Golgi Complex, Endoplasmic Reticulum Exit Sites, and Microtubules in Skeletal Muscle Fibers Are Organized by Patterned Activity

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The Golgi complex of skeletal muscle fibers is made of thousands of dispersed elements. The distributions of these elements and of the microtubules they associate with differ in fast compared with slow and in innervated compared with denervated fibers. To investigate the role of muscle impulse activity, we denervated fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles of adult rats and stimulated them directly with patterns that resemble the impulse patterns of normal fast EDL (25 pulses at 150 Hz every 15 min) and slow SOL (200 pulses at 20 Hz every 30 sec) motor units. After 2 weeks of denervation plus stimulation, peripheral and central regions of muscle fibers were examined by immunofluorescence microscopy with regard to density and distribution of Golgi complex, microtubules, glucose transporter GLUT4, centrosomes, and endoplasmic reticulum exit sites. In extrajunctional regions, fast

pattern stimulation preserved normal fast characteristics of all markers in EDL type IIB/IX fibers, although inducing changes toward the fast phenotype in originally slow type I SOL fibers, such as a 1.5-fold decrease of the density of Golgi elements at the fiber surface. Slow pattern stimulation had converse effects such as a 2.2-fold increase of the density of Golgi elements at the EDL fiber surface. In junctional regions, where fast and slow fibers are similar, both stimulation patterns prevented a denervation-induced accumulation of GLUT4. The results indicate that patterns of muscle impulse activity, as normally imposed by motor neurons, play a major role in regulating the organization of Golgi complex and related proteins in the extrajunctional region of muscle fibers.

Key words: muscle; Golgi complex; microtubules; plasticity; patterned activity; denervation

Skeletal muscle consists of a heterogeneous population of multinucleated fibers. The molecular basis for their functional diversity is the expression of specific isoforms of most of the proteins involved in muscle contraction and relaxation. Fibers are classified based on contraction speed and other physiological properties but predominantly, today, according to specific myosin heavy chain (MyHC) isoforms (I, IIA, IIB, and IIX; for review, see Schiaffino and Reggiani, 1996). Muscle fiber diversification occurs in several stages during development, both before and after muscle innervation. To which degree intrinsic factors (lineage) or innervation (trophic factors or electrical activity) contribute to fiber diversification remains a subject of intense interest.

Whereas trophic factors play an important role at the neuromuscular junction (NMJ) (Sanes and Lichtman, 1999), the importance of patterned electrical activity for the whole fiber has been shown by the demonstration of muscle plasticity. Fast prop-

erties can be induced in slow muscles and slow properties in fast muscles, as shown initially by experimental cross-innervation of a slow muscle with the nerve from a fast muscle and vice versa and, later, by stimulation of the nerves, or of the muscles after denervation (reviewed in Pette and Vrbová, 1985). Several properties respond to activity-dependent transformation, including myosin gene expression and metabolic enzyme activities (Buonanno and Fields, 1999 and references therein). However, intrinsic differences between fast and slow muscle fibers appear to limit the degree to which such transformation can occur (Westgaard and Lomo, 1988).

A potential basis for limits to plasticity is structural: the different fiber types differ both in content and geographical distribution of intracellular membrane systems such as T-tubules (Luff and Atwood, 1971) and sarcoplasmic reticulum (Eisenberg and Salmons, 1981), and subcellular organelles such as mitochondria (Gauthier and Padykula, 1966; Eisenberg, 1983). Only a few studies (Eisenberg and Salmons, 1981) have examined the plasticity of the muscle membrane systems.

We have recently discovered that the extrajunctional organization of the Golgi complex and of microtubules is fiber type-dependent in muscle fibers (Ralston et al., 1999). After denervation, the Golgi complex distribution is similar in all fibers and resembles the distribution observed in innervated slow-twitch fibers. At the NMJ, the distribution of the Golgi complex is not fiber type-dependent. These results suggested that the distribution of the Golgi complex in muscle is plastic and may be subject to neural control by trophic factors at the NMJ and electrical activity elsewhere.

To test whether Golgi complex organization responds to changes in patterned electrical activity, we have examined its

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distribution and that of related protein systems in the fast extensor digitorum longus (EDL) and slow soleus (SOL) muscle of adult rats, after denervation and direct stimulation with stimulus patterns that resemble the normal firing patterns of EDL and SOL motor neurons (Hennig and Lomo, 1985). We now report that we find all to be sensitive to patterned activity.

