Aggregation-related Association of Lipid with the Cytoskeleton of Rabbit and Human Platelets Prelabeled with [^{3}H]Palmitic Acid

Similar Effects of Adenosine Diphosphate- and Thrombin-induced Aggregation

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Abstract

To investigate the association of lipid with the cytoskeleton of platelets during aggregation, rabbit and human platelets were isolated and labeled with [^{3}H]palmitic acid; lipid extraction showed ~ 80% in phospholipid. Limited aggregation was induced with ADP or thrombin, and the cytoskeleton was isolated after lysis with 1% Triton X-100, 5 mM EGTA. Cytoskeleton from unactivated platelets had ~ 0.03% of the total label in the platelets, but after aggregation with ADP (2 μM) or thrombin (0.1 U/ml) for 20–30 s, 1.5–8% of the label was with the cytoskeleton. Fibrinogen enhanced aggregation and the association of label with the cytoskeleton; incorporation of label increased exponentially as aggregation proceeded, decreased exponentially during deaggregation, and appeared to be related to the number of sites of contact. Inhibitors that increase cyclic AMP inhibited aggregation and cytoskeletal labeling, but aspirin had no effect. Some experiments were done with DNase I and Ca^{2+} in the Triton X-100 lysis medium to cause actin depolymerization, under conditions in which the Ca^{2+}-dependent protease activity was inhibited. This greatly reduced the association of label with the cytoskeleton at early time points, but when aggregation had proceeded further, a large proportion of the label was not dissociated by this treatment. These findings, electron microscopy, and the enrichment of the cytoskeleton of aggregated platelets with only some of the membrane proteins that were labeled by the ^{125}I-lactoperoxidase method, indicated that with limited aggregation, the ^{3}H-labeled lipid was mainly associated with the cytoskeleton and not with trapped membrane fragments resulting from incomplete lysis. Since the pattern of cytoskeleton labeling ([^{3}H]palmitate) and the selective association of some membrane proteins with the cytoskeleton/lipid complex was the same with ADP and thrombin, the reactions must be dependent on aggregation and not on events associated with the release of granule contents.

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Introduction

The composition and organization of the platelet cytoskeleton has been extensively studied and several reviews have appeared in recent years (1–6). The cell fraction that is insoluble in 1% Triton X-100 is termed the cytoskeleton; it contains polymerized, but not monomeric, actin and a number of other associated proteins. Monomeric actin (G-actin) is soluble in Triton whereas polymerized, filamentous actin (F-actin) is insoluble. Platelets contain large amounts of actin and in unstimulated platelets ~ 40% of the actin is filamentous actin, which is concentrated in a submembranous layer (7). The filaments are associated with glycoprotein Ib in the plasma membrane in an interaction mediated by actin-binding protein (7–10).

When platelets are stimulated to change shape, aggregate, and release their granule contents, their content of F-actin increases from ~ 40% to 70–80% of the total actin and reorganization of the actin filaments occurs (6). Proteins that associate with actin take part in this reorganization. The phosphorylation of myosin light chain by myosin light-chain kinase in a reaction mediated by Ca^{2+} stabilizes the association of myosin with the cytoskeleton when platelets are stimulated with aggregating and release-inducing agents (6, 11–14). Actin-binding protein cross-links actin filaments into networks and may regulate the polymerization of actin filaments (6). When platelets have been aggregated, actin-binding protein is hydrolyzed by the calcium-dependent protease (9, 15) and gelsolin severs actin filaments (1, 16). It has been suggested that in platelets that have been aggregated with thrombin or concanavalin A, actin filaments are linked to the membrane glycoprotein IIb/IIIa complex (17, 18), either directly or through intermediate proteins such as ankyrin, spectrin, α-actinin, or vinculin (6).

Although the proteins of the platelet cytoskeleton have been the subject of numerous studies, very few reports have focused on the lipids associated with cytoskeletons (5, 19, 20). Schick and his colleagues (19) found that the lipid/protein ratio of platelet cytoskeletons is similar to that of whole platelets, but the composition of the cytoskeleton lipids is specific and distinctly different from that in whole platelets. Burn and his co-workers (21) reported that a supramolecular complex is formed in vitro between α-actinin and actin in the presence of diacylglycerol and palmitic acid and that the complex is structurally similar to the microfilament bundles observed in vivo. These investigators observed α-actinin-lipid complexes in platelets prelabeled with [^{3}H]palmitic acid when the platelets were aggregated with thrombin. They concluded that α-actinin may be one of the proteins involved in structures connecting the cytoskeleton to the membrane and that diacylglycerol and

certain fatty acids (such as palmitic acid) in a 1:1 molar ratio may take part in this connection to the platelet membrane.

The present study was initially based on the experimental system used by Burn and his colleagues (21) who used human platelets prelabeled with [^{3}H]palmitic acid. Experiments were designed to compare the effect on the formation of lipid-cytoskeleton complexes of a strong agonist (thrombin) that causes aggregation, the release of granule contents, thromboxane A_2 formation (22) and the degradation of phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate and 1,2-diacylglycerol (23), with a weak agonist (ADP) that causes aggregation without appreciable release of granule contents, formation of thromboxane A_2 or formation of inositol trisphosphate by rabbit platelets (24), and probably little formation of diacylglycerol. Another reason for comparing the effects of ADP and thrombin was that the association between myosin and F-actin that occurs when platelets are stimulated with thrombin has been found by some investigators (12), but not by others (11), to also occur when the platelets are stimulated with ADP. Rabbit and human platelets were compared because they differ in their dependence on added fibrinogen for ADP-induced aggregation (25) and in the ease with which they can be deaggregated (26). Association of the label with the cytoskeleton was studied during the initial shape change, during the early stages of aggregation before large aggregates formed, and during deaggregation. The effects of inhibitors were examined, and the effects of proteolysis and actin depolymerization were investigated. Because membrane lysis by Triton X-100 may be incomplete when large platelet aggregates have formed (20), several experimental approaches were used to determine the effect of the extent of aggregation on the presence of membrane fragments and on the amount of label in the isolated cytoskeleton. A comparison was made between cytoskeletons prepared from large and small aggregates by examining the effect of depolymerization of actin filaments, by electron microscopy, and by studying the ^{125}I-labeling pattern of the external proteins of intact platelets and the cytoskeletons of unstimulated platelets and platelets aggregated by ADP or thrombin.

Methods

Materials. Lyophilized human fibrinogen was grade L from AB Kabi, Stockholm, Sweden; it was dialyzed against 0.14 M NaCl before use. Albumin was Pentex, fraction V, from Miles Laboratories, Elkhart, IN. Bovine thrombin was from Parke Davis Co., Detroit, MI. Apyrase was prepared from potatoes by the method of Molnar and Lorand (27) and used as described previously (28). Hirudin, ADP, adenosine, 5′-Cl-5′-deoxyadenosine, forskolin, acetylsalicylic acid (aspirin), ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetra-acetic acid (EGTA), *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (Hepes), PMSF, leupeptin, benzamidine, and lactoperoxidase (80–100 U/mg protein) were purchased from Sigma Chemical Co., St. Louis, MO. Prostaglandin E_1 (PGE_1) was from Upjohn Co., Kalamazoo, MI. Sepharose 2B was obtained from Pharmacia, Dorval, Quebec. Glycine-L-proline-L-arginine-L-proline (GPRP)[1] was from Vega Biochemicals, Tucson, AZ. Triton X-100 was molecular biology grade, ultra-pure reagent, International Biotechnologies, New Haven, CT. DNase I (grade II) was from Boehringer Mannheim, Dorval, Quebec. Glycoprotein IIb and IIIa standards were kind gifts of Dr. D. R. Phillips, Gladstone Foundation Laboratories, San Francisco, CA, and Dr. S. Niewiarowski, Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA. Soluene-350 and Hionic-fluor were obtained from Packard Instrument Co., Mississauga, Ontario. Labeled palmitic acid ([^{3}H]9,10-palmitic acid; 10–30 Ci/mmol) and Na^{125}I (carrier free, NEZ 033L) were from Du Pont, Canada, NEN Products, Lachine, Quebec.

