Subaxolemmal Cytoskeleton in Squid Giant Axon. I. Biochemical Analysis of Microtubules, Microfilaments, and Their Associated High-Molecular-Weight Proteins

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Abstract. Using the squid giant axon, we analyzed biochemically the molecular organization of the axonal cytoskeleton underlying the axolemma (subaxolemmal cytoskeleton). The preparation enriched in the subaxolemmal cytoskeleton was obtained by squeezing out the central part of the axoplasm using a roller. The electrophoretic banding pattern of the subaxolemmal cytoskeleton was characterized by large amounts of two high-molecular-weight (HMW) proteins (260 and 255 kD). The α,β-tubulin, actin, and some other proteins were also its major constituents. The 260-kD protein is known to play an important role in maintaining the excitability of the axolemma (Matsumoto, G., M. Ichikawa, A. Tasaki, H. Murofushi, and H. Sakai, 1983, *J. Membr. Biol.*, 77:77–91) and was recently designated "axolinin" (Sakai, H., G. Matsumoto, and H. Murofushi, 1985, *Adv. Biophys.*, 19:43–89). We purified axolinin and the 255-kD protein in their native forms and further characterized their biochemical properties. The purified axolinin was soluble in 0.6 M NaCl solution but insoluble in 0.1 M NaCl solution. It co-sedimented with microtubules but not with actin filaments. In low-angle rotary-shadowing electron microscopy, the axolinin molecule in 0.6 M NaCl solution looked like a straight rod ~105 nm in length with a globular head at one end. On the other hand, the purified 255-kD protein was soluble in both 0.1 and 0.6 M NaCl solution and co-sedimented with actin filaments but not with microtubules. The 255-kD protein molecule appeared as a characteristic horseshoe-shaped structure ~35 nm in diameter. Furthermore, the 255-kD protein showed no cross-reactivity to the anti-axolinin antibody. Taken together, these characteristics lead us to conclude that the subaxolemmal cytoskeleton in the squid giant axon is highly specialized, and is mainly composed of microtubules and a microtubule-associated HMW protein (axolinin), and actin filaments and an actin filament–associated HMW protein (255-kD protein).

EVIDENCE has accumulated that the cytoskeletal network underlying the plasmalemma plays an important role in regulating the activities of the membrane in various types of cells. In this respect, the physiological roles of axonal cytoskeleton in the excitation process of the axolemma appear to be of interest. To analyze this problem, correlative physiological, biochemical, and morphological studies are required, and the squid giant axon offers an advantageous model for this purpose. The pioneer works dealing with this problem using squid giant axon have led to the idea that the cytoskeleton underlying the axolemma (subaxolemmal cytoskeleton) may contain some specific proteins indispensable to the excitability of the axolemma (4, 20, 24, 38). Recently, using squid giant axons, Matsumoto and his colleagues have demonstrated that membrane excitability is destroyed by the intraaxonal perfusion of microtubule-disrupting reagents such as colchicine, podophyllotoxin, vinblastine, and Ca ions, and is restored by further perfusion of the microtubule polymerization buffer containing tyrosinated tubulin and a high-molecular-weight (HMW)[1] (260,000-mol-wt) protein isolated from squid axons (7, 14–18, 27). Based on these experimental results, a model has been proposed in which the cytoskeletal network consisting of microtubules and 260-kD proteins plays an important role in generating the sodium currents (13). The following questions naturally arise: How do the microtubules and 260-kD proteins interact with sodium channels? Are the other cytoskeletal components such as actin filaments or other HMW proteins involved in the membrane excitation? It seems likely that an understanding of the molecular basis of the above physiological findings will require further biochemical

[1] *Abbreviations used in this paper:* HMW, high-molecular-weight; MAP, microtubule-associated protein.

and morphological analyses of the molecular organization of the subaxolemmal cytoskeleton in the squid giant axon.

Recently, Murofushi et al. (22) have partially purified the 260-kD protein, called "axolinin" by Sakai et al. (28), from the crude extract of squid nerves and reported that the axolinin interacted with microtubules to make bundles in vitro. Information on the in vitro nature of the other constituents in the subaxolemmal cytoskeleton is insufficient (see reference 2), mainly because it is difficult to obtain enough fresh squid nerves for the biochemical analysis. In the present study, we first analyzed biochemically the molecular organization of the subaxolemmal cytoskeleton of squid giant axons, and found evidence that its major constituents were actin, tubulin, axolinin, and an HMW (255,000-mol-wt) protein designated here as "255-kD protein." Secondly, axolinin and 255-kD protein were purified in their native forms and their biochemical properties were elucidated in vitro.

Materials and Methods

Squid Nerves

Squid, *Dorytheuthis bleekeri*, were collected in Sagami Bay and kept alive in a fish preserve in Misaki Marine Biological Station, or transported and maintained in a small circular and closed-system aquarium tank in the Electrotechnical Laboratory at Tsukuba (12, 19). The nerve containing a giant axon was dissected and rinsed with a cold solution consisting of 0.75 M glucose, 5 mM EGTA, and 25 mM Na-MES buffer (pH 6.8). The nerve was put on a transparent rubber, one end was cut off, and the axoplasm of the giant axon was gently squeezed out using a rubber-coated roller. The extruded axoplasm was collected and stored at −80°C. After extrusion, the subaxolemmal axoplasm of giant axon, ~20 μm thick (see Fig. 1), remained. We called such a subaxolemmal axoplasm-enriched preparation the "axonal sheath." Axonal sheaths were stored at −80°C until used for purification of axolinin and 255-kD protein. Fin nerves containing a large number of small axons were also dissected out, rinsed in the above solution, and stored at −80°C.

Intraaxonal Perfusion of Squid Giant Axon

After the central part of the axoplasm was squeezed out, the giant axon was intraaxonally perfused with the following solutions successively and the perfusates were analyzed. Intraaxonal perfusion was performed according to the method described previously (14). The giant axon was first perfused with a solution containing 355 mM KF and 25 mM K-Hepes buffer (pH 7.3) for 10 min, then with a solution of 355 mM KI and 25 mM K-Hepes buffer (pH 7.3) for another 10 min, and finally with a solution of 588 mM KI, 8 mM $CaCl_2$, and 12 mM K-Hepes buffer (pH 7.3) for the last 10 min. Perfusates were collected for every 2.5 min in succession and analyzed by SDS PAGE using a 7.5% gel. The gel was stained with silver reagents (Bio-Rad Laboratories, Richmond, CA).

Purification of Axolinin

Axolinin was soluble in 0.6 M NaCl but insoluble in 0.1 M NaCl solution. Taking advantage of this solubility, axolinin was able to be purified with high recovery. All steps were done at 0–4°C. 50 axonal sheaths (0.3 g wet weight) were minced with scissors and incubated for 10 min in 1 ml of buffer A containing 0.6 M NaCl. Buffer A was composed of 10 mM Na-MES buffer (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM GTP, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml pepstatin A, and 0.01 mg/ml leupeptin. The sample was then centrifuged at 37,000 *g* for 30 min, and the pellet was discarded. The supernatant (S1 fraction; 1 ml), containing ~7 mg of protein, was diluted with 5 vol of buffer A to lower the NaCl concentration and centrifuged at 3,000 *g* for 5 min. The pellet (P2 fraction) was used for further steps to purify the axolinin, while the supernatant (S2 fraction) was saved for the purification of the 255-kD protein. The P2 fraction was dissolved with 1 ml of buffer A containing 0.6 M NaCl, and centrifuged at 200,000 *g* for 60 min. The supernatant (S3 fraction) was diluted again with 5 vol of buffer A. The purified axolinin was pelleted as P4 fraction by centrifugation at 3,000 *g* for 5 min.

