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Effect of the Thiol Group Inhibitor Monobromobimane and Other Inhibitors on the Composition of the Platelet Cytoskeletal Core and Its Association With Glycoprotein IIIa

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SDS-polyacrylamide gel electrophoresis was used to study the effects of the thiol inhibitor monobromobimane (MB), EDTA, and prostaglandin E_1 (PGE_1) on the formation and composition of the platelet cytoskeletal core (Triton-insoluble residue) and its association with glycoprotein (GP) IIIa. Stimulation or aggregation of platelets in response to ADP or thrombin increased the amount of Triton-insoluble myosin. Aggregation resulted in incorporation of [^{125}I]GP IIIa and a new band at about 210 kDa into the cytoskeletal core. EDTA and PGE_1 caused little disaggregation of platelets that were aggregated in PRP with ADP and that had secreted the contents of their granules. In contrast to EDTA, PGE_1 decreased the amount of Triton-insoluble residue and its association with GP IIIa. MB added after ADP-induced aggregation caused an increase in the amount of cytoskeletal core despite marked disaggregation and a substantial decrease in core-associated GP IIIa. With aspirin-treated platelets that had not secreted, EDTA, PGE_1, and MB all caused disaggregation and loss of cytoskeletal GP IIIa. MB diminished, but did not reverse, thrombin-induced aggregation of washed platelets and arrested GP IIIa incorporation into the cytoskeletal core. Concanavalin A (Con A) cross-links glycoproteins on a single platelet and induces incorporation of GP IIIa into the Triton-insoluble residue in the absence of platelet aggregation. This induction was not inhibited by MB, although this reagent, as well as aspirin, inhibited Con A-induced secretion. Since GP IIIa incorporation caused by ADP-induced aggregation differs from that caused by Con A in its susceptibility to MB, it seems unlikely that thiol groups are directly involved in the association of GP IIIa with the cytoskeletal core.

Key words: platelet glycoproteins, assembly of triton-insoluble residue, reversal of platelet aggregation, platelet fibrinogen receptors

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The thiol-disulfide status of platelets affects a variety of platelet functions [1–3]. Zucker and Mauss [3] found that low concentrations of the thiol inhibitor monobromobimane (MB) (30–100 μM) abolished thromboxane A_2-induced secretion and aggregation caused by collagen or ADP in citrated platelet-rich plasma (PRP). Higher concentrations of MB (1–2 mM) abolished ADP-induced primary aggregation, whereas platelet shape change was resistant to this inhibitor. In washed platelets suspended in a medium containing fibrinogen, as little as 100 μM MB prevented primary ADP-induced aggregation and fibrinogen binding. Since MB does not react with the fibrinogen receptor glycoproteins (GP) IIb/IIIa, inhibition is attributed to prevention of ADP-induced "exposure" of GP IIb/IIIa [3]. MB reacts with GP Ib through a free thiol group on the beta-chain [4] and with many cytoskeletal proteins such as actin, actin-binding protein, myosin, and alpha-actinin [3]. When platelets are activated, these proteins assemble into a highly organized network insoluble in 1% Triton X-100. This cytoskeletal core contains GP IIb/IIIa when it is prepared from platelets that have aggregated [5–8].

Kinlough-Rathbone et al. [9] found that EDTA and prostaglandin E_1 (PGE_1) reversed ADP-induced aggregation of platelets that had not secreted their granule contents, but did not reverse aggregation when secretion had taken place. In view of the relationship between platelet stimulation and assembly of the cytoskeletal core, as well as the relationship between platelet aggregation and the association of GP IIIa with this core, we examined the effects of MB, EDTA, and PGE_1 on these parameters using platelets that were and were not able to secrete. We also investigated the effect of MB on the response to Concanavalin A (Con A) because this lectin cross-links glycoproteins on a single platelet and causes surface glycoproteins to associate with the cytoskeletal core [10,11].

MATERIALS AND METHODS

Reagents

Monobromobimane (MB) was obtained from Calbiochem-Behring Diagnostics, San Diego, CA (trade name, Thiolyte); bovine thrombin, from Upjohn Co., Kalamazoo, MI; acetonitrile, from Pierce, Rockford, IL; [side chain-2-^{14}C]-5-hydroxytryptamine creatinine sulphate (serotonin, 5-HT; 56 mCi/mmol), from Amersham, Arlington Heights, IL; collagen, from Helena Laboratories, Beaumont, TX; Gel-Bond, from FMC BioProducts, Rockland, ME; p-(amidinophenyl)methanesulfonyl fluoride (APMSF), from California Medicinal Chemistry Corp., San Francisco, CA; reagents for SDS-polyacrylamide gel electrophoresis (PAGE), from Bio-Rad Laboratories, Richmond, CA; and other reagents, from Sigma Chemical Co., St. Louis, MO or Fisher Scientific Co., Springfield, NJ.

MB was stored at 4°C as a 300 mM solution in acetonitrile, and further dilutions were made so that platelet suspensions contained no more than 0.5% acetonitrile. APMSF was kept at 4°C as a 10 mM solution in methanol. The following were stored at −20°C: ADP (10 mM), acetylsalicylic acid (ASA) (1 mM), thrombin (100 U/ml), and apyrase grade I, (10 mg/ml) in isotonic saline, and prostaglandin E_1 (PGE_1) (2.8 mM) in 95% ethanol.

Preparation of Platelets

Blood was obtained from healthy normal adults who had given informed consent according to the Helsinki declaration. The study was approved by our Human

Experimentation Committee. The donors claimed not to have ingested aspirin or other drugs for a week. Blood was drawn through a 19-gauge needle attached by plastic tubing to a syringe containing one-ninth volume of 0.109 M sodium citrate. For secretion experiments, [^{14}C]serotonin was added to the syringe for a final concentration in blood of 0.5μM. Platelet-rich plasma (PRP) was separated, and washed platelets were prepared as previously described [6] except that the final suspension medium was buffered to pH 7.4 with 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and contained 0.1 mg/ml apyrase, 2 mg/ml bovine serum albumin, and no added $CaCl_2$. For some studies, a small portion of the platelets was resuspended without apyrase or albumin and labeled with ^{125}I using lactoperoxidase [12]. The labeled platelets were then diluted in Hepes-Tyrode solution containing 5 mM EDTA, centrifuged, washed twice with this solution, and added to PRP or to washed platelets so that they represented about 5% of the platelets. The preparation was kept at 37°C for at least 30 min before it was used.

