STUDIES ON PLASMA FACTOR INVOLVED IN 5-HYDROXYTRYPTAMINE RELEASE FROM BLOOD PLATELETS BY BACTERIAL ENDOTOXIN

III. MECHANISM OF ACTION OF 5-HYDROXYTRYPTAMINE RELEASING FACTOR

Yasuyuki NOMURA, Toshikazu OKADA and Hiroshi TAKAGI
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto

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It has been well established that 5-hydroxytryptamine (5HT) release from blood platelets by bacterial endotoxin (lipopolysaccharide, LPS) requires the presence of plasma factor and calcium ion (1-4). Recently, separation and purification of plasma factor with 5HT releasing activity has been reported by Nomura and Takagi (5,6). They estimated that this factor could be mainly prothrombin-like protein, however, the mechanism of 5HT release by this factor is unknown. Thrombin has been considered an enzyme with 5HT releasing activity from the blood platelets and mechanism of its action was studied by Gainter et al. (7), Pletscher et al. (8) and Markwardt et al. (9-11).

The purpose of the present experiment is to investigate the mechanism of action of 5HT releasing factor purified using a previous method (6), as well as making a comparison with thrombin.

METHODS

1) Preparation of 5HT releasing factor: The factor was purified from rabbit plasma by a combination of chromatographies previously described (6).

2) Preparation of platelet suspension: Rabbit platelets isolated by similar methods previously described (4) were suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing isotonic salt solution, the composition of which is as follows: NaCl, 8.33 g; KCl, 0.35 g; CaCl2• 2H2O, 0.37 g; MgCl2• 6H2O, 0.24 g; glucose, 2.0 g in 1,000 ml of distilled water.

3) Assay of 5HT releasing activity: The purified 5HT releasing plasma factor (0.5 ml) or human thrombin solution (0.5 ml) and an equal volume of various drugs dissolved in saline solution were incubated for 10 min at 37°C and platelet suspensions added. The mixture was again incubated for 10 min at 37°C under nitrogen atmosphere with gentle shaking (50/minutes) then centrifuged at 1,200 g for 30 min at 4°C. Contents of 5HT in the supernatant and the pellet were determined spectrophotofluorimetrically by methods described by Bertler (12) and Bogdanski et al. (13).

4) Determination of protein concentration: Protein concentration was expressed as
absorbancy at 280 m\textmu m in a 1 cm cuvette of a Shimazu MR-31 spectrophotometer.

5) Preparation of brain thromboplastin solution: Rabbits of either sexes and weighing about 2.5 kg were sacrificed by bleeding from the carotid artery, cerebrum and brain stem were removed and adequately washed with cold saline solution. Brain tissue was squeezed into the mixture of 10 ml of the cold acetone and 0.1 ml of 0.2 M sodium citrate solution and the supernatant discarded. Brain tissue was squeezed and homogenized with 10 ml of the cold acetone three times. Acetone powder was dried at 37°C for 30 min. Acetone powder (0.2 g) suspended in 5.0 ml of saline solution was incubated at 50°C for 10 min. The supernatant of this suspension was used as a thromboplastin solution.

6) Electron microscopy: Fixation of platelets was carried out by methods of Tranzer et al. (14); Platelets were fixed for 4 hrs in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and then soaked overnight in cold 0.1 M sodium phosphate buffer (pH 7.4) containing 7% sucrose. Samples were overfixed further for one hr. in 2% osmium tetroxide diluted in 0.1 M sodium phosphate buffer (pH 7.4). After fixation, samples were processed through a series of graded alcohols, immersed in the propylene oxide and embedded in Epon resin. Ultrathin sections were prepared with a Porter-Blum Ultramicrotome and stained with uranyl acetate and lead monoxide. Electron microscopic observations were made using Nihondenshi JEM 7A electron microscope.

7) Materials used were: Endotoxin (lipopolysaccharide) of Pseudomonas fluorescens ("T.T.G.", Fujisawa Pharmaceutical Co., Ltd.), 5-hydroxytryptamine creatinine sulfate (Nakarai Chemicals Ltd.), thrombin (human thrombin, Midorijyuji Co., Ltd.), trasylol (Bayer Co., Ltd.), DFP (Sumitomo Chemicals Co., Ltd.), tosyl-arginine methyl ester hydrochloride (TAME, Nakarai Chemicals Ltd.) and prothrombin which was purified from bovine plasma by Dr. K. Fujikawa, Institute for Protein Research, Osaka University.

