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ACETYLCHOLINE RECEPTORS ON THE CELL BODY MEMBRANE OF GIANT INTERNEURONE 2 IN THE COCKROACH, *PERIPLANETA AMERICANA*

BY I. D. HARROW*

Department of Zoology, University of Cambridge, Downing Street, Cambridge, U.K.

AND D. B. SATTELLE

A.R.C. Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge, U.K.

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SUMMARY

1. Ionophoresis of acetylcholine (ACh) onto the cell body membrane of an identified giant interneurone (GI 2) in the central nervous system of the cockroach *Periplaneta americana* induced a depolarizing response at resting potential which was attributed to a population of extrasynaptic ACh receptors.

2. The sensitivity of the cell body membrane of GI 2 to ionophoresis of ACh was determined.

3. Perfusion of $1{\cdot}0 \times 10^{-6}$ M neostigmine, an inhibitor of acetylcholinesterase, potentiated the ACh sensitivity of the cell body membrane of GI 2. This indicated that a high acetylcholinesterase activity was present in the periphery of the sixth abdominal ganglion (A6).

4. The nicotinic antagonist, α-bungarotoxin (at a concentration of $1{\cdot}0 \times 10^{-7}$ M) was found to block the ACh response of the cell body membrane of GI 2. By contrast, the muscarinic antagonist, quinuclidinyl benzilate, (at concentrations up to $1{\cdot}0 \times 10^{-5}$ M) had no detectable effect on the ACh response.

5. It is suggested that an extrasynaptic nicotinic type of ACh receptor is present on the cell body membrane of GI 2.

INTRODUCTION

Acetylcholine (ACh) is a strong candidate as a neurotransmitter in mechanosensory and chemosensory neurones of arthropods (Barker, Herbert, Hildebrand & Kravitz, 1972; Florey, 1973; Pitman, 1971; Gerschenfeld, 1973; Callec, 1974; Sattelle, 1980). The pharmacological investigation of single identified neurones enables a systematic analysis of responses to a putative neurotransmitter (Ascher & Kehoe, 1975). In the present study the sensitivity to ACh and related pharmacological agents of the cell

*Present address: Department of Biological Sciences, Columbia University, New York, N.Y., 10027, U.S.A.

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body membrane of giant interneurone 2 (GI2) of the cockroach *Periplaneta americana* has been investigated.

In the cockroach central nervous system seven bilateral pairs of giant interneurones can be readily identified from their morphology (Harrow, Hue, Pelhate & Sattelle, 1980*a*; Daley, Vardi, Appignani & Camhi, 1981). These cells receive sensory input from filiform hair mechanoreceptors on the cerci (Callec, Guillet, Pichon & Boistel, 1971; Westin, Langberg & Camhi, 1977). The available physiological evidence suggests that a monosynaptic connection exists between the filiform hair mechanoreceptors and those giant interneurones (GIs 1,2,3) with the largest diameter axons (Callec *et al.* 1971; Callec, 1972). Pharmacological findings strongly suggest that cholinergic synapses mediate the cercal afferent, giant interneurone pathway and that the postsynaptic ACh receptors are of the nicotinic type (Shankland, Rose & Donniger, 1971; Callec, 1974; Sattelle, McClay, Dowson & Callec, 1976; Sattelle, 1978).

The cercal afferent, giant interneurone synapses are sited deep within the neuropile of the sixth abdominal (A6) ganglion (Farley & Milburn, 1969; Daley *et al.* 1981). This poses severe technical difficulties for microelectrode recording from postsynaptic processes. The location of the postsynaptic membranes also impedes the access of pharmacological agents. A further complicating factor, common to pharmacological studies of all synapses, is the possibility of drug actions on the presynaptic membrane. If it can be demonstrated that a giant interneurone has extrasynaptic (cell body) receptors which show comparable sensitivity to synaptic receptors on the same cell, with respect to specific cholinergic receptor ligands, then these cell body receptors may serve as a useful model for synaptic receptors (cf. Ascher & Kehoe, 1975). In the present study the extrasynaptic ACh receptors located on the accessible cell body of GI2 were investigated. ACh sensitivity was quantified and the effects of the pharmacological agents, neostigmine, α-bungarotoxin (α-BGTX) and quinuclidinyl benzilate (QNB) on ACh sensitivity were investigated.

MATERIALS AND METHODS

Animals and salines

Adult male cockroaches, (*Periplaneta americana*) were used throughout this study and all experiments were performed at room temperature (21–23 °C). The saline was designed for desheathed insect nervous tissue (Pitman, 1975), and had the following composition (in mM): NaCl, 214; KCl, 3·1; $CaCl_2$, 9·0; N-Tris-(hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES), 10·0; pH 7·0 (adjusted dropwise with saturated NaOH).

