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LACK OF NICOTINIC SUPERSENSITIVITY IN FROG SYMPATHETIC NEURONES FOLLOWING DENERVATION

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SUMMARY

1. The sensitivity of bull-frog sympathetic neurones to nicotinic, cholinergic agonists has been studied in both normal (control) and surgically denervated ganglia.

2. Using gross extracellular recording, the sensitivity to acetylcholine (ACh) increased 18-fold following denervation, while that to carbachol (CCh) was unchanged. Normal ganglia showed a similar sensitivity increase after inhibition of cholinesterase. This suggests that the rise in ACh sensitivity is due to reduced cholinesterase activity, not to true supersensitivity.

3. There was no significant difference in resting membrane potential or input resistance between normal and denervated neurones.

4. Neurones denervated for 7–50 days showed no significant change in sensitivity to ACh or CCh applied iontophoretically at a distance of 10 μm from the cell surface.

5. In control ganglia, localized iontophoretic application of ACh revealed an uneven distribution of sensitivity which is attributed to the localization of receptors to synaptic areas.

6. Fourteen days after denervation, the geometric mean sensitivity to focally applied ACh was not significantly different from that found in control ganglia. The variation in sensitivity to focally applied ACh at randomly chosen sites on denervated neurones was as great as that found in control ganglia.

7. It is concluded that denervation does not cause frog sympathetic neurones to become supersensitive to ACh. The apparent increase in nicotinic ACh sensitivity observed using extracellular recording from whole ganglia is due not to a change in the number or distribution of ACh receptors, but to a decrease in cholinesterase activity.

INTRODUCTION

In normal adult skeletal muscle, nicotinic acetylcholine (ACh) receptors are localized to the motor end-plate. A similar heterogeneous distribution of nicotinic ACh receptors also occurs on both parasympathetic and sympathetic neurones of the frog (Harris, Kuffler & Dennis, 1971; Marshall, 1981). Following denervation of skeletal muscle, there is an over-all increase in sensitivity of the tissue to applied ACh, i.e. 'denervation supersensitivity'. This is due primarily to an increase in the number of receptors in the extrasynaptic membrane (Thesleff, 1960; Fambrough, 1979). However, a reduction in cholinesterase activity following denervation may produce a further increase in ACh sensitivity (McConnel & Simpson, 1976).

Although most of our knowledge of denervation supersensitivity has come from the study of smooth and skeletal muscle, this phenomenon has also been observed in the cardiac ganglia (Kuffler, Dennis & Harris, 1971; Roper, 1976; Dennis & Sargent, 1979).

The occurrence of denervation supersensitivity is not, however, ubiquitous. In crayfish muscle, denervation does not give rise to an increased sensitivity to the excitatory amino acid glutamate (Frank, 1974). In the rat C.N.S. cholinergic denervation of the hippocampus results in an increased sensitivity to ACh but not to carbachol (CCh), due to a decrease in cholinesterase activity (Bird & Aghajanian, 1975).

In mammalian sympathetic ganglia, denervation has been reported to increase the sensitivity to ACh (Cannon & Rosenblueth, 1936; Perry & Reinert, 1954; Ambache, Perry & Robertson, 1956; Chien, 1960). However, in these early experiments, the contraction of the nictitating membrane produced by application of ACh to the superior cervical ganglion was measured, and the increase in sensitivity of the nictitating membrane was not always taken into account. Furthermore, no attempt was made to distinguish between changes in cholinesterase activity and changes in sensitivity of the neuronal membrane. Brown (1969) reported an increase in the sensitivity of cat sympathetic ganglia to ACh following denervation which was due to a decrease in cholinesterase acitivity, since the sensitivity to the cholinesterase-resistant agonist carbachol was unchanged. Other workers have reported a decrease in sensitivity of sympathetic neurones following denervation (Volle & Koelle, 1961; Green, 1969; Dun, Nishi & Karczmar, 1976).

We have examined the cholinergic sensitivity of bull-frog sympathetic neurones to look for any change in sensitivity following denervation which could be produced by a decrease in cholinesterase activity, a change in the number or distribution of ACh receptors, or by some other mechanism. Although denervation produced a dramatic increase in the sensitivity of ganglia to bath-applied ACh, when measured using gross extracellular recording, this appeared to be due to the loss of cholinesterase activity. Using iontophoretic application of ACh, we have been unable to detect a significant change in the sensitivity to ACh, and this suggests that the acquisition of supersensitivity may not be a general rule for denervated neurones.

METHODS

Denervation. Bull-frogs (*Rana catesbiana*) were anaesthetized by immersion in a solution of tricaine (0·1 %, w/v) for 1 h. The 9th and 10th sympathetic ganglia were denervated by surgical section of the afferent pathways (Hunt & Nelson, 1965). This procedure was performed by either removing a section of the interganglionic nerve between the 6th and 7th ganglia, and severing the 7th and 8th rami, or by removing the interganglionic nerve between the 8th and 9th ganglia (in some cases, either the 7th or 8th ganglion was completely removed). Since there was evidence of reinnervation by 20–30 days after surgery, the operation was repeated on some frogs during this time to produce longer periods of denervation. While it is generally accepted that the rami of the 9th and 10th ganglia contain only post-ganglionic axons (Langely & Orbeli, 1910; Hunt & Nelson, 1965), it has been reported that these rami may also carry a few preganglionic fibres (Pick, 1957). Although we considered this possible uncertainty, these rami were left intact in order to focus our investigation on the effects of afferent denervation alone, and to avoid the changes produced by axotomy of ganglion cells (reviewed by Purves & Lichtman, 1978).

