J. Physiol. (1977), **270**, *pp.* 75–88
With 6 text-figures
Printed in Great Britain

EFFECTS OF INNERVATION ON ACETYLCHOLINE SENSITIVITY OF DEVELOPING MUSCLE *IN VITRO*

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(*Received 27 September 1976*)

SUMMARY

1. Chick embryo skeletal muscle fibres were grown in culture. The acetylcholine (ACh) sensitivity of non-innervated fibres was compared with that of fibres innervated *in vitro* by chick embryo ciliary ganglion neurones.

2. The general pattern of ACh sensitivity was unchanged by innervation: ACh hot spots were superimposed on a background of uniform ACh sensitivity.

3. Quantitative comparisons revealed two differences between non-innervated and innervated fibres. First, hot spots were encountered about one third more often on innervated fibres. Secondly, about one-third of the hot spots on innervated fibres had significantly higher ACh sensitivities than the remainder, which were similar to those on control fibres.

4. A possible explanation of these results is that nerves which form synapses induce the appearance of end-plates which have higher ACh sensitivities than the pre-existing ACh hot spots.

INTRODUCTION

The formation of synapses in primary cultures containing embryonic skeletal muscle and cholinergic neurones has been demonstrated with a variety of preparations, and the nature of synaptic transmission resembles that in muscles which develop *in vivo* (Crain, 1964; Fischbach, 1970; Robbins & Yonezawa, 1971; Hooisma, Slaaf, Meeter & Stevens, 1975; Betz, 1976*a*). These results have naturally raised questions about subsequent *in vitro* effects of innervation on muscle fibre properties. Of particular physiological interest is the distribution of acetylcholine (ACh) receptors on the surface of the muscle fibres. Muscle fibres innervated for

a few days possess the same qualitative pattern of ACh sensitivity as non-innervated fibres, with small patches of high ACh sensitivity (ACh hot spots) superimposed on a surface with uniform background sensitivity (Fischbach & Cohen, 1973). Recently, it was shown that there is an increase in the average ACh sensitivity of muscle fibres after innervation, although it was not clear whether this was due to an increase in background sensitivity, hot spot sensitivity, or hot spot frequency (Fishbach, Berg, Cohen & Frank, 1976). The experiments described in the present paper confirm and extend these observations. The results suggest that, in innervated fibres, background sensitivity remains unchanged, hot spots are encountered more often, and the mean hot spot sensitivity is elevated.

METHODS

The techniques for culturing nerve and muscle were identical to those described earlier (Betz, 1976*a*). Briefly, 10–11 day chick embryo pectoral muscle was dissociated and plated and 4–6 days later 6–7 day chick embryo ciliary ganglia were added to the cultures. Experiments were performed 2–4 days after ganglia were plated. Since synapses form as early as 1 day after a ganglion is explanted (Hooisma *et al.* 1975; Betz, 1976*a*), no synapses were more than 3 days old in this study.

Electrophysiological experiments were performed as described earlier (Betz, 1976*a*), with the preparation mounted on the stage of a Reichert inverted microscope fitted with Nomarski optics. The preparation was viewed at $\times 780$ magnification (field diameter = 200 μm). Innervated fibres lay within 300 μm of the ganglion, and usually the field of view selected for ACh mapping of an innervated fibre included several nerve processes in contact with the fibre. Some control (non-innervated) fibres studied were in culture which contained a ganglion, but were several millimeters away from the ganglion. The remaining control fibres were studied in muscle cultures without added ganglia. Intracellular microelectrodes were filled with 4 M potassium acetate and had resistances of 50–150 MΩ.

Iontophoretic pipettes were filled with 3 M ACh chloride (Sigma), and had resistances of 150–400 MΩ. After a fibre was impaled with the recording electrode, the ACh pipette was lowered to the surface and the braking current was adjusted to the lowest possible level at which no measurable depolarization of the fibre could be detected (del Castillo & Katz, 1955; Dreyer & Peper, 1974). This level varied from 1–3 nA with different ACh pipettes. The good visibility allowed the ACh pipette to be positioned accurately at the muscle fibre surface. Current was monitored as the output of an operational amplifier which clamped the bath potential at virtual ground. Iontophoretic pulses were 1–4 msec in duration.

In the statistical analyses which follow, all values are mean ± S.E. unless noted otherwise.

RESULTS

All experiments were performed on 6–10 day old cultures, and no consistent differences due to age were noted. All comparisons discussed below were therefore made between non-innervated (control) and innervated fibres. Innervated fibres were identified by the presence of spontaneous synaptic potentials (Hooisma *et al.* 1975; Betz, 1976*a*).

