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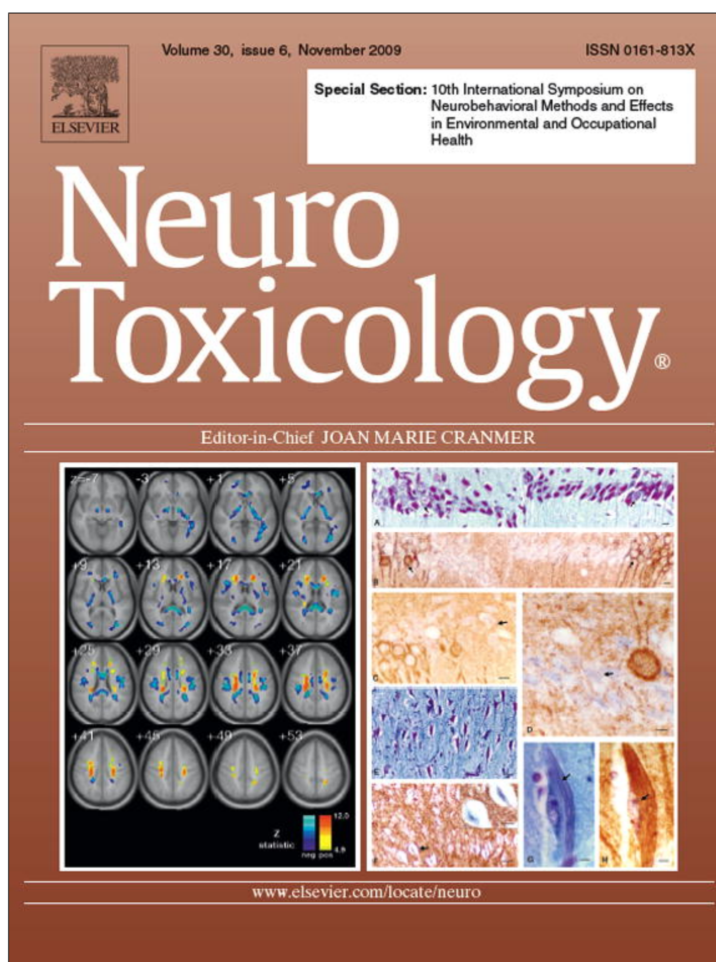

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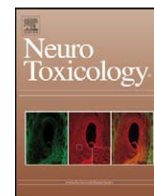

Agonist actions of clothianidin on synaptic and extrasynaptic nicotinic acetylcholine receptors expressed on cockroach sixth abdominal ganglion

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ABSTRACT

Clothianidin is new neonicotinoid insecticide acting selectively on insect nicotinic acetylcholine receptors (nAChRs). Its effects on nAChRs expressed on cercal afferent/giant interneuron synapses and DUM neurons have been studied using mannitol-gap and whole-cell patch-clamp techniques, respectively. Bath-application of clothianidin-induced dose-dependent depolarizations of cockroach cercal afferent/giant interneuron synapses which were not reversed after wash-out suggesting a strong desensitization of postsynaptic interneurons at the 6th abdominal ganglion (A6). Clothinidin activity on the nerve preparation was characterized by an increased firing rate of action potentials which then ceased when the depolarization reached a peak. Clothianidin responses were insensitive to all muscarinic antagonists tested but were blocked by co-application of specific nicotinic antagonists methyllicaconitine, α-bungarotoxin and d-tubocurarine.

In a second round of experiment, clothianidin actions were tested on DUM neurons isolated from the A6. There was a strong desensitization of nAChRs which was not affected by muscarinic antagonists, pirenzepine and atropine, but was reduced with nicotinic antagonist α-bungarotoxin. In addition, clothianidin-induced currents were completely blocked by methyllicaconitine suggesting that (1) clothianidin acted as a specific agonist of nAChR subtypes and (2) a small proportion of receptors blocked by MLA was insensitive to α-bungarotoxin. Moreover, because clothianidin currents were blocked by d-tubocurarine and mecamylamine, we provided that clothianidin was an agonist of both nAChRs: imidacloprid-sensitive nAChR1 and -insensitive nAChR2 subtypes.

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1. Introduction

The economic and social importance of insects as agricultural pests and vectors of human and animal diseases led to consider the interactions of insecticides with their target and the mechanisms of insect resistance. Neonicotinoid insecticides represent a relatively new group of chemicals that includes clothianidin ((*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-mitroguanidine). The first major neonicotinoid was imidacloprid (Imi), followed soon thereafter by clothianidin (Clo) (Tomizawa and Casida, 2003; Ford and Casida, 2006; Honda et al., 2006; Jeschke and Nauen, 2008). They exerted insecticidal effects on a broad range of insect pests (Nauen et al., 2003), including species with a long history of resistance to earlier-used products, as cockroach. It has been noted that these effects reflected their action on the insect central nervous system (CNS), especially on cholinergic synapses (Schroeder and Flattum, 1984). In fact, application of imidacloprid on cercal afferent/giant interneuron synapses localized in the abdominal sixth ganglion (A6), induced dose-dependent depolarizations and the blocking of the compound excitatory postsynaptic potentials (cEPSPs) evoked by electrical stimulation of cercal nerve XI (Buckingham et al., 1997). These effects were attributable to its selectivity on nicotinic acetylcholine receptors (nAChRs) because the responses to Imi were insensitive to muscarinic antagonist atropine (Atr) but completely blocked by the nicotinic antagonist mecamylamine (Meca) (Buckingham et al., 1997). Similar Imi effects were seen using isolated central nervous system of Colorado potato beetle, *Leptinotarsa decemlineata* (Tan et al., 2008). Imi increased the frequency of action potentials and this neuroexcitatory action was blocked by co-application of methyllicaconitine (MLA) (Tan et al., 2008).

Synaptic nicotinic receptors mediate fast excitatory neurotransmission in the central nervous system of insect species and are ligand-gated ion channels permeable to Na^+, K^+ and Ca^{2+}. Several nAChR subunits have been cloned and the sequencing of entire insect genomes has revealed all the genes encoding nAChR subunits in *Drosophila melanogaster* (Sattelle et al., 2005), *Anopheles gambiae* (Jones et al., 2005), *Tribolium castaneum* (Jones and Sattelle, 2007) and *Apis mellifera* (Jones et al., 2006) demonstrating that distinct subtypes of native nAChRs are present

