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PHYSIOLOGICAL PROPERTIES OF JUNCTIONS BETWEEN NERVE AND MUSCLE DEVELOPING DURING SALAMANDER LIMB REGENERATION

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SUMMARY

1. Physiological properties of developing nerve–muscle junctions were studied in regenerating limbs of adult salamanders.

2. During the period of synapse formation the muscle fibres had diameters of 4–10 μm, resting potentials of -90 to -100 mV and input resistances of 10–50 MΩ. Some, but not all, pairs of adjacent muscle fibres were electrically coupled.

3. At the stage when muscle fibres could first be identified, some of them were not innervated, at least as determined by electrophysiological criteria.

4. During muscle innervation the neuromuscular synapses were encountered in several intermediate phases of maturity.

(i) At the least mature junctions small spontaneous synaptic potentials occurred, but stimulation of the motor nerve trunk did not evoke synchronous transmitter release.

(ii) At other junctions maximal nerve stimulation evoked only a single end-plate potential of low quantum content.

(iii) More mature fibres received synaptic input from as many as four motor neurons, which could be distinguished by their discrete stimulus thresholds.

5. During this period of synapse development the fibres lacked an action potential but often showed a prolonged response to depolarization.

6. Fibres in normal adult muscles had from one to three synaptic inputs, were not electrically coupled, and responded to depolarization with an action potential.

INTRODUCTION

A series of characteristic physiological changes occurs during the reinnervation of adult muscles and ganglia (see reviews by Fishbach, 1974; Harris, 1974; Guth, 1968) and during the development of synaptic contacts

in tissue culture (see reviews by Fishbach, 1974; Shimada & Fishman, 1973). At present there is little information about the events that occur as tissue is innervated during normal development. In skeletal muscle fibres of neonatal rats, Diamond & Miledi (1962) found that acetylcholine sensitivity became restricted to the end-plate region some time after synaptic transmission developed. Also there are indications that the number of motor axons innervating a single mammalian skeletal muscle fibre becomes reduced from several to one during the first few weeks after birth (Redfern, 1970; Bagust, Lewis & Westerman, 1973). During regeneration of the walking leg of a crab, those synapses which are present in the most immature muscles tend to be small, with fluctuating responses of low quantal content and little facilitation, whereas the synapses which develop later are relatively large and show greater facilitation (Govind, Atwood & Lang, 1973). However, it is not clear what transient physiological stages appear prior to assumption of adult synaptic behaviour in that system.

In the present study I have developed a system which can be used to further examine the appearance and maturation of synaptic contacts *in vivo*, and to determine the influence of this innervation on the physiology of the post-synaptic cell. Urodele amphibia are capable of regenerating limbs by development from undifferentiated cells (Thornton, 1938). The regenerating salamander limb thus provides a source of developing muscle fibres which have not been previously innervated. It is shown here that one can record from such fibres at several stages of synaptic maturation.

METHODS

Aquatic newts, *Notopthalmus viridescens*, were obtained from Mr Glen Gentry, Donelson, Tennessee. They were held in tanks of tap water containing 5% frog Ringer and fed pieces of liver once or twice weekly. Animals of either sex were anaesthetized in tap water containing 1% ether, and the right foreleg cut off at the shoulder. After the operation they were immediately returned to their tanks and maintained as usual while the new limb developed.

At the appropriate stage of regeneration the animal was pithed and the entire new limb was removed along with the central trunks of the 3rd, 4th and 5th nerve. The skin was dissected off to reveal the immature fibres of the humero-antibrachialis muscle (Francis, 1934), in close contact with the developing humerus. The muscle, with bone, was mounted in a glass bottom chamber, and the trunks of the three nerves sucked into one electrode for stimulation. The muscle was viewed with Zeiss–Nomarski optics, using 12·5× oculars and a 40× water immersion objective which had been electrically isolated from the microscope body.

The muscle was bathed in frog Ringer of the following composition (in m-mole/l.): NaCl, 120; KCl, 2; $CaCl_2$, 1·8; glucose, 10. Buffer was usually omitted to permit addition of $LaNO_3$ to the medium without precipitation, although a 4 mM sodium phosphate buffer, pH 7·2, was sometimes included. The calcium concentration was usually raised, as indicated in Results.

Electrical recording from the muscle fibres was accomplished with glass micro-

pipettes, filled with 4 M potassium acetate, whose resistance was 100–300 MΩ. The signals were fed through W.P. Instruments M4A amplifiers, 1 × gain, and into a Tektronix 565 oscilloscope. Current injection through a recording electrode was accomplished by a circuit in the amplifier.

RESULTS

Gross morphology of developing muscles

Following the amputation of an adult forelimb at the shoulder there is a period of one or several weeks before outgrowth of a new limb bud begins, during which there is dedifferentiation of tissue immediately proximal to the lesion (Thornton, 1938; Hay, 1959). When the new bud appears it grows out for a relatively short distance, develops an elbow, grows a small amount more and begins to form digits (Iten & Bryant, 1973). The result is an appendage which has the general external features of the adult limb but which is much reduced in size. Thus, the region between the shoulder and elbow, spanned by the humero-antibrachialis muscle used in these studies, may be 1 mm in length as compared with 6 mm on the normal limb from the same animal. This miniature limb then grows to normal size.

There is considerable variability in the rate of limb regeneration among different animals. This seems to be, at least in part, a function of age; younger adults regenerate more quickly. A consequence of this variability is that one cannot use the time elapsed since limb amputation to predict when any one muscle will appear. It was therefore necessary to use external morphological features of the developing limb as such indicators.