MATERIALS AND METHODS

Antibodies and reagents. A rabbit antibody against the *cis*-Golgi protein GM130 (Nakamura et al., 1996) was donated by Dr. G. Warren (Yale University, New Haven, CT); a mouse monoclonal antibody against GM130 was obtained from BD Biosciences/Transduction Laboratories (San Diego, CA). The mouse monoclonal anti α -tubulin DM1a was purchased from Sigma (St Louis, MO). The rabbit anti p137, the mammalian homolog of the COPII complex protein Sec31p (Shugrue et al., 1999), was a gift from Dr. F. Gorelick (Yale University). The M8 rabbit anti-pericentrin antibody was received from Dr. S. Duxsey (University of Massachusetts, Worcester, MA). The rabbit anti-GLUT4 antibody P-1 has been described previously (Ploug et al., 1998). The mouse anti-slow MyHC NOQ7.5.4.D (Draeger et al., 1987) was purchased from Sigma. In our hands, it is the only available antibody to a specific adult MyHC that stains tissues fixed for more than a few minutes. Hybridomas BA-D5, specific for MyHC I (Schiaffino et al., 1989), and SC-71 specific for MyHC IIA (Bottinelli et al., 1991), were gifts from Dr. S. Schiaffino (University of Padova, Padova, Italy). Biotinylated α -bungarotoxin and Alexa-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR); Cy5-conjugated streptavidin was purchased from Vector Laboratories (Burlingame, CA). Hoechst 33342 (bis-benzimide) was purchased from Sigma, as were other reagents.

Rat muscle denervation and stimulation. Young adult male Wistar rats weighing ~250 gm were used. All surgical operations were done under deep anesthesia with Equithesin (42.5 mg chloral hydrate and 9.7 mg pentobarbitone in 1 ml solution, 0.4 ml/100 gm body wt, i.p.). SOL and EDL in one leg were denervated by resecting a 5-mm-long segment of the sciatic nerve in the thigh. Electrodes on SOL or EDL were implanted, and chronic stimulation was applied as described in detail in Windisch et al. (1998). Both muscles received either 25 square pulses at 150 Hz every 15 min (fast pattern) or 200 pulses at 20 Hz every 30 sec (slow pattern). For the sake of brevity, we will occasionally refer to these patterns as “150 Hz stimulation” or “20 Hz stimulation”. Each pulse was bipolar, lasted 0.4 msec, and passed 8–10 mA in either direction through the muscle. Identical experiments have been inspected and approved by the Norwegian Experimental Board and Ethical Committee for Animal Experiments on several occasions. The present experiments were overseen by the veterinarian responsible for the animal house. The animals were checked daily. The flexible tube extending from the animal's head to rotating contacts overhead allows free movements within the cage. Apart from one leg being denervated and contractions being visible during stimulation, the animals did not show obvious abnormal behavior or signs of pain.

Cryostat sections and staining. Because no antibody against any adult type II MyHC works on the well fixed whole fibers, we also prepared muscle sections to confirm that stimulation produced the expected changes in fiber types. SOL and EDL muscles were frozen in isopentane at freezing point and kept at -80°C until use. Transverse 10- μm -thick sections were cut in a cryostat, mounted on slides, and fixed with 2% paraformaldehyde for 10 min. They were then blocked in 10% NGS and 1% BSA in 0.01 M PBS, pH 7.4, and incubated for 1 hr with primary antibodies (P-1 anti-GLUT4, NOQ 7.5.4.D anti-MyHC I, BA-D5 anti-MyHC I and SC-71 anti-MyHC IIA) diluted in 3% NGS and 1% BSA in 0.01 M PBS, pH 7.4, washed and incubated in the same buffer for 1 hr with rhodamine- or fluorescein-conjugated secondary antibodies at room temperature.

Single muscle fiber preparations and staining for immunofluorescence. Muscles fixed by perfusion as described in Ploug et al. (1998) were dissected and kept in fixative at room temperature for an additional 30 min and then overnight at 4°C . After several rinses in PBS, small bundles of one to three fibers were separated by manual teasing with fine forceps and transferred to 50 mM glycine, 0.25% bovine serum albumin, 0.04% saponin, and 0.05% sodium azide in PBS for blocking and permeabilization for at least 30 min. They were then incubated overnight at room temperature with the primary antibodies and with biotinylated α -bungarotoxin (1:4000) in blocking buffer supplemented with 200 $\mu\text{g}/\text{ml}$ goat IgG. After three washes of 15 min each in PBS-0.04% saponin, they were incubated for 2 hr with Alexa 488-conjugated goat anti-rabbit F(ab)₂ fragments (1:250), Alexa 568-conjugated goat anti-mouse (1:250),

and Cy5-conjugated streptavidin (1:1000) in blocking buffer, then after three washes again with Hoechst 33342 (0.5 $\mu\text{g}/\text{ml}$) in blocking buffer. Fibers were mounted in Vectashield (Vector Laboratories) in two columns of parallel horizontal fibers on a glass slide. Each primary antibody combination (GM130-GLUT4, α -tubulin-GLUT4, GM130- α -tubulin, and MyHC I-GLUT4) was used on fibers from at least two different animals for each muscle and each treatment. Additional immunofluorescent staining was performed 1 week later on fibers newly teased from muscle fragments that had been kept in 50% glycerol at -20°C . We had previously tested (E. T. Ploug and E. Ralston, unpublished data) that the immunofluorescent staining for GLUT4 of fibers prepared from muscle preserved in glycerol for up to 1 month is undistinguishable from that of fibers prepared from freshly fixed muscle. Again, each primary antibody combination (Sec31p- α -tubulin, Sec31p-GM130, pericentrin-GM130, pericentrin- α -tubulin) was used at least twice.

