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Effects of diamide and dibucaine on platelet glycoprotein Ib, actin-binding protein and cytoskeleton

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During extraction of platelets by 1% Triton X-100, the actin-binding protein (platelet filamin) and a 230 kDa protein are degraded by a calcium-activated thiol protease. Occurrence of degration products of M_r 190000 (HF-1) and 90000 (HF-2) is a sensitive indicator of this proteolysis, and can be used to decide whether reduced amounts of the actin-binding protein in extracts are due to proteolysis or to incorporation in the Triton-insoluble (cytoskeletal) fraction. Diamide, which is a sulfhydryl-oxidizing protein cross-linker, inhibits the calcium-activated protease, polymerizes the actin-binding protein and the 230 kDa protein, increases the incorporation of glycoprotein Ib into the cytoskeletal fraction, and inhibits platelet agglutination induced by bovine von Willebrand factor. Inhibition of platelet agglutination by pretreatment with diamide is partly reversed by dibucaine which activates the calcium-activated protease. These observations are in accordance with a working hypothesis that interactions of glycoprotein Ib with cytoskeleton affect, and possibly regulate, its receptor function in the intact platelet.

Introduction

Binding of the plasma protein called von Willebrand factor (factor VIII-related protein) to the surface of the platelet is considered an important step in the adhesion of platelets to subendothelial structures in the damaged vessel wall during the process of hemostasis. In vitro, binding of von Willebrand factor to platelets induces a platelet agglutination. The membrane glycoprotein ** denoted glycoprotein Ib is the putative receptor for the von Willebrand factor (for review, see Ref. 1). Glycoprotein Ib is an amphiphilic protein [2–4] consisting of two disulfide-linked polypeptide chains, the α-chain of 143 and the β-chain of 22 kDa, respectively [5]. Through the action of one or more calcium-activated, thiol-dependent proteases present inside the platelets, glycoprotein Ib is proteolytically degraded during lysis of the platelets yielding the water-soluble product glycocalicin [2,3]. Recent observations indicate that glycoprotein Ib is a transmembrane protein [6–9]. Glycocalicin represents the highly glycosylated, externally oriented end of the α-chain [9].

Coller [10] found that incubation of platelets with dibucaine led to an increase, followed by a decrease in von Willebrand factor-induced platelet agglutination, and that this was accompanied by proteolysis of glycoprotein Ib. In a previous paper we have described how incubation of platelets with dibucaine leads to a continuous alteration of the mobility of glycoprotein Ib as studied by crossed immunoelectrophoresis of Triton X-100 extracts of platelets using antiserum to purified glycocalicin

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** The nomenclature for platelet membrane glycoproteins (abbreviated GP on illustrations) is adapted from Phillips and Poh Agin [5] with the exception that glycoprotein III is referred to as glycoprotein IIIa.

[11]. One possible explanation of this finding was that glycoprotein Ib might be complexed to an intracellular component acting as a substrate for the calcium-activated protease [11]. In a subsequent paper we demonstrated an association of glycoprotein Ib with the Triton-insoluble material prepared under conditions where this is considered to represent the cytoskeletal fraction of the platelets [9].

The protein cross-linking agent diamide is known to polymerize certaincytoskeletal proteins in the platelet through oxidation of sulfhydryl groups [12–14]. In the present study we have used this agent to further study the glycoprotein Ib-cytoskeleton interaction showing that incubation of platelets with diamide increases the incorporation of glycoprotein Ib into the Triton-insoluble fraction and inhibits agglutination of the platelets induced by bovine von Willebrand factor. Further, we show that incubation with dibucaine, which leads to proteolysis of the actin-binding protein and a 230 kDa protein, also gives a partial reversal of the inhibitory effect of diamide on the platelet agglutination.

Materials and Methods

Commercial materials. Diamide, Triton X-114, Nonidet P-40, Tris(hydroxymethyl)aminomethane (Tris), *o*-dianisidine, Coomassie brilliant blue G and R, 2-mercaptoethanol, lauryl sulfate (SDS) and EGTA were purchased from Sigma Chemical Company, St. Louis, MO, USA. Dibucaine (cinchocaine hydrochloride) from CIBA, Horsham, U.K.; Triton X-100 from Koch-Light Laboratories, Ltd., Colnbrook, Bucks., U.K.; concanavalin A and WGA-Sepharose 6MB from Pharmacia Fine Chemicals, Uppsala, Sweden; agarose HSA from Litex, Denmark; acrylamide and bisacrylamide from Bio-Rad, CA, U.S.A; ammoniumperoxidisulfate and EDTA from Merck, AG, Darmstadt, F.R.G.; TEMED from Fluka AG, Switzerland or Bio-Rad; the Schiff's reagent (Feulgen) from Raymond A. Lamb, London, U.K.; gelatin from Difco Laboratories, MI, U.S.A.; leupeptin from Protein Research Foundation, Japan; and nitrocellulose membrane BA85 from Schleicher and Schüll, Dassel, F.R.G. Diamide was dissolved at 5 mM in the standard Tris-buffered saline (pH 7.4) immediately before use. Dibucaine was dissolved in the same solution at 10 mM by addition of the minimal amount of HCl required and readjustment with NaOH to neutral pH. One tenth dilution in the same buffer gave pH 7.4.

Washing solutions and standard buffers. The solution used for washing of platelets consisted of 148 mM NaCl, 5 mM glucose, 0.6 mM EDTA and 20 mM Tris-HCl (pH 7.4, 280 mosM), and the Tris-buffered saline used for resuspensions of the platelets contained 148 mM NaCl and 20 mM Tris-HCl (at pH 7.4). The Triton X-100-containing solution used for extraction of platelets for the crossed immunoelectrophoresis studies, and for the SDS-polyacrylamide gel electrophoresis of Fig. 2, was composed of 38 mM Tris, 100 mM glycine (pH 8.7) and 1% (v/v) of Triton X-100. For extraction of platelets as an initial step in purification of glycoprotein Ib applying the phase separation technique of Bordier [15], the same buffer was used containing 1% Triton X-114 instead of Triton X-100. The standard Nonidet P-40-containing buffer used for immunoblotting consisted of 145 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.25 g/l gelatin and 0.05% Nonidet P-40 (pH 7.4). For the preparation of the cytoskeletal fraction, the platelet suspension medium and the extraction buffer were according to Jennings et al. [16]. The first consisted of 138 mM NaCl, 2.9 mM KCl, 12 mM NaH_2CO_3, 0.36 mM Na_3PO_4, 5.5 mM glucose and 1 mM EDTA (at pH 7.4), whereas the latter contained 2% Triton X-100, 10 mM EGTA and 100 mM Tris (at pH 7.4). During extraction these were mixed 1 : 1 (v/v). Standard SDS solution for sample preparation after a 1 : 1 dilution of the sample consisted of 500 μl 1 M sucrose, 100 μl 20% SDS, 19 μl 0.2 M NaH_2PO_4, 81 μl 0.2 M Na_2HPO_4, 20 μl Bromphenol blue and 280 μl H_2O per ml. For preparation of reduced samples this solution also contained 6% 2-mercaptoethanol.

