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SYNAPTIC PROTEINS

Characterization of Tubulin and Actin and Identification of a Distinct Postsynaptic Density Polypeptide

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ABSTRACT

The major proteins in isolated synaptic junctions (SJs) and postsynaptic densities (PSDs) have been compared to actin, tubulin, and the major neurofilament (NF) protein by two-dimensional gel electrophoresis and tryptic peptide map analysis. These studies show: (a) tubulin is present in SJ and PSD fractions and is identical to cytoplasmic tubulin, (b) actin in these fractions is very similar to the γ - and β -actin found predominantly in nonmuscle cells, and (c) the major PSD protein is distinct from all other known fibrous proteins.

KEY WORDS two-dimensional electrophoresis · peptide maps · synapse · postsynaptic density

Synapses in the central nervous system (CNS) display on the internal surface of the postsynaptic membrane a prominent structure called the postsynaptic density (PSD). This structure is a specialized matrix where fibrous proteins interdigitate into the junctional region of the postsynaptic membrane. Little is known about the function of the PSD. In numerous non-neural cell types, fibrous proteins such as microtubules and microfilaments are seen in close proximity to plasma membranes and are believed to be involved in structural stability, regulating the lateral mobility and distribution of integral and surface membrane proteins, and perhaps in controlling the shape of cell surfaces (25). It is tempting, therefore, to ascribe similar functions to the fibrous proteins at CNS synapses. However, before a rigorous functional analysis can be made, the molecular identity of these fibrous proteins must first be determined.

Recent observations have shown that the PSD contains appreciable amounts of polypeptides which co-migrate with tubulin (17, 16, 13, 30) and cross-react with antisera to tubulin (38, 31), a

small amount of polypeptide with the same apparent molecular weight as muscle actin (9, 13, 16, 17, 30). In addition there is a component with a molecular weight similar to the major neurofilament (NF) protein (17) (also called 100-Å intermediate filaments [7]) which weakly cross-reacts with antisera to NF (38). Tryptic peptide maps have also revealed similarities between tubulin and major PSD polypeptides, and one possibility suggested was that the major PSD protein was a modified form of tubulin (13). At present, however, the identity of the major PSD protein remains unknown. Thus, the aim of the present study is to determine whether the major PSD protein is distinct or whether it is related to NF or tubulin and to compare in detail the cytoplasmic fibrous proteins actin, tubulin, and NF to their presumptive counterparts in PSD and synaptic junction (SJ) fractions (Fig. 1).

We have employed two-dimensional gel electrophoresis (26) to compare polypeptides by two independent criteria: isoelectric points and apparent molecular weights. We have also compared two-dimensional patterns of peptide fragments produced by the action of trypsin on purified proteins. These methods allow comparisons of

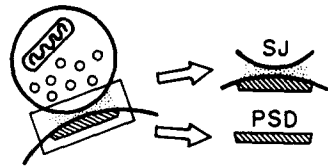


FIGURE 1 Nerve endings (or synaptosomes) consist of presynaptic membrane and occluded cytoplasmic organelles (e.g. mitochondria and synaptic vesicles) joined to a postsynaptic membrane by the SJ (enclosed area). The cytoplasmic surface of the postsynaptic membrane at the SJ is distinguished by the presence of an electron-opaque membrane specialization (hatched area) called the PSD. SJ fractions are purified from synaptosomes by virtue of their insolubilities in Triton X-100, and PSDs are purified by their insolubility in *N*-lauryl sarcosinate.

charge, apparent molecular weight, and primary structure of proteins and so provide an accurate index of the degree of similarity between proteins. The results expand our present knowledge concerning the biochemical distinctness of the major fibrous protein in isolated PSDs and provide the first characterization of the actin isoproteins associated with SJ and PSD fractions and the unambiguous identification of SJ- and PSD-associated α - and β -tubulin.

MATERIALS AND METHODS

Subcellular Fractionation

Sprague-Dawley male rats (60–100 days old) purchased from Simonsen Laboratories (Gilroy, Calif.) were used in these experiments. Subcellular fractions were prepared from forebrains obtained following decapitation and freehand dissection of brains rostral to the superior colliculi. Purified synaptic plasma membrane (SPM), SJ, and PSD fractions were prepared as previously described (17). The PSD fraction is >80% pure, on the basis of percent volume of electron-opaque material.

Purification of Tubulin, Actin, and NFs

Tubulin was purified from bovine brain by repeated cycles of polymerization as described by Shelanski et al. (28). Partially purified actin was obtained from rabbit skeletal muscle as described by Szent-Györgyi (29). NFs were isolated and purified from calf brain by the method of Yen et al. (37) and were a generous gift from Ronald Liem and Michael Shelanski (Department of Pharmacology, New York University Medical School). A portion of these purified protein preparations as well as synaptic fractions were solubilized in sodium dodecyl sulfate (SDS) (2.3%, wt/vol), and protein determinations were carried out by the method of Lowry et al. (21) and ap-

propriate controls were included for fractions containing formazan as previously described (10).

Protein Iodination

Partially purified proteins and synaptic fractions were radioiodinated by the [125 I]iodine monochloride method of McFarlane (23) after initial solubilization in 1% SDS. Proteins subjected to tryptic peptide analysis were radioiodinated in gel slices by the chloramine T method (see below). Radioactivity was measured in a Nuclear Chicago Unilux II liquid scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.).