Preparation of suspensions of platelets labeled with [^{3}H]palmitic acid. Suspensions of washed platelets from rabbits were prepared from blood anticoagulated with acid citrate dextrose according to the method of Ardlie et al. (25, 29). They were labeled with [^{3}H]palmitic acid (5 μCi/ml of platelet suspension at a platelet count of ~ 500,000/μl) in the third washing fluid (modified Tyrode solution, pH 7.35, with no added Ca^{2+}, no albumin, but containing apyrase). Unless otherwise stated, the incubation time at room temperature with [^{3}H]palmitic acid was 15 min. At the end of the incubation, 3.5% albumin was added to give a final albumin concentration of 0.35%. The platelets were recovered by centrifugation and resuspended for aggregation studies at a platelet count of 300,000/μl in a modified Tyrode-albumin solution containing 1.8 mM Ca^{2+}, 0.5 mM Mg^{2+}, 5 mM Hepes buffer, pH 7.35, and apyrase.

Suspensions of human platelets were prepared by gel filtration (30) from blood anticoagulated with acid citrate dextrose. Anticoagulated platelet-rich plasma that had been enriched in platelets by centrifugation at 2,300 *g* for 15 min to obtain a platelet count of ~ 1.5×10^6/μl was passed through a column of Sepharose 2B equilibrated with a modified Tyrode solution containing 5 mM Hepes, 1 μM PGE_1, and apyrase, but no added Ca^{2+} (pH 7.35). Platelets in the eluate were labeled with [^{3}H]palmitic acid as described above and the suspension was then passed through a second Sepharose 2B column equilibrated with a modified Tyrode solution, pH 7.35, containing 0.35% albumin, apyrase, 5 mM Hepes, but no added Ca^{2+}. The platelet count of the eluted suspension was adjusted to 300,000/μl.

Aggregation and isolation of cytoskeletons. For aggregation studies, the concentration of Ca^{2+} in samples of human platelets was adjusted to 1.8 mM by the addition of a $CaCl_2$ solution. In some experiments, 0.1 mg/ml of human fibrinogen was also added before the addition of ADP. Platelet shape change and aggregation were studied at 37°C by recording light transmission through 1 ml samples of platelet suspension stirred at 1,100 rpm in a cuvette in an aggregometer (Payton Associates, Scarborough, Ontario). The sensitivity of the aggregometer was adjusted so that the oscillations in light transmission characteristic of disc-shaped platelets could be observed. The initial decrease in light transmission after the addition of an aggregating agent and the reduction in the oscillations in light transmission indicate a change in shape of the platelets from discs to a more rounded form with pseudopods (31). Aggregation was measured as the percentage change in light transmission through the platelet suspension, with 0% defined as the lowest point of the light transmission curve after addition of the aggregating agent and 100% defined as light transmission after addition of Triton X-100-EGTA. The aggregating agents used were ADP and thrombin. In some experiments, inhibitors were added 1 min before the aggregating agent; the inhibitors were adenosine, 5′-Cl-5′-deoxyadenosine, forskolin, aspirin, and PGE_1. For studies of deaggregation after aggregation by thrombin, 0.4 mM GPRP was added before thrombin, and 2 U/ml hirudin was added 90 s after thrombin, before PGE_1 (20 μM). The amounts of all materials added to the platelet suspensions are expressed as final concentrations after all additions. Aggregation was stopped by the addition of a lysis solution containing 10% Triton X-100 and 50 mM EGTA, pH 7.35, to give final concentrations of 1% Triton and 5 mM EGTA, unless otherwise specified. The material that is insoluble in this medium is commonly defined as cytoskeleton (1). Cytoskeletons were isolated as described by Phillips et al. (17) and Burn et al. (21). Briefly, the samples were stirred for 45 s, cooled in ice, transferred quantitatively to an Eppendorf centrifuge tube and centrifuged for 4 min at 12,000 *g*. The supernatant was removed by suction and the pellet was washed twice without disturbing

1. *Abbreviations used in this paper:* CS, cytoskeleton; GPRP, glycine-L-proline-L-arginine-L-proline.

it with a modified Tyrode solution containing 1% Triton, 5 mM EGTA, but no Ca^{2+}. After a final wash in Ca^{2+}-free Tyrode solution, the tip of the centrifuge tube was cut into a liquid scintillation counting vial and the contents solubilized with 0.9 ml of Soluene-350. After the addition of 10 ml of Hionic-fluor, the amount of ^3H in the samples (cytoskeletons) was determined and expressed as a percentage of the total counts per minute in the platelet pellet. In some experiments, the Triton X-100-EGTA lysis solution was supplemented with various combinations of leupeptin, benzamidine, and PMSF. To depolymerize the cytoskeletons, EGTA was omitted from the Triton X-100 solution and/or DNase I (1 mg/ml) was included.

Lipid extraction. Lipids were extracted from whole platelets and from isolated cytoskeletons by the method of Bligh and Dyer (32) and separated as described by Burn et al. (21) by two-dimensional chromatography on high performance Merck TLC silica gel 60 plates (Mandel Scientific, Rockwood, Ontario). Lipid-containing spots were located by staining with iodine vapor, and phospholipids were located by comparison with the position on a second plate of standard phospholipids stained with Vaskovsky spray (33). The spots were scraped into liquid scintillation counting vials for determination of radioactivity.

Protein determinations. Protein was measured by the method of Lowry et al. (34).

Labeling and separation of ^{125}I-labeled proteins by SDS-PAGE. Membrane glycoproteins of human platelets were labeled with ^{125}I by the lactoperoxidase-H_2O_2 method of Phillips and Agin (35) with minor modifications. After the first gel filtration, 8 ml of platelet suspension ($10^6/\mu l$) was mixed with 1 mCi of $Na^{125}I$ and 80 μl lactoperoxidase (2 nmol); five additions of 10 μl of 3 mM H_2O_2 were made at 30-s intervals. Labeled platelets were isolated by passage through the second gel filtration column as described above. The method of Phillips et al. (17) was used to separate the proteins by SDS gel electrophoresis in 7.5% polyacrylamide gels and to determine the distribution of radioactivity on the gels. The purpose of these experiments was solely to determine whether the labeling pattern of the cytoskeletal proteins resembled that of intact platelets (which would be indicative of trapping of membrane fragments), or whether the pattern was distinctive (indicative of selective enrichment with some membrane proteins). No attempt was made to characterize the labeled proteins that were associated with the cytoskeleton.

Electron microscopy. Triton X-100-EGTA insoluble residues of platelets aggregated with 0.1 U/ml of thrombin for 30 s or 1 U/ml of thrombin for 3 min were fixed and processed for electron microscopy as described by Begg et al. (36).

Formaldehyde fixation. In a few experiments, platelets were fixed by the addition of 1% formaldehyde (37), examined microscopically, and the number of individual particles was counted with a Coulter counter, model Z_F, with a 70-μm diam orifice. As aggregation proceeds, the number of single particles decreases as the platelets form aggregates of 2 or 3 platelets that are counted as single particles and then form aggregates that are large enough to be excluded.