Platelets. Human platelets were obtained and washed by repeated centrifugations and resuspensions as described in detail elsewhere [9]. Except for the experiments given in Figs. 8 and 9, where 2-day-old platelets from platelet concentrates were used, the platelets were from 40 ml samples of blood drawn as described [9] and immediately processed for the experiments. The platelets were counted in a Thrombocounter model C.

Formaldehyde-fixed platelets. Fixation of washed platelets in formaldehyde was performed by incubation with 1.8% formaldehyde at pH 6.4 for 2 h followed by three further washings. However, to increase the sensitivity of the platelets they were incubated with 1 mM dibucaine for 4 min at 37°C prior to the addition of the formaldehyde solution. (For principles see Refs. 10 and 17.)

Triton X-100 extracts for crossed immunoelectrophoresis. These were prepared as described previously [9].

Antisera. Rabbit antisera to human glycocalicin (and thus to glycoprotein Ib), and to whole platelet proteins were prepared as previously described [2,18]. Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins in 0.01 M phosphate-buffer saline (pH 7.2) was purchased from DAKO immunoglobulins AS, Denmark.

The cytoskeletal fraction of platelets. This was prepared according to Jennings et al. [16], except that a higher concentration of platelets was used. In essence, washed platelets were suspended to 10^{10} cells/ml in the medium given above and diluted in an equal volume of the 2% Triton X-100-containing extraction buffer stated above, and the extraction was performed at room temperature as described previously [9].

Glycocalicin and glycoprotein Ib. Glycocalicin was purified as previously described [11]. A partially purified preparation of glycoprotein Ib used in the immunoblotting experiment was obtained as follows. Platelets from 2 units of 1-day-old platelet concentrates were washed and extracted at 4°C using the Triton X-114-containing buffer described above. After centrifugation at $100\,000 \times g$ for 45 min, the extract (supernatant) was incubated over-night at 4°C with 10 mg/ml of concanavalin A dissolved directly in the extract. After recentrifugation as above to remove concanavalin A-precipitated proteins (including glycoproteins IIb and IIIa), the extract was incubated at 37°C for 10 min followed by centrifugation at $8000 \times g$ for 4 min whereby a separation into a detergent phase and a water phase became evident. The water phase was isolated, cooled on ice, and Triton X-114 was added again, this time to a concentration of 2%, and the phase separation procedure was repeated. Whereas residual glycoprotein IIb-IIIa complex partitioned into the detergent phase, glycoprotein Ib was present in the water phase. This was used for lectin affinity chromatography at 4°C on a column of wheat germ lectin coupled to Sepharose 6 MB. The column was flushed with the same kind of Triton X-114-containing buffer as used for the initial extraction supplemented with 0.2 mM EDTA and 0.1% sodium azide. Glycoprotein Ib was eluted as a partially purified preparation using the same buffer which in addition contained 113 mM *N*-acetyl-D-glucosamine. The elution was monitored by Laurell rocket immunoelectrophoresis of each fraction against antiserum to glycocalicin. A water phase from the glycoprotein Ib-containing fractions were prepared as described above. The partly purified glycoprotein Ib used in Fig. 5 was obtained at this stage. A further purification, as judged by SDS-polyacrylamide gel electrophoresis, could be obtained by affinity chromatography of this material on thrombin-Sepharose 4B performed essentially as described elsewhere [11].

Bovine von Willebrand factor. Highly purified bovine von Willebrand factor (factor VIII related protein) was purified as previously described [19]. The commercial material used for platelet agglutination was outdated for clinical use (antihaemophilic globulin factor VIII bovine, Speywood Laboratories Ltd., Nottingham, U.K.). It was dissolved in the standard Tris-buffered saline and extensively dialysed against the same buffer. For agglutination, this was used at a dilution corresponding in agglutinating activity approximately to that of 8 μg/ml of the highly purified material.

Crossed immunoelectrophoresis. This was performed with 1% Triton X-100 present in the agarose to keep amphiphilic proteins in solution as described previously [18].

Rocket immunoelectrophoresis. The technique of Laurell [20] was used as modified by Weeke [21].

SDS-polyacrylamide gel electrophoresis. Slab gel electrophoresis using 3% stacking gel and 7% separating gel was performed with the method of Laemmli [22] essentially as modified by LeStourgeon and Beyer [23]. Staining of the gels with Coomassie brilliant blue G for total proteins or the periodic acid-Schiff's reagent for glycoproteins was performed essentially as described previously for cylindrical gels [24]. Standard concentration of 2-mercaptoethanol for reduction was 3%. Prior to

electrophoresis the samples were heated at 100°C for 5 min. Molecular weights were estimated by the use of a marker protein kit from Bio-Rad containing myosin (200 000), β-galactosidase (116 250), phosphorylase B (92 500), bovine serum albumin (66 200) and ovalbumin (45 000).

Total protein. This was determined by the Bio-Rad assay essentially as described by Bradford [25] using human serum albumin as standard. For determination of total protein in the Triton-insoluble fractions these were first dissolved in 1% (w/v) sodium deoxycholate in 0.2 M NaOH.

