Transmembrane Potential Polarization, Calcium Influx, and Receptor Conformational State Modulate the Sensitivity of the Imidacloprid-Insensitive Neuronal Insect Nicotinic Acetylcholine Receptor to Neonicotinoid Insecticides

Béatrice Bodereau-Dubois, Olivier List, Delphine Calas-List, Olivier Marques, Pierre-Yves Communal, Stéeve H. Thany, and Bruno Lapied

Laboratoire Récepteurs et Canaux Ioniques Membranaires (B.B.-D., O.L., D.C.-L., S.H.T., B.L.) and Laboratoire d’Études Environnementales des Systèmes Anthropisés (P.-Y.C.), Faculté des Sciences, Université d’Angers, Angers, Cedex France; and Groupement Interprofessionnel de Recherche sur les Produits Agropharmaceutiques, Beaucouzé, France (O.M., P.-Y.C.)

Received September 14, 2011; accepted January 27, 2012

ABSTRACT

Neonicotinoid insecticides act selectively on insect nicotinic acetylcholine receptors (nAChRs). Recent studies revealed that their efficiency was altered by the phosphorylation/dephosphorylation process and the intracellular signaling pathway involved in the regulation of nAChRs. Using whole-cell patch-clamp electrophysiology adapted for dissociated cockroach dorsal unpaired median (DUM) neurons, we demonstrated that intracellular factors involved in the regulation of nAChR function modulated neonicotinoid sensitivity. DUM neurons were known to express two α-bungarotoxin-insensitive nAChR subtypes: nAChR1 and nAChR2. Whereas nAChR1 was sensitive to imidacloprid, nAChR2 was insensitive to this insecticide. Here, we demonstrated that, like nicotine, acetamiprid and clothianidin, other types of neonicotinoid insecticides, acted as agonists on the nAChR2 subtype. Using acetamiprid, we revealed that both steady-state depolarization and hyperpolarization affected nAChR2 sensitivity. The measurement of the input membrane resistance indicated that change in the acetamiprid-induced agonist activity was related to the receptor conformational state. Using cadmium chloride, ω-conotoxin GVIA, and (R,S)-(3,4-dihydro-6,7-dimethoxyisoquinoline-1-yl)-2-phenyl-N,N-di-acetamide (LOE 908), we found that inhibition of calcium influx through high voltage-activated calcium channels and transient receptor potential γ (TRPγ) activated by both depolarization and hyperpolarization increased nAChR2 sensitivity to acetamiprid. Finally, using N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), forskolin, and cAMP, we demonstrated that adenylyl cyclase increased nAChR2 sensitivity to acetamiprid. Similar TRPγ-induced modulatory effects were also obtained when clothianidin was tested. These findings bring insights into the signaling pathway modulating neonicotinoid efficiency and open novel strategies for optimizing insect pest control.

Introduction

Neonicotinoids are an important class of insecticides used worldwide for eliminating and controlling sucking and biting insect pests (aphids, thrips, and whiteflies) (Tomizawa and Casida, 2005; Jeschke and Nauen, 2008). They were not derived from natural products but rather from the discovery of synthetic nitromethylene heterocycles (Kagabu, 1997). The first commercialized member of this class of insecticides was imidacloprid (IMI), which is used in foliar application (Condor, Bayer CropScience, Diegem, Belgium) and seed treatment (Gaucho, Bayer CropScience). Additional major classes of neonicotinoids such as acetamiprid (ACT) (Takahashi et al., 1992) and clothianidin (CLO) (Ohkawara et al., 2002; Tomizawa and Casida, 2003, 2005) have been commercialized since the discovery of IMI (Jeschke and Nauen, 2008). Based on their chemical structures, which are similar to that of nicotine (Nic), previous electrophysiological studies have
reported that neonicotinoids have agonist actions on insect nicotinic acetylcholine receptors (nAChRs), including partial and full agonist activities (Buckingham et al., 1997; Matsuda et al., 2005; Tan et al., 2007). Because it has been shown that neonicotinoids are more selective for insect nAChRs than vertebrate nAChRs, these properties made their use safer (Elbert et al., 2008). Despite this specific activity, two major problems may threaten the future of neonicotinoids: 1) the development of resistance in pests associated with neonicotinoid-specific metabolites (Gorman et al., 2007) and 2) an alteration of neonicotinoid efficacy by cellular and molecular factors involved in the regulation of nAChRs targeted by this class of insecticides (Courjaret and Lapied, 2001).

The emergence of insect resistance to neonicotinoids has been observed for a few insect species (Nauen and Denholm, 2005; Alyokhin et al., 2007; Gorman et al., 2007; Mota-Sanchez et al., 2008). The first well described resistance mechanism is an increase of neonicotinoid detoxification (Honda et al., 2006; Casida, 2011). In the whitefly, *Bemisia tabaci*, an increase of neonicotinoid metabolism is correlated with overexpression of the cytochrome P450 monoxygenase CYP6CM1 (Karunker et al., 2009). Overexpression of this gene decreases insecticide efficacy by facilitating its elimination from the organism. Another mechanism involves the nAChR subtypes. It is demonstrated that the subunit composition of nAChR subtype determines the pharmacological profile and neonicotinoid sensitivity (Lansdell and Millar, 2000; Yixi et al., 2009; Li et al., 2010). In addition, the point mutation Y151S in loop B of α1 and α3 nAChR subunits of the rice brown planthopper, *Nilaparvata lugens*, is also correlated with insect resistance to neonicotinoids. This mutation induces a decrease of nAChR sensitivity to neonicotinoid insecticides (Liu et al., 2005; Yixi et al., 2009). Finally, modification of nAChRs has been identified in the β1 subunit from *N. lugens*. The adenosine-to-inosine RNA editing in β1 nAChR subunits, which transforms the amino acid asparagine to aspartate in loop E, decreases IMI sensitivity without any effect on acetylcholine potency (Yao et al., 2009).