Controls. As a test for possible entrapment of [^3H]palmitic acid within the platelet aggregates, unrelated to the cytoskeletons, [^3H]-palmitic acid was added to aggregating unlabeled platelets (3×10^8/ml) at various times (2–20 s after thrombin; 0.1 U/ml). The amounts of label retained in the cytoskeleton (0.03–0.07% of the added label) were similar to the amounts retained in the cytoskeletons of unstimulated, labeled platelets (~ 0.03%), and many fold less than the amounts retained when the labeled platelets were aggregated. When [^3H]-palmitic acid was added immediately after lysis with Triton X-100-EGTA, 0.02% of the added label was retained in the cytoskeletons. Likewise, the addition immediately after lysis of a ^3H-lipid extract prepared from platelets that had been labeled with [^3H]palmitic acid, resulted in very little retention (0.04% or less) of the added label in the isolated cytoskeletons. In another control, platelets were incubated with both [^3H]palmitic acid and [^{14}C]glucose for 30 min and cytoskeletons were prepared from aggregating platelets. Relative to the label in unactivated, washed platelets, the cytoskeletons contained 100-fold less ^{14}C than ^3H. All these controls indicate that the ^3H label recovered in the cytoskeletons of aggregating platelets does not result from labeled material trapped during the preparation of the cytoskeletons. These controls, however, do not address the possibility that the cytoskeletons isolated from aggregated platelets may contain unlysed membrane fragments; this possibility was explored by electron microscopy, experiments with DNase I and Ca^{2+} in the Triton lysis medium, and by labeling membrane proteins by the lactoperoxidase method (see Results).

Results

Labeling of platelets with [^3H]palmitic acid. Burn and his colleagues (21) used a 2-h incubation period of platelets with [^3H]palmitic acid in their studies of the association of labeled lipids with the cytoskeleton of thrombin-activated platelets. Fig. 1 shows, however, that when platelets were incubated with [^3H]palmitic acid, the percentage of the label associated with the platelets reached a maximum of ~ 45% by 15–30 min; the amount of ^3H in the platelets was only slightly decreased (to ~ 40%) by the subsequent washing procedure in a solution containing 0.35% albumin, followed by two additional washes with Tyrode-albumin, or by gel filtration through a Sepharose 2B column equilibrated with an albumin-containing solution. Lipid extracts of samples of the labeled platelets, isolated by centrifugation of samples during the course of the incubation of the platelets with [^3H]palmitic acid, indicated that [^3H]-palmitic acid was rapidly incorporated into phospholipids (Fig. 1). The lipid extract of intact platelets prepared 1 h after the completion of labeling (either 15 or 120 min) was found to have about 80% of its label in phospholipids.

In all the experiments in which the platelets were aggregated and the cytoskeletons were isolated, a 15-min labeling time was used. After the platelets had been labeled, the preparation of the platelets for aggregation studies, including washing and resuspension or gel filtration, cell count and incubation for 15 min at 37°C required ~ 1 h.

Formation of labeled cytoskeletons. Material that was insoluble in Triton X-100-EGTA (cytoskeleton) from unactivated platelets, labeled with [^3H]palmitic acid, contained 0.03% or less of the total label in the platelets. However, the

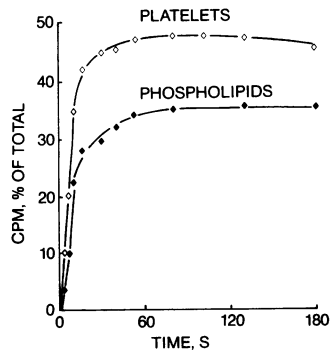

Figure 1. Rate of incorporation of [^3H]palmitic acid (170 nM) into rabbit platelets and into phospholipids. Platelets (500,000/μl) in a modified Tyrode solution (no Ca^{2+}, no albumin) were incubated with [^3H]palmitic acid for the times indicated. Samples were centrifuged and the supernatants counted to determine (by difference) the amount of radioactivity associated with the platelet pellet. Platelets were extracted by the method of Bligh and Dyer (32), the lipids were separated by TLC as described in Methods, and the radioactivity in the phospholipids was determined. Both the label in the platelets and the label in the phospholipids were expressed as a percentage of the total radioactivity in the platelet suspension. Typical of four experiments with rabbit platelets and two experiments with human platelets.

cytoskeleton from rabbit or human platelets that had been induced to aggregate by the addition of 2 μM ADP incorporated 1.5–3.5% of the label within 20 to 30 s when the change in light transmission was only 15–20% and oscillations typical of large aggregates had not begun (Table I). The effects of several aggregation conditions on the labeling of the cytoskeleton are summarized in Table I. With ADP as an inducer, the cytoskeleton labeling, like aggregation, showed greater dependence on added fibrinogen with human platelets than with rabbit platelets. Slow stirring (200 rpm) greatly reduced the amount of label that was incorporated into the cytoskeleton (Table I). When the platelets were aggregated with thrombin (0.1 U/ml), 3–8% of the label was incorporated into the cytoskeleton within 20 to 30 s.

Fig. 2 shows a representative time course of the incorporation of ^3H into cytoskeleton of aggregating platelets. Shown are: the aggregometer tracing of gel-filtered human platelets activated by 0.1 U/ml thrombin and the CPM associated with cytoskeletons separated at different times during aggregation. While the platelets underwent shape change, the label did not become associated with the cytoskeleton, but as aggregation proceeded, the incorporation of the label into the cytoskeleton increased exponentially. The pattern depicted in Fig. 2 was observed with both human and rabbit platelets. Rabbit platelets that were isolated by gel filtration by the method used for human platelets gave similar results. Aggregation of rabbit platelets induced by ADP (1–10 μM) or by thrombin (0.05–1 U/ml) caused the incorporation of the label into the cytoskeleton, although the incorporation of label in response to thrombin was 1.5 to 3 times greater than in response to ADP at the same extent of change in light transmission.

The number of platelets present in the assay greatly affected the extent of labeling of the cytoskeleton, as shown in Fig. 3. Rabbit platelets were induced to aggregate by stimulation with either 2 μM ADP or 0.1 U/ml thrombin for 30 s. The extent and rate of the change in light transmission through the platelet suspension was less affected by the platelet number than was the extent of labeling of the cytoskeleton, which was enhanced over 40-fold when the number of platelets was increased 4.6-fold (from 1.5×10^8 to 6.9×10^8 per ml) (Fig. 3).

The effects of EDTA on the aggregation of rabbit platelets and on the amount of ^3H in the cytoskeleton are illustrated in Fig. 4. Addition of 5 mM EDTA (in molar excess of Mg^{2+} and Ca^{2+} in the medium) before ADP or thrombin eliminated aggregation and virtually prevented the incorporation of radioactivity into the cytoskeleton, although the platelets changed shape. Addition of EDTA 30 s after the inducer not only caused deaggregation, but greatly reduced the labeling of the cytoskeleton. Loss of label preceded the fall in light transmission indicative of deaggregation. The effect of EDTA on human platelets aggregated by ADP in the presence of fibrinogen was similar to that shown for rabbit platelets in Fig. 4; that is, the platelets deaggregated and the labeling of the cytoskeleton fell to < 0.3%. However, when thrombin was used as the aggregating agent for human platelets, EDTA, added at 25 s, did not cause deaggregation and had only a limited effect on the cytoskeleton labeling within the following 30-s period. (Addition of EDTA with the Triton X-100-EGTA lysis medium did not affect the amount of label recovered with the cytoskeleton.)

Inhibitors. The effects of several inhibitors of platelet aggregation were investigated on the amount of ^3H that became associated with the cytoskeleton 30 s after the addition of ADP or thrombin. Inhibitors which decreased aggregation of rabbit platelets in response to 2 μM ADP or 0.1 U/ml of thrombin by 80% or more, almost completely inhibited the incorporation of

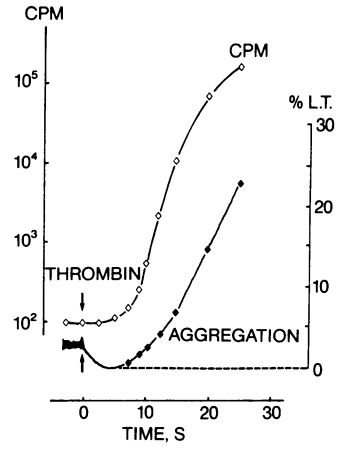

Figure 2. Aggregation of human platelets induced by 0.1 U/ml thrombin, and association of ^3H with isolated cytoskeletons. The platelets had been prelabeled with [^3H]-palmitic acid. The amounts of ^3H in the cytoskeletons isolated at various points on the aggregation curve are shown as counts per minute per sample. (Total counts per minute in a sample of the platelet suspension of the same volume as those from which the cytoskeletons were prepared was 1.24×10^6.) Aggregation is shown as percentage change in light transmission (L.T.). Similar results were obtained with rabbit platelets in two additional experiments.

