

THE EFFECTS OF AXOTOMY UPON THE EXTRASYNAPTIC
ACETYLCHOLINE SENSITIVITY OF AN IDENTIFIED
MOTONEURONE IN THE COCKROACH
PERIPLANETA AMERICANA

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SUMMARY

The effects of axotomy on the sensitivity of the fast coxal depressor motoneurone (D_7) of the cockroach (*Periplaneta americana*) to applied acetylcholine (ACh) and carbamylcholine (CCh) have been investigated.

ACh and CCh applied to the soma membrane either by bath perfusion or by iontophoresis caused depolarization; repeated application of large doses of these agonists resulted in a relatively rapid desensitization and depression of the response.

Axotomy performed 3-10 days before recordings were made caused an approximately threefold increase in the sensitivity to ACh but had no effect upon the sensitivity to CCh. The resting potential, input resistance and membrane time-constant remained within the normal range. In addition there was no change in the rise-times of the responses or of the ACh reversal potential, or in the apparent number of ACh molecules needed to combine with individual cholinergic receptors to produce a response.

The anticholinesterases physostigmine and neostigmine potentiated the ACh response at relatively low concentrations (10^{-7} M- 10^{-8} M). This potentiation was significantly greater in the normal cells than in the axotomized cells. It is therefore concluded that the increase in ACh sensitivity of this motoneurone results at least partly from a fall in the activity of cholinesterase in the region of the applied drug.

INTRODUCTION

When axotomized, many vertebrate neurones show a progressive decline in acetylcholinesterase (AChE) activity associated with their cell bodies and dendrites (see Lieberman, 1971). This may be expected to result in an increase in the sensitivity of the neurones to acetylcholine (ACh).

Investigations in which the sensitivity of axotomized neurones has been judged by their response to synaptic activation are limited because the concentration of ACh in the synaptic cleft may become elevated as a result of a local reduction in the AChE

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activity (McLennan, 1954). The results obtained using such preparations are further complicated by 'somatic stripping' which seems to accompany axotomy; presynaptic terminals appear to be either physically displaced by an invasion of glial processes into the synaptic clefts (Blinzinger & Kreutberg, 1968; Hillman, 1970) or by a 'retraction' of the dendritic tree (Sumner & Watson, 1971; Sumner & Sutherland, 1973). Brenner & Martin (1976) were able to overcome these problems by ionophoretically applying ACh directly on to the soma membrane of chick ciliary ganglion cells. Using this method they were able to show that although axotomy reduces the AChE activity of avian ciliary ganglion cells (Szentagothai, 1957), it also results in a reduction of the ACh sensitivity. They suggested that this reduction in sensitivity might be caused by a reduction in the number of ACh receptors in the postsynaptic membrane.

The experiments described in this paper were designed to test, on an identified insect neurone, whether or not axotomy was accompanied by a decrease in the level of cholinesterase (ChE) and if so, to what extent this affected the sensitivity of the cell to externally applied ACh. The complications associated with studies on synaptic receptors mentioned above were avoided by investigating a population of purely extrasynaptic receptors.

METHODS

All recordings were made by impalement of the soma of the 'fast' coxal depressor motoneurone (termed D_1 by Pearson & Iles, 1971; this is identical to cell 28 of Cohen & Jacklet, 1967) in the metathoracic ganglion of adult male cockroaches (*Periplaneta americana*) using previously described methods (Pitman, 1975). The soma of this neurone may be easily visualized because of its position and large size, while its axon may be severed with minimal surgery by transecting the fifth segmental nerve (Pipa & Cook, 1959) through a small incision in the cuticle at the base of the leg. It should be noted that this procedure could also remove some of the sensory input to D_1 since this nerve trunk carries sensory nerves from the leg in addition to the axon of this neurone.

Drugs were applied either to the bath or locally on to the soma membrane through an ionophoretic microelectrode filled with a 0.1 M solution of the appropriate drug (electrical resistance 60–100 M Ω).

RESULTS

Unless otherwise stated experiments were performed 4–10 days after cutting the ipsilateral fifth nerve trunk.