One-Dimensional Gel Electrophoresis

The discontinuous SDS-buffer system of Laemmli (20) was used. Proteins were solubilized in SDS and mercaptoethanol and electrophoresed in exponential-linear gradient slab gels as previously described (18). Both one- and two-dimensional gels were fixed and stained with Coomassie Blue as previously described (18).

Two-Dimensional Gel Electrophoresis

Isoelectric focusing followed by SDS gel electrophoresis was performed according to O'Farrell (26), except for a minor modification in protein sample preparation. Samples were first solubilized in 1% SDS and 5% mercaptoethanol, and then 9 vol of focusing buffer (9.5 M urea, 1.6% (wt/vol) pH 5–7 ampholytes, 0.4% (wt/vol) pH 3–10 ampholytes, 4% (vol/vol) Nonidet P-40, and 5% (vol/vol) β -mercaptoethanol) were added. All solubilization steps were conducted at 22°–25°C. Presolubilization in SDS increased the amounts of synaptic proteins that entered the isoelectric focusing gels without noticeably affecting protein isoelectric points. Protein sample volumes for focusing gels were \sim 100 μ l.

N-Terminal Amino Acid Analysis

The N-terminal amino acid of the major PSD protein purified by one-dimensional electrophoresis was determined by the method of Weiner et al. (33). Briefly, after localization by Coomassie Blue staining, the major PSD protein in gel slices was exhaustively washed in water to remove possible interfering substances (see reference 33). The protein was then eluted in 0.1% (wt/vol) SDS, 0.1% (wt/vol) β -mercaptoethanol, and concentrated by lyophilization. Samples were then resuspended in 0.2 M NaHCO₃ (pH 9.8) and reacted with dansyl chloride (5 mM final concentration; Pierce Chemical Co., Rockford, Ill.) in the presence of SDS (1%, wt/vol). The dansylated protein was precipitated in TCA, washed with acetone, and subjected to acid hydrolysis (6 N HCl) for 16 h at 95°C in the dark. After the initial electrophoresis, care was taken to protect samples from excessive exposure to light. Approximately 5 μ g (0.1 nmol) of purified major PSD protein was used for each determination. The dansylated reaction products were analyzed by ascending

chromatography in two dimensions in the first three solvents as previously described (33). Chromatography of the dansyl amino acid standards was conducted simultaneously but on opposite sides of two-sided polyamide sheets (Pierce Chemical Co.). This permitted easy visual identification, under a UV lamp, of the various dansyl amino acid derivatives.

Tryptic Peptide Mapping of Radioiodinated Proteins

The method of peptide mapping of radiolabeled proteins in single polyacrylamide gel slices described by Elder et al. (11) was used with minor modifications. Briefly, individual protein components from either one- or two-dimensional gels were sliced from dried gels and radioiodinated with 200 μ Ci of 125 I (New England Nuclear, Boston, Mass., sp act \sim 17 Ci/mg) in the presence of chloramine T. After removal of unbound 125 I by repeated washing, the gel slices were dried, and 0.1 ml of 50 μ g/ml of trypsin (Worthington TPK trypsin, Worthington Biochemical Corp., Freehold, N. J., 202 U/mg) in 0.05 M NH_4HCO_3 buffer (pH 8.0), 1 mM dithiothreitol was added to each sample. Samples were incubated at 37°C for 4–6 h, then 0.4 ml of buffer/dithiothreitol was added, and the incubation was continued overnight. Under these conditions, >95% of the radioiodinated tryptic peptides diffused from the gel slices. Electrophoresis (1 kV for 30 min) and chromatography in butanol:pyridine:acetic acid:water (32.5:25.5:20) on cellulose-coated thin-layer chromatography TLC plates (EM Laboratories, Inc., Elmsford, N. Y.) were performed as previously described (11). The plates were dried and analyzed by autoradiography using Kodak X-ray film.

Materials

Reagents used in electrophoresis and isoelectric focusing were purchased from Bio-Rad Laboratories (Richmond, Calif.). SDS was obtained from BDH Chemicals Ltd. (Poole, England), and Ultrapure urea was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.).

RESULTS

One-Dimensional Electrophoretic Analysis of SPM, SJ, and PSD Polypeptides

All synaptic fractions contain polypeptides with mol wt of 56,000, 54,000, 52,000, and 45,000 (Fig. 2A), although their relative concentrations vary considerably among fractions. The 52,000 mol wt polypeptide is notably enriched in the PSD fraction while the 56,000, 54,000, and 45,000 mol wt polypeptides are more prominent in SJ

and SPM fractions. Side-by-side comparisons of synaptic fractions, tubulin, muscle actin¹ and the major NF protein as well as mixing experiments suggest that tubulin (both α - and β -subunits) and actin are present in SPM, SJ, and PSD fractions. Moreover, the major PSD and NF proteins (Fig. 2A) have similar but not identical molecular weights because mixing experiments at low protein concentrations² (Fig. 2B) reveal that the major PSD and NF polypeptides have different electrophoretic mobilities. One-dimensional electrophoretic analysis cannot assign unambiguous molecular identities to polypeptides of such similar molecular weights and which comprise such diverse morphological structures (i.e., microtubules, NFs, PSDs, SJ, and muscle contractile elements). Therefore, additional physical properties of these polypeptides were examined.

