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MEMBRANE-BOUND TUBULIN IN HUMAN PLATELETS

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Platelet membranes contain colchicine-binding activity which is not due to cytoplasmic contamination. This activity could be solubilized, at least partially, by Triton X-100 and to a lesser degree by Lubrol PX. Nonidet P-40 solubilized more colchicine-binding activity than was apparent in the intact, non-detergent-treated membranes, indicating that some of the protein was available for binding in the latter. Normal membranes had a far higher binding capacity for the alkaloid vinblastine than for colchicine, both of which were measured under equilibrium conditions. Membrane tubulin was also identified by acrylamide gel electrophoresis and immunologic precipitation. The protein had characteristics similar to the cytoplasmic form but was found to be more heat resistant. These findings establish with a high degree of certainty the presence of tubulin in the platelet membrane.

Introduction

Microtubules constitute an important part of the cytoskeleton of platelets. Their function appears to be the stabilization of the discoid shape of normal, non-stimulated platelets [1]. In response to aggregation stimuli, microtubules rapidly dissociate [2]. Within a few seconds repolymerization supervenes, presumably at a more central location than their usual submembrane site. Electron microscopy has given evidence of connections between membrane structures and microtubules in platelets [3]. For these reasons we have speculated that tubulin or tubulin-like proteins may exist in the plasma membrane of platelets.

In this report we show that the surface membrane of human platelets contains a small amount of tubulin-like protein which was identified by a variety of methods. These findings do not allow us to draw any definitive conclusion regarding the role of such structures in normal platelets, but do raise interesting questions about their function in membranes.

Methods and Materials

Preparation of platelet membranes and cytoplasmic tubulin

Membranes of human platelets were prepared as previously described [4]. Glycerol-laden platelets were lyzed in the presence of 0.5 mM *N*-Cbz-α-L-glutamyl-L-tyrosine and 0.4 mM toluenesulfonyl isocyanate. Suspended in phosphate buffer, pH 7.2 containing 0.14 M NaCl, the protein concentration of such membrane preparations was adjusted to 50 μg/ml. The purity of the membranes was determined by measuring the specific activity of several membrane or organelle characteristic enzymes. All membrane preparations were washed twice at 4°C to free them of any loosely adherent cytoplasmic microtubule protein and were finally suspended in 0.25 M sucrose/10 mM $MgCl_2$/10 mM phosphate buffer (pH 6.5) and 0.1 mM GTP. Tubulin was isolated from human platelets as previously described [5].

Assay of colchicine-binding activity

The binding of [^{3}H]colchicine was determined by an adaptation of the DEAE filter paper method of Weisenberg et al. [6]. Briefly, suspensions of washed platelet membranes, 50 μg/ml, were incubated at 37°C for 60 min with 0.2 μCi [^{3}H]colchicine (spec. act. 10 Ci/mmol) at a final concentration of colchicine that varied between 0.1 and 2 μM. The binding reaction was stopped by placing the samples on ice, followed by filtration of a 0.1-ml aliquot through two layers of DEAE cellulose filter paper discs. The latter was first treated with nonradioactive colchicine (1 ml of a 10 μM solution). After passage of the radioactive sample through the filter stack, additional (5 vol. of 4 ml each) saturating solution of colchicine was passed through the filter. The filter papers were then counted by the use of Aquasol (New England Nuclear, MA). All experiments were performed in triplicate. Controls consisted of incubation mixtures containing no membrane protein that were subjected to the same incubation conditions as the test samples. The subsequent filtration assay was identical to that described above. The radioactivity of these blanks was deducted from that of the test samples.

In other experiments the binding of [^{3}H]vinblastine to membranes was determined by a filtration assay. The assay was essentially as outlined above. [^{3}H]Vinblastine, 0.6 μCi of specific activity 8.4 Ci/mmol were used together with a variable concentration of nonradioactive alkaloid to obtain a range from 0.2 to 5 μM.

