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THE DISTRIBUTION OF ACETYLCHOLINE RECEPTORS ON MUSCLE FIBRES OF REGENERATING SALAMANDER LIMBS

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SUMMARY

1. Muscle fibres developing during limb regeneration were examined for responsiveness to acetylcholine (ACh) applied iontophoretically along the membrane.

2. Fibres which were uninnervated as well as those with non-transmitting synapses had high over-all sensitivities, with only minor variations along their length.

3. Functionally innervated fibres in which depolarization did not yet elicit action potentials had high over-all sensitivities, even when the synaptic potentials had amplitudes of 40–50 mV. In these the membrane in the vicinity of synapses tended to have sensitivities above the background level.

4. Upon the appearance of action potentials, several weeks after fibre innervation, the responsiveness to ACh began to decline in synapse free regions of the membrane. In mature muscle the sensitivity to ACh is restricted to sites of synaptic contact.

INTRODUCTION

On normal skeletal muscle fibres ACh receptors are localized in the vicinity of synapses, whereas on denervated and developing fibres they are distributed over the entire surface. Most investigations of the means by which innervation influences the post-synaptic sensitivity have focused either on changes in receptor distribution caused by denervation and reinnervation of adult muscle (for recent reviews see Harris, 1974; Lømo & Westgaard, 1975*b*; Purves, 1976; Gutmann, 1976) or on the influence of innervation on muscle fibres developing in culture (Shimada & Fischman, 1973; Fambrough, 1974; Fischbach, 1974; Fischbach, Berg, Cohen & Frank, 1975). The intent of the present study was to ask when during the course of development and innervation of muscle fibres *in vivo* the ACh

receptors assume their adult disposition. The particular developmental system that we used, regenerating limbs of adult newts, has the virtue that a period of 2–3 weeks intervenes between the time at which functional synapses develop on the fibres and the time at which they begin to develop action potentials (Dennis, 1975). The temporal separation of these two events permitted us to ask whether one or the other had a primary influence over the distribution of receptors on the fibre surface. We found that the decline of extrajunctional ACh receptors began at about the time that action potentials appeared.

METHODS

The regenerating muscle fibres studied were those of a forelimb flexor (m. humeroantibrachialis) from the eastern red-spotted newt *Notopthalmus viridescens*. The techniques of amputation, animal care, dissection, nerve stimulation, intracellular recording and ACh iontophoresis were described previously (Dennis, 1975; Dennis & Ort, 1975).

ACh sensitivity mapping. Electrodes for iontophoresis were filled with 2 M-ACh chloride and had resistances of 200–500 MΩ. ACh was applied by passing 2 msec positive current pulses, the amplitudes of which were measured by a monitor that held the bath at virtual ground. When necessary a small negative backing current (about 10^{-9} A) was applied to prevent diffusion of ACh from the electrode tip.

The focal ACh sensitivity of individual fibres was measured at intervals of 10–25 μm along a length of 400 μm, which is the diameter of the visual field with the optics used. The segments to be mapped were selected according to where this length of any one fibre was accessible, which could occur in either the middle or end regions. Occasionally longer segments of a single fibre were mapped by withdrawing the recording electrode, shifting the field of view and repenetrating the fibre.

No compensation has been made in our analysis for differences in resting potential or input resistance between fibres; however, measurements were made only on fibres with resting potentials of −90 mV or greater. With the high resistance ACh pipettes and brief iontophoretic pulses used, the minimum sensitivity that we could reliably resolve was 2 mV/nC; all sensitivity values of less than 2 mV/nC have been plotted between 1 and 2 mV/nC. The spatial resolution of this mapping technique was estimated to be 5–10 μm.

Stages of fibre innervation. The various phases of innervation of fibres examined in this study have been described previously (Dennis, 1975) and are summarized here. When muscle fibres first appear some seem by physiological criteria to be uninnervated. The first sign of junction formation is the appearance in the fibre of small spontaneous synaptic potentials of low frequency, which usually occurs before development of stimulus-evoked synaptic potentials; this is referred to as the non-transmitting stage of innervation. Later in development, stimulation of the motor nerve trunk elicits a single end-plate potential of low quantal content. Still more mature fibres receive synaptic input from as many as four motor neurones, with large excitatory junctional potentials (e.j.p.s).

