

REDUCTION IN EXTRASYNAPTIC ACETYLCHOLINE SENSITIVITY OF AXOTOMIZED ANTERIOR PAGODA NEURONES IN THE LEECH

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Summary

1. The effects of axotomy on the sensitivity of the leech anterior pagoda (AP) neurone to acetylcholine (ACh) and carbamylcholine (CCh) have been studied 1–5 days after axon interruption.
2. Hyperpolarizing responses to ionophoretically applied ACh and CCh have been recorded intracellularly from desheathed cell bodies of normal and axotomized neurones. The electrical properties of the membrane have also been measured in the same neurones.
3. Axotomy produced a progressive loss of sensitivity to both ACh and CCh with a similar percentage reduction.
4. No significant changes have been found in the time to peak and in the reversal potential of the responses to agonists, or in the number of drug molecules needed to combine with a single receptor to produce a response.
5. Interruption of nerve roots and connectives which do not contain the AP axon did not induce the alterations of ACh sensitivity observed after axotomy.
6. It is concluded that the loss of ACh sensitivity following axotomy is due to a reduction in density of functional ACh receptors (AChRs).

Introduction

Several findings show that axotomy affects the electrophysiological properties of the soma-dendritic membrane of both vertebrate (Kuno, 1975; Sernagor *et al.* 1986; Titmus and Faber, 1986) and invertebrate neurones (Pitman *et al.* 1972; Goodman and Heitler, 1979; Kuwada, 1981). The changes reported in various preparations show differences in intensity, time course and domain of membrane involved. The alterations can all be viewed as a result of the abnormal accumulation of some types of ion channels in the soma-dendritic membrane due to lack of an axon target and also possibly to an increased rate of channel synthesis (Sernagor *et al.* 1986). The question therefore arises as to whether various types of ion channels are differentially expressed in the membrane of the same neurone during regeneration.

Key words: axotomy, acetylcholine sensitivity, leech.

A leech central neurone identified as an AP cell (Muller *et al.* 1981) responds to axotomy by developing the ability to produce action potentials in the cell body (Pellegrino *et al.* 1984; Matteoli *et al.* 1986). This change is due to an increase in the functional expression of voltage-gated sodium channels in the somatic membrane (Pellegrino and Matteoli, 1984; Pellegrino *et al.* 1986).

The purpose of the experiments described in this paper was to study, by using an ionophoretic technique, the functional expression of extrasynaptic ACh receptors in the membrane of the leech AP neurone during the early phase of the axotomy reaction.

Materials and methods

Experiments were performed on specimens of *Hirudo medicinalis* obtained commercially (Ricarimpex, France). The neurone selected for this study, identified as an AP cell, lies in the anterolateral packet on each side of the segmental ganglia and sends just two axonal processes to the contralateral roots (Muller *et al.* 1981; Pellegrino *et al.* 1984). Operations were carried out under chlorobutanol anaesthesia (0.15%). This procedure does not affect the response to axotomy, as shown elsewhere (Pellegrino *et al.* 1984). In the majority of experiments axotomy was produced by isolating single segmental ganglia and maintaining them in organ culture for 1–5 days at 25°C. Three ganglia were kept in each Petri dish (Falcon 3001, 35 mm) in 4 ml of Leibowitz 15 medium (Gibco), supplemented with 2% foetal calf serum (Boehringer), 100 µg ml⁻¹ gentamicin and 0.6% glucose. The culture medium was replaced every other day.

To validate the results obtained from the axotomized neurones of cultured ganglia, axotomy was also performed *in vivo* by cutting the roots on both sides of ganglia VIII and XII. Since postsynaptic potentials can be produced in each AP cell by electrically stimulating one of the four nerve roots or one of the two connectives of the segmental ganglion (A. Bigiani and M. Pellegrino, unpublished observations), we studied the effects of a partial deafferentation on intact AP neurones. Thus, in a few animals we cut the roots on only one side of the ganglion and crushed the anterior and posterior connectives. After the operation the leeches were maintained in diluted leech saline (10% in water) at 20°C for 5 days.

