of electrode, it was not detected by the threshold criterion. Changes were observed, however, by appropriate analysis of voltage waveforms, in the polarization impedance of the platinum electrodes and in the pore resistance of the capacitor electrodes. Both of these quantities rose slowly between stimulation sessions and returned toward their original values during stimulation. The changes of the polarization impedance of the platinum electrodes were interpreted as accumulation and then oxidative removal of organic surface contaminants (*6*). Increase in the pore resistance of the capacitor electrodes was probably due to tissue ingrowth or accumulation of other organic material. The reason for decrease of this resistance during stimulation is yet to be explained. The long-term pore resistance stabilized at a level two to three times higher than that measured immediately after implantation.

In conclusion, conventional metal electrodes generate oxidation-reduction products during stimulation of excitable tissue. Use of stimulation waveforms with zero net charge transfer may be sufficient in some cases to reduce these products to acceptable levels. However, large arrays of densely packed electrodes, such as those proposed for neural prosthetic devices, may require the extra margin of safety provided by capacitor electrodes.

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5. We thank M. Markarian and D. R. Ogden of Sprague Electric Company and J. Piper of Union Carbide Corporation for invaluable technical information and for providing experimental electrodes.
6. We thank B. Brummer of Tyco Laboratories, Inc., for advice on electrochemical matters.
7. Micrograph courtesy of P. Johnson and L. Hench of the University of Florida.

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Regulation of Muscle Acetylcholine Sensitivity by Muscle Activity in Cell Culture

Abstract. *Muscle in tissue culture provides a good system for studying long-term changes in surface membrane acetylcholine sensitivity. Muscle fibers stimulated intermittently over prolonged periods are less sensitive to iontophoretically applied acetylcholine and bind less ^{125}I-labeled α-bungarotoxin than inactive fibers.*

There is some evidence that the degree of muscle activity can influence the distribution of acetylcholine (ACh) sensitivity of the cell surface membrane. Direct electrical stimulation of denervated muscle can prevent the appearance of extrajunctional sensitivity that normally ensues (*1*). Muscle fibers that develop in culture from dissociated myoblasts are extremely sensitive to iontophoretically applied ACh. If it can be shown that ACh sensitivity is dependent on the degree of activity in vitro, then a tissue culture system may help elucidate the complex cellular mechanisms responsible for the change.

We previously noted that the chemosensitivity of several fibers (both innervated and uninnervated) that exhibited spontaneous twitches and action potentials was much lower than the mean of other inactive fibers (*2*). We now report additional experiments in which we have attempted to regulate activity over relatively long periods of time.

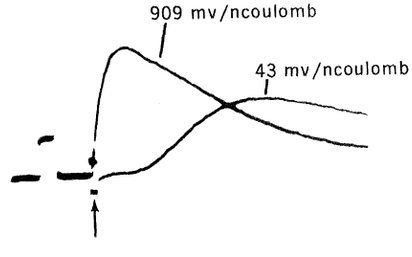

Fig. 1. Superimposed ACh potentials evoked at the same spot before and after a small displacement of the ACh electrode following a large current pulse. The slow response that began after a long delay (evoked with 0.219 ncoulomb) was converted to a rapidly rising response that began without a detectable latency (evoked with only 0.019 ncoulomb). Calibration of the square wave pulse, 5 mv and 10 msec, precedes the ACh response. Similar calibration pulses appear on oscilloscope traces in Fig. 2. The bar at the arrow indicates the duration of the ACh current pulse.

Muscle fibers were grown from myoblasts obtained from embryonic chick pectoral tissue by methods already described except that the drug cytosine arabinoside (ara-C) was not employed to eliminate fibroblasts (*2*). In older cultures, the background connective tissue anchors the muscle fibers and is more effective than a simple collagen layer in preventing them from pulling off the dish during periods of vigorous contraction. The techniques for intracellular microelectrode recording and ACh iontophoresis and for autoradiography have also been described (*2*).

In dense cultures, after an iontophoretic pulse the ACh response often started after a significant latency, an indication that the ACh pipette tip was located some distance from the receptors (*3*). Although some delay may have been due to a patchy distribution of receptors, the most significant factor was the diffusion barrier presented by the connective tissue. A more rapidly rising, larger response (Fig. 1) that occurred without a detectable delay followed a small vertical displacement of the ACh electrode tip [see (*4*)]. Only responses with less than a 5-msec latency were accepted.

