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INHIBITION OF THROMBIN BINDING AND SEROTONIN SECRETION FROM PLATELETS BY A 74,000 DALTON PROTEIN

Pankaj Ganguly and Nancy G. Fossett

Department of Biochemistry
University of Tennessee Center for the Health Sciences
Memphis, Tennessee 38163

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A 74,000 dalton protein has been isolated from solubilized human platelet membranes by affinity chromatography with a nonagglutinating derivative of wheat germ agglutinin. The protein as well as the lectin derivative blocked platelet aggregation by thrombin while aggregation induced by several other agents was unchanged. This protein inhibited the binding of thrombin to platelets in a competitive manner. The secretion of serotonin from platelets by thrombin was also blocked by the protein. These effects of the protein were observed when platelets were suspended in either plasma or buffer. This protein may have functional significance in the activation of platelets by thrombin.

Thrombin reacts with platelets to induce their aggregation and secretion of specific materials from intracellular organelles (1). Several aspects of this reaction cannot be explained using a simple proteolytic model and an agonist-receptor equilibrium reaction has been proposed (2,3). The first step in this interaction is thought to be binding of thrombin to putative receptors on the platelet surface (2-5). This binding exhibits considerable specificity, with no binding observed with prothrombin or the intermediates of prothrombin activation, and appears to correlate with thrombin-induced platelet secretion. Although there have been many studies on the binding of thrombin to platelets and the nature of the thrombin receptor, the fundamental questions still remain unanswered. Recently, we have prepared a nonagglutinating derivative of wheat germ agglutinin which strongly inhibited activation of human platelets by thrombin while platelet aggregation by a number of other agents remained unchanged (6). Lectin affinity chromatography of solubilized platelet membranes utilizing this new

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inhibitor led to the isolation of a protein of 74,000 daltons which blocked platelet aggregation by thrombin with considerable specificity (6). We show that this protein competes for thrombin binding to platelets and inhibits the secretion of serotonin from platelets by thrombin.

MATERIALS AND METHODS: Details of materials and methods utilized in this study have been reported in a number of papers (1,5-7,14). Platelets were isolated from human blood and were suspended in 0.125M NaCl, 0.025M Na_2HPO_4/NaH_2PO_4, 0.002M EDTA and 0.5% serum albumin, pH 7.2 The isolated protein has a strong tendency to reassociate with platelets and the presence of albumin was essential for the experiments. Thrombin (2500 NIH U/mg) was labeled by the chloramine T method and the free label was removed by gel filtration. Binding of [^{125}I]thrombin to platelets was measured by the oil centrifugation method (3,6). The reaction was initiated by adding 125 µl of the platelet suspension (2.5 x 10^8/ml) to 125 µl of the thrombin-protein mixture. In initial experiments, we established that there was little difference in the platelet-associated radioactivity at 30 sec and at 5 min. After 2 min at room temperature, 200 µl of the suspension was rapidly layered on 0.4 ml of Versilub in a conical polypropylene tube which was centrifuged for 2 min at 12,000 g. The supernatant was carefully aspirated, the tip of the tube which contained the platelet pellet was cut off and counted for radioactivity. Platelet secretion was measured with [^{14}C]serotonin (7) and the values were expressed as percent of the control in the absence of the protein. Platelet aggregation was measured in a dual channel aggregometer (1,5,6). The experimental sample was analyzed in one channel while a control was run in the other. The nonagglutinating derivative was prepared by cyanogen bromide treatment of wheat germ agglutinin in 65% formic acid for 20 hr at room temperature. Affinity chromatography of detergent-solubilized platelet membranes was carried out through plastic columns of the lectin derivative coupled to Sepharose (6). In gel electrophoresis, the isolated material showed a major band with an apparent molecular weight of 74,000 and occasionally, a trace contaminant of about 55,000 daltons (6). Protein concentrations were determined by the dye binding method (BioRad) with serum albumin as standard.

