Muscle Activity and Muscle Agrin Regulate the Organization of Cytoskeletal Proteins and Attached Acetylcholine Receptor (AChR) Aggregates in Skeletal Muscle Fibers

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Abstract. In innervated skeletal muscle fibers, dystrophin and β-dystroglycan form rib-like structures (costameres) that appear as predominantly transverse stripes over Z and M lines. Here, we show that the orientation of these stripes becomes longitudinal in denervated muscles and transverse again in denervated electrically stimulated muscles. Skeletal muscle fibers express nonneural (muscle) agrin whose function is not well understood. In this work, a single application of ≥10 nM purified recombinant muscle agrin into denervated muscles preserved the transverse orientation of costameric proteins that is typical for innervated muscles, as did a single application of ≥1 μM neural agrin. At lower concentration, neural agrin induced acetylcholine receptor aggregates, which colocalized with longitudinally oriented β-dystroglycan, dystrophin, utrophin, syntrophin, rapsyn, and β2-laminin in denervated unstimulated fibers and with the same but transversely oriented proteins in innervated or denervated stimulated fibers. The results indicate that costameres are plastic structures whose organization depends on electrical muscle activity and/or muscle agrin.

Key words: agrin • acetylcholine receptor • cytoskeleton • electrical activity • costameres

Introduction

The primary function of skeletal muscles is to generate mechanical forces for movement and stabilization. These forces are generated in sarcomeres and transmitted longitudinally to myotendinous junctions and laterally to extracellular matrix, and therefore to tendons (Street, 1983; Monti et al., 1999). Lateral forces are transmitted by chains of proteins, one of which consists of F-actin and dystrophin underneath the sarcolemma, β-dystroglycan and associated proteins in the sarcolemma, and α-dystroglycan and laminin-2 outside the sarcolemma (Ervasti and Campbell, 1993a). Immunolabeled dystrophin appears predominantly as transverse stripes over sarcomeres, forming a rib-like lattice, which together with other similarly organized cytoskeletal proteins are called costameres (Craig and Pardo, 1983; Straub et al., 1992). Lack of dystrophin, as in mdx mice, disrupts this lattice not only for dystrophin but also for F-actin and other costameric proteins (Rybakova et al., 2000). Mdx mice have focal sarcolemmal defects that render the fibers permeable to extracellular molecules and cause muscular dystrophy (Straub et al., 1997; Williams and Bloch, 1999). Gene mutations affecting other proteins in the chain result in other types of muscular dystrophies (Straub and Campbell, 1997). Thus, costameric proteins not only transmit mechanical forces but also provide necessary structural integrity in contracting and mechanically loaded muscle fibers.

In general, cytoskeletal proteins form dynamic structures that respond to mechanical and other signals by undergoing short- or long-term changes in shape (Ingber, 1997). For skeletal muscle fibers, little is known about the plasticity of cytoskeletal proteins other than the myofibrillar proteins that make up the contractile machinery (Schiaffino and Reggiani, 1996). In one recent study, denervation had minor or no effect on costameric appearance of β-spectrin and dystrophin in fast and slow muscles of adult rats (Williams et al., 2000). In another study, denervation caused the microtubular network to change to a predominantly longitudinal orientation (Boudriau et al., 1996). In a third study, the organization of subsarcolemmal microtubules were shown to be different in fast and slow muscles and sensitive to patterns of electrical muscle activity (Ralston et al., 2001).

Here, we have studied costameres in normal, denervated, and denervated plus electrically stimulated soleus (SOL)[1] muscles of the adult rat. We had noticed earlier

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[1]*Abbreviations used in this paper:* AChE, acetylcholine esterase; AChR, acetylcholine receptor; NMJ, neuromuscular junction; SOL, soleus; Rh-BuTx, TRITC-α-bungarotoxin.

(Bezakova et al., 2001, page 1441, this issue) that acetylcholine receptor (AChR) aggregates induced by recombinant neural agrin appeared as transverse strings in innervated fibers, but as longitudinal strings in denervated fibers. The transverse strings formed in innervated muscle reminded us of costameres, and therefore we looked more closely at costameres also in denervated muscles. We now report that dystrophin and other costameric proteins appear as longitudinal stripes in denervated muscles, and that electrical muscle stimulation through implanted electrodes causes these stripes to change back to their normal transverse orientation. Furthermore, in muscles injected with neural agrin, the induced AChR aggregates appear as transverse or longitudinal stripes in electrically active and inactive muscles, respectively, that colocalize to correspondingly oriented costameric proteins. Thus, the organization of costameres is plastic, depends on muscle activity, and is of importance for the organization of neural agrin–induced AChR aggregates.

We also had noticed earlier that high concentrations of neural agrin (≥ 1 μM) induced transversely oriented strings of AChR aggregates on denervated fibers. Neural agrin not only aggregates AChRs but also binds to α-dystroglycan and laminin (Gesemann et al., 1996; Denzer et al., 1997). Similarly, muscle agrin binds to α-dystroglycan but with 10 times higher affinity than neural agrin (Gesemann et al., 1996). To test the hypothesis that neural agrin had changed the organization of costameric proteins by binding to α-dystroglycan, we examined also the effect of muscle agrin on the organization of costameres, but at lower concentration than neural agrin. We now report that externally applied muscle agrin preserved the transverse stripes of dystrophin and other costameric proteins in denervated muscles at the concentration that was two orders of magnitude lower than the effective concentration of neural agrin, suggesting that muscle agrin acts in an activity-dependent and autocrine way to organize the subcortical cytoskeleton of skeletal muscle fibers. In addition, it presumably stabilizes the postsynaptic apparatus at neuromuscular junctions (NMJs).

Materials and Methods

Purification of Recombinant Agrin

Recombinant full-length chick neural and muscle agrin were purified from the conditioned media of stably transfected HEK 293 cells, as previously described (Bezakova et al., 2001, page 1441, this issue).

Surgical Procedures and In Vivo Stimulation

Adult male Wistar rats ($\sim$250 g body weight) were used. All surgical procedures were done under general anesthesia by Equithesin (0.4 ml/100 g body weight) injected i.p. SOL muscles were denervated by removing $\sim$5 mm of the sciatic nerve in the thigh. For stimulation, uninsulated ends of two wires (AS 632; Cooner) were placed across the SOL and connected to a stimulator above the animal's cage, as described (Bezakova et al., 2001, page 1441, this issue). Stimulation started 1 h or 7 d later and consisted of 60, 0.4-ms, bipolar square pulses at 100 Hz every 60 s for $\leq$28 d. The experiments were conducted in conformity with the laws and regulations for experiments on live animals in Norway and overseen by the veterinarian responsible for the animal house.

