The Actin-Driven Movement and Formation of Acetylcholine Receptor Clusters

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Abstract. A new method was devised to visualize actin polymerization induced by postsynaptic differentiation signals in cultured muscle cells. This entails masking myofibrillar filamentous (F)-actin with jasplakinolide, a cell-permeant F-actin–binding toxin, before synaptogenic stimulation, and then probing new actin assembly with fluorescent phalloidin. With this procedure, actin polymerization associated with newly induced acetylcholine receptor (AChR) clustering by heparin-binding growth-associated molecule–coated beads and by agrin was observed. The beads induced local F-actin assembly that colocalized with AChR clusters at bead–muscle contacts, whereas both the actin cytoskeleton and AChR clusters induced by bath agrin application were diffuse. By expressing a green fluorescent protein–coupled version of cortactin, a protein that binds to active F-actin, the dynamic nature of the actin cytoskeleton associated with new AChR clusters was revealed. In fact, the motive force generated by actin polymerization propelled the entire bead-induced AChR cluster with its attached bead to move in the plane of the membrane. In addition, actin polymerization is also necessary for the formation of both bead and agrin-induced AChR clusters as well as phosphotyrosine accumulation, as shown by their blockage by latrunculin A, a toxin that sequesters globular (G)-actin and prevents F-actin assembly. These results show that actin polymerization induced by synaptogenic signals is necessary for the movement and formation of AChR clusters and implicate a role of F-actin as a postsynaptic scaffold for the assembly of structural and signaling molecules in neuromuscular junction formation.

Key words: acetylcholine receptor cluster • actin • neuromuscular junction • latrunculin • jasplakinolide

Introduction

Innervation of the skeletal muscle signals differentiation of the sarcolemma at sites of nerve–muscle contact. This leads to the formation of acetylcholine receptor (AChR) clusters at the postsynaptic membrane. These receptors are initially distributed in a diffuse manner in the muscle membrane and become clustered to extremely high density during the formation of the neuromuscular junction (NMJ; Sanes and Lichtman, 1999). The signal transduction mechanism involved in AChR clustering during NMJ formation has been extensively studied both in vitro and in vivo. Agrin, a heparan-sulfate proteoglycan, is released by the motoneuron growth cone to induce postsynaptic differentiation (Bowe and Fallon, 1995; Ruegg and Bixby, 1998; Gautam et al., 1996). Recent works from our laboratory have also suggested the involvement of heparan-sulfate proteoglycan–bound growth factors in the signaling mechanism (Rauvala and Peng, 1997; Daggett et al., 1996a). Agrin, together with other yet to be unidentified muscle-derived signals, appears to activate the receptor tyrosine kinase (RTK) MuSK (muscle-specific kinase) to initiate the signal transduction cascade (Sanes and Lichtman, 1999; Ganju et al., 1995; Glass et al., 1996). Steps following MuSK activation are largely unknown. Recent studies have shown that MuSK, similar to other RTKs, can be activated by dimerization (Hopf and Hoch, 1998; Xie et al., 1997). A recurring theme in signal transduction involving RTKs is that their activation leads to the assembly of a cortical filamentous actin (F-actin) cytoskeleton through highly regulated polymerization of globular actin.
Materials and Methods