Resting potentials were the same in both groups of fibres, about -60 mV (control fibres $= -60{\cdot}3 \pm 2{\cdot}2$ mV ($n = 37$); innervated fibres $= -60{\cdot}3 \pm 2{\cdot}3$ mV ($n = 29$)). This lack of effect of innervation on resting potential confirms results of Hooisma *et al.* (1975), although their mean resting potential values were about 10 mV more negative. Input resistances were somewhat lower in innervated fibres, the difference being significant at the 5% confidence level (control fibres $= 4{\cdot}7 \pm 0{\cdot}4$ MΩ ($n = 27$); innervated fibres $= 3{\cdot}5 \pm 0{\cdot}5$ MΩ ($n = 30$)).

ACh sensitivity. Each muscle fibre was mapped at least 2 times with 5–10 μm between successive steps of the ACh micropipette, usually over a total length of about 200 μm. On any given fibre, identical iontophoretic pulses were used, with the dose adjusted at the outset to produce a few mV depolarization (background response). When an ACh hot spot was encountered, it was mapped in smaller, 2–3 μm steps. Several criteria were used to identify hot spots, and in nearly all cases they were easily distinguishable from background. First, hot spots were defined as responding with a peak depolarization at least 2·5 times larger than background. The response histograms in Fig. 1 show examples of the clear separation between responses at hot spots and background areas. Mean background sensitivity was calculated from responses obtained at points away from hot spots (open bars in Fig. 1). In addition, hot spots could be identified by the faster rise time of the ACh potential (Fig. 2*A*) and by the fact that ACh potential rise time at hot spots did not depend on ACh pulse amplitude as much as it did at background areas. Examples of dependence of rise time on ACh dose are illustrated in Fig. 2. The difference in rise times at hot spots and background areas probably reflects a lower background receptor density. Thus, with larger ACh doses, receptors under the iontophoretic pipette tip at background areas became saturated, and ACh diffused to and activated neighbouring receptors. The diffusion delay resulted in a prolonged rise time of the ACh potential. These additional tests were used only for hot spot confirmation; the necessary condition for hot spot identification was a reproducible peak depolarization at least 2·5 times greater than background.

While background sensitivities measured on individual fibres were quite uniform and reproducible with duplicate runs, greater variability was introduced when comparisons were made between different fibres. For instance, in Fig. 3 mean background response amplitude (ordinate) is plotted against iontophoretic dose (abscissa) for control (open circles) and innervated (filled circles) fibres. The graph surprisingly shows no relation between the two variables for either fibre group; linear regression correlation coefficients were 0·05 (control fibres) and 0·09 (innervated fibres). On any individual fibre, of course, response amplitude always increased

with ACh dose. Since many different ACh pipettes were used in this study, probably uncontrolled random variability in ACh pipette transfer characteristics produced the large amount of scatter in Fig. 3. Consistent with this explanation was the observation that background responses measured on different fibres mapped with the same ACh pipette