Application of Recombinant Agrin

Intramuscular Injection. SOL muscles were injected with 70 μl of 0.5 μM recombinant chick neural agrin, denervated immediately (see above) or

left innervated. Injected muscles were excised at different times thereafter, incubated with TRITC-α-bungarotoxin (Rh-BuTx) for 30 min, washed with PBS, fixed with 1.5% paraformaldehyde, and teased into $\sim$30 thin bundles each containing 50–100 muscle fibers. These bundles were then incubated with appropriate antibodies for immunolabeling of costameric and other proteins.

Bathing of Muscle. SOL muscles were exposed, dissected free from surrounding tissue except at tendons and entry zones for nerve and blood vessels, and immersed in PBS solution containing different concentrations of agrin for 2 h, as described (Bezakova et al., 2001, page 1441, this issue). Treated muscles were excised 7 d later, labeled with Rh-BuTx, washed with PBS, and fixed with 1.5% paraformaldehyde. Thin bundles of superficial fibers that had been directly in contact with agrin were then dissected out and immunolabeled with a monoclonal antibody against dystrophin.

Immunocytochemistry

Bundles from treated SOL muscles were permeabilized with 1% Triton X-100 for 15 min, incubated for 10 min with 100 mM glycine, blocked for 30 min with 1% BSA in PBS, incubated overnight at 4°C with primary antibodies, washed 3 times in 1 h with in PBS containing 1% BSA, and incubated 1 h with FITC-conjugated anti–rabbit or anti–mouse secondary antibodies (Sigma-Aldrich) diluted with 1% BSA in PBS. The bundles were then examined with a confocal laser–scanning microscope (TCS-SP; Leica) equipped with Ar$^+$ Kr$^+$ ion laser. Excitation was at 488 and 568 nm. The spectrometer settings (width and positions of the slits in front of the photomultiplier tubes) were selected in order to minimize cross-bleeding between the FITC and the TRITC channels.

Antibodies

Monoclonal antibodies against β-dystroglycan (8D5) and utrophin (12B6) were provided by Dr. C. Slater (University of Newcastle, Newcastle, UK) and diluted 1:200. Monoclonal antibodies against rapsyn (1579), dystrophin (1808), and syntrophin (1351) were provided by Dr. S. Froehner (University of Washington, Seattle, WA) and used at 16 μg/μl. Monoclonal antibody against β2-laminin chain was provided by Dr. J. Sanes (Washington University, St. Luois, MO) and diluted 1:200. Polyclonal antibody against α2-laminin chain was provided by Dr. R Timpl (Max-Planck Institute, Martinstried, Germany) and diluted 1:1000. Polyclonal antibody against acetylcholine esterase (AChE) was provided by Dr. J. Massoulie (CNRS, Paris, France) and diluted 1:500.

Results

Organization of Neural Agrin–induced AChR Microaggregates: Dependence on Innervation

We injected purified recombinant neural agrin into adult rat SOL muscles to induce aggregation of AChRs in the extrajunctional areas. Without such injections, AChR aggregates were not observed (Bezakova et al., 2001, page 1441, this issue). The organization of these aggregates was strikingly different on innervated and denervated muscle fibers (Fig. 1). On innervated muscles, the aggregates consisted of smaller microaggregates that appeared as transverse strings across the fibers (Fig. 1, bottom). In contrast, on denervated muscles, the microaggregates appeared as longitudinal strings (Fig. 1, top). This switch in orientation was striking already 3 d after denervation, the earliest time point examined.

Organization of Costameres: Dependence on Electrical Muscle Activity

The distribution of AChRs in Fig. 1 suggested that cytoskeletal proteins, to which AChRs are linked, might be similarly affected by denervation. We therefore performed confocal immunofluorescence analysis using specific monoclonal antibodies against proteins that define costameres in innervated muscles. As illustrated in Fig. 2, the labeled
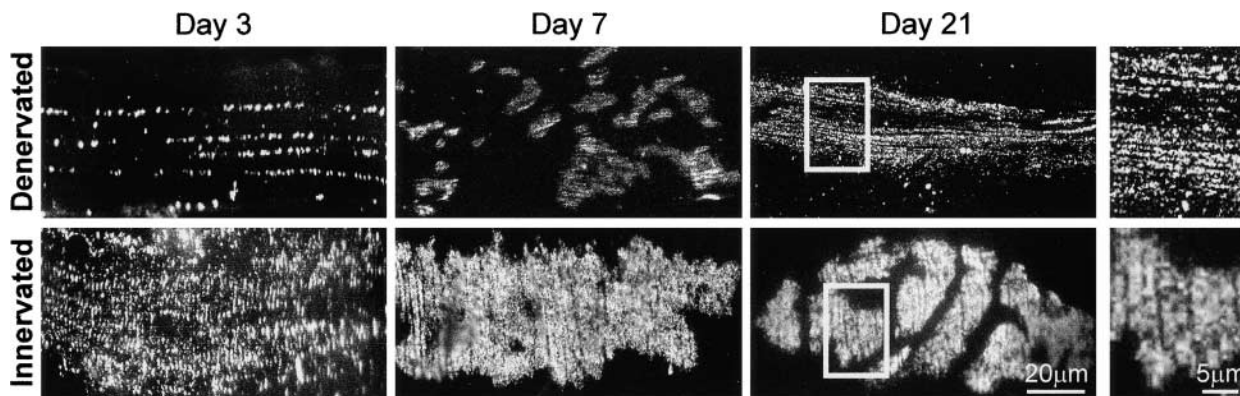
Figure 1. Distribution of neural agrin–induced AChR aggregates depends on innervation. 70 μl of 0.5 μM neural agrin was injected into denervated or innervated SOL muscles and examined at different days thereafter, as indicated. Denervation was performed at the time of injection. Note that the AChR microaggregates induced by neural agrin are longitudinally oriented on denervated muscle fibers and transversely oriented on innervated fibers.

proteins appeared as transverse stripes in innervated fibers and longitudinal stripes in denervated fibers, corresponding to the switch in orientation observed for AChR aggregates after denervation.

To examine if this switch in orientation is reversible and depends on electrical muscle activity or some other nerve-derived influence, we stimulated denervated muscles chronically through implanted electrodes. The stimulation started 7 d after the denervation when proteins present in costameres created already longitudinally oriented stripes. After 7 and 28 (not shown) d of stimulation, the labeled proteins displayed their normal transverse orientation (Fig. 2 A), whereas they retained their longitudinal orientation in unstimulated muscles (Figs. 1 and 3). Thus, the changes in the organization of cytoskeletal proteins induced by denervation are both reversible and dependent on electrical muscle activity.

Laminin-2, which binds to α-dystroglycan and thence to costameric proteins, also appeared as transverse stripes in innervated fibers (Fig. 2 B). In contrast to the longitudinal stripes of the underlying costameric proteins, however, its orientation remained transverse after denervation (Fig. 2, A and B). This result suggests that organization of laminin-2 is structurally more stable and less sensitive to changes in activity than costameric proteins. In addition, it suggests that denervation somehow uncouples the connection between laminin-2 and dystroglycan complex.

