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ACTIONS OF CHOLINERGIC PHARMACOLOGICAL AGENTS ON THE CELL BODY MEMBRANE OF THE FAST COXAL DEPRESSOR MOTONEURONE OF THE COCKROACH (*PERIPLANETA AMERICANA*)

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SUMMARY

The pharmacological properties of cholinergic receptors on the cell body membrane of the fast coxal depressor motoneurone (D_f) of the cockroach (*Periplaneta americana*) have been investigated. Parallel dose-response curves were obtained for the depolarizing actions of four bath-applied agonists, with the following order of effectiveness: nicotine > acetylcholine (ACh), in the presence of $1{\cdot}0\times10^{-7}$ M neostigmine > carbamylcholine > tetramethylammonium. By contrast, dimethyl-4-phenyl piperazinium, suberyldicholine, D,L-muscarine, oxotremorine, acetyl-β-methylcholine and sebacinylcholine were practically ineffective. Of the three putative receptor-specific ligands used to date in binding studies on insect CNS tissues, α-bungarotoxin (α-BGTX) was much more effective ($I_{50}=6{\cdot}4\times10^{-8}$ M) in blocking the depolarization resulting from ionophoretic application of ACh, than either quinuclidinyl benzilate (QNB) ($I_{50}=1{\cdot}6\times10^{-4}$ M) or decamethonium ($I_{50}=2{\cdot}8\times10^{-3}$ M). The order of effectiveness of ligands that were particularly effective in blocking ACh depolarization was α-BGTX > α-cobratoxin (α-COTX) > mecamylamine > dihydro-β-erythroidine > benzoquinonium. Less potent and almost equally effective were atropine, *d*-tubocurarine, pancuronium and quinuclidinyl benzilate. Even less effective were hexamethonium, gallamine, decamethonium and succinylcholine, all requiring concentrations of $\sim 1{\cdot}0\times10^{-3}$ M and higher to produce a significant block of the ACh response. Not all reversibly acting antagonists were equally effective in preventing irreversible block of the ACh-induced depolarization by α-BGTX. Whereas α-COTX protected the receptors, mecamylamine did not. With the cell body of D_f voltage-clamped, the degree of antagonism of the ACh-induced current was assessed at potentials in the range −120 mV to −60 mV. α-BGTX, dihydro-β-erythroidine, benzoquinonium, QNB and decamethonium appeared to be voltage-independent over this potential range, whereas *d*-tubocurarine and atropine were strongly voltage-dependent in their blocking actions. Sites of action of cholinergic antagonists at the insect ACh receptor/ion channel complex are discussed.

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Key words: Acetylcholine receptors, voltage-clamp, identified insect motoneurone.

INTRODUCTION

Although abundant electrophysiological evidence exists for the acetylcholine (ACh) sensitivity of many vertebrate (Krnjevic, 1974) and invertebrate (Leake & Walker, 1980) central neurones, in very few cases has a detailed pharmacological profile of the acetylcholine response been described. In insects, the radiolabelled putative cholinergic receptor ligands ^{125}I-α-bungarotoxin, [^{3}H]quinuclidinyl benzilate and [^{3}H]decamethonium have enabled characterization of three pharmacologically distinct binding sites in CNS particulate extracts (see Sattelle, 1980), but the actions of these and other cholinergic ligands on synaptic and extrasynaptic membranes of insect neurones are less well understood.

Considerable physiological evidence has accumulated for a neurotransmitter role for ACh at cercal afferent, giant interneurone 2 (GI 2) synapses in the sixth abdominal ganglion of the cockroach *Periplaneta americana* (see Callec, 1974; Sattelle, 1981). α-Bungarotoxin (α-BGTX), the potent antagonist at nicotinic receptors of electroplax (Changeux, 1975), vertebrate muscle (Chang, 1978) and one type of vertebrate central cholinergic synapse (Barnard *et al.* 1979), is the most effective of the receptor ligands tested at these insect synapses, and is about four orders of magnitude more effective than the muscarinic antagonist quinuclidinyl benzilate (Sattelle, David, Harrow & Hue, 1980; Harrow, David & Sattelle, 1982; Sattelle *et al.* 1983). ACh receptors on the cell body membrane of GI 2 are also much more sensitive to α-BGTX than to quinuclidinyl benzilate (Harrow & Sattelle, 1983). Receptors of a nicotinic cholinergic type, and sensitive to α-BGTX, are present at the synapses between trochanteral hair-plate afferents and the slow coxal depressor motoneurone (D_s) in the cockroach metathoracic ganglion (Carr & Fourtner, 1980). By contrast, dorsal unpaired median (DUM) neurones of the grasshopper (*Schistocerca nitens*) metathoracic ganglion, although sensitive to nicotinic agonists, are insensitive to α-BGTX (Goodman & Spitzer, 1980). Also, the ACh response of DUM neurones in the cockroach metathoracic ganglion is rather insensitive to α-BGTX, indicating that the possible existence of multiple ACh receptors in insects should be explored further (Lane, Swales, David & Sattelle, 1982). However, dose-response data for a wide range of cholinergic ligands have not been obtained for acetylcholine receptors of any identified insect neurone. Furthermore, no attempt has been made to assess whether or not particular antagonists of insect ACh receptors are acting on the open or closed receptor/ion channel complex. In this study we have examined, therefore, the actions of a range of cholinergic pharmacological agents on the cell body membrane of the fast coxal depressor motoneurone (D_f) of the cockroach *Periplaneta americana*.

Preliminary pharmacological experiments on the membrane of the D_f cell body have indicated that ACh receptors sensitive to α-BGTX are present (Sattelle *et al.* 1980). This large diameter cell body in the metathoracic ganglion is located without difficulty and is sensitive to bath-applied and ionophoretically-applied ACh. It is normally electrically inexcitable, and is readily impaled by microelectrodes (Pitman, Tweedle & Cohen, 1972; Pitman, 1975). The cell is therefore well suited to detailed pharmacological investigations. In the present study all three of the probe molecul[illegible] used in ligand-binding characterizations of putative insect cholinergic receptors ar[illegible]

tested on the cell body membrane of D_f. In addition current-clamp and voltage-clamp recording techniques, in conjunction with bath application and ionophoretic application of test molecules, have been used to determine an agonist profile together with the relative potency and voltage-dependence of a wide range of cholinergic antagonists.

MATERIALS AND METHODS

SDS-gel electrophoresis

Electrophoresis of α-BGTX in the presence of sodium dodecyl sulphate (SDS) was carried out in gels of 12·5 % (w/v) total acrylamide concentration (2·6 % of this as methylene bisacrylamide) as described by Barrett, Brown & Sayers (1979), using the 2-amino-2-methyl-1,3 propanediol/glycine/chloride buffer system developed by Wyckoff, Rodbard & Schrambach (1977). Molecular weight calibration of gels was performed using standard proteins spanning a range of molecular weights (3300–94 000), including cytochrome c (11 700) and aprotinin (6500).