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(Thany et al., 2007). But the pharmacological profiles of native nAChR subtypes were limited and correlated to hybrid receptors, in which Imi and related compounds appeared as full or partial agonists (Ihara et al., 2003) and recently, as antagonist (Zhang et al., 2008). Structurally, Clo has a 2-chloro-1,3-thiazol-5-yl as the aryl group and an acyclic guanidine moiety, while Imi has a 6-chloro-3-pyridyl group and a cyclic guanidine moiety. These structural differences could lead to distinct affinity and selectivity for insect nAChRs (Tomizawa and Casida, 2003). In fact, using cockroach *Periplaneta americana*, Ihara et al. showed that the inward currents evoked by Clo in the neurons from the terminal abdominal ganglia (TAG) were smaller than those induced by ACh suggesting that it was a partial agonist of native nAChRs while its agonist efficacy was higher than of Imi (Ihara et al., 2006). Similarly, using neurons from the three thoracic ganglia, Tan et al. found that Clo was a more effective agonist producing 60–100% of the maximum ACh currents (Tan et al., 2007). In both cases, Clo currents were blocked reversibly by Meca (Ihara et al., 2006) and MLA (Tan et al., 2007), suggesting that it acted as agonist of distinct nAChR subtypes expressed on cockroach neurons. This was exemplified by the finding that a desensitized (nAChRD) and a non-desensitized (nAChRN) α-Bgt-sensitive nAChRs have been identified in the three thoracic ganglia of adult cockroaches (Nauen et al., 2003; Salgado and Saar, 2004). Imi selectively inhibited the nAChRD while MLA specifically inhibited the nAChRN (Salgado and Saar, 2004). Interestingly, Clo was more selective than Imi for nAChD and activated nAChRN currents continuously, suggesting that Clo interacts with the two subtypes (Salgado and Saar, 2004). Moreover, cockroach dorsal unpaired median (DUM) neurons, located along the dorsal midline of the TAG, expressed two distinct α-Bgt-insensitive nAChR subtypes (Courjaret and Lapied, 2001). The α-Bgt-insensitive nAChR2 subtype blocked by Meca was shown insensitive to Imi while nAChR1 subtype, blocked by d-TC, was sensitive (Courjaret and Lapied, 2001). This result suggested that Imi and related compounds could acts as agonist of nAChR1 while nAChR2 could be insensitive to these ligands.

In this paper, we studied the agonist action of Clo on cockroach cercal afferent/giant interneuron synapses and DUM neuron nAChRs. Our data suggested that Clo acted as a specific agonist of nAChR subtypes and the agonist of both nAChR1 and nAChR2 subtypes previously identified. Finally, our results indicated the coexistence of at least three distinct nAChR subtypes on DUM neurons which are sensitive to clothianidin.

2. Materials and methods

All experiments were performed using adult male cockroaches *P. americana* reared at room temperature 29 °C on 12:12 h light/dark cycle.

2.1. Mannitol-gap experiment

2.1.1. Dissection of the abdominal nerve cord

Adult male cockroaches were dissected and opened along the longitudinal dorsal-median line. A fine pair of forceps was used carefully to remove the alimentary canal and overlying muscle and tracheae. The abdominal nerve cord, one circus and the corresponding cercal nerve XI were isolated and immediately flooded 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 (Buckingham et al., 1997). The preparation was then removed and transferred to the recording chamber and continuously superfused with saline and mannitol solution (87 g/l). The subdivisions of the chamber contained: the cerci mounted in air on raised platform followed by the cercal nerve under saline, the sixth abdominal ganglion under saline or test solution, a portion of the connective linking the fifth

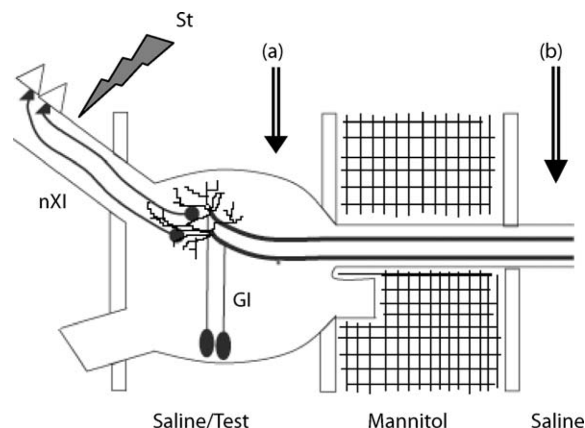

Fig. 1. Schematic representation of the cercal nerve and giant-fiber synapses in the chamber for mannitol-gap recordings. GI, giant interneurons; nXI, cercal nerve XI. The presynaptic cercal nerve XI may be electrically stimulated (St) eliciting an evoked-EPSP and postsynaptic action potential which was recorded by means of two electrodes (a and b). Under this condition, changes in the polarization of the saline/test compartment, during drug action, were measured on the assumption that the polarization of the other compartment remained unchanged (Callec and Sattelle, 1973).

and sixth abdominal ganglia under perfusion by a mannitol solution and the remainder of the ventral nerve cord under saline (Fig. 1). The disposition of the preparation within the chamber facilitated electrical stimulation of the cercal nerve and allowed to preserve the cEPSP, the action potentials and the postsynaptic polarization (Callec and Sattelle, 1973). Recordings were made at room temperature.

2.1.2. Measurement of synaptic activity

For pharmacological experiments, the sixth abdominal ganglion was carefully desheathed to facilitate penetration of bath-applied drugs. The recording electrodes were connected to the input of high-impedance amplifier, whose outputs were passed to a numeric oscilloscope (Hameg, Germany) and a chart recorder (Kipp and Zonen, BD 111, Holland). Variation of postsynaptic polarization was monitored on a chart recorder and the cEPSPs were evoked by electrical stimulation of the ipsilateral cercal nerve XI using a dual pulse stimulator (Campden 915, USA).

2.1.3. Perfusion and drug applications

Clo was applied during 3 min (Bunckingham et al., 1997) with a Micropump fast perfusion (Harvard Apparatus) that produced a constant solution exchange (500 μl/min). All antagonists were bath-applied for at least 20 min before a single perfusion of Clo.

2.2. Patch-clamp recordings

2.2.1. DUM neuron preparation

Recordings were performed on adult DUM neuron cell bodies isolated from the sixth abdominal ganglion, following enzymatic treatment and mechanical dissociation as previously described (Lapied et al., 1989, 1990). The A6 were removed from the nerve cord in the extracellular solution (see below). They were incubated 40 min at 37 °C in the same saline solution supplemented with collagenase (type IA, 2 mg/ml, Worthington Biochemical). They were then transferred back in saline solution supplemented with fetal calf serum (5% by volume, GIBCO-BRL, France), 50 IU/ml penicillin and 50 μg/ml streptomycin (GIBCO-BRL, France). The ganglia were mechanically dissociated by very gentle triturations using fire-polished Pasteur pipettes. The isolated DUM neurons were allowed to settle and adhere to the poly-D-lysine hydrobromide (MW, 70,000–150,000; Sigma Chemicals, France) coating

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the bottom of tissue culture Petri dishes and incubated at 37 °C. All preparations were made under semi-sterile conditions, i.e. all equipment was sterilized with ethanol and solutions were filtered. Cells were used for electrophysiological measurements after 24 h.