Materials

Jasplakinolide, rhodamine-conjugated phalloidin (Rh-phalloidin), and rhodamine-conjugated α-bungarotoxin (R-BTX) were purchased from Molecular Probes. Oregon green-conjugated BTX (OG-BTX) was a gift from Dr. Richard Rotundo (University of Miami, Miami, FL). Ltn A was purchased from Molecular Probes and from Biomol. Monoclonal anti-PY antibody 4G10 was purchased from Upstate Biotechnology. Fluorescence-conjugated secondary antibodies were obtained from Organon-Teknika Cappel. Recombinant heparin-binding growth-associated molecule (HB-GAM), kindly provided by Dr. Hekki Rauvala (University of Helsinki). The cortactin-EGFP construct was then subcloned into pCS2 cassette was then subcloned into pCS2+ vector in polyclinker 1 region downstream from SP6 promoter. To make synthetic mRNA of the cortactin-EGFP chimical protein, the plasmid was linearized and transcribed with SP6 RNA polymerase using a kit from Ambion, Inc., following the manufacturer’s instructions. The mRNA was injected into blastomeres at 1–2-cell stage with a Drummond oocyte injector (Drummond Scientific Co.). 1 to 2 ng mRNA in 4.6 to 9.2 nl was injected into each embryo. Embryos that showed green fluorescent protein (GFP) expression were used to prepare muscle cultures. Cultured muscle cells identified by GFP fluorescence were examined for the localization of the chimical protein after agrin or bead treatment.

Results

Induction of Actin Polymerization by Synaptogenic Signals

The F-actin localization at AChR clusters was examined by labeling muscle cells with Rh-phalloidin. The abun-
dance of myofibrillar F-actin in skeletal muscle cells made detection of its localization at AChR clusters difficult except at thin, lamellar areas. As shown in Fig. 2, A and B (see below), F-actin was concentrated at spontaneously formed AChR hot spots. Despite their colocalization, the F-actin patch was not congruent with the AChR cluster and they often existed in nearly complementary patterns.

To examine new F-actin assembly induced by AChR clustering stimuli, we experimented with jasplakinolide to mask the myofibrillar phalloidin labeling. This marine sponge toxin binds to F-actin with high specificity and is

Figure 1. Masking of preexistent F-actin structures by jasplakinolide pretreatment. (A–C) Competition between jasplakinolide and phalloidin for F-actin binding. (A) Rh-phalloidin labeling of myofibrils. (B and C) Pretreating the culture with 1 μM jasplakinolide followed by Rh-phalloidin blocked myofibril labeling, thus showing that these two toxins compete for F-actin binding. (D–G) Jasplakinolide pretreatment does not abolish new F-actin assembly in motile muscle cells. This cell was treated with 10 μM jasplakinolide for 3 h and rinsed with drug-free medium. The lamellipodia developed after jasplakinolide removal (compare D and E) exhibited F-actin assembly as shown by Rh-phalloidin labeling (G) at the end of the 24-h period. D and E are live images and F and G are fixed cell images. Phase–contrast of the cell at 24 h is shown in F. (H–K) In contrast to motile cells, this nonmotile cell showed no new F-actin assembly after jasplakinolide pretreatment. H and I are live images and J and K are fixed cell images.
cell permeant. It competes with phalloidin for F-actin binding as shown in Fig. 1 (A–C). After pretreating live muscle cells with micromolar concentrations of jasplakinolide, subsequent rhodamine-phalloidin labeling of preexisting F-actin structures, most notably myofibrils and AChR hot spots, was greatly diminished. However, this treatment did not prevent new actin polymerization associated with muscle cell motility such as lamellipodial extension as in the example shown in Fig. 1, D–G. In contrast, cells which did not exhibit such motility showed little new actin assembly (Fig. 1, H–K). This demonstrates the utility of this jasplakinolide application in masking preexisting F-actin to allow visualization of new actin assembly in muscle cells. Thus, this procedure was applied to examine actin polymerization induced by two stimuli known to induce AChR clustering. Latex beads coated with HB-GAM have been shown to induce discrete AChR clusters at bead-muscle contact sites in cultured Xenopus muscle cells (Peng et al., 1995). On the other hand, bath application of agrin, in particular the neuronal A4B8 form containing inserts of 4 and 8 amino acids at A and B positions, respectively, induces AChR clustering in a diffuse manner over the entire Xenopus muscle cell (Daggett et al., 1996b).

