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TIME RESOLVED ANALYSIS OF TUBULIN PHOSPHORYLATION DURING PLATELET ACTIVATION

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Summary: Tubulin phosphorylation was analyzed during the different phases of platelet activation. Platelets preloaded with [^{32}P]-phosphate were stimulated with collagen. Tubulin was immunoprecipitated from serial samples obtained during the activation process. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographs analyzed by laser densitometry. Agonist induced dephosphorylation of platelets occurred after the onset of shape change at the time of initiation of the secretory release. The dephosphorylation was selective affecting specific peptides. © 1989 Academic Press, Inc.

Microtubules, which are generally considered to maintain the discoid shape of platelets, are phosphorylated in multiple positions on the alpha and beta subunits of tubulin (1). Thrombin induced stimulation of platelets was found to produce dephosphorylation of tubulin (2). Cyclic AMP, a potent inhibitor of platelet activation, has been previously shown by us to bind to tubulin and to promote polymerization of microtubule protein (3). Cyclic AMP-dependent protein kinases are present in many cells including human platelets (4-8) and are able to phosphorylate platelet tubulin (9).

We are now reporting on an investigation that relates the time sequence of the platelet activation process to the agonist induced reduction of tubulin phosphorylation. We have used collagen, a platelet activator which may show a considerable lag time between the induction of shape change and the onset of the release reaction provided its concentration is judiciously chosen. The different phases of platelet activation were evaluated in relation to changes in tubulin phosphorylation. Our results show that dephosphorylation of tubulin occurs after the shape change has taken place at or before the start of the secretory phase.

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METHODS AND MATERIALS

Preparation of Platelets

Blood was obtained from normal volunteers who had abstained for a minimum of 10 days from any medication interfering with platelet function. Blood was collected into 1/10 vol of 3.8% sodium citrate. Platelets were isolated as described before (10) and after two washing steps, they were depleted of phosphate (11). Platelets were resuspended in 15 mM Tris-HCl buffer containing 0.14 M NaCl and 5.5 mM glucose, pH 7.4 (TBS) at a concentration of 3-4 x 10^9 platelets/ml and [^{32}P]-phosphoric acid (5-6 mCi/l-2 x 10^9 platelets) adjusted to pH 7.5 was added. The platelet suspensions were incubated for 2 hours at room temperature with occasional mixing. After adjusting the pH to 6.5 with acid-citrate-dextrose (ACD, U.S.P.), the platelets were pelleted and the supernatant removed. The platelets were then resuspended in TBS and their concentration adjusted to 1 x 10^9/ml.

Platelet Activation

Platelet suspensions (0.2 ml) were diluted with platelet poor plasma (PPP, 0.080 ml) obtained from the same donor as the platelets. The platelets were stimulated with bovine tendon collagen, usually 12.5 µg/ml. Before using [^{32}P]-phosphate loaded platelets, identically treated non-radioactive platelet suspensions were tested for their aggregability. The dosage of the stimulant was adjusted so that there was a 1.0-1.5 min lag time between shape change and the onset of aggregation. In spite of the acid solubilized collagen solution and the high concentration of agonist, the final pH of the platelet suspension was not lowered by the addition of the collagen solution. All platelet suspensions were temperature equilibrated to 37°C prior to the addition of the agonist. Only platelets capable of a 55-60% deviation from the baseline 4 min after the addition of collagen, using the customary turbidimetric assay (12), were accepted for these studies. Shape change was verified by phase microscopy as well as by the method of Patscheke et al. (13). The release reaction was documented in a lumi-aggregometer measuring ATP by the luciferin-luciferase method (14).

To block platelet secretory release, potential platelet donors were asked to ingest 0.5-1 g acetylsalicylic acid on the night before blood collection. Platelet suspensions obtained from these donors were further incubated with 20 mM indomethacin and 100 µM UK 38485 (a potent thromboxane synthetase inhibitor; Pfizer). Complete inhibition of platelet release reaction was confirmed by measuring ATP release.

