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IMIDACLOPRID ACTIONS ON INSECT NEURONAL ACETYLCHOLINE RECEPTORS

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Summary

The neonicotinoid insecticide Imidacloprid acts at three pharmacologically distinct acetylcholine receptor (AChR) subtypes in the cockroach (*Periplaneta americana*) nervous system, but is ineffective on muscarinic receptors. Imidacloprid (3–100 μmol l^{-1}) induced dose-dependent depolarizations at cockroach cercal afferent/giant interneurone synapses. These responses were insensitive to 20 μmol l^{-1} atropine but were completely blocked by the nicotinic antagonist mecamylamine (50 μmol l^{-1}). Similarly, Imidacloprid-induced depolarizations of cultured cockroach dorsal unpaired median (DUM) neurones dissociated from the same (terminal abdominal) ganglion were also completely blocked by 100 μmol l^{-1} mecamylamine. However, two components of the response could be distinguished on the basis of their differential sensitivities to 0.1 μmol l^{-1} α-bungarotoxin (α-BTX), which selectively blocks AChRs with 'mixed' nicotinic/muscarinic pharmacology in this preparation. This indicates that Imidacloprid affects both AChRs sensitive to α-BTX and α-BTX-insensitive nicotinic acetylcholine receptors (nAChRs). Thus, in the cockroach, Imidacloprid activates α-BTX-sensitive synaptic nAChRs in giant interneurones, α-BTX-insensitive extrasynaptic nAChRs in DUM neurones, and a recently characterized DUM neurone 'mixed' AChR that is sensitive to both nicotinic and muscarinic ligands. Imidacloprid does not act on muscarinic acetylcholine receptors (mAChRs) present on DUM neurone cell bodies and at the cercal afferent/giant interneurone synapses. This study shows that Imidacloprid can act on pharmacologically diverse nAChR subtypes.

Key words: Imidacloprid, nitroguanidine insecticide, nicotinic acetylcholine receptor, DUM neurones, cholinergic synaptic transmission, cockroach, *Periplaneta americana*.

Introduction

Imidacloprid {1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine} (Fig. 1) is a neonicotinoid insecticide with low soil persistence, high insecticidal potency and relatively low mammalian toxicity (Mullins, 1993; Nauen, 1995; Zwart *et al.* 1992, 1994). The insecticidal potency of Imidacloprid (Fig. 1) and related compounds (referred to collectively as neonicotinoid insecticides and including nitroguanidines and nitromethylenes) correlates with their capacity to cause excitation in cockroach nerve cords (Nishimura *et al.* 1994). Early work indicated that the principal site of action of neonicotinoids was on cholinergic synaptic transmission, based on pharmacological studies using extracellular electrophysiological recordings from the cockroach (*Periplaneta americana*) terminal abdominal ganglion (Schroeder and Flattum, 1984). This action was insensitive to α-bungarotoxin (α-BTX), an antagonist of many, though not all (Goodman and Spitzer, 1980; Lane *et al.* 1982), nicotinic acetylcholine receptors (nAChRs) of insects (Breer and Sattelle, 1987), and sensitive to atropine, which blocks insect muscarinic acetylcholine receptors (mAChRs) (Abdallah *et al.* 1991; Dudai, 1981), but also certain nAChRs (David and Sattelle, 1984). Liu *et al.* (1993) and Liu and Casida (1993) found that both muscarinic and nicotinic ligands displaced bound [^{3}H]Imidacloprid from housefly (*Musca domestica*) head membranes. While these studies suggested that certain nitromethylenes may act on both nAChRs and mAChRs, evidence is accumulating that their primary site of action is at nicotinic acetylcholine receptors. For example, radioligand binding (Tomizawa and Yamamoto, 1992, 1993), patch-clamp (Leech *et al.* 1991; Cheung *et al.* 1992; Zwart *et al.* 1992, 1994) and two-electrode voltage-clamp (Bai *et al.* 1991; Benson, 1989, 1992; Buckingham *et al.* 1995; Sattelle *et al.* 1989) electrophysiological studies on insects have provided support for this view. Also, a recombinant nAChR composed only of the α subunit (αL1) of a locust (*Schistocerca gregaria*) can be expressed in *Xenopus laevis* oocytes and mimics several aspects of the pharmacology of certain native insect nAChRs, including sensitivity to the nitromethylene 2-(nitromethylene)tetrahydro-1,3-thiazine (Leech *et al.* 1991;

