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Trophic Regulation of Acetylcholine Sensitivity of Muscle: Effect of Electrical Stimulation

Abstract. *Denervation of skeletal muscle results in a spread of acetylcholine sensitivity over the entire surface membrane. Electrical stimulation, programmed to mimic the normal activity pattern, was applied continuously to the denervated rat diaphragm in vivo. After 4 days, the acetylcholine sensitivity was far less in the stimulated diaphragms than in denervated controls. Muscle activity may account for "neurotrophic" regulation of the acetylcholine sensitivity.*

Denervation of skeletal muscle increases its sensitivity to acetylcholine (ACh) (*1*). In a normally innervated muscle fiber, only the motor endplate region is highly sensitive to ACh; after section of the motor nerve, the sensitivity of the remainder of the surface membrane increases more than one-thousandfold until it equals that of the endplate (*2*).

The mechanism by which the motor nerve regulates the ACh sensitivity of the muscle membrane is not known. It has been generally held that activity or usage of muscle could not account for this and other "trophic" influences of the motor nerve (*3*). However, evidence has appeared suggesting that usage of muscle might be responsible for neurotrophic regulation of chemosensitivity (*4–7*). We have studied the effect of direct electrical stimulation of denervated mammalian muscle on chemosensitivity to ACh. To optimize the effectiveness of the stimulation, we used programmed stimulus patterns designed to mimic the normal action of the nerve; these patterns were applied continuously throughout the period of denervation. In this way, we tested the possible "trophic" effect of depolarization and contraction of muscle on one well-defined denervation phenomenon, ACh sensitivity.

Sprague-Dawley rats weighing 100 to 150 g were used. Denervation of the left hemidiaphragm was carried out when the animal was under chloral hydrate anesthesia (0.4 g/kg, intraperitoneally). The phrenic nerve was snared with a sterile crochet hook inserted through a left thoracic incision, and was severed as close as possible to the diaphragmatic surface. In the control rats, no further procedure was carried out until the testing of ACh sensitivity. In the experimental animals, the insertion of wire electrodes for stimulation was done aseptically. A midline incision was made from the midabdomen to the xiphisternum and was carried laterally along the left costal margin. Flexible multistrand stainless steel wire, stripped of its Teflon insulation for 1 to 1.5 cm and knotted at the tip to prevent slipping, was sutured to the abdominal surface of the diaphragm just below the costal margin, or at the level of the entry of the phrenic nerve. A second wire lead was sutured to the diaphragmatic tendon (Fig. 1). The wires were led out through the animal's flank and the surgical incision was closed. The rat's limbs and tail were fastened to a Lucite board with masking tape, and the head was restrained with a U clamp. Food and water were placed within easy reach, and the animals were encouraged to eat and drink.

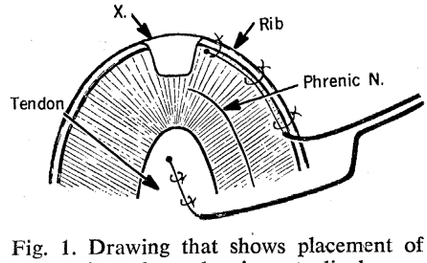

Fig. 1. Drawing that shows placement of stimulating electrodes in rat diaphragm. In four rats (not illustrated), the outer electrode was fixed near the site of the phrenic nerve (*X.*, position of the xiphisternum; *N.*, nerve).

Electrical stimulation was provided by a Grass SD5 or S48 stimulator through a capacitance-coupled stimulus isolation unit. The stimulus variables were chosen to produce vigorous contractions of the diaphragm at a rate slightly slower than the normal respiratory rate. This was achieved by delivering trains of 6 shocks each, 72 trains per minute. The stimulus was 7 to 9 volts (monitored by oscilloscope) and its duration was 0.25 to 0.3 msec.

