Mini-Review

The Submembrane Machinery for Nicotinic Acetylcholine Receptor Clustering

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THE segregation of membrane proteins into specialized domains is important for many aspects of cell function, especially those involving cell–cell interaction and communication. Of particular interest to cellular and molecular neurobiologists are the highly specialized distributions of ion channels at synapses. Both the pre- and postsynaptic membranes exhibit organized arrays of ion channels that are important for synaptic transmission. At the neuromuscular junction, voltage-activated calcium channels are clustered at presynaptic active zones (62), the sites of acetylcholine release. Directly across the synaptic gap from active zones are regions of the postsynaptic membrane containing extraordinarily high concentrations $(8–10,000/\mu^2)$ of nicotinic acetylcholine receptors (AChR)[1] (24). A few microns away from the synapse, the concentration of receptors abruptly decreases to very low levels. Typically, >90% of the AChR in a skeletal muscle fiber are found at the synapse, an area that occupies <0.1% of the total muscle membrane. The AChR is a pentameric complex of four different transmembrane subunits that is inherently capable of diffusion within the plane of the membrane. Understanding the molecular mechanisms responsible for anchoring AChR at postsynaptic sites has been a major issue for researchers interested in neuromuscular synaptogenesis.

Clustering of AChR occurs early during rat neuromuscular synapse formation, within hours of contact between the growing nerve and the muscle fiber (6). Subsequently, the receptor density and the size of the cluster continues to increase as the synapse matures (4, 71). In developing muscle, each postsynaptic site is innervated by several presynaptic terminals. During the second week after birth, most of these terminals are eliminated such that only one neuronal input remains at each postsynaptic site (60). Recent evidence suggests that terminal retraction is preceded by the loss of AChR from postsynaptic sites directly beneath those terminals destined for elimination and that this loss may occur by diffusion of receptors away from these sites (61). Thus, the regulation of AChR clustering is a key event, not only in the formation of the neuromuscular junction, but possibly also in synapse elimination and remodeling.

A clue to the molecular mechanisms by which AChR are anchored at synaptic sites came from studies conducted almost 10 years ago on *Torpedo* electric organ postsynaptic membranes. The electrocyte synapse is very similar to the neuromuscular junction and has served as an excellent system for biochemical studies of the AChR and other synaptic molecules. Postsynaptic membranes purified from the electric organ contain high densities of immobilized AChR (70). Removal of peripheral membrane proteins by alkaline extraction (53), most notably one of M_r 43,000 (43K protein), is accompanied by an increase in the mobility of AChR (5, 20, 48, 64) without affecting the functional properties of the receptor (53). These results suggest that the proteins removed by the alkaline pH treatment are involved in anchoring AChR at synaptic sites.

Further studies have, in most cases, supported a role for the 43K protein in AChR clustering. The AChR and 43K protein share a common distribution in the postsynaptic membrane of the electrocyte (66) and neuromuscular synapses (30, 32), and in AChR clusters on cultured myotubes (9, 14, 18, 56). In the electrocyte and in cultured myotubes, the AChR and 43K protein are present in approximately equimolar concentrations (45, 46). Chemical crosslinking studies (15) and freeze fracture immunoelectron microscopy (13) support the view that the 43K protein associates with cytoplasmic domains of the AChR, but direct biochemical evidence for such interaction is lacking. Solubilization of the AChR with detergents such as Triton X-100, sodium cholate, and deoxycholate causes dissociation of the 43K protein from the receptor. To date, conditions under which this putative complex can be isolated intact have not been discovered.