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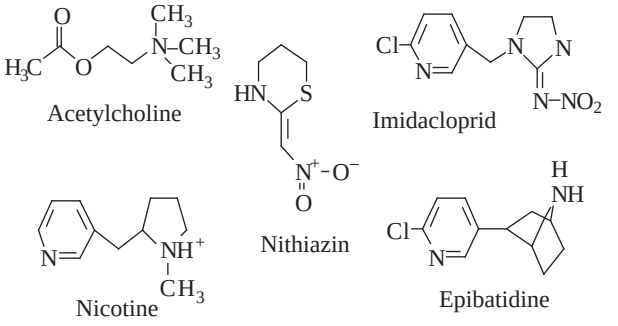

Fig. 1. The structure of Imidacloprid compared with the natural neurotransmitter acetylcholine and the nicotinic receptor agonists nicotine, Nithiazin and epibatidine. The structure of Imidacloprid closely resembles that of epibatidine, a potent nicotinic agonist isolated from the venom of the tree frog *Epipedobates tricolor*.

Marshall *et al.* 1990) (Nithiazin, Fig. 1). Nevertheless, in view of the displacement of [^{3}H]Imidacloprid binding by both nicotinic and muscarinic ligands (Liu *et al.* 1993; Liu and Casida, 1993), it may be that more than one type of AChR is affected by Imidacloprid.

The aim of the present study was to compare the actions of Imidacloprid on pharmacologically distinct populations of AChRs in the same preparation. In the central nervous system of the cockroach *Periplaneta americana*, there is evidence for monosynaptic cholinergic synaptic transmission between cercal mechanosensory neurones and giant interneurones in the terminal abdominal ganglion (TAG) (Callec, 1974; Sattelle *et al.* 1983; Blagburn and Sattelle, 1987). α-BTX-sensitive nAChRs are present on the postsynaptic neuronal membranes of these synapses (Sattelle *et al.* 1983), and presynaptic muscarinic receptors (mAChRs) are also present (Le Corronc and Hue, 1993). DUM neurones, located along the midline on the dorsal surface of the same ganglion, innervate the accessory gland and heart chambers in the male cockroach (Sinakevitch *et al.* 1994, 1996) and express three pharmacologically distinct types of AChR (Lapied *et al.* 1990; Tribut and Lapied, 1994). In addition to mAChRs (Lapied *et al.* 1992), there are two other classes of AChR present: an α-BTX-insensitive nAChR, and a separate class of AChRs termed 'mixed' because of their sensitivity to both nicotinic and muscarinic ligands. Thus, the cockroach terminal ganglion is well suited to studies of Imidacloprid–AChR interactions.

Materials and methods

All experiments were performed at room temperature (20–23 °C) using adult male cockroaches (*Periplaneta americana* L.) reared at 29 °C on a 12 h:12 h light:dark cycle.

DUM neurone preparation

Electrophysiological recordings were performed on isolated DUM neurone cell bodies obtained after enzymatic treatment and mechanical dissociation from the TAG under sterile conditions (Lapied *et al.* 1989; Grolleau and Lapied, 1995). Normal saline for cell isolation contained (in mmol l^{-1}): NaCl, 200; KCl, 3.1; $CaCl_2$, 5.0; $MgCl_2$, 4.0; sucrose, 50; Hepes, 10; pH adjusted to 7.4 with NaOH. Cells were continuously perfused with saline (0.1 ml min^{-1}) supplied through one of an array of parallel outlet tubes placed close (≈100 μm) to the cell. Putative antagonists were applied in the perfusing saline by displacing the array of tubes laterally so that the cell was adjacent to another tube through which the test solution flowed with a solution exchange time of less than 1 s. A Na^+ channel blocker tetrodotoxin (TTX) was added to the saline at a final concentration of 100 nmol l^{-1} to block action potentials. Imidacloprid was applied by pressure-ejection (80 ms pulses) from a pipette placed close (<75 μm) to the cell body using a pneumatic pressure system (Miniframe, Medical Systems Corporation, USA), allowing almost instantaneous applications of the drug.

The patch-clamp technique (Hamill *et al.* 1981) was used in the whole-cell, current-clamp configuration to record membrane potentials. Patch electrodes were pulled from borosilicate capillary tubes (Clark Electromedical Instruments, Reading, UK) with a PP-83 electrode puller (Narishige, Japan) and had resistances of 1–3 MΩ when filled with a pipette solution of the following composition (in mmol l^{-1}): KCl, 170; NaCl, 10; $MgCl_2$, 1; ATP–Mg, 3; Hepes, 10; pH adjusted to 7.4 with KOH. The liquid junction potential between the pipette medium and the superfusing solution was always corrected before seal formation (⩾10 GΩ). The resting membrane potential was recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) and displayed on a digital oscilloscope (310, Nicolet Instrument Corporation, Madison, WI, USA), stored on a DTR 1202 (Biologic, Claix, France) and continuously monitored on a chart recorder for off-line analysis.

