# Nerve-Muscle Interaction In Vitro

## Role of acetylcholine

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ABSTRACT    Nerve and muscle cells from clonal lines interact in vitro, resulting in the association on the muscle surface of an area of increased acetylcholine sensitivity with a site of nerve-muscle contact. This localization of acetylcholine sensitivity on the muscle cell to a site of contact between nerve and muscle was found to occur when acetylcholine receptors on the muscle had been blocked with $\alpha$-neurotoxin. Localization was also found to occur when the nerve cell had been prevented from releasing acetylcholine. It is concluded that neither the presence of active acetylcholine receptors on the muscle, nor the release of acetylcholine from the nerve, was required for the events leading to the localization of acetylcholine sensitivity in vitro.

## INTRODUCTION

Nerve and muscle cells from clonal lines interact in vitro, resulting in an area of increased acetylcholine sensitivity on the muscle membrane around some sites of contact between nerve and muscle cells (Harris et al., 1971). This observation is reminiscent of the distribution of acetylcholine sensitivity on the surface of innervated skeletal muscle cells (e.g., Peper and McMahan, 1972). The study of localization of acetylcholine sensitivity in vitro has the great advantage that there is increased control over experimental conditions. For example, clones of cells may be chosen on the basis of their biochemical characteristics, and cells may be grown in controlled experimental media. These aspects of culture technique can be exploited to provide insight into the nature of the processes that result in localization.

This report is concerned with the requirements for the events that subsequently result in the topological association of a contact between a nerve and muscle and an area of increased acetylcholine sensitivity on the surface of that muscle. It is possible that some aspect of the cholinergic transmission

system might be required; for instance, the release of acetylcholine from the nerve (Thesleff, 1960; Drachman, 1967) or the preexistence of high levels of acetylcholine sensitivity on the muscle (Katz and Miledi, 1964). In the present report, two specific questions are asked. First, do the events that result in localization require the presence of functional acetylcholine receptors on the muscle? This was studied by culturing nerve and muscle cells in the presence of $\alpha$-neurotoxin, which specifically and reversibly binds to and inactivates acetylcholine receptors. Second, do the events require the release of acetylcholine from the nerve? This was studied by choosing a line of neuroblastoma with very low levels of choline acetyltransferase activity, then culturing nerve and muscle in the presence of an inhibitor of that enzyme after any stores of acetylcholine had been depleted. In brief, neither aspect of the cholinergic transmission system was required for the events that result in localization.

## MATERIALS AND METHODS

### Cell Culture

The cells were from clone L6 of rat skeletal muscle myoblasts (Yaffe, 1968) originally provided by Dr. D. Yaffe, The Weizmann Institute of Science, Rehovoth, Israel, and clone N18 of the mouse C1300 neuroblastoma (Amano et al., 1972), provided by Dr. R. Rosenberg, University of California, San Diego. Cells were grown in modified Eagle's medium (Vogt and Dulbecco, 1963) supplemented with 10 % fetal calf serum, vol/vol (this solution will hereafter be called "medium"), at 36°C in an atmosphere of 88 % air, 12 % $CO_2$.

Myoblasts were grown in 60-mm plastic tissue culture dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) and were transferred before the cells became confluent, since after reaching confluency myoblasts fuse to produce multinucleate fibers. To transfer cells, we removed and replaced the medium with serum-free medium plus 0.5 % Viokase, vol/vol (Grand Island Biological Co., Grand Island, N. Y.). The cells were incubated for 10 min at 36°C, after which medium was added to stop the action of Viokase on the cells. Myoblasts were seeded at $5 \times 10^4$ cells per dish in 5 ml of medium. Cells to be used for electrophysiology were grown in the same way, but two glass cover slips (25-mm diameter) were placed in the bottom of each dish. Cover slips were used to facilitate handling the cells, since they could be washed by transferring the slips from one solution to another. The membrane electrophysiological properties of myoblasts and muscle fibers grown under identical conditions have been studied in this laboratory (Kidokoro, 1973).

Clone N18 of the mouse C1300 neuroblastoma was maintained in suspension culture in 60-mm plastic petri dishes (Falcon). Before culturing neuroblastoma and muscle cells together, the neuroblastoma was predifferentiated by seeding $10^5$ cells in a plastic tissue culture dish in 5 ml of medium plus 1 % or 2 % serum (Schubert et al., 1971). After 5–10 days, when the cells had developed long processes, they were removed from the dish with Viokase (0.5 % vol/vol) and seeded at $1$–$5 \times 10^4$ cells

per dish on top of fused muscle fibers grown on cover slips. The nerve and muscle cells were cultured together in medium for 10–15 days, and the medium was not changed during that time.

