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NEREISTOXIN: ACTIONS ON A CNS ACETYLCHOLINE RECEPTOR/ION CHANNEL IN THE COCKROACH *PERIPLANETA AMERICANA*

BY D. B. SATTELLE, I. D. HARROW*, J. A. DAVID, M. PELHATE†, J. J. CALLEC‡

A.F.R.C. Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge CB2 3EJ, U.K.

J. I. GEPNER

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

AND L. M. HALL

Department of Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, NY 10461, U.S.A.

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SUMMARY

Nereistoxin hydrogen oxalate (NTX), at low concentrations (in the range $2{\cdot}0\times10^{-8}$–$1{\cdot}0\times10^{-6}$ mol l^{-1}), induced a dose-dependent partial block of transmission at cercal afferent, giant interneurone synapses in the terminal abdominal ganglion (A6) of the cockroach *Periplaneta americana* which was not accompanied by changes in either membrane potential or input resistance of the postsynaptic membrane. At a concentration of $1{\cdot}0\times10^{-7}$ mol l^{-1}, NTX suppressed, in a voltage-dependent manner, acetylcholine-induced currents recorded from voltage-clamped cell bodies of both giant interneurone 2 (GI 2) in A6, and the fast coxal depressor motoneurone of the metathoracic ganglion (T3). At higher concentrations (in the range $1{\cdot}0\times10^{-5}$–$1{\cdot}0\times10^{-3}$ mol l^{-1}) depolarization of the postsynaptic membrane was observed. Axonal depolarization was noted at concentrations above $1{\cdot}0\times10^{-4}$ mol l^{-1}. Voltage-clamp experiments showed that the axonal actions of NTX included suppression of sodium and potassium currents and an increase in the membrane leak current. The concentrations of NTX (in the range $1{\cdot}0\times10^{-5}$–$1{\cdot}0\times10^{-3}$ mol l^{-1}) which show the postsynaptic depolarizing effect are in the same range as the NTX concentrations ($1{\cdot}7\times10^{-4}$ and $6{\cdot}6\times10^{-5}$ mol l^{-1}) required for 50% inhibition of the binding of ^{125}I-α-bungarotoxin to

*Present address: Animal Health Biology Department, Pfizer Central Research, Sandwich, Kent, CT13 9NJ. U.K.

†Present address: Faculté de Médecine, Université d'Angers, rue Haute de Reculée, Angers, 49045, France.

‡Present address: U.E.R. Sciences Biologiques, Université de Rennes, Rennes, France.

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Periplaneta abdominal nerve cord extracts and *Drosophila* head extracts, respectively. Thus a potent, voltage-dependent, blocking action of NTX is detected at the CNS acetylcholine receptor/ion channel complex of the cockroach. This, possibly together with the synaptic and axonal depolarizing effects noted at much higher concentrations, may contribute to the mechanism of action of this natural invertebrate neurotoxin which is also the active form of the synthetic insecticide Cartap.

INTRODUCTION

Nereistoxin (NTX), a naturally occurring substance which is active at cholinergic synapses (cf. Narahashi, 1973), was first isolated from the marine annelid *Lumbriconereis heteropoda* by Nitta (1934). Okaichi & Hashimoto (1962*a*,*b*) synthesized the toxin and determined its chemical structure as 4-*N*,*N*-dimethylamino-1,2-dithiolane (Fig. 1). Much of the interest in the mechanism of action of NTX stems from the high toxicity to insects of the natural toxin (Sakai, 1964, 1966*a*) and synthesized analogues such as the commercial insecticide 'Cartap' (the active form of which is NTX, Sakai & Sato, 1971). Nevertheless, little is known of the mechanism of action of NTX on insect excitable tissues. The present study, therefore, examines the effects of NTX on identified neurones in the central nervous system of the cockroach *Periplaneta americana*.

Three distinct synaptic actions of NTX were reported in the earlier studies on a variety of vertebrate preparations. First, a *d*-tubocurarine-like antagonism of nicotinic cholinergic receptors was demonstrated (Nitta, 1941; Sakai, 1966*b*; Deguchi, Narahashi & Haas, 1971). Secondly, the frequency of miniature end-plate potentials (MEPPs) recorded from frog muscle decreased in the presence of NTX (Deguchi *et al.* 1971), indicating an additional inhibitory effect of NTX on the mechanism of acetylcholine release. Finally, a number of stimulatory effects of NTX were reported in several mammalian tissues containing muscarinic cholinergic receptors (Nitta, 1941). Recently, Eldefrawi *et al.* (1980) have applied a multidisciplinary approach to the study of synaptic actions of NTX on vertebrate muscle and *Torpedo* electroplax tissues. They could find no evidence for a presynaptic effect of NTX on acetylcholine release. In the same investigation, electrophysiological experiments on the neuromuscular junctions of frog (sartorius) and rat (diaphragm) muscle were performed in parallel with receptor binding studies using ^{125}I-α-bungarotoxin, and binding to the ion channel using [^{3}H]perhydrohistrionicotoxin.

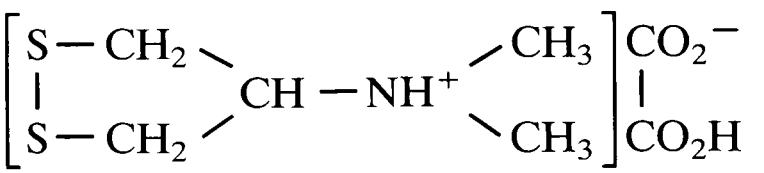

Fig. 1. The chemical structure of nereistoxin hydrogen oxalate.

NTX failed to inhibit binding of [^{3}H]perhydrohistrionicotoxin and did not modify either the linearity of the current voltage relationships, or the time-course of the end-plate current. It did, however, inhibit the binding of ^{125}I-α-bungarotoxin to *Torpedo* membranes and inhibited both postsynaptic and extrajunctional acetylcholine sensitivity of muscle. It was concluded that NTX inhibition of vertebrate neuromuscular transmission was due to an action at the receptor rather than the ion channel of the acetylcholine receptor/ion channel complex.