Chemicals

Solutions of acetylcholine chloride, neostigmine methyl sulphate (Sigma Chemical Co. Ltd., U.K.) and α-bungarotoxin (α-BGTX) (Miami Serpentarium Ltd., U.S.A.) were prepared in normal saline. Quinuclidinyl benzilate (QNB) was first dissolved in absolute alcohol, at a concentration of $1{\cdot}0 \times 10^{-2}$ M, and final concentrations were prepared by diluting aliquots of this stock solution into normal saline. All other chemicals were obtained from B.D.H. Chemicals Ltd., U.K.

Dissection and recording

Under saline, the cerci and abdominal nerve cord were dissected from the abdomen as described previously (Sattelle & Callec, 1977). The isolated nerve cord was mounted under saline on a Sylgard (Dow Corning) gel slab (2 mm thick) with A6 secured on its side by 'minuten' pins. The ganglion was desheathed using fine forceps. Often the stumps of nerve VIII and the large lateral tracheole remained, which aided the location of the cell body of GI 2. After desheathing A6, the abdominal nerve cord was cut just posterior to A3 and the left connective linking A6 and A5 was severed. The Sylgard slab was removed to the Perspex experimental chamber and mounted at 45 °. Acute-angle illumination usually enabled the relatively large cell body of GI 2 to be seen.

The severed connective projecting from A4 was stimulated using a suction electrode. All pharmacological experiments were performed with a single recording microelectrode (containing 2 M potassium acetate: 25–35 MΩ) located in the cell body of GI 2. Microelectrodes filled with 1 M cobaltous nitrate were employed in staining experiments and 50 nA, 0·5 s, positive, square pulses were applied at 1 Hz for 30–60 min. Cobalt-injected GI 2 preparations were treated with ammonium sulphide and silver intensified in whole-mount (Tyrer & Bell, 1974; Bacon & Altman, 1977; Harrow *et al.* 1980*a*). The recording microelectrode was connected to the input of a high impedance, d.c. preamplifier. The indifferent electrode (a chloridized silver wire) was immersed in 3 M-KCl and linked to the experimental bath via a saline-agar bridge. The output from the preamplifier was monitored on an oscilloscope and was also fed via an analogue-to-digital converter into a computer [North Star Horizon, 32 K Byte random access memory (RAM), with a single (180 K Byte) mini-floppy disc drive]. The stored events were retrieved from the disc and displayed on a digital X,Y plotter (Houston).

Application of pharmacological agents

Bath-applied pharmacological agents were perfused through the experimental chamber for 3 min at a rate of 6 ml min^{-1} (volume with preparation in place 1 ml) and then allowed to remain in the chamber for the desired length of time. Dye-clearance tests showed that with this protocol the final concentration was reached after perfusion for 2 min. The liquid in the chamber was circulated throughout all experiments by an air stream directed at the surface. The ionophoretic microelectrode, filled with 1 M-ACh (5–10 MΩ), was connected to the positive side of an isolated d.c. stimulator (Digitimer) via a resistor (10 MΩ). The negative side of the stimulator was led to ground. Ionophoretic current was measured with a virtual-earth circuit. Ionophoretic charge was used as an index of the agonist concentration ejected from the electrode tip (cf. Curtis, 1964; Purves, 1979). A negative d.c. retaining current (typically ~40 nA) prevented background leakage of ACh.

RESULTS

Identification of giant interneurone 2 (GI 2)

When the sixth abdominal ganglion was desheathed, the cell body of GI 2 could be recognized by its relatively large diameter (approximately 70–80 μm) and characteristic