Denervated preparations were examined by light microscopy (25 ×) to confirm that all afferent pathways had been cut, and that reinnervation had not occurred. If any preganglionic pathways appeared intact, the preparation was discarded. Despite these measures, in five of sixteen preparations, synaptic responses were recorded in approximately 10 % of the neurones impaled when the nerve between the 8th and 9th ganglia was stimulated. Any neurones responding to preganglionic nerve stimulation or exhibiting spontaneous miniature excitatory post-synaptic potentials (e.p.s.p.s) were excluded from the study.

Preparation. The lumbar sympathetic ganglia were removed from control and denervated frogs, and pinned out in the recording chamber. Following 5 min incubation with collagenase (1 mg/ml, Worthington, class CLSPA) the connective tissue over the 9th and 10th ganglia was dissected away. All experiments were carried out at room temperature (21–23 °C), in Ringer solution (115 mM-NaCl, 2 mM-KCl, 3·6 mM-$CaCl_2$, 1 mM-Na HEPES, pH 7·2) containing 0·5 μM-atropine sulphate (Sigma) to prevent activation of muscarinic receptors. At this concentration, atropine has no effect on the nicotinic response (MacDermott, Connor, Dionne & Parsons, 1980)

Extracellular recording. A single ramus from the 9th or 10th ganglion was cut close to the spinal nerve, and drawn up into a tightly fitting suction electrode. This electrode and a similar bath reference electrode were connected to a high gain (DC) differential amplifier.

The bath was perfused with Ringer solution at a rate of 2 ml/min. Drugs were applied by changing the input to the perfusion pump to the selected Ringer solution containing drug at the required concentration. To correct for the difference in response amplitude due to the variation in the size of the rami between different preparations, responses were normalized with respect to the response produced by 100 μM-γ-aminobutyric acid (GABA). This was found to be more consistent than normalizing to the depolarization produced by 6–12 mM-KCl.

Intracellular recording. Intracellular recordings were made from the large (B-type) neurones under 800 × Hoffman modulation contrast optics. Glass micro-electrodes were filled with 4 M-K acetate and had resistances of 90–100 MΩ. In some experiments, a conventional bridge balance circuit was used for passing current through the recording micro-electrode to measure the input resistance of the neurone.

Diffuse application of agonists. Micropipettes were filled with 2 M-acetylcholine chloride (ACh) or 2 M-carbamylcholine chloride (carbachol, CCh) and had resistances of 30–40 MΩ. These were carefully positioned at 10 μm above the surface of the impaled neurone by noting the micrometer movement required to change the focus from the upper surface of the cell soma to the tip of the pipette. A negative braking current of 6–12 nA was applied to the iontophoretic pipettes to prevent leakage of the drugs.This braking current was increased until a rapid approach to the cell with the pipette did not produce a noticeable depolarization. Agonists were ejected from the pipettes iontophoretically, using positive current pulses of 30 ms duration.

Focal application of agonists. The mapping of ACh sensitivity was carried out as described by Harris *et al.* (1971). For these experiments, finer micropipettes having a tip resistance of 100–300 MΩ were used. The iontophoretic pipette was pushed against the surface of the impaled cell, and 10 ms current pulses were passed through the pipette, until a 'current response' was observed. The pulse width was then reduced to 1 ms for the measurement of the ACh sensitivity. To restrict the effective diffusion radius, low iontophoretic currents, giving responses of less than 10 mV were employed.

Once a ganglion cell was impaled with the recording electrode, as many as five sites were examined for ACh sensitivity, however, impalements were often lost after only one or two sites had been studied. In some experiments on normal ganglia, ACh sensitivity was measured at the site of visible boutons, and at sites away from boutons. Boutons were recognized on neurones located near the edges of the ganglia where the tissue is thin and the optical resolution is quite high (see photomicrographs in Kuffler, 1980 and Dodd & Horn, 1983), comparable to that attained with cardiac neurones (McMahan & Kuffler, 1971).

Measurement of agonist sensitivity. For both diffuse and focal drug application, sensitivity (in mV/nC) was determined from the slope of graphs of peak response *vs.* iontophoretic charge (Kuffler & Yoshikami, 1975).

RESULTS

Extracellular recording

Some of the earliest experiments demonstrating the phenomenon of 'denervation supersensitivity' involved studying the response of intact tissues to exogenously applied agonists. Our study began with a similar approach by recording the gross depolarization of whole sympathetic ganglia produced by the bath application of ACh and CCh (Fig. 1 *A*). In non-denervated (control) ganglia, CCh was about 20 times more potent than ACh. After 14 days denervation, there was an 18-fold increase in the sensitivity of ganglia to ACh, while the sensitivity to CCh remained unaltered. As a result, ACh and CCh were almost equipotent in denervated ganglia (Fig. 1 *B*).

