J. exp. Biol. 144, 303–337 (1989)
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EFFECTS OF NICOTINIC AND MUSCARINIC AGENTS ON AN IDENTIFIED MOTONEURONE AND ITS DIRECT AFFERENT INPUTS IN LARVAL *MANDUCA SEXTA*

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Accepted 13 March 1989

Summary

The principal locomotory appendages of larval *Manduca sexta*, the prolegs, bear at their tips an array of mechanosensory hairs (the planta hairs). Each of the single sensory neurones associated with a planta hair sends an axon into the ganglion of the same segment where the afferent terminals make synaptic contact with interneurones and motoneurones. Electrical stimulation of a single afferent elicits a monosynaptic excitatory postsynaptic potential (EPSP) in PPR, one of the motoneurones controlling the prolegs. We have used this synapse to study the pharmacology of sensory transmission in *M. sexta*. The following observations were made.

1. The EPSP was reversibly inhibited in a dose-dependent fashion by the cholinergic antagonists *d*-tubocurarine, atropine and mecamylamine, indicating that the planta hair afferent neurones use acetylcholine (ACh) as a neurotransmitter. α-Bungarotoxin (αBGTX) also suppressed the EPSP but required concentrations above 1×10^{-6} mol l^{-1}.

2. PPR depolarized in response to ionophoretic or bath application of cholinergic agonists, but compared to motoneurones of a non-nicotine-resistant insect such as the cockroach, PPR was relatively insensitive to nicotine.

3. Application of *N*-methyl nicotinamide (NMN) to the nerve cord to inhibit putative alkaloid pumps in the central nervous system (CNS) only weakly potentiated PPR's response to nicotine. This suggests that such pumps do not markedly contribute to PPR's nicotine resistance.

4. PPR's responses to nicotinic agents showed several pharmacological differences from those reported for other insects, indicating that the nicotinic ACh receptors of *M. sexta* may be specifically adapted to accommodate a nicotine-rich diet.

5. During the application of muscarinic agonists to isolated abdominal ganglia, the firing rate of motoneurones, as monitored in the ventral nerve, increased

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Key words: *Manduca sexta*, insect CNS, cholinergic receptors, nicotine, muscarinic.

dramatically. PPR responded to muscarinic agents even during synaptic blockade, suggesting that muscarinic receptors may be present on PPR itself. The main effect of muscarine on PPR was to lower its spike threshold.

6. Bath-applied muscarinic agents also affected the afferent-evoked EPSP in a manner consistent with the presence of another group of receptors that, when stimulated, act presynaptically to inhibit the release of ACh from the sensory terminals.

These apparent pre- and postsynaptic actions of muscarinic agents are the first reported findings of muscarinic physiology in an identified insect neurone and its synaptic inputs.

Introduction

Sensory neurones that project to the central nervous system (CNS) of insects use acetylcholine (ACh) as the primary neurotransmitter. In the cockroach and locust, excitatory responses of interneurones and motoneurones to ACh are apparently mediated by nicotinic acetylcholine receptors (nAChRs). These receptors, which are agonist-gated channels, have been characterized pharmacologically and partially characterized biochemically (Breer, 1981; Breer & Sattelle, 1987). Although sharing many pharmacological and physiological properties with vertebrate neuronal and muscle nAChRs, it is clear that the insect nAChRs have marked differences in their subunit organization and toxin sensitivity (Filbin *et al.* 1983; Breer *et al.* 1986; Chiappinelli *et al.* 1987). It is also becoming apparent that, like vertebrate neuronal nAChRs, insect nAChRs are not a homogeneous class. For example, identified neurones within a single insect ganglion express cholinergic receptors with seemingly different sensitivities to the cholinergic blocking agent α-bungarotoxin (αBGTX) (Goodman & Spitzer, 1979, 1980; Lane *et al.* 1982; Lees *et al.* 1983). Although there is evidence that the αBGTX-binding component from locust nervous tissue may be a homo-oligomeric AChR (Breer *et al.* 1985), the isolation of cDNA clones from *Drosophila* that code for apparently non-α subunits of the AChR (Hermans-Borgmeyer *et al.* 1986) and for α-like subunits that do not bind αBGTX (Bossy *et al.* 1988), indicates that hetero-oligomeric AChRs may also exist in some insect species.

In addition to activating nAChRs, ACh also stimulates slower, modulatory changes in neurones and muscles of vertebrates. These effects are independent of ion fluxes through the nAChRs and are mediated by muscarinic acetylcholine receptors (mAChRs). These receptors require the presence of specific GTP-binding proteins and exert their actions by regulating a variety of second messengers (Nathanson, 1987). They can be further differentiated using pharmacological (Hammer *et al.* 1980), molecular (Kubo *et al.* 1986; Peralta *et al.* 1987) and functional (Peralta *et al.* 1988) criteria. In the mammalian brain, mAChRs appear to modulate ion channels, thereby altering the membrane characteristics or responsiveness of individual neurones (Krnjevic, 1974). Binding sites for particular mAChR ligands, which presumably correspond to mAChRs, are much mor

numerous than nAChRs in the vertebrate brain (Birdsall & Hulme, 1976). In the insect CNS these same muscarinic ligands generally reveal a low number of putative mAChRs which are less numerous than the nAChRs (Haim *et al.* 1979; Shaker & Eldefrawi, 1981; Lummis & Sattelle, 1985, 1986), although some studies have demonstrated quite high densities of muscarinic binding sites (Meyer & Edwards, 1980; Meyer & Reddy, 1985). Until very recently there were no reports of muscarinic responses in insect neurones, but Benson and collaborators (Benson & Neumann, 1987; Benson, 1988) have now obtained evidence for muscarine-induced conductance changes in dissociated locust neurones and, based on studies of neurotransmitter release from synaptosomes, it has also been proposed that mAChRs may modulate ACh release from insect nerve terminals (Breer & Knipper, 1984).

With these issues in mind, we have been studying the pharmacological responses of an identified motoneurone, and its direct afferent input, in larvae of the nicotine-resistant insect *Manduca sexta*. The synapse on which we have focused is part of the pathway mediating reflexive withdrawal of the abdominal prolegs. This reflex is elicited by tactile stimulation of long mechanosensory hairs (the planta hairs) located on the tip of each proleg (Weeks & Jacobs, 1987). The withdrawal is initiated by contraction of the principal planta retractor muscle (PPRM), which is innervated by a single motoneurone, PPR (Weeks & Truman, 1984). The reflex is sufficiently strong that displacement of a single planta hair is capable of eliciting a contraction in PPRM. Intracellular recordings from the soma of PPR reveal that afferent action potentials evoked by movement of a planta hair cause short-latency excitatory postsynaptic potentials (EPSPs) in the motoneurone which, by several criteria, appear to be monosynaptic (Weeks & Jacobs, 1987). Direct sensory-to-motoneurone connections such as these have not been widely reported in insects, with most sensory synapses being made instead on interneurones (discussed in Weeks & Jacobs, 1987). Cholinergic neurotransmission between mechanosensory afferents and interneurones has been extensively studied in the cockroach terminal ganglion (Callec *et al.* 1971; Callec, 1974; Sattelle, 1980; Harrow & Sattelle, 1983; Sattelle *et al.* 1983; Blagburn *et al.* 1986) and a monosynaptic connection between trochanteral hairplate afferents and a motoneurone (Ds) in the metathorax of the cockroach has also been shown to be cholinergic (Carr & Fourtner, 1980).

The experiments reported here describe the initial pharmacological characterization of cholinergic transmission in larval *M. sexta* using the planta hair afferent-to-motoneurone synapse. This system proved ideal because of the ease of controlling presynaptic elements while recording from the postsynaptic cell. Evidence is presented that the nAChRs of *M. sexta* may be specifically modified to resist the action of nicotine. In addition, we provide the first report of muscarinic responses in an identified insect neurone. These results suggest that, in addition to its role in fast synaptic transmission, ACh may also modulate neuronal responsiveness in *M. sexta*. Some of these results have been presented in abstract form (Trimmer & Weeks, 1987, 1988).

Materials and methods

Experimental animals

Larvae of the tobacco hornworm *Manduca sexta* were reared in individual containers on an artificial diet (Bell & Joachim, 1976) at 27°C on a 17 h: 7 h L: D photoperiod. Larvae of both sexes were used for experiments on the third or fourth day of the fifth instar. In some experiments, larvae were reared from hatching on tobacco plants (*Nicotiana tabacum*) or on an artificial diet containing DL(−)nicotine hydrogen tartrate at an alkaloid concentration similar to that found in leaves of the tobacco plant ($6\,mg\,g^{-1}$: Jones & Collins, 1958).

Midway through the fifth larval instar, a 'commitment pulse' of ecdysteroids in the blood triggers 'wandering' behaviour, the cessation of feeding and a purging of the gut (Dominick & Truman, 1985), and is followed by distinctive morphological changes leading to pupal ecdysis. In a few experiments we used these prepupal larvae which were at the 'dorsal-bar' stage of development (Truman *et al.* 1980).

Electrophysiological techniques

The 'isolated proleg' preparation was used as described by Weeks & Jacobs (1987) with some modifications (Fig. 1A). Briefly, this preparation consisted of the distal portion of a proleg from abdominal segment A4 or A5, dissected from the body wall together with its associated ganglion. The proleg was pinned on one side of a divided chamber, with the inner surface facing upwards to expose the hair sockets. The ganglion, connected to the proleg by the ventral nerve (VN) which contains axons of both the planta hair afferents and the retractor muscle motoneurones, was placed in the other half of the chamber. The two compartments of the chamber were electrically isolated with silicone grease. The ganglion side of the chamber was perfused rapidly with saline, to which pharmacological agents could be added by means of a multi-inlet valve (Holder & Sattelle, 1972). The entire solution bathing the ganglion was exchanged 2–3 times a minute. The proleg was maintained in a physiological saline (Weeks & Truman, 1985) and a single planta afferent was stimulated by means of a glass suction electrode placed over the socket of the largest hair in the middle, distalmost region of the planta array (the arrangement of the planta hairs is described in Peterson & Weeks, 1988). Fig. 1B illustrates the characteristic terminal arbor of planta hair afferents located in this position on the planta (see also Peterson & Weeks, 1988). The stimulus voltage applied at the hair socket was adjusted to be suprathreshold for the single sensory neurone associated with the hair (square pulses of 0·02 ms, 4–9 V); each stimulus evoked an EPSP in PPR. Activity in the VN was recorded extracellularly between electrodes on each side of the grease bridge and amplified *via* a.c.-coupled preamplifiers (Grass Instrument Co., Quincy, MA). In some experiments a suction electrode was used to stimulate electrically the anterior branch of the VN (VN_a), which carries the axons of the planta hair afferents (Weeks *et al.* 1989).