### Drugs Used

α-Neurotoxin ("toxin"), a protein isolated from the venom of the Indian cobra (*Naja naja*), was used to block the response of the muscle fibers to applied acetylcholine. It has been shown that toxin binds specifically and reversibly to the acetylcholine receptor on these muscle fibers (Patrick et al., 1972). Toxin was used at $3 \times 10^{-7}$ M (2 μg/ml) in all the experiments reported here. At $3 \times 10^{-7}$ M, toxin reduces the acetylcholine sensitivity of muscle cells to an undetectable level below 0.1 mV/nC, or less than 0.1 % of the average level found before the addition of toxin. Muscle cells do not become resistant to the action of toxin, for cells grown for 2 wk in toxin had undetectable acetylcholine sensitivity unless the cells were extensively washed in toxin-free medium. Further, toxin is stable in culture conditions, since if that toxin-containing medium was removed and placed over untreated muscle fibers, the acetylcholine sensitivity on those fresh cells was reduced to an undetectable level.

*N*-hydroxyethyl-4-(1-naphthylvinyl)-pyridinium bromide ("Pyridinium" hereafter; Calbiochem, Los Angeles, Calif.) was used at $5 \times 10^{-5}$ M to inhibit choline acetyltransferase activity (White and Cavallito, 1970). Pyridinium reduced the incorporation of [$^{14}$C]choline into acetylcholine in intact neuroblastoma cells by at least 99 % (for methods, see Hildebrand et al., 1971). It was stable for at least 2 wk in medium in which cells were growing (Schubert, unpublished observations; footnote 1). At $5 \times 10^{-5}$ M, it did not affect the growth rate of either L6 or N18 cells, and did not affect choline uptake or pool size. It reversibly blocked the acetylcholine response of muscle fibers with an $I_{50}$ of about $5 \times 10^{-6}$ M (footnote 1).

N18 cells were grown for at least five generations in $5 \times 10^{-5}$ M Pyridinium to reduce any stores of acetylcholine by division and hydrolysis. They were predifferentiated in Pyridinium and Pyridinium was added to muscle cultures before adding the nerves.

Cycloheximide was used to inhibit protein synthesis. Cycloheximide at $2.7 \times 10^{-4}$ M (50 μg/ml) inhibited protein synthesis in L6 muscle cells more than 99 %, as determined by techniques described earlier (Schubert et al., 1971).

### Experimental Protocol

Toxin and Pyridinium had to be removed before acetylcholine sensitivity could be assayed. The experimental protocol is shown in Fig. 2. Each "wash" was done at room temperature by taking the cover slip with attached cells out of the initial solution, touching the edge to absorbent paper to remove any remaining liquid, then immersing the slip in 2–5 ml of the wash solution for 1 min. This procedure was repeated to give a total of eight immersions in the wash solution. Incubations were done at 36°C in a 12 % $CO_2$/88 % air atmosphere. Solutions were made up in medium; the following concentrations of drugs were used: toxin, $3 \times 10^{-7}$ M; Pyridinium, $5 \times 10^{-5}$ M; *d*-tubocurarine, $1 \times 10^{-3}$ M; cycloheximide, $2.7 \times 10^{-4}$ M.

*Iontophoresis*

The distribution of acetylcholine sensitivity on the muscle cells was determined electrophysiologically. Micropipettes for intracellular recording were filled with 3 M KCl and had resistances of 70–150 MΩ; those for iontophoresis were filled with 2.7 M acetylcholine and had resistances of 100–300 MΩ. In some experiments, the acetylcholine was applied by using a feedback circuit (Nakajima and Cnodera, 1969; footnote 1); otherwise, a simple iontophoretic apparatus was used. A modified upright microscope (McBain Instruments, Inc., Chatsworth, Calif.) was used, with a 40× water immersion phase-contrast objective. All electrophysiology was done in medium. The pH of the medium was maintained by blowing a stream of water-saturated $CO_2$ across the surface of the medium. The cover slip with attached cells was placed on the bottom of a chamber that was warmed by passing warm water through a jacket surrounding it. The temperature of the fluid in the chamber varied from 35°C at the outside to 32°C in the center. In long experiments, some loss of liquid was noted, which was compensated for by adding distilled water to the chamber. Resting potentials of muscle fibers varied from −45 to −60 mV.