Two-Dimensional Electrophoretic Analysis of Synaptic Polypeptides

The two-dimensional electrophoretic system recently developed by O'Farrell (26) allows the separation of proteins by isoelectric focusing in the first dimension and then by discontinuous SDS slab gel electrophoresis (20) in the second dimension. In these two-dimensional gels, differences in spot positions in the horizontal plane represent differences in protein isoelectric points, and in the vertical plane differences in apparent molecular weights.

Because the major comparisons with synaptic proteins will be made to tubulin (α - and β -subunits), actin and the major NF proteins, Fig. 3A shows a two-dimensional gel of a mixture of these three partially purified protein fractions. The major NF protein, previously observed as a single component on SDS slab gels (Fig. 2A, B), is resolved as a series of three recognizable doublets, decreasing in intensity while displaying more acidic isoelectric points. These multiple NF protein spots are probably the result of artifactual charge heterogeneity of a single protein (see reference 26). All of the isoelectric points of the major NF proteins are more basic than those of

¹ The muscle actin preparation used in these experiments contained significant quantities of myosin (mol wt \sim 200,000).

² In SDS (see Fig. 2), the major NF protein displayed an electrophoretic mobility which increased in relation to increasing quantities of protein (>1–2 μ g).

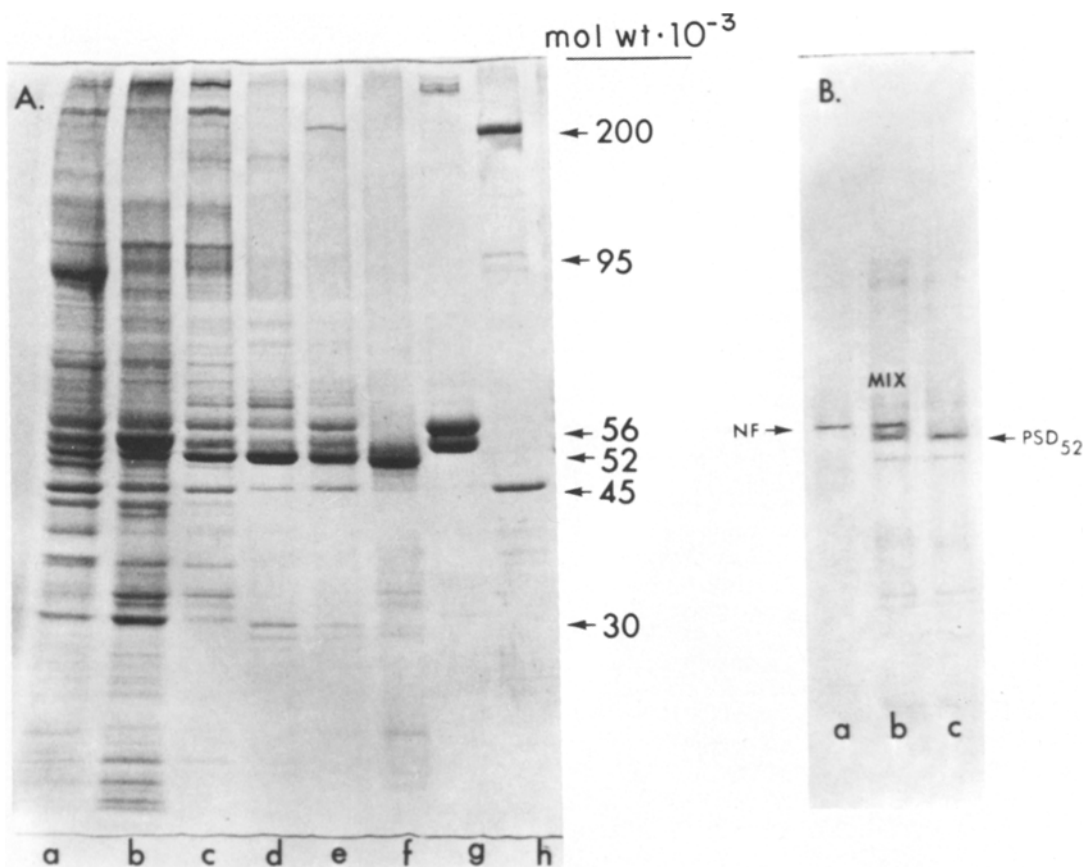


FIGURE 2 (A) One-dimensional SDS gel of subsynaptosomal fractions and purified fibrous proteins: (a) SPM, 45 μg ; (b) SJ prepared without *p*-iodonitrotetrazolium violet (INT) treatment (see reference 15), 45 μg . This fraction contains large amounts of a protein with a molecular weight similar to that of β -tubulin which is due to a major mitochondrial protein which contaminates synaptic fractions prepared without INT; (c) SJ prepared with INT treatment, 40 μg ; (d) PSD, 40 μg ; (e) a mixture of PSD, 20 μg ; actin, 2 μg ; major NF protein, 5 μg ; tubulin, 2 μg ; (f) major NF protein, 8 μg ; (g) tubulin, 6 μg ; (h) actin, 5 μg . (B) One-dimensional SDS-gel of the co-electrophoresis of PSD and NF polypeptides: (a) NF, 0.5 μg ; (b) a mixture of PSD, 4 μg , and NF, 0.5 μg ; (c) PSD, 4 μg . Gels were 8–20% exponential-linear gradients (17).

