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Pharmacological characterization of *cis*-nitromethylene neonicotinoids in relation to imidacloprid binding sites in the brown planthopper, *Nilaparvata lugens*

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Abstract

Neonicotinoid insecticides, such as imidacloprid, are selective agonists of the insect nicotinic acetylcholine receptors (nAChRs) and extensively used in areas of crop protection and animal health to control a variety of insect pest species. Here we describe that two *cis*-nitromethylene neonicotinoids (IPPA152002 and IPPA152004), recently synthesized in our laboratory, discriminated between the high and low affinity imidacloprid binding sites in the brown planthopper, *Nilaparvata lugens*, a major insect pest of rice crops in many parts of Asia. [^{3}H]imidacloprid has two binding sites with different affinities (*K*d value of 0.0035 ± 0.0006 nM for the high-affinity site and 1.47 ± 0.22 nM for the low-affinity site). Although the *cis*-nitromethylene neonicotinoids showed low displacement ability (*K*i values of 0.15 ± 0.03 μM and 0.42 ± 0.07 μM for IPPA152002 and IPPA152004, respectively) against [^{3}H]imidacloprid binding, low concentrations (0.01 μM) of IPPA152002 completely inhibited [^{3}H]imidacloprid binding at its high-affinity site. In *Xenopus* oocytes co-injected with cRNA encoding Nlα1 and rat β2 subunits, obvious inward currents were detected in response to applications of IPPA152002 and IPPA152004, although the agonist potency is reduced to that of imidacloprid. The previously identified Y151S mutation in Nlα1 showed significant effects on the agonist potency of IPPA152002 and IPPA152004, such as a 75.8% and 70.6% reduction in *I*max, and a 2.4- and 2.1-fold increase in EC_{50}. This data clearly shows that the two newly described *cis*-nitromethylene neonicotinoids act on insect nAChRs and like imidacloprid, discriminated between high and low affinity binding sites in *N. lugens* native nAChRs. These compounds may be useful tools to further elucidate the pharmacology and nature of neonicotinoid binding sites.

Keywords: *cis*-nitromethylene neonicotinoid, nicotinic acetylcholine receptors, *Nilaparvata lugens*, imidacloprid, binding site.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are neurotransmitter-gated ion channels that mediate fast cholinergic synaptic transmission in insect and vertebrate nervous systems (Sattelle, 1980; Matsuda *et al.*, 2001). In vertebrates, nAChRs are expressed at both the neuromuscular junction ('muscle-type' nAChR) and within the central and peripheral nervous system ('neuronal' nAChRs). In insects, nAChRs play an important role in the insect central nervous system (CNS), where acetylcholine is the major excitatory neurotransmitter (Breer & Sattelle, 1987). The structural model of the agonist site consists of amino acids from three distinct regions of the α-subunits (referred to as loop A, B, and C) and from at least three regions of the non-α (β, γ or δ) -subunits (loop D, E, and F) (Prince & Sine, 1998; Arias, 2000; Corringer *et al.*, 2000; Brejc *et al.*, 2001; Grutter & Changeux, 2001; Smit *et al.*, 2001).

Insect nAChRs have long been recognized as potential targets for insecticidal compounds, and in the past three decades this potential has been realised by the development of highly potent and selective agents that collectively offer effective control of the majority of insect pests of

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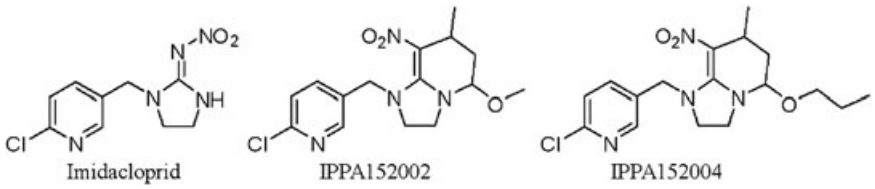

Figure 1. Chemical structure of imidacloprid and two *cis*-nitromethylene neonicotinoids (IPPA152002 and IPPA152004). Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine; IPPA152002, 1-((6-chloropyridin-3- yl)methyl)-1,2,3,5,6,7-hexahydro-5-methoxy-7-methyl-8-nitroimidazo[1,2-α]pyridine; IPPA152004, 1-((6-chloropyridin-3-yl)methyl)-1,2,3,5,6,7-hexahydro-7-methyl-8- nitro-5-propoxyimidazo[1,2-α]pyridine.

agricultural, veterinary and medical importance (Millar & Denholm, 2007). Neonicotinoid insecticides, such as imidacloprid, are selective agonists of insect nAChRs and are used extensively in areas of both crop protection and animal health (Matsuda *et al.*, 2001; Nauen & Denholm, 2005; Millar & Denholm, 2007). Neonicotinoid insecticides act selectively on insect nAChRs, accounting at least in part for the selective toxicity to insects over vertebrates (Matsuda *et al.*, 2001, 2005; Tomizawa & Casida, 2005). Neonicotinoid insecticides possess either an electron withdrawing nitro (–NO_2) or cyano (–CN) group, which have been postulated to contribute directly to their selectivity (Matsuda *et al.*, 2001; Tomizawa & Casida, 2005). The –NO_2 or –CN group in all commercial neonicotinoids is in *trans*-configuration. Recently, some potential neonicotinoids with *cis*-configuration have been synthesized in our laboratory and show insecticidal activity in a wide range of insects (Fig. 1; Tian *et al.*, 2007; Shao *et al.*, 2008). In addition, some potential *cis*-neonicotinoids showed higher activity than imidacloprid against the imidacloprid-resistant population of the brown planthopper (*Nilaparvata lugens*), an economically important insect pest of rice crops in many parts of Asia (Shao *et al.*, 2008).

