An Unusual \(\beta\)-Spectrin Associated with Clustered Acetylcholine Receptors

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Abstract. The clustering of acetylcholine receptors (AChR) in the postsynaptic membrane is an early event in the formation of the neuromuscular junction. The mechanism of clustering is still unknown, but is generally believed to be mediated by the postsynaptic cytoskeleton. We have identified an unusual isoform of \(\beta\)-spectrin which colocalizes with AChR in AChR clusters isolated from rat myotubes in vitro. A related antigen is present postsynaptically at the neuromuscular junction of the rat. Immunoprecipitation, peptide mapping and immunofluorescence show that the \(\beta\)-spectrin in AChR clusters resembles but is distinct from the \(\beta\)-spectrin of human erythrocytes. \(\alpha\)-Spectrin appears to be absent from AChR clusters. Semiquantitative immunofluorescence techniques indicate that there are from two to seven \(\beta\)-spectrin molecules present for every clustered AChR, the higher values being obtained from rapidly prepared clusters, the lower values from clusters that require several minutes or more for isolation. Upon incubation of isolated AChR clusters for 1 h at room temperature, \(\beta\)-spectrin is slowly depleted and the AChR redistribute into microaggregates. The \(\beta\)-spectrin that remains associated with the myotube membrane is concentrated at these microaggregates. \(\beta\)-Spectrin is quantitatively lost from clusters upon digestion with chymotrypsin, which causes AChR to redistribute in the plane of the membrane. These results suggest that AChR in clusters is closely linked to an unusual isoform of \(\beta\)-spectrin.

The accumulation, or clustering, of acetylcholine receptors (AChR) in the postsynaptic membrane of embryonic skeletal muscle cells is one of the first morphological changes involved in the formation of the vertebrate neuromuscular junction (e.g., references 2, 3, 8, 17, 26, 29; see references 16, 65 for reviews). Although extracellular factors other than the motor neuron can influence the distribution of AChR in sarclemma (reviewed in 16, 20, 66), cytoskeletal proteins appear to be most important in binding to and immobilizing receptors to form clusters (16, 20, 31). Several of these proteins have been identified at AChR-rich membrane in vivo (15, 18, 30, 32, 36, 67, 68, 75) and in vitro (12-14, 19, 32, 47, 59). Actin is one such protein that is present postsynaptically (12, 36) and that appears to be involved in the formation or maintenance of AChR clusters in muscle cells in vitro (12, 27, 28, 47).

Microfilaments underlie the AChR clusters of muscle cells cultured in vitro (58; Pumplin, D. W., manuscript submitted for publication; Bloch, R. J., M. Velez, J. Krikorian, and D. Axelrod, manuscript submitted for publication), but it is not yet clear how these structures influence AChR clustering.

At the AChR clusters of cultured rat myotubes, bundles of actin filaments are readily identified using fluorescent phalloidin derivatives (Bloch, R. J., M. Velez, J. Krikorian, and D. Axelrod, manuscript submitted for publication), and by ultrastructural techniques (Pumplin, D. W., manuscript submitted for publication). These microfilament bundles are not closely associated with the AChR-rich domains of these clusters, but are located over "contact domains," regions within the cluster that are poor in AChR and close to the tissue culture substrate (Pumplin, D. W., manuscript submitted for publication; Bloch, R. J., M. Velez, J. Krikorian, and D. Axelrod, manuscript submitted for publication). These same regions are enriched in several cytoskeletal proteins, including talin, vinculin, and \(\alpha\)-actinin (14; Bloch R. J., M. Velez, J. Krikorian, and D. Axelrod, manuscript submitted for publication), that are normally present where stress fibers terminate at the cell membrane (reviewed in 22, 33). All the cytoskeletal proteins underlying contact domains can be removed from AChR clusters without disturbing their overall structure (11), suggesting that these proteins and the associated microfilamentous structures are not closely linked to clustered receptors.

In addition to the large microfilamentous structures at contact domains of AChR clusters, actin also overlies "AChR domains," the regions within the cluster that are rich in receptor (12). Ultrastructural studies reveal few microfilaments.
in this region (Pumplin, D. W., manuscript submitted for publication). It has been suggested that actin at AChR domains may be present in a membrane-bound lattice resembling that of the human erythrocyte (12, 61). If this model is correct, then a protein resembling human erythrocyte spectrin should be closely linked to AChR at AChR domains. The experiments presented in this paper support this prediction.

We demonstrate that the clustered AChR of cultured rat myotubes are associated with an unusual isoform of the β subunit of human erythrocyte spectrin. This isoform, which we refer to simply as β-spectrin, appears to be present at AChR clusters in significant amounts without a corresponding α-spectrin subunit. β-Spectrin remains associated with the cluster membrane under conditions that do not remove the AChR-associated polypeptide, 43K (or v; references 30, 35, 56). AChR and β-spectrin appear to be closely linked, however, as they redistribute together in the muscle membrane as clusters are disrupted. Our results strongly suggest that an unusual isoform of β-spectrin is bound, either directly or indirectly, to the AChR, and that this binding is important for the clustering of receptors. Based on our findings, we present a model of AChR clusters that postulates a complex of the AChR and the 43K protein bound to oligomers of β-spectrin; these complexes may in turn be cross-linked by actin.

