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ORIGINAL PAPER

Quinuclidine compounds differently act as agonists of Kenyon cell nicotinic acetylcholine receptors and induced distinct effect on insect ganglionic depolarizations

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Abstract We have recently demonstrated that a new quinuclidine benzamide compound named LMA10203 acted as an agonist of insect nicotinic acetylcholine receptors. Its specific pharmacological profile on cockroach dorsal unpaired median neurons (DUM) helped to identify alpha-bungarotoxin-insensitive nAChR2 receptors. In the present study, we tested its effect on cockroach Kenyon cells. We found that it induced an inward current demonstrating that it bounds to nicotinic acetylcholine receptors expressed on Kenyon cells. Interestingly, LMA10203-induced currents were completely blocked by the nicotinic antagonist α-bungarotoxin. We suggested that LMA10203 effect occurred through the activation of α-bungarotoxin-sensitive receptors and did not involve α-bungarotoxin-insensitive nAChR2, previously identified in DUM neurons. In addition, we have synthesized two new compounds, LMA10210 and LMA10211, and compared their effects on Kenyon cells. These compounds were members of the 3-quinuclidinyl benzamide or benzoate families. Interestingly, 1 mM LMA10210 was not able to induce an inward current on Kenyon cells compared to LMA10211. Similarly, we did not find any significant effect of LMA10210 on cockroach ganglionic depolarization, whereas these three compounds were able to induce an effect on the central nervous system of the third instar *M. domestica* larvae. Our data suggested that these three compounds could bind to distinct cockroach nicotinic acetylcholine receptors.

Keywords 3-Quinuclidinyl benzamide · 3-Quinuclidinyl benzoate · Neurons · Nicotinic receptors · Insect

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Introduction

Insect nicotinic acetylcholine receptors (nAChRs) play a central role in rapid neurotransmission and are the major targets of neonicotinoid insecticides (Matsuda et al. 2005; Tomizawa and Casida 2005; Thany et al. 2007). Pharmacological studies performed in several insect neurons demonstrated that they expressed different nAChR subtypes with different profiles (Barbara et al. 2005, 2008; Brown et al. 2006; Oliveira et al. 2011). Interestingly, these pharmacological studies were accompanied by an active search of new compounds such as asperparalines and sulfoximines, which targeted insect nAChRs (Fig. 1) (Hirata et al. 2011; Watson et al. 2011; Oliveira et al. 2011). Recently, it was proposed that asperparaline A is a noncompetitive antagonist of *Bombix mori* nAChRs. It blocked the ACh-induced currents that were sensitive to the nicotinic antagonist mecamylamine (Hirata et al. 2011). Moreover, using co-expression of *Drosophila melanogaster* Dα2 and Dα1 and vertebrate β2 subunits, it was shown that the sulfoximine insecticide sulfoxaflor was a full agonist of Dα2/β2 nAChRs. Sulfoxaflor elicited significantly higher amplitude currents than all the compounds tested except clothianidin (Watson et al.

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2011). In others studies, sulfoxaflor and two novel 6-chloropyridine sulfoximine analogs were evaluated on the stick insect, *Carausius morosus* nAChRs (Oliveira et al. 2011). These sulfoximine insecticides induced small current amplitudes showing that they have agonist activity. In the same way, it was proposed that they have differential actions on stick desensitizing nAChR subtypes (Oliveira et al. 2011). Thus, these new compounds identify specific nAChRs in insect species.

Recently, electrophysiological studies performed with the cockroach *Periplaneta americana* proposed that different nAChRs existed with specific pharmacological profile (Courjaret and Lapied 2001; Thany et al. 2008; Calas-List et al. 2012). We demonstrated that several nAChRs were expressed in dorsal unpaired median (DUM) neurons such as the alpha-bungarotoxin (α-Bgt)-insensitive nAChR1 and nAChR2 (Courjaret and Lapied 2001; Courjaret et al. 2003; Thany et al. 2008). In addition, we have prepared and studied a benzamide compound named LMA10203 that could be regarded as a quinuclidine analog of tropisetron (Fig. 1). We have found that LMA10203 was able to act as a specific agonist of DUM neuron α-Bgt-insensitive nAChR2 (Mathé-Allainmat et al. 2012). These data confirmed our findings that specific compounds could help to identify nAChR subtypes in cockroach DUM neurons.

To go further in our investigations, we selected and prepared two novel 3-substituted quinuclidine derivatives. The first one, named LMA10210, is the strict 2-hydroxy analog of LMA10203. The second one, named LMA10211, is a quinuclidine analog of Tropisetron, characterized as a potent nAChR partial agonist (Macor et al. 2001). The present experiments were undertaken to determine whether LMA10203 and its analogs LMA10210 and LMA10211 were able to act as agonists of nAChRs expressed on cockroach Kenyon cells. In addition, we have compared their effects on *Musca domestica* central nervous system.

Materials and methods

Chemistry

All solvents and reagents were obtained from commercial sources without further purification. All reactions were performed under argon atmosphere. ^{1}H NMR and ^{13}C NMR spectra were recorded on a *BRUKER Avance I* (^{1}H = 300 MHz, ^{13}C = 75 MHz), in deuterated solvents using TMS as internal standard; chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hertz (Hz). Splitting patterns are designed as follows: s, singulet; dd, double doublet; t, triplet; m, multiplet. Flash column chromatography was performed on SDS alumina neutral (63–200 μm). Low-resolution mass spectra (MS) were performed on a Thermo-Finnigan DSQII quadripolar spectrometer in chemical ionization (CI) at 70 eV with NH_3 gas. High-resolution mass spectrometry (HRMS) analyses were performed at the "Centre Commun de Spectrométrie de Masse" in Lyon (France), on a Micro-TOFOII Thermofischer Scientific for electrospray ionization (ESI) measurements. Analytical high-pressure liquid chromatography (HPLC) was performed on CHIRALCEL® OD-H column (250 × 4.6 mm) with a flow rate of 0.5 mL/min (shipping solvent: *n*-hexane/ethanol solvent mixture 90:10 v/v), under UV detection. LMA 10211 was prepared following the procedure earlier described (Langlois et al. 1993).