Pharmacological agents

α-Bungarotoxin (α-BGTX) was obtained from the Miami Serpentarium Laboratories, U.S.A. (lots, BMα8-5Z, BMα9-2 and BMα9-27) and Boehringer-Mannheim GmBh (batch no. 117304). Cobratoxin (α-COTX) was obtained from the Miami Serpentarium Laboratories, Florida, U.S.A. (lot NSα73-1) and a sample was also kindly provided by Dr R. Hider of the Chemistry Department, Essex University, U.K. Benzoquinonium chloride was the gift of Dr G. R. Daniel of Sterling-Winthrop Research and Development Co. Dihydro-β-erythroidine was provided by Merck Sharp and Dohme Ltd, and pancuronium bromide was the gift of Dr D. Savage of Organon Laboratories Ltd. Dr J. F. Donnellan of the Shell Biosciences Laboratory, Sittingbourne, U.K., generously provided a sample of quinuclidinyl benzilate. Sebacinylcholine diiodide and suberyldicholine were gifts from Professor E. V. Zeimal of the Sechenov Institute, Academy of Sciences, Leningrad, U.S.S.R.

Acetylcholine chloride (ACh), acetyl-β-methylcholine chloride, atropine sulphate, carbamylcholine chloride, *d*-tubocurarine chloride, gallamine triethiodide, hexamethonium bromide, D,L-muscarine chloride, neostigmine methyl sulphate, oxotremorine, pilocarpine HCl and succinylcholine chloride were obtained from Sigma Chemical Co., Ltd. Other cholinergic ligands used in this investigation were decamethonium bromide (Koch-Light Laboratories, Ltd), dimethyl-4-phenyl piperazinium (DMPP) (Fluka, Ltd), nicotine bitartrate (ICN Pharmaceuticals, Ltd) and tetramethylammonium chloride (TMA) (BDH Chemicals, Ltd).

Microelectrode recordings from motoneurone D_f

The cell body of the fast coxal depressor motoneurone (D_f) was visually located in isolated, desheathed, metathoracic ganglia of the cockroach *Periplaneta americana*. The desheathed ganglion was mounted under saline in a Perspex experimental chamber (total volume 2 ml) and the cell body of D_f was impaled by two microelectrodes filled with 2·0 M potassium acetate, of resistance (R) 20–25 MΩ. Changes in membrane potential and membrane resistance were recorded as described elsewhere

(David & Pitman, 1982). Current passing through the experimental chamber was monitored using a virtual-earth circuit. Acetylcholine, and nicotine were applied ionophoretically to the surface of D_f from micropipettes filled with 1·0 M solutions of these cholinergic ligands (R = 5–15 MΩ). In all cases a retaining current of 45 nA was used to prevent leakage of the drug from the ionophoretic pipette. Cells were bathed in normal saline for 60 min prior to any drug application. In the construction of dose-response curves, successive, increasing doses of ionophoretically-applied ligands were ejected at 2-min intervals to minimize desensitization (David & Pitman, 1982). A range of cholinergic agonists were bath-applied to the cell and the peak of the induced membrane depolarization was noted. To compare antagonists quantitatively, the effects of a 60-min bath-application of each drug on the dose-response relationship to ionophoretically-applied acetylcholine were examined. In voltage-clamp studies of the actions of antagonists, the clamp current was recorded directly from a Dagan 3500 voltage-clamp amplifier. The gain of the voltage-clamp was set between $4–6\times10^3$. To assess the voltage-dependence of the blocking actions of cholinergic antagonists, ACh was applied from an ionophoretic micropipette located ~30 μm from the surface of the desheathed ganglion. ACh was delivered continuously until a steady-state response was obtained. The membrane was then jumped to a series of more negative potentials. The normal saline used throughout these experiments had the following composition: 214·0 mM NaCl; 9·0 mM $CaCl_2$; 3·1 mM KCl; 10·0 mM TES (pH 7·2, adjusted with 1·0 M NaOH). A mixture of 95 % O_2 and 5 % CO_2 was employed to circulate and oxygenate the saline in the experimental chamber.

RESULTS

Actions of bath-applied cholinergic agonists

The actions of a range of pharmacological agents known from other preparations to act as cholinergic agonists were tested on the cell body membrane of the fast coxal depressor motoneurone (D_f) by bath-application, and dose-response curves are given in Fig. 1. The inset shows the depolarization induced by the application of $5·0\times10^{-7}$ M nicotine. Parallel, sigmoid, dose-response curves were obtained for nicotine, ACh (in the presence of $1·0\times10^{-7}$ M neostigmine), carbamylcholine and TMA. Hill plots for these four agonists revealed a slope of approximately unity, indicating that in each case a single ligand molecule binds to one receptor molecule in mediating the response. In addition, as can be seen in Fig. 1, the maximum depolarization (V_{max}) induced by each of these four agonists approached the value for the ACh reversal potential (−34 mV, obtained by extrapolation; David & Pitman, 1982).

By contrast, DMPP, suberyldicholine, D,L-muscarine, oxotremorine, acetyl-β-methylcholine and sebacinylcholine were highly ineffective as agonists only giving significant depolarizing effects at concentrations in the range $1·0\times10^{-3}$ M–$1·0\times10^{-2}$ M (Fig. 1). The dose-response curves constructed for these six ligands were not parallel to those obtained for the previous group tested. The absence of dose-dependent effects for these six molecules points to a non-specific site of action. The relative potency (pD_2 – cf. Ariëns, 1964) of the four most active agonists tested was