The site of action of NTX in insects is not clear. They are rapidly paralysed by NTX but subsequently revive from the poisoning. Using extracellular, hook-electrode recording techniques, Sakai (1967, 1970) showed that concentrations above $2{\cdot}0 \times 10^{-6}$ mol l^{-1} NTX suppressed postsynaptic discharges at cockroach cercal-afferent, giant-interneurone synapses. Microelectrode recordings from giant axons in the same preparation revealed that $1{\cdot}0 \times 10^{-5}$ mol l^{-1} Cartap blocked synaptic transmission and depolarized the postsynaptic membrane (Bettini, D'Ajello & Maroli, 1973). By contrast, neuromuscular transmission in the femur of the hindleg of *Periplaneta* was not affected by $2{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX (Sakai, 1970).

Although the excitatory neurotransmitter at the cercal afferent, giant interneurone synapses has not been established, acetylcholine is the strongest candidate (Pitman, 1971; Callec, 1974; Sattelle, 1980). In support of this suggestion are experiments which show that of all molecules tested, α-bungarotoxin and other nicotinic cholinergic ligands are the most effective on excitatory postsynaptic potentials and acetylcholine-induced depolarizations of giant interneurones (Shankland, Rose, & Donniger, 1971; Callec, 1974; Sattelle, 1978; Sattelle, David, Harrow & Hue, 1980; Sattelle *et al.* 1983; Harrow, Hue, Pelhate & Sattelle, 1979; Harrow, David & Sattelle, 1982). An α-bungarotoxin-binding component exhibiting a similar pharmacological profile to that of postsynaptic receptors at cercal afferent, giant interneurone synapses has been characterized in extracts of abdominal ganglia of the same insect (Gepner, Hall & Sattelle, 1978). Interpretation of the action of NTX on insects would be facilitated by the use of experimental material such as the CNS of the cockroach *Periplaneta americana* that is well suited to the application of both electrophysiological and radiolabelled ligand-binding techniques.

In the present investigation electrophysiological methods are used to quantify the actions of NTX on synaptic, cell body and axonal membranes of GI 2 in the sixth abdominal ganglion of *Periplaneta americana*. In addition, the capacity of NTX to inhibit ^{125}I-α-bungarotoxin binding to abdominal nerve cord extracts of the same species is tested. Two separate ways in which NTX interacts with an insect acetylcholine receptor/ion channel complex are detected. A brief account of some of these findings has appeared in Conference proceedings (Sattelle & Callec, 1977; Sattelle, 1981).

MATERIALS AND METHODS

Adult male cockroaches (*Periplaneta americana*) were used in all physiological experiments and for binding experiments. *Drosophila melanogaster* extracts were

prepared from heads of Canton-S wild-type flies. Nereistoxin hydrogen oxalate (MW 239), from Takeda Chemical Industries Ltd, was used in all the present investigations and was bath-applied in saline in all physiological experiments.

Voltage-clamp and current-clamp of isolated giant axons

The actions of NTX on the isolated axonal membrane of giant interneurone 2 (GI 2) were studied using the oil-gap, single-fibre method. Details of the axon dissection, experimental chamber and the electrical circuitry for current-clamp and voltage-clamp have been reported elsewhere (Pelhate & Sattelle, 1982). Electrical responses of the axon were monitored on a storage oscilloscope and simultaneously stored using a Histomat S multichannel analyser which enabled display of the ionic currents after correction for leakage and capacity currents by the method of Hille & Campbell (1976). The physiological saline used for the axon was as follows (in $mmol\,l^{-1}$): NaCl, 200·0; KCl, 3·1; $CaCl_2$, 5·4; $MgCl_2$, 5·0; $NaHCO_3$, 2·0; Na_2HPO_4, 0·1 (pH 7·2). Most experiments were performed at 12 °C to prolong the viability of the isolated axon, although a number of experiments were also performed at 22–24 °C.

Electrophysiology of cercal afferent, giant interneurone synapses

The actions of NTX on transmission at the cercal afferent, giant interneurone synapses were monitored using an oil-gap, single-fibre synaptic recording technique (Pichon & Callec, 1970) and a sucrose-gap method (Callec & Sattelle, 1973). A preparation comprising the cerci, the cercal nerves, the terminal (sixth) abdominal ganglion and the remainder of the abdominal nerve cord was first excised from the animal, and the sixth ganglion was desheathed to facilitate the access of perfusing fluids to the synapses. Preparations of this type were used for sucrose-gap experiments. For single-fibre experiments, one giant axon (GI 2) was dissected from one of the connectives linking the fifth and sixth abdominal ganglia and the other connective was cut. Electrical and mechanical stimulation of several sensory neurones and the identification of GI 2 were performed as described previously (Sattelle *et al.* 1983). The experimental chamber for recording changes in membrane potential, input resistance and excitatory postsynaptic potentials, together with the electrical circuitry have been described elsewhere (Pichon & Callec, 1970; Callec, 1972). The ionic composition of the physiological saline used for experiments on synaptic transmission was as follows (in $mmol\,l^{-1}$): NaCl, 208·6; KCl, 3·1; $CaCl_2$, 5·4; $NaHCO_3$, 2·0; Na_2HPO_4, 0·1 (pH 7·2). All experiments were performed at 21 °C.

Electrophysiology of cell body membranes

The response to ionophoretically-applied acetylcholine of the cell body membrane of GI 2 in the sixth abdominal ganglion and D_f (the fast coxal depressor motoneurone) in the metathoracic ganglion were recorded as described earlier (Sattelle *et al.* 1980; David & Pitman, 1982; Harrow & Sattelle, 1983). The actions

of NTX on acetylcholine-induced current were monitored using voltage-clamp techniques described elsewhere (David & Sattelle, 1984).

