ISOLATION OF TUBULIN FROM THE NERVOUS SYSTEM OF *APLYSIA CALIFORNICA*

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1. Introduction

The marine mollusc Aplysia is very suitable for the study of the biochemical basis of neutral activity because its nervous system is organised as a set of ganglia joined by large connectives, one of which, the abdominal ganglion, contains large individually identifiable cells. The organisation and function of these neurons has been studied intensively by Kandel and Tauc and their colleagues [1–4]. Strumwasser [5], Peterson and Kernal [6] and Wilson [7] have observed correlations between electrophysiological activity and RNA and protein synthesis in individual cells of the abdominal ganglion. However, Schwartz et al. [8] have recently shown, by using inhibitors of protein synthesis, that many of the basic electrophysiological functions of some of the neurons, over a period of up to 30 hr, are not dependent on total protein synthesis. For more detailed studies however, the synthesis of specific identified components of the nervous system must be measured together with concomitant electrophysiological activity. For this reason, we have chosen to study the neurotubule subunit, tubulin (colchicine binding protein) [9], because of its suggested role in neuronal processes such as axonal flow [10] and neurite formation [11].

Recent work has shown that vinblastine sulphate precipitates aggregates of tubulin from 100,000 *g* supernatants of pig brain [12], cultured neuroblastoma cells [13], chick embryo brain [14] and cultured sympathetic ganglia [15]. These aggregates bind colchinine and consist of subunits with similar properties to the highly purified colchicine binding protein isolated by Weisenberg [9] from pig brain.

In this study, we describe the preparation of tubulin from Aplysia ganglia by extraction with PMG buffer. The crude protein was then purified from the resulting 100,000 *g* supernatant by either vinblastine precipitation or DEAE-Sephadex chromatography [9]. The protein was then further characterised as tubulin by its colchicine binding and electrophoretic mobility on SDS polyacrylamide gels.

2. Material and methods

Aplysia californica were obtained from the Pacific Biomarine Corporation, Venice, California, USA. The animals were kept in aerated aquaria containing artificial seawater (hw-Meeressalz, H. Wiegandt, Krefeld, W. Germany.) at 15° for between 2–14 days before use. For each preparation, 2–4 mature animals were dissected in artificial seawater at 15° and all the large ganglia (i.e. cerebral, pedal, pleural, visceral and, in most cases, buccal ganglia) removed with or without the large connectives. All subsequent operations were done at 4°. The material was washed twice in 10 mM sodium phosphate buffer pH 6.5 containing 10 mM $MgCl_2$ and 0.24 M sucrose, and once with this buffer containing 0.1 mM GTP (PMG–sucrose buffer). The ganglia and connectives were finely minced by hand with curved scissors in a watch glass, suspended in 2–5 ml of PMG–sucrose and homogenised in a glass homogeniser. The homogenate was centrifuged at 100,000 *g* for 1 hr and the supernatant used for preparing tubulin. The 100,000 *g* supernatant (approx. 10 mg protein) was made 5×10^{-4} M in vinblastine (Velban, Eli Lilly and Co.) and heated for 60 min at