At the end of each experiment (91 to 99 hours), the rat was anesthetized with chloral hydrate, and the abdominal incision was reopened. The diaphragm was observed while it was stimulated, and in all instances but one responded with vigorous contractions. (In the exceptional case, the diaphragm responded weakly, presumably because of poor contact between the electrodes and the muscle fibers.) The diaphragm was then removed, and the full-length segment that had been in contact with the electrodes was mounted, thoracic surface upward, on a silicone rubber platform. The preparation was bathed in Trowell's T-8 medium (*8*) and equilibrated with 5 percent CO_2 and 95 percent O_2.

To avoid any possible effect of fibrosis around the electrode wires, we used the thoracic surface of the diaphragm for sensitivity testing, whereas the electrodes had been applied to the abdominal surface. Fibrosis was not notable in the stimulated diaphragms.

The ACh sensitivity of fibers was tested in each diaphragm by a method similar to that used by others (*9*). Trans-

membrane potentials were monitored by intracellular glass microelectrodes filled with 2*M* KCl (resistance, 7 to 15 megohms). The signals were amplified by a Grass P16 d-c amplifier and displayed on one channel of a Hewlett-Packard 132A oscilloscope. The ACh was applied iontophoretically by a micropipette containing a 4*M* solution of ACh chloride. Outward diffusion of ACh was prevented by a bucking current supplied by an adjustable source. Since inaccurate placement of the ACh pipette may distort the results, the position of the ACh pipette and the current pulse were adjusted to obtain a maximal amplitude of response with a minimal latency (usually less than 3 msec and always less than 10 msec). In fibers with low sensitivities (5 mv/ncoulomb or less) the accurate placement of the micropipettes was confirmed by advancing the ACh pipette into the fiber, passing current, and observing electrotonic pulses (Fig. 2D).

The charge passed through the ACh pipette was calculated from the potential drop across a 15K resistor in the iontophoretic circuit and the duration of the applied pulse. Sensitivity to ACh was expressed as millivolts of response per nanocoulomb of charge. Since the amount of ACh released is proportional to the charge, the calculated value gives an indication of ACh sensitivity. In each diaphragm, sensitivity was tested at various distances from the end plate region, ranging from 0.25 to 5.9 mm. For calculations the values were combined, but this did not materially affect the results.

Results indicated that electrical stimulation prevented the spread of ACh sensitivity of denervated skeletal muscle to a major extent. The mean sensitivity of the fibers of the control diaphragms was 46.4 mv/ncoulomb (S.D. = 4.12), in keeping with values previously reported for diaphragms denervated for 4 days (*10*). However, the mean sensitivity of the stimulated diaphragms was 4.18 mv/ncoulomb (S.D. = 0.74) or, if the weakly stimulated one is excluded, 1.92 mv/ncoulomb (S.D. = 0.30). The difference between the experimental and control values is highly significant ($P \ll .001$).

Although the sensitivity values of individual fibers varied, all the control denervated fibers were sensitive, with only two fibers having values lower than 5 mv/ncoulomb. In contrast, 114 of the stimulated fibers had sensitivity values less than 1 mv/ncoulomb, and of these fibers, 51 had values that were less than 0.1 mv/ncoulomb or were undetectable (Fig. 2).

Table 1. Acetylcholine sensitivities of stimulated and control denervated diaphragms.

Denervated diaphragm	Fibers tested (No.)	Sensitivity (mean ± S.D.) (mv/ncoulomb)
Stimulated	199	4.18 ± 0.74
Control	97	46.4 ± 4.12

This notable effect of electrical stimulation could not be attributed to a nonspecific harmful effect of the experimental procedure itself. The resting potentials of the stimulated muscle fibers and the controls were comparable, and only fibers with membrane potentials greater than 60 mv were included. Further, there was no significant difference in the diameters of the stimulated and control denervated muscle fibers. Measurements were made of the diameters of 200 surface fibers in two denervated control diaphragms and of 350 surface fibers in six stimulated denervated diaphragms, by means of a Leitz Ortholux microscope equipped with objectives for long working distance. The average fiber diameter was 26.04 μm (S.D. = 6.014) in the stimulated diaphragms and 24.52 μm (S.D. = 6.09) in the control diaphragms.