The isolation of cDNA clones encoding both the muscle AChR (12) and the 43K protein (28, 31) provided the tools needed for a direct examination of the role of the 43K protein in AChR clustering. Functional AChR are expressed on the surface of *Xenopus* oocytes injected with RNA encoding the mouse muscle receptor α, β, γ, and δ subunits. These AChR are distributed uniformly in the surface membrane of the oocytes (34). In contrast, oocytes injected with AChR subunit RNA and RNA encoding the mouse muscle 43K protein express surface AChR in a clustered distribution (34; also see Fig. 1). The 43K protein appears to act directly in the clustering process since these AChR clusters, which are 1–2 μ in diameter, also contain the 43K protein. Finally, of particular importance is the observation that the 43K protein forms clusters of similar size and shape, even when expressed in oocytes in the absence of AChR (34). This result suggests

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptors; DRP, dystrophin-related protein.
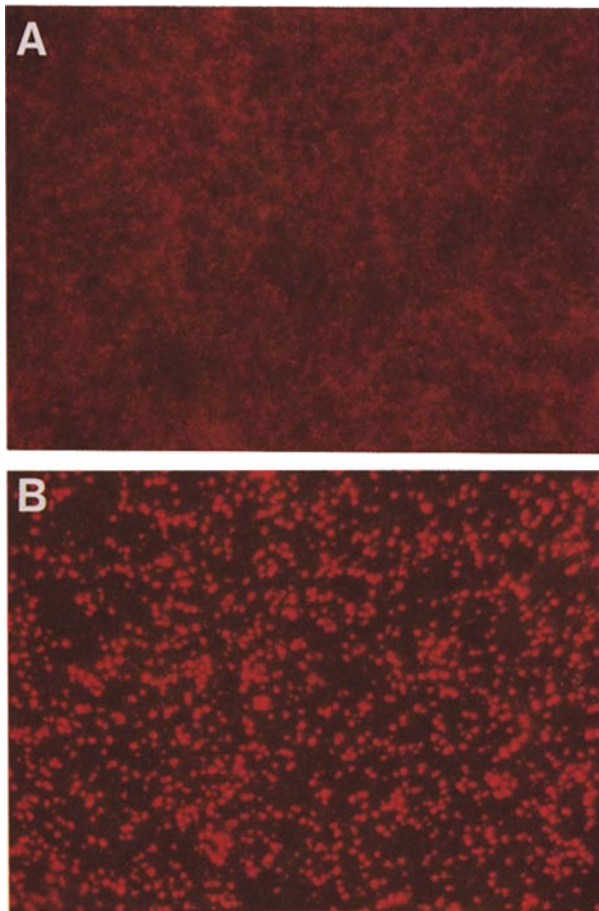
Figure 1. Clustering of AChR is induced by coexpression with 43K protein in *Xenopus* oocytes. Oocytes were injected with in vitro-synthesized RNA encoding the α, β, γ, and δ subunits of mouse muscle AChR alone (*A*) or in combination with RNA encoding the mouse muscle 43K protein (*B*). After 3 d, the distribution of AChR was visualized by sequential labeling with rhodamine α-bungaro-toxin, rabbit anti-α-bungarotoxin, and rhodamine goat anti-rabbit IgG. A small region of the surface of an oocyte is viewed en face. When expressed alone, AChR are distributed uniformly. Coexpression with the 43K protein causes the formation of AChR clusters of 1-2 μ in diameter. For details, see Froehner et al. (34).

a model in which the key event is the regulation of 43K clustering, and that the distribution of AChR is governed in large part by its interaction with the 43K protein.

Similar results were obtained subsequently with quail fibroblast cells stably transfected with cDNA encoding the mouse muscle AChR subunits (58). The surface AChR in these cells were uniformly distributed. Transient transfection with cDNA encoding the 43K protein caused a reorganization of AChR into clusters of 1-10 μ in diameter. Clustering occurred with both the adult ($\alpha_2\beta\epsilon\delta$) and the embryonic ($\alpha_2\beta\gamma\delta$) forms of AChR. As in the oocytes, parental fibroblast cells transfected with 43K cDNA express clustered 43K protein in the absence of AChR.