After masking preexistent F-actin by treating cells with 2.5–10 μM jasplakinolide for 3 h followed by extensive rinsing, the stimulus (beads or agrin) was applied, and 24 h later new F-actin was labeled by Rh-phalloidin and new AChR clusters were detected by OG-BTX. As shown in Fig. 2 (C–D and F–H), F-actin assembly was discretely localized at bead-induced AChR clusters. In the second example (Fig. 2, F–H), new actin polymerization was seen at bead-induced AChR clusters as well as at sites of lamellipodial and filopodial activity at the periphery of this muscle cell. At higher magnification, one can see that F-actin induced by beads existed in two configurations: a punctate pattern in the central region of the bead–muscle contact and a circumferential filament bundle surrounding the central region (Fig. 2, I–J). Interestingly, bead-induced AChR clusters also conformed to these two F-actin domains (Fig. 2, D, G, and J). However, the F-actin and AChR patterns were not congruent with each other. The majority of beads that induced AChR clusters also generated F-actin assembly (Fig. 2 E). Only rarely did we observe beads with only one type of specialization but not the other. In addition, no statistically significant difference was seen in the percentage of beads that induced AChR clustering after jasplakinolide pretreatment as compared with control cultures (percentage of beads with AChR clusters = 86 ± 2% after pretreatment versus 85 ± 2% in control).

On the other hand, bath application of agrin induced a more diffuse F-actin assembly and AChR clustering (Fig. 3). In general, F-actin patches induced by agrin were not closely associated with AChR clusters (Fig. 3, C–D). However, good colocalization of these two specializations was also detectable among clusters in an unpredictable manner as shown in Fig. 3 A and B (circles). Even in this case, a lack of congruency between F-actin and AChR clustering patterns is obvious. Similar to the bead-stimulated process, jasplakinolide pretreatment did not affect the number of agrin-induced clusters as compared with control cultures.

These data show that synaptogenic stimuli such as HB-GAM–coated beads and agrin induce the assembly of new F-actin. In contrast, the lack of phalloidin labeling at hot spots after jasplakinolide pretreatment indicates an absence of new F-actin assembly at these preexisting AChR clusters.
Cortactin–EGFP Localization at Newly Formed AChR Clusters

F-actin structures such as myofibrils in muscle cells are stable and undergo relatively little elongation and shrinkage (Littlefield and Fowler, 1998). In contrast, F-actin meshwork within the leading edge of motile cells is highly dynamic and retains polymerization and depolymerization activities (Condeelis, 1993). Cortactin is an F-actin binding protein that binds differentially to active F-actin meshwork at the leading edge and thus can be used as a marker to reveal the dynamic nature of its assembly (Wu and Parsons, 1993). To assess the extent of actin polymerization within the cytoskeleton at newly formed AChR clusters, we expressed cortactin–EGFP in cultured muscle cells and examined its distribution in relationship to receptor clusters induced by beads and agrin.

In quiescent cells, cortactin-EGFP was concentrated at lamellar structures that are present predominantly at the ends of muscle cells (Fig. 4 A). AChR hot spots generally were not associated with cortactin–EGFP (Fig. 4, A and B). In situations when it was detected at hot spots, the cortactin patch was usually smaller than the AChR cluster (data not shown). After HB-GAM bead treatment, cortactin–EGFP became reliably associated with bead-induced AChR clusters (Fig. 4, C and D, arrows), although they were not congruent with each other. In response to bath agrin application, cortactin–EGFP also became membrane associated (Fig. 4 E). However, unlike bead-treated cells, agrin-induced cortactin distribution was more diffuse. Although cortactin–EGFP was not precisely colocalized with agrin-induced AChR clusters, a general coexistence of these two molecules could be observed (Fig. 4, E and F). AChR clusters were more likely to be found along cortactin–EGFP enriched membrane areas.

Thus, HB-GAM beads induce a discrete localization of cortactin, whereas agrin induces a more diffuse membrane association of this protein. These two contrasting patterns of cortactin distribution are in agreement with those of F-actin distribution described above. The cortactin localization suggests an ongoing, dynamic F-actin assembly induced by beads and agrin.

