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NEUROMUSCULAR JUNCTIONS AND α-BUNGAROTOXIN-BINDING SITES IN DENERVATED AND CONTRALATERAL CAT SKELETAL MUSCLES

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SUMMARY

1. The distributions of cholinesterase (ChE) activity and acetylcholine (ACh) receptors were studied in normal and denervated cat hind-limb fast-twitch skeletal muscles and in muscles contralateral to denervated muscles.

2. On normal muscle fibres almost all receptors were confined to the immediately post-junctional membrane, although a perijunctional gradient of increased ACh receptor density was found on both fast- and slow-twitch fibres. After denervation, the extrajunctional ACh receptor density increased greatly and remained high for at least 10 months. Ectopic regions staining for ChE activity and having a high density of ACh receptors appeared in denervated muscle. The number of junctional ACh receptors decreased slowly after denervation, with a half-time of about 140 days.

3. Fast-twitch muscles contralateral to denervated muscles also showed changes, including an increase in junctional size and a small but significant increase in extrajunctional ACh receptor density.

INTRODUCTION

The physiology and biochemistry of skeletal muscle are influenced by its innervation and activity (for review see Harris, 1974 and Fambrough, 1979). In this and a subsequent paper (Eldridge, Liebhold & Steinbach, 1980), the effects of prolonged denervation and disuse on the morphology of the neuromuscular junction and on the number and distribution of acetylcholine (ACh) receptors are reported. This paper presents results of studies on fibres from normal cat skeletal muscles, from denervated muscles and from muscles contralateral to denervated muscles.

Three major objectives were pursued. The first was to obtain data for comparison with those from disused muscles. The second was to examine the effect of prolonged denervation on muscle ACh receptors. It has been reported that after denervation of rat skeletal muscle a high density of extrajunctional ACh receptors is produced, but that the extrajunctional receptor density may decrease after prolonged denervation (Albuquerque & McIsaac, 1970; Hartzell & Fambrough, 1972). Further, the number of junctional ACh receptors has been reported to decrease slowly after denervation (Frank, Gautvik & Sommerschild, 1975). Accordingly, the number of

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junctional ACh receptors and the density of extrajunctional receptors were determined after periods of denervation up to 327 days. It has also been reported that denervated mouse muscle fibres produce ectopic regions which have a high density of ACh receptors (Ko, Anderson & Cohen, 1977). It was of interest to determine whether such regions could be found on denervated cat skeletal muscle fibres. Finally, in frogs the denervation of one cutaneous pectoris muscle results in an increase in the incidence of multiply innervated fibres in the contralateral muscle (Rotshenker & McMahon, 1976). Comparable studies have not been reported for mammals, so muscles contralateral to denervated muscles were examined.

METHODS

Young adult cats (2–6 years) of either sex were used. Cats were anaesthetized with pentobarbitone for all procedures. Hind limbs were denervated either by ventral root section or by removal of a 2 cm length of the sciatic nerve in the sciatic notch. The success of the denervation was confirmed at the end of the denervation period by stimulating the sciatic nerve and the individual muscle nerves. In no case was any muscle contraction seen under a dissecting microscope.

Cholinesterase activity

An acetylthiocholine method was used to demonstrate cholinesterase activity histochemically (Karnovsky & Roots, 1964). The reaction was performed in acetate buffer at pH 5·2. A clearer staining pattern was obtained by reducing the substrate concentration to one-third the amount given by Karnovsky & Roots (1964) and by cooling the staining solution. Acetylthiocholine and butyryl thiocholine (both iodide salts) were obtained from Calbiochem (San Diego, Ca); ethopropazine was a gift of the Warner-Lambert Research Institute (Morris Plains, N.J.) and BW 284C51 a gift of Wellcome Research Laboratories (Beckenham, Kent).

Fluorescent α-bungarotoxin

Purified α-bungarotoxin was provided by Jim Patrick, the Salk Institute. It was conjugated with tetramethyl rhodamine isothiocyanate (Anderson & Cohen, 1974), and the mono-conjugated derivative (R-α-BT) isolated by ion-exchange chromatography (Ravdin & Axelrod, 1977). R-α-BT was used at a concentration of 5 μg/ml. in Dulbecco modified Eagle's medium, buffered to pH 7 with 15 mM-*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid and containing 1% heat-inactivated fetal calf serum ('medium'). Portions of muscles were incubated with R-α-BT for 2 hr at room temperature on a rocking table, then rinsed three times with medium and washed for 2 hr with changes of medium every 15–20 min. Tissue was then fixed with 4% (w/v) paraformaldehyde in 100 mM-Na-phosphate buffer (pH 7·0) for 60–120 min at room temperature. The tissue was rinsed with medium and sometimes stained to demonstrate cholinesterase activity. Tissue was stored in either 70% (v/v) ethanol/water or glycerol. Teased fibres or small bundles of fibres were cleared and mounted in glycerol and viewed with a Zeiss microscope.

Radio-iodinated α-bungarotoxin

Di^{125}iodo-α-bungarotoxin (I-α-BT) was prepared and purified by the methods of Vogel, Sytkowski & Nirenberg (1972). Portions of muscle were incubated in 2×10^{-8} M-I-α-BT in medium for 2 hr at room temperature. Control tissue was pre-incubated in 10^{-3} M-D-tubocurarine (curare) for 15–30 min, then incubated with 2×10^{-8} M-I-α-BT for 2 hr in the continued presence of 10^{-3} M-curare. Tissue was then rinsed three times with medium (with or without curare) and washed for 2 hr with changes of medium (with or without curare) every 15–20 min, fixed in 4% paraformaldehyde for 60–120 min at room temperature and stained for ChE activity to locate junctions.

