THE α-SUBUNIT OF TUBULIN IS PREFERENTIALLY ASSOCIATED WITH BRAIN PRESYNAPTIC MEMBRANE

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1. Introduction

Tubulin, the subunit protein of microtubules, is a heterodimer composed of two polypeptides α- and β- of ~55 000 M_r each [1,2]. The α- and β-subunits show microheterogeneity and both have been resolved into several components [3–7]. We have recently shown that cytoplasmic tubulin microheterogeneity is most prominent in the brain and increases with rat brain maturation [8]. Tubulin is not only confined to the cytoplasm, as it is found to be associated with various membranes [9–13] including the presynaptic membranes [14,15]. It was therefore of interest to determine the presence and properties of membrane bound tubulin from brain and compare it to tubulin from the cytoplasmic fraction.

By the criteria of molecular weight, isoelectric point, peptide mapping and vinblastine binding we show, in the present study, that synaptosomal membranes isolated from rat cerebral cortex contain significant amounts of tubulin. Furthermore, we find that the α- and β-tubulins differ in their association with these membranes.

2. Materials and methods

Ficoll-400 was obtained from Pharmacia, NP40 from British Drug Houses, DEAE-cellulose (DE-52) from Whatman, *Staphylococcus aureus* V8 protease (36-900-1) from Miles, Triton X-100 from Sigma and ampholine carrier ampholytes were from LKB. Vinblastine sulfate was a gift from Eli Lilly.

2.1. *Preparation of synaptosomal membrane from rat cerebral cortex*

Rat brain synaptosomes were prepared on isotonic Ficoll density gradients. The synaptosomes were exposed to osmotic shock and membranes were isolated by sucrose gradient centrifugation [16].

2.2. *Preparation of rat brain tubulin*

Rat brain tubulin was purified using DEAE-cellulose column chromatography as in [8].

2.3. *Gel electrophoresis*

For SDS–polyacrylamide gel electrophoresis samples were mixed with buffer to yield final conc. 10% glycerol/5% 2-mercaptoethanol/3% SDS/0.0625 M Tris–HCl (pH 6.8)/0.001% bromophenol blue, and heated at 100°C for 5 min. Samples were subjected to electrophoresis on 0.75 mm thick slab gels containing either 10–20% gradient of polyacrylamide gels in 0.1% SDS or 5% polyacrylamide gel containing 8 M urea and 0.1% SDS at 20°C [8,17]. For quantitative evaluation, the photographic transparencies of the Coomassie brilliant blue R stained gels were scanned at 560 nm in a Gilford 2400 S spectrophotometer.

2.4. *Isoelectric focusing and two-dimensional gel electrophoresis*

Samples were diluted with water to final conc. 2 mg protein/ml, then further diluted in a 1:1 ratio with a buffer containing 9.5 M urea, 2% v/v NP40, 5% 2-mercaptoethanol and 2% ampholines comprised of 1.6% pH 5–8 and 0.4% pH 3–10. Aliquots (10–100 μl) were loaded onto the isoelectric focusing

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polyacrylamide gels containing 1.6% ampholines pH 5–8 and 0.4% pH 3–10. The electrophoresis was performed according to [18]. When the gels were subjected to electrophoresis on a second dimension the second gel contained 5% polyacrylamide, 0.1% SDS and 8 M urea.

2.5. *Vinblastine precipitation of membrane-bound tubulin*

The membrane suspensions were brought to final conc. 1 mg protein/ml and 0.5% Triton X-100. The mixtures were then incubated for 1 min at room temperature and then centrifuged for 2.5 min at 12 000 × *g* at 24°C. The supernatant solutions were adjusted to contain 10 mM $MgCl_2$, 10 mM sodium phosphate buffer (pH 7.4) 0.2 mM GTP and 1 mg/ml vinblastine sulfate. The mixtures were incubated at 37°C for 30 min and then centrifuged for 2 min at 12 000 × *g*. The pellets were thereafter dissolved in the appropriate electrophoresis buffer.

