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Degradation of Two AChR Populations at Rat Neuromuscular Junctions: Regulation *in vivo* by Electrical Stimulation

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The effect of electrical stimulation on the stability of junctional ACh receptors (AChR) on soleus muscles of Wistar rats was compared to that of denervation and reinnervation. Denervation causes the degradation rate of the slowly degrading AChRs (R_s) at the neuromuscular junction to accelerate and be replaced by rapidly degrading AChRs (R_r), while reinnervation restabilizes the accelerated R_s. Electrical stimulation initiated at the time of denervation prevented the acceleration of the R_s. It could not, however, reverse the effect of denervation if initiated after the AChRs became destabilized, nor could it slow the degradation rate of the R_r. We conclude that electrical stimulation of denervated muscle downregulates the expression of the R_r and prevents the destabilization of R_s.

[Key words: ACh receptor, degradation, electrical stimulation, neuromuscular junction, denervation, muscle]

Studies on regulation of ACh receptor (AChR) stability in mouse muscle have shown that the neuromuscular junction can contain two types of AChRs with respect to metabolic stability: a slowly degrading population (R_s), present at mature innervated junctions ($t_{½} \sim 10$ d); and a rapidly degrading population (R_r), predominant at denervated end plates ($t_{½} \sim 1$ d) (reviewed in Salpeter and Loring, 1985; Salpeter, 1987b). Following denervation, the preexisting R_s loses some of its stability in the postsynaptic membrane, acquires an intermediate half-life of 2–4 d (Levitt and Salpeter, 1981; Stanley and Drachman, 1981; Brett et al ., 1982; Bevan and Steinbach, 1983) and, as it degrades, is replaced by R_r, still maintaining a constant site density at the neuromuscular junction (Shyng and Salpeter, 1989). The degradation rates of the two junctional AChR populations are regulated differently by the nerve. Following reinnervation of a denervated muscle, any original R_s still remaining in the membrane regain their metabolic stability (Salpeter et al., 1986), while the degradation rate of any R_r in this membrane is unaffected (Shyng and Salpeter, 1990). Since the long-term effect of reinnervation is complete stabilization of AChRs at the end plate, it appears that innervation, in addition to stabilizing R_s, inhibits expression of new R_r.

Since innervation leads to both muscle activity and release of trophic factors, the relative role of these two responses in regulating AChR stabilization is of great interest. Muscle activity has been shown to regulate AChR degradation (Avila et al., 1989; Brenner and Rudin, 1989; Fumagalli et al., 1990; Rotzler et al., 1991). Long-term inactivity of innervated muscle caused by a TTX cuff on the nerve (Fumagalli et al., 1990) or other paralytic agents (Avila et al., 1989) was shown to be equivalent to denervation in inducing acceleration of receptor degradation, whereas long-term (15 d) electrical stimulation of denervated muscles caused the appearance of stable AChRs (Fumagalli et al., 1990).

To obtain more insights into the mechanisms by which the metabolic stability of junctional AChRs is controlled and the possible role played by muscle activity, we have used rat soleus muscles to study the effects of electrical stimulation *in vivo* on degradation of R_r and R_s at the neuromuscular junction. This study did not address the degradation of extrajunctional AChRs appearing after denervation. Our results indicate that neither R_r nor the accelerated form of R_s can be stabilized by electrical muscle stimulation. However, muscle stimulation initiated at time of denervation prevented R_s acceleration. The findings support a model in which muscle activity suppresses the expression of R_r and inhibits the mechanism responsible for R_s acceleration.

Materials and Methods

The experiments were done on male Wistar rats from Møllegaards Breeding Centre Ltd. (Skensved, Denmark) and, in one series of denervations, on female Sprague–Dawley rats from Camm Research (Wayne, NJ). The Wistar rats were all about 300 gm except in one series of denervation experiments when they were about 100 gm, as were the Sprague–Dawley rats. Surgical operations were performed under Equithesin (Rikshospitalet, Oslo, Norway) anesthesia (0.4 ml/100 gm, i.p.). Soleus muscles were chronically denervated by cutting, ligating, and deflecting the sciatic nerve in the thigh. In the reinnervation experiments the axons to the soleus muscle were crushed once just distal to the point where the axons to the soleus and the lateral gastrocnemius muscle branch from the tibial nerve in the fossa poplitea. In the muscle stimulation experiments electrodes were implanted on each side of the denervated soleus muscles and connected to external stimulators as previously described (Westgaard and Lømo, 1988). The stimulation patterns were (1) 60 pulses at 100 Hz every 60 sec, (2) 200 pulses at 20 Hz every 15 sec, or (3) 100 pulses at 100 Hz every 100 sec (Rotzler et al., 1991). For the AChR degradation studies, soleus muscles were injected with 30 μl (100 gm rats) or 50 μl (300 gm rats) of 1 μM ^{125}I-α-bungarotoxin (^{125}I-BTX) (>200 Ci/mmol; Amersham) either just before denervation