Microscopy and image analysis. Conventional microscopy of sections or whole fibers was done with a Leica (Deerfield, IL) DMRD microscope. Digital images were collected with a Sensys CCD camera (Photometrics, Tucson, AZ) controlled by IPLab (Signal Analytics Corporation, Vienna, VA) run on a Macintosh G4 computer. Confocal images of whole fibers were obtained on Zeiss LSM 410 and 510 at the National Institute of Neurological Disorders and Stroke Light-Imaging Facility. Images were transferred to a Macintosh computer and analyzed with NIH Image (written by W. Rasband at the United States National Institutes of Health and available from the Internet at <http://rsb.info.nih.gov/nih-image/>).

To record systematic series of images or of Z-series, fibers were first localized at low magnification, using the Hoechst nuclear counterstain. The microscope was focused on the top fiber of either left side or right side of the slide. Images were then collected with a 63 $\times$ numerical aperture (NA) 1.4 objective lens from each successive fiber. Lateral movement of the stage was only as much as was necessary to avoid areas with structural damage or an accumulation of nonmuscle cells.

To compare the distribution of Golgi elements between the surface and the core of the fibers, the surface image was recorded from the plane that contains the nuclei, next to the plasmalemma. The core image was obtained by averaging a Z-series of six optical sections 1 μm apart starting 2–3 μm inside the fiber. The six images were combined by maximal projection. Each image was opened in NIH Image, inverted, thresholded, and made binary. An area covering most of the fiber but excluding nonmuscle Golgi complexes or occasional staining dirt was drawn. Its total area was measured as well as the number of particles it contained (the number the Golgi elements) and the surface of the summed Golgi elements. For the core images, the results were divided by 6 to obtain the number of Golgi elements or Golgi surface per optical section. To compare the size of individual elements in different conditions, a smaller area also excluding nuclei was drawn. The size of all particles >5 pixels (to exclude possible background staining) was measured. Results were imported from NIH Image to Microsoft Excel for calculations. Fiber dimensions were measured in the Zeiss 410 as described in Ralston et al. (1999), and fiber section surfaces were calculated assuming an elliptical section.

To localize the NMJ, Hoechst-counterstained fibers were examined at low magnification (10 $\times$ or 16 $\times$ objective lens) under UV fluorescence to localize junctional nuclei or with a Cy5 filter to localize bungarotoxin staining directly.

Images were adjusted for contrast with Photoshop 5.5 and printed from a Macintosh computer on a Pictography 3000 digital printer (Fuji, Elmsford, NY).

RESULTS

Changes in MyHC expression are observed after 2 weeks of stimulation of denervated fibers

We decided to examine the results after 2 weeks of stimulation. At that time, a partial phenotype switch has been shown in response to cross-stimulation (Windisch et al., 1998). We could thus rule out that Golgi complex changes may simply be a consequence of complete MyHC transformation. To confirm earlier findings in the present set of experiments, we stained single fibers with an anti-MyHC I antibody (NOQ 7.4.5.D), the only antibody to an adult MyHC isoform that stains fixed muscle. In control SOL muscle, which contains on the average 97% of type I fibers (Ausoni et al., 1990), 97% of the fibers examined ($n = 102$) were

SOL / ctrl **EDL / 20 Hz**

Figure 1. Partial transformation of MyHC expression in EDL fibers denervated and stimulated with the 20 Hz pattern for 2 weeks. Fibers from control SOL (*a*, *b*) and from EDL stimulated with a 20 Hz pattern (*c*, *d*) were stained with anti-MyHC I. Digital images were recorded on a conventional fluorescence microscope. Exposure time and image treatment were identical for the four panels. In control SOL, 97% of the fibers show a bright staining (*a*), whereas 3% are unstained (*b*). In the stimulated EDL, a few very bright fibers (*c*) likely represent the original type I fibers, whereas 21–34% of the fibers (see Results) appear to express a low level of MyHC I (*d*), as expected from partially transformed fibers. Scale bar, 50 μ m.

MyHC I-positive. In control EDL muscle which, in one material, contained on the average 45% type IIB, 29% type IIX, 23% type IIA, and 3% type I fibers (Windisch et al., 1998), 8% of fibers ($n = 95$) were positive for MyHC I. In both muscles, the staining was all-or-none, with relatively little variation along the fibers (Fig. 1*a–c*). After 2 weeks of denervation and stimulation with the 150 Hz pattern defined in the Materials and Methods, the proportion of MyHC I-positive fibers in the EDL remained at 8% ($n = 62$), but after stimulation with the 20 Hz pattern, the proportion had increased to 21% ($n = 115$) when only fibers stained from end to end were counted or to 34% when partially stained fibers were included. Digital photography showed that the staining intensity was lower than in the original type I fibers and varied more along the fibers (Fig. 1*d*). Fibers from SOL muscle stimulated with the 150 Hz pattern remained positive for MyHC I, but the staining intensity was lower than in control muscle (data not shown). Staining of SOL and EDL muscle sections from both control and stimulated muscles (data not shown) confirmed the

results obtained on whole fibers. We concluded, therefore, that 2 weeks were sufficient to observe the initiation of a fiber type transformation. Because we did not observe more than an occasional central nucleus in the fibers, there was no apparent muscle fiber regeneration, and we were observing true transformation of the original fibers.