Aggregation and Disaggregation (Reversal) Studies

PRP was incubated at 37°C for 30 min with 1/10th vol of isotonic saline or 1.0 mM ASA. Platelet aggregation in 0.6 ml of PRP was recorded at 37°C with an aggregometer (Payton Associates, Buffalo, NY, or Chrono-Log Corp., Havertown, PA). PRP was stirred with an inhibitor or its vehicle for 1 or 2 min before the agonist was added. Inhibition was expressed as the percentage decrease in maximum light transmission of the experimental (MB) curves compared to the control (vehicle) curves of aggregation. In reversal experiments, inhibitor was added 1–10 min after aggregation had begun. Reversal was calculated as the decrease in light transmission caused by the agonist. In some of these reversal studies, platelets aggregated with ADP were centrifuged after 4 min, then resuspended either in their own supernatant plasma or in fresh citrated plasma. Since the preparations that were returned to the aggregometer after centrifugation exhibited greater light transmission than was recorded at maximal aggregation, they were stirred in the aggregometer for 1 min to partially disperse the aggregates before EDTA was added. Percent reversal was calculated as the decrease in light transmission between the second and fourth minutes after addition of EDTA and expressed as a percentage of the increase in light transmission caused the agonist. In some experiments, including those with Con A, platelets were stimulated but not aggregated; that is, PRP or washed platelets were gently mixed but not stirred with the agonist.

In studies of [^{14}C]serotonin secretion, the labeled PRP or washed platelets were stirred or incubated in the presence of the agonist at 37°C for 4 min. Neutralized EDTA (final concentration of 5 mM) was added, and the samples were chilled and centrifuged at 12,000*g*. The difference between the concentration of [^{14}]serotonin in the supernatant of samples with stimulated and unstimulated platelets was expressed as a percentage of platelet-bound serotonin in the platelet-rich sample.

Preparation of Cytoskeletal Cores

At various times after the addition of the agonist to PRP or washed platelets, the suspension was mixed and vortexed with an equal volume of 2% Triton X-100, 100 mM Tris, and 10 mM EGTA at room temperature [5], kept at 4°C, and spun 30 min later in an Eppendorf centrifuge (12,000*g*). The small sediment was washed twice by resuspending it in the above Triton solution diluted with an equal volume of

isotonic saline. In some experiments, the lysis buffer contained 50 μM leupeptin and 100 μM APMSF. The sedimented cytoskeletal preparation was dissolved in a solution containing 2% sodium dodecylsulfate (SDS), 20% glycerol, 100 mM Tris, 5 mM *N*-ethylmaleimide, and 3 mM EGTA. The samples were placed in a boiling water bath for 3–5 min with 50 mM dithiothreitol and frozen.

SDS-PAGE

Samples were electrophoresed according to Laemmli [13] on 7.5% polyacrylamide (PA) gels (4.5% PA in the stacking gel) cast on GelBond film and stained with Coomassie Brilliant Blue. Gels that contained ^{125}I-labeled platelets were dried and exposed for radioautography [6]. Each gel represents results on cytoskeletal preparations from a single experiment; equal volumes were applied to each lane. Each experiment was done at least three times.

RESULTS

Reversal of Aggregation

ADP-induced aggregation in PRP. Table I summarizes the effect of adding MB, EDTA, or PGE_1 to PRP in which the platelets were aggregated for 3 min with ADP. While MB caused considerable disaggregation of platelets that had secreted, its vehicle, acetonitrile, had no effect. There was less reversal with MB when aggregation was allowed to continue beyond 3 min; for example, in one experiment (not shown), MB added at 3 min caused 78% reversal; at 4 min, 67%; at 6 min, 57%; and at 10 min, 40%. EDTA caused little disaggregation, and the response to PGE_1 was variable, but always less than the response to MB. A typical experiment showing the effect of MB, EDTA, and PGE_1 on aggregated platelets that had secreted is illustrated in Figure 1a. Figure 1b shows that all three inhibitors reversed the aggregation of ASA-treated, nonsecreting platelets, although as with non-ASA-treated platelets, EDTA was the least effective.

Although EDTA only slightly reversed the aggregation of platelets that had secreted, significant reversal was found when the aggregated platelets were sedimented and resuspended in fresh plasma instead of in the plasma in which they had aggregated (paired analysis, $P = 0.02$, $n = 6$).

Thrombin-induced aggregation of washed platelets. Addition of 200 μM MB 1 or 2 min after thrombin did not reverse aggregation but reduced its extent by about 20% at 6 min. Figure 2 illustrates this effect and also shows that aggregation was inhibited by 50% when the platelets were pretreated for 1 min with 200 μM MB.

TABLE I. Disaggregation of Platelets by MB, EDTA, or PGE_1

	Non-ASA PRP		ASA PRP	
Inhibitor	No.	% disaggregation (mean ± S.E.M.)	No.	% disaggregation (mean ± S.E.M.)
1 mM MB	12	67 ± 4.5	8	97 ± 2.8
5 mM EDTA	13	16 ± 2.8	8	66 ± 7.4
14 μM PGE_1	8	48 ± 9.8	7	94 ± 3.7

Platelets in citrated platelet-rich plasma, some of which had been incubated with ASA, were aggregated with 10 μM ADP. Inhibitors were added 3 min later.

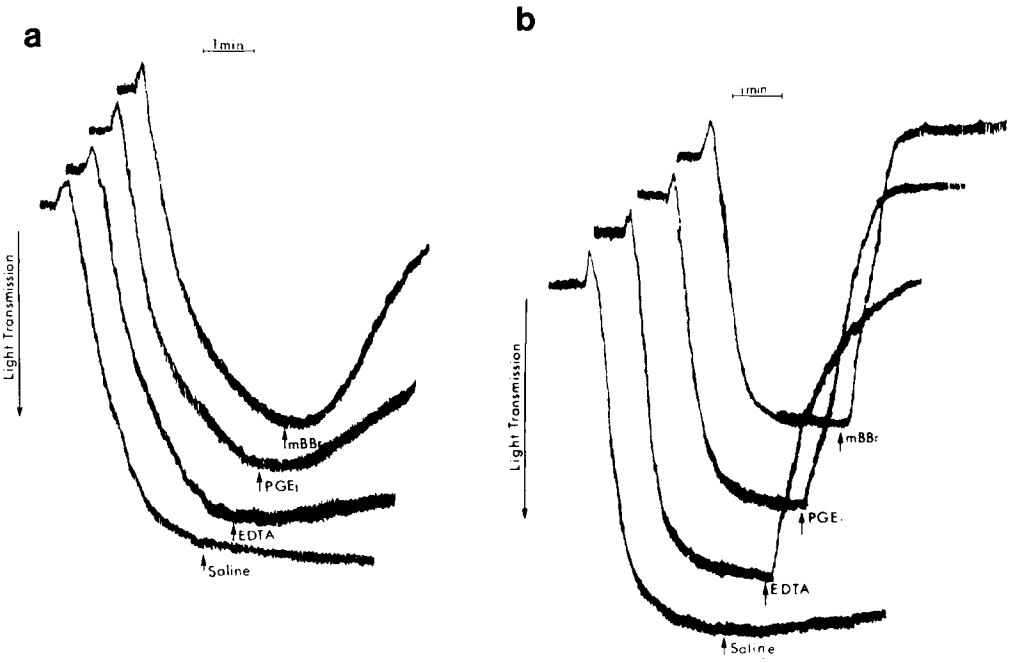

Fig. 1. Aggregation curves of a mixture of PRP and ^{125}I-labeled platelets (about 5%). The platelets were aggregated with 10 μM ADP and 3 min later, 1 mM MB, 5 mM EDTA, or 14 μM PGE_1 was added. At 6 min, Triton-insoluble residues were prepared. **a,** Non-ASA-treated platelets. **b,** ASA-treated platelets. Addition of acetonitrile (final concentration 0.5%) did not cause disaggregation.

