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THE CHARACTERIZATION OF ACTIN ASSOCIATED WITH POSTSYNAPTIC MEMBRANES FROM TORPEDO CALIFORNICA

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SUMMARY: SDS-polyacrylamide gel electrophoresis of acetylcholine receptor from Torpedo californica electroplax membrane fragments shows, in addition to the four receptor subunits of 40,000, 50,000, 60,000 and 65,000 daltons, other components of apparent molecular weights 43,000, 47,000 and 90,000 daltons. In this study deoxyribonuclease I inhibitory activity has been used to identify actin in Torpedo californica receptor-enriched membranes and affinity chromatography on a deoxyribonuclease I agarose column has been used to purify this protein from the membrane preparations. In addition the membrane protein components have been analyzed by electrophoresis on a series of SDS-polyacrylamide gels of varying acrylamide concentrations. Evidence is presented that actin is a component of most preparations of receptor-enriched membrane fragments, having an apparent molecular weight of 47,000 daltons, and is distinct from the 43,000 dalton protein.

INTRODUCTION

In addition to its well documented function in muscle contraction, actin has been shown to be a component of many types of non-muscle cells, where there is evidence for its involvement in motility and other cellular processes (for reviews, see 1-3). In many cases, actin has been found associated with cell membranes, although the mechanism of association is not known (3-6). It has also been identified at the synapse in several species (7), where again its function has not been determined. One of the unusual properties of actin is its ability to act as a specific inhibitor of deoxyribonuclease I (8); DNase I

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Abbreviations used: DNase I, deoxyribonuclease 1; SDS, sodium docecylsulfate; AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; CBB, Coomassie Brilliant Blue; DNA, deoxyribonucleaic acid.

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causes the depolymerization of F-actin by forming a complex with a stoichiometry of 1:1 with G-actin (9).

Recently, the question of the association of actin with the acetylcholine receptor (AcChR) has been raised. Purified Torpedo AcChR has been found to be composed of four subunits with molecular weights of 40,000, 50,000, 60,000 and 65,000 daltons (10-16), although there are some reports of fewer subunits (17,18). In addition SDS gels of AcChR-enriched membrane fragments contain a strongly staining band migrating at 43,000 daltons; the function of this protein and its relationship to the AcChR have been examined in several laboratories (16-20). In particular, there has been speculation that this 43,000 dalton protein may be actin due to their similar molecular weights and to the growing knowledge that actin is a common protein in non-muscle systems. Karlin et al (21) have stated that all or part of the 43,000 dalton species is composed of actin, based on anti-actin antibody precipitation and on gel electrophoresis patterns. Sobel et al, however, have observed that actin does not co-migrate with the 43,000 dalton component on SDS gels of AcChR-enriched membrane preparations from the related species Torpedo marmorata (17).

In the studies described here we have utilized the DNase I inhibition properties of actin, as well as electrophoresis in several SDS gel systems, to determine the relationship between actin and the 43,000 dalton protein. Evidence is presented that actin is a component of most preparations of AcChR-enriched membranes, but one that is distinct from the 43,000 dalton protein, which has been previously shown to be a peripheral membrane component (18,19).

MATERIALS AND METHODS

Crude membrane fragments were prepared from Torpedo californica electroplax by the method of Reed et al (22). Membrane fragments enriched in AcChR, prepared by either the method of Reed et al (22), or that of Elliott et al (16), bound 1.3 - 2.0 nmol α-BuTx per mg of protein. The concentration of α-BuTx sites was determined according to Schmidt and Raftery (23) using DEAE-cellulose filter discs and ^{125}I-α-BuTx (24,25). Protein concentrations were determined by the method of Lowry et al (26) using bovine serum albumin as the standard. SDS gel electrophoresis was done according to Laemmli (27) with the acrylamide

concentrations varied as presented in the text. After electrophoresis, gels were stained with Coomassie Brilliant Blue in 25% methanol, 10% acetic acid. Analysis for 3-methylhistidine was done on a Beckman model 120C amino acid analyzer using System A from Keuhl and Adelstein (28) and Dowex-50 resin.