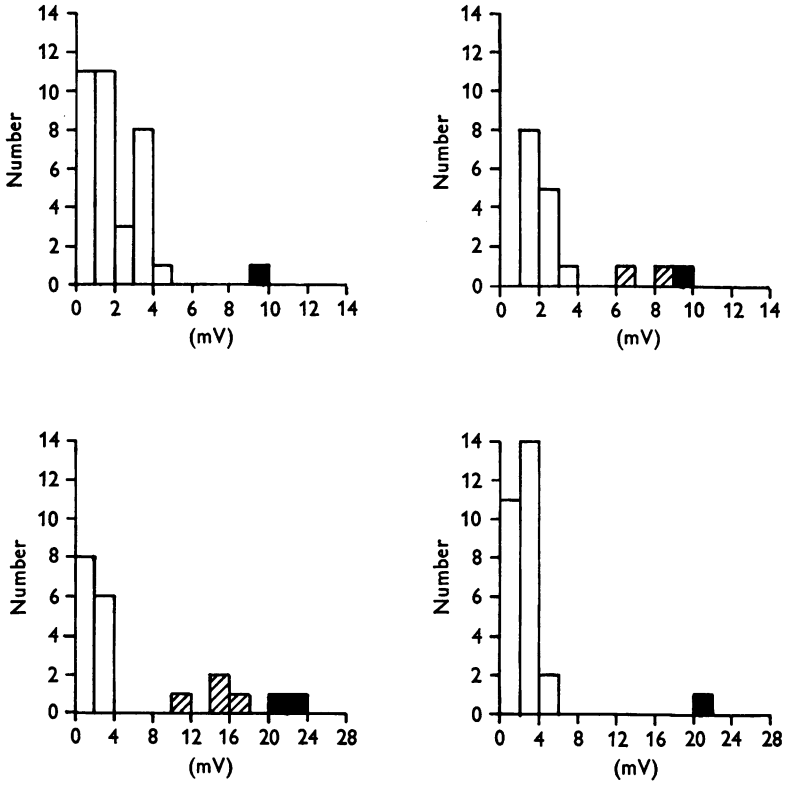

Fig. 1. Histograms showing the distribution of amplitudes of ACh potentials (abscissae) obtained at different locations on four myotubes, two of which were not innervated (upper histograms), and two innervated (lower). For each fibre, identical ACh pulses were used. Filled bars represent hot spot responses. Hatched bars represent responses obtained near hot spots, which were not used to calculate either background or hot spot sensitivities. Open bars represent background responses.

varied by only 1–2 mV, which is much less than the over-all variability illustrated in Fig. 3. Furthermore, ACh sensitivity depends critically on the amount of braking current (Dreyer & Peper, 1974), and inaccuracies in the conventional technique used to adjust braking current (see Methods) could have contributed to the variability seen in Fig. 3. Whatever the

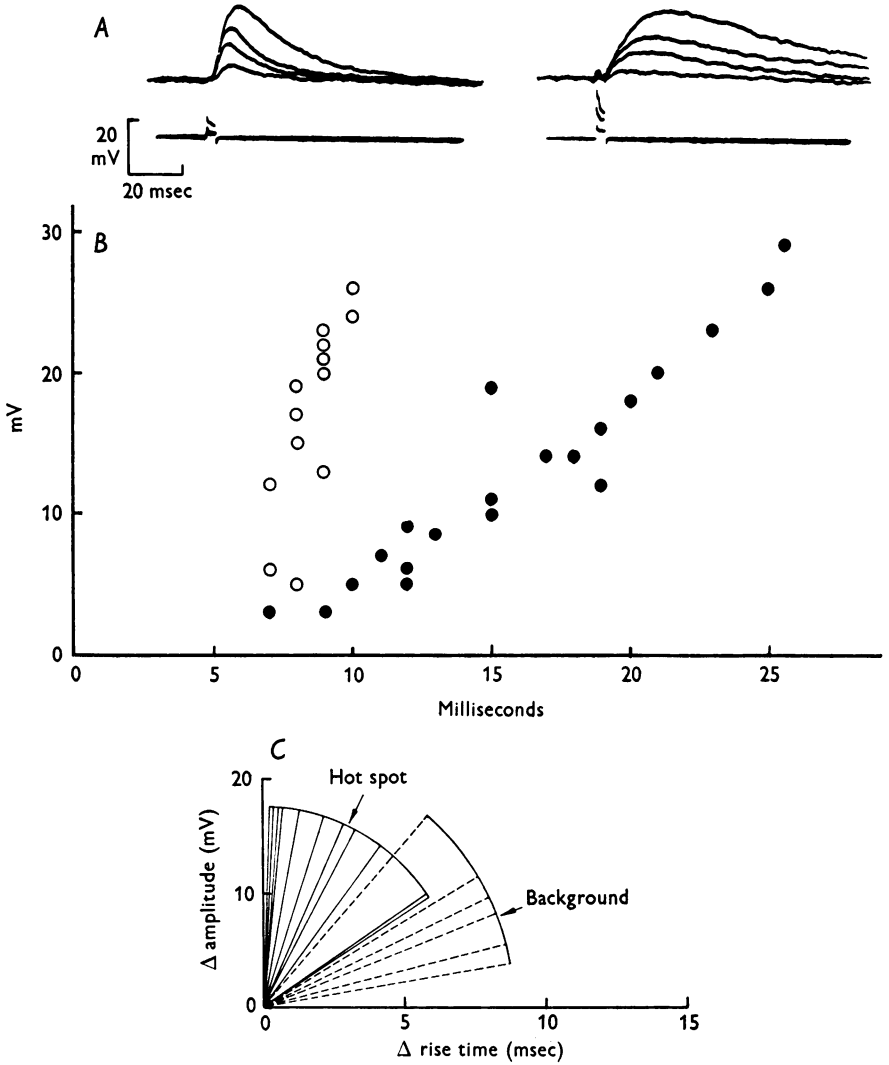

Fig. 2. Background and hot spot responses differentiated on the basis of time to peak measured from the beginning of the iontophoretic pulse. *A*, superimposed oscilloscope traces of responses obtained at a hot spot (left) and background area (right) on one fibre. Scale: 20 mV, 20 msec. *B*, complete results plotted from the experiment illustrated in *A*. Peak response amplitude (ordinate) is plotted against time to peak (abscissa). Open circles: hot spot responses; filled circles: background responses. *C*, collected results from the above and other experiments. For each experiment, a graph like that in *B* was made, and the points were fitted by eye with a straight line. The lines were given a common origin in *C* by plotting *change* in amplitude (ordinate) against *change* in rise time (abscissa) from minimum values. Continuous lines represent data obtained at hot spots, and dashed lines represent background data. Arrows mark mean values.