Electrical properties of normal and axotomized neurones

There was no significant difference in the resting potential recorded from the cell body of normal neurones (-64.9 , s.e. ± 1.4 mV; $n = 38$) and axotomized neurones (-66.7 , s.e. ± 1.9 mV; $n = 30$; ($P > 0.9$). Similarly, the mean input resistance (4.7 , s.e. ± 0.5 M Ω ; $n = 33$) was not significantly different from that of axotomized cells (4.9 , s.e. ± 0.5 M Ω ; $n = 30$). The current-voltage relationship was approximately linear from -50 mV to $+20$ mV with respect to the resting potential (Fig. 1b). In the majority of the control neurones depolarization by more than 20 mV produce

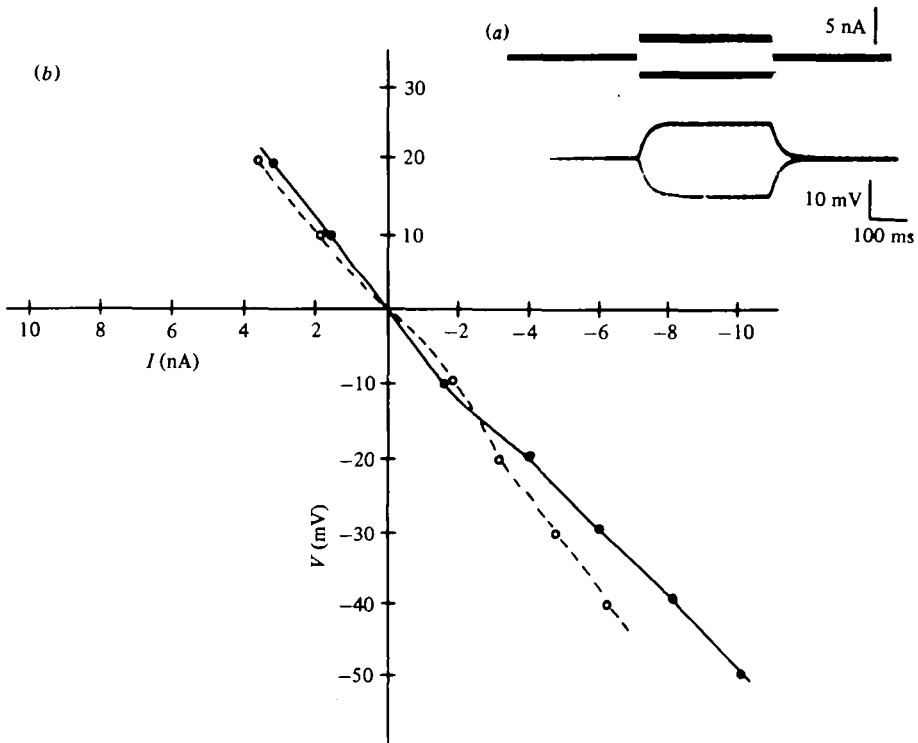


Fig. 1. Current-voltage relationship: (a) record of voltage change (lower trace) produced by positive and negative current pulses (upper trace) passed through the second electrode; (b) current-voltage relationship for a normal (●) and an axotomized (○) cell. Membrane response was nearly linear with input resistance of approximately $5 \text{ M}\Omega$.

damped membrane oscillations. After axon section, relatively small membrane depolarizations (10–15 mV) gave rise to overshooting action potentials. The membrane time-constant for normal cells was 13.1 , S.E. ± 1.2 ms ($n = 14$) and was not significantly altered by axotomy ($n = 14$; $P > 0.2$).

Bath application of drugs

The drug concentrations quoted are those of the drug applied to the bath and so represent a maximum estimate of the concentration reaching the neuronal membrane.

Acetylcholine

A standard concentration of 10^{-4} M-ACh was applied to the bath; the sensitivity of D_1 was estimated from the peak response to a single drug application since repeated applications produced desensitization and consequent depression of the response. The mean depolarization for 22 control (unoperated) cells was 6.0 , S.E. ± 0.9 mV. The sensitivity of axotomized cells was significantly elevated; 10^{-4} M-ACh produced an average depolarization of 13.9 , S.E. ± 2.2 mV ($n = 19$; $P < 0.005$).

