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Quantitative Assessment of Polymerized and Depolymerized Platelet Microtubules: *CHANGES CAUSED BY AGGREGATING AGENTS*

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Quantitative Assessment of Polymerized and Depolymerized Platelet Microtubules

CHANGES CAUSED BY AGGREGATING AGENTS

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ABSTRACT The equilibrium between assembled and disassembled microtubules was studied in human platelets exposed to aggregating agents. Soluble and insoluble tubulin were "frozen" by addition of a glycerol-dimethyl sulfoxide-containing medium. The two pools were estimated by measuring the colchicine binding activities of total and polymerized tubulin. Resting platelets were found to contain an average of 56.2 μg tubulin/1 $\times$ 10^9 cells of which 56.7% was in polymerized form. Platelet aggregation induced by thrombin, ADP, epinephrine, or collagen produced a transient decrease in the pool of polymerized tubulin which was evident within 15 s after addition of the aggregating agent. A return to base-line values occurred within 1–4 min depending upon the specific aggregating agent used. Neither secretory release nor aggregation of platelets were found to be prerequisites for the temporary disturbance of the equilibrium between soluble and polymerized tubulin. With thrombin as the aggregating agent a clear threshold concentration could be demonstrated above with a dose-dependent dissociation response of microtubules was evident. We conclude that microtubules exist in a dynamic equilibrium between polymerized and depolymerized forms in human platelets, which is transiently disturbed by their interaction with aggregating agents.

INTRODUCTION

Microtubules in platelets consist of a bundle of tubular structures that are located under the plasma membrane surrounding the platelet body in its equatorial plane. Their major, if not only, function is to maintain the discoid shape of platelets in which they seem to be assisted by structural microfilaments and submembrane filaments (1, 2). The most striking morphological difference between resting, nonstimulated, and activated, adhering or aggregating platelets is a change in shape from a flattened disk form to spherical configuration with multiple sharp and bulbous pseudopods. Electronmicroscopy has detailed the structural changes attending these morphological alterations (3–6). Pseudopods are associated with prominent microfilaments and often also with microtubules parallel to their long axis. At a later stage of aggregation, when platelets have undergone a secretory release, microtubules can be observed surrounding the centralized granules. Changes in the equilibrium between polymerized and depolymerized forms of structural proteins, which seem to accompany the platelet response to stimuli promoting aggregation or adhesion, are difficult to quantify by electronmicroscopy.

We have investigated the effect of aggregating agents on the equilibium between assembled and disassembled microtubule protein in human platelets. The interaction of aggregating agents with platelets leads to a rapid but transient disassembly of microtubules independent of aggregation but dependent upon the concentration of aggregating agent used.

METHODS

All studies were conducted with human platelets that were isolated from fresh blood anticoagulated with 1/10 vol of 3.8% sodium citrate. Platelet-rich plasma obtained by centrifugation at 147 *g* for 12 min at 22°C was used without adjustment of platelet number for aggregation studies. The platelet counts varied between 3.8 and 4.9 $\times$ 10^8 platelets/ml. Aggregation was induced by ADP, collagen, bovine thrombin, or epinephrine. These aggregating agents were added to platelet-rich plasma that was continuously agitated by a small magnetic stirring bar at 1,000 rpm in a temperature-controlled cell holder maintained at 37°C. Reactions were stopped by

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addition of 3 vol of microtubule-stabilizing medium (MSM)[1] that consisted of 66.7% glycerol, 13.3% dimethyl sulfoxide, and the remainder of 10 mM phosphate buffer, pH 6.8 with 5 mM $MgCl_2$, and 1 mM guanosine triphosphate (modified from Filner and Behnke [7]). The platelets were rapidly mixed with MSM and were left at 37°C for exactly 5 min before they were sonicated. In other experiments platelets were subjected to sonication as soon as MSM had been admixed. The time delay between addition of MSM and start of sonication in these experiments varied between 5 and 8 s. The time intervals at which incubations were stopped were the same as those used for the other method of termination described above.