RESULTS

1) Time course of 5HT releasing action of purified plasma factor

The purified plasma factor (dose: A_280 = 0.005) resulted in approx. 97% release of 5HT from platelets within 10 min. Similar rapid 5HT release also observed by 0.3 units/ml of thrombin (Fig. 1).

2) Dose-response curve of this factor

The dose-response curves of 5HT release by this factor and thrombin were shown in Fig. 2. As the dose of plasma factor increased, the amount of 5HT release markedly increased. This effect was greatly potentiated by addition of thromboplastin. Thrombin induced a marked 5HT release by approx. twice the amount.
Fig. 2. Dose-response curves of 5HT releases by the plasma factor and thrombin from platelets. A_{50} of 1.0 unit/ml thrombin was 0.0175. The 5HT releasing plasma factor was preincubated with brain thromboplastin for 10 min at 37 C. Incubation : 10 min at 37 C.

Fig. 3. Effect of calcium ion on 5 HT releasing actions of the plasma factor (A_{50} = 0.004) and thrombin (0.15 units/ml) from platelets suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing saline solution. Incubation : 10 min at 37 C.

3) Calcium requirement for 5HT release by this factor

Neither this factor nor thrombin induced 5HT release from platelets suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing isotonic salt solution but both samples exhibited marked effects under the presence of 2.7 mM calcium ion (Fig. 3).

4) Effect of thromboplastin

The preparation of the purified plasma factor used in this experiment was an active
The effect of thromboplastin on 5HT releasing actions of the plasma factor and bovine prothrombin. After preincubation of thromboplastin with the plasma factor or prothrombin for 10 min at 37°C, platelet solutions were re-incubated with these drugs for 10 min at 37°C. TP: thromboplastin, PTh: prothrombin.

Fig. 4. Effect of thromboplastin on 5HT releasing actions of the plasma factor and bovine prothrombin. After preincubation of LPS or heparin with the plasma factor or thrombin for 10 min at 37°C, platelets solutions were re-incubated with these drugs for 10 min at 37°C.

In the dose-response curve in Fig. 4, potentiation of the thromboplastin to the 5HT releasing effect of this preparation was clearly shown.

Fraction of hydroxylapatite chromatography. Specific activity of this preparation was less than that obtained by DEAE-cellulose chromatography.

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Thromboplastin or purified bovine prothrombin did not induce a significant 5HT release, however a combination of both samples markedly induced 5HT release (Fig. 4).
5) **Effect of LPS and heparin**

The effects of LPS and heparin to 5HT releasing activity of the plasma factor prepared by hydroxylapatite chromatography were investigated under the presence of thromboplastin. Heparin (2 units/ml or 5 units/ml) inhibited 5HT release by this factor and its inhibition was antagonized by the addition of LPS ($10^{-4}$ g/ml) (Fig. 5). LPS ($10^{-1}$ g/ml) alone produced a slight increase in 5HT releasing effect of this factor. Regarding the 5HT releasing effect of thrombin, heparin and LPS showed similar effect to that of this factor.

6) **Effects of DFP, trasylol and TAMe**

The 5HT releasing effect of this factor was slightly inhibited by DFP ($10^{-1}$ M and $10^{-4}$ M) or TAMe ($10^{-5}$ g/ml and $10^{-4}$ g/ml), but not by trasylol (500 KIE/ml and 50 KIE/ml) (Fig. 6).

![Graph](https://example.com/graph.png)

**Fig. 6** Effects of DFP, trasylol and TAMe on 5HT releasing action of the plasma factor. After preincubation of DFP, trasylol or TAMe with the plasma factor for 10 min at 37°C, platelets solutions were re-incubated with these drugs for 10 min at 37°C.

![Image](https://example.com/image.png)

**Fig. 7** An electron micrograph of platelets treated with saline for 10 min at 37°C. Scale: 1.0 micron.
Fig. 8. An electron micrograph of platelets treated with the plasma factor (A, 0.02). Incubation: 10 min at 37°C. Decrease of number of dense body granules and appearance of vacuoles and pale cytoplasm in part of the platelets can be observed. Scale: 1.0 micron.

Fig. 9. An electron micrograph of platelets treated with the thrombin (0.001 unit/ml). Incubation: 10 min at 37°C. Similar alterations to Fig. 7 are observed. Scale: 1.0 micron.