2.2.2. Electrophysiological recordings

Clo-induced currents were recorded using the patch-clamp technique in the whole-cell recording configuration under voltage-clamp mode with an axopatch 200B (Patch-clamp amplifier, Axon Instruments, Foster City, CA). Signals were digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments) and axoscope 10.2 software (Axon Instruments). The Petri dish containing isolated cell bodies was placed onto the inverted microscope (CK2: Olympus), and continuously bathed with the standard extracellular solution (in mM: NaCl 200, KCl 3.1, $MgCl_2$ 4, $CaCl_2$ 5, sucrose 50, HEPES 10, pH 7.4 adjusted with NaOH) using a gravity perfusion system positioned within 100 μm from the cell body. In all experiments DUM neurons capacitance ranged between 200 and 250 pF to ensure reproducible current amplitudes between cells.

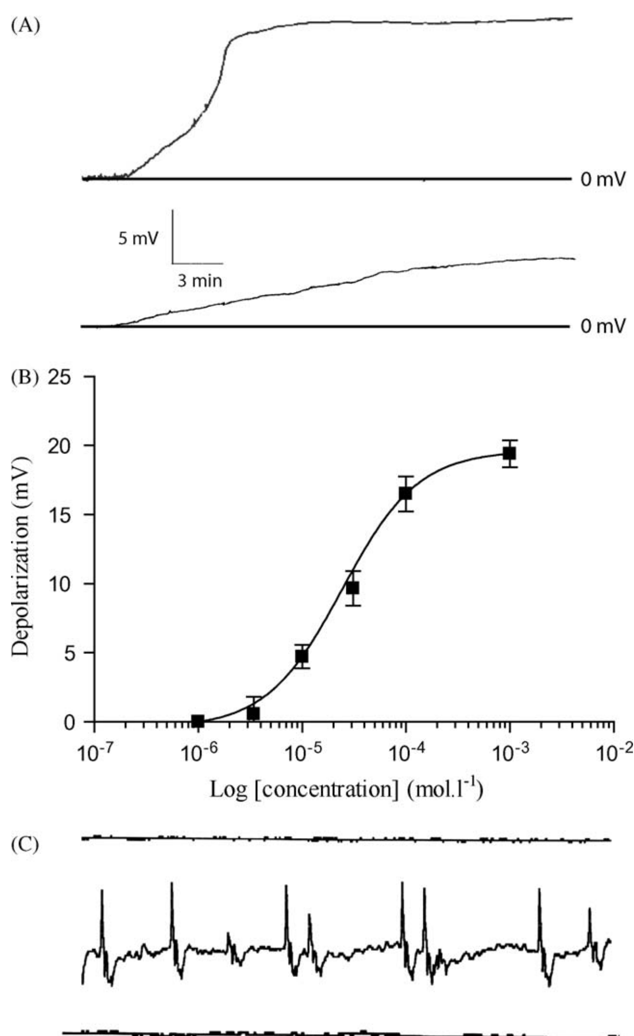

Fig. 2. Clothianidin actions at cholinergic synapse using the mannitol-gap recording. (A) Bath-application of Clo (100 μM, upper trace and 10 μM lower trace) for 3 min evoked a depolarization which did not reversed after wash-out. (B) Dose–response curve of Clo-induced depolarizations. Data are means values of the amplitude of the peak depolarization. (C) Before Clo application (100 μM), no spontaneous activity was observed (upper trace) but 20 s after application a marked increase in frequency of action potentials was found (middle trace). This effect completely disappeared at the depolarization peak (lower trace).

2.2.3. Solutions

Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with internal solution containing (in mM): K-D-gluconic acid, 160; NaCl, 10 mM; $MgCl_2$, 1 mM; $CaCl_2$, 0.5; K-fluoride, 10; ATP Mg, 3; EGTA 10; HEPES, 20 and pH adjusted to 7.4 with KOH. Pipettes had resistances ranging from 0.9 to 1 MΩ when filled with internal solutions. Clo (10 μm) was applied by pneumatic pressure ejection (15 psig, 100 ms. Miniframe, Medical System Corporation, USA). The pressure ejection was made through a glass micropipette, resistance 1.8 MΩ, positioned in solution within 100 μm from the isolated cell body.

2.3. Chemicals

Clo was made in dimethyl sulfoxide (DMSO) with the final concentration of DMSO always less than 0.01%. At this concentration, DMSO did not affect the nerve activity and neuron function. Clo was generously provided by Sumitomo Chemical Company (Tokyo, Japan). All other compounds tested were purchased from Sigma Chemical Company (France).

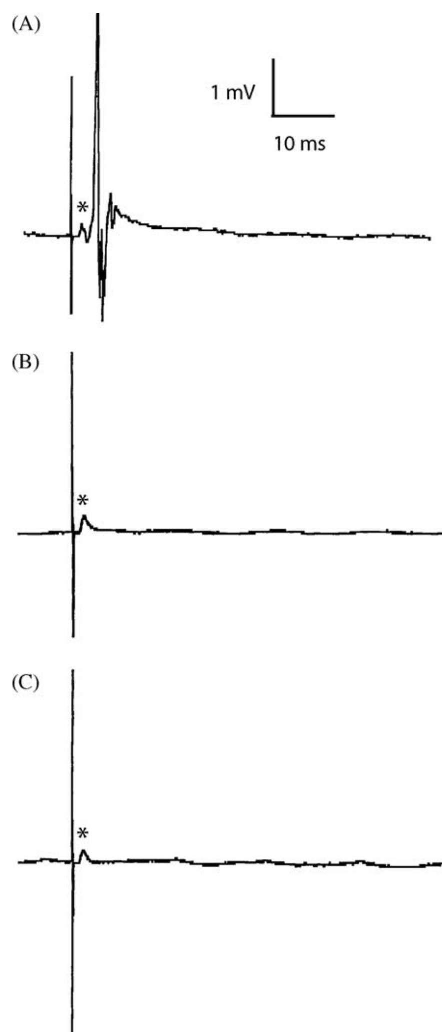

Fig. 3. Effect of clothianidin on electrical stimulation. (A) Excitatory postsynaptic potentials and action potential were observed in response to electrical stimulation of cercal nerve XI. (B) Action potentials and cEPSPs evoked by electrical stimulation were completely blocked by bath-application of 100 μM Clo (3 min). (C) The Blocking action was still visible 1 h after wash-out. We noted that the presynaptic action potential was still visible after Clo effect (indicated by the asterisk).

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2.4. Statistics

Statistical analysis was performed with analysis of variance 'ANOVA' using Prism program (GraphPad Software, San Diego, CA). To compare current amplitudes, the peak amplitudes were normalized ($I/I_{\max}$). The dose–response curve was derived from the fitted curve following the equation:

$$y = I_{\min} + \frac{I_{\max} - I_{\min}}{1 + 10^{(\log(EC_{50} - X)H)}}$$

where Y is the normalized response, $I_{\max}$ and $I_{\min}$ are the maximum and minimum responses, H is the Hill coefficient, EC_{50} is the concentration giving half the maximum response and X is the logarithm of the compound concentration.