Development of action potentials in regenerating fibres. It was noted previously (Dennis, 1975) that immature muscle fibres exhibit a long slow depolarizing potential change, lasting hundreds of milliseconds, in response to synaptic or direct depolarization (Fig. 1*A*). This response sometimes has an amplitude as great as 60 mV above rest, yet does not resemble the action potentials elicited in normal adult

fibres. The ion flux(es) which gives rise to this response is not known. During a maturation period which takes 2–4 weeks, the long depolarizing response gives way to a normal-looking action potential (Fig. 1 *D*). In adult fibres the action potentials have thresholds 15–25 mV above the resting level and amplitudes ranging from 80 to 110 mV. The change from depolarizing response to action potential is not a discrete one, and occasionally developing fibres are encountered which give an intermediate response (Fig. 1 *B* and *C*), suggesting that the membrane is undergoing a

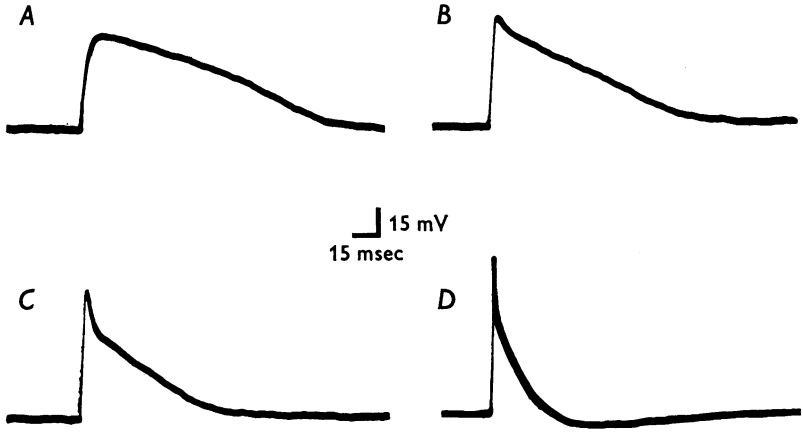

Fig. 1. Intracellularly recorded responses of muscle fibre membrane to synaptic depolarization. *A*, long lasting e.j.p. recorded in immature fibre. *B*, long lasting e.j.p., but shorter in duration and with more pronounced peak than in *A*. *C*, action potential (AP) with residual depolarization recorded in more mature fibre. *D*, action potential recorded from normal adult fibre. Calibration marks indicate 15 mV (vertical) and 15 msec (horizontal).

transition in the character of its potential-dependent ionic channels. Because of these intermediate responses, fibres could not easily be categorized as having or not having an action potential. Furthermore, the maximum rate of rise of depolarization did not change markedly from immature to mature fibres, and neither immature nor mature electrical responses were sensitive to tetrodotoxin. For the purpose of correlating the electrogenic activity of a muscle fibre with the distribution of ACh receptors on its surface, the rate of repolarization of the response from its initial peak proved a useful means of identifying fibres that gave action potentials. The chief disadvantage of this criterion is that there always occurs some residual depolarization from the synaptic potential, even in adult fibres. Thus, a fibre was said to give an action potential if the response fell by one third from its maximum value within 10 msec (as in Fig. 1*C* and *D*). All responses having a slower initial rate of repolarization were considered to have an immature electrical response (as in Fig. 1*A* and *B*).

RESULTS

Focal application of ACh at any point along a developing muscle fibre or near the end-plate region of an adult fibre resulted in a monophasic depolarization of the muscle membrane. Neither multiphasic nor

hyperpolarizing responses were ever observed (see Steinbach, 1975). These focal ACh responses, as well as the nerve evoked responses, were reversibly blocked by addition of D-tubocurarine (10^{-5} g/ml.) to the bathing medium, and irreversibly blocked by the addition of 4×10^{-6} g/ml. of α-bungarotoxin.