The molecular details of 43K clustering and its interaction with AChR are not known. Comparison of the amino acid sequences of the *Torpedo* (17, 27), mouse (28, 31), and *Xenopus* 43K proteins (3), however, reveals a number of conserved regions that may be important for cluster formation (Fig. 2). The amino terminus of the 43K protein is highly conserved,

with the first 19 residues being almost completely identical among the three species (only four nonidentities out of 57 residues). This high degree of conservation may be due in part to the recognition sequence required for myristylation of the amino-terminal glycine, a modification that has been demonstrated by direct biochemical tests (19, 52). The myristyl group may anchor the 43K protein to the membrane (59) by intercalation into the lipid bilayer. The consensus sequence for myristylation, however, requires only an NH_2-terminal glycine and small, uncharged residues at positions 2 and 5 (72). Thus, the highly conserved NH_2-terminal sequence may mediate some other aspect of 43K protein function. It may, in conjunction with the myristyl group, interact with other proteins, such as the AChR (19).

Three other conserved regions of the sequence of the 43K protein deserve attention. A potential leucine zipper structure, located approximately one fourth of the length of the polypeptide from the NH_2-terminus, may be important for dimerization of the 43K protein or for interactions with other proteins. Near the COOH terminus, a cysteine-rich region, originally identified as having homology with the regulatory region of protein kinase C (31), may fold into zinc finger structures. This sequence fits the CCCH-HCCC two finger sequence modified from Freemont et al. (29) and may mediate interactions of 43K protein with other proteins of the postsynaptic membrane. Finally, the residues surrounding serine-406 meet the requirements for both cyclic AMP-dependent (RRSS) and protein kinase C (SMK)–mediated phosphorylation. It should be noted that the 43K protein appears not to be a kinase, as once proposed (35), since it contains none of the other structural characteristics common to this family of enzymes (28). For now, the importance of these structural elements remains speculative, but the application of site-directed mutagenesis and deletion analysis should be instructive in determining their roles, if any, in the clustering of the 43K protein and its interaction with the AChR and with other proteins.

Although it has been known for some time that the 43K protein and the AChR are closely associated, high-resolution analysis of the *Torpedo* receptor in its membrane-associated state has afforded a more detailed view of the interaction between these two proteins. Unfortunately, the results provided by two laboratories do not agree. Toyoshima and Unwin (73) used three-dimensional image reconstruction of helical arrays of receptors in tubular membranes to arrive at a structure at 17Å resolution. Their results place a protein density attributed to the 43K protein directly beneath the AChR very near the inner mouth of the central ion channel. In contrast, Stroud and co-workers (51), using a combination of reconstruction of electron microscopic images and x-ray diffraction on similar preparations, place the 43K protein in close association with the bilayer beside the AChR in a position that could link two receptors together. These conflicting observations clearly suggest quite different modes of action of the 43K protein. In addition, it should be kept in mind that the assignment of a region of the density maps to the 43K protein is based on its absence from membranes extracted with alkaline pH, a treatment that may directly alter AChR structure (44). While it seems quite likely that the protein identified in these studies is the 43K protein, the contribution of other less abundant proteins in these membranes remains unknown. Thus, unambiguous identification of the 43K pro-

tein in these images, perhaps by antibody binding, and resolution of the conflicting views of its location with respect to the AChR await further experimentation.

At what stage of neuromuscular synaptogenesis does the 43K protein associate with the AChR to influence its distribution? One possibility is that the 43K protein is involved in the initial step of cluster formation that occurs soon after nerve–muscle contact. The observation that all nerve-induced AChR clusters on cultured muscle cells contain the 43K protein is consistent with this idea (14). Other studies, however, suggest that the initial nerve-induced clusters form by a different mechanism and that the 43K protein associates at a later stage, possibly to stabilize the clusters. *Torpedo* electrocytes are derived from musclelike precursor cells during electric organ development (26). Before innervation, the oval-shaped precursor cells accumulate AChR on the ventral surface. These receptor aggregates do not have 43K protein associated with them, as judged by immunofluorescence microscopy (42, 47). Subsequently, the cells flatten into thin disks, and become innervated on the ventral surface. At this stage, the co-distribution and equimolar stoichiometry of AChR and the 43K protein characteristic of adult electrocytes is already evident (47). A disparity between AChR clusters and the 43K protein distribution has also been found in chick muscle myoblasts (74). Some myoblasts exhibit clusters of