Materials and Methods

Cell Culture

Rat myotubes were cultured on glass coverslips as described (9, 14). Briefly, cells were seeded onto coverslips and grown for 4 day in Dulbecco-Vogt modified Eagle's medium supplemented with 10% FCS. On day 4, this medium was replaced with medium containing 20 μM tyrosine and actinomycin D. Myotube cultures were used for experiments 2-4 d later. For immunofluorescence experiments, cultures were usually labeled with monoclonal rhodamine-a-bungarotoxin (R-BT; 61) before further processing. For immunofluorescence studies, we used method I of reference 11, and method II, which omitted serum albumin but added protease inhibitors (11). Samples were rocked gently on a Luckham 802 suspension mixer (Tekmar Co., Cincinnati, OH) for 5-10 min, until the bulk of the cellular material was shed from the cover glass, usually for 5-10 min. After extraction, cover glasses with attached AChR clusters were either extracted with detergent, for immunoprecipitation, or fixed immediately, for immunofluorescence. For some immunofluorescence experiments, samples were transferred serially through two to four beakers containing PBS or buffer A (0.05% Triton X-100 in PBS, 0.2% sodium azide, 0.02% sodium chloride, pH 7.8). After correction for background fluorescence, the signal arising from the anti-β-spectrin was produced in rabbits by injection and repeated boosting with purified human erythrocyte β-spectrin, prepared as described (51, 72). The antibody was affinity purified by passing it over a column of cyanogen bromide-activated Sepharose 4B coupled to erythrocyte β-spectrin, and eluting with 1 M acetic acid followed by dialysis into PBS. It was visualized using rhodaminylated goat anti-rabbit IgG (RGAR; Cappel Laboratories). Controls showed no species cross-reaction of the FGAM and RGAR.

Quantitative analysis of the fluorescence signal arising from AChR clusters was performed as described (12, 13). Briefly, the fluorescence arising from a 5-μm circle of membrane was sampled through a pinhole aperture with a photomultiplier, and the signal was read by an I-to-V converter. After correction for background fluorescence, the signal arising from the FGAM (F) was divided by the signal arising from R-BT (R) to bound to the same area of membrane. The ratio (F/R), usually varying by ±10% (SEM) or less, was comparable from one sample to another. F/R did not change when we used higher concentrations of FGAM, indicating that this reagent was in excess.

Immunoprecipitation and Peptide Mapping

Cultures were metabolically radiolabeled with [35S]methionine (see above). On day 7 of culture, clusters were isolated by extraction with saponin, followed by method II of reference 11. The material isolated from 12 coverslips (25 mm diameter) was extracted at room temperature with 0.05% Triton X-100 in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). AChR and β-spectrin are solubilized under these conditions. The detergent extract was lyophilized, redissolved in 500 μl of precipitation buffer (5 mM EDTA, 10 mM Tris, 0.02% sodium azide, 30 μM PMSF, pH 7.6), centrifuged at 30,000 g for 5 min to remove insoluble material, and incubated for 10 min at room temperature with 50 μl of a 1:10 suspension of washed and fixed Staphylococcal aureus cells (Panisorb, Calbiochem-Behring Corp., San Diego, CA). After centrifugation for 2 min, the cleared supernatant from this incubation was divided into two equal aliquots and incubated for 30 min at room temperature with 40 μl of VIIF7 anti-β-spectrin, or with a nonimmune serum. For these and all subsequent dilutions, NET buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 30 μM PMSF, 0.02% sodium azide, pH 7.4) was used. Affinity-purified rabbit anti-mouse IgG antibody (20 μl; 0.7 μg/ml) was then added to enhance the capture of the immune complexes by the Staphylococcal cells, and the incubation was continued for an additional 30 min. Finally, 25 μl of fresh Staphylococcal cells were added to each sample and incubated for 20 min. The samples were underlaid with 150 μl of 1 M sucrose and centrifuged for 10 min at 30,000 g at 4°C. The immunoprecipitates were solubilized at 90°C with sample buffer (42) and applied to SDS-PAGE, performed according to Laemmli (42).

2. After solubilization under these conditions, AChR chromatographs with monomeric AChR (~250 kD) in gel filtration (Baetscher, M., and R. J. Bloch, unpublished data). Detergent extraction therefore dissociates AChR from any cytoskeletal proteins with which it might interact in situ.
The bands corresponding to the high molecular mass peptide immuno-
precipitated by VIIF7 or to human erythrocyte β-spectrin were excised from
the gel and labeled with $^{125}$I (Amersham Corp., Arlington Heights, IL)
using chloramine T (Sigma Chemical Co., St. Louis, MO), following previ-
ously described procedures (72). Each gel slice was labeled with 1 mCi of
$^{125}$I. After the iodination reaction, unbound radioactive iodine was re-
moved by exhaustive washing with 10% methanol containing 5 mCi tyrosine
and 10 μM KI. The iodinated gel slices were digested at 37°C for 24 h with
50 μg of α-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ),
in 1 ml of 50 mM NH₄HCO₃, pH 8.0. The supernatant was lyophilized,
redissolved in 20 μl of acetic acid/formic acid/water (15:5:80; [vol/vol/vol])
and chromatographed in two dimensions using Merck cellulose sheets
(Macalaster Bicknell Co., New Haven, CT). Other conditions were as de-
scribed (72).