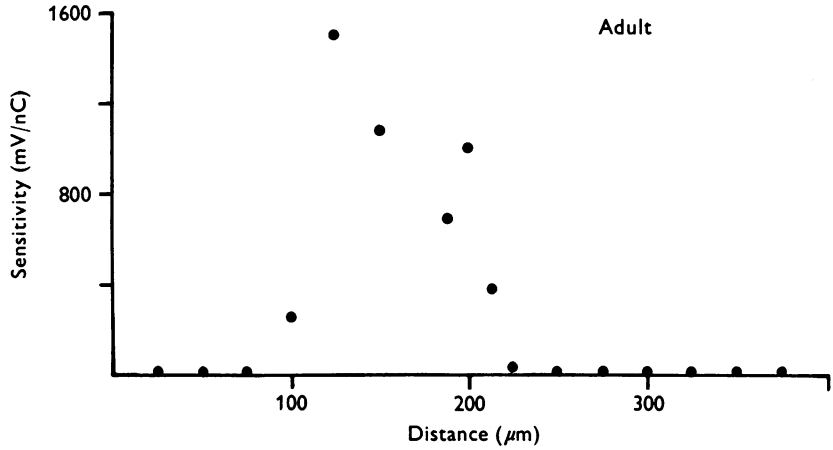

Fig. 2. ACh sensitivity distribution along a normal adult fibre. Abscissa: distance along the fibre. Points indicate sensitivity of the membrane at each locus to iontophoretically applied ACh. The region from 100 to 200 μm has high sensitivity characteristic of regions of neuromuscular junction.

Normal adult fibres. Regions of high ACh sensitivity on adult fibres were confined to the area of the neuromuscular junction. The distribution of values measured on a typical normal fibre is illustrated in Fig. 2. In the vicinity of a motor nerve ending, as judged by the termination of the myelin sheath, the maximum junctional sensitivity was 1500 mV/nC, while away from the nerve ending it fell below 2 mV/nC. The region of high sensitivity was approximately 100 μm in length. The sensitivities measured along seventeen normal adult fibres are presented in Fig. 3*D*. The large number of positions with sensitivities of less than 2 mV/nC on these mature fibres reflects the low density of ACh receptors of the extra-junctional region.

Fibres without transmitting synapses. The ACh sensitivity of fibres without functional synaptic transmission, including both those uninnervated and those with non-transmitting synapses, was generally high over the entire membrane surface (Fig. 3*A*). There was some variability of maximum sensitivity from fibre to fibre, in the range of 1000–7000 mV/nC, presumably due in part to variation in their input resistances. The lowest sensitivity found (70 mV/nC) was considerably higher than the lowest

sensitivities encountered in mature fibres. There was also variation of sensitivity along the length of individual fibres over a roughly fourfold range, as can be seen by comparing the values within individual columns of Fig. 3*A*. Even if all of this variability in sensitivity were due to heterogeneity in the distribution of ACh receptors, it is not as extreme as that observed on uninnervated myofibres developing in tissue culture; there the sites of highest responsiveness ('hotspots') are up to ten times more sensitive than the other areas of the membrane (Fischbach & Cohen, 1973; Fischbach *et al.* 1975; Prives, Silman & Amsterdam, 1976). The variation we observe must result at least in part from inaccuracies inherent in the measurement technique, and to that extent cannot be assumed to reflect quantitatively variation in receptor density.

Fibres with e.j.p. only. Fibres in which maximal nerve stimulation elicited an immature electrical response, e.j.p. without an action potential, had generally high ACh sensitivities. The sensitivities measured along thirty such fibres are shown in Fig. 3*B*, and the spatial distribution of sensitivities along a typical fibre is presented in Fig. 4. Many points showed very high sensitivity (> 1000 mV/nC), while few loci had values below 300 mV/nC. The amplitudes of the e.j.p.s in these fibres ranged from 10 to 65 mV, and those over 30 mV usually had prolonged time courses (as in Fig. 1*A*). As was the case in fibres without transmission, there was some variation in sensitivity along the length of individual fibres. Thus fibres at this stage of maturation show no generalized reduction of ACh sensitivity correlated with the establishment of functional neuromuscular junctions.