A series of variables that might effect the assay were studied using calf brain tubulin as test material and bovine serum albumin as control. The amount of vinblastine retained increased by about 20% as the number of filter discs was doubled from 1 to 2. Further increases in the number discs up to 5 per stack showed only a slight rise in alkaloid retained ($\leq$ 3%). Because of the reduction in flow rate with increasing filter stack height, two filter discs were finally chosen for the standard assay. Varying the time of absorption of the protein to the filter stack between 0 to 30 min did not change the retention of protein-bound ^{3}H-labeled alkaloid. Increasing the volume of washing buffer to more than 15 ml did not result in further removal of free [^{3}H]vinblastine. The background, i.e. the nonprotein bound counts retained by the filter were $\leq$ 0.5% of the total radioactivity applied. The reproducibility of the assay was quite satisfactory. Replicate determinations of the binding by calf brain tubulin and by platelet membranes showed a standard deviation of $\pm$3.6% of the mean.

Equilibrium binding studies of colchicine and vinblastine with platelet membranes

Gel filtration through Sepharose-2B was used in an adaptation of the method originally developed by Hummel and Dreyer [7] and modified by Fairclough and Fruton [8]. The instability of the tubulin-colchicine or tubulin-vinblastine complex precludes the use of conventional equilibrium dialysis. This method has been used before with success for measuring the interaction of vinblastine with brain microtubule protein [9]. 1.5 $\times$ 18 cm columns of the gel equilibrated with the appropriate solutions of colchicine or vinblastine were maintained at 37°C. The concentration of colchicine used were between 0.2 and 3 μM, those of vinblastine between 0.5 and 5 μM. The membrane suspensions contained the same concentrations of these two ligands as were in the columns. The flow rate was adjusted to 30–45 ml/h. Fractions of 1.5 ml each were collected and the absorbance measured at 354 nm for colchicine and at 320 nm for vinblastine. Protein was estimated from absorbance measurements at 280 nm. The procedure for determining the amount of ligand bound was that of Fairclough and Fruton [8]. The baseline was taken as the mean value of a series of fractions following the ending of the absorbance through. The latter was considered to have ended when the absorbance of a particular fraction was equal to or less than the average deviation from the mean baseline. The number of mols of vinblastine bound was calculated according to Lee et al. [9] with a molar absorption coefficient of 4750 for the alkaloid in 10 mM phosphate buffer, pH 6.8 containing 0.14 M NaCl and 0.1 mM GTP. For colchicine the molar absorption coefficient was 16400. Between 3–5 mg membranes protein was utilized for each run. Protein was measured according to Lowry et al. [10] using crystalline bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Membranes or platelet tubulin were dissolved in an equal volume of 20 mM Tris-HCl, pH 6.8 containing 2 mM EDTA and 5% sodium dodecyl sulfate (SDS). The protein concentrations were kept between 50 and 75 μg/ml. All samples were electrophoresed on 5–20% polyacrylamide (acrylamide/bis-acrylamide, 100 : 3) gradient gels containing 0.1% SDS and 0.375 M Tris-HCl, pH 8.8 [11]. The gels were subsequently stained with silver using the method of Sammons et al. [12].

Materials

Tritiated colchicine was a product of New England Nuclear (MA) and [^{3}H]vinblastine sulfate was obtained from Amersham Corp. (IL). Nonidet P-40 was purchased from Particle Data Laboratories; Triton X-100, Lubrol PX and sodium dodecyl sulfate from Sigma Chemical CO. (MO).

Results and Discussion

The glycerol lysis technique [13] which was used for the preparation of platelets allows gentle lysis of platelets whose membranes can be readily collected on a cushion of 27% sucrose. The soluble fraction of platelets and their subcellular granules are easily separated from the membrane vescicles which collect in a sharp double band a short distance into the sucrose cushion. Contamination of membrane bands with soluble and subcellular platelet granule contents was determined in twice washed membrane preparations by measuring certain enzyme activities characteristic of the major platelet subfractions. These results are presented in Table I. There was less than 0.1% contamination with platelet granule contents and less than 0.05% contamination with soluble platelet fraction.

