Differential Release of Calcium, Magnesium and Serotonin by Rabbit and Human Platelets

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Calcium and magnesium are extracellularly released by platelets during the release reaction. The characteristics of the release of these components by rabbit and human platelets were compared with the release of serotonin. When rabbit platelets were activated by thrombin which induces maximum response, approximately 80% of the platelet serotonin and calcium, but only 50% of platelet magnesium were released. It was suggested that only about one-half of the amount of platelet magnesium was localized in the releasable compartments in the cells. In response to a lower concentration of thrombin, calcium and magnesium were released to a greater extent than was serotonin. When phorbol myristate acetate was added to the rabbit and human platelet suspension serotonin was slowly but calcium and magnesium were rapidly released into extracellular fluid. Platelet factor-4 was also more rapidly released than serotonin by human platelets. The release of calcium and magnesium was analogous to the release of platelet factor-4.

It is conceivable that possible pools containing calcium and magnesium but little or no serotonin exist in the platelets and that their contents are more susceptible to mild stimuli than granular serotonin.

**Keywords** — platelet release reaction; calcium; magnesium; serotonin; thrombin; phorbol ester

**Introduction**

During platelet release reaction platelet granular contents are released into extracellular medium. The release has been divided into three types; (1) release of substances stored in dense granules (serotonin and non-metabolic adenine nucleotides), (2) release of acid hydrolases which occurs more slowly than the release of dense granule substances, and (3) release of alpha-granule substances (several characteristic platelet proteins). It has been recognized that calcium and magnesium are contained in the dense granules and that they are extracellularly released together with serotonin and the nucleotides. Species differences were significant in content and in characteristics of the release reaction of calcium and magnesium. In the present report, the characteristics of the release of calcium and magnesium were examined, and the release of these substances was compared with the release of serotonin by rabbit and human platelets. The release of platelet factor-4 (PF-4), a platelet alpha-granule protein, by human platelets was also examined.

**Materials and Methods**

**Chemicals** — Bovine thrombin (600 NIH units/mg protein) and 4-phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Company, St. Louis Mo., U.S.A., and 5-hydroxy side-chain-2-¹⁴C tryptamine creatinine sulfate (57.4 mCi/mmol) from Amersham Japan. Fatty acid-free bovine albumin was prepared from bovine albumin (powder fraction V, Wako Chemicals) according to the method of Chen. All other chemicals used were of reagent grade.

**Determination of Release Reaction of Platelets Suspended in a Tris-Buffered Saline** — Rabbits were anesthetized with pentobarbital and blood was collected by carotid catheterization into a plastic beaker containing 0.1 volume of 77 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 7.4). The platelets were isolated by differential centrifugation, essentially according to the method reported previously. The platelet rich plasma, which was obtained by centrifugation at 150 × g for 15 min, was centrifuged at 650 × g for 15 min. The sedimented
platelets were washed twice with centrifugation at 450 × g for 10 min, first in a medium containing EDTA (0.139 M NaCl - 0.0123 M Tris - HCl buffer pH 7.4 - 0.00154 M EDTA-Na-salt pH 7.4) and second in a medium without EDTA (0.139 M NaCl - 0.0154 M Tris - HCl buffer pH 7.4). The washed platelets were suspended in the second washing medium. All procedures were carried out at 4 °C. The platelet suspension (3 mg platelet protein/ml) in a volume of 0.9 ml including imipramine to inhibit re-uptake of serotonin7 was preincubated in a plastic vial with a diameter of 15 mm for 5 min at 37 °C, then 50 μl of the stimulant were added and further incubated with vigorous shaking (Taiyo incubator M-1, 240 strokes/min, horizontal shaking with an amplitude of 50 mm). The reaction was terminated with 50 μl of 30% formaldehyde solution,8 then the platelets were sedimented at 7000 × g for 1 min (Fisher centrifuge model 59). Serotonin, calcium and magnesium in the supernatant fluids were determined. Platelet release was expressed as the amount estimated in the supernatant fluid over that estimated in the whole platelet preparation before stimulation. When extra- and intracellular enzyme activities in the suspension were determined, the reaction was terminated by cooling the reaction mixture in an ice-water bath. The platelets were sedimented, and the activities in the supernatant fluid and in the sediment were determined. In order to estimate the activity in the sediment and in the whole platelet preparation, sonicated samples were used.