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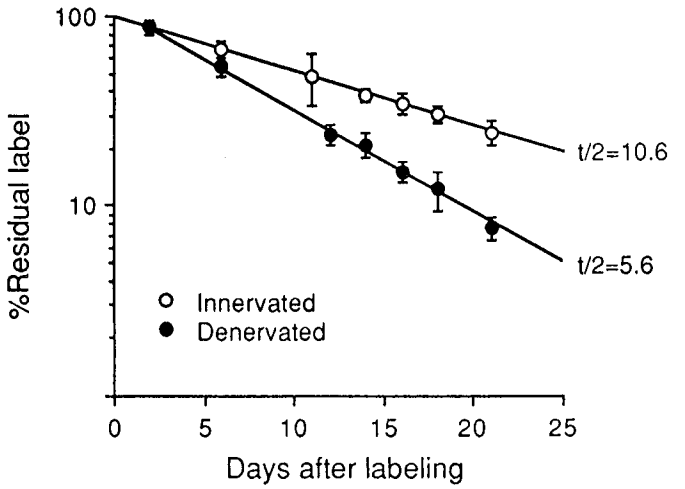

Figure 1. The degradation rate of the R_s accelerates after denervation. R_s receptors were labeled by injection with ^{125}I-BTX at the time of denervation. End plate–specific radioactivity was measured by gamma counting as described in Materials and Methods and expressed as residual label. The control (o) data were first fitted by linear regression assuming a single exponential. The value extrapolated at day 0 was then set to 100% and used to normalize the data on the assumption (affirmed experimentally) that all muscles had the same label at the time of the radioactive toxin injection. The data points on day 0 in this and Figures 3 and 4 are thus extrapolated and not experimental values. Half-life values were obtained by linear regression and are shown at the end of the curve. The regression line of the denervated muscles (●) starts to diverge from normal (O) 2–5 d after denervation. Values are means ± SD obtained from three to eight animals at each time point.

for the studies on R_s, or 10–25 d after denervation for the studies on R_r (see Results and the figure captions for more detailed descriptions of the protocols).

At different times after the injections of ^{125}I-BTX, three or four animals per time point were anesthetized and perfused through the aorta with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Muscles were then removed and kept in the same fixative solution for 2 hr. After staining for acetylcholinesterase, end plate–specific radioactivity was determined either per fiber (Fumagalli et al., 1990) or per muscle (Salpeter et al., 1986). Briefly, the first method consisted of teasing out thin bundles of muscle fibers (stained for cholinesterase), cutting the bundles into end plate–containing and end plate–free segments, and then gamma counting their radioactivity as well as counting the number of end plates to obtain the specific activity per end plate. The second method consisted of cutting the whole muscle (stained for cholinesterase) into end plate–containing and end plate–free parts, gamma counting the radioactivity of the end plate–containing part, and correcting for the activity of the end plate–free parts on a per weight basis. The degradation rates of the labeled junctional AChR were then calculated by the rate of loss of the end plate–specific radioactivity. Since both methods gave comparable results, the particular method used in each experiment is not further indicated. The gamma counter (Cobra 500, Packard Instruments, Meriden, CT) automatically corrects for the decay rate of the ^{125}I. No correction was included for unbinding of the BTX.

The experiments involving chronic muscle stimulation and local application of paralytic toxins have been inspected and permitted by the Norwegian Experimental Board and Ethical Committee for Animal Experiments and were overseen by the veterinarian responsible for the animal house. During the experiments the animals did not appear to suffer pain.

Results

We first determined how the basic characteristics of AChR degradation in rat soleus muscle compare to those previously established for several mouse muscles.