3. Results

3.1. Clothianidin actions at cholinergic synapse

We first examined the agonist action of Clo on cercal afferent/giant interneuron synapses in the TAG. Bath-applied Clo (100 μM or 10 μM) (during 3 min) induced a strong depolarization of postsynaptic ACh receptors expressed on 6th abdominal ganglion (Fig. 2A). The Clo-induced depolarization was not reversed after wash-out and this effect was also observed 1 h after application. In some cases a poor reversibility was seen, 3 or 4 h after wash-out. A number of such experiments were performed in which varying doses of Clo were applied to the sixth ganglion. By recording the depolarization in each case a dose–response curve was constructed according to the equation described in Section 2 (Fig. 2B).

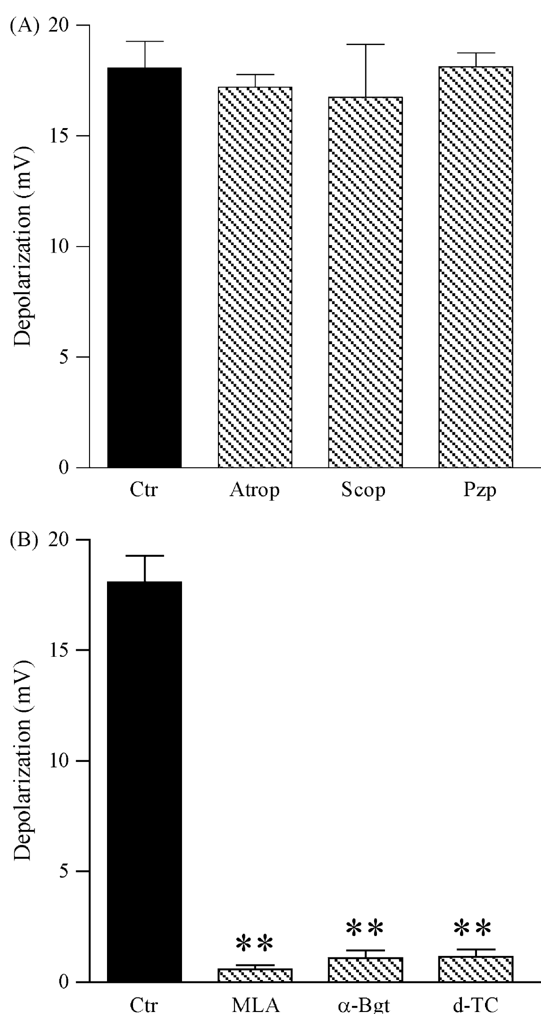

Fig. 4. Effects of cholinergic antagonists on clothianidin-induced depolarizations. (A) The amplitude of Clo responses were not reduced by atropine (50 μM), scopolamine (50 μM) and pirenzepine (50 μM). While (B) it was blocked by methyllicaconitine (50 μM), α-bungarotoxin (50 μM) and d-tubocurarine (50 μM). All antagonists were bath-applied for at least 20 min before a single application of 100 μm clothianidin during 3 min. Methyllicaconitine, MLA; α-bungarotoxin, α-Bgt; atropine, Atr; pirenzepine, Pzp; scopolamine, Scop; d-tubocurarine, d-TC; control, Ctr (one-way ANOVA, $^*p < 0.001$).

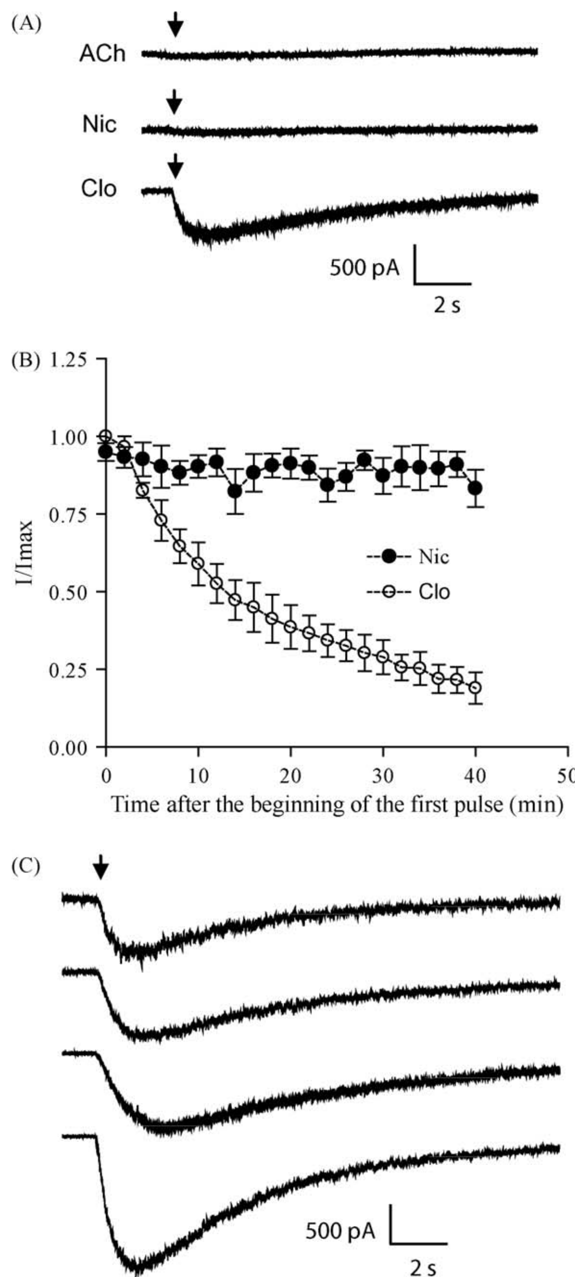

Fig. 5. Clothianidin-induced currents in isolated dorsal unpaired median (DUM) neurons. (A) Comparative effect of acetylcholine (ACh), nicotine (Nic) and clothianidin (Clot) on DUM neurons, at a holding membrane potential of −50 mV. For each molecule the concentration tested was 10 μM. Arrows indicate 100 ms pulse duration (15 psig). (B) DUM neuron responses to consecutive 100 ms pulse durations. Clo-induced current amplitudes (10 μM) decreased after the beginning of the first pulse while pressure application of nicotine (1 mM, Nic) was not variable. Repeated applications of Clo or Nic were made with 2 min interval duration between the end of one application and the beginning of the next. Each current was normalized to the maximum current amplitude obtained in the same condition. (C) Clo-induced currents from different DUM neurons. Demonstration that Clo could act as full and super agonists of insect nAChR depending to the cells, nAChR subtypes or intracellular pathways. Arrow indicates 100 ms pulse duration.

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Moreover, application of Clo was accompanied by a marked increase in frequency of action potentials, which then ceased when the depolarization reached a peak (Fig. 2C). This loss or decrease of functional response upon exposure to Clo was associated to desensitization of postsynaptic receptors and was further confirmed using electrical stimulation of the XI nerve before, during and after Clo application (Fig. 3). Clo completely blocked the cEPSPs evoked by electrical stimulation of cercal nerve XI and this effect was not reversed after wash-out (Fig. 3B and C). The maximum blocking was co-incident with the point of maximum depolarization showing a strong desensitization of postsynaptic acetylcholine receptors following Clo application. A small presynaptic spike, which was related to cercal nerve activity, was still visible, suggesting that as previously shown, the blocking action did not result from a failure of presynaptic action potentials (Buckingham et al., 1997).