The distribution of the Golgi complex is activity-dependent

The Golgi complex of muscle fibers is made of thousands of individual elements that are dispersed throughout the fibers. The distribution of these elements was shown to be fiber type-dependent in two respects: the relative distribution of the elements between the surface and the core of the fibers and the specific pattern within each of these domains (Ralston et al., 1999). In SOL type I fibers, 74% of Golgi complex elements were within the outer 1–2 μ m layer of cytoplasm, whereas only 27% of Golgi elements were at the surface of the type IIB fibers in the tensor fascia latae (TFL). Each nucleus of type I fibers was surrounded by an average of 13 Golgi elements, whereas nuclei of type IIB fibers were surrounded by only two elements, generally found at the nuclear poles. Golgi elements in the core of type I fibers were organized in chains, whereas they were more uniformly distributed in the core of type IIB fibers.

Because the EDL used as the fast muscle in the present work contains a small proportion of type I and IIA fibers, we first made sure that we could distinguish them and that the Golgi complex pattern of the EDL type IIB or IIX fibers resembles that observed in the TFL. On the basis of staining of muscle sections for MyHCs and GLUT4 (data not shown), we identified type IIB and IIX fibers in the EDL as large fibers (2137 μ m² average cross-section) in which GLUT4 was, indeed, found in single elements at the nuclear poles and dispersed in the fiber core. Type I and IIA fibers were smaller (1008 and 1125 μ m² average cross-section, respectively). GLUT4 staining in the type I EDL fibers resembles that in SOL type I fibers (data not shown), although it lacks the spectacular regularity found in SOL fibers (Ploug et al., 1998; Ralston et al., 1999). Unless otherwise mentioned, images shown are from the larger type IIB or IIX fibers, which we could not distinguish from one another.

The distribution of the Golgi complex was followed with an antibody against the *cis*-Golgi protein GM130, and double-staining with an antibody against the glucose transporter GLUT4 was routinely performed because it allows us to distinguish the muscle Golgi elements, which are all associated with GLUT4 (Ploug et al., 1998), from the nonmuscle (fibroblast, Schwann cells etc.) Golgi elements that are GLUT4-negative.

To assess the effects of denervation and of stimulation on the Golgi complex, at least 12 sets of confocal images, each from a different fiber, were recorded from each muscle (EDL and SOL, control, denervated, denervated and stimulated), from two different animals. Representative surface and core images from SOL fibers are shown in Figure 2. In denervated fibers stimulated for 2 weeks with the 20 Hz pattern, most fibers (27 of 30) were indistinguishable from control fibers: each nucleus was surrounded by several Golgi elements and, in the fiber core, the Golgi elements were grouped into linear stretches. In fibers stimulated with the 150 Hz pattern, in contrast, most (52 of 55) fibers showed decreased perinuclear staining, and the stretches of Golgi elements were lost from the fiber cores (Fig. 2). For the

Figure 2. The distribution of the Golgi complex is sensitive to patterned activity. Fibers from SOL and EDL were stained with anti-GM130. At least 12 series of confocal images were recorded for each muscle in each condition. The panels show representative confocal images focused on the nuclei (surface) or 5- to 8- μ m-deep in the fiber (core). For both muscles, stimulation with a pattern that mimics that originally provided by their motor neurons preserves the original Golgi complex distribution, whereas stimulation with the other pattern changes the Golgi complex distribution. Notice, at the surface, the perinuclear elements in control and in 20 Hz-stimulated SOL and EDL but not in control EDL or in 150 Hz-stimulated SOL or EDL. In the core, notice the rows of elements in control and 20 Hz-stimulated SOL. In some panels, arrows indicate the positions of nuclei. In one panel, an arrowhead points to the Golgi complex of a nonmuscle cell that remained associated with the muscle fiber. Scale bar, 10 μ m.