Immunoprecipitation of Platelet Tubulin

Reactions were terminated by addition of 1/10 vol of solubilizing buffer (100 mM Tris, 50 mM EDTA and 20% Triton X-100, pH 7.4) containing protease and phosphatase inhibitors (10 mM EGTA, 10 mM phenylmethylsulfonylfluoride, 10 mg% aprotinin, 100 mM NaF and 0.5 mM sodium orthovanadate). The samples were placed on ice for 10 min with occasional vigorous mixing. Solubilized preparations were then centrifuged at 15,000 x g for 30 min at 4°C. More than 85% of the total platelet tubulin was recovered in the supernatant (15). Tubulin was estimated as previously described. The supernatants were removed and were absorbed with bovine serum albumin (BSA) conjugated to Sephadex beads (50% suspension) (16). Each platelet lysate was absorbed with 0.3 ml of this suspension for 1 hour at room temperature with continuous mixing. After that, the beads were sedimented by centrifugation and to the supernatant was added monoclonal antibody to alpha or beta tubulin at a final dilution of 1:100. The immune reaction was allowed to proceed overnight at 4°C. Antigen antibody complexes were then precipitated with anti-mouse IgG (100 µg/reaction tube). After a 2 hour incubation the precipitates were removed by centrifugation at 12,000 x g for 15 min. The precipitate was washed twice with washing buffer and was then solubilized by addition of 1/10 vol of solubilizing buffer (0.625 M Tris, 10 mM EDTA and 10% SDS).

Electrophoresis and Autoradiography

Reduced and alkylated (1) immunoprecipitates were analyzed by SDS polyacrylamide gradient gel electrophoresis and occasionally by 2 dimensional eltrophoresis (17). The gels were electrophoretically transblotted onto polyvinylidene difluoride membranes (PVDF; Immobilon) at a constant voltage of 70 V. After 8-12 hours the PVDF membranes were dried and autoradiographs prepared using X-Omat AR films which were exposed at -80°C. The films were scanned by a laser densitometer (LKB Instruments). Western blots were stained with Coomassie blue. At times blots were reacted with monoclonal antibodies to alpha and beta tubulin to verify the identity of the radioactive bands. Each gel also contained a set of molecular weight standards comprising myosin (M_r 200,000), beta-galactosidase (M_r 116,250), phosphorylase B (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,400).

Peptide Analysis

[^{32}P]-phosphorylated tubulin monomers, electroblotted to the PVDF membranes and stained with Coomassie blue, were excised and reacted in situ with CNBr as described by Scott et al (18). After elution of the CNBr fragments from the PVDF membranes using a buffer containing 1% Triton X-100, 2% SDS and 50 mM Tris, pH 9.3 for 90 min at room temperature, the peptides were analyzed on 16% polyacrylamide gels (18). Electroblots and autoradiographs were prepared as described above.

Materials

[^{32}P]-orthophosphoric acid (spec. act. 185 TBq/mmol) was obtained from New England Nuclear, Boston, MA. Polyvinylidene difluoride transfer membrane sheets (Immobilon) were a product of Millipore Corp., Bedford, MA. Monoclonal antibodies to alpha (DM1A) and beta (DM1B) tubulin of chick brain (mouse IgG) were purchased from Amersham Corp., Arlington Heights, IL. X-Omat AR film was obtained from Eastman Kodak, Rochester, NY. Aprotinin, sodium orthovanadate, phenylmethylsulfonylfluoride and indomethacin were obtained from Sigma Chemical Co., St. Louis, MO. Ampholytes were purchased from BioRad, Richmond, CA. Solubilized bovine tendon collagen, 100 µg/ml was obtained from Helena Laboratories, Beaumont, TX.

RESULTS

[^{32}P]-phosphate loaded platelets were aggregated with collagen (Fig. 1). The shape change was easily separable from the release reaction. The first sampling after T_0 (the time at which the agonist was added) was done at a time at which there was no recognizable secretory release as manifested by the absence of any ATP secretion by the platelets. In fact, the more than 30 sec lag time between this sampling and the onset of ATP secretion assured that T_1 (15 sec after the addition of agonist) is representative of platelets that have undergone shape change as the only manifestation of activation. The immunoprecipitate of platelet tubulin, whether by monoclonal antibody to alpha or beta tubulin included several phosphorylated peptides ranging from M_r 14,000 to M_r 180,000 (Fig. 2a). Reduced and alkylated platelet tubulin was easily visible as clearly separated alpha and beta monomer subunits. 2-D electrophoresis of [^{32}P]-phosphorylated immunoprecipitate of platelet tubulin conformed to the characteristic pI of tubulin. There was no recognizable dif-

