Topographical Segregation of Old and New Acetylcholine Receptors at Developing Ectopic Endplates in Adult Rat Muscle

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ABSTRACT We have used radioautographic methods to examine the topography of addition and removal of acetylcholine receptors (AChRs) within receptor clusters at developing ectopic synapses in adult rat soleus muscle. After AChRs within a cluster had been pulse-labeled with ^{125}I-α-bungarotoxin (^{125}I-α-BuTx), the area that they occupied within the cluster shrank with time. Thus the old receptors at new endplates occupy a continually decreasing area of the growing receptor cluster. To localize newly added AChRs, we pretreated the muscles with unlabeled α-BuTx, thus blocking the old receptors, and then labeled newly added receptors with ^{125}I-α-BuTx 1 or 2 d later. In radioautographs, AChR clusters from these muscles appeared as annuli or "doughnuts," unlike control (unpretreated) clusters, which were more nearly uniformly labeled. This visual impression was confirmed by analyzing the radial grain density distribution. Thus growth and turnover of AChR clusters at ectopic endplates takes place by the addition of receptors at the periphery of the clusters. Our data are most consistent with a model in which receptor removal occurs by endocytosis randomly throughout the cluster.

One of the most striking features of the motor endplates of vertebrate muscles is the high density of acetylcholine receptors (AChRs) clustered in the postsynaptic membrane under the nerve terminal. At the endplate, AChRs are concentrated at the crests of the postsynaptic folds where they are closely packed at a density of 20,000–30,000/μm^2; in contrast, the densities are much lower in the extrajunctional membrane of embryonic and denervated adult muscle fibers (100–800/μm^2) and of normal adult muscles (<20/μm^2) (14, 16). During endplate formation, clusters of AChRs form under nerve terminals shortly after nerve-muscle contact (3, 9, 17). As the endplate matures, the cluster grows larger, and the number of AChRs at the endplate increases (6, 7).

The clustered AChRs at endplates undergo continual turnover (20, 24, 30). In newly formed clusters the degradation rate for AChRs is relatively rapid ($t_{1/2}$ = 1 d), but as the endplate matures the degradation rate slows dramatically ($t_{1/2} \geq 10$ d) (7, 8; footnote 1). During endplate maturation when the total number of receptors at the endplate is increasing, the rate of receptor addition is greater than the rate of removal, but in the adult the number of receptors at each endplate is constant and thus the clustered AChRs are in a metabolic steady state. The biochemical mechanisms by which receptors are added to and removed from AChR clusters at endplates are unknown. We report here experiments on newly formed ectopic endplates in rat soleus muscles that demonstrate that older receptors are found in the center of a cluster and that new receptors are added to a cluster at its periphery.

MATERIALS AND METHODS

Ectopic endplates were produced on soleus muscles in 100- to 200-g rats as described previously (18). The fibular nerve was implanted on an endplate-free region of the soleus muscle and, 2–3 wk later, the tibial nerve, which normally innervates the soleus, was transected in the thigh. Within a few days the fibular nerve formed new endplates on the soleus (1, 13, 19, 21, 29). Receptors in the soleus were labeled to 80–90% saturation by injecting a dose (10–12 μg/100 g body weight) of ^{125}I-α-bungarotoxin (^{125}I-α-BuTx), prepared as described previously (5), into the femoral artery of a rat anesthetized with ether. Under these conditions, receptors in the diaphragm were much less completely labeled and artifical respiration of the animals was not required. Because free toxin in the blood is virtually eliminated within a few hours after injection, only those receptors present on the soleus muscle fibers at the time of injection were labeled.[1]

[1] C. G. Reiness and C. B. Weinberg. Manuscript submitted for publication.