Effect of ADP and Inhibitors on Formation of Platelet Cytoskeletal Core of Unstimulated and Stimulated Platelets in PRP

Unstimulated platelets. The Triton-insoluble residues made from unstimulated platelets in PRP, with or without ASA, were small and contained almost no myosin (heavy chain, 200 kDa) or actin-binding protein, 260 kDa (Fig. 3, lane 2; Fig. 4b, lane 5). Addition of 1 mM MB or 5 mM EDTA 2 or 6 min before the Triton solution increased the amount of myosin markedly (Fig. 3, lanes 3,4; Fig. 4b, lane 6), while acetonitrile had no effect (not shown). In agreement with earlier results [6], addition of EDTA increased cytoskeletal myosin equally well whether it was added before or after the Triton (Fig. 3, lane 6), whereas MB had essentially no effect when it was added after lysing the platelets (Fig. 3, lane 5). A prominent band at 235 kDa was seen in 40% of the Triton-insoluble residues from unstimulated MB-treated platelet preparations (Fig. 4b, lane 6).

Stimulated platelets. The amount of cytoskeletal myosin and actin-binding protein was increased by ADP (Fig. 4b, lanes 1 or 2 compared to 5). The myosin was further increased when EDTA or MB was added before the ADP (Fig. 4a, lanes 3–6), whether or not the platelets had been incubated with ASA (Fig. 4b, lanes 3,4, 7). Addition of leupeptin and APMSF to the lysing medium did not change the amounts or proportions of the high molecular proteins of the cytoskeletal core. Furthermore, addition of EDTA to the lysing medium increased the amount of cytoskeletal myosin despite the presence of leupeptin and APMSF (not shown). Thus,

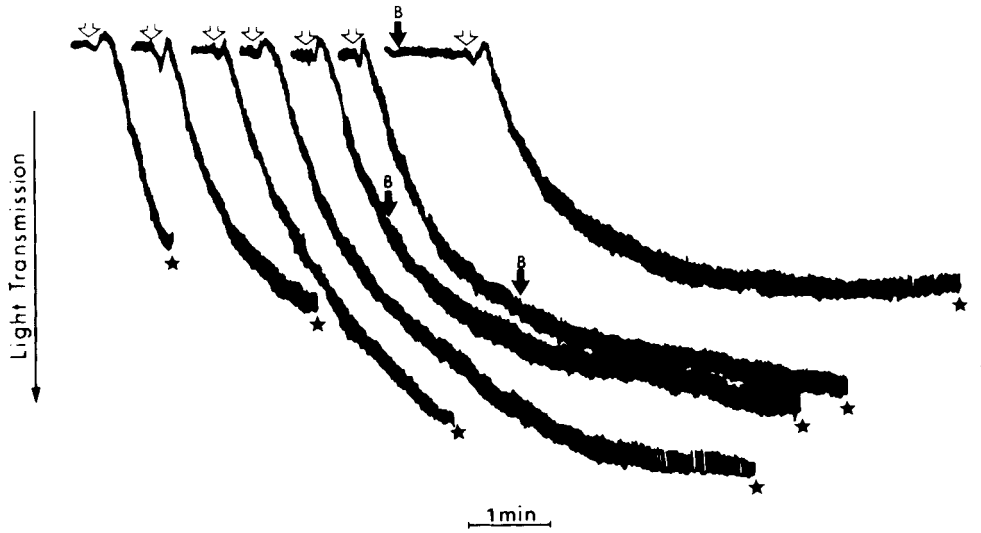

Fig. 2. A mixture of ^{125}I-labeled and unlabeled washed platelets in Hepes-Tyrode's containing apyrase and albumin was aggregated for 1,2,3, or 6 min with 0.3 U/ml thrombin (open arrows). MB (200 μM) was added before the thrombin or at 1 or 2 min after (closed arrows). Triton-insoluble residues were prepared at 1, 2, 3, or 6 min (stars).

the increase in the band at 200 kDa could not be attributed to proteolysis of proteins of higher molecular weight such as actin-binding protein. The band at 235 kDa was present in some MB-treated preparations (e.g., Fig. 4a, lane 6, Fig. 4b, lanes 3,4) but not in EDTA-treated preparations (Fig. 4b, lane 7).

Most of the cytoskeletal protein bands seen in gels derived from stimulated platelets in PRP were also present in gels prepared from stimulated washed platelets suspended in Tyrode's solution containing BSA and thus do not represent carryover of plasma proteins. The band seen at 68 kDa in the Triton-insoluble residue of PRP was albumin, judging by its apparent M_r of 54,000 when the sample was not reduced. Furthermore, most of it was probably derived from plasma, since it was prominent in autoradiographs prepared from PRP that had been mixed with ^{125}I-labeled albumin (not shown).

Effect of Inhibitors on the Cytoskeletal Core of Aggregated Platelets and the Association of GP-IIIa With the Core

ADP-aggregated platelets: cytoskeletal core proteins. After 3 min of aggregation, the amount of Triton-insoluble residue was increased compared with that in unstimulated platelets and did not show a marked increase during the next 3 min (Fig. 5a, lanes 1,2,6). After aggregation, a small band at about 210 kDa was frequently observed (Fig. 5a, lanes 2,3,6; Fig. 6a, lane 2), which was lost when aggregation was reversed (Fig. 5a, lane 5; Fig. 6a, lanes 3,5). Cytoskeletal cores prepared from ADP-aggregated platelets that were preincubated with 50 μM leupeptin also contained the 210-kDa protein band whether or not the lysing buffer contained 50 μM leupeptin and 100 μM APMSF (not shown).