For electrophysiological measurements, single ganglia were pinned ventral side up to the bottom of a recording chamber and perfused with leech saline (115 mmol l⁻¹ NaCl, 4 mmol l⁻¹ KCl, 7.5 mmol l⁻¹ CaCl₂, 10 mmol l⁻¹ Tris-maleate buffer, pH 7.4, osmolarity corrected with 130 mmol l⁻¹ glucose). AP somata, identified by size and position in the ganglion, were desheathed by cutting the connective tissue capsule, and then cleaned by flushing with leech saline using a Pasteur pipette.

Control AP neurones were obtained from ganglia which had been isolated from normal leeches just before the recording.

Intracellular recordings were carried out using conventional techniques. Micro-electrodes with a resistance of 20–40 MΩ were pulled from 1 mm capillary tubing

(Clark) and filled with 3 mol l^{-1} potassium acetate. A single microelectrode was used both to record voltage and to inject current with a bridge circuit. Negative capacitance control and bridge balance were adjusted to minimize errors in measurements of input resistance and membrane potential. In each neurone, resting potential and input resistance were measured, as well as duration, amplitude and maximal rate of depolarization of the action potential. Input resistance was found to depend on the membrane potential. To compare data from different cells, we measured input resistance by applying hyperpolarizing current steps (0.3 nA) at a reference membrane potential of -60 mV .

Ionophoretic applications were performed using glass micropipettes filled with filtered 1 mol l^{-1} acetylcholine chloride or 1 mol l^{-1} carbamylcholine chloride solutions ($40\text{--}70 \text{ M}\Omega$ resistance). Braking current was adjusted by noting the smallest current required to prevent membrane hyperpolarization by agonist leakage from the pipette tip when this was nearest to the cell surface. The closeness of the pipette tip to the cell membrane was estimated by using 'current responses', according to Harris *et al.* (1971). Ejection pulses of a fixed duration (50 ms) were delivered, and changes in the intensity were performed in a random sequence.

To measure the response to ionophoretically applied agonists under standard conditions the membrane potential was held at a reference value of -60 mV by injecting a steady current. This value suppressed the spontaneous activity of the neurones. The traces shown in Fig. 1A,B were recorded in a preliminary experiment, at a membrane potential of -50 mV . This cell was not used for quantitative analysis. The sensitivities to ACh and CCh were determined using the method described by Kuffler and Yoshikami (1975): at each chosen spot on the cell body surface the dose-response curve was constructed and sensitivity was expressed in mV nC^{-1} as the slope of the linear portion of the dose-response plot. Comparisons of data from different cells were made by correcting each sensitivity value by the input resistance measured at a membrane potential of -60 mV and, accordingly, the sensitivity was expressed in $\text{mV nC}^{-1} \text{ M}\Omega^{-1}$.

The Hill coefficient was used to estimate the mean number of agonist molecules required to activate each AChR. In accordance with Rang (1971), this coefficient was evaluated by measuring the slope of $\log(E/E_{\text{max}} - E)$ versus $\log d$ plots, where E is the response produced (in mV), E_{max} is the maximal response and d is the dose applied (in nC).

Results

Responses to ACh and CCh of normal and axotomized AP neurones

Acetylcholine, applied to the soma membrane of normal AP neurones either by bath perfusion or by ionophoresis, produced hyperpolarizing responses associated with an increase in membrane conductance (Fig. 1A,B). These responses, due to extrasynaptic receptors, showed no desensitization and were mainly mediated by Cl^- (Pellegrino and Simonneau, 1984).