causes of this scatter, the important point is that it occurred about equally with control and innervated fibre groups, for the range and mean sensitivities of the two groups were very similar. Statistical analysis revealed no significant difference between the two groups (mean sensitivities = 275 mV/nC (control) and 322 mV/nC (innervated); $0{\cdot}1 < P < 0{\cdot}25$, see also Table 1).

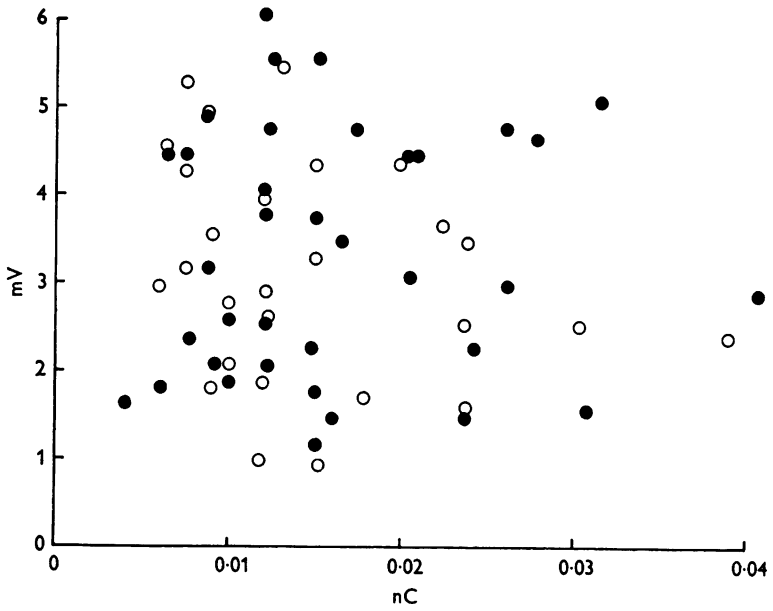

Fig. 3. Mean background response amplitude (ordinate) is plotted against iontophoretic dose (abscissa) for control (open circles) and innervated (filled circles) fibres. The appearance of random scatter for both groups of fibres was confirmed by statistical analysis: linear regression correlation coefficients were 0·05 (control) and 0·09 (innervated). The scatter probably reflects variability in ACh pipette transfer characteristics.

The simple definition of sensitivity described above is subject to several well known sources of error. First, voltage change does not mirror faithfully the underlying conductance change, so that a large response saturates electrically and leads to an underestimate of sensitivity. This problem was corrected for each fibre according to Martin (1955), assuming a reversal potential of 0 mV (Betz, 1976*a*). The resulting sensitivity values are given in Table 1 (correction *A*). A second source of error concerns the fact that innervated fibres had lower input resistances than control fibres. This also would lead to a relative underestimate of sensitivity of innervated fibres, and was corrected by accounting for differences in input resistance

for each fibre. Both corrections noted above were made by calculating the actual conductance change according to this equation:

$$g = \frac{v}{R(V_0 - v)},$$

where g = mean peak conductance change produced by ACh, v = mean peak depolarization, R = input resistance and V_0 = electrical driving

TABLE 1. Summary of ACh sensitivity measurements. Data expressed as mean ± S.E. (number of observations)

	Control fibres	Innervated fibres	*t* test results (control *vs.* innervated)
Background sensitivity			
Observed (mV/nC)	275 ± 30 (42)	322 ± 46 (37)	n.s. ($P > 0{\cdot}10$)
Corrected[a] (mV/nC)	295 ± 34 (41)	346 ± 51 (36)	n.s. ($P > 0{\cdot}10$)
Corrected[b] (μmho/nC)	1·47 ± 0·30 (20)	1·84 ± 0·35 (23)	n.s. ($P > 0{\cdot}05$)
Hot spot sensitivity			
Observed (mV/nC)	1277 ± 118 (35)	1876 ± 150 (46)	$P < 0{\cdot}005$
Corrected[a] (mV/nC)	2060 ± 291 (33)	3334 ± 331 (45)	$P < 0{\cdot}005$
Corrected[b] (μmho/nC)	10·0 ± 1·3 (16)	23·0 ± 2·7 (24)	$P < 0{\cdot}005$
Hot spot/background ratio			
Observed	5·4 ± 0·4 (32)	6·4 ± 0·5 (43)	$P < 0{\cdot}025$
Corrected[a]	7·0 ± 0·6 (32)	11·8 ± 1·3 (45)	$P < 0{\cdot}005$
Corrected[b]	6·8 ± 1·1 (16)	12·5 ± 2·1 (23)	$P < 0{\cdot}005$

