Is a Nicotinic Influence Involved in Denervation-induced Depolarization of Muscle?¹

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Abstract

The neurotrophic role of acetylcholine (ACh) in the denervation-dependent decline of muscle resting membrane potential (RMP) was evaluated. Freshly dissected rat hemidiaphragms with short or long (2 cm) nerve stumps attached ("–N" and "+N" preparations, respectively) were incubated in organ culture in the presence or absence of the nicotinic blockers, α -bungarotoxin (α -BTX) or *d*-tubocurarine (curare). Subsequently, RMPs and miniature endplate potentials (MEPPs) of the junctional region were measured.

Spontaneous MEPPs disappeared with a half-life of 12 and 20 hr in –N and +N preparations, respectively. A 10- to 15-mV depolarization of RMP was observed between 15 and 20 hr in –N muscles and between 24 and 28 hr in +N muscles. This time course of disappearance of spontaneous potentials and of membrane depolarization agrees well with that observed *in vivo*.

Although nicotinic transmission was blocked from the initiation of the incubation period in α -BTX- or curare-treated muscles, no acceleration of RMP decline in –N muscles *in vitro* was observed. Moreover, in +N preparations the effect of the nerve stump in delaying RMP depolarization persisted despite the continuous presence of α -BTX or curare.

Since excess ACh triggers a lysosomal proteolytic response at the nerve-muscle junction and since this may occur early in denervation, the possible role of a nicotinic-induced proteolytic mechanism was tested *in vitro* with the potent protease inhibitor leupeptin. This inhibitor did not delay or prevent the denervation-dependent alterations.

Thus, with respect to neuronal regulation of RMP, neither the effects of denervation nor of the long nerve stump are entirely explicable by a nicotinic mechanism, or by a leupeptin-sensitive proteolytic mechanism.

Denervated skeletal muscle undergoes numerous morphological and electrophysiological alterations (see reviews by Harris, 1974; Gutmann, 1976; Purves, 1976; Fambrough, 1979). The earliest post-denervation physiological events are the instant cessation of nerve-evoked action potential and muscle activity, followed by failure of miniature endplate potentials (MEPPs) at about 12 hr (Miledi and Slater, 1970; Card, 1977) and a decline of the muscle resting

membrane potential (RMP) at about 20 to 24 hr (Slater, 1966; Redfern and Thesleff, 1971; Card, 1977; Drachman et al., 1982), or possibly earlier (Albuquerque et al., 1971).

Although activity plays a role in neuronal regulation of various muscle properties (Drachman and Witzke, 1972; Cohen and Fishbach, 1973; Shainberg and Burstein, 1976; Hall and Reiness, 1977; Betz and Changuex, 1979), several observations have given rise to the hypothesis that a neurotrophic influence prevents or delays denervation-induced alterations, and are consistent with the suggestion that cholinergic transmission provides the above neurotrophic influence. First, both spontaneous quantal and non-quantal acetylcholine (ACh) release decline before RMP begins to depolarize (cf. references above, and Linden et al., 1983). Second, increasing the length of the nerve stump delays the denervation-dependent failure of neuromuscular transmission, the time of failure of spontaneous quantal ACh release, the loss of nerve terminal ACh and ACh synthesis (Miledi et al., 1981, 1982) and the depolarization of RMP (Miledi and Slater, 1970). The production of denervation characteristics when ACh receptors are blocked by α -bungarotoxin (α -BTX) further supports the idea of a cholinergic involvement (Drachman et al., 1982).