2.6. *Protease digestion of tubulin*

Samples were digested as in [19] with slight modifications [8].

3. Results and discussion

3.1. *Detection of tubulin in the presynaptic membrane*

Isolated synaptosomes from rat cerebral cortex were found to contain a 55 000 M_r protein which represents 4% of the total protein content. This protein comigrated with tubulin on SDS–polyacrylamide gel electrophoresis (fig.1). A higher content of the presumptive tubulin was observed in synaptosomal membranes (7% of the membrane protein content). This protein could be partially extracted from the membranes using a non-ionic detergent, Triton X-100, and thereafter be selectively precipitated by vinblastine, a plant alkaloid which interacts with tubulin [20]. Electrophoretic separation of the vinblastine precipitated proteins showed that the presumptive tubulin represents 27% of the total proteins applied on the SDS–polyacrylamide gels. Thus, both by the criteria of molecular weight as well as alkaloid binding this presynaptic membrane-associated protein appears to be tubulin.

In order to eliminate the possibility that the association of tubulin with the presynaptic membrane arose from artefactual binding of cytoplasmic tubulin during the isolation procedure, the following experiment was performed. [^{35}S]Methionine labeled tubulin from mouse neuroblastoma N18TG-2 cells [12] was added to synaptosomal suspension and the mixture was exposed to osmotic shock. Insignificant amounts of radioactivity were detected in the isolated membranes (< 0.5% of the total radioactivity added which amounts to < 0.3 μg labeled tubulin/mg membrane protein). Similar results were obtained when the [^{35}S]methionine-labeled tubulin was added during the initial homogenization of the cerebral cortex.

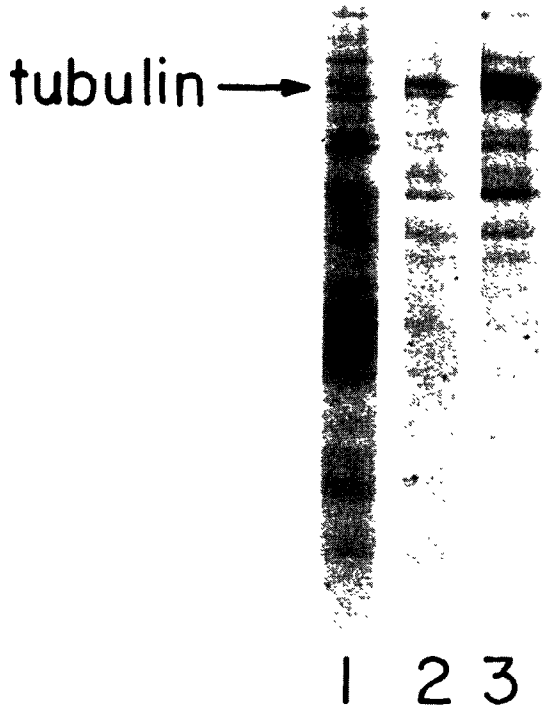

Fig.1. Electrophoretic separation of rat brain synaptic membrane proteins on SDS–polyacrylamide gels: (1) Synaptosomes; (2) synaptosomal membranes; (3) vinblastine precipitate from Triton X-100-extracted synaptosomal membranes.