Movement of AChR Clusters Propelled by Actin Polymerization

We next sought to understand the function of the F-actin assembly induced by AChR clustering stimuli. Sustained actin polymerization has been shown to be the mechanism of generating plasma membrane protrusions such as lamellipodia and filopodia (Condeelis, 1993). It also provides motile force for certain intracellular pathogens such as Listeria and Vaccinia to move in the cytoplasm of infected cells (Theriot et al., 1992; Cudmore et al., 1995). We thus examined whether newly formed AChR clusters induced by beads could undergo movement. AChR clusters were first induced by 5-μm HB-GAM beads. 24 h after bead treatment, when the clusters were well formed as shown by their bright R-BTX labeling, they were followed by fluorescence microscopy under low-light illumination. As shown in Fig. 5, AChR clusters underwent displacement on the cell surface over a distance of ~10 μm during the 40-h recording period. During this period of time, there was little cell movement as shown by (i) the constant distance between markers on and outside the cell (arrows in Fig. 5 B) and (ii) neighboring clusters often moved independently in both direction and velocity despite their proximity to each other (compare clusters 1 and 2 with 3 in Fig. 5 A and beads 3 and 4 in Fig. 6, A and C). Corresponding phase-contrast images showed that beads always moved with their associated AChR clusters as one unit. Thus, bead tracking offered a simple and less invasive means to study the cluster movement. As shown in Fig. 6, A and B, four bead-associated AChR clusters were followed for 24 h by recording bead positions at 10-min intervals. Their trajectories are plotted in Fig. 6 C. The beads moved in either a directional or zigzagged manner for a distance of ~10 μm during the recording period. To study the role of actin polymerization in this AChR cluster movement, the bead movement was followed in the pres-
ence of Ltn A, which sequesters G-actin and prevents its polymerization into F-actin (Spector et al., 1983; Coue et al., 1987). As shown in Fig. 6 D, except for a small degree of diffusional drift (see below), the bead movement was completely abolished.

To quantify the bead movement, mean square displacement (MSD) was calculated at each time point according to the formula (Lee et al., 1991; Dai and Peng, 1996).

$$\text{MSD}(n_{t_0}) = \frac{1}{N-n} \sum_{i=1}^{N-n} [(x_{n+i} - x_i)^2 + (y_{n+i} - y_i)^2]$$

where \((x_{n+i}, y_{n+i})\) is the position of the bead at time \(n_{t_0}\) \((t_0 = \text{interval between measurements})\) after starting at position \((x_i, y_i)\). \(N = \text{total number of positions recorded}\). \(n\) ranges from 1 to \(N-1\). Example MSD values in control and Ltn A–treated cultures are plotted in Fig. 6 E. MSD plots from Ltn A–treated cells were largely linear, consistent with the notion that the small amount of bead movement is diffusion-limited. By fitting the MSD plots from Ltn A–treated cells with the following formula, the diffusion coefficient of bead movement was calculated.

$$\text{MSD}(t) = 4Dt$$

where \(D\) is the diffusion coefficient and \(t\) is the elapsed time interval. A mean diffusion coefficient of \(3 \times 10^{-14} \text{cm}^2/\text{s}\) was obtained. This value was used to estimate the mean velocity of actin-driven bead movement from plots such as the one shown in Fig. 6 E by fitting the MSD data with the following formula:

$$\text{MSD}(t) = 4Dt + \nu^2 t^2$$

where \(\nu\) is the mean speed of actin-driven movement. From this calculation, a mean speed of 0.12 nm/s was obtained. As shown in Fig. 6 E (solid line), this calculation gave very good fit to the data (\(R\) coefficient = 0.99). This value is consistent with those obtained from the trajectories shown in Fig. 6 C.