At about the time that the limb is in the palette or early digit stage (Iten & Bryant, 1973) individual fibres of the humero-antibrachialis muscle begin to appear. In the earliest phase at which the muscle can be detected there are few fibres, sometimes less than ten, which are narrow ($\sim 5\ \mu$m diameter) and short (~ 1 mm length) (Pl. 1*A*). These are embedded among spindle-shaped myoblasts, unspecified cells, and connective tissue. In such immature limbs extreme care was required during dissection because the fibres can easily be missed or damaged. This stage of development was found to occur any time between 3 and 8 weeks after limb amputation.

Pl. 1*A* shows a micrograph of living fibres from an early stage muscle. With the optics used it was not always possible to ascertain whether the most immature fibres had developed striations; the resolution varied inversely with the density of connective tissue and non-specific cells in the vicinity. Pl. 1*B* shows a micrograph of typical adult fibres viewed at the same magnification.

Described below are a number of physiological stages which were detected in developing muscles. In any one muscle, fibres were often at

different stages of maturation, although there was a greater probability of encountering less mature physiological stages in the smallest muscles.

Electrical recording from normal adult fibres

Before describing the physiology of developing muscle fibres it is helpful to note several features of the fibres in the normal adult muscle. In a sample of 180 fibres from fifteen muscles the resting potentials were in the range of -90 to -105 mV. In the presence of a normal calcium concentration (1·8 mM) supramaximal stimulation of the motor input evoked propagated action potentials in sixty of sixty-two fibres examined in four muscles. The amplitudes of these action potentials fell in the range of 70 to 100 mV; that is, some overshot zero membrane potential while others did not. Direct depolarization of the muscle fibres by a second, current injection electrode also elicited action potentials of similar amplitude. The threshold for such initiation was in the range of 15–25 mV positive, with respect to the resting potential. Neither synaptic nor direct depolarization of these normal fibres was ever observed to elicit a prolonged electrical response like that described below for the developing fibres.

To check for multiple innervation muscles were curarized (with D-tubocurarine, $1–4 \times 10^{-6}$ g/ml.), single fibres penetrated and the motor nerve bundle stimulated repeatedly with progressively increasing intensity: of eighty-six fibres, thirty-eight had a single synaptic input, forty-five received two inputs and three had triple input. These synaptic contacts appeared to be localized at discrete areas of the fibres, rather than spread along the length as in tonic muscle, because in curarized muscles focal synaptic potentials could be recorded only in localized areas. There was no evidence of electrical coupling between muscle fibres.

In this species of salamander tetrodotoxin (4×10^{-7} g/ml.) did not abolish the action potential in nerve or muscle.

Electrical recording from developing fibres

Resting potential. The resting potentials recorded in developing fibres were in the range of -90 to -105 mV. With poor penetrations, the potential would drop almost immediately to a level of approximately -50 mV, where it usually remained stable. Sometimes the micro-electrode could be withdrawn and reinserted into the same fibre to record a larger potential (-90 to -100 mV). Penetrations were more successful after increasing calcium in the bathing medium from 1·8 to 4 or 8 mM. Most experiments were therefore performed in elevated calcium, although some were also repeated in normal calcium, as noted in the appropriate sections.

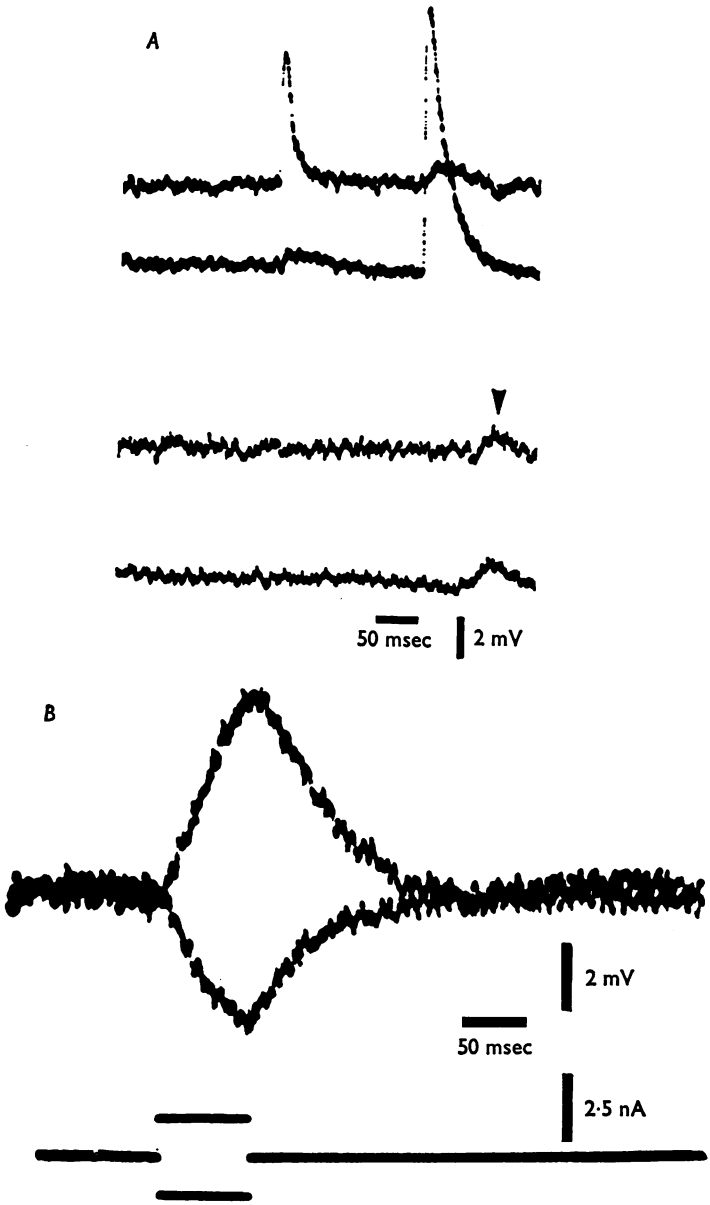
There did not appear to be any systematic change in the resting potential with maturation of the fibres, as has been suggested for muscle fibres

developing in culture (Fishbach, Nameroff & Nelson, 1971; McDonald & DeHaan, 1973). At the earliest stages, when the fibres were of relatively small diameter, the probability of a successful penetration was lower than in older muscles. However, in even the most immature fibres stable recordings of -95 mV or more were obtained. If there are systematic potential changes in the muscle fibres of this preparation they must occur at earlier developmental stages.

