release reaction, in the course of which the contents of special storage organelles are extruded into the external medium, is similar in many respects to secretory processes in other cells [4]. Since in many secretory cells the presence of Ca^{2+} is required for release to take place [5] it would appear that this cation also plays a dominant role in thrombin-stimulated platelets. The observation that ionophores for divalent cations induce the release reaction in platelets suggests that the activation of platelets is mediated by an increase of the concentration of Ca^{2+} in the cytoplasm [6–11]. The ionophores render membranes permeable to Ca^{2+} and thereby allow the passage of the cation from the outside or from Ca^{+}-storing organelles into the cytoplasm. In the resting platelet the cytoplasmic Ca^{2+}-concentration must be very low since the contractile protein actomyosin is in the relaxed state; Cohen and de Vries [12] have reported that contractile activity is observed at a Ca^{2+}-concentration of 10^{-6} M or higher. The existence of microtubules in the resting platelet also points to a

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

very low Ca^{2+}-concentration in the cytoplasm since these structures are depolymerized by Ca^{2+} [13, 14].

The most obvious explanation for these observations would be that thrombin, which interacts exclusively with the platelet surface, induces release by increasing the cytoplasmic Ca^{2+}-concentration by means of an increased influx of this cation through the altered plasma membrane. However, in contrast to many secretory cells [4, 5, 15] the induction of the release reaction in platelets does not require external Ca^{2+} and can even proceed in the presence of Ca^{2+}-chelators in the suspending medium [16, 17]. This makes it likely that Ca^{2+} is also mobilized, in thrombin-stimulated platelets, from internal storage organelles. The question then arises as to which of the two processes, internal release or influx from the outside is determinant for the different manifestations of platelet reactivity to thrombin. In order to answer this question we have studied more closely the relationship between the two processes.

METHODS AND MATERIALS

Human blood platelets

These were isolated within 20 h after collection from citrated blood collected for the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross in Berne [18]. The buffy coats were syphoned into a buffered glucose solution; the resulting platelet suspension ("buffered platelet concentrate") contained about 20 mM glucose, 12 mM phosphate (pH 6.8), 60–70 vol. % of citrated plasma and $3–4 \cdot 10^9$ platelets per ml. In some experiments the buffered platelet concentrate was used directly, while in others the platelets were thrice washed by repeated centrifugation and resuspension as described earlier [7]. Platelets were finally suspended in a buffered salt solution (see below) to $4–6 \cdot 10^9$ platelets per ml.

Measurement of the uptake of Ca^{2+} and of the release reaction

Samples of the platelet suspension were pipetted into 1 ml polypropylene centrifuge tubes containing 0.2 ml of separating oil (see below), buffer (with an inhibitor added if required), ^{45}Ca and [^{3}H]acetylated albumin, and were warmed to 37 °C. Thrombin or another stimulant was added and the mixture was incubated at 37 °C for a given time. The platelets were separated from the supernatant by sedimentation through the layer of separating oil in a microcentrifuge at $11\,000 \times g$ for 15 s. The sediment was solubilized in 1 ml of 0.5 % Triton X-100 and added to 10 ml of scintillator. The samples were counted in a dual channel liquid scintillation spectrometer. The amount of ^{45}Ca taken up by the platelets was calculated by correcting the ^{45}Ca-count rate for the extracellular space (albumin space) by means of the ^{3}H-count rate (see ref. 7), and the number of μmol Ca^{2+} taken up by platelets calculated on the basis of the specific activity of the ^{45}Ca in the incubation mixture.