Individual abdominal ganglia from segments A4 and A5 were desheathed afte

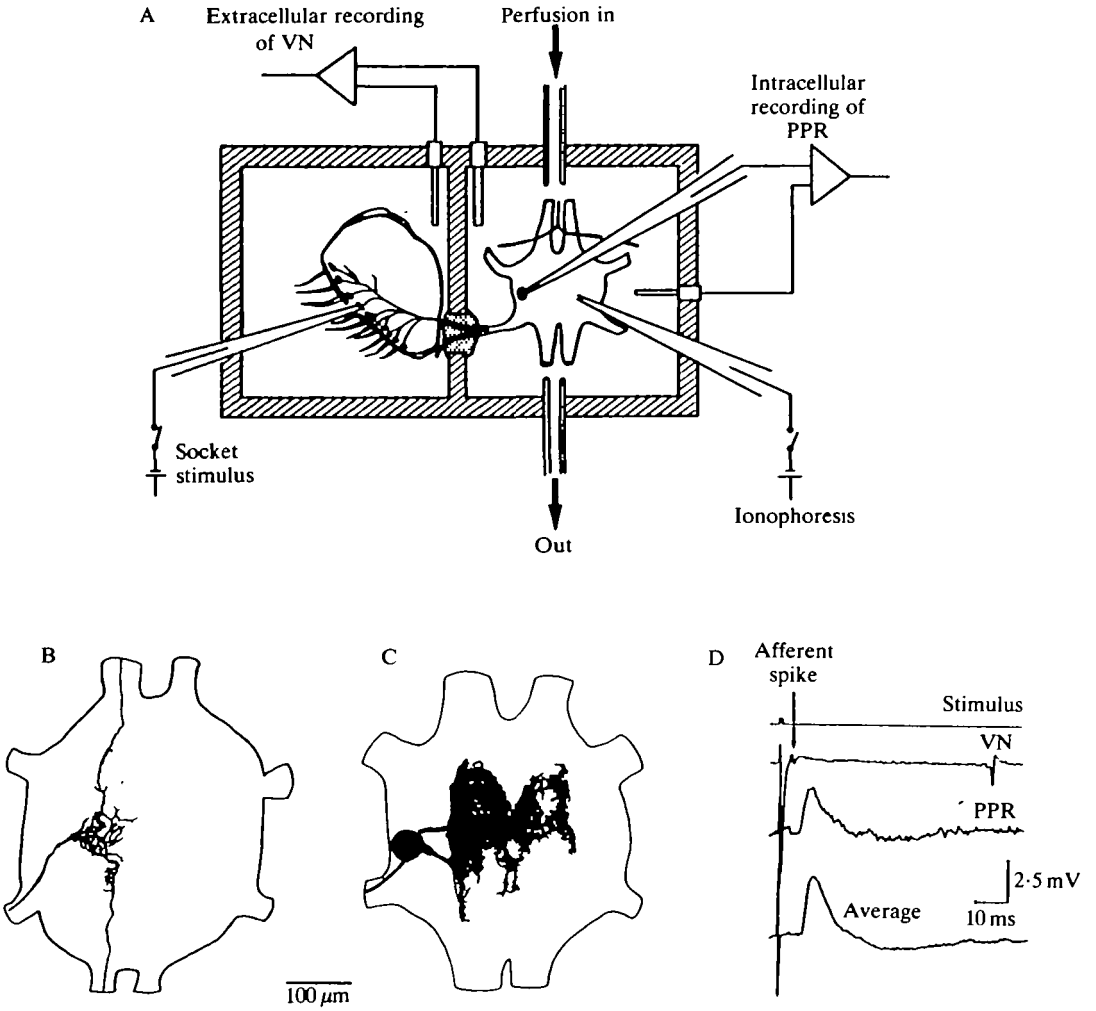
treatment with 3% collagenase–dispase, as described previously (Weeks & Jacobs, 1987), and maintained in a high-sodium saline (see below). Intracellular voltage recordings were made from the cell body of PPR (Fig. 1C) using glass micropipette electrodes filled with 4 mol l^{-1} potassium acetate (resistance 18–30 MΩ) and connected to a Dagan 8800 amplifier (Dagan Corp., Minneapolis, MN). Recordings were made in bridge mode or, for current injection, in single-electrode current-clamp (SEC) mode, after carefully balancing the electrode resistance and capacitance. Resting potentials were then measured relative to the extracellular potential in the bathing saline, using the high-impedance bath amplifier circuit of the Dagan 8800. The input resistance (R_{in}) of PPR was determined in SEC mode from the slope of the current–voltage (I–V) curve obtained by injecting 500 ms hyperpolarizing current pulses and measuring the potential achieved at equilibrium. PPR exhibits strong rectification during depolarizing current injection, so input resistance was determined for hyperpolarizing responses only. All intracellular and extracellular voltage signals, together with the current monitor, were recorded on magnetic tape. Triggered EPSPs were also analysed on-line by a computer using the analogue-to-digital hardware and signal-averaging software of RC Electronics (Santa Barbara, CA). For resting potential measurements during application of pharmacological agents, high-frequency signals were filtered out and the remaining low-frequency voltage changes digitized at a low sample rate. Although the EPSP can be facilitated (Weeks & Jacobs, 1987) and depressed (B. A. Trimmer & J. C. Weeks, in preparation) by different stimulation paradigms, the EPSP was stable for several hours when stimulated at a frequency of 1·5 Hz. All the EPSP measurements reported here were made at a stimulus rate of 1·5 Hz averaged over 60 sweeps (digitized at 10 kHz; Fig. 1D).

Identification of PPR

Motoneurone PPR was identified by its large spike in the VN and by the presence of a time-locked monosynaptic EPSP evoked by planta hair afferent stimulation (Weeks & Jacobs, 1987). This identification was confirmed in many preparations by re-impaling the neurone with an electrode containing cobaltic hexammine chloride (0·1 mol l^{-1}) which was ionophoresed into the cell. The cobalt was precipitated and the fill intensified using a modification of the technique of Bacon & Altman (1977). PPR was readily identified by its definitive contralateral arbor (Weeks & Truman, 1984; Fig. 1C). In prepupae, PPRM was dissected together with the ganglion, and PPR was identified by the occurrence of potentials recorded in PPRM fibres that were time-locked to the motoneurone spikes (Weeks & Truman, 1985).

Ionophoresis

Ionophoretic micropipettes were made using glass microelectrodes similar to those used for intracellular recordings. These were filled at the tip with the required pharmacological agent at concentrations of 0·1 or 1 mol l^{-1}, dissolved in



water. Resistances of 50–100 MΩ were typical with the lower concentration. A holding current of 2–15 nA was usually required to prevent leakage of the agent. The pipette was inserted into neuropile regions known to contain PPR's arbor, either ipsilateral or contralateral to the cell body, depending on the experiment (see Results). Drugs were expelled either by briefly switching off the holding current or by applying a small amount of positive current.

Saline solutions and drugs

The physiological saline used for desheathed ganglia was modified from that of Miyazaki (1980), and contained (in mmol l^{-1}): NaCl, 140; KCl, 5; $CaCl_2$, 4; glucose, 28; and Hepes, 5; pH 7·4. In some experiments a high-divalent saline was used to suppress spiking activity (Cohen *et al.* 1978; Weeks & Jacobs, 1987). This saline contained 20 mmol l^{-1} $CaCl_2$ and 20 mmol l^{-1} $MgCl_2$, with the NaCl concentration reduced correspondingly to maintain osmolarity. To block synapti

Fig. 1. The isolated proleg preparation. (A) Intracellular recordings from the soma of PPR were made in an isolated abdominal ganglion (right chamber) while stimulating a single sensory cell at the base of the largest planta hair (left chamber). Afferent and efferent spikes in the ventral nerve (VN) were recorded with differential electrodes placed on either side of a silicone grease barrier (stippled area). Drugs were applied to the ganglion by bath perfusion or by ionophoresis into the neuropile. (B) Planta hair afferent morphology. A *camera lucida* drawing of the terminal arbor of an afferent innervating the type of hair stimulated in these experiments is shown. The afferent enters the ganglion *via* the VN and sends axon collaterals to the adjacent ganglia. Anterior is up. The afferent was visualized by cobalt uptake as described in Peterson & Weeks (1988). (C) Morphology of PPR. A *camera lucida* drawing of motoneurone PPR stained by intracellular injection of cobalt is shown. PPR's soma is located on the dorsal surface at the lateral margin of the ganglion and it is the only VN-projecting motoneurone with a contralateral arbor (Weeks & Truman, 1984). Direct monosynaptic connections with the ipsilateral planta hair afferents are made on a ventral projection of PPR's ipsilateral arbor (Weeks & Jacobs, 1987), which is not visible in this dorsal view. The ganglion is oriented as in B. (C) A single electrical stimulus (0·02 ms, +6 V) applied to a planta hair socket (upper trace) evokes an afferent spike recorded *en passant* in the VN (second trace). This small spike immediately follows the stimulus artefact visible at the start of all the traces. For comparison, an unrelated motoneurone spike is also visible near the end of this record. The third trace shows a short-latency EPSP evoked in PPR by the afferent spike. EPSPs can be driven at 1·5 Hz for an hour or more without a detectable change in amplitude (see Materials and methods). For precise quantification of EPSP size, 60 successive stimulations were signal-averaged at a stimulus frequency of 1·5 Hz. An example of an averaged EPSP obtained from this preparation is shown in the lower trace. The amplitude was measured as the difference between the resting voltage immediately before the response and the voltage attained at the peak of the EPSP.

transmission, physiological saline containing 20 mmol l^{-1} $MgCl_2$ and 0 mmol l^{-1} $CaCl_2$ (with the NaCl suitably adjusted) was used (Weeks & Jacobs, 1987). The following pharmacological agents were obtained from Sigma Chemical Co. (St Louis, MO): acetylcholine chloride (ACh), atropine sulphate, α-bungarotoxin (αBGTX), carbamylcholine chloride (carbachol), decamethonium bromide, hexamethonium bromide, hippuric acid, mecamylamine hydrochloride, DL-muscarine chloride, neostigmine bromide, nicotine hydrogen tartrate, *N*-methyl nicotinamide (NMN), oxotremorine, scopolamine bromide, scopolamine methyl bromide, trimethylammonium chloride (TMA) and *d*-tubocurarine chloride (curare). In addition, a sample of neuronal bungarotoxin (also called κ-bungarotoxin, toxin F, and fraction 3·1) was kindly supplied by Dr R. Loring (Harvard Medical School, Boston). All these agents were dissolved in saline to the required concentration on the day of the experiment.

Results

Nicotinic agonists

In feeding fifth-instar larvae, PPR had a mean resting potential of −43·5 mV (±4·6 S.D., $N = 73$) and a mean R_{in} of 12·7 MΩ (±3·3 S.D., $N = 24$). Ionophoresis

of nicotine (Fig. 2Ai) or carbachol (Fig. 2Bi) either onto the cell body or into the ipsilateral neuropile caused PPR to depolarize transiently. The size of this response depended upon the total amount of charge delivered from the pipette. Depolarization could still be elicited when synaptic transmission was blocked in 0 mmol l^{-1} $CaCl_2$/20 mmol l^{-1} $MgCl_2$ saline, indicating that the response was mediated by receptors on PPR itself (data not shown). The response to carbachol outlasted the response to nicotine (Fig. 2); for instance, for the same mean depolarization of 3·5 mV, the R_{50} (the time taken for the potential to decay to half of its peak value) of carbachol was 2·9 s (±1·0 S.D., $N = 9$), whereas that of nicotine was 1·6 s (±0·7 S.D., $N = 7$). Bath application of the nicotinic antagonist, mecamylamine (5×10^{-5} mol l^{-1}) completely abolished the response to nicotine

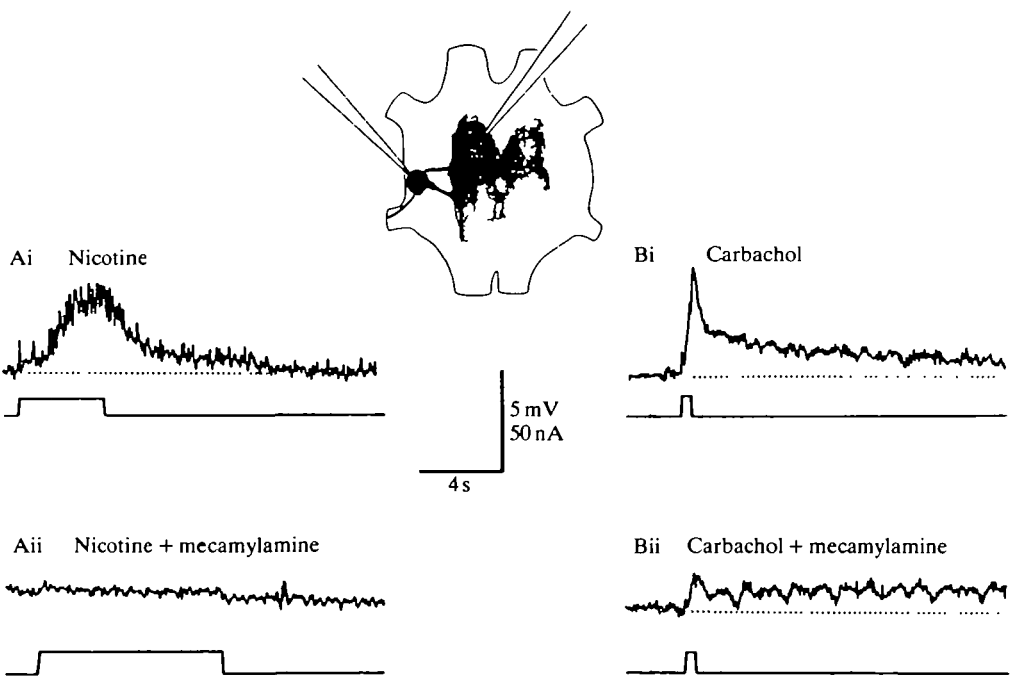


Fig. 2. Ionophoretic responses to nicotine and carbachol. (A) Response of PPR to nicotine, applied ionophoretically into the ipsilateral neuropile. (i) A 4 s pulse of nicotine delivered by a 2 nA current pulse caused a 3·5 mV depolarization. (ii) This response was abolished during bath application of the nicotinic antagonist mecamylamine (5×10^{-5} mol l^{-1}), so that even a prolonged pulse (8 s) at the same current was without effect. Both traces are from the same preparation and PPR was hyperpolarized slightly (to −50 mV, −0·3 nA) throughout these recordings to prevent spiking. The rapid potential fluctuations in Ai are spontaneous EPSPs. (B) In a different preparation, carbachol (i) applied ionophoretically to the ipsilateral neuropile (0·4 s, 3·4 nA) caused a depolarization in PPR of similar amplitude but longer duration than that seen with nicotine (compare Fig. 2Bi to Fig. 2Ai). (ii) In the same preparation, bath application of mecamylamine blocked most of the response but consistently left a small, prolonged depolarization. PPR was held hyperpolarized (−51 mV, −0·3 nA) to prevent spiking.