Spontaneous potential amplitude. All fibres penetrated except those which were uninnervated (see below), had spontaneous synaptic potentials. One of the most striking features of these spontaneous events in immature fibres was their large amplitude, which was often greater than 10 mV. Examples of this can be seen in Text-figs. 1*A*, 2 and 4*A*, and an amplitude histogram of those recorded in one fibre is shown in Text-fig. 4*B*. The explanation for their large size was found to be the high input resistance of the developing fibres. The resistance was determined by injecting small hyperpolarizing current pulses through one micro-electrode and measuring the resultant potential change with a second (see Text-fig. 6*A*). Input resistances of these fibres were in the range of 5–50 MΩ. When a plot of average spontaneous potential amplitude vs. input resistance was made (cf. Katz & Thesleff, 1957) the points from developing and normal muscles fell along the same line.

Electrical coupling between muscle fibres. In some innervated muscle fibres one saw two distinct types of spontaneous potential: one which was relatively large ($\geqslant$ 1 mV) and fast rising and the other which was small and had a rise time roughly equal to the decay time (Text-figs. 1*A*, 2). At first it was thought that these different potentials resulted from spontaneous transmitter release at different distances from the recording electrode with distant potentials reduced and slowed by the passive electrical properties of the muscle fibres. However, it was found that the observations were satisfactorily explained by coupling of muscle fibres. Thus, if neighbouring fibres were impaled it was sometimes found that the small slow events in one fibre were correlated with large fast spontaneous potentials in the other fibre (Text-fig. 1*A*). When such a relationship was seen, the reciprocal also occurred; that is, fast events in the first fibre caused small slow potentials in the second. Since spontaneous events arise from random release of single packets of transmitter at one junction, simultaneous release in two fibres would occur quite rarely. Therefore, it was concluded that these fibres were electrically coupled.

The largest coupling ratio observed when comparing the amplitude of the coupling potential to that of the min.e.p.p. of origin was approximately 1/15. However, the use of min.e.p.p.s as the source of the voltage pulse was not satisfactory when trying to quantitate the degree of coupling,



Text-fig. 1. *A*, simultaneous recording from two developing fibres which are electrically coupled. In the upper pair of traces one spontaneous synaptic potential in each fibre causes a small and slow electrotonic potential in the other. In the bottom pair of traces small coupling potentials occur simultaneously in both fibres (arrow), suggesting common coupling to a third fibre. *B*, potential changes evoked in one fibre during injection of current into a second fibre, coupled to the first. Upper traces are sequential responses evoked by equal current pulses of opposite polarity. Lower trace monitors current pulse. As the current electrode was withdrawn from its fibre the evoked potential disappeared.

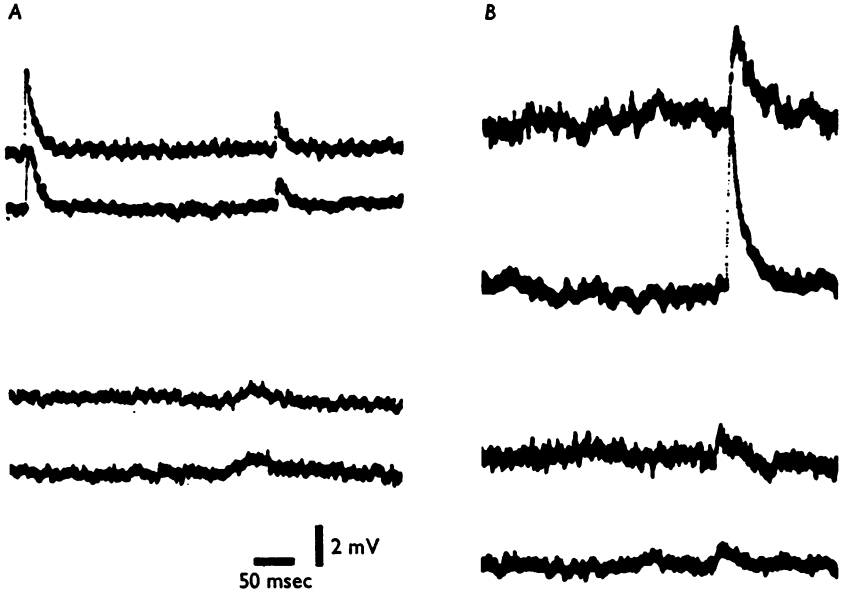
since much of the initial interfibre current generated by the min.e.p.p. would be expended in discharging the capacity of the following fibres. This no doubt explains why the rate of rise of such coupling potentials often seemed as slow as the rate of decay (cf. Text-figs. 1 and 2). As a further check of coupling, current was passed through the recording electrode in one of a pair of fibres and the resultant potential change recorded in the second. When the pair of penetrated fibres appeared to be coupled by the criteria described above it was also found that current injected into one fibre did evoke a potential change in the other (Text-fig. 1*B*). Such current flow showed no rectification. Owing to my inability to accurately balance the bridge in the current injection circuit, it was not possible to also monitor the potential change at the site of current injection. This coupling potential immediately disappeared when the current passing electrode was withdrawn from the fibre it had been in. Pairs of fibres which did not show coupling by the criteria above also did not show any sign of current flow measured in this manner.

In some pairs of coupled fibres one observed small slow spontaneous potentials which occur simultaneously in both (Text-fig. 1*A*, arrow). This presumably resulted when both of the observed fibres were electrically coupled to a third.