Frozen Sections
Frozen sections of rat diaphragm were cut as described previously (15).

Figure 1. β-Spectrin at isolated AChR clusters. Rat myotubes were labeled with R-BT' and either extracted with saponin (E-H; method
I of reference 11) or treated briefly with ZnCl₂ and then sheared
(A-D). Samples were immediately fixed in paraformaldehyde and labeled
with VIIF7 anti-β-spectrin followed by FGAM. A, C, E, and G show R-BT fluorescence; B, D, F, and H show β-spectrin immuno-
fluorescence. (A and B) Sheared sample at low magnification showing anti-β-spectrin labeling at AChR clusters (arrowheads) and in filamen-
tous structures of both myotubes and fibroblasts (double arrowheads). (C and D) Sheared sample at higher magnification showing that VIIF7
labels AChR domains (arrowheads), but not contact domains (double arrowheads). (E and F) Sample isolated with saponin at low mag-
nification, showing that the only sites of enrichment of VIIF7 labeling coincide with AChR clusters (arrowheads). (G and H) Sample iso-
lated with saponin, at higher magnification, showing that VIIF7 labels AChR domains (arrowheads), but not contact domains (double ar-
rowheads). The asterisks (C, D, G, and H) indicate limited areas that label brightly for AChR but only slightly for β-spectrin. Bars: (F)
25 μm; (H) 10 μm.
Results

Our aim in the experiments presented here was to determine if spectrin is associated with clustered AChR in cultured rat myotubes, and, if so, to characterize this association further. In our preliminary experiments, we therefore screened a library of monoclonal and polyclonal antibodies against erythrocyte spectrin and brain spectrin (fodrin) to learn if they bound to AChR clusters. In this, as in later experiments, all immunolocalization of spectrin was performed using fluorescence techniques. High levels of generalized cytoplasmic fluorescence in detergent-permeabilized myotubes made it difficult to study spectrin at the AChR clusters of whole cells. We therefore used two different methods to remove the bulk of the cytoplasm and expose the intracellular surfaces of the clusters to antibody. Both rely on the fact that the AChR clusters of cultured rat myotubes are localized at sites of membrane-substrate attachment (5, 14, 43), and are left behind on the cover glass when the bulk of the muscle cell is removed. The first technique, shearing the myotube cultures with a stream of buffer (14), yields AChR clusters together with associated cytoplasmic structures, such as stress fibers and coated vesicles (Pumplin, D. W., manuscript submitted for publication), as well as fragments of contaminating fibroblasts. The second method, extraction for 5–10 min with a buffered solution of saponin, yields AChR clusters purified ~100-fold, still contaminated with substrate-attached membrane from fibroblasts, but essentially free of contamination from cytoplasmic structures (11).

Spectrin at AChR Clusters

When we exposed AChR clusters prepared by shearing or by extraction with saponin to a battery of anti-spectrin antibodies, we obtained significant labeling with two: VIIF7, a mouse monoclonal antibody specific for the β I domain of human erythrocyte β-spectrin (reference 38; see Fig. 1), and an affinity-purified preparation of one rabbit anti-β-spectrin (see Fig. 9). VIIF7 also labels filamentous structures in fragments of myotubes and fibroblasts that remain after shearing (Fig. 1, A and B). Upon closer examination of AChR clusters, we found that VIIF7 labeled the AChR domains of clusters, but not the contact domains, which are poor in AChR (Fig. 1, C and D). Similarly, β-spectrin was not enriched at the focal contacts of fibroblasts (not shown), consistent with reports that the latter are devoid of spectrin (23). These observations suggested that a spectrin-like molecule is present in the AChR domains of AChR clusters, and in filamentous structures in both myotubes and fibroblasts.

In contrast to samples prepared by shearing, myotube cultures extracted with saponin show very little spectrin-like material in fibroblast fragments and in areas of myotubes lacking clusters (Fig. 1, E and F). Observation at higher magnification (Fig. 1, G and H) confirmed that VIIF7 labeled the AChR domains (arrowheads) and not the contact domains (double arrowheads) of AChR clusters.

Although the correlation between the distribution of AChR and β-spectrin in isolated clusters was strong, we did find limited regions of some clusters, prepared either by shearing or by extraction with saponin, that lacked significant amounts of β-spectrin (Fig. 1, C, D, G, and H, asterisks). The AChR in these areas was often stained to a lesser extent with R-BT and was less well-organized than receptors in other parts of the cluster, suggesting that these areas were in the process of accumulating or losing AChR (see Discussion). Whatever causes the occasional absence of immunolabeling, our results clearly establish that the spectrin-like material obtained after extraction with saponin is highly enriched at the AChR domains of clusters, and is not present in significant amounts in any other structures. We therefore used the saponin-extracted preparation to study the cluster-associated spectrin further.