Polymerized and total tubulin content of platelets were measured by the method of Rubin and Weiss (8). Minor modifications adapted their technique to platelets. To measure the total pool of tubulin, $7-8 \times 10^8$ platelets were washed with 50 mM phosphate buffer, pH 7.2 that contained 5 mM $MgCl_2$, and 100 mM NaCl (phosphate-buffered saline [PBS]) and were then suspended in 10 mM phosphate buffer, pH 6.8 that contained 5 mM $MgCl_2$, 200 mM NaCl, and 1 mM guanosine triphosphate (colchicine-binding buffer [CBB]). This platelet suspension was exposed to 20 kHz ultra sound produced by an ultrasonic system (Biosonik IV) of Bronwill Scientific, Rochester, N. Y. The 2.8-cm^2 probe was placed just beneath the surface meniscus of the cell suspension. The apparatus was operated at the 70% intensity setting with the "Hi-Lo" input switch in "Lo" position for a continuous interval of 30 s at room temperature. These operating conditions were selected after preliminary experiments established a plateau level of tubulin at this energy output and time interval. Varying the time of sonication from 10 s to 40 s in 10-s intervals yielded 17.3, 32.5, 55.8, and 53.6 μg total tubulin/1×10^9 platelets (measured by colchicine binding activity), respectively (means of three experiments). A slight disruptive effect on polymerized tubulin was noticeable at the conditions of sonication utilized. The fraction of platelet tubulin recovered in polymerized form at the above power output setting of the ultrasonic generator was 64.3% at 10 s, 60.1% at 20 s, 57.0% at 30 s, and 45.8% at 40 s sonication (means of three experiments). Complete dissociation of microtubules was obtained by leaving the sonicate for 30 min at 4°C. After removing particulate matter by centrifugation at 60,000 g for 30 min at 4°C, the supernate was used for determination of total platelet tubulin, i.e., that derived from the pool of polymerized and depolymerized microtubule protein.

Polymerized tubulin was measured in control and aggregated platelet suspensions to which MSM had been added to freeze the respective pools of soluble and polymerized tubulin. The platelets were disrupted by sonication and microtubules separated by centrifugation at 95,000 g for 60 min at 22°C. The sedimented microtubules were suspended in CBB and were disassembled by exposure to 4°C for 30 min. After recentrifugation, the dissociated polymerized tubulin fraction contained in the supernate was determined by measuring its colchicine binding activity. The latter was assayed by the method of Wilson (9) with DEAE 81 filter disks used to trap the colchicine-binding protein. Samples were incubated with 10 μM [^3H]colchicine (270 mCi/mmol) for 90 min at 37°C. To determine nonspecific binding, duplicate blanks that contained labeled colchicine but no protein were carried out with each experiment and processed as the experimental samples. The radioactivity of the washed disks (9) was measured in a liquid scintillation spectrometer (10). All counts were corrected for nonspecific binding. As colchicine-binding protein in CBB undergoes an aging process whereby its ability to bind colchicine decays according to first-order kinetics (9) all results were extrapolated along the decay line to the time of addition of CBB.

The basic technique to fix and hold polymerized and depolymerized tubulin unchanged by addition of MSM has been sufficiently well documented in the literature (8, 11–13). Therefore, we omitted detailed confirmatory proof of the accuracy of the individual steps of the method in this paper. Each phase of the isolation procedure for total and polymerized platelet tubulin has been checked, however, in our laboratory before this study was begun. Specifically, we have tested the stabilizing effect of MSM on microtubules in our system, we have determined the completeness of dissociation of the pelleted microtubules resuspended in cold CBB, and we have studied the recovery of preformed microtubules and soluble tubulin added during the sonication step. The preservation of microtubules in MSM at temperatures ranging from 4°C to 37°C and for time intervals up to 90 min was shown by turbidimetric measurements (14) of in vitro polymerized platelet tubulin, by electronmicroscope studies and by determination of the colchicine binding activity of total and soluble (nonprecipitable) tubulin fraction of in vitro polymerized platelet microtubules. Depolymerization under these experimental conditions did not exceed 10% at 4°C and was <7% at 22°C and 37°C (means of three experiments). Complete depolymerization of microtubules sedimented from MSM was demonstrated by the absence of any colchicine binding activity from the second pellet obtained after recentrifugation of the microtubules, which were resuspended in ice-cold CBB and allowed to stand for 30 min at 4°C. Addition of measured amounts of platelet colchicine-binding protein in polymerized and depolymerized form to intact platelets just before sonication yielded a recovery which ranged from 97 to 118% of the calculated amount of total tubulin and from 86 to 104% of the polymerized form of microtubule protein (range of values obtained in three experiments).

