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LOSS OF EXTRASYNAPTIC ACETYLCHOLINE SENSITIVITY UPON REINNERVATION OF PARASYMPATHETIC GANGLION CELLS

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SUMMARY

1. The extrasynaptic acetylcholine sensitivity of frog cardiac ganglion cells was measured both after denervation and during the early stages of reinnervation by preganglionic axons. Sensitivity was measured by ionophoretic application of acetylcholine (ACh) to randomly chosen sites on ganglion cell bodies.

2. Extrasynaptic sensitivity rose gradually following denervation and after 3 weeks reached a mean value of approximately 1000 mV/nC.

3. Reinnervation of the cardiac ganglion began about 3 weeks after nerve crush. The ACh sensitivity of ganglion cells fell markedly during the 23–31 day period, to a mean of 184 mV/nC. None of forty-three neurones studied during that period received synaptic inputs sufficient to generate action potentials.

4. Twenty-nine of the forty-three neurones examined 23–31 days after nerve crush had not yet received detectable synaptic inputs, yet even these cells had markedly reduced ACh sensitivity.

5. When reinnervation of cardiac ganglia was delayed by resecting the preganglionic nerves, ACh sensitivity was reduced slightly (43 %) between 14–21 and 23–31 days after surgery. Thus, most of the sixfold reduction in sensitivity that occurs during this time after nerve *crush* is a specific effect of reinnervation.

6. We conclude that loss of extrasynaptic receptors coincides with, or may even precede, the earliest physiological signs of synapse formation. Restoration of action potential activity in the ganglion cells is not essential to initiate this loss.

INTRODUCTION

Considerable attention has been focused on the long-term influences that neurones exert over their targets (reviewed by Purves, 1976). The best studied neuronal influence is that exerted upon extrajunctional acetylcholine (ACh) receptors of skeletal muscle fibres; there the density of ACh receptors outside the synaptic area is normally low but increases by orders of magnitude upon loss of innervation (Axelsson & Thesleff, 1959; Miledi, 1960*b*; Fambrough, 1974). Several general mechanisms have been proposed to explain this and other denervation-induced changes in the properties of muscle fibres. The explanation first advanced by Miledi

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(1960*b*) suggests that normal motor nerve terminals release some trophic substance which prevents the development of extrajunctional receptors. The loss of innervation would remove this trophic influence and thus allow extrajunctional receptors to develop. The converse possibility is that degenerating nerve terminals release some substance that produces an inflammatory response and that induces the appearance of extrajunctional receptors (Jones & Vrbová, 1974; Lømo & Westgaard, 1975). A quite different explanation is that motoneurones exert their influence through control of muscle activity (Jones & Vrbová, 1970; Lømo & Rosenthal, 1972; Drachman & Witzke, 1972); such a mechanism does not invoke the release of any substance other than the synaptic transmitter. Consideration both of trophic factor(s) and of activity may be necessary for a complete understanding of denervation-induced changes in muscle fibre properties.

In neurones denervation also produces supersensitivity to transmitter (Cannon & Rosenblueth, 1936). Recently, Kuffler and his colleagues demonstrated that denervation of parasympathetic ganglion cells in the frog results in an increase in extrasynaptic ACh sensitivity (Harris, Kuffler & Dennis, 1971; Kuffler, Dennis & Harris, 1971; see also Roper, 1976). Denervation results both in the elimination of preganglionic synaptic terminals and in the loss of ganglion cell activity, thus raising the question of which of these factors is responsible for the change in receptor distribution. We have addressed this question by studying the loss of extrasynaptic sensitivity during the reinnervation of ganglion cells. We find that the high over-all sensitivity of denervated neurones is markedly reduced during the earliest stage of reinnervation, well before action potentials are elicited by newly formed synapses. We conclude that the loss of extrasynaptic ACh receptors on ganglion cells does not require resumption of nerve impulse activity.

METHODS

Experiments were performed on parasympathetic ganglion cells in the frog heart. *Rana pipiens* (5 cm body length) of either sex were obtained from a supplier in the northeastern United States. There were no obvious seasonal variations in ACh sensitivity. Frogs were fed twice weekly with beef liver and were housed at 24 °C in chambers where they had access both to a dry platform and to a reservoir of running tap water (14–18 °C).