Neighbouring muscle fibres whose membranes were in apparent physical contact were not necessarily coupled. In some instances each of two adjacent fibres were coupled to one or more other fibres, but not to each other. This suggested that this electrical coupling in developing muscles was not so extensive as to form a syncytium of linked fibres.

The finding that some of the small-slow spontaneous potentials could be attributed to electrical coupling did not eliminate the initial assumption that the electrode might also be recording focal and non-focal spontaneous potentials, both of which arose in the same fibre, one close to and the other distant from the recording pipette. This explanation was excluded by impaling a given muscle fibre simultaneously at two sites, as far apart as possible. The procedure used was difficult because the experiment had to be done with a lower power (16×) objective, to increase the field of view, which thereby decreased the visual resolution. Consequently, double impalement of the same muscle fibre was accomplished by trial and error. Nevertheless, six single fibres were successfully studied. In all cases the interelectrode distances were 800–1000 μm in fibres that were up to 2 mm long; each electrode was approximately one quarter of the fibre length from one end. In Text-fig. 2*A* and *B* are examples of two typical double recordings. The spontaneous potentials which were relatively large and fast at one electrode were also the largest and fastest at the other; similarly, the small and slow events were so at both sites. Furthermore, in the fibre

of Text-fig. 2*A* there was little discrepancy in absolute potential amplitudes between the two electrodes. In some of these dual penetrations there was a difference between the absolute amplitude recorded at the two sites; the extreme being illustrated in Text-fig. 2*B*. Even there the relative size of large and small events was still preserved at the two recording sites.



Text-fig. 2. Simultaneous recording from two points 800 μm apart in one muscle fibre. *A*, fibre in which the absolute amplitude of spontaneous potentials was approximately equal at the two recording loci. *B*, fibre in which the relative amplitude of large and small events was maintained at the two loci, but the absolute amplitude of the large events was not the same. See text for further details of protocol.

This observation that small-slow potentials appeared the same near both ends of the developing fibres argued against the possibility that some of them were simply non-focal min.e.p.p.s, at least in the fibres studied by this method. The length constant of the muscle fibres was long enough for one electrode to detect all of the potential changes arising at any point along the fibre. This agrees with the observations of Diamond & Miledi (1962) who noted such relatively long length constants in the fibres of neonatal rat diaphragm.

Spontaneous potential frequencies. Due to the existence of electrical coupling between developing muscle fibres and the consequent coupling potentials resulting from large spontaneous events in other fibres, one could not readily determine the absolute frequency of spontantous poten-

tials that arose in one fibre. If one counted all spontaneous potentials, the estimate would be too great, because the coupling potentials are included. On the other hand, measurement of min.e.p.p. frequency in normal adult fibres might err in the opposite direction, if the length constant were relatively short and the fibres multiply innervated (see Burke, 1957). Despite these sources of error, counting all events the mean min.e.p.p. frequency in developing fibres ($f = 0{\cdot}18 \pm 0{\cdot}15$/sec, measured in seventy-seven fibres from twenty-eight muscles) was lower than in normally innervated adult fibres ($f = 0{\cdot}4 \pm 0{\cdot}15$/sec, sixty fibres from five muscles). These measurements were made in the presence of 8 mM calcium, although changes in calcium concentration did not significantly alter the min.e.p.p. frequency in individual fibres. It should be noted that there is considerable variation in frequency of spontaneous potentials between different fibres in developing muscles (0·02–0·85/sec).

Stages of innervation

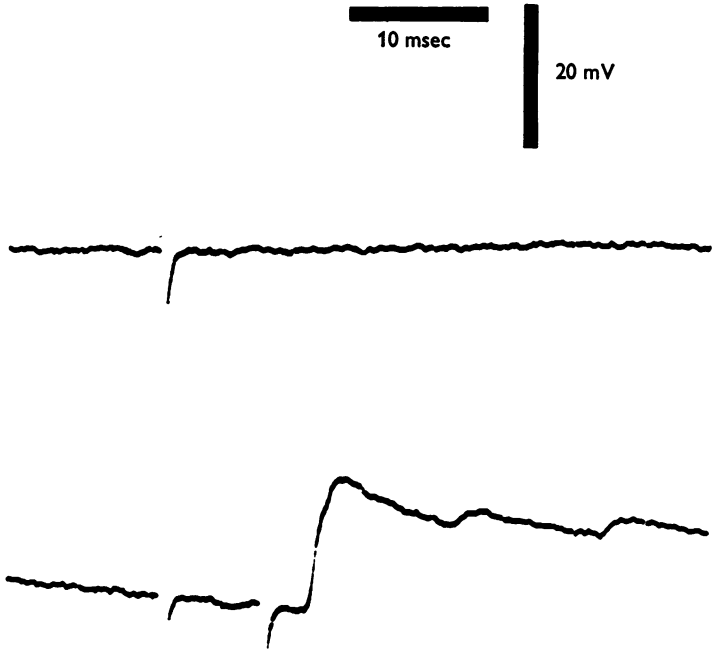
Uninnervated. In the most immature muscles some fibres were penetrated which showed no physiological signs of innervation: there were no spontaneous potentials, and no response to stimulation of the nerve. Furthermore, the addition of 1 mM lanthanum never evoked spontaneous potentials (seven fibres in six muscles); lanthanum increases the frequency of spontaneous potentials at the normal frog neuromuscular junction (Blioch, Glagoleva, Liberman & Nenasher, 1968; Heuser & Miledi, 1971; DeBassio, Schnitzler & Parsons, 1971) and at innervated fibres in this preparation (see below). Due to the usual large amplitude of the spontaneous synaptic potentials in this preparation (see above), it seems unlikely that they would have been missed if they did occur.

The present physiological criteria for lack of innervation, although very suggestive, do not exclude the possibility that a nerve terminal has come into contact with the fibre but is not yet active. Axons were certainly in the vicinity because often other fibres in the same muscle did show signs of innervation.