The degradation rate of rat soleus R_s accelerates after denervation

In one series of experiments ^{125}I-BTX was injected into both soleus muscles of 300 gm male Wistar rats and the sciatic nerve on the right side was cut. The soleus muscles were removed 2–18 d thereafter and end plate–specific radioactivity determined (see Materials and Methods). The degradation rate of the slowly degrading AChRs (R_s) present at the junction before denervation was 10.6 ± 1.4 d (±SD; range, 9.4–12.6 d; data from 4 experiments, 3–5 time points per experiment, 3 or 4 animals per time point). About 2 d after denervation the R_s accelerated to a half-life of 5.8 ± 0.7 d (±SD; range, 5.0–6.8 d; data from 6 separate experiments, 3–5 time points per experiment, 3 or 4 animals per time point) (Fig. 1).

Similar results were obtained in 5-week-old 100 gm rats (data not shown). At denervated and innervated end plates of these young rats the mean half-life of the innervated and accelerated R_s was 10.3 d and 5.0 d, respectively (data from one experiment, four time points, four animals per time point). The acceleration again occurred about 2 d after denervation, as in the older rats, but the number of binding sites per end plate was less than half the corresponding values in the older rats, indicating that the kinetic behavior of R_s was well established even before end plates had reached their full size.

These results show that the R_s accelerate earlier but reach a slower degradation rate after denervation in male Wistar rats than in most mouse muscles (Levitt and Salpeter, 1981; Stanley and Drachman, 1981; Bevan and Steinbach, 1983; Wetzel and Salpeter, 1991). To establish whether this difference is related only to a species difference, we performed similar experiments using female Sprague–Dawley rats. The denervation caused the R_s to accelerate from a half-life of about 7 d to about 3.8 d by 7–9 d after denervation (data not shown). These values are closer to those reported for the mouse. Thus, the time of acceleration and the degradation rate of R_s vary between animal strains and, as reported previously, even between different muscles (Bevan and Steinbach, 1983; Wetzel and Salpeter, 1991). The overall response of R_s to denervation, however, is very similar in all rodent muscles tested to date. All subsequent experiments were performed only on the 300 gm Wistar rats.

Innervation stabilizes rat soleus R_s

For studies on the effect of reinnervation on R_s degradation, the axons to the soleus muscle were crushed immediately after they branch off from the tibial nerve in the fossa poplitea. The time course of reinnervation was determined in a separate series of experiments in which the soleus muscles with the nerve attached were removed from the animal 10–17 d after the crush and placed in a perfusion chamber. We then examined 30–50 surface fibers with intracellular electrodes for evoked endplate and/or action potentials in response to stimulation of the soleus nerve. No signs of innervation could be detected until day 10 after the crush. On day 12, end-plate potentials and/or action potentials were evoked in about 40% of the fibers, and on day 17, the reinnervation was nearly complete (>90%).

The effect of reinnervation on the stability of R_s was then determined by injecting ^{125}I-BTX into the soleus muscles 2 hr before the nerve was crushed and removing muscles at various time points after nerve regeneration, that is, between days 18 and 36 after the crush. Control animals had the nerve cut, ligated, and deflected in the thigh to prevent reinnervation. The

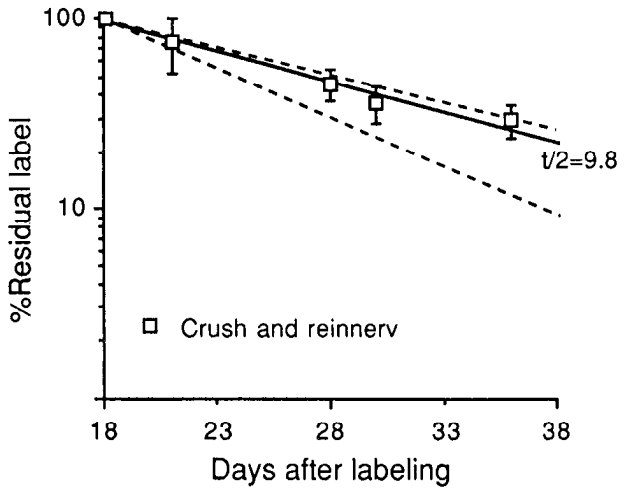

Figure 2. Reinnervation restores stability of the R_s. Muscles were labeled at time of denervation by nerve crush. Residual end plate–specific label (%) for all groups was normalized to their value obtained experimentally on day 18 after the crush, when more than 90% of the end plates were reinnervated (as judged by the presence of nerve-evoked end-plate or action potentials in the muscle fibers). Values are mean ± SD obtained from three to five animals at each time point. *Dashed lines* give the degradation curves for the R_s in innervated and denervated control end plates (see Fig. 1).

data, normalized to the value obtained at day 18 for each experimental group (Fig. 2), demonstrate that, just as in the mouse (Salpeter et al., 1986), reinnervation induces stabilization of the postdenervation accelerated R_s ($t_{1/2}$ of R_s at reinnervated muscles = 9.8 d; data from 22 rats).