Surgery and recordings

Frogs were anaesthetized by immersion in 0·05 % (w/v) tricaine, and the cardiac ganglion was denervated by crushing or resecting the vagosympathetic nerves bilaterally (Kuffler *et al.* 1971). The site of nerve crush was about 1·2 cm from the heart.

In acute experiments ganglion cells were visualized at a magnification of 500 × using interference phase contrast optics (McMahan & Kuffler, 1971; Dennis, Harris & Kuffler, 1971). The extent of reinnervation was determined by recording intracellularly from ganglion cells and stimulating vagosympathetic nerves via a suction electrode. Intracellular recordings were made at room temperature (20–23 °C) in Leibovitz-15 medium (Pacific Biological) diluted to 40% and supplemented with salts, glucose and buffer to give the following concentrations (mM): NaCl, 114; KCl, 2·1; $CaCl_2$, 3·6; $MgCl_2$, 0·8; glucose, 5; HEPES, 5 (pH 7·2). The calcium concentration used was twice that of frog Ringer, which served both to increase the size of evoked synaptic potentials and to enhance the stability of intracellular recordings. The calculated osmolarity of our modified L-15 formula is 6% greater than that of frog Ringer. Extracellular recording from the nerve trunks in the ganglion were made in frog Ringer supplemented with glucose (114 mM-NaCl, 2·0 mM-KCl, 1·8 mM-$CaCl_2$, 5 mM-glucose, 5 mM-HEPES, pH 7·2).

Intracellular recordings were made with glass micro-electrodes filled with 4 M-potassium acetate and bevelled to a resistance of 50–100 MΩ (50–90 % of the starting value) on a 0·3 μm alumina surface. Only neurones with resting potentials more negative than −40 mV were studied. Input resistance was measured with a single micro-electrode using a bridge circuit (M4A amplifier, W-P Instruments).

Ganglion cells penetrated by micro-electrodes were never spontaneously active. To discover if activity was suppressed *in vitro* either by the presence of the micro-electrode or by the raised extracellular calcium concentration (Frankenhaeuser & Hodgkin, 1957), we searched for spontaneous ganglion cell activity by extracellular recording in normal Ringer solution. Most ganglion cells are situated along one of two major nerve trunks in the interatrial septum and send axons into the ventricle by way of these trunks. We recorded from these trunks at the ventricular edge of the septum with tight-fitting suction electrodes. Signals were fed into an oscilloscope through a Grass P15 AC preamplifier. Under good conditions ganglion cell axons generated impulses with peak-to-peak amplitudes of 10–25 μV.

Ionophoretic application of acetylcholine

The distribution of receptors on the surface of ganglion cells was estimated by focal ionophoretic application of ACh (Miledi, 1960*a*; Kuffler & Yoshikami, 1975). Ionophoretic micropipettes were back-filled with Millipore-filtered 2 M-acetylcholine chloride and had resistances of 100–200 MΩ. Acetylcholine was ejected from the pipette electrophoretically with a 1 msec pulse of positive current. Diffusion of ACh from the pipette tip was prevented by the application of a steady braking current of 0–4 nA. The magnitude of braking current was adjusted by noting the smallest current required to prevent a slow depolarization of the ganglion cell when the pipette tip was immediately outside the neurone. The sensitivity of a given site on the surface of a ganglion cell was measured as peak depolarization divided by charge applied to the ionophoretic pipette (mV/nC; Miledi, 1960*a*). Ionophoretic current was measured by an operational amplifier which held the bath at virtual ground.

As with skeletal muscle (Kuffler & Yoshikami, 1975) ganglion cell surfaces often showed a non-linear relationship between dose of ACh and peak depolarization at the smallest ACh doses. An example of a typical dose–response curve is shown in Fig. 1. The non-linearity illustrated in Fig. 1 must be considered when comparing the sensitivities of different sites. We have accounted for the non-linearity in a crude way by determining sensitivities, when possible, for responses in the 10–15 mV range.