In fibres with transmitting synapses, such as in Figs. 3*B* and 4, it was of interest to know whether the spots of high sensitivity occurred at sites of synapse formation. Unfortunately, it was not possible to locate the nerve terminals by visual means. However, in some cases the location of functional synapses could be determined by making use of the fact that increases in bath osmolarity increase spontaneous transmitter release from nerve terminals (Fatt & Katz, 1951). A pipette filled with 2·5 M sucrose, broken to a tip diameter of 3–6 μm and attached to a pressure injection apparatus was lowered on to the fibre surface. Brief application of pressure ejected sucrose and produced a localized, transient rise in osmolarity. Sucrose so applied over the end-plate region of a normal muscle fibre produced an immediate increase in the frequency of spontaneous miniature junctional potentials. Similar osmolarity increases produced no effect in the extrajunctional region of adult fibres or over large regions of membrane of intermediate stage fibres. Using the criterion of a response within 500 msec of the onset of pressure, the spatial resolution of this osmolarity mapping was about 20–25 μm. In fibres tested by

this technique and mapped for ACh sensitivity, the sites of positive response to sucrose were always the same as areas of high ACh sensitivity (seventeen sites on eleven fibres). One example of this is presented in Fig. 4. On the other hand, some spots of high ACh sensitivity showed no

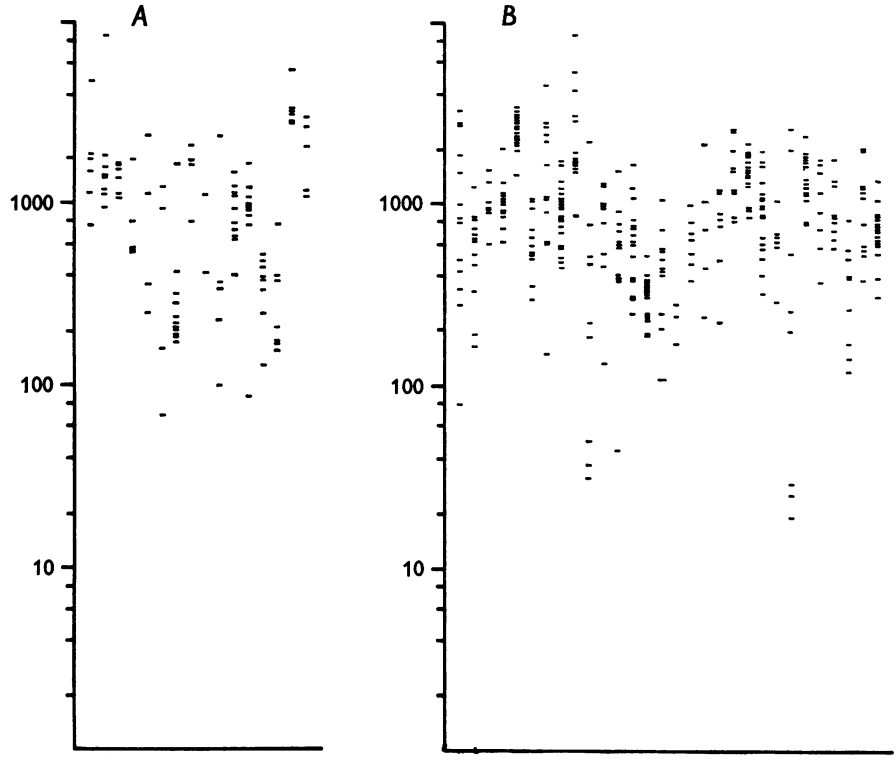

Fig. 3. ACh sensitivities of individual muscle fibres at various developmental stages. Ordinate: sensitivity in mV/nC on a log scale. Each vertical column represents sensitivities measured along a single muscle fibre. Loci with values below 2 mV/nC are indicated at the bottom of the column. *A*, sensitivities of developing fibres in which maximal nerve stimulation evoked no response. The first eleven fibres were uninnervated, the last five were in the non-transmitting stage of innervation. *B*, sensitivities from developing fibres in which maximal nerve stimulation elicited an e.j.p. without an action potential. *C*, values from developing fibres in which nerve stimulation elicited an action potential. *D*, sensitivities from normal adult fibres.

signs of innervation by this test, indicating that not all the areas of high ACh sensitivity are associated with synapses.