As in unstimulated or stimulated but unstirred platelets, addition of EDTA or MB increased the amount of cytoskeletal myosin compared with that in platelets to

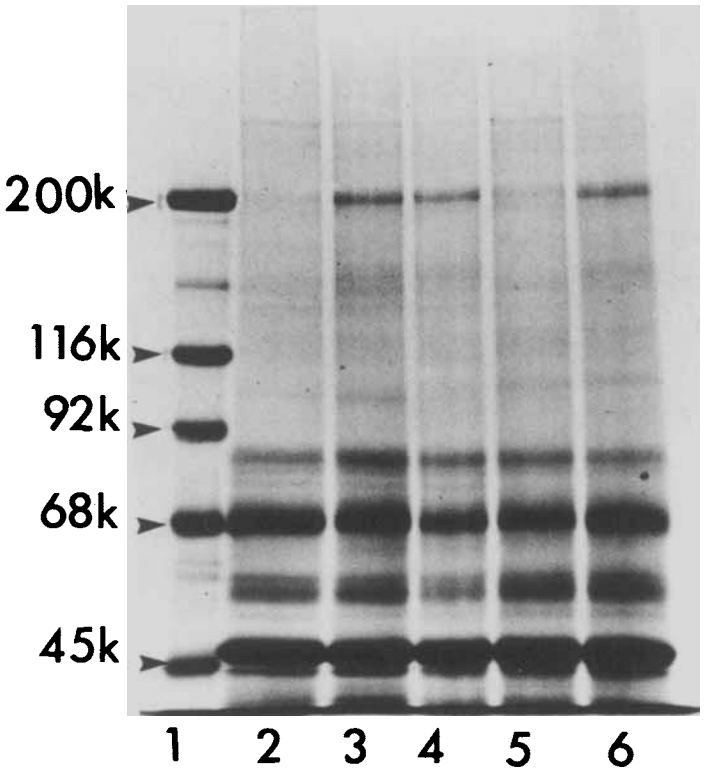

Fig. 3. SDS-PAGE of cytoskeletal cores prepared from PRP; effect of MB on cytoskeletal myosin, before and after addition of Triton. Lane 1, molecular weight standards: myosin, 200,000; β-galactosidase, 116, 250; phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000. Cytoskeletal cores: lane 2, from unstirred PRP; lane 3, from PRP incubated for 2 min with 1 mM MB; lane 4, from PRP incubated with 5 mM EDTA; lanes 5 and 6, from PRP to which Triton, and 1 min later, 1 mM MB and 5 mM EDTA, respectively, were added.

which no inhibitor had been added (Fig. 5a, lanes 3,5). The apparent quantity and number of the other major cytoskeletal bands were not affected. After addition of PGE_1, the cytoskeletal myosin band was smaller (Fig. 5a, lane 4). In samples treated with MB before ADP, the band at 235 kDa was evident in most of the experiments (Fig. 5a, lanes 7,8).

ADP-aggregated, ^{125}I-labeled platelets: autoradiography of surface-labeled proteins associated with the cytoskeletal core. Platelets that were processed as for radiolabeling, except for the omission of ^{125}I, and incubated for 30 min at 37°C in citrated plasma aggregated normally with 10 μM ADP, indicating that labeled platelets behave like platelets in PRP.

Figure 5b shows an autoradiograph of the gel reproduced in Figure 5a. GP IIIa, the prominent band noted at M_r 95,000, was present in the Triton-insoluble residue of the aggregated platelets at 3 and 6 min. The number of radioactive bands associated with the cytoskeletal core increased markedly between 3 and 6 min after aggregation was initiated (Fig. 5, lanes 2,6; Fig. 6, lanes 2,6).

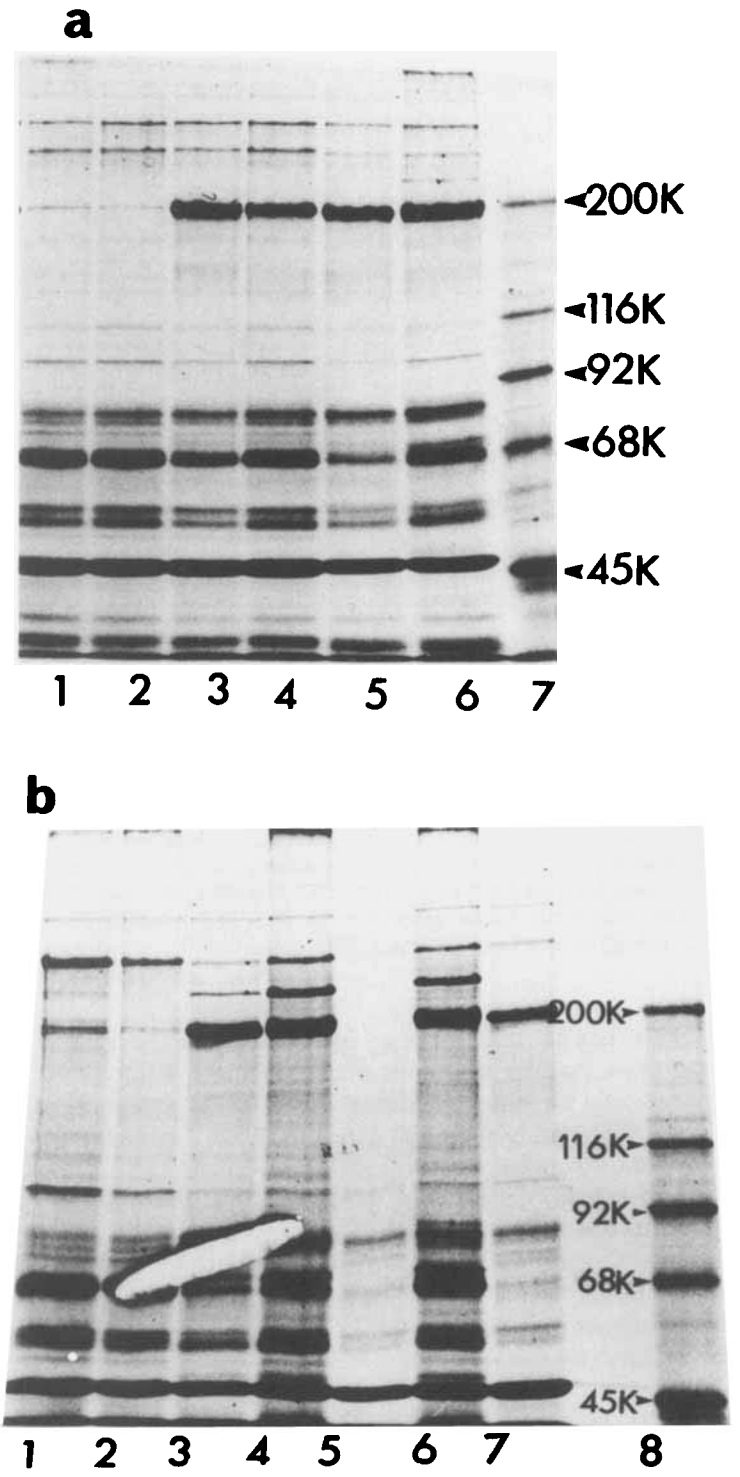
a
200K
116K
92K
68K
45K
1 2 3 4 5 6 7
b
200K
116K
92K
68K
45K
1 2 3 4 5 6 7 8

Figure 4.