Data indicate that neonicotinoid actions also depend on different molecular and cellular factors including: 1) the phosphorylation/dephosphorylation process involving cAMP-dependent protein kinase and protein phosphatase 1/2A, which are known to decrease IMI efficiency on insect nAChR subtypes; and 2) the conformational state of nAChR (Courjaret and Lapied, 2001). In the latter case, previous findings have reported that in insect neurosecretory cells identified as dorsal unpaired median (DUM) distinct nAChR subtypes (named nAChr1 and nAChr2) present different sensitivity to the neonicotinoid insecticide IMI. Although nAChr1 is sensitive to IMI, nAChr2 is insensitive to this insecticide, whereas the insect has never been exposed to such a molecule (Courjaret and Lapied, 2001). Furthermore, it seems that the uncommon conformational state of nAChr2 (i.e., open at the resting state and closed upon cholinergic agonist application) (Courjaret and Lapied, 2001; Courjaret et al., 2003; Thany et al., 2008) could be responsible for the different neonicotinoid sensitivity observed in these two nAChR subtypes. These data clearly demonstrate that the neonicotinoid mode of action on insect nAChRs is more complex than expected.

Because insecticide efficacy is closely related to both the chemical structure and conformational state of nAChR, we performed electropharmacological studies on IMI-insensitive nAChr2 by using another type of neonicotinoid, ACT (Takahashi et al., 1992; Okazawa et al., 2000), which differs structurally from IMI. Indeed, the use of ACT, which is characterized by having a N-cyano-amidine pharmacophore, whereas the chemical structure of IMI is coupled with a N-nitroguanidine pharmacophore (Elbert et al., 2008), allows us to bring insights into molecular and cellular factors involved in the modulation of neonicotinoid efficacy. Although ACT is the active ingredient of different insecticide formulations [e.g., Supreme (Certis Europe, Guyancourt, France), Roseclear Ultra (Scotts France SAS, Ecully, France), INSYST 20 SG (Nisso Chemical Europe GmbH, Düsseldorf, Germany), Polysect Ultra (Scotts France, SAS)], and except for a few studies on the toxicological effects of this insecticide (Kiriyama et al., 2003; Mo et al., 2005), there are no data available on its precise mode of action at both the cellular and molecular levels on insect nAChRs. In this study, we have revealed, for the first time, that 1) ACT exerts an agonist activity on IMI-insensitive nAChr2; 2) different cellular factors such as membrane polarization and calcium influx through TRP-γ and high voltage-activated (HVA) calcium channels may decrease or increase the sensitivity of insect nAChr2 to ACT through the modification of the receptor conformational state; and 3) the involvement of such cellular factors on nAChr2 sensitivity is not particular to ACT because similar effects have also been observed with another neonicotinoid, CLO.

**Materials and Methods**

**Cell Preparation.** Experiments were carried out on DUM neuron somata isolated from the midline of the terminal abdominal ganglion (TAG) of the nerve chord of adult male cockroaches (*Periplaneta americana*). Cockroaches were obtained from our RCIM laboratory stock colony and maintained at 29°C on 12-h light/dark cycle. Animals were immobilized ventral side up on a dissection dishes. The ventral cuticle and the accessory gland were removed to allow access to the TAG, which was carefully dissected under a binocular microscope and placed in normal cockroach saline containing 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES, and 50 mM sucrose, and pH was adjusted to 7.4 with NaOH. Isolation of adult DUM neuron somata was performed under sterile conditions by using enzymatic digestion by collagenase (type IA, 300 IU/ml; Worthington Biochemicals, Freehold, NJ) at 29°C for 35 min. Then, a mechanical dissociation through fire-polished Pasteur pipettes was used to isolate DUM neurons from the TAG (Lapied et al., 1989). DUM neuron somata were maintained at 29°C for 24 h before electrophysiological experiments were carried out.

**Electrophysiological Recordings.** ACT-, CLO-, IMI-, and Nic-induced currents were recorded by using the patch-clamp technique in the whole-cell recording configuration under voltage-clamp mode. Membrane potential and input membrane resistance were recorded under current-clamp mode. Ejection pipettes and patch-clamp electrodes were pulled from borosilicate glass capillary tubes (GC150T-10; Harvard Apparatus Inc., Holliston, MA) by using a P-97 model puller (Sutter Instrument Company, Novato, CA). Patch pipettes had resistances ranging from 1 to 1.2 MΩ when filled with internal pipette solution (see composition below). The liquid junction potential between extracellular and intracellular solutions was always corrected before the formation of a gigahm seal (>1 GΩ). Signals were recorded with an Axopatch 200A (Molecular Devices, Sunnyvale, CA). Ionic currents induced by Nic and neonicotinoids were displayed on a computer with software control pClamp (version 10.0; Molecular Devices) connected to a digitizer (DIGIDATA 1322; Mo-
DUM neuron somata were voltage-clamped at a steady-state holding potential of ~−50 mV (except where otherwise stated). Experiments were carried out at room temperature.

**Curves Fitting and Data Analysis.** We performed data analysis, including fitting procedures, by using the software Prism 4 (GraphPad Software, Inc., San Diego, CA). Data are presented as the mean ± S.E.M. Information regarding which pairs of means were significantly different and which were not was determined by Student’s t test for multiple comparisons. In this case, statistical analysis was expressed as nonsignificant for *p > 0.05 and significant for *p < 0.05, **p < 0.01, and ***p < 0.001. The equation used to fit the monomeric sigmoid curve was:

\[
I = \frac{I_{\text{max}}}{1 + (ED_{50}/x)^{nH}}
\]

(1)

where \(I\) is the percentage of agonist-induced inward current, \(I_{\text{max}}\) is the maximal current value, \(ED_{50}\) is the pressure ejection duration that produces 50% activation of the maximal agonist-induced current, \(x\) is the pressure ejection duration of agonist, and \(nH\) is the Hill coefficient. The biphasic dose-response curves were fitted by using a “double” Hill equation:

\[
I = \frac{\left[I_{\text{max},1}/1 + (ED_{50,1}/x)^{nH,1}\right] + \left[I_{\text{max},2}/1 + (ED_{50,2}/x)^{nH,2}\right]}{1}
\]

(2)

where \(I\) is the percentage of agonist-induced inward current, \(I_{\text{max},1}\) is the maximal current value for the first receptor subtype, \(I_{\text{max},2}\) is the maximal response for the second receptor subtype, \(ED_{50,1}\) is the pressure ejection duration that produces 50% activation of the maximal agonist-induced current for the first receptor subtype, \(ED_{50,2}\) is the pressure ejection duration that produces 50% activation of the maximal agonist-induced current for the second receptor subtype, \(x\) is the pressure ejection duration, \(nH,1\) is the Hill coefficient for the first receptor subtype, and \(nH,2\) is the Hill coefficient for the second receptor subtype.

