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MEDIATION OF THE PHYSIOLOGICAL RESPONSE OF PLATELETS BY INTERACTIONS OF SPECTRIN AND PROTEIN 4.1 WITH THE CYTOSKELETON

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Summary: Spectrin and protein 4.1 became associated with the Triton-insoluble cytoskeletons during platelet activation. Inhibition of platelet activation by a PGI_2 analogue resulted in release of the proteins from the cytoskeletons. Changes in subcellular distributions of the proteins preceded changes in the state of platelet aggregation. These results suggest that interactions of spectrin and protein 4.1 with the cytoskeletons are involved in mediating the physiological response of the platelet.

Spectrin and protein 4.1 are the major structural proteins of the erythrocyte membrane skeleton, along with actin (1-3). Protein 4.1 is present in the erythrocyte membrane skeleton in equimolar concentrations with spectrin dimers and promotes the spectrin-actin association (4-7). Spectrin and protein 4.1 are essential for preservation and regulation of normal erythrocyte morphology (8, 9). Proteins immunologically similar to erythrocyte spectrin and protein 4.1 are also present in

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Abbreviation used in this paper: STA_2, 9,11-epithio-11,12-methano-thromboxane A_2.

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platelets (10, 11). By analogy with erythrocytes, spectrin and protein 4.1 are thought to have a role in membrane-cytoskeletal attachment in platelets. However, it is not established whether the proteins may be involved in platelet activation.

During the early stages of platelet activation, actin filaments become crosslinked into structures that sediment from Triton X-100 lysates at low g forces (15,600 g for 4 min) (12). The platelet cytoskeleton probably interacts with the inner surface of the plasma membrane, thereby accounting for its contractile properties (13). In the present work, we investigated the role of membrane skeletal proteins in platelet aggregation.

Materials and Methods

Purification of Proteins--- Human erythrocyte ghosts obtained from bloods of healthy donors were isolated as described by Bennett and Branton (14). Spectrin was extracted from these ghosts at 37 ^{o}C in a low-ionic-strengh buffer and was further purified by the method of Cohen and Foley (4). Human erythrocyte protein 4.1 was prepared as described by Ohanian and Gratzer (15).

Antibody Production and Affinity Purification--- Purified spectrin was electrophoresed on SDS-polyacrylamide gels containing 5 % acrylamide. The gel was stained with Coomassie brilliant blue, and two major bands corresponding to the alpha and beta chains of spectrin were cut out of the gel. Spectrin was then electrophoretically eluted from the gel strips using Amicon Centrilutor (Amicon, MA). Anti-spectrin sera were raised in two rabbits by subcutaneous injections of erythrocyte spectrin (total of 200 μg per rabbit). Anti-spectrin antibody was affinity purified by Sepharose 4B coupled with erythrocyte spectrin. Erythrocyte protein 4.1 excised from 7.5 % polyacrylamide gels was used for immunization. Two rabbits received 20-30 subcutaneous injections of erythrocyte protein 4.1 (total of 50 μg per rabbit). After 6 weeks, one of the rabbits produced serum which reacted with erythrocyte protein 4.1. Affinity purification of anti-protein 4.1 antibody was carried out using Sepharose 4B coupled with a C-terminal polypeptide of human erythrocyte protein 4.1 (V^{574}TKVVVHQETEIADE^{588}, amino acids in single letter code) (16), as described above.

Immunoblotting--- Erythrocyte ghosts and platelet proteins were electrophoresed and transferred to polyvinylidene difluoride paper. Electroblots were stained with anti-spectrin antibody (0.6 μg/ml) and anti-protein 4.1 antibody (28 μg/ml), and counterstained with 1:1000 peroxidase conjugated anti-rabbit IgG (Bio-Rad) as described elsewhere (17).

Preparation of Platelet Suspensions and Subcellular Fractionation--- Venous blood was freshly drawn from healthy

donors. Platelets were finally resuspended at $2x10^8$ cells/ml in a modified Tyrode-HEPES buffer containing a final concentration of 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 % dextrose, 3.75 mM NaH_2PO_4, and 15 mM HEPES, pH 7.5 (suspension buffer). An aliquot of platelet suspension was lysed by adding an equal volume of ice-cold x2 Triton extraction buffer (2 % Triton X-100, 10 mM EGTA, 1 mg/ml leupeptin, 2 mM phenylmethyl sulfonyl fluoride, and 0.1 M Tris, pH 7.5). Triton lysates were first centrifuged at 15,600xg at 4 °C for 4 min. The bulk of the actin filaments (cytoplasmic actin filaments) sedimented at these g forces. The membrane-bound filaments remained in the supernatant and were subsequently isolated by centrifugation at 100,000xg at 4 °C for 3 hr.