Purification of 255-kD Protein

The S2 fraction obtained during axolinin purification was the starting material for the 255-kD protein purification. All steps were done at 0–4°C. 50 ml of the S2 fraction containing 35 mg of protein was applied to a DEAE-cellulose column (1 × 13 cm) equilibrated with buffer A. The proteins were eluted with a linear gradient created by 50 ml each of 0.1 and 0.6 M NaCl in buffer A. The flow rate was 20 ml/h, and 5-ml fractions were collected. Each fraction eluted from the column was analyzed by SDS PAGE. The 255-kD protein–enriched fraction (fraction 6 in Fig. 9*A*) containing 4.4 mg protein was saved and further fractionated by gel filtration through TSK-Gel G4000SW (0.75 × 60 cm). 0.5-ml fractions were collected and each fraction was analyzed by SDS PAGE. Three fractions enriched in the 255-kD protein (fractions 28–30 in Fig. 9*B*) were pooled, concentrated to 0.5 ml by ultrafiltration through Diaflo PM30 membrane (Amicon Corp., Danvers, MA) and passed through Bio Gel P-10 (Bio-Rad Laboratories) column (0.8 × 10 cm) equilibrated with a reassembly buffer consisting of 0.1 M Na-MES buffer (pH 6.8), 1 mM EGTA, 0.5 mM $MgSO_4$, and 1 mM dithiothreitol, 0.2 mM GTP, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01 mg/ml each of pepstatin A and leupeptin.

Preparations of Microtubule Proteins, Purified Tubulin, and Actin

Microtubule proteins were prepared by two or three cycles of temperature-dependent assembly and disassembly from squid axoplasm (27), squid optic ganglia (6), and rat brain (29). In the case of squid axoplasm, ~0.5 mg of microtubule protein was obtained from 30 mg of axoplasmic protein which was provided by 40 squid. To prepare rat brain microtubule proteins, 4 M glycerol was added to the reassembly buffer during only the first assembly. The purified tubulin was prepared from microtubule proteins obtained from rat brain as described elsewhere (6).

Actin was prepared from rabbit skeletal muscle according to the method developed by Spudich and Watt (30).

Co-sedimentation Experiments of Axolinin or 255-kD Protein with Microtubules and Actin Filaments

Tubulin (2 mg/ml) or actin (1.5 mg/ml in the case of axolinin; 0.7 mg/ml in the case of 255-kD protein) was polymerized in the presence or absence of the purified axolinin (0.2 mg/ml) or 255-kD protein (0.1 mg/ml) by incubation for 30 min at 30°C in 0.1 ml of a solution consisting of 0.45 M Na-glutamate, 10 mM Na-MES buffer (pH 6.8), 10% dimethyl sulfoxide, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.2 mM GTP, 0.05 mM ATP, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml pepstatin A, and 0.01 mg/ml leupeptin. The samples were then centrifuged for 30 min at 100,000 *g*, and the supernatant and pellet were analyzed by SDS PAGE.

Purification of Myosin, Spectrin, Fodrin, and Filamin

Myosin, spectrin, fodrin, and filamin were purified from rabbit skeletal muscle, human erythrocyte, rat brain, and chicken gizzard, respectively, according to the methods previously reported (3, 26, 35, 37).

Deoxyribonuclease I (DNase I) Column

DNase I (Sigma Chemical Co., St. Louis, MO) was covalently bound to agarose as described by Lazarides and Lindberg (9) using CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). 3 mg of DNase I was immobilized per 1 ml of resin.

To identify axoplasmic actin, the axoplasm was analyzed using this column. All steps were done at 4°C. Squid axoplasm (0.05 ml) was homogenized with 1 ml of a buffer B consisting of 1 mM Tris-HCl (pH 7.2), 1 mM EGTA, and 0.05 mM ATP. The homogenate was dialyzed against the buffer B for 24 h. The dialysate was applied to DNase I column (0.5 × 2.5 cm). The column was washed successively with 1 ml each of (*a*) buffer B, (*b*) 0.1 M NaCl in buffer B, (*c*) 0.6 M NaCl in buffer B, (*d*) 0.6 M NaCl and 2 mM $CaCl_2$ in buffer B, and (*e*) 3 M guanidine-HCl. Axoplasm and each fraction eluted from the column was analyzed by SDS PAGE.

SDS PAGE

The method developed by Laemmli (8) was used with stacking gel containing 3% polyacrylamide and with separation gel containing 3–12% linear gradient of polyacrylamide. The gels were stained with Coomassie Brilliant Blue R-250.

Immunological Methods

Antisera to the purified axolinin were elicited in rabbits. Approximately 0.2 mg of antigen in 0.5 ml of 0.6 M NaCl and 10 mM Na-MES buffer (pH 6.8) was emulsified with an equal volume of complete Freund's adjuvant, and intracutaneously injected at multiple sites along the backs of two rabbits. The second and third injections were repeated 4 and 5 wk after the first injection, respectively. The rabbits were bled a week after the third injection. The IgG fraction was further purified from the antisera using DEAE-cellulose ion-exchange column chromatography.

Immunoblotting was performed by SDS PAGE followed by electrophoretic transfer to nitrocellulose sheets as described by Vaessen et al. (36). Nitrocellulose sheets were treated with the anti-axolinin antibody followed by horseradish peroxidase-conjugated antibodies (Miles Laboratories, Inc., Elkhart, IN). The sheets were then treated with 4-chloro-1-naphtol solution to localize the peroxidase.

Protein Assay

Protein concentrations were determined according to the protocol of Lowry et al. (11), using bovine serum albumin as a standard. For rapid assay of protein concentrations of column elutes, the Bradford procedure (1) was used with tubulin as a standard.

Low-angle Rotary-shadowing Electron Microscopy

To study the morphology of the isolated axolinin and 255-kD protein molecules, we used the low-angle rotary-shadowing technique, mainly according to the method developed by Tyler and Branton (34). 200 μl of a solution containing axolinin (0.025 mg/ml), 0.6 M NaCl, 50% glycerol, and 10 mM Na-MES buffer (pH 6.8) or a solution containing 255-kD protein (0.035 mg/ml), 0.1 M NaCl, 50% glycerol, and 10 mM Na-MES buffer (pH 6.8) was sprayed onto freshly cleaved mica. The droplets on the mica were then dried at room temperature under vacuum (1×10^{-6} Torr) in an Eiko freeze-etch device, FD-2 (Eiko Engineering, Mito, Japan), for 10 min. Platinum was then rotary-shadowed at an angle of 5° followed by a coating from above with carbon. The replicas were floated off on the distilled water and picked up on the formvar-filmed grids. They were examined in a Hitachi 11-DS electron microscope at an accelerating voltage of 75 kV. Electron microscope negatives were contact-reversed and printed as negative images.

Results

Protein Composition of Subaxolemmal Cytoskeleton in Squid Giant Axon

After the central part of the axoplasm of a squid giant axon was squeezed out, the peripheral axoplasm, ~20 μm thick, remained under the axolemma (Fig. 1). This subaxolemmal axoplasm–enriched preparation was called the "axonal sheath." First, we compared the protein composition of the axonal sheath with that of the extruded axoplasm by the use of SDS PAGE (Fig. 2). We faced a little difficulty in the analysis of the axonal sheath proteins; some connective tissues were by no means solubilized even with a solution containing 8 M urea, 2% SDS, and 5% 2-mercaptoethanol, and disturbed the electrophoreogram (see Fig. 2, lane *4*). To avoid this difficulty, a solution containing 0.6 M NaCl, 10 mM $CaCl_2$, and 10 mM Na-MES buffer (pH 6.8) was used according to the method of Sakai and Matsumoto (27). This solution solubilized almost the same proteins as the above urea–SDS solution without any disturbance of the electrophoresis system (compare lane *3* in Fig. 2 with lane *4*). As a result, it became clear that the axonal sheath was rather simple in its protein composition when compared to the central axoplasm. The electrophoretic banding pattern of the axonal sheath was characterized by large amounts of the two HMW polypeptides designated 255 kD and 260 kD. Judging from its molecular mass, the 260-kD polypeptide was considered to be identical to the "axolinin" designated by Sakai et al. (28). The 255-kD protein and axolinin accounted for 1.7 and 1.5% of the total protein in extruded axoplasm of a giant axon, and 5.2 and 8.9% of that in the axonal sheath extract, respectively. Considering that the protein composition of the whole axonal sheath solubilized with a solution containing 8 M urea and 2% SDS highly resembles that of the axonal sheath extract (see Fig. 2), we can conclude that both 255-kD protein and axolinin were highly concentrated in the subaxolemmal axoplasm. In addition to these proteins, the 45-kD, 53-kD, and 55-kD polypeptides were also predominant in the extracts of axonal sheaths, which might correspond to actin, β-tubulin, and α-tubulin, respectively.