The Orientation of Costameres and AChR Aggregates Is Similarly Affected by Muscle Activity

To examine if costameres and AChR aggregates at NMJs are similarly affected by denervation, we compared their distributions at NMJs in innervated and denervated muscles. As illustrated in Fig. 3, a longitudinal orientation of colocalized AChR aggregates and dystrophin is evident on denervated fibers. In innervated muscles, the transverse orientation of dystrophin present outside the junction cannot be resolved inside using confocal fluorescence microscopy (Fig. 3), presumably because the structural complexity at normal NMJs distorts the regular pattern seen outside.

To explore further the relation between AChR aggregates and cytoskeletal proteins, we subsequently focused on neural agrin–induced aggregates outside the NMJ. On innervated fibers, neural agrin induced AChR aggregates that were few, relatively large and uniform in size, and predominantly located near myotendinous junctions (Bezakova et al., 2001, page 1441, this issue). The microaggregates within these larger aggregates appeared along transverse stripes, which colocalized precisely to β2-laminin, rapsyn, and utrophin (Fig. 4). They also colocalized with dystrophin and β-dystroglycan, but whereas these two proteins appeared as transverse stripes along the entire fiber, β2-laminin, rapsyn, and utrophin were restricted to the sites of AChR aggregates. AChE also colocalized with AChR aggregates but extended for a limited distance beyond them (Fig. 4).

In denervated fibers, the distributions of all the proteins described above were strikingly different. The AChR microaggregates appeared as longitudinal stripes, which colocalized to the same proteins as they did on innervated fibers except that the stripes were now longitudinal rather than transverse (Fig. 5). The different staining patterns obtained with different primary antibodies (β-dystroglycan, β2-laminin, rapsyn, utrophin, dystrophin), using the same secondary antibody (Figs. 4 and 5), further indicated the specificity of labeling. These results confirm that the organization of AChR aggregates on muscle fibers in vivo depends on the way these aggregates link to proteins that are known to connect the cytoskeleton to the extracellular matrix.

Muscle Agrin Restores Costameres to Their Normal Orientation in Denervated Muscle Fibers

In experiments reported elsewhere (Bezakova et al., 2001, page 1441, this issue), we applied neural agrin at different concentrations to denervated rat SOL and noticed that neural agrin caused the appearance of AChR microaggregates that formed longitudinal stripes at low concentrations but transverse stripes at higher (1 and 10 μM) concentrations. Here, we have combined this observation with immunolabeling of dystrophin to show that, at low concentrations (≤0.1 μM), neural agrin induces AChR microaggregates on denervated fibers that colocalize to longitudinal stripes of dystrophin (Fig. 6, top) and, at higher concentrations (≥ 1 μM), to transverse stripes of dystrophin (Fig. 6, second
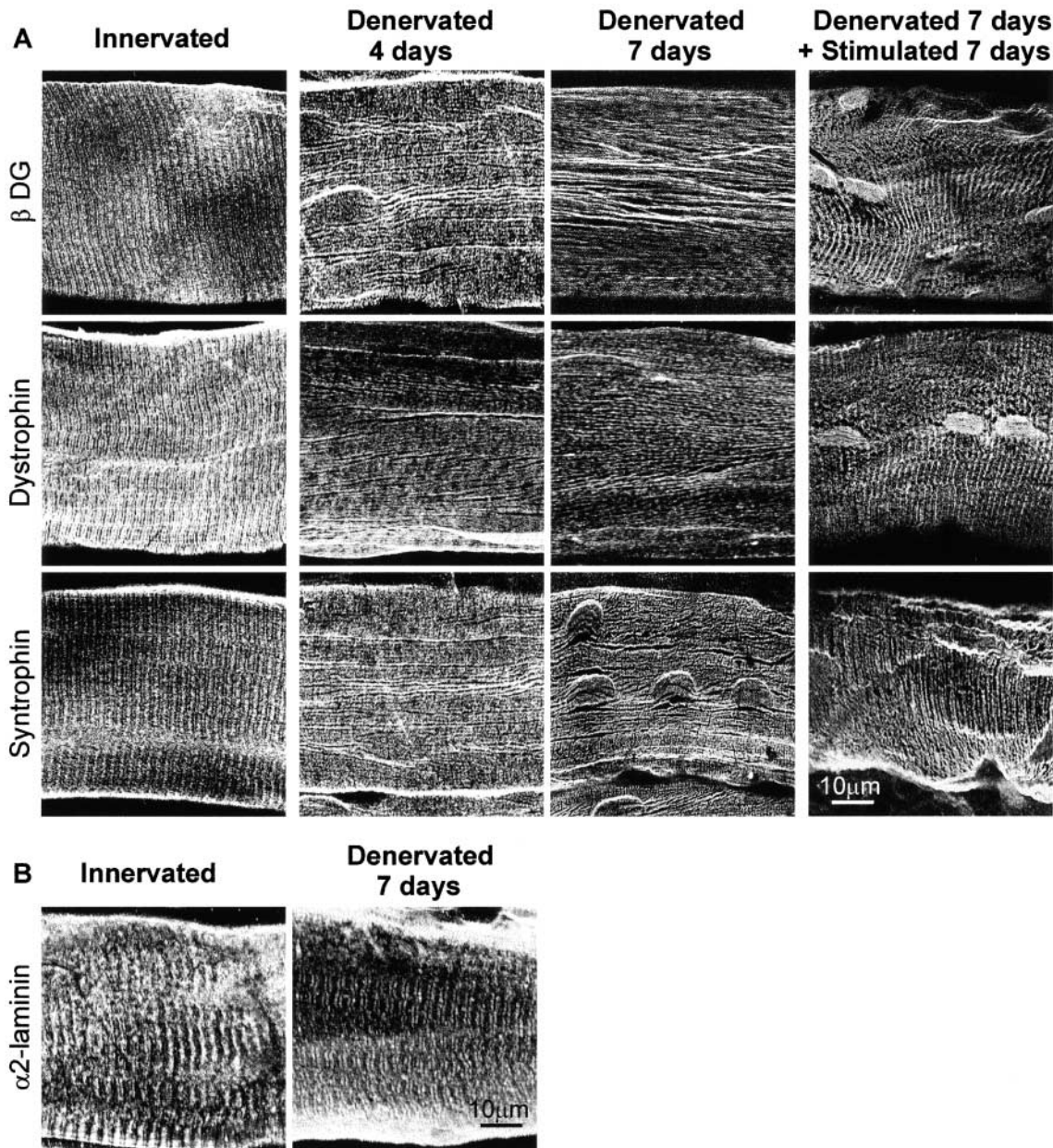
Figure 2. Distribution of cytoskeletal proteins and laminin in differently treated SOL muscles. Immunolabeled β-dystroglycan (β-DG), dystrophin, syntrophin, and α2 chain of laminin-2 were examined in innervated muscles or in muscles that had been denervated for 4 or 7 d, or denervated for 14 d and electrically stimulated during the last 7 d, as indicated. Note that β-DG, dystrophin, and syntrophin are transversely oriented in innervated and denervated stimulated muscles but longitudinally oriented in denervated unstimulated muscles (A). In contrast, laminin-2 identified by imunolabeling of α-2 chain remains transversely oriented in denervated muscles (B).

row). These results show that not only electrical muscle activity but also neural agrin alone can preserve a normal transverse costameric pattern in denervated muscle fibers.

