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A Thrombin-Sensitive Protein of Human Platelet Membranes

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ABSTRACT **The action of thrombin on intact human platelets has been studied with the aid of polyacrylamide gel electrophoresis in sodium dodecylsulfate. A single major membrane protein band with a molecular weight of 190,000 disappears after thrombin treatment, while a new membrane protein with a molecular weight of 107,000 appears. This may represent hydrolysis of the thrombin-sensitive protein. When platelets are disrupted or when the thrombin-sensitive protein is solubilized from membranes prior to thrombin treatment, no hydrolysis occurs. The effect of thrombin on the platelet membrane protein is complete within 2 min which suggests that hydrolysis of this membrane protein may trigger the physiological effects of thrombin on platelets.**

Platelets are anucleate circulating particles, with a diameter of 2–4 μm in man, which function in hemostasis. These "cells" are metabolically active and catalyze the formation of fatty acids, phospholipids, glycogen, and even protein (1). Both glycolytic and Krebs cycle oxidative pathways are active in these cells.

Blood platelets are rapidly converted from free floating cells to an aggregated mass of intact platelets during the process of hemostasis or of thrombus formation. This obvious change in the platelet surface is accompanied by a variety of morphological and biochemical changes which have been termed "viscous metamorphosis" (1). The most potent agent known to induce this process is the proteolytic enzyme thrombin, which is generated from reactions of the coagulation pathway. Characterization of the role of thrombin in producing viscous metamorphosis is integral to understanding thrombus formation and may also provide some insight into the membrane specification of cell–cell interactions.

Previous work done in our laboratory (2) has shown that thrombin causes a 7-fold increase in the rate of incorporation of glycerol into phosphatidyl serine in the platelet membrane. This burst in the rate of lipid synthesis is maximal within 2 min of the addition of thrombin and such synthesis returns to the pre-thrombin rate by 20 min. Studies of the mechanism of this thrombin effect indicate that proteolysis is involved, since the effect can be mimicked by trypsin and inhibited by prior incubation of trypsin with soybean trypsin inhibitor. Intact cells are required for the reaction, and disruption of platelets by homogenization, freeze-thawing, or sonication abolishes the thrombin effect. The time course, the requirement for intact platelets, and the involvement of proteolysis suggests that hydrolysis of a membrane protein may trigger the effects of thrombin on phospholipid synthesis in platelets.

This report presents experiments that demonstrate a thrombin-sensitive protein which is hydrolyzed when thrombin is added to intact platelets. Although we refer to the action of thrombin as hydrolysis of the thrombin-sensitive protein, we have not demonstrated this directly. This protein has been located in the membrane fraction of the cell.

Abbreviation: SDS, sodium dodecylsulfate.

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MATERIALS AND METHODS

Sodium dodecylsulfate (SDS) obtained from Sigma Chemical Co. was recrystallized by the method of Burgess (3). Human thrombin was a gift of Dr. Kent Miller and was assayed as described previously (4). Reagents used for acrylamide electrophoresis were purchased from Canalco.

Human platelets, collected and isolated as described previously (2), were washed twice in a pH 6.5 isotonic buffer containing 0.113 M NaCl, 0.0043 M K_2HPO_4, 0.0043 M Na_2HPO_4, 0.0244 M NaH_2PO_4, and 1 mg/ml glucose. After washing, platlets were resuspended in either 0.154 M NaCl–0.154 M Tris·HCl (pH 7.4) 9:1 with 1 mg/ml glucose, or in a pH 7.5 buffer containing 0.109 M NaCl, 0.043 M K_2HPO_4, 0.0106 M Na_2HPO_4, 0.0083 M NaH_2PO_4, and 1 mg/ml glucose.

Platelets were incubated at 37°C at 1–2 $\times$ 10^9 cells/ml in resuspension buffer with or without 1 U/ml thrombin and 2.5 mM $CaCl_2$. The cells were disrupted by no-clearance-pestle homogenization (5), glycerol loading, and hypotonic lysis (6), or (routinely) by sonication for 15 sec at 70% intensity with a Biosonik sonifier. Centrifugation in a Sorvall RC2-B centrifuge for 30 min at 50,000 $\times$ g pelleted the membrane fraction, which was then examined directly or washed one or more times in the resuspension buffer.