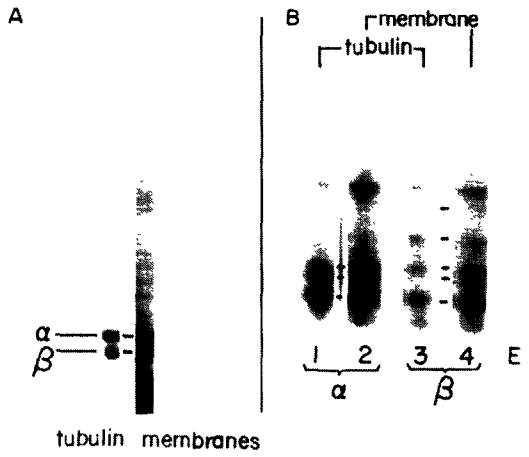

Fig.2. (A) Electrophoretic analysis of synaptic membrane proteins on urea–SDS polyacrylamide gels. The position of the tubulin bands was identified by using as a marker cytoplasmic rat brain tubulin purified by DEAE-cellulose chromatography (left slot). (B) Protease cleavage peptides of tubulin isolated from synaptic membranes. Synaptic membrane preparations were subjected to urea–SDS polyacrylamide gel electrophoresis. The tubulin bands were excised from the Coomassie brilliant blue stained gels and inserted into sample slots of 15% polyacrylamide slab gels in a buffer containing *Staphylococcus aureus* protease as in [8,19]. (1) Brain cytoplasmic α-tubulin; (2) membrane associated α-tubulin; (3) brain cytoplasmic β-tubulin; (4) membrane associated β-tubulin; (E) enzyme alone.

3.2. *Detection of α- and β-tubulin subunits in the presynaptic membrane*

Urea–SDS polyacrylamide gel electrophoresis of the membrane bound preparations indicated the presence of both the α- and β-tubulin subunits (fig.2A). The degree of relation between the membrane associated tubulins and cytoplasmic rat brain α- and β-tubulin subunits was further analyzed by peptide mapping analysis. The results in fig.2B show a close similarity in the overall distribution of the proteolytic peptide chains derived from the cytoplasmic or the membrane-associated α- and β-tubulin subunits.

While the amount of cytoplasmic α-tubulin equals that of the β-subunit, membranes contain more α- than β-tubulin. Quantitative analysis of the densitometric tracing of the urea–SDS polyacrylamide gels reveals that the α/β ratio is 1.6/1 for the membrane associated tubulin. The decreased amount of the β-subunit in the membrane fraction raised the possibility that this subunit is loosely associated with the presynaptic membrane and partially lost during its purification.

3.3. *α-Tubulin is preferentially associated with the presynaptic membrane*

Further characterization of the membrane-bound tubulin was achieved by isoelectric focusing. When the membrane preparation was subjected to isoelectric focusing followed by urea–SDS polyacrylamide gel electrophoresis on a second dimension (fig.3A) it appeared that in contrast to the β-tubulin very little of the α-tubulin migrated in both dimensions on the gel (α/β = 0.3/1). This could be explained by the fact that most of the α-subunit and only a small amount of the β-subunit remained at the origin of the isoelectric focusing gels. However, this material migrated on the second dimension allowing the resolution of the α- and β-tubulins (α/β = 4/1). Since under the isoelectric focusing conditions the membranes were suspended in 4.25 M urea containing 1% NP40 and ampholines, while in the second electrophoretic dimension SDS is included, we conclude that SDS is required for the complete dissociation of tubulin and especially α-tubulin from the membranes. The limited solubility of the membrane α-tubulin in urea NP40 is apparently also manifested by the heavy streaking of this subunit (fig.3A), which was not evident on two dimensional electrophoresis of soluble brain tubulin [8].

It should be noted that brain tubulin is resolved by isoelectric focusing into several components, but that electrophoresis of these isoelectric focusing gels on urea–SDS polyacrylamide gels in a second dimension results in overlapping of the bands ([8] and fig.3A). In order to examine the pattern of membrane isotubulins one has therefore to inspect the first dimension isoelectric focusing gels. It has been shown that cytoplasmic tubulin from adult brain is resolved under these conditions into nine components in which isotubulins 1–4 comprise the α-subunit 5–9 the β-subunit [8]. Figure 3B shows the densitometric tracings of the isoelectric focusing gel of cytoplasmic cerebral cortex tubulin which was resolved into 9 components. The relative proportions of the various isotubulins differed from that of cytoplasmic tubulin from whole brain [8]. In addition, the separation of the cerebral cortex bands 2–4 was less pronounced