Preparation of Actin:

Chicken smooth muscle actomyosin was purified from a gizzard muscle homogenate by the method of Driska and Hartshorne (29). Pure actin was isolated from the actomyosin fraction by preparative SDS gel electrophoresis on gels with dimensions of 11 × 13.5 × 0.6 cm, using a 12.5% acrylamide, 0.1% methyline-bis-acrylamide separating gel and a 3% acrylamide, 0.08% bis-acrylamide stacking gel. After staining wtih CBB, the actin band was cut out and the protein eluted from the gel by the method of Lazarides (30).

DNase Inhibition Assay:

DNase inhibition activity of membrane fragments was determined by a modification of the method of Lindberg (31). Membrane fragments were solubilized in 1% Triton X-100 for 30 min and the insoluble material pelleted by centrifugation at 15,000 xg for 10 min. Aliquots of the supernatant were incubated for 5 min with 1 µg of bovine panceratic DNase I (Sigma), then mixed by vortexing with 3 ml of DNA substrate solution (0.04 mg/ml DNA (calf thymus-Sigma), 4 mM Mg^{++}, 2 mM Ca^{++}, pH 7.5) for 10 sec and the hyperchromicity upon DNA depolymerization monitored immediately as an increase in absorbance at 260 nm. This result was compared to the absorbance increase when 1 µg DNase I alone was added to the DNA substrate solution. One inhibition unit was defined by Lindberg (31) as the amount of inhibitor causing a decrease of 0.001 OD/min in 1 µg of DNase.

DNase I Affinity Chromatography:

DNase I-agarose was prepared by the method of Lazarides and Lindberg (32), using cyanogen bromide-activated Sepharose 2B (33). Membrane fragments were solubilized in 1% Triton and clarified by centrifugation before affinity chromatography, which was performed according to Lindberg and Erikson (32). The actin was eluted from the column with 3 M guanidine HCl, then dialyzed extensively against water and lyophilized.

RESULTS

The ability of actin to inhibit DNase I activity provided a means for determining the presence of actin in fractions from Torpedo californica electric organ. Using this assay, actin was found in the supernatant after the first centrifugation of electric organ homogenates, in isolated crude membrane extracts, and in most, but not all, membrane preparations enriched in AcChR (see Figure 1A). The amount of actin in these AcChR-enriched membrane fragments varied from zero to 90 DNase I inhibition units per mg of protein, or up to 90 inhibition units per nmole of α-BuTx binding sites. Approximately 1-3 µg of purified chicken smooth muscle actin are required to completely inhibit I µg of DNase I (8); on the basis of this information, it can be calculated that AcChR-enriched membrane preparations contain approximately

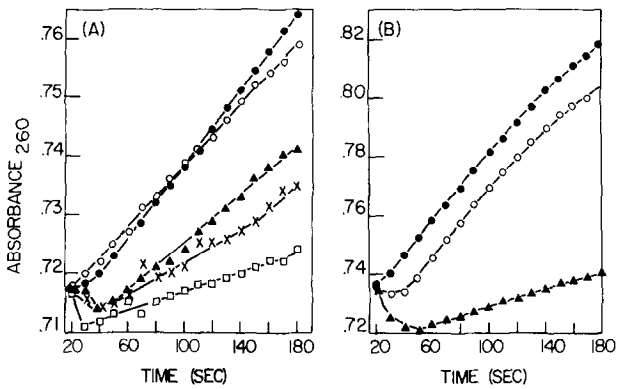

FIGURE 1: (A) DNase I inhibition assay of increasing amounts of Triton-solubilized AcChR-enriched membrane fragments: 0.12 mg of protein (●), 0.24 mg (△), 0.30 mg (✕) and 0.45 mg of protein (□) show increasing inhibition of DNase I activity over the control with no membrane protein, (○). 0.45 mg of membrane protein caused 62% inhibition of 1 µg of DNase I.
(B) DNase I inhibition assay of Triton-solubilized AcChR-enriched membrane fragments before (○) and after (▲) DNase I affinity chromatography. Before treatment, the membrane preparation caused 60% inhibition of 1 µg of DNase I; afterwards, the DNase I activity was the same as that of the control (●).