We then injected muscle agrin to see if this agrin isoform, like neural agrin, could preserve a transverse costameric pattern in denervated muscles. As illustrated in Fig. 6 (middle), muscle agrin had a similar effect, but at the concentration that was two orders of magnitude lower than neural agrin and, as expected, without inducing AChR aggregates. Muscle agrin applied at higher concentrations together with neural agrin affected the aggregating activity of neural agrin, since addition of neural agrin now induced AChR aggregates that were less discrete and

less intensely labeled than in the absence of muscle agrin. The costameres, however, were preserved (Fig. 6, fourth row). Finally, when denervated muscles treated with 0.1 μM neural agrin were electrically stimulated, both the costameric cytoskeletal proteins and the colocalized AChR aggregates switched to a transverse orientation (Fig. 6, bottom), although the same concentration of neural agrin alone had no such effect (Fig. 6, top). These results show that muscle agrin, like much higher concentrations of neural agrin (100-fold), and like electrical muscle activity alone, can restore costameric orientation of cytoskeletal proteins and colocalized AChR aggregates to their normal transverse orientation in denervated fibers.
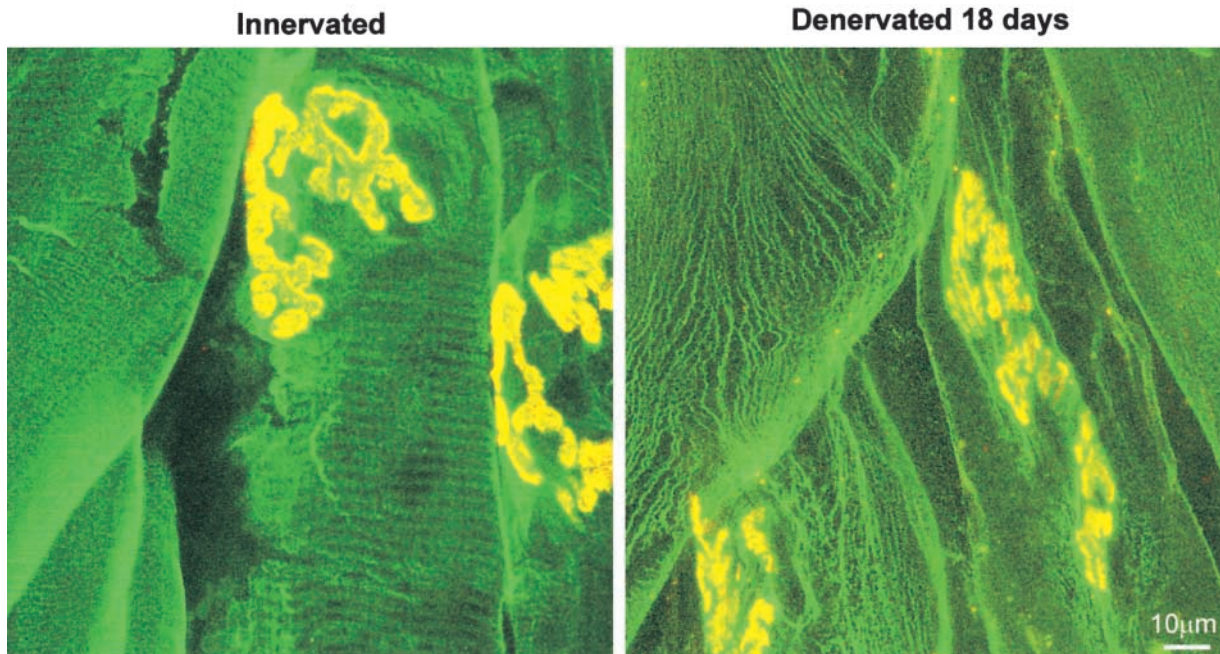
Figure 3. Distribution of AChR aggregates and dystrophin at NMJs and nearby regions. At innervated junctions, dystrophin is transversely oriented just outside the junction, whereas no distinct pattern can be seen inside. Immunolabeled dystrophin (green) and colocalized AChRs (red, but here yellow due to colocalization) are predominantly longitudinally oriented inside and outside denervated (18 d) junctions.

Discussion

Costameres Show Activity-dependent Plasticity

In this work, we have identified costameres in adult rat SOL fibers by immunolabeling dystrophin, syntrophin, and β-dystroglycan. We have shown that the costameric proteins, which appear in innervated fibers as predominantly transverse stripes over Z and M lines, become longitudinally oriented in denervated muscles and then transverse again if the denervated muscle is stimulated electrically. Accordingly, costameres are plastic structures whose organization depends on electrical muscle activity. Such plasticity and activity dependence of costameres have not been previously described, and the underlying mechanisms are unclear.

Costameres are believed to stabilize the plasma membrane of muscle fibers during contraction or stretch. This stabilization involves dystrophin, which attaches strongly to actin filaments underneath the membrane (Rybakova et al., 2000) and to β-dystroglycan that spans the membrane (Jung et al., 1995; Straub and Campbell, 1997). α-Dystroglycan then connects β-dystroglycan to laminin-2 (Ervasti and Campbell, 1993a,b; Henry and Campbell, 1999), which is critical for assembling and maintaining the basal lamina (Ryan et al., 1996). In the present experiments, laminin-2, like costameres, appeared as transverse stripes in normal SOL fibers. Unlike costameres, however, laminin-2 retained its transverse orientation after denervation. Therefore, denervation appears to uncouple laminin-2 from the dystroglycan complex. Reduced glycosylation of α-dystroglycan and lower affinity of α-dystroglycan to laminin, as observed after denervation (Leschziner et al., 2000), might contribute to such uncoupling.

When applied to the surface of cultured myotubes in sufficiently high concentration, laminin-2 polymerizes into extensive polygonal networks that drive underlying dystroglycans, dystrophin and other costameric proteins, into corresponding networks (Colognato et al., 1999). Furthermore, if laminin-2 is defective in polymerization, the organization of underlying cytoskeleton fails (Colognato and Yurchenco, 1999). Whether laminin-2, however, directs the organization of costameres in adult muscle fibers is not clear. In adult fibers of mice or humans with similar defects in laminin-2, the sarcolemma and costameres appear normal despite severe muscle dystrophy (Straub and Campbell, 1997; Rybakova et al., 2000).