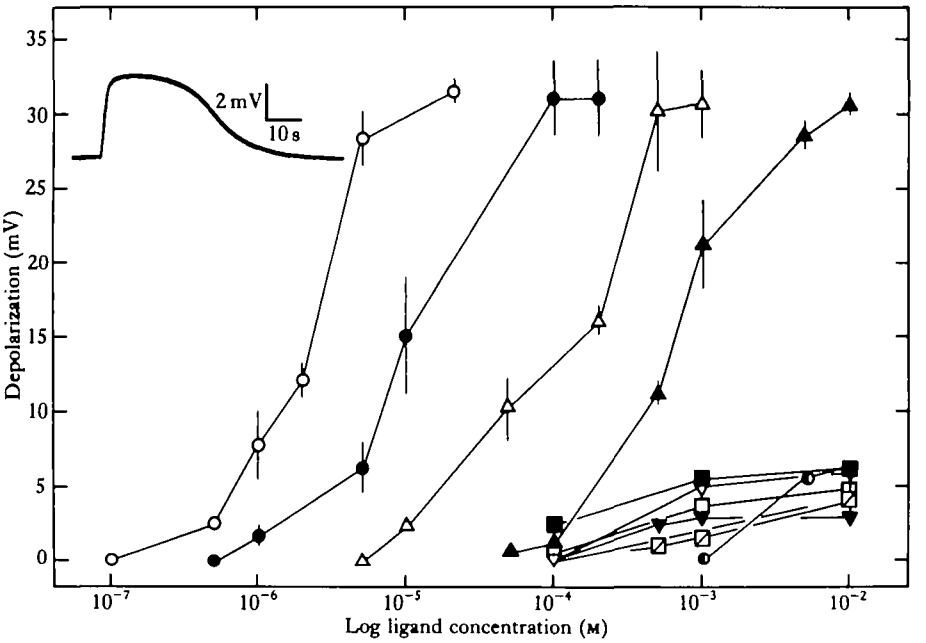

Fig. 1. Dose-response curves for a range of cholinergic agonists bath-applied to the cell body membrane of D_f. Amplitude of depolarization (mV) is plotted against the logarithm of ligand concentration. Each preparation was challenged once with a selected concentration of ligand. Data points therefore represent the mean depolarizations derived from several preparations. Standard errors are shown as vertical bars. Inset shows the depolarization of the cell body membrane in response to bath-application of $5{\cdot}0\times10^{-7}$ M nicotine. (○), Nicotine; (●), acetylcholine (in the presence of $1{\cdot}0\times10^{-7}$ M neostigmine); (△), carbamylcholine; (▲), tetramethylammonium; (■), dimethyl-4-phenyl piperazinium; (▽), suberyldicholine; (▼), muscarine; (⊠), oxotremorine; (□), acetyl-β-methylcholine; (◐), sebacinylcholine.

as follows: nicotine, $5{\cdot}22\pm0{\cdot}31$ ($N=5$); ACh (in the presence of $1{\cdot}0\times10^{-7}$ M neostigmine), $5{\cdot}00\pm0{\cdot}40$ ($N=6$); carbamylcholine, $3{\cdot}79\pm0{\cdot}38$ ($N=5$); TMA, $3{\cdot}10\pm0{\cdot}02$ ($N=5$), where N = number of cells tested.

Ionophoretic application of acetylcholine (ACh) on to the cell body membrane of D_f

Detailed consideration of the depolarizing actions of ionophoretically-applied ACh on the cell body membrane of D_f are provided elsewhere (David & Pitman, 1982). Here we have tested whether or not the ACh response that is the basis for all the pharmacological studies involving antagonists is a local, cell body response, the time-course of which is governed by the diffusion of ACh. Use is made of the observation that potassium-sensitive electrodes are also highly sensitive to ACh (Purves, 1981). By placing a potassium electrode approximately the same distance from the ionophoretic electrode as the recording electrode, the time-course of changes in extracellular ACh was followed simultaneously with membrane current changes during the ionophoretic application of ACh (Fig. 2). The potassium electrode was not responding to an ACh-induced potassium efflux since the amplitude of the response was not noticeably altered when the membrane potential was varied between −60 mV and −110 mV or when the cell response to ACh was completely blocked by $5{\cdot}0\times10^{-5}$ M

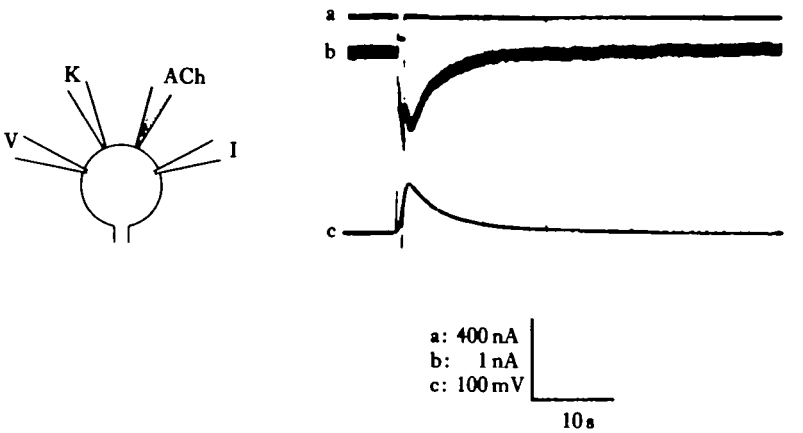

Fig. 2. Ionophoretic application of acetylcholine (ACh) on to the cell body membrane of D_f. (a) Ionophoretic current; (b) intracellular recording of the ACh-induced current under voltage-clamp; (c) simultaneous extracellular recording from a potassium-sensitive microelectrode which also detects changes in extracellular ACh (see text). A schematic representation of the arrangement of the microelectrodes is shown: V, voltage recording electrode; K, potassium/ACh-sensitive electrode; ACh, micropipette for ionophoretic application of ACh; I, current injecting electrode.

mecamylamine. The close similarity of these time-courses is a clear demonstration that the ACh response investigated in this study is purely a cell body response.

Actions of bath-applied cholinergic antagonists on the response to ionophoretically-applied acetylcholine (ACh)

The competence of a range of cholinergic antagonist molecules to modify the response of the cell body membrane of D_f to ionophoretically-applied ACh was tested. No agonist action was observed for any of these molecules.

α-Bungarotoxin (α-BGTX)

Polypeptide fractions purified from the venom of the krait *Bungarus multicinctus* include α-BGTX, which at nanomolar concentrations is an essentially irreversible antagonist at most peripheral and some central nicotinic cholinergic receptors of vertebrates (Schmidt, Hunt & Polz-Tejera, 1979). At concentrations above $1{\cdot}0\times10^{-8}$ M, α-BGTX inhibited the response of D_f to ionophoretically-applied ACh. The toxin samples used in this study each appeared as a single band on SDS gels (Fig. 3). No differences in effectiveness were noted between the various batches of toxin. The effects of $5{\cdot}0\times10^{-7}$ M α-BGTX on the dose-response curve for ionophoretically-applied ACh are illustrated in Fig. 4A. A highly effective blockade of the ACh response was seen which did not reverse even after prolonged (100 min) washing of the preparation with normal saline.

Quinuclidinyl benzilate

Quinuclidinyl benzilate, a potent antagonist at muscarinic receptors of vertebrates (Yamamura & Snyder, 1974*a*,*b*), was effective in inhibiting depolarization of the cell body membrane of D_f resulting from the ionophoretic application of ACh. Antagonist

concentrations above $1{\cdot}0\times10^{-5}$ M resulted in some degree of suppression of the response to ACh. As shown in Fig. 4B, a 60-min exposure to $1{\cdot}0\times10^{-4}$ M quinuclidinyl benzilate (ethanol 0·1 %) suppressed by almost 50 % the ACh-induced depolarization of the cell body membrane. Control experiments showed that ethanol (0·1 %) did not account for this effect. No reversibility of the blocking action was detected during prolonged washing (90 min) with normal saline.