Extraction of Activated Platelets--- Washed platelet suspensions were extracted with 1 % Triton X-100 after treatment with 0.1 unit/ml of thrombin or 1 μM of 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2, a kind gift from Ono Pharmaceutical Co., Ltd., Osaka, Japan) at 25 °C for various times. Triton-soluble and -insoluble fractions from platelets were prepared by treating the suspended platelets for 5 min with a 1/4 volume of ice-cold x4 Triton extraction buffer and isolated by centrifugation at 15,600xg at 4 °C for 4 min. The fractions were solubilized in 2 % SDS sample buffer with brief sonication. Spectrin and protein 4.1 in the samples were detected by immunoblotting, and actin was visualized by Coomassie brilliant blue staining. The amounts of proteins were determined from scans of the stained blots and gels using a Bio-Rad model 620 video densitometer. The area of individual bands was measured above background in densitometric tracings and expressed as OD x mm as described elsewhere (18). Platelet aggregation was monitored photometrically and quantified, as described (18,19).

In the inhibition assay for platelet aggregation, the platelets were first incubated with 0.2 μM of STA_2 for 15 sec, then were added 0.2 μg/ml of 5(E)-6,9α-methylene-15-cyclopentyl-16,17,18,19,20-pentanor-PGI_2 (OP-41483, a kind gift from Ono Pharmaceutical Co., Ltd., Osaka, Japan). The samples were then treated as described above.

Results and Discussion

Anti-spectrin and anti-protein 4.1 antibodies were produced in rabbits immunized with spectrin and protein 4.1 purified from human erythrocytes. Affinity-purified antibodies were tested for reactivities against spectrin and protein 4.1 by Western blotting of erythrocyte ghosts and platelet proteins. Figure 1 shows that affinity purified anti-spectrin antibody reacted with the alpha and beta chains of erythrocyte spectrin (Fig. 1, lane a). The antibody reacted with a doublet in platelet proteins (Fig. 1, lane b). The doublet had mol wts. of 240,000 and 235,000 corresponding to those of platelet spectrin subunits (10,20). Affinity purified anti-protein 4.1 antibody reacted with

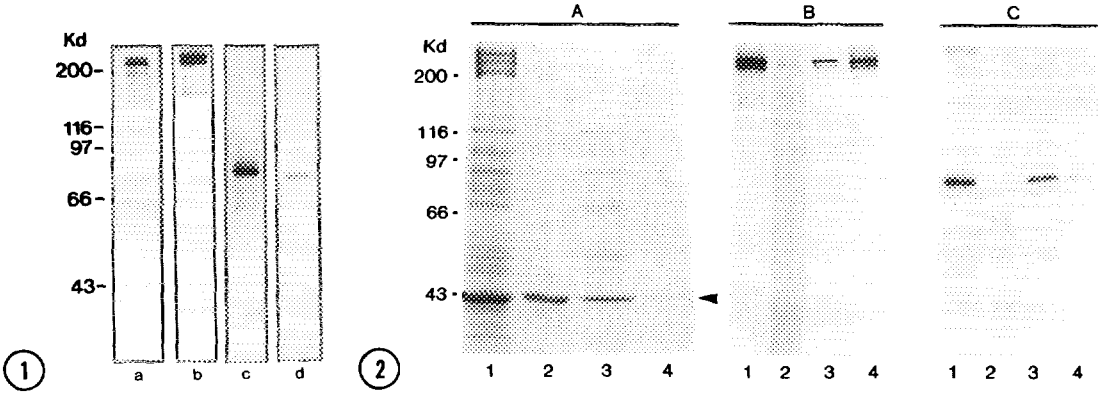

Fig. 1. Specificities of anti-spectrin and anti-protein 4.1 antibodies. Erythrocyte ghosts (1 μg of protein) (lanes a and c) and washed platelet suspensions (10 μg of protein) (lanesb and d) were each loaded on 7.5 % SDS-PAGE and transferred to blotting paper. The blots were incubated with affinity purified antibodies against erythrocyte spectrin (0.6 μg/ml, lanes a and b) and erythrocyte protein 4.1 peptide (28 μg/ml, lanes c and d). The antibodies bound to the paper were detected with peroxidase conjugated anti-rabbit IgG.