Synthesis of *N*-1-azabicyclo[2,2,2]oct-3-yl-2-fluorobenzamide (**(±)-1**-LMA10203)

The dihydrochloride salt of (±)-3-aminoquinuclidine (0.79 g, 4 mmol) was dissolved in dry chloroform (15 mL) under argon in the presence of Et_3N (1.1 mL, 8 mmol) and pyridine (1 mL) during 45 min. To a solution of

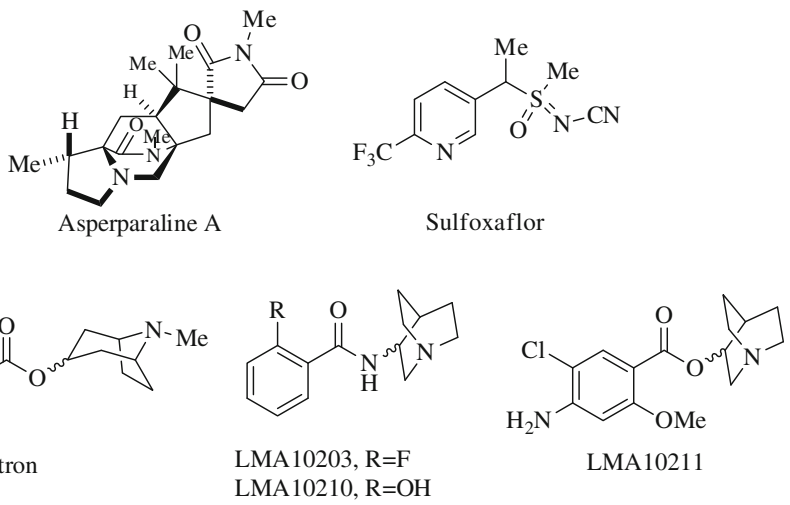

Fig. 1 Molecules identified as nicotinic acetylcholine receptors ligands (asperparaline A, sulfoxaflor and tropisetron) and novel 3-quinuclidinyl benzamide (LMA10203 and LMA10210) and benzoate (LMA10211) compounds studied

2-fluorobenzoyl chloride (0.5 mL, 4 mmol) in chloroform (10 mL) was added dropwise at 0 °C and under argon to the previously prepared free aminoquinuclidine solution. The reaction mixture was then stirred at room temperature during 18 h and quenched with 2.5 N solution of NaOH. The aqueous phase was extracted twice with chloroform, and the organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The residue was then purified by flash chromatography on neutral alumina column (1–5 % MeOH in $CHCl_3$) to give 0.87 g (88 %) of benzamide **(±)-1** as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.08$ (ddd, 1H, $J = 7.94$ and 1.89, ArH), 7.26 (m, 1H, ArH) (td, 1H, $J = 7.68$ and 1.1, ArH), 7.125 (ddd, 1H, $J = 12.25$, 8.27 and 1.1, ArH), 4.18 (m, 1H, CH), 3.445 (dd, 1H, $J = 14.2$ and 9.9, CH_2), 2.86 (m, 4H, 2xCH_2), 2.61 (dd, 1H, $J = 14.2$ and 4.8, CH_2), 2.03 (m, 1H, CH), 1.71 (m, 3H, CH_2), (1.53 (m, 1H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 162.98$ (CO), 160.61 (d, $J = 244$, CF), 133.22 (d, $J = 9$, Car), 131.98 (Car), 124.85 (Car), 121.01 (d, 11.2, C_{IV}ar), 115.93 (d, $J = 24$, Car), 56.33 (CH_2), 47.38 (CH_2), 47.19 (CH), 46.61 (CH_2), 25.76 (CH_2), 25.6 (CH), 20.28 (CH_2). HRMS (CI): $C_{14}H_{18}FN_2O$ $[M + H]^+$ *m/z*, calcd 249.1398, found 249.1399. HPLC: T_R min, 25.435 (48 %) and 29.92 (52 %).

Synthesis of *N*-1-azabicyclo[2,2,2]oct-3-yl-2-hydroxybenzamide (**(±)-3-LMA10210**)

The dihydrochloride salt of (±)-3-aminoquinuclidine (1.2 g, 6 mmol) was dissolved in dry chloroform (15 mL) under argon in the presence of Et_3N (1.67 mL, 12 mmol) and pyridine (1 mL) during 45 min. 2-Methoxybenzoyl chloride was prepared by refluxing the corresponding carboxylic acid (0.91 g, 6 mmol) in toluene (10 mL) in the presence of oxalyl chloride (2.3 mL, 30 mmol, 5 eq.) during 3 h and then concentrated to dryness. The residue was taken up in chloroform (10 mL), and this solution was added dropwise at 0 °C and under argon, to the previously prepared free aminoquinuclidine solution. The reaction mixture was then stirred at room temperature during 18 h and quenched with 2.5 N solution of NaOH. The aqueous phase was extracted twice with chloroform and the combined organic layers were dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The residue was then purified by flash chromatography on neutral alumina column (1–5 % MeOH in $CHCl_3$) to give 1.22 g (78 %) of benzamide **(±)-2** which was derived to its hydrochloride salt **(±)-2**. HCl. ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 10.8$ (sl, 1H), 8.43 (d, 1H, $J = 6.49$ Hz, NH), 7.56 (dd, 1H, $J = 1.68$ and 7.55, ArH), 7.46 (td, 1H, $J = 1.7$ and 8.9, ArH), 7.13 (d, 1H, $J = 8.3$ ArH), 7.02 (t, 1H, $J = 7.3$, ArH), 4.28 (m, 1H, C*H*NH), 3.86 (s, 3H, OMe), 3.59 (t, 1H, $J = 10.05$, NCH_2CH), 3.36–3.00 (m, 5H, NCH_2CH and NCH_2), 2.17 (m, 1H, C*H*CHNH), 2.02 (m, 1H, CH_2), 1.88 (m, 2H, CH_2), 1.74 (m, 1H, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 165.72$ (CO), 156.54 (Car-OMe), 131.85 (Car), 129.58 (Car), 124.02 (C_{IV}ar), 120.29 (Car), 115.83 (Car), 55.80 (OMe), 50.89 (CH_2), 45.15 (CH_2), 44.75 (CH_2), 44.14 (CH), 24.25 (CH), 21.29 (CH_2), 17.03 (CH_2).