Laminin-2, but not costameres, retained the normal transverse pattern after denervation. Thus, in adult fibers, the basal lamina seems more stable than the subcortical cytoskeleton. In keeping with this observation, the basal lamina of adult muscle fibers survives prolonged denervation and, at synaptic sites, retains components that can instruct the formation of an underlying postsynaptic apparatus in fibers regenerating in the absence of the nerve (McMahan, 1990). In contrast, the basal lamina of cultured myotubes shows pronounced activity-dependent plasticity, increasing in amount and organization with the level of activity (Sanes and Lawrence, 1983).

The marked effects of denervation on costameres probably reflect a drastic reduction of mechanical stress in denervated paralyzed fibers. As a first step to clarify the underlying mechanisms, one would like to know the time course of the activity-dependent changes in costamere structure with a view to whether they primarily involve short-term enzymatic processes and/or longer-term gene transcription-dependent processes. In addition, one would like to know whether costameres are differently organized
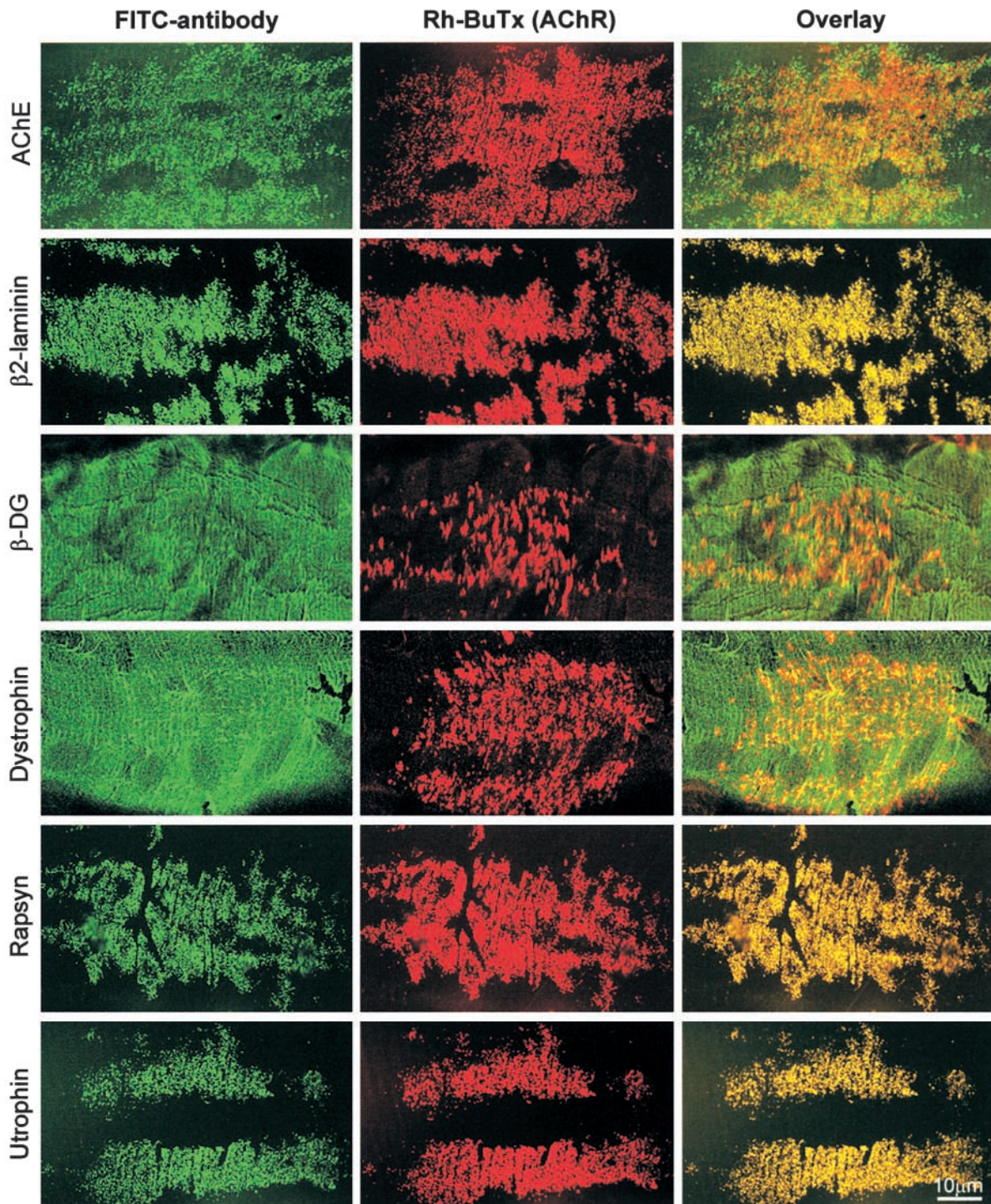
Figure 4. Neural agrin–induced AChR aggregates colocalize to costameric and synapse-specific proteins in innervated SOL muscle fibers. The indicated proteins were immunolabeled 10 d after an intramuscular injection of neural agrin (0.5 μM). Note that neural agrin–induced AChR microaggregates, as revealed by Rh-BuTx, colocalize to transversely oriented costameric (β-dystroglycan, [β-DG] and dystrophin) and synapse-specific (AChE, β2-laminin, rapsyn, and utrophin) proteins. In the overlay to the right, AChRs are red and labeled proteins green. Yellow indicates colocalization.

in fibers generating different types of activity, such as fast and slow muscle fibers. Recently, subsarcolemmal microtubules have been shown to be differently organized in fast and slow muscle fibers and subject to regulation by electrical muscle activity (Ralston et al., 2001).