either tubulin subunit or muscle actin. As previous experiments would predict (14, 22), the α - and β -tubulin subunits produced two characteristic spots after two-dimensional electrophoresis. Closer examination of the α - and β -tubulin spots reveals a more complex but reproducible staining pattern. The β -tubulin spot (the more acidic and lower molecular weight) actually consists of a doublet. The lower molecular weight and more acidic spot of this β -tubulin doublet possesses a noticeable trailing edge on its acidic isoelectric side. The α -tubulin subunit spot (more basic and higher molecular weight) also contains a trailing

edge extending from its acidic isoelectric side. The α -tubulin subunit frequently splits by molecular weight into a narrowly spaced doublet (Fig. 3A). This two-dimensional pattern of tubulin subunits is very similar to that recently reported by Berkowitz et al. (2). Partially purified rabbit muscle actin produced one major spot (Fig. 3A). Three actin isoproteins differing only in isoelectric points have been recently reported in skeletal muscle, cultured calf muscle, chick embryo fibroblasts (27, 35), and a variety of mammalian cell lines (15). The predominant isoprotein in muscle is designated α -actin whereas the other two primarily

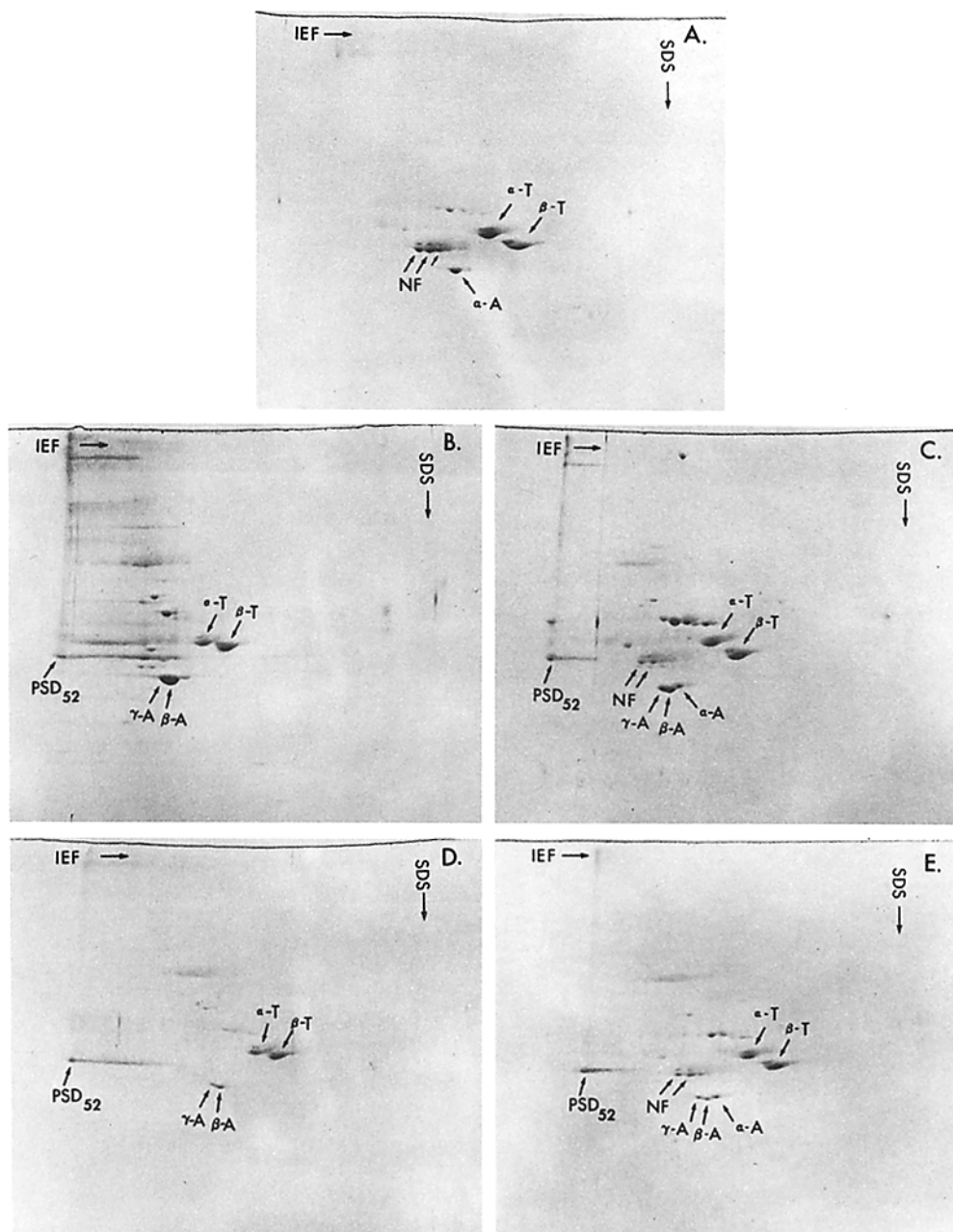


FIGURE 3 Two-dimensional gels of SJ and PSD fractions and purified fibrous proteins (basic isoelectric points are to the left [IEF, isoelectric focusing] and acidic to the right). (A) Mixture of tubulin (*T*), 5 μg ; actin (*A*), 2 μg ; NF, 5 μg ; α -*A*, β -*A*, and γ -*A* denote the actin isoproteins (see Results) and α -*T* and β -*T* denote the two subunits of cytoplasmic tubulin; (B) SJ, 50 μg ; (C) Mixture of SJ, 20 μg ; tubulin, 2 μg ; NF, 2.5 μg ; α -actin, 1 μg ; (D) PSD, 50 μg ; (E) Mixture of PSD, 25 μg ; tubulin, 2 μg ; NF, 2.5 μg ; α -actin, 1 μg . Gradient gels were the same as in Fig. 1. The pH gradients in focusing gels were very similar to those used by O'Farrell (26).