To demonstrate that axolinin, 255-kD protein, tubulin, and actin were really located in the subaxolemmal axoplasm, a

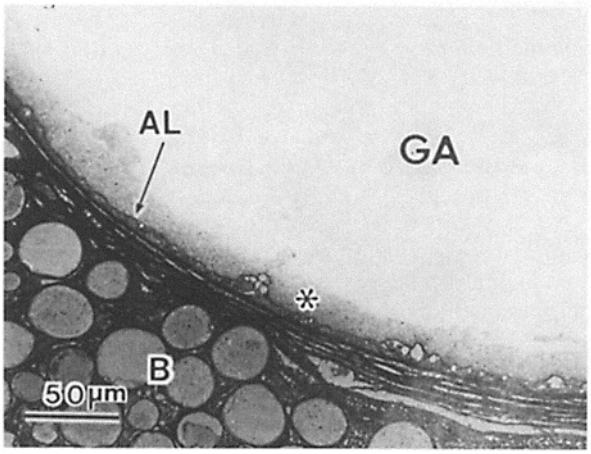

Figure 1. Light micrograph of transverse section of the squid nerve containing a giant axon (*GA*) after the central part of axoplasm has been squeezed out followed by intraaxonal perfusion. The giant axon is surrounded by the bundle of small axons (*B*). After the intraaxonal perfusion, the subaxolemmal axoplasm (*), ~20 μm thick, remains under the axolemma (*AL*).

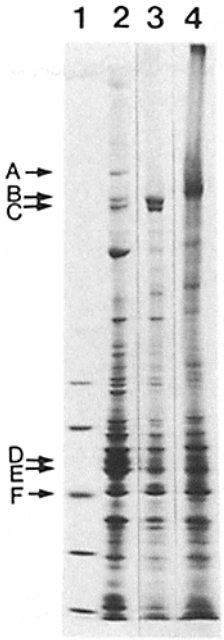

Figure 2. SDS PAGE of the extruded axoplasm of the squid giant axon and the axonal sheath extract. (Lane *1*) Standard proteins used as mobility markers consisting of phosphorylase b (92 kD), bovine serum albumin (68 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), and cytochrome *c* (12 kD); (lane *2*) axoplasm extruded from squid giant axon by a roller; (lane *3*) axonal sheath proteins extracted with 3 vol of 0.6 M NaCl, 10 mM $CaCl_2$, and 10 mM Na-Hepes buffer (pH 7.2) for 10 min at 0°C; (lane *4*) axonal sheath proteins extracted with 3 vol of 8 M urea, 2% SDS, 5% 2-mercaptoethanol, and 0.1 M Tris-HCl buffer (pH 6.8) for 2 min at 100°C. Note that, among HMW polypeptides (*A*, 320 kD; *B*, 260 kD; C, 255 kD), the axolinin (*B*) and 255-kD protein (*C*) are enriched in the axonal sheath extract, while the 320-kD protein (*A*) is detected only in the extruded axoplasm. Tubulin (*D* and *E*) and actin (*F*) are major constituents of both the central and subaxolemmal axoplasm.

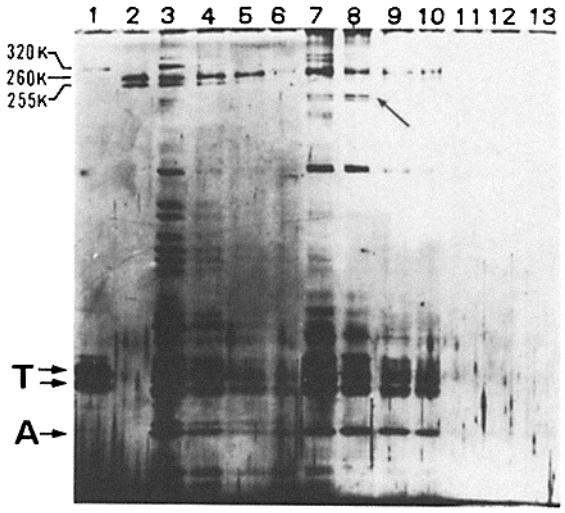

Figure 3. SDS PAGE of the proteins released from the interior of a squid giant axon during intraaxonal perfusion, first with the KF solution for 10 min, then with the KI solution for another 10 min, and finally with the KI-Ca solution for the last 10 min (see Materials and Methods for details). Perfusates were collected for every 2.5 min in succession and analyzed by SDS PAGE combined with silver staining method. (Lane *1*) Microtubule proteins prepared from squid optic ganglia; (lane *2*) mixture of the purified axolinin and 255-kD protein; (lanes *3–6*) perfusates of KF solution; (lanes *7–10*) perfusates of KI solution; (lanes *11–13*) perfusates of KI-Ca solution. It is clearly shown that the KF solution releases large amounts of axolinin (*260K*) and 255-kD protein (*255K*) together with tubulin (*T*) and actin (*A*). Note that an appreciable amount of actin is released together with "fodrin" (arrow) after the perfusion with KI solution.

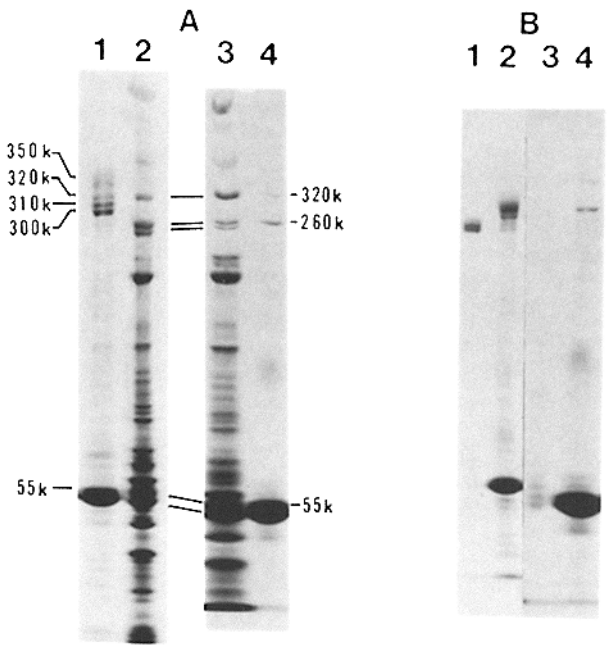

Figure 4. SDS PAGE of microtubule proteins prepared from rat brain and squid axoplasm. (*A*) Electrophoretic banding pattern of isolated microtubules. The HMW-MAPs of squid axoplasmic microtubules (lane *4*) is rather simple, consisting of axolinin (*260K*) and a 320-kD protein (*320K*), while the rat brain microtubules (lane *1*) contain four polypeptides ranging from 300 to 350 kD as HMW-MAPs. Lanes *2* and *3* show axoplasm of squid giant axon. (*B*) Heat treatment of microtubule proteins at 100°C for 2 min. In rat brain microtubule preparations, 320-kD and 350-kD MAPs are precipitated together with tubulin after heat treatment followed by centrifugation (lane *2*), while 300-kD and 310-kD MAPs are heat resistant and recovered in supernatant (lane *1*). In the case of squid axoplasmic microtubules, both axolinin and 255-kD protein were precipitated by heat treatment (lane *4*), and no MAPs are recovered in supernatant (lane *3*).