The objective of the present study was to evaluate the neurotrophic role of nicotinic ACh transmission in the development of the denervation-dependent depolarization of the muscle resting membrane. If spontaneous (quantal or non-quantal) or action potential-evoked ACh release by the motor nerve were the major or sole requirement for RMP maintenance, then the denervation-induced depolarization of RMP should be accelerated by the "instant denervation" induced by nicotinic blockers. An *in vivo* test of this prediction would not be decisive, since nicotinic receptor blockers are reversible and difficult to regulate *in vivo* (e.g., by *d*-tubocurarine) or are relatively short-lived in functional effect. For instance, transmission block induced by *in vivo* α -BTX lasts as little as 12 hr because the recovery of only 26% of unbound receptors is enough to support impulse transmission (Rochel and Robbins, 1984). Therefore, in order to ensure that complete and continuous block of nicotinic transmission is maintained during the critical period, an *in vitro* system, in which several denervation phenomena occur approximately "on schedule," was used. Furthermore, emphasis was placed on the first 27 hr after denervation, since in this and in previous reports, loss of ACh release as well as onset of membrane depolarization occurred within this period. In this system, the findings provided evidence against the cholinergic hypothesis.

Acetylcholine in high concentrations induces a leupeptin-sensitive proteolysis at the endplate region by a nicotinic mechanism (Leonard and Salpeter, 1979). In addition, the proteolytic inhibitor, leupeptin, was previously shown to prevent two denervation phenomena—muscle atrophy (Libby and Goldberg, 1980) and decrease of acetylcholinesterase (Fernandez and Duell, 1980; Cater and Brimjoin, 1981). Given reports of a transient increase in spontaneous ACh release just before failure of the denervated nerve terminal (e.g.,

Received June 4, 1984; Revised February 8, 1985;
Accepted March 4, 1985

¹ This work was supported by National Institutes of Health Grants NS 07118 and NS 18694 and a National Amyotrophic Lateral Sclerosis Foundation grant to S. R. We wish to thank Diana Guegel for technical help.

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Figure 1. MEPPs and RMP after *in vivo* denervation. The right branch of the left phrenic nerve was sectioned, while the left branch was kept intact as an internal sham control. MEPP failure (A) and resting potential (B) of denervated (○) and sham (□) muscles are presented as a function of time after denervation. The length of the nerve within the muscle was between 2 and 6 mm from the site of nerve section. Each data point represents the mean of 60 fibers from two or three muscles. Bars are standard error of the mean.

Figure 2. Time course of denervation-induced MEPP failure *in vitro*. Diaphragms were incubated *in vitro* as described under "Materials and Methods." The percentage of fibers with MEPPs in diaphragms with short nerve stump (●) or long nerve stump (○) is plotted as a function of time after denervation. MEPP failure showed a $t_{1/2}$ of 12 hr in -N strips and 19.5 hr in +N strips. Each data point is the average of 60 fibers from three preparations.

Figure 3. Time course of denervation-induced depolarization of RMP *in vitro*. The RMP of diaphragms with short nerve stump (●) and long nerve stump (○) are plotted as a function of time. Each data point is the average of 60 fibers from three preparations. *, Pooled data at these and later time points show RMP significantly different ($p < 0.01$) from control (fresh dissected or preceding times *in vitro*).

A. SHORT NERVE STUMP SYSTEM

B. LONG NERVE STUMP SYSTEM

Figure 4. Effect of cholinergic blockers on denervation-induced depolarization of membrane potential. α -Bungarotoxin ($0.6 \mu\text{M}$) or curare ($4 \mu\text{M}$) were present throughout the incubation period. The RMPs of diaphragms with short nerve stump (A) or long nerve stump (B) are plotted as a function of time after denervation. Solid bars, control; hatched bars, α -BTX (*) or curare (□).

Figure 5. Effect of the protease inhibitor leupeptin on RMP after denervation. Leupeptin (4 or 20 μ M) was present throughout the incubation period. The RMPs are plotted as a function of time. Solid bars represent short nerve stump; hatched bars represent short nerve stump with leupeptin. Each data point is the average of 20 fibers from one preparation.

Card, 1977; Komatsu et al., 1984), it was of interest to determine whether this type of nicotinic proteolytic mechanism played a role in denervation-induced membrane depolarization.

Materials and Methods

Materials. α -Bungarotoxin was obtained from Miami Serpentarium, and *d*-tubocurarine and leupeptin were from Sigma Chemical Co. Male Sprague-Dawley rats weighing 90 to 120 gm were obtained from Hilltop Laboratory Animals, Inc.