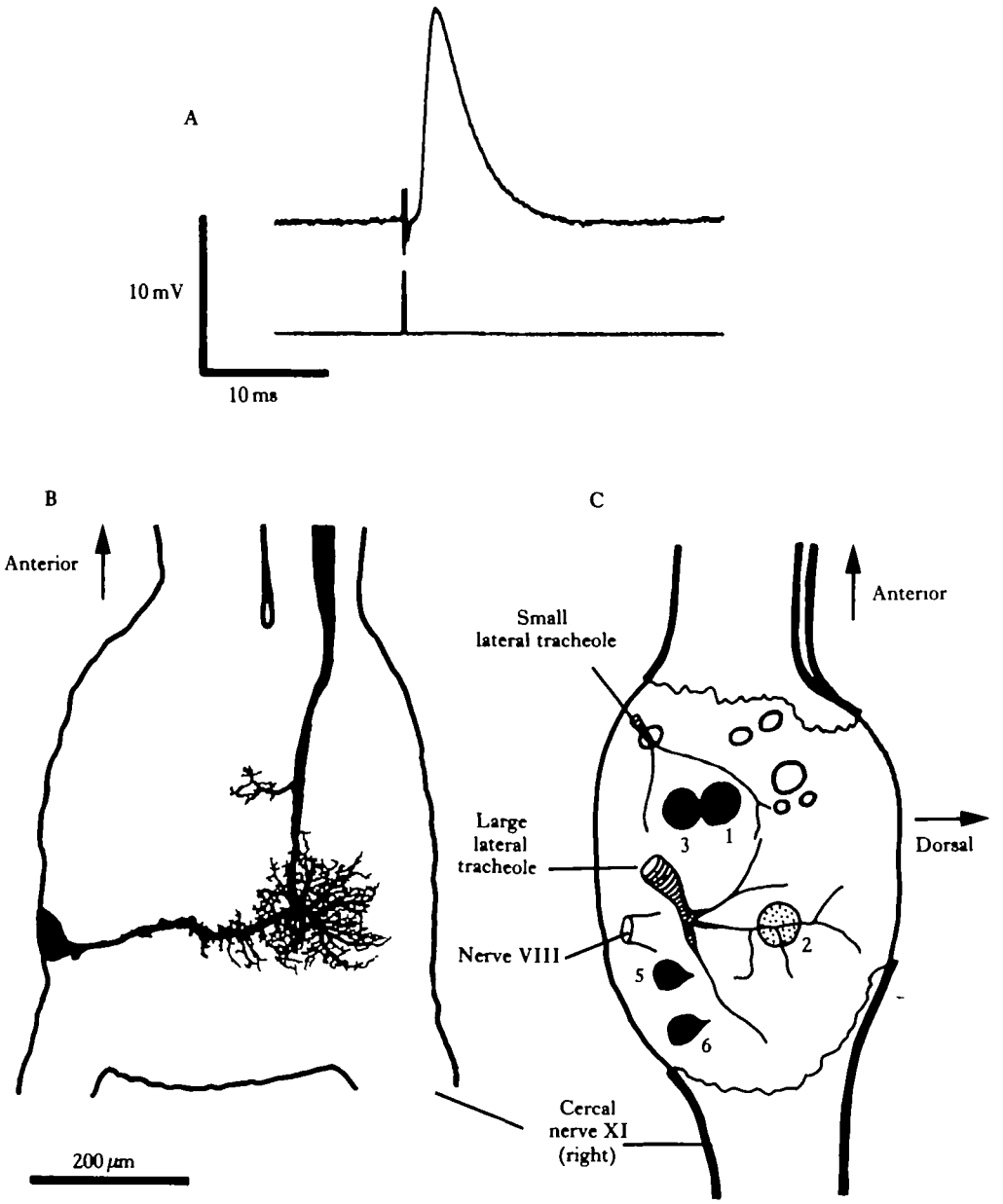

Fig. 1. Identification of giant interneurone 2 (GI 2). (A) A short-latency, antidromic spike was recorded from the cell body of GI 2 (the microelectrode contained 2 M potassium acetate). Stimulus marker is shown in the lower trace. (B) Using a microelectrode filled with 1 M cobaltous nitrate, cobalt ions were ionophoretically injected to reveal the characteristic morphology of GI 2 (cf. Harrow, Hue, Pelhate & Sattelle, 1980*a*; Daley, Vardi, Appignani & Camhi, 1981). A dorsal view of the sixth abdominal ganglion is shown. (C) After detection of the antidromic spike shown in (A), the cell body was damaged with the tip of the microelectrode by a controlled stabbing action. When the contralateral connective linking the fifth and fourth abdominal ganglia was backfilled with cobaltous chloride (2% w/v), the cell body of GI 2 was unstained and scarcely detectable in its characteristic location (dotted cell body). By contrast, cell bodies of all the other (numbered) giant interneurones were stained. The sixth abdominal ganglion is shown in a side view (in this orientation the cell bodies of GI 4 and GI 7 are not visible).

location (cf. Harrow *et al.* 1980*a*; Daley *et al.* 1981) close to the dorsal bifurcation of the large lateral tracheole and just posterior to nerve VIII (nomenclature of Roeder, Tozian & Weiant, 1960). A resting potential of $-80{\cdot}8 \pm 0{\cdot}5$ mV (mean ± 1 S.E., $N = 140$) was recorded from the cell body of GI 2. Electrical stimulation of the contralateral connective linking the fifth and fourth abdominal ganglia elicited an antidromic spike (Fig. 1A) of amplitude $13{\cdot}2 \pm 0{\cdot}4$ mV (mean ± 1 S.E., $N = 140$). The short latency (2–3 ms) of the antidromic spike indicated a high axonal conduction velocity (approximately 8 m s^{-1}) as would be predicted for a giant interneurone.