Although the experimental design precluded significant amounts of aggregating agents to remain in the solutions that were finally used for the colchicine binding assay, experiments were carried out to determine whether the exposure of MSM-treated microtubule protein to aggregating agents after sonic disruption of platelets could affect its distribution between polymerized and depolymerized forms and alter its colchicine binding activity. Platelet tubulin was extracted and purified by three cycles of temperature-dependent polymerization-depolymerization (10). To measured amounts of polymerized and depolymerized tubulin was added 3 vol of MSM or CBB, respectively, that contained 0.8 mg bovine serum albumin and either ADP, epinephrine, collagen, or thrombin in ¼ the concentration in which these agents were used to induce platelet aggregation. Polymerization or depolymerization were accomplished by adjusting the temperature to 37°C or 4°C, respectively. After sonication, polymerized and soluble tubulin were prepared for colchicine binding assay, which was performed in the usual manner. When colchicine binding activity was expressed as micrograms of protein, the radioactive counts recovered on the DEAE disks and corrected for nonspecific adsorption were converted to tubulin equivalents by assuming a 1:1 molar ratio of colchicine bound to microtubule protein and a molecular weight for tubulin of 110,000 (15). Protein concentrations were measured according to Lowry et al. (16) with bovine serum albumin as standard.

To measure in vitro polymerization of tubulin we extracted

[1] *Abbreviations used in this paper*: CBB, colchicine-binding buffer ($MgCl_2$, NaCl, and guanosine triphosphate); MSM, microtubule-stabilizing medium; PBS, phosphate-buffered saline ($MgCl_2$ and NaCl).

and purified platelet microtubule protein by three cycles of temperature-dependent polymerization-depolymerization (10) Platelet tubulin was suspended in 0.1 M piperazine-*N,N'*-bis(2-ethane sulfonic acid) buffer, pH 6.9 that contained 4 mM EGTA, 2 mM $MgSO_4$, and 1 mM guanosine triphosphate at a concentration of 1–1.5 mg protein/ml. Polymerization was measured by a turbidimetric method (14).

Bovine topical thrombin (Parke, Davis & Co., Detroit, Mich.) was purified according to the method of Lundblad (17). The specific clotting activity of the preparation was 2,100 National Institutes of Health (NIH) U/mg protein. Fibrinogen clotting activity was determined as described by Lundblad (17) and protein concentrations were measured by the method of Lowry et al. (16) with bovine serum albumin as standard. Thrombin was stored in small samples at −70°C.

RESULTS

The total tubulin content of platelets averaged 56.2 ±2.7 $\mu g/1 \times 10^9$ platelets (mean of 12 experiments ±SEM) of which 56.7±2.8% (31.0±1.6 $\mu g/1 \times 10^9$ platelets) was in polymerized form.

Aggregation of platelets by 0.5 U thrombin/ml produced a significant reduction in polymerized tubulin within 15 s after addition of the aggregating agent. The temporal course of change in this pool of tubulin over a period of 5 min is shown in Fig. 1. There was no change in the total content of platelet tubulin during this time interval, thus indicating that the pool of depolymerized tubulin which was not directly measured, increased transiently during the course of platelet aggregation (Table I). Heat-denatured thrombin used in the same concentration did not produce these changes. Aggregation induced by ADP (Fig. 2) and by collagen and epinephrine (Fig. 3) also had a dissociating effect on platelet microtubules similar to that of thrombin. Addition of these agents to extracted platelet tubulin in the final concentrations in which they were present in MSM was without effect on the colchicine binding activity of polymerized or depolymerized microtubule protein (Table II). A technical problem of some concern was the slow penetration of platelets by glycerol. Together with dimethyl sulfoxide this substance forms