TABLE I

PURITY OF PLATELET MEMBRANE PREPARATIONS

Enzyme		Activity [a]	Percent of activity in membrane preparation
Purine nucleoside phosphorylase (cytosol)		0.090 ± 0.016 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein	0.04
Reduced NAD dehydrogenase (microsomes)		0.630 ± 0.049 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein	8.5
Isocitrate dehydrogenase (mitochondria)		0.051 ± 0.001 $mmol \cdot min^{-1} \cdot mg^{-1}$ protein	0.1
$(Na^+ + K^+)$-ATPase (membrane)		0.237 ± 0.042 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein	100
Adenylate cyclase (membrane)	Basal	7.4 ± 0.9	100
	$+PGE_1$	59.1 ± 8.5 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein	

[a] Mean ± S.D. of five determinations.

To determine whether cytoplasmic tubulin could be extracted together with the platelet membrane fraction, *N*-[^{14}C]ethylmaleimide alkylated cytoplasmic platelet tubulin was added during platelet lysis and carried through the regular process of membrane preparation. The radioactive counts isolated with the washed membrane preparation were less than 0.05% of those originally added.

Cochicine binding to platelet membranes was measured over the concentration range 0.1–2 μM. Analysis of the results by double reciprocal plot revealed a $K_{diss} = 1 \cdot 10^{-6}$ M (Fig. 1). The colchicine binding activity showed a temperature op-

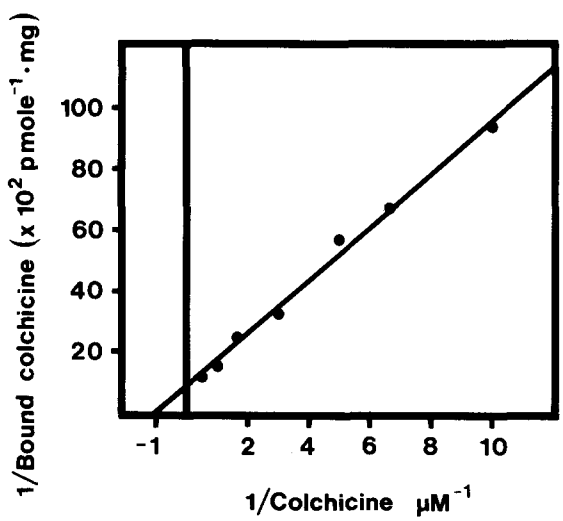

Fig. 1. Lineweaver-Burk plot of colchicine binding to platelet membranes. Suspensions of platelet membranes were incubated with [^{3}H]colchicine in the indicated concentrations for 60 min at 37°C.

timum at about 47°C when measured in the temperature range from 4–55°C. Peak binding activity was found at pH 6.9. It was dependent on the amount of protein present. Up to 2.5 mg/ml, the maximum tested, a straight linear relationship between binding activity and protein content was found. The presence of sucrose in the medium enhanced the stability of the binding activity whose half life of decay was 9 h while it was slightly less than 150 min in its absence.

The binding of the alkaloid vinblastine was found to have a $K_{diss} = 2.9 \cdot 10^{-6}$ M (Fig. 2). The number of binding sites was evaluated under equilibrium conditions using the gel filtration method described under Methods. Analysis of the binding data by Scatchard plot [14] revealed at least two types of binding sites (Fig. 3). High affinity sites had a maximal binding capacity of 0.28 nmol/mg protein with a binding constant $K_B = 4.05 \cdot 10^6$ M^{-1} whereas low affinity sites had a binding capacity of 1.06 nmol/mg protein with a binding constant $K_B = 3.9 \cdot 10^5$ M^{-1}. Based on the finding that the tubulin dimer has two binding sites for vinblastine [9] and on the speculative interpretation that the high affinity site represents primarily tubulin, one can calculate that approx. 1.5% of the platelet protein is tubulin.