Fig. 2. Subcellular distributions of spectrin and protein 4.1 in resting platelets. Washed platelet suspensions were solubilized directly into SDS sample buffer (lane 1 in each panel) or lysed by addition of an equal volume of x2 Triton extraction buffer. Lysates were centrifuged at 15,600 g for 4 min, and the Triton X-100 supernatant was centrifuged at 100,000 g for a further 3 hr as described in "Methods". The sediments obtained at low-speed (lane 2 in each panel) and high-speed (lane 4 in each panel), and the supernatant obtained at high-speed (lane 3 in each panel) were analyzed on 7.5 % SDS-PAGE. Polypeptides were detected with Coomassie brilliant blue stain (A), and spectrin (B) and protein 4.1 (C) were detected by Western blotting. Actin is indicated by an arrowhead.

erythrocyte protein 4.1 (Fig. 1, lane c). The antibody reacted with a band of a relative molecular mass 78,000 in the platelet lysates, findings consistent with that of platelet protein 4.1 reported by Davies and Cohen (10) (Fig. 1, lane d). To determine whether spectrin and protein 4.1 are associated with actin filaments in resting platelets, washed platelets were lysed with Triton X-100 and actin filaments were sedimented at a low g force (15,600 g for 4 min) and then at a high g force (100,000 g for 3 hr). It is well established that most cytoplasmic actin filaments in platelets can be sedimented from Triton X-100 lysates by low-speed centrifugation while membrane-bound

filaments require high-speed centrifugation to be sedimented (21, 22 and also see Fig. 2A). Figure 2B shows that little spectrin was associated with the actin filaments that could be sedimented from Triton lysates of resting platelets at the low g forces (Fig. 2B, lane 2) as reported by Fox et al. (20). About 60 % of the total spectrin was associated with actin filaments requiring high-speed centrifugation to be sedimented by density measurements of the immunoblots (Fig. 2B, lane 4). While little protein 4.1 was associated with the cytoplasmic actin filaments, most did not cosediment with the membrane-bound filaments (Fig. 2C, lanes 2, 3 and 4).

Incorporation of spectrin and protein 4.1 into cytoplasmic actin filaments during platelet activation was examined following low-speed centrifugation. Most of spectrin and protein 4.1 were Triton-soluble in unstimulated platelets as demonstrated above (Fig. 3, 0"). Activation of platelets with 0.1 U/ml of thrombin led to a dramatic increase in the amounts of spectrin, protein 4.1, and actin in the Triton-insoluble fraction. Increased amounts of the three proteins in the Triton-insoluble form were accompanied by corresponding decreased concentrations in the supernatant (Fig. 3). The amounts of myosin and actin-binding protein in the Triton-insoluble material were increased by the

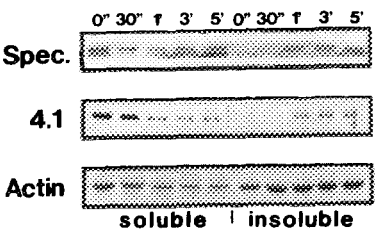

Fig. 3. Change of subcellular distributions of spectrin, protein 4.1, and actin during thrombin activation of platelets. Platelets in suspension buffer were extracted with 1 % Triton X-100 after treatment with 0.1 U/ml of thrombin for various times. Spec., Spectrin; 4.1, protein 4.1.

thrombin activation as reported by Jennings et al. (23) (data not shown). Activation of platelets by STA_2 induced the same changes (see below). Time-dependent changes in the amounts of spectrin, protein 4.1, and actin in the Triton-insoluble cytoskeletons are shown in Fig. 4A. In unstimulated platelets, 7 % and 1 % of the total platelet spectrin and protein 4.1, respectively, were associated with the Triton-insoluble cytoskeletons. Within 3 min after the activation, these values increased 6 and 10 fold for spectrin and protein 4.1, respectively, and reached a plateau. Fig. 4A also shows that increase in the amounts of three proteins associated with the cytoskeletons preceded platelet aggregation.