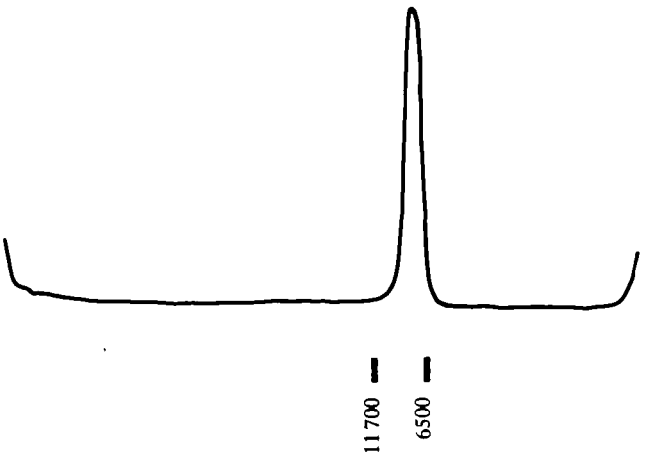

Fig. 3. SDS gel electrophoresis of α-bungarotoxin (Miami Serpentarium batch No. BMα9-27). Densitometer trace of track containing α-bungarotoxin. The positions of standard MW markers (cytochrome c M_r, 11 700 and aprotinin, M_r 6500) in an adjacent track of the same slab gel are indicated.

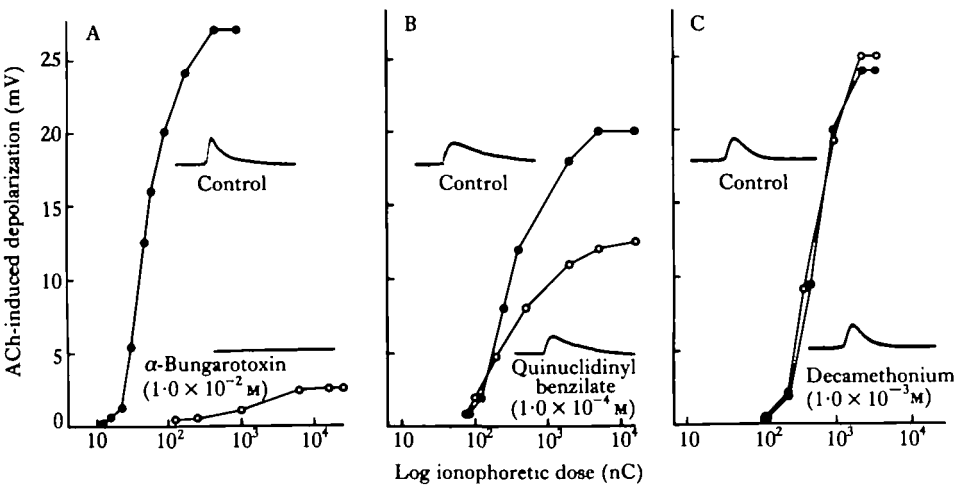

Fig. 4. Dose-response curves for ACh applied by ionophoresis to the cell body membrane of D_f in normal saline (filled circles) and in the presence of bath-applied putative receptor ligands (open circles) which have been used in radiolabelled ligand binding studies (for review see Sattelle, 1980): (A) α-bungarotoxin (α-BGTX) $5{\cdot}0\times10^{-7}$ M; (B) quinuclidinyl benzilate (QNB) $1{\cdot}0\times10^{-4}$ M; (C) decamethonium $1{\cdot}0\times10^{-3}$ M. Each drug was bath-applied for 60 min. Insets show ACh-induced depolarizations recorded before and after drug application.

Decamethonium

This bisquaternary compound induces depolarization and block at neuromuscular junctions on vertebrate skeletal muscle (del Castillo & Katz, 1955) and the antagonism is voltage-dependent (Neher & Sakmann, 1975; Adams & Sakmann, 1978). A reversible voltage-dependent block of ACh-induced depolarization has been observed in studies on the actions of decamethonium on *Aplysia* neurones (Ascher, Marty & Neild 1978). At concentrations above $1{\cdot}0\times10^{-4}$ M, a degree of inhibition of the ACh response of D_f was sometimes detected but complete block was only achieved at $1{\cdot}0\times10^{-2}$ M decamethonium. In the experiment depicted in Fig. 4C, for example, exposure to a concentration of $1{\cdot}0\times10^{-3}$ M decamethonium for 60 min failed to modify significantly the ACh dose-response curve. The effects of decamethonium were reversible at concentrations up to $1{\cdot}0\times10^{-2}$ M.

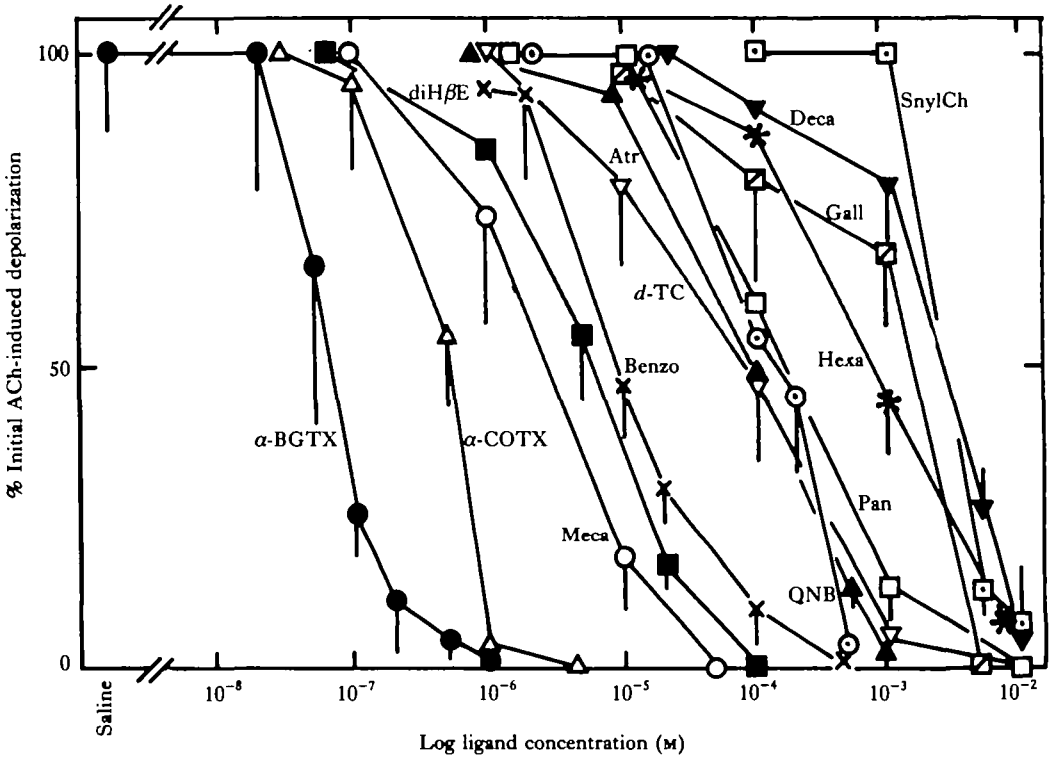

Fig. 5. The relative potency of a range of cholinergic antagonists on the response of D_f to ionophoretically-applied ACh. The percentage inhibition by the antagonist of a depolarizing response to a standard dose (d) of ACh was calculated (where d is the dose resulting in half-maximal depolarization estimated from the ACh dose-response curve determined for each cell prior to antagonist application). Each preparation was challenged for 60 min with one concentration of a particular antagonist. Data points therefore represent the means from several preparations. Vertical bars represent one standard error. Abbreviations: α-BGTX (●), α-bungarotoxin; α-COTX (△), α-cobratoxin; Meca (○), mecamylamine; diHβE (■), dihydro-β-erythroidine; Benzo (×), benzoquinonium; *d*-TC (▽) *d*-tubocurarine; Atr (▲), atropine; Pan (□), pancuronium; QNB (⊙), quinuclidinyl benzilate; Hexa (✱), hexamethonium; Gall (▨), gallamine; Deca (▼), decamethonium; SnylCh (⊡), succinylcholine.