In some experiments the platelets were previously labeled with [^{3}H]serotonin (1 h at 18°) and the tritiated albumin was omitted. The release of serotonin was then calculated from the ^{3}H-count rate of the sediment; in these experiments the calcium content was not corrected for the extracellular space. In other experiments twin samples were prepared: one set, designed to measure Ca^{2+}-uptake, with unlabeled platelets to which ^{45}Ca and tritiated albumin were added, and the other with platelets labeled with [^{14}C]serotonin; release of serotonin was calculated from the count rate

of the supernatant of these samples as described earlier [7, 19]. If the corrected calcium uptake and release of serotonin had to be measured in the same sample, the platelets were incubated with ^{45}Ca and tritiated albumin. The amount of serotonin released was estimated by fluorimetric assay of serotonin in the supernatant of the incubation mixture, after reaction with *o*-phthaldialdehyde [20].

Aggregation

Platelet aggregation was measured by a turbidimetric method using buffered platelet concentrate in an aggregometer (Labintec, Montpellier, France); in order to measure calcium uptake following aggregation the centrifuge tubes mentioned above were used instead of the regular glass tubes.

Materials

Buffered salt solution contained 130 mM NaCl, 3 mM KCl, 0.06 mM $CaCl_2$, 0.02 mM $MgCl_2$, 26 mM TES-NaOH buffer (pH 6.8) and 25 mM glucose. Separating oil: a mixture of 10 vol. of dinonylphthalate and 25 vol. of dibutylphthalate ([21]; adapted for platelets by P. Bally, personal communication). Scintillator: 3 g/l Permablend III (Packard) in a mixture of 2 vol. of xylene and 1 vol. Triton X-100. Bovine thrombin: Hoffmann-La Roche, Basle (Switzerland), 50 N.I.H. units per mg, dissolved in saline. Human thrombin: gift from D. L. Aronson, Rockville, Md., (U.S.A.). Aggregated γ-globulin: human immunoglobulin G coupled with bisdiazo-benzidine [22], gift from S. L. Pfueller. Collagen: Kollagenreagens Horm, gift from Hormonchemie, München (G.F.R.). Adrenalin: bitartrate, Fluka, Buchs (Switzerland). Indomethacin: Sigma. Phenylbutazone: gift from Ciba-Geigy, Basle (Switzerland). Amitriptyline: gift from Hoffmann-La Roche, Basle (Switzerland). Prostaglandin E_1: gift from J. E. Pike, Upjohn Co, Kalamazoo, Mich. (U.S.A.). Isobutylmethylxanthine: Aldrich-Europe, Janssen Pharmaceuticals, Beerse (Belgium). Antimycin A: Calbiochem, Lucerne (Switzerland). [^{3}H]serotonin: TRA 223, The Radiochemical Centre, Amersham (U.K.). [^{14}C]serotonin: CFA 170, The Radiochemical Centre. ^{45}Ca: Ca-A-1, Eidgenössisches Institut für Reaktorforschung, Würenlingen (Switzerland), made up in saline to contain 10 μCi/ml and 10^{-4} M $CaCl_2$. [^{3}H]acetylated bovine serum albumin: prepared from [^{3}H]acetic-anhydride, NET-018 A, New England Nuclear, Dreieichenhain (G.F.R.) [7]. All reagents used were of analytical grade.

RESULTS

Effects of thrombin

The thrombin-induced uptake of Ca^{2+} is a fast and limited process (Fig. 1). The amount of Ca^{2+} taken up by platelets corresponds to about 1 % of the Ca^{2+} present in the suspending medium and to about 2 % of the total Ca of the platelets. In the controls, a fast binding of ^{45}Ca which presumably occurs at the cell surface is followed by a slow and continuous increase in ^{45}Ca-activity.

The preparation of bovine thrombin used in these experiments is impure. In order to exclude the possibility that the observed uptake of Ca^{2+} is an artifact due to a contaminant of thrombin, the experiment described in Fig. 1 was performed also with purified human thrombin which was diluted to the same clotting activity as the