Muscle fibers in 7- to 10-day cultures generate action potentials and twitch in response to a depolarizing stimulus (Fig. $2A_1$). Many fibers twitch spontaneously, but the amount of activity varies considerably from culture to culture and within a single culture over long periods of time. To regulate the activity we stimulated electrically one group of cultures through relatively large electrodes fastened in each plate (*5*).

Stimulated muscle fibers were compared to a group of fibers made inactive by the addition of 2.0×10^{-7} g of tetrodotoxin (TTX) per milliliter to the medium. The TTX, which blocks active Na^+ currents in other tissues (*6*), abolished spontaneous and stimulus-evoked action potentials within 1 minute at a concentration as low as 5×10^{-8} g/ml (Fig. $2A_2$).

In our experiments, stimulation (and TTX treatment) was begun on days 7 to 9 and continued for 2 to 6 days.

The cultures were then washed with a balanced salt solution, and the ACh sensitivity was assayed immediately thereafter. The main result is shown in Fig. 2B which compares ACh potentials from a stimulated (Fig. $2B_1$) and a TTX-treated (Fig. $2B_2$) culture. The slow response of the stimulated fiber was evoked with 0.494 ncoulomb, whereas only 0.006 ncoulomb evoked a faster, larger response from the inactive fiber. We tested the sensitivities of five to ten points chosen at random on each stimulated and TTX-treated fiber; histograms of all values in both groups are shown in Fig. 2C. Such a comparison is valid because variation between fibers within each group was small compared to differences between the groups. There was little overlap in the sensitivities expressed in terms of millivolts of depolarization per nanocoulomb of charge ejected (mv/ncoulomb). Geometric means of approximately 200 values from 26 active and 30 inactive fibers were, respectively, 11.8 ± 1.1, with $N = 196$; and 456 ± 60, with $N = 221$ ($P < .001$).

The larger ACh response of inactive fibers is probably due to a greater change in membrane permeability caused by the drug. However, fiber input resistance (R_{in}) and membrane potential (V_m) are also important determinants of the size of potential change (*7*) and must be considered in making a quantitative comparison. In turn, R_{in} depends on fiber diameter and after several days of inactivity fibers are, on the average, thinner than controls. We selected fibers from both groups to cover a comparable range (12 to 45 μm) in diameter. The means (20 μm, active; 23 μm, inactive) were not significantly different. The mean V_m (39.4 mv, active; 41.6 mv, inactive) were not different either.

TTX did not affect the size of the ACh potential immediately after addition to the bath (*8*), and it seems unlikely that the increased ACh sensitivity after 2 to 6 days is a direct drug effect. Fibers exposed to lidocaine (5.0×10^{-5} g/ml) stop twitching and also become extremely sensitive to ACh after 2 to 5 days (some of these values are included in the histograms of Fig. 2C).

Distinct peaks of sensitivity similar to those found in cultures treated with ara-C (*2*, *9*) were often detected over inactive, sensitive fibers. We have not identified similar peaks over active,