Two types of experiment were used to test the reliability of using cell body size and location, together with the detection of a short-latency, antidromic spike, for routine identification of GI 2. In the first of these a microelectrode filled with cobaltous nitrate was used to impale the cell body and the short-latency, antidromic spike was evoked. Then cobalt ions were injected into the cell body of the neurone under investigation, which always revealed the characteristic morphology of GI 2 (Harrow *et al.* 1980*a*) in a whole-mount preparation of the sixth abdominal ganglion (Fig. 1B). Another test involved mechanical disruption of the cell body with the tip of a recording microelectrode after detection of the short-latency, antidromic spike. When the contralateral connective was backfilled with cobalt chloride (2% w/v), all the cell bodies of the giant interneurones were stained except for that of GI 2 (Fig. 1C). Cell body diameter, position, and detection of a short-latency, antidromic spike were therefore used as criteria for the routine identification of the cell body of GI 2. If these criteria were not satisfied the preparation was rejected.

Acetylcholine sensitivity

Ionophoresis of ACh on to the cell body of GI 2 produced a depolarizing response at resting potential which was dose-dependent (Fig. 2). From the relationship between log dose and the amplitude of depolarization, ACh sensitivity was estimated for a particular cell by calculating the gradient of maximum slope. By this means the ACh sensitivity was found to be $0{\cdot}092 \pm 0{\cdot}019$ mV nC^{-1} (mean ± 1 S.E., $N = 15$) a value identical to that reported by David & Pitman (1982) for the cockroach fast coxal depressor motoneurone (D_f). By pooling the data from 15 GI 2 cells, a single dose-response curve for ACh was generated (Fig. 2). The resting potentials recorded from these cells prior to the application of ACh were in the range -75 to -85 mV. The minimal quantity of ACh required to induce a 1 mV depolarizing response was estimated from the equation,

$$Q = \frac{1}{F} C.H$$

where Q = amount of ACh released; F = the Faraday; C = coulombs passed; H = the transport number for acetylcholine (0·1 after Dionne, 1976). For GI 2, a value for Q of $5{\cdot}0 \times 10^{-15}$ mol was calculated. The peak maximum concentration of ligand (ACh) reaching the receptors was estimated from the equation of Carslaw & Jaeger (1959):

$$L_c = \frac{Q}{8(\pi DT)^{1{\cdot}5}} \exp\left(\frac{-r_2}{4DT}\right)$$

where D = the diffusion coefficient of acetylcholine ($6{\cdot}11 \times 10^{-6}$ cm^2 s^{-1}, cf. Dionne,

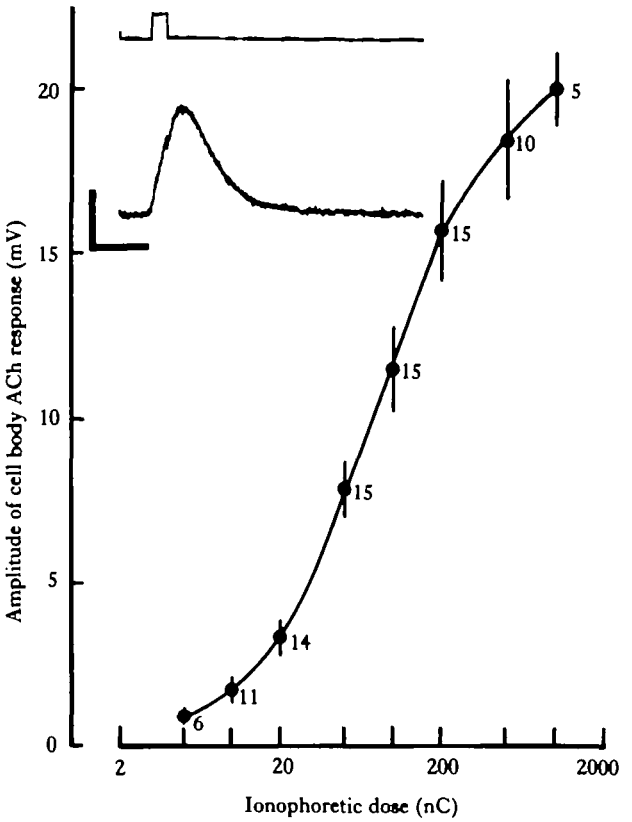

Fig. 2. ACh sensitivity of the cell body of GI 2. A semi-logarithmic dose-response curve was plotted. Each point on this curve is the mean response to a particular dose of ACh. The vertical bar drawn through each point represents ± the standard error of the mean and the number of contributing preparations is indicated beside each bar. The inset shows a typical depolarizing response to ionophoresis of ACh onto the cell body of GI 2. The ionophoretic current is shown in the upper trace and the resulting ACh response in the lower trace. Calibration: vertical, (upper) 120 nA; (lower) 5 mV; horizontal, 2 s.