The number of extrajunctional and junctional I-α-BT binding sites was determined by teasing five small bundles of fibres from each labelled muscle. The number of muscle fibres in each bundle was counted, the bundles were cut into measured lengths of junction-containing and extrajunctional muscle, and the amount of bound radioactivity determined by gamma counting. The extrajunctional binding per fibre per unit length was calculated and subtracted from the binding to junction-containing regions to estimate the number of junctional receptors. The specific binding of I-α-BT to ACh receptors was calculated by subtracting the binding per fibre per unit length in the presence of curare. To convert the extrajunctional toxin binding to surface binding site densities, the mean fibre circumference was calculated. In some cases this was done by embedding tissue in soft plastic (Fambrough, 1974), cutting cross-sections and determining the mean diameter and circumference of 50–100 fibres on enlarged photomicrographs using a digitizer connected to a desk-top computer (Hewlett-Packard). A more rapid method was used in most cases. The mean fibre width for fifty fibres was estimated at 400 × in the light microscope. The mean fibre width and mean diameter agreed fairly well (within 15%, $P > 0{\cdot}10$ by the t test) when fibres from the same muscle were analysed. In the cross-sectioned samples the mean circumference was found to be 3·3 × the mean diameter (mean ratio = $3{\cdot}27 \pm 0{\cdot}10$, mean ± S.D., $n = 14$). The circumference was calculated from the width by multiplying by this factor.

The average curare-protectable percentage of junctional toxin binding was 88 ± 4% (mean ± S.D., $n = 11$, range 76–92%). On denervated muscles the extrajunctional binding was 95 ± 3% ($n = 4$, range 86–99%) curare-protectable. In one experiment the degree of saturation of I-α-BT binding sites was examined by incubating portions of a normal muscle for 2 or 4 hr at room temperature. The junctional binding in the presence of curare increased approximately threefold with the longer incubation, while the binding in the absence of curare was not significantly changed. In an additional experiment, Dr James Boulter kindly analysed solubilized extracts of normal cat muscle labelled *in vitro* using sucrose gradient centrifugation (Boulter & Patrick, 1977). He found that when extracts were re-labelled with the same batch of I-α-BT, approximately 30% more radioactivity was included in the 9S peak, suggesting that approximately 75% of the I-α-BT binding sites had been occupied during the 2 hr *in vitro* labelling period. Since only undamaged surface fibres or bundles were used for autoradiography or gamma counting it appears likely that more than 75% of the available I-α-BT binding sites were labelled by the protocol used.

Autoradiographs were prepared by the methods of Bevan & Steinbach (1977), using NTB-2 emulsion (Kodak, Rochester, N.Y.) diluted 1:1 with deionized water. Grain densities were converted to binding site densities by assuming that the emulsion had an efficiency of 0·5 grains per gamma emission (Ada, Humphrey, Askonas, McDevitt & Nossal, 1966; Burden, 1977).

Silver staining

Nerve fibres were stained by the method of Namba, Nakamura & Grob (1967), adapted to *en bloc* staining. Tissue was fixed in 4% paraformaldehyde in 100 mM-Na-phosphate buffer, pH 7, then stained lightly for ChE activity as described above. The stained tissue was stored in 70% ethanol/water (v/v) at 4 °C for 1–14 days. It was rehydrated in distilled water and bundles of less than 1 mm diameter teased out. These bundles were incubated in 0·25% (w/v) K_3FeCN_6 for 15 min at 37 °C, then washed in distilled water and dehydrated with 70, 90, 95% ethanol (5 min each step) and finally absolute ethanol (Goshgarian, 1977). They were rehydrated briefly then incubated in the silver impregnation solution (10% $AgNO_3$, 0·05% $CuSo_4.5H_2O$, all w/v in distilled water) at 37 °C for 2 hr. The silver solution was poured off, distilled water added (about an equal volume) and the tissue incubated another 2 hr at 37 °C. This wash reduced background muscle fibre and connective tissue staining. The silver was reduced in 2·5% sodium sulphite and 0·25% hydroquinone (w/v in distilled water; this solution must be fresh). Reduction was carried out for 3–5 min with swirling or rocking of the dish. Tissue was washed in distilled water (2–24 hr) and stored in glycerol. The tissue was easily teased. This staining method is relatively rapid and gave consistent results. The background staining was variable but generally low. The impregnation stains myelinated branches and major terminal branches clearly, but it is possible that it does not show the finest terminal branches.