Binding of ^{125}I-α-bungarotoxin to CNS particulate extracts

Membrane extracts, containing the particulate form of a putative nicotinic cholinergic receptor, were prepared by differential centrifugation as described elsewhere for *Drosophila melanogaster* (heads) (Schmidt-Nielsen, Gepner, Teng & Hall, 1977) and *Periplaneta americana* (abdominal nerve cords) (Gepner *et al.* 1978). Aliquots of the extract [equivalent to 5 mg heads (*Drosophila*) starting material, or 20 mg wet weight *Periplaneta* abdominal nerve cords] were preincubated for 30 min at 21 °C in Krebs original Ringer phosphate buffer pH 7·4 (Dawson, Elliott, Elliott & Jones, 1969) containing 1·7 times the final concentration of NTX in a total volume of 70 μl. Then 50 μl of 4 nmol l^{-1} ^{125}I-α-bungarotoxin (specific activity 27 Ci $mmol^{-1}$) in Krebs-Ringer buffer containing 0·1 % bovine serum albumin was added and the mixtures were incubated for an additional 30 min. The mixtures were then centrifuged for 30 min at 30 000 × ***g*** and the amount of ^{125}I-α-bungarotoxin bound was determined by a centrifugal assay. The centrifugal assay and preparation of ^{125}I-α-bungarotoxin have been described by Schmidt-Nielsen *et al.* (1977).

RESULTS

Axonal resting potential, input resistance and delayed rectification

At concentrations of $1{\cdot}0 \times 10^{-4}$ mol l^{-1} and below, no effects of bath-applied NTX were detected on the resting potential of the isolated axon. At higher concentrations, a dose-dependent depolarization of the axonal membrane was observed (Fig. 2A). The depolarization induced by $2{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX was accompanied by a drop in input resistance as shown by the application of hyperpolarizing current pulses (Fig. 3). The membrane depolarizations induced by the application of depolarizing current pulses were increased in the presence of NTX ($2{\cdot}0 \times 10^{-4}$ mol l^{-1}), a possible explanation for which is a change in the delayed rectification of the axon. NTX at $5{\cdot}0 \times 10^{-3}$ mol l^{-1} depolarized the axon by 15–25 mV. Similar NTX-induced depolarizations were observed in the presence of tetrodotoxin (TTX) at $5{\cdot}0 \times 10^{-7}$ mol l^{-1}, a concentration which completely blocks axonal sodium channels of cockroach giant axons (Sattelle, Pelhate & Hue, 1979).

Axonal action potentials

Action potentials of normal amplitude (90–100 mV) were observed under current-clamp conditions even after a 30-min exposure to $1{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX. In the concentration range $2{\cdot}0 \times 10^{-4}$–$5{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX (six experiments), a slowing down of the repolarization phase was noted (Fig. 4). At these concentrations NTX did not suppress action potentials even after a 30-min application. In

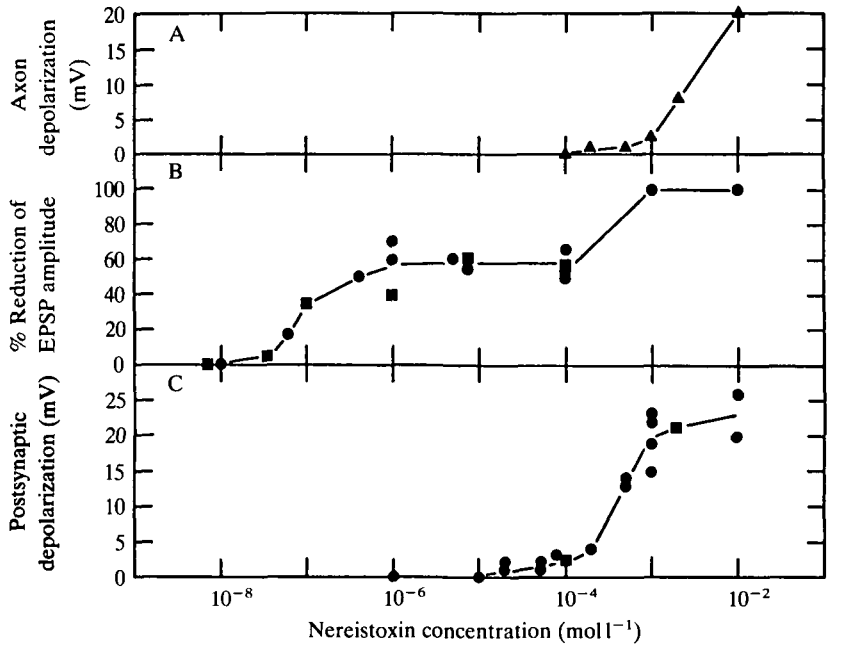

Fig. 2. Dose-response curves for the actions of nereistoxin (NTX) on potentials recorded from axonal and synaptic membranes in the cockroach abdominal nerve cord. All data are obtained 15–20 min after commencing bath-application of NTX to the preparation under test. (A) Axonal membrane potential changes recorded from the isolated axon of giant interneurone 2 (GI 2) using the oil-gap, single-fibre technique (▲). The axon was isolated from a connective linking the fourth and fifth abdominal ganglia. (B) Excitatory postsynaptic potential (EPSP) amplitude, plotted as percentage reduction of the EPSP amplitude recorded in normal saline. Data from sucrose gap (●) and oil-gap, single-fibre (■) experiments on GI 2, are shown. (C) Postsynaptic membrane potential changes. Data from sucrose-gap (●) and oil-gap, single-fibre (■) experiments are shown.

fact after a 30-min exposure at 22–24 °C to $2{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX, a repetitive firing of action potentials was detected in response to long-lasting depolarizing current pulses. In contrast, at higher NTX concentrations of $1{\cdot}0 \times 10^{-3}$ mol l^{-1}, it was usually impossible to elicit action potentials from fibres after 15 min of perfusion.

Axonal currents

Using voltage-clamped axons, NTX in the concentration range $1{\cdot}0 \times 10^{-6}$–$1{\cdot}0 \times 10^{-4}$ mol l^{-1} had no effect on membrane currents after 30 min of bath application (five experiments). However, at $2{\cdot}0 \times 10^{-4}$ mol l^{-1} and $5{\cdot}0 \times 10^{-4}$ mol l^{-1} NTX, all the currents were slowly and irreversibly modified. Both the steady-state outward (potassium) current (I_K) and the peak inward (sodium) current (I_{Na}) were reduced, whereas the leak current (I_L) was increased. The actions of NTX ($1{\cdot}0 \times 10^{-3}$ mol l^{-1}) on axonal currents are shown in Fig. 5.