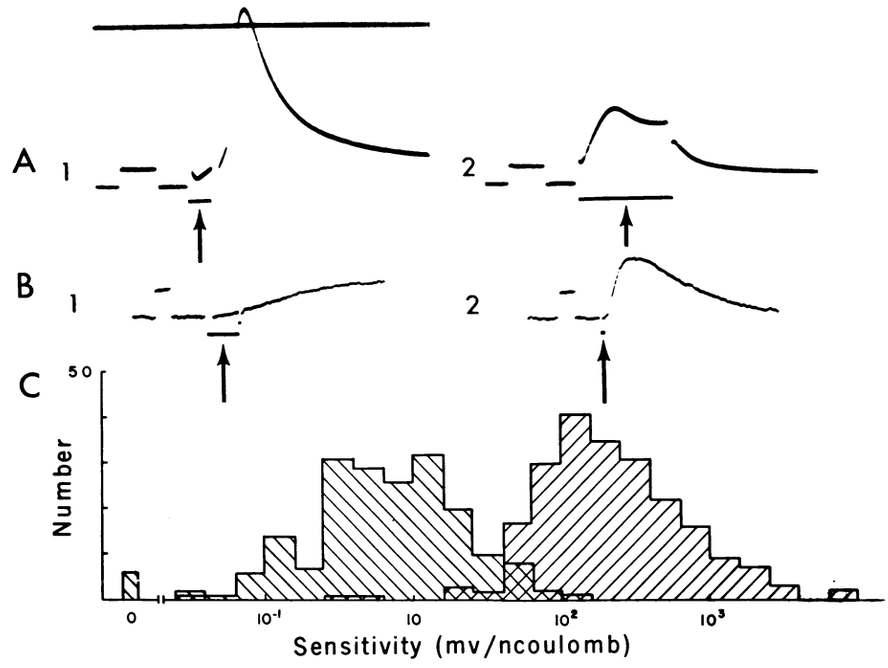

Fig. 2. (A) Block of action potential by TTX. A_1 is a spike evoked by a brief (bar at arrow) current pulse (42 na); the initial membrane potential was 85 mv; the upper trace indicates the zero reference potential; the calibration was 10 mv, 2 msec (also applies to A_2). A_2 represents failure of an even larger and longer (bar) current pulse to evoke an action potential in presence of 5.0×10^{-8} g of TTX per milliliter. (B) ACh potentials from active and inactive muscle fibers. B_1 is the response of a cell in a 9-day culture that had been stimulated for 68 hours. B_2 is the response of a cell in a 14-day culture exposed to TTX (2.0×10^{-7} g/ml) for 137 hours. Bars beneath traces represent the duration of the ACh pulse; calibration: 5 mv, 10 msec in both. Measured sensitivities were 13.7 and 1943 mv/ncoulomb, respectively. (C) Histograms of all ACh responses from 26 stimulated (\\\\) and 30 TTX- or lidocaine-treated (////) fibers. The sensitivity is shown in millivolts per nanocoulomb on a log scale. There is nearly no overlap between the two populations.

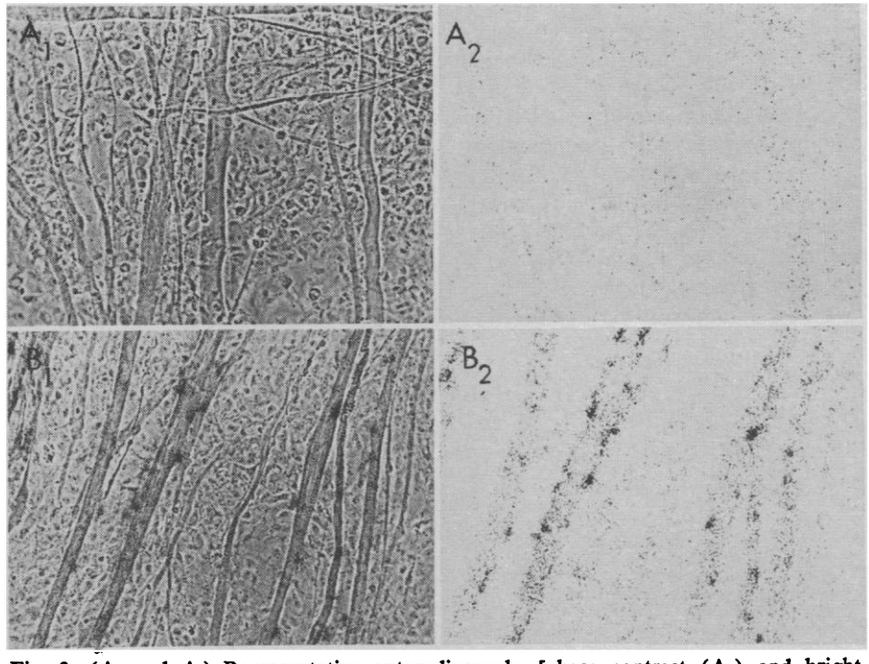

Fig. 3. (A_1 and A_2) Representative autoradiographs [phase contrast (A_1) and bright field illumination (A_2)] of 14-day cultures electrically stimulated (*8*) for 6 days prior to fixation. (B_1 and B_2) Representative autoradiographs [phase contrast (B_1) and bright field illumination (B_2)] of 14-day cultures grown in TTX for 6 days before fixation. The paucity of grains in the active fibers should be compared with the heavy labeling in the inactive fibers. There are focal accumulations of grains in the inactive fibers.

relatively insensitive cells, but a more thorough search is required.