Other antagonists

The relative potency of a range of cholinergic antagonists on the response of D_f to ionophoretically-applied ACh was assessed. Following a 60-min exposure to a particular concentration of an antagonist, the suppression by the antagonist of the peak amplitude of a depolarizing response to a standard ionophoretic dose (d) of ACh was calculated. The dose (d) resulting in half-maximal depolarization (V_{50}) in normal saline was obtained from the ACh dose-response curve determined for each cell prior to bath-application of the antagonist under test. By testing a range of antagonist concentrations in this way, dose-response curves were constructed (Fig. 5). The concentration of ligand estimated to inhibit by 50% the ACh-induced depolarization (I_{50}) was obtained from such dose-response curves. The order of effectiveness of the most potent ligands was as follows (I_{50} values shown in brackets). α-BGTX ($6{\cdot}4\times10^{-8}$ M) > α-COTX ($5{\cdot}0\times10^{-7}$ M) > mecamylamine ($2{\cdot}5\times10^{-6}$ M) > dihydro-β-erythroidine ($5{\cdot}6\times10^{-6}$ M) > benzoquinonium ($1{\cdot}0\times10^{-5}$ M). Rather less potent and almost equally effective were atropine ($1{\cdot}0\times10^{-4}$ M), *d*-tubocurarine ($8{\cdot}0\times10^{-5}$ M), pancuronium ($1{\cdot}5\times10^{-4}$ M) and quinuclidinyl benzilate ($1{\cdot}6\times10^{-4}$ M). Even less effective were hexamethonium ($8{\cdot}0\times10^{-4}$ M), gallamine ($1{\cdot}5\times10^{-3}$ M), decamethonium ($2{\cdot}8\times10^{-3}$ M) and succinylcholine ($2{\cdot}8\times10^{-3}$ M).

Effects of pretreatment with a reversible antagonist on the blocking action of α-bungarotoxin (α-BGTX)

The capacity of certain reversible antagonists to protect the ACh receptor from the irreversible antagonist α-BGTX was assessed. As shown in Fig. 6, pretreatment with $1{\cdot}0\times10^{-6}$ M α-COTX (60 min), followed by a further 60 min in which both α-COTX ($1{\cdot}0\times10^{-6}$ M) and α-BGTX ($1{\cdot}0\times10^{-6}$ M) were applied together did not

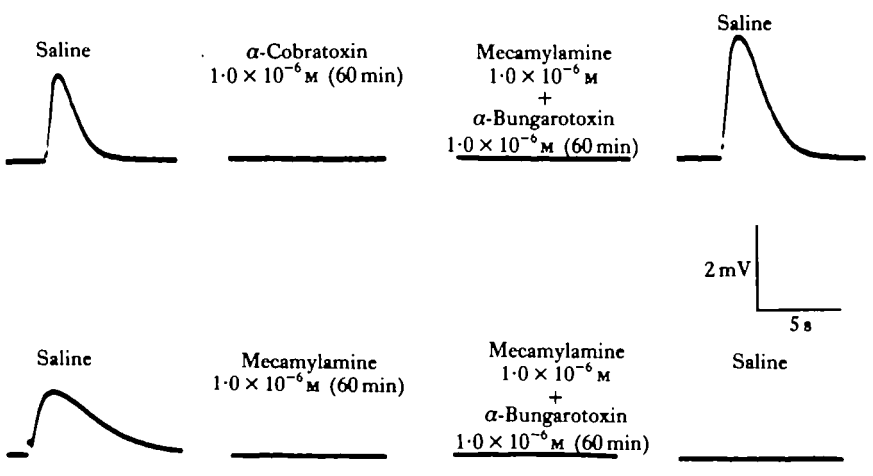

Fig. 6. The effects of pretreatment with reversible antagonists (α-cobratoxin and mecamylamine) on the ability of α-bungarotoxin to block the depolarization of the cell body membrane of D_f induced by ionophoretic application of ACh. The reversible antagonist was applied for 60 min prior to and throughout the addition of $1{\cdot}0\times10^{-6}$ M α-bungarotoxin for a further 60 min. Finally the preparation was washed in saline.

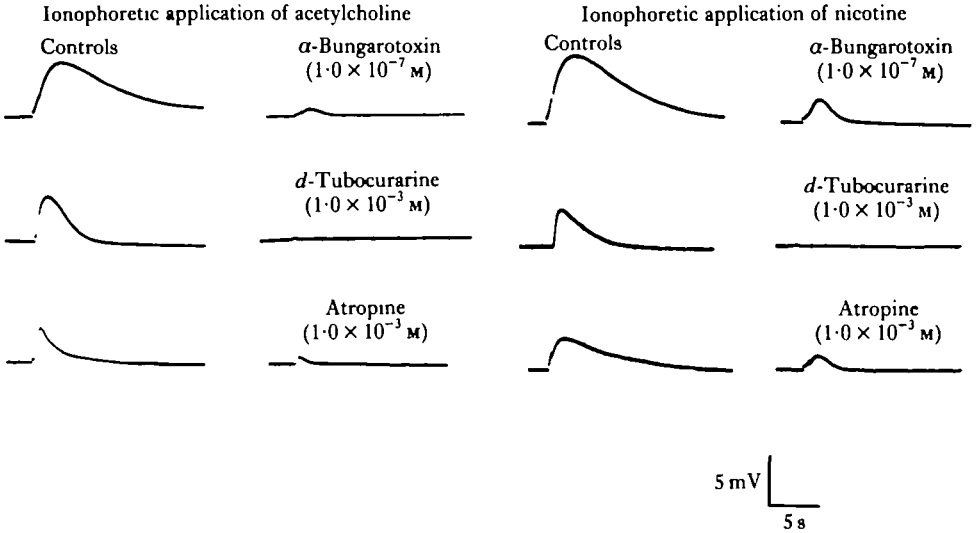

Fig. 7. Comparison of the ability of bath-applied (60 min) α-bungarotoxin, *d*-tubocurarine and atropine to block the depolarization resulting from the ionophoretic application of ACh and nicotine.