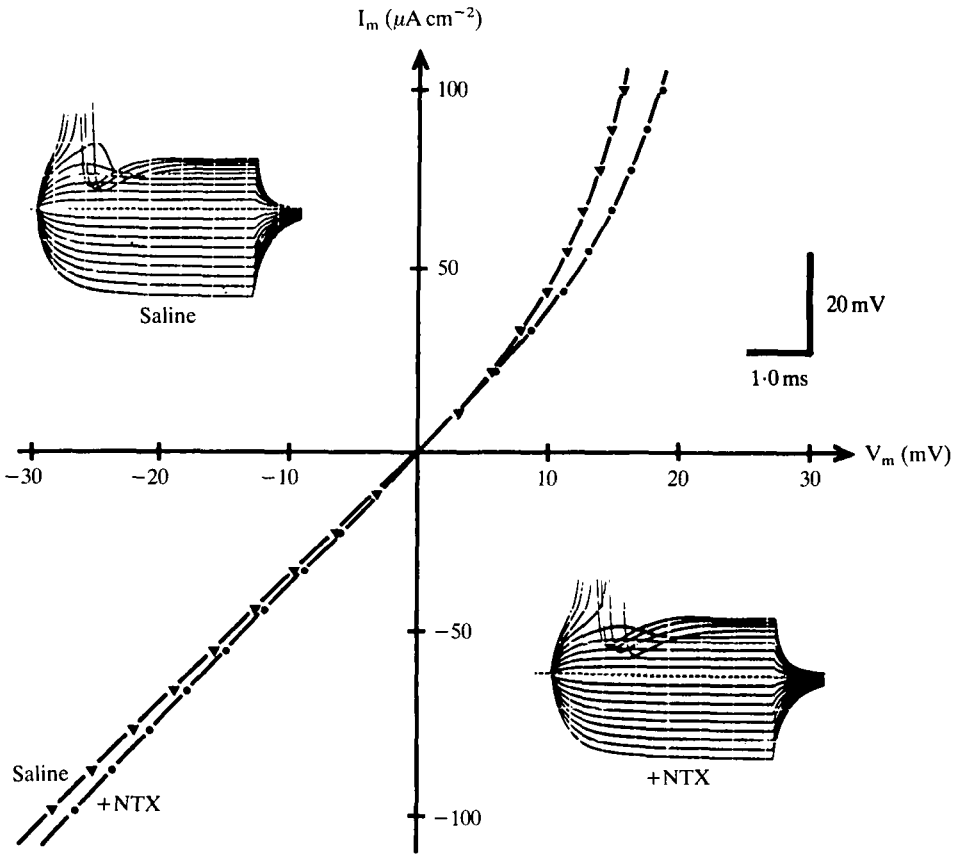

Fig. 3. Results of an oil-gap, single-fibre experiment showing the current-voltage relationships for a current-clamped cockroach axon bathed in normal saline (▼), and following a 15-min exposure to saline containing $2{\cdot}0 \times 10^{-4}$ mol l^{-1} nereistoxin (NTX) (●). Insets show voltage changes, in response to a range of current pulses. I_m, membrane current; V_m, membrane potential. Membrane current amplitudes were measured during the steady (plateau) phase. A 6 % decrease in input resistance was noted and the delayed rectification was reduced by 17 %. Between the record obtained in saline and the record obtained in the presence of NTX the axonal membrane depolarized by 0·5 mV. The duration of the applied current pulse was 3·5 ms.

Synaptic transmission at cercal afferent, giant interneurone synapses

Sucrose-gap experiments

Compound EPSPs were recorded from cercal afferent, giant interneurone synapses by the sucrose-gap method, following electrical stimulation of cercal nerve X1. Following a 15- to 20-min exposure to NTX at $3{\cdot}0 \times 10^{-8}$ mol l^{-1} and higher concentrations, compound EPSPs were reduced in amplitude (Fig. 2B). A reduction of the EPSP amplitude to approximately 40 % of its normal value in physiological saline (3–8 mV) was achieved at $1{\cdot}0 \times 10^{-6}$ mol l^{-1} NTX. Increasing the toxin concentration to $1{\cdot}0 \times 10^{-4}$ mol l^{-1} produced no further reduction of EPSP amplitude (Fig. 2B). The EPSP attenuation was observed after 15 min

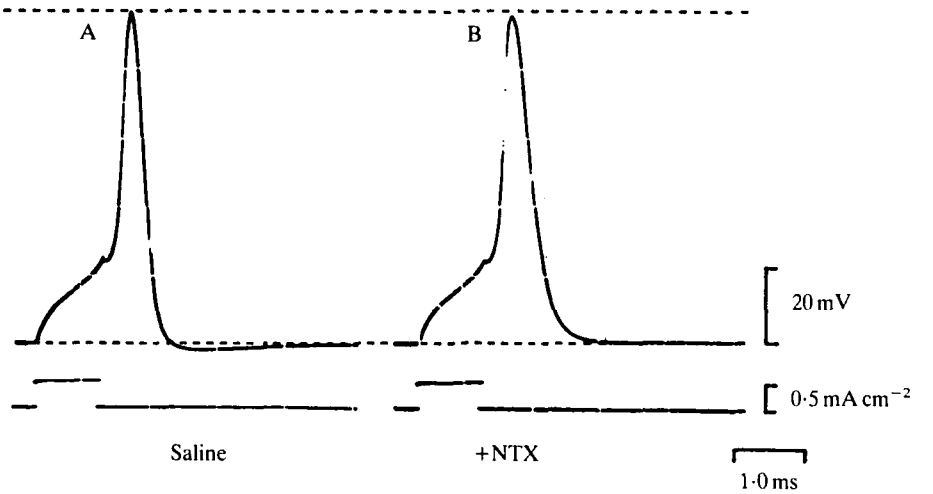

Fig. 4. Action potentials recorded from a cockroach axon (GI 2) (A) in normal saline and (B) after a 30-min exposure to saline containing $2{\cdot}0 \times 10^{-4}$ mol l^{-1} nereistoxin (NTX). The duration of the action potential was increased following NTX treatment. The action potential undershoot was suppressed by NTX.