Although neither EDTA nor PGE_1 added at 3 min reversed aggregation in non-ASA-treated platelets, they had different effects on the incorporation of labeled glycoproteins into the Triton-insoluble residue. EDTA caused little decrease, whereas PGE_1 caused a marked reduction. MB added 3 min after ADP-induced aggregation caused both disaggregation and loss of radioactive bands from the cytoskeleton (Fig. 5b, lanes 3,4,5). MB added before ADP prevented both aggregation and incorporation of GP IIIa or other radioactive glycoproteins into the Triton-insoluble residue (Fig. 5b, lanes 7,8). In ASA-treated platelets, GP IIIa was lost from the cytoskeletal core after addition of EDTA, PGE_1, or MB (Fig. 6a, lanes 3,4,5), while aggregation was reversed virtually completely by PGE_1 and MB, but only about 60% by EDTA.

Reversal of aggregation by 2 mM MB was greater when the time after addition of ADP was shorter. Thus, disaggregation was 50% when MB was added 3 min after aggregation had been initiated with 10 μM ADP, whereas it was 44 and 34% when it was added 4½ and 6 min after ADP. Figure 7 shows an autoradiograph of the cytoskeletal proteins in this experiment in which Triton was added 7 min after the MB. When aggregation reversed 50%, no GP IIIa remained in the Triton-insoluble residue (lanes 2,3), whereas with less disaggregation, GP IIIa was only partially lost (lanes 4,5).

Thrombin-aggregated platelets. The Triton X-100-insoluble residue of washed platelets aggregated for 1 min with 0.3 U/ml of thrombin contained actin-binding protein, myosin heavy chain, actin, and other proteins. Bands at about 100 and 210 kDa (Fig. 8a, lanes 1–4) and labeled GP IIIa (Fig. 8b, lanes 1–4) became more prominent as aggregation progressed.

Pretreatment of the washed platelets with 100 or 200 μM MB drastically decreased the amount of GP IIIa associated with the cytoskeletal core (Fig. 8b, lanes 5,6,7), although aggregation was only decreased about 50% (Fig. 2). When 200 μM MB was added 1 min after thrombin, aggregation continued but at slower rate (Fig. 2). Some GP IIIa had become incorporated into the cytoskeletal core at 1 min (Fig. 8b, lane 1), but further association was arrested by the MB (Fig. 8b, lanes 8,9,11).

Con A-stimulated platelets. Incubation of washed platelets with Con A-induced secretion of [^{14}C]serotonin that was inhibited by incubating the platelets with 100 μM ASA or by addition of 30 μM MB 1 min before the agonist (Table II). This response to inhibitors resembles that of secretion induced by low concentrations of collagen or by platelets aggregated with ADP in an environment low in calcium [14]. Con A also induced marked incorporation of GP IIIa into the cytoskeletal core of unstirred washed platelets. Incubation of the platelets with 200 μM MB for up to 3 min before addition of Con A failed to decrease the amount of GP IIIa incorporated

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Fig. 4. SDS-PAGE of Triton-insoluble residues prepared from ADP-stimulated non-ASA and ASA-treated PRP, stained with Coomassie Blue. **a,** Cytoskeletal cores: lanes 1 and 2, prepared at 1 min and 6 min after addition of 10 μM ADP (nonstirred) to acetonitrile-treated PRP; lanes 3 and 4, prepared at 1 and 6 min after addition of ADP to EDTA-pretreated PRP; lanes 5 and 6, prepared at 1 and 6 min after addition of ADP to 1 mM MB-pretreated PRP; lane 7, molecular weight standards. **b,** Cytoskeletal cores: lanes 1 and 2, prepared at 1 and 6 min after addition of 10 μM ADP (nonstirred) to ASA-treated PRP to which acetonitrile had been added; lanes 3 and 4, prepared at 1 and 6 min after addition of ADP to 1 mM MB-pretreated ASA-treated PRP; lanes 5 and 6, prepared at 6 min from unstimulated ASA-PRP preincubated with NaCl and 1 mM MB, respectively; lane 7, prepared 6 min after addition of ADP to 5 mM EDTA-preincubated ASA-treated PRP; lane 8, molecular weight standards.