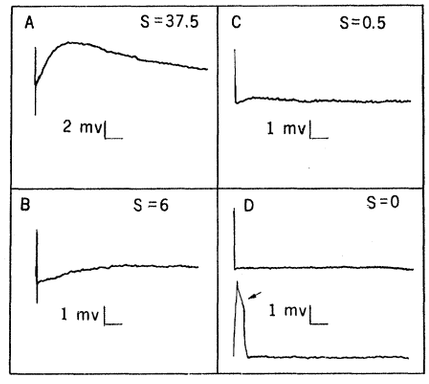

Fig. 2. Potentials evoked by iontophoretic application of ACh to muscle fibers. Sensitivities (*S*), computed from response amplitudes and charges passed through ACh pipette, are given in millivolts per nanocoulomb. The time calibration is 50 msec. Potentials are shown for (A) control denervated muscle at 96 hours (charge, 0.13 ncoulomb), (B) moderately sensitive fiber of a stimulated diaphragm (charge, 0.13 ncoulomb), (C) slightly sensitive fiber of a stimulated diaphragm (charge, 0.8 ncoulomb), and (D) insensitive fiber of a stimulated diaphragm. Here, response was recorded in the upper trace. The ACh pipette was advanced slightly, and an electrotonic response (arrow) indicated penetration of the muscle fiber by the pipette.

These results indicate that electrical stimulation inhibits the spread of ACh sensitivity of denervated muscle. Therefore, electrical stimulation may be capable of substituting for the normal "trophic" influence of the motor nerve in regulating ACh sensitivity of the muscle membrane.

Since the effect of stimulation was less than complete in some fibers, the possibility that an additional "trophic factor" may be needed for the full regulation of ACh sensitivity cannot be excluded. However, the simplest explanation is that the stimulation may have fallen short of maximal effectiveness in activating some fibers. This explanation is supported by the substantial degree of sensitivity (28.4 mv/ncoulomb) found in the one diaphragm that responded weakly to the same stimulus that caused vigorous contractions in all others. In the experimental preparation, the stimulus undoubtedly does not activate all fibers of all muscle preparations equally.

We chose to study the effect of purely muscular events in the absence of neuromuscular transmission. Precautions were taken to eliminate any residual effect of the severed nerve. The nerve stump was cut short to limit the persistence of its effect, and the denervation period was prolonged to assure loss of the nerve's ability to transmit ACh (*11*). Stimulation was applied to the muscle directly, by electrodes placed in most instances as far from the nerve stump as possible, at opposite ends of the muscle fibers. Under these conditions of direct muscle stimulation, marked prevention or limitation of ACh hypersensitivity took place. Precisely which muscular event—depolarization, excitation-contraction coupling, or muscle contraction and energy utilization—may be responsible for the effect remains to be determined.

Our data add to a growing body of evidence that activity of muscle is related to the "trophic" control of membrane chemosensitivity. Under normal circumstances, activation of skeletal muscle is triggered by transmission of ACh from the motor nerve. Interruption of motor nerve impulse conduction or neuromuscular transmission of ACh would be expected to result in enlargement of the area sensitive to ACh. Indeed, when impulse conduction or transmission is blocked by the use of local anesthetic agents (*5*) or botulinum toxin (*12*), enlargement of the area sensitive to ACh occurs as it does in denervation. Similarly, in one strain of

mice, defective impulse transmission is associated with increased ACh sensitivity of the muscle membrane (*6*). Conversely, electrical stimulation of a severed but still functioning motor nerve has been reported to restrain the development of ACh hypersensitivity in denervated muscle (*7*).

As judged by the present evidence, it seems likely that the role of neurally released ACh in regulating the sensitivity of the muscle membrane is mainly, if not entirely, that of triggering the chain of events which constitutes muscle activity. This provides support for the concept (*13*) that ACh is important in mediating neurotrophic influences on muscle.