exposure to $1{\cdot}0 \times 10^{-6}$ mol l^{-1} NTX and was not further modified by continued perfusion of NTX for another 15 min. This rapid, partial suppression of EPSP amplitude was not accompanied by changes in either input resistance or membrane potential. At concentrations of NTX above $2{\cdot}0 \times 10^{-5}$ mol l^{-1}, depolarization of the postsynaptic membrane was observed (Fig. 2C). Using 15 min exposure to saline containing NTX, maximum depolarization was achieved at concentrations in the range $1{\cdot}0 \times 10^{-3}$ to $1{\cdot}0 \times 10^{-2}$ mol l^{-1} NTX. Depolarization of the postsynaptic membrane was accompanied by a block of the EPSP and a reduction in input resistance.

Oil-gap, single-fibre experiments

The oil-gap, single-fibre technique was used to test the effects of NTX on EPSPs recorded from GI 2 following mechanical stimulation of a single cercal mechanoreceptor, and corresponding in amplitude to the unitary EPSPs previously recorded from this pathway (Callec, 1974). The amplitudes of such EPSPs were in all cases ($N=18$) progressively reduced to about 30–40 % of the value in normal saline by NTX at concentrations in the range $1{\cdot}0 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-4}$ mol l^{-1}. As shown in Fig. 6, the suppression by $2{\cdot}0 \times 10^{-5}$ mol l^{-1} NTX of the EPSP resulting from the stimulation of a single cercal mechanoreceptor was only partially reversed by bathing in normal saline. Compound EPSPs, recorded by the oil-gap, single-fibre technique from GI 2 in response to electrical stimulation of many cercal afferents, were partially blocked by NTX at concentrations similar to those reported in sucrose-gap experiments where the response is derived from many postsynaptic fibres (see Fig. 2B). The residual component of the EPSP that was not blocked by NTX was rapidly blocked by *d*-tubocurarine or atropine ($1{\cdot}0 \times 10^{-4}$ mol l^{-1}).

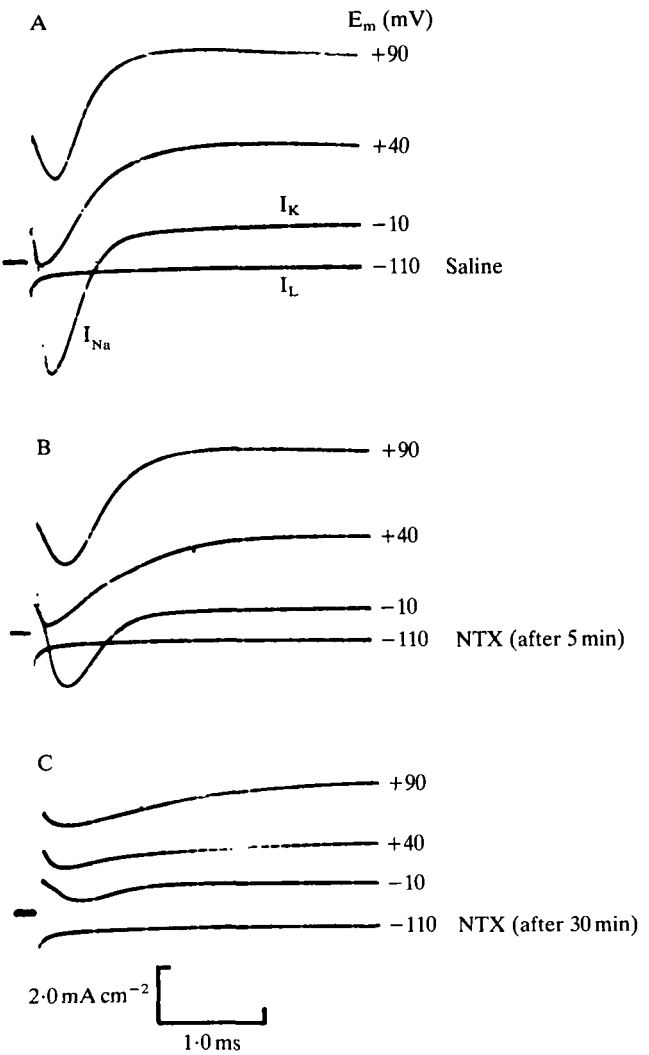

Fig. 5. Actions of $1{\cdot}0 \times 10^{-3}$ mol l^{-1} nereistoxin (NTX) on ionic currents recorded from a cockroach giant axon (GI 2). Voltage-clamp recordings of axonal membrane currents corresponding to square voltage steps from the holding potential ($E_h = -60$ mV) to the potentials (E_m) indicated on each trace. (A) Normal saline; (B) and (C) saline with the addition of $1{\cdot}0 \times 10^{-3}$ mol l^{-1} NTX for 5 min and 30 min, respectively. NTX suppressed both the peak inward (sodium) current (I_{Na}) and the steady-state, outward (potassium) current (I_K), which have been characterized in detail in earlier pharmacological studies (Pelhate & Sattelle, 1982). The leak current (I_L) increased in the presence of NTX.