**Determination of Release Reaction of Platelets Suspended in a Modified Tyrode Solution** — Rabbit blood was collected as described above. Human blood was taken from a forearm vein. In order to prepare 14C-serotonin-labelled platelets, a 10 ml portion of human platelet rich plasma, which was obtained by centrifugation at 150 × g for 15 min, was incubated at 37 °C for 30 min with 5 μCi of the radioactive serotonin creatinine sulfate. Rabbit and human platelets were isolated by differential centrifugation essentially according to the method of Mustard et al.9 The first washing medium included 137 mM NaCl, 2.7 mM KCl, 1.2 mM NaHCO3, 0.36 mM NaH2PO4, 5.5 mM glucose, 0.2 mM ethyleneglycol-bis-(β-aminoethyl ether)tetra-acetic acid (EGTA), 5 mM N-2-hydroxyethyl-piperazine-N'-2-ethane-sulphonic acid (HEPES) buffer (pH 7.4) and 0.35% albumin. EGTA was omitted from the second washing medium and the suspending medium. The centrifugation was carried out according to the method described above. The platelet suspension (2 × 10⁶ cells/ml in rabbit, and 0.7–1.0 × 10⁶ cells/ml in human platelet suspension) in a volume of 0.5 ml including imipramine (2 μM) and PMA (100 ng/ml) in a Fisher centrifuge tube was incubated at 37 °C. The tube was tightly stoppered, fastened in a horizontal position to a shaking rack, and the rack vigorously shaken in water (Taiyo incubator M-1). Immediately after the incubation, the suspension was centrifuged at 7000 × g for 1 min in the Fisher centrifuge, and the supernatant fluid was separated from the sediment. To the sediment, 0.5 ml of deionized water was added and the cells were disintegrated with sonication. Calcium, magnesium, serotonin and PF-4 in the supernatant fluid, in the resuspended sediment, and in the whole platelet suspension before incubation were determined. Platelet release was expressed as described above. The non-releasable portion was also expressed as the amount estimated in the sediment over that estimated in the whole platelet suspension.

**Determination of Calcium, Magnesium, Serotonin, PF-4, Lactate Dehydrogenase (LDH) and Protein** — Calcium and magnesium were determined with an atomic absorption spectrophotometer (Shimadzu AA 640–12). Serotonin was determined by measuring the natural fluorescence of the substance at 335 nm when excited at 301 nm in near-neutral solution with a fluorospectrophotometer (Aminco Bowman) in the experiments using rabbit platelets.10 Serotonin-release by human platelets was determined essentially according to the method of Holmsen et al.11 The radioactivity of serotonin was determined by counting 50 μl of the sample in 3 ml of ACSII (Amersham) in a liquid scintillation counter (Aloka LSC-751). PF-4 was determined with a RIA kit (PF-4 RIAKIT, Abbott Laboratory), and LDH activity according to the method described by Bergmyer and Bernt.12 Platelet protein was determined ac-
According to the method described by Layne.130

Results

Thrombin-Induced Rabbit Platelet Release Reaction in Tris-Buffered Saline

The contents of serotonin, calcium and magnesium in rabbit platelets were 48.4 ± 2.5 (the mean of 16 experiments ± standard error of the mean), 17.8 ± 1.0 (10 experiments) and 60.2 ± 4.1 (14 experiments) nmol/mg platelet protein, respectively.

Figure 1 shows the time course of thrombin-induced platelet release of serotonin, calcium and magnesium. The release reached plateau levels within 1 min after thrombin addition. The levels were maintained during the observation. At the high concentration of 2.0 units/ml thrombin, about 80% of platelet serotonin and calcium were released, while only 50% of platelet magnesium was released. At the low concentration of 0.2 units/ml thrombin, the platelets released only 30% of the total platelet serotonin and 50% of the total platelet calcium.