To identify the ACh receptors underlined this effect, we used different ACh receptor antagonists. Clo-induced depolarizations were not reduced in the presence of 50 μM atropine (17.2 ± 0.6 mV; $p > 0.05$; $N = 5$), 50 μM scopolamine (16.8 ± 2.4 mV; $p > 0.05$; $N = 5$) and 50 μM pirenzepine (18.3 ± 0.6 mV; $p > 0.05$; $N = 5$) compared to control condition (18.1 ± 1.2 mV) (Fig. 4A). However, the amplitude of Clo responses was blocked by 50 μM α-Bgt (1.07 ± 0.4 mV; $p < 0.001$; $N = 5$), 50 μM MLA (0.57 ± 0.2 mV; $p < 0.001$; $N = 5$) and 50 μM d-TC (1.14 ± 0.3 mV) (Fig. 4B). These results suggested that the main action of Clo on cholinergic synapses was on postsynaptic nicotinic receptors.

3.2. Actions of clothianidin on DUM neuron nicotinic acetylcholine receptors

Pressure application of CLO (10 μM) on DUM neurons induced an inward current at a holding membrane potential of −50 mV. The amplitude of the inward currents was variable and dependent to the cell size. Cells could be categorized as cells with membrane capacitance (Cm) < 250 pF (61%), cells with Cm between 250 and 350 pF (23%) and cells with Cm > 350 pF (12%). For accurate kinetic measurements the Clo-induced currents were studied in cells with Cm between 200 and 250 pF and the pulse duration adjusted to 100 ms. In this condition, compared to nicotine and acetylcholine evoked currents, Clo acted as a full agonist of DUM neuron ACh receptors since its potency to elicit inward currents was higher than that of ACh (10 μM) and nicotine (10 μM) (Fig. 5A). In addition, the current amplitudes evoked by successive pulses decreased strongly during continuous exposure to Clo while nicotine currents remained stable (Fig. 5B). We suggested that this process reflected a strong desensitization of DUM neuron receptors as shown with postsynaptic nAChRs (Quick and Lester, 2002). As we discussed above, Clo currents could be due to a second component, identified as muscarinic (Salgado and Saar, 2004) or 'mixed' nicotinic/acetylcholine receptor (Lapied et al., 1990). But, in our case, the maximal Clo evoked currents (0.76 ± 0.15 nA; $N = 8$) were not affected by 1 μM Pzp (−0.95 ± 0.13 nA; $p > 0.05$; $N = 8$) and 1 μM Atr (−0.87 ± 0.18 nA; $p > 0.05$; $N = 8$) in the bath (Fig. 6A) confirming that as shown with mannitol-gap experiment (see above), Clo actions occurred only through activation of nAChR subtypes. Note that, imidacloprid-induced currents were reduced

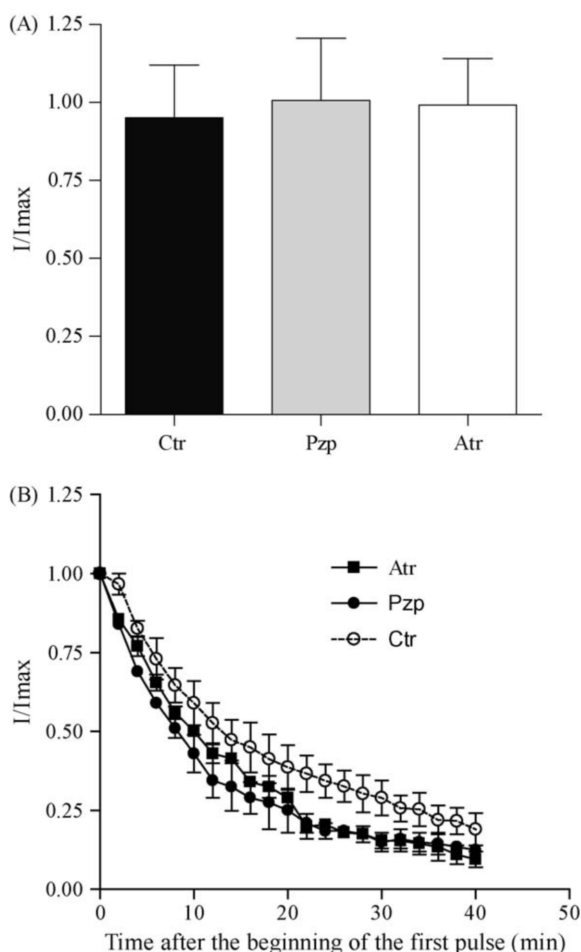

Fig. 6. Clothianidin action after bath-application of muscarinic antagonists. (A) Comparative histogram of the Clo-induced currents after 1 μM pirenzepine (Pzp) and 1 μM atropine (Atr). Each column represents the value of $N = 8$ different cells. Data were mean ± S.E.M. (B) The Clo-induced current amplitudes decreased also after bath-application of 1 μM pirenzepine and 1 μM atropine. Points correspond to the amplitude of Clo currents measured on the same cell body and were normalized to the maximum Clo current obtained in the same condition. In each case, $N = 8$.

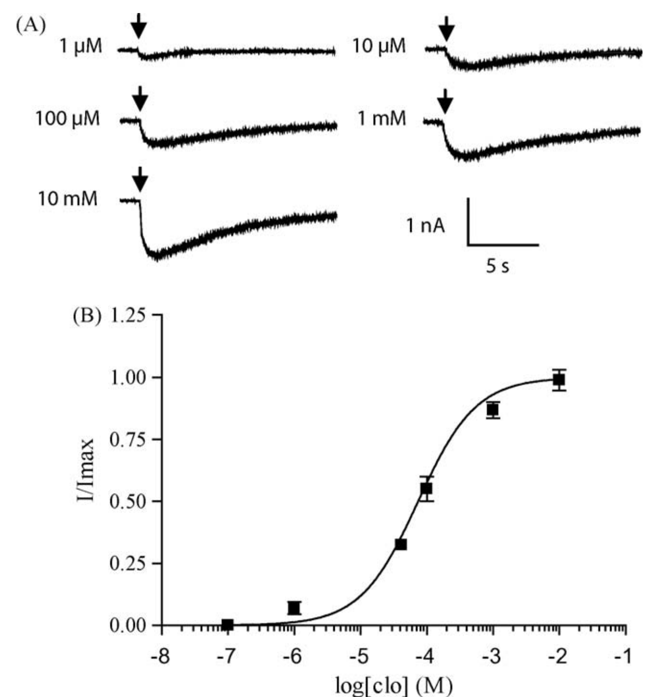

Fig. 7. Concentration–response relationships for clothianidin. (A) Examples of the clothianidin-induced currents at different concentrations (pulse duration 100 ms; at −50 mV holding membrane potential). (B) Peak current induced by each Clo concentration was measured and normalized to the maximum elicited current (relative to the current induced by 10 mM Clo). Curve was fitted to the data point using the Hill equation (see Section 2). Points are mean ± S.E.M. and in each case, $N = 8$.