Table I. Conditions Influencing the Amount of ^3H in the Isolated Cytoskeletons of Platelets Prelabeled with [^3H]Palmitic Acid

	Label in cytoskeletons cpm (% total)	
Assay condition	Human platelets	Rabbit platelets
1 Complete*	2.53	3.40
2 No fibrinogen	0.05	0.94
3 No ADP	0.03	0.03
4 As 1, but stirring at 200 rpm	0.03	0.02

* 1-ml assay: 3×10^8 platelets, stirred at 1,100 rpm, 37°C, Tyrode-albumin solution with 1.8 mM Ca^{2+}, 0.5 mM Mg^{2+}, 5 mM Hepes, pH 7.35, and apyrase. Aggregation induced with 2 μM ADP in the presence of 110 μg/ml fibrinogen. Lysis was induced 25 s after addition of ADP by addition of Triton X-100-EGTA. Typical of three experiments.

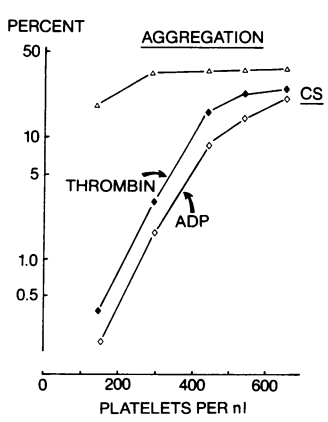

Figure 3. Effect of the number of rabbit platelets in suspension on the extent of aggregation 30 s after the addition of 2 μM ADP or 0.1 U/ml thrombin, and on the amount of ^3H associated with the cytoskeletons (thrombin, ♦; ADP, ◇). The radioactivity in the cytoskeletons (CS) is expressed as a percentage of the total radioactivity in the platelet pellet. Aggregation is shown as percentage change in light transmission. Typical of two experiments.

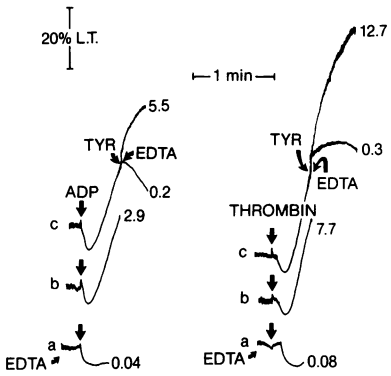

Figure 4. Effect of EDTA (5 mM) on rabbit platelet aggregation induced by 2 μM ADP or 0.1 U/ml thrombin. The percentage of the total ^3H in the platelets that was associated with the isolated cytoskeletons is shown beside the curves. (*a*) EDTA added before aggregating agent, Triton X-100-EGTA added 24 s after aggregating agent; (*b*) no EDTA, Triton X-100-EGTA added 30 s after aggregating agent; (*c*) EDTA or Tyrode (TYR) added 30 s after the aggregating agent, Triton X-100-EGTA added at 48 s. A 20% change in light transmission (L.T.) is shown by the bar. Typical of four experiments with rabbit platelets. Similar results were obtained when ADP was added to human platelets. However, when thrombin was added to human platelets, EDTA slowed and stopped aggregation, but little or no deaggregation occurred and there was only a small loss of label from the cytoskeleton within 30 s.

^3H into the cytoskeletons of platelets prelabeled with [^3H]-palmitic acid; these inhibitors were ones that act through adenylate cyclase to raise the concentration of cyclic AMP, namely, adenosine (30 μM), PGE_1 (3 μM), and forskolin (30 μM). In contrast, aspirin (100 μM) had no inhibitory effect on aggregation induced by ADP or thrombin and did not inhibit the incorporation of the label into the cytoskeleton.

Reversible aggregation and cytoskeleton labeling. During deaggregation of rabbit platelets that had been aggregated with ADP, the labeling of the cytoskeleton decreased at a faster rate than the decline in light transmission shown by the aggregation tracing (Fig. 5). When a second wave of aggregation was induced by the addition of a second, larger dose of ADP, a second wave of cytoskeleton labeling occurred in parallel with aggregation (Fig. 6). When apyrase was omitted from the suspending medium so that the platelets remained aggregated, the labeling of the cytoskeleton did not diminish (data not shown).

Rabbit platelets that have been induced to aggregate by the addition of thrombin can be deaggregated by PGE_1 in the presence of GPRP and hirudin (26). Fig. 7 shows that following the addition of PGE_1 to rabbit platelets aggregated by 0.1 U/ml thrombin, a rapid decline in the labeling of the cytoskeleton occurred, clearly preceding the change in light transmission. The exponential nature of the decline in labeling is indicated by the linearity of the plot of the *logarithm* of the fraction of maximal counts per minute in the cytoskeleton as a function of time.

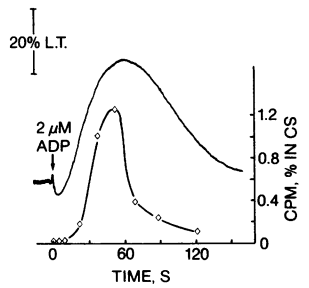

Figure 5. Aggregation and deaggregation of rabbit platelets after the addition of 2 μM ADP (*upper curve*) and association and dissociation of ^3H with the cytoskeleton of platelets prelabeled with [^3H]-palmitic acid (*lower curve*, ◇). The counts per minute in the cytoskeleton (CS) are expressed as a percentage of the total radioactivity in the platelet pellet. A 20% change in light transmission (L.T.) is shown by the bar. Typical of four experiments.

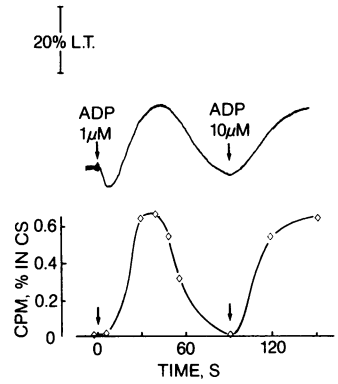

Figure 6. Aggregation, deaggregation and reaggregation of rabbit platelets after the addition of 1 μM ADP, followed by 10 μM ADP at 90 s (*upper curve*), and association, dissociation and reassociation of ^3H with the cytoskeleton of platelets prelabeled with [^3H]-palmitic acid (*lower curve*, ◇). The counts per minute in the cytoskeleton (CS) are expressed as a percentage of the total radioactivity in the platelet pellet. A 20% change in light transmission (L.T.) is shown by the bar. Typical of two experiments.

Effect of proteolysis and actin depolymerization. As shown in Table II, the exclusion of EGTA from the lysis medium greatly diminished the label recovered in the Triton-insoluble residue. When EGTA was omitted, Ca^{2+} was present during lysis because the platelet suspending medium contained 1.8 mM Ca^{2+}. Ca^{2+} may affect the cytoskeletons in at least two ways: (*a*) as a cofactor for the Ca^{2+}-dependent platelet protease, capable of hydrolyzing proteins associated with the cytoskeleton (9, 10, 15, 38); (*b*) by a Ca^{2+}-dependent depolymerization of actin filaments by gelsolin (1, 16). To evaluate these possibilities, the effect on the labeling of the cytoskeletons of including several protease inhibitors in the lysing solution was studied. Then, the effect of DNase I, known to depolymerize actin filaments (8), was examined in the presence of the protease inhibitors.

