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THROMBIN INDUCED SURFACE CHANGES OF HUMAN PLATELETS

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SUMMARY

Membrane proteins of both control and thrombin-treated human platelets were labeled by (^{3}H)-sodium borohydride reduction of Schiff bases formed between pyridoxal phosphate and protein amino groups. Fluorographic analysis of solubilized platelet proteins disclosed a substantial difference between the labeling patterns of control vs. thrombin-treated cells. Whereas the normal platelets showed a single intensely labeled protein band, cells exposed to thrombin showed about ten labeled polypeptides. When thrombin-induced serotonin release from platelets was blocked by 3',5'-adenosine diphosphate, the protein labeling pattern on the fluorograph resembled that of control platelets. These data suggest that serotonin release from platelets by thrombin involve major changes in the architecture of proteins of platelets surface.

INTRODUCTION

Thrombin, a proteolytic enzyme with limited specificity, is a potent inducer of platelet "release reaction" (1,2) and its site of action has been shown to be the platelet surface (3-8). Despite extensive study (9-14), however no generally accepted hypothesis has emerged to explain the role of membrane proteins in the thrombin-induced "release reaction" of platelets. Part of the difficulty lies in identifying specific inducer-stimulated surface changes on intact platelets. We have demonstrated that pyridoxal phosphate (PALP) forms a Schiff base with the amino groups of platelet membrane proteins (15,16). Subsequent reduction of these complexes with tritiated sodium borohydride (NaB^3H_4) incorporates a tritium label of high specific radioactivity into membrane proteins of a wide variety of cells including platelets (15-20). Because PALP reacts mainly with the amino group of lysines, it can be used to

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*It should be noted that minor protein components of high specific radioactivity may overlap within the gels with abundant but unlabeled proteins and thereby incorrectly assign a label to a particular protein.

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gain information about the surface accessibility of an amino acid other than those (tyrosine and histidine) labeled by lactoperoxidase-catalyzed iodination (21-26).

The purpose of this investigation was to exploit PALP as a chemical probe for the detection of thrombin-induced alterations in the membrane surface of platelets suspended in plasma (PRP). Platelets were examined for their interaction with PALP before and after thrombin treatment in the presence and absence of 3',5'-adenosine diphosphate (3'5'-ADP), a potent inhibitor of platelet function (27).

MATERIALS AND METHODS

Venous blood was obtained from normal healthy volunteers who had not taken any medication for 2 weeks preceding blood collection. One volume of 4% sodium citrate was added to every nine volumes of blood and the samples were then centrifuged at room temperature at 300 x g for 10 minutes to prepare PRP. The platelet count of isolated PRP was adjusted to 300,000/μl with autologous platelet-poor plasma. 3'5'-ADP was used as an inhibitor of thrombin-induced platelet aggregation of PRP and serotonin release (27). Commercial bovine thrombin was purified by ion-exchange chromatography (28) to a minimum specific activity of 2000 NIH units of activity per mg of protein. Thrombin-induced "release reaction" was monitored by the release of [^{14}C] serotonin from platelets prelabeled with this amine (29). Aliquots of 0.5 ml PRP were used to study [^{14}C] serotonin release induced by varying amounts of thrombin (10-100 milliunits of activity). The amount of [^{14}C] serotonin released into the supernatant was measured and expressed as a percentage of the total radioactivity present in nonaggregated platelets. In a separate experiment, surface proteins of both control and thrombin-treated human platelets suspended in PRP were labeled by NaB^3H_4 reduction of Schiff bases formed between PALP and platelet protein amino groups (15). The labeled platelets were solubilized in a sample buffer containing 5% sodium dodecylsulfate and 1% β-merceptoethanol (30). Platelet protein (50 μg) were separated by electrophoresis on 7.5% polyacrylamide slab gels and then stained for protein bands with Coomassie blue (30). The gels were subsequently impregnated with 2,5-diphenyloxazole for fluorographic detection of radioactive protein bands (31). The minimum molecular weights of labeled protein bands were estimated from a calibration curve obtained with purified proteins of known molecular weights. The migration of each standard protein was determined in relation to 1% bromophenol blue marker.