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after bath-application of 1 μM Pzp (data not shown). Moreover, we observed that current amplitudes were also decrease after Pzp and Atr application indicating that Clo-induced desensitization was not affected by muscarinic or 'mixed' nicotinic/muscarinic receptors (Fig. 6B). Because, Clo-induced currents decreased during repeated application, the establishment of concentration–response curve was performed in independent cells (Fig. 7A and B). The EC_{50} value was derived from the fitted curve and was estimated to 1.7 ± 0.11 μM.

The ionic current induced by Clo could be due to activation of α-Bgt-sensitive and -insensitive nAChRs, including both nAChR1 and nAChR2 subtypes previously identified (Courjaret and Lapied, 2001). Consequently, Clo-induced currents were tested after bath-application of 0.5 μm α-Bgt. The peak amplitude of the inward current, obtained in independent cells, decreased linearly between −90 and −30 mV before increasing again between −30 and +20 mV (Fig. 8A and B). A statistically significant difference was found for α-Bgt compared to control group. The mean current amplitudes were: −0.33 ± 0.05 nA and −0.76 ± 0.15 nA, at −50 mV holding membrane potential ($p < 0.05$; $N = 8$). Interestingly, d-TC and Meca, identified as antagonist of both nAChR1 and nAChR2 (Courjaret and Lapied, 2001), significantly reduced the Clo currents when they were co-applied with 0.5 μM α-Bgt (Fig. 8C). We noted that 20 μM d-TC reduced the current amplitudes (−0.13 ± 0.02 nA; $p < 0.05$; $N = 8$) while 5 μm Meca completely blocked the current (−0.06 ± 0.01 nA; $p < 0.001$; $N = 8$) evoked by pressure application of Clo suggesting that, at this holding membrane potential, the global inward current caused by Clo was determined in part by its affinity to nAChR2 subtypes sensitive to Meca (Fig. 8C). It was interesting to note that the desensitization of nAChRs was reduced following α-Bgt application (Fig. 8D) confirming that (1) this process was not due to run-down and (2) this desensitization occurred through activation of α-Bgt-sensitive nAChRs and consequently, could differ across the nAChR subtypes found in the DUM neurons as shown with the locust αL1 subunit (Amar et al., 1995). Fig. 9A showed the antagonistic effects of MLA on Clo-induced current amplitudes. Bath-application of 0.5 μM MLA completely blocked Clo currents showing that the α-Bgt-sensitive and -insensitive nAChR subtypes are both blocked by MLA. This blocking action occurred despite that the Clo dose was elevated by progressively raising the length of the pressure ejection pulse (Fig. 9B).

4. Discussion

4.1. Agonist actions of clothianidin on synaptic nAChRs

Several studies have shown that ACh was an excitatory transmitter at the synaptic transmission between cercal mechanoreceptor afferent neurons and the giant interneurons (Callec, 1974). Cholinergic antagonists such as snake neurotoxins reduced responses to directly applied ACh as well as EPSP evoked by electrical stimulation of nerve XI showing that they all blocked postsynaptic nAChRs at sub-micromolar concentration (Sattelle et al., 1983). We shown here that bath-application of Clo resulted in dose-dependent depolarization of postsynaptic interneurons which was not reversed after wash-out. This effect was different to those previously described with Imi. In fact, Imi induced a rapid depolarization of postsynaptic neurons which reversed after wash-out (Buckingham et al., 1997). We attributed this effect to a strong desensitization of postsynaptic nicotinic receptors because: (1) Clo actions were not affected by muscarinic antagonists such as Atr, Pzp and Scop and (2) this desensitization was characterized by a loss or reduction of biological response: the inhibition of

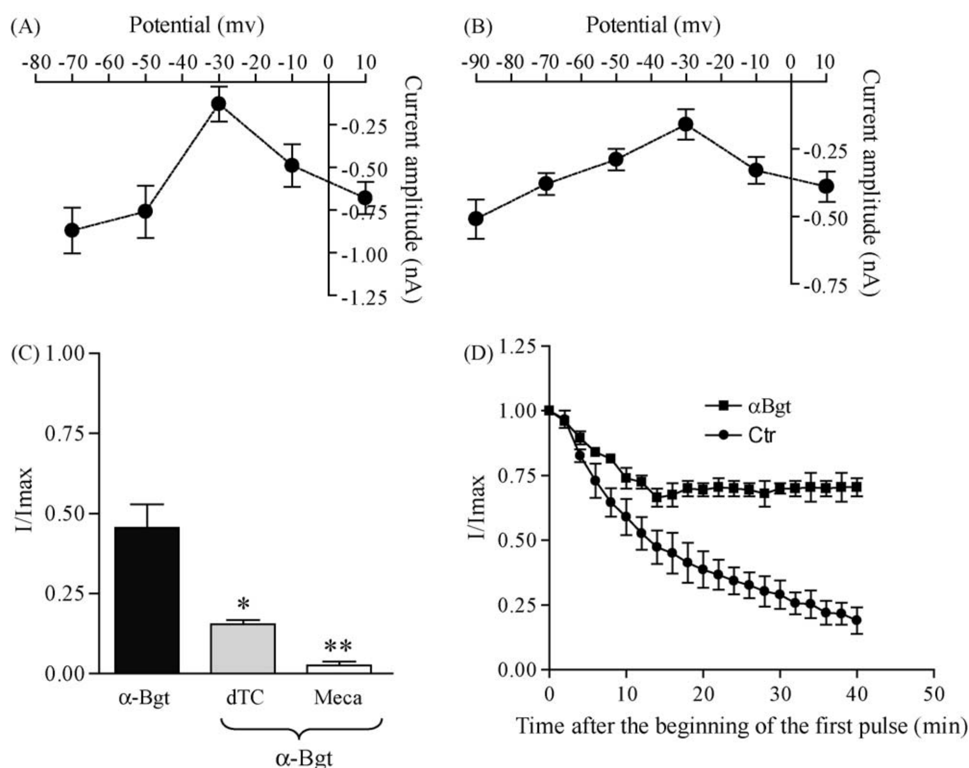

Fig. 8. Clothianidin actions after bath-application of 0.5 μM α-bungarotoxin. (A) Current–voltage relationships of Clo-induced current amplitudes plotted as a function of steady state holding potentials. (B) Clo-induced currents under bath-application of 0.5 μM α-bungarotoxin (α-Bgt). Each point corresponds to independent cells. (C) Histograms illustrating the decrease of clothianidin currents. Application of d-tubocurarine (d-TC) and mecamylamine (Meca) in the presence of 0.5 μM α-Bgt also decrease the current amplitudes. Data are normalized to the maximum current amplitude in control condition (10 μM clothianidin; at −50 mV holding membrane potential; 100 ms pulse duration). Each column is mean ± S.E.M. of 8 cells (*$p < 0.05$; **$p < 0.001$). (D) Clo-induced desensitization was blocked by bath-application of 0.5 μM α-Bgt, 15 min after the beginning of the first pulse. Repeated applications of Clo were made with 2 min interval duration between the end of one application and the beginning of the next. Each current was normalized to the maximum current amplitude obtained in the same condition ($N = 8$).