Cell body acetylcholine-induced responses

The actions of NTX on the cell body membrane of GI 2 and the fast coxal depressor motoneurone (D_f) were examined. In voltage-clamp experiments on the cell body of D_f, currents resulting from ionophoretic application of acetylcholine were recorded at membrane potentials (E_m) in the range −120 mV to −60 mV, in normal saline, and following a 25-min exposure to $1{\cdot}0 \times 10^{-7}$ mol l^{-1} NTX

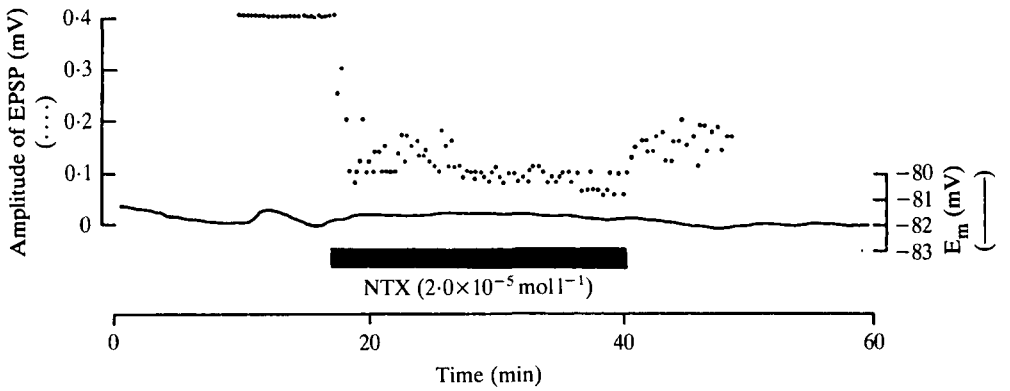

Fig. 6. Actions of $2{\cdot}0\times10^{-5}$ mol l^{-1} nereistoxin (NTX) on the amplitude of the EPSP recorded from giant interneurone 2 (GI 2) in response to mechanical stimulation of a single cercal mechanoreceptor. Saline containing NTX was bath-applied during the period indicated by the horizontal bar. No significant modification of the resting potential (E_m) of GI 2 was noted during the partial blocking action of NTX.

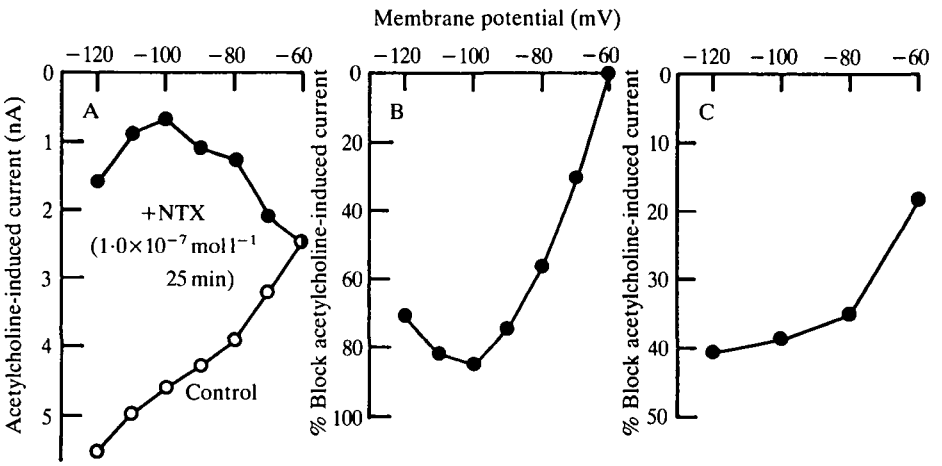

Fig. 7. Voltage-dependence of the block by nereistoxin (NTX) of the acetylcholine-induced current in the cell body membranes of giant interneurones 2 (GI 2) and of the fast coxal depressor motoneurone (D_f). (A) Acetylcholine-induced currents recorded from D_f as a function of membrane potential in normal saline (●) and following the addition of $1{\cdot}0\times10^{-7}$ mol l^{-1} NTX (○). (B) Using the data from A, the percentage block of the acetylcholine-induced current is plotted as a function of membrane potential. (C) Data from GI 2 showing the voltage-dependence of the percentage block of the acetylcholine-induced current by $1{\cdot}0\times10^{-6}$ mol l^{-1} NTX.

(Fig. 7A). The degree of inhibition of the acetylcholine-induced inward current increased with membrane hyperpolarization up to −100 mV and thereafter (−110 mV and −120 mV) declined somewhat, indicating a voltage-dependence of the action of NTX (Fig. 7B) with a maximum block at −100 mV. The threshold concentration for an action of the toxin on the cell body membrane of D_f was in the range $1{\cdot}0\times10^{-7}$ to $1{\cdot}0\times10^{-6}$ mol l^{-1}.

The cell body membrane of GI 2 exhibited an increased percentage block of

acetylcholine-induced current with membrane hyperpolarization over the potential range (−120 mV to −60 mV) when exposed to $1{\cdot}0\times10^{-6}$ mol l^{-1} NTX. (Fig. 7C). Both GI 2 (Harrow & Sattelle, 1983) and D_f (Sattelle *et al.* 1980; David & Sattelle, 1984) possess α-bungarotoxin-sensitive nicotinic acetylcholine receptors on their cell body membranes. For both cells, inhibition of the acetylcholine-induced current by NTX is voltage-dependent in the range −120 mV to −60 mV. At concentrations of $1{\cdot}0\times10^{-4}$ mol l^{-1} NTX and higher, the cell body membranes of D_f and GI 2 were depolarized.

Inhibition of ^{125}I-α-*bungarotoxin binding*

The inhibition by NTX of ^{125}I-α-bungarotoxin binding to extracts from *Drosophila* (heads) and *Periplaneta* (abdominal nerve cords) was investigated (Fig. 8). Each point on the binding curves represents the average of three