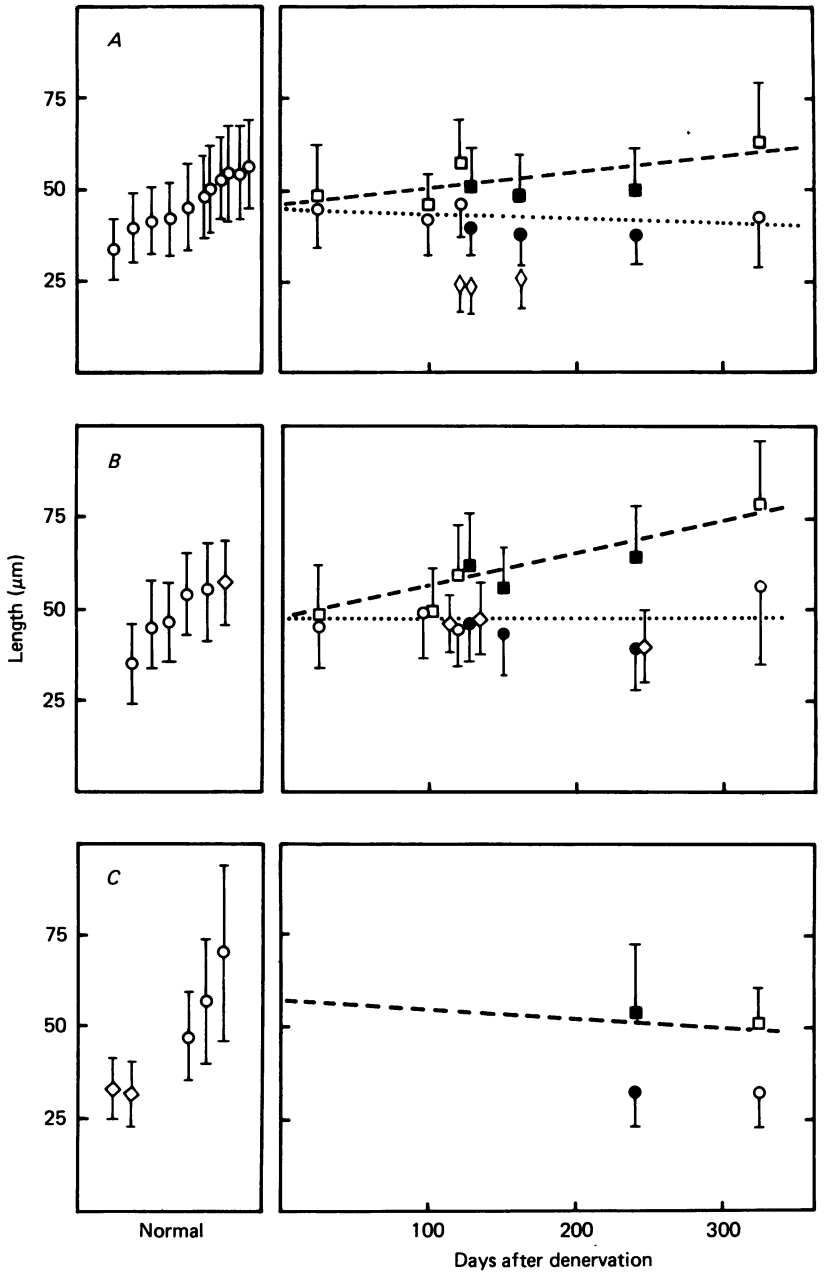

Fig. 1. Lengths of cholinesterase-staining regions in normal and denervated fast-twitch muscles. The arithmetic mean ChE-staining lengths ± S.D. are shown for p.l. (*A*), e.d.l. (*B*) and Sol. (*C*) muscles. Lengths for normal muscles are shown on the left, those for denervated (circles) and contralateral (squares) muscles are shown as a function of time after denervation on the right of each panel. The open symbols indicate that the muscles were denervated by ventral root section, the filled symbols indicate that the sciatic nerve was cut. In *A* the diamonds show mean lengths for ectopic ChE-staining regions on denervated p.l. muscles; in *B* the diamonds show the mean ChE-

RESULTS

Most observations were made on muscles of the peroneal group (peroneus longus, (p.l.) and tertius (p.t.)) and extensor digitorum longus (e.d.l.) muscles, all fast-twitch muscles located in the calf. Some observations were made on the soleus (sol, a slow-twitch calf muscle) and the vastus intermedius (v.i., also called crureus, a slow-twitch lower thigh muscle). In a few cases the extensor digitorum carpi (e.d.c., a fast-twitch forelimb muscle) was examined.

The size of an individual junction was measured as the length which showed ChE activity. This measure was chosen because it was readily obtained and because it did not suffer from the problem of estimating the 'width' of a junction on the curved muscle fibre (cf. Anzenbacher & Zenker, 1963).

The extrajunctional ACh receptor density was quantitated in two ways. The curare-protectable I-α-BT binding per unit fibre length was obtained as described in the Methods. To provide an indication of the spatial distribution of extrajunctional receptors, autoradiographs were made of muscle fibres teased from muscles after incubation with I-α-BT. For simplicity the phrase 'ACh receptor' will be used, although the I-α-BT binding site number or density was actually determined.

Muscles from normal cats. As can be seen in Fig. 1 there was a range of mean junctional length in the same muscle in different cats. The differences in mean length are great enough to indicate that different cats have different mean junctional sizes, at least for fast-twitch muscles. A one-way analysis of variance showed that the mean lengths on different cats were not due to random sampling variations from a single homogeneous population of junctional lengths ($P < 0{\cdot}005$ for p.l. and $P < 0{\cdot}025$ for e.d.l. muscle). In three cats the two junctional lengths for contralateral p.l. muscles were determined; in each case the mean lengths were essentially identical (Fig. 1).

Normal cat muscles had a dense accumulation of ACh receptors at the neuromuscular junction (Pl. 1; Fig. 3*A*). There was no curare-protectable I-α-BT binding in the distant extrajunctional region (Fig. 3*C*). However, autoradiographs of fibres

staining length on a forelimb muscle (e.d.c.). The following points should be noted. There was no significant change in the mean ChE-staining length on denervated p.l. (*A*) or e.d.l. (*B*) muscles. The dotted lines show linear regressions of mean length on time after denervation. In *A* the slope is $b = -0{\cdot}015$ μm/day, in *B* $b = 0{\cdot}006$ μm/day. In neither case is this slope significantly different from 0 (t test on b, $P > 0{\cdot}5$). Further, the populations of mean lengths on normal or denervated p.l. or e.d.l. muscles were not significantly different by the Wilcoxon rank test ($P > 0{\cdot}2$). The mean ChE-staining lengths on contralateral p.l. or e.d.l. muscles increased as a function of time after denervation. The dashed line in *A* has $b = 0{\cdot}045$ μm/day, in *B* $b = 0{\cdot}087$ μm/day. These slopes are significantly different from 0; for the line in *A*, $P < 0{\cdot}025$; *B*, $P < 0{\cdot}001$. There was no change in mean length on a forelimb muscle after hind-limb denervation (*B*). In three normal cats the mean ChE-staining lengths were determined for the two p.l. muscles (paired points on the left in *A*). The junctional lengths did not differ between the two sides of the animal (by the t test, $P > 0{\cdot}5$). Lengths for normal v.i. (diamonds) and Sol. (circles) muscles are shown on the left in *C*. The right side shows mean lengths on denervated (circles) and contralateral (squares) Sol. muscles as a function of time after denervation. (The dashed line through the squares has slope $b = -0{\cdot}025$ μm/day, this is not significantly different from a slope of 0, $P > 0{\cdot}5$). Each mean was calculated from 50–150 individual length measurements.

teased from normal muscles incubated in I-α-BT showed a gradient of extrajunctional receptors around the junction (Fig. 2; see Pl. 1). The gradients were similar on fibres from fast-twitch or slow-twitch muscles (Fig. 2). The I-α-BT binding in the gradient was specific, since the extrajunctional grain density about 150 μm from the junction was 90% protectable by 10^{-3} M-curare in fibres teased from one cat whereas the junctional binding determined by gamma counting in this cat was 75% protectable.