We characterized the β-spectrin-like molecule associated with AChR clusters further by immunoprecipitation and peptide mapping. Immunoprecipitation of material from receptor cluster preparations which had been metabolically radiolabeled with [35S]methionine showed that a polypeptide with an apparent mass of 220,000 D reacted with the VIIF7 anti-β-spectrin (Fig. 2). No other polypeptide was specifically precipitated by the antibody. Thus the immunofluorescence labeling obtained with anti-β-spectrin was due to the presence at AChR domains of a molecule of the same apparent polypeptide chain molecular weight as human erythrocyte β-spectrin (M, = 220,000; Fig. 2 A). Significantly, even under the low stringency conditions for immunoprecipitation employed here, no peptide corresponding to α-spectrin (240,000–M,) was precipitated.

We prepared two-dimensional cellulose 125I-peptide maps precipitates were analyzed by SDS-PAGE. (Lane a) Coomassie Blue-stained gel of human erythrocyte ghosts containing BSA and cytochrome C as molecular mass standards. The α and β subunits of human erythrocyte spectrin are marked (240,000 and 220,000 M, respectively). The two arrows indicate the position of BSA and actin (68,000 and 43,000 M, respectively). (Lanes b–d) Autoradiograms of the immunoprecipitates of the samples that had been metabolically radiolabeled with [35S]methionine. (Lane b) Precipitation by VIIF7 anti-β-spectrin, showing a high molecular mass polypeptide which migrates near the position of the beta subunit of erythrocyte spectrin. (Lane c) Precipitation by nonimmune rabbit serum, used as a control. No high molecular mass peptide is evident. A mouse monoclonal antibody that reacted specifically with rat brain synapsin I also failed to precipitate the high molecular mass polypeptide (not shown). (Lane d) Material precipitated by the Streptococcus coccus alone, during the prewashing procedure. Only the VIIF7 anti-β-spectrin precipitates a high molecular mass polypeptide.
of the band precipitated by VIIF7 anti-β-spectrin to compare it with other spectrin isoforms. Our results (Fig. 3) indicate that the cluster-associated polypeptide is similar, but not identical to the β subunit of human erythrocyte spectrin: 39 of the 48 discrete spots (81%) on the map of muscle β-spectrin correspond closely to those for human erythrocyte β-spectrin. Similar comparison of muscle β-spectrin with the 235-kD β subunit of brain spectrin (37) showed significantly less homology (not shown). The peptide maps are therefore consistent with the idea that preparations of AChR clusters from rat myotubes contain a form of β-spectrin that is similar but not identical to the β subunit of human erythrocyte spectrin. For brevity, we refer to this molecule simply as β-spectrin.

As mentioned above, several other polyclonal and monoclonal antibodies to human erythrocyte and pig brain spectrins failed to label AChR clusters (Table I). We examined the monoclonal antibodies on this list further, to determine if they reacted in immunoblots of spectrin from rat erythrocytes. We also tested their ability to react with spectrin still bound to human erythrocyte membranes (Ursitti, J. A., and R. J. Bloch, unpublished results). Most were active by both criteria; the sole exception was mAb VIIIC7, which bound to rat erythroid β-spectrin in immunoblots but did not label membrane-bound spectrin. Thus, these antibodies were active and (with the exception of VIIIC7) able to bind to membrane-associated spectrin. These results support the conclusion, reached from peptide mapping, that the isoform of β-spectrin at AChR clusters is similar, but distinct from, β-erythroid spectrin, and that it is not associated with an α subunit.

**Semiquantitative Measurements**

We next measured, semiquantitatively, how much spectrin was associated with AChR in clusters. To do so, we determined the intensity of the fluorescein fluorescence due to VIIF7 and FGAM binding and compared this to the intensity of R-BT fluorescence from the same patch of membrane (12, 13). The amount of fluoresceinated antibody bound varied linearly with the amount of R-BT (Fig. 4, inset). Thus, at any given concentration of antibody, the ratio of fluorescein (F) to rhodamine (R) fluorescence was constant. By varying the concentration of VIIF7, we obtained a binding isotherm (Fig. 4) that saturated at a F/R ratio of 2.0 (mean in four experi-