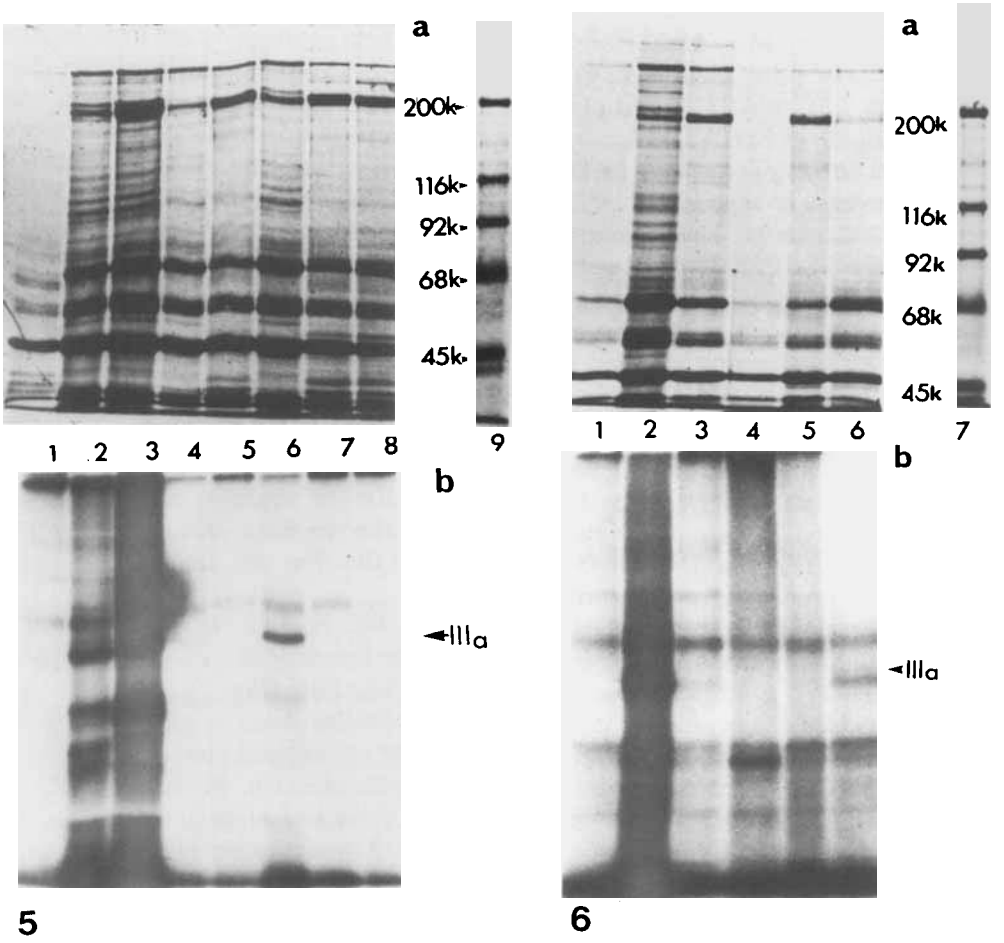

Fig. 5. Effect of MB on the assembly of the cytoskeletal core and its association with GP IIIa in ADP-aggregated PRP. **a**, SDS-PAGE of Triton-insoluble residues prepared from PRP containing 5% ^{125}I-labeled platelets stirred at 37°C in an aggregometer. Lane 1, from stirred control PRP; lanes 2,6, prepared at 6 and 3 min, respectively, after initiating aggregation with 10 μM ADP; lanes 3,4,5 5mM EDTA, 14 μM PGE_1 or 1 mM MB, respectively, added 3 min after ADP, and Triton-insoluble residues prepared 6 min after initiation of aggregation; lanes 7, 8, preincubated with 1 mM MB for 2 and 1 min before ADP; cytoskeletal cores prepared 6 min after ADP; lane 9, molecular weight standards. **b**, Autoradiograph of gel shown in panel a.

Fig. 6. Effect of MB on the assembly of the cytoskeletal core and its association with GP IIIa in ADP-aggregated ASA-treated PRP. **a**, SDS-PAGE of cytoskeletal preparations: lane 1, from control ASA-PRP, stirred; lanes 2, 6, prepared at 6 and 3 min, respectively, from ADP-aggregated ASA-PRP; lanes 3,4,5, from ADP-aggregated ASA-treated PRP to which 5 mM EDTA, 14 μM PGE_1, and 1 mM MB, respectively, were added at 3 min and Triton-insoluble residues were prepared 6 min after initiation of aggregation; lane 7, molecular weight standards. **b**, Autoradiograph of gel shown in panel a.

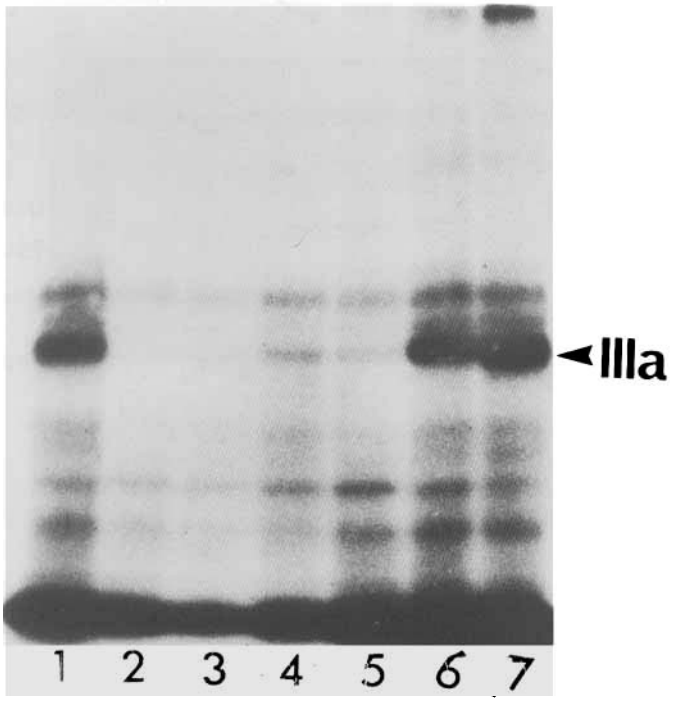

Fig. 7. Autoradiograph of SDS-PAGE of cytoskeletal cores from ^{125}I-labeled platelet illustrating the effect of MB added at varying intervals after ADP-induced aggregation in PRP on the association of GP IIIa with the Triton-insoluble residue. Lanes 1,6,7, prepared from PRP 3, 6, and 13 min after aggregation with 10 μM ADP; lanes 2,3, prepared from PRP aggregated with ADP for 3 min and treated with 1 and 2 mM MB for 7 min (disaggregation 50 and 52%); lanes 4,5, prepared from PRP aggregated with ADP for 4½ and 6 min treated with 2 mM MB for 7 min (disaggregation 44 and 34%).

into the cytoskeletal cores of non-ASA (Fig. 9b, lanes 2,3) and ASA-treated platelets (lanes 7,8). In the presence of this agonist, in contrast to ADP and thrombin, pretreatment with MB reduced the quantity of the myosin band (Fig. 9a, lanes 2,3,7,8).

DISCUSSION

Assembly of a cytoskeletal core, that is, formation of a Triton X-100-insoluble residue, is one of the platelet responses to stimulation [5–8,10,15]. Only a small amount of core protein is assembled in unstimulated platelets in PRP, and the core contains almost no myosin when it is examined in SDS-polyacrylamide gels. Stimulation with ADP, with or without aggregation, increases the amount of Triton-insoluble actin and myosin, with a smaller increase in actin-binding protein. In ADP-treated samples, the protein band at about 68 kDa is mostly albumin, derived at least in part from plasma, and the bands seen at 65, 56, and 47 kDa may represent the fibrinogen Aα, Bβ, and γ-chains. Since the association of these extracellular proteins with the Triton-insoluble residue must occur following lysis, the reported association of secreted platelet proteins such as coagulation factor V with these residues [16] should be interpreted cautiously. Bands characteristic of albumin and fibrin, formed