The gradients probably did not result from diffusion of bound α-BT along the membrane or from internal diffusion of radioactive degradation products for three reasons. First, incubations in I-α-BT were usually performed at room temperature, at which the mobility of aggregated α-BT binding sites (Axelrod, Ravdin, Koppel, Schessinger, Webb, Elson & Podleski, 1976), and the rate of extrajunctional receptor degradation (Devreotes & Fambrough, 1975) are both small. Secondly, similar gradients were seen on fibres teased from muscles incubated with I-α-BT and washed at 4 °C, at which temperature the non-specific uptake of α-BT is low (Libelius, 1975). Finally, in one cat the muscle was first fixed with 4% paraformaldehyde, then incubated with I-α-BT and fibres autoradiographed. These fibres also showed gradients, although they were not quantitated. An estimate of the number of ACh receptors in the perijunctional gradient can be made by fitting exponential curves to the data. The length constant was about 200 μm, the peak extrapolated density about 50 ACh receptors/μm^2 and the fibre circumference about 150 μm. These values give an estimate of 3–4 $\times$ 10^6 ACh receptors in the gradient, or 5–10% of the number at the junction.

In a few cases it was possible to tease muscle fibres with the tendon end intact. In these cases fibres from the v.i. (a slow-twitch muscle) showed a peak of receptor density at the muscle–tendon junction (Fig. 2*C*). The peak at the tendon end was lower than the perijunctional gradient. (For five fibres, the peak density near the tendon was 0·5 $\pm$ 0·5 (mean $\pm$ S.D.) times the density 100 μm from the junction.) Ten fibres with intact tendon insertions were teased from the v.i. muscle and all showed a raised extrajunctional ACh receptor density at the tendon end, while only two of the four fibres teased from the p.l. did.

Denervated muscles. The mean ChE-staining lengths on individual fibres were determined on muscles after different periods of denervation (Fig. 1). Cholinesterase staining could be found at junctional regions even 327 days following denervation, although the stain was slower to appear and the pattern was distorted (Pl. 1). In fast-twitch muscles the mean ChE-staining length did not decrease up to 327 days after denervation (Fig. 1). The lack of change in length of the ChE-staining region on fast-twitch muscles is surprising. However, the slopes of the dotted regression lines in Fig. 1 are not significantly different from zero. Furthermore, the mean ChE-staining lengths for normal or denervated muscles appear to be drawn from the same population ($P > 0{\cdot}5$ by the Wilcoxon rank test). In sol. muscle the mean lengths on both denervated muscles were significantly reduced from the grand mean of all lengths on normal sol. muscles ($P < 0{\cdot}01$ for either period by the t test).

There were accumulations of ACh receptors at denervated junctional regions on p.l. muscles (Pl. 1), although the number of junctional ACh receptors decreased with time after denervation (Fig. 3*A*). In a semilogarithmic plot the decrease in the number of junctional ACh receptors had a time constant of 200 days, with a 95% confidence interval ranging from 100 to 800 days.

Following denervation the extrajunctional ACh receptor density increased greatly

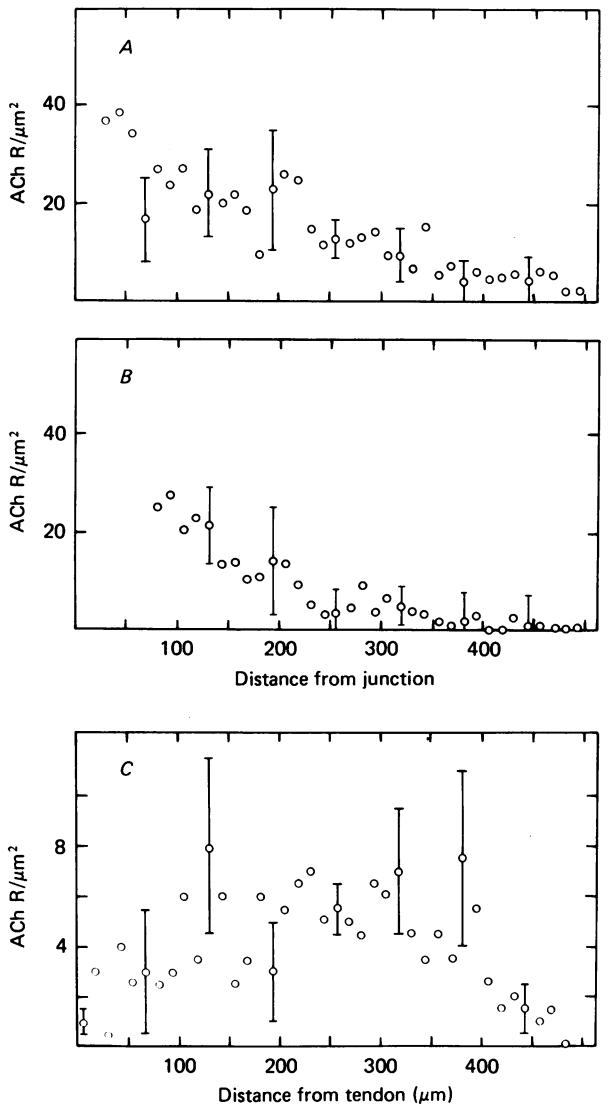

Fig. 2. Distribution of extrajunctional ACh receptor density on autoradiographs. The calculated extrajunctional ACh receptor (ACh R) density is shown as a function of distance from the junction (*A* and *B*). The mean density is shown, with ± 1 S.D. shown on every fifth point. *A* shows the mean gradient on four fibres from normal p.t. muscle, and *B* the mean gradient on four normal v.i. fibres. When a single exponential curve was fitted to the data, a length constant of 158 μm was obtained for the data in *A* and 118 μm for the data in *B*. For three additional similar averaged gradients on fibres from normal muscles values of 196 μm (p.t.), 142 μm (e.d.l.) and 102 μm (Sol.) were calculated for length constants. *C* shows the mean density of ACh receptors from the tendon end on three normal v.i. fibres. The grain density in autoradiographs was determined along the fibre. The distant extrajunctional density was taken as the non-curare-protectable level and subtracted from the gradient values. The gradient could not be reliably quantitated close to the junction, because the grain density in the emulsion perpendicular to the dense junctional grain accumulation also was above background. The grain densities were converted to site densities by using the known specific activity of the I-α-BT and assuming that each disintegration produced 0·5 grains (see Methods).