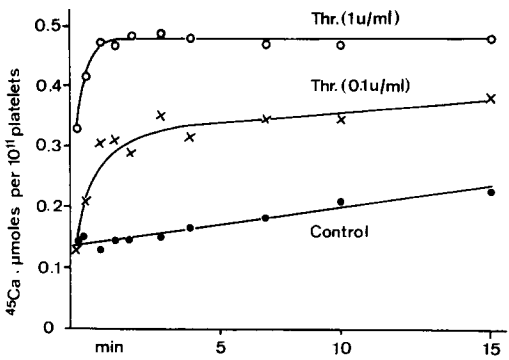

Fig. 1. Thrombin-induced uptake of ^{45}Ca by washed human platelets. The platelets were incubated at 37 °C for various times with 0, 0.1 or 1 unit/ml of thrombin. Final concentrations: 34 mM Tris · HCl + 8 mM TES (pH 7.4 at 37 °C); 0.3 mM $CaCl_2$; 0.5 mM KCl; NaCl to isotonicity; 4 mM glucose; 1 mg/ml albumin; 0.7 µCi/ml ^{3}H and 0.04 µCi/ml ^{45}Ca; 10^9 platelets per ml. Vol. = 0.6 ml.

bovine thrombin preparation. The pure preparation induced release of serotonin and uptake of Ca^{2+} to the same extent as bovine thrombin.

The time course of the Ca-uptake in relation to the release of serotonin is shown in Fig. 2; in this experiment uptake of ^{45}Ca and release of serotonin were measured simultaneously in each sample after different incubation periods. Both processes start immediately after the addition of thrombin, proceed in parallel and come to an end within 1 min.

The high rate and limited extent of the uptake of Ca^{2+} might indicate that Ca^{2+} does not penetrate an intracellular compartment but rather is bound to extracellular sites which become available by the action of thrombin. Measuring the uptake at various calcium concentrations allows the distinction between these two possibilities: in a plot of the amount of Ca^{2+} taken up versus the external Ca^{2+}-concentration, binding would yield a saturation curve, whereas a straight line passing through

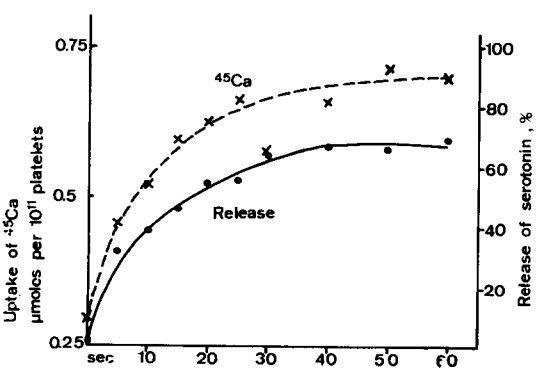

Fig. 2. Time course of the uptake of ^{45}Ca and of the release of serotonin by platelets, induced by thrombin. Platelets were labeled with [^{3}H]serotonin and then incubated at room temperature with ^{45}Ca and thrombin. Final concentrations as in Fig. 1, but 0.8 unit/ml thrombin; ^{3}H-labeled albumin was omitted and the uptake was not corrected for the external ^{45}Ca.