The previously prepared salt **(±)-2**.HCl (0.53 g, 1.78 mmol) was dissolved in chloroform (6 mL) and a solution of BBr_3 in chloroform (4 mL) was added dropwise at 0 °C. The reaction middle was stirred during 0.5 h at 0 °C and another 0.5 h at room temperature and then quenched with a 1 N NH_4OH solution (10 mL). The aqueous phase was extracted with chloroform and the combined organic layers were dried over $MgSO_4$ and filtered. The expected compound **(±)-3** was purified by flash chromatography on neutral alumina column (1–10 % MeOH in $CHCl_3$) to give 0.29 g (67 %) of a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 12.3$ (sl, 1H, OH), 9.10 (d, 1H, $J = 6.08$ Hz, NH), 8.11 (dd, 1H, $J = 1.5$ and 7.97, ArH), 7.40 (td, 1H, $J = 1.3$ and 7.25, ArH), 7.13 (d and t, 2H, ArH), 4.35 (m, 1H, C*H*NH), 3.59 (t, 1H, $J = 10.05$, NCH_2CH), 3.4–3.00 (m, 5H, NCH_2CH and NCH_2), 2.20 (m, 1H, C*H*CHNH), 2.08 (m, 1H, CH_2), 1.88 (m, 2H, CH_2), 1.71 (m, 1H, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 169.28$ (CO), 159.75 (Car-OH), 133.83 (Car), 128.41 (Car), 118.48 (Car), 117.20 (Car), 115.12 (C_{IV}ar), 50.06 (CH_2), 45.20 (CH_2), 44.85 (CH_2), 44.53 (CH), 24.27 (CH), 21.34 (CH_2), 17.15 (CH_2). MS (CI) : $C_{14}H_{19}N_2O_2$ $[M + H]^+$ *m/z*, 247.

Cell culture

The cockroach brain was removed from the head capsule and placed in saline solution (in mM: NaCl 200, KCl 3.1, $MgCl_2$ 4, $CaCl_2$ 5, sucrose 50, HEPES 10, pH 7.4 adjusted with NaOH). Then, the mushroom bodies (MBs) were dissected out from the brain and collected in a hemolyse tube 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 MBs were mechanically dissociated by gentle triturations using fire-polished Pasteur pipettes (Benzidane et al. 2011). The isolated Kenyon cells were allowed to settle and adhere to the poly-D-lysine hydrobromide (MW, 70,000–150,000; Sigma Chemicals, France) coating the bottom of tissue-culture Petri dishes and were then incubated at 37 °C. Cells were used for electrophysiological measurements after 24 h.

Patch-clamp recordings

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 saline solution, using a gravity perfusion system positioned within 100 µm of the cell body.

Solutions

Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with an internal solution containing (in mM) the following: K-D-gluconic acid, 160; NaCl, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; K-fluoride, 10; ATP Mg, 3; EGTA 10; HEPES, 20; and pH adjusted to 7.4 with KOH. Pipettes had resistances of 9 MΩ when filled with internal solution. Compounds were applied by pneumatic pressure ejection (15 psig, 200 ms. Miniframe, Medical System Corporation, USA). The pressure ejection was carried out through a glass micropipette, resistance 1.6 MΩ, positioned in the solution within 100 µm of the isolated cell body. All compounds were diluted in DMSO to 1,000× the desired test concentration, giving a final DMSO concentration of 0.01 %.

Dissection of the cockroach abdominal nerve cord

Adult male cockroaches were dissected and opened along the longitudinal dorsal-median line. A fine pair of forceps was used to remove the alimentary canal and overlying muscle and tracheae carefully. The abdominal nerve cord, one cercus 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 following: the cerci mounted in air on a 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 and sixth abdominal ganglia under perfusion by a mannitol solution, and the remainder of the ventral nerve cord under saline (Thany 2009). The arrangement of the preparation within the chamber facilitated electrical stimulation of the cercal nerve and preserved the excitatory postsynaptic potentials (EPSPs), the action potentials and the postsynaptic polarization (Callec and Sattelle 1973).

Measurement of synaptic activity

The sixth abdominal ganglion was carefully desheathed to facilitate the penetration of bath-applied drugs. The extracellular recording electrodes were connected to the input of a high-impedance amplifier whose outputs were passed to a numeric oscilloscope (Hameg, Germany) and an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) connected to a computer with the pClamp software control (pClamp 10.0, Axon Instruments, Foster City, USA). Variation in postsynaptic polarization was recorded with pClamp 10.0. The EPSPs were evoked by electrical stimulation of the ipsilateral cercal nerve XI using a dual pulse stimulator (Campden 915, USA).

Perfusion and drug applications

Drugs were applied for 3 min (Buckingham et al. 1997) with a Micropump fast perfusion (Harvard Apparatus) that produced a constant solution exchange (500 µL/min).

Animals

Experiments were performed with adult male cockroaches *Periplaneta americana* from Arbiotech (Saint Gilles, France) and reared under 12 h:12-h day/light conditions.

CNS preparations from the third instar *M. domestica* larvae

Musca domestica larvae were provided by Dr. Phil Kaufman (cultures maintained in the Department of Entomology and Nematology, Medical Entomology Laboratory, University of Florida, Gainesville, FL, USA).

Suction electrode recordings

Suction electrode recordings were performed on the central nervous system of third instar *M. domestica* larvae and were based on the methods described in Bloomquist et al. (Bloomquist et al. 1991). Electrodes/pipettes were pulled from borosilicate glass capillaries with filament (outer diameter 1.0 mm, inner diameter 0.8 mm; Sutter Instrument, Novato CA, USA) on a P-1000 Flaming/Brown micropipette puller (Sutter Instrument). The recordings were performed with saline (in mmol l^{-1}): NaCl, 140; $CaCl_2$, 0.75; KCl, 5; $MgCl_2$, 4; $NaHCO_3$, 5; HEPES, 5; pH 7.25.

The CNS was manually transected posterior to the cerebral lobes to disrupt the blood–brain barrier and enhance the chemical penetration into the CNS. After dissection was complete, nerve trunks were drawn into a recording suction electrode, allowing amplification and digitization of the electrical activity originating from the CNS. The signal was

fed into an amplifier that quantified individual spikes and converted them into a rate, expressed in Hz (MacLab, ADInstruments, Colorado Springs, CO, USA). Activity was monitored using LabChart 7 for a 10-min time period to establish a constant baseline firing rate.

Drug applications

After a constant baseline was established, the CNS preparation was directly exposed to the compound by adding 200 µL of the chemical to the bath containing 200 µL of saline. The final concentration of solvent in the bath was 0.1 % DMSO. Each concentration was recorded for at least 3 min or longer to allow stabilization of firing frequency, prior to the addition of the next inhibitor concentration.