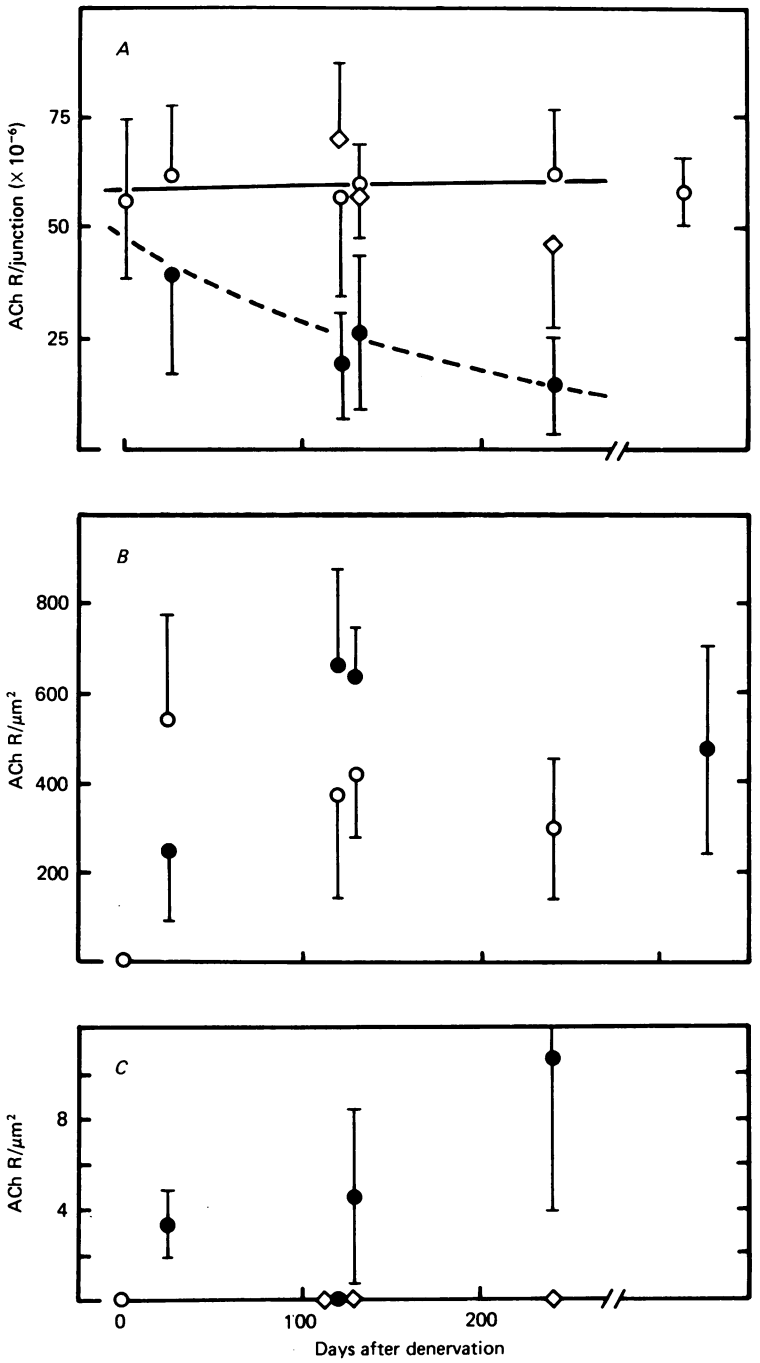

Fig. 3. ACh receptors on denervated and normal muscles. *A* shows estimates of the mean number (± 1.S.D.) of ACh receptors per junction on muscle fibres. Open circles show estimates on normal (left axis) or contralateral p.l. muscles as a function of time after denervation, filled circles show estimates on denervated muscle fibres, while diamonds show estimates on forelimb (e.d.c.) fibres. There is no significant change in the number of junctional ACh receptors on contralateral p.l. or on e.d.c. fibres after

(Fig. 3*C*). However, there was a suggestion from both the gamma counting and autoradiographic estimates that the extrajunctional receptor density decreased somewhat after prolonged denervation (Fig. 3*C*). A semilogarithmic plot of the gamma-counting estimates as a function of time gave a time constant of 356 days for decrease of extrajunctional receptor density, but the 95% confidence limits on the time constant were 190–3000 days. The autoradiographic data gave slopes for either linear or semilogarithmic regressions of receptor density on time which were not significantly different from zero.

Although the general extrajunctional ACh receptor density increased after denervation, no perijunctional gradient was apparent (Pl. 1). The extrajunctional receptor density was estimated on autoradiographs of twelve denervated fibres from three denervated cats at two regions: near, about 100 μm from the previous junctions and far, about 600 μm from the junction. The average ratio of near to far receptor densities was $1{\cdot}11 \pm 0{\cdot}26$ (mean $\pm$ S.D.). This ratio is not significantly different from 1 ($P > 0{\cdot}1$), confirming the qualitative impression that the perijunctional gradient was absent on denervated fibres.

Ectopic ChE-staining regions were seen in muscles denervated 100 or more days. These deposits were generally rather simple in appearance, although the deposit occasionally showed stripes or striations (Pl. 2). Portions of denervated p.l. and e.d.l. muscles from a cat denervated 140 days previously were incubated using acetylthiocholine, acetylthiocholine plus ethropropazine (10^{-4} M) to demonstrate acetylcholinesterase or butyrylthiocholine plus BW284-C51 (5×10^{-5} M) to demonstrate butyrylcholinesterase activity (Silver, 1974). Both denervated junctions and ectopic regions had predominantly AChE activity, as has been found for normal cat neuromuscular junctions (Nystrom, 1968*b*). The ectopic ChE-staining regions appeared to be randomly distributed along the muscle fibre length whereas the previous innervation band could still be readily found because of the aligned deposits of reaction product. The mean length of ectopic ChE-staining regions was also less than that of denervated junctions (Fig. 1*A*).