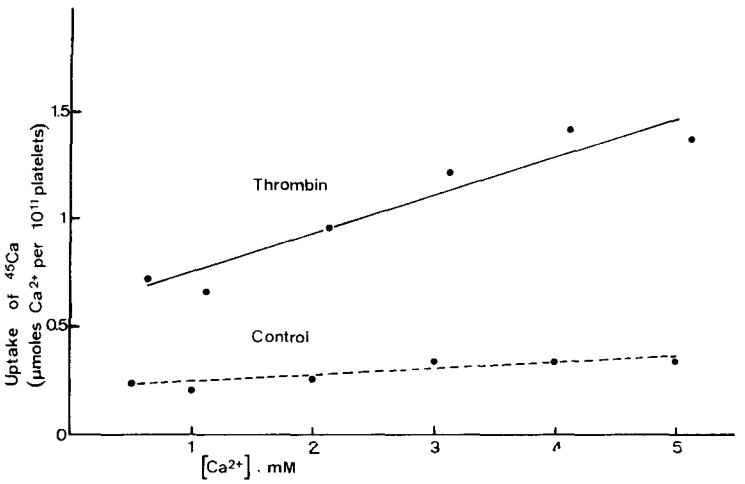

Fig. 3. Thrombin-induced ^{45}Ca-uptake of platelets; dependence on the Ca^{2+}-concentration. Samples of a platelet suspension containing ^{45}Ca, [^{3}H]acetylated albumin and different concentrations of $CaCl_2$ were incubated for 20 min at 37 °C with or without 1 unit/ml thrombin. The platelets were then centrifuged through separating oil; the sediments were analyzed for ^{45}Ca and ^{3}H. Final concentrations: 36 mM TES (pH 6.8); 0.5–5 mM $CaCl_2$; 0.4 mM KCl; NaCl to isotonicity; 4 mM glucose; 2 mg/ml albumin; 0.5 μCi/ml ^{3}H and 0.16 μCi/ml ^{45}Ca; $5 \cdot 10^{8}$ platelets per ml; vol. = 0.7 ml.

the origin of such a plot would be expected if a compartment is freely penetrated by the ions, since the internal concentration would be proportional to the external concentration. Fig. 3 shows that the experimental results are accounted for by a combination of both types of curves. Apparently thrombin causes the appearance of new binding sites which are capable of binding about 0.4 μmol of Ca^{2+} per 10^{11} platelets (based on extrapolation of the two curves to the y-axis). In addition, thrombin causes the influx of Ca^{2+} into a compartment with an apparent volume, based on the Ca^{2+}-concentration of the medium, of about 0.15 ml per 10^{11} platelets; this is expressed by the increase of the slope of the curve in the presence of thrombin.

In order to discriminate between net uptake and exchange of Ca^{2+}, platelets were loaded with ^{45}Ca for 24 h and the influence of thrombin on the efflux was measured. Fig. 4 shows the result of such an experiment: before addition of thrombin a constant efflux of ^{45}Ca is observed. The addition of thrombin is followed by a burst of ^{45}Ca and thereafter the efflux is increased with respect to the control. The burst probably represents ^{45}Ca which had been taken up into the dense bodies during the loading period and is released from them in the course of the release reaction [2]. The increased efflux can be interpreted as an increased permeability of the plasma membrane to Ca^{2+} which allows an increased Ca^{2+}-flux in both directions. Alternatively, it may result from the activation of exchange diffusion as a consequence of the increased cytoplasmic calcium concentration. In this case the efflux is reduced when the external Ca^{2+}-concentration is lowered as this process requires the presence of the transported species on both sides of the membrane. Fig. 5, however, shows that this is not the case: the efflux of ^{45}Ca is the same irrespective of whether the outside medium contains 0.06 mM Ca^{2+} or 1 mM EDTA. In a similar experiment, ^{45}Ca-loaded platelets were suspended in media containing 0.06 and 1 mM Ca^{2+}, respectively.

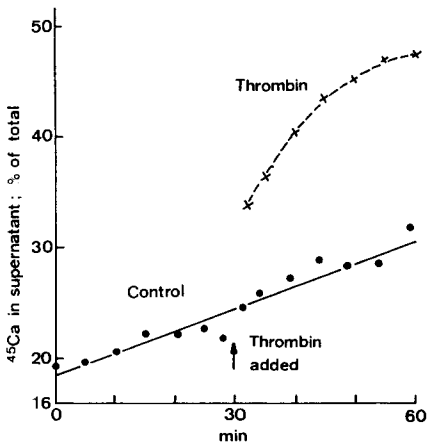

Fig. 4. Efflux of ^{45}Ca from platelets and its stimulation by thrombin. Buffered platelet concentrate ($4 \cdot 10^9$ platelets per ml) was labeled with ^{45}Ca (0.5 μCi/ml) for 24 h at 18 °C. The platelets were centrifuged and washed 3 times as described and suspended in buffered salt solution ($3.8 \cdot 10^9$ platelets per ml). Samples of 0.6 ml were centrifuged at various times through separating oil and the supernatant analyzed for ^{45}Ca. At 30 min 1 unit/ml thrombin was added to one half of the suspension and further samples of both halves were processed for another 30 min in the same way.