(Fig. 2Aii), yet only partially blocked the response to carbachol (Fig. 2Bii), leaving a small and long-lasting depolarization. This finding will be discussed below.

When bath-applied to the ganglion, nicotine, carbachol and TMA caused a depolarization of PPR which led to an increase in spiking activity and, at higher concentrations, to the eventual blockade of all activity. These effects are illustrated in Fig. 3A, using nicotine as the agonist. The depolarization was maintained at a plateau level throughout the drug application (at least 15 min); upon washout, PPR repolarized rapidly and then exhibited a marked hyperpolarization lasting for several minutes. This phenomenon probably reflects increased sodium-pump activity that typically follows prolonged depolarization (e.g. Jansen & Nicholls, 1973). During agonist-induced depolarization, the afferent-evoked EPSP was reversibly abolished (Fig. 3B) and PPR's R_{in} measured at the soma decreased (Fig. 3C). Carbachol and TMA both produced effects similar to those shown for nicotine in Fig. 3.

When administered alone, ACh had no effect on PPR, even when perfused over the ganglion for 20 min at 1×10^{-2} mol l^{-1} ($N=3$, data not shown). However, if the ganglion was exposed to the acetylcholinesterase inhibitor neostigmine (5×10^{-7} mol l^{-1}) before and during ACh application, the same concentration of ACh then elicited a 7 mV depolarization ($N=2$; data not shown). Higher concentrations of neostigmine caused an extremely variable and unstable resting potential and prevented further quantification of ACh sensitivity.

The magnitude of PPR's depolarization was related to the concentration of bath-applied agonists, allowing their relative potencies to be assessed. Each ganglion was treated with a single concentration of agonist, unless a very low concentration was applied initially, in which case a second, higher, concentration was sometimes applied after extensive washing of the ganglion. This protocol minimized the possibility of desensitization (Sattelle *et al.* 1976). Dose–response curves for different agonists are shown in Fig. 4. Nicotine and carbachol were the most potent agonists, with decamethonium and TMA progressively less effective. Apart from decamethonium, which in the cockroach has antagonistic rather than agonistic actions (see below), the rank potency order of these agents is the same as that reported for the cockroach motoneurone Df (David & Sattelle, 1984). Furthermore, the absolute potencies are also similar, with the marked exception that nicotine is approximately 60 times less potent in *M. sexta* than in the cockroach *Periplaneta americana*; the concentration producing a half-maximum effect, D_{50}, is 91 μmol l^{-1} in *M. sexta* (Fig. 4) and 1·5 μmol l^{-1} in *P. americana* (David & Sattelle, 1984). The lengthy incubations (typically 15–20 min) used in these measurements, and the rapid responses to agonists (within 30 s, Fig. 3A), make it unlikely that a selective permeability barrier is responsible for this relative insensitivity to nicotine in *M. sexta*.

Alkaloid pumps

One possible cause of the reduced potency of nicotine in *M. sexta* might be

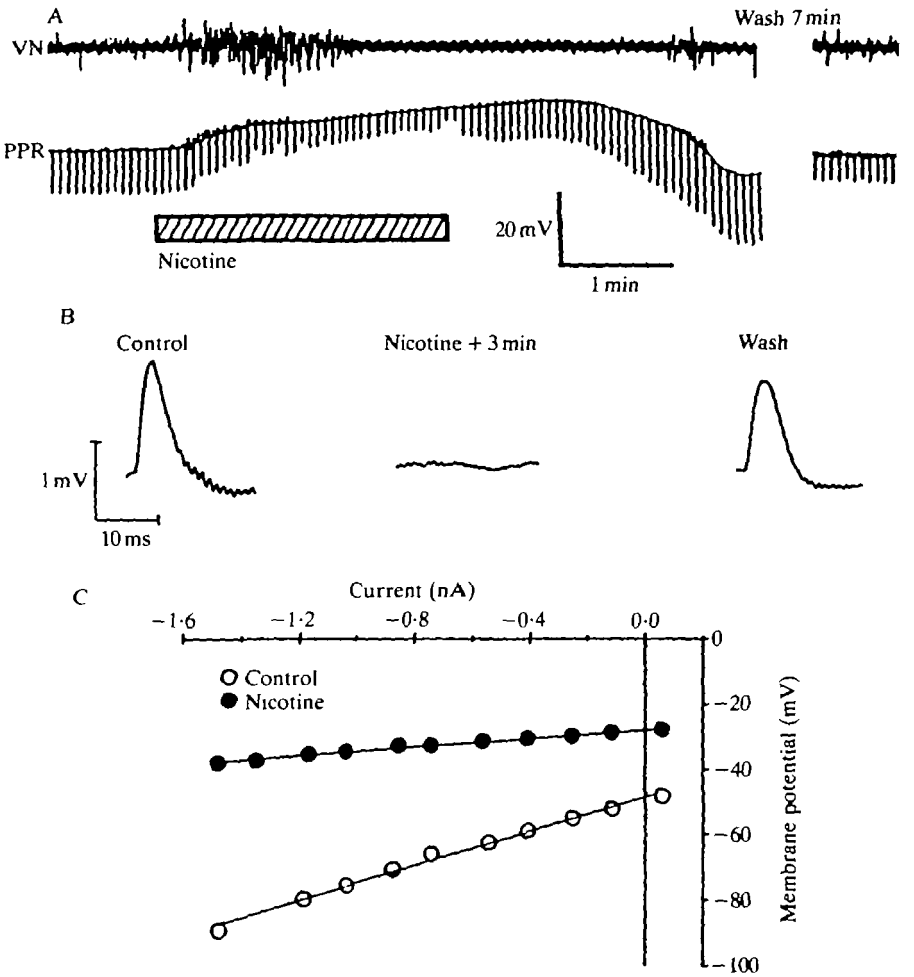


Fig. 3. PPR's responses to nicotine. (A) Bath application of nicotine (1×10^{-4} mol l^{-1}; hatched bar) to a ganglion caused PPR to depolarize (lower trace) and was accompanied by spike activity in the VN (upper trace). Action potentials in PPR are filtered so that changes in resting potential can be seen more easily. The downward deflections in the lower trace are voltage changes in response to current pulses (−0·5 nA) injected through the recording electrode; their size is proportional to PPR's input resistance (R_{in}). (B) During nicotine application the afferent-evoked EPSP is reversibly abolished. These averaged EPSPs were acquired during a subsequent application of nicotine to the same preparation as that used in A. The control and wash examples were obtained by slightly hyperpolarizing PPR to prevent spiking and the middle trace was acquired at the plateau of the depolarization caused by nicotine after spike blockade. The disappearance of the EPSP during nicotine application was not due to afferent spike failure but, presumably, resulted from the depolarization and decreased membrane resistance of PPR. (C) A plot of the current–voltage (I–V) relationship of PPR before and during the application of nicotine (1×10^{-4} mol l^{-1}). Each point is a single determination of the membrane potential during the application of a 500 ms current pulse. In this preparation, which is different from that used in A and B, the resting potential (zero current) was reduced from −48·4 to −27·9 mV, and the R_{in} (slope of the regression line) decreased from 26 to 6·5 MΩ.

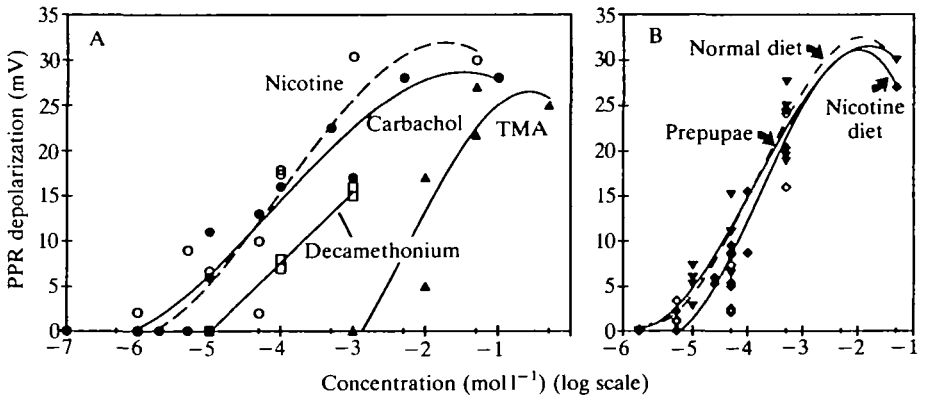


Fig. 4. Dose–response curves of bath applied cholinergic agonists. (A) Agonists were bath-applied to PPR in the same manner as that shown for nicotine in Fig. 3A. The depolarization evoked by each drug was measured at the plateau of the response for each application to an individual ganglion. Each point is a single determination for an individual ganglion. (B) The nicotine response of PPR from larvae fed a normal diet (curve taken from A; dashed line) is compared with the nicotine responses of PPR from prepupae (▼; solid line) and from larvae fed on diet containing nicotine (◆; solid line). Data points are also shown for PPR from larvae fed on tobacco (◇). The lines are third-order polynomial curve-fits (except for decamethonium which is a first-order linear regression) with *r*-coefficients above 0·9. Half-maximal responses were: nicotine (○), 91 μmol l^{-1}; carbachol (●), 117 μmol l^{-1}; decamethonium (□), 880 μmol l^{-1}; TMA (▲), 13 800 μmol l^{-1}. The estimated half-maximal response to nicotine for PPR from nicotine-fed larvae was 120 μmol l^{-1} and for prepupae 96 μmol l^{-1}.

specialized pump mechanisms that protect synapses from toxic compounds. It is possible that alkaloid pumps, similar to those described in the Malpighian tubules of insects (Nijhout, 1975; Maddrell & Gardiner, 1976), are also present in the CNS to provide nicotine resistance. We therefore attempted to interrupt the working of these putative transport systems. Previously it has been shown that alkaloid pumps in insect Malpighian tubules will transport a variety of bases, such as atropine or histamine, thereby competitively inhibiting excretion of nicotine (Maddrell & Gardiner, 1976). However, since both these bases are pharmacologically active in the CNS, they were unsuitable for use as pump inhibitors in our experiments. Instead, a similar base, *N*-methyl nicotinamide (NMN), which is actively transported by organic cation pumps (Sperber, 1948) and is reported to be pharmacologically inactive in *M. sexta* (Morris, 1984), was used to inhibit the alkaloid pumps competitively. In these experiments, a ganglion was exposed to a certain concentration of nicotine, washed and then perfused with 1×10^{-3} mol l^{-1} NMN. This concentration of NMN alone caused only a slight and reversible change in PPR's resting potential (1·8 mV ± 1·6 S.D., $N = 11$). When the resting potential was stable, the nicotine application was repeated in the continued presence of NMN. As shown in Fig. 5A, a given concentration of nicotine