AChR soon after plating. Approximately one-third of these clusters exhibit no fluorescence staining with anti-43K antibodies. Furthermore, the ratio of staining intensity for AChR and 43K protein varied among clusters. Although these results demonstrate that not all clusters of AChR require the 43K protein for formation, the relevance of these clusters in electrocyte precursor cells and myoblasts to those that form during synaptogenesis is not clear.

The simplicity of purified *Torpedo* postsynaptic membranes, in which the AChR and the 43K protein are the only major proteins, and the ability of the 43K protein to induce receptor clustering suggest that the interaction of these two proteins is sufficient to anchor AChR. This is undoubtedly an oversimplification. The 43K induced-clustering seen in oocytes and fibroblasts may use proteins provided by the recipient cells. In fact, the clusters formed in oocytes are much smaller than mature clusters in muscle cells and thus may represent only the first in a series of steps necessary for the formation of the AChR accumulations characteristic of adult neuromuscular junctions. A number of proteins associated with the postsynaptic membrane have been identified with antibodies against known cytoskeletal proteins and with antibodies to alkaline extractible proteins from *Torpedo* electric organ membranes (summarized in Table I). A current goal in this field is to determine which of these proteins are direct players
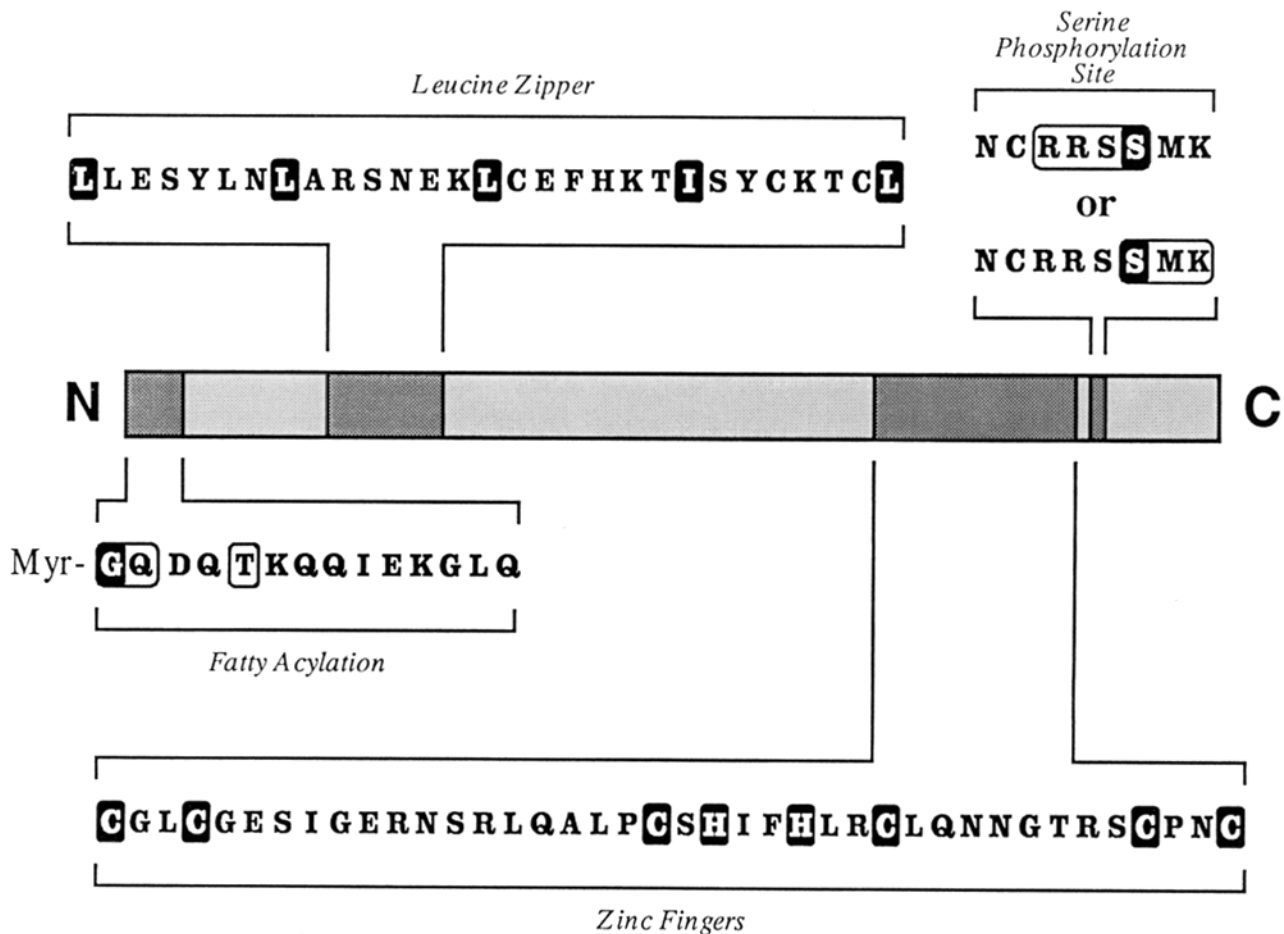