Each ectopically innervated muscle was fixed in 0.8% glutaraldehyde and 2% formaldehyde in 150 mM NaCl, 3 mM $CaCl_2$, 30 mM HEPES buffer at 4°C, and the segment of muscle under the foreign nerve (a region devoid of original endplates) was removed. To obtain single fibers, we homogenized the segment in 10–15 ml of distilled water in a VirTis homogenizer (Virtis Co., Inc., Gardiner, N. Y.) at settings 30–50 for several seconds and then at setting 20 for 10–30 s. Gelatin-coated slides containing a few drops of the fiber suspension were air-dried, dipped in a mixture of two parts Kodak NTB-2 emulsion to one part 3% glycerol at 40°C, and exposed in the dark at 4°C. The slides were then developed at 20°C and examined under dark-field illumination. Receptor clusters, which were larger than those seen in denervated soleus muscle,[2] were assumed to be at ectopic endplates, as they first appeared 2 d after denervation of implanted muscles and were seen only in the area of the muscle containing the implanted nerve.[1] The areas of endplates were measured by plotting them on a sheet of graph paper with the aid of a drawing tube, cutting out the endplates, and weighing them. The area occupied by each endplate could be determined accurately because there was at least a 10-fold difference in receptor density between the receptor cluster and the surrounding membrane. The radial distriubtion of receptors within a cluster was determined by plotting the silver grains on a sheet of polar coordinate paper with the aid of the drawing tube. Each cluster was centered by eye on the polar coordinate paper, which had been calibrated with a stage micrometer. The grains were counted in concentric 2-μm rings; in each ring the grain density was calculated by dividing the number of grains by the area of the ring (see Fig. 3).

The grain density distribution at large distances from the center of a cluster is distorted by this analysis, because endplates tend to be elliptical or irregular and only portions of them extend beyond 8–10 μm from the center. Thus the grain density in our figures fall off more rapidly at large distances than the actual density of grains within the cluster; however, the central portion of interest is unaffected by this distortion.

RESULTS

If a foreign nerve is implanted in an endplate-free zone of adult rat soleus muscles, removal of the original innervation results in formation of ectopic endplates. Within 2 d after cutting the original nerve, clusters of AChRs can be detected near the implanted foreign nerve;[2] within 3 d functional transmission is established (25, 26). During the course of studies on the growth of these clusters and the degradation of receptors within them, we found that when the receptors in a cluster were pulse-labeled the area occupied by the labeled receptors decreased with time.

Ectopically innervated muscles were labeled in vivo with ^{125}I-α-BuTx and subsequently removed for radioautography. Clusters of receptors at ectopic endplates were located, and the area containing labeled receptors was measured. The results of these experiments are shown in Fig. 1. In muscles labeled 2 d after the original nerve was cut, there is a rapid exponential decline in the area of the cluster that contains labeled receptors. This does not reflect a decrease in the total area occupied by the cluster, which in fact increases during this period. This can be seen in Fig. 1 by comparison of the initial points on the curves for muscles labeled at different times. The shrinkage cannot be accounted for by folding of the membrane, because postsynaptic folds are not seen at ectopic endplates until the 2nd and 3rd wk after denervation (22). Thus, within AChR clusters at newly formed ectopic endplates, old receptors occupy an ever-decreasing area. We have observed a similar shrinkage of the area occupied by pulse-labeled AChRs within clusters at developing neuromuscular junctions in embryonic rat diaphragms (our unpublished experiments).

At endplates labeled later than 2 d after denervation, the area occupied by labeled receptors shrinks more slowly (Fig. 1). By 17 d after denervation, as at adult endplates in normal muscles, the area occupied by labeled receptors does not decrease at all but remains constant; however, this may be misleading because adult endplates have a complex structure composed of many receptor clusters (2), and a decrease in the area of each one of these would not significantly change the overall pattern of labeling observed over the entire endplate.