[a], corrected for non-linear summation; [b], corrected for non-linear summation and input resistance. See text for explanations of corrections.

force (reversal potential-resting potential). Sensitivity was then expressed as μmho conductance change/nC iontophoretic charge (Table 1, correction *b*). Both corrections tended to increase the small difference in observed background sensitivity, but even after these corrections, the difference still did not reach the 5% significance level. Some uncertainty remains, however, since the correction factors are not entirely accurate (Martin, 1976), and other potential sources of error, such as chemical receptor saturation (cf. Kahn & Yaouane, 1971), were not taken into account. Despite these uncertainties, it seems safe to conclude that innervation had little, if any, short-term effect on background ACh sensitivity.

Measurements obtained at ACh hot spots were subjected to the same analysis as described above for background, and clear differences were observed between control and innervated fibres. As shown in Table 1, hot spot sensitivities were higher on innervated fibres, and the difference was significant at the 0·5% level for observed values and also for both corrected values of sensitivity.

A potential complication in this analysis arose with the observation that control fibres with high background sensitivities tended to have hot spots with high sensitivities, and those with low background sensitivities had hot spots with low sensitivities. This is shown in Fig. 4, where background

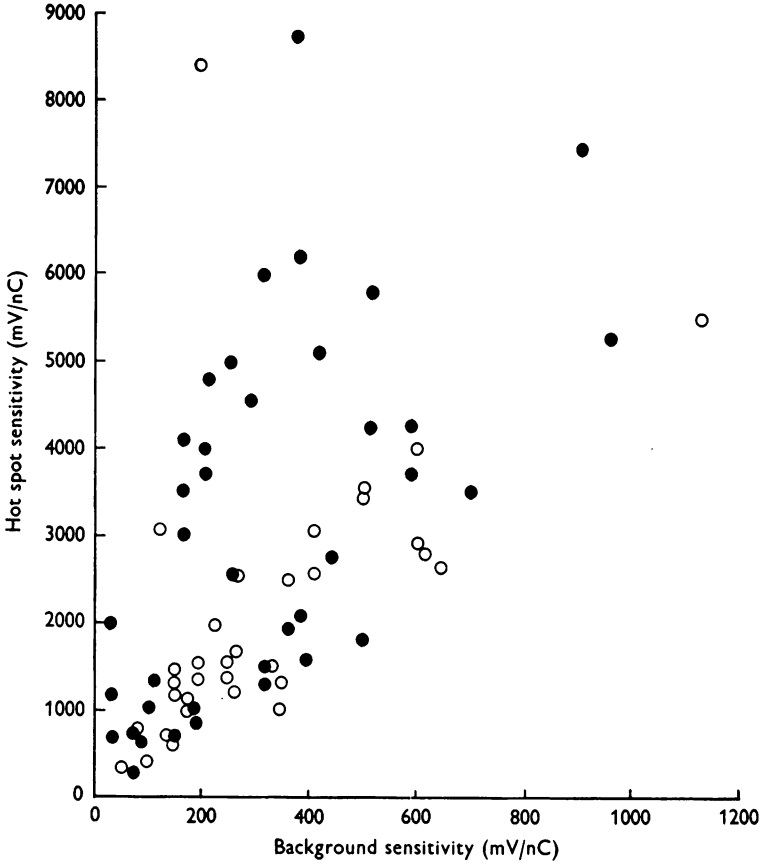

Fig. 4. Hot spot ACh sensitivity (ordinate) is plotted against background sensitivity (abscissa) for control (open circles) and innervated (filled circles) fibres.

(abscissa) and hot spot (ordinate) sensitivities are plotted for control (open circles) and innervated (filled circles) fibres. The reason for this relationship is not known with certainty, but it is likely that variation in iontophoretic braking current in different experiments is at least partly responsible. In order to avoid this complication, the ratio of hot spot sensitivity to background sensitivity was calculated for each fibre. When these values were compared (Table 1), the difference between innervated

and control fibres persisted, and was significant at the 2·5% level (uncorrected values) and 0·5% level (corrected values).