Mannitol-gap recordings

The abdominal nerve cord, one cercus and the corresponding cercal nerve XI were isolated. The TAG was carefully desheathed to facilitate penetration of bath-applied drugs and ionophoretic micropipettes. The preparation was mounted in a mannitol-gap recording chamber described in detail elsewhere (Callec and Sattelle, 1973), and the TAG compartment was continuously superfused (1 ml min^{-1}) with saline of the following composition (in mmol l^{-1}): NaCl, 208; KCl, 3.1; $CaCl_2$, 5.4; $NaHCO_3$, 2; sucrose, 26; pH 7.4. The recording electrode was connected to the input of a high-impedance amplifier, whose output was passed to a digital oscilloscope, a chart recorder and a personal computer for off-line analysis. Data were digitized using an analogue-to-digital converting interface (Hameg, Germany) and stored on computer. Data were analysed using software developed in our own laboratory. These conditions allowed the recording of unitary excitatory postsynaptic potentials (uEPSPs) resulting from the activity of cercal mechanoreceptors. Compound EPSPs (cEPSPs) were evoked by electrical stimulation of the ipsilateral cercal nerve XI (presented as the mean of at least four recordings), eliciting action potentials in the connective. Variation of postsynaptic polarization was monitored on a chart recorder. The direct responses of postsynaptic cholinergic

receptors were studied by ionophoretic injections of 1 mol l^{-1} carbamylcholine chloride (CCh) using an IP-2 ionophoresis pump (Medical Systems Corp. NY, USA). The tip of the microelectrode was introduced into the region of the dendritic fields of the giant interneurones and CCh was injected with a positive current (20–30 ms duration, 400 nA amplitude). Statistical significance was assessed by a standard Bonferroni's test using Instat software (GraphPad, UK). Data were expressed as the mean ± one standard error of the mean (S.E.M.). Dose–response data were fitted to the equation:

$$y = \frac{\text{max}}{1 + 10^{(\log EC_{50})H}}$$

where max is the maximum response obtained and H is the Hill slope.

All compounds were purchased from Sigma Chemicals except Imidacloprid, which was kindly supplied by colleagues at DuPont Agricultural Products, Newark, DE, USA.

Results

Imidacloprid actions at a cholinergic synapse

Agonist actions of bath-applied Imidacloprid were examined on cercal afferent/giant interneurone synapses in the TAG. Perfusion of the experimental chamber for 3 min with saline containing Imidacloprid (0.1–100 μmol l^{-1}) resulted in dose-dependent depolarizations of postsynaptic interneurones which reversed upon wash-out (Fig. 2A). For concentrations up to 3 μmol l^{-1}, the Imidacloprid-induced depolarization was accompanied by a marked increase in frequency of action potentials, which then ceased as the depolarization reached a peak (Fig. 2B). These effects resemble the actions of acetylcholine and certain nicotinic ligands on synaptic transmission in the TAG (Callec, 1985). Fig. 2C shows the dose–response curve for Imidacloprid. For longer applications (>3 min), and for the highest concentration used (100 μmol l^{-1}), reversibility was poor even after prolonged wash-out.

Bath-application of Imidacloprid (10 μmol l^{-1}) also reduced the amplitude of cEPSPs evoked by electrical stimulation of cercal nerve XI. Only partial recovery was usually observed even after a 20 min saline wash (Fig. 3A), after which an increase in the level of electrical stimulation was necessary to restore cEPSP amplitude (results not shown). Even when cEPSPs were completely blocked by Imidacloprid, presynaptic action potentials (recorded electrotonically) evoked by electrical stimulation were still visible, suggesting that this blocking action did not result from a failure of presynaptic action potentials. The CCh-induced depolarization recorded before and after bath-application of Imidacloprid (Fig. 3B) was affected in a similar way to the cEPSP. The CCh response was abolished during the Imidacloprid-induced depolarization, and a large reduction of CCh potential amplitude was still observed even after complete recovery of ganglionic polarization. This suggests that postsynaptic cholinergic receptors are desensitized by Imidacloprid.