In vivo denervation. Rats were anesthetized with ether and a small incision was made in the peritoneal wall along the lower border of the left rib cage, exposing the abdominal surface of the diaphragm. The intradiaphragmatic right branch of the left phrenic nerve was sectioned about 1 mm beyond its entrance into the diaphragm. The abdominal wall was then sutured, and the rat was allowed to recover. The muscle strip innervated by the intact left branch served as an internal sham control. At appropriate times, the diaphragms were removed and electrophysiologic investigation was performed, as described below.

Organ culture. Left hemidiaphragms were removed within 5 min after death from rats killed by head concussion. The muscle was then cut into paired strips, one with and one without a 2-cm phrenic nerve stump ("+" and "-", respectively), and was incubated as previously described (Robbins, 1981; Olek and Robbins, 1981). Briefly, the strips were placed for various times in organ culture (36°C, oxygenated Medium 199) with continuous rocking (1.5 min atmosphere, 0.5 min immersed). In the indicated experiments, a nicotinic blocker, α -BTX (0.6 μ M) or *d*-tubocurarine (4 μ M), or the protease inhibitor leupeptin (4 or 20 μ M) was added to the medium. Upon completion of the incubation period, the diaphragm strips were placed in oxygenated Krebs solution (25°C), and the RMPs and MEPPs of surface muscle fibers were recorded, using glass microelectrodes filled with 3 M KCl (15 megohms DC resistance, tip potential less than 2 to 3 mV) as previously described (Robbins, 1981). At the conclusion of the electrophysiology, some of the ("interim") preparations were reincubated and reused for electrophysiological measurements at later time points. RMPs at each time represent the average of two interim and two terminal recordings. There was no statistical difference between observations in muscles from terminal and repeatedly sampled organ cultures.

Results

Denervation-induced RMP depolarization in vivo. In hemidiaphragm denervated *in vivo* with a short nerve stump (2 to 7 mm), MEPPs disappeared between 8 and 10 hr after denervation (with half-life of 9 hr), and the depolarization of the RMP occurred several hours later (Fig. 1). In the control portions of diaphragms, MEPPs were maintained in 100% of the muscle fibers, and RMP was unchanged. These results are similar to those reported in extensor digitorum longus (EDL) and soleus muscles (Card, 1977).

Time course of denervation-induced MEPP failure in vitro. Rat left

hemidiaphragms with short or with long nerve stumps were incubated for various times, and the percentage of fibers with MEPPs was determined upon termination of the incubation (Fig. 2). Following denervation, MEPPs disappeared with a half-time of 12 hr in -N muscles (with 0- to 5-mm nerve stump) and 19.5 hr in +N muscles with a 2-cm stump. Thus, as reported previously (Robbins, 1981), the denervation-dependent disappearance of MEPPs as well as the nerve stump effect delaying MEPP failure occurred *in vitro* as it does *in vivo* (Miledi and Slater, 1970) and with similar time course.

Time course of denervation-induced depolarization of RMP in vitro. Rat left hemidiaphragms were maintained in organ culture for various periods of time after the denervation entailed in removing the muscles from the animal. Resting membrane potentials, as determined upon termination of the culture, declined between 12 and 19.5 hr after denervation in -N muscles and between 20 and 27 hr in +N muscles (Fig. 3). The *in vitro* delay of RMP depolarization produced by the nerve stump closely resembles the previously described *in vivo* nerve stump effect on RMP (Card, 1977).

Effect of cholinergic blockers on denervation-induced depolarization of membrane potential. The cholinergic blockers, α -BTX (0.6 μ M) or curare (*d*-tubocurarine, 4 μ M), produce "instant cholinergic denervation" and, thus, should accelerate the onset of denervation effects if nicotinic ACh transmission plays a role in maintenance of normal RMP. The most critical time points for observations of this acceleration are those prior to the time of expected membrane depolarization (i.e., before 12 and 22 hr in short- or long-stump preparations, respectively) and also those still earlier times when ACh might still be released at denervated endplates (see "Discussion"). Therefore, all studies were focused only on time periods up to about 27 hr after denervation. Nicotinic transmission in the treated muscles was blocked by α -BTX or curare from the initiation of the culture: no MEPPs, indirect contractions, or endplate potentials could be recorded in freshly dissected blocked preparations. An *in vitro* analysis was essential since, *in vivo*, α -BTX both wears off and cannot be administered safely at sufficiently high concentration to produce complete and continuous inhibition of AChR.