The Schwann cells which envelope the cardiac ganglion cells (McMahan & Kuffler, 1971) provide a substantial diffusion barrier to ACh (Harris *et al.* 1971). It was thus essential in these experiments to establish criteria for determining when the tip of the ionophoretic pipette was immediately outside the neuronal membrane. We found the 'current response' criterion described by Harris *et al.* (1971) to be adequate for this purpose: when the ionophoretic pipette tip is immediately outside the neuronal membrane, passage of a large, long current pulse (50 nA, 10 msec) often results in the flow of current directly into the neurone, which is depolarized only for the duration of the current pulse. This response observed with the 10 msec pulse may arise from a transient impalement of the cell, or from dielectric break-down of the neuronal membrane immediately opposite the pipette tip. When we applied this current response criterion to sensitivity measurements on denervated cardiac neurones, which are highly sensitive (Kuffler *et al.* 1971), it invariably yielded data indicative of high sensitivity. Thus, none of 208 sites examined on neurones denervated for 14–21 days showed a sensitivity of less than 185 mV/nC when the current response indicated propinquity of the ionophoretic pipette tip to the neuronal membrane. The consistency with which the current response standard yielded measurements of high sensitivity for *these* neurones suggests that the criterion can be applied with confidence to less sensitive neurones.

The sites to which ACh was ionophoretically applied were chosen randomly. About 5% of the surface area of a normally innervated ganglion cell is covered by preganglionic axon terminals (McMahan & Kuffler, 1971). Consequently, randomly chosen sites will usually be extrasynaptic. Although some lateral diffusion of ionophoretically ejected ACh probably occurs, we shall assume in the present study that the sensitivity of randomly chosen sites approximates extrasynaptic sensitivity.

Once a ganglion cell was penetrated with a recording electrode as many as six randomly chosen

sites (mean, 3·1) were assayed for ACh sensitivity. Measurements were made at a total of 930 sites on 294 cells. In the analysis each sensitivity measurement was weighted equally.

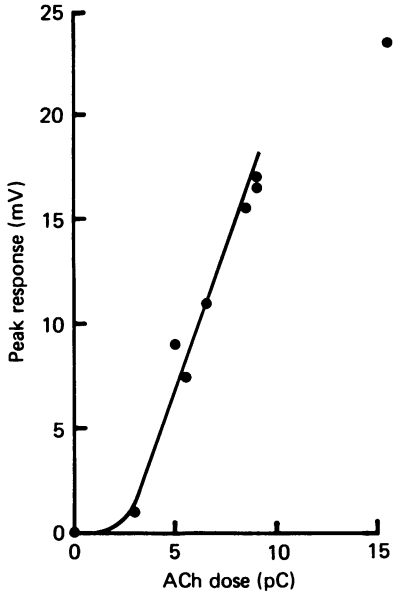

Fig. 1. Dose–response curve for acetylcholine ionophoresis. This Figure shows the non-linear relation between the dose of ACh, measured as charge applied to the ionophoretic pipette, and the response of the ganglion cell, measured as peak depolarization. Data taken from a ganglion cell denervated for 18 days by nerve crush. The line was drawn by eye.

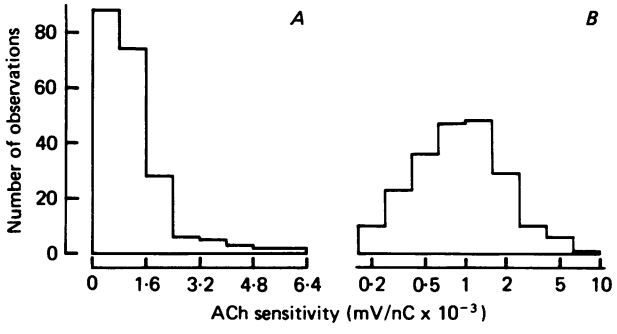

Fig. 2. Distribution of sensitivities of denervated neurones. The same set of sensitivity measurements are displayed arithmetically (*A*) and geometrically (*B*). Data consist of 208 measurements taken from sixty cells in eight preparations denervated for 14–21 days. The arithmetic distribution shows pronounced skewness, whereas the geometric distribution has a nearly normal appearance.

Statistical comparisons (Student's *t* test) were made between the *geometric* means of measured sensitivity rather than between arithmetic means. The justification for this procedure is based on the distribution of sensitivity measurements from 'super-sensitive' neurones (denervated 14–21 days). The amplitude histogram of 208 such measurements (taken from fifty-nine cells), shown in Fig. 2*A*, is skewed rather than normal. The mean sensitivity, 1212 mV/nC, is considerably larger than the median value, 957 mV/nC. Furthermore, the scatter in values (range 186–6400 mV/nC) is such that the standard deviation exceeds the mean. When the 208 sensitivity

measurements are displayed geometrically, i.e. according to their (natural) logarithms, the amplitude histogram has a normal appearance (Fig. 2*B*). The geometric mean sensitivity (904 mV/nC) is approximately equal to the median value, 957 mV/nC.