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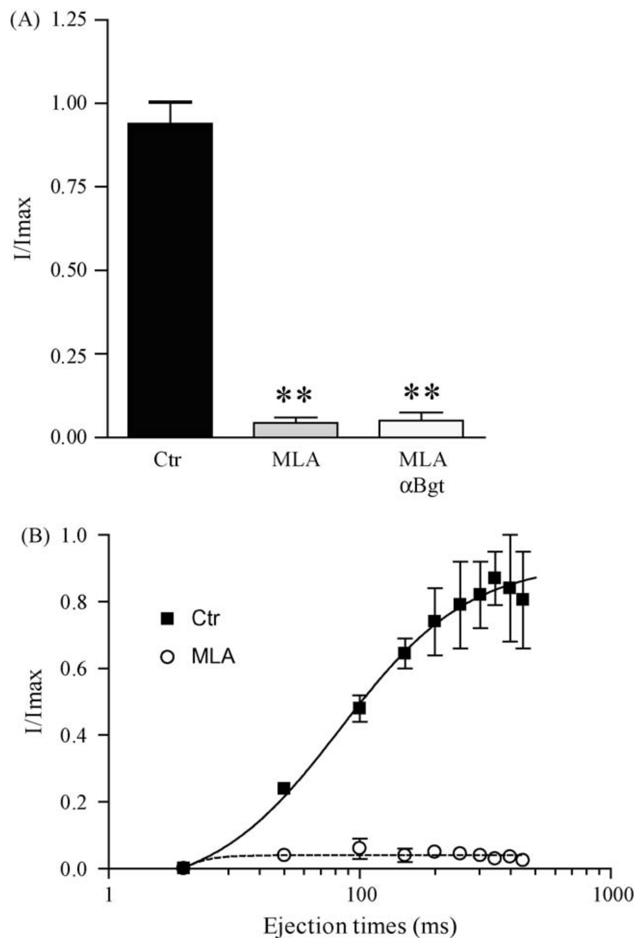

Fig. 9. Effects of 0.5 μM methyllicaconitine (MLA) on clothianidin-induced currents. (A) Histogram showing the blocking action of MLA on Clo-induced currents. Data are mean ± S.E.M. (one-way ANOVA; $^*p < 0.001$; $N = 8$). (B) Clo dose was elevated by progressively raising the length of the pressure ejection pulse (Alix et al., 2002). The amplitude of the inward current was completely blocked by 0.5 μM MLA. Data were normalized to the maximum Clo currents in response to 400 ms pulse duration (at −50 mV holding membrane potential).

spontaneous activities and electrical stimulations. As shown with Colorado potato beetle, *L. decemlineata* (Tan et al., 2008), Clo had also two distinct effects: the first action was an excitatory action characterized by a strong increase of the number of action potentials and the second stage was the inhibition of this burst of action potentials. In fact, the cEPSP evoked by electrical stimulation of the nerve XI was completely blocked after Clo application suggesting that as with Imi, it altered the postsynaptic nicotinic receptors (Buckingham et al., 1997). We noted that these effects on cockroach CNS was observed at the same concentration and could be associated to its insecticidal activity. The lethal dose of Clo and others neonicotinoids was characterized by a biphasic activity: a period of hyperexcitation followed by a partial paralysis, with weak and diminishing uncoordinated movements. We concluded that the decrease or inhibition of synaptic activity and action potentials could explain the neurotoxicity effect of Clo to the insect CNS. Our results were in accordance with previous studies showing that lethality in cockroaches is more closely related to the CNS inhibitory effect of nicotinic agonists on nAChRs (Tan et al., 2008).

4.2. Clothianidin currents associated with the existence of different nAChRs subtypes

Investigations performed on cockroach native neurons have identified two distinct α-Bgt-sensitive and -insensitive nAChR subtypes. The α-Bgt-sensitive subtypes included the 'mixed' nicotinic/muscarinic receptor (Lapied et al., 1990) and both nAChRD and nAChRN receptors (Salgado and Saar, 2004); while the other insensitive was characterized by the nAChR1 and nAChR2 subtypes (Courjaret and Lapied, 2001). Pressure application of Imi on DUM neurons suggested that it could bind to the 'mixed' nicotinic/muscarinic receptors (Buckingham et al., 1997). By contrast, the present data demonstrated that Clo did not acted as its agonist because Clo-induced currents were not affected by Pzp and Atr contrary to ACh and nicotine (Lapied et al., 1990). Nevertheless, we have shown that a second nAChR subtype insensitive to α-Bgt could bind to Clo because MLA completely blocked Clo currents. This was persistent with the finding that a small proportion of receptors labelling MLA could be insensitive to α-Bgt (Davies et al., 1999). These α-Bgt-insensitive nAChRs could include both nAChR1 and nAChR2 subtypes which were blocked by d-TC and Meca (Courjaret and Lapied, 2001). All these results suggested that heterogeneity existed within the cockroach nicotinic receptors previously assumed to be both α-Bgt and MLA sensitive. In fact, previously, it has been shown that the maximal currents induced by Clo in the cockroach TAG were smaller than those induced by ACh, indicating that it was a partial agonist of native nAChRs (Ihara et al., 2006) while, Tan et al., showed that Clo was a full agonist (Tan et al., 2007). This discrepancy between partial and full agonist action, identified in other insects (Deglise et al., 2002; Brown et al., 2006), was probably due to difference in the subunits composing the nAChRs tested and/or distinct nAChR subtypes. In the terminal abdominal ganglion, DUM neurons are arranged along the dorsal midline in three clusters: anterior, median and posterior which innervate specific targets (Sinakevitch et al., 1995). It was difficult to identify these clusters following mechanical dissociation; nevertheless, we found that Clo could act as 'super' agonist of some DUM neurons (see Fig. 5C). Consequently, we confirmed that Clo acted as partial, full and super agonist depending to its affinity to nAChRs expressed on cockroach neurons.