After stimulation with thrombin, no influence of the external Ca^{2+}-concentration on the efflux of the cation was observed.

Therefore it is more probable that thrombin increases the passive permeability of the plasma membrane for Ca^{2+}.

The limitation of the influx of Ca^{2+} can be caused either by the small capacity

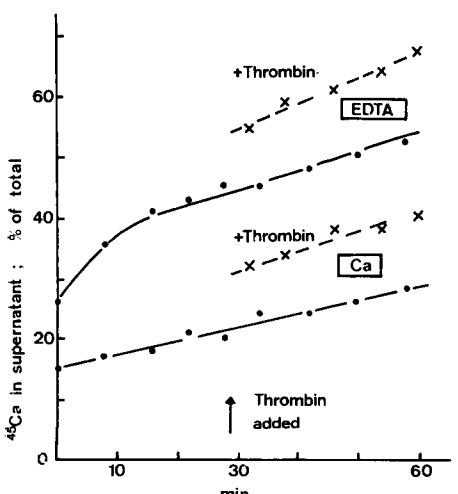

Fig. 5. Efflux of ^{45}Ca from platelets and its stimulation by thrombin; influence of EDTA. The experiment was performed as indicated in Fig. 4, but with two different suspension media for the ^{45}Ca-labeled platelets; one contained 0.06 mM $CaCl_2$ and the other 1 mM EDTA (one of two experiments with similar results).

of the compartment into which Ca^{2+} is taken up or by a temporal limitation of the permeability increase. Foreman and Garland [23] have shown that the Ca^{2+}-dependent release reaction of mast cells stimulated by immunological reaction requires the presence of external Ca^{2+} at the time of stimulation, and that the response of the cells ceases if Ca^{2+} is added a few minutes later. Foreman suggested that the stimulus opens Ca-gates for a short time only. The possibility that the permeability increase of the platelet membrane lasts for a short time only was tested in an experiment similar to the one described in Fig. 1, except that ^{45}Ca was added either prior to or up to 10 min after thrombin addition. The amount of ^{45}Ca taken up was found to be independent of the time of its addition; therefore it is unlikely that the Ca-gates opened under the influence of thrombin quickly close again.

Effects of other stimulants

Ca^{2+} is also taken up when platelets are stimulated with various other agents, as shown in Table I: in addition to thrombin, aggregated γ-globulin, collagen, ADP, and adrenalin cause an uptake of Ca^{2+} under the conditions under which they stimulate the release reaction. Fig. 6 shows the time course of the release of serotonin and of the uptake of Ca^{2+} during the aggregation of platelets by ADP: both uptake and release begin with the onset of the second wave of aggregation and finish at the end of aggregation. No uptake of Ca^{2+} takes place in the early stages of aggregation, i.e. during the rapid shape change (not discernible in this aggregation curve) and the first wave of aggregation.

TABLE I

RELEASE OF SEROTONIN AND UPTAKE OF Ca^{2+} INDUCED BY VARIOUS REAGENTS

(a) Samples of 0.7 ml of a platelet suspension, prelabeled with [^{3}H]serotonin and containing ^{45}Ca, were incubated with either aggregated γ-globulin or thrombin. They were then centrifuged through separating oil and the sediments analyzed for ^3H and ^{45}Ca. Final concentrations: see Fig. 1; 2 μM of imipramine were added to reduce reuptake of serotonin. (b–d) Buffered platelet concentrate was stirred at 37 °C in the cuvette of an aggregometer; the aggregating agent was added and the aggregation was recorded by turbidimetry. After completion of the aggregation the platelets were centrifuged through separating oil. The sediments were analyzed for Ca-uptake and the supernatants for serotonin released (fluorimetrically). Final concentrations: 34 mM Tris, pH 7.4; 3 mM KCl and 2 mM $MgCl_2$ added; 28 vol.% citrated plasma; NaCl to isotonicity; $1.5 \cdot 10^9$ platelets per ml; 0.8 μCi/ml ^3H; 0.2 μCi/ml ^{45}Ca. Vol. = 0.35 ml.