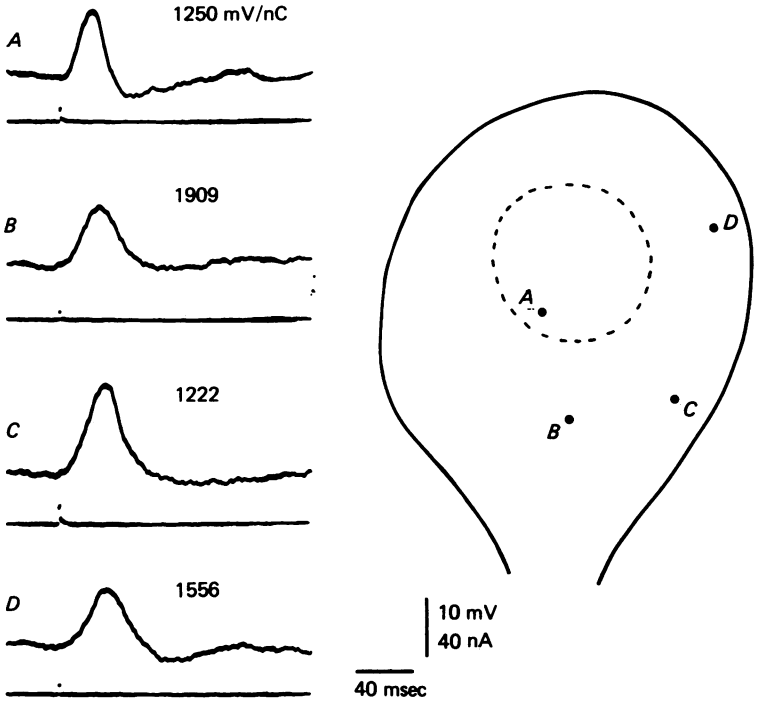

Fig. 3. Depolarization of a ganglion cell by ionophoretic application of ACh. ACh was applied to four sites (*A–D*) on the surface of a ganglion cell denervated for 18 days by nerve crush. The outline of the cell and position of its nucleus are indicated. The upper trace of each of the four records indicates membrane potential, and the lower trace indicates ionophoretic current (braking current not indicated). The sensitivity of each response is given above the voltage trace.

RESULTS

Time course of reinnervation following nerve crush

When the vagosympathetic nerves were crushed, synaptic transmission in the cardiac ganglion was abolished in 2–4 days. The earliest sign of reinnervation was seen about 3 weeks after the operation. An account of the time course of ganglion cell reinnervation can be found in Dennis & Sargent (1978; Text-fig. 2 from that work is reproduced in part here as Fig. 4*B*).

ACh sensitivity of ganglion cells after nerve crush

Denervated ganglia. The development of supersensitivity to acetylcholine during the first week of denervation has been documented by Kuffler *et al.* (1971). In the present study we examined the sensitivity during the second and third weeks. At 12–13 days after nerve crush the geometric mean sensitivity was 508 mV/nC (fifty sites examined from twelve cells), and at 17–18 days after nerve crush the mean sensitivity was 1049 mV/nC (sixty sites examined from seventeen cells, significantly

different than 12–13 day data, $P < 0{\cdot}001$). These measurements and a more complete set taken after nerve resection (see below) indicate that the extrasynaptic sensitivity of neurones to ACh rises continuously until about 14 days after denervation, at which time a plateau of approximately 1000 mV/nC is attained. Typical sensitivity measurements taken from a cell examined at this stage are shown in Fig. 3.