Non-transmitting junctions. A second category of muscle fibres was encountered which showed spontaneous end-plate potentials, but maximal stimulation of the motor nerve bundle did not give rise to an evoked end-plate potential (e.p.p.). This was regarded as an intermediate phase of synapse development.

It could be argued that the failure of such junctions to respond to nerve stimulation resulted from nerve damage during dissection. However, this assumption seemed unlikely because there were usually other fibres in the same muscles which did have transmitting junctions; sometimes this was the case in immediately adjacent fibres. Another argument against the

damage hypothesis was that occasionally a fibre was found which did not respond to a single nerve stimulus, but gave a multi-quantal e.p.p. when the nerve was stimulated twice with a 10 msec interval between pulses (Text-fig. 3).



Text-fig. 3. Response at an immature synapse to stimulation of the motor nerve bundle. Single stimuli, top trace, evoked no response. When the nerve was stimulated with a second pulse of the same intensity 8 msec later, bottom trace, a multiquantal end-plate potential was evoked. Note that two spontaneous potentials appear during the decaying phase of the e.p.p. Ringer solution contained 8 mM calcium.

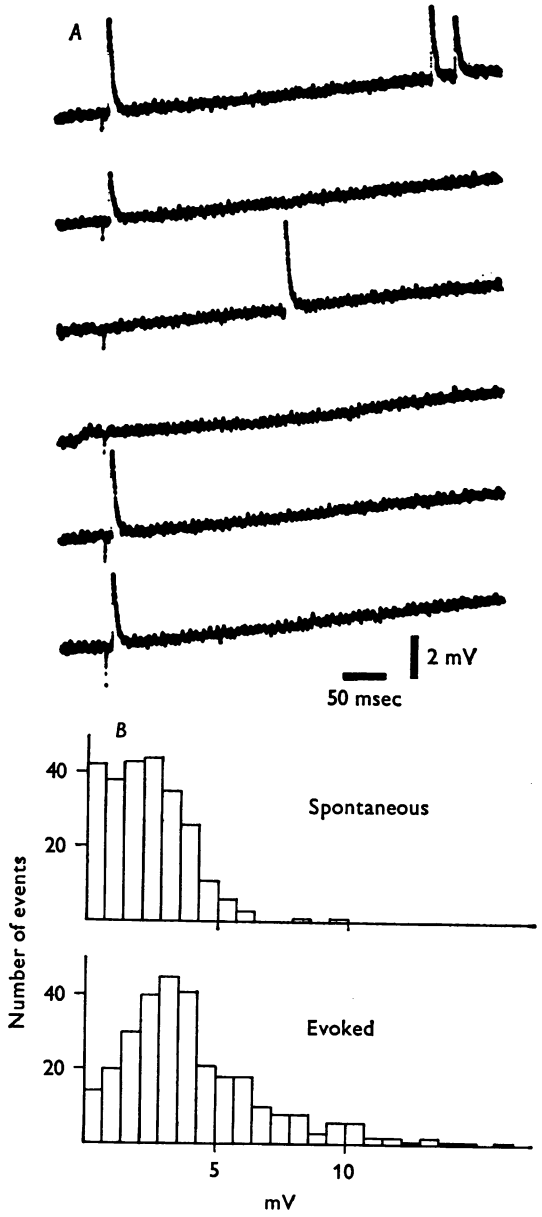
To check that the spontaneous events at non-transmitting junctions did in fact result from release of transmitter by nerve terminals, the preparations were subjected to manipulations which are known to increase spontaneous release. In normal motor nerve terminals miniature end-plate potential frequency is consistently increased by raising both osmolarity (Furshpan, 1956) and, as mentioned above, by the addition of lanthanum. Such changes do not increase the release of acetylcholine from Schwann cells at denervated adult junctions (Birks, Katz & Miledi, 1960; Bevan, Grampp & Miledi, 1973). When a non-transmitting fibre was impaled, and 1 mM lanthanum nitrate subsequently added to the bath there was invariably a very striking increase, often as much as several hundredfold, in the frequency of spontaneous potentials in non-transmitting fibres

(tested on nine fibres in seven muscles). Also increasing osmolarity by addition of 40 mM sodium sulphate to the bath consistently caused the potentials in non-transmitting fibres to increase in frequency between four- and forty-fold (six fibres in four muscles).

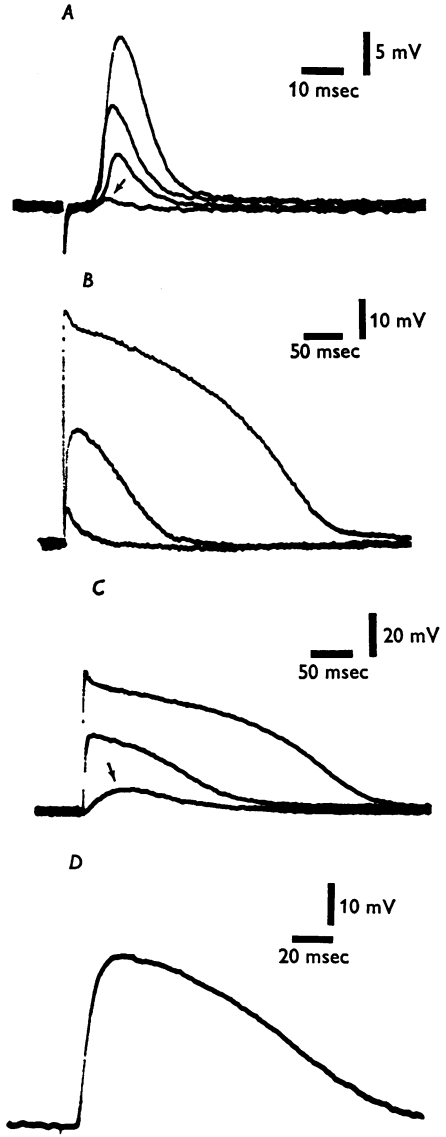
A phase of non-transmitting innervation, such as this, has also been observed during reinnervation of adult skeletal neuromuscular junctions in other animals (Miledi, 1960; Bennett, Pettigrew & Taylor, 1973; Dennis & Miledi, 1974*a*). There the failure to release transmitter seems to result from a block of the nerve impulses which prevents their propagation into the presynaptic nerve terminal (Dennis & Miledi, 1974*a*). Since the present observations of non-transmitting junctions were made in elevated calcium, which is known to increase the threshold for action potential propagation in frog myelinated nerves (Frankenhaeuser & Meves, 1958), it seemed that the failure of transmission might result from calcium-induced blockage of impulse propagation. This was not always the case, however; in several muscles a number of non-transmitting fibres was observed when the bathing fluid had a normal (1·8 mM) calcium concentration.