squid giant axon was intraaxonally perfused with several kinds of solutions after its central part of the axoplasm was squeezed out, and the perfusate was collected and analyzed by the use of SDS PAGE combined with the silver staining method (Fig. 3). As shown in lanes *3–6* of Fig. 3, appreciable amounts of axolinin and 255-kD protein were released together with other proteins containing tubulin by the perfusion of a solution consisting of 355 mM KF and 25 mM K-Hepes buffer (pH 7.3). This solution was routinely used as the internal solution in physiological experiments to maintain the excitability of the axolemma. After the perfusion solution was switched to the solution composed of 355 mM KI and 25 mM K-Hepes buffer (pH 7.3), large amounts of axonal proteins containing tubulin, actin, and axolinin were released (Fig. 3, lanes *7–10*). It should be noted that after the KI solution, a great deal of actin came off together with two HMW polypeptides (235- and 240-kD) which might correspond to the polypeptides called "fodrin" by Morris and Lasek (21). This seemed to indicate that actin was located very close to the axolemma or that actin filament was stabilized inside axons compared to microtubules. Almost no polypeptides were detected in the perfusate obtained by perfusion with a solution composed of 355 mM KI, 8 mM $CaCl_2$, and 25 mM K-Hepes buffer (pH 7.3).

These findings have led us to conclude that the subaxolemmal axoplasm was mainly composed of axolinin (8.9% of total protein), 255-kD protein (5.2%), α,β-tubulin (8.0%), actin (5.4%), and some other polypeptides. Next, we have studied some biochemical properties of these proteins isolated from the squid giant axon.

Isolation of Microtubule Proteins from Axoplasm of Squid Giant Axon

Microtubule proteins from the extruded axoplasm of squid giant axons were prepared by two cycles of temeprature-dependent assembly and disassembly as described by Sakai and Matsumoto (27). Approximately 0.5 mg of microtubule proteins was obtained from 30 mg of axoplasmic proteins which was provided by 40 squid. Microtubules prepared from squid axoplasm contained two main components of HMW microtubule-associated proteins (HMW-MAPs) of 320 kD and 260 kD (Fig. 4*A*, lane *4*), both of which were precipitated by heat treatment for 2 min at 100°C (Fig. 4*B*, lane *4*). The 260-kD MAP was considered to be axolinin. As shown in Fig. 2, the 320-kD MAP was localized only in the central axoplasm but not in the subaxolemmal axoplasm. For comparison, microtubules were prepared from rat brain using the same isolation procedure. The rat brain microtubules were shown to contain four polypeptides ranging from 300 to 350 kD as HMW-MAPs (Fig. 4*A*, lane *1*). The 300- and 310-kD MAPs were heat resistant (Fig. 4*B*, lane *1*), while the 320- and 350-kD MAPs were heat sensitive (Fig. 4*B*, lane *2*). Although it is difficult to simply compare the HMW-MAPs of squid giant

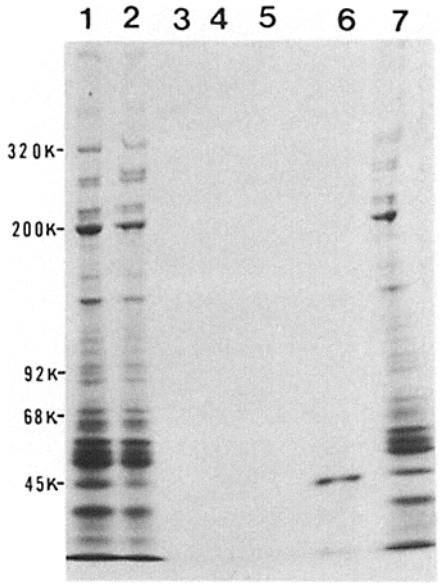

Figure 5. Identification of actin in the axoplasm of squid giant axon by the use of DNase I column. (Lanes *1* and *7*) Extract of axoplasm applied to DNase I column (see Materials and Methods for details); (lane *2*) combined fraction of flow-through and low salt wash; (lane *3*) 0.1 M NaCl eluate; (lane *4*) 0.6 M NaCl eluate; (lane *5*) 0.6 M NaCl-2 mM $CaCl_2$ eluate; (lane *6*) 3 M guanidine-HCl eluate. The 45-kD protein is specifically adsorbed on and eluted from the column (lane *6*), indicating that this 45-kD protein is actin.

axon with those of rat brain, it is safe to say that the HMW-MAP family of squid giant axon is rather simple, and that the central and peripheral parts of the axoplasm contain different types of HMW-MAPs, the 320-kD MAP and axolinin, respectively.

Identification of Actin in Axoplasm of Squid Giant Axon

To study whether the 45-kD polypeptide in the axoplasm was really actin, the extruded axoplasm was analyzed using the DNase I affinity column according to the method developed by Lazarides and Lindberg (9) (Fig. 5). As a result, the 45-kD polypeptide was specifically adsorbed on and eluted from the DNase I column under the same condition as actin, indicating that the 45-kD polypeptide was actin. Actin was identified in large amounts in both central and peripheral axoplasm (see Fig. 2). In the condition used here, no actin-binding protein was trapped by the DNase I column.

Purification of Axolinin from Axonal Sheaths

We found that axolinin was soluble in 0.6 M NaCl solution but insoluble in 0.1 M NaCl solution. Because of this characteristic, only successive centrifugations separated axolinin from other proteins with high recovery (Fig. 6). In the crude extract (S1 fraction; see Materials and Methods for details) containing 0.6 M NaCl from axonal sheaths, axolinin, 255-kD protein, tubulin, actin, and some other proteins including neurofilament 60-kD protein were prominent (Fig. 6, lane *2*). When the extract was diluted to 0.1 M NaCl solution followed by centrifugation, in the pellet (P2 fraction) the axolinin was strikingly enriched (Fig. 6, lane *4*) and the 255-kD proteins and other proteins were recovered in the supernatant (S2 fraction; Fig. 6, lane *3*). The P2 fraction was dissolved in 0.6 M NaCl solution, and the precipitation–centrifugation procedure was repeated two more times. As a result, the axolinin was obtained in a high degree of purity (Fig. 6, lane *8*). Approximately 1 mg of the purified axolinin was obtained from 50 giant axonal sheaths. Fin nerves were also a good source to purify the axolinin (22). Approximately 8 mg of the axolinin was purified from 50 fin nerves (1 g wet weight).

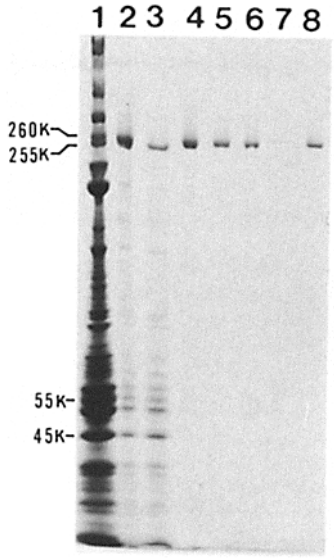

Figure 6. Purification of axolinin. The protein composition of each fraction in the steps of the purification was analyzed by SDS PAGE. For details of the purification procedure, see Materials and Methods. (Lane *1*) Axoplasm; (lane *2*) S1 fraction, 0.6 M NaCl extract of axonal sheath; (lane *3*) S2 fraction, 0.1 M NaCl-soluble fraction from S1; (lane *4*) P2 fraction, 0.1 M NaCl-insoluble fraction from S1; (lane *5*) S3 fraction, supernatant after centrifugation of P2 dissolved in 0.6 M NaCl; (lane *6*) P3 fraction; (lane *7*) S4 fraction, 0.1 M NaCl-soluble fraction from S3; (lane *8*) P4 fraction, 0.1 M NaCl-insoluble fraction from S3. The axolinin (*260K*) is purified with high recovery as P4 fraction (lane *8*). Note that the 255-kD protein is enriched in S2 fraction (lane *3*).