Thus, two features of the receptor distribution at this stage are noteworthy. Firstly, there seems to have been no decline in sensitivity of the fibre membrane away from sites of synaptic contact, even though the

transmission is often well developed. Secondly, synapses are associated with regions of membrane with the highest sensitivity. The latter conclusion at first appears to conflict with the observation in the preceding section, that uninnervated fibres show no great heterogeneity in receptor

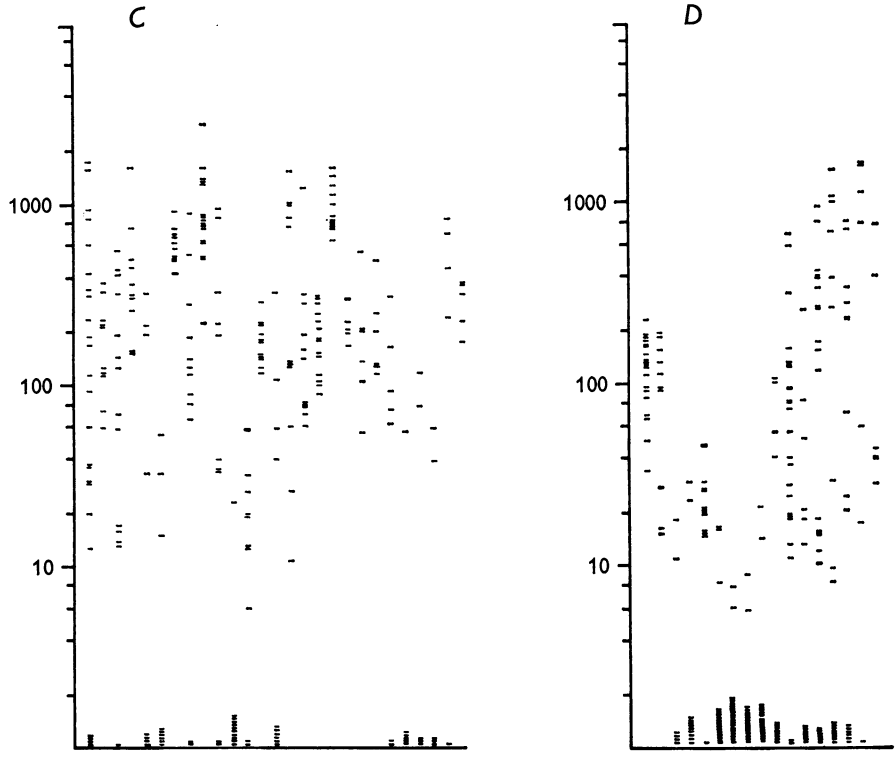

Fig. 3*C* and 3*D*. For legend see facing page.

distribution. However, recent work has shown lateral mobility of ACh receptors in the membranes of cultured *Xenopus* muscle fibres, with accumulation of those receptors in the vicinity of newly formed synapses (Anderson & Cohen, 1976). It seems likely that such a process may also occur during innervation of the fibres studied here.

Fibres with action potentials. The pattern of ACh sensitivity on fibres which had matured sufficiently to give action potentials was, in most instances, different from the earlier stages in that the sites of high sensitivity were frequently restricted to discrete regions (Fig. 3*C*). On such fibres a greater proportion of the sites tested had sensitivities below 300 mV/nC in comparison to less mature fibres, and many had values below 10 mV/nC. When these fibres were tested with focal sucrose application,

the synaptic sites were again always associated with regions of high sensitivity.