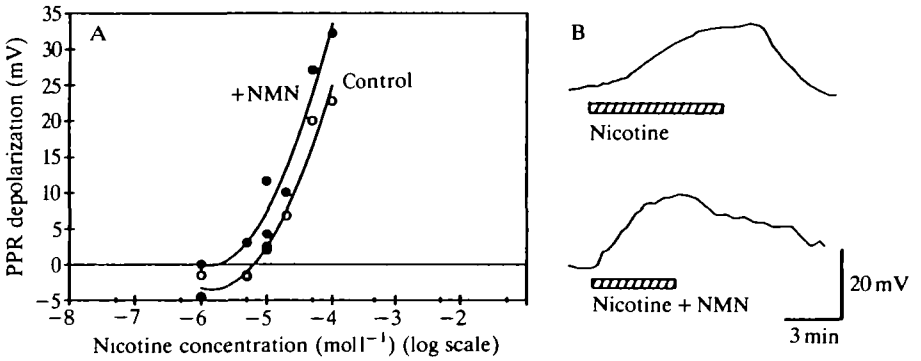


Fig. 5. *N*-Methyl nicotinamide (NMN) influences the size and rate of PPR's response to nicotine. (A) Each ganglion was tested with a single concentration of nicotine, first without NMN and then, after a 20 min wash, with NMN at a concentration of 1×10^{-3} mol l^{-1}. Each point is a single measurement. The depolarization evoked by nicotine was measured from the resting potential immediately before nicotine application to the plateau value achieved at approximately 7 min after the start of the response. The threshold of the response was apparently unaffected by NMN, but the response at higher concentrations was slightly potentiated. (B) During nicotine application to PPR, the rate of depolarization was increased and the rate of repolarization decreased by the presence of NMN. These examples show the response of PPR to 5×10^{-5} mol l^{-1} nicotine before (upper trace) and during (lower trace) exposure to NMN (1×10^{-3} mol l^{-1}) and were taken from a continuous record from a single cell. These traces were made by filtering spikes and other high-frequency changes and then measuring the membrane potential at 10 s intervals. The times taken for the depolarization to decay to half its peak value are 64 s (upper trace) and 151 s (lower trace).

produced a slightly greater depolarization when NMN was present. In addition, in the presence of NMN, the rate of depolarization in response to nicotine increased, and the rate of repolarization decreased (Fig. 5B). These observations are consistent with the presence of protective basic pumps that help to remove nicotine from pharmacologically sensitive regions of the nerve cord. However, even in the presence of NMN, *M. sexta* neurones were not as sensitive to nicotine as the cockroach motoneurone Df has been shown to be (David & Sattelle, 1984); the D_{50} of PPR's response to nicotine in the presence of NMN is estimated to be 18 μmol l^{-1} (Fig. 5A). Furthermore, NMN did not affect the absolute threshold of PPR's response to nicotine (Fig. 5A), implying that alkaloid pumping is not the only factor involved in *M. sexta*'s nicotine-resistance.

In contrast to the effects of NMN, it was observed that the acidic pump inhibitor, hippuric acid (Nijhout, 1975), had no effect on the threshold for nicotine-induced depolarization or on the response to suprathreshold concentrations of nicotine, even at a concentration of 1×10^{-1} mol l^{-1} (data not shown). PPR's resting potential and its response to afferent stimulation were completely unaffected by the application of hippuric acid.

Nicotine-resistance in prepupae

It has been reported that prepupal *M. sexta*, 60 h after the gut purge in the fifth instar, have little or no capacity to transport organic dyes across either the midgut or the Malpighian tubule epithelia (Nijhout, 1975). This inability suggests that alkaloid pumps become inoperative as larvae begin the pupal transformation. We tested the nicotine-sensitivity of PPR in these prepupal insects and found it to be the same as that for pre-wandering larvae (Fig. 4B).

Nicotine-fed larvae

Since our *M. sexta* colony is reared on an artificial diet without exposure to nicotine (or other alkaloids), we tested the nicotine-sensitivity of hatchling larvae taken from our colony and reared to the fifth instar on tobacco plants or on a diet containing nicotine (see Materials and methods). The nicotine dose–response curve of PPR in these animals was very slightly shifted to the right of that of our diet-reared animals, indicating a lower nicotine-sensitivity, but was within the normal range (Fig. 4B). This finding suggests that inducible nicotine-resistance does not play a role in PPR's nicotine-insensitivity within a single generation.

Lobeline

Lobeline, the active alkaloid constituent of Indian tobacco (*Lobeliaceae* sp.), is reported to be a nicotinic agonist in vertebrate ganglia (Volle & Koelle, 1970; Lambert *et al.* 1980) and in *M. sexta* (Morris, 1984). However, lobeline's effects on PPR were quite distinct from those of the other nicotinic agonists that we tested. Exposure of PPR to lobeline at concentrations of 5×10^{-4} mol l^{-1} and higher had no consistent effect on the R_{in} and had very little effect on the resting potential of PPR (six cells showed no depolarization, five others had a mean depolarization of $5{\cdot}6\pm2{\cdot}5$ mV S.D., Fig. 6A). The most striking effect of lobeline was to cause spikes in PPR to lengthen and to occur in bursts until they became plateau depolarizations lasting several seconds (Fig. 6B). During this response, spike activity in the VN disappeared. The changes in action potential duration were reversible (Fig. 6B). Lobeline (5×10^{-4} mol l^{-1}) also caused immediate suppression of the afferent-evoked EPSP in all the preparations ($N=4$), an effect that was not reversible (Fig. 6C). Concentrations of lobeline lower than 1×10^{-5} mol l^{-1} had no detectable effects.

Decamethonium

Bath-applied decamethonium, at concentrations higher than 5×10^{-5} mol l^{-1}, caused PPR to depolarize in a concentration-dependent manner (Fig. 4), but in all preparations this depolarization was accompanied by slow oscillations of the membrane potential around the new level ($N=6$; Fig. 6D,E). The afferent-evoked EPSP, although diminished during depolarization, was never completely suppressed and recovered as the decamethonium was washed out and PPR repolarized (Fig. 6F). Concentrations higher than 1×10^{-3} mol l^{-1} produced unstable membrane potentials (including sudden long-lasting jumps) that prevented

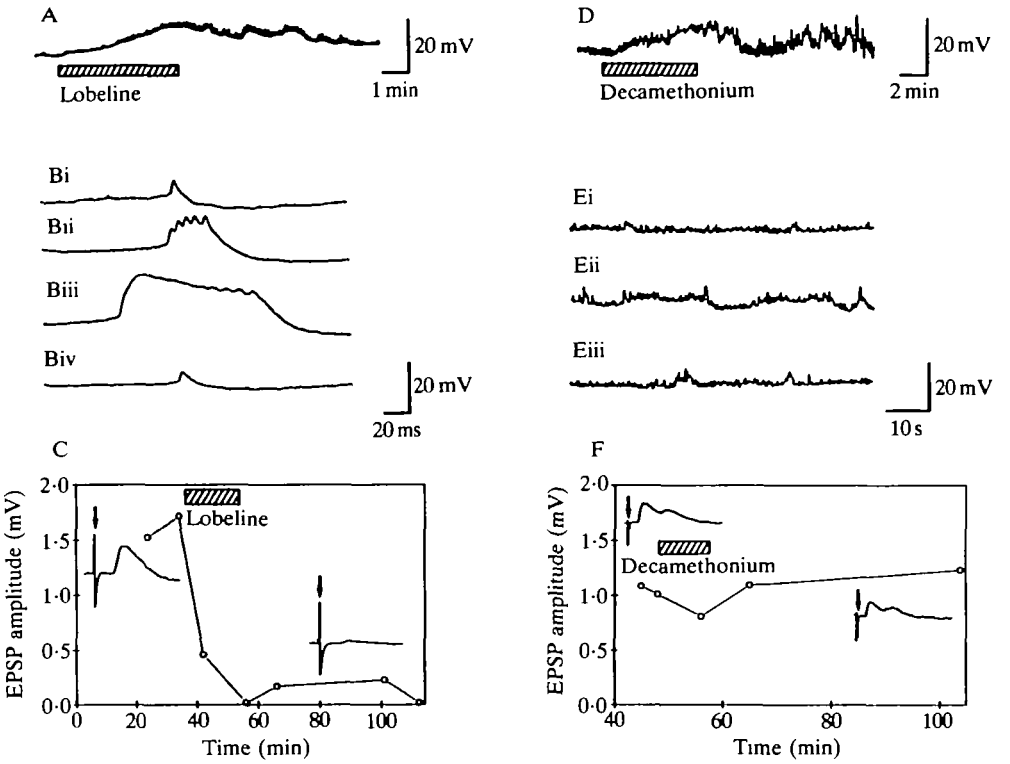


Fig. 6. The effects of bath-applied lobeline and decamethonium on PPR. (A) A high concentration of lobeline (5×10^{-4} mol l^{-1}) caused PPR to depolarize slightly in some preparations. The example shown here is a record of the membrane potential changes evoked by lobeline (filtered to remove spike activity). In more than half the preparations tested, lobeline had no measurable effect on the resting potential of PPR. This example was the largest depolarization seen (see text). (B) The main effect of lobeline was to cause repetitive spiking leading to sustained depolarizations lasting 100 ms or more. (i) A control action potential recorded in the soma of PPR at low gain. (ii, iii) The progressive lengthening of PPR's action potential in the presence of 5×10^{-4} mol l^{-1} lobeline (the trace iii was taken after 5 min exposure). Trace iv was taken after washing the ganglion for 20 min. (C) Lobeline (5×10^{-4} mol l^{-1}) completely suppressed the afferent-evoked EPSP, which could not be fully recovered even after prolonged washing. The insets show examples of averaged EPSPs before and after lobeline application. Arrows mark stimulus artefacts. (D) During exposure to decamethonium (1×10^{-4} mol l^{-1}) PPR depolarized and began to show membrane potential fluctuations. The recording has been filtered such that the small, rapid voltage transients consist mainly of spontaneous EPSPs.(E) The oscillations shown in D are shown here on an expanded time scale. The first trace was taken before exposure to decamethonium, the middle trace at the plateau of the response (5 min) and the third trace after 7 min of washing. (F) During exposure to decamethonium the afferent-evoked EPSP is reversibly inhibited. The insets show examples of averaged EPSPs before and during decamethonium (1×10^{-4} mol l^{-1}) treatment. The hump on the repolarization part of the initial monosynaptic response results from additional polysynaptically mediated input from this afferent (Weeks & Jacobs, 1987). Arrows mark stimulus artefacts.

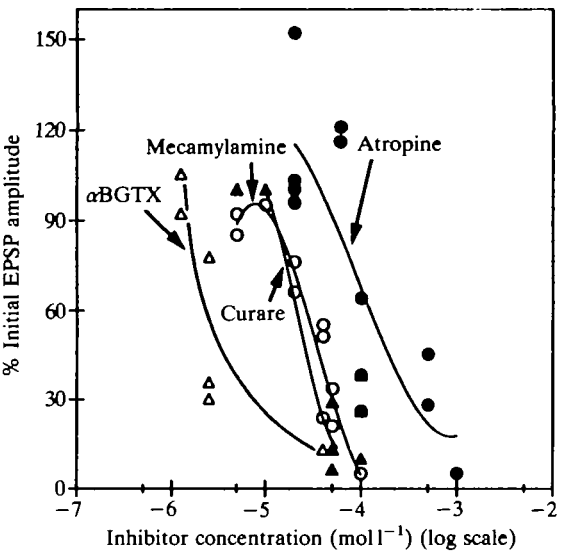


Fig. 7. The effect of cholinergic antagonists on EPSP size. The size of the EPSP is expressed as a percentage of its initial size before drug application. All the measurements were the average peak amplitude of 60 EPSPs. Averages were determined 10 min after drug application except for α-bungarotoxin (αBGTX) where determinations were made 40 min after application. αBGTX (△); mecamylamine (○); *d*-tubocurarine (▲); atropine (●). The lines drawn through the points are fitted by eye.

estimation of EPSP amplitude or the depolarized resting potential. This observation contrasts with the reported antagonistic actions of decamethonium in the cockroach *P. americana* (Carr & Fourtner, 1980; David & Sattelle, 1984) and in the crab *Cancer pagurus* (Marder & Paupardin-Tritsch, 1978, 1980).