Immunoblotting (Western). This was done essentially according to Towbin et al. [26]. After the SDS-polyacrylamide gel electrophoresis the 7% slab gel was incubated for approx. 45 min at room temperature in a 2.5 mM borate buffer (pH 9.2), and the proteins were electrophoretically transferred to a sheet of nitrocellulose membrane overnight in the same buffer at approx. 30 volts. Thereafter, unreacted groups on the nitrocellulose sheet were blocked by collagen (gelatin), whereafter this was washed three times in the standard Nonidet P-40-containing buffer described above. It was then incubated for approx. 18 h with the rabbit antiserum to human glycocalicin diluted in the same buffer 1 : 200 as compared to the concentration of the antiserum used for crossed immunoelectrophoresis. This solution thus served as the source of the primary antibodies. This incubation was followed by three washings, each time soaking the membrane in the Nonidet P-40-containing buffer for 5 min. Thereafter, the nitrocellulose membrane was incubated over-night with the commercial peroxidase-conjugated swine anti-rabbit immunoglobulin solution diluted 1 : 600 in the same buffer, as the secondary antibody preparation. Finally, it was washed five times as above and used for a peroxidase-catalyzed conversion of *o*-dianisidine by incubation of the paper with a mixture of 250 µl of 1% *o*-dianisidine, 250 µl of 3% H_2O_2 and 50 ml of H_2O at room temperature until suitable colour production had occurred. Finally, the nitrocellulose membrane was soaked in H_2O followed by 0.9 M acetic acid, and dried

Platelet agglutination. This was monitored in a Payton aggregometer equipped with a Tarkan W + W recorder 600. In the standard procedure 450 µl of platelet suspension was stirred at 400 revolutions/min in the aggregometer at 37°C for 3 min. The recorder was started, and after 25 s to establish the baseline, 50 µl of the bovine von Willebrand factor were added. After an immediate increase in light transmittance due to dilution, platelet agglutination was recorded as a continuous increase in transmittance. The aggregometer was calibrated in such a way that a difference in platelet number between that of the undiluted suspension (usually $3 \cdot 10^8$ cells/ml) and a 1 : 1 dilution of this ($1.5 \cdot 10^8$ cells/ml) corresponded to a change in the recorder input of 5 mV corresponding to deflection from 10 to 60 chart divisions on the recorder.

Results

Isolated, EDTA-washed, blood platelets dissolved directly in SDS and subjected to SDS-polyacrylamide gel electrophoresis on 7% polyacrylamide gels demonstrate the presence of three predominant Coomassie blue staining proteins in the 250 000–200 000 M_r range (Fig. 1A). These are also seen when the platelets are first extracted in 1% Triton X-100 in the presence of leupeptin as inhibitor of the calcium-activated platelet protease(s) (Fig. 2A and C) and the same patterns are seen with unreduced (Fig. 1) and reduced samples (Fig. 2). The largest of these proteins (M_r 250 000) represents the actin-binding protein, and the smallest (M_r 200 000) the myosin heavy chain, whereas the middle one, denoted the 230 kDa protein, was characterized only recently [27] and has been less studied. Corresponding electrophoreses of Triton X-100 extracts prepared without protease inhibitors demonstrate a near to total absence of the actin-binding protein and the 230 kDa protein (Fig. 2 B and D). However, electrophoresis of the small amount of protein that is insoluble in 1% Triton X-100 under these conditions showed that this fraction consistently contains some undegraded actin-binding protein (data not shown). Concomitantly with the disappearance of the above-mentioned proteins from the extract, new bands appear, two of which are easily observed with Coomassie brilliant blue (Fig. 2). These are denoted HF-1 (M_r 190 000) and HF-2 (M_r 90 000), respectively (Fig. 2).

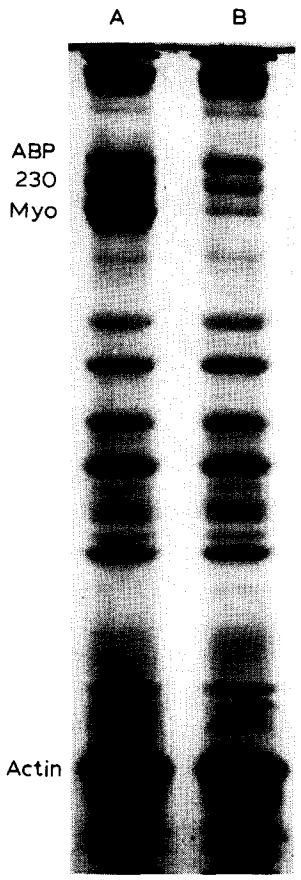


Fig. 1. SDS-polyacrylamide gel electrophoresis of whole platelet protein from (A): control platelets and (B): diamide-treated platelets. Unreduced samples to demonstrate the general protein pattern and the reduction in the amounts of SDS-soluble proteins in the 200–250 kDa range due to diamide-induced polymerizations. One portion of washed platelets (corresponding to B) was incubated at 37°C for 10 min with 0.5 mM diamide in Tris-buffered saline (at pH 7.4) (10^9 cells/ml), and another portion (corresponding to A) with buffer only. Thereafter, the platelets from both portions were sedimented by centrifugation and resuspended in the Tris-buffered saline to $5 \cdot 10^9$ cells/ml. An aliquot from each suspension was diluted in an equal volume of the standard SDS-solution without reducing agent, solubilized, and subjected to SDS-slab gel electrophoresis. The gel (7% polyacrylamide) was stained by Coomassie brilliant blue. Each lane represents $7.5 \cdot 10^7$ platelets. ABP, actin-binding protein; 230, 230 kDa protein; Myo, myosin heavy chain.

