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Denervated Skeletal Muscle Fibers Develop Discrete Patches of High Acetylcholine Receptor Density

Abstract. *Denervated skeletal muscle fibers of mice develop discrete patches of high acetylcholine receptor density. The patches vary in size from less than 1 micrometer up to 30 micrometers, depending on the muscle and the period of denervation. Within the patches the acetylcholine receptor density is some 20 times greater than elsewhere along the muscle fiber and probably approaches that in the subsynaptic membrane.*

A current problem in the study of synapse formation concerns the manner in which receptors become preferentially localized at sites of synaptic contact during the course of development. Recent observations on the distribution of acetylcholine (ACh) receptors in cultures of embryonic chick muscle cells are particularly interesting in this respect. During the first few days in culture, the cells display a relatively uniform distribution of receptors along their entire length, but after about 1 week they develop "ACh hot spots," discrete patches of membrane where the number of receptors is considerably greater than elsewhere on the cell (*1–3*). Estimates based on the uptake of α-bungarotoxin, which binds specifically and with high affinity to the receptors, indicate that the receptor density in these patches approaches that in the subsynaptic membrane of normal adult skeletal muscle (*1*). These findings have therefore raised the possibility that the patches might be targets for growing nerve fibers (*1, 2*). Consistent with this suggestion is the finding that the development of the patches is not dependent upon previous innervation of the cells (*4*).

So far, the occurrence of such discrete patches of ACh receptors has been demonstrated only on cultured embryonic muscle cells. The question therefore arises whether their development is somehow related to the artificial conditions of the culture situation or whether they also develop in vivo. It is also pertinent to know if their development is restricted to embryonic muscle. To answer these questions we have examined the distribution of ACh receptors on adult denervated muscle fibers. It is well known that after denervation, muscle fibers acquire ACh receptors along their entire length (*5*). Estimates, based on the uptake of α-bungarotoxin, indicate that these extrajunctional receptors attain a mean density which is about one-tenth of that in the subsynaptic membrane (*6*). There is evidence, however, that the distribution of the receptors is not uniform (*7*). This conclusion is extended by the present study which indicates that many denervated muscle fibers develop discrete patches of high ACh receptor density analogous to those which have previously been observed in cultures of embryonic muscle.

The experiments were carried out mainly on the plantaris muscle of adult CBA and Swiss mice weighing 20 to 25 g and 30 to 35 g, respectively. The mice were anesthetized and the muscles were denervated by removing a 4- to 8-mm segment of the appropriate nerve remote from its entry into the muscle. The animals were then maintained for various periods of time up to 3 weeks. In order to examine receptor distribution the receptors were stained with fluorescent conjugates of α-bungarotoxin as previously described (*8*). This technique is especially suitable for visualizing regions of high receptor density. In brief, muscles