**Solutions and Drug Application.** Bath solution superfusing the cells contained 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl\(_2\), 4 mM MgCl\(_2\), and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. Patch pipettes were filled with solution containing 160 mM K*/t-glucuronate, 10 mM KF, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 3 mM ATP, 0.1 mM cAMP, 10 mM EGTA, and 20 mM HEPES; pH was adjusted to 7.4 with KOH.

ACT (1 mM), CLO (1 mM), Nic (10 mM), and IMI (0.1 and 1 mM) were applied by pneumatic pressure ejection (15 psig) (Lapied et al., 1990; Courjaret and Lapied, 2001; Alix et al., 2002) with a pneumatic pressure system (Mini frame; Medical Systems Corporation, Green Valley, NY) to minimize receptor desensitization resulting from bath application of agonists. The pressure ejection was made through a controlled calibrated patch pipette geometry obtained according to the protocol described above (with a resistance of 1.8 M\(\Omega\) when filled with agonists) positioned in extracellular solution within 50 \(\mu\)m from the isolated neuronal cell body. When droplets were ejected under oil (Macherey-Nagel, Düren, Germany) was used. Mobile phase A was ultra-pure water/glacial acetic acid (100:0.05 v/v) + 5% methanol filtered on nylon filter (0.20 \(\mu\)m), and mobile phase B was methanol/glacial acetic acid (100:0.05 v/v). Detection was made with tandem mass spectrometry (multiple reaction monitoring mode), and the calibration was made as follows. Standard solutions were realized in physiological saline. A calibration curve was calculated and plotted by linear regression (peak area expressed in counts, versus the concentration expressed in \(\mu\)g/liter). The predicting mathematical model to determine the concentration was calculated by using Varian software (version 6.9.2).

The steady-state recordings were made 5 min after establishment of the whole-cell configuration and repeated application of ACT, IMI, and Nic. Isolated neuron somata were continuously bathed with saline (see composition above) by using a speed-controlled gravity perfusion system (0.1 ml/min) supplied through one of an array of parallel outlet tubes placed within 100 \(\mu\)m of the cell body. Repeated applications of Nic, CLO, and ACT were made at 2-min intervals. IMI, CLO, and ACT stock solution (100 mM) were prepared in dimethyl sulfoxide. Final dilution never contained more than 0.1% dimethyl sulfoxide. This concentration of solvent was not found to have without any effect on the electrophysiological properties of DUM neurons. Pharmacological agents such as α-bungarotoxin (α-bgt; 0.5 \(\mu\)M), d-tubocurarine (d-TC; 50 \(\mu\)M), tetrodotoxin (TTX; 0.1 \(\mu\)M), cadmium chloride (CdCl\(_2\); 0.5 mM), ω-conotoxin GVIA from Conus geographus (ω-CgTx; 0.1 \(\mu\)M), and (R,S)-3,4-dihydro-6,7-di-methoxy-isouquinoline-1-yl)-2-phenyl-N,N-di-acetamide (LOE 908; 40 \(\mu\)M) were added to external solution. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (0.5 mM), 1,2-bis(2-aminophenoxyethane-N,N,N’N’-tetraacetic acid (10 mM), and cesium chloride (CsCl; 10 mM) were added in the internal pipette solution immediately before use. All compounds were purchased from Sigma (St. Louis, MO) except LOE 908, which was purchased from Torcs Bioscience (Bristol, UK).

**Results**

**Effects of Nicotine, Imidacloprid, and Acetamiprid on α-Bungarotoxin-Insensitive nAChRs Expressed in DUM Neurons.** As demonstrated previously, DUM neurons expressed both α-bgt-sensitive and “mixed” nAChRs, which were blocked by extracellular application of 0.5 \(\mu\)M α-bgt (Lapied et al., 1990; Courjaret and Lapied, 2001) (Fig. 2B). In this condition, pressure ejection application of Nic (10 mM, 100 ms) onto DUM neuron somata evoked a transient inward current (Fig. 3A). As reported previously (Courjaret and
Lapied, 2001; Courjaret et al., 2003; Thany et al., 2008), when the peak amplitude of the inward currents was plotted against the steady-state holding potentials a biphasic aspect was observed (Fig. 3B). The peak amplitude decreased linearly between −90 and −30 mV and then increased between −30 and +30 mV. This unusual biphasic aspect indicated that Nic acted on two different subtypes of nAChR1 and nAChR2 (Fig. 2B) (Courjaret and Lapied, 2001; Courjaret et al., 2003; Thany et al., 2008). It is noteworthy that after pressure application of IMI (0.1 mM, 100 ms) we never observed a biphasic current-voltage curve (Fig. 3C) (Courjaret and Lapied, 2001), whereas ACT (1 mM, 200 ms) induced a similar effect as that of Nic (Fig. 3D). These last results indicated that: 1) like Nic and CLO (Thany 2009), ACT was able to act on both nAChR1 and nAChR2 and 2) confirmed that nAChR2 was insensitive to IMI (Courjaret and Lapied, 2001).

**Acetamiprid Acts as an Agonist on Both DUM Neuron nAChR1 and Imidacloprid-Insensitive nAChR2 Subtypes.** As indicated in Materials and Methods, pressure ejection application of ACT was made through a glass pipette positioned in solution within 50 μm of the isolated neuron.