The major findings (Fig. 4) were that α -BTX did not accelerate RMP depolarization in denervated muscle fibers with short nerve stump, nor did α -BTX or curare accelerate the depolarization of muscle fibers supplied by a long nerve stump. Since inhibitors of nicotinic receptors did not alter the delaying effect of the nerve stump on RMP decline, the nerve stump effect on RMP cannot result from a nicotinic cholinergic mechanism. There was only one statistically significant difference between blocked and denervated muscles, namely, at 20 hr in -N cultures (Fig. 4A). However, the absolute difference was only 5 mV and the blocked muscles had greater membrane potentials (i.e., the opposite sign from that predicted if ACh release were important in maintenance of RMP). Furthermore, in +N preparations, which should even more clearly demonstrate an acceleratory effect of nicotinic blockers on membrane depolarization, there were no differences with or without the nicotinic blockers, even when (at 27 hr), RMP began to diminish (Fig. 4B).

Effect of the protease inhibitor, leupeptin, on resting potential and MEPP alteration. If leupeptin-sensitive proteolytic processes are involved in denervation changes (see the introduction), leupeptin should prevent or delay such changes. Therefore, left hemidiaphragm strips were incubated in the presence or absence of leupeptin (4 μ M, or 20 μ M) for 12, 14, 26, or 27 hr, and RMP and MEPPs were recorded. The presence of the leupeptin in the medium did not prevent or delay presynaptic MEPP failure or the denervation-induced membrane depolarization (Fig. 5). Thus, cellular processes underlying membrane depolarization do not include a leupeptin-sensitive protease component.

Discussion

As explained in the introduction, it was essential in these experiments to ensure that nicotinic receptors were entirely blocked throughout the post-denervation period in which ACh release de-

clined and membrane depolarization began. Since an organ culture system was used to attain this objective, it is also important to establish that this system sufficiently reproduces post-denervation events *in vivo*. This was shown here and previously for several parameters.

In organ culture, the time course of the disappearance of MEPPs and of the depolarization of the RMP closely resembled those reported for the corresponding changes observed *in vivo* (Miledi and Slater, 1970; Card, 1977). Other denervation phenomena that showed similar time courses *in vivo* and *in vitro* included increase of extrajunctional ACh receptor (Olek and Robbins, 1981) and increase of glucose 6-phosphate dehydrogenase (G6PD) activity (Robbins, 1981). Finally, delay of onset of denervation phenomenon by the presence of a long nerve stump was also reproduced *in vitro* in respect to timing of MEPP failure, depolarization of membrane potential, and increase of G6PD (present study; Robbins, 1981). In all of these cases of close similarity between *in vivo* and *in vitro* phenomena, the only difference in the two conditions was a shift of 3 to 4 hr in time of appearance of certain denervation phenomena. Small temperature differences (36°C *in vitro* versus 38°C *in vivo*) and an initial equilibration period could account for these minor disparities. Furthermore, the maintenance of normal RMP in muscles with long nerve stumps argues for the viability of muscle under these *in vitro* conditions. Thus, there is strong evidence that this system is suitable for *in vitro* study of neurotrophic regulation of muscle RMP.