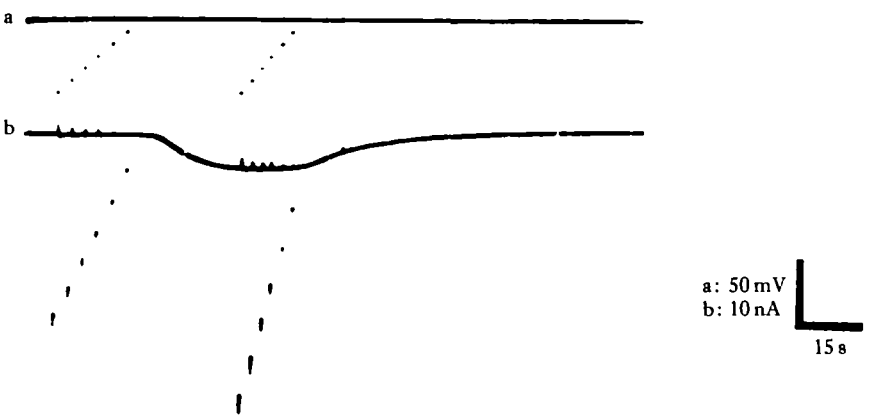

Fig. 8. Measurement of the ACh-induced current, recorded from the voltage-clamped cell body membrane of D_f, at a series of membrane potentials. Upper trace, (a), clamp potential; lower trace, (b), clamp current. The membrane potential was held at −50 mV and jumped to a series of more negative potentials (−120 mV to −70 mV in 10 mV steps) both in the absence and presence of ionophoretically-applied ACh.

lead to irreversible block of the response of D_f to ionophoretically-applied ACh. Washing the preparation in saline at the end of the experiment resulted in a full recovery of the amplitude of the ACh response. By contrast, pre-exposure to $1{\cdot}0\times10^{-6}$ M mecamylamine (60 min) followed by exposure to both mecamylamine ($1{\cdot}0\times10^{-6}$ M) and α-BGTX ($1{\cdot}0\times10^{-6}$ M) for 60 min did not lead to a recovery of the ACh response when the preparation was rebathed in saline.

Comparison of the blocking actions of cholinergic antagonists on depolarizations induced by ionophoretic applications of nicotine and ACh

The depolarization resulting from ionophoresis of nicotine on to D_f was completely

blocked by α-bungarotoxin ($1{\cdot}2\times10^{-5}$ M). Atropine and *d*-tubocurarine at $1{\cdot}0\times10^{-3}$ M significantly reduced the nicotine depolarizations. As shown in Fig. 7, α-BGTX, atropine and *d*-tubocurarine exerted comparable, blocking actions, at similar concentrations, for the depolarizing responses to both nicotine and ACh, when these agonists were ionophoretically applied to the cell body membrane of D_f.

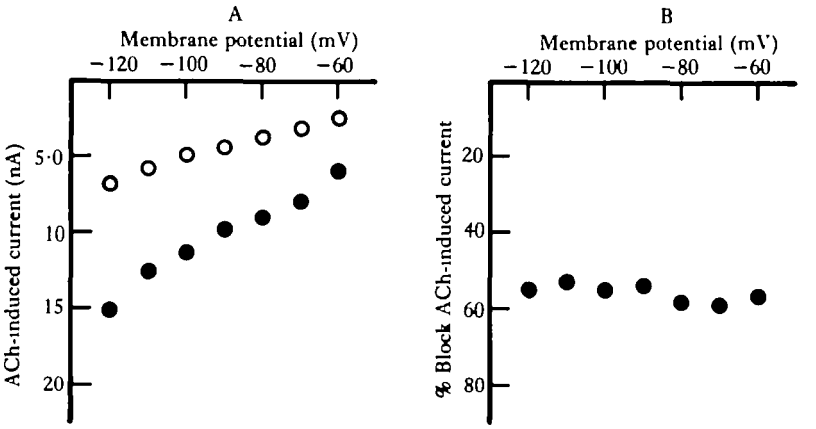

Fig. 9. The dependence on membrane potential of the block by α-bungarotoxin of the ACh-induced current. (A) The ACh-induced current as a function of membrane potential in saline (●) and in the presence of $5{\cdot}0\times10^{-7}$ M α-bungarotoxin (○). (B) The percentage block of the ACh-induced current by α-bungarotoxin at different membrane potentials.

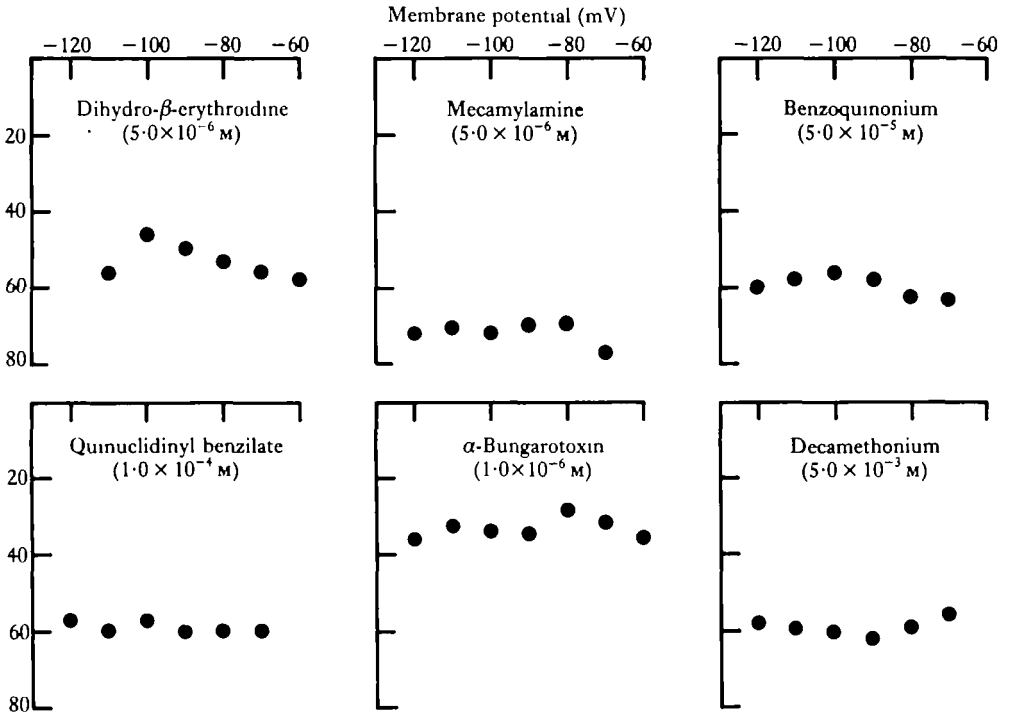

Fig. 10. The dependence on membrane potential of the block by six cholinergic antagonists of the ACh-induced current in the cell body membrane of D_f.