Muscle Agrin Regulates the Organization of Costameres

The second main result of this work is that muscle agrin applied to the surface of denervated muscle fibers preserved the transverse orientation of costameric cytoskeletal proteins typical for innervated muscles. Electrical stimulation
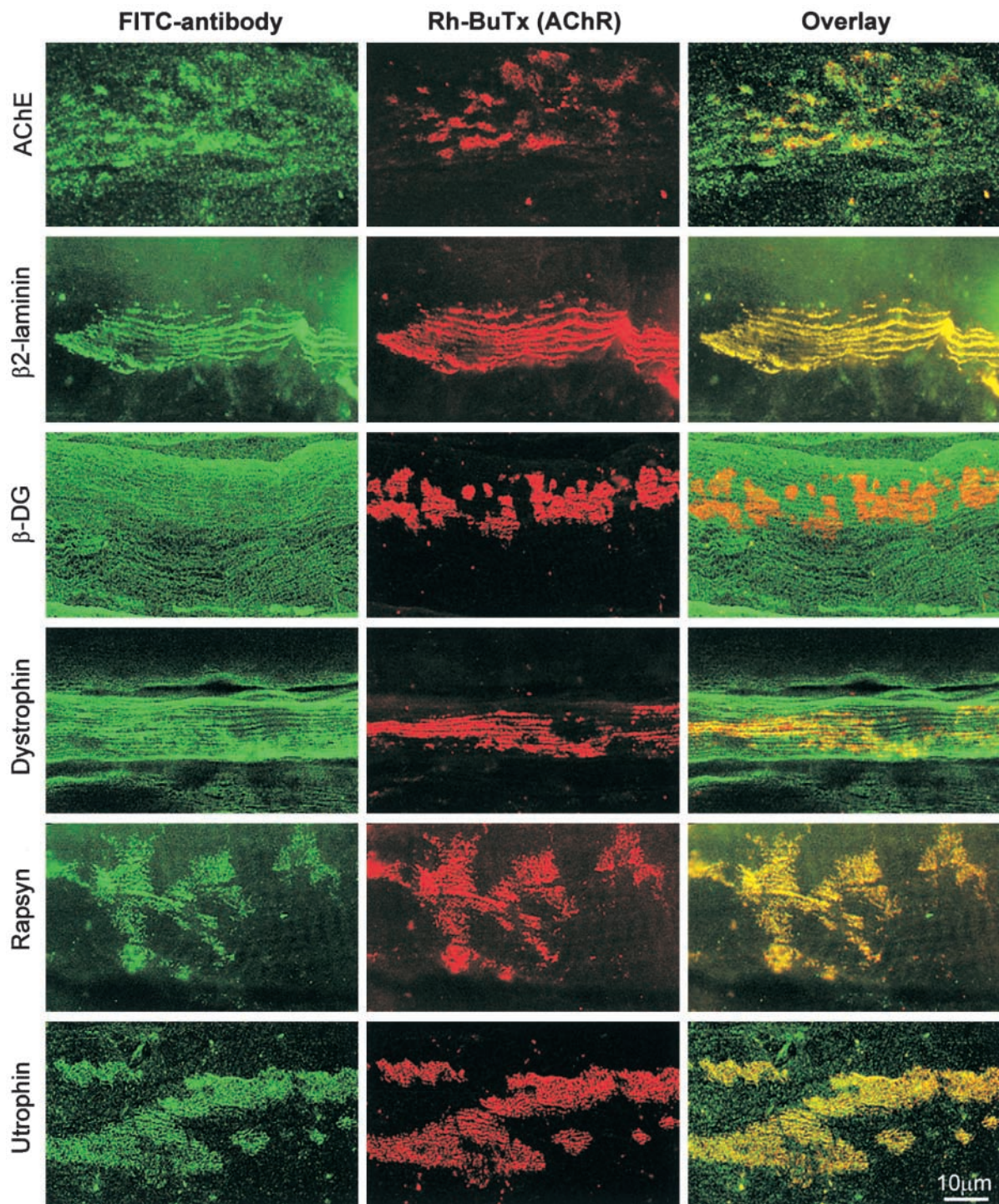
Figure 5. Neural agrin–induced AChR aggregates colocalize to costameric and synapse-specific proteins in denervated SOL muscle fibers. The indicated proteins were immunolabeled 10 d after denervation and intramuscular injection of neural agrin (0.5 µM). Note that neural agrin–induced AChR microaggregates, as revealed by Rh-BuTx, colocalize to longitudinally oriented costameric (β-DG, dystrophin) and synapse-specific (AChE, β2-laminin, rapsyn, and utrophin) proteins. In the overlay to the right, AChRs are red and labeled proteins green. Yellow indicates colocalization.

had the same effect, suggesting that muscle agrin may mediate the regulatory effects of muscle activity on costameres by acting extracellularly in an autocrine or paracrine way.

Muscle inactivity, as after denervation, could alter the organization of costameres in several ways (Fig. 7 A). (a) It could reduce expression or secretion of muscle agrin and/or

alter muscle agrin posttranslationally so that its affinity to α-dystroglycan and/or laminin is reduced. (b) It could alter α-dystroglycan and/or laminin posttranslationally so that these proteins can no longer interact effectively and/or bind muscle agrin. (c) It could alter downstream effectors of α-dystroglycan necessary for building competent links be-
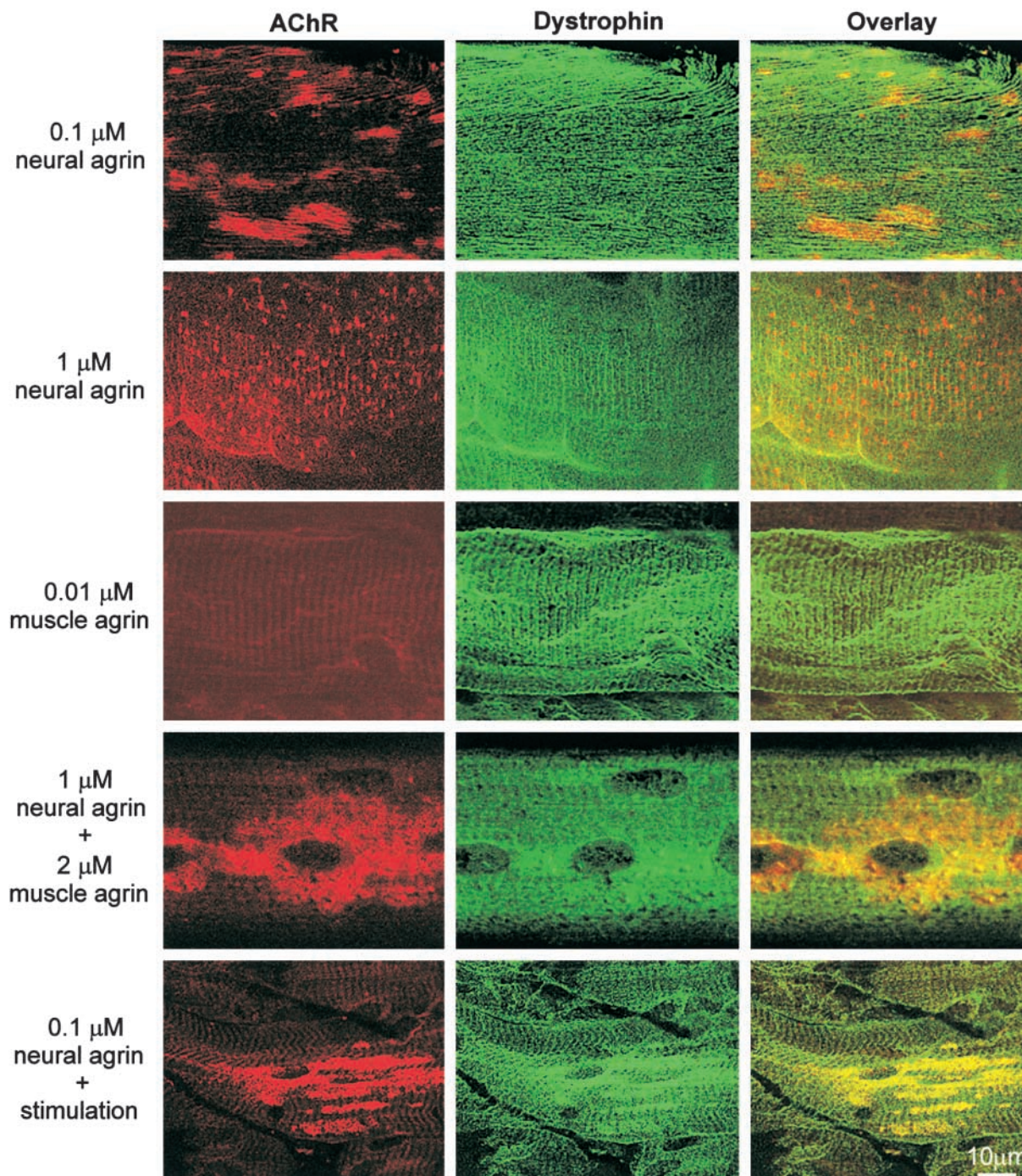
Figure 6. Distribution of AChR aggregates and dystrophin in SOL muscles after treatments with neural agrin, muscle agrin, and/or electrical muscle stimulation. Muscles were denervated and bathed (see Materials and Methods) with different concentrations of neural or muscle agrin, as indicated. Muscles were examined 7 d later for immunolabeled dystrophin and Rh-BuTx-labeled AChRs. Some muscles were in addition stimulated electrically during the 7-d period after denervation. Note that (i) AChR aggregates colocalize to longitudinally oriented dystrophin after application of 0.1 μM neural agrin and to transversely oriented dystrophin after application of 1 μM neural agrin, (ii) muscle agrin at 0.01 μM induces the normal transverse orientation of dystrophin without aggregating AChRs, (iii) muscle agrin (2 μM) affects the AChR aggregation by neural agrin (1 μM), and (iv) electrical muscle stimulation causes dystrophin and colocalized neural agrin–induced (0.1 μM) AChR aggregates to adopt a transverse orientation. In the overlay to the right, AChRs are red and labeled proteins green. Yellow indicates colocalization.