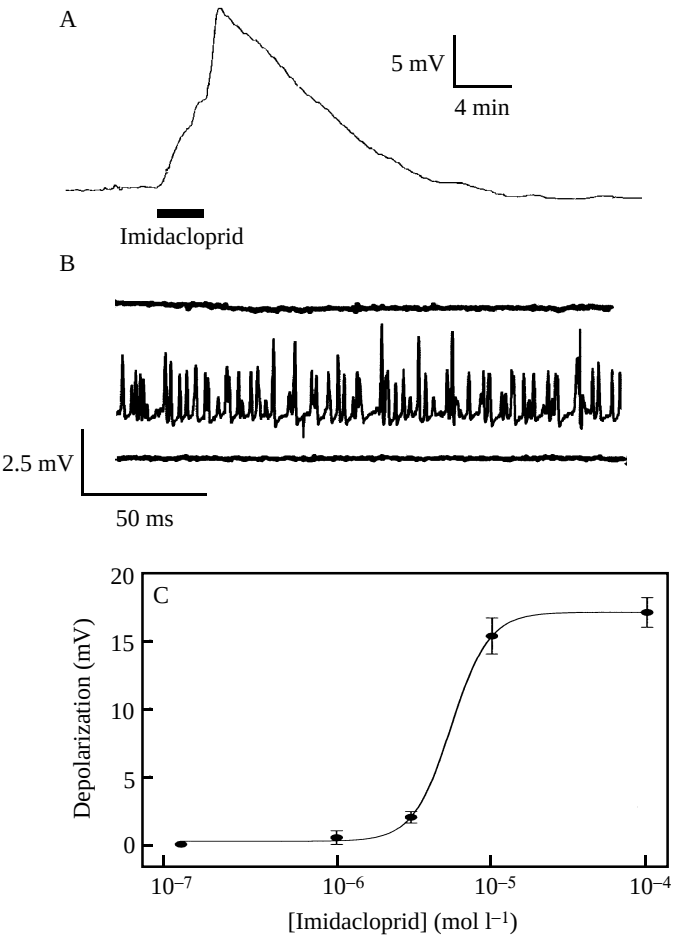

Fig. 2. Representative effects of Imidacloprid on postsynaptic interneurones recorded using the mannitol-gap technique. (A) Bath-application of 10 μmol l^{-1} Imidacloprid for 3 min (indicated by the horizontal bar) evoked a large-amplitude depolarization with rapid onset that completely reversed upon wash-out in normal saline. (B) On an expanded time-scale, and before the application of Imidacloprid (upper trace), little or no spontaneous background activity (unitary EPSPs or action potentials) was observed. Imidacloprid evoked action potentials and compound EPSPs (middle trace) during the rising phase of the depolarization. As the depolarization progressed, however, background activity declined and eventually ceased (lower trace). (C) A semi-logarithmic dose–response curve for Imidacloprid. Mean values of the amplitude of peak depolarization are plotted. Data were pooled from 3–5 preparations. Error bars represent ± 1 S.E.M. In one case, the error bars fall within the size of the plotted point. The curve represents the best fit to the data using the equation given in the Materials and methods section.

To characterize the pharmacology of the Imidacloprid-induced depolarization, its sensitivity to a nicotinic antagonist (mecamylamine) and a muscarinic antagonist (atropine) were examined. Preliminary experiments indicated that Imidacloprid could induce desensitization of postsynaptic nicotinic receptors, so repeated applications of Imidacloprid at higher (>3 μmol l^{-1}) concentrations were avoided. However, although responses to repeated applications (at 40 min intervals) of 10 μmol l^{-1}