The hot spot sensitivity measurements described above were obtained with single-strength ACh pulses, which for each fibre were the same as that used to measure background sensitivity. More thorough measurements were obtained at some hot spots by applying varying amounts of ACh, and constructing a dose–response graph for each. For instance, in Fig. 5, response (ordinate) is plotted against iontophoretic dose (abscissa) corrected for electrical saturation and input resistance, for six hot spots, three on control fibres (open symbols) and three on innervated fibres (filled symbols). The maximum slope of each plot provides an estimate of ACh sensitivity (Kuffler & Yoshikami, 1975), and it is clear that the hot spots on innervated fibres were more sensitive than those on controls. A total of seventeen control and twenty-six innervated fibre hot spots were studied in this fashion. Maximum dose–response slopes were estimated by eye, and mean $\pm$ S.D. values were: control = $32{\cdot}8 \pm 59{\cdot}8$ μmho/nC, innervated = $237{\cdot}9 \pm 390{\cdot}0$ μmho/nC. These results confirm the observation that mean hot spot sensitivity was elevated on innervated fibres.

There are several possible mechanisms which could produce the elevated hot spot sensitivity on innervated fibres. One of these, an increase in hot spot area, was examined by measuring the spatial decrement of sensitivity from the point of maximum sensitivity at hot spots. The ACh pipette was moved in 2–3 μm steps in all four compass directions away from the hot spot centre. The fall-off was symmetrical on both fibre types, and the hot spot sizes were similar, the sensitivity falling to about 50% of its maximum at $3{\cdot}5 \pm 1{\cdot}9$ μm ($n = 34$) on control fibres and at $3{\cdot}7 \pm 0{\cdot}4$ μm ($n = 43$) on innervated fibres. These measurements probably overestimate the actual size of hot spots, since diffusion of ACh from the pipette tip will activate receptors several microns away. The error will occur on both control and innervated fibres, however, so it seems reasonable to conclude that hot spot size and shape are unchanged by innervation. Consequently, the higher sensitivity of hot spots on innervated fibres probably reflects a higher density of ACh receptors in the hot spot area, or a qualitatively different type of receptor.

The increased mean hot spot sensitivity on innervated fibres may not have been due to a generalized increase in the sensitivity of all hot spots. This is suggested by results illustrated in Fig. 6 which shows histograms of hot spot/background sensitivity ratios (corrected for electrical saturation) Ratios from control fibres (Fig. 6*A*) are distributed fairly uniformly about the mean, but ratios from innervated fibres (Fig. 6*B*) are distributed quite differently, with one major group in the same region as the control fibres, and a second, smaller group representing more sensitive hot spots. An interpretation of this highly skewed distribution is given below.

Hot spot frequency. Evidence was given earlier that hot spots were about the same size on innervated and control fibres, about 5–10 μm diameter circular patches. Since the ACh micropipette during mapping runs was moved in 5–10 μm increments, it is likely that few, if any hot spots in the