RESULTS

The thrombin-induced release of [^{14}C] serotonin from platelets showed a dependence on concentration of the enzyme. At each thrombin concentration tested, [^{14}C] serotonin was rapidly released from platelets during the first minute after the addition of the enzyme and very slowly, if at all, thereafter. When PRP was preincubated with 3',5'-ADP (60-100 μM) for 15 minutes at 37°,

thrombin-induced [^{14}C] serotonin release was completely blocked (27). In the present study, PALP was used to determine if thrombin-induced [^{14}C] serotonin release affected the membrane topography of intact platelets. A typical polyacrylamide gel electrophoresis pattern of solubilized platelet proteins is shown in Fig. 1. This distribution pattern of Coomassie blue-stained proteins remained unchanged even when the platelets were exposed to thrombin (10-100 milliunits of activity) or treated with PALP (or both); however, comparison of the distribution of radioactivity in control vs. thrombin treated platelet proteins disclosed a distinct difference (Fig. 2). The fluorograph of control PRP showed a single intensely labeled protein band (VIII) with a molecular weight of 69,000 (Fig. 2A). When the PRP was exposed to 10 milliunits of thrombin activity, there was about 10% of [^{14}C] serotonin release and the fluorograph of such a preparation was similar to that of control PRP (c.f. Figs. 2A and 2B) except that one more protein band (II, mol wt 200,000) was labeled with a higher intensity. However, exposure of PRP to 30 milliunits of thrombin produced about 50% of [^{14}C] serotonin release from platelets and such a preparation showed three intensely labeled protein bands (Fig. 2C) on the fluorograph*: II (mol wt 200,000), VII (mol wt 90,000) and VIII (mol wt 69,000). Several moderately labeled polypeptides were also detected: III (mol wt 180,000), IV (mol wt 150,000), V (mol wt 130,000), VI (mol wt 110,000) and IX (mol wt 54,000). Each of these polypeptides was intensely labeled when PRP was treated with higher amounts of thrombin (60 milliunits of activity) which resulted in about 80% of the [^{14}C] serotonin release (Fig. 2D). Such a treatment of PRP also caused the appearance of another labeled protein band (X, mol wt 43,000) on the fluorograph which comigrated with platelet actin (X) as indicated in Fig. 1. The number, as well as the intensity of labeling of proteins by PALP were found to be related to the amount of [^{14}C] serotonin released from platelets. Accordingly, the fluorograph of PRP incubated with 3',5'-ADP (60 μM) prior to the addition of thrombin (60 to 100 milliunits of activity) resembled that of control PRP (c.t. Figs. 2A and 2E). These data indicate that PALP interact