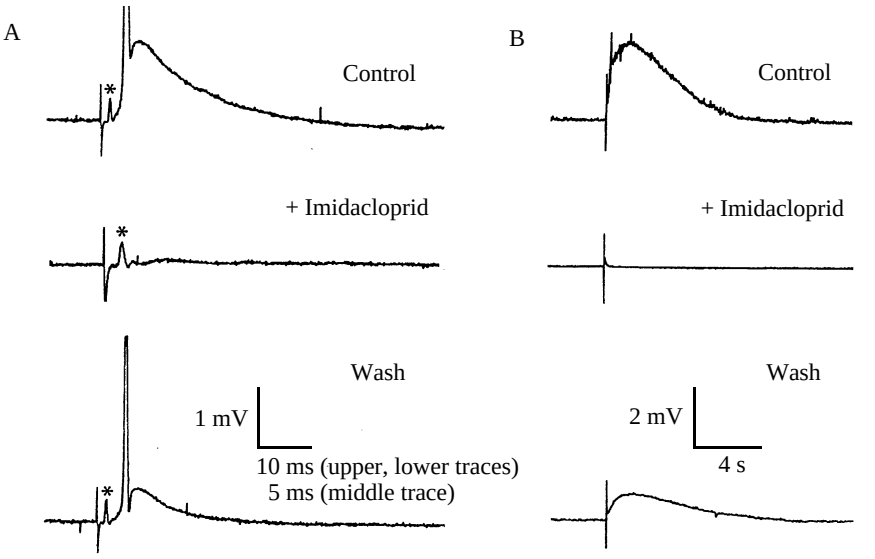

Fig. 3. Actions of 10 μmol l^{-1} Imidacloprid on compound EPSPs and the carbamylcholine (CCh)-induced depolarization. (A) Stimulation of nerve XI evoked a compound EPSP (control, upper trace), which was blocked by the bath-application for 3 min of Imidacloprid (middle trace). The presynaptic action potentials were still visible (indicated by the asterisk), even when the postsynaptic response was completely blocked. To emphasise the presynaptic spikes, the time scale was enlarged for the middle trace. The effect was partially reversed after a 20 min wash-out (lower trace). Note the presence of action potentials superimposed on some compound EPSPs; the top of spikes was cut off by the limitation of the oscilloscope excursion. (B) CCh-induced potentials evoked by ionophoretic injection of 1 mol l^{-1} CCh into the neuropile. The CCh response was abolished during the Imidacloprid-induced depolarization and the CCh-induced potential did not fully recover even after a 30 min wash. This implies that the postsynaptic nicotinic receptor/channel complex is the major site of Imidacloprid action and that it is desensitized by the insecticide. The traces shown in A and B are from two different preparations.

Imidacloprid declined in amplitude and became slower in onset (not shown), responses to repeated applications of 3 μmol l^{-1} Imidacloprid were stable (Fig. 4A). The mannitol-gap technique permits continuous recordings for 4–5 h without reduction in cEPSP amplitude or alteration of the postsynaptic membrane potential (see also Callec and Sattelle, 1973), suggesting that the decline of postsynaptic depolarization was not attributable to either non-specific actions or deterioration in the preparation. Since the amplitude of the postsynaptic depolarization was not stable when applications of Imidacloprid were repeated, antagonists were bath-applied for at least 25 min before a single application of Imidacloprid (10 μmol l^{-1}). In all cases, control experiments were alternated with those of each test group. Responses to Imidacloprid were greatly reduced by 50 μmol l^{-1} mecamylamine from a control value of 14.7±1.3 mV (N=5) to 2.4±0.2 mV (N=3) in the presence of mecamylamine, and to 2.2±0.2 mV(N=3) in the presence of both mecamylamine and atropine (Fig. 4B). These differences were significant (P<0.001, Bonferroni's test) when comparing blocking protocols *versus* controls, but there was no significant difference between the two blocking protocols (P>0.05, Bonferroni's test). However, the amplitude of the Imidacloprid response was