than that of whole brain cytoplasmic tubulin [8]. Figure 3B also shows the densitometric tracing of the isoelectric focusing gel of Triton X-100 extracted membrane tubulin which was further purified by vinblastine precipitation. The loosely-bound membrane β-tubulin seems to contain isotubulins: 6–8 and traces of 9. Moreover, the relative proportions of the membrane isotubulins differs from that of the cytoplasmic cerebral cortex fraction. As noted before the tightly-bound membrane tubulin could only be eluted with SDS, a detergent that interfered with resolution by isoelectric focusing.

The finding of tubulin in the presynaptic membrane may point to the existence of microtubules in this region. Alternatively, the tighter association of the α-tubulin with the membrane may indicate, a non-microtubular structure. Perhaps, the membrane-associated tubulin functions by ways other than polymerization into microtubules and thus pharmacological agents affecting microtubule assembly will not necessarily interfere with its presumptive role in synaptic transmission [21].

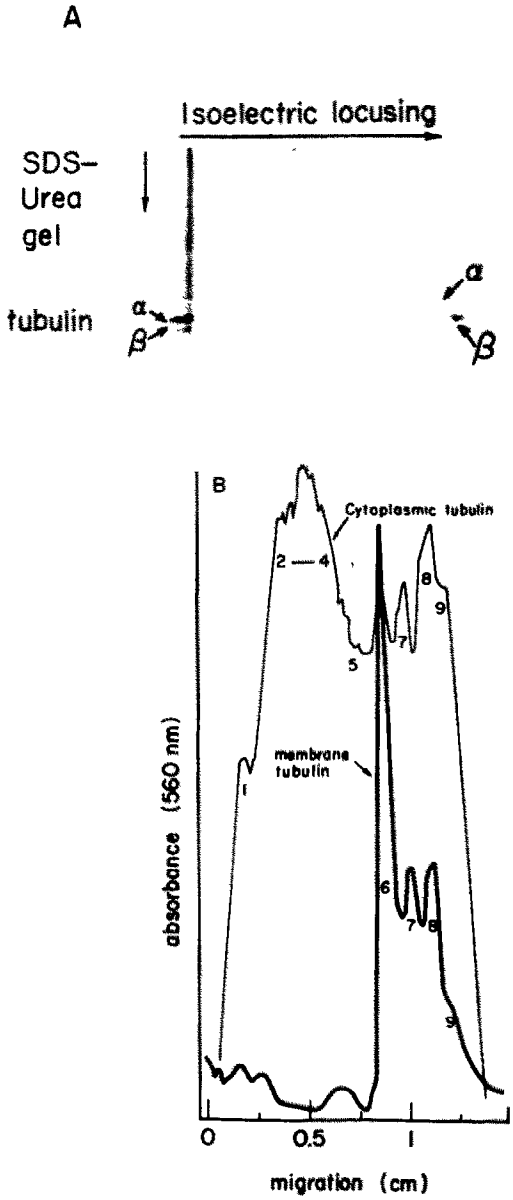

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Fig.3. (A) Two-dimensional gel electrophoresis of proteins from synaptic membranes. The tubulin position was identified by using as a marker cytoplasmic rat brain tubulin purified by DEAE-cellulose chromatography. The tubulin bands appearing on the left side of the gel are derived from the material that remained at the origin of the isoelectric focusing gel. (B) One-dimensional isoelectric focusing of synaptic membrane tubulin. The picture shows the densitometric traces of the photographic transparencies of the Coomassie brilliant blue-stained isoelectric focusing gels. Synaptic membrane tubulin was isolated from synaptic membranes after Triton X-100 extraction and vinblastine precipitation. It was compared to DEAE-cellulose purified tubulin from rat cytoplasmic cerebral cortex. The isotubulin positions were determined using a DEAE-cellulose purified cytoplasmic brain tubulin as a marker [8].

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