These data show that AChR clusters with their associated beads undergo actin-driven movement in the plane of the membrane. As receptors within the cluster are connected with the bead via an extracellular matrix linkage and are linked with other cytoskeletal proteins such as the dystrophin complex, as we have shown previously (Peng and Chen, 1992; Rochlin et al., 1989), the AChR cluster movement actually involves an entire transmembrane protein complex. In contrast to bead-induced AChR clusters, there is no evidence that preexistent hot spots, which are not associated with dynamic actin assembly, undergo movement (our unpublished observation).

**The Effect of Ltn A and Jasplakinolide on AChR and PY Clustering**

The colocalization of F-actin with AChR clusters also suggests a role of actin polymerization in their formation. This
premise was tested by interfering with F-actin assembly with Ltn A. Muscle cultures were pretreated with Ltn A for 30 min and then presented with beads or agrin in the continued presence of this toxin. Ltn A, at a concentration as low as 1 μM, reduced F-actin assembly induced by beads (Fig. 7, A and B). AChR clustering induced by beads was also reduced as shown by a decrease in the intensity of R-BTX labeling associated with beads (Fig. 7 C). At higher Ltn A concentrations, cluster formation was abolished in a dose-dependent manner with half-inhibitory concentration for complete blockade at 20 μM (Fig. 8, A–D and I). An example of this inhibition is shown in Fig. 8, A–D. At these concentrations, cytochalasin D had little effect on bead-induced AChR clustering (Fig. 8 I).

In addition to inducing new AChR clusters, HB-GAM beads invariably cause the dispersal of preexisting hot spots (Dai and Peng, 1998). This effect is similar to nerve-induced dispersal of hot spots (Moody-Corbett and Cohen, 1982b; Kidokoro and Brass, 1985). In the presence of Ltn A, the dispersal of hot spots induced by beads was also inhibited (Fig. 8 C, arrow). Thus, Ltn A, for the duration of these experiments that last for 24 h, does not affect the stability of preexisting AChR clusters.

Ltn A also inhibited agrin-induced cluster formation in a dose-dependent manner (Fig. 8, E–I). Hot spots, evidenced by their larger size as compared with the smaller agrin-induced clusters, also underwent dispersal in response to agrin treatment. Similar to the bead situation, they were also retained in agrin-treated cultures in the presence of Ltn A (Fig. 8, G–H, arrows). Thus, F-actin assembly is also a necessary step involved in agrin-induced AChR clustering. Due to the fact that Ltn A inhibited neurite outgrowth, we were unable to assess the effect of Ltn A on NMJ formation in vitro.

To assess whether Ltn A’s inhibition on clustering was due to deleterious effects on cell viability, the reversibility of this phenomenon was studied. Cells were treated with 40 μM of this toxin for 24 h, returned to normal medium, and then stimulated with beads or agrin. As shown in Fig. 8 J, near complete recovery of AChR clustering was observed. Thus, the viability of the cells was not compromised by Ltn A treatment for the duration of these experiments.

In addition to Ltn A, the effect of jasplakinolide on AChR cluster formation was also studied. Although at low concentrations and for short durations this F-actin binding toxin did not inhibit AChR clustering as described above, prolonged treatment at higher concentrations did inhibit cluster formation as shown in Fig. 9. Both bead- and agrin-induced clustering processes were inhibited in a dose-dependent manner by jasplakinolide (Fig. 9 G). Clusters that still formed in the presence of high concentrations of jasplakinolide (50 μM) were much smaller than control clusters (Fig. 9, E and F). Despite its effect on new cluster formation, hot spots remained stable in the presence of jasplakinolide (Fig. 9 C).

As tyrosine kinase activation is pivotal to AChR clustering, we sought to understand the relationship between actin polymerization and PY accumulation induced by beads. Consistent with our previous finding (Baker and Peng, 1993), PY was accumulated at bead–muscle contacts (Fig. 10, A–D). Similar to its effect on AChR clustering,
Ltn A also blocked bead-induced PY accumulation (Fig. 10, E–G). However, PY accumulation was unchanged at hot spots by Ltn A treatment (Fig. 10, E–F, arrows). These data suggest that the accumulation of tyrosine-phosphorylated proteins at sites of postsynaptic stimulation requires F-actin assembly. The persistence of PY at hot spots is consistent with the relatively stable F-actin cytoskeleton at these specializations implicated by the results described above.