7) Morphological changes of platelets treated by this factor and thrombin

The ultrastructures of platelets treated by this factor (A, 0.02), thrombin (0.001 unit/ml) and saline were observed by electron microscopically (Figs. 7-9). In these treatments, 5HT releases were 15.6%, 19.4%, and 7.6% respectively.

Ultrastructure of normal platelets showed the same observation as the results reported by Kuruma and Takagi (15). After addition of the plasma factor platelets decreased the number of osmiophilic dense granules (its decrease was about 69.1%, compared to number
of dense body granules of control platelets) and increased the number of vacuoles and occurrence of platelets in which cytoplasm became pale. Similar changes in ultrastructures of platelets were observed after addition of thrombin (Fig. 9). Moreover, both this factor and thrombin induced slight aggregation of platelets.

DISCUSSION

Chemical nature

In our previous paper (6) it was suggested that our purified factor was prothrombin-like protein. In the present experiment, it was found that there were some similarities between this factor (active form) and thrombin in several respects: 1) the speed of 5HT release was very high, 2) both required calcium ion for 5HT release, 3) there were similarities in both does-response curves, 4) there was also similarity in ultrastructural change of platelets, and 5) thromboplastin potentiated both the 5HT release of this factor and bovine prothrombin.

These results also support the suggestion that the purified plasma factor might be prothrombin-like protein which is converted to thrombin-like protein by the combined effect of calcium ion and thromboplastin or its related co-factor.

Mechanism of 5HT release

If the active form of this factor is a thrombin-like enzyme, there appears the possibility that the action of this factor might be mediated through the clotting process which results in release of 5HT from the platelets. However, this possibility is refuted by the following: 1) 5HT release by thrombin is not related to its clotting mechanism (11), 2) bacterial endotoxin antagonized the heparin-induced inhibition on the 5HT release by this factor, but not on the clotting action (Fig. 5 and our previous paper (4). 3) 5HT release by this factor or thrombin was observed in low concentrations that did not cause notable morphological changes such as disruption of the membrane and degranulation.

On the role of calcium ion in the 5HT release by this factor, the following two actions are to be considered: one is activation of this factor (from prothrombin-like protein to thrombin-like protein), and

![Diagram](image-url)
to another is the effect on the 5HT storage granule in the platelets.

Markwardt (11) suggested that thrombin could make the membrane permeable to calcium ion, thereby an influx or mobilization of calcium occurs and the platelet ATPase (thrombosthenin) becomes activated, leading to an extrusion of the contents of 5HT storage granule outside of the cell. But there is no definite evidence on the role of thrombosthenin.

Recently, Wolfe and Shulman (17) reported that thrombin induced a rapid release of calcium from human platelets suspended in the calcium-free medium. In the present experiments thrombin did not induce 5HT release from platelets suspended in calcium-free medium. These results suggest that thrombin might release calcium from the platelet membrane, thereby the platelet membrane becoming permeable to calcium ion. When concentration of intracellular calcium reaches a certain critical level, 5HT release may occur, since the 5HT release from the 5HT storage granule requires certain concentrations of calcium ion. (8, 16).

Pletscher et al. (8) have demonstrated that thrombin is capable of releasing 5HT from platelets but not from the isolated 5HT storage granule.

From the foregoing, the following can be concluded: the active form of the 5HT releasing factor could induce a change in the permeability of platelet membrane, thereby facilitating entry of calcium ion into the platelet which in turn releases 5HT from the storage granule (Fig. 10).

SUMMARY

1. Mechanism of 5HT release from blood platelet by the purified 5HT releasing factor was studied in comparison with that of thrombin.
2. Speed of 5HT release by the 5HT releasing factor was rapid as was thrombin.
3. Potency of active form of this factor was approx. double than that of thrombin.
4. Neither this factor nor thrombin induced 5HT release from platelets in the calcium-free medium.
5. Thromboplastin potentiated 5HT release by this factor.
6. Heparin inhibited 5HT release by this factor or thrombin. Bacterial endotoxin (LPS) antagonized heparin-induced inhibition.
7. 5HT release by this factor was inhibited by DFP and TAMe but not by trasylol.
8. Platelets treated with this factor or thrombin showed decrease in dense body granules and increase in vacuoles and platelet aggregation according to electron microscopic observations.
9. The results mentioned above suggest that 5HT releasing factor was probably prothrombin-like protein which is converted into the active form having thrombin-like properties and an activated factor could induce an increase in the permeability of platelet membrane, thereby inducing influx or mobilization of calcium which in turn released 5HT from the storage granule.
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