During the time in which the area occupied by labeled receptors is shrinking, the density of the labeled receptors decreases slightly (5–10%/d for all cases, data not shown). Thus the shrinkage is not accompanied by an increase in receptor density that might have been expected if the receptors moved centripetally but were not removed. Because the density of labeled receptors changes only slightly after labeling, and at similar rates for ectopic endplates labeled at different times, the changes in the rate of shrinkage parallel the changes in degradation rate that we have described previously.[1]

That older receptors occupy a steadily decreasing area of developing endplates suggests that new receptors may be preferentially localized at the periphery of the clusters. To determine where newly added receptors are found, we blocked AChRs in ectopically innervated muscles with unlabeled α-BuTx and, 1–2 d later, reacted them with ^{125}I-α-BuTx. Because the cold toxin bound to virtually all the AChRs in the muscle and because the dissociation of toxin from receptor is very slow ($t_{1/2} \geq 2$ wk) (20, 30), this procedure labeled principally the receptors added to the cluster after the initial reaction with cold toxin. Most of the ectopic endplates in radioautographs of fibers from such muscles appeared as annuli or "doughnuts," whereas most ectopic endplates from control muscles that had not been pretreated with cold toxin appeared to have labeled receptors throughout the cluster: only a few of them could be described as annuli. Several examples of ectopic endplates from experimental and control muscles are shown in Fig. 2. The resolution of radioautographs with ^{125}I is at best 1 μm (31). Thus some of the grains in the central regions of clusters in the experimental muscles are produced by labeled receptors at the periphery and the inhomogeneity in the actual receptor distribution is probably even greater than is seen in these experiments (Fig. 2).

To obtain a more quantitative description of the distribution

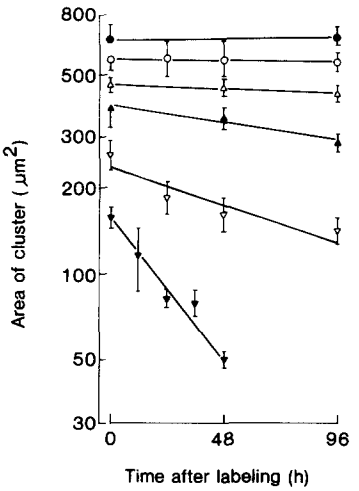

FIGURE 1 Decrease in area occupied by old AChRs. The area containing labeled AChRs within a cluster is shown as a function of the time after labeling the AChRs at ectopic endplates with ^{125}I-α-BuTx. AChRs were labeled at various times after denervation: ▼, 2 d; ▽, 4 d; ▲, 6 d; △, 17 d; ●, 40 d; ○, normal. Values are shown as mean ± SEM (n = 4–8).

[2] C. B. Weinberg, J. R. Sanes, and Z. W. Hall. Manuscript submitted for publication.

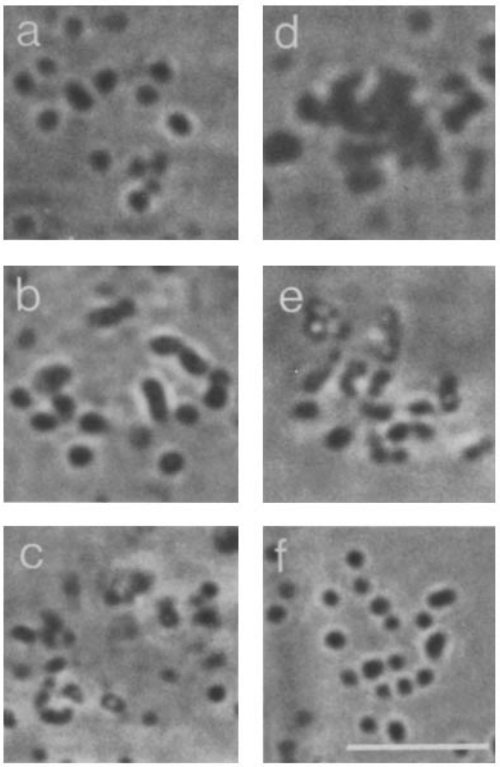

FIGURE 2 Localization of new AChRs in growing clusters. Radioautographs of ectopic endplates labeled with ^{125}I-α-BuTx 6 d after cutting the original nerve: AChRs on experimental muscles (*a–c*) had been blocked with unlabeled α-BuTx 2 d before labeling, whereas control muscles (*d–f*) were not pretreated with cold toxin. (Because the clusters do not lie in one plane of focus, many grains are distorted or difficult to see at high magnification.) Bar, 15 μm. × 1,250.