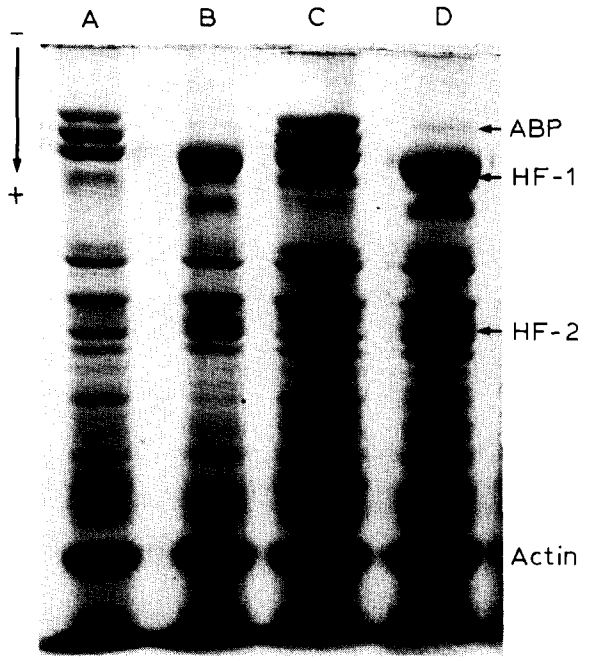


Fig. 2. Degradation of the actin-binding protein (ABP) and the 230 kDa protein by solubilization of platelets in 1% Triton X-100 in the absence of added inhibitors of the calcium-activated platelet protease(s). Washed platelets were extracted in 1% Triton X-100 in the standard Tris-glycine buffer (pH 8.7) (10^9 cells/ml) in (A and C): the presence of leupeptin (4.2 mM), or (B and D): in the absence of added inhibitors. Aliquots for SDS-polyacrylamide gel electrophoresis of reduced proteins were diluted one third in the standard SDS-solution containing 2-mercaptoethanol. The gel was stained with Coomassie brilliant blue. Each of lanes A and B represents $3 \cdot 10^7$ platelets, whereas lanes C and D represent $7.5 \cdot 10^7$ platelets each.

Crossed immunoelectrophoresis of platelets solubilized in Triton X-100 in the absence of added inhibitors of the calcium-activated protease give immunoprecipitate patterns as shown in Fig. 3 A and C, where the second-dimension electrophoresis have been performed with rabbit antibodies to human whole platelet proteins or to glycocalicin, respectively. Incubation of the platelets with 0.5 mM diamide at 37°C for 10 min prior to solubilization and crossed immunoelectrophoresis, demonstrated two phenomena. First, the immunoelectrophoresis patterns changed (Fig. 3 B and D) and became essentially like those observed with known inhibitors of the calcium-activated protease (see Fig. 6 and Ref. 11). Secondly, the amount of extracted glycoprotein Ib was strongly reduced as judged from the area below the glycoprotein Ib-related immunoprecipitates (Fig. 3D compared to Fig. 3C). These observations indicate that diamide can function as an inhibitor of the

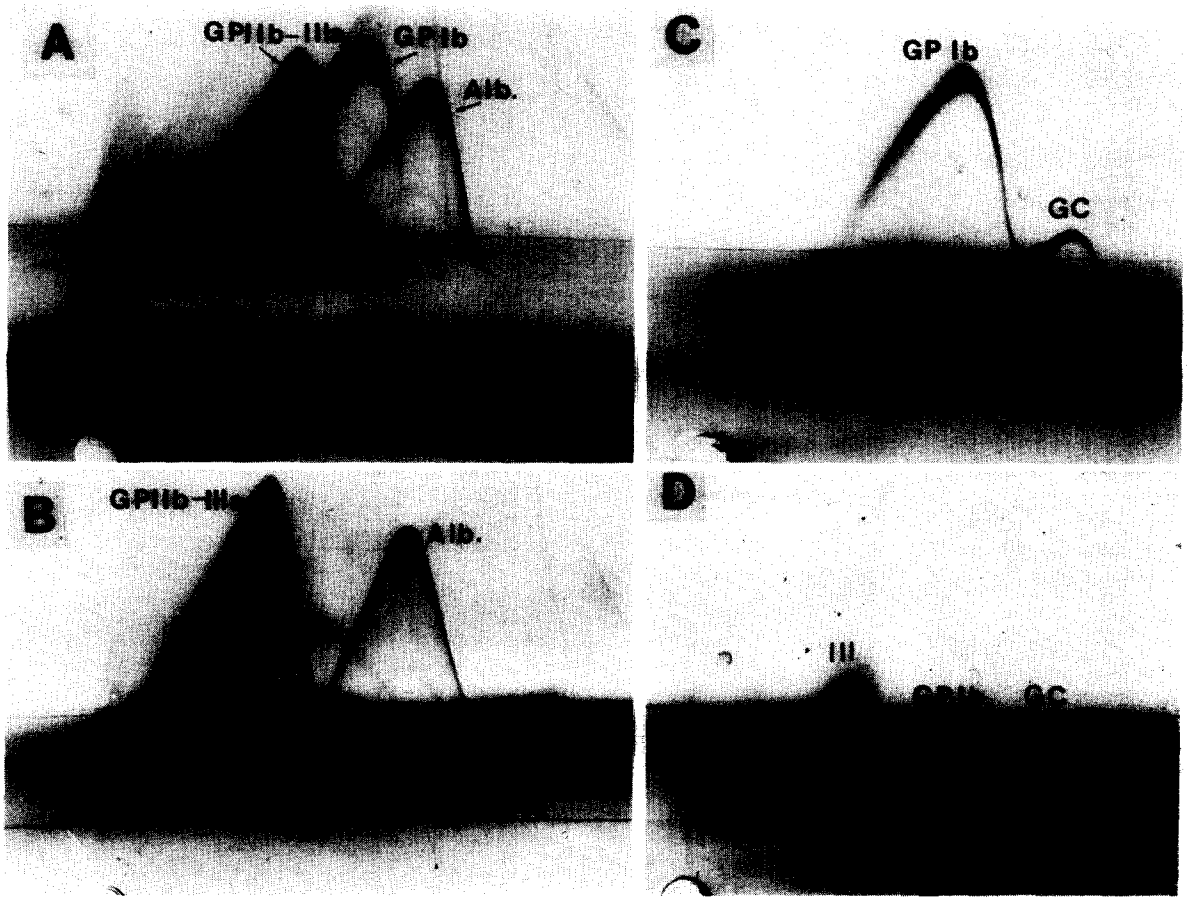


Fig. 3. Effects of prior incubation of platelets with diamide on the immunoprecipitate patterns observed after crossed immunoelectrophoresis of platelet extracts prepared with Triton X-100 using (A and B): antibodies to whole platelet proteins, and (C and D): antiserum to glycocalicin. Washed platelets were incubated with either (B and D): diamide (0.5 mM, 37°C, 10 min), or (A and C): buffer only, as described in the legend to Fig. 1. Thereafter, the platelets of both portions were extracted with 1% Triton X-100 in the standard extraction buffer at 10^{10} cells/ml. Each electrophoresis represents material from 10^8 platelets. GC, glycocalicin; Alb, albumin.

calcium-activated protease, and points to an incorporation of glycoprotein Ib in the Triton-insoluble fraction. Comparisons to the areas below the immunoprecipitates representing albumin or the complex of the membrane glycoproteins IIb and IIIa, indicate that the decreased extraction of glycoprotein Ib represents a specific phenomenon as these areas were not reduced in size by diamide (Fig. 3B compared to Fig. 3A). The inhibitory effect of diamide on the calcium-activated protease could also be seen from the fact that HF-1 and HF-2 was not observed after SDS-polyacrylamide gel electrophoresis of Triton X-100 extracts of diamide-treated platelets (data not shown).