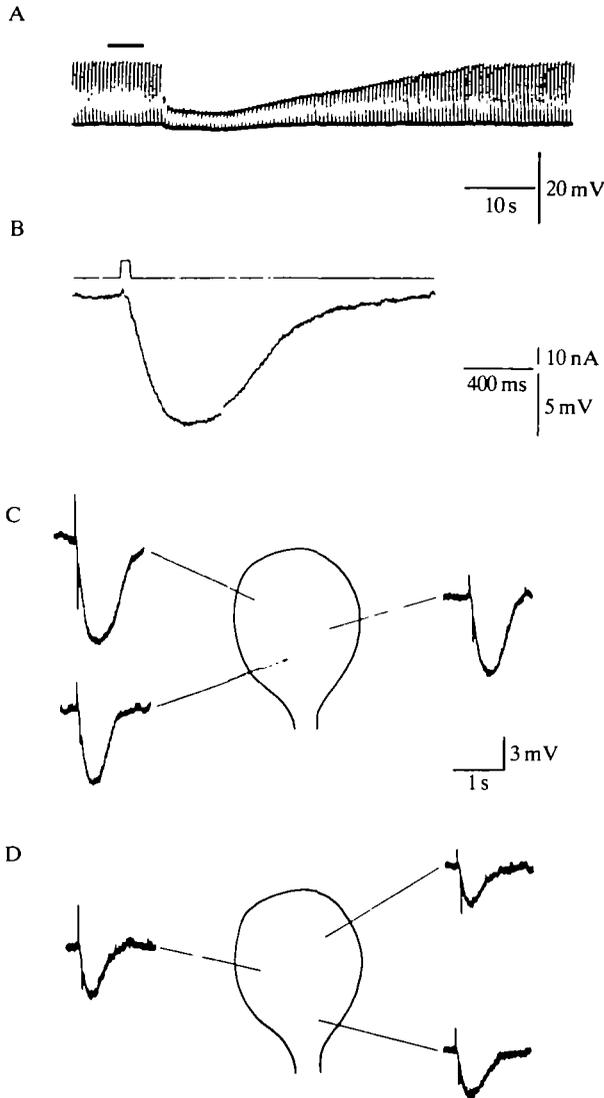


Fig. 1. (A, B) Effect of acetylcholine (ACh) on a normal AP neurone at a membrane potential of -50 mV. (A) Hyperpolarizing response to bath application of ACh (10^{-3} mol l $^{-1}$). The membrane resistance, measured as the potential change produced by a test hyperpolarizing current pulses, decreases during the response. (B) Response to ACh focally applied on the cell body membrane by ionophoresis (10 nA, 50 ms). (C, D) Uniform distribution of the ACh sensitivity on the soma membrane of a normal (C) and an axotomized (D) neurone. The dose applied is the same in both experiments (10 nA, 50 ms). The membrane potential was held at -60 mV in both cells.

ACh sensitivity of the AP soma membrane appeared quite uniform within the spatial resolution of our ionophoretic pipettes. Fig. 1C illustrates responses to ACh obtained by applying the same dose of ACh to three different areas of a

normal cell body. The amplitude and the time course of the response do not differ significantly from site to site.

Axotomized AP neurones retained all the qualitative features of the ACh-induced response observed in normal cells. Fig. 1D shows three responses to the same dose of ACh applied to different sites on the cell body of an axotomized AP neurone (in ganglia cultured for 4 days). The amplitude of the responses to ACh changed consistently and progressively after axotomy. The reduction in the peak amplitude of the hyperpolarizing responses to ionophoretically applied ACh, which was detectable as early as the first day after isolation in culture, became more pronounced with time. Fig. 2A illustrates three groups of responses to the same doses of ACh recorded from a normal cell (top traces) and from two cells of ganglia maintained in culture for 3 and 5 days (middle and bottom traces, respectively). The time course is comparable in all groups of traces, but the peak amplitude progressively diminishes with time.

To express quantitatively the ACh sensitivity, dose-response plots were constructed and the slopes of their linear portions were estimated. The dose-response curves of the three examples illustrated in Fig. 2A are shown in Fig. 2B. The ACh sensitivity is reduced to 30% of the control value 3 days after isolation and to 15% of the control value 5 days after isolation.

Similar experiments have been performed on AP neurones axotomized *in vivo*, to rule out the possibility that the reduction in ACh sensitivity was associated with the culture procedure. Fig. 2C illustrates a comparison between representative samples of responses to ionophoretically applied ACh recorded from a neurone which had been axotomized 5 days earlier (lower traces) and those from a control cell (upper traces). The dose-response curve for the cell axotomized *in vivo*, shown in Fig. 2D, is indistinguishable from that obtained in culture.