Table II shows the effect of leupeptin, known to inhibit the Ca^{2+}-dependent platelet protease activity (39), benzamidine, PMSF and EGTA, in various combinations. While PMSF was inactive, the inclusion of leupeptin or benzamidine in the lys-

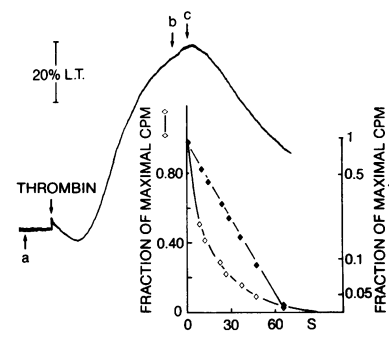

Figure 7. Aggregation and deaggregation of rabbit platelets after the addition of 0.1 U/ml thrombin (*upper curve*) and association and dissociation of ^3H with the cytoskeleton of platelets prelabeled with [^3H]-palmitic acid. Additions: (*a*) 0.4 mM GPRP; (*b*) hirudin, (2 U/ml); (*c*) PGE_1 (20 μM). A 20% change in light transmission is shown by the bar. The amount of ^3H associated with the isolated cytoskeletons is shown both linearly (◇) and logarithmically (♦) as a fraction of the maximal counts per minute associated with the cytoskeletons at 90 s (before the addition of PGE_1 to cause deaggregation). Typical of three experiments.

Table II. Effects of Protease Inhibitors and EGTA Added to the Triton X-100 Lysis Medium on the Amount of ^3H in the Isolated Cytoskeletons of Rabbit Platelets Prelabeled with [^3H]Palmitic Acid

Inclusion in Triton X-100 lysis medium	Amount of label in cytoskeleton
	*cpm (% total)**
1 None	0.06±0.03
2 PMSF (2 mM)	0.06±0.04
3 Benzamidine (50 mM)	0.29±0.03
4 Leupeptin (0.5 mM)	0.18±0.01
5 EGTA (5 mM)	5.52±0.38
6 PMSF + benzamidine	0.29±0.05
7 PMSF + benzamidine + leupeptin	0.41±0.01
8 PMSF + benzamidine + EGTA	5.19±0.16
9 PMSF + benzamidine + EGTA + leupeptin	7.47±0.49

* Mean±SD of 3 experiments. Similar results were obtained with human platelets.
Assay conditions: 3×10^8 platelets/ml, 0.1 U/ml thrombin for 19 s at 37°C.
Final concentration of Triton X-100, 1%.

ing solution significantly increased the labeling of the cytoskeleton. A much larger effect was exerted by EGTA, and the highest labeling was observed when all tested compounds were included in the lysing medium. The impact of leupeptin is illustrated by its significant effect in the presence of benzamidine (treatment 7 vs. 6) or EGTA (treatment 9 vs. 8). The same pattern shown in Table II was also observed with rabbit platelets aggregated by ADP (2 μM) and with human platelets aggregated by thrombin (0.1 U/ml).

The effect of DNase I was studied in the presence of leupeptin, benzamidine, and PMSF. Table III shows that the inclusion of DNase I in the lysis medium lowered the cytoskeleton labeling when aggregation was induced by either thrombin or ADP. A further decrease occurred when DNase I was added in the presence of Ca^{2+}; this result is consistent with the known stimulatory effect of Ca^{2+} on DNase I (40) and with the combined effect of DNase I and Ca^{2+} on depolymerization of platelet actin (8).

On the basis of these results it appears possible to utilize the lowering of the amount of label in the Triton-insoluble residue by the inclusion of DNase I and Ca^{2+} in the lysis medium as a measure of the label associated specifically with the cytoskeleton, distinct from the contribution of insoluble membranes, possibly trapped in the aggregates (20). It would be anticipated that the effect of DNase I and Ca^{2+} would be diminished under conditions in which fragments of membranes are present in the Triton-insoluble fraction.

Fig. 8 shows the recording of the light transmission changes during rabbit platelet aggregation induced by thrombin (*A*) and the temporal distribution of label in the DNase I-Ca^{2+}-sensitive and Ca^{2+}-resistant fractions of the Triton-insoluble residue (*B*). Three stages are apparent: shape change; the Triton-insoluble residue contained no label. Early stages of aggregation, no oscillations (up to 15 to 25 s after the addition of thrombin): the label in the Triton-insoluble residue was most sensitive to the depolymerizing effects of DNase I and Ca^{2+}. Aggregation at advanced stages, typified by wide oscillations of the light transmission tracing and, concomitantly, a gradual increase in the amount of label in the Triton-insoluble residue that was resistant to DNase I and Ca^{2+}, while the amount of label in the sensitive residue declined.

To explore further the possibility of trapping pieces of insoluble membrane in the Triton-insoluble residue, two aggregation conditions were selected for human platelets: (*a*) a condition leading to large aggregates (1 U/ml thrombin for 3 min) for which the presence of membranes was described by Zucker and Masiello (20), and (*b*) the condition used for the standard assays in the present study (0.1 U/ml thrombin for 20 s). Mi-

Table III. Effect of DNase I and Ca^{2+} in the Triton X-100 Lysis Medium on the Amount of ^3H in the Isolated Cytoskeletons of Rabbit Platelets Prelabeled with [^3H]Palmitic Acid

Addition to lysing solution*	Relative labeling of cytoskeletons (%)‡	
	ADP	Thrombin
None	100	100
DNase I (1 mg/ml)	38±9	40±12
Ca^{2+} (1.6 mM)§	24±15	23±17
DNase I, Ca^{2+}§	10±3	8±6

* The standard lysing solution gave final concentrations of 1% Triton X-100, 5 mM EGTA, 2 mM PMSF, 50 mM benzamidine, and 0.5 mM leupeptin. Lysis was induced 20–28 s after the addition of 2 μM ADP and 19 to 26 s after the addition of thrombin (0.1 U/ml).
‡ Mean±SD of seven values from three different experiments. Percent total radioactivity in platelets that was in the Triton X-100 insoluble residue: ADP 1.6 to 7.8%; thrombin 2.3 to 8.3%.
§ Omission of EGTA resulted in a solution containing Ca^{2+} derived from the platelet suspending medium.
Similar results were obtained with human platelets.

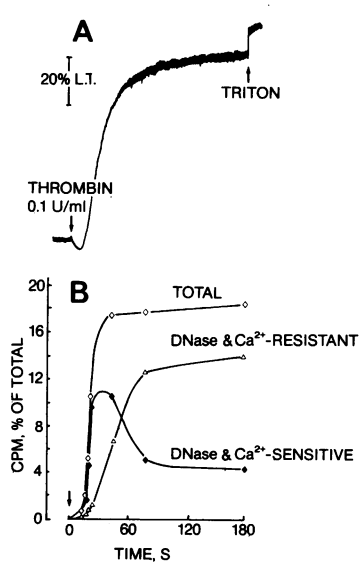

Figure 8. Effect of DNase I and Ca^{2+} in the Triton X-100 lysis solution (which contained 2 mM PMSF, 50 mM benzamidine, and 0.5 mM leupeptin) on the amount of ^3H in the isolated cytoskeletons of rabbit platelets prelabeled with [^3H]palmitic acid. (*A*) The platelets were aggregated with 0.1 U/ml thrombin. A 20% change in light transmission is shown by the bar. (*B*) Radioactivity is expressed as a percentage of the total counts per minute in the platelets. Total: platelets lysed with Triton X-100-EGTA in standard manner ◇; DNase and Ca^{2+}-resistant: platelets lysed with Triton X-100 (no EGTA) containing DNase I to give a final concentration of 1 mg/ml △; DNase and Ca^{2+}-sensitive: values obtained by subtracting DNase and Ca^{2+}-resistant values from total ♦.

