FURTHER CHARACTERIZATION OF BRAIN ACTIN BY ELECTRON MICROSCOPY

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ABSTRACT

The physical state of actin in nerve ending preparations and its relationship to the membranes was studied at the ultrastructural level by negative staining with uranyl acetate before and after treatment with muscle heavy meromyosin (HMM). Actin prepared from synaptosomal or synaptic membrane preparations did not polymerize to fiber formation as readily as did striated muscle actin under the same conditions. Treatment of these brain actin preparations with HMM, however, resulted in formation of fibers characteristically decorated with arrowheads which were quite similar to those formed with muscle actin. Treatment of the synaptosomal or synaptic membrane fractions themselves with HMM caused the formation of numerous decorated fibers although fibers were not evident before HMM treatment. This did not occur with the presynaptic vesicle fraction. The studies suggest that at least part of the actin is associated with synaptic membranes and is in a partially polymerized or non-polymerized state; polymerization can be induced by HMM.

INTRODUCTION

Actin has been prepared from bovine (3, 24, 28) and chick (11, 19) brain as well as cultures of chick sympathetic ganglia (11). It has also been isolated from nerve ending preparations of rat brain (4, 5, 29), as well as the membrane fraction separated from such synaptosomal preparations (4, 5).

Based on biochemical evidence that there is a disparate distribution of the actin-like and myosin-like proteins between membrane and vesicle enriched fractions, respectively, the hypothesis was proposed that these contractile-like proteins may play a role in transmitter release via a chemo-mechanical transduction at sites of interaction between presynaptic vesicles and membranes (4). Such a mechanism for transmitter release would depend in part on the manner and the state in which the actin is associated with the mem-

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398
branes of the nerve endings. Other possible functions have been discussed in a review article (2).

Actin characteristically interacts with fragments of myosin (subfragment 1, S1 and heavy meromyosin, HMM) which are visualized as arrowhead configurations at the electron microscope level (14, 16). Actin filaments isolated from chick brain have been decorated with S1 (6). Filaments decorated with heavy meromyosin (HMM) have been demonstrated in cultures of neuroblastoma (7, 8) and brain tissue sections (21, 23). In these studies, based on the techniques developed by Huxley (14) and Ishikawa et al. (16), the preparations are extracted with glycerol before treatment with the HMM for the formation of filaments decorated with arrowhead configurations. The preparations are then fixed, embedded, cut and stained before examination in the electron microscope.

In the present study subcellular brain fractions, as well as the actin isolated from such fractions, were examined directly on the grid by negative staining with uranyl acetate. The negative staining technique is a more rapid and direct approach and usually gives more distinct arrowhead decorations. Since actin can be isolated directly from such subcellular fractions the intent of this study was to see whether information could be obtained as to the state of the actin in these nerve endings and the relationship of the actin to the membrane.

METHODS

Muscle Proteins:

Muscle actin and myosin were prepared from rabbit skeletal muscle using the method of Rees and Young (30) for actin and a modification of the procedure of Richards et al. (31) for myosin. Heavy meromyosin was prepared from myosin utilizing the procedure of Ikemoto et al. (15). The proteins were characterized by SDS gel electrophoresis (37) and measurement of ATPase activity as previously described (3). The actin was monitored by examining its ability to enhance the Mg$^{2+}$-stimulated ATPase activity of myosin. Heavy meromyosin was tested for its Ca$^{2+}$-stimulated ATPase activity as well as its ability to interact with actin to generate Mg$^{2+}$-stimulated activity.

Brain Proteins:

Synaptosomal fractions were prepared utilizing a discontinuous Ficoll gradient (29). The procedure of DeRobertis et al. (10), as previously modified (4, 29), was utilized to obtain synaptic membrane, synaptic vesicle and osmotic shock supernatant fractions. Actin was prepared from an acetone powder of intact synaptosomal and membrane fractions. The synaptosomes or membranes were treated with ice-cold acetone (10–20 volumes), filtered on a Buchner funnel and dried in vacuo. The acetone powder was then extracted with 20 volumes of 0.5 mM ATP, 0.5 mM mercaptoethanol, 0.2 mM CaCl$_2$, made pH 7.5 with NaOH (Solvent A) and centrifuged at 100,000 x g for 1 hour. The supernatant was made 0.1 M in KCl and 1 mM in MgCl$_2$ and polymerized at room temperature for 2 hours and then stored at 4° overnight. It was then centrifuged for 3 hours at 100,000 x g. The resulting pellet was suspended in 0.1 M KCl, 1 mM MgCl$_2$, 0.01 M Tris, pH 7.5 (Buffer B) with gentle homogenization. Actin was obtained directly from the supernatant fraction by generating conditions favorable for the polymerization of actin (0.1 M KCl, 1 mM MgCl$_2$, 0.5 mM ATP), and then after 2 hr. at room temperature and overnight at 4°C, it was centrifuged for 3 hrs. at 100,000 x g to pellet the protein (25).
Electron Microscopy:

Actin preparations (0.2–0.3 mg/ml) were examined on 400 mesh carbon-formvar coated grids. One drop of material to be studied was placed on the grid for 60 seconds. The grid was flooded with Buffer B and the excess drawn off with filter paper. In some preparations, HMM (0.4–0.5 mg/ml) was reacted with the actin another 60 seconds before flooding the grid with HMM solvent buffer (0.1 M KCl, 0.05 M Tris, pH 7.2). These preparations were negatively stained with 1% aqueous uranyl acetate for 90 seconds, excess stain drawn off with filter paper, dried in air and examined in a Hitachi HU-11F electron microscope.

Synaptosomal, membrane and vesicle preparations were examined in a similar fashion.

RESULTS

Actin prepared from rabbit skeletal muscle and rat brain synaptosomes by similar methods showed different characteristics upon inspection in the electron microscope following negative staining. Polymerized muscle F-actin, shown in Figure 1, formed fibers 70Å ± 10Å in diameter. In contrast, the actin prepared from rat brain synaptosomes showed few fibers, although this actin was prepared under conditions which favor polymerization of muscle actin. The brain actin consisted of amorphous aggregates with no apparent regularity of size or structure (Figure 2A).

Treatment of this brain actin preparation with HMM directly on the electron microscope grid (followed by negative staining) resulted in formation of decorated fibers (Figure 2B, C). These decorations were comparable to the acto-HMM complex of muscle (compare Figure 2 and 1); the brain acto-HMM had a diameter of 80Å ± 10Å and a periodicity of 350Å ± 20Å. The arrowheads on any single fiber were unidirectional.

Attempts were made to produce polymerized brain actin fibers by varying the isolation conditions. The absence of calcium from the extraction and polymerizing medium did not affect the ability of the actin to polymerize or become decorated by HMM. Warming the actin to 37°C also did not induce polymerization.

Synaptosomal preparations negatively stained with uranyl acetate are shown in Figure 3. In the top figure several synaptic vesicles are seen (arrows), clearly delineated and close to the synaptosomal membrane. Although actin could be extracted from this synaptosomal preparation, no fibers were evident. When HMM was added to grids of synaptosomes, numerous structures like those shown in the bottom figure appeared; characteristically decorated fibers were evident, associated with synaptosomes. The fibers again were 80Å ± 10Å in diameter and showed typical arrowhead decorations with a periodicity of 350Å ± 20Å. Fibers were never seen in synaptosomal preparations unless HMM was added. Most of the fibers were clustered around synaptosomes; however, some fields of decorated fibers did not contain synaptosomal structures.