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Iodine-125–Labeled Antibody to Viral Antigens: Binding to the Surface of Virus-Infected Cells

Abstract. *The specific binding of iodine-125–labeled antibody to viral antigens can be used to detect newly synthesized viral antigens and determine the time of appearance of these antigens on the surface of infected cells. Incubation of infected cells with unlabeled antibody to viral antigens specifically blocks the attachment of labeled antibody, and by this inhibition technique the titer of unlabeled antibody to viral antigens can be calculated. The attachment of the labeled antibody to virus-infected cells offers an objective and sensitive method of detecting viral antigens and measuring antibody to virus.*

Immunofluorescence has been used to detect viral antigens in the cytoplasm and nucleus of infected cells (*1*), and membrane immunofluorescence has been used to detect viral antigens on the surface of infected cells (*2*). Although these techniques have proved useful for many virus studies, they are difficult to quantitate and are time-consuming. Moreover, interpretation is subjective and often hampered by high background activity. In theory, certain of these difficulties could be overcome by the use of isotopically labeled antibody. We now report that the binding of ^{125}I-labeled antiviral antibody to the surface of virus-infected cells could be used for detecting viral antigens and measuring antibody to virus.

Monolayers of primary rabbit kidney (PRK) cells and vaccinia virus (strain CVI-79) were prepared (*3*, *4*). Serums from rabbits hyperimmunized with vaccinia were the source of antibody to virus (*5*). The serum titers at which plaque formation was reduced by 50 percent were approximately 1:2000. Serums from unimmunized rabbits served as controls, and all serums were heated at 56°C for 30 minutes before use. Serums from immunized and control rabbits were fractionated on Sephadex G-200 (*3*), and the immunoglobulin G (IgG) fractions were labeled with ^{125}I by the chloramine-T method (*6*).

In a typical experiment, confluent monolayers containing approximately $10^{6.0}$ PRK cells were inoculated with infectious virus (virus/cell ratio, 2.0). Except where stated, the virus was allowed to adsorb to the cells for 2 hours. The inoculum then was removed, the monolayers were washed three times, and fresh medium was added. Uninfected cells served as controls. At given times thereafter, the medium was removed, and the monolayers were washed three times and then incubated for 1 hour at 37°C with 0.5 ml of a 1:64 or 1:100 dilution of [^{125}I]IgG from immunized or unimmunized animals. The medium then was aspirated and the monolayers were washed six times. The amount of ^{125}I that remained bound to the cells was determined by removing the cells and assaying them for radioactivity (Packard Auto-Gamma spectrometer). Data are expressed as the number of counts per minute (count/min) bound to the monolayers, and each number represents the average for three monolayers.

The first experiment was designed to study the time of appearance of viral antigens on the surface of vaccinia-infected cells. Within 1 hour after inoculation of the virus, three to four times more antibody had attached to infected cells than to uninfected cells (Fig. 1A). In the next several hours the binding of antibody increased gradually, but 5 hours after infection the binding of antibody began to increase precipitously. At 12 hours after infection, 33 times more antibody had attached to infected cells than to uninfected cells. In contrast, [^{125}I]IgG from unimmunized animals did not bind to either infected or uninfected cells. These results suggest that the binding of labeled antibody early in the infection was due to the attachment of antibody to virus particles that had adsorbed to the surface of cells, whereas the binding of antibody later was due to the appearance of newly synthesized viral antigens (*7*).

To study further the interaction of [^{125}I]IgG with viral antigens that had adsorbed to the surface of cells, we inoculated monolayers with partially purified virus or soluble viral antigens (*8*). Approximately 25 times more virus was used in this than in the first experiment. Lysates from uninfected cells served as controls. Within 5 minutes after addition of the virus, binding of antibody to monolayers exposed to virus was more than 6000 count/min, compared to 500 count/min on monolayers that had not been exposed to virus (Fig. 1B). The large increase in uptake of labeled antibody in the first 40 minutes, compared to the relatively small increase in the next 80 minutes, suggests that most of the virus had adsorbed to the monolayers shortly after inoculation. Soluble viral antigens also adsorbed rapidly to the monolayers but, in contrast to infectious virions, the noninfectious soluble viral antigens did