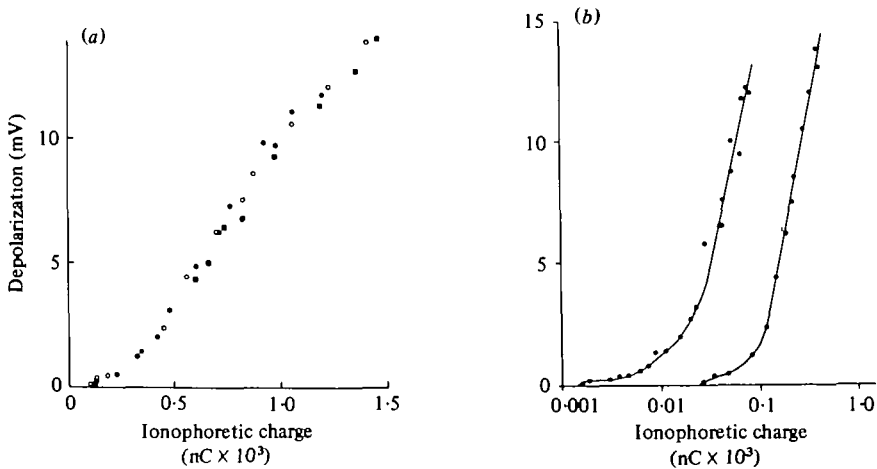


Fig. 2. Ionophoretic application of ACh on to the cell body membrane: increasing dose. (a) ACh dose-response curve obtained by varying the pulse width at constant current amplitude, pulse durations (s) ■, 1.0; ○, 0.5; ●, 0.1. Response was independent of pulse durations for durations between 0.1 and 1.0 s. (b) ACh dose-response curve for a normal (●) and axotomized (○) cell obtained by increasing current strength of constant duration (0.5 s) pulses.

Carbamylcholine

Carbamylcholine (CCh) acts as an agonist on both nicotinic and muscarinic receptors in vertebrate tissues (Jenkinson, 1960; Thron & Waud, 1968). It has been shown to mimic the effect of ACh on insect neurones (Kerkut, Pitman & Walker, 1969). The mean response of 10^{-4} M-CCh was 9.7, s.e. ± 1.2 ($n = 7$) in normal cells and 11.6, s.e. ± 3.3 mV ($n = 5$) in axotomized cells. There was no significant difference between these two values ($P > 0.6$) indicating that the increase in ACh extra-synaptic sensitivity of axotomized neurones may be a result of a fall in ChE activity.

Ionophoretic application of drugs

To overcome the problem of desensitization, ACh and CCh were also applied to the soma membrane by iontophoresis. In most experiments 100 ms ejecting pulses were used; for less sensitive cells the pulse duration was increased to 500 ms. An interval of 2 min was allowed between successive doses. A retaining current of 15 nA was used to prevent drug leakage between ejection pulses.

Acetylcholine

For a given charge the depolarization produced by ionophoretically applied ACh was independent of pulse duration over the range 0.1–1.0 s (Fig. 2a). The charge necessary to produce a given response was considerably smaller in the axotomized cells than in the controls. Fig. 2b shows the relationship between the ionophoretic charge and membrane depolarization for a normal and an axotomized cell. The maximum slopes of such dose-response curves were taken as measures of the ACh sensitivity. The mean sensitivity of 33 normal cells was 0.077, s.e. ± 0.020 mV/nC compared with a mean of 0.246, s.e. ± 0.039 for 20 axotomized cells ($P < 0.001$).