Protein was determined by the method of Lowry *et al.* (7) or by a microbiuret assay (8). Sialic acid was assayed according to Warren (9). Membranes were extracted with pyrophosphate buffer by the method of Cohen *et al.* (10).

SDS solubilization and gel electrophoresis

Samples were solubilized in 1–3% SDS–1% β-mercaptoethanol –0.1 M sodium phosphate pH 7.5 overnight at room temperature at a protein concentration of 2–3 mg/ml. Electrophoresis in 5% polyacrylamide gels 20 cm in length, containing 0.1% SDS and 0.1 M sodium phosphate pH 7.5, was carried out for 11 hr at 8 mA/gel. The gels were stained and destained by standard techniques using Coomassie brilliant blue (11, 12).

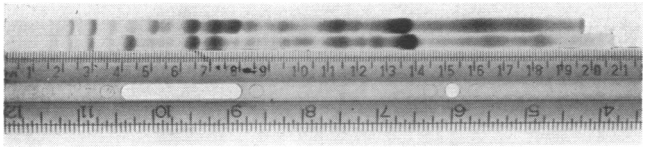

FIG. 1. SDS–polyacrylamide gel electrophoresis of membranes from control (lower gel) and thrombin-treated platelets (upper gel). Note difference at 42 mm.

RESULTS

Thrombin-sensitive membrane protein

SDS–polyacrylamide electrophoresis of SDS-solubilized membranes discloses a large number of bands encompassing a broad range of molecular weights (Fig. 1). A previous study (13) of platelet membranes by SDS–polyacrylamide electrophoresis failed to demonstrate the number of proteins shown here, but that work used 10% acrylamide gels, which are unable to resolve the high molecular weight proteins contained in platelet membranes. One of the major membrane bands, occurring at a distance of 42 ± 2 mm from the gel origin, is missing in membranes derived from thrombin-treated platelets. A new electrophoretic band at 78 ± 3 mm appears in the membranes from thrombin-treated platelets, which is not present in control platelet membranes. Unlike the thrombin-sensitive band, this band stains with variable intensity. In some experiments it appears to be more prominent relative to the thrombin-sensitive band, while in others it is much less prominent. Other minor differences are noted on these gels but these are not consistently found, while the changes in the two major bands are constant. The thrombin-sensitive protein band at 42 mm and the new 78-mm band remain in membranes of control and thrombin-treated platelets, respectively, after five washes, which suggests that these components are integral parts of the platelet membrane rather than adsorbed serum proteins or contaminants from the soluble fraction of the cell. When platelets are disrupted, the thrombin-sensitive protein and the new 78-mm band always appear in the membranous fraction of the cell contents, never in the soluble supernatant fraction, even after sonication (Fig. 2).

Molecular weights of membrane proteins

Molecular weights of platelet membrane proteins were determined by subjecting standard proteins of known molecular weights to electrophoresis in SDS–polyacrylamide gel in parallel with solubilized platelet membranes (11, 12). Fig. 3 shows a plot of log molecular weight versus electrophoretic mobility. A molecular weight of 190,000 was found for the thrombin-sensitive protein. The new band appearing at 78 mm in the membranes of thrombin-treated platelets has a molecular weight of approximately 107,000.

Molecular weight data for the thrombin-sensitive protein indicate that it is not fibrinogen, since human fibrinogen fractioned by this technique yielded three subunits of MW 73,000, 60,000 and 53,0000 as described previously (14). Platelet-poor plasma centrifuged at 48,200 × g to remove all platelets contains no protein corresponding to the 190,000 molecular weight thrombin-sensitive protein when fractionated by SDS–polyacrylamide electrophoresis.

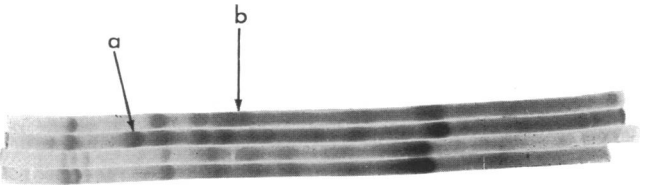

FIG. 2. SDS–polyacrylamide gel electrophoresis of membranes and soluble proteins from control and thrombin-treated platelets. The gels, from lower to upper, are as follows: supernatant protein from control platelets; supernatant protein from thrombin-treated platelets; membrane from control platelets; and membrane from thrombin-treated platelets. *a*, thrombin-sensitive protein; *b*, the band that appears after thrombin treatment.