The synaptosomal preparation was subjected to osmotic shock and further separated into a membrane fraction, a fraction containing predominantly presynaptic vesicles, and a supernatant fraction (10). Figure 4 shows membrane ghosts prepared from synaptosomes; no fibers were observed in this preparation until HMM was added, as shown in the middle and bottom
Fig. 1. Electron micrograph of negatively stained preparation of actin prepared from rabbit skeletal muscle. Top: The actin polymerizes spontaneously to form fibers which are 70Å in diameter (Bar = 450Å). Middle: When heavy meromyosin is added to the actin fibers directly on the grid, the fibers become decorated in an arrowhead configuration (arrow) (Bar = 265Å). Bottom: At higher magnification it is seen that all arrowheads on a single fiber point in the same direction (arrows, Bar = 190Å).
Fig. 2. Actin prepared from rat brain synaptosomes and negatively stained with 2% aqueous uranyl acetate. A. The actin does not spontaneously form fibers but consists of amorphous material made up of granules of varying size (Bar = 70Å). B. When heavy meromyosin is added to this actin directly on the grid, decorated fibers are formed (Bar = 200Å). C. As with muscle actin, the arrowheads on a single fiber (arrows) point in the same direction (Bar = 120Å).
Fig. 3. Negatively stained preparations of synaptosomes prepared from rat brain. Top: Synaptosome with presynaptic vesicles (arrows). The surrounding area is free of fibers (Bar = 175 Å). Bottom: Synaptosomes when heavy meromyosin is added directly on the electron microscope grid. Decorated fibers are seen surrounding the synaptosome structure (Bar = 280 Å).
Fig. 4: Electron micrographs of negatively stained preparations of membranes prepared from rat brain synaptosomes. Top: Membranes form empty synaptosomal ghosts. No fibers are evident in the preparation (Bar = 225Å). Middle: Membrane preparation with heavy meromyosin added directly on the electron microscope grid. Decorated fibers are present around the membranes (Bar = 450Å). Bottom: At higher magnification, the arrowhead structure can be seen in the fibers (Bar = 160Å).
figures. Clusters of characteristically decorated fibers appeared around the membranes. In addition there were decorated fibers formed with no apparent association with membranes. The dimensions of the decorated fibers were the same as those observed with the synaptosomal preparation and the actin isolated from it.

Similar to actin from synaptosomes, actin prepared from this membrane fraction did not polymerize into fibrous structures (Figure 5). Again, treatment with HMM resulted in the formation of numerous decorated fibers which were evident in every field studied (Figure 5, middle). The addition of 0.1 mM ATP to the brain acto-HMM complex on the grid resulted in the loss of decorations as well as a marked reduction in the number of polymerized fibers (Figure 5, bottom). The remaining undecorated fibers were of smaller diameter than fibers of muscle actin (50Å ± 10Å).

The vesicle fraction prepared from the synaptosomal pellet showed no fibers. When HMM was added to this fraction, decorated fibers were seen only when fragments of membrane were also present in the field. The vesicles themselves were free of fibers even when HMM was added.

Actin obtained directly from the supernatant fraction remaining after the separation of membrane and pre-synaptic vesicles also did not show fibers unless treated with HMM.

DISCUSSION

In the presence of low concentrations of KCl, MgCl₂ and ATP, globular actin of striated muscle origin polymerizes to the fibrous state. Under similar conditions, actin isolated from synaptosomal preparations did not show distinct fiber formation. However, in the presence of HMM, the brain actin was induced to form fibers which were decorated with arrowhead configurations. The interaction of the brain actin with HMM could not be distinguished from the interaction of the polymerized muscle actin with HMM. Similar to the muscle preparation the interaction between the brain actin and HMM was reversed by ATP. It is unlikely that the HMM stabilized the brain actin fibers and merely prevented the actin polymers from being destroyed during negative staining since the muscle actin fibers were well preserved under these conditions. Since the brain actin was sedimented from the polymerization buffer to a transparent pellet at 100,000 xg for 3 hr., the protein was probably in a partially polymerized or aggregated state. Similar results were obtained with actin isolated from an acetone powder of synaptosomal membrane preparation; this actin did not polymerize to fiber formation as readily as did striated muscle actin under the same conditions. A similar problem in polymerization was encountered in studies with actin from slime mold and has been discussed in detail by Adelman (1).

Repeated cycles of polymerization and depolymerization are utilized for purification of muscle actin. This procedure did not purify brain actin to a similar extent. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate always revealed the presence of proteins of greater than 200,000 daltons (Fig. 6). This protein is not myosin since the actin preparation
Fig. 5: Actin prepared from synaptosome membranes and examined by negative staining in the electron microscope. Top: Actin from membranes does not form fibers but remains as amorphous subunits similar to the actin from the synaptosomes (Figure 2, top) (Bar = 80Å). Middle: Membrane actin to which heavy meromyosin was added directly on the grid. Arrowhead decorations are evident on the fibers which have formed (Bar = 480Å). Bottom: Membrane actin reacted with heavy meromyosin and then exposed to 0.1 mM ATP. The arrows point to short segments of polymerized brain actin (Bar = 380Å).
ELECTRON MICROSCOPY OF BRAIN ACTIN

Fig. 6: SDS polyacrylamide gel electrophoresis in a 5% gel. Left gel, rat brain actin; right gel, rabbit striated muscle actin. a, actin band. Arrow points to protein band >200,000 daltons.

does not have ATPase activity. The protein appears to be intimately associated with the brain actin since they copurify and emerge together in the void volume when chromatographed on Sephadex G-200. It is possible that this protein interferes with the formation of the fibrous state in the absence of HMM. Whether the brain actin is associated with these proteins in the membrane is not known.