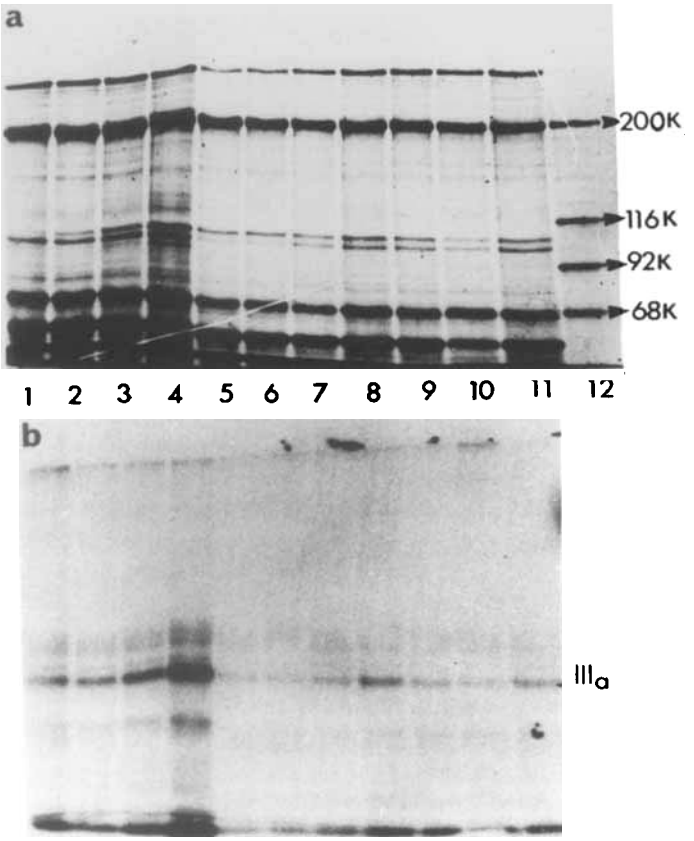

Fig. 8. Effect on the assembly of cytoskeletal proteins and their association with GP IIIa when MB is added before and after thrombin-stimulated aggregation of washed platelets. **a**, SDS-PAGE of cytoskeletal cores (CY) prepared as indicated; lane 1, 1 min after thrombin (all preparations 0.3 U/ml except lane 10); lane 2, 2 min after thrombin; lane 3, 4 min after thrombin; lane 4, 6 min after thrombin; lane 5, 200 μM MB 2 min *before* thrombin, CY 6 min after thrombin; lane 6, 200 μM MB 1 min *before* thrombin, CY 6 min after thrombin; lane 7, 100 μM MB 1 min *before* thrombin, CY 6 min after thrombin; lane 8, 100 μM MB 1 min *after* thrombin, CY 6 min after thrombin; lane 9, 200 μM MB 1 min *after* thrombin, CY 6 min after thrombin; lane 11, 200 μM MB 2 min *after* thrombin, CY 6 min after thrombin; lane 10, CY 6 min after 0.1 U/ml thrombin; lane 12, molecular weight standards. **b**, Autoradiograph of gel shown in panel a.

from platelet fibrinogen [17], are also evident in residues prepared from thrombin-stimulated washed platelets suspended in Hepes-Tyrode's solution containing albumin.

As in other studies [6,15], we noted that the amount of cytoskeletal myosin increases markedly when EDTA is added to suspensions of unstimulated or stimulated platelets before or after lysis. As postulated by Rosenberg et al. [15], the increase probably results from rigor bonds formed between myosin and actin filaments after chelation of Mg^{2+}. Cytoskeletal cores prepared in the presence of MB also had increased myosin and often contain a 235-kDa protein that may be talin [18]. These proteins are not increased when MB is added after the Triton X-100. The effects of MB are doubtless complex since virtually all of the platelet proteins involved in the contractile process contain thiol groups, as judged by their ability to fluoresce with

TABLE II. Effect of ASA or MB on Secretion of [^{14}C]Serotonin Incubated With Con A or Stirred With Collagen or ADP

	Percent platelet-associated [^{14}C]serotonin secreted							
	0.5 mg/ml Con A			6–12 μg/ml collagen			10 μM ADP	
Experiment	Alone	ASA	MB	Alone	ASA	MB[a]	Alone	MB[a]
1	25	3		14	3			
2	42	7		43	4			
3	28	7	0	25	0	1		
4	43	2	7					
5				47		0		
6				23		0		
7				40		3		
8				36		6		
9							78	0
10							33	1
11							36	0

Platelets were allowed to take up labeled serotonin. Con A was incubated for 4 min with washed platelets, some of which had been incubated with 100 μM ASA; collagen or ADP were stirred with control or ASA-treated PRP. MB (30 μM) was added to non-ASA-treated washed platelets or PRP 1 min before the agonist.

[a]Aggregation decreased in extent and became reversible.

this reagent [3]. Even in vitro, the effects of thiol group reactions on myosin are very complex [19].

We and other [6,8,10,20] found that stimulation of platelets with Con A increased cytoskeletal myosin. However, the mechanism for cytoskeletal assembly with Con A differs from that in ADP-stimulated platelets since addition of Con A to MB-pretreated platelets decreases rather than increases the amount of cytoskeletal myosin.

When EDTA is added to platelets that have undergone the release reaction, we find little or no disaggregation, in agreement with Kinlough-Rathbone et al. [9]. While EDTA dissociates fibrinogen from its receptor GP IIb/IIIa in unaggregated platelets [21], it may not have this effect in platelets that have aggregated and undergone the release reaction. The inability of EDTA to reverse aggregation following secretion may be attributed to the small portion of thrombospondin that binds to the platelet surface in the absence of ionized calcium [22–24] or, as suggested [9], to bonds other than thrombospondin or fibrinogen. The slight disaggregation caused by EDTA can be augmented significantly if the aggregated platelets are sedimented and resuspended in fresh plasma rather than in plasma in which aggregation had taken place. This suggests that the absence of released products in the fresh plasma affects the equilibrium of bound and unbound secreted proteins.

Others [9] have noted that PGE_1 fails to dissociate aggregates of platelets that have secreted their granule contents; in the present studies the effects of PGE_1 were variable, showing occasional partial disaggregation. Unlike EDTA-treated samples, these platelets exhibit a decrease in cytoskeletal myosin and other proteins, as would be expected from the ability of PGE_1 to raise cyclic AMP and thus reverse responses to excitation [25].