Statistical analysis

Statistical analysis was performed using one-way ANOVA, and statistical significance was assessed by a standard Bonferroni's test using GraphPad software. Results were expressed as mean ± SEM. The dose–response curves were derived from the fitted curve following the equation:

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

where Y is the normalized response, $I_{\max}$ and $I_{\min}$ are the maximum and minimum responses, "H" is the Hill coefficient, and EC_{50} is the concentration giving half the maximum response. For mannitol-gap experiments, $I_{\min}$ and $I_{\max}$ were replaced by V and Vmax, respectively. For electrophysiological experiments using third instar *M. domestica* larvae, mean spike frequencies ($N = 3$–5) for each concentration were used to construct the dose–response curves. Mean spike frequencies were determined through analysis of the complete three-minute time period unless the preparation exhibited nerve block within the same concentration that induced neuroexcitation. This effect was observed with LMA10211 as 100 µM induced neuroexcitation followed immediately by block. In this instance, the mean spike discharge frequency was determined through averaging the spike frequencies from the addition of the drug, through the excitatory phase, and just prior to the onset of nerve block. Dose–response curves were used to determine the EC_{50} values and were calculated by nonlinear regression (variable slope) using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA).

Results

Synthetic chemistry

The 3-quinuclidinyl benzamides described in this paper were prepared according to the synthetic scheme (Fig. 2) starting from the corresponding commercial or synthesized, ortho substituted benzoyl chloride (Fig. 2a). The dihydrochloride salt of (±)-3-aminoquinuclidine was first suspended in chloroform in the presence of an excess of Et_3N and pyridine to liberate the free amine. This solution was then treated with the selected benzoyl chloride derivative to give, after purification, the racemic 2-fluoro **(±)-1** (named LMA10203) or 2-methoxy-benzamide **(±)-2**. Demethylation of **(±)-2** in the presence of boron tribromide gave than access to the corresponding 2-hydroxy analog **(±)-3** (named LMA10210). The hydrochloride form of 3-quinuclidinyl benzoate **(±)-4** (named LMA10211) was obtained by esterification of the *N*-protected and acid-activated 4-amino-5-chloro-2-methoxybenzoic acid, with 3-quinuclidinol, followed by acidic treatment (Fig. 2b) (Langlois et al. 1993).

Effects of LMA10203 on cockroach Kenyon cells

We have first tested the ability of LMA10203 to induce ionic currents on cockroach Kenyon cells. The amplitude of the LMA10203-elicited currents was concentration-dependent. The EC_{50} was 203.9 µM and 110 µM for

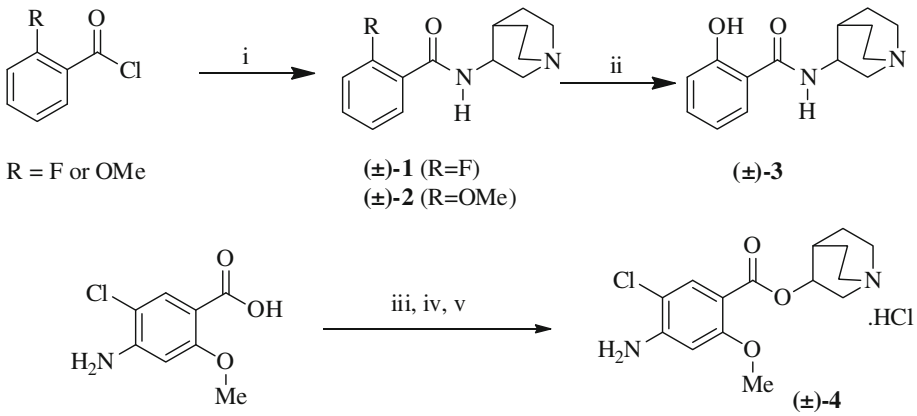

Fig. 2 Reagents and conditions: (*i*) 3-aminoquinuclidine dihydrochloride, Et_3N/pyridine, $CHCl_3$, r.t, 18 h, 88 % (**1**; R = F) and 78 % (**2**; R = OMe); (*ii*) BBr_3, $CHCl_3$, 0 °C than r.t., 1 h, 67 %; (*iii*) trityl chloride, CH_2Cl_2, pyridine, rt; 18 h, 86 %; (*iv*) carbonyldiimidazole, THF, 3 h, reflux, 74 %; (*v*) 3-quinuclidinol, DBU, THF and then $CHCl_3$, conc. HCl, 2 h, 36 % on the two steps

LMA10203 and nicotine, respectively. We found also that the apparent maximal response of LMA10203 was high compared to nicotine (Fig. 3a), demonstrating that at high concentration, LMA10203 was more potent than nicotine. The mean current amplitudes were −0.440 ± 0.03 nA and −0.243 ± 0.02 nA at 1 mM (1 s pulse duration, 15 psig) for LMA10203 and nicotine, respectively. In addition, we found that 0.5 μM α-Bgt completely blocked LMA10203-induced current amplitudes (Fig. 3b, c), whereas it partially blocked nicotine current amplitudes (−40 ± 3.8 pA, Fig. 3d, e). Thus, we suggested that LMA10203 is an agonist of Kenyon cell α-Bgt-sensitive nAChRs. We have previously demonstrated that LMA10203 induced a specific current–voltage curve in which the current amplitudes increased from −90 to +20 mV, demonstrating that it acted as an agonist of the α-Bgt-insensitive nAChR2 (Mathé-Allainmat et al. 2012). In the present study, the current–voltage curve obtained at different holding potentials shows a linear curve that decreased from −90 to +20 mV holding potential (Fig. 4a) that reinforced the finding that LMA10203 acted as an agonist of α-Bgt-sensitive receptors other than nAChR2.