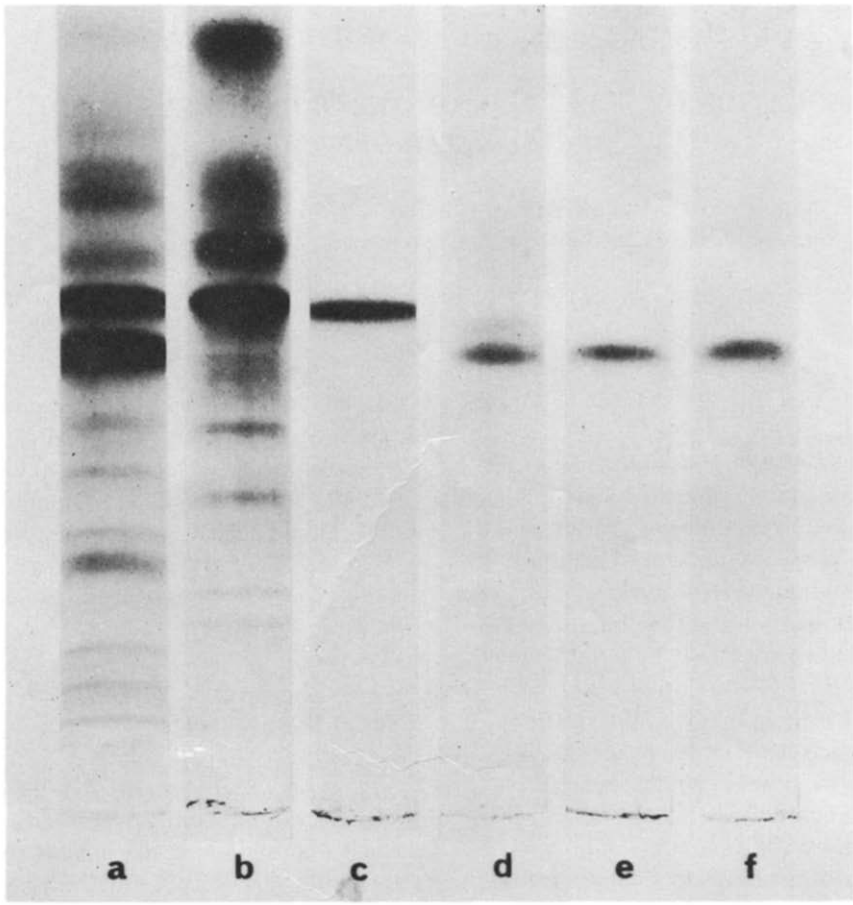

Fig. 1. Comparison of partially purified Aplysia ganglion tubulin with highly purified pig brain tubulin by SDS polyacrylamide gel electrophoresis. 5% Acrylamide 0.13% BIS, phosphate buffer pH 7.1, I = 0.1. Brain tubulin was prepared by the method of Weisenberg et al. [9] and Aplysia ganglion tubulin by DEAE-Sephadex chromatography [9]. a) 100,000 *g* supernatant of Aplysia ganglia. b) Supernatant after precipitation of a) with 5×10^{-4} M vinblastine. c) Actin from rabbit skeletal muscle (gift from H. Baker). d) Vinblastine-precipitated protein from 100,000 *g* supernatant of Aplysia ganglia. e) Aplysia ganglion tubulin. f) Brain tubulin.

37° to precipitate the tubulin. The precipitate (1 mg) was readily dissolved in PMG buffer at 0° and aliquots assayed for colchicine binding. Protein was estimated by the method of Lowry [16] after removing vinblastine [14].

A highly purified preparation of tubulin from pig brain was prepared by ammonium sulphate precipitation and DEAE-Sephadex A-50 chromatography [9]. The latter step was also used for preparing tubulin from Aplysia ganglia without prior ammonium sulphate fractionation.

Assays for colchicine binding were done by the DEAE filter method or by gel filtration [9].

Samples for gel electrophoresis were made 2% in SDS and 3% in mercaptoethanol and heated at 100° for 3 min, then dialysed against 1% SDS–1% mercaptoethanol overnight to remove vinblastine. SDS–polyacrylamide gel electrophoresis was carried out using a phosphate buffer system pH 7.1 [18] (5% acrylamide, 0.13% BIS) or the discontinuous, Tris glycinate system pH 8.9 [18] (7.5% acrylamide, 0,2% BIS), both buffers with 0.1% SDS in the reservoir and gel buffers. Gels were stained with 0.25% Coomasie Blue G.250 (Serva, Herdelberg) and destained with

methanol–acetic acid–water (1:1.5:17.5). Polypeptide chain weights were estimated from a calibration curve of proteins of known size [18].