of grains at ectopic endplates from experimental and control muscles, we projected the endplates onto polar coordinate paper and determined the radial distribution of grains. This procedure is illustrated in Fig. 3 for two of the clusters shown in Fig. 2 (*b* and *e*). We analyzed all of the ectopic endplates that could be seen *en face* in a set of three experimental muscles (15 endplates) and two control muscles (14 endplates) and averaged the distributions found in each case. The average grain density distributions obtained are shown in Fig. 4. This analysis confirmed our visual impression: in endplates from control muscles, the silver grains were distributed throughout the clusters, whereas in the experimental muscles the grains were located almost exclusively at the periphery of clusters. Thus, newly added AChRs are preferentially localized at the periphery of developing endplates.

DISCUSSION

The results that we present here show that in developing ectopic endplates there is a topographical segregation of new and old AChRs within clusters. New receptors are located at the cluster periphery, whereas older ones occupy a continually decreasing area in the center. These results would not have been observed if receptors were freely exchangeable between the center and the periphery of a cluster. Thus the mobility of receptors within a cluster is severely restricted. A similar conclusion was reached by Axelrod et al. (4) for receptors within clusters on uninnervated myotubes in primary cell culture.

Our results show that new receptors are added to growing endplates at the periphery of the AChR clusters. Addition of receptors could occur either by insertion into the surface membrane at the periphery or by recruitment of receptors from extrajunctional regions (12) where AChRs, unlike those in clusters, are freely diffusible (4). Experiments showing that ingrowing neurons can induce preexisting receptors on the myotube surface to cluster at nerve-muscle contacts (3) are consistent with the latter idea. In adult frogs and in both adult and embryonic rats, there is a relatively high extrajunctional receptor density in the vicinity of endplates (11, 15, 27–29), and it is attractive to suppose that these perijunctional receptors might represent newly added AChRs before their accumulation at endplates.

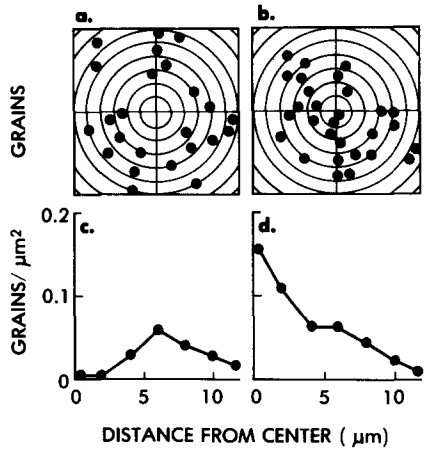

FIGURE 3 Analysis of radial grain density distribution. Polar coordinate projections of radioautographs of ectopic endplates were made with the aid of a drawing tube. Endplates are shown from (*a*) an experimental muscle (Fig. 2 *b*) and (*b*) a control muscle (Fig. 2 *e*). Each radial division is 2 μm. (Grains can be located more precisely on such projections than from photographs, because the observer can adjust the plane of focus during plotting.) Grain densities were determined for each 2-μm ring by dividing the number of grains in the ring by its area. Grain density distributions for the plots in *a* and *b* are shown in *c* and *d*, respectively.

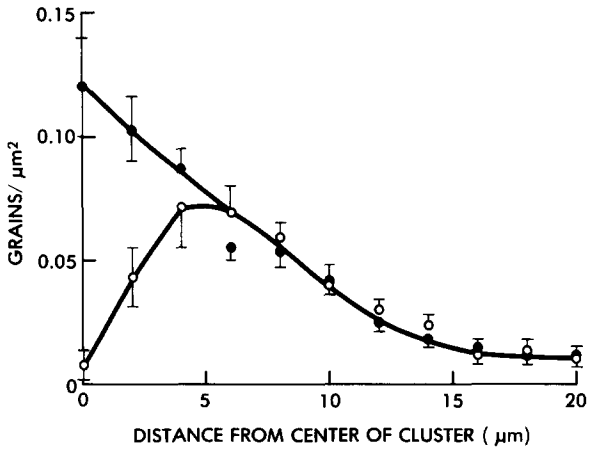

FIGURE 4 Radial grain density distributions for ectopic endplates. Radial grain density distributions were averaged for all the ectopic endplates that could be found in (○) three experimental muscles (n = 15) and in (●) two control muscles (n = 14). Values are shown as mean ± SEM.