Our experiments were designed to test the hypothesis that ACh released from nerve terminals is a neurotrophic factor producing its effect through nicotinic receptors (Drachman et al., 1982). The present experiments with nicotinic blockers can only be evaluated after discussing the time course of ACh release after denervation. Acetylcholine transmission (spontaneous quantal release) in muscles with short or long nerve stumps failed with half-lives of 12 and 20 hr after denervation, respectively, in general agreement with previous and the current reports based on *in vivo* denervations (Slater, 1966; Redfern and Thesleff, 1971; Card, 1977; Drachman et al., 1982). Also, non-quantal ACh release, which may comprise most spontaneous ACh release, declines significantly by 11 hr (to 75%) and to 50% of control by 18 hr after denervation (Linden et al., 1983); choline acetyltransferase follows a similar time course (Miledi et al., 1981, 1982). If quantal or non-quantal ACh were a major trophic influence preventing depolarization, then nicotinic blockers should have accelerated the depolarization of RMP by 8 to 11 hr, an effect which would have been readily detected in our system. Yet, blockade of nicotinic transmission did not advance the time at which the RMP declined in muscles with either short or long nerve stumps. Hence, the neuronal regulation of RMP is not entirely explicable by a nicotinic mechanism.

The result of the nerve stump experiments combined with those with nicotinic blockers further strengthen the case against ACh as a critical trophic factor. First, if any form of ACh release (but especially spontaneous quantal or non-quantal ACh) were involved in the delaying effect of the nerve stump on RMP depolarization, it would be necessary that this influence persist for 8 hr longer in the case of the long nerve stump preparation. Yet, even in muscles cultured with long nerve stumps, the timing of membrane depolarization was unaffected by the continuous presence of nicotinic blockers. Second, it might be proposed that nicotinic blockers had no accelerating action on membrane depolarization solely because a minimum time (equal to that obtained with a short nerve stump) is required for development of the denervation effect. However, the delay of the effects of denervation by the presence of a nerve stump and the lack of effect of nicotinic blockers in this condition as well rules out this possibility.

Our results are at odds with those of Bray et al. (1982), in which rat diaphragm placed in organ culture showed a nerve stump-dependent depolarization after only 3 hr in culture, and in which cholinergic blockers accelerated depolarization in preparations with

longer nerve stumps. First, a depolarization in 3 or 6 hr has not been observed *in vivo* by most workers (Miledi and Slater, 1970; Card, 1977; Drachman et al., 1982; present report). In our case, we used a transabdominal *in vivo* denervation to mimic closely the short length of nerve stump in the organ-cultured muscles. Second, as noted above, the time courses of the changes seen in our preparation *in vitro* closely conform to those of a number of denervation events *in vivo*, whereas this is not clear in the cited work (and certainly not so for membrane depolarization). Therefore, we suggest that the unusually rapid membrane depolarization noted by Bray et al. (1982) may reflect peculiar culture conditions (e.g., insufficient oxygenation, an effect of Trowell's T-8 medium in which spontaneous contractions occur in the first hours of incubation (unpublished observations)), or conditions in which a nicotinic proteolytic mechanism is provoked.

In conclusion, the studies described in this paper provide evidence for a neurotrophic influence independent of cholinergic transmission. Thus, as an overview, the neuronal influence on muscle appears to consist of three functions: (1) stimulation of muscle activity (Drachman and Witzke, 1972; Lømo and Rosenthal, 1972; Shainberg and Burstein, 1976; Hall and Reiness, 1977; Betz and Changeux, 1979), (2) nicotinic influence as supported by the tetrodotoxin experiments (Lavoie et al., 1976; Pestronk et al., 1976) combined with results of experiments with α -BTX (Drachman et al., 1982), and (3) a non-activity-dependent non-nicotinic function, as indicated by the present study (see also Tobias and Masukawa, 1983, for non-activity effects). Our data provide no indication of the nature of this latter function. A non-nicotinic trophic action of ACh, trophic factor(s) other than ACh, or an interaction between nerve, muscle, and synaptic matrix are all possible trophic mechanisms consistent with our data.

Another hypothesis to explain the third mechanism would have to do with nerve terminal degeneration products initiating the early response of denervation. In this event, leupeptin, a lysosomal protease inhibitor, might delay denervation effects or delay the onset of a denervation phenomenon such as resting membrane depolarization. However, leupeptin did not delay MEPP failure or alter denervation-induced changes in RMP. Although leupeptin apparently does prevent or delay loss of acetylcholinesterase activity (Fernandez and Duell, 1980) and muscle atrophy (Libby and Goldberg, 1980) after denervation, the neural regulation of RMP may have a different mechanism.

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