The data presented in Fig. 3 suggest that a pattern of ACh sensitivity like that of the adult begins to emerge at the time that muscle fibres

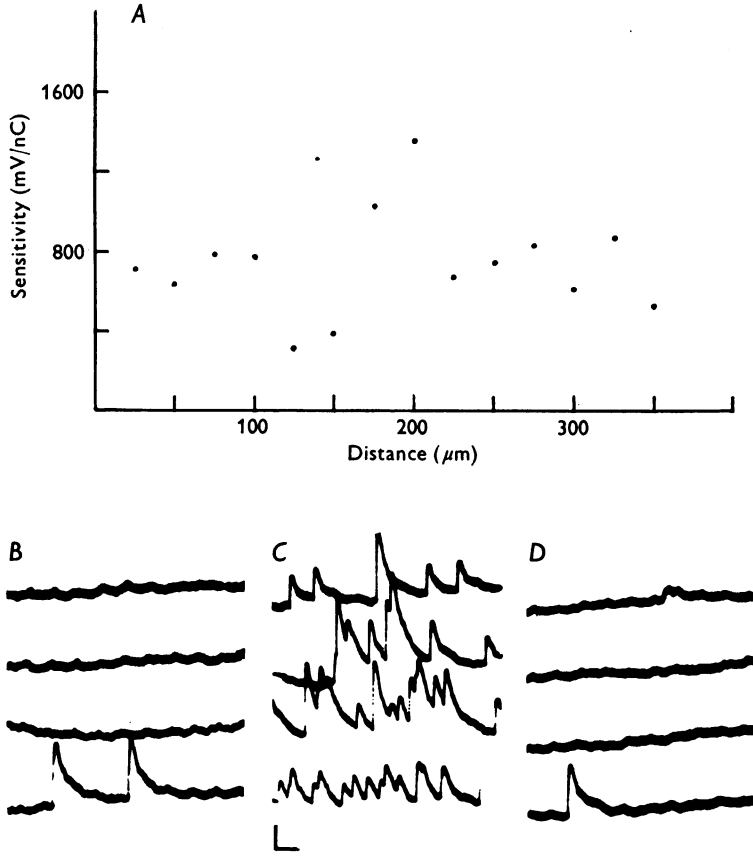

Fig. 4. Correlation of ACh sensitivity distribution and synapse location on a developing muscle fibre in which maximal nerve stimulation evoked a 45 mV e.j.p. with no action potential. *A*, map of ACh sensitivity *vs.* distance along fibre, with conventions as in Fig. 2. *B–D*, successive oscilloscope traces showing frequency of spontaneous miniature synaptic potentials. *B*, normal frequency of spontaneous potentials. *C*, frequency of spontaneous potentials during focal application of sucrose at the 200 μm position of the map shown in *A*. This response to sucrose application indicates a functional synapse is located within 25 μm of the tip of the sucrose pipette. *D*, frequency of spontaneous potentials during focal increase in osmolarity at the 325 μm position of the map in *A*. The frequency here is essentially the same as in control, indicating the absence of a superficial synapse near this point of sucrose application. Calibration marks indicate 2 mV (vertical) and 20 msec (horizontal).

develop the ability to generate action potentials. This change is more obvious when the sensitivity measurements from Fig. 3 are grouped into histograms, as in Fig. 5. Before the development of the normal action potential mechanism the post-synaptic membrane showed generally high ACh sensitivity (> 1000 mV/nC). At the time of development of action potentials, in contrast, areas of low sensitivity (< 100 mV/nC) appear.