In the course of metabolic turnover, AChRs are continually being removed from clusters. Recent experiments on myotubes in cell culture have shown that unclustered receptors may be removed by coated vesicles, which then fuse with lysosomes where the receptors are degraded (10, 23). The mechanism by which AChRs are removed from the receptor clusters at endplates, however, is not known. If removal of AChRs from synaptic receptor clusters also takes place by endocytosis of small patches of receptor-containing membrane ($<0.1\ \mu m^2$ for 500- to 600-Å vesicles), then the surrounding membrane would be pinched together as each patch is removed, causing the area occupied by the remaining receptors to shrink. Random removal of patches throughout a prelabeled cluster would cause an exponential decline in the area occupied by the labeled receptors, because a constant fraction of the labeled membrane would be removed per unit of time.

Two other possible explanations for our results should be considered. The first is localized removal of receptors at the center of each cluster by endocytosis. Such a process would cause the area occupied by pulse-labeled receptors to shrink, but a constant rate of removal would produce a linear decrease in area rather than the exponential decrease that we observe. A second possibility is that removal of receptors occurs throughout the cluster but is much more rapid in a peripheral zone than at the center. This model, however, does not explain the continued progressive decline in area occupied by pulse-labeled receptors, unless there were a continuously graded decrease in turnover time from the periphery to the center. In other experiments, we have shown that in 2-d ectopic endplates at least 75% of the receptor population turns over with a single $t_{1/2}$ of 24 h.[1] This model thus does not adequately explain the results at very young endplates where receptor turnover is rapid.

In summary we conclude that at developing ectopic endplates in adult rats, receptors are added to clusters at their periphery and remain segregated within the cluster by age. Our results are most easily explained by a model of receptor removal by endocytosis of membrane at sites distributed randomly throughout the cluster.