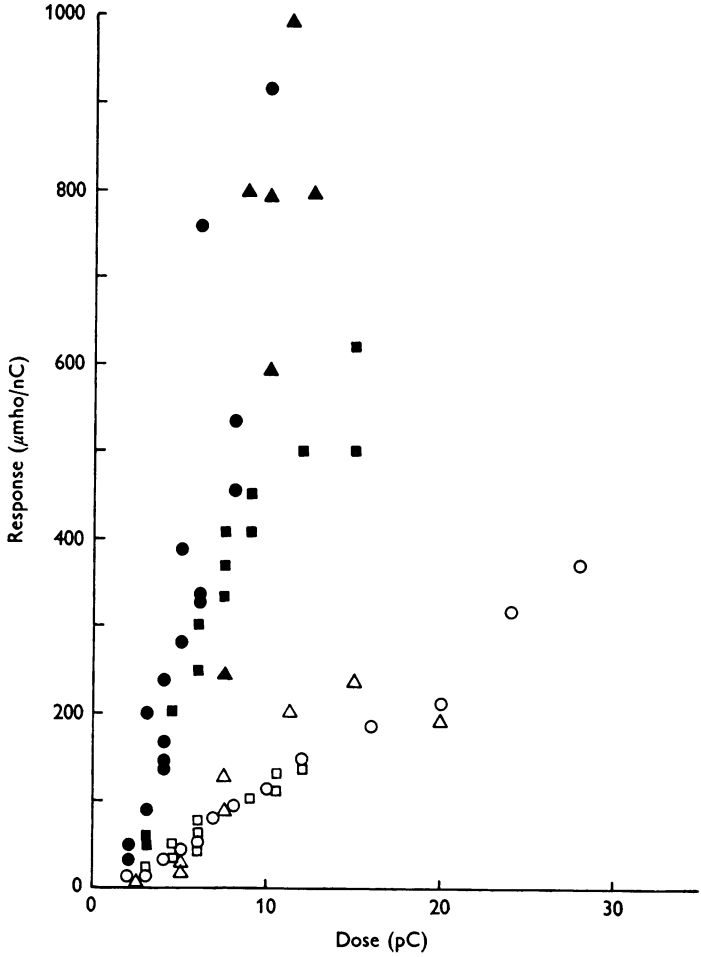

Fig. 5. Dose–response curves obtained at hot spots on three control fibres (open symbols) and three innervated fibres (filled symbols). Responses were corrected for electrical saturation and input resistance, as described in the text. Symbols of the same shape represent experiments performed on fibres in one culture with the same iontophoretic pipette.

fields being mapped escaped detection. Thus it was possible to estimate with some confidence the frequency of hot spots on the two fibre types. For control fibres, on the average, one hot spot was found every thirty-six electrode placements. For innervated fibres, the mean frequency was one

every twenty-five electrode placements. Thus, hot spot frequency on control fibres was only 69 % of that on innervated fibres. It is interesting that, in the hot spot/background sensitivity ratio histogram of Fig. 6*B* (innervated fibres), the dominant group (lower sensitivity) of hot spots contains

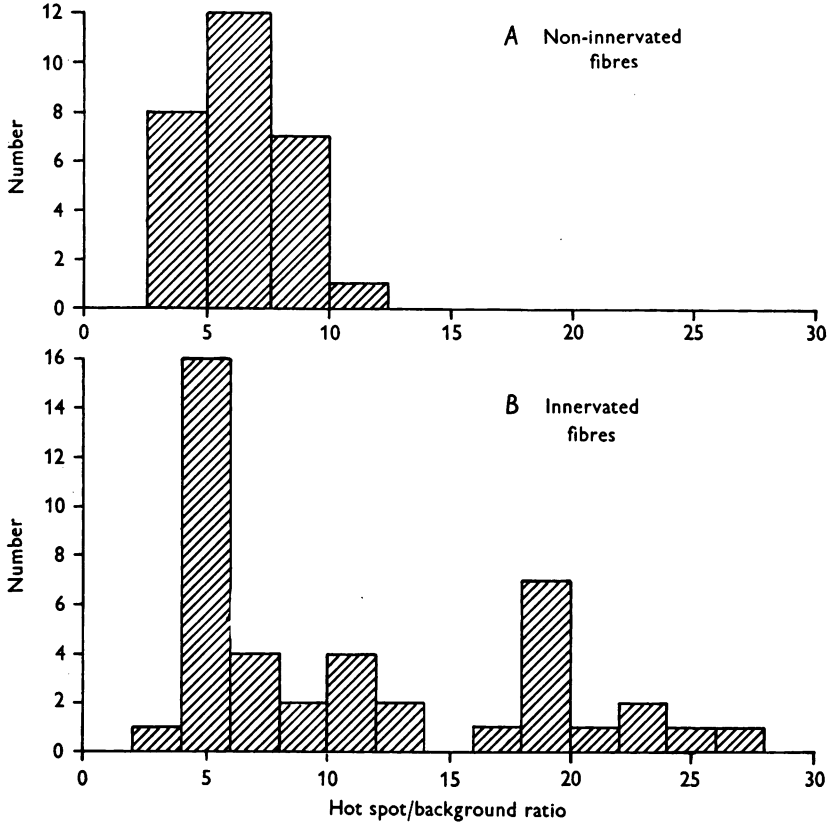

Fig. 6. Histograms of the distribution of ratios of hot spot sensitivity ÷ background sensitivity for control fibres (*A*) and innervated fibres (*B*). The control fibre histogram in *A* is uniformly distributed about the mean, but the innervated fibre histogram in *B* is highly skewed. The difference between the histograms is due to a difference in hot spot sensitivities, since background sensitivity is not affected by innervation. The innervated fibres with ratios greater than 15 (i.e. greater than that observed on any control fibre) represent about one third of the total number of hot spots found on innervated fibres.