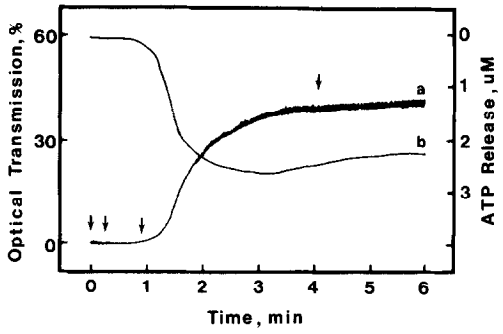

Figure 1. Collagen induced aggregation of human platelets which were pre-loaded with [^{32}P]-phosphate. Immediately before aggregation was induced with 12.5 µg collagen/ml the [^{32}P]-phosphate loaded platelet suspension was diluted with PPP as described under METHODS. Aggregation (curve a) and ATP release (curve b) were measured in a lumi-aggregometer. The arrows indicate the times at which immunoprecipitates of platelet tubulin were prepared.

ference in the phosphorylation between T_0 and T_1. The first decrease in tubulin phosphorylation became manifest at T_2 (45 sec after addition of agonist), a point in the platelet activation sequence at which release reaction was clearly in progress (Fig. 2b). The last sample point (T_3) at the height of the aggregation response showed only a minor further decline in the phosphorylation of tubulin.

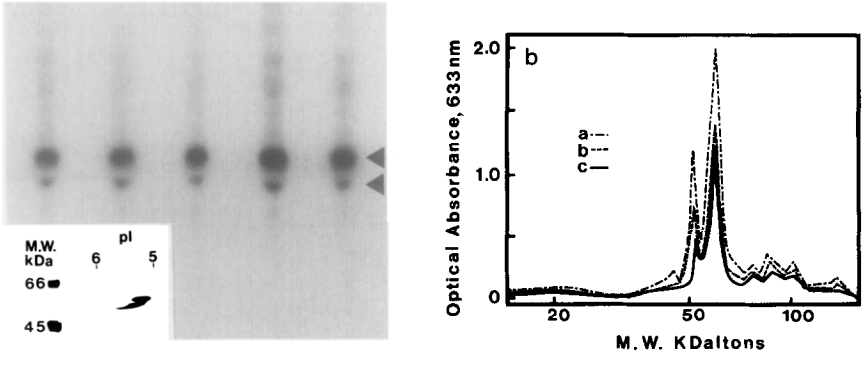

Figure 2a. Immunoprecipitates of platelet tubulin sampled at different times (T_0-T_3) during platelet aggregation. Antibody to alpha tubulin was used to prepare these precipitates whereas the left unlabeled band was precipitated with antibody to beta tubulin. The arrows indicate the position of completely reduced and alkylated alpha and beta tubulin subunits. A 2-D elecrophoretic analysis of the tubulin precipitate is shown in the inset.

Figure 2b. Laser densitometric scans of autoradiographs of immunoprecipitated platelet tubulin. Sequential measurements are shown of platelets sampled at T_0 (curve a) at T_2 (curve b) and at T_3 (curve c).

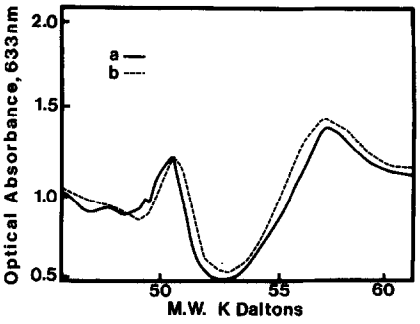

Figure 3. Laser densitometric scan of immunoprecipitated tubulin extracted from aspirinized platelets that were treated in vitro with indomethacin and UK 38485. Curve a represents platelets extracted at T_0, curve b platelets extracted at T_3.

Inhibition of the release reaction completely abolished the dephosphorylation of tubulin associated with platelet stimulation (Fig. 3). Agonist induced shape change which occurs under these circumstances could be verified. Sodium pyrophosphate, a known inhibitor of phosphatase activity was found to exert a potent reduction in platelet aggregation (Table 1). Stimulation of sodium pyrophosphate preincubated platelets by agonists induced no change in the phosphorylation of the platelets.

To determine the specificity of the dephosphorylation of tubulin, we analyzed the electrophoretic distribution of phosphopeptides produced by CNBr treatment of tubulin immoblized on PVDF membranes and compared those obtained from inactive platelets (T_0) with those of aggregated platelets (T_2) (Fig. 4). Platelet activation was associated with an asymmetric loss of [^{32}P]-phosphate from certain α -tubulin peptides. CNBr peptides of β-tubulin showed no change.