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REFERENCES

1. Aitken, J. T. 1950. Growth of nerve implants in voluntary muscle. *J. Anat.* 84:38–49.
2. Anderson, M. J., and M. W. Cohen. 1974. Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J. Physiol. (Lond.).* 237:385–400.
3. Anderson, M. J., M. W. Cohen, and E. Zorychta. 1977. Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.).* 268: 731–756.
4. Axelrod, D., P. Ravdin, D. E. Koppel, J. Schlessinger, W. W. Webb, E. L. Elson, and T. Podleski. 1976. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle fibers. *Proc. Natl. Acad. Sci. U. S. A.* 73:4594–4598.
5. Berg, D. K., R. B. Kelly, P. B. Sargent, P. Williamson, and Z. W. Hall. 1972. Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. Natl. Acad. Sci. U. S. A.* 69:147–151.
6. Bevan, S., and J. H. Steinbach. 1977. The distribution of α-bungarotoxin binding sites on mammalian skeletal muscle developing *in vivo*. *J. Physiol. (Lond.).* 267:195–213.
7. Burden, S. 1977. Development of the neuromuscular junction in the chick embryo: the number, distribution, and stability of acetylcholine receptors. *Dev. Biol.* 57:317–329.
8. Burden, S. 1977. Acetylcholine receptors at the neuromuscular junction: developmental change in receptor turnover. *Dev. Biol.* 61:79–85.
9. Chow, I., and M. W. Cohen. 1978. Distribution of acetylcholine receptors in the myotomes of *Xenopus laevis* during normal development. *Soc. Neurosci. Abstr.* 4:368.
10. Devreotes, P. N., and D. M. Fambrough. 1976. Turnover of acetylcholine receptors in skeletal muscle. *Cold Spring Harbor Symp. Quant. Biol.* 40:237–251.
11. Dreyer, F., and K. Peper. 1974. The spread of acetylcholine sensitivity after denervation of frog skeletal muscle fibres. *Pfluegers Arch. Eur. J. Physiol.* 348:287–292.
12. Edwards, C., and H. L. Frisch. 1976. A model for the localization of acetylcholine receptors at the muscle endplate. *J. Neurobiol.* 7:377–381.
13. Elsberg, C. A. 1917. Experiments on motor nerve regeneration and the direct neurotization of paralyzed muscles by their own and foreign nerves. *Science (Wash. D. C.).* 45:318–320.
14. Fambrough, D. M. 1974. Acetylcholine receptors: revised estimates of extrajunctional receptor density in denervated rat diaphragm. *J. Gen. Physiol.* 64:468–472.
15. Feltz, A., and A. Mallart. 1971. An analysis of acetylcholine responses of junctional and extrajunctional receptors of frog muscle fibres. With an appendix by R. Kahn and A. Le Yaouanc. *J. Physiol. (Lond.).* 218:85–100.
16. Fertuck, H. C., and M. M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-α-bungarotoxin binding at mouse neuromuscular junctions. *J. Cell Biol.* 69:144–158.
17. Frank, E., and G. D. Fischbach. 1979. Early events in neuromuscular junction formation in vitro. *J. Cell Biol.* 83:143–158.
18. Frank, E., J. K. S. Jansen, T. Lømo, and R. H. Westgaard. 1975. The interaction between foreign and original motor nerves innervating the soleus muscle of rats. *J. Physiol. (Lond.).* 247:725–743.
19. Guth, L., and A. A. Zalewski. 1963. Disposition of cholinesterase following implantation of nerve into innervated and denervated muscle. *Exp. Neurol.* 7:316–326.
20. Heinemann, S., J. Merlie, and J. Lindstrom. 1978. Modulation of the acetylcholine receptor in rat diaphragm by anti-receptor sera. *Nature (Lond.).* 274:65–68.
21. Jansen, J. K. S., T. Lømo, K. Nicolaysen, and R. H. Westgaard. 1973. Hyperinnervation of skeletal muscle fibers: dependence on muscle activity. *Science (Wash. D. C.).* 181:559–561.
22. Korneliussen, H., and H. Sommerschild. 1976. Ultrastructure of the new neuromuscular junctions formed during reinnervation of rat soleus muscle by a "foreign" nerve. *Cell Tiss. Res.* 167:439–452.
23. Libby, P., S. Bursztajn, and A. L. Goldberg. 1980. Degradation of the acetylcholine receptor in cultured muscle cells: selective inhibitors and the fate of undegraded receptors. *Cell.* 19:481–491.
24. Linden, D. C., and D. M. Fambrough. 1979. Biosynthesis and degradation of acetylcholine receptors in rat skeletal muscles. Effects of electrical stimulation. *Neuroscience.* 4:527–538.
25. Lømo, T., and C. R. Slater. 1976. Control of neuromuscular synapse formation. *In* Synaptogenesis. L. Tauc, editor. Naturalia & Biologia, Jouy en Josas, France. 9–30.
26. Lømo, T., and C. R. Slater. 1978. Control of acetylcholine sensitivity and synapse formation by muscle activity. *J. Physiol. (Lond.).* 275:391–402.
27. Miledi, R. 1960. The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol. (Lond.).* 151:1–23.
28. Miledi, R. 1960. Junctional and extra-junctional acetylcholine receptors in skeletal muscle fibres. *J. Physiol. (Lond.).* 151:24–30.
29. Miledi, R. 1963. Formation of extra nerve-muscle junctions in innervated muscle. *Nature (Lond.).* 199:1191–1192.
30. Reiness, C. G., C. B. Weinberg, and Z. W. Hall. 1978. Antibody to acetylcholine receptor increases degradation of junctional and extrajunctional receptors in adult muscle. *Nature (Lond.).* 274:68–70.
31. Rogers, A. W. 1967. Techniques of Autoradiography. Elsevier/North Holland Biomedical Press, Amsterdam. 53–57.