Nicotinic antagonists

In vertebrates, nAChRs expressed in muscle or in different parts of the nervous system can be distinguished by their sensitivity to various antagonists. Thus, in most vertebrates, curare and αBGTX are potent antagonists of the muscle nAChRs (Bovet *et al.* 1959; Changeux *et al.* 1970) and the vertebrate brain nAChR is preferentially blocked by mecamylamine. In contrast, the ganglionic nAChR is particularly sensitive to hexamethonium (Taylor, 1980).

In *M. sexta*, curare, mecamylamine and very high concentrations of αBGTX all inhibited the monosynaptic EPSP in PPR (Fig. 7). Atropine, which is considered to be a general muscarinic blocking agent in most vertebrates, was also quite effective at inhibiting the EPSP. This inhibition of fast cholinergic neurotransmission by atropine has also been demonstrated in other insect species (Carr & Fourtner, 1980; Sattelle, 1980). For low concentrations of curare, mecamylamine and atropine, the inhibition was at least partially reversible; i.e. the EPSP typically returned to 80 % of its control value after 20 min of washing (data not shown). The

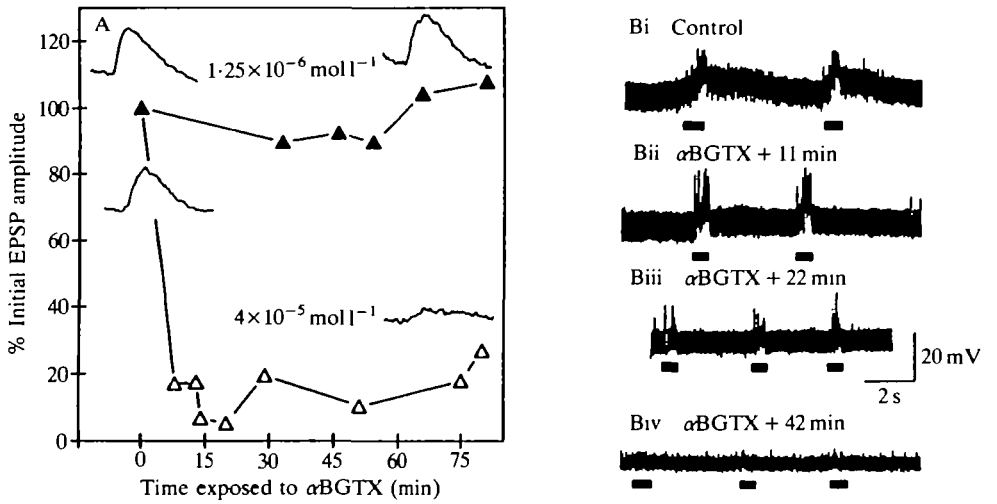


Fig. 8. Effects of α-bungarotoxin (αBGTX) on EPSP size and sensory responsiveness. (A) Prolonged exposure of two preparations to two concentrations of αBGTX. At a concentration of $1{\cdot}25\times10^{-6}$ mol l^{-1} there was no consistent decline in the averaged EPSP (▲). The insets above the top trace show averaged EPSPs taken before application of the toxin and after 80 min of continuous perfusion of the toxin over the ganglion. The lower trace (△) shows the rapid but incomplete blockage of the EPSP by 4×10^{-5} mol l^{-1} αBGTX. The lower pair of insets are averaged EPSPs taken before application of the toxin and after 80 min of continuous perfusion of toxin. The inhibited EPSP at 80 min is small but still detectable. (B) Effect of αBGTX on sensory responsiveness. The bar beneath each trace indicates the approximate duration of a light stroke applied to the planta hair array with a small probe. (i) This stimulus causes a prolonged depolarization of PPR and an increased rate of spiking (see also Weeks & Jacobs, 1987). (ii–iv) High concentrations of αBGTX progressively reduce this response. Traces ii–iv were taken after 11, 22 and 42 min in 4×10^{-5} mol l^{-1} αBGTX. Spike size decreased during this long experiment, but (iv) the resting potential, spike frequency and R_{in} were normal.

potencies of these agents are slightly less than those required to block ACh-induced responses in the cockroach motoneurone Df (David & Sattelle, 1984; see Discussion). The vertebrate ganglionic blocking agent hexamethonium was a very poor antagonist, requiring concentrations higher than 1×10^{-2} mol l^{-1} to produce even partial blockade of the EPSP ($N = 3$, results not shown). Surprisingly, very low concentrations of atropine sometimes caused a potentiation of the EPSP (Fig. 7) and this finding will be discussed below.

An important difference between the responses of cockroach motoneurone Df and the hornworm motoneurone PPR is in the relative insensitivity of PPR to αBGTX. At a concentration of 1×10^{-6} mol l^{-1}, there was no detectable block of the EPSP, even after 2 h of continuous perfusion ($N = 3$; data not shown). At concentrations higher than 1×10^{-5} mol l^{-1}, αBGTX suppressed both the unitary EPSP (Fig. 8A) and the compound synaptic response caused by lightly brushing the planta hair array (Weeks & Jacobs, 1987; Fig. 8B). Inhibition of the EPSP

occurred within 30 min, but blockade was incomplete and could be partially reversed by high-frequency stimulation of the afferent (B. A. Trimmer & J. C. Weeks, unpublished observations). This relative insensitivity may have resulted from a slow penetration of the high molecular weight toxin, so we attempted to remove possible diffusion barriers by treating the desheathed ganglion with collagenase–dispase (3 %; w/v) for 15 min before applying the toxin (see Discussion). Although this procedure caused some instability of the long-term recordings, the EPSP was still detectable after perfusing toxin (1×10^{-6} mol l^{-1}) over the ganglion for 2 h ($N = 3$).

The very high doses of αBGTX needed to inhibit the EPSP were in a range where both non-specific lipase contamination (Chang & Lee, 1963; Tu, 1977) and the presence of neuronal bungarotoxin (also called κ-bungarotoxin, toxin-F and Bgt 3.1) in the sample could have produced the blockade (Ravdin & Berg, 1979). However, exposure to 2×10^{-6} mol l^{-1} neuronal bungarotoxin for periods up to 45 min also failed to suppress the afferent-evoked EPSP ($N = 4$, data not shown).

Muscarinic agents

No functional role has been established for putative muscarinic receptors in the cockroach. In a study of the synapse between filiform hair sensory neurones and giant interneurone 3 in the cockroach, bath application of several muscarinic agonists or antagonists failed to evoke any detectable effect on the pre- or postsynaptic cells or on the afferent-evoked EPSP (Blagburn & Sattelle, 1987). In *M. sexta*, however, PPR appears to be sensitive to a variety of muscarinic agents. Bath application of the archetypal muscarinic agonist DL-muscarine at concentrations higher than 1×10^{-6} mol l^{-1} caused membrane oscillations and bursts of action potentials in PPR (Fig. 9A). These effects were not accompanied by any consistent change in the R_{in} of PPR at the cell body (Fig. 9B; see also Fig. 12A).

Bath application of the muscarinic agonist oxotremorine caused an increased firing rate of neurones in the VN (and the dorsal nerve) at extremely low concentrations (threshold at 1×10^{-9} mol l^{-1}; Fig. 9C). This effect was prevented by cotreatment with atropine (5×10^{-5} mol l^{-1}; results not shown), which suggests that it was receptor-mediated. When spiking in the ganglion was reduced with high-divalent saline, application of oxotremorine elicited membrane oscillations in PPR similar to those evoked by muscarine. The physiological basis of oxotremorine's actions was hard to determine since the general level of activity in the ganglion increased to such an extent that it became very difficult accurately to measure the resting potential or R_{in} after this drug had been applied.

Ionophoresis of muscarine into the ipsilateral or contralateral neuropile regions elicited a small depolarization of PPR, followed by a prolonged period of spike activity (Fig. 10A). Muscarine produced essentially the same increased tendency to repetitive spiking when synaptic transmission was blocked with 0 mmol l^{-1} Ca^{2+}/20 mmol l^{-1} Mg^{2+} saline (Fig. 10B). Synaptic blockade in these experiments was monitored by the loss of transmission at the afferent-to-PPR synapse and by the complete absence of spontaneous EPSPs in PPR. During this synaptic

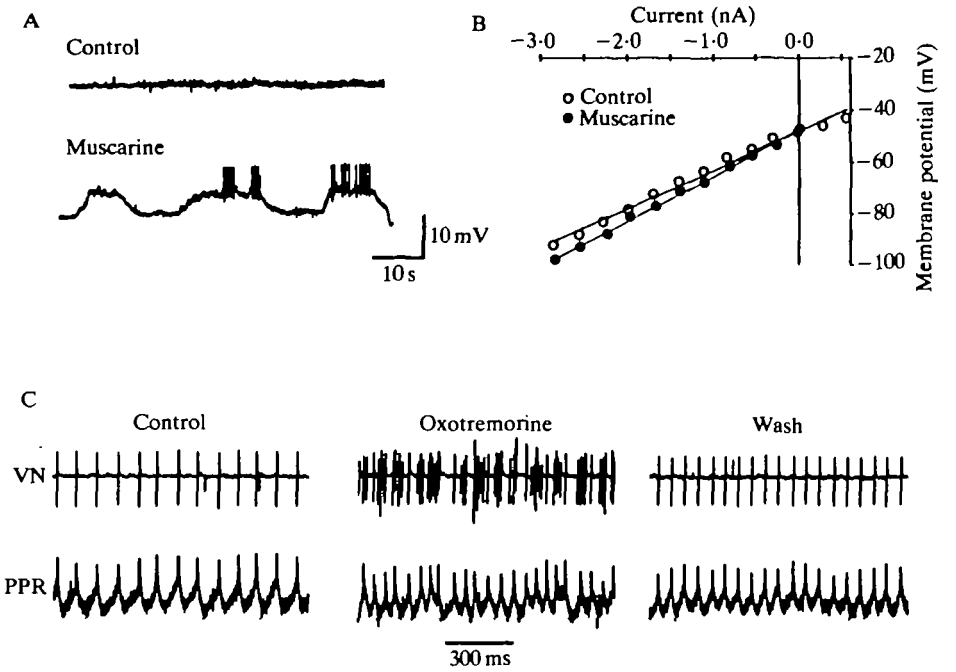


Fig. 9. The effects of bath-applied muscarinic agonists on PPR. (A) Bath applied muscarine (1×10^{-5} mol l^{-1}) caused oscillations in PPR's membrane potential. The upper trace is a control showing the stable resting potential (−46 mV) before applying muscarine. The second trace was taken 10 min after muscarine application and shows the rhythmic oscillations in the membrane potential that persisted for as long as the agonist was present. (B) During muscarine application there was no detectable decrease in PPR's input resistance (R_{in} for control = 15·1 MΩ, R_{in} for muscarine = 17·7 MΩ), nor was there any change in its resting potential (resting potential for control, −48·2 mV; resting potential in muscarine, −47·8 mV, measured between the periodic depolarizations at zero current). All the data points are from the same preparation. (C) Extracellular recordings of the VN (upper trace) show that bath application of the muscarinic agonist oxotremorine (1×10^{-9} mol l^{-1}) to the ganglion dramatically increases the firing rate of VN motoneurones. Intracellular recordings from PPR (lower trace) show that PPR also increases its firing rate with very little change in resting potential. The oxotremorine trace was recorded after 5 min of exposure to the drug and the effect was reversed after 20 min of perfusion in regular saline (wash). Note that PPR's action potential recorded in the VN is time-locked to the intracellular spike.

blockade, the addition of the nicotinic antagonist mecamylamine, at a concentration (4×10^{-4} mol l^{-1}) sufficient completely to suppress responses to ionophoretically applied nicotine (Fig. 2A) and to block afferent-evoked EPSPs (Fig. 7), also failed to prevent muscarine-induced spiking (Fig. 10B).