---

**Fig. 2.** A, structure of the nicotinic receptor agonist nicotine (upper left) compared with imidacloprid (upper right), clothianidin (lower right), and acetamiprid (lower left). B, comparative electrophysiological and pharmacological properties of nAChR1 and nAChR2 expressed by the cockroach DUM neurons (from Courjaret and Lapied, 2001; Thany et al., 2008).
In this situation, the logarithmic concentration of agonists, at any point of the cell body, will be proportional to the pulse duration of the cholinergic agonist applications (at constant pressure) as reported previously (Lapied et al., 1990; Courjaret and Lapied, 2001; Di Angelantonio and Nistri, 2001; Alix et al., 2002). Pressure ejection application of 1 mM ACT onto isolated DUM neuron somata produced a dose-dependent inward current (Fig. 4, A and B). When mean values of the peak inward current amplitude were plotted against the logarithm of the noncumulative pressure ejection duration, a biphasic dose-response curve was obtained (Fig. 4A). According to the “double” Hill equation (see eq. 2 under Materials and Methods), an apparent maximum was obtained at approximately 200- to 400-ms ejection duration of ACT, but it was only a plateau between the two parts of the curve. For pressure ejection duration longer than 500 ms, the ACT-induced current further increased before reaching maximum amplitude at approximately 2 s. For comparison, pressure application of 1 mM IMI induced only a monophasic inward current amplitude between 0.1 nA; holding potential of 30 mV, compared with the Nic-induced current, which was well correlated with the first component of the biphasic curve observed with ACT (Fig. 4A). The sigmoid curve corresponded to the best fit according to the Hill equation (see eq. 1). Based on these findings and because we reported previously that IMI exerted only an agonist activity on nAChR1 (Courjaret and Lapied, 2001; this study), the biphasic aspect of the dose-response curve observed with ACT might be explained by an agonist action on both nAChR1 and IMI-insensitive nAChR2 (Fig. 4C). To substantiate this hypothesis, we measured the input membrane resistance, reflecting the selective conformational state of nAChR1 and nAChR2, at two different pressure ejection durations of ACT (300 and 1000 ms; Fig. 4D). Under current-clamp condition (membrane potential of −50 mV), a decrease in DUM neuron input membrane resistance in response to a hyperpolarization current pulse was observed after pressure application of ACT for 300 ms (Fig. 4D, middle trace). In contrast, the input membrane resistance was increased when ACT was pressure applied for 1000 ms (Fig. 4D, top trace). These results indicated that the first component of the dose-response curve for ACT corresponded to the activation of nAChR1, whereas the second one resulted in an inhibition of nAChR2-mediated spontaneous potassium conductance. Furthermore, it was possible to show that nAChR1 was more sensitive to ACT (ED50 of 186 ± 27 ms; n = 4) than nAChR2 (ED50 of 729 ± 39 ms; n = 4).

High Concentration of d-Tubocurarine Allows the Study in Isolation of the Effects of Cholinergic Agonists, Acetamiprid and Clothianidin, on Imidacloprid-Insensitive nAChR2. We indicated previously that relatively low concentrations of d-TC (10–20 μM) induced an inhibition of nAChR1 without any effect on the second component of the biphasic current-voltage relationship corresponding to nAChR2 activation (Courjaret and Lapied, 2001; Thany et al., 2008). It is noteworthy that when Nic was applied in the presence of a higher concentration of d-TC (50 μM) we observed an unexpected important increase of the inward current amplitude between −30 and −70 mV currents (from −1.13 ± 0.12 to −3.36 ± 0.30 nA; holding potential of −70 mV; n = 5; p < 0.001) and reduced the current between −30 and +20 mV, compared with the Nic-induced inward current amplitude recorded without d-TC (Fig. 5, A and B). It should be noted that the inward current amplitude measured after nAChR2 activation resulted, in fact, from an
loss of the spontaneous potassium conductance. Because nAChR2 was known to be selectively blocked by the application of cesium chloride (Courjaret and Lapied, 2001), we performed experiments in the presence of this potassium channel blocker. Indeed, under 50 μM d-TC treatment the increased current amplitude was almost completely reduced (from −3.36 ± 0.30 to −0.38 ± 0.10 nA; n = 4; p < 0.001) with 10 mM cesium chloride intracellularly applied (Fig. 5B). Finally, pressure ejection of IMI did not produce any effect on DUM neurons pretreated with 50 μM d-TC, confirming that the agonist action of ACT occurred through nAChR2 activation (data not shown). Furthermore, as illustrated in Fig. 6A, pressure application of ACT onto isolated DUM neuron somata pretreated with 50 μM d-TC and 0.1 μM TTX to inhibit spontaneous sodium-dependent action potentials produced a transient depolarization with an amplitude smaller than that of produced by ACT without d-TC (from 32.8 ± 0.2 to 18.1 ± 0.5 mV; p < 0.001; n = 4; Fig. 6B). This effect was consistent with an increase in input membrane resistance measured in response to a 350-ms hyperpolarization current pulse in the presence of 50 μM d-TC (Fig. 6B). Finally, like Nic, the current-voltage relationship established with 50 μM d-TC revealed that ACT produced an important increase of the peak inward current amplitude for potentials more negative than −10 mV (from −0.08 ± 0.01 nA in control condition to −0.48 ± 0.07 nA for a steady-state holding potential of −70 mV; n = 5; p < 0.001; Fig. 6C). Based on these results, all the following experiments were performed in the presence of 50 μM d-TC.

**Variation of the Membrane Potential Affects the Sensitivity of nAChR2 to Neonicotinoids.** To express more quantitatively the sensitivity of nAChR2 to ACT, isolated DUM neuron somata were exposed to various pressure ejection durations of ACT in the presence of 50 μM d-TC (Fig. 7A; holding potential of −50 mV). Mean values for percentage peak current amplitudes were plotted against the logarithm of the noncumulative pressure ejection duration of ACT. In these conditions, a monophasic dose-response curve was observed, shifted significantly to the left (the ED50 decreased from 729 ± 39 to 125 ± 5 ms; n = 4; p < 0.001) with no significant change in the maximum effect (maximum current amplitudes −0.50 ± 0.08 and −0.48 ± 0.02 nA in control and in the presence of 50 μM d-TC, respectively; n = 4; Fig. 7A). To study the voltage dependence of the sensitivity of nAChR2 to ACT, the data for ACT obtained at −50 mV were compared with the dose-dependent action of ACT at both depolarized (−30 mV) and hyperpolarized (−70 mV) steady-state holding potentials. The dose-response curve established at −30 mV was slightly shifted to the left with an ED50 lower than that for −50 mV (Fig. 7B; Table 1). In contrast, the sensitivity of nAChR2 to ACT was strongly decreased for a holding potential of −70 mV with a dose-response curve shifted significantly to the right on the logarithm of the pressure ejection duration axis (Fig. 7B; Table 1; n = 5; p < 0.001). Another interesting point indicated by these data concerned the evolution of the ACT-induced current amplitude measured at −30 and −70 mV compared with −50 mV (Table 1). According to the specific biophysical property of nAChR2 explained above, the decrease and increase in ACT-induced current amplitude measured at −30 and −70 mV, respectively (−0.18 ± 0.03 and −0.48 ± 0.07 nA, respectively; n = 4; 200 ms in duration) compared with that mea-
sured at −50 mV (−0.31 ± 0.02 nA; n = 4) indicated that nAChR2 conformational state depended on the membrane potential. In other words; nAChR2 seemed to be less open at more hyperpolarized (−70 mV; Fig. 8B) than at −30 mV under resting conditions. This was confirmed by measuring, under current-clamp condition, the DUM neuron input membrane resistance in response to a 200-ms hyperpolarization current pulse before and after application of ACT (200 ms in duration). As illustrated in Fig. 7C, the increase in DUM neuron membrane input resistance observed after ACT application was more important when the steady-state holding potential was hyperpolarized from −50 mV (resting control condition) to −70 mV (n = 4; p < 0.001). These results indicated that the increased ACT-induced current amplitude observed at more hyperpolarized potential correlated well with the high value of the input membrane resistance observed in the presence of ACT. This suggests that nAChR2, before agonist application, was in a more open conformational state at steady-state hyperpolarized membrane potential, which thereby decreased the sensitivity of nAChR2 to ACT.