1976); T = time to peak of the response (0·25 s); r = distance between the pipette tip and the receptors. Taking $r^2 = 6DT$ (cf. del Castillo & Katz, 1955) gives an estimate for r = 30 μm. In this way a value for L_c of $1{\cdot}3 \times 10^{-8}$ M was estimated. Prior to the bath-application of pharmacological agents the stability of the ACh response was established by applying a fixed ionophoretic dose of ACh at 5 min intervals for 20–30 min.

Effects of neostigmine

Following exposure to the anticholinesterase neostigmine ($1{\cdot}0 \times 10^{-6}$ M), the ACh sensitivity of the cell body membrane of GI 2 increased to 0·145 mV nC^{-1} (mean, $N = 2$). Fig. 3 shows dose-response curves for ACh prior to and following a 10 min exposure to $1{\cdot}0 \times 10^{-6}$ M neostigmine. The ~50 % increase in sensitivity to ACh resulting from neostigmine treatment is much less than the increase (~300 %) reported in comparable experiments on the cockroach motoneurone D_f (David & Pitman, 1982).

Effects of α-bungarotoxin

α-Bungarotoxin (α-BGTX) isolated from the venom of the snake, *Bungarus multicinctus*, is a potent, essentially irreversible antagonist of nicotinic ACh receptors of mammalian skeletal muscle (Chang, 1978), fish electroplax (Changeux, 1975) and some central nervous system (CNS) receptors of vertebrates (Barnard *et al.* 1979; Freeman, Schmidt & Oswald, 1979; Oswald, Schmidt, Norden & Freeman, 1979). A putative nicotinic type of cholinergic receptor has been characterized in CNS extracts of several insect species using radiolabelled α-bungarotoxin as a specific probe (see Sattelle, 1980 for review). When tested at a concentration of $1{\cdot}0 \times 10^{-6}$ M in six experiments, α-BGTX blocked the response of the cell body of GI2 to ionophoretically-applied ACh (Fig. 4). Block of the ACh response typically occurred after 60 min without any change in membrane polarization or loss of the antidromic spike. After toxin-induced block, prolonged washing with saline