Table 1

PLATELET AGGREGATION RESPONSE TO SODIUM PYROPHOSPHATE

Agonist	Concentration	Addition	Δ O.T.(%)†	Slope *
Thrombin	0.25 U/ml	-	69 ± 4.2	3.7 ± 0.1
		$Na_4O_7P_2$	9 ± 1.3	0.2 ± 0.01
Collagen	0.8 µg/ml	-	66 ± 3.8	2.3 ± 0.3
		$Na_4O_7P_2$	8.5 ± 0.8	0.2 ± 0.01
ADP	2.5 µM	-	59 ± 3.7	2.8 ± 0.2
		$Na_4O_7P_2$	12 ± 1.1	0.4 ± 0.02
CaIonophore	2.8 µM	-	52 ± 2.6	2.4 ± 0.2
		$Na_4O_7P_2$	10 ± 0.7	0.2 ± 0.02

† Change in Optical Transmission
* Mean ± 1 SD of 4 experiments

Platelet suspensions were preincubated without (control) or with sodium pyrophosphate (4 mM) for 30 min at 20°C without agitation. All differences between control and $Na_4O_7P_2$- preincubated platelet suspensions were significant at $p < 0.001$.

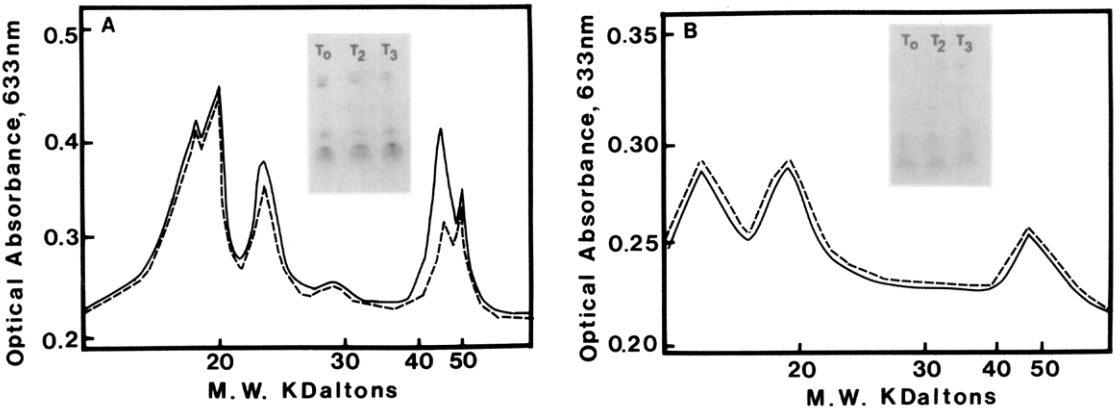

Figure 4. Laser densitometric scans and autoradiographs (insets) of CNBr treated alpha (A) and beta (B) tubulin. Peptides of platelets at T_0 (solid line), and at T_3 (interrupted line).

DISCUSSION

A considerable number of [^{32}P]-phosphorylated proteins were found to co-precipitate with tubulin. However, they did not interfere with the unequivocal identification of tubulin, which after reduction and alkylation was clearly separable into alpha and beta heterodimers. Tubulin was identified by reacting Western blots of the immunoprecipitate with monoclonal antibodies whose specific binding was recognized by a gold conjugated second antibody. Further confirmation of the identity of tubulin was obtained by 2-D electrophoresis and N-terminal amino acid sequencing which provided the final and definitive proof of the identity of tubulin in the electrophoretic transblots (data not shown).

We opted for collagen as the agonist to induce platelet aggregation in preference to thrombin as it facilitated sequential sampling of the platelet preparation. The rapid evolution of the aggregation process with thrombin as the agonist makes it difficult to separate shape change from the ensuing secretory process of the platelet. Although the depletion of phosphate in the platelets and the subsequent incubation with [^{32}P]-phosphate reduced the aggregability of the platelets, an increase in the concentration of agonist was able to overcome this deficiency.

The time resolved analysis of changes in tubulin phosphorylation showed that dephosphorylation which occurs during platelet activation (2), is delayed well beyond the initiation of shape change. Inhibition of the release reaction completely blocked the dephosphorylation step. Sodium pyrophosphate completely blocked the agonist induced dephosphorylation of tubulin and was found to be a potent inhibitor of platelet aggregation induced by various agonists. We interpret these findings as evidence that some event leading to secretory release activates tubulin dephosphorylation in platelets. The de-

phosphorylation of tubulin during the activation process involves specific peptides of both subunits, primarily of the alpha monomer.

We believe that our results give convincing evidence that the agonist induced change in phosphorylation of tubulin occurs after the initiation of the shape change and before or at the beginning of the release reaction.

ACKNOWLEDGMENT

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