“nonmuscle” isoproteins with more basic isoelectric points are called β - and γ -actin (27, 35).

Figs. 3B and D show two-dimensional gels of rat brain SJ and PSD fractions, respectively. As would be expected, these gels resolve more polypeptides because many proteins differing in isoelectric point have nearly identical molecular weights. The most apparent conclusion from the comparison of SJ and PSD fractions is that PSD polypeptides appear to be a subset of those present in SJ. These gels also demonstrate that certain proteins do not form distinct spots but instead produce somewhat variable streaks or smears. This has been observed in other eukaryote systems (26) and is likely a result of membrane protein insolubility and/or artifactually produced protein charge heterogeneity.

Two-dimensional gels of SJ and PSD fractions display Coomassie Blue spots which appear very similar in their two-dimensional positions to α - and β -tubulin. More important, however, is the fact that these spots of both SJ and PSD fractions possess the same doublet composition and shape complexity as purified cytoplasmic tubulin. Also present in SJ and PSD fractions is a very closely spaced pair of spots of approximately equal intensities which migrate to the same general region as α -actin. These spots possess isoelectric points slightly more basic than α -actin.

The relative amounts of α - and β -tubulin and actin in SJ and PSD fractions in two-dimensional gels are very similar to their relative amounts and enrichments in one-dimensional gels (compare Fig. 2A to 3B and D). There is, however, no significant counterpart of the major NF proteins in either SJ or PSD fractions. The major PSD protein (52,000 mol wt) is present as a pronounced streak 2–5 cm in length which extends from directly below the top (basic end) of the isoelectric focusing gel to the more acidic regions of the gels. The amount of the major PSD protein appears relatively less in two-dimensional gels when compared to its amount on one-dimensional gels of SJ and PSD fractions (compare Fig. 2A to 3B and D).

A more precise comparison between these specific synaptic proteins and purified actin, tubulin, and NF proteins was achieved by mixing experiments (Fig. 3C and E). Approximately equal amounts of tubulin and actin were mixed with their presumptive synaptic counterparts so that, if co-electrophoresis of certain proteins did not occur, the resulting intensities of adjacent spots

would be approximately equal in magnitude. A small amount of purified NF proteins was also included.

On the basis of discrete protein spots, the major NF proteins had no significant counterparts in the PSD fraction (Fig. 3E). It seemed possible, however, that SJs might contain small amounts of the major NF proteins (Fig. 3B and C; see Discussion). Both α - and β -tubulin unambiguously co-electrophoresed with their equivalent SJ and PSD polypeptides. The purified muscle α -actin displayed a very similar but distinguishable two-dimensional electrophoretic pattern when compared to SJ and PSD counterparts. The pair of actin spots in both SJ and PSD fractions have nearly identical molecular weights compared to muscle α -actin; however, both spots possess more basic isoelectric points than muscle α -actin.

To examine further the nature of these SJ and PSD polypeptides whose apparent molecular weights are similar to that of skeletal muscle α -actin but whose isoelectric points are dissimilar, electrophoretic comparisons to actins from non-muscle cells were performed. Actin, purified by two cycles of polymerization-depolymerization, was prepared from adrenal fibroblasts and smooth muscle. These two non-neural tissues have been shown to contain predominantly two actin isoproteins (β - and γ -actin) which possess more basic isoelectric points than skeletal muscle α -actin (15, 27, 35). β - and γ -actins from either fibroblast or smooth muscle produced staining patterns similar to those of the actin polypeptides in SJ and PSD fractions (Fig. 4A). α -Actin when co-electrophoresed with either fibroblast or smooth muscle β - and γ -actins (Fig. 4B) produced staining patterns similar to those obtained in comparable mixing experiments with SJ or PSD fractions and α -actin (Fig. 3C and E). Moreover, when mixtures of either fibroblast or smooth muscle actins, α -actin, and PSD polypeptides were electrophoresed, co-electrophoresis of β - and γ -actins with their synaptic counterparts resulted (Fig. 4C). In view of the known differences in isoelectric points of actins from different cell types, we believe that the actins present in PSD and SJ fractions are the β - and γ -actins found in nonmuscle cells (15, 27, 35).

Electrophoretic Analysis of ^{125}I Proteins

SJ and PSD fractions, purified NF, actin, and tubulin were solubilized in SDS and iodinated as described in Materials and Methods. After iodi-