tween the cytoskeleton and extracellular matrix via the dystroglycan complex. The second mechanism (b) is suggested by the observation that α-dystroglycan shows reduced glycosylation and affinity for laminin-2 after denervation (Leschziner et al., 2000). However, since applied recombinant muscle agrin alone effectively preserved transverse pattern of costameres in denervated muscle fibers, the former mechanism (a) seems the more likely or important one.
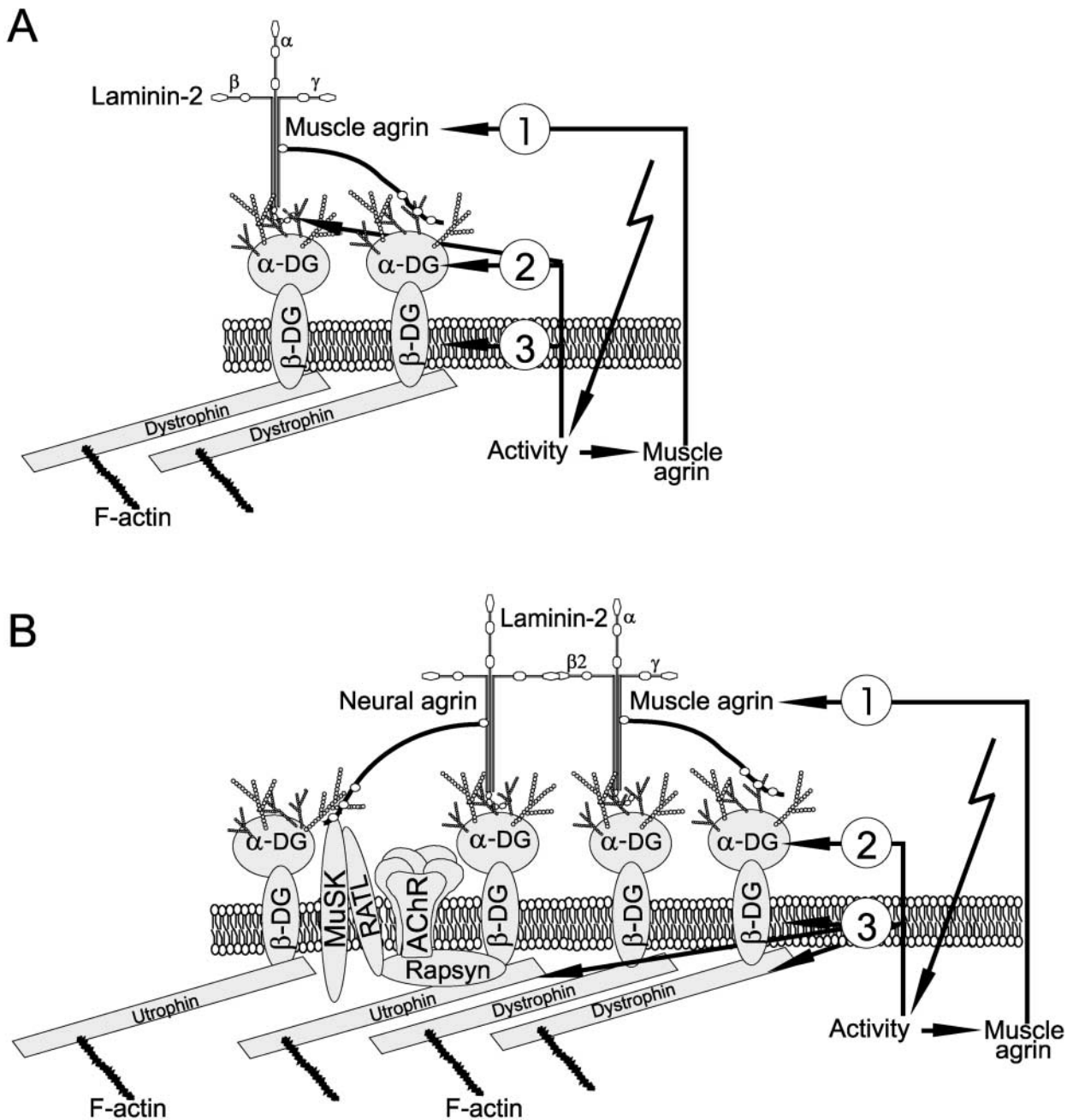
Figure 7. Hypothetical model suggesting how muscle activity and muscle agrin may participate in organization of the cytoskeleton of muscle fibers outside (A) and inside (B) NMJs. (A) Muscle activity regulates expression, secretion, and/or processing of muscle agrin. Muscle agrin then binds in an autocrine way to α-dystroglycan (1). Activity may also affect α-dystroglycan itself (2), or downstream effectors of α-dystroglycan (3). Through these effects on the dystroglycan complex that involve electrical and mechanical signals in the muscle, links between the extracellular matrix and the cortical actin network become stabilized. (B) At NMJs, similar mechanisms operate with several additions. Here, neural agrin, not present outside, binds to a receptor complex containing muscle-specific kinase (MuSK) and induces the appearance of synapse-specific aggregates of AChRs, β2-laminin, rapsyn, and utrophin. These proteins (and others) then become linked to the cortical actin network and are stabilized by muscle activity and muscle agrin as in A. α-and β-DG, α- and β-dystroglycans; RATL, rapsyn-associated transmembrane linker (hypothetical protein).