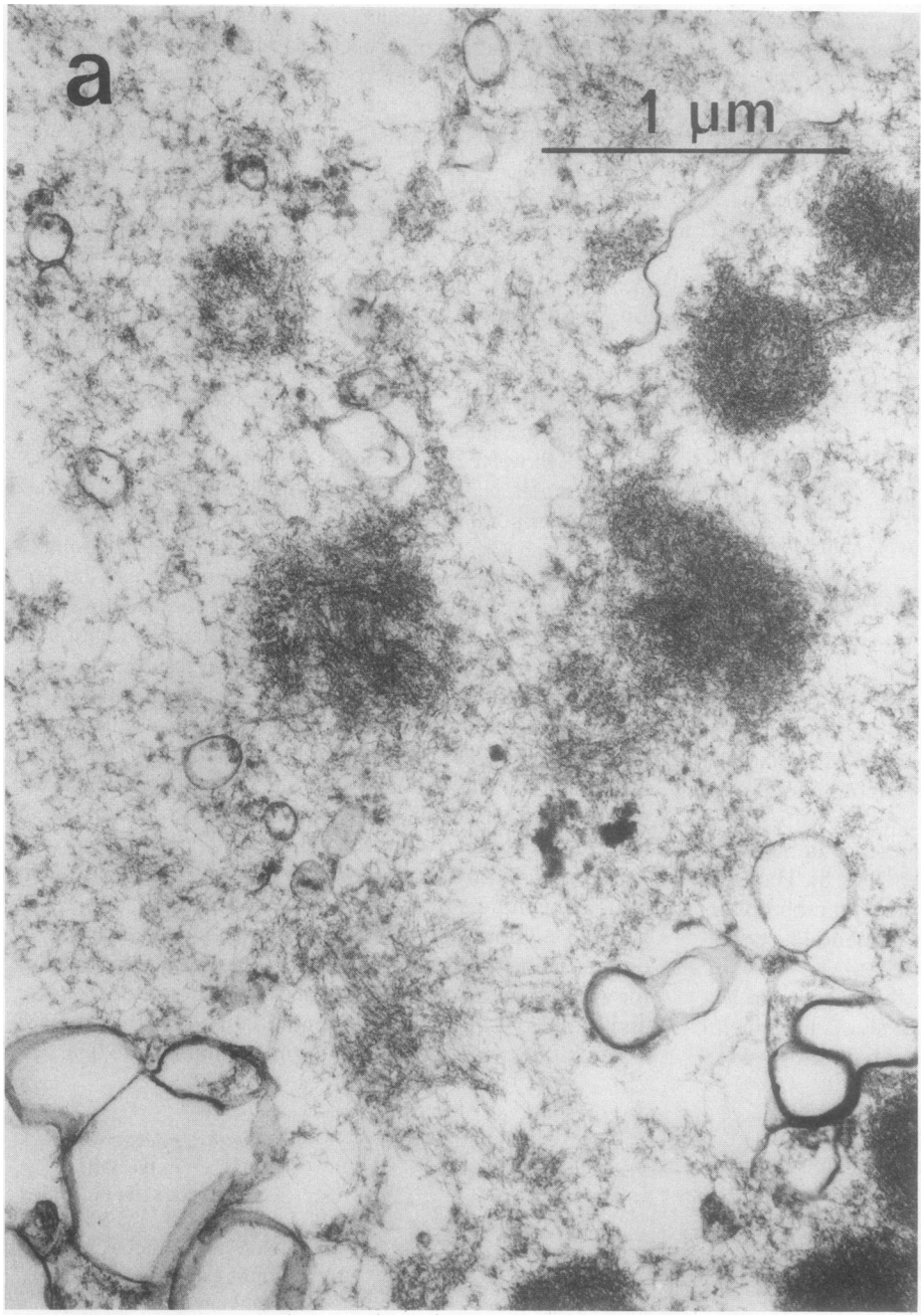

Figure 9. Electron micrographs of thin sections of Triton X-100-EGTA-insoluble residues prepared from human platelets aggregated with (*a*) 1 U/ml thrombin for 3 min (*b*) 0.1 U/ml thrombin for 20 s. Bar equals 1 μm.

croscopic examination after 1 U/ml of thrombin for 3 min showed platelet aggregates that were too large to permit an estimate of the number of platelets they contained; the Coulter counter showed a drop in the number of single particles to 3% of the prestimulation value. In contrast, the small aggregates formed after limited aggregation with 0.1 U/ml of thrombin contained only 2–10 platelets and the number of single particles was reduced to 20% of the prestimulation value. Under the conditions leading to large aggregates, the amount of label in the Triton-insoluble fraction was 27% of the label in the platelets, and DNase I and Ca^{2+} did not lower the amount of label in this fraction. In contrast, at 20 s, when small aggregates had been induced with 0.1 U/ml of thrombin, DNase I and Ca^{2+} reduced the amount of label in the cytoskeleton to less than one-tenth of that found when DNase I and Ca^{2+} were not present in the lysis medium.

Extent of membrane lysis. Because of the conclusion of Zucker and Masiello (20) that membrane lysis by Triton may be incomplete so that the cytoskeleton isolated from aggregated platelets may contain membrane fragments, several other experimental approaches were used to determine whether the labeled palmitic acid was actually associated with the cytoskeleton, or present in membrane fragments isolated with it. In addition to the experiments with DNase I in the presence of Ca^{2+} described above, these approaches involved electron microscopy of isolated cytoskeletons and comparison of the profile of ^{125}I-labeling of external proteins in intact platelets and in the isolated cytoskeleton.

Electron micrographs of Triton-insoluble residues prepared from the large aggregates formed when the platelets were aggregated with 1 U/ml thrombin for 3 min revealed the presence of numerous, large membrane fragments (Fig. 9). How-

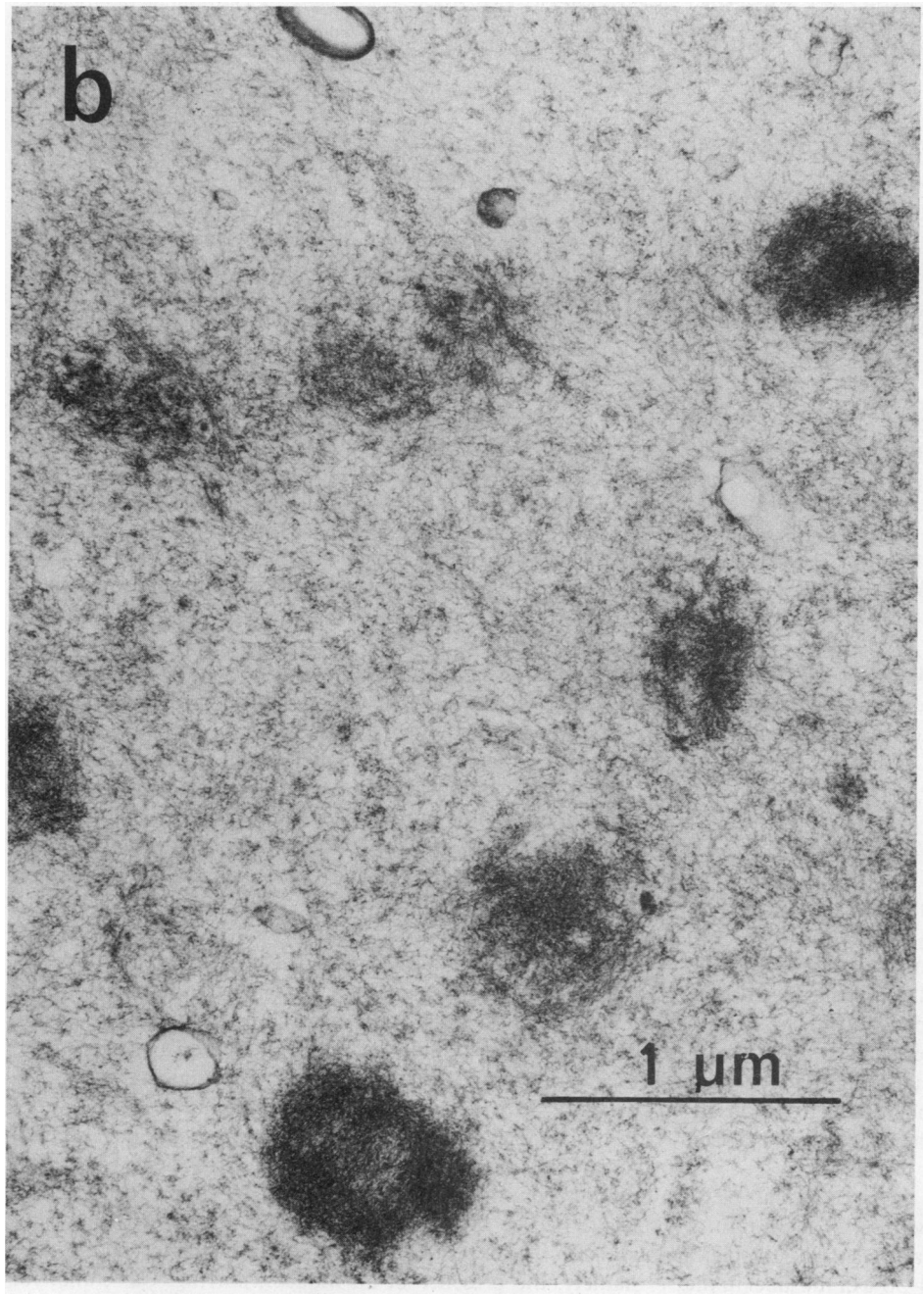

Figure 9 (*Continued*)