Electrical stimulation prevents acceleration of R_s

Once we had established that the basic characteristics of AChR degradation in rat soleus muscle resembled that of mouse muscle, we examined the effect of electrical muscle activity on this system. R_s degradation rate was measured in muscles that were stimulated (60 pulses at 100 Hz every 60 sec) from the time of denervation until they were removed. No difference was observed between the denervated plus stimulated muscle in one leg and the innervated muscle in the opposite leg (Fig. 3*A*). These results indicate that R_s acceleration does not occur if muscles are kept active from the time of denervation (3 time points, 10 rats total).

Electrical stimulation does not stabilize accelerated R_s

To determine the effect of muscle activity on the degradation rate of accelerated R_s, electrical stimulation started 10 d after denervation and continued until the muscles were removed. The stimulation had no effect on the degradation rate of the accelerated R_s (Fig. 3*B*). The half-life was 5.8 ± 0.3 d (±SD; average from 2 experiments, 3 time points per experiment, 3 or 4 animals per time point) and was thus the same as the half-life of accelerated R_s in denervated, nonstimulated muscles, as given in Figure 1.

Effect of electrical stimulation on R_r degradation rate

Both sciatic nerves were cut, ligated, and deflected in the thigh to avoid regeneration. Twenty-five days later, when most of the R_s had degraded and almost all of the receptors at the neuromuscular junction were of the rapidly degrading (R_r) type, radioactive toxin was injected into both muscles. At the same

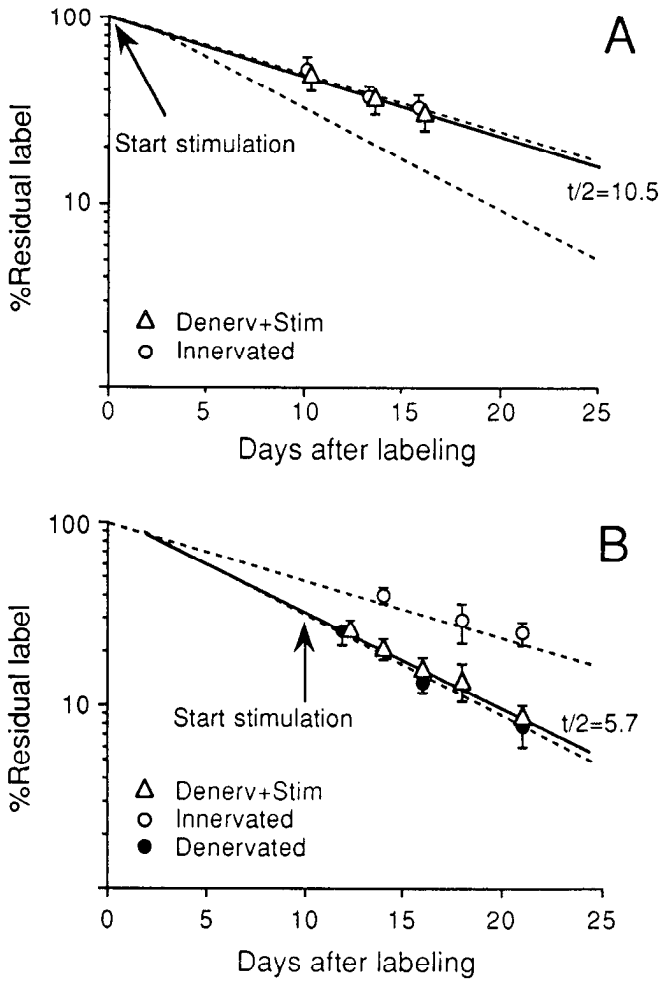

Figure 3. Electrical stimulation prevents acceleration of the R_s but does not restabilize the accelerated R_s. Muscles were injected with ^{125}I-BTX before denervation. The muscles in the opposite control leg were either intact (○) or denervated (●). The experimental muscles (△) were electrically stimulated with 60 pulses at 100 Hz every 60 sec, the stimulation starting either <1 hr after denervation (*A*), or 10 d later (*B*). In *A* the degradation rate is comparable to that in the intact control muscles (*upper dashed line*), and in *B*, to that in the denervated control muscles (*lower dashed line*).