Inducer	Incubation Time, (min)	Release of serotonin (%)	Uptake of Ca^{2+} μmol per 10^{11} platelets
(a) Control	4	0	0.33*
Thrombin (1 unit/ml)	1	82	0.63*
Aggregated γ-globulin (50 μg/ml)	4	68	0.54*
(b) Control	5	0	0.08
Collagen (1 μg/ml)	5	34	0.16
(c) Control	5	1	0.04
ADP (3.6 μM)	5	22	0.21
(d) Control	5	1	0.06
Adrenalin (1 μM)	5	45	0.25

* Not corrected for external space.

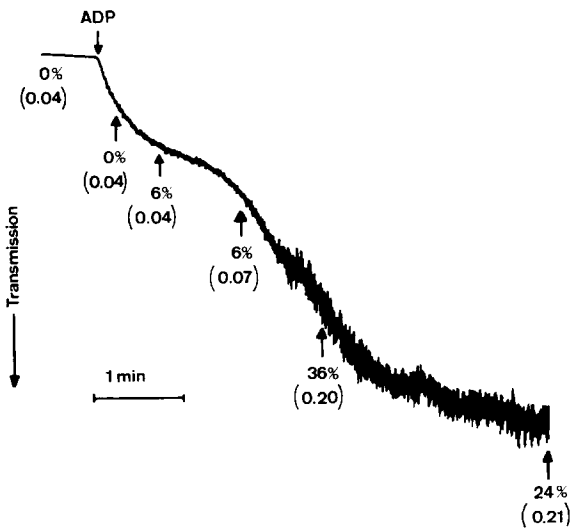

Fig. 6. Time course of the uptake of Ca^{2+} and of the release reaction during aggregation of platelets by ADP. Buffered platelet concentrate was stirred at 37 °C in the aggregometer in the presence of ^{45}Ca. 3.6 μM ADP was added and the aggregation was measured. At various times the sample was centrifuged through separating oil. The uptake of ^{45}Ca and the release of serotonin were measured (see Table I, b–d). The curve is a superposition of aggregation curves interrupted at the time indicated by the arrow. The percentages under the arrows represent % release of serotonin (measured fluorimetrically), the figures in brackets Ca^{2+} taken up corrected for the Ca^{2+} in the external space and expressed in μmol per 10^{11} platelets.

When platelets are aggregated by collagen or adrenalin the release reaction and the uptake of Ca^{2+} also start together and proceed simultaneously.

Effects of inhibitors of the release reaction

Many compounds are known to inhibit the activation of platelets (for a review, see ref. 24). As shown in Table II, representatives of various classes of drugs, if added in concentrations which are just sufficient to block completely collagen-induced aggregation and release of serotonin, inhibit at the same time also the uptake of Ca^{2+} by collagen-stimulated platelets.

The thrombin-induced uptake of Ca^{2+} is inhibited also in parallel with the release reaction when the energy supply of the platelets is interrupted by incubation with antimycin and deoxyglucose or when the temperature is lowered to 0 °C, as is shown in Table III.

DISCUSSION

The experiments described indicate that the release reaction of human platelets is invariably accompanied by an influx of Ca^{2+}. Thereby only a limited amount of Ca^{2+} is taken up. It has been shown that this limitation cannot be due to closure of membrane calcium gates after a short time as the influx is not decreased if ^{45}Ca is added some time after thrombin addition; therefore the limit to the influx of Ca^{2+}

TABLE II

EFFECTS OF VARIOUS DRUGS ON AGGREGATION, RELEASE OF SEROTONIN AND UPTAKE OF ^{45}Ca INDUCED BY COLLAGEN

Samples of buffered platelet concentrate were preincubated with or without one of the drugs for 5 min at 37 °C. The samples were then stirred in the aggregometer, collagen was added and the transmission recorded for 5 min. The concentration of drug just sufficient to suppress aggregation completely was selected. The samples were centrifuged through separating oil. The release of [^{14}C]-serotonin and the uptake of ^{45}Ca were assayed in twin samples (see Methods). The figures are the mean of at least 2 experiments. Final concentrations: see Table I (b), but with 2.8 μg/ml collagen.