To test whether changes in cholinesterase activity were involved in the change of ACh sensitivity observed in axotomized neurones (David and Pitman, 1982), carbamylcholine was used instead of ACh. Both normal and axotomized AP neurones were responsive to the agonist CCh; the hyperpolarizing responses had roughly the same time course, though their amplitude was about 70% of that produced by ACh. Nevertheless, sensitivity to CCh also decreased after axotomy. Fig. 2E illustrates typical CCh responses from normal and axotomized neurones and Fig. 2F shows the dose-response curves.

Fig. 3A summarizes the data for ACh sensitivity from 26 cells of normal ganglia (plotted at time zero), 54 cells of ganglia cultured for 1–5 days (each point refers to 10–12 cells) and 16 cells axotomized *in vivo* 5 days beforehand. In the same diagram the data for CCh sensitivity from 13 control cells and 11 cells from ganglia cultured for 3 days are reported. Each slope was corrected for input resistance measured in the corresponding neurone.

The results illustrated in Fig. 3A, expressed as means \pm s.e.m., demonstrate a progressive decrease in the ACh sensitivity of AP cells in cultured ganglia, the mean value becoming significantly lower than the control ($P < 0.01$) at 2 days and reaching about 15% of the control value after 5 days. A similar value for ACh

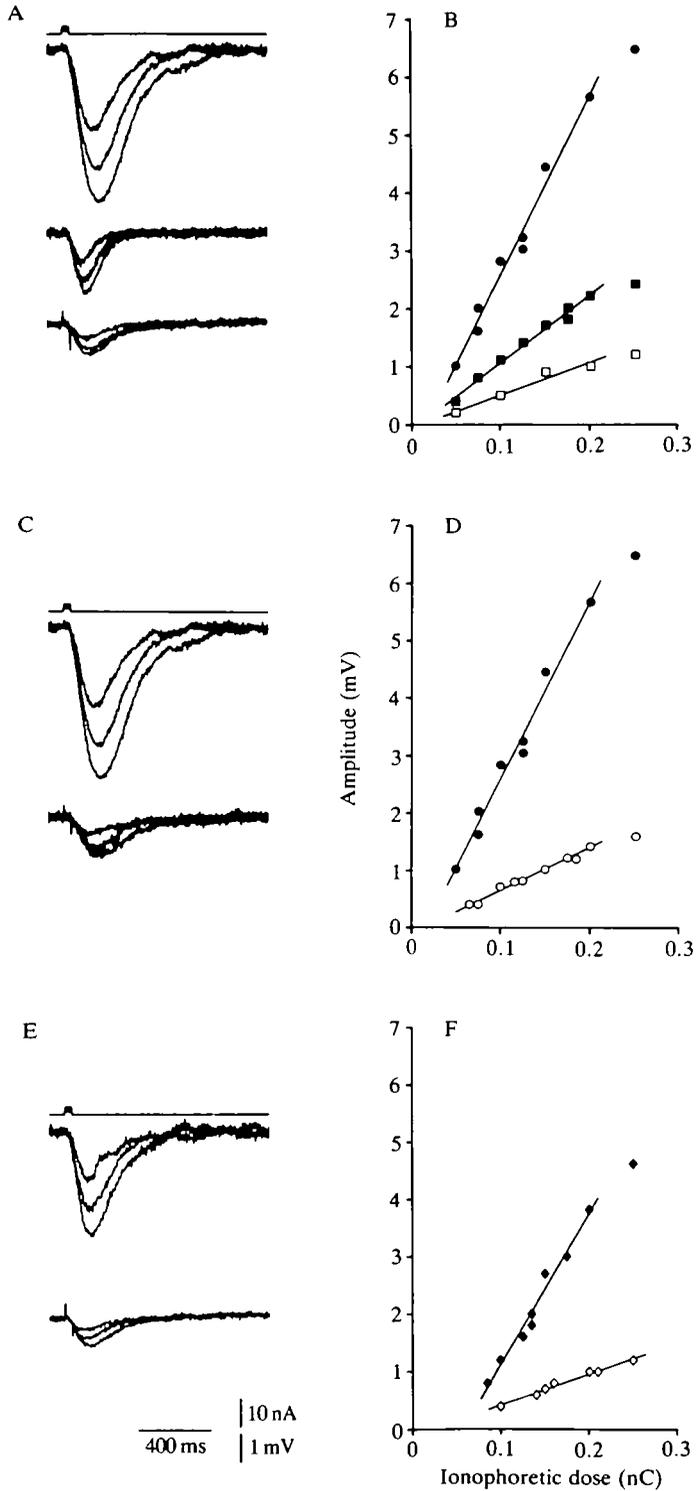


Fig. 2

Fig. 2. (A) Examples of responses to ionophoretic application of three doses of ACh recorded from a normal cell (upper traces) and two cells from ganglia maintained in culture for 3 (middle traces) and 5 (lower traces) days. (B) Dose-response curves for the neurones shown in A (● normal cell, ■ 3 days, □ 5 days in culture). The plots were constructed by increasing current strength of pulses of constant duration (50 ms). The linear portions of the dose-response plots were fitted with regression lines of a slope of 30.8 mV nC^{-1} (control), 11.6 mV nC^{-1} (3 days) and 5.6 mV nC^{-1} (5 days). (C) Representative responses to the same doses of ionophoretically applied