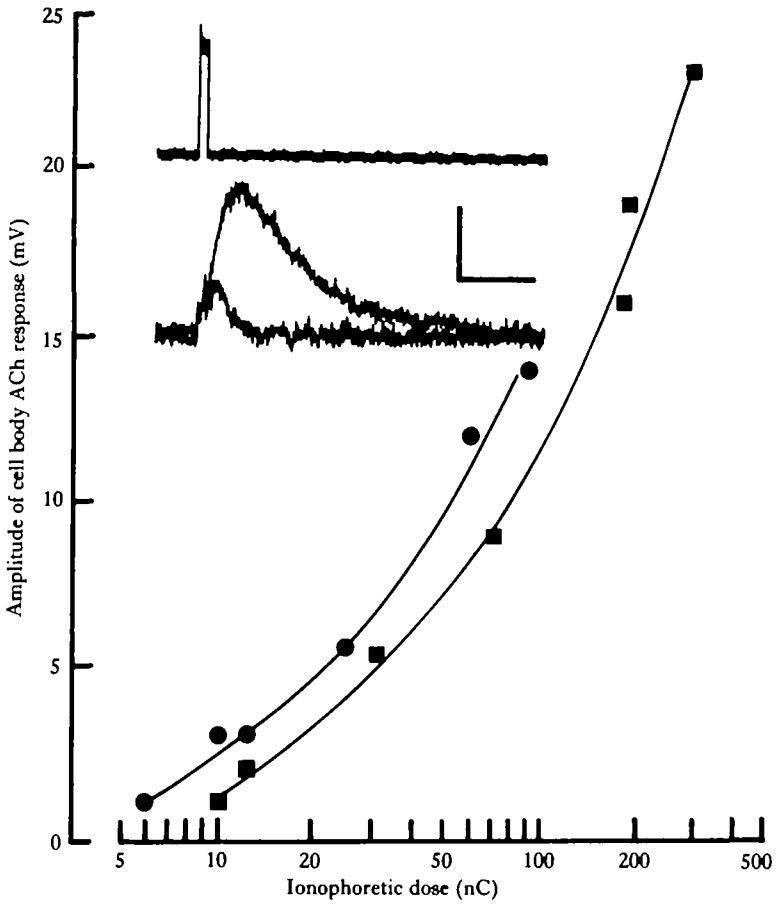

Fig. 3. Dose-response curves for ACh before (■) and after (●) perfusion of $1{\cdot}0 \times 10^{-6}$ M neostigmine. The inset shows ACh responses evoked by the same ionophoretic dose in the absence and presence of $1{\cdot}0 \times 10^{-6}$ M neostigmine (10 min exposure). The ionophoretic currents are shown in the upper trace and the resulting ACh responses are shown in the lower trace. Calibration: vertical (upper) 35 nA, (lower) 1·5 mV; horizontal 2 s.