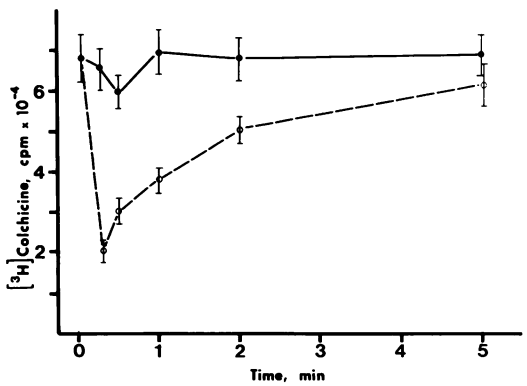

FIGURE 1 Changes in polymerized tubulin during aggregation of platelets induced by thrombin. Platelets isolated from citrated blood were washed once with PBS and were then resuspended in this buffer at a concentration of 5.0×10^8 cells/ml. Platelet suspensions, 1 ml in volume, were incubated at 37°C for 5 min. Agitation with a magnetic stirring bar rotating at 1,000 rpm was then begun and 0.5 U purified bovine thrombin that contained Ca^{2+} (0.125 mM final concentration) was added. Suspensions were incubated for time intervals ranging from 15 s to 5 min. Reactions were stopped by rapid admixture of 3 ml of MSM after which incubation was continued without agitation for another 5 min at 37°C. The suspensions were then sonicated, the polymerized tubulin fraction isolated, and its colchicine binding activity determined as described in the text. All binding assays were performed in triplicate. Open circles indicate means of five experiments in which aggregation was induced by 0.5 U thrombin. Full circles indicate the means of four experiments in which heat-denatured (10 min at 56°C) thrombin, 0.5 U/ml, was added to platelet suspensions. The vertical lines enclosed by horizontal bars denote the extent of 2 SD. The total pool of platelet tubulin did not change during aggregation by thrombin (Table I).

TABLE I

Assay of Total Tubulin in Platelets Exposed to Various Aggregating Agents

Aggregating agent	Concentration	Total tubulin				
		Time after addition of aggregating agents, *s*				
		0*	15*	30‡	60*	120‡
		μg/5 × 10⁸ platelets				
None	—	28.1±1.4	27.9±1.5	27.8	28.1±1.5	28.7
ADP	2 μM	27.6±1.5	27.3±1.6	28.3	27.6±1.6	27.4
Epinephrine	5 μM	28.5±1.6	28.0±1.5	27.8	28.2±1.6	27.3
Collagen	75 μg/ml	27.8±1.6	27.1±1.6	28.0	27.7±1.5	27.1
Thrombin	0.5 U/ml	27.2±1.6	27.6±1.6	28.2	27.3±1.6	27.6

Total tubulin was assayed as described under Methods. The values listed were obtained on the same platelets whose changes in polymerized tubulin are shown in Figs. 1–3 and in Table II.

* Means of seven experiments±1 SD are shown.

‡ Means of three experiments each are listed.

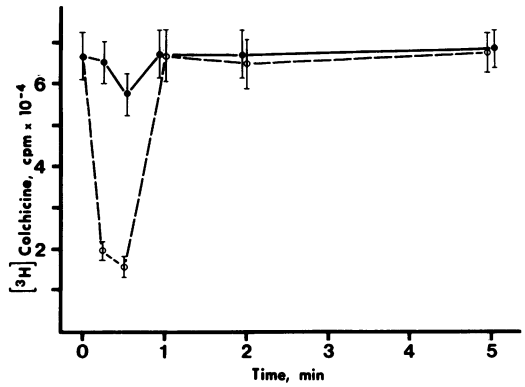

FIGURE 2 Changes in polymerized tubulin during ADP-induced aggregation of platelets. Platelet suspensions were prepared as described in the text. Aggregation was induced by addition of ADP in a final concentration of 2 μM (open circles). Other platelet suspensions were preincubated for 10 min at 37°C with 40 μM 2-chloroadenosine (final concentration) after which ADP, 2 μM was added (full circles). The reactions were terminated and the colchicine binding activity of polymerized and total tubulin measured as described in legend to Fig. 1 and in the text. The means of four experiments each are shown with the extent of 2 SD indicated. All colchicine binding data shown in this figure have been adjusted to 5.0×10^8 platelets. The total pool of platelet tubulin did not change during aggregation by ADP (Table I).

the principal part of the microtubule stabilizing medium which freezes the equilibrium between polymerized and depolymerized tubulin. In an effort to eliminate the uncertainty of the exact time sequence of