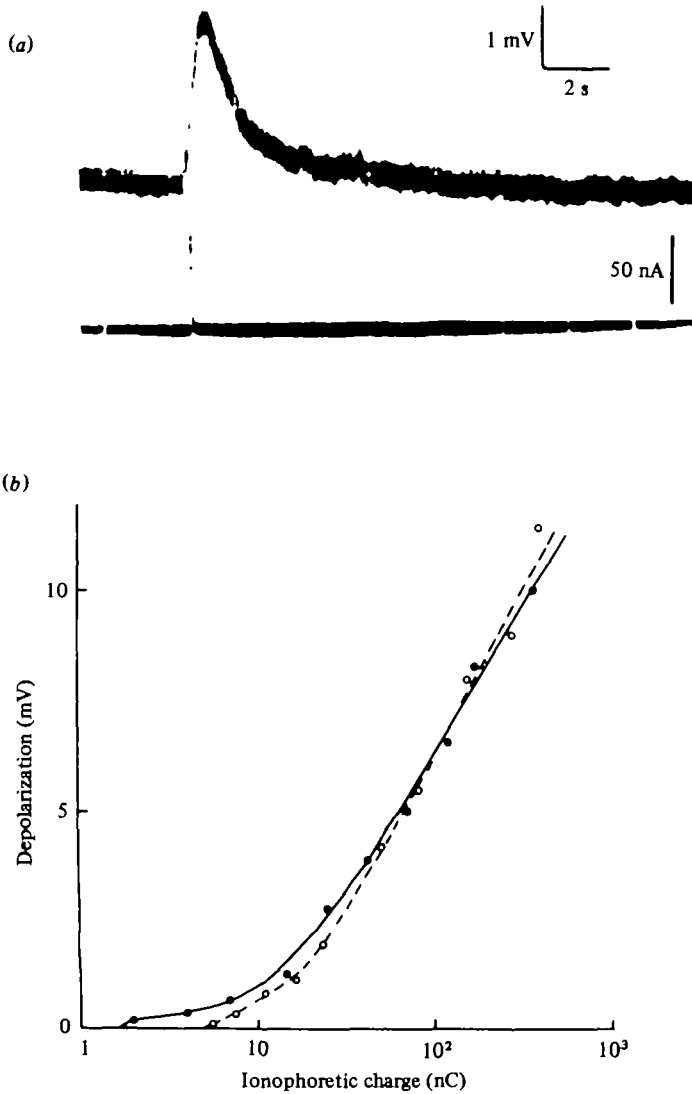


Fig. 3. Ionophoretic application of CCh on to the cell body membrane. (a) Upper trace, membrane depolarization; lower trace, ionophoretic current. (b) Dose-response curve for a normal (●) and axotomized (○) cell.

Carbamylcholine

Plotting the relationship between log ionophoretic charge and maximum response for CCh (Fig. 3) revealed that the threshold dose was much lower than for ACh applied to normal cells while the maximum slope of the graph was not as steep. Since the kinetics of drug release and diffusion from ionophoretic microelectrodes are essentially the same for ACh and CCh (Dionne, 1976; Dreyer, Peper & Sterz, 1978) the relatively low threshold is probably due to the resistance of CCh to cholinesterase, enabling a greater proportion of the drug to reach receptors. CCh dose-response

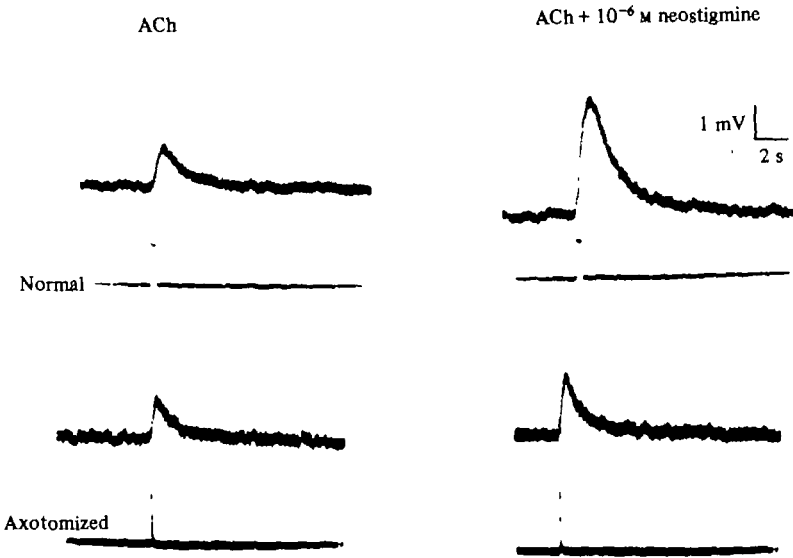


Fig. 4. Potentiation of ACh response by physostigmine. Left, response of a normal and an axotomized cell to ionophoretically applied ACh. Upper trace, membrane depolarization, lower trace, ionophoretic current (axotomized cell, 45 nA; normal cell, 160 nA). Right, response of same cells after bathing preparations in 10^{-6} M physostigmine for 2 min.