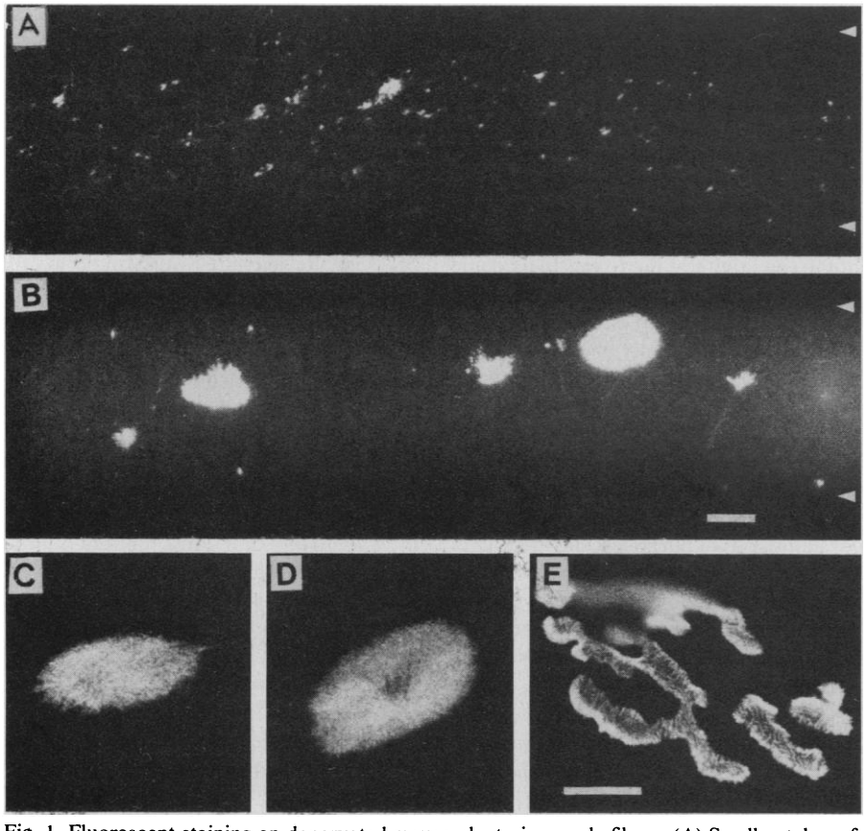

Fig. 1. Fluorescent staining on denervated mouse plantaris muscle fibers. (A) Small patches of stain on a muscle fiber denervated for 4 days. Arrowheads indicate edges of fiber. (B) Larger patches on a fiber 10 days after denervation. (C and D) Higher magnification views of single patches 9 and 14 days after denervation. (E) Fluorescent staining of an endplate on a fiber denervated for 9 days. Scale bars, 10 μm. The endplate shows bright outlining and fine bright lines whereas the patches show a relatively uniform staining intensity. In all examples except B the muscles were stained while alive. In B, the muscle was fixed with 4 percent paraformaldehyde for 90 minutes at 37°C before being stained. Such fixation did not affect the staining patterns.

were removed from the animals and bathed for 1 hour in a physiological solution containing 5 percent calf serum and 10^{-5} g/ml of fluorescein- or tetramethylrhodamine-labeled α-bungarotoxin. After being rinsed for 1 to 2 hours the muscles were fixed in 95 percent ethanol (−16°C) or in 10 percent neutral formalin (4°C). Individual muscle fibers were then dissected, mounted on glass slides, and examined in a Zeiss microscope with incident-light fluorescence optics and with phase contrast optics. For some experiments, ACh receptor distribution was also studied by autoradiography with ^{125}I-labeled α-bungarotoxin, prepared as described by Berg *et al.* (*9*). Mouse plantaris muscles were exposed to the radioactive toxin (10^{-6} g/ml for 1 hour), rinsed, and then fixed with 2.5 percent glutaraldehyde. Individual muscle fibers were dissected, dehydrated in ethanol, and air-dried at room temperature on glass slides. After they were coated with Kodak NTB-2 emulsion, the slides were kept in the dark at 4°C for up to 7 days, developed at 18°C, and fixed.

Figure 1, A to D, illustrates typical examples of discrete patches of fluorescent stain on denervated mouse plantaris muscle fibers. Patches were generally circular or oblong, but in some instances doughnut shapes were also observed. The size and number of patches varied according to the period of denervation. By 4 days individual fibers contained many patches which were usually less than 5 μm in diameter and often less than 1 μm (Fig. 1A). After longer periods, individual muscle fibers displayed fewer and larger patches (Fig. 1, B to D), some of which extended for up to 30 μm. Patches were observed on 15 out of 39 fibers (three muscles) which had been denervated for 4 days, on 50 out of 102 fibers (nine muscles) which had been denervated for 9 to 14 days, and on 36 out of 87 fibers (six muscles) which had been denervated for 16 to 20 days. They occurred most frequently in regions midway between the endplate and tendons and were never seen at the ends of muscle fibers. Occasionally they were found in the immediate vicinity of the endplate.

Discrete patches of fluorescent stain were also seen in other denervated muscles that were examined, including the soleus and extensor digitorum longus of the mouse and the plantaris and soleus of the rat, but some differences were noted with regard to size. For example, although small patches were detected in the mouse soleus 4 days after denervation, they did not increase in size as much as in the mouse plantaris. Even after 8 to 16 days the patches on soleus muscle fibers were rarely greater than 10 μm. Similar differences were also observed between the rat plantaris and soleus. In addition, patches in the extensor digitorum longus were comparable in size to those in the plantaris. These observations suggest that patch size varies according to whether the muscle is fast or slow. Other characteristics such as intensity, location, and frequency of occurrence, were generally similar for all of the muscles examined.