The distribution of acetylcholine sensitivity on single muscle fibers was determined by penetrating the cell with a KCl-filled micropipette, then applying iontophoretic pulses of acetylcholine to the muscle membrane. The acetylcholine sensitivity is defined as the peak membrane voltage response (in millivolts) divided by the quantity of charge (in nanocoulombs) passed through the acetylcholine pipette to deliver the acetylcholine that produced the response (mV/nC; see Miledi, 1960 a). The minimal spatial resolution of the iontophoretic technique is not known, but differences in sensitivity could be demonstrated at sites separated by less than 10 μm (see also Peper and McMahan, 1972). High resistance acetylcholine micropipettes must be used to obtain this high resolution, and the backing current must be carefully adjusted on each pipette (usually $1-2 \times 10^{-9}$ A was used) to prevent desensitization.

It should be emphasized that the assay for acetylcholine sensitivity only defines the relative sensitivities across the surface of a single fiber, for when the sensitivities of two fibers in the same culture dish are compared they may vary by 100-fold. For example, the absolute values for sensitivities varied from 16 to 960 mV/nC (peak-localized sensitivities), from 3 to 100 mV/nC (average sensitivities on fibers away from localization), and from 1 to 500 mV/nC (average sensitivities on muscle fibers grown alone). The variation is at least partly due to variations in fiber input impedances. This degree of variability prevented the comparison of "extra-contact" sensitivities on fibers to, for instance, the average sensitivity on muscle fibers that had not been cultured with nerve cells.

It was sometimes necessary to compare the acetylcholine sensitivities on different muscle fibers in a culture, in which case fibers of similar morphology were tested. When fibers of 20–40 μm diameter, 50–150 μm length, were selected, the variation in sensitivity between fibers was only 5–10-fold. Each point in Figs. 4 and 5 gives the average sensitivity of one muscle fiber in a culture. The difference in average sensitivities between similar fibers in a given culture can be estimated by examining the spread of points in each treatment group in Figs. 4 and 5.

## RESULTS

### Muscle Cells in the Absence of Nerve Cells

Before examining the distribution of acetylcholine sensitivity on muscle fibers cultured with nerve cells, it was necessary to establish the distribution on muscle fibers that had not been cultured with nerves. The results of one such mapping can be seen in Fig. 3 A. The average variation of sensitivity on the surface of a fiber that had not been cultured with nerve cells was about two-fold (Table I). In view of this variation, the minimal criteria for localization

TABLE I

VARIATION IN ACETYLCHOLINE SENSITIVITY BETWEEN POINTS ON THE SURFACE OF SINGLE MUSCLE CELLS IN VITRO

The column headed "cell group" gives the conditions in which the cells were grown. The "number of cells" given for muscle fibers alone is the total number of cells examined; for muscles cultured with nerve it is the number that showed localization of acetylcholine sensitivity (see Results). The "ratio of sensitivities" given is the ratio of the highest sensitivity found on a cell to the background sensitivity on that cell (mean ± standard deviation). The "number of sites per cell" is the number of different locations on the surface of a single cell that were tested with acetylcholine. "Success" is the ratio of the number of nerve-muscle pairs in a given cell group that were shown to have localized sensitivity to the number of pairs in that group that were chosen for study. (The site of contact between nerve and muscle had to be clearly visible, and the nerve process had to be traceable back to the cell body. The contact had to be situated in such a place that the sensitivity could be determined completely around the site; see Fig. 1.)

| Cell group | Number of cells | Ratio of sensitivities | | Number of sites tested/cell | | "Success" |
| | | Mean | Range | Mean | Range | |
| --- | --- | --- | --- | --- | --- | --- |
| L6 alone, group 1 | 75 | 1.9 ± 1.0 | 1.0-5.5 | 3 | 2-6 | — |
| L6 alone, group 2 | 28 | 1.8 ± 0.5 | 1.1-3.0 | 19 | 8-31 | — |
| L6/N18 in toxin | 2 | 13 | 6,20 | 20 | 14,25 | 1.0 |
| L6/N18 in Pyridinium | 7 | 13 | 5,40 | 13 | 9,17 | 0.7 |
| L6/N18 in toxin and Pyridinium | 11 | 10 | 5-30 | 17 | 6-26 | 0.6 |

of acetylcholine sensitivity were that there had to be a clear gradient of sensitivity on the surface of the muscle fiber, with a peak which showed at least a fivefold increase over the background sensitivity and which was located at a nerve-muscle contact (Fig. 1).

Nerve and muscle interaction was shown by a nonuniform distribution of acetylcholine sensitivity across the surface of the muscle fiber (e.g., Miledi, 1963 *a*). The possibility that the nonuniform distribution detected was a technical artifact was eliminated by the following observations and procedures. The close physical contact between nerve and muscle cells might block the dilution of acetylcholine by diffusion and produce a larger response.