Actin extracted from rabbit alveolar macrophages is also associated with a high molecular weight protein (13). This actin-binding protein has properties similar to spectrin, the structural protein of erythrocyte membranes. Macrophage actin can be dissociated from the binding protein with 0.6 M KCl.
Erythrocyte actin copurifies with spectrin and the latter has been shown to bind with rabbit muscle actin (26, 36). The spectrin seems to be associated with the actin in erythrocytes forming an anastomosing network beneath the membrane (36). The interaction between spectrin and actin results in a decrease in polymerization of G-actin induced by low salt concentrations (36). However, spectrin has been reported to stimulate polymerization of G-actin under other conditions (26). Proteins similar to spectrin have also been found in sperm (35) and sea urchin eggs (17). Whether the high molecular weight protein associated with the brain actin is also a spectrin-like protein, remains to be established. Preliminary studies indicate that in 0.6 M KCl these proteins may be induced to dissociate and the brain actin to polymerize.

The synaptosomal pellet suspended in sucrose-buffer medium and examined by negative staining did not reveal the presence of microfibrils. However, following application of HMM to the synaptosomes on the grid, arrowhead-decorated fibers were surprisingly abundant. It is extremely unlikely that the actin was present in the medium in which the synaptosomes were suspended since the latter were purified on a discontinuous Ficoll gradient and the synaptosomal layer washed in sucrose-buffer medium. No doubt the polymerized actin was drawn from the synaptosomal structures. Similar to the results obtained with the synaptosomal preparation, the synaptosomal membranes did not show fibers unless treated with HMM. With both of these preparations, the decorated fibers were usually associated with membranous structures. It is of interest that the "fluffy" layer above the synaptic membrane fraction of the osmotic shock preparation contained membrane fragments which did not react with HMM. Therefore, it is not likely that the actin was merely contaminating the membrane fraction.

These studies suggest that at least part of the actin in these preparations is associated with the membranes. It exists in a partially polymerized or perhaps non-polymerized state, and can be induced to polymerization by HMM. The synaptic vesicle fraction did not reveal actin fibers either before or after treatment with HMM. The low content of actin in the vesicle fraction and the association of the actin with the membrane, as well as the cytosol fractions, has also been established by biochemical studies (4, 5). In addition there is biochemical evidence for the presence of actin in membranes from mouse fibroblast and Hela cells (12) as well as blood platelets (34) and amoeba (27).

The ability of myosin or HMM to stimulate the polymerization of actin is well established. Szent-Gyorgyi (32) has observed that myosin stimulates the instantaneous polymerization of muscle actin in the presence of low salt concentrations (0.1 M KCl). A number of studies have shown that HMM also stimulates the polymerization of G-actin (9, 18, 21, 33, 38). Stimulation of fiber formation by HMM has been noted in neuroblastoma cultures (7, 8) and in brain tissue preparations (21, 23). Lazarides raised the question whether the actin in fibroblasts is present in two biochemically distinct forms, one of which can assemble into filaments and one which cannot, or whether the same pool of actin can exist in both forms (20). Bray has suggested that non-muscle actin has a higher threshold for polymerization than muscle actin and
that in the cell much of it is in a monomeric form (personal communication).

The physiological importance of the state of the actin in synaptosomes and its response to HMM as reported here, as well as the nature of its association with the membrane, requires further study. Such information as well as the location of the myosin, is basic to the understanding of the physiological role of contractile systems in this as well as other non-muscle systems. Genetic defects, aging or abnormal biochemical or drug-induced alterations in this complex system in nervous tissue could contribute to any of a variety of neurologic disorders. Szent-Gyoryyi (32) has remarked, "The myosin catalysis of the G-F transformation is very striking and can not fail to have its bearing on the muscle in which actin and myosin are in closest proximity". This process may be of even greater importance for the non-muscle systems.

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