Discussion

In this work, we have documented the roles of actin polymerization induced by two NMJ stimuli with three main findings. (i) By masking preexisting F-actin structures with jasplakinolide, the new F-actin cytoskeleton induced by AChR clustering stimuli was clearly visualized. (ii) Actin polymerization at sites of AChR clustering is highly dynamic and generates enough force to propel the entire cluster complex to move along the membrane. (iii) F-actin assembly is necessary for the formation of AChR and PY clusters. These results indicate that actin polymerization triggered by synaptogenic signals is a pivotal event in AChR clustering and motility.

Relationship between F-Actin and AChRs

Our results indicate that actin polymerization can take place in two distinct configurations in response to synaptogenic signals. Responding to spatially discrete stimuli such as beads, muscle cells assemble F-actin locally and the resultant cytoskeleton conforms to the AChR cluster. The fact that we did not see a one-to-one relationship between F-actin and bead-induced AChR cluster can be explained by the fact that the former is a filamentous polymer whereas the latter is a globular protein. Thus, a single F-actin filament may accommodate multiple AChRs. On the other hand, bath application of agrin, which induces diffuse AChR clustering, results in a more global F-actin assembly that encompasses the region of AChR clusters but only occasionally colocalizes with them. On this point, we would like to offer two possible explanations. First, the
newly polymerized F-actin at sites of AChR clusters may be variable and not extensive enough to be detected reliably with the method used. Second, AChR clusters induced by agrin may form on preexisting, localized F-actin and may bind directly to F-actin (Walker et al., 1984). A ChR subunits (Sanes, 1997; Porter and Froehner, 1983; Maimone and Merlie, 1993; Ramarao and Cohen, 1998) and may bind directly to F-actin (Walker et al., 1984). A recent study has shown that AChR clustering in skeletal muscle is abolished in rapsyn-null mice (Gautam et al., 1996; Qu et al., 1996). Thus, rapsyn is capable of organizing membrane domains on its own, perhaps through its interaction with the cortical actin cytoskeleton. Local F-actin assembly during synaptogenesis may recruit rapsyn and its associated AChR to cluster (Burden, 1985; Wallace, 1989; Peng and Froehner, 1985). Recent study has also shown that rapsyn can interact with MuSK through a putative transmembrane linker that interacts with rapsyn intracellularly and with MuSK's ectodomain extracellularly (Apel et al., 1997). It is thought that AChRs become clustered as a consequence of MuSK clustering. Thus, MuSK cluster could serve as a scaffold for AChR clustering. The fact that AChR clustering induced by agrin which acts through MuSK is abolished after Ltn A treatment suggests that F-actin assembly may also underlie the formation of the MuSK scaffold.

Recent studies have shown that cortical F-actin meshwork assembly induced by RTK activation is mediated by small GTPases Rac and Cdc42 (Ridley and Hall, 1992; Machesky and Hall, 1996; Ridley et al., 1992). The requirement of Rac and Cdc42 in agrin-induced AChR cluster formation in myotubes has recently been reported (Weston et al., 2000). Thus, this finding also implicates the role of actin polymerization in agrin-induced AChR clustering. Although our results have shown a diffuse F-actin assembly as a result of bath agrin application, local deposition of agrin during motor innervation (Cohen et al., 1994) would presumably result in localized actin polymerization similar to that induced by beads.