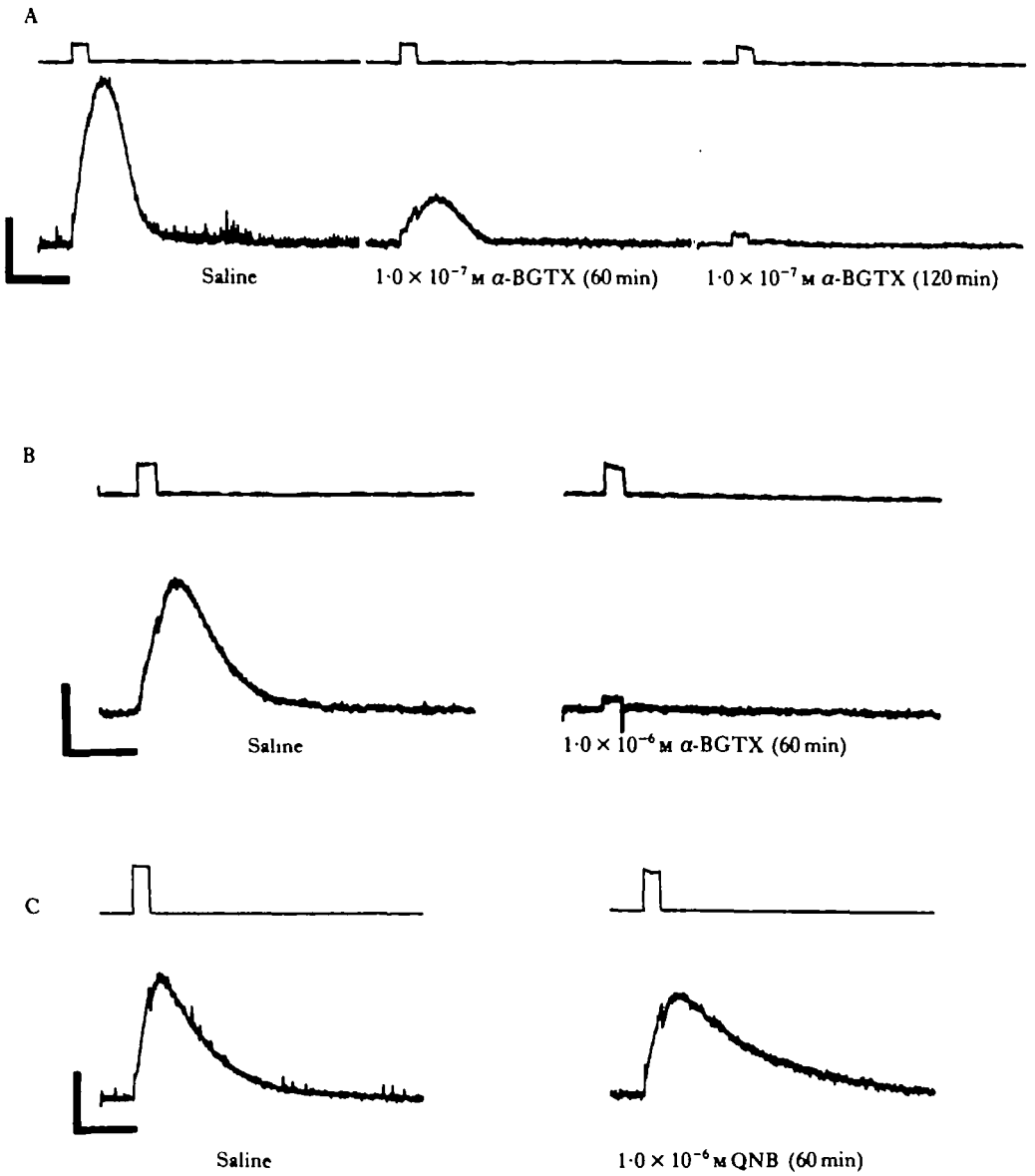

Fig. 4. Effects of α-bungarotoxin (α-BGTX) and quinuclidinyl benzilate (QNB) on the response to ionophoretically-applied ACh of the cell body of GI2: (A) $1{\cdot}0 \times 10^{-7}$ M α-BGTX (120 min), ionophoretic dose throughout the experiment was 50 nC; (B) $1{\cdot}0 \times 10^{-6}$ M α-BGTX (60 min), ionophoretic dose throughout the experiment was 25 nC; (C) $1{\cdot}0 \times 10^{-6}$ M QNB (60 min), ionophoretic dose throughout the experiment was 115 nC. Calibration: (A) vertical (upper) 400 nA, (lower) 4 mV, horizontal, 2 s; (B) vertical (upper) 120 nA, (lower) 5 mV, horizontal, 2 s; (C) vertical (upper) 300 nA, (lower) 2·5 mV, horizontal, 2 s.

never led to recovery of the ACh response, indicating irreversible antagonism.

In a single experiment, α-BGTX was tested at a concentration of $1{\cdot}0 \times 10^{-7}$ M. After 60 min of exposure, the amplitude of the response to ionophoretically-applied ACh was reduced by approximately 50 %. Complete block of the ACh response was achieved after 120 min (Fig. 4).

Effects of quinuclidinyl benzilate

Quinuclidinyl benzilate (QNB) has been used as a specific probe for the muscarinic ACh receptors of vertebrate smooth muscle (Yamamura & Snyder, 1974*a*) and brain tissue (Yamamura & Snyder, 1974*b*). A putative muscarinic type of cholinergic receptor has recently been characterized in CNS extracts of several insect species (cf. Sattelle, 1980), using radiolabelled QNB. When tested at $1{\cdot}0 \times 10^{-6}$ M (in the presence of 0·01 % ethanol) in two experiments, QNB had no effects on the amplitude of the ionophoretic ACh response, membrane polarization or the antidromic spike (Fig. 4). A ten-fold higher concentration ($1{\cdot}0 \times 10^{-5}$ M) of QNB (0·10 % ethanol present) appeared to block the ionophoretic ACh response, but this action was accompanied by changes in membrane polarization (hyperpolarization followed by depolarization). In another experiment a similar complex action was also observed during a 60 min exposure to 0·10 % ethanol. This finding indicated that ethanol, rather than QNB, was primarily responsible for the effects observed when the muscarinic antagonist was tested at $1{\cdot}0 \times 10^{-5}$ M in the presence of 0·10 % ethanol.

DISCUSSION

In the present study, the cell body of an identified giant interneurone (GI 2) in the central nervous system of the cockroach *Periplaneta americana* was found to exhibit depolarizing responses to the ionophoretic application of ACh. No chemical synapses on to cell bodies have been detected in the sixth abdominal ganglion of the cockroach (Smith & Treherne, 1965). In fact, ultrastructural studies to date have failed to reveal presynaptic terminals associated with cell bodies in the central nervous system of any species of adult insect. Therefore, a population of extrasynaptic ACh receptors, located on the cell body membrane of GI 2, appears to mediate the response to ionophoretically-applied ACh.

Cell body depolarizing responses to ACh ionophoresis have been reported for numerous other central neurones in various species of insect: unidentified neuronal cell bodies on the dorsal-midline of the sixth abdominal ganglion of the cockroach (Kerkut, Pitman & Walker, 1969); the fast coxal depressor motoneurone in the metathoracic ganglion of the cockroach (David & Pitman, 1979, 1982); dorsal unpaired median (DUM) cells in the metathoracic ganglion of the cockroach (Sattelle, David, Harrow & Hue, 1980; Lane, Swales, David & Sattelle, 1982); DUM cells in the metathoracic ganglion of the grasshopper embryo (Goodman & Spitzer, 1980); and unidentified, isolated neurones from the metathoracic ganglion of the locust (Usherwood, Giles & Suter, 1980). In all cases to date, as observed for the cell body membrane of GI 2, the ionophoretic ACh response is depolarizing at resting potential. In addition, the quantities of ACh (Q) required to produce a minimal depolarization of the cell body membrane are comparable for cockroach neurones. The estimate of $Q = 5{\cdot}0 \times 10^{-15}$ mol for GI 2, using an ACh transport number of 0·1, is close to the value of $1{\cdot}31 \times 10^{-13}$ mol calculated by Kerkut *et al.* (1969) for unidentified neuronal cell bodies of the dorsal midline of the metathoracic ganglion using a value for the ACh transport number of 0·5. This value for dorsal midline cells is equivalent to a value of $2{\cdot}62 \times 10^{-14}$ mol when the same transport number (0·1 after Dionne, 1976) used