69 % (29 of 42) of the total number of hot spots found. This group also has a similar sensitivity profile to that of the control fibres (Fig. 6*A*). In other words, the higher frequency of hot spots found on innervated fibres matches the apparent distribution of hot spots into low- and high-sensitivity groups. One might, therefore, suppose that those in the high-sensitivity

group represent the population of hot spots induced by innervation, although of course the quantitative similarity might simply be coincidental.

Finally, it should be noted that most hot spots on innervated fibres were at sites of nerve contact, although not every nerve contacted a hot spot. The few hot spots that were not contacted by nerve processes fell into the low-sensitivity group. Synaptic sites were not identified, although it was shown previously (Betz, 1976*b*) that many nerve-muscle contacts do not form synapses in these cultures.

DISCUSSION

The present experiments have confirmed earlier observations that shortly after innervation of chick embryonic muscle *in vitro*, no large scale changes occur in the pattern of ACh sensitivity on the muscle fibres (Fischbach & Cohen, 1973; Fischbach *et al.* 1976). Isolated hot spots are still found scattered across an otherwise uniformly sensitive muscle surface. However, detailed examination revealed several quantitative differences between ACh sensitivities of control and innervated fibres. While background sensitivity was unchanged, mean hot spot sensitivity was higher on innervated fibres, and hot spots were encountered more often on innervated fibres than on controls.

It would be of interest to know whether innervation caused an increase in the sensitivity of all hot spots on a muscle fibre, or whether the effect was restricted, for instance to sites of synapse formation. The histogram of hot spot to background sensitivity ratios changed markedly after innervation, becoming skewed towards higher ratios (Fig. 6). About two thirds of the ratios overlap those on control fibres, while the remaining one third are higher, indicating increased hot spot sensitivity. This might reflect a localized effect of innervation, with most hot spots remaining unaffected. Alternatively, the skewness may have resulted from a delayed effect of innervation. That is, if the sensitivity of all hot spots increased after innervation, but with a delay, then those fibres only recently innervated at the time of the experiment would not have exhibited the effect. This possibility could be tested by measuring the sensitivities of many hot spots on single fibres, or by measuring the time of innervation in long term experiments on single fibres. Both of these experiments are difficult technically, requiring repeated impalements of muscle fibres. The only other observation pertinent to this question is that the hot spots (on innervated fibres) which were not contacted by nerve processes had sensitivities in the lower range (i.e. like those on control fibres); all hot spots with sensitivities in the higher range were contacted by nerves. This suggests that the effect was localized to points of nerve contact.

Another difference between control and innervated fibres was that hot spots were encountered more often on innervated fibres than on controls. Frequencies were one per twenty-five positions (innervated) and one per thirty-six positions (controls), a difference of about one third, which suggests that new hot spots form after innervation. This conclusion rests on the assumption that no hot spots escaped detection, or more generally that the fraction of existing hot spots detected was the same for both groups. This assumption seems reasonable because mapping experiments were performed in a consistent fashion, the ACh electrode being moved in 5–10 μm steps, and because hot spots were shown to be about the same size and shape (5–10 μm diameter, circular patches) on both control and innervated fibres. An alternative explanation is based on the fact that fields for mapping innervated fibres were usually selected to include visible nerve processes in contact with the muscle. If hot spots move in the plane of the membrane and are 'captured' by contacting nerve processes, then the observed increase in hot spot frequency would not reflect an increase in the absolute number of hot spots on innervated fibres, but rather a shift in their spatial distribution. Experiments in which hot spot positions have been monitored for up to a day have not revealed any movement (unpublished observations). While the appearance and disappearance of hot spots on non-innervated fibres has been reported, these changes may reflect changes in turnover rate of ACh receptors rather than movement of hot spots within the membrane (Fischbach *et al.* 1976).

The two changes caused by innervation discussed above share a quantitative similarity. To restate them, (1) about one third of the hot spots on innervated fibres had sensitivities higher than those on control fibres, and (2) hot spots were encountered about one third more often on innervated fibres than on controls. The quantitative coincidence suggests the possibility of a causal connexion between the two observations. For instance, both observations might be explained by supposing that nerves which form synapses induce the formation of new hot spots (end-plates), and that these acquire a higher ACh sensitivity than the pre-existing population of hot spots. While other explanations cannot be ruled out with certainty, this hypothesis seems to explain most simply the observed results. Finally, it should be noted that a somewhat puzzling aspect of the hypothesis is that it affords no obvious role for the pre-existing ACh hot spots in the process of synapse formation.

Expert technical assistance was provided by Henry Hart and Phil Morgan. Supported by N.I.H. grant HS 10207. M.O. was supported on N.I.H. training grant no. 5 T01 GM-02118-05.

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