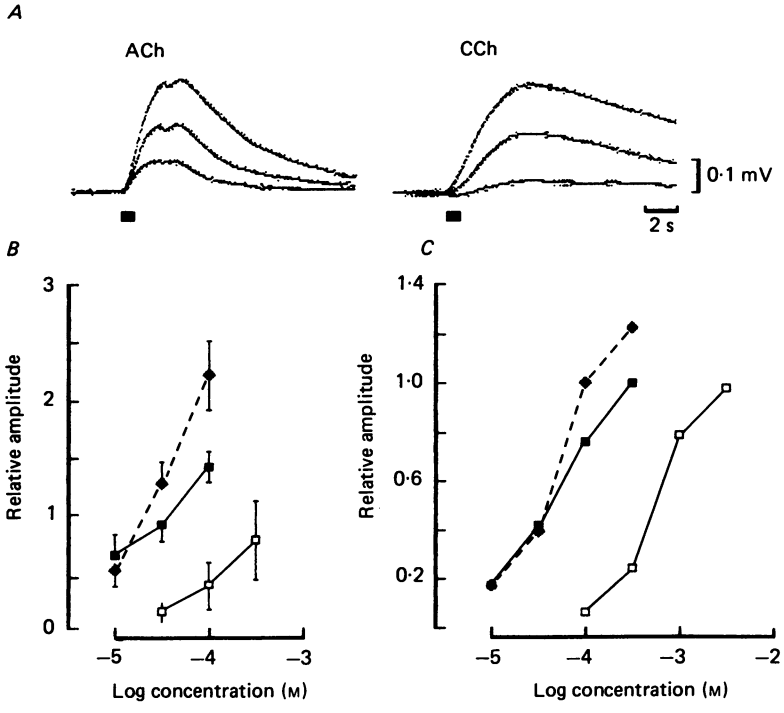

Fig. 1. Extracellular recording of whole ganglion depolarization produced by bath-applied ACh and CCh. *A*, representative responses to increasing concentrations of ACh (0·3, 1·0 and 3·0 mM) and CCh (10, 30 and 100 μM) applied during the time indicated by the bars below. *B*, log dose–response curves for extracellularly recorded ganglion depolarization produced by ACh in normal ganglia (open squares) and ganglia denervated for 14 days (filled squares). Denervated ganglia had significantly higher ACh sensitivity ($P < 0{\cdot}01$ by Student's t test). The responses to CCh (dashed line) were not significantly different in control and denervated ganglia, and have been combined for clarity. Each point represents the mean ±S.E. from three ganglia. *C*, log dose–response curves for ACh recorded from a single control ganglion, in the absence (open squares), and the presence of 10 μM-edrophonium (filled squares). Responses to CCh (dashed line) were unchanged by the presence or absence of edrophonium. Responses were normalized with respect to the peak depolarization produced by 100 μM-GABA (see Methods). The cause of slight depressions in the digitized responses to ACh in *A* is not known.

Denervation of cholinergically innervated tissues causes a decrease in acetylcholinesterase activity (Guth, 1968), and this alone might produce an increase in the sensitivity to ACh. When control ganglia were treated with the rapidly reversible anticholinesterase agent edrophonium (Tensilon), there was a marked increase in the sensitivity to ACh, while no significant change was detected in the response to the cholinesterase-resistant agonist CCh (Fig. 1*C*). Although it is not possible to come to a firm conclusion from this experiment alone, it seems likely that this selective increase in sensitivity to ACh produced by denervation can be accounted for by a decrease in cholinesterase activity. Rather than study this phenomenon in more detail at the extracellular level, we examine the ACh sensitivity of individual neurones.

Intracellular recording

Passive membrane properties. It is possible that a change in the passive electrical properties of the neurones following denervation might obscure any change in the ACh sensitivity. For this reason, in some experiments we determined the input resistance of neurones in denervated and non-denervated (control) ganglia. Neurones in control ganglia had a resting membrane potential of $54{\cdot}2 \pm 10{\cdot}6$ mV and an input resistance of 63 ± 35 MΩ (mean ±S.D. from fifteen cells). In ganglia denervated for 14 days, the membrane potential was $49{\cdot}5 \pm 5$ mV and the input resistance was $77{\cdot}6 \pm 67$ MΩ (mean ±S.D. from twelve cells). The values obtained in denervated and non-denervated ganglia were not significantly different ($P > 0{\cdot}05$, by Student's *t* test).

Spontaneous activity. Spontaneous impulse activity was observed in three of the twenty-four neurones examined after 30 days of denervation. These action potentials occurred at regular intervals, although the frequency declined from approximately 1 to 0·1 Hz over a period of 20–30 min. These neurones had membrane potentials greater than −45 mV, and no spontaneous miniature e.p.s.p.s were observed. Spontaneous impulse activity was never seen in any neurones of the non-denervated ganglia examined. The mechanism behind this spontaneous activity is unclear, but it may be analogous to the fibrillation observed in skeletal muscle following denervation (Salafsky, Bell & Prewitt, 1968).

Diffuse application of agonists

Sensitivity. Iontophoresis of ACh or CCh from a micropipette positioned 10 μm from the neurone surface produced responses that rose to a peak in approximately 50 ms then declined over a period of 0·5–1 s (Fig. 2*A*). The peak amplitude of responses to ACh applied in this manner were comparable to those produced by bath application of 5–100 μM-ACh, which is about a 5- to 10-fold lower concentration than required for whole ganglion depolarizations recorded extracellularly.

For both diffuse and focal drug application, sensitivity was determined as described by Kuffler & Yoshikami (1975). A series of responses to increasing iontophoretic currents were recorded, and the total iontophoretic charge and peak membrane depolarization were measured. Graphs of peak response *vs.* iontophoretic charge were constructed and the sensitivity in mV/nC was determined from the slope of the graph (Fig. 2*B*). These plots were usually linear over the range of iontophoretic currents used. For experiments using diffuse application of ACh and CCh, graphs of peak response *vs.* log iontophoretic charge were also constructed. On these semilogarithmic

graphs, the curves for ACh and CCh were approximately parallel, so the relative potency (dose ratio) was calculated from the lateral separation of the curves (Fig. 2*C*).