ACh recorded from a normal cell (upper traces) and from a cell axotomized *in vivo* 5 days earlier (lower traces). (D) Dose-response curves for the two neurones shown in C (● normal cell, ○ 5 days). Slope of the regression lines: 30.8 mV nC^{-1} (normal) and 7.7 mV nC^{-1} (axotomized). (E) Examples of responses to the same dose of ionophoretically applied CCh obtained in a normal (upper traces) and axotomized (lower traces) cell. (F) Dose-response curves for the cells shown in E (◆ normal cell, ◇ 3 days). Slope of the regression lines: 26.2 mV nC^{-1} (normal) and 5.4 mV nC^{-1} (axotomized).

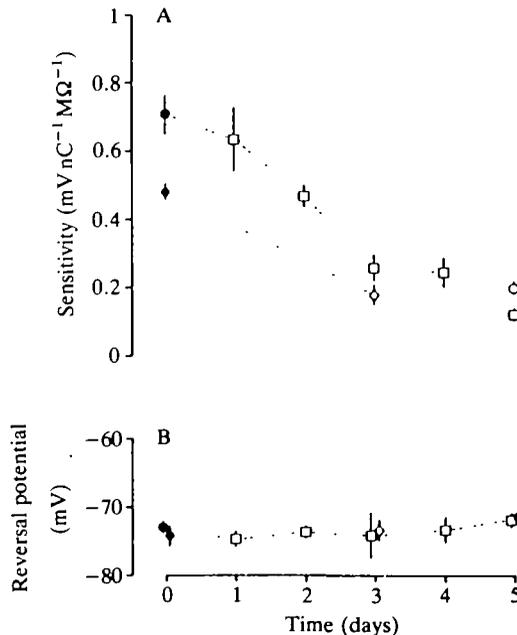


Fig. 3. (A) Time course of changes in sensitivity to ACh and CCh of cells maintained in culture and the change in sensitivity to ACh of cells axotomized *in vivo* 5 days earlier. The points represent mean \pm s.e.m. All the mean values of sensitivity from 2 to 5 days are significantly lower than the control value ($P < 0.01$). (●, ACh; ◆, CCh) normal cells; (□, ACh; ◇, CCh) cells from cultured ganglia; (○, ACh) cells axotomized *in vivo*. (B) Relationship between reversal potential (mean \pm s.e.m.) and time after axotomy, for the cells represented in A. Symbols as in A. The number of measurements in each experiment is given in the text.

Table 1. Comparison of the effects of deafferentation and axotomy on the membrane properties of AP neurones

	AP _r	AP _l	N
Resting potential (mV)	-42.0±1.3	-50.0±2.4	(5)
Input resistance (mΩ)	35.6±1.3	34.8±2.0	(5)
Action potential duration (ms)	4.9±0.1	21.2±4.4	(5)
Action potential amplitude (mV)	5.1±0.6	14.3±5.7	(5)
Maximum rate of depolarization (Vs ⁻¹)	2.8±0.4	6.0±1.9	(5)
ACh sensitivity (mV nC ⁻¹ MΩ ⁻¹)	0.77±0.13	0.19±0.05	(4)
Reversal potential (mV)	-73.8±1.4	-71.1±2.5	(2)

AP_r, partially deafferented; AP_l, axotomized and partially deafferented.
Values are mean±s.e.m., N=numbers of cells.

sensitivity was also found in AP cells which had been axotomized *in vivo* 5 days earlier. Furthermore, by 3 days the percentage loss of sensitivity to CCh was similar to that for ACh.