Figure 2. Conserved structural features of the 43K protein. The relative positions of four, highly conserved elements are indicated on the central bar representing the linear sequence of the 43K protein. *N*, amino terminus; *C*, carboxy terminus; *Myr*, myristyl. See text for details.

in AChR clustering and which have functions in other aspects of postsynaptic membrane structure. The remainder of this review will consider this issue.

Antibodies have been used to compare the distribution of each of these proteins with the distribution of AChR at the neuromuscular junction and in clusters that occur spontaneously on aneural myotube cultures. AChR clusters on rat myotubes are comprised of two interdigitating membrane domains, one rich in AChR and the other AChR poor (7). The latter, which is most closely apposed to the substratum, is also called the contact domain and is probably analogous to the deeper regions of the postjunctional folds in the mature junction. The AChR-rich zones correspond to the crests of the folds. The structural resolution achieved by this system has been very important in sorting out which of the many proteins found at the neuromuscular junction are in the AChR-rich domains and therefore likely to be involved in receptor clustering, or at least in some AChR-related function.

Several postsynaptic cytoskeletal proteins are probably not directly involved in AChR clustering. Vinculin (10, 23), talin (67), paxillin (75), filamin (10, 23), α actinin (10, 23), ankyrin (25), and dystrophin (22, 38, 69) are all present throughout the sarcolemmal membrane but are concentrated at the neuromuscular junction. Some, and possibly all, of this apparent concentration may arise from the folding of the postsynaptic membrane, which would cause a uniformly distributed antigen to appear severalfold more concentrated at the endplate. The conclusion that these proteins are not involved directly in clustering is based on three criteria, at least one of which is fulfilled by each protein. First, in myotubes, they are localized primarily in the contact domains of AChR clusters. Second, the AChR remain clustered when these proteins are removed. Finally, although concentrated at endplates, their distribution does not coincide with the AChR. Apparently normal clustering of AChR in human Duchenne muscle (65) (in which disruption of the dystrophin gene abrogates dystrophin expression) further supports the contention that dystrophin is not obligatory for cluster formation. Nevertheless, this group of cytoskeletal proteins may have other important functions at the neuromuscular junction, possibly involving the formation of postjunctional folds or the stabilization of other proteins restricted to this region of the synaptic membrane.