To evaluate whether the influence of the membrane potential on the sensitivity of nAChR2 was particular to ACT, comparative additional experiments were performed with CLO, another neonicotinoid insecticide that is known to act as an agonist on nAChR2 (Thany, 2009). As illustrated in Fig. 7D, when mean values for percentage peak current amplitudes were plotted against the logarithm of the noncumulative pressure ejection duration of CLO a monophasic dose-response curve was observed (ED50 of 66 ± 3 ms; holding potential −50 mV; n = 4; Table 1). The results for CLO obtained at −50 mV were then compared with the dose-dependent action of CLO at both depolarized (−30 mV) and hyperpolarized (−70 mV) steady-state holding potentials. As was observed with ACT, the sensitivity of nAChR2 to CLO was decreased for a holding potential of −70 mV with a dose-response curve shifted significantly to the right on the logarithm of pressure ejection duration axis (Fig. 7D; Table 1; n = 4; p < 0.001). In contrast, steady-state holding potential depolarization to −30 mV did not produce any significant shift of the dose-response curve as was observed with ACT (Table 1).

**Calcium Influx through HVA Calcium Channels and TRPγ Modulates the Sensitivity of nAChR2 to Neonicotinoids.** We indicated previously that 1) variation of DUM neuron membrane potential is often associated with change in intracellular calcium concentration and 2) change in intracellular calcium concentration modulates nAChR2 functional properties (Grolleau and Lapied, 2000; Wicher et al., 2001; Thany et al., 2008). If relatively high calcium was introduced into the DUM neuron somata by adding 0.5 mM calcium without EGTA in the patch pipette, the dose-response curve established at −50 mV was shifted to the right, yielding the estimated ED50 from 125 ± 5 ms (control condition) to 304 ± 45 ms (data not shown). In contrast, as illustrated in Fig. 8, A–C, the semilogarithmic dose-response curves constructed, in the presence of 0.5 mM CdCl2, at more depolarized (i.e., −30 mV; Fig. 8A) and hyperpolarized (i.e., −70 mV; Fig. 8C) steady-state holding potentials than in control (−50 mV; Fig. 8B), all were shifted to the left with the same maximum reached. The corresponding ED50 values together with the ACT-induced current amplitudes measured in each experimental condition are summarized in Table 2. As expected from the results presented in Fig. 7, B and C, the decrease in ACT-induced current amplitudes observed at −50 and −70 mV, for instance, correlated well with the decrease in DUM neuron input membrane resistance in
implication of cellular factors on nAChR2 sensitivity 333

response to a 200-ms hyperpolarization current pulse before and after application of ACT (200 ms in duration) and 0.5 mM CdCl2 (p < 0.01 for −50 mV; p < 0.001 for −70 mV; n = 4; Fig. 8D). These results suggested that inhibiting calcium influx into DUM neuron somata allowed nAChR2 to be maintained in a less open conformational state, which thereby rendered nAChR2 more sensitive to ACT.

Based on these results and because previous findings reported that calcium influx in DUM neurons might be accomplished by 1) HVA calcium channels known to be functional in a positive membrane potential range (Wicher et al., 2001; Grolleau et al., 2006) and 2) TRPγ activated by hyperpolarization (Wicher et al., 2006), additional experiments were first performed with ω-conotoxin GVIA, one of the more potent N-type HVA calcium channel blockers in DUM neurons (Grolleau et al., 2006; Gautier et al., 2008). As shown in Fig. 9A, the semilogarithmic curve established at a holding potential of −30 mV in the presence of 0.1 μM ω-conotoxin GVIA was shifted to the left, yielding a lower ED50 of 53 ± 1 ms than in control (84 ± 5 ms; n = 5; p < 0.05). These results indicated that calcium influx through the activation of HVA calcium channels decreased nAChR2 sensitivity to ACT at depolarized membrane potential.

The involvement of TRPγ activation was confirmed by studying the effect of LOE 908 (40 μM), a specific blocker of TRPγ in DUM neurons (Wicher et al., 2004; Grolleau et al., 2006; Gautier et al., 2008). The data for LOE 908 were compared with the dose-dependent action of ACT tested alone (Fig. 9B) at a holding potential of −70 mV. The LOE 908 dose-response curve was shifted significantly to the left in a parallel manner. The estimated ED50 (205 ± 27 ms; n = 4) was more than 2-fold lower than the value estimated in the control condition (567 ± 58 ms; n = 5), and the current amplitude decreased from −0.48 ± 0.07 nA to −0.14 ± 0.02 nA for the 200-ms ejection time (n = 4; p < 0.05; Table 3). For comparison, parallel experiments were performed with CLO. Because depolarization did not induce a significant effect on nAChR2 sensitivity to CLO (Fig. 7D), we only performed experiments at hyperpolarized steady-state holding potentials. As illustrated in Fig. 9C, the semilogarithmic curve established at a holding potential of −70 mV in the presence of 40 μM LOE 908 was shifted to the left, yielding a lower ED50 of 51 ± 4 ms than in control (162 ± 8 ms; n = 4; p < 0.001; Table 3).