RESULTS: The 74,000 dalton protein partially blocked the binding of labeled thrombin to washed platelets (Fig. 1). At high concentrations of the protein, the binding curve reached a plateau where the binding of labeled thrombin was similar to that obtained in the presence of excess unlabeled thrombin. This residual level of thrombin binding may represent the non-saturable fraction, generally referred to as "nonspecific" binding. Since platelets function in plasma and the protein could block thrombin-induced platelet aggregation with considerable specificity when added to plasma (6), we considered it desirable to repeat the thrombin binding measurements with platelets in plasma. The binding of thrombin to these platelets prepared without pelleting and washing was again blocked by the isolated protein and

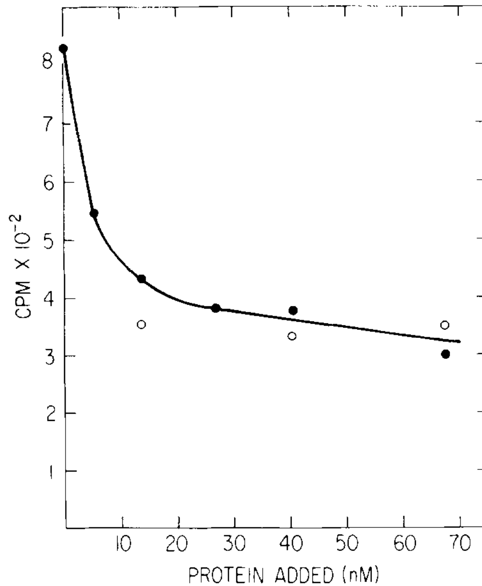

Fig. 1. Effect of the 74,000 dalton protein on the binding of thrombin to washed platelets. A constant amount of [^{125}I]thrombin (0.25 nM) was incubated for 10 min at room temperature with varying concentrations of the protein as indicated. Platelets (2.5 x 10^8/ml) in buffer was then added to each tube and the incubation was continued for another 2 min. The cells were separated by oil centrifugation as described under Materials and Methods. ●——●, protein plus labeled thrombin; O——O in the presence of 0.5 μM unlabeled thrombin.

the nature of the curve was similar to that of figure 1 (Fig. 2). To explore the nature of this inhibition, thrombin binding measurements to washed

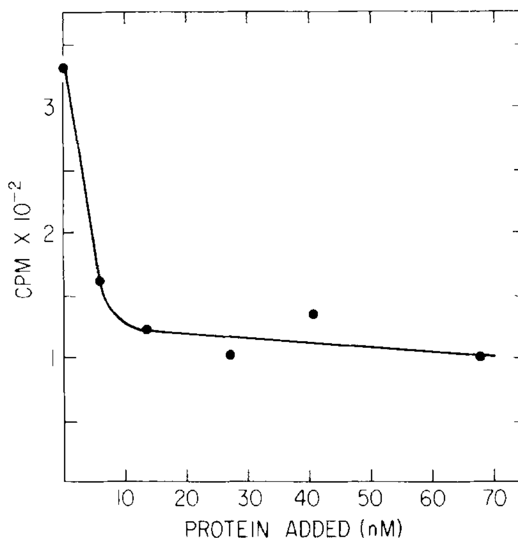

Fig. 2. Effect of the 74,000 dalton protein on the binding of thrombin to platelets. This experiment was done as in fig. 1 except that platelets were not washed and they were suspended in their physiological plasma medium.

platelets were carried out in the absence and presence of the protein at different concentrations of thrombin and the data were plotted as double reciprocals by the method of Steck and Wallach (8). In the absence of the protein, thrombin bound to approximately 600 sites/platelet with an apparent dissociation constant of 1.5 nM which are consistent with the values reported in the literature (2-5). In the presence of the protein, thrombin binding to platelets was lower and the lines intersected at the same point on the ordinate (Fig. 3). Thus, it appears that the reduction in thrombin binding to platelets by the isolated protein fits a model of competitive inhibition.