Drug	Release of serotonin (%)	Uptake of ^{45}Ca, μmol per 10^{11} platelets
Control (with collagen)	44[a]	0.18[b]
Blank (without collagen)	6[c]	0.08[d]
0.04 mM indomethacin	1	0.12
0.2 mM acetylsalicylic acid	2	0.04
1 mM phenylbutazone	2	0.11
0.1 mM amitriptyline	7	0.07
0.1 mM chlorpromazine	16	0.11
0.2 mM *N*-ethylmaleimide	0	0.06
0.4 μM prostaglandin E_1	3	0.06
0.4 mM papaverine	6	0.08
0.5 mM isobutylmethylxanthine	1	0.11

[a] $n = 15$, S.D. = 4
[b] $n = 14$, S.D. = 0.08
[c] $n = 4$, S.D. = 5
[d] $n = 13$, S.D. = 0.04

TABLE III

ENERGY AND TEMPERATURE DEPENDENCE OF THE RELEASE OF SEROTONIN AND OF THE Ca-UPTAKE INDUCED BY THROMBIN

Washed platelets, suspended in buffered salt solution with glucose omitted were incubated for 1 h at 18 °C with glucose (25 mM) or with inhibitors (1 μM antimycin + 50 mM deoxyglucose). Release of [^{14}C] serotonin and uptake of ^{45}Ca were assayed in twin samples (see Methods). The samples were incubated with thrombin (1 unit/ml) for 1 min at 37 °C or 0 °C and centrifuged. The temperature-dependence was measured in a separate experiment. Final concentrations as in Fig. 1.

	Release of serotonin (%)			Ca-uptake, μmol per 10^{11} platelets		
	glucose (37 °C)	inhibitors (37 °C)	glucose (0 °C)	glucose (37 °C)	inhibitors (37 °C)	glucose (0 °C)
Blank	4	4	0	0.16	0.21	0.13
Thrombin	68	5	1	0.32	0.21	0.13

must be determined by the limited capacity of the compartment accessible to Ca^{2+} following stimulation.

During the first minute after stimulation several rapid movements of Ca^{2+} have been shown or presumed to occur: (i) The release of Ca^{2+} from Ca-accumulating organelles into the platelet cytoplasm; this process presumably provides the Ca^{2+} necessary for, among others, the decay of microtubules which occurs during the rapid shape change even in the presence of external EDTA [25]; (ii) the release of Ca^{2+} from the dense bodies to the outside medium [2, 26]; (iii) the uptake of Ca^{2+} from the external medium, which is the process measured when ^{45}Ca is added to the suspending medium. This uptake consists of a saturable component which probably reflects binding to the membrane, and a component which seems to reflect the penetration of Ca^{2+} into an internal space.

Considering the multiplicity of these simultaneous movements it cannot be expected that the ^{45}Ca taken up by platelets in short time experiments is a true measure for the influx of Ca^{2+} since its specific activity might not remain constant during uptake proper. However, in the experiments represented in Fig. 3, the platelets have been incubated for 20 min with ^{45}Ca and thrombin; it would be expected that after this time the compartment which is made accessible by thrombin has, with respect to ^{45}Ca, reached isotopic equilibrium with the suspending medium. This compartment takes up the amount of Ca^{2+} contained in 0.15 ml of medium (expressed per 10^{11} platelets). The internal volume of 10^{11} platelets is 1 ml ("internal water space", see ref. 27). However, electron micrographs show that a large part of the platelet volume is occupied by organelles which might not be accessible for Ca^{2+}. Therefore it is conceivable that the compartment made accessible during stimulation is indeed identical to the cytoplasm, which consequently would comprise about 15 % of the total platelet volume and, upon stimulation, would acquire a Ca^{2+}-concentration equal to the one of the suspending medium.