Several findings are consistent with this model. First, myofibers secrete muscle agrin (Lieth et al., 1992), which binds to α-dystroglycan (Gee et al., 1994) and laminin (Denzer et al., 1995, 1997) on the outside of the fibers. Muscle agrin can therefore act in an autocrine manner. Second, muscle agrin has 10 times higher affinity for α-dystroglycan than neural agrin (Sugiyama et al., 1994;

Gesemann et al., 1996; Hopf and Hoch, 1996) and, as shown here, also affects the organization of costameres at much lower concentration than neural agrin (~one hundredth). Third, chick agrin overexpressed under a muscle specific promoter in laminin-2–deficient (dy^w) mice strongly reduces the dystrophic pathology (Moll, J., P. Barzaghi, E. Engvall, T. Meirer, and R.A. Ruegg. 2000.

Soc. Neurosci. Abstr. 91.1), showing that muscle derived agrin can compensate for defective laminin-2 and suggesting that muscle agrin stabilizes the dystroglycan complex.

Non-neural agrin (A0B0) and α-dystroglycan are expressed by cells in kidney and lung but not liver and co-localized at basal membranes around glomeruli and alveoli (Gesemann et al., 1998; Groffen et al., 1998; Raats et al., 1998). Thus, agrin A0B0 may be expressed where cells must withstand mechanical stresses arising for example from movements in muscle and lung or water transport in kidney.

Organization of AChR Aggregates: Dependence on Costameres and Muscle Activity

A final main result of this work is that the AChR aggregates induced by neural agrin colocalize to costameric proteins, whether these proteins display their normal transverse orientation in innervated fibers and denervated stimulated fibers or their longitudinal orientation in denervated unstimulated fibers. The synapse-specific proteins β2-laminin, rapsyn, and utrophin appeared only at sites of neural agrin–induced AChR aggregates where they and the AChRs became precisely colocalized. Also these proteins switched orientations after denervation or during stimulation, along with costameric dystrophin and β-dystroglycan. These results indicate that costameric proteins organize the postsynaptic-like apparatus of neural-agrin induced AChR aggregates in an activity dependent way.

This conclusion seems likely to hold also for the postsynaptic apparatus at NMJs. There, AChRs and costameric proteins (revealed by dystrophin labeling) were precisely colocalized and after long-term denervation displayed the longitudinal pattern typical of neural-agrin induced AChR aggregates in denervated fibers. At innervated NMJs, the transverse costameric pattern was observed up to but not inside the junction itself. The reason for this, we suggest, is that the complex postsynaptic structure of the mature NMJs distorts the regular pattern seen outside the junction. If so, the coupling of AChRs to the cytoskeleton is probably not principally different at neural agrin–induced and nerve-induced AChR aggregates.

Muscle agrin and electrical muscle stimulation had similar effects on costameres and AChR distribution at neural agrin–induced AChR aggregates, suggesting that muscle agrin plays a role in the organization of postsynaptic specializations and their attachments to the cytoskeleton. Several findings are consistent with this view. Muscle agrin accumulates at nerve- and neural agrin–induced AChR aggregates and is thought to play a role in their maturation and stabilization (Lieth and Fallon, 1993). AChRs at denervated NMJs and at NMJs in mdx mice lacking dystrophin (Xu and Salpeter, 1997) turn over much faster than at innervated NMJs. At sufficiently high concentrations, neural agrin alone fully stabilizes the AChRs at neural agrin–induced AChR aggregates in denervated muscles (Bezakova, G., I. Rabben, G. Fumagalli, and T. Lømo, submitted for publication). Since muscle agrin affects the organization of dystrophin and other costameric proteins much more effectively than neural agrin, muscle agrin could be primarily responsible for AChR stabilization. Muscle stimulation prevents the destabilization of AChRs observed at acutely denervated NMJs (Andreose et al., 1993) and stabilizes AChRs at long-term denervated junctions (Fumagalli et al., 1990). Both effects could be mediated by muscle agrin, given that muscle stimulation and muscle agrin have similar effects on costameres. Thus, neural and muscle agrin seem to have complementary roles at NMJs. Neural agrin aggregates AChRs but has low affinity for α-dystroglycan and affects costameres only at high concentrations. Muscle agrin, on the other hand, does not aggregate AChRs but has high affinity for α-dystroglycan and affects costameres at low concentrations. Accordingly, neural agrin may initiate NMJ formation, whereas muscle agrin ensures subsequent stabilization by linking the junction to the cytoskeleton.

Fig. 7 B presents a model of possible events at NMJs. Neural agrin activates receptor complex containing muscle-specific kinase, aggregates AChRs and sets the scene for impulse transmission and nerve evoked muscle activity, as described (Sanes and Lichtman, 1999). Muscle activity and accompanying mechanical stresses regulate the expression and/or processing of muscle agrin. Muscle agrin binds to α-dystroglycan in an autocrine way and causes the cytoskeleton to adjust in accordance with the electrical and mechanical signals. AChRs become attached to the cytoskeleton through synapse-specific rapsyn and utrophin. Synapse specific β2-laminin, which like rapsyn and utrophin switch orientation in active and inactive fibers, participates in the process. Links between β2-laminin, dystroglycans, rapsyn, utrophin, and F-actin thus serve to anchor the NMJ to the cytoskeleton of the muscle fiber in an activity- and muscle agrin–dependent way. Dystrophin and syntrophin, not shown in Fig. 7 B, overlapped with rapsyn and utrophin and responded similarly to muscle activity and muscle agrin. Dystrophin and syntrophin are concentrated underneath the plasma membrane in the depth of postsynaptic folds where also sodium channels are concentrated (Flucher and Daniels, 1989). Dystrophin and syntrophin may therefore help in anchoring sodium channels to the cytoskeleton at the depths of synaptic folds (Gee et al., 1998), as rapsyn and utrophin presumably do for AChRs at the tips of the folds. The effective concentration of neural and muscle agrin in the synaptic cleft is not known. Therefore, the extent to which neural agrin may compensate for any lack of muscle agrin is also not known. Not included in this model are proteins such as sarcoglycans, sarcospans, dystrobrevins, and others, which complement the formation of the postsynaptic apparatus (Duclos et al., 1998a,b; Peters et al., 1998; Crosbie et al., 1999). Here, however, the purpose is to suggest how muscle agrin may participate in securing the mechanical integrity of the NMJ in a stable yet flexible activity–dependent way.

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