6 µg of active actin per mg of protein. The membrane fragments were solubilized in 1% Triton for the DNase I inhibition assays; control experiments showed that intact membrane preparations did not inhibit the enzyme and that all the DNase I inhibitor extractable from the membranes was contained in the solubilized supernatant after extraction with Triton. Further controls showed no effect on DNase I activity by Triton alone or by any of the other components of the buffers used. In addition, no relationship was found between the level of DNase I inhibition and the ligand affinity state of the AcChR molecule (34,35).

DNase I affinity chromatography was used to isolate actin from Triton-solubilized membrane preparations enriched in AcChR. As shown in Figure 1B, all DNase I inhibitory activity can be removed from the membrane fragments by this method. After elution from the affinity column with 3 M guanidine HCl, the actin no longer acted as a DNase I inhibitor, presumably as a result of the destruction of its DNase binding site. SDS gel electrophoresis on this material showed it to co-migrate with both purified chicken smooth muscle

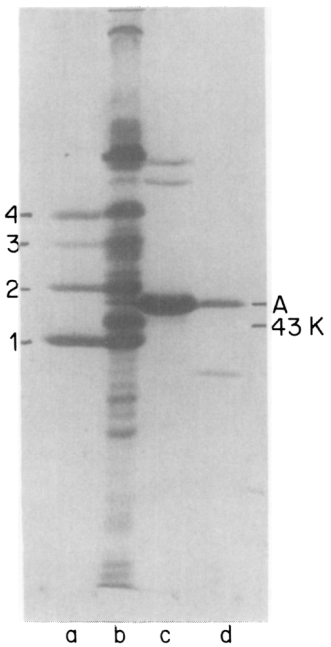

FIGURE 2: SDS-polyacrylamide gel (12.5% acrylamide, 0.1% bis-acrylamide) on (a) Triton-solubilized, purified AcChR, (b) AcChR-enriched membrane fragments, (c) DNase I affinity column purified actin from AcChR enriched membrane fragments, (d) chicken smooth muscle actin. The four AcChR subunits (1 = 40,000 daltons, 2 = 50,000, 3 = 60,000 and 4 = 65,000 daltons) are marked as well as the 43,000 dalton band and actin (A). It can be seen that actin and the 43,000 dalton band are distinct from each other.

actin and with a minor component of the membrane preparations with a molecular weight of ~47,000 daltons (see Figure 2). This band was distinct from the major 43,000 dalton band observed on the gels.

Confirmation that the 43,000 dalton polypeptide was not actin was provided by analysis of this band (cut from a preparative SDS gel of the AcChR-enriched membrane fragments and determined to be pure by re-electrophoresis) for the amino acid 3-methylhistidine. 3-methylhistidine, a relatively rare amino acid, is found as a component of actin from several species (36-39). Under conditions where less than one residue per molecule would be readily detected, no 3-methylhistidine was found in the 43,000 dalton protein.

When AcChR-enriched membrane fragments were electrophoresed on SDS gels of varying acrylamide concentrations, the CBB staining pattern of the proteins