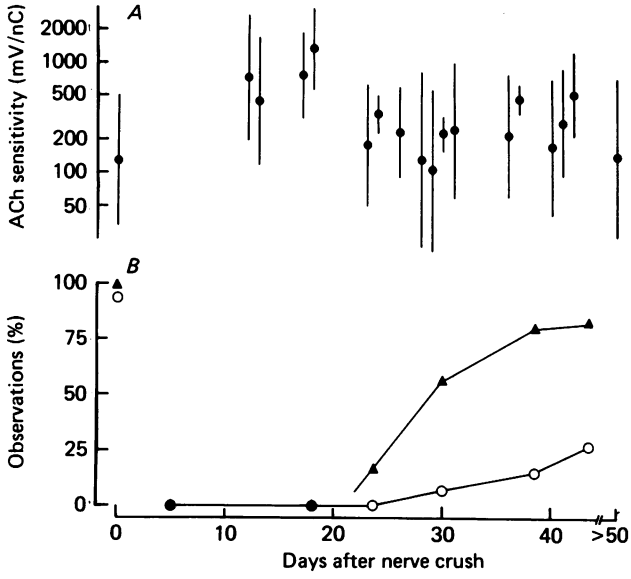

Fig. 4. ACh sensitivity (*A*) and degree of reinnervation (*B*) of ganglion cells as a function of time after nerve crush. *A*, each point between 12 and 42 days indicates the geometric mean and standard deviation of 8–34 sensitivity measurements (mean, 20) taken from an average of six cells in a single cardiac ganglion. The mean sensitivity at '0' days refers to normally innervated neurones (three preparations); the mean sensitivity at '> 50' days was taken from five preparations examined 52–123 days after nerve crush, when reinnervation is nearly complete (Dennis & Sargent, 1978). All means at $\geqslant$ 23 days are significantly different from combined 17–18 day data ($P < 0{\cdot}001$ for all comparisons except 37 day and 42 day, where $P < 0{\cdot}01$). *B*, both the fraction of ganglion cells reinnervated (filled triangles) and the fraction with suprathreshold inputs (open circles) are indicated (Dennis & Sargent, 1978). The mean sensitivity of ganglion cells is reduced as soon as reinnervation of the ganglion begins.

Newly reinnervated ganglia. Reinnervation of cardiac ganglion cells begins about 21–22 days after nerve crush (Dennis & Sargent, 1978). We measured extrasynaptic ACh sensitivity in seventeen ganglia examined 23–123 days after nerve crush; the values from these measurements are presented in Fig. 4*A*, which gives the mean extrasynaptic sensitivity as a function of time after nerve crush. In Fig. 4*B* the time course of reinnervation is shown. One striking feature of the data is that the mean extrasynaptic sensitivity is markedly reduced when reinnervation first begins. The data taken from 23–31 days after nerve crush are of special interest since few of these neurones received synaptic inputs adequate to generate action potentials. Nevertheless, the mean sensitivity during the 23–31 day period was 184 mV/nC (n = 123 sites), approximately sixfold less than the sensitivity of the 2–3 week denervated cells ($P < 0{\cdot}001$). Thus, extrasynaptic sensitivity is significantly reduced

early in reinnervation, well before the parasympathetic neurones are activated through ganglionic synapses. In fact, at least some ganglion cells lose extrasynaptic ACh sensitivity before any synaptic potentials can be evoked from them. Of the forty-three neurones examined 23–31 days after nerve crush, twenty-nine were apparently not innervated, yet had significantly reduced sensitivity (254 mV/nC, sixty-three sites) relative to neurones examined at 17–18 days ($P < 0{\cdot}001$). Thus, in newly reinnervated ganglia sensitivity is reduced even for those neurones which have no detectable synaptic inputs. The mean sensitivity of 23–31 day neurones that did receive synaptic inputs was lower still, 132 mV/nC ($n = 60$, $P < 0{\cdot}01$).

TABLE 1. Passive electrical properties of ganglion cells

Operation	Time after operation (days)	No. of ganglia examined	No. of cells examined	Input resistance (MΩ)	Resting potential (−mV)
Crush	14–21	3	50	106 ± 46	49 ± 6
Crush	23–31	6	64	117 ± 58	52 ± 7
Resection	14–21	6	69	114 ± 69	51 ± 8
Resection	23–31	4	50	113 ± 51	52 ± 6

Values of input resistance and resting potential are given as mean ± S.D. None of the means was significantly reduced at 23–31 days ($P > 0{\cdot}9$).

The lower ACh sensitivity seen at the early stages of reinnervation implies a reduced density of ACh receptors. We can exclude the possibility that the reduction in sensitivity was due to changes in passive electrical properties of the cells, since neither the input resistance nor the resting potential changed significantly between 14–21 and 23–31 days (Table 1).