time electrodes were implanted and the soleus in the right leg stimulated until both soleus muscles were removed. The stimulation pattern was either 60 pulses at 100 Hz every 60 sec (Fumagalli et al., 1990) or 200 pulses at 20 Hz every 15 sec. These patterns bear some resemblance to the natural motor unit patterns in fast and slow rat muscles (Hennig and Lømo, 1985; Eken and Gundersen, 1988). Figure 4, *A* and *B*, shows that control R_r had a half-life of 2–3 d (somewhat longer than the corresponding values in mouse muscle; Shyng and Salpeter, 1989; Shyng et al., 1991) and that neither stimulation pattern affected the degradation rate of the R_r.

In the two experiments just described, the stimulation pattern was started long after denervation, when the muscle was considerably atrophied and the number of junctional AChRs was considerably reduced (Frank et al., 1975; Steinbach, 1981; Fumagalli et al., 1990). Therefore, in a third experiment we started the stimulation much earlier, when fibers were less atrophic and the number of junctional AChRs was still normal (Frank et al., 1975; Loring and Salpeter, 1980; Fumagalli et al., 1990). To avoid contaminating our labeled receptors with not yet degraded

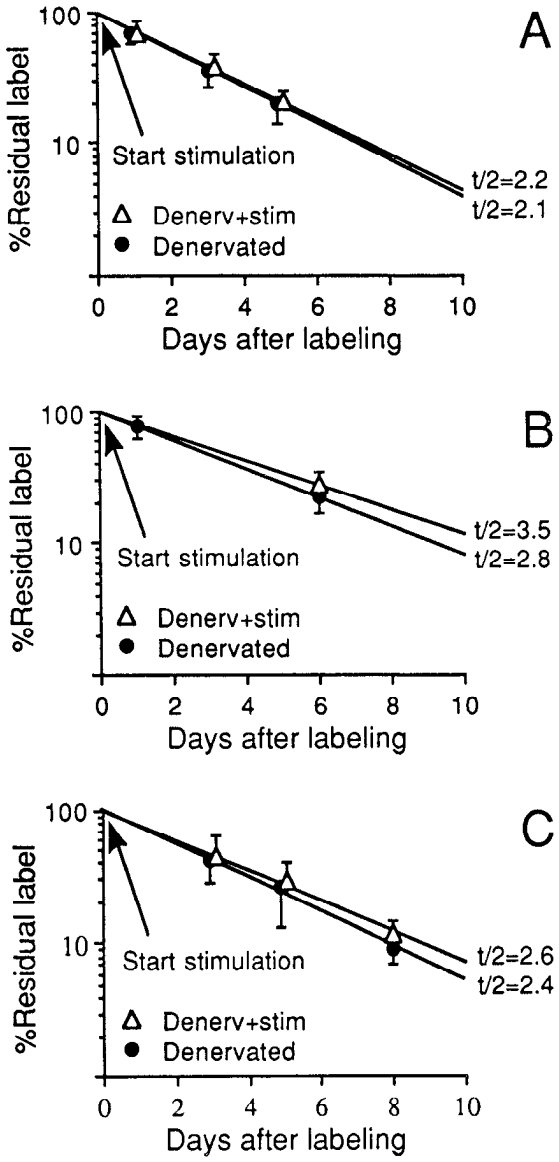

Figure 4. Electrical stimulation does not stabilize the R_r. Soleus muscles in the right and left legs were denervated and injected with ^{125}I-BTX either 25 d (*A* and *B*) or 10 d (*C*) later. Electrical stimulation started (as marked by the *arrow*) <1 hr after the injection. The values are means ± SD from at least three muscles per time point and represent percentage of end plate–specific radioactivity remaining, normalized to 100% on the day of labeling. The half-lives were obtained by linear regression and are shown at the end of the curve for denervated–stimulated (△) and denervated–nonstimulated (●) muscles, respectively. *A*, Twenty-five day denervated muscles were injected with ^{125}I-BTX and stimulated with 60 pulses at 100 Hz every 60 sec. *B*, Same as *A* but the stimulation pattern was 200 pulses at 20 Hz every 15 sec. *C*, Soleus muscles of both legs were denervated and 4 d later were injected with nonradioactive BTX followed 6 d later with ^{125}I-BTX. The soleus on the right side was then stimulated with 100 pulses at 100 Hz every 100 sec while the contralateral muscle acted as a nonstimulated control.