These last results demonstrated that the activation of TRPγ decreased the sensitivity of nAChR2 to ACT, CLO, and also to a cholinergic agonist such as nicotine, because similar effects were observed when the semilogarithmic curves were established at a holding potential of −50 and −70 mV and in the presence of 40 μM LOE 908 (ED50 of 78 ± 7, 285 ± 12, and 90 ± 9 ms, respectively; n = 4; Fig. 9D).

We demonstrated previously that TRPγ activation contributes to the regulatory process of ion channels and membrane receptors via intracellular calcium-dependent pathways (Wicher et al., 2004, 2006; Grolleau et al., 2006; Gautier et al., 2008). Consequently, we then investigated the regulatory role of the calcium/CaM complex in the sensitivity of nAChR2 to ACT via the calcium entry through TRPγ activation. Experiments were carried out at a more hyperpolarized holding steady-state potential (−70 mV) than the resting potential (−50 mV) (Wicher et al., 2006). To investigate further the modulatory role of the calcium/CaM complex, the data obtained in control (−70 mV) were compared with the dose-dependent action of ACT recorded with the calmodulin inhibitor W7. The dose-response curve obtained with 0.5 mM W7, applied intracellularly, was shifted significantly to the left (Fig. 10A). The estimated ED50 (193 ± 20 ms; n = 4) was approximately 5-fold lower than for ACT applied alone (p < 0.01; n = 4). In parallel, the input membrane resistance, reflecting the conductance property of nAChR2, was measured before (when whole-cell configuration was established) and after W7 application. As expected from voltage-clamp experiments, W7 produced a statistically significant increase of the input membrane resistance from 95 ± 15 to 187 ± 8 MΩ (n = 4; p < 0.01) in response to a hyperpolarizing current pulse (200 ms in duration) measured under current-clamp conditions (not illustrated). Taken together, these results suggested that the calcium/CaM complex was an important factor accounting for the increased sensitivity of nAChR2 to
TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>ACT</th>
<th>CLO</th>
<th>ACT</th>
<th>CLO</th>
<th>ACT</th>
<th>CLO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED50, ms</td>
<td>84 ± 5</td>
<td>62 ± 4</td>
<td>125 ± 5</td>
<td>66 ± 3</td>
<td>567 ± 58</td>
<td>162 ± 8</td>
</tr>
<tr>
<td>I, nA</td>
<td>-0.18 ± 0.03</td>
<td>-0.03 ± 0.01</td>
<td>-0.31 ± 0.02</td>
<td>-0.09 ± 0.02</td>
<td>-0.48 ± 0.07</td>
<td>-0.21 ± 0.04</td>
</tr>
</tbody>
</table>

ACT via a modification of conformational spontaneous channel opening of nAChR2.

Effect of Intracellular cAMP Concentration Rise on nAChR2 Sensitivity to Acetamiprid and Clothianidin.

In DUM neuron cell bodies, the calcium/CaM complex is an important signal that regulates adenyl cyclase activity, which thereby influences intracellular cAMP concentration (Courjaret and Lapied, 2001). We then examined the effect of forskolin on nAChR2 sensitivity to ACT. Figure 10B demonstrates that in the presence of 1 μM forskolin the dose-response curve was slightly shifted to the right, indicating a small decrease of nAChR2 sensitivity to ACT (p < 0.05; n = 4). Because the calcium/CaM complex plays a crucial role in the activation of adenyl cyclase, we investigated the effect of forskolin with 0.5 mM W7, which blocked CaM. In this condition, the dose-response curve was not statistically different from the control (Fig. 10C). In other words, W7 counteracted the effect of forskolin (n = 4; p > 0.05). These results suggest that the calcium/CaM complex modulated the efficacy of ACT on nAChR2 via the regulation of activation of adenyl cyclase, which thereby regulated the level of cAMP accumulation. To substantiate this hypothesis, DUM neurons were perfused by using a pipette solution containing a higher cAMP concentration (0.3 mM) than in the control condition (0.1 mM). In this case, the ED50 was 55 ± 2 ms, a value approximately 10-fold lower than the control value, 567 ± 58 ms (n = 4; p < 0.001; Table 3). It is noteworthy that similar effects were observed with CLO when DUM neuron cell bodies were dialyzed with an internal pipette solution containing 0.3 mM cAMP (Fig. 10E). The estimated ED50 was 43 ± 4 ms, compared with the control ED50 of 162 ± 8 ms estimated with 0.1 mM cAMP (n = 4; Table 3).

These results confirmed the involvement of cAMP in direct and/or indirect modulation of the sensitivity of nAChR2 to ACT and CLO. Finally, it has been demonstrated that calcium influx via TRPγ activated at hyperpolarized membrane potential depends on the level of intracellular cAMP because increasing cAMP concentration reduces TRPγ activity (Wicher et al., 2006). When DUM neurons were superfused with LOE 908, we never observed any effect of 0.3 mM cAMP, applied intracellularly, on nAChR2 sensitivity to ACT and CLO (Fig. 10, D and F; n = 4; p > 0.05; Table 3). These results confirmed that high cAMP level inhibited TRPγ activation, reducing calcium influx, which thereby increased the sensitivity of nAChR2 to ACT and CLO.

Physiological Significance of the Modulation of the Sensitivity of nAChR2 to Acetamiprid. As reported in this study, membrane potential variations influence the sensitivity of nAChR2 to ACT via changes in intracellular calcium concentration. To demonstrate the physiological importance of such modulation, we performed additional experiments with GABA, which is known to mediate fast inhibitory transmission in the insect central nervous system by increasing the flow of chloride ions through ionotropic GABA receptors (Alix et al., 2002; Zhao et al., 2003; Dupuis et al., 2010; Janssen et al., 2010). Under current-clamp conditions, pressure ejection application of ACT (1 mM; 200 ms) onto DUM neuron soma (membrane potential −50 mV) produced a transient membrane depolarization (14.1 ± 1.1 mV; n = 4; Fig. 11). When DUM neuron soma were super-
fused by the inhibitory neurotransmitter GABA (10 μM), a hyperpolarization of the membrane potential (14.9 ± 1.1 mV; Fig. 11A, lower trace; n = 4) was observed. In this condition, the depolarization amplitude observed with pressure application of ACT (200 ms) was strongly reduced (from 14.1 ± 1.1 to 7.7 ± 0.9 mV; Fig. 10B; n = 4; p < 0.05).