To determine if the inhibition of thrombin binding to platelets by the isolated protein is reflected in its biological action, we measured platelet secretion. The secretion of serotonin from platelets by a constant concentration of thrombin was progressively blocked by increasing amounts of the protein. At higher concentrations of the protein, the release reaction was completely inhibited (Fig. 4). This inhibition of thrombin-induced sero-

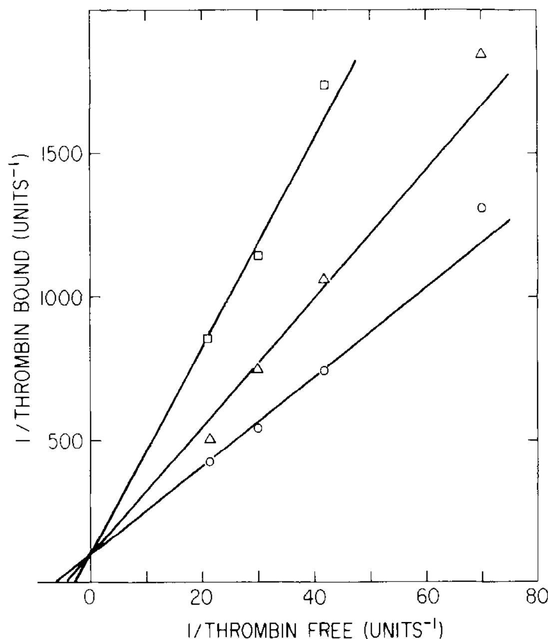

Fig. 3. Binding of thrombin to platelets and its competitive inhibition by the 74,000 dalton protein. Experiments were conducted as in fig. 1 with different concentrations of [^{125}I]thrombin in the absence or presence of the protein and data plotted as double reciprocals. 0—0 control △—△ 5 nM protein and □—□ 40 nM protein.

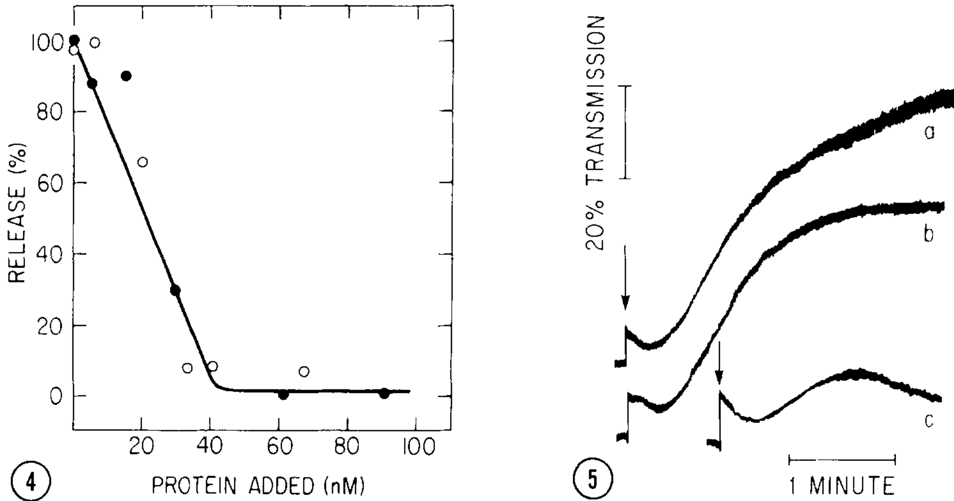

Fig. 4. Effect of the 74,000 dalton protein on thrombin-induced secretion of [^{14}C] serotonin from platelets. Platelets in plasma were loaded with serotonin, washed and resuspended in phosphate-buffered saline. Thrombin (2 nM) was incubated with different amounts of the protein for 10 min at room temperature and then serotonin-loaded platelets (2.5×10^8/ml) were added in a final volume of 200 µl. After 2 min, the reaction was stopped by adding 50 µl of 10% formaldehyde. The tubes were centrifuged and the radioactivity in an aliquot of the supernatant was measured and expressed as percent with respect to appropriate controls. Data from two experiments utilizing different protein, thrombin and platelet preparations are shown.