**Figure 3.** The protein precipitated by VIIF7 has a peptide map similar to that for erythrocyte β-spectrin. The gel bands corresponding to the high molecular mass peptide immunoprecipitated by VIIF7 (b) or to human erythrocyte β-spectrin were excised and labeled with 125I using chloramine T, extensively digested with α-chymotrypsin, and chromatographed in two dimensions (see Materials and Methods). (a) Autoradiogram showing the map of the 220,000-D polypeptide specifically immunoprecipitated by VIIF7. (b) Map of erythrocyte β-spectrin. (c) Cartoon depicting the features of the maps shown in a and b. Shared spots are solid. Peptides present in the myotube β-spectrin but absent in erythrocyte β-spectrin are open. Peptides present in erythrocyte β-spectrin but absent in the myotube β-spectrin are dotted. Overall, 81% (39 of 48) of the spots in the map of myotube β-spectrin correspond with spots in the map of erythrocyte β-spectrin.
VIIF7 has rather low affinity for the ~-spectrin at AChR
ments, 2.35 ± 0.55 SEM). Using purified VIIF7, we found
of 1.95. The curve is a rectangular hyperbola with F/R~x = 2.0 and [VIIF7]I/2 = 3.2. In experiments using purified VIIF7, half-maximal labeling was obtained with ~1 µM VIIF7. (Inset) Fluorescence ratio, F/R, at near-saturating concentra-
4. The solid circle indicates nonspecific labeling, which was very low for samples prepared by either method. The results show that labeling by VIIF7 declines upon incubation of isolated AChR clusters. Values are means ± SEM (n = 10). Symbols lacking error bars represent values for which the SEM was too small.
were stained with different antibodies to erythrocyte spectrin and brain fodrin. Only VIIF7 and a polyclonal antiserum to human erythrocyte beta spectrin be labeled with one of a panel of antibodies to erythrocyte spectrin and brain fodrin. The specificities of the monoclonal antibodies are described in references 36 and 77.
ments, 2.35 ± 0.55 SEM). Using purified VIIF7, we found
that the concentration of antibody needed to reach half-maximal saturation was ~1 µM (not shown), suggesting that VIIF7 has rather low affinity for the ~-spectrin at AChR clusters.
When we studied the spectrin at AChR clusters prepared by shearing, we obtained somewhat different results. The immu
fluorescence ratio, F/R, at near-saturating concentra-
tions of VIIF7 was 4.9 ± 0.58 (mean ± SEM n = 5; range 3.5 to 6.5), significantly greater (p < 0.05 by t test) than that obtained with AChR clusters isolated by extraction with saponin from parallel cultures. Clusters isolated by shearing are prepared and fixed in <15 s; those isolated by extraction with saponin require 5-10 min of incubation before fixation. We therefore investigated the possibility that ~-spectrin was lost from clusters during longer incubations. The data in Fig. 5 show that this is indeed the case. Clusters prepared by shearing and immediately fixed gave F/R ratios significantly greater (p < 0.005) than those that were sheared and incubated for 5 min in PBS or in the presence of saponin. The latter gave values that were not significantly different (p > 0.8) from those obtained with AChR clusters isolated using sapo-
in alone. These results suggest that some of the ~-spectrin at AChR clusters is only loosely associated with the myotube membrane.
We subjected AChR clusters to several different treatments to learn if we could deplete more of the spectrin from AChR clusters, and if this could be correlated with changes in the organization of AChR in the membrane. Treatment with buffer A (2 mM Tris-HCl, 0.2 mM ATP, pH 8.0; reference 73) did not cause further loss of spectrin from saponin- isolated AChR clusters. Extraction at pH 11 or treatment with chymotrypsin eliminated nearly all specific labeling by VIIF7, however (Fig. 6 A). Similarly, VIIF7 labeling of sheared AChR clusters was drastically reduced by digestion with chymotrypsin, but not by extraction with buffer A (Fig. 6 B).
The fluorescence images of clusters treated with buffer A or chymotrypsin are shown in Fig. 7. As expected (12; see above), these samples showed significant changes in both AChR and ~-spectrin (Fig. 7, C–F). Receptors in both samples were more evenly distributed in the plane of the membrane, with little evidence of the membrane domains seen in controls. ~-spectrin labeling was still bright in clusters treated with buffer A, but, like AChR, was redistributed across the membrane plaque (Fig. 7 D). Microaggregation of AChR was often observed in the samples treated with chymotrypsin (Fig. 7 E). In agreement with the semiquantitative data in

**Table I. Few Anti-Spectrin Antibodies Label Isolated AChR Clusters**

| Antibody | Specificity | Immunofluorescence of AChR Clusters |
|----------|-------------|-----------------------------------|
| Monoclonals | | |
| VIIF7 | Spectrin, ~ domain | Bright |
| IIF8 | Spectrin, ~ domain | Poor |
| VD4 | Spectrin, ~ domain | Poor |
| IIIC7 | Spectrin, ~ domain | Poor |
| IIID2 | Spectrin, ~ domain | Poor |
| Polyclonals | | |
| Anti-~ Spectrin beta chain | Bright |
| RAS C | Spectrin alpha chain | Poor |
| RAF A | Fodrin | Poor |