The sensitivities, determined from the slope of the dose–response curve, did not have a normal distribution, but showed a skewed distribution (Fig. 3*A*; Dennis & Sargent, 1979). However, when these data were displayed geometrically (i.e. on a logarithmic scale), an apparently normal distribution was observed (Fig. 3*B*). For this reason, all statistical analysis was carried out on the geometric means rather than the arithmetic means.

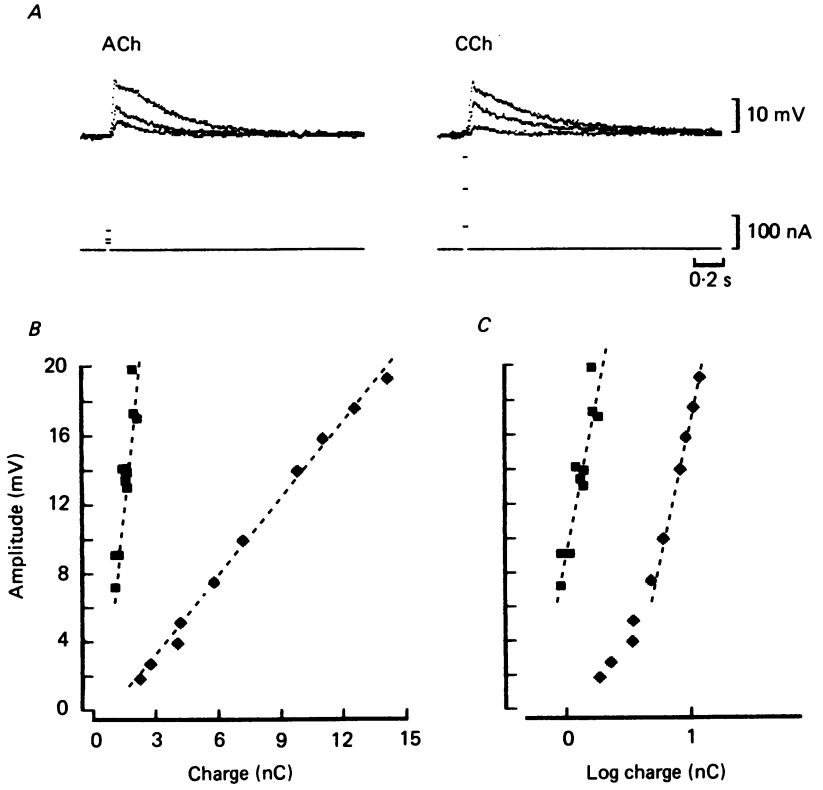

Fig. 2. Measurement of nicotinic ACh sensitivity. *A*, responses of a single control neurone to iontophoretic application of ACh and CCh at a distance of 10 μm from the cell surface. Linear (*B*) and semilogarithmic (*C*) plots of iontophoretic charge *vs.* peak response to ACh (squares) and CCh (diamonds). The slopes of these plots in *B*, determined by least-squares linear regression, yielded sensitivity values for ACh and CCh of 11·9 and 1·8 mV/nC respectively. For the plots in *C*, the dose ratio between ACh and CCh on this cell was found to be 5·5. Amplitude scale in *B* applies to *C*.

In neurones from control ganglia, the geometric mean sensitivities for ACh and CCh were 5·37 (4·17–6·92) mV/nC and 1·62 (1·26–2·09) mV/nC (mean and 95 % confidence limit) respectively (Fig. 4*A* and *C*). For neurones denervated for 7–50 days, the sensitivities to ACh and CCh were 5·01 (4·17–6·03) mV/nC and 1·28 (1·07–1·55) mV/nC respectively. These values were not significantly different from those obtained from innervated neurones ($P > 0{\cdot}05$ by Student's t test).

Furthermore, least-squares regression analysis of graphs of sensitivity *vs.* time after denervation gave slopes of zero, indicating that there was no progressive change in sensitivity with time after surgery (Fig. 4*B* and *D*). In one ganglion, after 20 days denervation, the sensitivity to ACh and CCh was significantly higher than controls, but in this preparation, three of the fifteen cells examined were innervated (see Methods) and these cells had similar sensitivities to the denervated cells. In another

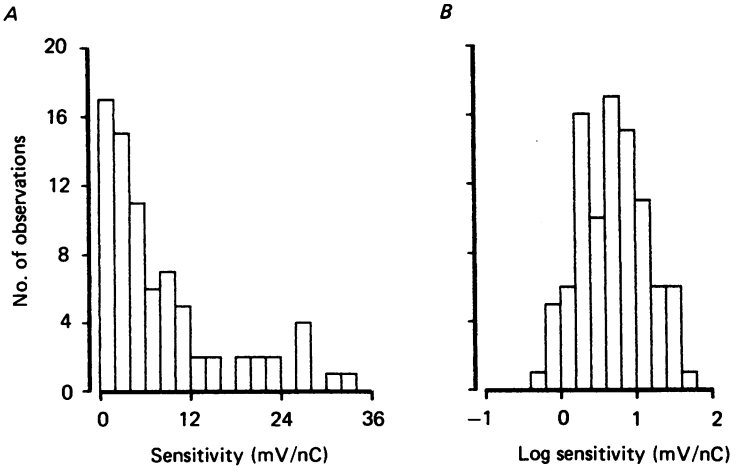

Fig. 3. Frequency distribution of ACh sensitivities of seventy-eight individual neurones from nine denervated ganglia. When displayed linearly (*A*), the data shows a markedly skewed distribution, but when plotted on a semilogarithmic scale (*B*), a more normal distribution is observed. Vertical scale in *A* applies to *B*.

preparation 24 days after denervation, the sensitivity was significantly lower than the control value. It is unclear why, in these two preparations, the sensitivities should have been so different, but it seems unlikely to be a result of denervation.