When diamide-treated platelets were dissolved directly in an SDS-containing buffer, and the proteins were electrophoresed in their unreduced states on polyacrylamide gels followed by Coomassie brilliant blue staining, the intensities of the aforementioned bands in the 200–250 kDa region were clearly diminished in comparison to those from untreated platelets (Fig. 1). No obvious differences were observed when such comparisons were done using reduced samples (not illustrated).

Glycoprotein Ib is incorporated into the

Triton-insoluble sediment when platelets are extracted in 1% Triton X-100 under conditions where this material is considered to represent the cytoskeletal fraction of the platelets [9]. SDS-electrophoresis of Triton X-100 extracts (supernatants) prepared under such conditions followed by staining of the gel for glycoprotein by the periodic acid-Schiff's reagent, showed that the Triton-soluble portion of glycoprotein Ib was strongly diminished by prior incubation of the platelets with diamide (Fig. 4) indicating that diamide leads to an increased incorporation of glycoprotein Ib in the cytoskeletal fraction. Again glycoprotein IIb and IIIa could serve as controls of the specificity of this incorporation as diamide apparently did not affect these proteins (Fig. 4). Further, when the cytoskeletal fraction (sediment) from the diamide-treated platelets was analyzed in the same way, and shown to contain glycoprotein Ib, only a very weak staining could be seen in the position of the glycoproteins IIb and IIIa relative to that of glycoprotein Ib (Fig. 4).

Western immunoblotting using the same rabbit anti human glycocalicin serum as used for the crossed immunoelectrophoreses as source of the primary antibody, further demonstrated the presence of glycoprotein Ib in this Triton-insoluble fraction, and established its identity immunologically (Fig. 5). As seen from Fig. 5, glycocalicin moves slightly faster than the glycoprotein Ib α-chain in this system, and comparisons to marker proteins gave molecular weights of 145 000 and 133 000 for the glycoprotein Ib α-chain and glycocalicin, respectively.

Experiments in which the platelets were sedimented and washed once subsequent to the incubation with diamide, demonstrated that the effect of diamide did not depend on its extracellular presence. In Fig. 6 this is shown for diamide and also for leupeptin, as regards the crossed immunoelectrophoresis patterns. However, the same was shown for diamide also for the other systems described in this paper. This probably means that diamide penetrates the membrane and acts intracellularly. One difference between diamide and leupeptin was noted as to the crossed immunoelectrophoresis patterns of the corresponding Triton X-100 extracts with anti glycocalicin (Fig. 6). Whereas a rocket-like immunoprecipitate at the application point is regularly observed after the platelets have been treated with leupeptin (Fig. 6 A and B, and Ref. 9), this was either not observed,

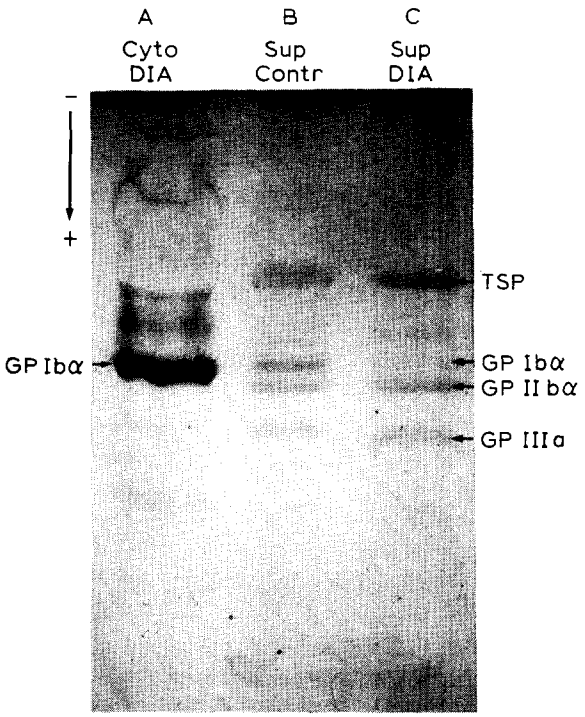


Fig. 4. Effect of diamide on the incorporation of glycoprotein Ib in the Triton-insoluble (cytoskeletal) fraction of platelets as studied with SDS-polyacrylamide gel electrophoresis and staining of glycoproteins with the periodic acid-Schiff's reagent. (A) Triton-insoluble sediment (cytoskeletal fraction) from diamide-treated platelets. (B) Triton-soluble supernatant from control platelets. (C) Triton-soluble supernatant from diamide treated platelets corresponding to (A). Washed platelets were incubated with diamide (0.5 mM, 37°C, 10 min) or with buffer only. After centrifugation at $1900 \times g$ for 10 min, the sedimented platelets were resuspended to 10^{10} cells/ml in the suspension buffer used for the preparation of the cytoskeletal fraction, and diluted in an equal volume of the extraction buffer used for the same purpose containing 2% Triton X-100 and 10 mM EGTA (at pH 7.4) as essential components. Thereafter, the platelets were solubilized by stirring. After centrifugation at $8000 \times g$ for 4 min, the supernatants were used for the subsequent electrophoresis after dilution of an aliquot in an equal volume of the standard SDS-solution. The sediments were washed and dissolved (corresponding to 10^{10} cells/ml) in the standard SDS-solution diluted 1:1 in 0.15 M NaCl. Reduced samples (2-mercaptoethanol) were electrophoresed on a 7% polyacrylamide slab gel. While each of the supernatant lanes (B and C) represent material from $1.25 \cdot 10^8$ platelets, the cytoskeletal material (lane A) was derived from $5 \cdot 10^8$ platelets. TSP, thrombospondin.