Innervation by one axon. A small proportion of the fibres responded to maximal intensity nerve stimulation with only a very small e.p.p. Text-fig. 4*A* shows the successive responses elicited in one such fibre by repetitive stimulation at 1/sec. Some stimuli failed to evoke any response, while the others caused only relatively small potentials, which were similar in amplitude to the spontaneous potentials recorded in the same fibre (see histograms of Text-fig. 4*B*). The fibre of Text-fig. 4 gave 307 responses to 693 stimuli, indicating a mean quantal content, m, of 0·58 (del Castillo & Katz, 1954). These features suggest that the small amplitude of responses evoked at such immature junctions is due to a low mean quantal release. When comparing the amplitude histograms of spontaneous and evoked events from one fibre, as in Text-fig. 4*B*, one cannot expect the means to be identical because most fibres have more than one motor axon terminating on them (see below); consequently, only a fraction of the spontaneous events may derive from transmitter release by the same nerve terminal as is being stimulated. Furthermore, since the fibres are electrically coupled it cannot be assumed that all of the spontaneous events are due to transmitter release on the fibre in question.

Multiple innervation. A common feature of these developing muscles was multiple innervation of single fibres. As one gradually increased the intensity of the stimulus to the motor nerve bundle during repetitive stimulation, increments in the response amplitude occurred at discrete stimulus intensities (Text-fig. 5*A*, *B* and *C*). Usually two or three inputs to one fibre could be detected by this method. As a rule one could also



Text-fig. 4. Potentials recorded from a fibre in which the evoked response had a low mean quantal content. *A*, consecutive traces repeated at a frequency of 1 sec^{-1}, with a nerve stimulus of constant intensity occurring 50 msec after each sweep initiation. Evoked responses appear within 50 msec after some of the stimuli. Spontaneous potentials appear randomly in several of the traces. *B*, amplitude histograms of spontaneous and evoked potentials recorded in the same fibre. 307 responses were evoked by 698 stimuli. Medium contained 1·8 mM calcium.



Text-fig. 5. Multiple synaptic input to single fibres, revealed by the fact that increasing the intensity of nerve stimulation with successive sweeps causes increments in end-plate potential amplitude. *A*, responses in fibre with synapses from three motoneurons. The very small depolarization appearing alone in the first trace (arrow) is probably an electrotonic potential caused by electrical coupling to another fibre. *B*, triple synaptic input. Note the change in wave form with successively larger responses. *C*, fibre with two synaptic inputs, eliciting large fast responses, which in addition is electrically coupled to a second fibre whose activation causes a smaller, slow rising potential (arrow). *D*, synaptically evoked response of long duration which shows no spike at the peak of depolarization. Note different calibrations.

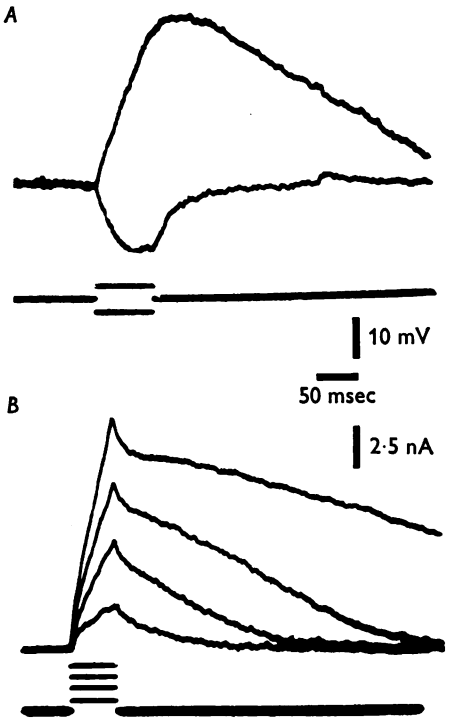
distinguish primary synaptic inputs on to the penetrated fibre from electrotonic potentials caused by coupling with other activated fibres. The coupling potentials had a slower rate of rise than the synaptic responses originating in the impaled muscle fibre (see above). For example, the three potentials shown in Text-fig. 5*A* all have a relatively fast rise, whereas the smallest potential in Text-fig. 5*C* rises much more slowly (note the difference in time scale). In some fibres it was difficult to decide whether a small increment in e.p.p. amplitude brought on by increasing stimulus intensity resulted from a primary synaptic potential or a coupling potential.

Long-lasting responses in muscle fibres. In many fibres, maximal nerve stimulation evoked a response which was unusual in both its duration and wave form (Text-figs. 5*B*, *C*, *D* and 6); after rising to a peak, the potential would decay very slowly, sometimes with a protracted plateau, occasionally lasting more than one second. In fibres giving these long responses, stimulationg at a lower intensity usually resulted in a smaller e.p.p. which had the same rate of rise but a more conventional exponential decay (Text-figs. 5*B*, *C* and 6*B*). Furthermore, the spontaneous potentials at such junctions also had a standard wave form (exponential decay), expected of synaptic potentials. The fact that the smaller amplitude events had a conventional wave form indicated that the prolongation of the large e.p.p. could not be ascribed to the passive electrical properties of the developing muscle membrane.