Molecular Shape of Axolinin Molecule

The molecular shape of purified axolinin molecules in 0.6 M NaCl solution was analyzed by means of low-angle rotary-shadowing electron microscopy. The axolinin molecule looked like a straight rod ~105 nm long with a globular head at one end (Fig. 7). This molecule tended to form a ring structure by head-to-tail association within one molecule (Fig. 7, *a*, *e*, and *f*). Occasionally, some molecules were split into two strands which were arranged parallel to each other (Fig. 7*d*), suggesting that the native axolinin might be a homodimer in 0.6 M NaCl solution.

Co-sedimentation Experiments of Axolinin with Microtubules and Actin Filaments

Binding ability of the purified axolinin to microtubules and actin filaments was studied by co-sedimentation experiments (Fig. 8). After the incubation of tubulin or actin with the purified axolinin at 30°C for 30 min, the samples were centrifuged to precipitate assembled microtubules or actin filaments. The proteins in the supernatant and pellet were then analyzed by electrophoresis. Lacking either tubulin or actin, about 16% of the total amount of axolinin was sedimented. However, in the presence of tubulin, the sedimented axolinin remarkably increased in amount, to 70% of the total axolinin. On the other hand, actin filaments did not induce the sedimentation of axolinin. Considering that the axolinin itself had no effects on the sedimentation properties of tubulin and actin, it was concluded that axolinin could bind to microtu-

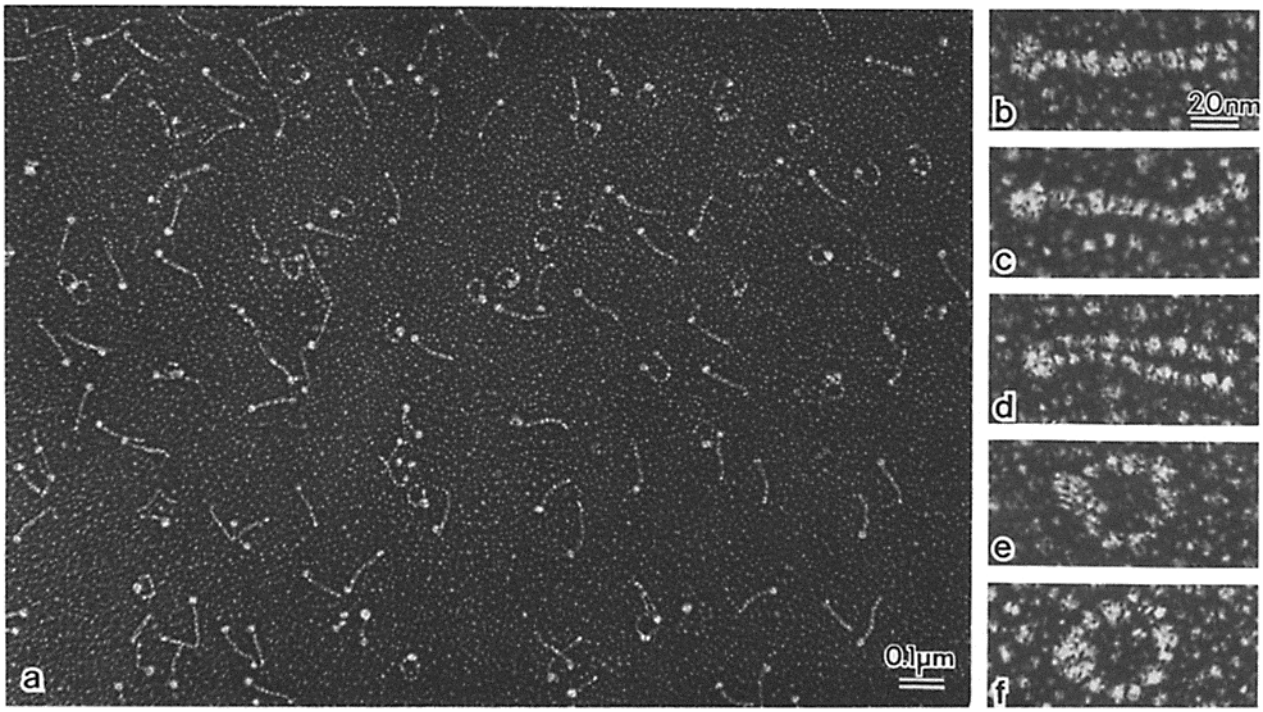

Figure 7. Morphology of axolinin molecule in 0.6 M NaCl solution in rotary-shadowed preparations. Axolinin molecule appears as a straight rod about 105 nm long with a globular head at one end (*a–c*). Some molecules are split into two thinner strands (*a* and *d*) and form ring structures (*a*, *e*, and *f*).

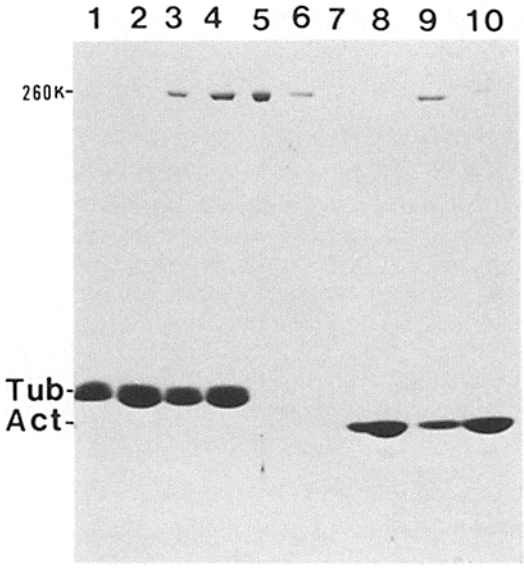

Figure 8. Co-sedimentation experiments on axolinin with microtubules and actin filaments. For details of experimental conditions, see Materials and Methods. Lanes *1*, *3*, *5*, *7*, and *9* represent the protein composition of the supernatant of each experiment, and lanes *2*, *4*, *6*, *8*, and *10* represent that of the pellet. (Lanes *1* and *2*) Only tubulin was incubated; (lanes *3* and *4*) axolinin and tubulin were incubated; (lanes *5* and *6*) only axolinin was incubated; (lanes *7* and *8*) only actin was incubated; (lanes *9* and *10*) axolinin and actin were incubated. Axolinin specifically co-sediments with microtubules.

bules but not to actin filaments in vitro. Addition of 2 mM $CaCl_2$ to the incubation medium had no effect on the co-sedimentation of the axolinin with actin filaments (data not shown). Recently, similar results have been obtained by Murofushi et al. using different conditions for the co-sedimentation experiments (22).

Purification of 255-kD Protein from Axonal Sheaths

The S2 fraction obtained during the purification of the axolinin was the starting material for the preparation of the 255-kD protein. Upon SDS PAGE of the S2 fraction, the 255-kD protein, α,β-tubulin, and actin were prominent, and some other polypeptides such as neurofilament 60-kD protein were also present (see Fig. 6, lane *3*). DEAE-cellulose column chromatography yielded a fraction enriched in the 255-kD protein (Fig. 9*A*). A fraction of fraction 6 shown in Fig. 9*A* was further fractionated by gel filtration (Fig. 9*B*). As a result, ~0.7 mg of the highly purified 255-kD protein was obtained from 50 axonal sheaths. In sharp contrast with axolinin, the purified 255-kD protein was soluble in both 0.1 and 0.6 M NaCl solution. When the purified 255-kD protein was co-electrophoresed with the purified axolinin, it was evident that these proteins were distinct in their molecular masses (Fig. 9*C*).