for calculations on GI 2 is adopted. The values of Q for insect neuronal cell bodies are similar to the estimate of $1{\cdot}0 \times 10^{-14}$ mol reported for nerve cell bodies in the visceral ganglion of the mollusc *Cryptomphallus aspersa* by Gerschenfeld & Stefani (1966), using a transport number of 0·3.

Neostigmine, an inhibitor of acetylcholinesterase was found to potentiate the ACh sensitivity of the cell body of GI 2. This finding agrees with previous histochemical evidence for a high acetylcholinesterase activity located in the periphery of the sixth abdominal ganglion (Smith & Treherne, 1965). The desheathed cell bodies of GI 2 (this study) and D_f (David & Pitman, 1982) normally exhibit the same sensitivity to ACh, whereas in the presence of $1{\cdot}0 \times 10^{-6}$ M neostigmine, the sensitivity of GI 2 increases by only 50 % compared to a 300 % increase in the sensitivity of D_f (cf. David & Pitman, 1979, 1982). It is concluded, therefore, that the cell body membrane of GI 2 is less sensitive, and by implication has a lower density of ACh receptors, than the cell body membrane of D_f. There also appears to be more peripheral acetylcholinesterase in the vicinity of the cell body of D_f compared to that of GI 2.

Electrophysiological and radiolabelled ligand binding studies provide complementary experimental approaches in the pharmacological analysis of ACh receptors in insects (Sattelle, 1980). An extract from the abdominal nerve cord of the cockroach, *Periplaneta americana*, contains an [^{125}I]-α-BGTX binding component possessing the properties expected for a nicotinic ACh receptor (Gepner, Hall & Sattelle, 1978). Electrophysiological investigations of synaptic transmission at cercal afferent, giant interneurone synapses demonstrate the presence of a synaptic ACh receptor with nicotinic properties (Shankland *et al.* 1971; Sattelle, 1978) which is sensitive to α-bungarotoxin (Harrow *et al.* 1980*b*). Recently, specific binding of [^{3}H]-QNB was reported for an extract derived from the terminal abdominal ganglion of the cricket, *Acheta domesticus* (Meyer & Edwards, 1980). In view of these findings it was of particular interest to compare the effects of α-BGTX and QNB on the cholinergic receptors of GI 2.

The extrasynaptic ACh receptors of GI 2 were found to be very sensitive to α-BGTX but less sensitive to QNB. The response to ionophoretically-applied ACh was completely abolished by exposure to $1{\cdot}0 \times 10^{-7}$ M α-BGTX which suggests the presence of a single population of extrasynaptic ACh receptors on the cell body membrane of GI 2. The extrasynaptic ACh receptors on the cell body of the fast coxal depressor motoneurone (D_f) in the metathoracic ganglion have also been shown to be particularly sensitive to α-BGTX and much less sensitive to QNB (Harrow, David & Sattelle, 1982). On the other hand, the ACh-induced depolarization of the cell bodies of DUM neurones both in a grasshopper *Schistocerca nitens* (Goodman & Spitzer, 1980) and a cockroach *Periplaneta americana* (Sattelle *et al.* 1980; Lane *et al.* 1982) has been reported to be relatively insensitive to α-BGTX. Considered together, these findings suggest that more than one type of extrasynaptic ACh receptor may be present in the central nervous system of insects. This emphasizes the need to select identified neurones for pharmacological experiments.

Synaptic transmission between cercal mechanoreceptors and GI 2 has also been found to be α-BGTX-sensitive and by contrast QNB is relatively ineffective (Harrow *et al.* 1982). Therefore, both the synaptic and extrasynaptic ACh receptors of an identified giant interneurone in the cockroach sixth abdominal ganglion have simil

pharmacological properties with respect to ligands tested to date. It is of interest in this context that Pitman & Kerkut (1970) noted similar reversal potentials and a comparable sensitivity to gallamine for both the extrasynaptic ACh responses and excitatory post synaptic potentials in an unidentified cell body of the dorsal region of the same ganglion.

The results presented here demonstrate that the cell body membranes of identified neurones such as GI 2 provide an accessible model system for pharmacological studies of insect ACh receptors.

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