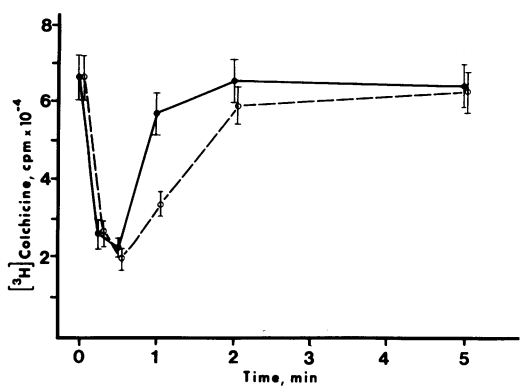

FIGURE 3 Changes in polymerized tubulin during platelet aggregation induced by collagen (open circles) and epinephrine (full circles). Preparation of platelet suspensions was described in the text. Platelets were aggregated by addition of 75 μg soluble skin collagen or 5 μM epinephrine (final concentration). Termination of platelet aggregation and quantification of colchicine binding activity of polymerized and total platelet tubulin were performed as described in legend to Fig. 1 and in the text. The means of four experiments each are presented. The extent of 2 SD is indicated. All colchicine binding data shown in this figure have been adjusted to 5.0×10^8 platelets. The total pool of platelet tubulin did not change during aggregation by collagen or epinephrine (Table I).

TABLE II
Effect of Aggregating Agents on Tubulin Assay

Addition	Concentration	Colchicine-binding protein	
		Polymerized	Soluble
		μg	
None	—	28.9	31.2
ADP	0.5 μM	28.5	30.5
Epinephrine	1.25 μM	29.1	31.0
Collagen	19 μg	28.9	29.7
Thrombin	0.125 U/ml	28.1	28.9

30 μg of polymerized or depolymerized platelet tubulin was exposed to the additions listed in the table and their colchicine binding activity determined. Means of three experiments each are listed.

microtubule dissociation that follows exposure of platelets to aggregating agents, experiments were performed in which addition of MSM was immediately followed by sonication of platelet suspensions. The results of these experiments are summarized in Table III. No significant shift in the temporal profile of microtubule depolymerization occurred as a consequence of the immediate sonication of MSM-treated platelets. Aggregation profiles for all four aggregating agents tested conformed to those normally seen with platelets from healthy donors who had been off any medication adversely affecting platelet aggregation for a minimum of 10 d.

Neither secretory release nor aggregtion of platelets were prerequisites for the transient disassembly of microtubules. The former was shown in experiments in which platelet-rich plasma was pretreated with 1 mM acetylsalicylic acid (10 min at 37°C) and was then exposed to 5 μM ADP. Whereas the secretory release,

TABLE III
Effect of Immediate Sonication on Changes in Polymerized Platelet Tubulin after Addition of Aggregating Agents to Intact Platelets

Aggregating agent	Concentration	Polymerized tubulin			
		Time after addition of aggregating agent, s			
		0	15	30	60
		μg/5 × 10⁸ platelets			
None	—	15.4	15.5	15.6	15.3
ADP	2 μM	15.6	4.7	4.4	14.4
Epinephrine	5 μM	15.8	6.2	4.8	13.0
Collagen	75 μg/ml	15.5	6.4	4.7	7.6
Thrombin	0.5 U/ml	15.8	4.8	6.9	8.7

* Means of three experiments each are listed.

normally induced by ADP, was completely blocked by the addition of acetylsalicylic acid (18), the disassembly of microtubules proceeded in a manner similar to that seen in the absence of the release inhibitor, both with respect to magnitude and time of occurrence of dissociation. Further studies showed that aggregation itself was not required for the disassembly of platelet microtubules. Collagen added to platelet-rich plasma in a concentration of 15 μg/ml, although causing shape change of platelets, failed to produce platelet aggregation. Disassembly of microtubules, however, did take place similar to that shown in Fig. 3. We suggest that the interaction of the aggregating agent with the platelet plasma membrane provides the stimulus for the depolymerization of a considerable portion of the platelet microtubules. Support for this contention was obtained from the results of an experiment in which platelets were preincubated with 2-chloroadenosine, followed by addition of ADP. Only a very minor decrease of the pool of polymerized tubulin was recorded. One hypothesis holds that adenosine or 2-chloroadenosine blocks ADP sites on the plasma membrane of platelets (19, 20). The aggregating agent-induced dissociation of tubulin appears dose dependent as shown in the experiment in which platelets were exposed to increasing concentrations of thrombin (Table IV). A minimal thrombin concentration of >0.05 but <0.1 U/ml was necessary to induce measurable depolymerization of platelet microtubules. The disassembly reached a maximum at about 1.0 U thrombin/ml.