Cholinesterase inhibition. In marked contrast to the gross extracellular response to ACh, inhibition of cholinesterase had only a small and variable effect on the intracellular response to iontophoretically applied ACh. In the presence of 5–20 μM-edrophonium some cells showed no detectable effect. In other cells there was an increase in the amplitude and duration of the response to ACh, while the responses to CCh were unaffected (Fig. 5*A*). Consequently, edrophonium produced a parallel shift in the log dose–response curve to ACh (Fig. 5*B*), but not to CCh (Fig. 5*C*). In thirteen neurones from control ganglia, the peak amplitude of a submaximal ACh response was increased by an average of 27 % (range 0–150 %). Potentiation of ACh responses by edrophonium was never observed in any neurones of the three denervated ganglia examined. Thus it appears that cholinesterase activity is much less important in limiting the amount of applied ACh that reaches neurones in the outer cell layer of the ganglion, than for the larger population of neurones within the ganglion (see Discussion).

Focal application of ACh

Distribution of sensitivity in normal neurones. It is possible that denervation could produce an increase in the sensitivity of the extrasynaptic membrane, which may not

be easily detected as a significant change in the over-all sensitivity to diffuse application of ACh. For this reason, we continued our study using the focal application of ACh (Harris *et al.* 1971) to look for changes in the distribution of ACh sensitivity over the ganglion cell surface following denervation. As with the cardiac ganglion, neurones of the frog sympathetic ganglia possess no dendrites and the synapses are confined mainly to the cell soma (Weitsen & Weight, 1977; Marshall, 1981).

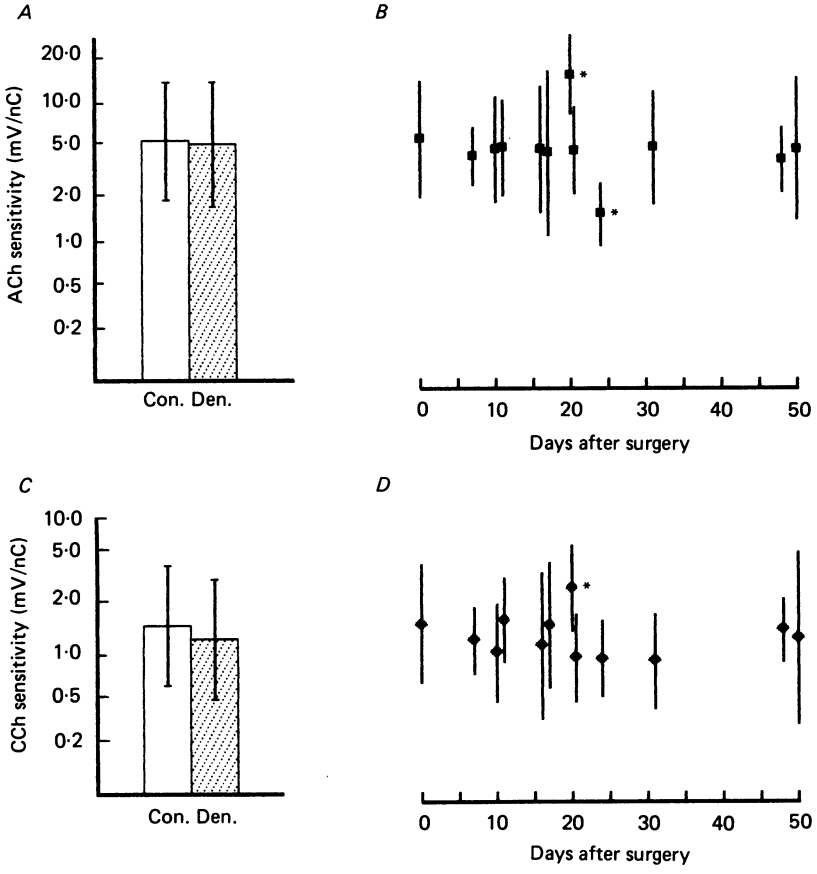

Fig. 4. Sensitivity of control and denervated neurones to diffuse iontophoretic application of ACh and CCh. The bar graphs show sensitivity to ACh (*A*) and CCh (*C*) in control (con.) and denervated (den.) ganglia. Bars represent geometric means ±S.D. of thirty-eight cells from six control ganglia and ninety-three cells from eleven denervated ganglia. The plotted graphs show the sensitivity to ACh (*B*) and CCh (*D*) at different times after denervation. Each point represents the geometric mean (±S.D.) of six to thirteen neurones from single preparations. A least-squares regression analysis of these graphs gave slopes of 0·0, and correlation coefficients of $< 0{\cdot}02$. * Significantly different from control sensitivities ($P < 0{\cdot}05$ by Student's *t* test).

Iontophoresis of ACh at sites on the surface of neurones evoked responses that reached a peak in 10–40 ms, then declined over a further 20–100 ms. Responses to ACh applied at identified synaptic boutons usually had shorter times to peak and showed higher sensitivity than responses from sites where boutons were clearly not present (Fig. 6*A*). The mean sensitivity of 447 mV/nC at synaptic sites (Fig. 6*B*) was

significantly higher ($P < 0{\cdot}005$ by Student's t test) than the mean of 195 mV/nC found at randomly chosen sites on the neurone surface (Fig. 6*C*). This is in agreement with the findings in the cardiac ganglion of the frog (Harris *et al.* 1971) and mudpuppy (Roper, 1976).