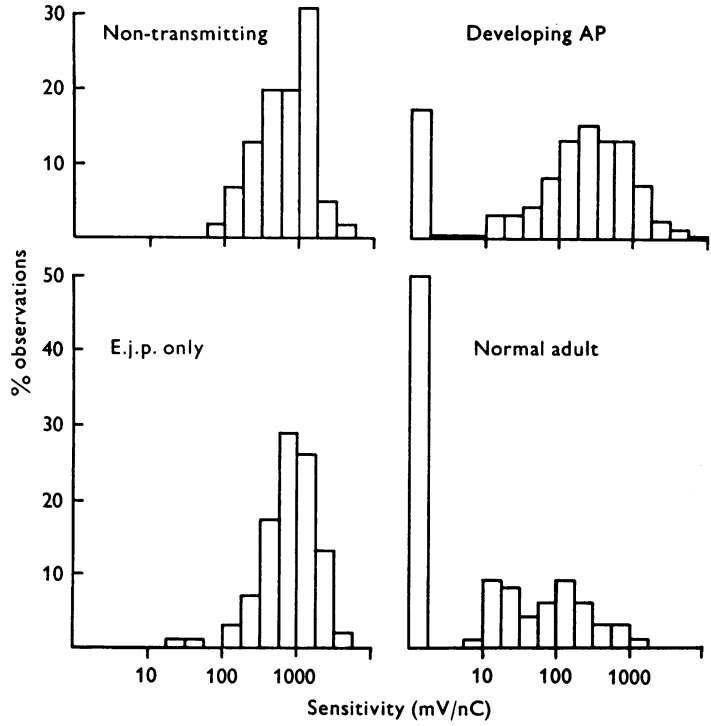

Fig. 5. Histograms of ACh sensitivities at four developmental stages. Data taken from Fig. 3. Ordinate: % total observations in each interval. Abscissa: sensitivity in mV/nC on a log scale. Note the trend toward lower sensitivity values in the stage in which action potentials are first appearing.

DISCUSSION

The experiments reported here were undertaken to determine the stage at which muscle fibres developing *in vivo* assume a transmitter sensitivity pattern characteristic of innervated adult muscle, and further to ask whether the maturation of this membrane property can be correlated with other membrane changes. We show a positive correlation between the loss of extrajunctional ACh receptors and the appearance of action potentials in the developing fibres. The strength of this correlation

is increased by the fact that other potentially relevant changes, specifically the establishment of nerve-muscle contact and the appearance of synaptic transmission, occur several weeks earlier than the first signs of recession of supersensitivity. The most plausible interpretation of our results is that the appearance of action potentials in the maturing muscle fibres occurs in association with the restriction of ACh receptors to the vicinity of the synapse. Action potential activity has similarly been indicated as the primary factor influencing membrane sensitivity in adult muscle (Lømo & Rosenthal, 1972; Drachman & Witzke, 1972; Cohen & Fischbach, 1973; Purves & Sakmann, 1974; Berg & Hall, 1975; Lømo & Westgaard, 1975*a*, *b*). An alternative interpretation of our observations is that the process of receptor recession is initiated upon innervation but takes a few weeks to be seen. Although our results cannot exclude this possibility, it seems unlikely, especially in light of the fact that recession of extrajunctional receptors begins within 2–3 days of direct electrical stimulation of denervated rat muscle (Lømo & Westgaard, 1975*b*).

The lack of temporal correlation between fibre innervation and sensitivity recession resembles events in several other developing systems. In neonatal rat diaphragm the loss of extrajunctional ACh sensitivity occurs in the first few weeks after birth, even though fibre innervation occurs at least 4 days before birth (Diamond & Miledi, 1962). Tadpole tail muscle transplanted to developing limb buds of bullfrogs first shows ACh sensitivity localization many days after functional reinnervation (Letinsky, 1975). During reinnervation of mouse skeletal muscle supersensitivity to ACh persists until nerve stimulation is capable of evoking action potentials in the muscle fibres (Tonge, 1974). All of these observations are consistent with the proposal that it is not the establishment of synaptic transmission but rather the development of some level of electrical activity in the muscle fibres which dictates the distribution of receptors on the membrane.

Another developmental question raised here is how the specific sites of synapse formation are determined. Synapses might form at random points of contact between nerve and muscle, or the nerve might grow to some specific locus on the muscle membrane such as a region of high transmitter sensitivity. The latter possibility was strengthened by the observation that uninnervated chick fibres developing in culture have regions where the ACh sensitivity is 5–10 times above background levels, referred to as 'hot spots' (Fischbach & Cohen, 1973; Prives *et al.* 1976). Here we observe some heterogeneity of sensitivity along uninnervated fibres, but the range of variation (two- to fivefold) is about at the level of reproducibility of our measurement technique. Thus in this system there is little indication of sites of particularly high receptor density on uninnervated

muscle fibres to which motor nerve terminals might grow. Subsequent to innervation muscle membrane in the vicinity of newly formed synapses does tend to have the highest levels of sensitivity.

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