ever, when aggregation was limited in extent, as it was in the present study because thrombin was used at a concentration of 0.1 U/ml for only 20–30 s, typical membrane structures could not be discerned; a few structures that may have been residual membrane material, were observed, but their numbers were similar in preparations in which Ca^{2+} and DNase I were either included in the lysis solution (to cause depolymerization) or not, despite the large decrease in the amount of label associated with the Triton-insoluble residue, caused by depolymerization (Table III).

In studies of the pattern of labeled proteins in the cytoskeletons, compared with intact platelets that had been labeled with ^{125}I by the lactoperoxidase method, the proteins were separated by SDS-PAGE. When the protein bands were stained with Coomassie Blue, the patterns that were obtained were typical of the cytoskeleton (2, 6, 12); they were enriched with actin, actin-binding protein, and a protein of molecular weight about 100,000 which other investigators have identified as alpha-actinin (4, 6, 41) (Fig. 10). Radioautographs of the gels were prepared to determine whether the labeling pattern of the cytoskeletal proteins resembled that of intact platelets (which would be indicative of trapping of membrane fragments) or whether the pattern was distinctive (indicative of selective enrichment with some membrane proteins). These radioautographs showed that although several radioactive bands that were present in intact platelets were also detectable in the cytoskeleton of stimulated platelets, there were labeled bands in the intact platelets that were not apparent in the cytoskeleton of stimulated platelets. Among these were bands in the positions of the glycoprotein IIb and IIIa standards and a band at a molecular weight of ~ 68,000. In addition, there were labeled bands in the cytoskeleton of aggregated platelets

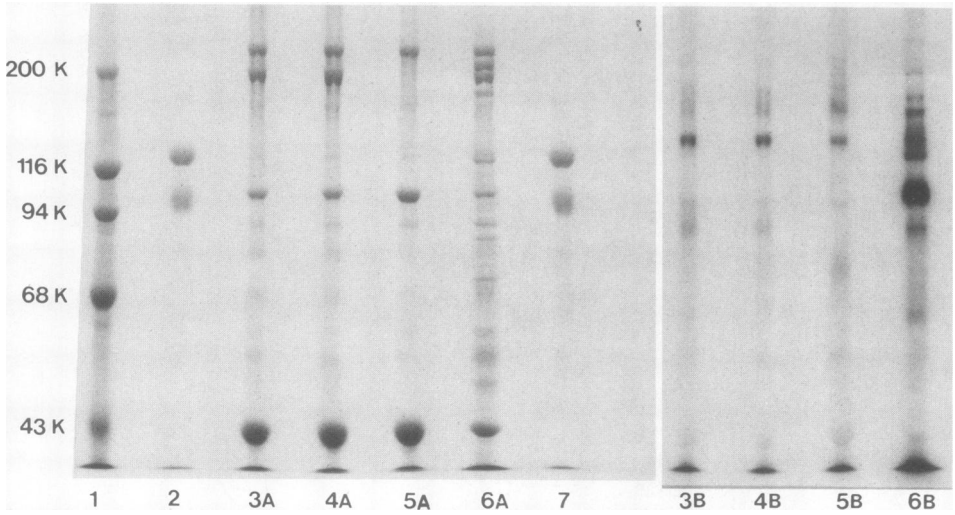

Figure 10. Distribution of proteins and of ^{125}I-labeled membrane glycoproteins in SDS gels prepared from cytoskeletons and intact platelets. Washed platelets from human donors were labeled with ^{125}I by the lactoperoxidase method, isolated, and resuspended as described in Methods. Platelets were aggregated with 5 μM ADP in the presence of 100 μg/ml fibrinogen, or with 0.2 U/ml thrombin, to the extent of 21 and 12% change in light transmission, respectively. Cytoskeletons were prepared using the standard lysing solution described in the footnote of Table III, and then solubilized in SDS and subjected to electrophoresis on SDS gels. Lanes *1–7:* Gel stained with Coomassie Blue. Lanes *3B* to *6B:* Autoradiogram of dried gel showing distribution of ^{125}I. The samples in the lanes are: lane *1*, molecular weight standards; lanes *2* and *7*, glycoprotein IIb and IIIa standard; lanes *3 A, B*, cytoskeleton after ADP-induced aggregation; lanes *4, A, B*, cytoskeleton after thrombin-induced aggregation; lanes *5 A, B*, cytoskeleton of unstimulated platelets; lanes *6 A, B*, unextracted, intact platelets (not treated with lysing solution—SDS treatment only). Typical of three experiments.

that were not present in the cytoskeleton of unstimulated platelets, most notably, a band at a molecular weight of 85,000 to 90,000 (Fig. 10). The pattern of the radioautographs was similar for platelets stimulated with ADP and platelets stimulated with thrombin (Fig. 10).

Discussion

In confirmation of the findings of Burn et al. (21), aggregation of platelets that had been prelabeled by incubation with [^{3}H]-palmitic acid led to the incorporation of the label into the platelet cytoskeleton. We further found that this association of the label with the cytoskeleton was dependent on aggregation and occurred with either thrombin or ADP and with both rabbit and human platelets. The association of the label with the cytoskeleton formed in conjunction with aggregation appears to be a specific event related to the interactions between platelets during aggregation.

The uptake of [^{3}H]palmitic acid by rabbit or human platelets was rapid, reaching completion within 15 to 30 min at room temperature. Thereafter the amount of label in the platelets remained essentially constant at 40–50% of the label in the medium. The apparent leveling off may represent the balancing effect of two opposing processes probably operating at a similar rate, so that further uptake of palmitic acid was compensated by its metabolism. Spector et al. (42) observed that 65% of labeled palmitic acid was in platelets after a 1-h incubation and Burn et al. (21) reported that 40% of the labeled palmitic acid added to a platelet suspension had been taken up by 2 h. In the present experiments, the [^{3}H]palmitic acid that was taken up was incorporated into phospholipids at a rate similar to that of its uptake. About 80% of the label in the platelets at the time when aggregation was studied was in phospholipids. Most other investigators have also observed that high percentages (55–79%) of labeled palmitic acid appear in platelet phospholipids (42–45), but Burn et al. (21) reported only 3% incorporated into phospholipids after 2 h. The reason for this marked discrepancy is not clear. In the present studies, short (15 min) and long (120 min) incubations with [^{3}H]-palmitic acid were compared and in both cases the pattern of an immediate and sizable incorporation of labeled palmitic acid into phospholipids was observed.