Pharmacological activities of LMA10210 and LMA10211 on cockroach Kenyon cells

We studied the novel compounds LMA10210 and LMA10211 that were selected as analogs of the benzamide compound LMA10203 and of the ester compound tropisetron, respectively. Patch-clamp studies were then performed to identify their effect on Kenyon cells. Pulse application of 1 mM LMA10210 did not have any effect on Kenyon cells, whereas 1 mM LMA10211 induced an ionic current (Fig. 4a). The mean current amplitude was −0.258 ± 0.02 nA at 1 mM (1 s pulse duration, 15 psig). We proposed that LMA10210 was a poor agonist of Kenyon cell nAChRs or that nAChRs sensitive to this compound are not expressed on Kenyon cells (Fig. 4b). In addition, we found that LMA10211-induced current amplitudes were partially blocked by 0.5 μM α-Bgt (−0.052 ± 0.0 nA; Fig. 5a, b) and completely by co-application of 0.5 μM α-Bgt and 5 μM mecamylamine (in each case, $N = 6$ and $p < 0.001$, Fig. 5c, d). Because LMA10210 did not activate Kenyon cells nAChRs, it was applied in the bath while observing its effect on 1 s test pulse of 1 mM nicotine. We found that it never blocked nicotine-induced currents (Fig. 6a). Similarly, nicotine had no effect on LMA10210 activity (Fig. 6b). We also noted that LMA10210 had no antagonist effect against LMA10203 and LMA10211 (data not shown). But bath application of 1 mM LMA10203 partially reduced nicotine-induced current amplitudes to 56.3 ± 4.9 % (Fig. 6c, d), while 1 mM nicotine bath applied reduced LMA10203

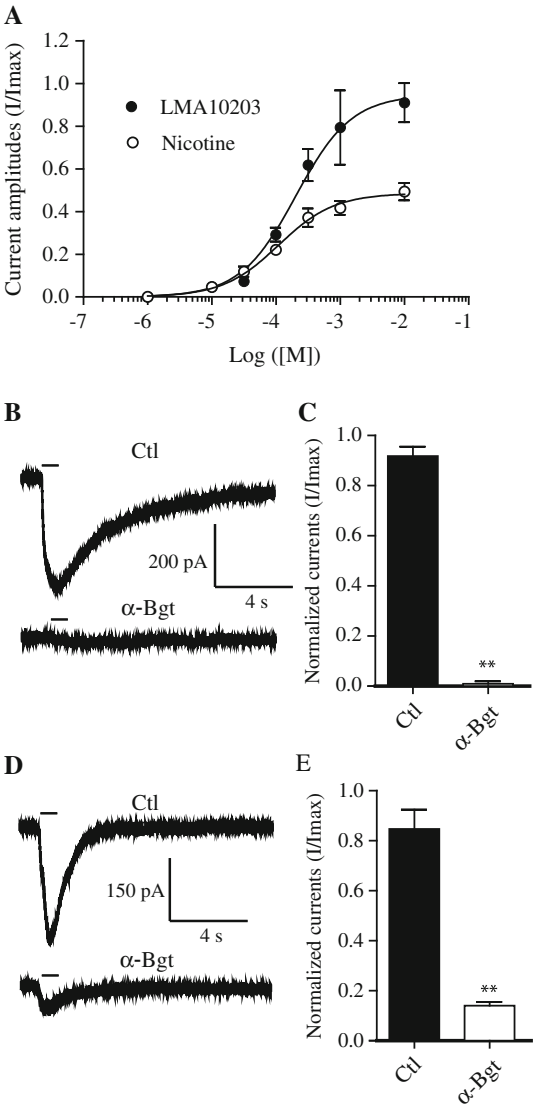

Fig. 3 Effect of LMA 10203 on Kenyon cells. **a** Dose–response curve of LMA10203 and nicotine on Cockroach Kenyon cells. Concentration–response relation obtained at −110 mV holding potential (1 s pulse duration, 15 psig). Data are mean ± SEM ($N = 8$). Data for each cell were normalized with the maximum LMA10203 current amplitudes. The dose–response curve was fitted by the Hill equation (see "Materials and methods"). **b**, **c** Currents and histograms illustrating the effects of 1 mM LMA10203 (control condition: Ctl) and under bath application of 0.5 μM α-Bgt. **d**, **e** Effect of 1 mM nicotine under control condition (Ctl) and with bath application of 0.5 μM α-Bgt. In each current, the horizontal bar indicates 1 s pulse application (15 psig). Data are mean ± SEM ($N = 8$). $^{**}p < 0.001$

current amplitudes to 10.3 ± 5 % (Fig. 6e, f), demonstrating that a competitive interaction could exist between nicotine and LMA10203. Indeed, 10 mM nicotine bath-applied completely blocked LMA10203-induced current amplitude (data not shown). In addition, successive applications of 1 mM LMA10203 and nicotine did not induce a decrease in current amplitudes (Fig. 7), demonstrating that

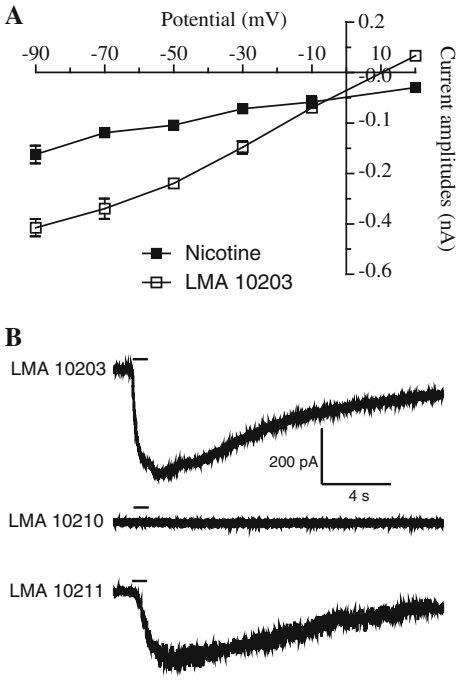

Fig. 4 Effect of LMA10203 on cockroach Kenyon cells. **a** Current–voltage relationships of 1 mM nicotine and LMA-induced current amplitudes on Kenyon cells. Currents are plotted as a function of steady state holding potentials. Data are mean ± SEM ($N = 8$). **b** Comparative effect of 1 mM LMA10203, LMA10210 and LMA10211 on Kenyon cells. *Horizontal bars* indicate 1 s pulse application (15 psig)