R_s, we inactivated such residual R_s by injecting a saturating dose of nonradioactive α-BTX 4 d after denervation. Six days later, ^{125}I-BTX was injected to label only newly inserted R_r, and muscle stimulation was started at that time. In this case, the stimulation pattern was identical to that used by Rotzler et al. (1991) (100 pulses at 100 Hz every 100 sec). The soleus muscles were removed 3, 5, and 8 d later. Again, the stimulation had no effect on the stability of R_r (Fig. 4*C*).

Since the half-life values were not affected by stimulation in any of the different protocols, we conclude that the degradation rate of the R_r is insensitive to modulation by muscle activity.

Discussion

The present study examines some of the mechanisms controlling the stability of AChR degradation rate at Wistar rat soleus neuromuscular junctions. The major results are that (1) denervation causes an acceleration of R_r degradation and a replacement of R_s by R_r, as previously described for mouse muscles; (2) reinnervation stabilizes the accelerated R_s; (3) electrical stimulation starting at the time of denervation keeps R_s metabolically stable; and (4) electrical muscle stimulation starting after R_s has accelerated does not restabilize R_s, nor does it stabilize R_r.

Denervation

The changes in metabolic properties of the junctional AChR after denervation have previously been described in detail in several mouse muscles (Loring and Salpeter, 1980; Stanley and Drachman, 1981; Levitt and Salpeter, 1981; Brett et al., 1982; Bevan and Steinbach, 1983; Salpeter et al., 1986; Avila et al., 1989; Shyng and Salpeter, 1990). Here we show that denervation of rat soleus muscle similarly accelerates the degradation rate of R_s and causes R_r to replace R_s at the neuromuscular junction. We found, however, that the exact time course of acceleration and the AChR degradation values in the muscles studied here were somewhat different from those reported previously. In the soleus muscle of male Wistar rat the estimated $t_{1/2}$ values for the R_r and accelerated form of R_s are 2–3 d and 5–6 d, respectively. These values are about twice as long as the corresponding values in mouse muscles and in the female Sprague–Dawley rat soleus muscle. However, even in mouse muscles there is a sizable range of R_s values (e.g., Bevan and Steinbach, 1983; Shyng and Salpeter, 1990; Wetzel and Salpeter, 1991). Furthermore, the larger $t_{1/2}$ for the R_r receptors reported here could be due in part to the inclusion of the small (10%) slow component synthesized in denervated muscle (Shyng and Salpeter, 1990). In general, many properties of the neuromuscular junction differ in detail among muscles, for example, the number of functional AChRs (Matthews-Bellinger and Salpeter, 1983; Sterz et al., 1983), end-plate size (Fahim et al., 1984; Salpeter, 1987a; Wärhaug, 1992), morphological stability (Lichtman et al., 1987; Wigston, 1989), and interval between time of denervation and onset of R_s acceleration. However, the overall response of junctional AChRs to denervation is strikingly similar among different muscles in different species.

Muscle activity and R_s

The fact that reinnervation stabilized the R_s motivated us to ask what role muscle activity might play in mediating this effect. Our finding that direct stimulation from the time of denervation prevented acceleration of R_s is not surprising since the opposite event (i.e., muscle paralysis) has been shown to accelerate R_s (Avila et al., 1989). In addition, it was previously shown that block of nerve conduction by TTX for 18 d causes the same drop in junctional AChR half-life (from 12 d to 3 d) as does denervation (Fumagalli et al., 1990). The surprising finding in the present work was that direct electrical stimulation, in contrast to reinnervation, failed to restore normal stability to accelerated R_s.