The effects of muscarine had marked consequences upon the response of PPR to afferent stimulation. In normal saline without nicotinic blocking agents, a brief (1 s) stimulation of the branch of the VN that carries planta-hair afferent axon

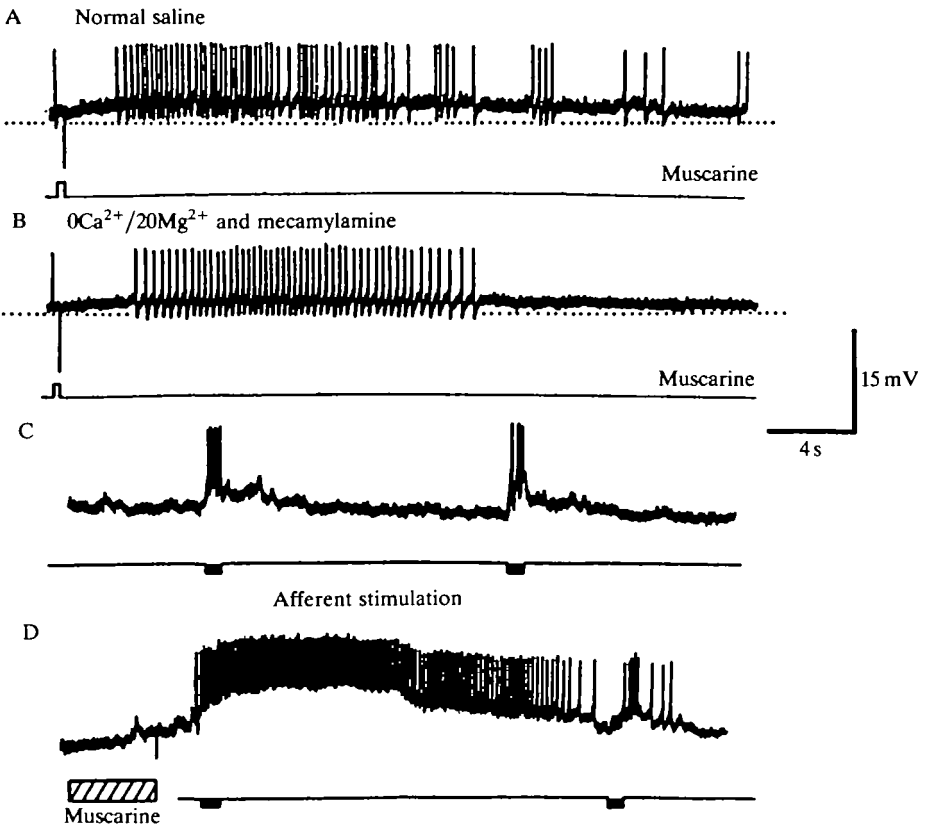


Fig. 10. Muscarine potentiates PPR's response to sensory stimulation. (A) Intracellular recordings from PPR show that in normal saline an ionophoretic pulse of muscarine into the ipsilateral neuropile (300 ms, 15 nA, lower trace) evokes a small, slow depolarization and a train of action potentials that gradually diminish in frequency. The dotted line below the intracellular record is a reference for the resting potential. (B) In the same preparation as A, suppression of synaptic activity with 0 mmol l^{-1} Ca^{2+}/20 mmol l^{-1} Mg^{2+} saline and complete blockade of all nicotinic responses with mecamylamine (5×10^{-5} mol l^{-1}) fails to prevent the muscarine-induced train of action potentials. Ionophoretic application of nicotine has no effect under these conditions. (C) Intracellular recordings from PPR in normal saline show PPR's response to a 1 s train of shocks (50 Hz, 10 V, 1 ms duration) delivered to the planta hair sensory nerve (VN_a). PPR was slightly hyperpolarized throughout the experiment to decrease spontaneous firing. During each stimulus (dark bar beneath each record) PPR depolarized and fired several action potentials. (D) D follows C and shows that ionophoresis of muscarine (3 s, 10 nA; hatched bar) immediately before the VN_a stimulus greatly potentiated PPR's response to the sensory stimulation. As the response decayed, a second VN_a stimulus elicited a response which was greater than the control (C) but much smaller than that evoked immediately after ionophoresis. Muscarine itself caused a depolarization (6 mV) of PPR but no action potentials (data not shown).

(VN_a) caused a brief depolarization of PPR and elicited a burst of action potentials (Fig. 10C). In Fig. 10D, an ionophoretic pulse of muscarine delivered prior to the VN_a stimulus greatly potentiated PPR's response to sensory stimulation. Approximately 20 s after ionophoresis stopped, a second VN_a stimulation evoked a response that was only slightly larger than the control (Fig. 10D). In the same preparation, delivery of the muscarine pulse alone evoked a 6 mV depolarization of PPR but no action potentials (data not shown). Thus, muscarine ionophoresis and VN_a stimulation combined to produce a much greater response than that elicited by either stimulus alone, i.e. PPR's response to sensory stimulation was potentiated.

The sensitivity of PPR to muscarine during synaptic blockade (Fig. 10B) suggests that muscarine's actions are exerted directly upon PPR (i.e. they are not mediated by other cells or neurotransmitters) and are independent of the nAChRs. This hypothesis was supported further by the finding that PPR's response to ionophoretic pulses of muscarine delivered in different regions of the neuropile corresponded to the location of its dendritic arbor (Fig. 11). PPR is the only motoneurone with an axon in the VN that has a dendritic arbor within the contralateral neuropile; the arbors of all other VN motoneurones remain ipsilateral to their cell bodies (Weeks & Truman, 1984; Weeks & Ernst-Utzschneider, 1989). Correspondingly, in the presence of mecamylamine and 0 mmol l^{-1} Ca^{2+}/20 mmol l^{-1} Mg^{2+} saline, a brief pulse of muscarine delivered into the ipsilateral neuropile evoked spiking activity simultaneously in PPR and a number of other VN motoneurones (Fig. 11A). In contrast, when muscarine was ionophoresed into the contralateral neuropile, PPR was consistently the first VN motoneurone to respond, with the other units being unaffected or (in two out of six experiments) responding after a delay of more than 20 s (presumably after diffusion of muscarine into the ipsilateral neuropile). This correlation between PPR's morphology and its responses to muscarine supports the notion that mAChRs are located on the motoneurone itself.

Spike threshold

PPR's increased spiking in the presence of muscarine appeared to be caused primarily by a lowered spike threshold. Although this effect could be seen in normal saline (Fig. 10A,D), it was most easily observed by increasing the spike threshold with high-divalent saline and simultaneously blocking nicotinic responses with mecamylamine. Under these conditions ionophoretic pulses of muscarine resulted in a small (5 mV) depolarization of PPR which lasted for many seconds (Fig. 12A). During this depolarization PPR's R_{in} (indicated by the size of the voltage deflections produced by constant current pulses) changed minimally (in the example shown in Fig. 12A, R_{in} was 11·5 MΩ before, and 10·4 MΩ during, the response). During the muscarine response PPR's spike threshold, measured by increasing current injection under current-clamp conditions, was markedly reduced (Fig. 12Bii) from the pre-muscarine level (Fig. 12Bi). Injection of hyperpolarizing current during the response, to restore PPR's membrane potential

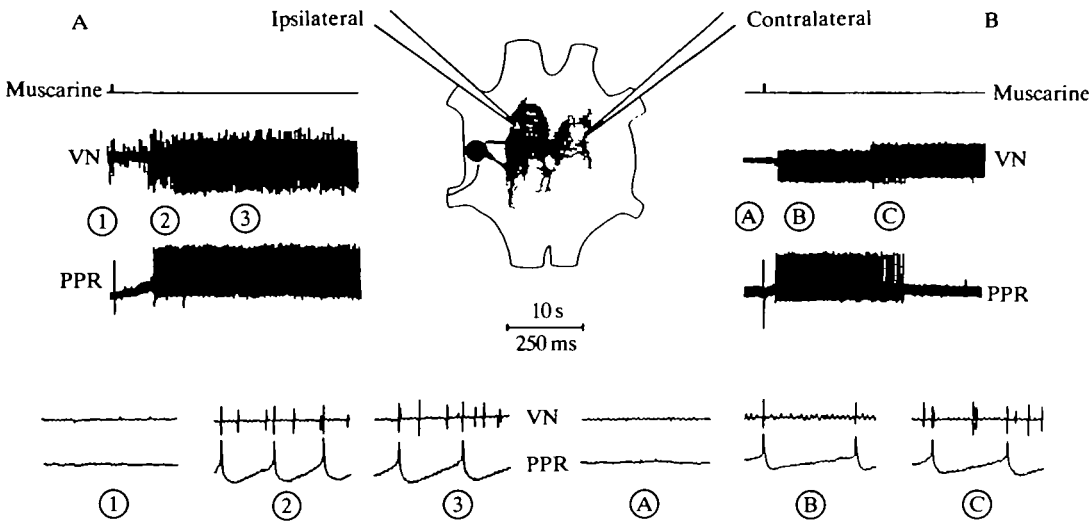


Fig. 11. PPR responds to muscarine ionophoresis in both the ipsi- and contralateral neuropile. PPR is the only motoneurone with an axon in the VN which also has a contralateral arbor. In this experiment ionophoresis of muscarine (indicated by the upper trace) into either the ipsilateral (A) or the contralateral (B) neuropile evoked a train of action potentials in PPR. Extracellular recordings from the ventral nerve (VN) show that motoneurones with axons in this nerve respond rapidly to ipsilateral application of muscarine at approximately the same time as PPR. However, contralateral ionophoresis rarely activates units other than PPR. When it does, as shown in this example, they begin to fire after a prolonged delay (20 s or more), presumably after diffusion of muscarine to the ipsilateral side. This experiment was carried out in 0 mmol l^{-1} Ca^{2+}/20 mmol l^{-1} Mg^{2+} saline to inhibit synaptic interactions. The same ionophoretic electrode was inserted first into the ipsilateral and then the contralateral neuropile. The lower records show parts of the traces [at times indicated by the numbers (ipsilateral) and letters (contralateral)] on an expanded time scale to demonstrate the motor units in the VN and the time-locked spikes of PPR.

to its level before muscarine application, did not block the muscarine-induced lowering of the spike-threshold (Fig. 12Biii), implying that the effect is independent of the change in potential measured at the soma. The original spike threshold was completely restored several minutes after the ionophoretic pulse (Fig. 12Biv). This muscarine-induced change in threshold could be blocked by 1×10^{-5} mol l^{-1} scopolamine methyl bromide (data not shown) suggesting that it is receptor-mediated. In contrast to the effects of muscarinic agents, ionophoretic pulses of nicotine, either in the presence or absence of mecamylamine, had no detectable effect on the subsequent spike threshold of PPR (data not shown).