ACh sensitivity of ganglion cells after nerve resection

The extrasynaptic sensitivity of neurones denervated by crush is reduced several-fold as reinnervation begins. To determine if reinnervation causes this reduction experiments were performed after nerve resection. The sensitivity of ganglion cells was measured in eighteen preparations denervated by nerve resection for 8–31 days (reinnervation does not commence until 50–60 days after the operation when 1 cm lengths of the vagosympathetic nerves are resected). The results, plotted as mean sensitivity *vs.* time after resection, are shown in Fig. 5. During the second week after resection the sensitivity rose gradually and reached a maximum of 872 mV/nC at 14–21 days (148 sites). In the 23–31 day interval there was an unexpected decline in mean sensitivity of some preparations. The apparent subdivision of preparations into two distinct populations is emphasized by noting that the mean from each 23–31 day experiment was either indistinguishable from the 14–21 day values ($P > 0{\cdot}2$) or else differed from them with a high significance ($P < 0{\cdot}001$, see Fig. 5). The over-all mean of 182 sites on seventy-three cells examined 23–31 days after nerve resection, 501 mV/nC, is significantly different from the mean for sites examined at 14–21 days ($P < 0{\cdot}001$). The basis for the slight fall in sensitivity of denervated neurones is unknown, although speculation is presented in the Discussion. The fall

in sensitivity is not explained by changes in passive electrical properties of neurones: the input resistance and resting potential of ganglion cells do not change significantly between 14–21 and 23–31 days after nerve resection (Table 1).

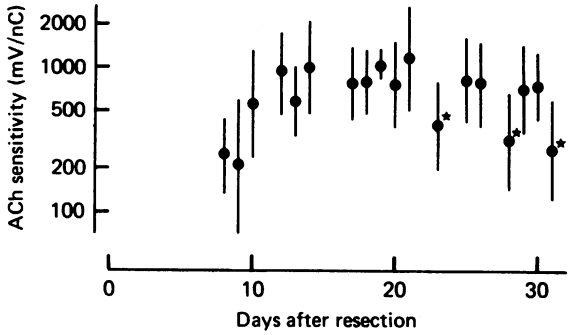

Fig. 5. Acetylcholine sensitivity of ganglion cells as a function of time after nerve resection. Each point represents the mean ± S.D. of 7–49 sensitivity measurements (mean, 26) taken from an average of eight cells in a single cardiac ganglion. Preganglionic reinnervation does not occur until about 50 days after nerve resection. Between 23 and 31 days after surgery the mean sensitivity of some preparations fell significantly below the plateau achieved at 14–21 days (stars). The standard deviations are considerably smaller for these data than for those shown in Fig. 4; the reason for this is not known.

Reinnervation-specific loss of extrasynaptic sensitivity

The change in ACh sensitivity produced by reinnervation can be appreciated by comparing data obtained after nerve crush with that from nerve resection (Fig. 6). Between 17 and 21 days of denervation there is little difference between the sensitivities of neurones denervated by the two methods. However, reinnervation of ganglia following nerve crush does cause a nearly threefold reduction in mean sensitivity.

Can spontaneous activity of ganglion cells contribute to loss of extrasynaptic ACh sensitivity?

The reduction in extrasynaptic ACh sensitivity of ganglion cells precedes by several weeks the stage when ganglionic synapses are mature enough to elicit action potentials. From this we conclude that the resumption of ganglion cell activity is unnecessary in bringing about the loss of extrasynaptic sensitivity. This conclusion requires assurance that ganglion cells are not active spontaneously during the initial stages of reinnervation. To this end we have considered whether cells are active after removal of the cardiac ganglion from the animal. We searched for spontaneous activity by recording extracellularly from nerve trunks containing ganglion cell axons. Recordings were made in normal frog Ringer containing 1·8 mM-calcium, and 0·0 mM-magnesium. Following an initial observation period during which no *intracellular* recordings were made an average of ten ganglion cells per septum were penetrated and stimulated via the intracellular electrode to demonstrate that individual action potentials could have been detected by the extracellular recording. No spontaneous activity was seen in three ganglia examined during the period of

maximal sensitivity, 14–17 days after nerve crush. Likewise, none was seen in three ganglia studied early in the period of reinnervation, 24–27 days after crush. At 28–30 days a small number of spontaneous impulses were observed in three of five ganglia. The activity appeared at approximately the same time as the reinnervating preganglionic axons reached the extracellular recording site; possibly then the recorded activity is from preganglionic axons. If the observed activity *were* to arise from ganglion cells it would be accounted for if each cell fired once every 1–2 min.