In autoradiographs of fibres teased from denervated muscles it was noticed that

denervation; the over-all mean of these data is $58 \pm 6{\cdot}6 \times 10^6$ ACh receptors/junction (open circle at right). The dotted line through the filled circles is the best fitting single exponential. On a semilog plot this line has a slope of $-0{\cdot}005$ days^{-1}. (This slope is significantly different from 0, $P < 0{\cdot}025$ by the t test.) The number of ACh receptors was estimated from the curare-protectable junctional I-α-BT binding, as described in Methods. No correction has been applied to allow for the ACh receptors in the perijunctional gradient. Such a correction would reduce the numbers of junctional ACh receptors in normal or contralateral junctions by $3–4 \times 10^6$ (see text), and would not alter the values for denervated muscles since the perijunctional gradient is absent after denervation (see text). *B* shows estimates of extrajunctional ACh receptor density on denervated p.l. fibres as a function of time after denervation (mean $\pm$ I.S.D.). The open circles are estimates from gamma counting, the filled circles from grain counts on autoradiographs. *C* shows estimates of extrajunctional ACh receptor density on normal and contralateral muscles. The circles show data from normal (open circle) or contralateral (filled circles) p.l. muscles. The diamonds show data from forelimb (e.d.c.) muscles. The estimates were obtained from gamma counting of bound I-α-BT. The binding to contralateral p.l. muscles in the absence of curare was significantly higher than in the presence of curare for the three points plotted above the abscissa ($P < 0{\cdot}025$ by the t test).

some individual fibres which showed more than one ChE deposit also showed multiple areas of high receptor density at the same sites. This point was studied further in four denervated muscles. In confirmation of the observations of Ko *et al.* (1977), fibres from denervated muscles had regions away from the original end-plate zone which stained with fluorescent α-BT (Pl. 2). In muscles denervated 100–150 days about 15–25 % of the fibres in bundles had such ectopic sites. The staining pattern with R-α-BT was clearly different from either normal or denervated junctions (Pls 1 and 2; see also Ko *et al.* 1977). Some denervated muscles were doubly stained for ChE activity and with R-α-BT. In these muscles, most sites staining for ChE activity also showed a high density of ACh receptors, although the association of the two stains did not appear to be absolute. 63 % of the ectopic regions stained for both ChE and ACh receptors, 7 % for ChE alone and 30 % for ACh receptors alone. (In all, 109 sites from four denervated p.l. muscles were examined.) It is not known whether the observation that some ectopic sites stained with only one or the other stain is significant. In the majority of the cases both stains occurred in the same region.

It is possible that these ectopic sites resulted from reinnervation of denervated fibres. This possibility is unlikely for three reasons. First, the muscles did not contract to stimulation of the sciatic nerve or individual muscle nerves. Secondly, silver stains of two muscles denervated by sciatic section showed no signs of nerve axons although ectopic sites were present. Finally, electron micrographs of junctional regions of one denervated muscle showed no signs of nerve processes (F. G. Klier, personal communication), although most reinnervation of denervated muscles occurs at previous junctional regions (see, for example, Bennett, McLachlan & Taylor, 1973).

Contralateral and forelimb muscles from denervated cats. Fast-twitch hind-limb muscles contralateral to denervated legs underwent some changes. The mean junctional lengths of p.l. and e.d.l. muscles increased with time following contralateral denervation (Fig. 1), and three out of four contralateral p.l. muscles had significant amounts of curare-protectable extrajunctional I-α-BT binding (Fig. 3*C*). These changes were not seen in forelimb (e.d.c.) muscles from denervated cats (Figs. 1*B* and 3*C*).

The increase in mean junctional length is unlikely to be a sampling artifact. The strongest argument is that the regression lines of mean length on time after denervation have a slope which is significantly different from zero. Other tests are more difficult to make in view of the variability among normal cats. If it is accepted that ChE-staining lengths on denervated p.l. and e.d.l. muscles did not change over this period of denervation, then the mean ChE-staining lengths on denervated muscles can be used as controls. In thirteen out of fourteen pairs the mean lengths of the contralateral muscles were longer. This has a $P < 0{\cdot}005$ of occurring randomly, by the binomial distribution. The increase in length occurred whether muscles underwent motor denervation (ventral root section) or total denervation (sciatic nerve section; see Fig. 1). Junctions on contralateral soleus muscles did not appear to increase in length (Fig. 1), although relatively few muscles were examined.

It has been reported that frog cutaneous pectoris muscles contralateral to denervated muscles have an increased incidence of multiply innervated fibres (Rotshenker

& McMahon, 1976; Rotshenker, 1978). This did not seem to occur in these cat muscles: of 340 junctions examined in four silver-stained normal muscles and 519 junctions in five silver-stained contralateral muscles, no evidence for multiple innervation of single junctions was seen. In two normal and two contralateral muscles fifty fibres were teased from ChE- and silver-stained preparations, and in no case was any evidence seen for separated sites of multiple innervation (for normal muscles, see also Tuffery, 1971; Brown & Matthews, 1960).

DISCUSSION

There is variability from cat to cat in the mean junctional length for a given normal muscle, as might be expected for an outbred population. It is difficult to compare directly the present data on junctional length with published observations, since other authors have used various measurements of junctional size (area: Mann & Salafsky, 1970; mean diameter: Nystrom, 1968*a*; Tuffery, 1971; Ip & Vrbova, 1973) and have studied muscles from only a few animals. In a study on rabbit muscle, Dias (1974) found that, in general, junctions in slow-twitch muscles had larger junctional areas than in fast-twitch muscles, although he also reported that there were considerable differences in mean areas between different muscles of the same type.

The distribution of ACh receptors on cat muscle fibres is similar to that on all normal skeletal muscle fibres studied to date. Most receptors are confined to the primary junctional fold while the density of extrajunctional receptors far from the junction is undetectably low. Both fast- and slow-twitch fibres from cat show perijunctional gradients of extrajunctional receptors (Axelsson & Thesleff, 1959; Johns & Thesleff, 1961). Such gradients are also present on adult rodent muscles (Dreyer, Muller, Peper & Sterz, 1976; Hartzell & Fambrough, 1972) but contain lower receptor densities. It is likely that only slow-twitch fibres have an appreciable density of receptors at the muscle–tendon junction (see also Miledi, Stefani & Zelena, 1968).

A high density of extrajunctional ACh receptors develops after denervation (see also Axelsson & Thesleff, 1959), and is maintained for at least a year. There may be a slow decrease in the extrajunctional receptor density after prolonged denervation. The perijunctional gradient disappears after denervation, as has been found for rat muscle (Albuquerque & McIsaac, 1970; Hartzell & Fambrough, 1972).