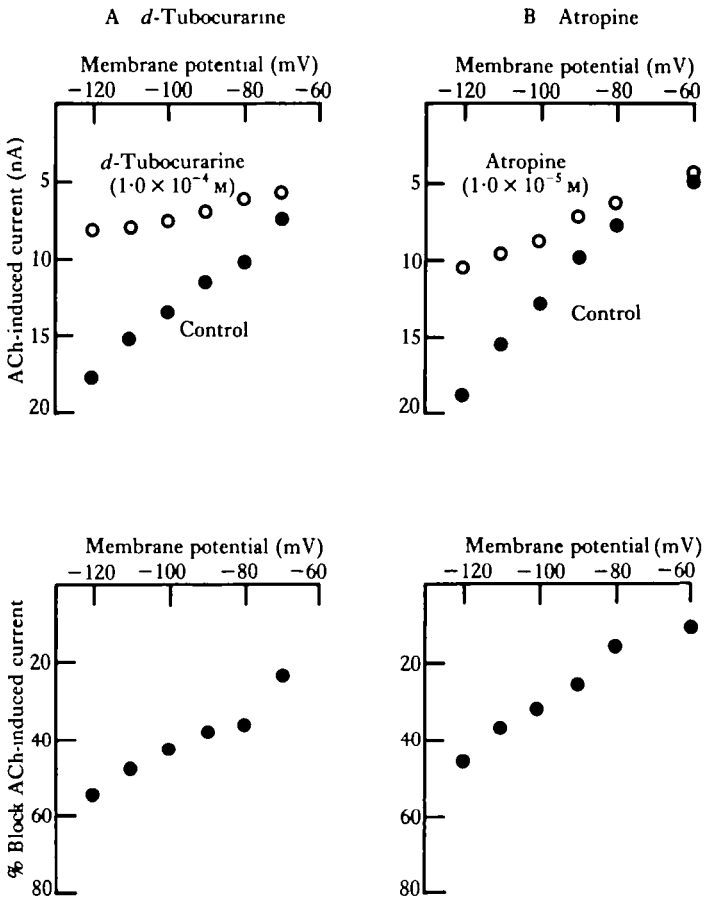

Fig. 11. The dependence on membrane potential of the blocking actions of *d*-tubocurarine ($1{\cdot}0\times10^{-4}$ M) and atropine ($1{\cdot}0\times10^{-5}$ M) on the ACh-induced current in the cell body membrane of D_f.

Membrane potential dependence of the actions of cholinergic antagonists

The membrane of the cell body of D_f was clamped at a series of different potentials between −120 mV and −60 mV. From an initial holding potential of −60 mV, the membrane was jumped to a series of successively less negative potentials (each jump lasting 700 ms), both before and during ACh application. In this way, the degree of antagonism of the ACh response at different membrane potentials was assessed (Fig. 8). The results are considered below.

Voltage-independent antagonists

A number of drugs tested revealed no dependence of their blocking action on membrane potential. For example, as shown in Fig. 9A, the peak inward ACh-induced current is plotted as a function of membrane potential in both the presence and absence of $5{\cdot}0\times10^{-7}$ M α-BGTX. From Fig. 9B, it is clear that the percentage block varies by less than 10% at all membrane potentials tested. Similar voltage-independent block of the ACh current was observed in the case of dihydro-β-erythroidine ($5{\cdot}0\times10^{-6}$ M), mecamylamine ($5{\cdot}0\times10^{-6}$ M), benzoquinonium ($5{\cdot}0\times$

10^{-5} M), quinuclidinyl benzilate ($1{\cdot}0\times10^{-4}$ M) and decamethonium ($5{\cdot}0\times10^{-3}$ M) (Fig. 10). Thus the most potent of the ACh antagonists tested in this study are voltage-independent in their blocking actions, over the membrane potential range −120 mV to −60 mV.

Voltage-dependent antagonists

d-Tubocurarine and atropine are both strongly voltage-dependent in their blockage of the ACh-induced currents in the cell membrane of D_f (Fig. 11). These results point to a possible site of action on the ion channel linked to the ACh receptor rather than with the receptor recognition site.

DISCUSSION

A range of cholinergic receptor ligands have been used to characterize the agonist and antagonist profiles of the ACh receptors of the cell body membrane of the fast coxal depressor motoneurone (D_f) of the cockroach (*Periplaneta americana*) metathoracic ganglion. The agonist profile reported here (nicotine > ACh > carbamylcholine > TMA) resembles that described for ACh receptors of unidentified dorsal midline cells (Kerkut, Pitman & Walker, 1969) and unspecified giant interneurones in the cercal afferent, giant interneurone pathway in the cockroach sixth abdominal ganglion (Callec, 1974; Sattelle, 1978). Nicotine also has potent excitatory effects on identified dorsal unpaired median neurones in the metathoracic ganglion of *Schistocerca nitens* (Goodman & Spitzer, 1979, 1980). Comparable findings have been observed in other arthropod neurones including unidentified central neurones in both the CNS of *Limulus polyphemus* (Walker & James, 1978) and the stomatogastric ganglion of *Cancer pagurus* (Marder & Paupardin-Tritsch, 1978). Similarly, nicotine, ACh and carbamylcholine all stimulate the two types of nicotinic receptors found on identified neurones of the mollusc *Aplysia californica* (Kehoe, 1972; Kehoe, Sealock & Bon, 1976). Using identified cells in another mollusc, *Helix aspersa*, two similar nicotinic receptors have also been characterized (Chad, Kerkut & Walker, 1979; Yavari, Walker & Kerkut, 1979). In Retzius neurones of the leech *Hirudo medicinalis*, nicotine also stimulates ACh receptors which depolarize certain identified cells (Woodruff, Walker & Newton, 1971; Sargent, Yan & Nicholls, 1977).

The view that the ACh receptors on the cell body membranes of D_f are predominantly of a nicotinic type is further evidenced by the ineffectiveness of the muscarinic agonists D,L-muscarine, acetyl-β-methylcholine and oxotremorine (cf. Koelle, 1975). However suberyldicholine and sebacinylcholine, which are effective nicotinic agonists on certain molluscan neurones (Vulfius, Veprintzev, Zeimal & Michelson, 1967; Ger & Zeimal, 1977) and a crustacean muscle (Marder & Paupardin-Tritsch, 1980) were ineffective on D_f. Similarly DMPP, which is an effective nicotinic agonist at vertebrate ganglionic receptors (Volle & Koelle, 1975) and *d*-tubocurarine-sensitive ACh receptors of *Aplysia* neurones (Kehoe, 1972) induced only small depolarizations of D_f at concentrations of $1{\cdot}0\times10^{-2}$ M.