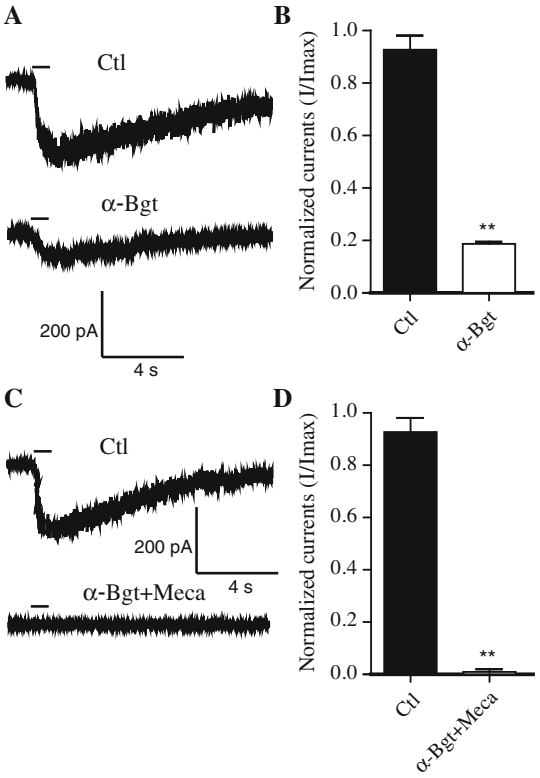

Fig. 5 Effect of LMA10211 on cockroach Kenyon cells. **a** Currents induced by 1 mM LMA10211 (control condition: Ctl) and in the presence of 0.5 µM α-Bgt (α-Bgt). **b** Histogram illustrating the effects of 0.5 µM α-Bgt on LMA10211. Data are normalized with the maximum LMA10211 current obtained in the same condition. **c**, **d** Currents and histogram illustrating the effects of 1 mM LMA10211 under co-application of 0.5 µM α-Bgt and 5 µM mecamylamine (Meca), bath applied. In each current, the *horizontal bar* indicates 1 s pulse application (15 psig). Data are mean ± SEM ($N = 8$). $^{**}p < 0.001$

there was no desensitization of LMA10203 and nicotine under our conditions.

Agonist effect of LMA 10210 and LMA 10211 on ganglionic depolarization of cockroach sixth abdominal ganglion

We have previously demonstrated that LMA10203 induced a depolarization of the sixth abdominal ganglion and the electrical stimulation of nerve XI was blocked when the ganglionic depolarization reached a peak (Mathé-Allainmat et al. 2012). In addition, LMA10203-induced depolarization was blocked by the nicotinic antagonist mecamylamine demonstrating that it also acted as an agonist of nAChR expressed at postsynaptic receptors (Mathé-Allainmat et al. 2012). In the present study, we tested the agonist effect of LMA10210 and LMA10211 on ganglionic depolarization. As previously demonstrated, LMA10211 induced a strong increase in spontaneous activity (Fig. 8a). We also found that 1 mM LMA10211 was able to induce a depolarization of sixth abdominal ganglion, whereas 1 mM LMA10210 did not behave so (Fig. 8b). The dose–response curve demonstrated that both LMA10203 and LMA10211 were agonists of the sixth abdominal ganglion with EC_{50} values of 326 µM and 489 µM, respectively (Fig. 8c). In addition, we found that electrical stimulation of the sixth abdominal ganglion was blocked by bath application of 1 mM LMA10211 when the ganglion reached a peak (Fig. 9a). This effect was not seen with 1 mM LMA10210 (Fig. 9b).

Effect of 3-quinuclidinyl compounds on CNS of *M. domestica* larvae

Finally, because these novel compounds had specific effects on cockroach ganglionic depolarization suggesting that they act on different nAChRs, we performed this supplementary study to understand their effect on other insect species. Experiments performed on the central nervous system of third instar *M. domestica* larvae demonstrated that the three compounds were all able to show a dose-dependent stimulatory activity of the central nervous system (Fig. 10). The three chemicals were found to be neuroexcitatory at low to mid micromolar concentrations.

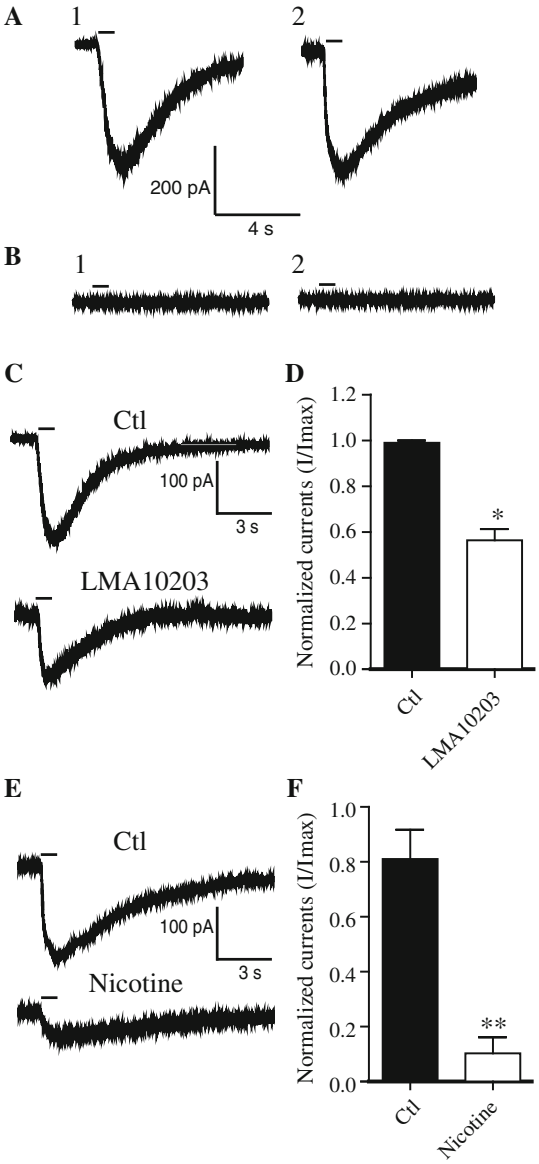

Fig. 6 Comparative effect of nicotine, LMA10203 and LMA10210 on Kenyon cells. **a** 1 mM nicotine pulse application (1 s, 15 psig) when cells were bathed with 1 mM LMA10210. *A1* nicotine-induced current before and *A2* during bath application of 1 mM LMA10210. **b** LMA10210 did not induce current when cells were bathed with 1 mM nicotine *B1* before bath application of 1 mM nicotine and *B2* during nicotine application. **c**, **d** Currents and histograms illustrating the effects of 1 mM nicotine, under bath application of 1 mM LMA10203. Control: Ctl represents 1 mM pulse application before bath application of LMA10203. *Horizontal bar* represents 1 s pulse application of 1 mM nicotine. Data are normalized with the maximum nicotine current amplitude and are mean ± SEM ($N = 6$). *$p < 0.05$. **e**, **f** Bath application of 1 mM nicotine. Control: Ctl represents 1 mM pulse application of LMA10203. *Horizontal bar* represents 1 mM LMA10203 pulse application (1 s, 15 psig). **$p < 0.001$. In all cases, $N = 5$

LMA10211 was found to be the most potent chemical by up to threefold. On the other hand, LMA10203 and LMA10210 were found to be equipotent with EC_{50} values