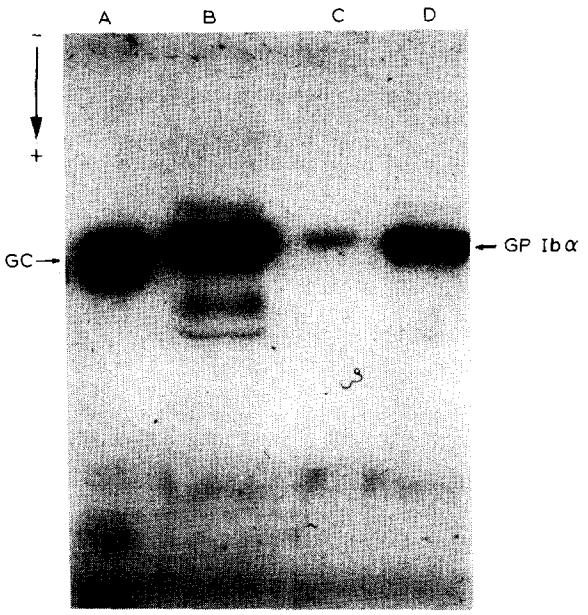
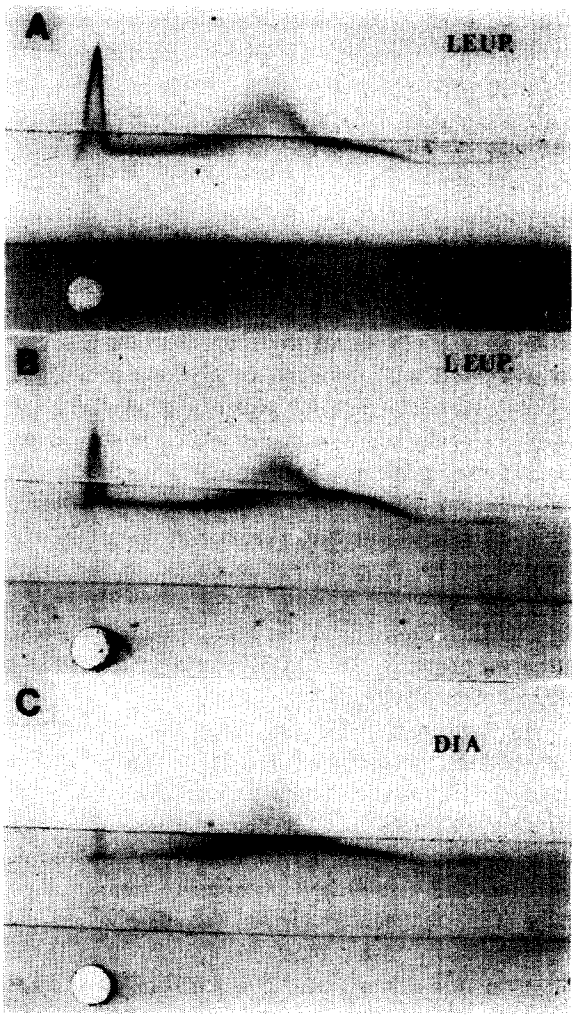


Fig. 5. Identification of glycoprotein Ib in the cytoskeletal fraction of platelets by Western immunoblotting. The lanes represent: (A) purified glycocalicin; (B) the cytoskeletal (Triton-insoluble) fraction from diamide-treated platelets; (C) partly purified glycoprotein Ib, and (D) whole platelet proteins. SDS-samples of whole untreated platelets and the cytoskeletal fraction of diamide-treated platelets were obtained as described in the legends to Figs. 1 and 4. Solutions of purified glycocalicin and glycoprotein Ib were diluted 1:1 directly in the standard SDS-solution. All samples were reduced by 2-mercaptoethanol. Following electrophoresis, the gel (7% polyacrylamide) was soaked in buffer and the proteins were transferred to a nitrocellulose membrane which was washed and incubated in rabbit antiserum to glycocalicin and further treated as given in Materials and Methods using peroxidase-conjugated porcine anti rabbit immunoglobulin as secondary antibody. Finally, the spots representing bound antibodies were stained by a mixture of H_2O_2 and *o*-dianisidine.

or was strongly reduced in height using extracts from diamide-treated platelets (Fig. 3D and 6C). This immunoprecipitate has been explained as representing glycoprotein Ib in association with actin filaments not sedimented during the low-speed centrifugation regularly used to sediment the cytoskeletal material [9]. Accordingly, this finding indicates that diamide also cross-links proteins present



diamide and leupeptin as to the rocket-like immunoprecipitate at the application well. Triton X-100 extracts were obtained from platelets that had been treated in the following ways: (A) incubated in buffer only, washed and extracted in a solution of Triton X-100 containing leupeptin; (B) incubated with leupeptin, washed, and extracted in the absence of added inhibitor, and (C) incubated with diamide, washed and extracted in the absence of added inhibitor. The pattern corresponding to a control with no inhibitor added at any stage, is shown in Fig. 3C. Leupeptin (4.2 mM), or diamide (0.5 mM), or buffer only, were added to identical portions of washed platelets. These were incubated at 37°C for 10 min and each portion was centrifuged at 1900 × *g* for 10 min. The sedimented platelets were then washed once by resuspension in the standard washing solution, and recentrifuged. The platelets from the portions which had been incubated with leupeptin or diamide, were extracted in the standard Triton X-100 extraction buffer without any additions, whereas the platelets which had been incubated with buffer only, were extracted in the same solution which in addition contained 4.2 mM leupeptin.

Fig. 6. Effect of washing of platelets after incubation with diamide or leupeptin, on the crossed immunoelectrophoresis pattern with antiserum to glycocalicin. Difference between

in such filaments allowing them to sediment at lower *g*-values. Also, the amount of total protein in the Triton-insoluble fraction increased by around 60% comparing extractions in 1% Triton X-100 of diamide-treated platelets to those of untreated control platelets extracted in the presence of leupeptin.

Incubation of washed human platelets with diamide inhibited the agglutination of these platelets by bovine von Willebrand factor (Fig. 7B). Such inhibition brought about by diamide was not observed using formaldehyde-fixed platelets (Fig. 7A). This suggests that the diamide-induced reactions affecting platelet agglutination, occur only with metabolically active platelets. Control experiments also showed that the same effect of diamide was observed whether the agglutination was induced by highly purified von Willebrand factor or by the commercial material. Further, the inhibition was observed whether or not EDTA was present in the platelet suspension during the incubation with diamide and the subsequent agglutination.