Focal sensitivity following denervation. Since synaptic sites cannot be identified on denervated neurones, we compared the sensitivity of sites chosen at random on neurones from control ganglia and ganglia which had been denervated for 14 days;

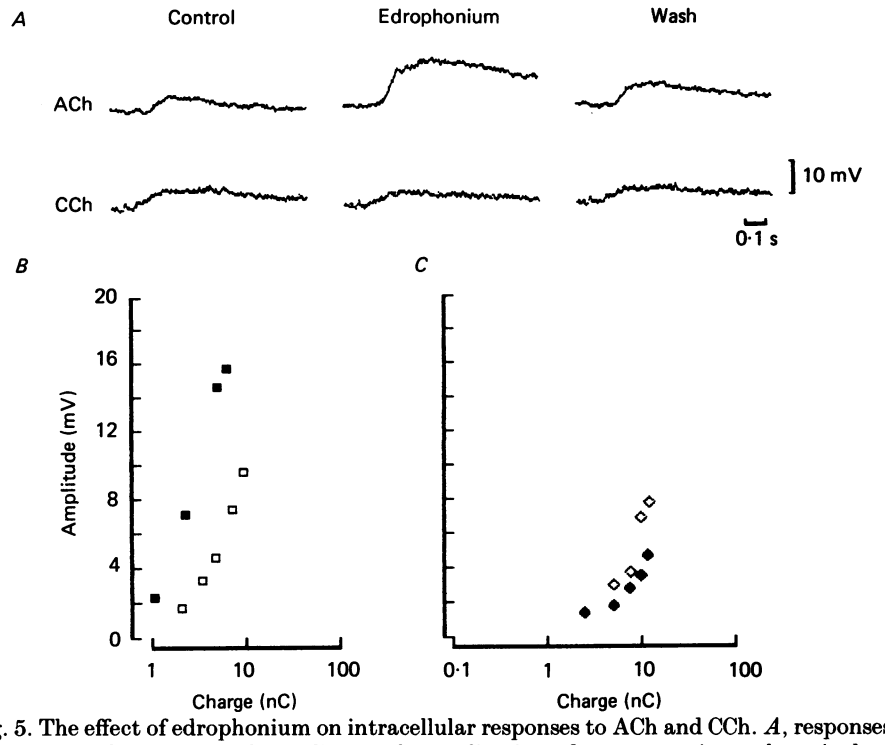

Fig. 5. The effect of edrophonium on intracellular responses to ACh and CCh. *A*, responses of a neurone from a control ganglion to the application of a constant iontophoretic dose of ACh and CCh before, in the presence of 10 μM-edrophonium, and after wash-out. Log dose–response plots for iontophoretic application of ACh (*B*) and CCh (*C*) in the absence (open symbols), and presence (filled symbols) of edrophonium (10 μM). Amplitude scale in *B* applies to *C*.

a time when one might expect changes to be apparent (Kuffler *et al.* 1971; Dennis & Sargent, 1979). ACh was applied to spots on the surface of the neurone, excluding the axon hillock region where nerve terminal boutons are densely packed. Boutons in the non-hillock region cover an average of 5·4 % of the cell surface (P. A. Fullwood & L. M. Marshall, unpublished), so most of these randomly chosen sites were probably on the extrasynaptic membrane. If denervation produced an increase in the density of ACh receptors in the non-synaptic membrane comparable to that seen in skeletal muscle, one would expect a significant increase in the mean sensitivity (see Discussion), a decrease in the range of sensitivities measured at random sites, and faster-rising responses (Kuffler *et al.* 1971).