When a fibre demonstrating long-lasting responses was penetrated with a second micro-electrode, the synaptically evoked responses could be mimicked by injection of brief depolarizing current pulses (Text-fig. 6). Small positive pulses gave small depolarizations which decayed exponentially, whereas larger pulses of the same duration and polarity evoked responses in the fibre which had a protracted time course. The responses to hyperpolarizing pulses did not undergo such a change in wave form with increasing pulse intensity; they always had a simple exponential onset and decay. This difference is illustrated in Text-fig. 6*A*, which shows the response to positive and negative current pulses of equal magnitude. From these observations it seems that the prolonged potential is due to an electric response of the muscle fibres, rather than a peculiarity of the synapses.

One simple explanation for this prolonged depolarization was that it could be a movement artifact resulting from muscle contraction. This possibility seemed to be discounted by three tests. (1) In very immature muscles maximal nerve stimulation sometimes caused no detectable movement when examined with the 200 × magnification of the Nomarski optics (see Pl. 1). In some such muscles I could record prolonged responses. (2) In more mature muscles sufficient D-tubocurarine was added to the

bath (*ca.* 4×10^{-6} g/ml.) to suppress all visible movement with maximal nerve stimulation. In this curarized state slow depolarizing responses were still detected. (3) When the prolonged response was evoked by injecting current into a single fibre, visual inspection of that fibre revealed no movement, even though the optics were adequate to watch the spacing between individual sarcomeres.



Text-fig. 6. Slow depolarizing responses evoked in developing fibres by direct current injection through a second electrode. *A*, voltage traces show superimposed responses to equal current pulses of opposite polarity. Note the pronounced difference in wave form of potential change, depending on polarity. *B*, change in wave form of depolarizing responses with increase in current intensity. Small responses have simple exponential decay whereas larger ones show prolonged depolarization after cessation of current.

In many of these developing fibres maximum synaptic stimulation gave an e.p.p. of 40 mV amplitude, yet an action potential was not evoked. The large synaptic responses seen here sometimes did have a small spike appearing at the top of the rising phase (e.g. Text-fig. 5*B*, *C*), whereas on other occasions there was no sign of any such peak (Text-fig. 5*D*). This small peak might be interpreted either as an abortive action potential, or

else as just the initial portion of the passive, exponential decay of the imposed depolarization, preceding the onset of the slow electrical response.

DISCUSSION

The regenerating salamander limb provides a system in which it is possible to study *in vivo* various stages of the developing nerve–muscle synapses. The immature synapses, as well as the muscle fibres themselves, differ physiologically from the normal adult structures in a number of ways. It is of interest to consider some of these properties in relation to what is known of junctions developing during reinnervation of adult muscle and of those formed in tissue culture.

A non-transmitting phase of innervation was encountered in which the axon terminal released transmitter spontaneously but stimulation of the nerve trunk did not evoke an end-plate potential. Furthermore, adding lanthanum or increasing the osmolarity of the bath always increased the frequency of spontaneous min.e.p.p.s. A similar non-transmitting phase has been observed during reinnervation of adult frog (Miledi, 1960; Elul, Miledi & Stefani, 1968), chicken (Bennett, Pettigrew & Taylor, 1973) and rat (Dennis & Miledi, 1974*a*) muscles. In reinnervated frog muscle a similar inability of the nerve impulses to liberate transmitter resulted from failure of the action potential to propagate into the presynaptic nerve terminal (Dennis & Miledi, 1974*a*). The same explanation may apply in the system described here.

Synapses developing within cultured explants of frog embryo neural plate (Cohen, 1972) at an early stage showed only marginal increases in min.e.p.p. frequency with increased bath osmolarity, even though nerve stimulation did evoke an e.p.p. This differs from the present observation that even at non-transmitting junctions the min.e.p.p. frequency is significantly accelerated by hypertonicity and lanthanum ions. The difference may be due to the immaturity of the presynaptic motoneurones in the neural plate culture. In the foetal rat diaphragm increased osmolarity also causes a striking (100–1000×) increase in min.e.p.p. frequency (Diamond & Miledi, 1962).

The relatively low frequency of min.e.p.p.s in these developing muscle fibres is reminiscent of observations made in foetal rat muscle (Diamond & Miledi, 1962), at reinnervated frog (Miledi, 1960; Dennis & Miledi, 1974*a*), rat (McArdle & Albuquerque, 1973), rabbit (Bennett, McLachlan & Taylor, 1973*a*) and avian (Bennett, Pettigrew & Taylor, 1973) muscle; also after autonomic innervation of skeletal muscle in frog (Landmesser, 1971) and rabbit (Bennett, McLachlan & Taylor, 1973*b*), and at synapses developing in culture (Fishbach, 1972). Related to this is the observation that in immature

fibres the e.p.p. often has a quantal content which is lower than normal, a situation which again is also seen at early stages of reinnervated adult end-plates (Dennis & Miledi, 1974*b*; Bennett *et al.* 1973*a*; see, however, Miledi, 1960) and at junctions formed in culture (Fishbach, 1972; Cohen, 1972; Robbins & Yonezawa, 1971). This subnormal rate of transmitter release, both spontaneous and evoked, at developing junctions may result from a reduced probability of quantal release per unit area of terminal membrane, which increases during maturation, as was suggested by observations made at reinnervated frog junctions (Dennis & Miledi, 1974*b*). However, another factor which could be involved is increase in the total synaptic surface area with time, by increase in either diameter or length of terminal axon, with quantal release in constant proportion to area (Kuno, Turkanis & Weakly, 1971; Kuno, Muñoz-Martinez & Randić, 1973). In muscles of regenerating crab leg, Govind *et al.* (1973) noted that the quantal content and synaptic surface area was smaller in the first synapses formed than in those which developed later, but they gave no indication whether a given synapse changes with maturation.