FIGURE 1.    The interaction between nerve and muscle cells in vitro. The contact between nerve and muscle cells can be seen in the photomicrograph (arrow); the distribution of acetylcholine sensitivity on the muscle surface is shown in the graph below. The distance scale is the same for both representations of the interaction, and the photograph and graph are correctly aligned. Thus, the highest sensitivity was found at the site of contact. The approximate location of other detected sensitivities is on the muscle fiber directly above the position plotted on the graph. (Note that this reduces a two-dimensional distribution across the fiber to a one-dimensional plot. The distribution was symmetrical about the contact point.) This localization developed in the presence of both Pyridinium and toxin. Photomicrograph, × 430.

However, it was possible to pull the neuroblastoma off the muscle and still demonstrate an enhanced response. Also, the increased response was not present at every place where a nerve came in contact with a muscle. It was occasionally possible to produce an increased response if the tip of the acetylcholine pipette was placed on the surface of the cover slip at the edge of the fiber. To avoid this, points at the extreme edges of fibers were not mapped, and a clear gradient of sensitivity across the surface of the fiber had to be demonstrated. Since the delivery characteristics of the high resistance micropipettes used for iontophoresis may change, the current passed and the input impedance of the iontophoretic pipette were monitored throughout an experiment and the pipette was discarded if changes were seen. A further control was to always use the same current pulse amplitude and duration on any one cell. Finally, on each cell the pipette was occasionally returned to a site that had been tested before. If the two responses at that site differed by more than 25%, the data from that cell were not used.

### Localization Can Occur when Acetylcholine Receptors Are Blocked by α-Neurotoxin

Muscle cells were cultured with nerve cells in the presence of α-neurotoxin, which specifically inactivates acetylcholine receptors (see Materials and Methods). After the cells were washed extensively in toxin-free medium by the procedure shown in Fig. 2, acetylcholine sensitivity could be detected. Examples of localization were found (Fig. 3 B and Table I).