The simultaneous increase in influx and efflux of ^{45}Ca indicates that thrombin increases the permeability of the plasma membrane to Ca^{2+} in both directions. This explanation is supported by two observations described earlier: (i) Mürer and Holme [2] reported that the ^{45}Ca taken up after thrombin treatment is washed out easily. (ii) Cohen and de Vries [12] reported that a clot made from platelet-rich plasma by treatment with thrombin develops tension when the external medium contains Ca^{2+} but relaxes when the external Ca^{2+} is chelated by EGTA. The cycle of retraction and relaxation can be repeated several times. Since actomyosin is located in the platelet cytoplasm these experiments indicate that external Ca^{2+} has indeed access to the cytoplasm of activated platelets. The release reaction as well as contractility (which includes morphological changes) depend on the internal Ca^{2+}-concentration. Since in the resting platelet the Ca^{2+}-concentration is extremely low compared to the external medium, it would seem logical that the observed increased permeability to Ca^{2+} of the plasma membrane should be directly related to the different calcium-dependent manifestations of platelet activity.

However, it is known that the influx of external Ca^{2+} is not necessary for activation, since various agents can activate platelets even in the presence of EDTA [16, 17]. Furthermore, the uptake of Ca^{2+} is inhibited whenever the release reaction is prevented, irrespective of the mode of action of the inhibitor. These findings indicate that the uptake of Ca^{2+} might be a consequence rather than the cause of the activation

of platelets. A similar conclusion has been reached by Robblee et al. [3] who investigated the uptake of ^{45}Ca by calf platelets and found it to be delayed until 30 s after the onset of the release reaction. Although, perhaps due to species differences, such a lag phase is not observed in our experiments, additional evidence speaks in favor of our conclusion that also in human platelets the influx of external Ca^{2+} is not required for the first manifestations of calcium-dependent activities: thus, it is noteworthy that the uptake of Ca^{2+} does not start at once when platelets are aggregated by ADP but begins only at the onset of the second wave of aggregation which is accompanied by the release reaction. ADP initially causes a rapid shape change, which must reflect the breakdown of microtubules and possibly the activation of part of the contractile system of the platelets. Since both these processes require Ca^{2+}, it is probable that the cytoplasmic Ca^{2+}-concentration rises at once and before release occurs after the addition of ADP. Le Breton and Feinberg [28] have indeed found that upon addition of ADP the intracellular Ca^{2+}-concentration increases without an influx of ^{45}Ca.

All these observations together suggest that the primary stimulus exerted on the platelet membrane generates, by a hitherto unknown mechanism, 4 groups of events which follow each other in a short time: (i) The liberation of Ca^{2+} from Ca-accumulating organelles into the cytoplasm, leading to an increased cytoplasmic Ca^{2+}-concentration. (ii) The decay of microtubules and restricted contractile activity, leading to the rapid shape change and to primary aggregation. (iii) A conformational change of the plasma membrane. This enables the membrane to fuse with organelle-membranes, with the release reaction and the second wave of aggregation as consequences. At the same time the permeability of the membrane for Ca^{2+} is increased, leading to the influx of Ca^{2+} into the cytoplasm which is described here in detail. (iv) The clot retraction which presumably results from the organization and activation of the main body of the platelet's actomyosin.

Whereas the three first groups of events, with the exception of aggregation and Ca^{2+}-influx, are independent of external Ca^{2+}, clot retraction depends entirely upon the Ca^{2+}-concentration of the suspending medium [12]. The increased permeability of the plasma membrane to Ca^{2+} obviously makes the platelet's contractile manifestations of longer duration independent of the limited availability of the cation from intracellular sources.

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