The junctional accumulations of ChE activity and ACh receptors appeared to be quite stable after denervation (Frank *et al.* 1975). The quantitative data on the number of ACh receptors at denervated cat junctions suggest that the accumulation of receptors actually outlasts the individual ACh receptor in the accumulation. The half-life of rodent junctional receptors has been estimated at 5–13 days (Berg & Hall, 1975; Merlie, Heinemann & Lindstrom, 1979; Linden & Fambrough, 1979). These estimates of receptor half-life would have to be incorrect by at least tenfold if the loss of junctional receptors from denervated cat junctions were solely due to slow degradation of individual receptors. The present data are consistent with the idea that the junctional receptor aggregating activity has a half-life of about 140 days at denervated cat neuromuscular junctions. Burden, Sargeant & McMahon (1979)

have recently shown that denervated frog junctional regions can induce the aggregation of ACh receptors on newly formed muscle fibres, and suggest that a stable substance deposited in the junctional basal lamina (or possibly the remaining Schwann cells) actually directs the accumulation of ACh receptors. Their observation shows that a receptor-aggregating activity can survive loss of both the nerve and muscle. It is not known that the same activity is involved in both situations, although this seems likely.

Following denervation small patches of receptor-rich membrane appear in extrajunctional regions of adult muscle fibres (Ko *et al.* 1977). Frequently these patches also stain for ChE activity, and the histochemical stain appears to result from AChE activity. It has not been reported previously that extrajunctional patches of ChE stain appear on denervated adult muscle, although Lubinska & Zelena (1966) found that denervated neonatal rat muscle did produce ectopic ChE deposits. No study was made of the temporal sequence for production of these ectopic regions in denervated cat muscles, so the period of denervation required for their production is not known. Based on the results in denervated mouse muscle (Ko *et al.* 1977), however, it seems likely that patches of receptor-rich membrane appear before the ChE activity is high enough to detect histochemically. Short term denervation of rodent muscle results in the loss of all detectable 16S (junctional specific) AChE from the muscle within 14 days (Vigny, Koenig & Rieger, 1976; Weinberg & Hall, 1979; Fernandez, Duell & Festaff, 1979), although an accumulation of AChE activity is demonstrable at denervated junctions for at least 56 days (Guth, Albers & Brown, 1964). If the results from rodent muscle are applicable to denervated cat muscle, the esterase staining on denervated cat muscle fibres is due to AChE activity, but probably the enzyme is not the 16S (junctional) form.

Fast-twitch muscle contralateral to denervated limbs showed several changes. It is possible that the changes resulted from relative disuse of contralateral muscles (Eldridge *et al.* 1980), since the cats were less active at climbing and leaping after denervation. The role of activity in stimulating the contralateral changes is uncertain, because the reduction in activity must have been relatively small, and studies have actually used contralateral denervation as a means of producing increased muscle use and hypertrophy (Drahota & Gutmann, 1962). Some general conclusions may be drawn, in any case. First, muscles in limbs contralateral to denervated limbs are not necessarily control muscles. Secondly, the stimulus for the changes is non-systemic, since forelimb muscles were not affected. Thirdly, the stimulus does not involve sensory feed-back from denervated muscles, since similar effects were observed whether ventral root (motor only) or sciatic nerve (total) denervation was performed. Possibly analogous observations have been made in frog skeletal muscle. Denervation (or total removal) of one cutaneous pectoris muscle results in an increase in multiple innervation of muscle fibres in the contralateral muscle (Rotshenker & McMahan, 1976; Rotshenker, 1978). Unfortunately, the results obtained in frogs do not elucidate the role of muscle use in these phenomena, since the activity of normal or contralaterally denervated cutaneous pectoris muscles is not known.

Other work has shown that contralateral denervation can produce alterations in sciatic nerve protein content (Luttges, Kelly & Gerren, 1976) and in proteins

synthesized and transported by sensory neurones (Theiler & McClure, 1978). There are some indications that muscle twitch times may be altered following contralateral denervation as well (Syrovy, Gutmann & Melichna, 1972; Kean, Lewis & McGarrick, 1974).

Several over-all conclusions may be drawn from these observations. (1) The specialization which directs the accumulation of ACh receptors and ChE activity at adult junctions is quite stable after denervation. (2) A high density of extrajunctional ACh receptors is maintained on denervated muscle fibres for a long period after denervation. (3) The development of regions on the muscle fibre which stain for both high densities of ACh receptors and ChE activity can occur in the absence of innervation. (4) A relatively mild stimulus (contralateral denervation) can result in an alteration of the size of neuromuscular junctions. This stimulus does not involve any direct treatment of the affected nerve or muscle, and the result raises questions about both the nature of the stimulus and the mechanism by which junctional size is regulated under normal circumstances.

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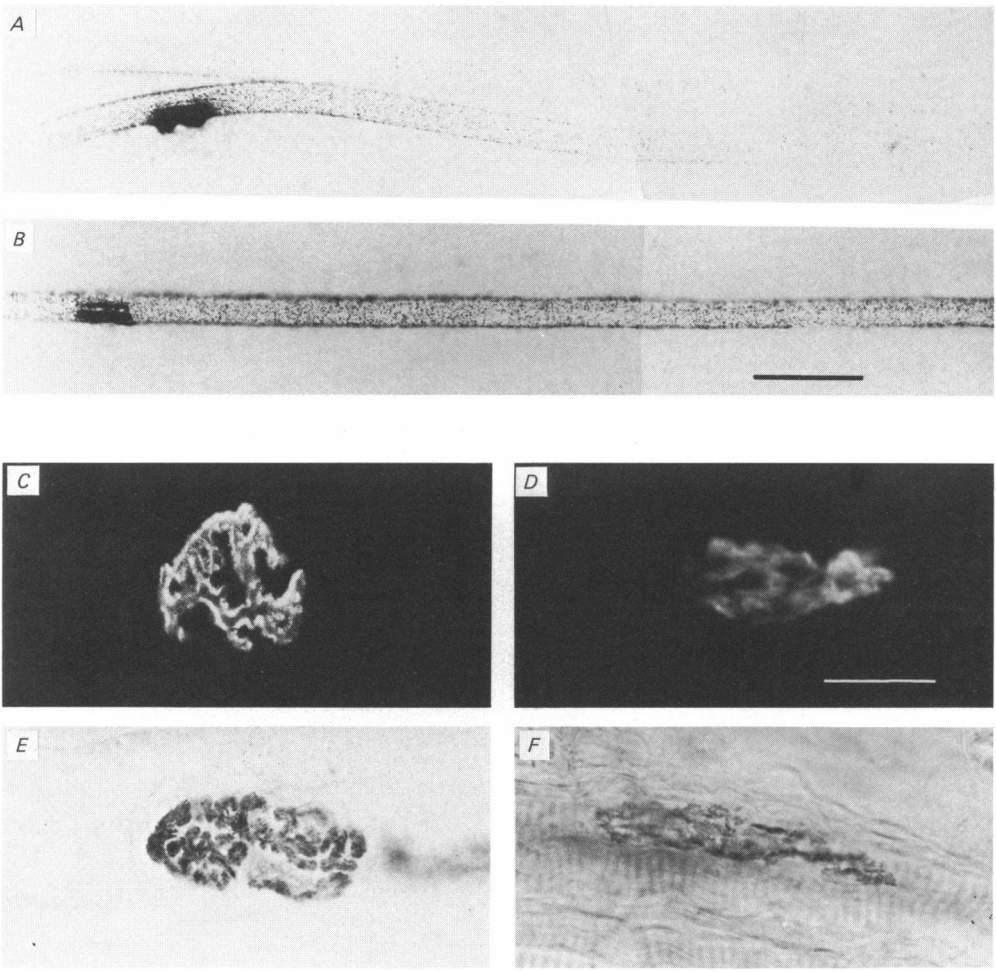
EXPLANATION OF PLATES