Three other cytoskeletal proteins, desmin, lamin B, and

Table I. Cytoskeletal and Peripheral Membrane Proteins Associated with AChR Clusters and the Neuromuscular Postsynaptic Membrane

Protein	References	Location and comments
43K Protein	(9, 13, 15, 34, 58, 66)	Associated directly with the AChR; coexpression causes clustering of AChR in oocytes and fibroblasts
Actin	(8, 23, 36)	AChR domains; removal alters AChR distribution; synaptic isoform distinct from myofibrillar actin
β-spectrin	(11)	AChR domains; removal alters AChR distribution; unique isoform
Vinculin	(10, 23)	Contact domains; concentrated at endplates; present extrasynaptically
Talin	(43, 63, 67)	Contact domains; concentrated at endplates; present extrasynaptically
Paxillin	(75)	Contact domains; concentrated at endplates; present extrasynaptically
Filamin	(10, 23)	Concentrated at endplates; present extrasynaptically
α-actinin	(10, 23)	Concentrated at endplates; present extrasynaptically
Tropomyosin 2	(1, 50)	Concentrated at endplates; present extrasynaptically; microinjection of antibodies blocks new cluster formation in cultured myotubes
58K Protein	(Butler, M. H., K. Douville, A. A. Murnane, R. Sealock, and S. C. Froehner. 1990. J. Cell Biol. 111:165a; and 18, 23, 33)	AChR domains; concentrated at endplates; present extrasynaptically; associated with dystrophin in sarcolemma
87K Protein	(18)	Overlaps but extends beyond AChR clusters; concentrated at endplates; present extrasynaptically
Dystrophin	(22, 38, 69)	Contact domains; concentrated at endplates but not in AChR domains; extrasynaptic sarcolemma; not required for AChr clustering
Dystrophin-related protein	(40, 49)	Domain unknown but more endplate specific than dystrophin
Desmin	(68)	Concentrated near the depths of the postsynaptic folds
α-tubulin	(39)	Acetylated microtubule arrays extending from subsynaptic nuclei towards the endplate
Lamin B	(21)	54-kD immunologically related protein localized at electrocyte innervated membrane; concentrated at muscle endplates
Ankyrin	(25)	Depths of the postsynaptic folds

tubulin are concentrated in the general region of the post-synaptic membrane, but not directly at AChR sites. In addition to being a component of Z-lines, desmin is also concentrated at the endplate, but near the depths of the folds, and occasionally surrounding synaptic nuclei (68). A 54-kD protein immunologically related to lamin B may be the site of insertion of intermediate filaments into the postsynaptic membrane (21). Microtubule arrays, which contain an acetylated form of tubulin, extend from subsynaptic nuclei towards the endplate membrane (39). That these cytoskeletal proteins may be involved in regulating synaptic nuclei specialized for AChR expression is an intriguing possibility, but they do not appear to be part of the cytoskeleton most intimately associated with AChR.

In addition to the 43K protein, a small group of other cytoskeletal proteins fulfill at least some of the requirements expected for AChR anchoring proteins. Actin (8, 36) and a unique muscle isoform of β-spectrin (11) are components of AChR-rich domains of cultured myotubes and are enriched at neuromuscular junctions. A role for actin and β-spectrin in AChR organization is supported by the observation that their removal from the membrane by relatively mild treatments is accompanied by disruption of AChR domains (8, 11). Models in which either actin (77) or β-spectrin (11) interact directly with the 43K protein have been proposed, but still remain to be tested.

Two other candidates for anchoring proteins, the M_r 58,000 (58K) and M_r 87,000 (87K) proteins (18, 30, 33), were first identified as alkaline extractable proteins of *Torpedo* postsynaptic membranes. Both are concentrated at skeletal muscle endplates and are associated with extrasynaptic membranes. The 87K protein overlaps but extends beyond the region of AChR clusters in chick myotubes (18). The 58K protein is also concentrated at AChR clusters on myotubes (33), where it is located primarily in AChR-rich domains (R.J. Bloch, personal communication).