The reduction in ACh and CCh sensitivities could be due to a positive shift in the reversal potential of the response to agonists. Accordingly, we measured this parameter in the various experimental groups. The reversal potentials in axotomized neurones were not significantly different from control values (Fig. 3B).

The Hill coefficient, a measure of the mean number of agonist molecules required to activate a single receptor, was determined from recordings performed in both normal and axotomized neurones. It was consistently found to have a value of about 2, and axotomized neurones did not differ significantly from control ones.

In five ganglia in which only the roots on the right side were cut and both anterior and posterior connectives had been crushed *in vivo* 5 days earlier, the AP cells of the two sides were differently affected. The neurones on the left side (AP_l), that were both axotomized and partially deafferented, gave the expected response with a decrease in ACh sensitivity and an increase in excitability, whereas the neurones on the right side (AP_r), that were partially deafferented but not axotomized, appeared normal in their properties. Table 1 shows the results of this experiment. It is worth noting that the ACh sensitivity of reactive neurones (the left side ones) was comparable to that of the cells illustrated in Fig. 3A at 5 days.

Electrical properties of normal and axotomized AP neurones

The electrical properties recorded from axotomized neurones changed both in cultured ganglia and in ganglia operated on *in vivo*. However, some differences have been observed in the two experimental conditions.

Normal AP cells had a resting potential of -44.4±0.5 mV (mean±s.e.m., N=44) and an input resistance, measured at -60 mV, of 35.5±0.8 MΩ. Fig. 4 illustrates the changes of passive membrane parameters in 97 AP cells from ganglia cultured for 1-5 days (values are the means from 15-30 cells) and in 27 cells of ganglia operated on *in vivo*. The resting potential of axotomized cells became

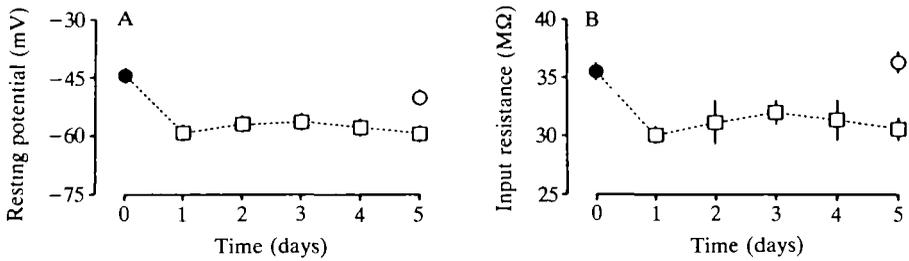


Fig. 4. Passive electrical properties of normal (represented at time zero) and axotomized AP cells. Each point represents the mean \pm S.E.M. of measurements from 44 normal cells (●), 97 cells from cultured ganglia (□) and 27 cells axotomized *in vivo* (○).

significantly more negative both in culture ($P < 0.001$ for all points) and *in vivo* ($P < 0.001$) (Fig. 4A), though the change measured *in vivo* was less pronounced. The input resistance did not change in cells axotomized *in vivo* ($P > 0.7$), whereas it decreased significantly in cultured axotomized cells ($P < 0.05$ for all points) (Fig. 4B).

Duration, amplitude and maximal rate of depolarization of the spontaneous action potentials increased in axotomized AP cells (see Table 1). These changes, which have been described elsewhere (Pellegrino *et al.* 1984, 1986), can be interpreted as an increased electrogenesis in the cell body membrane.

Discussion

In this study we report that AP neurones in the central nervous system of the leech respond to axotomy with a decrease in sensitivity of the somatic membrane to ACh and CCh. Neurones that have been axotomized *in vivo* and those in cultured ganglia show the same responses.