Muscle cells grown in the presence of toxin become sensitive to acetylcholine after being washed in toxin-free medium (Fig. 4). A number of possible mechanisms could account for this return of sensitivity. Toxin could dissociate from previously existing receptors. Alternatively, new receptors could be synthesized and inserted into the membrane or cryptic receptors could be activated (Hartzell and Fambrough, 1973). It is essential for the interpretation of the present results that the acetylcholine sensitivity detected after washing toxin-treated cells be due to receptors that had been present when the toxin was present. The procedure shown in Fig. 2 was designed to minimize the contribution of any newly synthesized or revealed receptors. Cycloheximide was used to reduce protein synthesis, and a curare wash was used to increase the rate of dissociation of bound toxin from the muscle fibers (Patrick et al., 1972). When this procedure was used, at least 90% of the sensitivity detected on cells grown in toxin resulted from toxin dissociating from receptors. This conclusion is based on the following data. It has been shown that toxin binds reversibly to the acetylcholine receptor (Patrick et al., 1972). The data shown in Fig. 4 demonstrate that acetylcholine sensitivity returned even when protein synthesis was blocked by cycloheximide. The return of sensitivity was slowed in the presence of cycloheximide, but eventually approximately equal
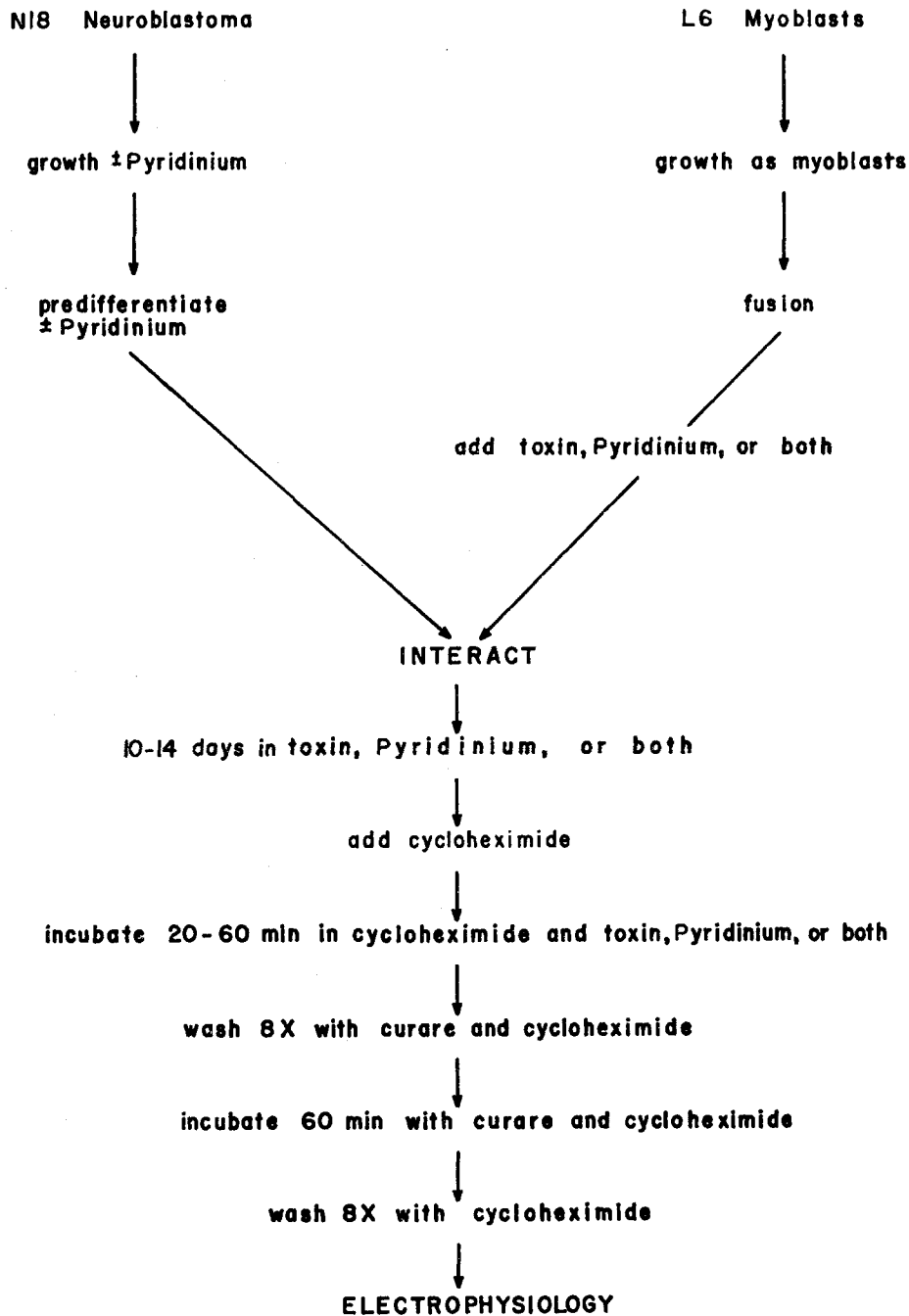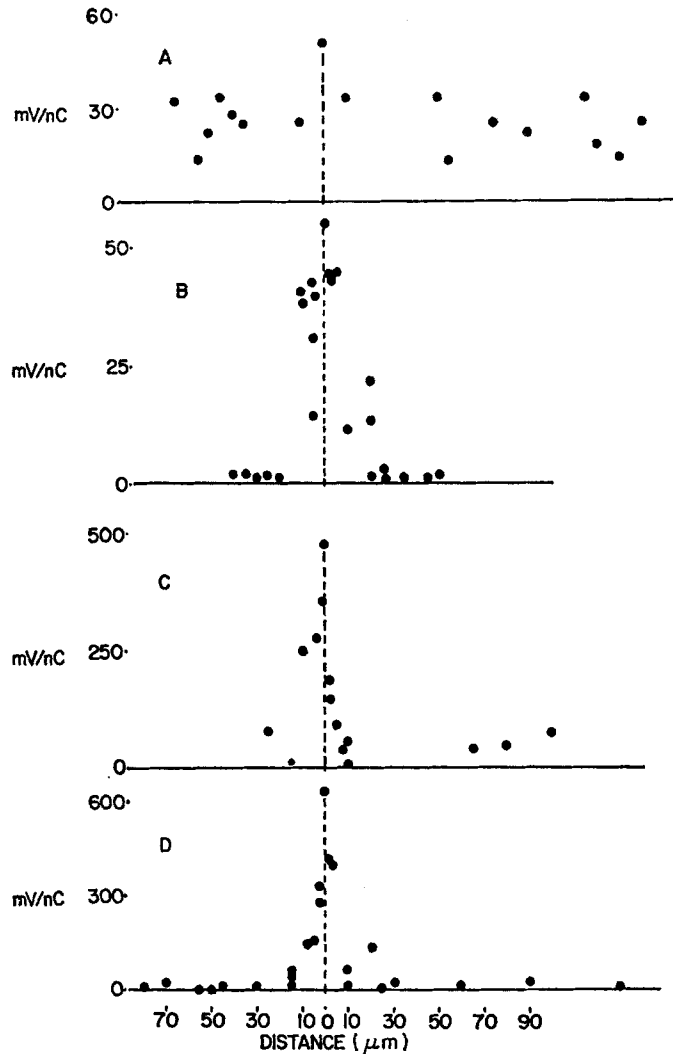
NI8    Neuroblastoma                                    L6    Myoblasts

growth ±Pyridinium                                   growth as myoblasts

predifferentiate                                          fusion
± Pyridinium

                    add  toxin, Pyridinium,  or  both

                              INTERACT

        10-14 days in toxin,  Pyridinium,   or  both

                          add  cycloheximide

incubate  20-60 min  in cycloheximide and  toxin, Pyridinium, or both

        wash 8X  with  curare  and  cycloheximide

    incubate  60 min  with  curare  and  cycloheximide

        wash 8X  with  cycloheximide

                      ELECTROPHYSIOLOGY

FIGURE 2.    The experimental protocol.