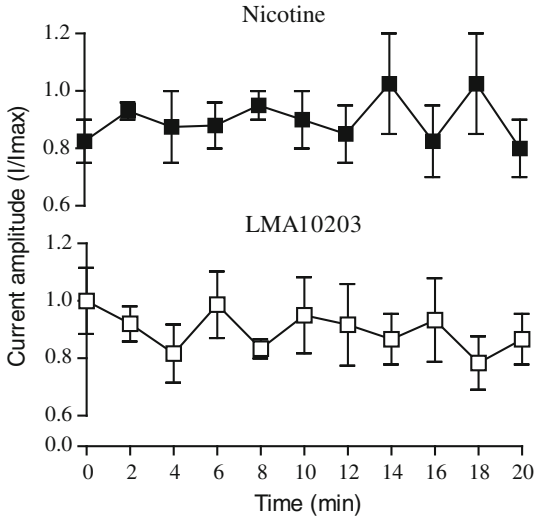

Fig. 7 Effect of LMA10210 and LMA10211 on cockroach postsynaptic interneurons. *Upper trace* no spontaneous background activity was observed after bath application of 1 mM LMA10203. *Lower trace* 1 mM LMA10211 evoked an increase in spontaneous activity

of approximately 300 µM. The EC_{50s} were 340 µM (294–395), 398 µM (310–512) and 398 µM (310–511), for LMA10203, LMA10210 and LMA10211, respectively (Fig. 11). Compared to previous data on cockroach ganglionic depolarization, we observed that they had a strong effect on CNS discharge rates of *M. domestica* larvae.

Discussion

Effect of 3-quinuclidinyl compounds on cockroach Kenyon cells

In the present study, we have evaluated the effect of LMA10203 and two other quinuclidine derivatives, LMA10210 and LMA10211, on Kenyon cells. Our experiments demonstrated that LMA10203 and LMA10211 were able to act as agonists of cockroach Kenyon cells, whereas LMA10210 had no effect. We proposed that LMA10203 effects occurred through α-Bgt-sensitive receptors. This result was different from our previous data demonstrating that it acted as an agonist of DUM neuron α-Bgt-insensitive nAChR2 (Mathé-Allainmat et al. 2012). Taking into account these data, we suggested that it differently acted as an agonist of nAChRs expressed on both DUM and Kenyon cells. The apparent differences in the current–voltage curve reinforced our finding that cockroach expressed different nAChR that were selectively sensitive to cholinergic ligands (Calas-List et al. 2012). Moreover, we found that novel compounds such as LMA10210 and LMA10211 have not the same affinity for nAChRs expressed on Kenyon cells. LMA10211 was an agonist of cockroach Kenyon cell

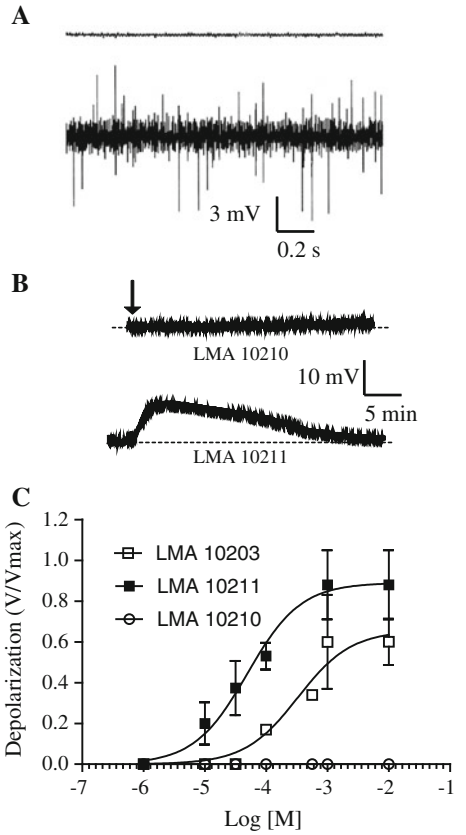

Fig. 8 Effect of LMA10210 and LMA10211 on the sixth abdominal ganglion. **a** Effect of LMA10210 and LMA10211 on spontaneous activities. *Upper trace* 1 mM LMA10210 did not increase spontaneous activity. *Lower trace* increase in spontaneous activity after bath application of 1 mM LMA10211. **b** Effect of LMA10210 and LMA10211 on ganglionic depolarization. *Arrow* indicates 3 min bath application of LMA10210 and LMA10211. **c** Dose–response curve induced by LMA10203, LMA10210 and LMA10211 on the sixth abdominal ganglion

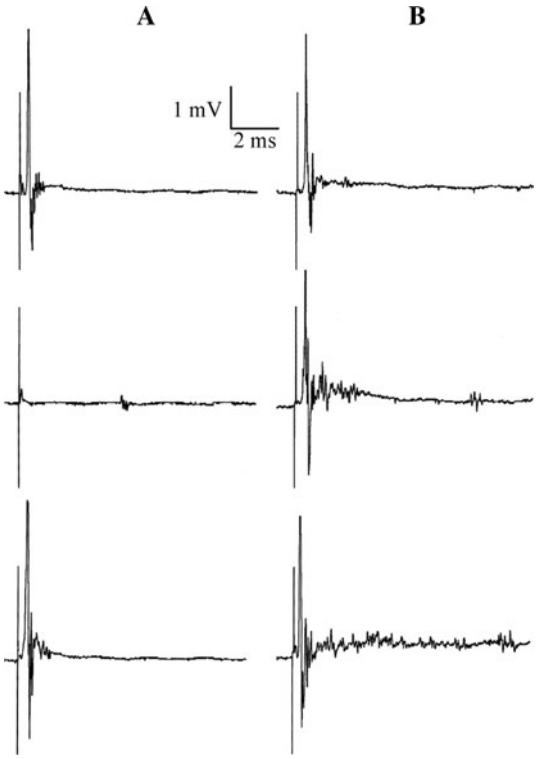

Fig. 9 Electrical stimulation of the nerve XI under bath application of 1 mM LMA10211 (**a**) and 1 mM LMA10210 (**b**). *Upper traces* (before bath application), *middle traces* (during drug application, when the depolarization reached a *peak*) and *lower trace* 10 min after bath application of the drugs

nAChRs. On the other hand, LMA10210 was not able to induce current amplitudes in comparison with its fluoro analog LMA10203. We proposed that nAChRs sensitive to this compound were not expressed or that it had a poor affinity for these receptors. Indeed, when bath applied, LMA10210 did not block nicotine currents, while bath application of LMA10203 reduced nicotine currents. Moreover, LMA10203 effect was completely blocked by high nicotine concentration demonstrating a competitive effect between nicotine and LMA10203. We argued that competitive effect occurred through activation of α-Bgt-sensitive nAChRs because α-Bgt completely blocked LMA10203-induced currents and partially nicotine currents.