It may appear paradoxical that muscle activity can prevent acceleration of junctional R_s (present study) and induce expression of new stable R_s (as seen in experiments where AChRs are labeled only after periods of electrical stimulation, i.e., Brenner and Rudin, 1989; Fumagalli et al., 1990) but not restabilize old R_s once their degradation has accelerated (present study). Our results show that muscle activity cannot fully mimic neural innervation in regulating R_s degradation, and suggest that restabilization of R_s may require a neurogenic trophic factor independent of muscle activity. At present the nature of such a neurotrophic factor(s) is not known. In nerve-free organ culture, restabilization of R_s occurs in the presence of increased levels of cAMP (Shyng et al., 1991). It would be of interest to determine whether cAMP is also involved in the stabilization of R_s during normal development *in vivo*, and whether cAMP and muscle activity interact in this process.

Muscle activity and R_r

Our finding that metabolic stability of R_r at denervated end plates is unaffected by direct stimulation of the muscle is inconsistent with the finding of Rotzler et al. (1991), who reported that less than 24 hr of muscle stimulation is sufficient to stabilize the junctional AChRs in rat soleus muscles denervated for 18–22 d. Although the Rotzler et al. (1991) study did not distinguish between R_s and R_r, the majority of the AChRs after about 20 d of denervation must have been R_r. The rapid changes in half-life reported by these authors strongly suggest that all the receptors present at time of labeling (the R_r and the new receptors inserted during the stimulation period) were stabilized by activity. At present, the reasons for the discrepancy with the results of Rotzler et al. (1991) are unclear. We can only point to differences in technique. Rotzler et al. determined the degradation rate of AChRs *in vitro* over a period that was short relative to the extrapolated half-life, whereas we counted the radioactivity for a considerably longer period in muscles that had been maintained *in vivo*. Yet in our experiments we could not stabilize the R_r with any of three stimulation patterns used, including a pattern identical to that used by Rotzler et al. (1991). Furthermore, electrical stimulation failed to stabilize R_r both at a time when the number of AChRs at the denervated end plate was constant (up to 18 d after denervation) and at a time when the number of AChRs declined (18–33 d of denervation; see Fumagalli et al., 1990), suggesting that our inability to change the degradation rate of R_r was not due to the length of the denervation period. We therefore conclude that the degradation rate of R_r at denervated neuromuscular junction is independent of muscle activity. This conclusion was also drawn for R_r on muscle cells in culture (see Salpeter et al., 1993).

A previous study had shown that muscle stimulation induces the appearance of slowly degrading R_s ($t_{1/2} \sim 13$ d) at long-term denervated end plates (Fumagalli et al., 1990). In that study there was no sign of R_r after 15 d of stimulation. Since our present study showed that neither the R_r nor the accelerated R_s present in the membrane at the time of labeling could be stabilized by electrical stimulation, we conclude that the stabilization of AChRs at the neuromuscular junction, as seen, for instance, by Fumagalli et al. (1990), involves the degradation of R_r molecules during the period of electrical stimulation and their replacement by stable R_s. Evoked muscle activity thus apparently blocks the expression of new R_r.

The relative role of neurotrophic factors and muscle activity has been examined for many aspects of neuromuscular organization. In the present study we pursued this question in connection with establishing and maintaining slowly degrading AChRs at vertebrate neuromuscular junction. Our study builds on previous studies that R_r could not be stabilized by either innervation (Salpeter et al., 1986) or cAMP (Shyng et al., 1991). It concludes that muscle activity plays a major role in producing a stable AChR population, not by stabilizing AChRs, but by replacing rapidly degrading receptors (R_r) with slowly degrading receptors (R_s) and by maintaining the stability of inserted R_s. Trophic factors, on the other hand, possibly acting via a mechanism similar to that seen for cAMP analogs, could be involved in stabilizing any accelerated R_s. (We do not address the additional aspect raised by Ramsay et al., 1992, regarding the stability of R_s at the time of insertion.) Several problems, such as the mechanisms whereby trophic factors exert their influence and whether they interact with muscle activity in maintaining a stable AChR population, remain to be solved.

Note added in proof

After this article was submitted, Coroni et al. (1993) reported stabilization of R_r after 6 hr of stimulation at 100 pulses per train at 100 Hz but not after 4.5 hr at that stimulation pattern, or after 6 hr at 60 pulses per train at 100 Hz, all *in vitro*. Since we stimulated for much longer periods (days) and obtained no stabilization of R_r with either of the above patterns *in vivo*, we have no explanation for why these small differences between a 4.5 hr and 6 hr stimulation or between 60 and 100 pulses per train could produce such dramatic differences when assayed *in vitro*.

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