Table 1. Quantitative changes in distribution of the Golgi complex after denervation and cross-stimulation of SOL and EDL

	SOL ctrl	SOL 150	EDL ctrl	EDL 20
Size of Golgi elements (μ m ²)	0.51 $\pm$ 0.06	0.46 $\pm$ 0.08	0.42 $\pm$ 0.06	0.42 $\pm$ 0.08
Golgi staining at the surface of the fiber (μ m ² /400 μ m ²)	8.61 $\pm$ 4.63	5.60 $\pm$ 1.75	2.30 $\pm$ 0.54	5.16 $\pm$ 1.58
Golgi staining in the core (μ m ² /400 μ m ²)	0.76 $\pm$ 0.43	0.91 $\pm$ 0.39	0.37 $\pm$ 0.14	0.92 $\pm$ 0.39
Surface to core ratio	12.5 $\pm$ 4.1	6.9 $\pm$ 2.7	6.7 $\pm$ 2.2	6.0 $\pm$ 2.1

Confocal images were recorded from 12 fibers for each condition. Staining at the surface of the fiber was recorded, then the focus was moved below the plane of the nuclei (2–3 μ m deep) and a series of six images, 1 μ m apart, was recorded. The surface image as well as the projection of the core images were analyzed with NIH Image as described in Materials and Methods. The results shown (average $\pm$ SD) are for control SOL and EDL as well as for SOL stimulated with a 150 Hz pattern and EDL stimulated with a 20 Hz pattern.

EDL, stimulation with the 20 Hz pattern resulted in an increase in perinuclear staining, which gave the fibers a type I look at the surface but not in the core, where stretches of Golgi elements were rare, most of them appearing scattered as in fast fibers. Stimulation of the EDL at 150 Hz led to the preservation of the original pattern as stimulation at 20 Hz did for the SOL.

Because an important difference between type I and IIB fibers (Ralston et al., 1999) is the relative distribution of the Golgi complex between fiber surface and fiber core, we quantitated some of the present recordings with NIH Image (Table 1). In the SOL, surface staining diminished, whereas core staining did not

change much after denervation and stimulation with the 150 Hz pattern. In EDL fibers, stimulation with a 20 Hz pattern led to an increased surface staining but also to increased core staining, possibly related to the decrease in the diameter of these fibers. The average size of the individual Golgi elements in the cytoplasm (excluding the nuclei) did not change.

These results thus demonstrate, qualitatively and quantitatively, that the Golgi complex organization responds to changes in patterned activity and suggest that activity is responsible for at least a major part of the fiber type-related differences in its organization.

Figure 3. Microtubule density and orientation respond to changes in patterned activity. Fibers from SOL and EDL controls (*top row*), 20 Hz pattern-stimulated (*middle row*), and 150 Hz pattern-stimulated (*bottom row*) were stained with anti- α -tubulin. Confocal images were recorded from at least 12 fibers for each condition. Typical examples of surface distributions are shown. In control SOL and in SOL and EDL stimulated with the 20 Hz pattern, there is a dense layer of long microtubules in practically all possible orientations. Also typical of type I fibers are the microtubule fascicles that join nuclei (in the SOL/20 Hz panel) and the dense perinuclear staining. In control EDL and EDL stimulated with the 150 Hz pattern, in contrast, the surface layer of microtubules is thinner, and they form an orthogonal lattice. Clear nucleation points are observed at some of the nuclear poles (*arrowheads*), including in 150 Hz-stimulated SOL fibers. In these, however, transformation is incomplete and microtubules appear mostly longitudinal. Scale bar, 10 μ m.

Figure 4. Pericentrin distribution is affected by activity. Control (*ctrl*) and cross-stimulated SOL and EDL fibers were stained with anti-pericentrin. The panels display single confocal images focused on the nuclei. Notice the accumulation of pericentrin at the nuclear poles of control EDL and, partially, of cross-stimulated SOL fibers compared with the more uniform distribution in control SOL and cross-stimulated EDL. In the control SOL fibers, there is a network of fine pericentrin dots between the nuclei, which is not found in the cross-stimulated EDL. *Small arrows* point to pericentrin dots that correspond to microtubule-nucleation sites, whereas *arrowheads* point to centrosomes associated with nonmuscle cells at the surface of the fibers, as determined by double-staining with anti-tubulin (data not shown). Scale bar, 10 μ m.

The distribution of microtubules is sensitive to patterned activity

If the fiber type-dependent distribution of the Golgi complex is linked to the organization of the microtubule cytoskeleton (Ralston et al., 1999), activity should affect microtubule distribution as well. SOL and EDL fibers, double-stained with anti- α -tubulin combined with anti-GM130 or anti-GLUT4, were examined (Fig. 3). In this figure, the image is focused on the plane between plasmalemma and nuclei. The SOL shows a dense layer of long microtubules, in all possible orientations. In some areas, this layer is so thick that the perinuclear staining does not show through it (data not shown). In SOL fibers stimulated with the 20 Hz pattern, this layer

of microtubules is preserved, although it appears thinner in some fibers. The SOL/20 Hz panel in Figure 3 also shows the long fascicles of microtubules that extend longitudinally between nuclear poles in type I fibers. Interestingly, a thick layer of microtubules is also found between plasmalemma and nuclei of 20 Hz-stimulated EDL fibers. Its disordered pattern contrasts with the orthogonal lattice of microtubules observed in control and 150 Hz-stimulated EDL. Although stimulation with the 150 Hz pattern preserves the original fast pattern of microtubules in the EDL, it does not seem to induce this pattern in SOL fibers. Their microtubules appear fewer than in control or 20 Hz-stimulated SOL, and they show some nucleation at the nuclear poles (*arrowheads*), but most of the microtubules are

longitudinal. Of 30 fibers examined, only one (not shown here) showed some transverse microtubules.