Table 1

	Mean sensitivity before antiChE (mV/nC)	Mean sensitivity after antiChE (mV/nC)	Potentiation (%)	
Physostigmine 10^{-6} M				
Normal	0.044 ± 0.034	0.349 ± 0.181	1034 ± 305	($n = 7$)
Axotomized	0.185 ± 0.059	0.446 ± 0.206	199 ± 27	($n = 7$)
Neostigmine 10^{-6} M				
Normal	0.092 ± 0.039	0.688 ± 0.316	687 ± 150	($n = 5$)
Axotomized	0.219 ± 0.043	0.661 ± 0.222	349 ± 53	($n = 7$)
Neostigmine 10^{-6} M				
Normal	0.092 ± 0.030	0.386 ± 0.138	408 ± 25	($n = 7$)
Axotomized	0.185 ± 0.048	0.435 ± 0.175	214 ± 32	($n = 6$)

curves were similar for normal and axotomized cells, while their sensitivities calculated from the maximum slopes were not significantly different (0.068 , S.E. ± 0.021 mV/nC, $n = 5$) and 0.075 , S.E. ± 0.035 mV/nC, $n = 5$) respectively.

Anticholinesterases

Because axotomy is not accompanied by an increase in extrasynaptic sensitivity to CCh, the enhanced ACh sensitivity may result from a reduction in the cholinesterase activity, which increases the effective drug concentration at the site of action. Ther

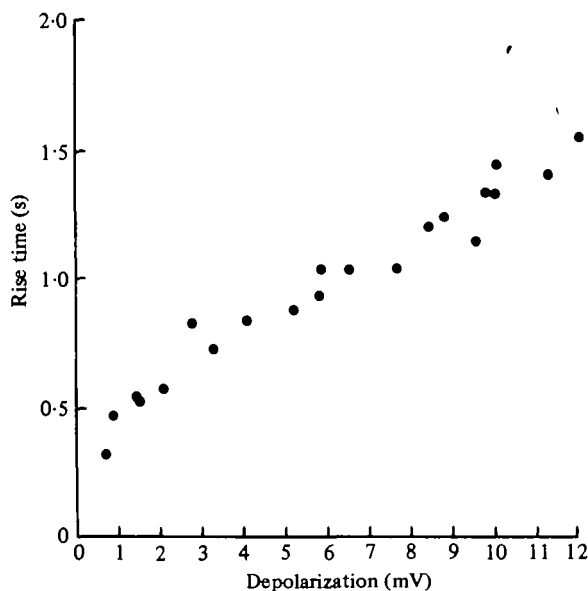


Fig. 5. Relationship between ACh response amplitude and rise-time for a normal cell. Time taken to reach maximum response increases approximately linearly with response amplitude.

fore, the effect of the anticholinesterases physostigmine (eserine) and neostigmine on the response to a given dose of ACh was studied.

ACh ionophoretic pulses were adjusted to give a depolarization of approximately 1 mV before treatment with an anticholinesterase. A second standard pulse was then applied 2 min after anticholinesterase administration. Fig. 4 shows the response to ionophoretically applied ACh before and after adding 10^{-6} M neostigmine. The degree of potentiation was taken as the percentage increase in the sensitivity to ACh. The results of these experiments are illustrated in Table 1. The degree of potentiation by both physostigmine and neostigmine was greater in normal than in axotomized preparations. This supports the proposal that a reduction in cholinesterase activity is at least in part responsible for the development of supersensitivity following axon section.