3. Results and discussion

The 100,000 *g* supernatant from Aplysia ganglia binds colchicine. Incubation with 5×10^{-4} M vinblastine at 37° precipitates approx. 90% of this activity in the form of crystalline aggregates similar to those seen by Marantz et al. [19] using negative staining. The precipitated protein can be made to dissolve and precipitate reversibly by changing the temperature between 0 and 37° as observed by Wilson [14]. The precipitated protein increased 6-fold in specific activity of colchicine binding compared with the 100,000 *g* supernatant and bound 0.05 moles of ^{3}H-colchicine/120,000 *g* of vinblastine purified protein. The protein-bound ^{3}H-colchicine is eluted from a G-200 Sephadex column with PMG buffer at the void volume and is a large aggregate of high molecular weight as reported by Olmsted et al. [13]. Polyacrylamide gel electrophoresis at pH 7.0 revealed that the unfractionated 100,000 *g* supernatant contained two major proteins of polypeptide chain weight 45,000 and 55,000 daltons (fig. 1). Vinblastine (5×10^{-4} M) precipitated the higher molecular weight protein and left most of the other protein in the supernatant. This latter protein migrated with muscle actin (fig. 1) and probably corresponds to an actin-like protein from the connectives joining the ganglia (Jacobs, unpublished result) and possibly the ganglia themselves [17]. The protein of chain weight 55,000 was identified as Aplysia tubulin since it migrated with the colchicine binding protein of Aplysia ganglia prepared by DEAE-Sephadex chromatography as well as with the highly purified brain tubulin [9] (fig. 1). Electrophoresis of column-purified Aplysia and the brain tubulins at pH 8.9 split both tubulins into two polypeptide chains of chain weights approx. 53,000 and 55,000 (fig. 2). Identical electrophoretic patterns were obtained when the protease inhibitor 1 mM β-phenyl sulphonyl chloride was included in the buffers used for the preparation and electrophoresis of tubulin. Non-identical polypeptide chains have been found similarly in tubulins from brain [21], neuroblastoma [22], cultured symphatic ganglia [15] and outer tubule doublets of Chlamydomonas flagella [13, 23] and Tetrahymena cilia [24] under similar electrophoretic conditions.

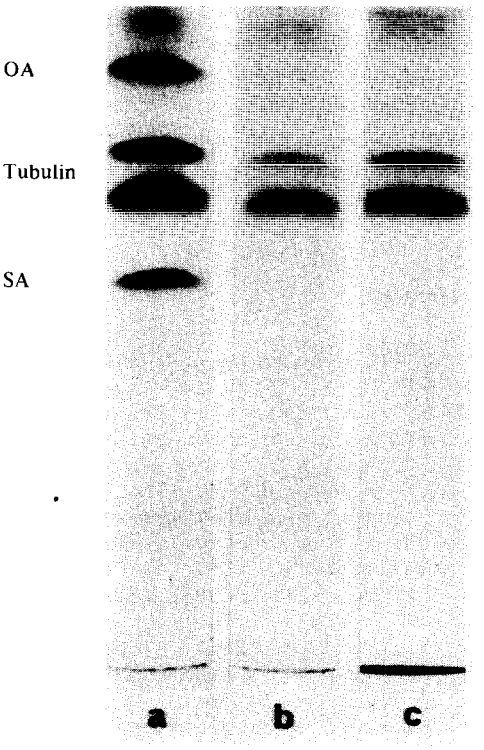

Fig. 2. SDS polyacrylamide gel electrophoresis of purified tubulin from pig brain and Aplysia ganglia. 7.5% Acrylamide 0.2% BIS, Tris glycinate discontinuous buffer system pH 8.9. a) Mixture of brain tubulin, Aplysia ganglion tubulin, serum Albumin (SA) and Ovalbumin (OA). b) Aplysia tubulin. c) Brain tubulin. Brain and Aplysia ganglion tubulin prepared as in fig. 1.

4. Summary

Tubulin, assayed by colchicine binding, was isolated from the 100,000 *g* supernatant of Aplysia ganglion homogenate by vinblastine precipitation or by DEAE-Sephadex column chromatography [9]. The protein was further characterised by SDS polyacrylamide gel electrophoresis at pH 7.1. The vinblastine precipitate contained tubulins of apparent molecular weight 55,000, and another major protein of molecular weight 45,000 thought to be actin-like. Purified Aplysia

tubulin was further resolved into two non-identical polypeptide chains by electrophoresis at pH 8.9.

Vinblastine precipitation, although yielding an impure preparation of tubulin, provides a very rapid method, in conjunction with electrophoresis, for isolating small quantities of the above proteins for following their synthesis.

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