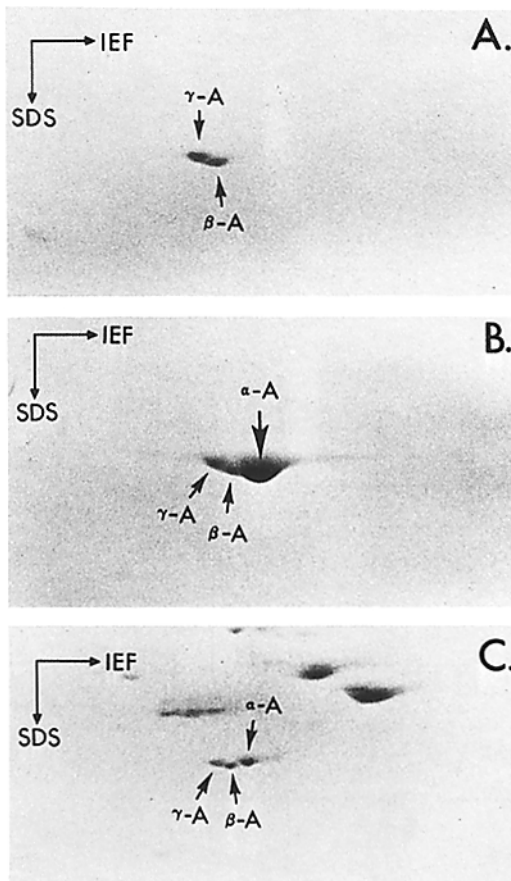


FIGURE 4 Two-dimensional gels (expanded views) of purified actins and PSD polypeptides. (A) Approximately 1 μg of actin purified from adrenal fibroblasts by the method of Yang and Perdue (36). (B) Mixture of fibroblast actin (1 μg) and α -actin (3–4 μg). (C) Mixture of fibroblast actin (0.5 μg), α -actin (1 μg), and PSDs (25 μg) (2 μg of NF protein was also added to this mixture).

nation, SJ and PSD fractions, and partially purified NF, tubulin, and actin preparations were electrophoresed on slab gels in the presence of SDS. Individual bands from these known fibrous ^{125}I proteins and their separately resolved synaptic counterparts were cut from gels, eluted, and subjected to two-dimensional electrophoresis. In general, the major NF proteins, the tubulin subunits and the major PSD protein show only minor changes in isoelectric points after iodination, whereas multiple isoelectric points are generated when purified muscle α -actin is iodinated (data not shown). Two-dimensional electrophoresis of a mixture of presumptive ^{125}I PSD actin and

^{125}I muscle actin produced a series of actin spots indistinguishable from those of ^{125}I muscle actin.

After two-dimensional electrophoresis of SJ and PSD fractions, a significant decrease in the amount of Coomassie Blue staining of the major PSD protein was seen. This decrease is more obvious when the major PSD protein streak in two-dimensional gels is compared to the respective band intensities in one-dimensional staining patterns for equivalent amounts of total protein for these fractions (compare Fig. 2A to 3B and D). To examine this apparent discrepancy, electrophoretically purified and iodinated major PSD protein was subjected to two-dimensional electrophoresis. Examination of the autoradiographs and liquid scintillation counting of residual first-dimension plus stacking gel and the second-dimension resolving gel revealed that a large percentage (>70%) of the major PSD protein remains immobilized in the isoelectric focusing gel during electrophoresis in the second dimension. Of the remaining radioactivity which migrates into the second dimension forming a streak in the 52,000 mol wt region, >95% is localized to isoelectric regions more basic than those for the major NF proteins. The exact nature of the insolubility of the major PSD protein in isoelectric focusing sample buffer (9 M urea, 5% mercaptoethanol, 4% NP-40 and 2% ampholytes) is currently under investigation. It is important to note, however, that under identical conditions, purified tubulin, actin, and NF preparations left <0.5% of the total radioactivity in the first-dimension remnant and stacking gels after two-dimensional electrophoresis.

N-Terminal Amino Acid Analysis of the Major PSD Protein

As a result of the apparent inability to completely resolve the major PSD protein by two-dimensional electrophoresis, it seemed possible that it was composed of multiple protein components with identical molecular weights. To address the question of protein heterogeneity, electrophoretically purified major PSD protein was subjected to the dansyl chloride N-terminal analysis method of Weiner et al. (33). After analysis, the major PSD protein produced only one N-terminal amino acid which was identified as tyrosine by comparison to a known dansyl-tyrosine standard (data not shown). Whether or not the major PSD protein contains multiple components which share a com-

mon N-terminal amino acid will be examined below (see Peptide Maps section).

Peptide Maps

To compare in greater detail the molecular identity of the fibrous proteins used as standards to their proposed counterparts in isolated SJ and PSD fractions, these radiiodinated proteins were digested with trypsin and the resulting peptide maps were analyzed by autoradiography (Fig. 5). The two-dimensional peptide mapping procedure used in these studies has been shown to be very sensitive in detecting similarities and differences in the primary structure of genetically and developmentally related membrane proteins (11).

In Fig. 5a the tryptic map of the major PSD protein is shown. This pattern is very reproducible and is the same whether the major PSD protein is obtained from gels of SJ or PSD fractions, or from one- or two-dimensional gels. The major PSD protein appears to have a very conserved primary structure because peptide maps of the protein obtained from bovine and human brains (data not shown) are very similar to those from rat. The large degree of similarity in primary structure between rat and bovine major PSD proteins is important because our NF protein standard is from bovine brain.

In the two-dimensional electrophoretic analysis described above, the major PSD protein did not resolve into a single spot. It produced a somewhat variable streak (Fig. 3). The peptide mapping technique used in these studies lends itself to an analysis of the possible protein heterogeneity which could produce the unsatisfactory isoelectric focusing of the major PSD protein. The streak region of the major PSD protein from a two-dimensional gel was divided horizontally into 2-mm segments and each segment was mapped separately. Comparisons of the tryptic maps from adjacent segments revealed that the protein comprising the streak had the same primary structure throughout this region of the gel (data not shown). Furthermore, these maps were identical to those obtained from the major PSD protein purified by one-dimensional electrophoresis. These results in addition to the finding of a single N-terminal amino acid in electrophoretically purified major PSD protein (see above) support our notion that this protein is homogeneous.