Rat myotubes were labeled with R-BT, extracted with saponin, and labeled with one of a panel of antibodies to erythrocyte spectrin and brain fodrin. Only VIIF7 and a polyclonal antiserum to human erythrocyte beta spectrin be labeled clusters. The specificities of the monoclonal antibodies are described in references 36 and 77.
Figure 7. β-Spectrin is lost upon treatment with chymotrypsin, but not upon extraction at low ionic strength. AChR clusters were labeled with R-BT and isolated from rat myotubes by extraction with saponin. Samples were then treated with buffered saline, buffer A, or chymotrypsin, as described in Fig. 6, labeled with VIIF7 and FGAM, and photographed under fluorescence illumination. A, C, and E show AChR, visualized with R-BT; B, D, and F show β-spectrin, visualized with VIIF7 and FGAM. (A and B) Control cluster, washed and incubated in PBS, showing codistribution of AChR and β-spectrin in AChR domains (arrowheads), but not in contact domains (double arrowheads). (C and D) Cluster washed and incubated in buffer A, which removes actin from the clusters (12), showing a more even distribution of AChR and β-spectrin. β-Spectrin is retained after extraction with buffer A. (E and F) Cluster washed in PBS and incubated in PBS plus chymotrypsin, showing a redistribution of AChR, with some microaggregation (E, arrowhead). β-Spectrin is lost upon exposure to chymotrypsin. Bar, 10 μm.

Fig. 6, β-spectrin was almost completely absent from these samples (Fig. 7 F). This effect of chymotrypsin was not observed if the enzyme was first inactivated with PMSF or by boiling (not shown). SDS-PAGE of samples treated with buffer A or with chymotrypsin were consistent with our immunofluorescence results. Control samples contained several bands with high apparent polypeptide chain molecular masses (200–250 kD), including a band near 220,000 M. These high molecular mass bands were unaffected by extraction with buffer A, but were all lost from chymotrypsin-treated samples, together with several other smaller polypeptides (not shown). The apparently rapid proteolytic degradation of β-spectrin observed here has also been observed in in vitro studies (71). These results are consistent with the idea that buffer A causes both AChR and β-spectrin to redistribute in the muscle membrane, and that proteolysis destroys β-spectrin and disrupts AChR organization.

Coaggregation of β-Spectrin and AChR

The experiments described above suggest that β-spectrin may redistribute with AChR in clusters treated with buffer A. We sought more definitive evidence that the distribution of these two proteins was closely linked, and so studied AChR clusters that were severely disorganized.

AChR clusters can be dispersed in intact myotubes by treatment with azide (9) or by withdrawing Ca²⁺ from the culture medium (10, 24). We exposed cells to both treatments, and then extracted them with saponin and labeled
Figure 8. β-Spectrin at dispersing or reforming AChR clusters. Rat myotube cultures were labeled with R-BT with or without treatment to disrupt AChR clusters. Clusters were isolated by shearing (I and J) or by extraction with saponin, and either fixed immediately (A–F) or incubated for 1 h in buffered saline before fixation (G–J). All samples were then labeled with VIIIF7 and FGAM. A, C, E, G, and I show R-BT label; B, D, F, H, and J show immunofluorescence of β-spectrin. (A and B) Sample treated for 4 h with 5 mM sodium azide, to disrupt AChR clusters. (C and D) Sample treated for 4 h in medium depleted of Ca²⁺, to disrupt AChR clusters. (E and F) Sample incubated for 6 h in azide, and then washed and incubated for 2.5 h in control medium, to allow AChR clusters to start to reform. Controls for the experiments in A–F showed that, after 6 h of treatment in azide or in medium lacking Ca²⁺, >90% of the AChR clusters present at the beginning of the treatment were dispersed. (G and H) Sample extracted with saponin and incubated for 1 h before fixation. (I and J) Sample prepared by shearing and incubated for 1 h before fixation. The results indicate that β-spectrin is associated with AChR even in highly disrupted clusters. Bar, 10 μm.

them with VIIIF7 and FGAM. The results (Fig. 8, A–D) show that, like actin (12), β-spectrin is lost from cluster membrane more or less in parallel with AChR: areas of clusters that have lost AChR are also poor in β-spectrin, whereas areas that still retain AChR also retain β-spectrin. In related experiments, we examined cells several hours after removal of azide, when AChR clusters reform (9). β-Spectrin was associated with all reforming AChR clusters in these samples (Fig. 8, E and F). The F/R ratios for VIIIF7 labeling of β-spectrin in reforming AChR clusters and in clusters dis-
Figure 9. Spectrin codistributes with actin within isolated receptor clusters. Rat myotube cultures were subjected to saponin extraction, and immediately fixed in paraformaldehyde. Samples were labeled with a monoclonal antibody to actin (HP249; reference 12) and FGAM (A) and, simultaneously, with an affinity-purified rabbit antibody to β-erythroid spectrin and RGAR (B). Actin and spectrin codistribute. Bar, 10 μm.

ruptured by azide or Ca2+ depletion are similar to those of control clusters (F/R = 2.5–4.0 in treated samples and controls). This suggests that β-spectrin is not lost from or added to clusters independently of AChR.

We investigated the linkage between AChR and β-spectrin further by studying clusters that reorganize spontaneously. AChR clusters that are isolated either by shearing or by extraction with saponin and then incubated for 1 h in buffered saline lose a significant portion of their β-spectrin (Fig. 5). They also fail to display the AChR-rich and AChR-poor membrane domains typical of freshly isolated structures. Instead, the receptors in these clusters redistribute into irregularly spaced microaggregates. Most of the β-spectrin remaining in the reorganized clusters is enriched at the AChR microaggregates (Fig. 8, G–J). Thus, β-spectrin and AChR remain associated even under conditions in which β-spectrin is slowly lost from cluster membrane and the AChR redistribute in the bilayer. These results strongly suggest that β-spectrin and AChR are closely associated.