The mean ACh sensitivity at random sites on neurones in denervated ganglia was

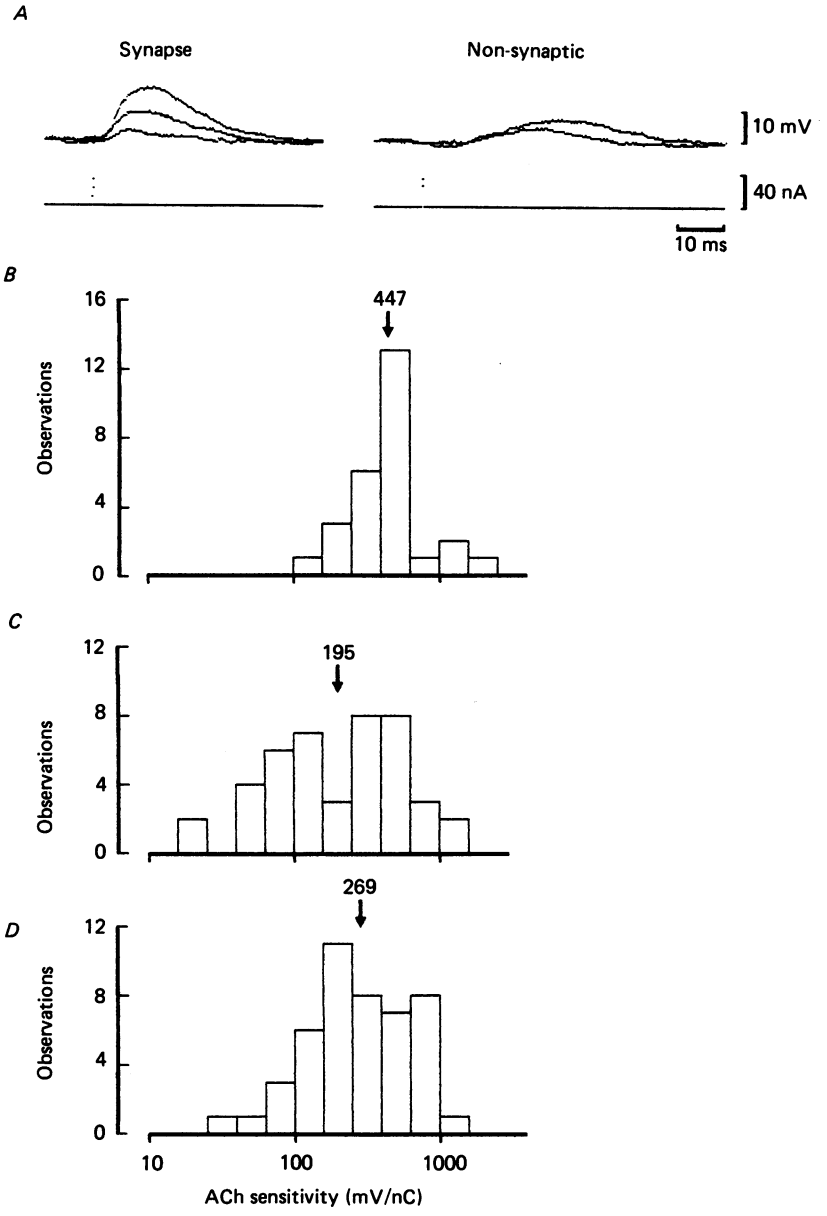

Fig. 6. Spatial distribution of ACh sensitivity. *A*, responses to focal iontophoretic application of ACh at a synapse and a non-synaptic site. The frequency distributions of ACh sensitivities recorded at identified synaptic sites on control cells (*B*), and at random sites on neurones from control (*C*) and 14-day denervated ganglia (*D*). The arrows indicate the geometric mean sensitivities. The mean value of 447 mV/nC in *B* was significantly different ($P < 0{\cdot}005$) from the means in *C* and *D*, while the mean value of 269 mV/nC in denervated ganglia (*C*) was *not* significantly different ($P > 0{\cdot}05$; Student's *t* test) from the 195 mV/nC found in control ganglia (*D*). Sensitivity scale in *D* applies to *B* and *C*.

269 mV/nC (95 % confidence limit, 226–351) as shown in Fig. 6*D*. Although this mean sensitivity was greater than the mean of 195 mV/nC (149–254) found in control ganglia (Fig. 6*C*), the increase was *not* statistically significant ($P > 0{\cdot}05$ by Student's *t* test). The ACh sensitivity at random sites on denervated neurones was significantly lower ($P < 0{\cdot}005$) than at identified boutons on control neurones (Fig. 6*B*). Also, the range in sensitivities observed at random sites on denervated neurones was as great as that in the controls. Furthermore, the mean time-to-peak of responses evoked at random sites on neurones from control and denervated ganglia were not noticeably different at $27{\cdot}8 \pm 13{\cdot}9$ ms (± S.D., twenty-one sites) and $28{\cdot}6 \pm 13{\cdot}8$ ms (± S.D., thirty-three sites), respectively. Therefore, we conclude that there was probably no appreciable change in the spatial distribution of ACh receptors following denervation.

DISCUSSION

Our original intention was to find a way to experimentally increase the number of functional ACh receptors in frog sympathetic ganglia as a prelude to possible studies of receptor regulation at the molecular level. We expected to find that sympathetic neurones of the frog, like muscle fibres and cardiac neurones, would become more sensitive to ACh after denervation, perhaps by increasing the density of ACh receptors in the extrasynaptic membrane. However, this study forces us to conclude that supersensitivity to ACh does not occur in frog sympathetic neurones during the period of 7–50 days following surgical denervation. Three principal findings support this interpretation: (1) the apparent increase in sensitivity to ACh but not to CCh, observed by extracellular recording from denervated ganglia, was found to be due to a loss of cholinesterase activity (Fig. 1); (2) individual neurones showed no significant increase in sensitivity to diffuse application of ACh (Fig. 4); and (3) focal application of ACh revealed no apparent change in the spatial distribution of sensitivity on the neuronal surface (Fig. 6).

Cholinesterase activity

Using gross extracellular recording, we observed a pronounced increase in the ACh sensitivity of the denervated ganglion, while the sensitivity to the cholinesterase-resistant agonist CCh was unchanged. This suggests that the increase in ganglion sensitivity to ACh resulted from a decrease in cholinesterase activity, a view supported by the ability of edrophonium to mimic the effect of denervation. A similar selective increase in the ACh sensitivity of cholinergically innervated tissues following denervation has previously been observed in the cat superior cervical ganglion (Brown, 1969), and in the hippocampus of the rat (Bird & Aghajanian, 1975).