Fig. 2. Thrombin Dose–Response Curves

Platelets suspended in Tris-buffered saline in the presence of 20 μM imipramine were incubated for 5 min and after the thrombin addition the platelets further incubated for 5 min with vigorous shaking. (A) Ordinate indicates release as percentage of the total content. Values represent means of 4 experiments. Error bars represent standard error of the mean. (B) Ordinate indicates release as percentage of the maximum release.
Figure 2A shows thrombin dose-response curves. The ordinate represents release of each substance during 5 min incubation as percent of total platelet content. When thrombin concentration was increased, the release of serotonin linearly increased and reached a plateau level of about 80% release of the total content at concentrations more than 2.0 units/ml. At lower concentrations of thrombin, the release of calcium was larger in extent than that of serotonin, although the maximum release of about 80%, as in serotonin-release, was achieved at concentrations higher than 2.0 units/ml. The percent release of total magnesium, like calcium, was larger than the percent release of serotonin at lower concentrations of thrombin. The release, however, was within 50% of the total content, even at thrombin concentrations higher than 2.0 units/ml, suggesting that only one-half of the amount of platelet magnesium was localized in releasable compartments in the cells. A significant difference in the extent of the releasable pool between magnesium and calcium or serotonin was observed. LDH recovered from the extracellular fluid after incubation in the absence and the presence of thrombin in a concentration of 2 units/ml was 1.8 and 2.0% of the activity in whole platelet suspension before stimulation, respectively. The result suggested that little cell lysis was induced by thrombin.

In Fig. 2B, in order to compare the characteristics of the release among these substances, thrombin-induced release was expressed as a percentage of the releasable pool. For the value of releasable pool, the release by thrombin at 2 units/ml at which the maximum release was achieved, was used. The release of magnesium showed a similar characteristics as the release of calcium, while serotonin release was significantly different.

**PMA-Induced Rabbit Platelet Release Reaction in Tris-Buffered Saline**

When PMA was added to the platelet suspension, serotonin, calcium and magnesium were slowly released. Also in the PMA-induced release reaction, the released magnesium did not exceed 50% of the total content, while the release of serotonin and calcium was about 80%. Differences in extents of the releasable pools between magnesium and the other two substances were confirmed also in the platelets activated by PMA (Fig. 3A). After 15 min incubation in the presence and the absence of PMA, about 3% of platelet LDH was detected in the extracellular fluid, and about 92% in the sediment. No difference was observed between the PMA-added and the control. Fig. 3B represents the release of serotonin as calcium and magnesium as percentages of their maximum release (% of the release at 15 min). At 10 min after the PMA addition, calcium- and magnesium-release reached near plateau levels, while serotonin-release was far below its maximum level. Also in the PMA-induced platelet release reaction, significant dif-

![Fig. 3. Time Course of PMA-Induced Release of Calcium, Magnesium and Serotonin in Tris-Buffered Saline](image)

Platelets were suspended in Tris-buffered saline in the presence of 20 μM imipramine and incubated with vigorous shaking. (A) Ordinate indicates release as percentage of the total content. Values represent means of 4 experiments. PMA (100 ng/ml) was added at the point indicated by an arrow. Dotted lines represent values without PMA. Error bars represent standard error of the mean. (B) Ordinate indicates release as percentage of the maximum release after subtracting the values at time 0.
ference in characteristics between release of calcium and magnesium and that of serotonin was observed.

**PMA-Induced Rabbit Platelet Release Reaction in Modified Tyrode Solution**

Figure 4 shows PMA-induced rabbit platelet release reaction in the modified Tyrode solution. The extracellular appearance of the substance was represented as sup, and the non-releasable portion as pt. The release of calcium and magnesium reached near plateau levels at 15 min after PMA addition. The released calcium and magnesium during incubation of 15 min with PMA amounted to approximately 70 and 40% of the total platelet contents, respectively. The release of serotonin lagged significantly behind calcium- and magnesium-release. Although the released serotonin during 15 min incubation with PMA amounted to 50% of the total content, the release did not reach any plateau level at 15 min after PMA addition. The sum of the amount of calcium recovered from the supernatant fluid and the sediment (sup + pt) was, on the average, 104% of the amount estimated in the whole suspension before incubation. Similarly, the recoveries of magnesium and serotonin were 92 and 95%, respectively. The values indicate the validity of the method for detection of the release reaction. The extracellular LDH before and after 15 min incubation could not be detected in either system with or without PMA, suggesting that cell lysis did not occur during the incubation.