Rise-times and receptor distribution

The increase in extrasynaptic sensitivity to ACh could be explained in terms of alterations in a diffusion barrier which limits drugs access to the receptor site on the neurone membrane. This was investigated by examining the rise-times of the ACh responses and the regional distribution of sensitive areas. Ejecting pulses of 100 ms duration were used in all of these experiments.

Rise-times of the ACh response appeared to increase linearly with the dose (Fig. 5) and were relatively slow when compared to ACh responses of junctional membranes (cf. Harris, Kuffler & Dennis, 1971; Feltz & Mallart, 1971). These characteristics of the ACh response rise-times are similar for extrajunctional receptors on frog muscle fibre (Feltz & Mallart, 1971). The effect of cholinesterases was eliminated by bathing the preparation in a 10^{-6} M solution of neostigmine bromide. In addition it was important to maintain the same dose from cell to cell because of the dose dependency

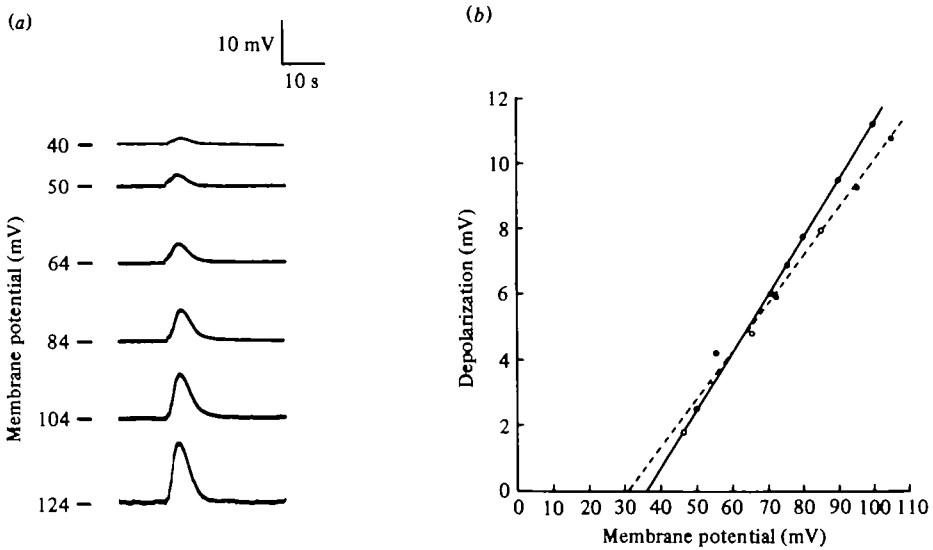


Fig. 6. (a) Effect of hyperpolarizing the neuronal membrane upon the ionophoretic ACh response. (b) Magnitude of ACh response plotted as a function of membrane potential; (●) normal cell, (○) axotomized cell. Linear regression line extrapolates to -32 mV (axotomized cell).

of the rise-time described above. The average times to peak of 14 normal and 10 axotomized cells were 0.85 S.E. ± 0.13 s and 1.09 S.E. ± 0.23 s respectively using a 10 nC ionophoretic pulse. These values were not significantly different ($P > 0.05$), indicating that after axotomy there are no changes in either the density of ACh receptors or in any diffusion barrier to ACh.

The ACh reversal potential

To test whether the increased sensitivity to ACh after axotomy results from a positive shift in the ACh reversal potential, this parameter was estimated by extrapolation. The amplitude of the response to a standard ionophoretic dose of ACh increased linearly with membrane potential over the range tested as shown in Fig. 6a,b. Extrapolation of this line should intersect the ordinate at the ACh reversal potential. However, this method of determination is subject to some error and may not represent the true ACh reversal potential (Mallart, Dreyer & Peper, 1976), although it should reveal any significant change in the value since the input resistance was similar in normal and axotomized cells. The reversal potentials obtained in this way for 5 normal and 5 axotomized cells were -35 , S.E. ± 2.4 and -33 , S.E. ± 2.9 mV respectively ($P > 0.05$) indicating that the increase in sensitivity after axotomy is not due to a change in the ACh reversal potential.