In contrast to EDTA and PGE_1, the thiol inhibitor MB causes marked reversal of ADP-induced aggregation in platelets that have undergone the release reaction. MB probably prevents ADP-induced aggregation by inhibiting the changes in GP IIb/

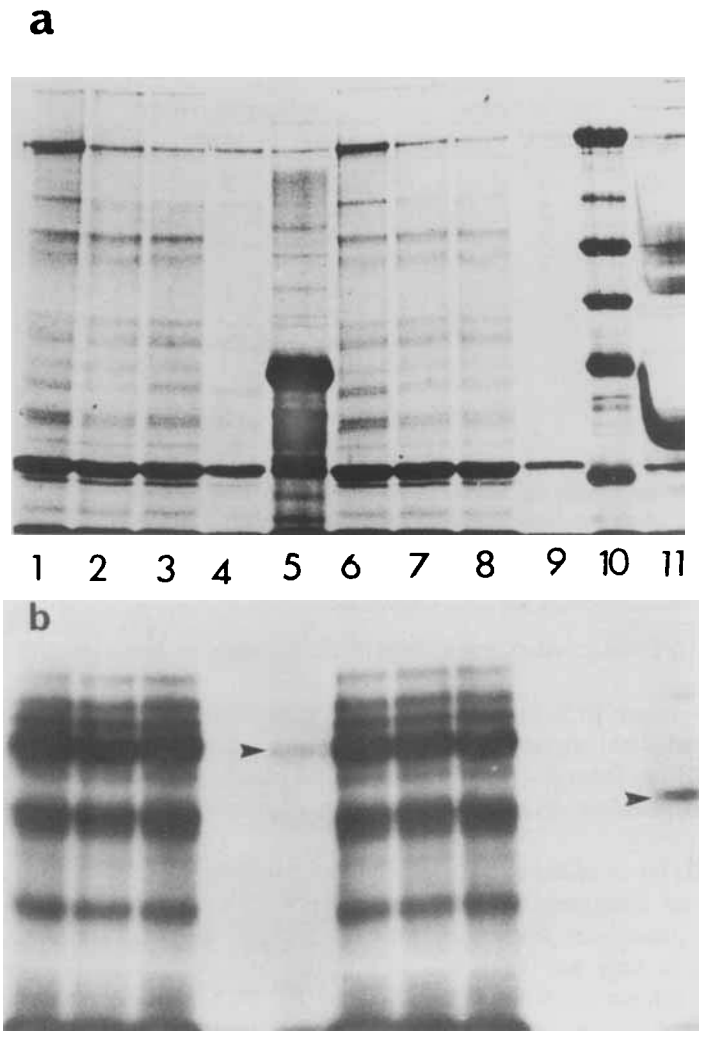

Fig. 9. Effect of MB on GP IIIa incorporation into the cytoskeletal core of Con-A-stimulated washed platelets. **a**, SDS-PAGE-reduced Triton-insoluble residue of ^{125}I-labeled platelets prepared 6 min after addition of : lane 1, Con A (0.1 mg/ml) to platelets; lane 2, Con A to platelets preincubated for 2 min with 200 μM MB; lane 3, Con A to platelets preincubated with 30 μM MB; lane 4, saline to platelets; lane 5, total platelets (reduced); lane 6, Con A to ASA-treated platelets; lane 7, Con A to ASA-treated platelets preincubated with 200 μM MB; lane 8, Con A to ASA-treated platelets preincubated with 30 μM MB; lane 9, saline to ASA-treated platelets; lane 10, molecular weight standards; lane 11, total platelets (unreduced). **b**, Autoradiograph of gel in panel a. Arrow indicates position of GP IIIa.

IIIa configuration that permit them to bind fibrinogen [3], whereas EDTA does not prevent those changes [21]. Incubation of washed platelets with MB before addition of thrombin decreases the extent of aggregation. Addition of MB after thrombin reduces the extent of aggregation but does not reverse it, perhaps because bonds dependent on thrombospondin or other secreted proteins are stabilized by thrombin [26].

Stimulation with ADP without stirring (hence, without aggregation) does not induce the incorporation of GP IIb/IIIa, confirming earlier findings [5–7], whereas the Triton-insoluble residue obtained from platelets aggregated with ADP contains GP IIIa. The less heavily labeled GP IIb and other radioactive proteins are also sometimes present. When aggregation is reversed, for example, when EDTA, PGE_1, or MB is added at 3 min to ADP-aggregated platelets that had not secreted (i.e., ASA-treated platelets), GP IIIa becomes dissociated from the cytoskeletal core.

The process by which GP IIIa is incorporated into the cytoskeletal core is not understood. Electron microscopic studies indicate that portions of the platelet membrane are not lysed when Triton X-100 is added to strongly aggregated platelets [6,27]. These unlysed fragments are probably responsible for the presence of some of the ^{125}I-labeled membrane glycoproteins [6] and [^{3}H]palmitate-labeled phospholipids [27] that are found in the Triton-insoluble residue of aggregated platelets several minutes after aggregation is initiated. The presence of such membrane fragments may account for the increase in the amount of protein and radiolabeled glycoproteins that we noted between 3 and 6 min after ADP-induced aggregation. Others have shown that when Triton was added when aggregation was only 10–20% complete, the [^{3}H]phospholipid that became Triton-insoluble was probably not present in unlysed membrane fragments, since it was released if actin was depolymerized [27]. However, under those conditions, GP IIIa was not incorporated into the cytoskeletal core [27]. The study of Wheeler et al. [7] probably provides the best evidence in the literature that membrane glycoproteins can be specifically incorporated into the cytoskeletal core and that GP IIb/IIIa were the only labeled proteins incorporated when Triton was added as soon as aggregation reached its peak. Our studies provide evidence that GP IIIa in the cytoskeletal core of aggregated platelets is specifically associated with the cytoskeletal proteins, rather than with membrane fragments, since it can be dissociated from the core despite persistence of substantial aggregation. For example, MB added 1 or 2 min after thrombin-induced aggregation arrested the incorporation of GP IIIa into the core without arresting or reversing aggregation. Similarly, PGE_1 added to platelets that had secreted reversed GP IIIa incorporation without appreciably reversing aggregation.

Cross-linking of surface glycoproteins IIb/IIIa with Con A causes them to associate with cytoskeletal proteins without aggregation [6,8]. Metabolic inhibitors fail to affect this association [24]. Cytochalasin B and PGE_1 are also ineffective although they decrease the amount of cytoskeletal actin and myosin [8]. We report here that MB also fails to decrease Con A-induced GP IIb/IIIa cytoskeletal association. Con A induces secretion [28–30], and we find that this secretion, like the thromboxane A_2-dependent secretion caused by collagen or ADP-induced aggregation [3,14], is readily abolished by ASA or by brief incubation with 30 μM MB.

The cytoskeletal core of ADP-aggregated platelets resembles the cytoskeletal core induced by Con A in that they both incorporate GP IIIa. Although occupancy of the fibrinogen receptors GP IIb/IIIa causes them to cluster [31], this is not sufficient for their incorporation into the cytoskeletal core. Perhaps aggregation, like the intraplatelet bonds induced by Con A, immobilizes the clustered fibrinogen receptors, leading to their association with the cytoskeletal core. Since MB reverses the association of GP IIIa caused by aggregation but does not prevent the association caused by Con A, its action cannot be directly on the attachment of GP IIIa to the cytoskeletal core. Rather, it probably acts by disrupting the configuration of GP IIb/IIIa necessary for fibrinogen binding, thus reversing clustering.

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