Molecular Shape of 255-kD Protein Molecule

In low-angle rotary-shadowing electron microscopic images, the 255-kD protein molecule appeared as a characteristic horseshoe-shaped structure with diameter of ~35 nm (Fig. 10). Neither in 0.1 nor in 0.6 M NaCl solution was there an indication that this protein formed aggregates.

Interaction of the 255-kD Protein with Microtubules and Actin Filaments

The microtubules isolated from the squid giant axon did not contain the 255-kD protein (see Fig. 4), indicating that the

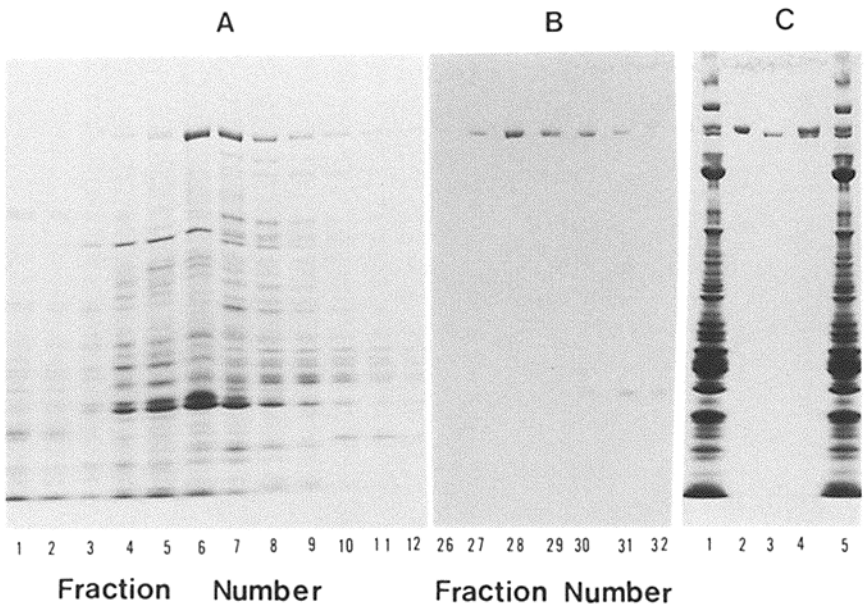

Figure 9. Purification of the 255-kD protein. (*A*) Fractionation by DEAE-cellulose column chromatography. The S2 fraction in Fig. 6 was applied on the column. Fraction No. 6 was further fractionated. (*B*) Fractionation by gel filtration through TSK-Gel G4000SW. Three fractions, fractions 28–30, were combined. (*C*) Comparison of the molecular mass of the purified axolinin (lane *2*) and the 255-kD protein (lane *3*). When these proteins are co-electrophoresed (lane *4*), two bands are clearly separated. Lanes *1* and *5* show the protein composition of axoplasm extruded from giant axon.

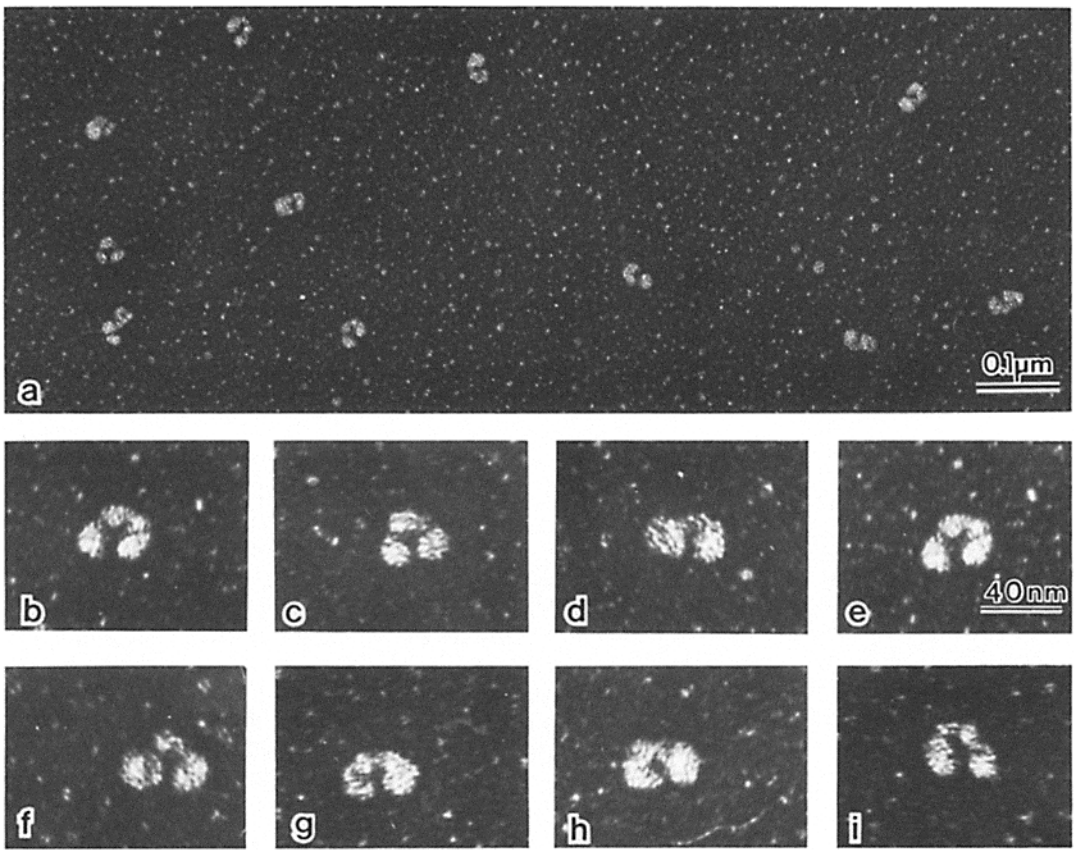

Figure 10. Molecular shape of the 255-kD protein molecule in 0.1 M NaCl solution. The 255-kD protein molecule appeared as a characteristic horseshoe-shaped structure ~30–35 nm in diameter.

255-kD protein might not bind to microtubules. Actually, when the purified 255-kD protein was incubated with microtubules followed by centrifugation, only 8% of the total amount of the 255-kD protein was sedimented. The same amount of 255-kD protein was precipitated in the absence of tubulin (Fig. 11). On the other hand, in the presence of actin filaments, 62% of the total 255-kD protein was sedimented. The presence of the 255-kD protein had no effect on the sedimentation properties of tubulin and actin. Taken together, it was concluded that the 255-kD protein was actin binding but not tubulin binding.