Extraction of thrombin-sensitive protein

The thrombin-sensitive protein is extractable from platelet membranes by sodium pyrophosphate buffer at low ionic strength (Fig. 4). Approximately 50% of this protein is removed in one cycle of extraction. Such pyrophosphate-extractable material contains a large amount of glycoprotein, since 25% of the total cell sialic acid is present in the pyrophosphate extracts shown in Fig. 4. This extract also possesses calcium-activated ATPase activity, which in platelets has been ascribed (10) to the contractile protein component thrombosthenin M.

Substrate requirement

The disappearance of this protein from the platelet membrane caused by thrombin requires intact platelets as the thrombin substrate. No change in the thrombin-sensitive electrophoretic band of sonicated platelets or of membrane pyrophosphate extracts occurs upon treatment with thrombin under conditions in which this band is eliminated from membranes of

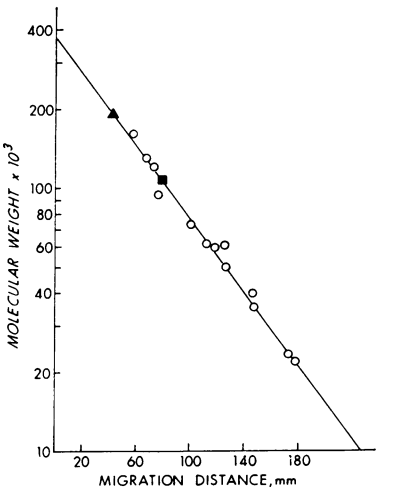

FIG. 3. SDS–polyacrylamide gel electrophoresis of standard proteins. Distance of migration is plotted against logarithm of molecular weight. The standards used are: 7S IgG gamma globulin 165,000, β-galactosidase 130,000, IgG heavy chain dimer 120,000, phosphorylase A 94,000, fibrinogen α-chain 73,000, IgG heavy chain 61,000, fibrinogen β-chain 60,000, catalase 60,000, fibrinogen α-chain 53,000, aldolase 40,000, pepsin 35,000, trypsin 23,000, IgG light chain 22,000. The thrombin-sensitive protein ▲ and the band which appears after thrombin treatment ■ are also shown.

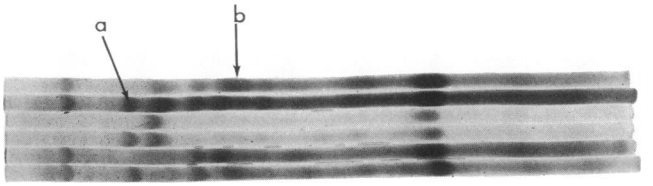

FIG. 4. SDS–polyacrylamide gel electrophoresis of membranes and pyrophosphate-extracted membranes from control and thrombin-treated platelets. The gels, from lower to upper, are as follows: pyrophosphate-extracted membranes from control platelets; pyrophosphate-extracted membranes from thrombin-treated platelets; pyrophosphate extract from control platelets; pyrophosphate extract from thrombin-treated platelets: unextracted membranes from control platelets; unextracted membranes from thrombin-treated platelets. *a*, *b*, as in Fig. 2.

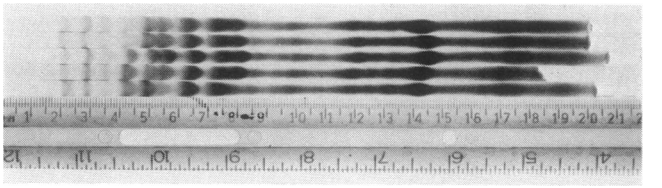

FIG. 5. SDS–polyacrylamide gel electrophoresis of membranes after various times of thrombin treatment of intact platelets. The gels, from lower to upper, are as follows: zero time; 15 sec; 30 sec; 2 min; 5 min.

intact platelets, nor did the thrombin-sensitive band in the pyrophosphate extract disappear after incubation up to 1 hr and thrombin levels to 3 U/ml.