Incubation of platelets with dibucaine led to degradation of the same proteins that disappear on Triton X-100 extraction of platelets in the absence of inhibitors of the calcium-activated protease, i.e. mainly actin-binding protein and the 230 kDa protein (Fig. 8). The degradation products of 190 kDa (HF-1) and 90 kDa (HF-2) were also formed. In addition, a band (denoted X in Fig. 8) at around 140 kDa was seen as a dominant new band on incubation with dibucaine. These time-

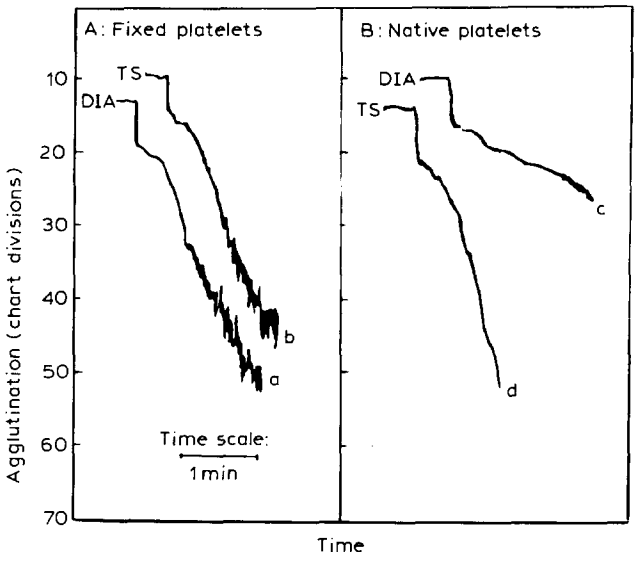


Fig. 7. Inhibition by diamide of the agglutination of human platelets induced by bovine von Willebrand factor. Lack of effect of diamide on platelets fixed in formaldehyde. (A) Formaldehyde-fixed platelets. A portion of 400 μl of fixed platelets ($3 \cdot 10^8$ cells/ml) were incubated with constant stirring in the aggregometer after addition of 50 μl of either a solution of 5 mM diamide or buffer only. After 10 min of incubation, the recorder was started, and after a further 24 s to establish the aggregometer base line, 50 μl of a solution of a commercial bovine von Willebrand factor were added, and the agglutination was monitored. (B) Regular washed platelets. Otherwise the same system as in (A). (b and d): platelets incubated with buffer only. (a and c): platelets incubated with diamide (0.55 mM).

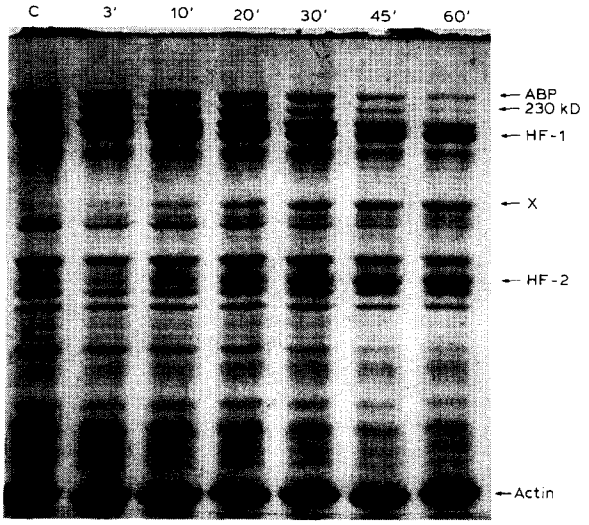


Fig. 8. Degradation of the actin-binding protein (ABP) and the 230 kDa protein during incubation of platelets with dibucaine as demonstrated by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The number above each lane represents the time of incubation in min, and C represents a control without dibucaine. For explanations of HF-1, X and HF-2 see text. Washed platelets ($4.5 \cdot 10^9$ cells/ml) were incubated in the standard Tris-buffered saline at pH 7.4 and 37°C without stirring with 1 mM dibucaine or buffer only. After 3, 10, 20, 30, 45 and 60 min of incubation aliquots were withdrawn and centrifuged at $8000 \times g$ for 4 min. The sedimented platelets were used for SDS-polyacrylamide gel electrophoresis on a 7% slab gel as reduced samples. Each lane represents $4.5 \cdot 10^7$ platelets. The supernatants were used for Laurell 'rocket'-immunoelectrophoresis against the antiserum to glycocalicin. At the same points during the incubation another aliquot was withdrawn and used for agglutination with bovine von Willebrand factor as described in Fig. 7.

dependent degradation processes were accompanied by significant effects on glycoprotein Ib and the platelet agglutination by bovine von Willebrand factor. As to the experiment in Fig. 8, the agglutination was unchanged or slightly increased at 10 min of incubation with dibucaine at which time the aggregometer base-line also was practically unaffected. From 20 min and onwards there was a steady decline in the agglutinability, and a corresponding lowering of the aggregometer base-line indicating morphological changes of the platelets. The control platelets were unaffected during the incubation (data not shown). From 20 min of incubation significant amounts of glycocalicin could be observed extracellularly by Laurell rocket immunoelectrophoresis of the supernatants with anti glycocalicin. The production of glycocalicin was almost complete between 45 and 60 min (data not shown). Fig. 9 demonstrates that the effect of dibucaine on platelet agglutination described above with an increased agglutination was observed even with platelets which had been pretreated with diamide to inhibit the agglutination (Fig. 9d compared to Fig. 9b). Even if dibucaine thus counteracted the inhibition brought about by diamide, an agglutination as strong as that obtained with platelets that had not been treated with any of the agents (Fig. 9a) was not observed. Even with the diamide-treated platelets, prolonged incubation with dibucaine led to morphological changes reflected in the aggregometer base-line and almost total loss of agglutinability (Fig. 9f) as well as splitting of glycoprotein Ib yielding extracellular glycocalicin and degradation of the actin-binding protein and the 230 kDa protein (data not shown).