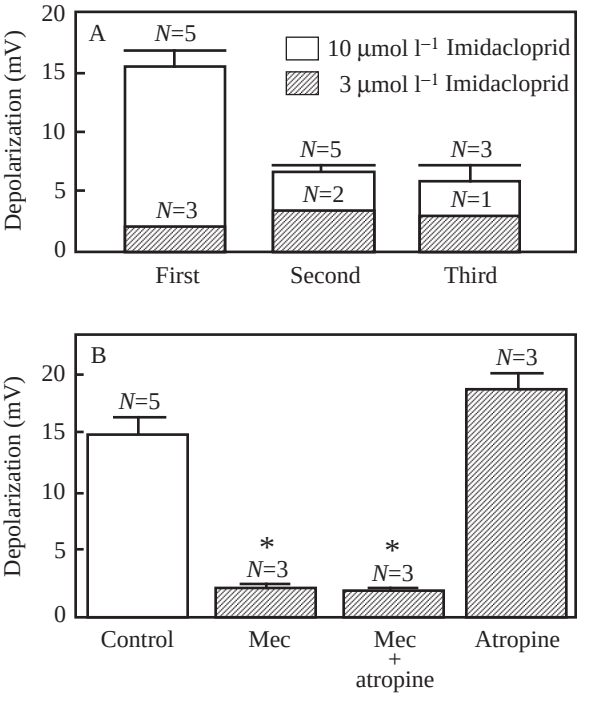

Fig. 4. Effects of repeated applications of Imidacloprid and some pharmacological properties of Imidacloprid-induced depolarizations recorded from the cercal afferent/giant interneurone preparation. (A) The amplitude of the responses to three successive exposures (40 min intervals) of the terminal abdominal ganglion to 10 μmol l^{-1} Imidacloprid (3 min application) declined in amplitude. However, responses to 3 μmol l^{-1} Imidacloprid were stable in amplitude over repeated doses. The number of experiments (N) is shown. (B) The response to bath-applied Imidacloprid (10 μmol l^{-1}; 3 min application) was insensitive to 20 μmol l^{-1} atropine (the amplitude of the response in the presence of atropine was not significantly different from the control value, P>0.05, Bonferroni test) but was blocked by the nicotinic antagonist mecamylamine (Mec, 50 μmol l^{-1}). The additional inclusion of both atropine (10 μmol l^{-1}) and mecamylamine (50 μmol l^{-1}) in the perfusate caused no further reduction in the amplitude of the response to Imidacloprid. Antagonists were applied at least 35 min before a single application of Imidacloprid (see text for further details). Values are expressed as mean + 1 S.E.M. for 3–5 experiments. The Bonferroni test was used to compare control values with values obtained from preparations treated with mecamylamine and with both mecamylamine and atropine. Values that were significantly different from the control value are indicated by an asterisk (P<0.001).

unaffected by 20 μmol l^{-1} atropine (P>0.05, Bonferroni's test, Fig. 4B). These results indicate that the main action of Imidacloprid is *via* synaptic nAChRs (not mAChRs) in the TAG of the cockroach.

Imidacloprid actions on DUM neurones

Dissociated, adult DUM neurones from the cockroach TAG were incubated in saline containing 5 μmol l^{-1} atropine and 100 nmol l^{-1} α-BTX, which have been shown to block selectively the α-BTX-sensitive 'mixed' AChRs present on DUM neurones (Lapied *et al.* 1990), leaving the α-BTX-insensitive nAChR response intact. Pressure-applied Imidacloprid (80 ms, 90 kPa, 100 μmol l^{-1} Imidacloprid in the pipette) resulted in large depolarizations (19.8±2.6 mV, N=5, Fig. 5A) which reversed rapidly. Subsequent responses to even larger doses of Imidacloprid were either much reduced in amplitude or absent, even after prolonged (>20 min) wash-out (Fig. 5A). The dose-dependence of the depolarizing action of Imidacloprid on α-BTX-insensitive nAChRs was therefore measured by pressure-application (2 s, 90 kPa) of various concentrations of Imidacloprid in the pressure pipette to cells not previously exposed to the compound (Fig. 5B).

An Imidacloprid-evoked depolarization was also seen following incubation of the cells in saline containing 100 nmol l^{-1} TTX in the absence of α-BTX or atropine (Fig. 6A). However, the depolarization of the membrane was more prolonged than that observed in the presence of α-BTX. The duration of the depolarization was greatly reduced when the isolated DUM neurone was superfused with saline containing 100 nmol l^{-1} α-BTX (known to block selectively 'mixed' receptors on DUM neurones, Lapied *et al.* 1990), which leaves the residual nAChR response intact (Fig. 6A,B). This indicates that a proportion of the total Imidacloprid response is also mediated by 'mixed' receptors.