A similar analysis with colchicine gave a binding capacity of 12.8 pmol/mg protein (Fig. 3). Assuming a molecular weight of 110000 for the colchicine binding protein, we can calculate that approx. 0.14% of the membrane protein had this characteristic. The marked discrepancy between these two tubulin ligands could be due to the less

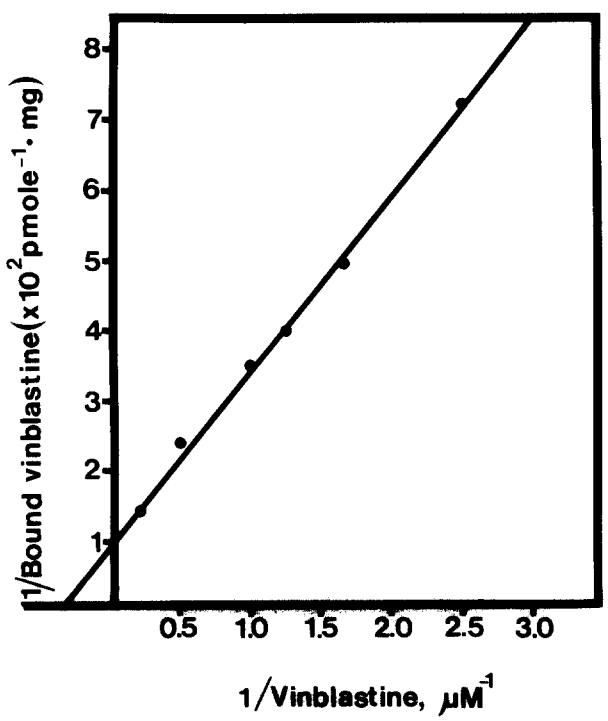

Fig. 2. Double reciprocal plot of vinblastine binding to platelet membranes. Suspensions of platelet membranes were incubated with [^{3}H]vinblastine in the indicated concentrations for 60 min at 37°C.

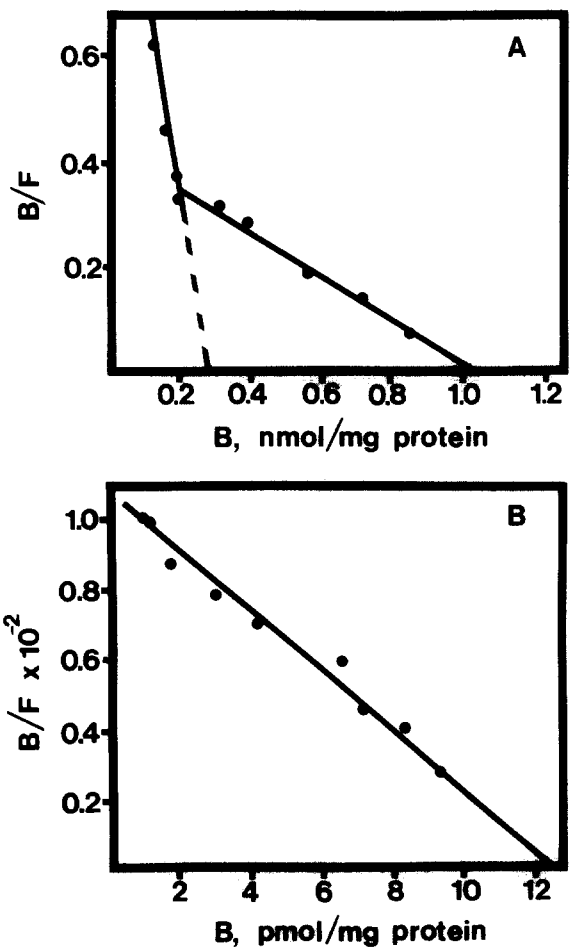

Fig. 3. Scatchard plot of gel filtration data of colchicine (A) and vinblastine (B) binding under equilibrium conditions. The tubulin-ligand complex was estimated from the optical absorbance tracings and the magnitude of binding determined as described under Methods. B, bound ligand; F, free ligand.

TABLE II

SOLUBILIZATION OF PLATELET MEMBRANE PROTEIN

Detergent	Concn. (%)	Protein solubilized (%)	Colchicine-binding activity solubilized (%)	Inhibition of colchicite-binding activity (%)
Nonidet P-40	0.1	41.3	100	15
	0.2	55.7	125	24
Lubrol PX	0.2	36.4	24	72
Triton X-100	0.1	35.1	32	65
	0.2	41.7	43	79
SDS	0.1	94.2	0	100