Since denervation produced such a marked increase in the gross extracellular response of the ganglion to bath-applied ACh, we were surprised that iontophoretic application of ACh to individual neurones did not show a similar change in the sensitivity. However, subsequent intracellular recordings in the presence of edrophonium revealed that cholinesterase activity had relatively little effect on the ACh response of neurones located on the surface of ganglia. Histochemical staining shows cholinesterase activity on the external surface of the preterminal and terminal portions of the preganglionic axons (Weitsen & Weight, 1977). The most plausible

explanation we can think of is that bath-applied ACh molecules penetrating deeper into the ganglion encounter more esterase activity through exposure to larger numbers of preganglionic nerve fibres. It follows that, unlike neurones located deep within the ganglia, the superficial neurones should require lower concentrations of applied ACh for depolarization. Therefore, even a large loss of cholinesterase activity from the ganglion following denervation may not be detected by examining the ACh sensitivity of neurones on the outer surface of the ganglion.

Comparison to skeletal muscle

The higher ACh sensitivity in denervated skeletal muscle is primarily due to increased ACh receptor density in the extrasynaptic membrane (see review by Fambrough, 1979). In view of the extreme structural differences between ganglion cells and muscle fibres, it is necessary to consider whether similar changes in the extrasynaptic membrane of these neurones could increase the total number of ACh receptors enough to raise the over-all ACh sensitivity. Using values of receptor density from skeletal muscle, we made the following estimates.

Boutons cover about 15% of the total surface of B-type sympathetic neurones including the axon hillock region (P. A. Fullwood & L. M. Marshall, unpublished observations). Since the subsynaptic membrane consists of small, receptor-rich patches (Marshall, 1981) which occupy about 4% of the area beneath a bouton (Nishi, Soeda & Koketsu, 1967), the total subsynaptic membrane covers approximately 0·6% of the neurone surface membrane. In normal frog muscle the density of ACh receptors at the synapse is about 500 times that in the extrasynaptic membrane (Matthews-Bellinger & Salpeter, 1978). If the density of extrasynaptic receptors increases by 10-fold following denervation, as reported for skeletal muscle by Pestronk, Drachman & Griffin (1976), each neurone would now possess three times the normal number of ACh receptors. A 100-fold increase in the extrajunctional receptor density as reported by Fambrough (1974) would increase the total number of receptors by nearly 25-fold.

For an increase in extrasynaptic receptor density in frog sympathetic neurones comparable to that in denervated skeletal muscle, we would expect *at least* a 3-fold increase in the total number of ACh receptors. We would also expect such an alteration to substantially increase the sensitivity to diffusely applied cholinergic agonists, but no significant change was found. Therefore, if there was an increase in the extrasynaptic receptor density, it must be much less than occurs on denervated skeletal muscle fibres.

Comparison to denervated cardiac ganglia

Neurones of frog parasympathetic and sympathetic ganglia have similar morphology; a single preganglionic axon distributes synaptic boutons to the initial segment of the axon (hillock) and over the surface of a cell body, 25–50 μm in diameter (McMahan & Kuffler, 1971; Weitsen & Weight, 1977; Marshall, 1981). Preliminary studies on B-type sympathetic neurones (P. A. Fullwood & L. M. Marshall, unpublished observations) show that boutons occupy 15% of the total cell surface; 10% of this area is a large densely packed cluster of boutons located near the axon hillock, and the remaining 5% consists of about thirty boutons widely dispersed over the non-hillock region. We restricted focal application of ACh to this non-hillock region

where the distribution and density of boutons was comparable to that of cardiac ganglia in which boutons occupy about 3% of the neuronal surface (McMahan & Kuffler, 1971).

Given the structural similarities of parasympathetic and sympathetic neurones, it was surprising to find results that contrast with previous studies on cardiac ganglia of the frog (Kuffler *et al.* 1971; Dennis & Sargent, 1979) and the mudpuppy (Roper, 1976). Kuffler *et al.* (1971) applied ACh focally to randomly chosen spots on neurones denervated for 2–28 days. Four to eight days after cutting the vagus nerve, the mean sensitivity rose from 164 mV/nC to a plateau level of 258 mV/nC and the mean rise time of the ACh response fell from 42 to 21 ms. Since the responses at random spots became nearly as large and as rapid as those obtained at identified synapses, the authors concluded that ACh sensitivity was now evenly distributed over the neuronal membrane surface.

In the present study, however, we found no noticeable change in the mean rise time of the ACh response and, due to the considerable overlap in sensitivity values observed before and after denervation (cf. Fig. 6*C* and *D*), the shift in mean sensitivity from 195 to 269 mV/nC was not statistically significant. Thus, our data argues against increased sensitivity. Looking back at the similar overlap in sensitivity values and the absence of statistical tests in the study by Kuffler *et al.* (1971) it is reasonable to question the strength of this earlier demonstration of denervation supersensitivity. However, their data do indicate a significantly faster rising response to ACh, and this remains as a clear difference from our finding that the mean rise time was essentially unchanged 14 days after denervation.

The issue of increased ACh sensitivity in denervated frog cardiac ganglion cells gained strong support when Dennis & Sargent (1979) reported a striking increase in mean ACh sensitivity from about 130 mV/nC to 1000 mV/nC by 2–3 weeks after denervation. Increased maximal sensitivity (cf. Kuffler *et al.* 1971) was attributed to improved techniques: sensitivity measurement in the linear portion of the ACh dose–response relation, precise adjustment of iontophoretic braking current, and careful impalement of cells to ensure higher input resistances. After taking all of these precautions in the present study (see Methods), we think it unlikely that the absence of such supersensitivity in sympathetic neurones is due to technical problems.

Although it is clear that our findings on sympathetic neurones differ from those reported on parasympathetic neurones, we see no obvious explanation for why two types of autonomic neurones would respond differently to denervation. Nevertheless, the present study does suggest that denervation supersensitivity may not be a general principle for neurones.

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