These experiments therefore show that activity plays a major role in microtubule organization, because a stimulation pattern similar to the endogenous one was sufficient to maintain the native distribution of microtubules in both SOL and EDL. However, the plasticity of microtubules in the present experimental setup was limited: stimulation with the 20 Hz pattern induced a slow type I pattern in EDL fibers but stimulation with the 150 Hz pattern only partially succeeded in inducing a type II pattern in SOL fibers.

To evaluate the possibility that changes in microtubule orientation are linked to changes in microtubule nucleation, we examined the distribution of the centrosomal protein pericentrin (Doxsey et al., 1994). During muscle differentiation, proteins of the pericentriolar material, such as pericentrin, become perinuclear (Tassin et al., 1985; our unpublished data). In control SOL fibers, pericentrin encircles most nuclei uniformly, whereas in control EDL pericentrin shows a polar distribution with heavier staining at the nuclear poles (Fig. 4). The distribution of pericentrin was affected by changes in activity patterns in both muscles: the fraction of nuclei with uniform distribution increased from 22% in control to 90% in 20 Hz-stimulated EDL fibers and decreased from 89% in control to 49% in 150 Hz-stimulated SOL fibers. In control SOL, pericentrin was also found in bands of small dots between the nuclei, which were not found in 20 Hz-stimulated EDL fibers. Pericentrin was found in larger dots which correspond to microtubule nucleation centers in control EDL, and were found in 150 Hz-stimulated SOL as well. We conclude that activity affects pericentrin and, presumably, microtubule nucleation.

Golgi elements are localized at the endoplasmic reticulum exit sites, the pattern of which is also activity-dependent

Proteins are exported from the endoplasmic reticulum (ER) to the Golgi complex in COPII-coated vesicles that assemble at the ER exit (or export) sites (Barlowe, 1998). In cells with a compact classic Golgi complex, the ER exit sites, labeled with antibodies against COPII proteins such as Sec31p (Shugrue et al., 1999) are uniformly distributed over the cell. We have recently shown that the ER exit site distribution changes during muscle differentiation and that Golgi elements in C2 myotubes are localized at the ER exit sites (Lu et al., 2001), because they are in cells with disrupted microtubules (Cole et al., 1996). The ER exit sites thus appear as important determinants of the localization of Golgi elements in muscle, but their distribution in mature fibers has never been determined.

SOL and EDL fibers were double-stained with anti-GM130 and with an antibody against Sec31p (Shugrue et al., 1999). Figure 5 shows confocal images from control and cross-stimulated SOL and EDL fibers. The figure demonstrates the remarkable degree of juxtaposition of the Golgi elements and of the ER exit sites in all conditions and the sensitivity of both to changes in patterned activity. Double-staining for α -tubulin and for Sec31p (Fig. 6) shows that most ER exit sites are aligned with microtubules and seem to be preferentially positioned at the node points where several microtubules cross. Therefore, activity may affect Golgi elements indirectly, by affecting the localization of the ER exit sites.

Activity affects the distribution of GLUT4 at the NMJ

Having observed that the Golgi complex organization at the NMJ appears independent of fiber type (Ralston et al., 1999), we assumed that nerve-derived trophic factors dominate the subcellular

Figure 5. Golgi complex and ER exit sites are closely associated in control and stimulated fibers. Fibers from SOL and EDL, control and cross-stimulated, were double-stained with anti-GM130 and with anti Sec31p and observed in the confocal microscope. Notice the practically identical pattern of the two markers in both control and cross-stimulated fibers, although there also is a lighter staining for Sec31p that has no corresponding GM130 staining. Each panel shows one nucleus and the area around it. Arrows indicate the position of the nuclei in control EDL and cross-stimulated SOL in which nuclei are not highlighted by perinuclear Golgi elements. Scale bar, 10 μ m.

localization and trafficking of membrane proteins at the NMJ. Accordingly, we did not observe any systematic changes in the distribution of the Golgi complex at the NMJ of stimulated fibers. We were, however, surprised to observe a striking GLUT4 accumulation at the NMJ of denervated but not of denervated and stimulated fibers (Figs. 7, 8). At high magnification (Fig. 7), the staining appears as a dense plaque that is present in practically all denervated SOL fibers (39 of 41). We have previously reported that, in contrast, the NMJ does not stand out in control SOL fibers stained for GLUT4 (Ralston and Ploug, 1996; Ralston et al., 1999). When we localized the NMJ (Fig. 8) by scanning SOL fibers at low magnification for junctional nuclei or for α -bungarotoxin staining, and then observed GLUT4 staining, we found it to stand out in 3 of 21 control fibers, 3 of 20 fibers stimulated with the 20 Hz pattern, and 11 of 20 fibers stimulated with the 150 Hz pattern. In the latter fibers, the intensity of GLUT4 staining was still considerably lower than in denervated unstimulated fibers. Denervated EDL fibers showed the same junctional accumulation of GLUT4 (in 18 of 21 fibers), and it was reduced in stimulated fibers as well (data not shown). Because