Although cytochalasins are widely used as inhibitors of F-actin assembly, they are ineffective in blocking AChR clustering, as reported here. Their effect on preexisting AChR hot spots is inconsistent. Hot spots in chick myotubes are disrupted by cytochalasins (Connolly, 1984), but those in cultured Xenopus muscle cells are not (Moody-Corbett and Cohen, 1982a). The mechanism underlying cytochalasins' inhibition of actin polymerization is different from that of Ltn A. The latter binds to G-actin and sequesters it from polymerization into F-actin (Spector et al., 1983, 1989; Coue et al., 1987), whereas the former binds to the barbed end of actin filament but does not inhibit monomer addition to the pointed end nor does it completely block addition to the barbed end of the elongating filament (Bonder and Mooseker, 1986). Thus, Ltn A is a much more potent inhibitor of actin polymerization (Coue et al., 1987; Spector et al., 1989). The action of jasplakinolide in inhibiting cluster formation is different from that of Ltn A. The latter blocks actin polymerization, whereas the former actually induces actin polymerization and/or stabilizes preexisting actin filaments due to its binding to F-actin (Bubb et al., 1994). Thus, it may promote F-actin assembly in the entire muscle cell, thereby leading to a decrease in AChR mobility as a result of its interaction with the cytoskeleton. This may cause a depletion of the mobile AChR pool for cluster formation. Alternatively, it may bind to and stabilize incompletely assembled actin filaments induced by synaptogenic signals to interfere with the dynamic cytoskeletal assembly necessary for

Figure 7. The effect of Ltn A on AChR clustering. (A and B) Rh-phalloidin labeling after jasplakinolide masking. Ltn A at 1 μM diminished bead-induced actin polymerization. (C) Change in fluorescence intensity of bead-induced AChR clusters as shown by OG-BTX labeling resulting from Ltn A treatment.
AChR cluster formation. The diminution in the size of AChR clusters that still form in the presence of jasplakinolide is consistent with the latter notion.

**Actin-driven Cluster Movement**

The novel actin-driven AChR cluster movement appears qualitatively similar to the “inductopodia” induced by polycationic beads on Aplysia growth cones (Forscher et al., 1992). In that case, F-actin assembly induced by submicron beads produces sufficient force to propel them to move on the growth cone surface. Growth cones also exhibit another kind of actin-based protrusive structure termed intrapodium, generated by a burst of actin polymerization resulting from the removal of cytoskeletal disrupting agents such as cytochalasin and nocodazole.
Actin-driven movements have also been well documented on intracellular pathogens such as *Listeria* and *Vaccinia* and on cytoplasmic Arp2/3 spots (Theriot, 1995; Cudmore et al., 1995; Schafer et al., 1998). In addition to AChRs, the cargo moved by polymerizing actin most likely includes other AChR-associated proteins. Our previous studies have shown that 1-d bead-induced AChR clusters similar to the ones studied here already have a basement membrane in the cleft space between the bead and the cell (Peng and Chen, 1992). In addition, these clusters are associated with cytoskeletal and signaling proteins including rapsyn, dystrophin complex (dystrophin, syntrophin, and dystroglycan), and focal adhesion kinase (Peng and Froehner, 1985; Chen et al., 1990; Baker et al., 1994; Peng et al., 1998). Furthermore, the AChR-rich membrane at bead–muscle contacts is already deeply invaginated at this time (Peng and Chen, 1992). Thus, the bead-associated cluster movement involves an entire transmembrane protein complex as well as complicated membrane topography.

Quantitatively, the velocity of the AChR cluster movement is three orders of magnitude slower than other actin-driven movements described above (Theriot et al., 1992; Schafer et al., 1998). A reason for this difference could be that the AChR cluster complex is tethered to a global cortical cytoskeleton and the connections have to be modified as the cluster moves along the membrane. The fact that these clusters move at all is indicative of the considerable propulsive force generated by actin polymerization induced by synaptogenic stimuli.

The physiological significance of this receptor cluster movement is not known. It could serve as a mechanism for coalescing small clusters into large ones during NMJ formation. Although the movement reported here is nondirectional, it is conceivable that during innervation, AChR patches can move in a preferred direction as a result of the directional neurite extension on the muscle cell. A directional cluster movement may serve to remodel or to enlarge the postsynaptic membrane. The movement of large transmembrane protein complex is also exemplified by the recent demonstration of focal adhesion translocation in stationary fibroblasts (Smilenov et al., 1999).