We also compared tryptic maps of the fibrous proteins used as standards to comparable SJ- and PSD-associated proteins resolved by two-dimen-

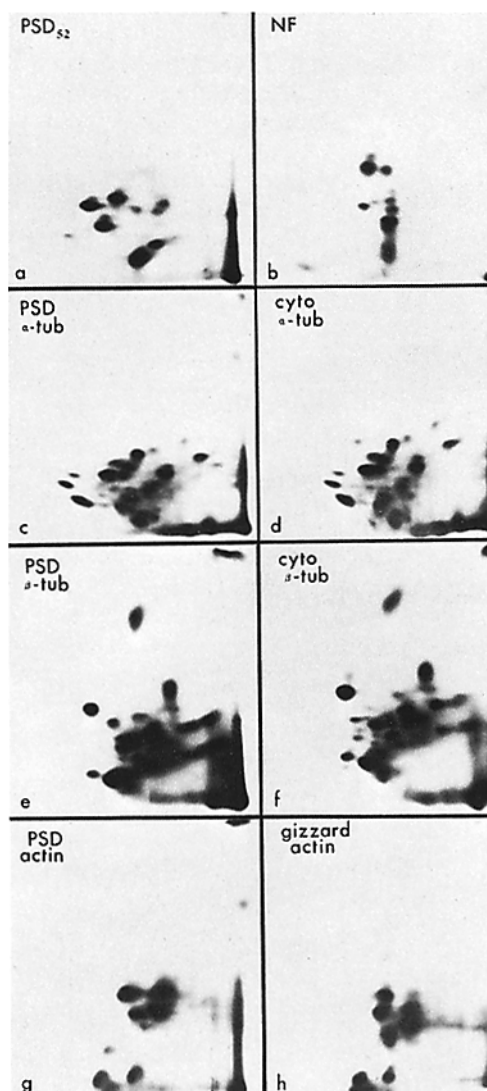


FIGURE 5 Comparison of tyrosine-containing tryptic peptides of various synaptic and fibrous proteins. (a) Major PSD protein; (b) major NF protein; (c) α -tubulin from PSDs; (d) α -tubulin from cytoplasmic microtubules; (e) β -tubulin from PSDs; (f) β -tubulin from cytoplasmic microtubules; (g) actin from PSDs; (h) actin from chicken gizzard. Each sample contained approximately 1×10^6 cpm and was applied to the TLC plate in the lower right corner (see darkened spots). Electrophoresis was from right to left, and chromatography was from bottom to top.

sional electrophoresis (Fig. 5). The tryptic maps of α -tubulin were the same whether this protein was isolated from purified microtubules, SJ (not shown), or PSD fractions (Fig. 5c and d). Very

similar tryptic maps were also observed for β -tubulin (Fig. 5e and f) obtained from the different subcellular origins. In comparing the α - and β -tubulin maps between the two different sources, small differences are apparent. These differences occur primarily with peptides that appear to incorporate small levels of radioactivity. Alternatively, these minor differences may represent charge variants of cysteine- and/or methionine-containing peptides since the chloramine T used for iodination can oxidize these amino acids. After short autoradiographic exposures, these spots appear in some maps and are absent in others. Longer exposures verified that these minor spots were present in each respective subunit although their intensities varied among experiments. The tryptic map of a mixture of β - and γ -actins obtained from smooth muscle (chicken gizzard) was quite similar to that of the actin in SJ (data not shown) and PSD fractions (Fig. 5g and h) although the gizzard actin contained two additional major spots. The tryptic map of the major NF protein (Fig. 5B) was very different from those of all other proteins examined. Tryptic maps of α - and β -tubulin, the major PSD and NF proteins, and actin were all quite different and, therefore, it is unlikely that the major PSD protein is related to any of the others.

DISCUSSION

Actin has been shown to be associated with the postsynaptic region *in situ* by heavy meromyosin-binding studies (24). Other studies have suggested that a protein resembling actin in molecular weight is associated with isolated PSDs (9, 17). Recently, Blomberg et al. (6) have shown on the basis of antisera reactivity and amino acid composition that a polypeptide from dog PSDs is very similar to skeletal muscle α -actin. Unless rigorously conducted, however, comparisons based on antisera reactivity and amino acid composition would probably not distinguish between protein isospecies as in the case of α -, β -, and γ -actins. In fact, our two-dimensional gels show that the major actin isoprotein of skeletal muscle is present in SJ and PSD fractions in very low concentrations, if at all. Instead, the β - and γ -actins predominantly found in "nonmuscle" tissues (35) are present in these synaptic fractions. β - and γ -actins are present in approximately equal quantities in either SJ or PSD fractions, although the PSD contains reduced quantities of both.

We can now state that isolated SJs and PSDs

contain a substantial amount of tubulin. Tubulin accounts for $\sim 7\%$ of the total protein in SJ fractions and 14% in PSD fractions (17). Two-dimensional electrophoresis reveals that SJ and PSD polypeptides comigrate with α - and β -tubulin and that these spots share the same complexity (see Fig. 3A). Two-dimensional tryptic maps of either α - or β -tubulin obtained from PSDs are indistinguishable from those of their respective subunit of cytoplasmic tubulin. We have also shown by radioimmunoassay that antisera to cytoplasmic tubulin reacts strongly with the tubulin bands from PSDs (38). Thus, PSD-associated tubulin appears unmodified in terms of average charge density, molecular weight, primary structure, and antigenicity.