The reduction of extrasynaptic ACh sensitivity is not caused by the interruption of the nerve trunks, which contain inputs to AP cells but do not contain their axons. Nor is it associated with changes in reversal potential of the responses. These results make it unlikely that alterations in equilibrium potential can account for the observed phenomenon. Moreover, the Hill coefficient is not affected by axotomy, suggesting that the number of agonist molecules required to activate a single receptor does not change.

Since the percentage decrease in sensitivity is similar for ACh and CCh, it cannot be attributed to changes in cholinesterase activity. The reduction in sensitivity after axotomy is more likely to be due to a progressive loss in density of functional AChRs in the soma membrane, either because of a reduced rate of insertion or because of a process of masking. Changes in receptor properties, such as single-channel conductance or affinity of a single receptor for ACh, cannot be ruled out from the available evidence. However, changes in the density of AChRs

after axotomy have already been reported in other neurones (Jacob and Berg, 1987, 1988).

It is worthwhile pointing out that our results are in agreement with those obtained, with the ionophoretic technique, for different ACh receptors by Brenner and Martin (1976) from chick ciliary ganglion cells. These authors suggested a reduction in the number of ACh receptors in the soma membrane after axotomy. Similar results were obtained by Fumagalli *et al.* (1978) and Fumagalli and De Renzis (1980) using binding techniques in both the chick ciliary ganglia and the rat superior cervical ganglia. Decreases in α -bungarotoxin binding components and AChRs has been confirmed by Jacob and Berg (1987, 1988) in the chick ciliary ganglia.

In contrast, an apparent increase in ACh sensitivity has been reported for identified motoneurons in cockroaches (David and Pitman, 1982); this was partly explained by a fall in acetylcholinesterase (AChE) activity. Our results in the leech cannot confirm either an increase in ACh sensitivity or an involvement of AChE. The results with ACh closely parallel those obtained with CCh, the AChE-resistant agonist. The cellular mechanisms underlying a progressive loss of functional AChRs after axotomy remain to be determined.

The input resistance decreased in culture but not *in vivo*. It is difficult, at present, to explain this difference. The most consistent alteration concerns the resting potential, which became more negative both in culture and *in vivo*. Experiments at the single-channel level have recently been performed on potassium leak conductance (Pellegrini *et al.* 1989) in axotomized AP cells, and clear-cut increases in the density of leak channels have been found (Simoni *et al.* 1990). The increase in the density of these channels might therefore account for the change in resting potential.

The present results and those previously reported for the same neurone (Pellegrino and Matteoli, 1984; Pellegrino *et al.* 1984, 1986) indicate that AP cells react to axotomy by expressing differentially voltage-gated ion channels and extrasynaptic ACh receptors. The physiological significance of these alterations is unknown.

The changes of excitability in axotomized neurones are not necessarily associated with sprouting. Neurones with spiking somata show minor changes (Kuwada and Wine, 1981; Gustafsson and Pinter, 1984; Gordon *et al.* 1987) or no changes in their electrical properties (Bannatyne *et al.* 1989) during regeneration. The electrophysiological changes are so dramatic in some neurones, such as the AP cells, that they probably reflect metabolic features, and represent, on an amplified scale, alterations that are more subtle in other neurones.

The insertion of functional ion channels in the soma membrane may be the first step in completing the maturation of the newly synthesized axolemma by a process of lateral diffusion (Strichartz *et al.* 1984). The conditions required to sustain sprouting, therefore, can be either to have a pool of inserted ion channels to send along the axon or to have action potentials in the cell body, to modulate the anabolic response (Offord and Catterall, 1989). Accordingly, neurones with

spiking somata might be considered as already equipped to sustain regeneration, whereas those with non-spiking somata would have to be able to spike before they could regenerate. The role of extrasynaptic receptors on cell bodies of leech neurones is not clear. Among other possibilities, their presence in the soma membrane might represent a transitional stage before their transfer to synaptic sites in the neuropile (Sawyer, 1986).

It is also interesting to point out that, besides changes in the rate of ion channel biosynthesis, alterations in the expression of certain neuronal genes providing growth-specific materials have been demonstrated in axotomized neurones (Willard and Skene, 1982). The differential expression of functional AChRs and voltage-gated ion channels might therefore be connected with different phases of axolemmal reorganization.

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