Figure 6. ER exit sites are positioned along microtubules. Control SOL and EDL fibers were double-stained with anti- α -tubulin (red) and with anti-Sec31p (green) and observed in the confocal microscope. In the SOL fiber, the perinuclear ER exit sites appear next to and inside the microtubule ring, whereas ER exit sites along longitudinal microtubules (*inset*) appear to be positioned on microtubules. ER exit sites are often found at microtubule nodes (*arrowheads*). Scale bar, 10 μ m.

junctional nuclei in fast fibers stand out even in control fibers (Ralston et al., 1999), we did not quantitate the NMJ staining in EDL fibers further. These results suggest a massive docking of GLUT4 vesicles at the plasmalemma, which is prevented by muscle activity but with less pattern dependence than the other fiber features examined in the present work.

DISCUSSION

The main results of the present work are that fast pattern stimulation of EDL and slow pattern stimulation of SOL prevented the changes in Golgi complex and related proteins induced by denervation. Furthermore, when the stimulation patterns were switched, EDL changed toward a slow phenotype and SOL toward a fast phenotype. These results indicate that the pattern of electrical muscle activity plays a major role in organizing the Golgi complex and associated protein structures.

The observed transformations were incomplete in terms of type I fibers acquiring the appearance of type IIB fibers and vice versa. Such completeness was not expected because the stimulation was of relatively short duration (2 weeks). In comparable studies of MyHC expression, even 2 months of stimulation were insufficient to replace type IIB with type I MyHC in the EDL (Windisch et al., 1998) and type I with type IIB MyHC in the SOL (Ausoni et al., 1990). Two to three weeks of stimulation are sufficient, however, to replace IIB and IIX fibers with IIA fibers in the EDL and pure type I fibers with hybrid fibers containing predominantly IIX and IIA but also small amounts of type I MyHC in the SOL. Golgi complex transformation appears then to start early in relation to fiber type switching. In EDL fibers stimulated with the 20 Hz pattern, the degree of type I appearance of the Golgi complex was independent of the level of expression of type I MyHC in the fiber (data not shown), suggesting that the Golgi complex and associated proteins may be affected by stimulation directly, rather than as a consequence of MyHC type change.

Different subcellular locations showed different degrees of transformation. For the Golgi complex, ER exit sites and pericentrin, it was most complete around the nuclei and least complete in the core of the fibers. In contrast, Eisenberg and Salmons (1981) observed an essentially complete fast to slow change in sarcoplasmic reticulum organization in low frequency-stimulated innervated rabbit muscle. It is likely that the ease of transformation of rabbit fibers compared with rat fibers manifests itself at the level of the Golgi complex as well. Although an explanation for fine spatial differences is not obvious, it is worth noting that several signaling pathways localize to the perinuclear region in skeletal or cardiac muscle. For example, Jaconi et al. (2000) have shown that release of inositol 1,4,5-trisphosphate in rat cardiomyocytes triggers a calcium release limited to the perinuclear region.

Nor did all markers respond to cross-stimulation uniformly. In the case of microtubules, for example, denervated EDL fibers stimulated with a 20 Hz pattern developed a layer of microtubules

Figure 7. GLUT4 accumulates at the NMJ of denervated fibers. Denervated, unstimulated SOL (*top row*), and EDL fibers (*bottom row*) were stained with biotinylated- α -bungarotoxin (α -butx; blue) and with anti-GLUT4 (red). An accumulation of GLUT4 is found at the NMJ. Fine dark ridges (*arrows*) interrupt the GLUT4 staining. In the EDL example, junctional nuclei showing the usual perinuclear staining can be seen around the plaque-like GLUT4 staining. Scale bar, 10 μ m.

Figure 8. The accumulation of GLUT4 at the NMJ is prevented by activity. SOL fibers were triple-stained with biotinylated- α -bungarotoxin (α -butx), anti-GLUT4, and anti-GM130. Single confocal images were recorded in identical conditions and treated identically for each muscle. Two NMJs (control and stimulated with the 150 Hz pattern) are viewed en face, whereas the other two NMJs are viewed sideways. In denervated and unstimulated fibers (*den*), there is a striking accumulation of GLUT4 at the NMJ, together with a fringe of increased GM130 staining. In control and stimulated fibers, GLUT4 is found around the junctional nuclei, some of which can be seen en face in the ctrl and 150 Hz examples, and also gives a diffuse background staining. Arrowheads in one panel (GM130, 150 Hz) point to Golgi complexes from nonmuscle cells. Scale bar, 10 μ m.