The actions of a range of cholinergic antagonists have been tested for their capacity to block the depolarization of the cell body membrane of D_f induced by ionophoretic application of ACh. Nicotinic cholinergic antagonists (α-bungarotoxin,

α-cobratoxin, mecamylamine, dihydro-β-erythroidine and benzoquinonium) were particularly effective. *d*-Tubocurarine and pancuronium were much less potent, showing about the same potency as the muscarinic antagonists atropine and quinuclidinyl benzilate. Hexamethonium, gallamine, decamethonium and succinylcholine were even less effective, requiring concentrations in excess of $1{\cdot}0\times10^{-3}$ M to induce a substantial block of the ACh response. Of the three ligands used in radiolabelled ligand binding studies of putative insect central ACh receptors (cf. Sattelle, 1980), α-BGTX is by far the most potent, blocking the response to ionophoretically-applied ACh at concentrations in the nanomolar range. From the specific binding of [^{125}I]-α-BGTX to *Periplaneta* CNS extracts an apparent K_D of $1{\cdot}0\times10^{-9}$ M was estimated (Gepner, Hall & Sattelle, 1978; D. B. Sattelle, J. I. Gepner & L. M. Hall, unpublished observations). A more precise value ($0{\cdot}6\times10^{-9}$ M) was determined using association and disassociation rates of specific binding of [^{125}I]-α-BGTX to *Drosophila melanogaster* (Dudai, 1978). Thus α-BGTX is effective on D_f at concentrations close to its K_D.

The irreversible ACh blocking action of α-BGTX on D_f is not voltage-dependent, suggesting interactions with the closed state of the receptor/ion channel complex. The action of this toxin together with that of the reversibly acting α-COTX compares with their blockade of vertebrate peripheral cholinergic receptors, which is also detected at nanomolar concentrations (cf. Lee, 1972). The potent blocking action of α-BGTX on D_f contrasts with its ineffectiveness on the Na^+- and K^+-mediated ACh responses of *Aplysia* neurones (Kehoe, 1972), although the Cl^--mediated ACh response of these molluscan cells is reversibly blocked. The ACh receptors of autonomic ganglia are also insensitive to α-BGTX (Carbonetto, Fambrough & Muller, 1978; Ravdin & Berg, 1979).

Quinuclidinyl benzilate, by contrast, is only effective on D_f at concentrations (50% block of ACh response at $1{\cdot}6\times10^{-4}$ M) that depart substantially from its K_D determined in binding studies: $0{\cdot}15$–$0{\cdot}7\times10^{-9}$ M for head extracts of *Drosophila melanogaster* (Haim, Nahum & Dudai, 1979); $5{\cdot}9\times10^{-9}$ M estimated for nerve cord extracts of *Acheta domestica*; (Meyer & Edwards, 1980) and $1{\cdot}0\times10^{-9}$ M determined for cockroach CNS extracts (S. C. R. Lummis & D. B. Sattelle, unpublished observations). At a concentration of $1{\cdot}0\times10^{-5}$ M, QNB blocks the channel site of the nicotinic receptor/ion channel complex of vertebrate peripheral ACh receptors (Schofield, Warnick & Albuquerque, 1981). However, on D_f, QNB was not voltage-dependent in its blocking action, though the high concentrations required make it unlikely that this represents a specific action at the receptor recognition site of a muscarinic type of receptor. Finally, decamethonium only blocked the ACh-induced response at $1{\cdot}0\times10^{-2}$ M, a concentration several orders of magnitude greater than the K_D values reported for [^{3}H]-decamethonium binding to *Musca domestica* head extracts: $5{\cdot}2\times10^{-7}$ M; $6{\cdot}7\times10^{-6}$ M (Cattell & Donellan, 1972; Donnellan, Jewess & Cattell, 1975). Decamethonium, though capable of inducing voltage-dependent block of ACh receptors at the frog neuromuscular junction (Neher & Sakmann, 1975) and *Aplysia* neurones (Ascher *et al.* 1978) was voltage-independent in its blocking action on D_f which also required extremely high doses ($>1{\cdot}0\times10^{-3}$ M). No agonist action of decamethonium was detected on D_f in contrast to the findings on vertebrate neuromuscular junctions (Adams & Sakmann, 1978). Thus of the three receptor

probes tested on D_f, α-BGTX is by far the most effective and the only one of the three to exert its blocking action at concentrations close to its K_D value determined in binding studies on insect CNS tissues.

Of the other antagonists examined, the vertebrate nicotinic antagonist dihydro-β-erythroidine is particularly effective and its blocking action does not depend on membrane potential. Mecamylamine, a ganglionic blocker (Volle & Koelle, 1975), is also voltage-independent in its actions on parasympathetic neurones (Ascher, Large & Rang, 1979) and crustacean muscle (Marder & Paupardin-Tritsch, 1980). Benzoquinonium, a nicotinic neuromuscular blocking drug with anticholinesterase properties (Volle & Koelle, 1975), appeared insensitive to membrane potential in the degree of suppression of the ACh current induced. All the antagonist actions so far discussed could be accounted for in terms of an interaction with the closed receptor/ion channel.

However, not all antagonists tested were voltage-independent. Both *d*-tubocurarine and atropine were strongly voltage-dependent in their blocking actions, and therefore probably interact with the open receptor/ion channel complex. *d*-Tubocurarine also induces a voltage-dependent reduction of the ACh response of *Aplysia* neurones (Marty, Neild & Ascher, 1976) and frog neuromuscular junctions (Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979). As in the case of D_f, atropine also appears to block the ion channel at vertebrate peripheral nicotinic receptors (Feltz, Large & Trautmann, 1977), and extrasynaptic ACh receptors in *Aplysia* (Ascher *et al.* 1978; Slater & Carpenter, 1982).

The ACh receptors on the cell body membranes of D_f show many similarities with the pharmacological profile reported from binding studies on several insect species using [^{125}I]-α-bungarotoxin (cf. Sattelle, 1980). There are one or two notable exceptions however. For example, *d*-tubocurarine is much less effective on D_f than in both binding studies and in blocking cercal afferent, giant interneurone synaptic transmission (Sattelle *et al.* 1983). This may reflect differences in sensitivity between synaptic and extrasynaptic ACh receptors, but a comparison of the actions of *d*-tubocurarine on these two populations of receptors of an identified neurone is needed to clarify this apparent discrepancy. Also as shown in the present study mecamylamine acts in a voltage-independent manner but at a different site to α-bungarotoxin, so that its effectiveness in binding studies may be a poor indicator of its actions on the receptor/ion channel complex. Differences have emerged between the pharmacological properties of cholinergic receptors on D_f and those reported for sympathetic neurones (Carbonetto *et al.* 1978; Kouvelas, Dichter & Greene, 1978), parasympathetic neurones (Ascher *et al.* 1979) and some central synapses (Miledi & Szczepaniak, 1975) in respect of sensitivity to α-BGTX and the mechanism of action of hexamethonium. Nevertheless, several similarities have emerged between the insect CNS toxin-sensitive receptor and the vertebrate CNS toxin-sensitive receptor (Freeman, Schmidt & Oswald, 1980; Oswald & Freeman, 1979; Barnard *et al.* 1979).

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