Effect of quinuclidine compounds on synaptic activity

Recently, we have studied the agonist effect of LMA10203 on cockroach postsynaptic receptors using the sixth abdominal ganglion. We have demonstrated that in addition to its agonist action on DUM neuron nAChRs, it acted as an agonist of postsynaptic receptors. Using the same method, we found that LMA10211 was also able to activate postsynaptic receptors and confirmed that it was an agonist of these receptors. LMA10210 was not able to depolarize the sixth abdominal ganglion which suggested that it had no effect on postsynaptic nAChRs. Moreover, our data demonstrated that LMA10211 was more potent than LMA10203 on cockroach sixth abdominal ganglion, whereas LMA10203 was more potent on Kenyon cell nAChRs. Interestingly, studies performed on *M. domestica* larvae demonstrated that LMA10210 could be an agonist of *M. domestica* nAChRs. Indeed, it induced a strong postsynaptic activity similar to both LMA10203 and LMA10211 demonstrating that LMA10210 is a good agonist of the *M. domestica* nAChRs. We proposed that they had different affinity for *M. domestica* nAChRs because LMA10211 was more potent than LMA10203 and LMA10210.

Distinct effect of LMA10203 between cockroach dorsal unpaired median neurons and Kenyon cells: expression of different nAChR subtypes

In the present study, we demonstrated that LMA10203 differently acted as an agonist of cockroach DUM neuron and

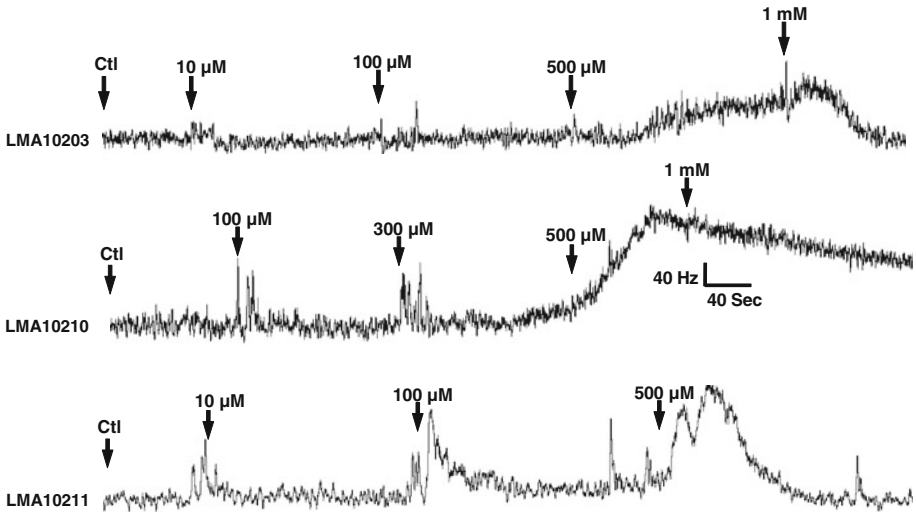

Fig. 10 Effect of quinuclidine compounds on *M. domestica* larvae. Potency of LMA10203, LMA10210 and LMA10211 on CNS nerve discharge of the third instar *M. domestica*

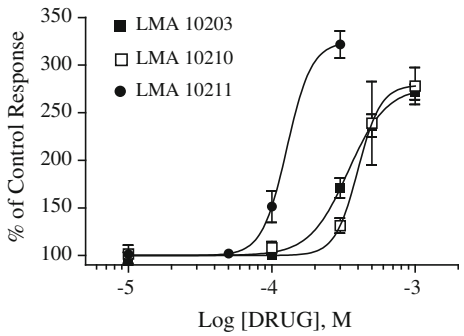

Fig. 11 Effect of LMA10203, LMA10210 and LMA10211 on *M. domestica* larvae. Dose–response curve representing nerve discharges of the CNS from *M. domestica* third instar larvae. Data points represent mean ($n \geq 3$) spike discharge frequencies, and *error bars* represent SEM

Kenyon cell nAChRs. These data were consistent with previous studies demonstrating the existence of different nAChR subtypes between synaptic and extrasynaptic nAChRs (Thany 2009, 2011; Calas-List et al. 2012). Moreover, we also found that LMA10203 was more potent than LMA10211 on Kenyon cells and the order was reversed on cockroach synaptic transmission where LMA10211 was more potent than LMA10203. All these data demonstrated that synaptic and extrasynaptic nAChRs expressed on cockroach *P. americana* and *M. domestica* larvae had different affinity for LMA10203, LMA10210 and LMA10211, respectively. These kinds of distinct effects were demonstrated in honeybee and stick insect nAChRs (Wustenberg and Grunewald 2004; Barbara et al. 2005, 2008; Watson et al. 2011; Oliveira et al. 2011). On antennal lobes, the pharmacology of nicotine is different to that of the Kenyon cells. On pupal Kenyon cells, nicotine had low efficacy in comparison with adult antennal lobe cells. This greater variability was associated with the existence of different nAChR subtypes (Barbara et al. 2005, 2008). Moreover, sulfoxaflor activated heterologously expressed α2β2 nAChR with high efficacy but had a very low efficacy on native nAChRs (Oliveira et al. 2011). Thus, we conclude that the pharmacological efficacy of 3-quinuclidinyl compounds could vary depending to the cell types.

Conclusion

The present study was focused to identify new compounds that could help identifying insect nAChR subtypes and their specific pharmacological profile. The synthesis and evaluation of quinuclidine benzamides demonstrated that a discrete chemical pharmaco-modulation could totally change the pharmacological profile of the tested compounds. These results could outline that different nAChRs were expressed in cockroach DUM and Kenyon cells, and probably, DUM neuron nAChR2 was not expressed in these cells, due to its specific pharmacological profile (Courjaret and Lapied 2001; Courjaret et al. 2003; Thany et al. 2008; Bodereau-Dubois et al. 2012). We suggest that the synthesis of these compounds could help to identify other insect nAChR subtypes taking into account that subunit composition of insect native nAChR subtypes still remains unknown.

Conflict of interest None.

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