Interaction of the 255-kD protein with actin was further studied by viscometry at a low-shear rate (Fig. 12). Solution containing 255-kD protein and G-actin was sucked up into a

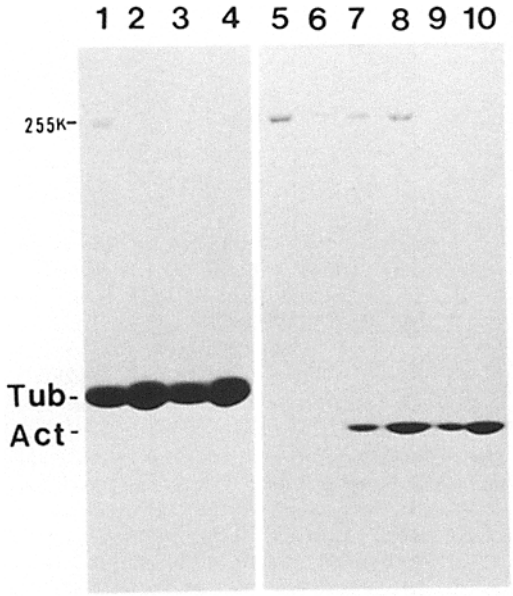

Figure 11. Co-sedimentation experiments of the 255-kD protein with microtubules and actin filaments. For details of the experimental conditions, see Materials and Methods. Lanes *1*, *3*, *5*, *7*, and *9* represent the protein composition of the supernatant, and lanes *2*, *4*, *6*, *8*, and *10* represent that of the pellet. (Lanes *1* and *2*) 255-kD protein and tubulin were incubated; (lanes *3* and *4*) only tubulin was incubated; (lanes *5* and *6*) only 255-kD protein was incubated; (lanes *7* and *8*) 255-kD protein and actin were incubated; (lanes *9* and *10*) only actin was incubated. 255-kD protein co-sediments with actin filaments, but not with microtubules.

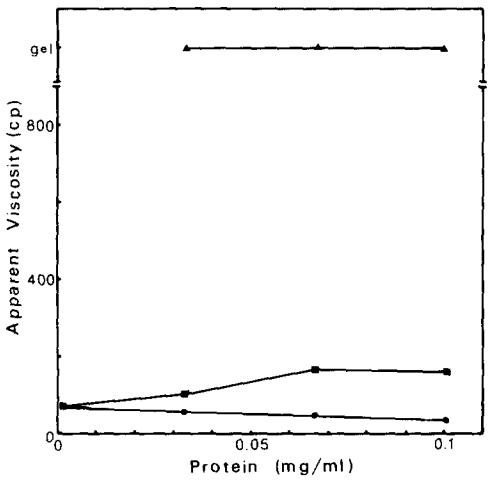

Figure 12. Interaction of the 255-kD protein (■), axolinin (●), or chicken gizzard filamin (▲) with actin filaments as studied by low-shear viscometry. The basic mixture consisted of 0.45 M Na-glutamate, 10 mM Na-MES buffer (pH 6.8), 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM ATP, and 0.2 mg/ml actin. Samples were incubated for 30 min at 37°C before measurement of viscometry. The viscosity of actin solution increases gradually with increasing concentration of the 255-kD protein, but its ability to gel the actin solution is very low compared to that of filamin. Axolinin does not increase the viscosity of the actin solution.

capillary pipette, and the falling ball assay was performed after 30 min at 37°C. As a result, with increasing concentration of the 255-kD protein, the viscosity of the actin solution increased gradually, but compared to the chicken gizzard filamin its ability to gel the actin solution was very low.

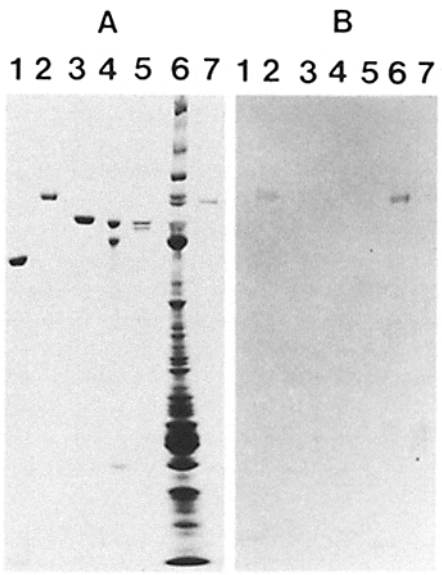

Figure 13. Immunological reactivity of anti-axolinin antibodies with several HMW proteins. (*A*) SDS polyacrylamide gels stained with Coomassie Brilliant Blue; (*B*) nitrocellulose replica stained with anti-axolinin. (Lane *1*) Rabbit skeletal muscle myosin; (lane *2*) purified axolinin; (lane *3*) chicken gizzard filamin; (lane *4*) human erythrocyte spectrin; (lane *5*) rat brain fodrin; (lane *6*) squid axoplasm; (lane *7*) purified 255-kD protein. This antibody exclusively reacts with axolinin.

Immunological Reactivity of Anti-Axolinin Antibodies with Squid Axoplasmic Proteins and Some Other HMW Proteins

To clarify the difference or similarity of axolinin with other HMW proteins, especially with the 255-kD protein, antisera to the purified axolinin were elicited in rabbits and the immunological properties of the axolinin were characterized by the immune blotting procedure using peroxidase (Fig. 13). When the squid axoplasm was analyzed by SDS PAGE followed by immune blotting, anti-axolinin stained exclusively the band of axolinin, but not the band of 255-kD protein (Fig. 13, lane *6*). The purified 255-kD protein showed no cross-reactivity with the anti-axolinin (Fig. 13, lane *7*), while this antibody strongly stained the band of the purified axolinin (Fig. 13, lane *2*). Since the axolinin molecule resembled a straight rod (see Fig. 7), the following HMW proteins showing rod-like molecular shape were purified and their reactivity to the anti-axolinin was analyzed: rabbit skeletal muscle myosin, chicken gizzard filamin, human erythrocyte spectrin, and rat brain fodrin. None of them showed any cross-reactivity (Fig. 13, lanes *1*, *3*, *4*, and *5*). Furthermore, this antibody did not stain any of the HMW-MAPs obtained from rat brain (data not shown).

Gel Filtration Profile of Axolinin and 255-kD Protein

To estimate the subunit configuration of the axolinin and the 255-kD protein, both proteins were passed through a TSK-gel G4000SW column together with rat brain spectrin (tetramer), rabbit skeletal muscle myosin (dimer), and chicken gizzard filamin (dimer) (Fig. 14). The axolinin in the 0.6-M

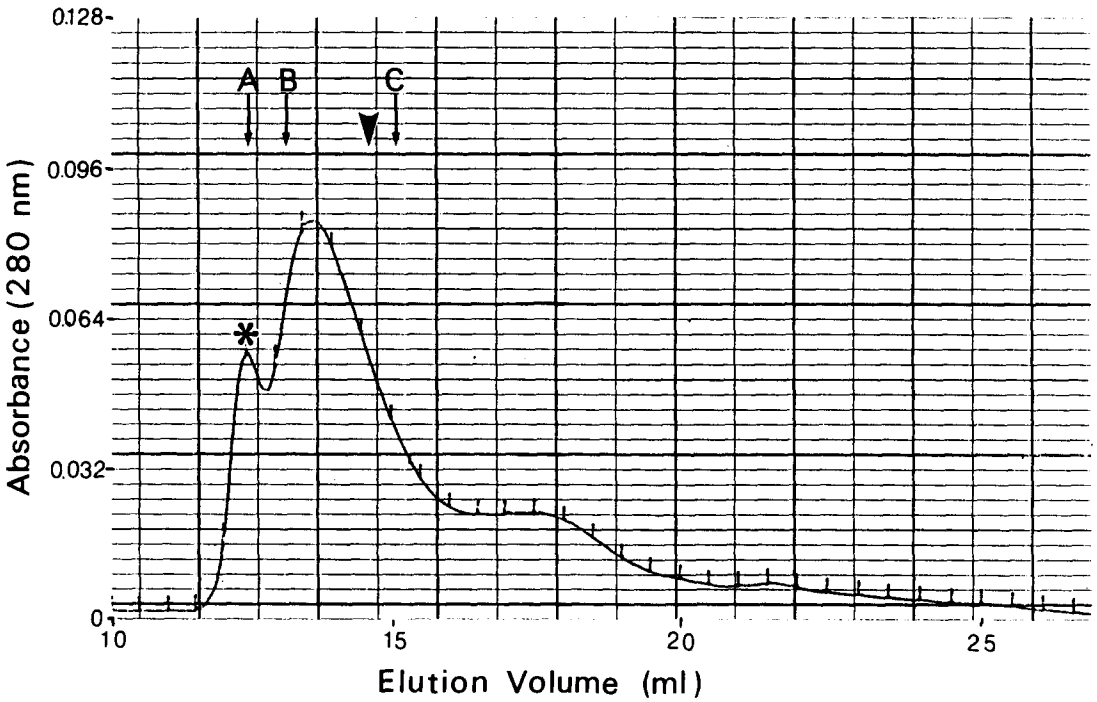

Figure 14. Gel filtration profile of purified axolinin. The purified axolinin in buffer A containing 0.6 M NaCl (see Materials and Methods) was passed through a TSK-gel G4000SW column equilibrated with buffer A containing 0.6 M NaCl. Absorbance at 280 nm of each fraction was monitored. For comparison, rat brain fodrin (tetramer), rabbit muscle myosin (dimer), and chicken gizzard filamin (dimer) were passed through the same column under the same condition. The peak positions of these proteins are indicated by *A*, *B*, and *C*, respectively. The 255-kD protein was shown to elute at the position indicated by arrowheads. *, flow-through fractions.