Investigations on new-born mammals have suggested that there is a reduction during maturation in the number of motoneurones ending on single muscle fibres (Redfern, 1970; Bagust *et al.* 1973). It is not clear from the present experiments whether such a change occurs in the regenerating salamander limb. The occurrence of single innervation seems more common in the normal adult muscle, but the presence of electrical coupling between muscle fibres, combined with the low quantal content and consequent large amplitude fluctuations of many of the e.p.p.s, make it difficult to decide the number of inputs in many developing fibres (e.g. Text-fig. 5*D*). The loss of electrical coupling which occurs during maturation could thus lead to a false impression of a reduction in innervation. Another factor which hampers judgement about changes with time is that the total number of fibres in the muscle continues to increase after the period studied here, and it is possible that those developing later have a reduced amount of multiple innervation. The result would be a decline in the proportion of multiply innervated fibres without any reduction in the multiplicity of innervation to a given fibre.

Long-lasting regenerative responses like those elicited by depolarization of the developing muscle fibres have been observed in cultures of cloned rat skeletal muscle (Kidokoro, 1973) and of dissociated chick skeletal muscle fibres (Kano & Shimada, 1973). In those systems the slow inward current, which seems to be carried at least in part by calcium ions, coexists with a faster sodium and potassium conductance increase which is more like that of the normal action potential. Experiments are presently under way to study the ionic basis of this slow regenerative response, and to

determine the time of appearance of the action potentials which are seen in mature muscle fibres.

A pressing question which these results raise is that of the fine structure of each of the various developmental stages. Although Lentz (1969) has described the anatomy of synapse development, there is no way at present of correlating my physiological findings with his morphological stages of development. It will be particularly useful to examine identified fibres whose physiological properties are known. One possibility of obvious interest is that after the nerve makes physical contact with a muscle fibre, it remains for some time before there are any physiological indications of transmitter release (see Fishbach, 1974).

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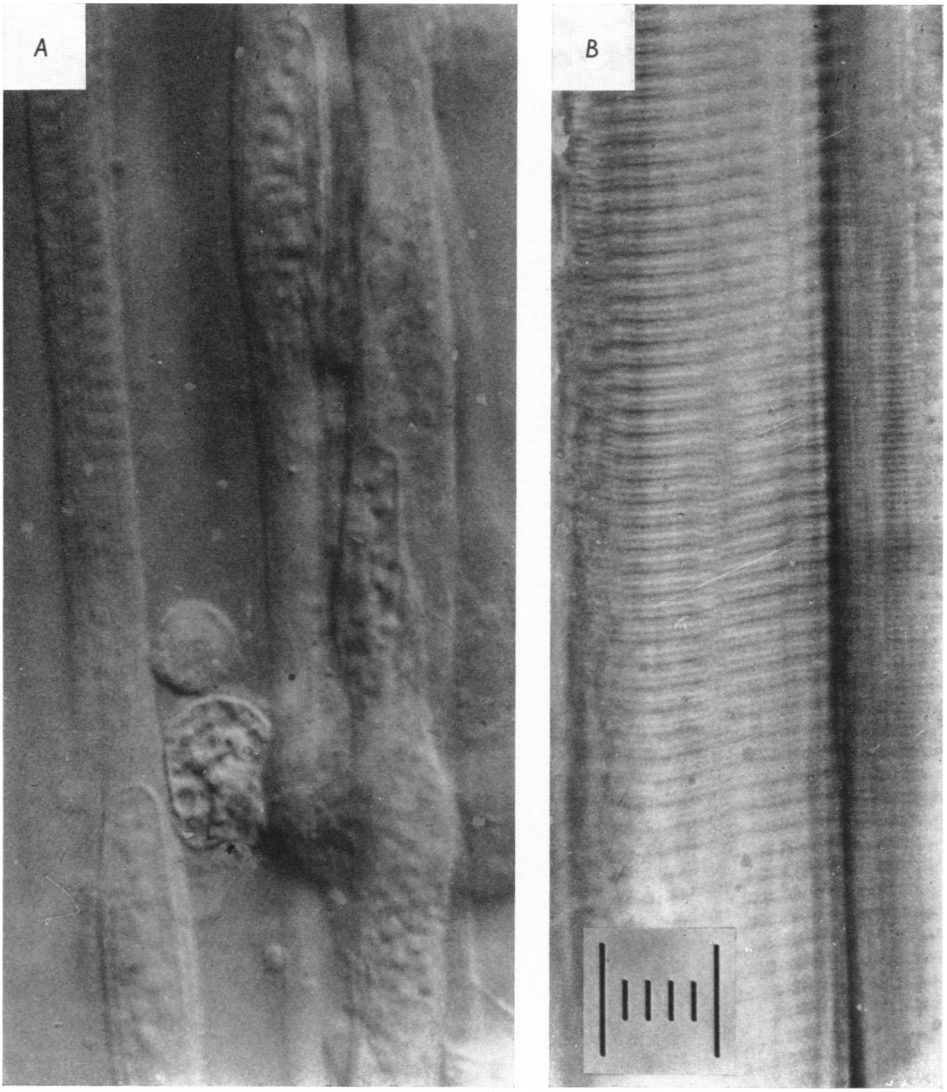
EXPLANATION OF PLATE

A, living fibres in a regenerating humero-antibrachialis muscle, viewed at the stage when synapses are forming. Note the striations in the fibre on the left, and the prominent nuclei. *B*, normal adult fibres from a control muscle. Both micrographs taken with Zeiss–Nomarski optics. Calibration, 2·5 μm per division.



(*Facing p.* 702)