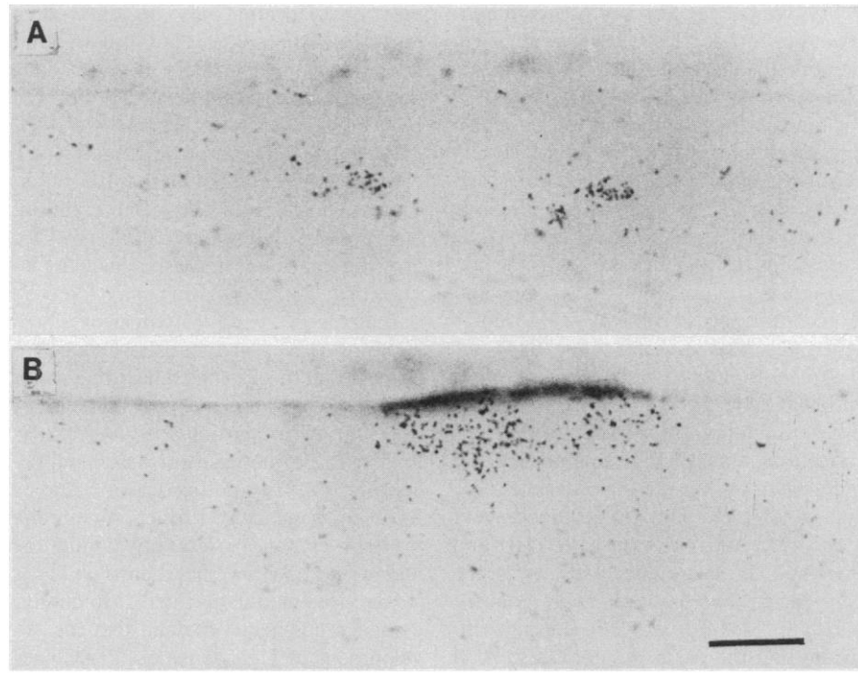

Fig. 2. Autoradiographs showing distribution of grains on a single mouse plantaris muscle fiber after exposure to ^{125}I-labeled α-bungarotoxin. The muscle had been denervated for 9 days. (A) Region not in area of endplate. Note the widespread distribution of grains as well as the patches of higher grain density. (B) Endplate region of same muscle fiber. A widespread distribution of grains is apparent together with a higher grain density at the endplate. The dark band is due to a dense accumulation of grains and indicates that part of the endplate is located on the edge of the fiber. The endplate can be distinguished from the patches on the basis of its larger size. Prior treatment of denervated muscles with unlabeled toxin (3 μg/ml for 1 hour) reduced grain densities by over 90 percent. Scale bar, 20 μm.

Fluorescent staining was entirely prevented by prior treatment of the muscles with unlabeled α-bungarotoxin (2 μg/ml for 1 hour) and was significantly reduced in intensity when carbachol chloride (3×10^{-4} g/ml) was present during exposure to the fluorescent toxin. The staining is thus specific for ACh receptors [see also (*8*)], and the patches reflect regions on the muscle fibers where the number of receptors is relatively high. Either the sarcolemma in these regions contains a relatively high density of receptors or else receptor density is the same as elsewhere but the sarcolemma is highly folded. That the first interpretation is correct is supported by the following observations. When viewed at high magnification the patches were found to follow the contours of the cell surface and had a very precise focus. In addition, as shown in Fig. 1, C and D, the intensity of the staining often appeared relatively uniform throughout individual patches. This is in contrast to the fluorescent staining at endplates (Fig. 1E) where one sees a bright outlining, which is attributable to staining of receptors associated with the walls of the junctional gutter, as well as finer bright lines which are attributable to staining of receptors associated with the junctional folds (*8*). Indeed, when stained endplates are viewed face-on, there is a continuous sequence of planes of focus as one passes from the top of the junctional gutter to the junctional folds. Thus the spatial resolution of the fluorescent staining technique is sufficiently great that folds in the sarcolemma of about a micrometer in depth would be detected if they were present. It is therefore unlikely that the patches were associated with any pronounced folding of the sarcolemma. Instead, the present results suggest that the patches reflect regions of sarcolemma where the receptor density is particularly high. A similar conclusion has also been reached for the discrete regions of high uptake of α-bungarotoxin on cultured chick myotubes (*3*).