The effect of muscarinic agents on the EPSP

During bath application of the muscarinic agonists oxotremorine and muscarine the averaged EPSP was markedly suppressed; this effect is illustrated for a very low concentration of oxotremorine (1×10^{-9} mol l^{-1}) in Fig. 13A. Because oxotre-

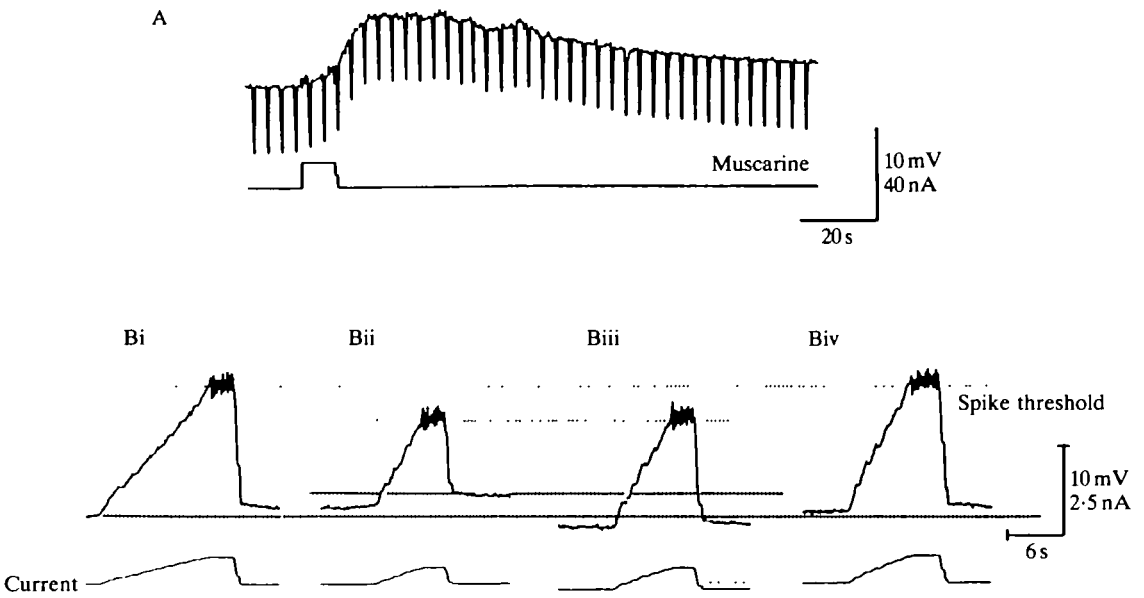


Fig. 12. Muscarine lowers the spike threshold of PPR. (A) Ionophoretic application of muscarine to the ipsilateral neuropile (indicated by the lower trace), evokes a small, prolonged depolarization of PPR. In this example, pulses of hyperpolarizing current (−0·6 nA, 500 ms) delivered every 3 s give an estimate of the R_{in}. This experiment was carried out in the presence of high-divalent saline (to suppress action potentials) and 5×10^{-5} mol l^{-1} mecamylamine (to block nicotinic responses). (B) In the same preparation as A, PPR's spike threshold was estimated before the ionophoretic pulse (i), during the depolarization response (ii, iii) and 3 min after the response (iv). The upper trace shows PPR's membrane potential during a ramp of depolarizing current shown in the lower trace. In the PPR trace the dotted lines indicate the critical potential for action potential production (spike threshold) and dashed lines are used to indicate the resting potential levels. During the muscarine response (ii) PPR depolarizes and the spike threshold is lowered (for this preparation the spike threshold was lowered 3·2+1·7 mV S.D.; $N = 6$ applications of muscarine). The change in spike threshold caused by muscarine does not depend on the muscarine-induced depolarization. This is demonstrated in trace iii (taken immediately after trace ii), in which PPR was hyperpolarized to hold its membrane potential below the control level before muscarine ionophoresis (the dotted line on the current trace of iii indicates the current level before this steady hyperpolarizing current was applied). This hyperpolarization of PPR had no effect on spike threshold (iii). Spike threshold returned to control levels after a minute or so (trace iv, taken 12 s after ionophoresis). All records were made under current-clamp conditions.

morine causes increased spike activity in the ganglion (Fig. 9C), this EPSP suppression could have resulted from a masking effect caused by many non-time-locked synaptic inputs, but this finding is also consistent with the postulated presence of presynaptic mAChRs that, when stimulated, inhibit the release of ACh from the afferent terminal (Breer & Knipper, 1984). In support of this possibility, the muscarinic antagonist scopolamine methyl bromide, applied a

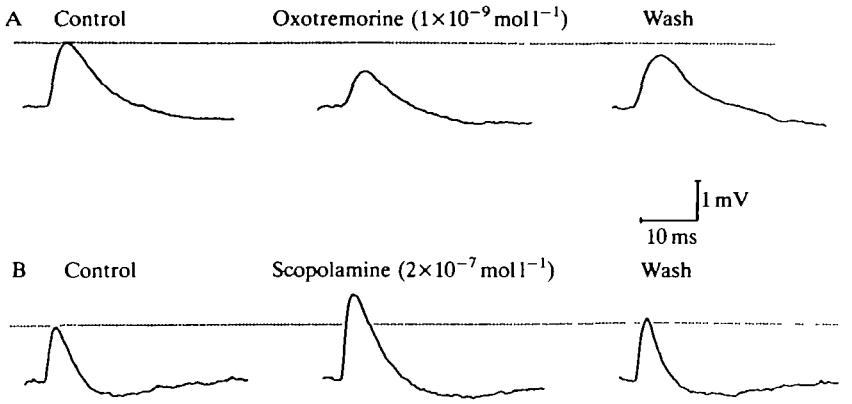


Fig. 13. Muscarinic agents reversibly influence EPSP size. (A) Oxotremorine at 1×10^{-9} mol l^{-1} caused suppression of the afferent-evoked EPSP. Each trace is an average of 60 EPSPs, before drug application (control), 5 min after applying oxotremorine, and after washing for 20 min (wash). (B) The muscarinic antagonist, scopolamine, when bath-applied at 2×10^{-7} mol l^{-1}, caused a reversible increase in the size of the averaged afferent-evoked EPSP. This concentration did not affect the firing rate of VN motoneurones, PPR's resting potential or its spontaneous firing rate. Concentrations higher than 1×10^{-4} mol l^{-1} caused a reversible inhibition of the EPSP (not shown).

very low concentrations (2×10^{-7} mol l^{-1}), was found to increase the amplitude of the averaged monosynaptic EPSP (Fig. 13B). Similar potentiation was also observed with low concentrations of atropine (see Fig. 7). The potentiation occurred with no measurable effect on the resting potential or R_{in} of PPR (data not shown). These observations are consistent with the possibility that presynaptic mAChRs serve to modulate the release of ACh from the afferent terminals.

Discussion

Nicotine-resistance

The main toxicity of nicotine in mammals results from its powerful agonistic action at the nAChR. Most insects are also sensitive to nicotine, which has led to its extensive use as an insecticide (David & Gardiner, 1953; Eldefrawi, 1985). One major site of its action in insects has been presumed to be central sensory synapses where the primary neurotransmitter is ACh (Sattelle, 1985). Interestingly, *M. sexta* is remarkably resistant to nicotine, even though ACh is used as a neurotransmitter at its sensory synapses. This role for ACh has been demonstrated in the antennal lobes of adult *M. sexta* (Sanes *et al.* 1977; Sanes & Hildebrand, 1976; Hildebrand *et al.* 1979) and the present work supports the same role for ACh in the larval afferent-to-PPR synapse. The nicotine-resistance of *M. sexta* larvae is evident from their ability to feed and grow normally on tobacco plants (Reynolds

et al. 1986) which are toxic to most insects. Various studies have shown that much of this chronic tolerance is achieved by the rapid excretion of the absorbed alkaloid by the Malpighian tubules (Self *et al.* 1964; Maddrell & Gardiner, 1976). However, the ability to excrete alkaloid is unlikely to be the only factor in *M. sexta's* resistance, since another lepidopteran, *Pieris brassicae*, is also capable of excreting unmetabolized nicotine, yet is easily killed by nicotine in its diet (Maddrell & Gardiner, 1976). In addition, injections of high concentrations of nicotine directly into the haemolymph do not incapacitate *M. sexta*, suggesting that the nervous system has its own means of protection (Self *et al.* 1964).

One possible mechanism for this nicotine-resistance could be a selective barrier preventing nicotine from reaching the receptors, but flux studies suggest that nicotine penetrates the nerve cords of *M. sexta* and the non-resistant cockroach *P. americana* equally well (Morris, 1983*a*). In related studies it was established that *M. sexta* nerve cords metabolize nicotine to a greater extent than do those of *P. americana*, forming water-soluble conjugates (Morris, 1983*b*). In addition, the kinetics of nicotine/metabolite efflux in *M. sexta* are much slower and less complicated than in *P. americana* (Morris, 1983*c*). This indicates that nicotine-processing in *M. sexta* differs from that of non-resistant insects.

Using 'global extracellular' recording methods, Morris estimated the nerve cord of *M. sexta* to be two orders of magnitude less sensitive to nicotine than that of *P. americana* (Morris, 1984). The intracellular recording methods used in the present study have enabled us to study this resistance in greater detail at the level of an identified motoneurone and its direct sensory input. We have found that even in desheathed preparations motoneurone PPR in *M. sexta* is far less sensitive to nicotine than the cockroach Df motoneurone (David & Sattelle, 1984). However, this effect is remarkably selective, since the dose-dependence of responses to other nicotinic agonists such as carbachol and TMA were similar in the two species. The rapid rate of perfusion used in the present study, together with the long incubation times, makes it seem unlikely that metabolic conversion accounts for all the protection. However, it should be noted that if the perfusion system is switched off during a nicotine-induced depolarization, a very gradual recovery of resting potential is seen, indicating that nicotine breakdown may occur (B. A. Trimmer & J. C. Weeks, unpublished observations).

Morris (1984) reported that NMN increased the sensitivity of nerve cords to nicotine. We have found that this increase is not caused by a direct agonistic action of NMN on PPR, but is consistent with an inhibition of alkaloid transport from the extracellular space. The application of NMN results in a slight increase in the magnitude of the depolarization induced by a particular concentration of nicotine, but its main effect is to increase the rate of response and to slow the rate of recovery. NMN is known to inhibit cationic transport in the kidney (Sperber, 1948) and would be expected to compete with nicotine for the organic cation pumps such as those in the Malpighian tubules (Maddrell & Gardiner, 1976). In contrast, hippuric acid, an inhibitor of acidic (anionic) transport in the Malpighian tubules and gut of *M. sexta* (Nijhout, 1975), is without effect on the nicotine response. Thi

observation suggests that the transport of acidic metabolites of nicotine is not involved in nicotine resistance.

The continued (although diminished) resistance to nicotine after NMN treatment and during a developmental stage (prepupal) when cationic pumps are inactive suggests that something other than active exclusion is involved in *M. sexta*'s adaptive resistance. Possibilities include a selective permeability barrier within the CNS or a modification of the nAChR itself. Morphological studies indicate that in *M. sexta* the tight junctions of the perineurium surrounding the CNS constitute the major blood–brain barrier from the late embryonic to the adult stage (Lane & Swales, 1979). Previous work has established that the perineurium also plays some role in nicotine-resistance (Morris, 1984). However, in the present study great care was taken to remove as much of the sheath as possible. Although there is almost certainly a further diffusion barrier to molecules penetrating the inner neuropilar regions, the rapid effectiveness (and reversibility) of most nicotinic antagonists at the deep afferent-to-PPR synapse (Weeks & Jacobs, 1987) suggests that this barrier is permeable to small molecules. A breakdown of the blood–brain barrier takes place during adult development (Lane & Swales, 1979) but PPR cannot be studied at this time because it undergoes a programmed death during the pupal stage (Weeks & Truman, 1985). However, another proleg motoneurone, the accessory planta retractor (APR), which also receives monosynaptic afferent input (Weeks & Jacobs, 1987), is cholinoreceptive (B. A. Trimmer, unpublished observations) and survives to adult eclosion (Weeks & Truman, 1984; Weeks & Ernst-Utzschneider, 1989), could be used for developmental studies of nicotine-resistance. The prepupal insects used in part of the present study were at a stage before the major changes occur in the fine structure of the neural lamella and perineurium of the connectives (McLaughlin, 1974) or in the ganglia themselves (Lane & Swales, 1979).

The emerging evidence for heterogeneity in neuronal nAChRs of vertebrates (Boulter *et al.* 1987) is consistent with the possibility that nicotine-resistance in *M. sexta* is achieved by receptor modification. There is evidence that different isoforms of the nAChR are present in nicotine-resistant and wild-type *Drosophila* (Hall *et al.* 1978). Expression studies using cDNA clones coding for mammalian neuronal nAChRs have established that combinations of different α-subunits with the β_2-subunit result in pharmacologically distinct subtypes of the functional nAChR (Heinemann *et al.* 1988; Papke *et al.* 1988; Wada *et al.* 1988; Boulter *et al.* 1987). It is conceivable that a similar genetically based alteration of the insect nAChR could be achieved by the production of a novel subunit type which gives rise to the pharmacological specializations seen in the *M. sexta* receptor. It is also possible that resistance is conferred by post-translational modification of an otherwise 'typical' insect nAChR. These sorts of changes could have arisen during evolution as an adaptation to an alkaloid-rich diet and may have given rise to the pharmacological specialization and apparent lack of desensitization found in the present study (see below). In either case, it is clear that resistance to nicotine is an intrinsic attribute of *M. sexta* rather than an inducible trait (e.g. Maddrell &

Phillips, 1978), since our insect colony has not been exposed to nicotine for at least 100 generations and we detected no further resistance in insects reared on tobacco plants or a nicotine-laced diet.