When platelets had been prelabeled by incubation with [^{3}H]palmitic acid, the label was not incorporated into the cytoskeleton during the initial shape change of the platelets. Thus polymerization of actin and elongation of the actin filaments that occur during shape change (12, 13) do not lead to binding of the label to the cytoskeleton. Aggregation in response to either thrombin or ADP caused the incorporation of label into the cytoskeleton, indicating that it is a consequence of aggregation, rather than being dependent on the induction of the release reaction since ADP causes very little release of the contents of the granules of rabbit or human platelets in the Ca^{2+}-containing medium in which the platelets were suspended (22). In addition, reactions involving the formation of thromboxane A_2 and inositol trisphosphate are not essential for the incorporation of label into the cytoskeleton since they do not occur during ADP-induced aggregation of rabbit platelets (24). The association of [^{3}H]palmitic acid with the cytoskeleton was dependent on the requirements for aggregation, namely, the presence of an inducer, stirring, Ca^{2+}, and, for ADP-induced aggregation of human platelets, fibrinogen. However, added fibrinogen does not appear to be essential for the association of the label with the cytoskeleton since rabbit platelets, which aggregate in response to ADP without added fibrinogen (25), incorporated the label into their cytoskeleton during ADP-induced aggregation. Despite this observation, it should be pointed out that the presence of fibrinogen markedly increased the amount of label that associated with the cytoskeleton of rabbit platelets aggregated with ADP. Human platelets are more dependent on added fibrinogen for extensive ADP-induced aggregation (46), and fibrinogen greatly increased the amount of label that became associated with the cytoskeleton of human platelets during ADP-induced aggregation.

Inhibitors of aggregation that raise the concentration of cyclic AMP in platelets and cause the sequestration of cytosolic Ca^{2+}, inhibited the association of label with the cytoskeleton. In contrast, the addition of aspirin, which blocks the for-

mation of thromboxane A_2 by platelets was without effect, although this is not surprising since neither ADP-induced aggregation in a medium containing 1–2 mM Ca^{2+}, nor thrombin-induced aggregation is dependent on the formation of thromboxane A_2 (22). When aggregation was blocked by chelating external Ca^{2+} with EDTA, association of label with the cytoskeleton did not occur.

As aggregation in response to either ADP or thrombin took place, the amount of label that was associated with the cytoskeleton increased exponentially. Deaggregation was accompanied by a decline in the amount of label associated with the cytoskeleton; this decline occurred more rapidly than deaggregation (as recorded by changes in light transmission) and at an exponential rate, indicating that it preceded the separation of the platelets from each other. This was observed following ADP-induced aggregation of both human and rabbit platelets, both of which deaggregate readily when apyrase is present in the suspending medium used in these experiments. It was also possible to study the effect on the amount of label associated with the cytoskeleton of rabbit platelets that had been aggregated with thrombin and then deaggregated since rabbit platelets, unlike human platelets, can be easily deaggregated if thrombin is inhibited with hirudin and PGE_1 is added (26). These experiments were done in the presence of GPRP to prevent polymerization of any fibrin that might have been formed from released fibrinogen (26). During deaggregation of rabbit platelets after thrombin-induced aggregation, the label dissociated from the cytoskeleton at an exponential rate and dissociation began before the decrease in light transmission shown by the aggregation tracing. A possible explanation for these exponential rates of association and dissociation of the label during aggregation and deaggregation may be that the label represents the number of sites of contact between neighboring platelets that would increase exponentially as platelet aggregates increase in size and decrease exponentially as the platelets deaggregate. In keeping with this concept is the finding that the amount of label associated with the cytoskeleton was increased over 40-fold by an increase of only 4.6-fold in the number of platelets.

Conditions which resulted in proteolysis of cytoskeleton proteins or in depolymerization of actin, led to a reduction of the amount of label from [^{3}H]palmitic acid in the cytoskeleton. When EGTA was not included in the lysis medium very little label was found in the cytoskeleton, probably because the Ca^{2+} that was present supported the ability of the Ca^{2+}-dependent protease to hydrolyze proteins such as actin-binding protein that are associated with the cytoskeleton (9, 10, 15, 38) and promoted the Ca^{2+}-dependent depolymerization of actin filaments by gelsolin (1, 16). Leupeptin and benzamidine also increased the amount of label that was isolated with the cytoskeleton.

In the present studies, we addressed the problem raised by Zucker and Masiello (20) that cytoskeletons isolated from aggregated platelets may contain membranes, due to inadequate lysis by Triton X-100. These authors studied large platelet aggregates, prepared by shaking suspensions of washed platelets with 1 U/ml thrombin for 3 min and based their conclusion on analysis of the Triton-insoluble fraction with respect to ^{125}I-labeled glycoproteins, phospholipid content, and electron microscopy. Our results are in agreement with those of Zucker and Masiello (20) for the conditions that they employed which led to the formation of large aggregates. We too observed membrane fragments when platelets were aggregated with 1 U/ml of thrombin over a 3-min period. In contrast, very few fragments were seen in the cytoskeleton prepared from platelets in the present experiments in which much lower concentrations of thrombin were used and the time was shorter (15–20 s) so that the extent of change of light transmission was limited to $\sim$ 15%. Several other lines of evidence, however, also indicate that the label associated with the cytoskeleton in the present study cannot be explained by inadequate membrane lysis: (*a*) Although the labeling of cytoskeleton was associated with aggregation, there were qualitative and quantitative differences between the patterns of aggregation and cytoskeleton labeling. Thus, the decline in cytoskeleton labeling clearly preceded deaggregation, so that labeling became minute while aggregation was still prominent. (*b*) During the early stages of aggregation (up to 30 s) before large aggregates formed, nearly all of the label in the Triton-insoluble residue was sensitive to the depolymerizing action of DNase I and Ca^{2+}, indicating that the label was probably associated, either directly or indirectly, with actin. However, when aggregation was more advanced, the proportion of the label in the Triton-insoluble residue that was resistant to DNase I and Ca^{2+} increased and became predominant; this label was probably largely present in fragments of membrane that were not specifically associated with the cytoskeleton, but were isolated with it because of incomplete lysis of the large aggregates. Since the experiments in the present studies were done during the early stages of aggregation, it seems likely that the label from [^{3}H]-palmitic acid was specifically associated with the cytoskeleton of aggregated platelets. (*c*) The pattern obtained upon radioautography of the cytoskeletons of platelets that had been labeled with ^{125}I by the lactoperoxidase method showed a selective enrichment of labeled proteins, compared with the pattern from intact platelets. If there had been large amounts of membrane fragments present in the isolated cytoskeletons, the patterns would have been similar. Although these observations do not eliminate the possibility of some contamination of the isolated cytoskeleton with membrane fragments, they do establish that the major part of the association of the label from [^{3}H]palmitic acid with the cytoskeleton is not due to contamination with membrane fragments.

The enrichment of the cytoskeleton of aggregated platelets with some of the labeled membrane proteins, compared with cytoskeleton of unstimulated platelets, indicates that membrane proteins became selectively associated with the cytoskeleton during aggregation, in agreement with the observations of other investigators (17, 18). It should be pointed out, however, that the enrichments we observed differ from those seen by Phillips et al. (17), probably because of major differences in the extent of aggregation, since Phillips et al. used 10 times the concentration of thrombin and left the platelets with the thrombin for 30 min instead of the 20–25 s used in the present experiments. Under the conditions of our experiments one would not expect association of glycoproteins IIb/IIIa with the cytoskeleton (5).

Since the patterns we observed were the same, regardless of whether the platelets had been aggregated with thrombin or ADP, it is evident that the association of labeled membrane glycoproteins with the cytoskeleton is dependent on aggregation and does not require the events that take place during the release reaction. This finding necessitates a reassessment of the reports that only strong platelet agonists, and not ADP, cause

the association of membrane glycoproteins with the cytoskeleton (5).

From our results we cannot deduce whether the labeled lipid is associated with membrane proteins in unstimulated platelets and becomes associated with the cytoskeleton because a complex forms between membrane proteins and the cytoskeleton, or whether the lipid is not associated with membrane protein and associates directly with the cytoskeleton during aggregation. Associations of lipids with membrane proteins have been reported in other types of cells (47). The incorporation of lipid into the cytoskeleton of stimulated platelets may be another example of the regulatory role of lipid in associations between membrane proteins and skeletal components (48).

Acknowledgments

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