Autoradiography of cultures exposed to ^{125}I-labeled α-bungarotoxin, a molecule that binds specifically to ACh receptors (*10*), supports the electrophysiologic findings (Fig. 3). The paucity of grains over active fibers, compared to grains over inactive cells, is striking. There are dense patches of grains over inactive fibers [see (*2, 11*)] which may correspond to the peaks of sensitivity detected by ACh iontophoresis.

In conclusion, we have demonstrated that activity of muscle fibers that develop in vitro from isolated myoblasts is sufficient to reduce the sensitivity of the surface membrane to ACh. It remains to be demonstrated that activity is also a necessary condition. The finding that fibers innervated in vitro by added spinal cord neurons but which remain relatively inactive are as sensitive as controls (*2, 9*) suggests that it is.

Our initial impression is that peaks as well as the "background" level of chemosensitivity are reduced or eliminated after stimulation of uninnervated fibers. It will be important to confirm this finding and to determine whether similar peaks that are found on innervated fibers near nerve terminals (*2, 9, 12*) are maintained under the same conditions.

It might be possible, in this in vitro system, to further analyze the roles played by the complex membrane phenomena and the muscle twitch associated with "activity" in altering membrane chemosensitivity by uncoupling excitation from contraction.

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13. We thank J. Whysner for supplying the ^{125}I-labeled α-bungarotoxin. Supported in part by NIH fellowship 1 F02 NS53870-01 to S.A.C.
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Mobility of Potassium Ion in Frog Muscle Cells, both Living and Dead

Abstract. *The diffusion coefficient of potassium ion (D_K) in frog muscles was studied by a new method. In normal cytoplasm D_K averaged one-eighth of the value in the free solution. Arrest of metabolism or injury caused an increase in D_K. In muscles killed with iodoacetate, D_K rose to three-quarters of the value in free solution. The data support the association-induction hypothesis.*

Widely cited in support of the membrane theory (*1*) is the demonstration by Hodgkin and Keynes (*2*) of a nearly normal self-diffusion coefficient of K^+ labeled with ^{42}K in giant squid axons. More recently, Kushmerick and Podolsky (*3*) measured the diffusion coefficients of K^+ and other solutes in small segments of single frog muscle fibers. They concluded that all of these solutes (except Ca^{2+}) are free in living frog muscle cells and that it is the mechanical barrier effect of intracellular proteins which impartially reduces virtually all diffusion rates by a factor of 2.

Since K^+ mobility is crucial in the controversy over what constitutes the physicochemical nature of a living cell, and since criticisms can be made of the above-mentioned experiments (*4, 5*), we have developed a technique to reinvestigate the mobility of K^+ in sartorius muscle cells of northern North American leopard frogs (*6*). At least a major portion of the K^+ in the sartorius muscle was exchanged with ^{42}K. This substantial exchange of cell K^+ with ^{42}K insured that the monitored K^+ mobility was truly representative of the bulk K^+ (*4*). The muscle, thus labeled with ^{42}K, was drawn through a close-fitting slit in a silicone rubber plug, which closed one end of a glass tube; the tube was filled either with a mixture of O_2 (95 percent) and CO_2 (5 percent) or with purified petroleum jelly. The tibial end of the muscle protruding beyond the plug was then cut off (*7*) at about 0.5 to 1 mm from the outer edge of the slit (see Fig. 1A). The muscle in the tube was approximately 20 mm long and was maintained in a straight but relaxed length. The extreme tip of the cutoff tibial end, about 2 mm, was cut off and discarded. The remainder of the cutoff end was analyzed for the initial ^{42}K content of the muscle. The cut end of the muscle in the tube was then exposed to a large amount (1 liter or more) of well-stirred, sterile Ringer-GIB medium (*8*) kept at 25° ± 1°C. After a specific length of time, the muscles were frozen in liquid nitrogen at their natural lengths and cut into 10 to 12 sections; their ^{42}K and water contents were then assayed. The concentration of labeled K^+, expressed as a fraction of the initial concentration, was then plotted against x/l, where x is the distance of the section from the cut end and l is the total length of the muscle (*9*) (Fig. 1).

The virtually straight line in Fig. 1A shows that before cutting and washing there is an even distribution of ^{42}K in the sartorius muscle except in the last two sections—at the extreme tibial and pelvic ends. From 13 June to 7 Decem-