In addition to its association with AChR-rich membrane, the 58K protein is one of several proteins complexed with *Torpedo* dystrophin (Butler, M. H., K. Douville, A. A. Murnane, R. Sealock, and S. C. Froehner. 1990. *J. Cell Biol.* 111:165a). Furthermore, the 58K protein associates very inefficiently with the extrasynaptic sarcolemma of *mdx* mouse, which lacks dystrophin (Butler, M. H., K. Douville, A. A. Murnane, R. Sealock, and S. C. Froehner. 1990. *J. Cell Biol.* 111:165a). Staining for 58K protein at the neuromuscular junction, however, is maintained in *mdx* muscle, suggesting that it is associated with some other member of the dystrophin/spectrin/α-actinin family of proteins at this site. Candidates include β-spectrin and the dystrophin-related protein (DRP), a protein with strong sequence homology to dystrophin but encoded by an autosomal gene (40, 49). Preliminary evidence with anti-peptide antibodies indicates that the DRP is highly concentrated at endplates, with only very weak staining of extrasynaptic membranes (S. C. Froehner, K. Douville, and R. Sealock, unpublished results). Further studies are needed to determine whether the 58K protein associates with the DRP and whether this complex has a role in AChR organization.

A completely different approach has led to the identification of a postsynaptic tropomyosin-like protein that may be involved in AChR clustering. Transformation of chick myotubes by Rous sarcoma virus prevents AChR clustering (2).

Biochemical analyses demonstrated that the infected myotubes were missing a 37-kD protein immunologically related to tropomyosin (1). An mAb to this protein, called tropomyosin 2, stained uninfected myotubes diffusely (1), and when injected into cells blocked the formation of new clusters, but did not disrupt preexisting clusters (50). The blocking action of the antibody may be because of interference with specific interactions that occur locally at the AChR cluster between tropomyosin 2 and other cluster-associated proteins. Alternatively, since tropomyosin 2 is a major myotube protein, antibodies to it may have widespread disruptive effects on the cytoskeleton and, thus, block AChR cluster formation indirectly.

The studies reviewed here have identified several membrane-associated proteins, in addition to the 43K protein, that may play a role in AChR clustering at the neuromuscular synapse. Examination of the effects of these proteins on cluster formation by coexpression with the AChR and/or 43K proteins is an important next step in understanding their functions in postsynaptic membrane assembly. In addition, biochemical analysis of the interactions that occur among these proteins will be necessary to test proposed models. Most importantly, we should now be in a position to begin to understand, at the molecular level, the regulation of postsynaptic cytoskeleton assembly and AChR clustering by the nerve. Several agents, including brain-derived soluble proteins (23, 41, 76), basal lamina components (54), basic fibroblast growth factor (57), and positively charged beads (55) induce AChR clustering on muscle cells, but the mechanisms by which these agents act remain largely unknown. Agrin, a basal lamina protein that promotes AChR clustering (54), has recently been shown to stimulate phosphorylation of AChR tyrosine residues, primarily on the beta subunit (78). An intriguing possibility is that this modification, which could also be stimulated by basic fibroblast growth factor, enhances the interaction of AChR with the 43K protein or other cytoskeletal proteins.

Finally, the regulation of the genes encoding these synaptic cytoskeletal proteins remains virtually unexplored. It is noteworthy that the isoforms of some postsynaptic cytoskeletal proteins (actin and β-spectrin, for example) and extracellular matrix proteins (s-laminin; 37) differ from those found at other sites in the muscle cells. This recurring theme of molecular specialization at the postsynaptic membrane may permit the nerve to regulate differentially the expression of these proteins in the muscle cell during synaptogenesis.

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