Codistribution with Actin
Actin has been shown to be present at the AChR domains, but not the contact domains, of AChR clusters isolated by extraction with saponin (12). We performed double immunofluorescence labeling experiments using a mouse monoclonal antibody to actin (12) and an affinity-purified rabbit antibody to β-spectrin to learn if β-spectrin and actin codistribute. Controls showed that all the labeling we observed was specific for the antigen probed (see Materials and Methods). Our results showed that β-spectrin codistributes with actin (Fig. 9). Comparison with samples labeled with R-BT and antibodies to β-spectrin, actin, or the 43K protein, showed that the types of labeling depicted in Fig. 9 were only obtained at AChR clusters.

The Neuromuscular Junction
We labeled frozen sections through the junctional region of rat diaphragm muscle to determine if β-spectrin is present there. Junctional regions, identified using R-BT, labeled brightly with VIIF7 and FGAM (Fig. 10, A and B, arrowheads), suggesting that β-spectrin is indeed a synaptic antigen. We also examined frozen sections of muscles that had been denervated for ~14 wk, and found bright labeling for β-spectrin at former endplate regions (Fig. 10, C and D, arrowheads). Extrajunctional regions were also labeled in both control and denervated muscles (double arrowheads). Labeling of junctional and extrajunctional regions by a control mouse IgG (mope 21) was insignificant (Fig. 10, E and F). These results suggest that β-spectrin is a junctional antigen, and, because presynaptic structures are lost from muscle denervated for more than 5 wk (49), is present in the post-synaptic region.

Discussion
Using the criteria of immunofluorescence, immunoprecipitation, and peptide mapping, we have found a protein in preparations of isolated AChR clusters that appears to be a new isofrom of β-spectrin. Although its polypeptide chain molecular mass, 220,000D is similar to that of human erythroid β-spectrin, the β-spectrin at AChR clusters has a different peptide map. This is probably not due to species differences alone, as maps of rat erythroid β-spectrin show several major spots that are not present in β-spectrin from AChR clusters (W. Knowles, personal communication). The β-spectrin at AChR clusters is also immunologically distinct, as it fails to bind several monoclonal antibodies that react with the rat erythroid isoform in immunoblots. Its mass further distinguishes the β-spectrin we have studied from the common β-spectrin isoforms of brain and other tissues, including skeletal muscle, which are larger (M, = 235,000; references 37, 44, 53, 63, 70). Thus the β-spectrin at AChR clusters resembles, but is distinct from, β-spectrin isoforms from brain and erythrocytes.

Another feature that distinguishes the β-spectrin at AChR clusters from other β-spectrins is the apparent absence of an associated α chain. In nearly all the cells where it has been found, spectrin is an heterodimer composed of α and β subunits which can be distinguished on the basis of their biochemical and antigenic properties (see references 7 and 48, and references therein). We have been unable to identify a second spectrin subunit resembling erythroid α-spectrin in preparations of isolated AChR clusters. A set of monoclonal and polyclonal, affinity-purified antibodies directed against the α subunits of human erythrocyte or human or pig brain spectrins failed to label AChR clusters. If β-spectrin in clusters exists as an heterodimer, its paired subunit, therefore,
may be a unique isoform. Different isoforms of spectrin have been extensively studied in avian cells (e.g., references 44, 45, 53, 54, 57) and mammalian isoforms have been reported in human skeletal muscle (70), mouse brain (63), rat Sertoli cells (80), and in Friend erythroleukemia cells (34). Alternatively, the β-spectrin associated with clusters may be present as homotetramers, reported to exist in chicken erythroid cells and to bind to the cytoplasmic face of erythrocyte membranes with low affinity (76).

Our experiments showing that AChR and β-spectrin redistribute together in cluster membrane suggest that these two proteins are closely linked. Of the proteins present at AChR clusters, only the 43K protein appears to be more closely associated with the AChR in situ (e.g., references 21, 56, 69). In the AChR clusters of rat myotubes, the 43K protein is approximately equimolar with AChR, and can only be removed from clusters by extraction at pH 11 or with lithium diiodosalicylate (13). It is not removed from clusters by chymotrypsin (13), which does, however, remove β-spectrin. Actin, on the other hand, is depleted from isolated AChR clusters by buffer A (12), which does not affect β-spectrin. Other cytoskeletal proteins of clusters, present over AChR-poor domains (14; Bloch, R. J., M. Velez, J. Krikorian, and D. Axelrod, manuscript submitted for publication), are removed almost quantitatively by extraction with saponin without disrupting cluster organization (11), and so are not directly associated with AChR. Thus, of the proteins we have studied, β-spectrin appears to be more readily dissociated from AChR than the 43K protein, but less readily dissociated than actin and some other cytoskeletal proteins.