Tubulin is not the major PSD polypeptide as was suggested by Walters and Matus (31) but is present as a substantial component. It is enriched in PSD over SJ fractions and is not removed by sodium lauryl sarcosinate treatment. The lack of classical microtubule profiles in SJ or PSD fractions, despite their large tubulin contents, suggests that this tubulin may exist in an altered morphological and/or polymerized state possibly as the ring structures observed by Bryan (8). We cannot yet state whether or not tubulin directly joins the membrane or whether an intermediate protein provides an anchorage site. Wang and Mahler (32) concluded from surface labeling studies that tubulin is exposed at the external surface of synaptic membranes but, because their fractions are primarily presynaptic and because their gel system did not resolve the major PSD protein from tubulin, their conclusions about the orientation of tubulin may be erroneous.

The idea that cytoplasmic tubulin is a membrane-bound protein at synapses is not new but has had its share of controversy. Early colchicine-binding studies provided initial evidence for the presence of tubulin in brain plasma membranes (4) and synaptosomal particulate fractions (12). Later, in studies paralleling the development of a method to isolate highly purified PSDs (10), work in this laboratory established that the major PSD polypeptide(s) had an electrophoretic mobility very similar to that of purified cytoplasmic tubulin, and led to the speculation that PSDs might contain tubulin (1). At about this time, electrophoretic analyses corroborated the presence of a tubulin-like protein in brain synaptosomes (5), and a number of reports appeared concerning the presence of tubulin in synaptic membranes (19) and

isolated PSDs (31). In a key immunohistochemical study, Walters and Matus (31) observed that antisera prepared against tubulin reacted with the PSD. Later, Westrum and Gray (34) observed microtubules associated with the PSD. Recent work in our laboratory (13, 16, 17, 38) and others (30) have suggested that tubulin is present in isolated PSDs, but its identity and relationship to the major PSD protein remained uncertain. The data presented in this paper establish that the major PSD protein is distinct from tubulin and that SJ- and PSD-associated tubulin is, by these analyses, identical to cytoplasmic tubulin. The molecular nature of the association of tubulin with synaptic complexes and PSDs can be only defined operationally at present: radiolabeled brain soluble proteins (12) and tubulin (4) are not adsorbed adventitiously to plasma membranes during tissue homogenization and subcellular fractionation, and tubulin, as well as actin, are present in SJ and PSD fractions in a form that resists solubilization by detergents.

What is the major polypeptide of the PSD? Our results from the tryptic maps and N-terminal analysis strongly support the contention that it is indeed a homogeneous entity. Furthermore, comparisons of the tryptic maps of actin, α - and β -tubulin and the major NF and PSD proteins (Fig. 5) show that, on the basis of primary structure analysis, the major PSD protein is unrelated to these other fibrous proteins.

Isolated PSDs contain very little, if any, major NF protein. The major PSD cross-reacts very weakly with antisera to NF and not with antisera to tubulin (38). NFs appear associated with the PSD *in situ* because immunohistochemical studies show significant reactivity *in situ*. It is likely that NFs in the PSD and SJ are not major integral components and that what is present *in situ* is largely lost during the isolation of these fractions (see below).

On the other hand, Blomberg et al. (6) have tentatively labeled the major protein in isolated dog PSDs as the "neurofilament" protein on the basis of similar molecular weights. They also reported that an antiserum prepared against axonal NF protein formed a precipitin line with the major component of dog PSDs, using double immunodiffusion (3). Until these results are presented in detail, however, it is not possible to determine the extent and specificity of cross-reactivity of PSD polypeptides with anti-NF sera, so that these data may in fact partially agree with

ours (38). We have observed, on occasion, minor bands in gels of SJ fractions in the molecular weight region between the major PSD protein and the β -tubulin subunit. We have mapped some of these minor bands, and one produces a tryptic pattern very similar to that of the major NF proteins. We have not observed this minor component in PSD fractions, and it is often absent from SJ fractions.

It should be pointed out, however, that by comparisons of protein composition and content of glycoproteins which bind concanavalin A, the PSD fraction prepared by Blomberg et al. (6) is more like our SJ than PSD fraction. Thus, it is important that comparisons of synaptic fractions prepared by different methods be based on multiple criteria, i.e., protein and glycoprotein composition in addition to electron microscope ultrastructure, before a fraction is finally designated SJ or PSD. Most important, however, is that our studies show that the major PSD and NF proteins are distinct from one another by a number of criteria.

The major component of the PSD is a protein of 52,000 mol wt which is unusually insoluble; it forms aggregates and appears largely insoluble in 9 M urea and 5% β -mercaptoethanol, even after presolubilization in SDS. Furthermore, this protein has an inherent propensity to spontaneously form intermolecular disulfide bonds with itself and probably other proteins (16). These properties make it a suitable candidate for the major structural component which forms the supramolecular network at the PSD of mature synaptic junctional complexes. We have recently been successful in isolating immature SJs from newborn rats. These SJ fractions have less than one-tenth the amount of major PSD protein in adult SJs, although the amounts of tubulin and actin are similar.³ It appears, therefore, that SJ-associated tubulin and actin may be more important in regulating the interactions between molecules during the formation of synaptic contacts and that the major PSD protein has a more important role at the mature synapse.

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