**Discussion**

It has previously been established that the sensitivity of insect nAChRs to neonicotinoid insecticides depends on 1) nAChR subtype subunit composition (Lansdell and Millar, 2000; Yixi et al., 2009; Li et al., 2010), 2) molecular modification of nAChRs by point mutation and post-transcriptional processes (Liu et al., 2005; Perry et al., 2008; Yao et al., 2009; Yixi et al., 2009); and 3) nAChR regulation by intracellular transient mechanisms such as the phosphorylation/dephosphorylation process (Courjaret and Lapied, 2001; Thany et al., 2007). In this study, we have demonstrated that the IMI-insensitive nAChR2, which differs from nAChR1 by uncommon functional properties (i.e., open at resting membrane potential and closed upon agonist application), is sensitive to other types of neonicotinoids, ACT and CLO (Thany 2009). Furthermore, the data presented in this study confirm that the identification of the cellular factors that render an insecticide more effective on a given target represents a fundamental step to adapting novel strategies in pest control.

As described below, our study demonstrates that the sensitivity of nAChR2 to ACT and CLO depends on the receptor conformational state closely related to complex cellular mechanisms involving transmembrane potential polarization, calcium influx through HVA calcium channels and TRPγ, and the intracellular calcium-dependent signaling pathway.

**Transmembrane Potential Polarization Variations Together with the Receptor Conformational State Modulate the Sensitivity of nAChR2 to Acetamiprid.**

We have reported in this study that transmembrane potential polarization affects the nAChR2 conformational state, which is confirmed by measuring the input membrane resistance. In fact, because nAChR2, open at the resting state, mediates a permanent spontaneous outward potassium conductance (Courjaret and Lapied, 2001; Thany et al., 2008), the inwardly directed current amplitude observed after cholinergic agonists and/or neonicotinoid insecticides correlate well with 1) the switch from the open to closed states of
nAChR2, 2) an increase in input membrane resistance, and 3) the consecutive inhibition of the spontaneous potassium conductance. These biophysical parameters seem to play crucial roles in the modulation of nAChR2 sensitivity to ACT and CLO. In this context, the proposed hypothetic two-state model of nAChR2 could be combined with a ternary complex model to generate a general qualitative model of the interaction between the nAChR2 conformational state and binding of ACT and CLO (Fig. 12A). In this ternary complex model, nAChR2 exists in different interconvertible states (R1–R4) that could be an intrinsic property of the receptor itself. R1 represents free open-state nAChR2 under the physiological condition. R4, which binds ACT or CLO (NEO in Fig. 12A), will exhibit lower or higher sensitivity to the neonicotinoid depending on nAChR2’s ability to be more or less open before the application of neonicotinoids. When the membrane is hyperpolarized, the equilibrium may shift toward R2 (i.e., the more open state), thereby revealing a lower sensitivity of R4 to ACT (Fig. 12A, A2) and a resulting increased current amplitude. In contrast, when the membrane is depolarized, the equilibrium shifts toward R3 (i.e., the less open state; Fig. 12A, A3) exhibiting a slight higher sensitivity to ACT and generating a consecutive decreased current amplitude. These findings have direct physiological consequences on the sensitivity of nAChR2 to ACT and CLO because any means that will produce depolarization (e.g., acetylcholine) or hyperpolarization (e.g., GABA; this study; Fig. 11) will affect neonicotinoid efficacy. Finally, in this hypothetic model, the interaction between ACT and CLO and nAChR2, which is driven by the transmembrane potential variation, is also driven by an additional parameter, intracellular calcium concentration, which influences the magnitude of nAChR2 sensitivity to neonicotinoids.

**Calcium Influx through HVA Calcium Channels and TRPγ Decreases the Sensitivity of nAChR2 to Acetamiprid.** Change in intracellular calcium concentration is known to modulate nAChR-mediated response to agonists (Liu and Berg, 1999; Voitenko et al., 2000; Thany et al., 2008). In this study, we have reported that depolarization affects intracellular calcium balance and nAChR2 sensitivity to mainly ACT through the activation of HVA calcium channels. In contrast, hyperpolarization and the consecutive activation of TRPγ will affect nAChR2 sensitivity to both ACT and CLO. Application of more positive membrane potential than resting condition (−50 mV) produces a limited displacement to the left of the dose-response curve for ACT (see Fig. 12A, dashed triangle). In contrast, hyperpolarization results in a limitless rightward shift of the dose-response curve for ACT and CLO on nAChR2. Using calcium channel blockers such as ω-CgTx GVIA and LOE 908, we have revealed that N-type HVA calcium channels and TRPγ, which are known to be activated at depolarized and hyperpolarized membrane potentials in DUM neurons, respectively (Wicher et al., 2004; Gautier et al., 2008), are the plasma membrane calcium channels generating extracellular calcium influx. It is interesting to note that the increase in sensitivity of nAChR2 to ACT observed for membrane potential more depolarized than −50 mV (i.e., physiological condition) is not as important compared with the decrease in sensitivity of nAChR2 to this neonicotinoid observed for hyperpolarization, although calcium influx occurs through both HVA calcium channels and TRPγ. This apparent different sensitivity might be explained...
by the fact that at a steady-state membrane potential of \(-30\) mV more than 60% of HVA calcium channels are inactivated (Grolleau et al., 2006), thus reducing the influence of calcium ions on nAChR2 sensitivity to ACT in this membrane potential range. In contrast, for hyperpolarization, calcium influx-induced decrease of the sensitivity of nAChR2 can be counteracted by LOE 908, which is known to block TRP\(^\gamma\) in DUM neurons (Wicher et al., 2004). Because similar effects are obtained in the presence of high internal cAMP concentration (i.e., 0.3 mM), it is tempting to suggest that the cAMP-dependent signaling pathway involved in the regulation of TRP\(^\gamma\), as described previously in the same preparation (Wicher et al., 2004; Grolleau et al., 2006), plays a key role in the modulation of the sensitivity of nAChR2 to ACT and CLO. As illustrated in Fig. 12B, the molecular events involved in such modulation can be summarized according to the hypothetic scheme. Hyperpolarization induces the activation of TRP\(^\gamma\), and the elevation of intracellular calcium concentration results in the formation of the calcium/calmodulin complex (Fig. 12B, ①). The consecutive increase in cAMP...
concentration via a calcium/calmodulin-sensitive adenyl cyclase (Courjaret and Laped, 2001) modulates the TRPγ negatively, which in turns reduces calcium influx (Fig. 12B, 2), as shown previously in the same preparation (Wicher et al., 2004, 2006), resulting in an increase of nACHr2 sensitivity to ACT and CLO. Although calcium allosteric modulation is a well known mechanism for modifying vertebrate pharmacological nACHr activity (Galzi et al., 1996; Changeux et al., 1998; Paterson and Nordberg, 2000; Dajas-Bailador and Wonnacott, 2004), this needs to be explored more deeply in such a context.