Figure 10. β-Spectrin at the neuromuscular junction. Frozen sections, 4 μm thick, were cut through the junctional regions of diaphragms from control rats (A, B, E, and F), or from rats that had been phrenecotomized 14 wk earlier (C and D). Sections were labeled, unfixed, with VIIF7 and FGAM, and with R-BT, to reveal the junctional regions (A-D). Additional sections were labeled with mopc 21, a control mouse IgG (E and F). A, C, and E show R-BT fluorescence; B, D, and F show immunofluorescence. The results indicate that, in the rat, β-spectrin is present in the postsynaptic region of the neuromuscular junction (A-D, arrowheads). Immunofluorescence due to β-spectrin was also apparent in extrajunctional regions (B and D, double arrowheads). Bar, 20 μm.
AChR clusters of rat myotubes, the relative ease of extracting these proteins from the AChR domains, the relative amounts of AChR, 43K, and β-spectrin estimated to be present. Higher oligomers of β-spectrin (hexamers, octamers) are not shown, but may be present. Evidence for the binding of the 43K protein to spectrin and the oligomeric nature of the actin is still lacking. Not drawn to scale.

The possibility that structural proteins at AChR domains are highly ordered prompted us to quantitate the amount of β-spectrin at clusters relative to AChR. This approach was made possible by the fact that VIIF7 is an IgG. Earlier semiquantitative work on AChR clusters with IgG mAbs against AChR and the 43K protein yielded fluorescein antibody/R-BT (F/R) ratios of ~1 for both antibodies, consistent with a stoichiometry of their respective antigens of 1:1 (13). If one assumes that FGAM reacts equally well with all IgG monoclonal antibodies, and that the number of ligand binding sites of the mAb that are occupied by the antigen is always either one or two, then the value of F/R obtained with VIIF7 should provide an estimate of the relative stoichiometry of β-spectrin and AChR at AChR clusters. Preliminary results in quantitative ELISA experiments suggest that the former assumption is correct (Resneck, W. G., and R. J. Bloch, unpublished data). If the latter assumption is wrong, then we may have to modify our estimate, but only by a factor of two. This twofold uncertainty does not concern us at present, as the range of the F/R ratios we have obtained using VIIF7 is of the same magnitude. The interpretation of our results is further complicated by the evidence that β-spectrin is lost from AChR clusters upon incubation. Nevertheless, shared AChR clusters, which should lose the least amount of antigen during their preparation, yield F/R values of 3.5–6.5. Thus, we propose that AChR clusters contain four to seven β-spectrin moieties for each molecule of AChR and 43K protein.

The simplest organization of these molecules that would be consistent with their stoichiometries and their relative ease of extraction from clusters is depicted in Fig. 11. A similar organization is found in the human erythrocyte, where spectrin, present largely as tetramers with some higher oligomers (6, 46, 50, 52), is bound to the membrane largely through interactions with ankyrin (for reviews, see references 7, 48). We propose that spectrin at AChR clusters is linked to AChR by the receptor-associated 43K protein. The model shown in Fig. 11 does not include interactions between the 43K protein and actin (74), or other postsynaptic proteins, such as the 58K and 300K proteins (32, 75). It may, therefore, have to be revised or discarded as we learn more about these proteins and their association with clustered AChR.

The presence of several β-spectrin molecules for each AChR and 43K protein may help to account for the loss of spectrin from clusters after their isolation. The shedding of β-spectrin could be due to gradual depolymerization of oligomeric and tetrameric β-spectrin to dimers and monomers, leaving bound to the membrane only those subunits that are closely associated with AChR. Spectrin depolymerization, reported to occur after solubilization of the human erythrocyte membrane (46), would be consistent with the apparent stoichiometry, deduced from the F/R ratios, of one β-spectrin homodimer for each AChR present in isolated clusters after long incubations (Fig. 5).

Our observation that some regions of clusters are not enriched in β-spectrin (Fig. 1, C and D, asterisks) may be explained by a reversible association of spectrin with the muscle membrane. The AChR in spectrin-poor regions are usually more poorly organized and less densely clustered than receptors associated with β-spectrin. This poor organization may reflect the dissociation of spectrin during cluster isolation, or the possibility that clusters were isolated as they were forming or dispersing. Stages when the binding of spectrin to the membrane may be more reversible. Changes in the extractability of membrane-bound spectrin occur in differentiating epithelial cells (55). Alternatively, the absence of β-spectrin from some regions of AChR clusters may indicate that β-spectrin is not absolutely required for AChR clustering.

Our results show that an isoform of β-spectrin is associated with the nicotinic AChR of cultured rat myotubes, but we believe that the association between spectrin and postsynaptic receptors may well be more general. Together with actin, spectrin has already been found at synaptic contacts in the Mauthner cells of the goldfish (41) and at postsynaptic densities in mammalian brain (25, 40, 78). Spectrin may be involved in the differentiation of muscle and nerve (44, 45, 53, 54, 64, 78, 79). The close association of β-spectrin with clustered AChR, and the similarities between clusters and the developing neuromuscular junction (16), suggest that the unusual isoform of β-spectrin that we have described here may play an important role in synaptic morphogenesis.

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