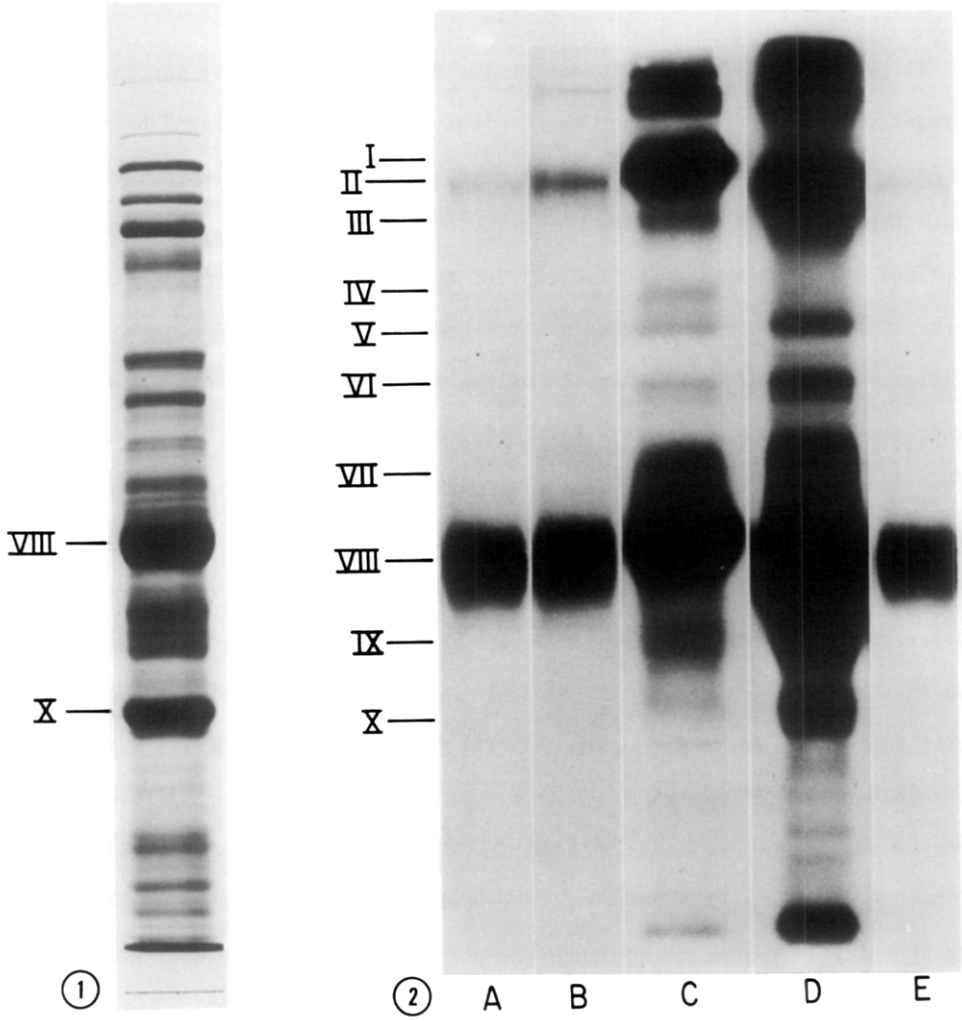

Figure 1. Electrophoretic pattern of Coomassie blue-stained proteins from control or thrombin-treated platelets. The platelet proteins were solubilized with dodecylsulphate and separated on 7.5% polyacrylamide slab gels. About 50 µg of the platelet protein sample was applied for electrophoretic separation. The polypeptide (X) denotes the actin band of human platelets with an apparent mol wt of 43,000. The protein staining pattern remained the same as the control even when the cells were exposed to thrombin (100 mu activity) or treated with PALP (3 mM) or both.

Figure 2. Relation of platelet protein labeling by PALP/ (^{3}H)$NaBH_4$ to concentration of thrombin in PRP. The fluorographs depict (A) control PRP or PRP exposed to thrombin activity of (B) 10 mu; (C) 30 mu; and (D) 50 mu. (E) A fluorograph of PRP preincubated with 3',5'-ADP (60 µM) for 15 min at 37°C before exposure of platelets to 100 mu of thrombin.

differently with cell membrane proteins depending on whether platelets are at rest or stimulated to cause a [^{14}C] serotonin release.

DISCUSSION

Thrombin has been shown to act at the platelet surface to initiate both aggregation and release (3) and the "release reaction" stimulated by thrombin has been proposed to involve conformational changes in the platelet surface (32). However, earlier studies using lactoperoxidase-catalyzed iodination of platelet outer surface proteins failed to differentiate appreciably the protein labeling pattern between control and thrombin-treated platelets (10-14). Moreover, these studies made use of washed platelets rather than PRP and utilized higher amounts of thrombin than actually needed to cause a "release reaction". In our studies, by using PRP instead of washed platelets and with a much lower amount of thrombin, we were able to identify the thrombin-induced surface changes in platelets as they might occur under physiological conditions. The appearance of several labeled protein bands on the fluorographs of thrombin-treated platelets indicate that the action of this proteolytic enzyme on platelets caused different proteins to emerge on the platelet surface, whereby became accessible to react with PALP. This increased availability of PALP-building proteins in thrombin-treated PRP might have resulted from the immediate action of the enzyme on the platelet surface or from subsequent release of serotonin from the platelets. It is not possible at present to favor either of these alternatives. The labeled polypeptide band IV (mol wt 150,000) was previously characterized as platelet glycoprotein I which was suggested to be a platelet receptor protein for thrombin (15,16). Based on the molecular weight, it may be reasonable to suggest that the labeled band II (mol wt 200,000) corresponds to platelet thrombosthenin M (33) and the protein band III (mol wt 180,000) represents the "thrombin sensitive protein" of human platelets (3). In view of the fact that several of the labeled polypeptides were not observed when the thrombin-induced "release reaction" was blocked by 3',5'-ADP, it may be suggested that the serotonin release from platelets involves alterations in the structural orientation of platelet membrane proteins. This is evidenced by the appearance of labeled actin on the fluorograph of thrombin-treated PRP where, 80% of total

[^{14}C] serotonin was released from the platelets (Fig. 2D). The labeling of actin by PALP could be due to the binding of coenzyme to actin during its exposure from inside to the outer surface of the cell.

Although platelets are known to contain several membrane glycoproteins with molecular weights ranging from 90,000 and 160,000, the exact identity of the labeled polypeptides identified in this study (Figs 2C and 2D) remain to be determined. The labeling procedure outlined in this study may help to determine whether or not other inducers of platelet function also cause similar surface changes on platelets as documented for thrombin.

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