Optimization of Neonicotinoid Insecticide Efficiency. Our results demonstrate that transmembrane potential polarization together with an increase of intracellular calcium concentration affect the sensitivity of nACHr2 to neonicotinoids via a modification of the nACHr2 conformational state. These new insights in this study could be taken into account in pest control strategy to optimize neonicotinoid insecticide efficiency. Among the novel strategies proposed, insecticide mixtures present the best practical advantage only if synergistic interactions may occur between different components used in combination. In this context, combined formulations of neonicotinoids with pyrethroids have been developed with the aim of broadening the insecticidal spectrum and avoiding cross-resistance (Elbert et al., 2008). However, no synergist effect has been observed for such combinations including, for instance, neonicotinoids (e.g., thiamethoxam, clothianidin) and pyrethroid type II (e.g., cyhalothrin) (Bradshaw et al., 2008; Peck et al., 2008). Based on our results reporting that membrane polarization combined with calcium influx activation plays a key role in reducing neonicotinoid efficacy, it is tempting to suggest that this absence of synergistic effect could be explained by the fact that pyrethroid type II causing a membrane depolarization accompanied by a suppression of the action potential (Du et al., 2009; Soderlund, 2010) might affect neonicotinoid effects. Furthermore, another interesting finding is that physiological impact of hyperpolarization-induced activation of TRPγ can be achieved after the release of inhibitory neurotransmitters such as GABA acting through ionotropic receptors (Alix et al., 2002; Dupuis et al., 2010; Janssen et al., 2010). Therefore, these physiological mechanisms will lead to reduced neonicotinoid effect. Based on our results, combinations of neonicotinoids with pentylenzamide insecticides such as fipronil, which blocks GABA and glutamate action on ionotropic receptors (Narahashi et al., 1996; Changeux et al., 1998; Paterson and Nordberg, 2000; Dajas-Bailador and Sattelle, 1997) Idem, may be a good strategy to counteract GABA-induced hyperpolarization and produce a synergistic effect. The expected results could be an increase of insecticide efficicy, while reducing the dose of each compounds used.

Authorship Contributions

Participated in research design: Bodereau-Dubois and Laped.
Conducted experiments: Bodereau-Dubois, List, Calas-List, Marques, and Communal.
Performed data analysis: Bodereau-Dubois, List, Calas-List, Marques, and Communal.
Wrote or contributed to the writing of the manuscript: Bodereau-Dubois, Thany, and Laped.

References

Buckingham S, Laped B, List M, and Calas-List M (1997) Neonicotinoids and pyrethroids type II (e.g., cyhalothrin) (Bradshaw et al., 2008). Based on our results reporting that membrane polarization combined with calcium influx activation plays a key role in reducing neonicotinoid efficacy, it is tempting to suggest that this absence of synergistic effect could be explained by the fact that pyrethroid type II causing a membrane depolarization accompanied by a suppression of the action potential (Du et al., 2009; Soderlund, 2010) might affect neonicotinoid effects. Furthermore, another interesting finding is that physiological impact of hyperpolarization-induced activation of TRPγ can be achieved after the release of inhibitory neurotransmitters such as GABA acting through ionotropic receptors (Alix et al., 2002; Dupuis et al., 2010; Janssen et al., 2010). Therefore, these physiological mechanisms will lead to reduced neonicotinoid effect. Based on our results, combinations of neonicotinoids with pentylenzamide insecticides such as fipronil, which blocks GABA and glutamate action on ionotropic receptors (Narahashi et al., 1996; Changeux et al., 1998; Paterson and Nordberg, 2000; Dajas-Bailador and Sattelle, 1997) Idem, may be a good strategy to counteract GABA-induced hyperpolarization and produce a synergistic effect. The expected results could be an increase of insecticide efficicy, while reducing the dose of each compounds used.

Optimization of Neonicotinoid Insecticide Efficiency. Our results demonstrate that transmembrane potential polarization together with an increase of intracellular calcium concentration affect the sensitivity of nACHr2 to neonicotinoids via a modification of the nACHr2 conformational state. These new insights in this study could be taken into account in pest control strategy to optimize neonicotinoid insecticide efficiency. Among the novel strategies proposed, insecticide mixtures present the best practical advantage only if synergistic interactions may occur between different components used in combination. In this context, combined formulations of neonicotinoids with pyrethroids have been developed with the aim of broadening the insecticidal spectrum and avoiding cross-resistance (Elbert et al., 2008). However, no synergist effect has been observed for such combinations including, for instance, neonicotinoids (e.g., thiamethoxam, clothianidin) and pyrethroid type II (e.g., cyhalothrin) (Bradshaw et al., 2008; Peck et al., 2008). Based on our results reporting that membrane polarization combined with calcium influx activation plays a key role in reducing neonicotinoid efficacy, it is tempting to suggest that this absence of synergistic effect could be explained by the fact that pyrethroid type II causing a membrane depolarization accompanied by a suppression of the action potential (Du et al., 2009; Soderlund, 2010) might affect neonicotinoid effects. Furthermore, another interesting finding is that physiological impact of hyperpolarization-induced activation of TRPγ can be achieved after the release of inhibitory neurotransmitters such as GABA acting through ionotropic receptors (Alix et al., 2002; Dupuis et al., 2010; Janssen et al., 2010). Therefore, these physiological mechanisms will lead to reduced neonicotinoid effect. Based on our results, combinations of neonicotinoids with pentylenzamide insecticides such as fipronil, which blocks GABA and glutamate action on ionotropic receptors (Narahashi et al., 2007), might be a good strategy to counteract GABA-induced hyperpolarization and produce a synergistic effect. The expected results could be an increase of insecticide efficicy, while reducing the dose of each compounds used.