FIGURE 3.    The distribution of acetylcholine sensitivity on muscle fibers grown in vitro. The ordinate indicates the sensitivity of the muscle membrane in millivolts per nanocoulombs; the abscissa indicates the distance in micrometers between the point tested on the muscle surface that demonstrated the highest acetylcholine sensitivity and the point that demonstrated the plotted sensitivity. (A) Muscle fiber cultured without nerve cells, (B) nerve-muscle pair cultured in toxin, (C) pair cultured in Pyridinium, (D) pair cultured in both toxin and Pyridinium.

average sensitivities were reached in the presence or absence of cycloheximide. Prolonged treatment with toxin and cycloheximide (6 h) before washing with cycloheximide solution did not block the return of acetylcholine sensitivity. Cycloheximide had no direct effect on sensitivity, since treatment of muscle cultures with cycloheximide for up to 11 h did not change the average sensi-

tivity of cells in the cultures. No conclusion can be made from the slowing of the return by cycloheximide, since this could result from several mechanisms, for example, an effect of cycloheximide on dissociation of toxin from receptors or the prevention of new receptor synthesis. However, since the return of acetylcholine sensitivity is not blocked by cycloheximide or cold (see later), it probably does not require protein synthesis and, hence, mechanisms requiring receptor synthesis are unlikely. Finally, incubating toxin-treated cells with curare in the absence of protein synthesis increased the sensitivity detected by 10–20-fold (Fig. 5). This is consistent with data showing that curare increases the observed rate of dissociation of toxin-receptor complexes (Patrick et al., 1972). When muscle cells were washed with curare and cycloheximide (no toxin) for 1 h, a slight decrease in acetylcholine sensitivity was seen, so a curare wash did not directly increase the acetylcholine sensitivity.

### Localization Can Occur when Acetylcholine Release Is Blocked

Acetylcholine release was prevented by inhibiting the synthesis of acetylcholine. Clone N18 was chosen because the level of choline acetyltransferase present in the cells is comparable to that in fibroblasts (Amano et al., 1972; Schubert and Jobe, unpublished observations). The synthesis of acetylcholine was further reduced by Pyridinium, and any stores of acetylcholine were depleted before nerve and muscle cells were cultured together (see Materials and Methods). Nerve and muscle cells were cultured together in Pyridinium, and then washed as shown in Fig. 2. Localization of acetylcholine sensitivity was found (Fig. 3 C and Table I).

Finally, nerve cells were treated with Pyridinium as described, and nerve and muscle cells were cultured together in the presence of both toxin and Pyridinium. The cells were washed as described, then examined for localization. Many examples of localization were found (Fig. 1, Fig. 3 D, Table I).

### Localization Is Likely To Have Occurred While Toxin and Pyridinium Were in the Medium

It is possible that both the signal and the ensuing localization could have occurred after toxin and Pyridinium were washed from the cultures but before the distribution was determined. It is reasonable to assume that an area of raised acetylcholine sensitivity on a muscle cell is due to the presence of an increased density of acetylcholine receptors in that part of the muscle surface membrane (e.g., Berg et al., 1972; Hartzell and Fambrough, 1972). Two basic mechanisms could produce such a localized increase in receptor density: there could be a local change in receptor metabolism or there could be a redistribution of previously existing receptors. Either of these general mechanisms should be slowed or blocked by lowering the temperature of the culture (Frye and Edidin, 1970; Conconi et al., 1966). Accordingly, nerve and muscle cells were cultured together in the presence of toxin and Pyridinium for 10–14 days.
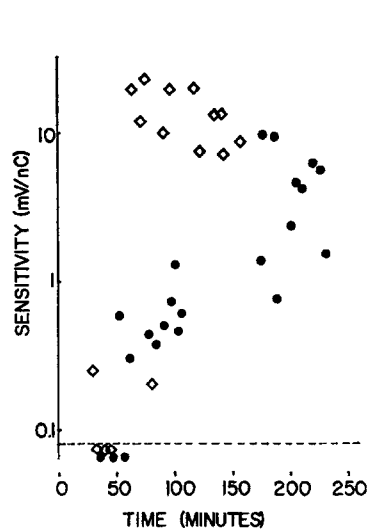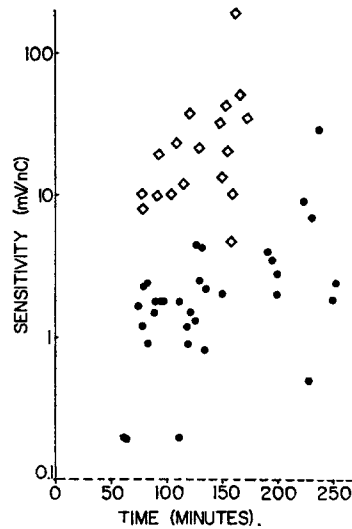
FIGURE 4
FIGURE 5