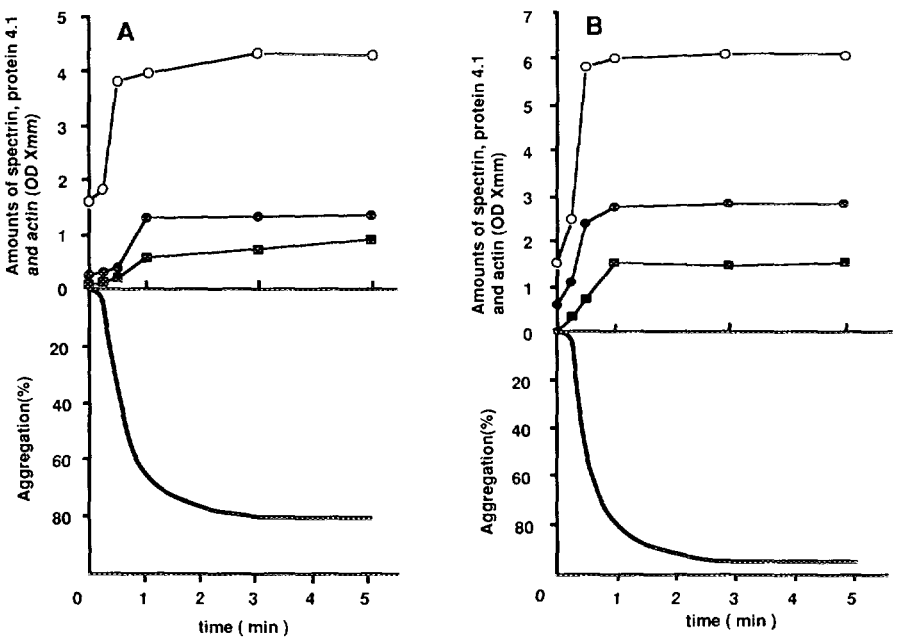

Fig. 4. Time-dependent changes in the amounts of spectrin, protein 4.1 and actin associated with platelet cytoskeletons, and in aggregation during activation. A, thrombin activation (0.1 U/ml); B, STA_2 activation (1 μM). upp, Sedimentable spectrin (●), protein 4.1 (■), and actin (o) in cytoskeletal preparations from control and the activated platelets. The amounts of proteins in individual bands (as shown in Fig. 3) were estimated by densitometric scans as described in "Methods". The values are the means of three different experiments. bottom, The aggregation was monitored photometrically and the responses were expressed as a percentage.

Activation of platelets with 1 μM of STA_2 resulted in the same changes as observed with thrombin activation (Fig. 4B).

To demonstrate whether association of spectrin, protein 4.1 and actin with the cytoskeletons was reversible, a PGI_2 analogue (OP-41483) was used. This analogue is a potent inhibitor of platelet aggregation induced by STA_2 (24). The inhibition of platelet aggregation by OP-41483 is mediated through specific membrane receptors which stimulate adenylate cyclase and increase

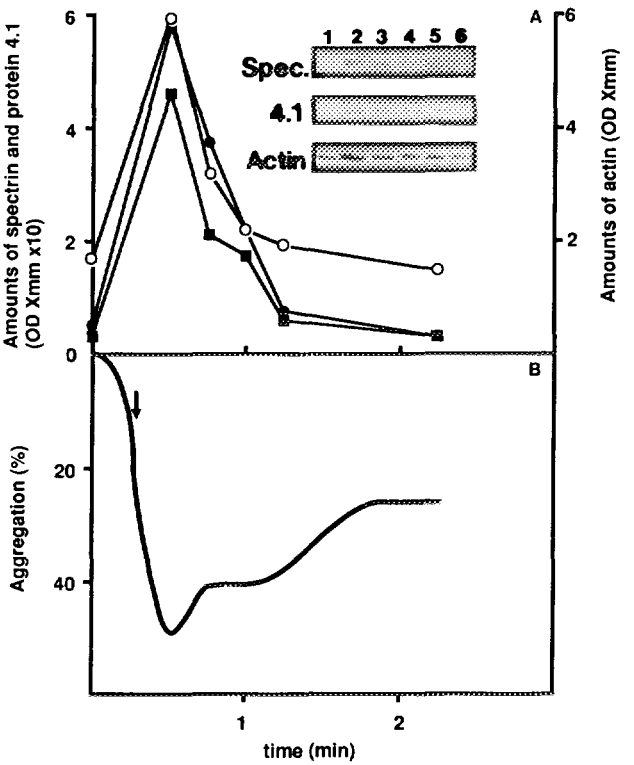

Fig. 5. Dissociation of spectrin, protein 4.1 and actin from platelet cytoskeletons mediated by OP-41483. Platelets were activated by STA_2 (0.2 μM) for 15 sec, then incubated with OP-41483 (0.2 μg/ml) for the times indicated, and processed as described in "Methods". A, Time courses of change in the amounts of spectrin (●), protein 4.1 (■) and actin (○) associated with the Triton-insoluble cytoskeletons. The amounts were estimated from the bands of spectrin (Spec.), protein 4.1 (4.1) and actin shown in inset by densitometric scan: 0 s, lane 1; 30 s, lane 2; 45 s, lane 3; 60 s, lane 4; 75 s, lane 5; 135 s, lane 6. B, Time course of the inhibition by OP-41483 of STA_2-induced aggregation. The arrow indicates the time when the inhibitor was added. Similar results were obtained in two other experiments.

cAMP production (24). Addition of the inhibitor to the STA_2-activated platelet suspensions produced a time-dependent release of the three proteins from the Triton-insoluble cytoskeletons (Fig. 5A, compare with Fig. 4B). The inhibition of aggregation was preceded by dissociation of the three proteins (Fig. 5A and B).

In the present study, the kinetics of incorporation of actin, spectrin, and protein 4.1 into the Triton-insoluble cytoskeletons were similar and in parallel to that of platelet aggregation. Furthermore, when the aggregation of platelet was inhibited, the three proteins dissociated from the cytoskeletons in parallel to the rate of inhibition. It is suggested that membrane skeleton-cytoskeletal interactions play a fundamental role in the process of platelet activation.

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