Multivalent ligands such as immunoglobulins and lectins can cause their receptors to aggregate (*10*). Although α-bungarotoxin is a univalent ligand (*11*), the question arises whether it may have a similar effect on ACh receptors. To test this possibility, we conducted experiments which were based on the previous finding that prior fixation with paraformaldehyde at 37°C prevents the ligand-induced aggregation of concanavalin A receptors on fibroblasts (*12*). Denervated plantaris muscles were fixed for up to 90 minutes with 4 percent paraformaldehyde at 37°C before being exposed to fluorescent α-bungarotoxin. Although this fixation caused a nonspecific increase in background fluorescence, the typical discrete patches of fluorescent stain were still observed (Fig. 1B) and they had the same specificity as in the case when live muscles were stained with fluorescent toxin. These results indicate that the toxin did not cause ACh receptors to aggregate into high-density patches, but rather that the patches existed before the muscles were exposed to the toxin.

Patches of ACh receptors were also observed when denervated mouse plantaris muscles were exposed to ^{125}I-labeled α-bungarotoxin and examined by autoradiography (Fig. 2A). The ratio of the grain densities in the patches compared to adjacent extrajunctional regions on the same fibers was 21.5 ± 3.1 (mean ± standard error; N = 11) after 9 days of denervation and 17.9 ± 4.5 (N = 9) after 20 days of denervation. The corresponding ratios for endplates (Fig. 2B) relative to extrajunctional regions (not including patches) were 26.0 ± 2.7 (N = 19) and 29.5 ± 4.2 (N = 18). Grain density should be directly proportional to receptor density in extrajunctional regions where the sarcolemma is not folded, and it therefore follows from the present findings that the density of ACh receptors in patches is about 20 times greater than along the rest of the fiber. Values for grain density at endplates are less readily converted to relative receptor density. On the one hand the branching patterns of endplates are not revealed by autoradiography and, as a result, relative receptor density will be underestimated. On the other hand, the folding of the sarcolemma at the endplate will lead to an overestimate. If one assumes that these opposing errors approximately balance each other, our autoradiographic measurements suggest that the receptor density in patches may be close to that in the subsynaptic membrane.

Patches of high ACh receptor density thus develop not only on embryonic muscle cells in culture, but also on adult denervated muscle fibers in vivo. Since denervated muscle fibers can become reinnervated at sites other than the original endplate (*13*), the present findings are consistent with the notion that the patches may be preferred sites of innervation. However, even if they are not targets for growing nerve fibers the mechanisms involved in their formation may likewise participate in the development of a high receptor density at synapses. One possibility is that the patches reflect regions of sarcolemma where the rate of receptor degradation is particularly low, as is known to be the case for junctional receptors (*14*). In addition, many studies have revealed that surface membrane proteins can move laterally within the membrane (*15*), and that their mobility is subject to modulation (*10*). Recently, evidence has been obtained that the development of high densities of ACh receptors in patches and at sites of innervation on cultured amphibian muscle cells involves a process of receptor redistribution (*16*). It is therefore reasonable to assume that ACh receptors can move within the sarcolemma and that at sites of high packing density their mobility is significantly restricted.

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Partial Denervation Affects Both Denervated and Innervated Fibers in the Mammalian Skeletal Muscle

Abstract. *Partial denervation of the rat extensor digitorum longus muscle was performed by sectioning only one of the sciatic nerve roots. Measurements of spike resistance to tetrodotoxin in individual muscle fibers revealed denervation changes not only in the denervated fibers but also in the adjacent innervated ones. The results support the concept that products of nerve degeneration play a role in the origin of muscle changes induced by denervation.*

Sectioning of the motor nerve to skeletal muscles is followed by a number of striking changes in the electrical properties of muscle fibers, such as the appearance of sensitivity to acetylcholine (ACh) in the extrajunctional membrane (*1, 2*), the development of resistance of the action potential to tetrodotoxin (TTX) (*3*), a decrease in resting membrane potential (*4*), and spontaneous spike activity (fibrillation) (*5*). Most of these changes occur shortly (about 1 day) after the onset of nerve degeneration which follows the separation of peripheral axons from their cell bodies. Denervation changes have been attributed either to the lack of a hypothetical "neurotrophic factor" for muscle (*2*) or to the absence of nerve impulses (*6–8*). That two factors must be involved in the origin of denervation changes is now well established. On the one hand, there is no doubt that nerve impulses play a prominent role, because continuous direct stimulation of denervated muscles prevents or represses denervation changes (*7–9*). On the other hand, the participation of a factor different from nerve impulses is strongly suggested by experiments of partial denervation of the