FIGURE 4. The effect of cycloheximide on the return of acetylcholine sensitivity on toxin-treated muscle fibers. Cultures of muscle fibers were treated with toxin for 1 h, then cycloheximide was added to the incubation medium of one culture. Both cultures were incubated 30 min longer, washed in toxin-free medium plus or minus cycloheximide, then the acetylcholine sensitivity of different muscle fibers was determined at various times after the wash (see Materials and Methods). The dotted line at a sensitivity of 0.08 mV/nC indicates the lowest detectable sensitivity. Solid circles, cells washed in the presence of cycloheximide; diamonds, cells washed in the absence of cycloheximide. FIGURE 5. The effect of washing toxin-treated muscle fibers with curare on the return of acetylcholine sensitivity. Cultures of muscle fibers were treated with toxin for 16 h, then cycloheximide was added to the incubation medium of both cultures. The cultures were incubated 30 min longer, then washed with toxin-free medium plus cycloheximide, and plus or minus curare. They were incubated in those respective solutions for 60 min, then washed with medium plus cycloheximide. The dotted line at 0.1 mV/nC indicates the lowest detectable sensitivity. Solid circles, cells washed and incubated without curare; diamonds, cells washed and incubated with curare.

Cycloheximide was added, the cultures were incubated 30 min longer, then washed at 4°C with medium containing cycloheximide and curare. They were incubated in that solution at 4°C for 2–4 h, then washed at 4°C with medium plus cycloheximide. The distribution of acetylcholine sensitivity was determined with cultures in medium containing cycloheximide cooled to 12°C. Under these conditions, localization was still found to occur (Fig. 6). Thus, it is likely that the localization found was established when the toxin and Pyridinium were in the medium.

DISCUSSION

The above results show that neither the presence of physiologically active acetylcholine receptors in the muscle, nor the release of acetylcholine from
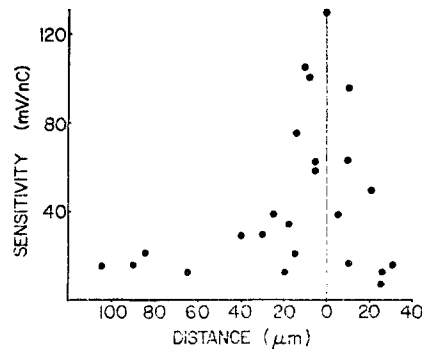
FIGURE 6. The distribution of acetylcholine sensitivity on a fiber washed and assayed at lowered temperature. The figure is similar to Fig. 3, except for the treatment of the culture during the time when toxin and Pyridinium were removed from the culture medium (see text for details).

the nerve cell, are required for the events that result in localization of acetylcholine sensitivity to a nerve-muscle contact in vitro.

The production of an area of raised acetylcholine sensitivity could precede or follow a contact between a nerve and that muscle. The possibility that there are muscle-determined sites for innervation of muscle has been raised (e.g. Miledi, 1960 b; Landmesser, 1972). For instance, during reinnervation of denervated adult muscle, the region around the old end plate appears to be preferentially innervated (Miledi, 1960 b; Csillik, 1967; Landmesser, 1972), but it is also possible to form new end plates away from the old sites (Guth and Zalewski, 1963; Fex et al., 1966; Saito and Zacks, 1969). Thus, the possibility of muscle-determined sites for innervation exists, but the sites are clearly not unique even on single terminally innervated muscle fibers. It has been suggested that a high degree of acetylcholine sensitivity might be required for a muscle to be innervated (Katz and Miledi, 1964) since the two phenomena are associated (e.g., Miledi, 1963 b; Fex et al., 1966). There is evidence that muscle fibers in primary cultures of chicken embryo tissue can generate areas of increased acetylcholine sensitivity (Cohen and Fischbach, 1971) or receptor density (Sytkowski et al., 1973) in the absence of nerve cells. There is no evidence at present that muscle fibers formed by the fusion of L6 myoblasts do generate such areas in the absence of nerve cells. Moreover, the present results show that if the muscle does generate such areas of raised sensitivity, the presence of physiologically active receptors is not required for the nerve to preferentially contact the muscle at that site. These results do not rule out the possibility that some other muscle specialization associated with a high acetylcholine sensitivity might result in such a preferential nerve contact.