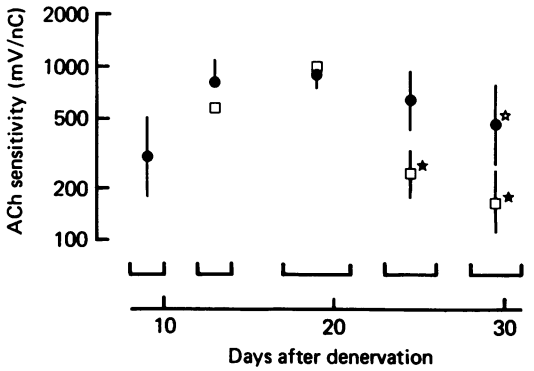

Fig. 6. Effect of reinnervation on ACh sensitivity of ganglion cells. The data from Figs. 4 (open squares) and 5 (filled circles) were combined to indicate the effect of reinnervation upon the ACh sensitivity of ganglion cells. Each mean from Figs. 4 and 5 was treated as a single point, and data falling within the time intervals indicated by brackets were pooled. Means with error bars ($\pm$ 1 S.D.) were generated from three to five experiments; means without error bars were generated from two experiments. Values indicated by filled stars are significantly different from resected data ($P < 0{\cdot}05$); values indicated by open stars significantly different from 17 to 21 day data ($P < 0{\cdot}05$). The ACh sensitivity of ganglion cells is significantly reduced by preganglionic reinnervation, which commences about 3 weeks after nerve crush.

DISCUSSION

Development of extrasynaptic sensitivity to ACh upon denervation

Previous results of Kuffler *et al.* (1971) indicate that extrasynaptic ACh sensitivity of cardiac ganglion cells increases between the second and fifth day after denervation, reaching a plateau of sensitivity of about 250 mV/nC. We did not analyse ACh sensitivity during the first week of denervation but found that during the second week sensitivity continued to rise. The mean values for cells denervated for 8–10, 12–13, and 14–21 days was 321, 584 and 920 mV/nC, respectively (crush and resection data combined). During the third week the sensitivity remained approximately constant (Fig. 5). Our results thus differ from those reported previously, both in the apparent rate at which denervated neurones attain maximal sensitivity and in the value of this sensitivity (compare 250 mV/nC with 920 mV/nC). The difference in the rate of development of extrasynaptic chemosensitivity might be explained by the temperature at which the frogs were housed. The frogs used in the present experiments were kept at a lower temperature, on the average, than frogs used by Kuffler *et al.* (1971). If the appearance of extrasynaptic sensitivity on ganglion cells is accompanied by the synthesis of new receptors, as for skeletal muscle (Brockes &

Hall, 1975; Devreotes & Fambrough, 1976), a temperature dependence for the development of sensitivity would be expected.

Our results also differ from those of Kuffler *et al.* (1971) in that we see a nearly fourfold greater mean sensitivity in denervated neurones. This discrepancy may be attributed to technical differences. First, the input resistance of ganglion cells was higher in the present study, presumably due to improved recording techniques; this would yield higher values of perceived sensitivity (Katz & Thesleff, 1957). Secondly, the measured value of sensitivity (equivalent to the 'chord sensitivity' of Fig. 1) depends upon the dose of ACh. Because the dose–response curve is sigmoid, sensitivities determined with responses of 10–15 mV, as in the present study, should generally exceed sensitivities determined with responses of 4–6 mV as used by Kuffler *et al.* 1971. Further, it is now appreciated (Kuffler & Yoshikami, 1975) that the amount of braking current applied to the ionophoretic pipette must be adjusted precisely to obtain maximum sensitivity.

Decline in extrasynaptic chemosensitivity in the absence of ganglion reinnervation

Some loss of sensitivity occurs during the fourth and fifth weeks after nerve resection, before the onset of reinnervation (Fig. 5). This loss is not explained by changes in passive electrical properties (Table 1), and presumably reflects a reduction in the density of ACh receptors. Similar findings have been reported for cardiac ganglion cells in *Necturus* by Roper (1976). Muscle fibres also undergo a reduction in extrajunctional ACh receptor density upon prolonged denervation (Albuquerque & McIsaac, 1970; Hartzell & Fambrough, 1972). It is unlikely that this loss of sensitivity is caused by spontaneous activity, for denervated ganglion cells show no tendency to fire spontaneously *in vitro*. In the prolonged absence of preganglionic innervation cardiac ganglion cells do form synaptic connexions with each other (Sargent & Dennis, 1977). However, the time course of appearance of these post-ganglionic collateral synapses is too slow to explain the present sensitivity loss; only 5% of the ganglion cells receive detectable post-ganglionic collateral inputs 42 days after resection (P. B. Sargent & M. J. Dennis, in preparation.)