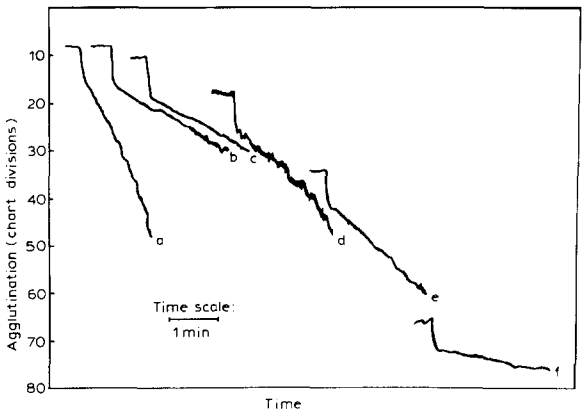


Fig. 9 Combined effects of diamide and dibucaine on the agglutination of human platelets by bovine von Willebrand factor. After a prior incubation with diamide, the platelets were washed once and incubated further with dibucaine for (c): 3 min, (d): 10 min, (e): 20 min and (f): 60 min. (a) represents a control with neither diamide nor dibucaine, whereas (b) represents a control with diamide alone. Washed platelets suspended in the standard Tris-buffered saline (pH 7.4) were incubated at 37°C for 10 min with 0.5 mM diamide ($0.9 \cdot 10^9$ cells/ml) or buffer only. The platelets were then washed once in the standard washing solution, resuspended in the Tris-buffered saline (pH 7.4), and further incubated without stirring at 37°C with 1 mM dibucaine ($4.5 \cdot 10^9$ cell/ml). At the times stated, during the incubation with dibucaine, aliquots were withdrawn, diluted in the Tris-buffered saline to $5 \cdot 10^8$ cells/ml, and used for agglutination with the commercial bovine von Willebrand factor as described in Fig. 7.

Discussion

In recent years, thiol-dependent, calcium-activated neutral proteases have been implicated in an increasing number of biological systems. Such protease activity was first described for platelets by Phillips and Jakabova [28], and further studied by several groups [28–31]. In particular, the degradation of the actin-binding protein and the 230 kDa protein was described [28–31], and we also demonstrated that the water-soluble protein called glycocalicin [32] was a degradation product of glycoprotein Ib resulting from the action of the protease during platelet lysis [2,3].

Truglia and Stracher [29] have demonstrated with purified proteins that the actin-binding protein is degraded to give products of 190 kDa (HF-1) and 90 kDa (HF-2), respectively, and Collier and Wang [27] have shown that the 230 kDa protein (protein P 235) is degraded to a stable product of 200 kDa. In a whole platelet system this product and HF-1 appear to overlap in the SDS-polyacrylamide electrophoretic analyses. Whereas the actin-binding protein is definitely an integral part of the cytoskeleton involved in the branching of actin filaments, it is uncertain to what degree the 230 kDa protein affects formation of cytoskeletal structures [27,33]. The degradation products, in particular HF-2 which on SDS-polyacrylamide gel electrophoresis appears as a sharp band at a position free of other lines, are sensitive indicators of proteolysis. This is of special interest

working with Triton X-100 extracts since a slight reduction in the amount of the actin-binding protein itself is difficult to observe, and in addition, disappearance of this protein may either be due to proteolysis or to its incorporation into the Triton-insoluble sediment.

The effects of diamide on platelet proteins have been studied extensively by Davies and Palek [12] and by Spangenberg and co-workers [14] who have shown that diamide polymerizes specific platelet proteins, in particular the actin-binding protein, the 230 kDa protein and myosin, as also seen in the present study. In this work we have shown that diamide, which appears to act intracellularly, also acts as an inhibitor of the calcium-activated protease probably due to its sulfhydryl-oxidizing capacity. This is shown both by its effects on the crossed immunoelectrophoresis patterns of Triton X-100 extracts of diamide-treated platelets, as well as by the absence of the degradation products HF-1 and HF-2 in such extracts as studied by SDS-polyacrylamide gel electrophoresis. Further, we have confirmed the polymerizing effect of diamide on the proteins in the 200–250 kDa range by SDS-polyacrylamide gel electrophoresis. The fact that the Coomassie brilliant blue staining of the corresponding bands was strongly diminished with unreduced, but not with reduced samples of SDS-dissolved diamide-treated platelets, clearly indicate that disulfide-linked polymers not penetrating the 7% gel were produced during the incubation with diamide. This phenomenon was used to study further the association of glycoprotein Ib with the cytoskeletal fraction [7–9]. The importance of this resides in the observation that diamide increases this interaction, thus permitting clear-cut controls showing that this association is specific for glycoprotein Ib compared to platelet membrane proteins in general. The use of diamide thus magnifies a phenomenon which also occurs without diamide [9]. However, an effect of diamide directly on glycoprotein Ib as the reason for its increased incorporation into the Triton-insoluble sediment, can not be excluded. It should be noted though, that Davies and Palek [12] did not observe covalently linked complexes of glycoproteins after treatment of platelets with diamide.

A central question concerning the association of glycoprotein Ib with the cytoskeletal material, is whether this connection is established during the extraction procedure, or reflects associations in the intact platelet. This is still unsolved as the increased incorporation of glycoprotein Ib into the cytoskeletal fraction after incubation with diamide may be compatible with either alternative. However, the observations that diamide and dibucaine affect von Willebrand factor-induced platelet agglutination with opposite results, is of interest, and may indicate functional importance of the observations as glycoprotein Ib is believed to act as a membrane receptor for von Willebrand factor.

Thus, increased interaction of glycoprotein Ib with cytoskeletal material is parallelled by inhibition of the agglutination, whereas splitting of the actin-binding protein is accompanied initially by an increased agglutination. It should also be mentioned that evidence has already been provided that glycoprotein Ib may exist as a transmembrane protein that can be phosphorylated at the inner surface of the membrane [6,9], and it has recently been demonstrated that glycoprotein Ib can be coprecipitated with the actin-binding protein during immunoprecipitation by antibodies to this protein [8]. This may mean that a complex between glycoprotein Ib and the actin-binding protein may establish a direct link between the plasma membrane and the platelet cytoskeleton when platelets become 'activated' and a cytoskeleton is formed, or the interaction may be mediated by an additional protein. Such a linkage might impose restrictions on the movement of the glycoprotein Ib molecule in the plasma membrane reducing its efficiency as receptor for the von Willebrand factor. A release of glycoprotein Ib from this linkage due to splitting of the actin-binding protein, might increase the efficiency of its receptor function.

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