Qualitatively, there is an increase in acetylcholine sensitivity on the surface

of a muscle at the neuromuscular junction in vivo, and at the site of contact between nerve and muscle in vitro, but the quantitative enhancement in vitro is less. Typically, the ratio of junctional to extrajunctional sensitivity on mammalian muscle in vivo is about $10^5$ (Albuquerque and McIsaac, 1970). The gradient of sensitivity across the muscle surface is extremely steep at the frog neuromuscular junction, falling by almost 100-fold within 10–20 $\mu$m from a branch of a nerve (Peper and McMahan, 1972). However, the gradient of sensitivity in vitro is steeper, and for the distances mapped, the ratio of localized to nonlocalized sensitivity is higher, than those reported for the distribution of acetylcholine sensitivity of fetal rat muscle (Diamond and Miledi, 1962). The situation in vitro may be analogous to the situation in embryo since the muscle fibers have never been innervated before, and have been cultured with nerves for a relatively short period of time.

There is no evidence at present for chemical transmission between nerve and muscle cells from clonal lines. However, Heinemann and Kidokoro[1] have shown that muscle fibers formed from L6 myoblasts can make cholinergic junctions with neurons from fetal rat spinal cord. In any case, the results given in this paper, and other results obtained in vivo (see below), indicate that cholinergic transmission is not required for the localization of acetylcholine sensitivity.

The events leading to localization can be placed in two phases: an interaction between the two cells that localizes the site of contact and a process in the muscle that localizes the acetylcholine sensitivity. The question as to which occurs first is left open. A number of likely candidates for the intercellular interaction phase can be proposed, and it is valuable to consider the present results in relation to these possible interactions.

First, there could be some diffusible substance released from the nerve that acts on a component in the muscle. It has been suggested that acetylcholine (Thesleff, 1960; Drachman, 1967) or some undefined "trophic substance" (Miledi, 1963 a) may serve in this type of interaction.

Second, the electrical activation of the muscle membrane by the nerve, or the resulting contractile activity, could mediate the interaction (Lømo and Rosenthal, 1972; Drachman and Witzke, 1972).

Third, it is possible that there is a surface component interaction between the nerve and the muscle cell. This general type of intercellular interaction is thought to occur in sponge cell reaggregation (Humphreys, 1963) and during the induction of the immune response (Kreth and Williamson, 1971).

The last alternative considered here is that the interaction takes the form of the passage of a diffusible substance between the two cells through a low resistance junction. Some metabolic coupling between fibroblasts in culture is mediated by such junctions (Gilula et al., 1972). Close junctions were seen in

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[1] Heineman, S., and Y. Kidokoro. Manuscript in preparation.

an electron microscope study of developing rat neuromuscular junctions (Kelly and Zacks, 1969). In an electron microscope study of developing junctions in cultures of chick embryo tissue, no tight junctions were seen (James and Tresman, 1969), but electrophysiological evidence has been presented showing that low resistance junctions can occur between chick nerve and muscle cells in primary cultures (Fischbach, 1972).

What conclusions can be made about the applicability of these proposed interactions to this in vitro system? The diffusible substance is not acetylcholine. The effect on the muscle membrane is not the activation of the acetylcholine receptor. Cholinergic synaptic activity is not required for localization, and further studies have ruled out muscle electrical or contractile activity as a requirement for the interaction.[2] If there is an interaction between surface components, it is probable that the acetylcholine receptor is not involved since it was blocked (although reversibly) by bound toxin. The role of gap junctions in the events leading to localization has not been studied, but electrical coupling between neuroblastoma and muscle cells has been noted in vitro (Harris et al., 1971).

These results obtained with cells from clonal lines are in agreement with other results which suggest that actual cholinergic transmission is not required in the formation of neuromuscular junctions (Cohen, 1972) or in the formation or maintenance of localized acetylcholine sensitivities (Miledi, 1960 b; Duchen and Stefani, 1971; Albuquerque et al., 1972; Drachman and Witzke, 1972; Lømo and Rosenthal, 1972).

In conclusion, no known aspect of the cholinergic transmission system is required for the events that result in the localization of acetylcholine sensitivity in vitro, and it is necessary to test other possible intercellular interactions.

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