The loss in sensitivity that occurs in the absence of reinnervation has, as yet, no satisfactory explanation.

The loss of extrasynaptic sensitivity produced by reinnervation

Ganglion cells undergo a five- to sixfold reduction in extrasynaptic ACh sensitivity at the earliest stage of reinnervation following nerve crush (Fig. 4), even though the newly formed synapses are not powerful enough to elicit action potentials. Thus, restoration of activity by preganglionic input is not crucial for loss of extrasynaptic ACh sensitivity. This conclusion is identical to one arising from similar experiments performed nearly 20 years ago on skeletal muscle by Miledi (1960*b*). To demonstrate that the loss of extrasynaptic sensitivity which occurs during the fourth week after nerve crush is *caused* by reinnervation, we have shown that only a relatively slight loss in sensitivity ($< 50\%$) occurs at this time when reinnervation is delayed by resecting rather than crushing the nerve.

The objections that can be raised to this conclusion fall into two categories. First, does the loss of extrasynaptic sensitivity signify a reduction in ACh receptor density? We used comparable

ionophoretic pipettes throughout, and in all experiments we used the 'current response' criterion to establish that the tip of the ionophoretic pipette was apposed to the neuronal membrane (see Harris *et al.* 1971). We have shown that the loss of sensitivity is not accompanied by changes in resting potential or input resistance (Table 1). We further think it unlikely that an increase in cholinesterase coincident with reinnervation could explain our results: first, anticholinesterases do not consistently enhance synaptic potentials in normally innervated ganglion cells (Dennis *et al.* 1971), suggesting that hydrolysis is not the primary means of transmitter inactivation; secondly, in skeletal muscle the effect of innervation upon cholinesterase levels depends upon activity (Lømo & Slater, 1976), whereas in the present experiments restoration of activity is preceded by changes in sensitivity. Thus we assume that reduced sensitivity does reflect a reduction in receptor density.

The second potential objection challenges our claim that ganglion cells are not active during the fourth week after nerve crush. One might suppose that newly formed ganglionic synapses are less powerful *in vitro* than they are in the animal. Although this possibility cannot be ruled out, we think it unlikely. Electrophysiological experiments *in vitro* were done in twice-normal calcium, which would increase synaptic potentials evoked by preganglionic nerve stimulation. In addition, there is substantial discrepancy between the time when sensitivity is reduced ($\leqslant$ 23 days) and the time when half of the neurones are activated through ganglionic synapses *in vitro* ($\geqslant$ 50 days, Fig. 4). We have shown that ganglion cells are not active *in vitro* when reinnervation first occurs, even in normal ionic conditions and without intracellular recording. Thus spontaneous activity is unlikely to explain the reduction in extrasynaptic ACh sensitivity.

In newly reinnervated ganglia individual neurones show reduced extrasynaptic ACh sensitivity whether or not they receive a detectable synaptic input. Most of the neurones examined 23–31 days after nerve crush that have not yet received an input will be innervated in the ensuing 2 weeks (Dennis & Sargent, 1978). Thus, loss of extrasynaptic sensitivity precedes the stage at which synaptic potentials can first be evoked from ganglion cells. It is possible, of course, that synaptic inputs have been made onto these cells but that they are fragile and have been lost during the acute experiment. Barring this possibility, there are less trivial explanations for how regenerating nerve terminals might act to reduce extrasynaptic sensitivity. The preganglionic nerve terminals might innervate most or all of the ganglion cells some time before functional synaptic transmission is established (cf. Dennis & Miledi, 1974). Some inductive interaction could occur between individual pre- and post-synaptic cells before maturation of the transmitter release mechanism. Alternatively, one-to-one contact between pre- and post-synaptic cells may not be necessary to initiate recession of supersensitivity. Perhaps some 'trophic' factor released by the regenerating axons influences the entire ganglion (e.g. Schmidt & Stefani, 1977). All ganglion cells could thereby be affected as soon as some preganglionic terminals find their way into the heart. Although this explanation is unorthodox, it best describes our results.

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