Figure 5 represents a comparison among the release of these substances. In order to compare the characteristics of the release among these substances, the PMA-induced release at each incubation time was expressed as a % of that at 15 min incubation (the maximum release during the observation). A significant difference was observed between the PMA-induced release of both calcium and magnesium and that of serotonin in the Tyrode solution.

**PMA-Induced Human Platelet Release Reaction in Modified Tyrode Solution**

![Figure 5](image_url)
Platelet Release of Calcium and Magnesium

Fig. 6. LDH Activity Recovered during Incubation
Human platelets suspended in the Tyrode solution were incubated in the presence (dotted column) and absence (open column) of PMA (100 ng/ml). Incubation; 0, 5, 10, 15 min at 37 °C with vigorous shaking. Ordinate indicates LDH activities recovered from the sediments as percentages of the total activity of the platelet suspension. Total activity; 1.70 µmol reduced nicotinamide adenine dinucleotide decreased/min/10^9 cells.

Intra- and extra-cellular LDH activity was detected in human platelet suspension during incubation (Fig. 6). The activities in the extracellular fluid during incubation with and without PMA could not be detected. No differences in the activity recovered from the sediment between the presence and the absence of PMA were observed, both before and after incubation, although a slight loss of the activity by incubation was found in both systems. It is conceivable that cell lysis was not induced by PMA also in human platelet suspension.

When PMA was added to the human platelet suspension, the release of calcium and magnesium reached near plateau levels at 10 min after PMA addition, while serotonin-release lagged significantly behind calcium- and magnesium-release (Fig. 7) as in the rabbit platelet suspension. The released calcium and magnesium

Fig. 7. Time Course of PMA-Induced Release of Calcium, Magnesium and Serotonin by Human Platelets in Modified Tyrode Solution
Abbreviations are same as in Fig. 4. Values represent means of 4 experiments and standard errors of the mean. Total content; calcium 121.9 ± 12.0 nmol/10^9 cells, magnesium 66.6 ± 2.7 nmol/10^9 cells.
during incubation of 15 min with PMA were approximately 70 and 20% of the total platelet contents, respectively. The released radioactivity of serotonin at 15 min incubation was about 60% of the total activity incorporated into the platelets before stimulation. The extracellular and intracellular amounts of calcium, magnesium and serotonin recovered after 15 min incubation without PMA were not significantly different from those before incubation, suggesting that no extracellular release was induced by incubation without PMA (data not shown).

Figure 8 shows PMA-induced PF-4-release by human platelets. PF-4-release reached a plateau level at 10 min after the addition of PMA, similar to the release of calcium and magnesium. The extent of the release at 15 min incubation was around 50% of the total contents. The recovery from the supernatant fluid plus sediment was 98% of the amount estimated in the whole platelet suspension.

Figure 9 represents PMA-induced human platelet release as a % of maximum release at 15 min incubation, as in Fig. 5. Similarity in characteristics of release of calcium, magnesium and PF-4 and the significant differences between the release of these three substances and that of serotonin were observed.

Discussion

The contents of calcium and magnesium found in rabbit platelets were comparable with those reported by Da Prada et al., however Meyer et al. reported a higher value of calcium content in rabbit platelets. It was reported that pig platelets released about 50% of their magnesium together with serotonin and 5'-phosphonucleotides when stimulated by thrombin, but without a marked release of calcium. Meyer et al. demonstrated that the releasable pools of rabbit platelet calcium and magnesium were 29% and 44% of total contents, respectively. In the present study, in contrast to their results, a marked release of calcium by rabbit platelets was observed when the platelets were stimulated by thrombin or PMA. In our system, EDTA was not included and the medium contained μM order of calcium. The presence of a trace amount of calcium ion in the medium may affect the extracellular release of platelet calcium. Releasable magnesium was approximately 50% of the total content in rabbit platelet preparation, similar to the results of Meyer et al.