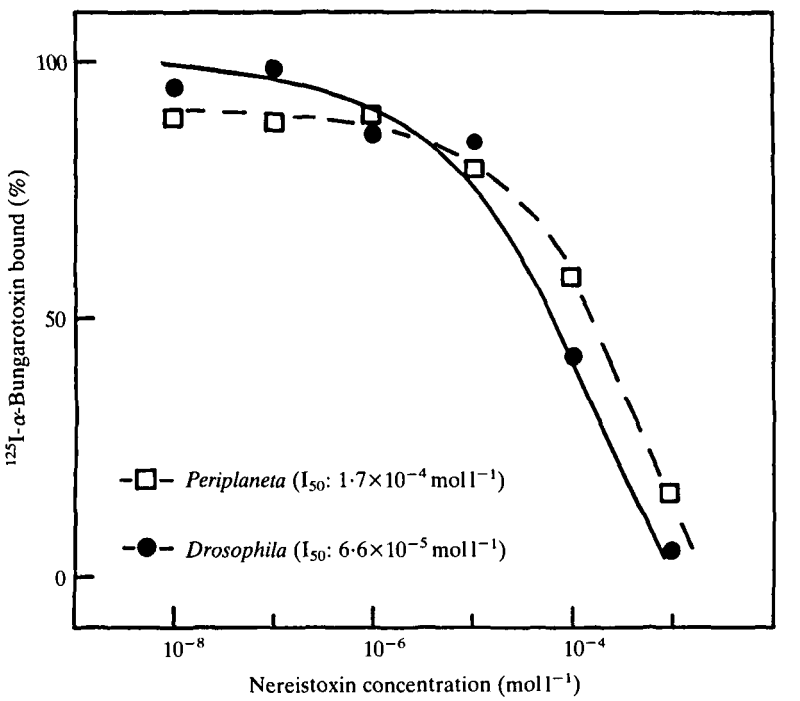

Fig. 8. Effects of nereistoxin (NTX) on the binding of ^{125}I-α-bungarotoxin to CNS crude membrane extracts prepared from cockroach abdominal nerve cords and *Drosophila* heads. Aliquots of CNS crude membrane extract (equivalent to 20 mg wet weight cockroach nerve cords and 5 mg *Drosophila* heads starting material respectively) were incubated for 30 min at 21 °C in Krebs original Ringer phosphate pH 7·4 (Dawson, Elliott, Elliott & Jones, 1969) containing 1·7 times the final concentration of NTX in a total volume of 70 μl. Then 50 μl of 4 nmol l^{-1} ^{125}I-α-bungarotoxin (specific activity 27 Ci mmol^{-1}) in Krebs original Ringer buffer containing 0·1 % bovine serum albumen was added and the mixtures incubated for a further 30 min. The mixtures were then centrifuged for 30 min at 30 000 × *g* and the amount of ^{125}I-α-bungarotoxin bound was determined by the centrifugal assay as described by Schmidt-Nielsen, Gepner, Teng & Hall (1977). Background binding to heat-inactivated extracts has been subtracted. 100 % binding was defined as the amount of ^{125}I-α-bungarotoxin bound in the absence of added NTX. The data shown represent triplicate incubation mixtures at each point.

incubation mixtures at that particular toxin concentration. From the *Drosophila* data, the concentration of NTX required to inhibit ^{125}I-α-bungarotoxin-binding by 50 % (I_{50}) was found to be $6{\cdot}6 \times 10^{-5}$ mol l^{-1}. In an independent repetition of this experiment on *Drosophila* extract the I_{50} for NTX was determined to be $5{\cdot}6 \times 10^{-5}$ mol l^{-1}. From the *Periplaneta* data, an I_{50} of $1{\cdot}7 \times 10^{-4}$ mol l^{-1} was estimated. Thus the ability of NTX to inhibit ^{125}I-α-bungarotoxin binding is similar for *Drosophila* and *Periplaneta* extracts. This inhibition is only seen at the higher concentrations of NTX where electrophysiological studies on *Periplaneta* neurones show depolarization.

DISCUSSION

The data presented here provide a quantitative description of the actions of NTX on axons, synapses and cell bodies of identified neurones together with its effects on an ^{125}I-α-bungarotoxin binding site in the central nervous system of the insect *Periplaneta americana*. It is possible to consider potential sites for the central actions of this toxin based on these results. These include: voltage-sensitive ion channels (e.g. sodium and potassium); non-specific membrane actions; acetylcholine release; acetylcholine receptor/ion channel.

The axonal membrane is relatively insensitive to NTX at concentrations below $1{\cdot}0 \times 10^{-4}$ mol l^{-1}. At concentrations higher than $2{\cdot}0 \times 10^{-4}$ mol l^{-1} several modifications of axonal membrane properties are detected including suppression of the sodium current, decrease in potassium current and increase in leak current. The decrease in potassium current is consistent with the repetitive activity sometimes induced during prolonged exposure to low concentrations of NTX. The increase in the leak current may explain the depolarizing axonal effects of NTX seen under current-clamp conditions. Depolarization in this case is not due to a specific increase in resting sodium conductance since it is noted in the presence of tetrodotoxin (TTX), a specific sodium channel blocker of cockroach axons (Pichon, 1974; Sattelle *et al.* 1979). Thus NTX at a concentration $1{\cdot}0 \times 10^{-3}$ mol l^{-1} blocks voltage-dependent sodium and potassium channels and induces a non-specific, irreversible increase in ion permeability of the axonal membrane.

NTX, a weak inhibitor of insect acetylcholinesterase (Sakai, 1966*c*) and choline acetyltransferase (A. C. Baillie, K. Wright & D. B. Sattelle, unpublished observations), nevertheless exhibits several potent actions at cercal afferent, giant interneurone synapses. An action of NTX as an antagonist at the postsynaptic acetylcholine receptor recognition site could account for changes in EPSP amplitude. However, this site is probed by α-bungarotoxin and the concentration of NTX producing half-maximal, non-depolarizing block of transmission (maximum suppression of EPSP amplitude = 60 %) is $8{\cdot}0 \times 10^{-7}$ mol l^{-1}, whereas 50 % inhibition of ^{125}I-α-bungarotoxin binding is produced by NTX concentrations of $1{\cdot}7 \times 10^{-4}$ mol l^{-1} (*Periplaneta americana* extract) and $6{\cdot}6 \times 10^{-5}$ mol l^{-1} (*Drosophila melanogaster* extract). In studies on fly head (*Musca domestica*) extracts, Eldefrawi & Eldefrawi (1980) showed that $1{\cdot}0 \times 10^{-5}$ mol l^{-1} NTX inhibited by

50 % the specific binding of ^{125}I-α-bungarotoxin. The discrepancy between the concentrations of NTX effective as a non-depolarizing blocking agent and as an inhibitor of ^{125}I-α-bungarotoxin binding suggests that if NTX is interacting with the receptor recognition site of the acetylcholine receptor/ion channel, the site of its blocking action is distinct from the α-bungarotoxin binding site.