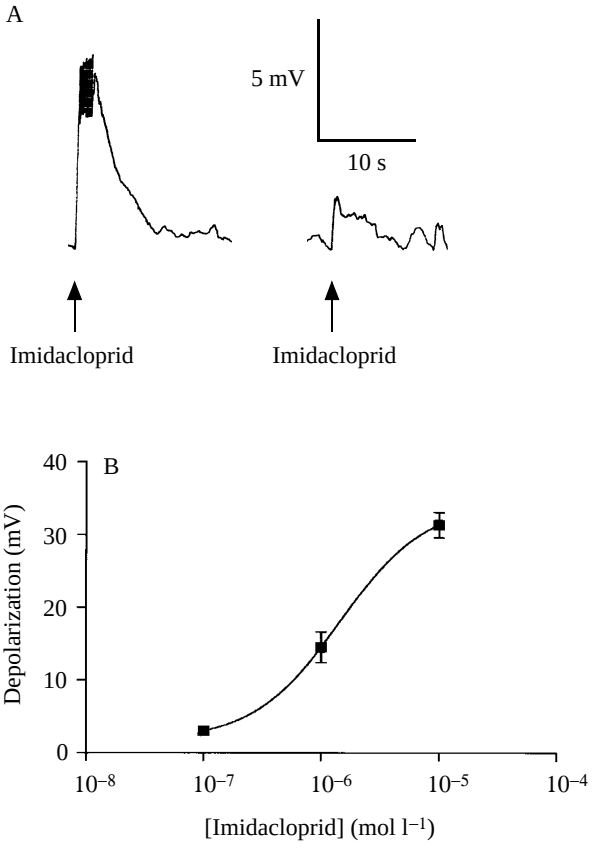

Fig. 5. Responses recorded from cell bodies of DUM neurones to pressure-applied Imidacloprid. Imidacloprid was applied by pressure-ejection from a patch pipette filled with saline containing Imidacloprid (100 μmol l^{-1} in A, and a test concentration in B) and placed within 75 μm of the cell. (A) Successive applications of Imidacloprid result in membrane depolarizations of diminishing amplitude. The application of the compound is indicated by the arrows. In the case illustrated, Imidacloprid was pressure-applied (80 ms pulses, 90 kPa) at 5 min intervals. Recovery of the responses to Imidacloprid was rarely observed even after a wash-out in normal saline of more than 20 min. (B) The dose–response curve for the action of Imidacloprid on isolated DUM neurones. Because the responses to successive, similar applications of Imidacloprid declined in amplitude, each point of the dose–response curve had to be determined on a separate preparation. Each data point represents the mean of at least three separate experiments, and error bars represent one standard error of the mean. The error bar for one point (10^{-7} mol l^{-1}) is smaller than the plotted point.

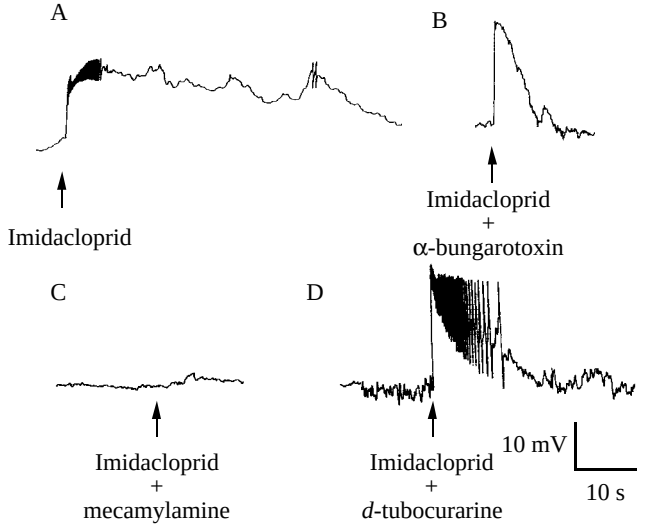

Fig. 6. The pharmacology of Imidacloprid-evoked depolarizations recorded in isolated DUM neurones. The recording conditions are as described for Fig. 5. Tetrodotoxin (TTX) (0.1 μmol l^{-1}) was present in the saline in all experiments and α-bungarotoxin (α-BTX) (0.1 μmol l^{-1}) and atropine (5 μmol l^{-1}) were present in the saline in the experiments illustrated in traces B, C and D. (A) The response to pressure-applied Imidacloprid (arrow) in the absence of α-BTX. When the ACh receptors of 'mixed' pharmacology are blocked by perfusion with atropine and α-bungarotoxin (B), the response to Imidacloprid reverses more rapidly, indicating that the response is mediated by more than one type of acetylcholine receptor. 100 μmol l^{-1} mecamylamine, however, completely blocks the Imidacloprid-evoked membrane depolarization (C). In contrast, 50 μmol l^{-1} *d*-tubocurarine, a competitive antagonist of nAChRs, fails to block the response (D).

The α-BTX-insensitive Imidacloprid-induced depolarization (evoked in the presence of 100 nmol l^{-1} α-BTX and 5 μmol l^{-1} atropine) was blocked by the inclusion of mecamylamine (100 μmol l^{-1}) in the perfusing saline (N=3) (Fig. 6C). However, no block of the response was observed when the cells were superfused in saline containing TTX (100 nmol l^{-1}), α-BTX (100 nmol l^{-1}), atropine (5 μmol l^{-1}) and *d*-tubocurarine (50–100 μmol l^{-1}) (N=4, Fig. 6D). This failure of *d*-tubocurarine to block the Imidacloprid-evoked depolarization was also seen when the cell was artificially hyperpolarized to −100 mV by the injection of current through the recording pipette, indicating that the failure of *d*-tubocurarine to block the Imidacloprid response was not due to the strong-voltage dependence of blocking action previously observed on cockroach nAChRs (David and Sattelle, 1984).