PLATE 1

A and *B* show autoradiographs of fibres teased from a normal muscle (*A*, two fibres seen) and a muscle denervated 25 days previously (*B*). Note the presence of a perijunctional gradient on the normal fibre, and its absence in *B*. The specific activity of the I-α-BT and the exposure time were such that one grain per unit area in *A* corresponds to one-fifth of the I-α-BT binding-site density as in *B*. Bar in *B* indicates 100 μm. *C* and *D* show junctions stained with R-α-BT; *C* shows a junction from a normal muscle while *D* shows a junction denervated 128 days previously. The denervated junction is not out of focus, the fluorescent staining of denervated junctions becomes progressively blurred and spreads. *E* and *F* show ChE staining at junctions denervated 128 days (*E*) and 327 days (*F*) previously. Notice the progressive loss of detail in the pattern and the increasing distortion with prolonged denervation. The bar in *C* indicates 50 μm for *C*–*F*.

PLATE 2

This plate shows ChE and R-α-BT stains of denervated muscle fibres. *A* and *B* show staining patterns for a junctional region denervated 128 days previously. *C* and *D* show an ectopic region on a fibre denervated 128 days previously, while *E* and *F* show an ectopic region on a fibre denervated 250 days previously. These ectopic regions were chosen because they showed a relatively high degree of morphological differentiation. Bar in *F* indicates 50 μm.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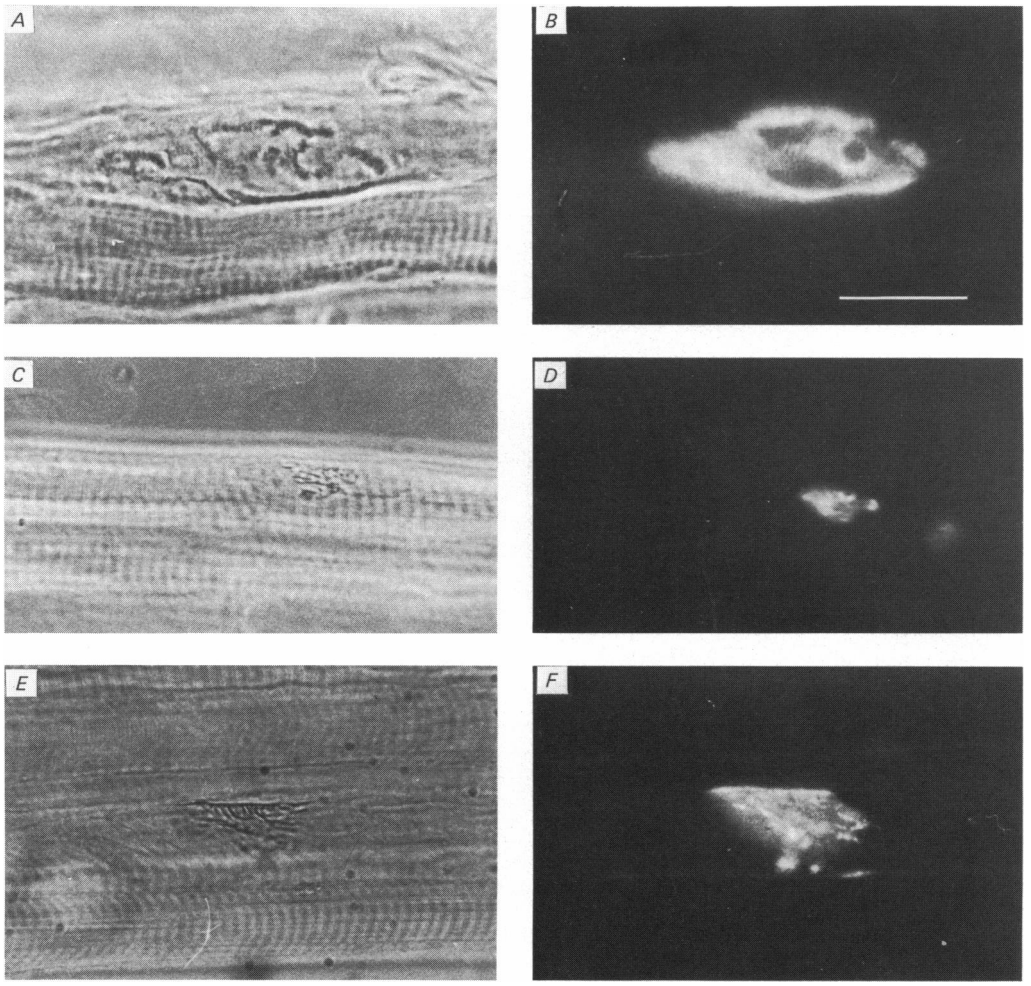
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