NaCl solution was eluted between myosin and filamin. Considering that myosin and filamin molecules as well as axolinin molecules appear as rod-shaped structures ~100–150 nm in length, it is safe to say that the axolinin molecule in the 0.6-M NaCl solution may be a dimeric form rather than a monomeric form. The 255-kD protein was eluted between axolinin and filamin. Taking into consideration that 255-kD protein molecule appears as a horseshoe-shaped structure with diameter of 35 nm and that a globular protein is eluted more slowly than rod-shaped proteins with similar molecular masses, it may well be that the purified 255-kD protein is also a dimeric form rather than a monomeric form.

Discussion

Our present results have clearly demonstrated that large amounts of two distinct unique HMW proteins, axolinin and 255-kD protein, are localized in the subaxolemmal cytoskeleton of squid giant axon together with tubulin and actin, and that the purified axolinin and 255-kD protein can bind to microtubules and actin filaments in vitro, respectively. These HMW proteins were also found in the central axoplasm extruded from squid giant axons, but only in small amounts. We suppose that these two proteins, together with microtubules and actin filaments, may form a densely packed cytoskeletal network tightly bound to the axolemma, since this subaxolemmal cytoskeleton is hardly extruded when the axon is squeezed with a roller (see Fig. 1). The morphological aspects of the subaxolemmal cytoskeleton will be described in the following paper (33).

When the microtubule proteins were prepared from the axoplasm of squid giant axons, the axolinin was co-purified with microtubules. In this sense, the axolinin can be categorized as HMW-MAPs. Actually, in vitro, the purified axolinin was co-sedimented with microtubules. Recently, Murofushi et al. have shown that the axolinin has no ability to promote the polymerization of purified tubulin, while MAP-2 prepared from porcine brain has this ability (22). Furthermore, the molecular shape of the axolinin molecule demonstrated in this study seems distinct from that of the MAP-2 molecule, which has been shown to look like a very thin flexible strand ~180 nm in length without a head-like globular structure on its end. The axolinin was heat sensitive, while MAP-2 was heat resistant. When these characteristics are taken together, it can be concluded that axolinin is not a MAP-2–like protein. Since our knowledge of the in vitro nature of the mammalian HMW-MAPs other than MAP-2 is still fragmentary, it is premature to further compare axolinin with the mammalian HMW-MAPs. However, it is interesting to speculate that some of the mammalian HMW-MAPs may resemble axolinin and constitute the subaxolemmal axoplasm in mammalian axons.

The 255-kD protein was also enriched in the subaxolemmal cytoskeleton of squid giant axon. Judging from its ability to bind to actin filaments, its molecular shape, and its solubility in 0.1 M NaCl solution, it can be said that the 255-kD protein is not a degradation product of the axolinin. This was confirmed by the fact that the 255-kD protein showed no cross-reactivity with anti-axolinin antibodies. The localization of the 255-kD protein in the squid giant axon, its binding property to actin filament, and its molecular mass of 255 kD have persuaded us to consider the relation of the 255-kD protein and spectrin-like proteins. Recently, a spectrin-like

protein, fodrin/calspectin, has been isolated from mammalian brain (3, 5, 32), and revealed to be localized just beneath the axolemma in mammalian myelinated axons (3, 10, 25). Rat fodrin was reported to be composed of two distinct HMW polypeptides (designated 235 kD and 240 kD) and to bind to actin filaments. However, the 255-kD protein should not be regarded as a spectrin-like protein, mainly for the following reasons. (*a*) The molecular shape of the 255-kD protein resembled a horseshoe-shaped globular structure ~35 nm in diameter, while the fodrin molecule looked like a flexible rod ~100 nm in length in a dimeric form. (*b*) Antibodies to chicken erythrocyte spectrin cross-reacted with rat brain fodrin, but not with the squid 255-kD protein (data not shown). (*c*) As Morris and Lasek have pointed out (21), two HMW polypeptides (235 kD and 240 kD) are present in the same amounts in the axoplasm of the squid giant axon, and their molecular masses are completely identical to the mammalian fodrin (for example, see Fig. 3). Therefore, at present, it is difficult to identify the HMW proteins in vertebrate cells that resemble the axolinin or the 255-kD protein. We believe that this difficulty does not represent a species difference and that the proteins similar to axolinin and 255-kD protein will be identified in vertebrate axons in the future, since at least axolinin is known to play an important role in the excitation of the axolemma in squid giant axon (14).

It remains to be elucidated whether the 255-kD protein and axolinin can bind to neurofilaments, but so far there is no data which suggests that these proteins are neurofilament-binding proteins. Pant et al. (23) analyzed the protein composition of the neurofilament-enriched fraction obtained from squid giant axons by SDS PAGE. Judging from their data, it is clear that only a trace of 260 kD and 255 kD proteins are co-purified with neurofilaments. Furthermore, a recent study by Zackroff and Goldman (39) has also shown that the neurofilaments purified from squid brain contain the 220-kD protein but neither the 260-kD nor the 255-kD proteins.

Tasaki and his colleagues have pointed out the importance of the protein layer beneath the axolemma of a squid giant axon as a reservoir of a specific protein indispensable to the electrical excitability of the axolemma (31). They have stressed that this electrical excitability deteriorates when a certain specific subaxolemmal protein is released from this protein layer. One of such proteins has been identified as a 12-kD protein. It may be, however, that this 12-kD protein is a component of the intraaxonally perfused pronase itself or a proteolytic product derived from the subaxolemmal proteins, as Tasaki et al. have stated themselves (31). It should be noted that the amount of the 12-kD polypeptide is not great either in the extruded axoplasm or in the axonal sheath. On the other hand, Matsumoto and his colleagues have suggested that axonal microtubules with the 260-kD protein (axolinin) may take a direct role in generating sodium currents in squid giant axon (7, 14–18, 27). Thus, it is possible to speculate that the 12-kD protein may be a degradation product of tubulin or axolinin. These studies have provided the crucial information on the physiological roles of the subaxolemmal cytoskeleton in membrane excitation, and the present study has clearly demonstrated the molecular organization of the subaxolemmal cytoskeleton in squid giant axon. However, the most important and fundamental problem remains to be elucidated: what kind of interaction occurs between sodium channels and subaxolemmal cytoskeletons at the molecular level during membrane excitation? There seem to be far more axolinin and 255-kD protein molecules than sodium channels in a squid giant axon. Taken together with the morphological data shown in the following paper (33), we can conclude that neither axolinin nor 255-kD protein works as a cross-linker between sodium channels and the subaxolemmal cytoskeleton. We have just begun to search for the cytoskeletal proteins which can directly interact with sodium channels. For this purpose, we believe the squid giant axon with its simple protein composition of the subaxolemmal cytoskeleton will continue to serve as an ideal model system.

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