It has been demonstrated that Tris-based buffers inhibit platelet aggregation and release reaction induced by a low concentration (0.083 units/ml) of thrombin. In the present experiments, rabbit platelets suspended in Tris-buffered saline dose-dependently released serotonin, calcium and magnesium within 1 min after the addition of higher concentrations of thrombin (0.2–2 units/ml), but the platelets did not aggregate at thrombin concentrations of less than 1 unit/ml in the absence of added calcium. Although platelet release reaction is independent from aggregation, the reaction is accelerated and amplified by several reactions during aggregation such as protein contraction. Some of the releasable components, which are coincidentally secreted at the maximum level in the presence of the aggregation, are secreted in a separate manner in the absence of the aggregation. Thus, detailed differences in the release pattern of calcium, magnesium and serotonin were clearly demonstrated in thrombin-induced release reaction under the present condition using Tris-buffered saline as the suspending medium. At lower concentrations of thrombin, larger extents of calcium- and magnesium-release than serotonin-release were observed. When the platelets were stimulated by PMA, at
first calcium and magnesium were released and, after a lag period, release of serotonin followed.

The release reaction using Tyrode solution containing albumin as a more physiological suspending medium than Tris-buffered saline was also examined. In order to determine extracellular release of calcium and magnesium, these cations were omitted from the medium. Furthermore, since the pH of Tyrode solution is unstable, HEPES buffer (pH 7.4) was added. The results, showing that rabbit platelets suspended in the Tyrode solution in the presence of PMA released calcium and magnesium more rapidly than serotonin, were similar to the results using platelets suspended in Tris buffered saline. Also in human platelets, calcium and magnesium were more rapidly released than serotonin. Since little of no platelet LDH was detected in the supernatant fluid after the reaction, the extracellular appearance of calcium and magnesium as well as serotonin could not be attributed to cell lysis. Platelets were found to take up extracellular serotonin, but not calcium and magnesium. Since imipramine was added to inhibit the re-uptake of released serotonin by the platelets, the differences in the release pattern among serotonin, calcium and magnesium cannot be explained by the differences in re-uptake among these substances.

In order to compare the release of these substances, the release should be expressed as a % of the releasable pool. When the release reached near plateau levels during incubation, the extent of the release is conceivably equal to the extent of the releasable pool. The values of calcium- and magnesium-release in Figs. 5 and 9 are approximately equal to % release of the releasable pool. On the other hand, since serotonin-release in the experiments with the Tyrode solution did not reach any plateau level, the extent of releasable pool of serotonin is expected to be larger than the extent of the release at 15 min incubation. If the release is expressed as a % of the releasable pool, the values of serotonin release would be lower than those represented in Figs. 5 and 9. Then the difference in the release of serotonin from that of calcium or magnesium would be more significant.

Although localization of high concentration of calcium in serotonin-organelles of human platelets has been confirmed, it is likely that subcellular structures other than dense granules contain calcium and magnesium. A mitochondrial and dense tubular system location for calcium has been recognized. Monoamine oxidase, a mitochondrial enzyme, and CDP-diacylglycerol:inositol transferase, a microsomal enzyme, were not extracellularly detected after thrombin- and PMA-induced platelet activation (unpublished observation) and it is unlikely that mitochondria and dense tubular systems were released during platelet activation. It has been suggested that the platelet activation is initiated by an increase in cytosolic calcium released from the dense tubular system. Evidence has been presented that thrombin induces an increase in permeability of the platelet membrane to calcium. The released calcium in this study may partially include diffusible cations attributed to dense tubular systems. On the other hand, there are several reports which suggested that alpha-granules contained calcium and magnesium. In human platelets, PF-4, an alpha-granule protein, was released more rapidly than serotonin. Kaplan et al. showed that the release of alpha-granule proteins was achieved by a milder stimuli than that required for serotonin-release. The characteristic of the release of calcium and magnesium was more like that of PF-4 than that of serotonin.

It has been considered that calcium and magnesium are the predominant cations in serotonin granules and that they are extracellularly released together with serotonin during platelet activation. The present results, however, suggest possible existence of calcium- and magnesium-pools, whose contents are more susceptible to mild stimuli than granular serotonin in rabbit and human platelets.

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References

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