specific nature of vinblastine binding sites in membrane-associated tubulin. The latter possibility was investigated by attempts at solubilizing membrane bound tubulin. Four different detergents were utilized. As summarized in Table II, Nonidet P-40 had the least suppressant effect on the colchicine binding activity, while SDS abolished any such activity. Similar observations were made by Bhattacharyya and Wolff [15]. The amount of total protein solubilized ranged from slightly less than 95% for SDS to 24% for Lubrol PX. Nonidet P-40 solubilized more colchicine binding activity than all other detergents tested and, corrected for its decrease of the binding activity, revealed a greater amount of tubulin in platelet membranes but still did not come close to the value of membrane bound vinblastine. The known lack of specificity of vinblastine presumably is responsible for the discrepancy in binding of the two tubulin ligands.

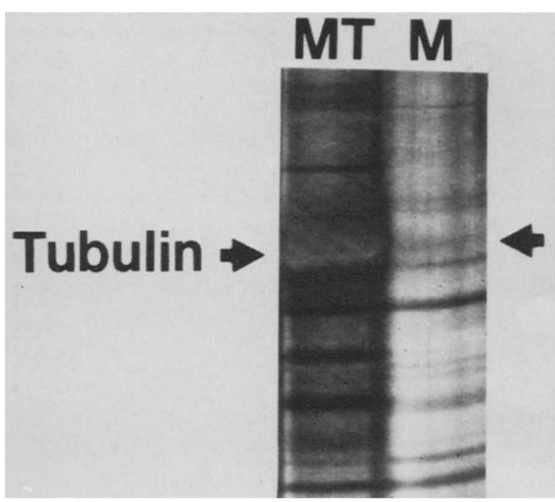

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of platelet membranes (M) and platelet microtubule protein (MT). Gels were stained with silver. A close up view of the area of tubulin monomer is presented.

Further tangible evidence for the presence of tubulin in platelet membranes was obtained by SDS-polyacrylamide gel electrophoresis. The highly sensitive silver stain [12] revealed a protein band in membranes coinciding with that of isolated cytoplasmic tubulin (Fig. 4). In addition, and possibly more convincing, the color properties of the two bands were identical, both staining slate gray. Several of the microtubule-associated proteins could be found in platelet membranes. This was determined by comparing the staining patterns obtained by polyacrylamide gel electrophoresis of platelet membranes and DEAE Sephadex-isolated [16] microtubulin-associated proteins of cytoplasmic platelet tubulin.

Finally, an antibody raised against platelet tubulin was able to abolish the colchicine binding activity of isolated platelet membranes and produced a precipitin line on Ouchterlony plates with solubilized membrane tubulin which was identical to that of the cytoplasmic microtubule protein.

The blocking of the colchicine binding of tubulin antiserum was dose-dependent. At maximal inhibition only 12% of the original binding activity remained. The specificity of this effect could be shown by the absence of any significant blocking

action (89% of the original colchicine-binding activity remained) when nonimmune IgG was used instead of the tubulin antiserum.

This study has shown that tubulin is an integral part of the platelet membrane. The membrane-associated microtubule protein had similar characteristics with respect to the binding of colchicine and vinblastine as its far more abundant cytoplasmic representative. The one exception noted was the high thermal stability. A similar observation was made by Bhattacharyya and Wolff [15]. Because of the small amount of tubulin present in the platelet membrane, a number of different techniques were brought to bear on demonstrating its identity and verifying its location. In view of the unrelated methods which showed tubulin to be part of platelet membranes, I feel confident that it is indeed an integral membrane protein and not a contaminant. Castle and Crawford [17] determining the subcellular distribution of [^{3}H]colchicine-binding activity in pig platelets, also found a small amount in the membrane but did not show that it represented a true component rather than a contaminant.

Whether the membrane based tubulin can interact with the cytoplasmic microtubule protein is not known but other investigators have shown that protein from guinea pig brain was able to polymerize [18]. In platelets as in other cells, microtubules are not seen to be in direct contact with membrane proteins. However, there is no doubt that modification of the state of polymerization of microtubules affects the behavior of platelets expressed through membrane-related functions. It, therefore, seems logical to postulate that membrane tubulin may form one of the sites of nucleation of cytoplasmic tubulin and thus fulfill an important role in connecting membranes to cytoskeletal protein.

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