Discussion

We have demonstrated that Imidacloprid acts on three distinct populations of AChRs present in the same ganglion of the cockroach: (a) a nicotinic AChR sensitive to α-BTX in the cercal afferent/giant interneurone synapse; (b) a nicotinic AChR resistant to α-BTX, and (c) a 'mixed' AChR sensitive to α-BTX (where b and c are both present on the cell body of DUM neurones). However, it must be noted that the irreversible decline in the amplitude of responses to Imidacloprid suggests that this compound may have diverse and complex actions, additional to the activation of AChRs, that may also contribute to its insecticidal action.

Synaptic nAChRs activated by Imidacloprid

At the cercal afferent/giant interneurone synapse in the cockroach TAG, Imidacloprid induced a rapid depolarization of postsynaptic neurones accompanied by the induction of action potentials. These effects are similar to those produced by nicotinic agonists, in that they are blocked by mecamylamine but not by atropine. Imidacloprid cross-desensitizes with carbachol. We therefore conclude that the depolarization caused by Imidacloprid is mediated through nicotinic acetylcholine receptors. To date, no responses with this time-course have been shown to result from the application of muscarinic agonists.

The Imidacloprid-induced inhibition of the EPSP evoked by excitation of nerve XI reversed poorly during wash-out in normal saline, and the postsynaptic depolarization declined in amplitude following repetitive application of Imidacloprid. This might be explained by competition between Imidacloprid and endogenous ACh or possibly by a reduction in the difference between the net ganglionic transmembrane potential and the reversal potential of the nicotinic response (approximately −40 mV to −20 mV). It could also be attributed to desensitization of postsynaptic nAChRs, similar to the desensitization observed for Imidacloprid actions on DUM neurones. Finally, we cannot exclude a presynaptic effect of Imidacloprid on afferent nerve terminals.

Cell body AChRs activated by Imidacloprid

DUM neurone responses to Imidacloprid include at least two components, one sensitive to α-BTX and another insensitive to α-BTX. The α-BTX-insensitive response is blocked by mecamylamine, suggesting that this component of the response is not mediated by muscarinic receptors. To detect a possible Imidacloprid action on mAChRs, the effect of Imidacloprid in the presence of both of α-BTX and atropine would have to be compared with its effect in the presence of α-BTX alone. Since atropine blocks both the muscarinic and the 'mixed' receptors, and α-BTX blocks only the DUM neurone 'mixed' receptors (Lapied *et al.* 1990), a difference in response amplitudes recorded under these two conditions would indicate a muscarinic component to the α-BTX-insensitive responses. However, it must be noted that the α-BTX-insensitive component of the Imidacloprid response (inferred from a comparison of the response in the presence of α-BTX and atropine with that recorded in their absence) differs from muscarinic responses in its time course. The response to muscarine is a slow depolarization and the response to McN-A-343, a muscarinic agonist, is a fast, transient, hyperpolarizing response followed by a slow depolarizing response (Lapied and Hue, 1991; Lapied *et al.* 1992). Our data therefore strongly suggest that Imidacloprid can act on two different populations of DUM neurone AChRs: an nAChR resistant to α-BTX and a 'mixed' AChR that is sensitive to α-BTX and has a 'mixed' pharmacology.

The failure of *d*-tubocurarine to block the response may be due to its competitive action (David and Sattelle, 1984), or it may reflect actions of Imidacloprid at a site distinct from the *d*-tubocurarine binding site(s) on the receptor.

Possible actions of Imidacloprid on muscarinic acetylcholine receptors

The Imidacloprid responses recorded from DUM neurones and those recorded from the cercal afferent/giant interneurone synapse preparation were insensitive to atropine but were completely blocked by mecamylamine. The mecamylamine-insensitive component of the Imidacloprid response at the synaptic preparation was not blocked by the further addition of atropine. This indicates that the action of Imidacloprid on these postsynaptic membranes is not muscarinic. Liu and Casida (1993) reported that both nicotinic and muscarinic ligands displaced Imidacloprid binding, although only one binding site was obtained. Very high concentrations of quinuclidinyl benzilate and dexetemide were necessary to displace Imidacloprid binding, and the EC_{50} values for these two muscarinic antagonists were very similar to those reported in the characterization of nicotinic acetylcholine receptors (David and Sattelle, 1984). Thus at high concentrations these muscarinic ligands may have been acting directly on nAChRs. These findings therefore support our conclusion that Imidacloprid acts upon nicotinic, but not muscarinic, receptors.

In conclusion, Imidacloprid acts on several AChRs including nicotinic receptors and a receptor of 'mixed' nicotinic/muscarinic pharmacology, suggesting that it exerts actions at multiple nAChRs.

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