**Dynamics of F-actin Assembly**

From both jasplakinolide masking and cortactin–EGFP localization results, the dynamic state of the F-actin cytoskeleton at new versus existing AChR clusters is contrasted. The use of cortactin to mark sites of new actin assembly is supported by findings that show its localization...
at leading edge as described above. In addition, cortactin also binds to dynamic Arp2/3-positive actin spots in fibroblasts (Kaksonen, M., H.B. Peng, and H. Rauvala, manuscript in preparation). It does not bind to stress fibers or myofibrils (Wu and Parsons, 1993; Peng et al., 1997). The cortactin–EGFP data presented here are consistent with our previous immunofluorescence study, which showed that endogenous cortactin is localized at newly formed AChR clusters induced by beads (Peng et al., 1997). As shown here, newly induced AChR clusters are associated with new actin assembly, which remains dynamic as shown by its association with cortactin. Preexisting hot spots have little newly polymerized F-actin and cortactin and thus the cytoskeleton at these sites seems stable. These results are consistent with the differential effects of Ltn A on new versus preexisting AChR clusters. Ltn A inhibits the formation of new clusters, but it has little effect on hot spots.

In contrast to preexisting AChR clusters in muscle cells, NMDA and AMPA receptor clusters located on cultured hippocampal neurons are dispersed after Ltn A treatment (Allison et al., 1998). In fact, Ltn A disrupts the F-actin cytoskeleton within dendritic spines on which these glutamate receptors are clustered. This suggests that the spine actin cytoskeleton is maintained in a dynamic state, probably similar to that at newly formed AChR clusters in cultured muscle cells. Consistent with this notion is the finding that cortactin is also enriched in the postsynaptic density associated with these central receptor clusters (Boeckers et al., 1999; Naisbitt et al., 1999). Thus, the actin-driven motility of the postsynaptic apparatus described here may also be applicable to central synapses, where it may play a role in synaptic plasticity.

The Actin Cytoskeleton As a Scaffold for Postsynaptic Signaling

In addition to cytoskeletal proteins, the F-actin specialization may also provide a mechanism for the anchorage of signaling molecules as evidenced by the necessity of its assembly in PY accumulation. The identity of tyrosine-phosphorylated proteins at the AChR cluster is unknown. Several studies have shown that the β-subunit of AChR is tyrosine-phosphorylated upon MuSK activation (Hopf and Hoch, 1998; Apel et al., 1997; Glass et al., 1997). However, recent data have shown that proteins other than the receptor are responsible for the early PY accumulation at sites of AChR clustering, and β-subunit tyrosine phosphorylation is not necessary for cluster formation (Baker and Peng, 1993; Meyer and Wallace, 1998). In addition to MuSK, several other kinases, such as focal adhesion kinase and Src, are known to be either concentrated or activated by synaptic signals (Fuhrer and Hall, 1996; Baker et al., 1994). AChR clusters are also associated with other signaling and structural proteins that are tyrosine kinase substrates such as Grb 2, dystrobrevin, and cortactin (Colledge and Froehner, 1997; Peng et al., 1997; Grady et al., 2000). These proteins are candidates for the observed PY accumulation at AChR clusters. Besides AChR cluster formation, our results suggest an additional role of the F-actin assembly in hot spot dispersal, since Ltn A treatment abolishes the dispersal of preexisting hot spots (Fig. 8). Our recent study has implicated the role of tyrosine phosphatases in preexisting AChR cluster dispersal (Dai and Peng, 1998). Thus, the actin cytoskeleton may also play a role in phosphatase signaling. In sum, actin polymerization induced by synaptogenic signals may lead to the assembly of a scaffold for both structural and signaling molecules necessary for the formation of the postsynaptic apparatus.

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