4.3. Functional implications in pest control and insect resistance: working hypothesis

Based on maximum inward currents, neonicotinoids have been divided into two groups. Those with heterocyclic ring (i.e. nicotine and imidacloprid) which were partial agonists, and the open chain compounds (i.e. clothianidin) which were more effective agonists (Tan et al., 2007). According to previous studies, we confirm that Imi and Clo differently act as agonist of nAChRs expressed on cockroach neurons. We propose that two distinct nAChRs with low and high affinity to neonicotinoids could exist in the insect. Consequently, the development of insect resistance to insecticide could be mediated by a decrease of the number of high affinity receptors or the increase of low affinity receptors. Thus, if the total number of receptors in the membrane is constant, phosphorylation–dephosphorylation mechanisms could account for the overall increase in the number of high affinity nAChRs sensitive to Clo or the decrease of low affinity receptors. In fact, it has been shown that phosphorylation–dephosphorylation mechanisms could preferentially stabilize a fraction of the total receptor population in one state that has distinct biophysical properties to the alternative (Buisson and Bertrand, 2002). In addition, it has been shown that increasing cAMP concentration decrease the amplitude of imidacloprid-induced currents suggesting that intracellular pathways differently regulated nAChR function (Courjaret and Lapied, 2001). Our future goal will be to investigate the involvement of intracellular pathways modulating neonicotinoid actions.

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Conflict of interest

Author declares that there are no conflicts of interest.

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References

Alix P, Grolleau F, Hue B. Ca^{2+}/calmodulin-dependent protein kinase regulates GABA-activated Cl-current in cockroach dorsal unpaired median neurons. J Neurophysiol 2002;87:2972–82.

Amar M, Thomas P, Wonnacott S, Lunt GG. A nicotinic acetylcholine receptor subunit from insect brain forms a non-desensitising homo-oligomeric nicotinic acetylcholine receptor when expressed in Xenopus oocytes. Neurosci Lett 1995;199:107–10.

Brown LA, Ihara M, Buckingham SD, Matsuda K, Sattelle DB. Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J Neurochem 2006;99:608–15.

Buckingham S, Lapied B, Corronc H, Sattelle F. Imidacloprid actions on insect neuronal acetylcholine receptors. J Exp Biol 1997;200:2685–92.

Buisson B, Bertrand D. Nicotine addiction: the possible role of functional upregulation. Trends Pharmacol Sci 2002;23:130–6.

Bunckingham SD, Le Corronc BL, Grolleau HF, Sattelle DB. Imidacloprid actions on insect neuronal acetylcholine receptors. J Exp Biol 1997;200:2685–91.

Callec JJ. Synaptic transmission in the central nervous system of insects. In: Treherne JE, editor. Insect Neurobiology. Amsterdam: Elsevier; 1974, pp. 119–185.

Callec JJ, Sattelle DB. A simple technique for monitoring the synaptic actions of pharmacological agents. J Exp Biol 1973;59:725–38.

Courjaret R, Lapied B. Complex intracellular messenger pathways regulate one type of neuronal alpha-bungarotoxin-resistant nicotinic acetylcholine receptors expressed in insect neurosecretory cells (dorsal unpaired median neurons). Mol Pharmacol 2001;60:80–91.

Davies AR, Hardick DJ, Blagbrough IS, Potter BV, Wolstenholme AJ, Wonnacott S. Characterisation of the binding of [^{3}H]methyllycaconitine: a new radioligand for labelling alpha 7-type neuronal nicotinic acetylcholine receptors. Neuropharmacology 1999;38:679–90.

Deglise P, Grunewald B, Gauthier M. The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. Neurosci Lett 2002;321:13–6.

Ford KA, Casida JE. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. Chem Res Toxicol 2006;19:1549–56.

Honda H, Tomizawa M, Casida JE. Insect nicotinic acetylcholine receptors: neonicotinoid binding site specificity is usually but not always conserved with varied substituents and species. J Agric Food Chem 2006;54:3365–71.

Ihara M, Brown LA, Ishida C, Okuda H, Sattelle DB, Matsuda K. Actions of imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors of American cockroach neurons and their relationships with insecticidal potency. J Pestic Sci 2006;31:35–40.

Ihara M, Matsuda K, Otake M, Kuwamura M, Shimomura M, Komai K, et al. Diverse actions of neonicotinoids on chicken alpha7, alpha4beta2 and *Drosophila*–chicken SADbeta2 and ALSbeta2 hybrid nicotinic acetylcholine receptors expressed in Xenopus laevis oocytes. Neuropharmacology 2003;45:133–44.

Jeschke P, Nauen R. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Manag Sci 2008;64:1084–98.

Jones AK, Grauso M, Sattelle DB. The nicotinic acetylcholine receptor gene family of the malaria mosquito, *Anopheles gambiae*. Genomics 2005;85:176–87.

Jones AK, Raymond-Delpech V, Thany SH, Gauthier M, Sattelle DB. The nicotinic acetylcholine receptor gene family of the honey bee, *Apis mellifera*. Genome Res 2006;16:1422–30.

Jones AK, Sattelle DB. The cys-loop ligand-gated ion channel gene superfamily of the red flour beetle, *Tribolium castaneum*. BMC Genom 2007;8:327.

Lapied B, Le Corronc H, Hue B. Sensitive nicotinic and mixed nicotinic–muscarinic receptors in insect neurosecretory cells. Brain Res 1990;533:132–6.

Lapied B, Malecot CO, Pelhate M. Ionic species involved in the electrical activity of single adult aminergic neurones isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana*. J Exp Biol 1989;144:535–49.

Nauen RE-K, Salgado U, Kaussmann VLM. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pestic Biochem Physiol 2003;76: 55–69.

Quick MW, Lester RA. Desensitization of neuronal nicotinic receptors. J Neurobiol 2002;53:457–78.

Salgado VL, Saar R. Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. J Insect Physiol 2004;50:867–79.

Sattelle DB, Harrow ID, Hue B, Pelhate M, Gepner JI, Hall LM. Alpha-bungarotoxin blocks excitatory synaptic transmission between cercal sensory neurones and giant interneuron 2 of the cockroach, *Periplaneta americana*. J Exp Biol 1983;107:473–89.

Sattelle DB, Jones AK, Sattelle BM, Matsuda K, Reenan R, Biggin PC. Edit, cut and paste in the nicotinic acetylcholine receptor gene family of *Drosophila melanogaster*. Bioessays 2005;27:366–76.

Schroeder ME, Flattum RF. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. Pest Biochem Physiol 1984;22:148–60.

Sinakevitch II, Geffard M, Pelhate M, Lapied B. Octopaminergic dorsal unpaired median (DUM) neurones innervating the colleterial glands of the female cockroach *Periplaneta americana*. J Exp Biol 1995;198:1539–44.

Tan J, Galligan JJ, Hollingworth RM. Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. Neurotoxicology 2007;28:829–42.

Tan J, Salgado VL, Hollingworth RM. Neural actions of imidacloprid and their involvement in resistance in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Pest Manag Sci 2008;64:37–47.

Thany SH, Lenaers G, Raymond-Delpech V, Sattelle DB, Lapied B. Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. Trends Pharmacol Sci 2007;28:14–22.

Tomizawa M, Casida JE. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu Rev Entomol 2003;48:339–64.

Zhang Y, Liu S, Gu J, Song F, Yao X, Liu Z. Imidacloprid acts as an antagonist on insect nicotinic acetylcholine receptor containing the Y151M mutation. Neurosci Lett 2008;446:97–100.