Pharmacological specialization

The pharmacological responses of PPR show several marked differences from those reported in other insects. For example, although decamethonium acts as a nicotinic antagonist in *P. americana* (David & Sattelle, 1984) it causes PPR to depolarize in *M. sexta*. Moreover, although decamethonium does not give rise to detectable muscarinic physiology in the cockroach (David & Sattelle, 1984; Blagburn & Sattelle, 1987), it causes membrane oscillations in *M. sexta* similar to those evoked by muscarine. These effects of decamethonium in *M. sexta* could be mediated by a 'mixed' receptor, such as that characterized biochemically (as high-affinity decamethonium binding sites) in houseflies (Harris *et al.* 1981). However, these binding sites have been neither identified nor associated with any physiology, and consequently their nature remains controversial (Sattelle, 1985). Indeed, since decamethonium has been shown to inhibit acetylcholinesterase (AChE) (Thesleff, 1955; Changeux, 1966) this binding may consist largely of AChE. The membrane potential oscillations seen in the present study could have resulted from such indirect effects on ACh metabolism by decamethonium. Morris (1984) reported that the AChE inhibitor, eserine, caused oscillations in the ganglionic potentials of *M. sexta* and, consistent with this observation, we found that another AChE inhibitor, neostigmine, also elicited oscillations in PPR's resting potential at concentrations above 5×10^{-7} mol l^{-1}. An additional possibility is that decamethonium activates both nicotinic and muscarinic-like receptors, but clearly further studies will be required to examine fully and distinguish among these possibilities.

Lobeline is a nicotinic agonist at the vertebrate neuromuscular junction. In extracellular recordings from *M. sexta* (Morris, 1984), the observed transient increase in spike activity of PPR caused by lobeline, followed by a failure of the spikes to propagate in the axons, resembles the stimulation and blockade typical of nAChR activation. At the cellular level, however, lobeline's action is clearly not nicotinic, since there is no sustained depolarization or decrease in R_{in}. Lobeline's actions on PPR resemble those of potassium channel inhibitors, such as tetraethylammonium (TEA), which cause extended action potentials by decreasing the repolarizing potassium flux through the delayed rectifier and other potassium channels (Hille, 1984). Blocking potassium channels with TEA also broadens action potentials in lepidopteran neurones (Miyazaki, 1980; J. C. Weeks & D. J. Sandstrom, unpublished observations).

The effective antagonist concentrations reported in the present study are somewhat higher than those reported to block ACh-induced responses in the cockroach motoneurone Df (David & Sattelle, 1984). This difference in potency may simply arise from a difference in the receptor density at the synapse used in the present study, and the cell body used in David & Sattelle's study. Th

extremely low potency of αBGTX in our study is, however, an order of magnitude less than that reported by David & Sattelle and is 50–5000 times less potent than that reported to block synaptic transmission between cercal sensory neurones and giant interneurone 2 in the cockroach (Sattelle *et al.* 1983). In addition, the evoked reflex from the trochanteral hairplate afferents to motoneurone Ds in the cockroach metathoracic ganglion is blocked within 30 min in the presence of 1×10^{-7} mol l^{-1} αBGTX and within 8 min in 1×10^{-6} mol l^{-1} αBGTX (Carr & Fourtner, 1980). The apparent insensitivity of *M. sexta* receptors to αBGTX may result from a penetration barrier since, unlike the small molecule antagonists discussed above, αBGTX is relatively large (approximately 7800 Da) and slow to diffuse (Freeman *et al.* 1980). Attempts were made to reduce the penetration barrier by enzyme treatment of the ganglion using collagenase–dispase. This enzyme is extremely effective at weakening the sheath prior to sheath removal (Weeks & Jacobs, 1987) and we found that continued exposure caused the ganglion to soften and cell bodies to separate from one another. PPR's mechanical instability within such treated ganglia made it extremely hard to maintain prolonged intracellular recordings and several hours after exposure to the enzyme the resting potential became unstable. Despite these difficulties, we were able to record from three preparations for 2 h in 1×10^{-6} mol l^{-1} αBGTX and could find no evidence for an increased sensitivity to αBGTX. Pinnock *et al.* (1988) found that a combination of collagenase and hyaluronidase significantly increased the rate of blockade of cockroach AChRs by snake toxins and it would be interesting to test these enzymes on *M. sexta*. It is highly unlikely that in *M. sexta* the receptor blockade seen with very high concentrations of toxin can be attributed to contamination by neuronal bungarotoxin, since purified neuronal bungarotoxin had no effect on synaptic transmission, even at 5×10^{-6} mol l^{-1} (by way of contrast, avian and murine ganglionic receptors are completely blocked by 4×10^{-8}–$1{\cdot}5\times10^{-6}$ mol l^{-1} neuronal bungarotoxin; Chiappinelli, 1985). Neuronal bungarotoxin has been reported to inhibit ACh responses of the cockroach motoneurone Df, where it is slightly less potent than αBGTX (Chiappinelli *et al.* 1987; Pinnock *et al.* 1988). We have not yet ruled out the possibility that some other minor and highly potent constituent(s) of the toxin sample may be involved in blockade at very high concentrations. It is also possible that αBGTX does not act upon the nAChRs of PPR. There is certainly a precedent for this insensitivity, since most mammalian neuronal nAChRs neither bind αBGTX nor are blocked by it (Conti-Tronconi *et al.* 1985). Certain cholinoreceptive neurones in orthopterans are unaffected by αBGTX (Goodman & Spitzer, 1979, 1980; Lane *et al.* 1982; Lees *et al.* 1983) and the nAChR α-like subunit (ALS) identified from genomic and cDNA clones of *Drosophila* apparently does not bind αBGTX (Bossy *et al.* 1988). It remains to be determined whether the αBGTX binding sites identified in adult *M. sexta* brains (Sanes *et al.* 1977; Hildebrand *et al.* 1979; Prescott & Perez, 1985; Perez & Prescott, 1987) represent the functional receptors studied here in the larval stage.

The failure of PPR to repolarize during the continued presence of a supramaxi-

mal concentration of nicotine (Fig. 3A) or carbachol suggests that the receptors desensitize very slowly, if at all. It is possible that this failure is an adaptation that allows rapid recovery of synaptic function after acute exposure to massive concentrations of nicotine. If this lack of desensitization, as assayed by the sustained depolarization, is truly reflected at the level of single receptors/channels, then it could greatly facilitate the study of insect nAChRs through patch-clamp techniques. Although the rate of desensitization of AChRs in other species can be modulated by several processes (Downing & Role, 1987; Middleton *et al.* 1988), none of these processes has been shown to result in the loss of desensitization (it is usually enhanced). The precise mechanism(s) of desensitization is still unknown, but is presumed to involve an allosteric transition which results in a change in the affinity of the agonist receptor site accompanied by a closing of the ionic pore (Changeux *et al.* 1984). This process is being studied in *Torpedo* at the level of the primary sequence and the tertiary folding of the extracellular portions of the nAChR subunits (Conti-Tronconi *et al.* 1988); a non-desensitizing nAChR from *M. sexta* could be most useful in such investigations.

There is no detailed description of desensitization in insect receptors. Although some studies have demonstrated desensitization (Sattelle *et al.* 1976), more recent work on reconstituted locust nAChRs in lipid bilayers has demonstrated that single-channel responses are slow to diminish, even at high agonist concentrations (Hanke & Breer, 1986; H. Breer, personal communication). Although this weak desensitization could result from the use of a reconstitution system, it may also suggest that non-desensitizing AChRs are present in other insect species.

Muscarinic responses

Comparison of the effects on PPR of ionophoretic pulses of nicotine and carbachol showed that there was a small depolarization elicited by carbachol which resisted blockade by mecamylamine (Fig. 2). This was never observed during nicotine application. In the vertebrate CNS, carbachol is a strong agonist at the mAChR, but in insects it stimulates nAChRs at low concentrations (Sattelle, 1985) and electrophysiologically appears to have no 'muscarine-like' effects. Our investigations revealed that muscarinic agonists cause PPR to depolarize and also alter its excitability and it is possible that part of PPR's response to carbachol is mediated by postsynaptic mAChRs. PPR's responses to muscarinic agents provide some insight into the potential physiological role of the 'muscarinic' scopolamine and quinuclidinyl benzilate (QNB) binding sites described in the insect CNS (Haim *et al.* 1979; Harris *et al.* 1981; Meyer & Edwards, 1980; Aguilar & Lunt, 1984; Meyer & Reddy, 1985; Lummis & Sattelle, 1985, 1986). It has long been assumed that these binding sites, as in vertebrate brains, represent functional mAChRs, but physiological evidence for this has been lacking. Recently, Benson and others described muscarinic responses (ACh_2-type responses) in isolated neuronal cell bodies of the locust (Benson & Neumann, 1987; Benson, 1988). These depolarizations were characterized by a conductance increase an

involved an inward current that decreased with hyperpolarization. The responses were relatively slow to develop and had a lengthy time course. Such effects are quite unlike the muscarine-activated hyperpolarization of vertebrate neurones in the thalamic nucleus reticularis (McCormick & Prince, 1986) or in the nucleus parabranchialis (Egan & North, 1986), and they cannot be attributed to the closing of M-channels (Brown & Adams, 1980). However, similar responses have been described in rabbit smooth muscle (Benham *et al.* 1985) and in the cardiac ganglion of the lobster (Freschi & Livengood, 1987).

In the present study we describe the first observations of muscarinic responses in an identified insect motoneurone. Our preliminary experiments suggest that, through muscarinic-type receptors on PPR itself, ACh can modify the effectiveness of incoming signals, primarily by lowering the spike threshold. Although we did not detect changes in the input resistance measured at the soma (Fig. 9), and we could not detect voltage changes that would be expected to accompany M-current relaxations during current-pulse injections, it is quite possible that resistance changes in the neuropile or close to the spike-initiation zone might have occurred that were not detectable by our measurements. Thus, it is possible that muscarine evokes its effect through one of the channel changes described above, without causing a consistent change in R_{in}. However, the generation of second messengers by mAChRs of vertebrate cells (Nathanson, 1987) and the emerging evidence for the induction of similar signals by muscarinic agents in insects (Trimmer & Berridge, 1985; Duggan & Lunt, 1986) certainly allows for the possibility of non-electrical modulation.

The potentiation of EPSPs by scopolamine may result from an additional class of mAChRs which act presynaptically to inhibit neurotransmitter release. These receptors would serve to modulate the effectiveness of particular sensory inputs. There is a great deal of evidence for this type of modulation in vertebrate systems (Muscholl, 1979; Raiteri *et al.* 1983), but the only example in insects comes from work on locust synaptosomes, in which muscarinic agonists inhibit the release of ACh and antagonists facilitate its release (Breer & Knipper, 1984). The results parallel our findings at the afferent-to-motoneurone synapse in *M. sexta* (Fig. 13).

It may be possible to dissect pharmacologically the pre- and postsynaptic actions of these proposed mAChRs in *M. sexta* using additional pharmacological and biochemical approaches. Since, in the vertebrates, there are several types of mAChR that can be distinguished by their pharmacology (Hammer *et al.* 1980), their associated G-proteins (Bourne, 1986), their cellular effectors (McKinney & Richelson, 1984; Nathanson, 1987; Peralta *et al.* 1988) and by their gene sequences (Kubo *et al.* 1986; Peralta *et al.* 1987), continued studies of insect mAChRs may help to shed light on the origins and functions of this class of receptors. We are presently characterizing the effects of muscarinic agents at the afferent-to-PPR synapse in an attempt to understand the role of modulatory receptors in sensory neurotransmission.

This research was supported by a Whitehall Foundation Research Grant.

Neuronal bungarotoxin was a gift from Dr R. Loring of Harvard Medical School. We thank Dr William Roberts, Dr Carol Miles, David Sandstrom and Carol Kazmer for helpful comments on the manuscript.

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