between nuclei and plasmalemma, as found in control SOL fibers, but denervated SOL fibers stimulated with the 150 Hz pattern did not present the orthogonal microtubule pattern found in all normal type II fibers; instead they showed a longitudinal pattern of microtubules similar to that of denervated unstimulated fibers (Ralston et al., 1999). The surface layer of microtubules in SOL fibers has been observed by others (Boudriau et al., 1993), as has the orthogonal pattern of microtubules in fast fibers (Rahkila et al., 1997). Similarly, pericentrin changed more completely in the EDL than in the SOL. Other examples have been observed previously. For example, transformation of the fast-twitch cat flexor digitorum longus (FDL) by cross-reinnervation with SOL motor neurons is complete after 30–50 weeks, whereas cross-reinnervation of SOL by FDL motor neurons is very incomplete (Dum et al., 1985 a,b). The mechanisms underlying these differences in plasticity are unknown. The effects of the 20 Hz pattern stimulation on the EDL may be reinforced by effects outside the muscle fibers. We found increased connective tissue in these fibers, in agreement with Brown et al. (1976), who reported increases in capillarization of rabbit tibialis anterior and EDL fibers stimulated at low frequency. In addition, the total amount of activity increased in the 20 Hz pattern-stimulated EDL but decreased in the 150 Hz pattern-stimulated SOL. Some properties may respond to the amount of activity more than to the specific pattern.

Muscle properties at the NMJ differ from those along the rest of the fibers by their dependence on nerve-derived trophic factors such as agrin or neuregulins and, as recently suggested, by mechanical factors as well (Marques et al., 2000). However, the NMJ may be affected by electrical activity as well. Electrical activity has been shown to affect the enzymatic activity of the junctional acetylcholinesterase (Lømo et al., 1985) in fast and slow muscles. It can also prevent denervation-induced reduction in acetylcholine receptor (AChR) stability and number at the NMJ (Andreose et al., 1993). Here, we show that denervation causes an accumulation of GLUT4 at NMJs in both SOL and EDL, which

is reduced or suppressed by both self-like and cross-stimulation. At the resolution of light microscopy we cannot determine whether GLUT4 is in the postsynaptic membrane or in vesicles docked at the membrane. The accumulation of GLUT4 may be linked to increased endocytosis and exocytosis around the endplates of denervated fibers (Vult von Steyern et al., 1993), although the endocytic markers do not label the NMJ itself. Whatever its origin, this staining depends on evoked electrical activity but not so much on its specific pattern. Similarly, the expression of extrajunctional AChRs is blocked by electrical stimulation regardless of the pattern (Lømo and Westgaard, 1975), emphasizing that muscle properties depend on electrical activity in different ways.

The present work demonstrates that centrosomal proteins, microtubules, ER exit sites, and Golgi elements are linked and affected by activity. Which one of them localizes the others is less clear, because very little is known, at this point, of the link between ER exit sites and microtubules. ER exit sites have been reported to be mostly immobile in HeLa and similar cell types (Hammond and Glick, 2000), but their organization changes during muscle differentiation (Lu et al., 2001). The observation that the response of microtubules to the 150 Hz pattern stimulation in the SOL is less complete than the response of the Golgi complex and ER exit sites themselves suggests the possibility that the ER exit sites may determine the course of the microtubules rather than the reverse. It is also likely that the static view provided by immunofluorescence of fixed fibers is insufficient to provide a full understanding of the organization of the highly dynamic microtubules.

Although we have focused on the role of microtubules as organizers of the Golgi complex, microtubules play a role in the localization of other subcellular organelles such as endosomes and mitochondria. Their organization may also affect muscle contraction. Microtubule stabilization has indeed been linked to contractile dysfunction in pressure overload cardiac hypertrophy (Sato et al., 1997). We have not looked for changes in microtubule

stability in the present work, because we found stable microtubules in both fast and slow fibers (Ralston et al., 1999). Microtubules can also mediate spatial organization of signal transduction (Gundersen and Cook, 1999), and of mRNAs, including the α -MyHC mRNA in cardiac myocytes (Perhonen et al., 1998). Their different organization in different fiber types could therefore affect protein localization and synthesis by routes not directly related to the Golgi complex, thereby broadening the potential impact of changes in activity.

At this point, we have no information on the pathways that transmit the effects of activity to the cytoskeleton and organelles. In neurons, there have been reports of microtubule regulation by electrical activity (Alvarez and Ramirez, 1979) but no mention of a pathway. Several of the effects of activity (MyHC switch, for example) take place at the level of transcriptional activation (Buonanno and Fields, 1999). At least some are post-transcriptional, for example the upregulation of hexokinase in rat fast-twitch muscle stimulated at low frequency (Hofmann and Pette, 1994). The recent demonstration of the involvement of Ras-MAP kinase signaling in the switch from a default fast fiber type to a slow fiber type in an *in vivo* regeneration model (Murgia et al., 2000) is suggestive because the Ras-GTPase superfamily is known to be involved in cytoskeleton organization. Similar experiments will hopefully allow us to uncover the pathways required to allow changes in the internal membrane systems during fiber transformation.

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