TABLE IV
Effect of Thrombin Concentration on Polymerized Platelet Tubulin

Thrombin	Polymerized tubulin [^{3}H]Colchicine bound	
U/ml	*cpm/5 × 10^8 platelets*	
0.0	65,475	68,450
0.01	62,783	63,278
0.05	59,211	57,254
0.1	52,407	50,122
0.2	31,455	33,480
0.5	21,033	22,391
1.0	17,612	19,225
2.0	18,184	18,926

The results of two individual experiments are shown. Conditions of incubation were identical to those described in legend to Fig. 1 except for the variation in the concentration of thrombin added. All incubations were terminated after 15 s by the rapid admixture of MSM. Separation of depolymerized tubulin and colchicine binding assay were performed as described in the text. The total amount of tubulin in thrombin-treated platelets did not differ from that of control platelets.

DISCUSSION

Approximately 3% of platelet protein was shown to be microtubule protein. This value is considerably higher than that reported recently by Pipeleers et al. (21). Differences in the method of disrupting platelets undoubtedly contributed most to the discrepant results. Completeness of ultrasonic disruption of platelets in our experiments is suggested by the leveling off of the recovery of colchicine binding protein after different intervals of sonication. As our experiments have shown, the increased recovery of tubulin is associated with a slight loss in the fraction of polymerized tubulin. At a maximal recovery of 64.3% of platelet tubulin as microtubules with a 10-s sonication interval our results are still somewhat lower than those reported by Pipeleers et al. (21) who found 89% of platelet tubulin in polymerized form. However, these results show general agreement that platelets have a far higher proportion of their microtubule protein in a polymerized state than other cells studied to this date (8, 11, 12, 21).

These studies also demonstrate that platelet tubulin similar to the microtubule protein of other cells exists in a dynamic equilibrium between polymerized and depolymerized forms. This equilibrium was drastically shifted toward the depolymerized form of tubulin by the interaction of aggregating agents with platelets. Our results suggest that this phenomenon may be related to the interaction of aggregating agents with their specific membrane sites. This is supported by the observations that neither platelet release nor aggregation were necessary for the transient disassembly of platelet microtubules, that denatured thrombin was ineffective and that a probable competitor for the ADP receptor site prevented the ADP-induced depolymerization of tubulin.

The time-course of disassembly and subsequent reassembly of platelet microtubules induced by aggregating agents was very rapid. In vitro experiments to clarify the kinetics of this process could not be done as the aggregating agents were without effect on tubulin in a cell-free system. However, experiments by Houston et al. (14) have shown that depolymerization induced by exposure of preformed microtubules to 5°C exceeded 50% within 20 s. Equally fast was the formation of microtubules from soluble tubulin on exposure to 37°C although a short lag period preceeds the polymerization (22). Temperature and concentration dependence of this process have been clearly shown (23). These observations indicate that the time interval for depolymerization-polymerization of platelet tubulin observed in our experiments is plausible.

A technical problem of this study concerned the time scale of the aggregation study and the sampling sequence for measuring polymerized tubulin. These two are not congruent as the MSM, which is added

to prevent any further change of soluble and precipitable pools of tubulin, does not penetrate platelets instantaneously. Glycerol especially has a delayed transport compared to dimethyl sulfoxide (24) and phosphate. Nevertheless, experiments in which we attempted to determine this effect by sonicating platelet suspensions immediately upon addition of MSM have failed to show a significant change from the results we obtained when platelet suspensions were allowed to stand for 5 min after admixture of MSM before sonication. In part, at least, this may be caused by the technical difficulty to reduce the time lag between admixture of MSM and start of sonication to <5–8 s. Also the disruption of platelets is far from complete even 10 s after exposure to ultrasound has begun. Thus the sampling at 15 s, at which time most aggregating agents already induced maximal or near maximal disassembly of microtubules, probably is representative of the situation existing 20–30 s after addition of aggregating agents.

These results represent a quantitative assessment of soluble and insoluble microtubule protein in resting and aggregating platelets. Combined with electron-microscopic observations our data suggest that major changes in the intracellular position of microtubules as occur during platelet shape change and aggregation are predicated on a temporary dissociation of these structures.

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