Time course of the reaction

The fact that thrombin does not act on the membrane protein in sonicated platelets allowed a time-course study of this phenomenon, since thrombin action on the platelets could be halted by sonication of the incubation mixture. Fig. 5 demonstrates the time course of the thrombin-induced changes in the platelet membrane. Within 15 sec after initiation of thrombin treatment the new electrophoretic band at 78 mm is beginning to appear; at 30 sec the thrombin-sensitive band at 42 mm is approximately half gone, and by 2 min it has almost totally disappeared.

DISCUSSION

In addition to causing rapid aggregation, thrombin produces several metabolic effects on platelets, including a stimulation of phosphatidyl serine formation, increased ATPase activity, and increased glycolysis, glycogenolysis, and glucose oxidation (for review see ref. 1). These effects all occur rapidly after thrombin addition and are essentially complete within 15 min. The observed time course of disappearance of the thrombin-sensitive membrane protein is sufficiently rapid to account for the physiological effects of thrombin. Although the close parallel between the time course of the various effects of thrombin on platelet function suggests that hydrolysis of the thrombin-sensitive protein might trigger these effects, it is possible that thrombin induces these changes in several independent ways. There are numerous possible mechanisms by which hydrolysis of a membrane protein could induce changes in a number of independent pathways. These include proteolysis-induced conformational changes in the cell membrane, or mediation by one or more "second messengers" such as cyclic AMP. A further possibility is that the thrombin-sensitive protein described here does not represent the primary substrate for thrombin in the platelet but may be hydrolyzed secondary to thrombin action on an undetected earlier substrate. This more complex reaction sequence might be disrupted by cell breakage.

The striking feature of the thrombin reaction is that the membrane protein appears to be a substrate for thrombin only when intact platelets are used. This property of the thrombin-sensitive protein distinguishes it from other thrombin-sensitive proteins, including fibrin-stabilizing factor, which is hydrolyzed by thrombin in solution (15–20). Furthermore, in many previous studies high concentrations of thrombin for prolonged time periods have been used, which raises the question of nonspecific proteolysis by thrombin. Cohen *et al.* (10) have described a thrombin-sensitive protein which is hydrolyzed from intact equine platelets after incubation with 10 U/ml thrombin for 1 hr. These authors found that the protein is extracted from platelets by dilute pyrophosphate buffer. They have suggested that the protein is thrombosthenin M, which is analogous to myosin. We have demonstrated that a pyrophosphate extract of human platelets is heterogeneous, containing not only thrombosthenin M but also 25% of the sialic acid of the cell. Since thrombosthenin is not a glycoprotein, it is clear that other proteins are contained in this fraction. Although standard disc gel electrophoresis of the pyrophosphate extract yields a single protein band, we believe that the band contains an aggregate of multiple membrane proteins. Electrophoresis in SDS–acrylamide gel of pyrophosphate extracts yields multiple bands, including the thrombin-sensitive protein (Fig. 4). Further studies are required to determine whether the thrombin-sensitive protein described here is derived from thrombosthenin. The molecular weight of the thrombin-sensitive protein is similar to the subunit molecular weight of myosin, which is consistent with this hypothesis.

The origin of the protein with a molecular weight of 107,000 which appears after thrombin treatment is unknown. The lack of constant correlation between the staining intensity of the thrombin-sensitive protein and the MW 107,000 band suggests that the latter protein may not be derived from the former. The initial product of the fibrin cross-linking reaction is a dimer of γ chains, with a molecular weight of 105,000 (14). Since platelet membranes contain fibrinogen, this intermediate in the formation of insoluble fibrin could represent the protein which appears in platelets after thrombin treatment.

It has not been possible to isolate plasma membranes from platelets. Indeed the "sponge-like" quality of platelets with their extensive surface-connected internal membrane system (21) raises a question as to the morphological equivalent of the plasma membrane in these cells. Most of our studies have utilized the total membrane material of the platelet; thus we are unable to define exactly where the thrombin-sensitive membrane protein is, although it is accessible to added thrombin.

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