The Hill coefficient

The neuronal ACh sensitivity would be increased if there was a reduction in the average number of ACh molecules required to activate an individual receptor. The number of drug molecules required to activate a single receptor is known as the Hill coefficient (n). The effect of ionophoretically applied ACh was expressed in terms

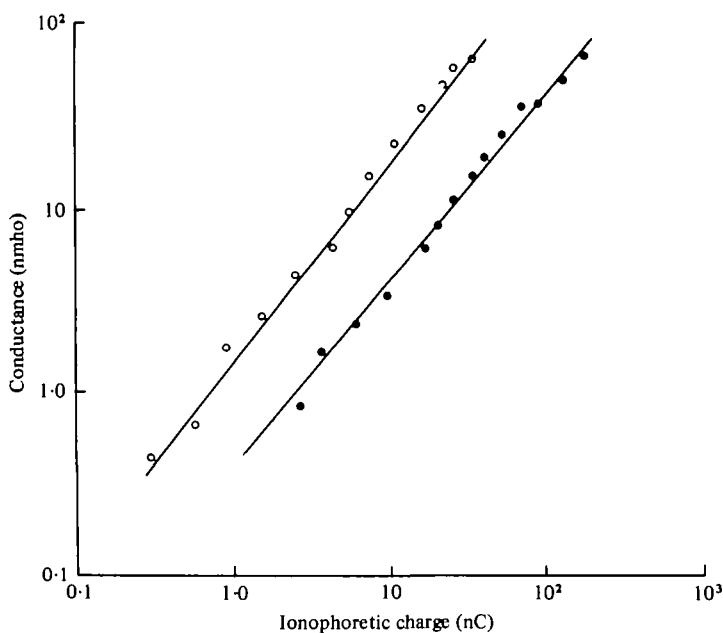


Fig. 7. Dose-response relationship for a normal (●) and axotomized (○) cell, double logarithmic plot. Ordinate, peak conductance change calculated from equation (1); abscissa, charge passed from ionophoretic pipette.

the change in membrane conductance. The peak conductance change was calculated from the equation:

$$\Delta g = \frac{\Delta V}{R(E - \Delta V)} \quad (1)$$

where ΔV was the peak depolarization, R the resting input resistance and E the difference between the resting potential and the reversal potential for the ACh response. The reversal potential was taken as -34 mV. Plotting $\Delta g/\Delta g_{\max} - \Delta g$ against $\log [ACh]$ should give a graph of slope equal to the Hill coefficient (Rang, 1973).

However, it was not possible to obtain an accurate value for Δg_{\max} by direct measurement since maximal doses of ACh normally produced a significant reduction in membrane resistance and membrane oscillations. In addition, many of the axotomized cells produced action potentials when depolarized more than about 10 mV. If Δg is small, $\Delta g_{\max} - \Delta g$ can be treated as a constant in the calculations (Kahn & Le Yaouanc, 1971; Dreyer, Peper & Sterz, 1978). Therefore with low doses of ACh, plotting $\log \Delta g$ against \log -dose should give a straight line of slope proportional to (although not equivalent to) n (Fig. 7). The mean value of the slope was 1.16, s.e. ± 0.15 for 11 normal cells and 1.13, s.e. ± 0.19 for 11 axotomized cells. These values were not significantly different ($P > 0.5$).

DISCUSSION

The cell body of the 'fast' coxal depressor motoneurone (D_1) is depolarized by ACh and its analogue CCh; its sensitivity is similar to unidentified dorsal unpaired median (DUM) neurones of the cockroach sixth abdominal ganglion (Kerkut, Pitman & Walker, 1969), locust neurones (Usherwood, Giles & Suter, 1980) and vertebrate skeletal muscle extrasynaptic receptors (Miledi, 1960; Feltz & Mallart, 1971; Fambrough, 1970). Nevertheless, the sensitivities of these cells are about two orders of magnitude lower (Dennis, Harris & Kuffler, 1971; Roper, 1976). The difference in the slopes of the ACh and CCh dose-response curves could result from receptor desensitization caused by relatively prolonged exposure to CCh; desensitization would increase progressively with an increase in the dose. Alternatively, CCh may have a lower affinity for the receptors than ACh (Eldefrawi, Eldefrawi, Seifert & O'Brien, 1972; Sattelle, McClay, Dowson & Callec, 1976).