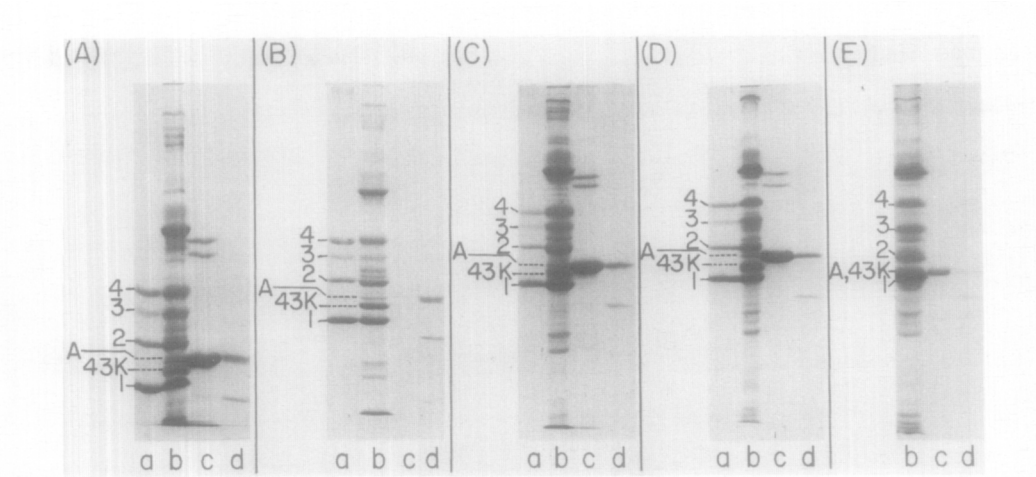

FIGURE 3: SDS-polyacrylamide gels on (a) Triton-solubilized, purified AcChR, (b) AcChR-enriched membrane fragments, (c) DNase I affinity column purified actin from AcChR enriched membranes, and (d) chicken smooth muscle actin. Laemmli SDS gels are: (A) 7.5% acrylamide, 0.2% bis; (B) 8.7% acrylamide, 0.23% bis; (C) 10% acrylamide, 0.26% bis; (D) 12.5% acrylamide, 0.1% bis; and (E) 12.5% acrylamide, 0.1% bis in half strength Laemmli buffer. The four AcChR subunits (1,2,3,4) are marked, as are the 43,000 dalton protein and actin (A). The separation of actin from the 43,000 dalton protein is very clear in gel D.

changed slightly from one gel to the next, as shown in Figure 3. As the concentration of acrylamide was increased from 7.5% to 12.5%, the separation between the 43,000 dalton protein and actin improved and became optimal using the 12.5% acrylamide, 0.1% bis-acrylamide gels. The separation worsened in gels run in half-strength Laemmli running buffer (Figure 3E) and in cases where electrophoresis was carried out at currents of >15 mA/cm^2 (data not shown).

DISCUSSION

Actin has been identified in the supernatant after the first centrifugation of homogenized Torpedo electric organ, in crude electroplax membrane fragment preparations, and in membrane fragments enriched in AcChR. This protein, which has been identified both by its ability to inhibit DNase I and by co-migration with purified actin on SDS gels, is present at higher concentrations in the original supernatant than in either of the membrane preparations, leading to the conclusion that the majority of actin in electroplax is a component of the cytoplasm. A portion, however, is associated with the mem-

branes sufficiently strongly to remain with them through sucrose density gradient centrifugation. This membrane-associated actin is freed to inhibit DNase I only upon solubilization of the membranes with Triton. The function of actin in these preparations and its relationship to the AcChR molecule remains to be determined.

SDS gel electrophoresis of the material bound to the DNase I affinity column shows it to co-migrate with actin purified from chicken smooth muscle and to correspond to a band in the membrane fragments migrating at ~47,000 daltons. The separation of this actin band from the 43,000 dalton component of membrane fragments was poor in all of the gels tried, except for the Laemmli gel using 12.5% acrylamide and 0.1% bis-acrylamide. This may account for the lack of agreement of these results with those of Hamilton et al, (20,21), who have stated that the 43,000 dalton protein co-migrates with actin. Since the four AcChR subunits are glycoproteins (15) and actin is not, their relative mobilities may be expected to vary in different gel systems. That actin co-migrates wtih the 47,000 dalton component of the AcChR-enriched membranes and with the DNase inhibitor purified from these membranes in all gel systems used here indicates that these proteins are identical.

The 43,000 dalton protein is a major component of AcChR-enriched Torpedo membrane preparations from several laboratories (16-21). It has been tentatively implicated in the binding of histrionicotoxin (17) and local anesthetics (40), although we have found (41) the histrionicotoxin binding site to be on one or more of the AcChR subunits instead. Furthermore, its removal from the membrane preparations by treatment at pH 11 has been shown to have no effect on AcChR function (17, 19). That this protein is not actin has been shown here by its lack of the amino acid 3-methylhistidine and by its failure to co-migrate with purified actin on SDS polyacrylamide gels. Actin has, instead, been identified as a separate component of AcChR-enriched membrane fragments migrating on SDS gels between the 43,000 dalton protein and the 50,000 dalton subunit of the AcChR. Whether actin has a structural or functional relationship to the AcChR molecule remains to be investigated.

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