Only partial blockade of both compound EPSPs and EPSPs evoked by stimulation of a single cercal mechanoreceptor, was achieved by micromolar NTX. In the case of compound EPSPs, increasing the toxin concentration by about two orders of magnitude failed to cause further block of synaptic potentials. Since even single cercal mechanoreceptor afferents terminating on GI 2 make many synaptic contacts (J. M. Blagburn, D. J. Beadle & D. B. Sattelle, unpublished observations), it is possible that some of the synapses between cercal afferents and GI 2 are insensitive to NTX. Alternatively, all synapses may be partially blocked. The underlying mechanism remains to be determined.

A possible explanation for the synaptic blocking effect is an inhibition of the mechanism of transmitter release from presynaptic terminals. Although Deguchi *et al.* (1971) noted an inhibitory effect of NTX on acetylcholine release at the frog neuromuscular junction, Eldefrawi *et al.* (1980), following an analysis of quantal content and frequency of miniature endplate potentials, failed to confirm this observation. No comparable studies of possible presynaptic actions on transmitter release mechanisms are available for the cockroach cercal afferent, giant interneurone synapses. This possibility of a presynaptic action in insects cannot be discounted at the present time, though it should be recalled that acetylcholine responses of cell body membranes are highly sensitive to NTX and synaptic inputs to neuronal cell bodies of adult cockroaches have not been detected (cf. Smith & Treherne, 1965).

The voltage-dependence of the suppression by NTX of acetylcholine-induced currents in the cell body membranes of GI 2 and D_f, at a concentration of $5{\cdot}0 \times 10^{-7}$ mol l^{-1}, indicates that the site of this action of NTX is either the channel site of the acetylcholine receptor/ion channel or another component of this complex that is membrane-potential sensitive. The less pronounced voltage-dependence of the block of acetylcholine-induced current at more negative (hyperpolarizing) potentials may result from the following possible actions of NTX: (a) an increase in lifetime of the acetylcholine receptor/ion channel; (b) a reduction in voltage-dependence of acetylcholine desensitization; (c) movement of positively charged NTX through the acetylcholine receptor/ion channel at large negative potentials. In preliminary experiments (cf. Sattelle & David, 1983) we have been unable to find evidence for voltage-dependent desensitization of acetylcholine responses in D_f. Further experiments are needed to distinguish between these possible explanations for the voltage-dependent behaviour of acetylcholine currents in the presence of NTX. In a recent study on D_f it was also shown that *d*-tubocurarine, an ion channel blocker at other neuronal nicotinic cholinergic receptors (see for example Ascher, Large & Rang, 1979), was voltage-dependent in its blocking of acetylcholine-induced currents, whereas α-bungaro-

toxin, a probe of the acetylcholine receptor recognition site (Heidmann & Changeux, 1978), was voltage-independent (David & Sattelle, 1984).

The finding that NTX exhibits a voltage-dependent block of the acetylcholine response of insect neurones at submicromolar concentrations contrasts with the observations on vertebrate peripheral cholinergic receptors. Eldefrawi & Eldefrawi (1980) provide clear evidence that NTX does not alter the linearity of the current-voltage relationship, nor does it affect the time-course of the end-plate current in frog sartorius muscle. In the same study, the binding of [^{3}H]perhydrohistrionicotoxin, a selective probe of the channel of the vertebrate nicotinic acetylcholine receptor/ion channel complex (Albuquerque *et al.* 1973; Masukawa & Albuquerque, 1978), was found to be unaffected by NTX at concentrations up to $1{\cdot}0 \times 10^{-3}$ mol l^{-1}.

The present study on *Periplaneta* reveals a postsynaptic depolarization by NTX accompanied by EPSP block, at concentrations of $2{\cdot}0 \times 10^{-5}$ mol l^{-1} and above, and axonal depolarization at concentrations of $2{\cdot}0 \times 10^{-4}$ mol l^{-1} and above. The concentrations effective in depolarizing the synaptic membrane are also effective in blocking the binding of ^{125}I-α-bungarotoxin to *Periplaneta* CNS extracts. One possible explanation is an agonist action on the recognition site of the insect nicotinic acetylcholine receptor/ion channel complex; however, actions at other voltage-sensitive channels and non-specific effects may also account for the postsynaptic depolarizing actions of NTX. NTX is a poor inhibitor (I_{50}: $1{\cdot}0 \times 10^{-3}$ mol l^{-1}) of flyhead acetylcholinesterase (Sakai, 1966*c*), so an action on this enzyme resulting in elevation of extracellular synaptic acetylcholine levels is unlikely to account for the postsynaptic depolarization. A depolarizing action of NTX has been observed at vertebrate neuromuscular junctions (Eldefrawi *et al.* 1980). The insect studies reveal a voltage-dependent blocking action at concentrations well below those required for depolarization.

The differences in the degree of voltage-dependence of the blocking actions of NTX at vertebrate peripheral nicotinic acetylcholine receptors and insect central nicotinic acetylcholine receptors provide further evidence for differences in the properties of these two receptors. Striking differences in the actions of decamethonium at these receptors have already been noted (cf. Neher & Sakmann, 1975; Schmidt-Nielsen *et al.* 1977; David & Sattelle, 1984). The disulphide bond of NTX may be reduced to a dithiol which could covalently react with the acetylcholine receptor/ion channel complex in a manner similar to that shown for dithiothreitol, which is known to modify cholinergic receptor properties (cf. Karlin, 1969). The structure of this invertebrate neurotoxin therefore lends itself to chemical manipulation in pursuing further the molecular basis of its action. From the data presented here, an acetylcholine receptor/ion channel complex of the insect CNS appears to be one site of action of nereistoxin.

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