The ACh reversal potential value reported here (approximately -30 mV) is similar to that obtained by extrapolation in cockroach DUM neurones (Pitman & Kerkut, 1970) and giant interneurones (Callec, 1974) but is more negative than the ACh reversal potential of grasshopper embryonic DUM neurones in which the value is in excess of $+20$ mV (Goodman & Spitzer, 1979). Many other preparations have more positive ACh reversal potentials than that reported here (cf. Ginsborg, 1967 for review). The relatively negative equilibrium potential observed in D_1 probably results from a relatively large increase in the membrane conductance to one or more ions with negative equilibrium potentials in addition to an increase in sodium conductance.

Both the slow rise-time and its dependence on dose can be explained by supposing that the density of extrajunctional receptors is relatively low. At such relatively insensitive areas the nearest receptor site would become quickly saturated and the response would develop by stimulation of progressively more distant receptors. Larger doses would saturate larger areas of the membrane and so the time to reach the maximum response would be prolonged. Since there was no detectable reduction in the response rise-time in axotomized neurones it is unlikely that the increase in ACh sensitivity results from an increase in extrasynaptic receptor density. The sensitivity of the synaptic membrane of parasympathetic neurones of the frog atrium to ionophoretically applied ACh is approximately 1.7 times that of the extrasynaptic membrane (Harris, Kuffler & Dennis, 1971), while the times to peak of these responses at synaptic regions are about twice as fast as those at extrasynaptic regions. These differences were attributed to a difference in the receptor density of the two regions. Two further indications that there is no change in the density of extrasynaptic ACh receptors in this insect motoneurone after axotomy are firstly that the sensitivity of normal and axotomized cells were the same in the presence of anticholinesterases and, secondly, that there was no difference in the sensitivity of these neurones to CCh. The apparent (calculated) Hill coefficient was also similar in normal and axotomized cells. Indeed, a relatively large change in the Hill coefficient (about 30%) would be required in order to produce a sensitivity change of the magnitude observed. We therefore conclude that the increase in soma ACh sensitivity produced by axotomy of this motoneurone results largely from a fall in the activity of cholinergic

esterase in the region of the applied drug. This is consistent with the decline in cholinesterase activity observed in whole cockroach metathoracic ganglia (David & Pitman, 1981) and frontal ganglia (Penzlin, Huther & Gundel; 1980) following nerve trunk section. Although the activity of ChE is reduced in axotomized preparations significant activity must still be present since the ACh response continued to be potentiated by anticholinesterases. These findings are consistent with histochemical and biochemical studies on axotomized vertebrate neurones. Postganglionic denervation of rat and cat autonomic ganglia produces a large but incomplete loss in the ChE activity of the ganglion cell bodies and dendrites (Brown, 1958; Taxi, 1961; Harkonnen, 1964; Fredricsson & Sjoqvist, 1962; Gromadzki & Koelle, 1965). Similar changes in ChE activity have also been reported in spinal motoneurones of the dog (Hard & Peterson, 1949, 1950), rat (Schwarzacher, 1958), bullfrog and common toad (Chacko & Cerf, 1960) and hypoglossal neurones of the rat (Lewis & Shute, 1965).

The reduction in ChE activity observed in axotomized vertebrate neurones (Schwarzacher, 1958; Taxi, 1961; Lewis & Shute, 1965) appears to occur during the period of increased RNA and protein production (Pearse, 1955; Harkonnen, 1964; Watson, 1968). This appears to be also true for cockroach neurones; the cell body of D₁ showed an increase in sensitivity to applied ACh, corresponding to a fall in ChE activity, 3–4 days after axotomy, at the same time as RNA synthesis begins (Cohen, 1967; Byers, 1970). The reduction in ChE activity associated with D_f is probably accelerated by orthograde axoplasmic transport of the enzyme. This has been shown to continue after nerve trunk section in the cockroach stomatogastric nervous system (Penzlin, Huther & Gundel, 1980).

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