Fig. 5. Inhibition of platelet ($\sim 3 \times 10^8$/ml) aggregation in plasma by the 74,000 dalton protein and its reversal with increased thrombin concentration. (a) Control pattern of aggregation in which 50 mU/ml of thrombin was added to platelets at the arrow. (c) Thrombin (50 mU/ml) was first incubated with 0.6 µg of the isolated protein for 10 min at room temperature and then added to platelets. (b) The protein (0.6 µg) was preincubated with a higher concentration of thrombin (60 mU/ml) and then added to platelets. Data shown are representative of three different experiments.

tonin secretion was observed when the platelets were suspended in either plasma or buffer. We have previously reported that this protein could block platelet aggregation by thrombin (6). At a constant concentration of the protein, the inhibition of aggregation could be overcome by increasing concentrations of thrombin (Fig. 5). Platelet aggregation induced by several other agents such as ADP, collagen or ristocetin was not affected by the protein (6). These results make it unlikely that the protein acts on platelets causing a general perturbation of the cells there by affecting their aggregation. It appears more likely that the protein acts on thrombin.

DISCUSSION: The thrombin-platelet interaction is complex and the functional significance of the thrombin binding phenomenon as well as the nature of the

putative thrombin receptor have remained unclear. It is known that intact platelets have thrombin inhibitors on the cell surface (9) and that most of the thrombin bound is associated with nonproductive sites of platelets (2-6). Thus, it is likely that the platelet surface contains a number of components which may react with thrombin but may not necessarily be involved in the physiological activation of platelets by thrombin. A direct in vitro search for the thrombin receptor may yield a nonfunctional, thrombin-reactive platelet component irrespective of the model assumed. The only approach which will have a reasonable assurance of succeeding in such a complex system will be a physiological approach which relies on biological expression and can distinguish between productive and nonproductive interactions. A similar situation exists in thrombin-induced mitogenesis of fibroblasts and B lymphocytes, and stimulation of prostacyclin formation and release by vascular endothelial cells (10-12). In each of these cells, thrombin can interact with a number of cell surface components, covalently and non-covalently, and it becomes difficult to determine which of these interactions may be involved for the physiological effect. Finally, platelets function in plasma and thrombin can activate platelets in plasma with a high degree of specificity and efficiency. Although this point has generally been over-looked in most thrombin-platelet studies, any putative receptor/substrate of thrombin must have the specificity to be able to function in a plasma medium to have any physiological significance. This sort of reasoning led to the development of the lectin derivative which competitively and specifically inhibited platelet activation by thrombin. Then utilizing this new inhibitor, we isolated the 74,000 dalton protein which seems to have some of the properties desirable for a thrombin receptor in platelets. The following points may be noted. (a) The protein blocked thrombin-induced platelet aggregation and serotonin secretion while aggregation by ADP, collagen, epinephrine, ristocetin or trypsin was not affected (6). Thus, there is considerable specificity in the action of this protein on platelet function. (b) The protein competitively inhibited thrombin binding to platelets. (c)

The effects of the protein were observed when the platelets were suspended in either plasma or buffer again showing its high degree of specificity for thrombin. (d) The lectin derivative, similar to the isolated protein, selectively blocked thrombin activation of platelets showing that the 74,000 dalton protein is unlikely to be a nonfunctional inhibitor of thrombin. Thus, the action of the wheat germ agglutinin derivative is in many ways similar to an antibody to the protein. (e) Cross-linking of the platelet-bound lectin derivative, presumably to this protein, led to aggregation-independent serotonin secretion which is characteristic of thrombin (13). Recent studies in this laboratory indicate that the 74,000 dalton protein is a part of a multiprotein complex which may modulate the action of thrombin in a complicated manner (14). These properties of the 74,000 dalton protein suggest that it may be involved in the physiological activation of platelets by thrombin.

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