In order to understand the action mode of these potential neonicotinoids with *cis*-configuration, the pharmacological characterization was performed in *N. lugens* native nAChRs and heterologously expressed hybrid receptors. The influence of Y151S mutation, found in *N. lugens* nAChRs α subunit, on the agonist potency of these potential neonicotinoids was also evaluated and compared with that of imidacloprid.

Results

Toxicity of cis*-nitromethylene neonicotinoids against* Nilaparvata lugens

In susceptible (Sus) and resistant (Res) strains, the toxicities of two *cis*-nitromethylene neonicotinoids, IPPA152002 and IPPA152004, were determined and compared with the commercial neonicotinoids imidacloprid and clothianidin (Table 1). IPPA152002 and IPPA152004 showed relatively lower toxicities against susceptible insects than imidacloprid and clothianidin. In Res strain, IPPA152002, IPPA152004 and imidacloprid showed similar toxicities, and resistant insects showed some cross-resistance to IPPA152002 and IPPA152004.

Radioligand binding to native nAChRs in Nilaparvata lugens

Membrane protein was extracted from *N. lugens* and [^{3}H]imidacloprid binding was performed. High-affinity specific binding of [^{3}H]imidacloprid was detected in whole-body membrane preparations and saturation radioligand binding studies revealed two high-affinity binding sites for imidacloprid (*K*d = 0.0035 ± 0.0006 nM and 1.47 ±

Table 1. Toxicity of different neonicotinoids against susceptible (Sus) and resistant (Res) strains of *Nilaparvata lugens*

Strain	Neonicotinoids	Insect number	Slope ± SE	LD50 (95% FL) (ng/pest)	Relative toxicity*	RR factor†
Sus	Imidacloprid	103	3.232 ± 0.271	0.151 (0.122–0.186)	1.00	1.00
	Clothianidin	88	2.673 ± 0.351	0.240 (0.207–0.279)	1.59	1.00
	IPPA152002	95	2.759 ± 0.322	1.816 (1.477–2.258)	12.03	1.00
	IPPA152004	96	2.556 ± 0.415	4.722 (4.201–5.330)	31.27	1.00
Res	Imidacloprid	85	1.793 ± 0.368	22.161 (14.585–36.227)	1.00	168.4
	Clothianidin	105	1.427 ± 0.276	5.664 (4.952–7.139)	0.26	23.6
	IPPA152002	114	1.611 ± 0.579	20.098 (13.101–29.442)	0.91	11.07
	IPPA152004	99	1.304 ± 0.316	33.552 (20.280–48.175)	1.51	7.11

*Relative toxicity was calculated by comparing LD_{50} values of other neonicotinoids and that of imidacloprid in Sus and Res strain, respectively. †RR (resistance ratio) factor was calculated by comparing LD_{50} values for each neonicotinoid against Res and Sus strains.

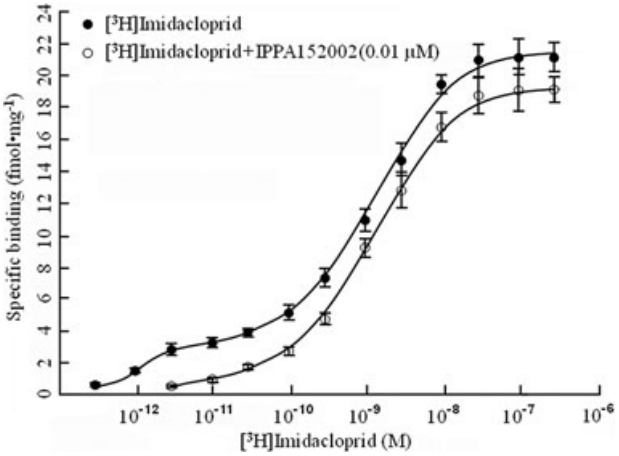

Figure 2. Equilibrium saturation binding of [^{3}H]imidacloprid on *Nilaparvata lugens* native nAChRs in the absence and the presence of 0.01 μM IPPA152002. Data points are means of at least three independent experiments.

0.22 nM; Fig. 2) to native nAChRs from *N. lugens*. In Scatchard plot (Fig. 3A), the plot clearly demonstrated the presence of two binding sites. The maximal binding capacity (*B*max) for two binding sites is 4.1 ± 0.6 fmol/mg and 18.3 ± 2.3 fmol/mg, respectively. These results are identical to our previous findings (Liu *et al*., 2005), and in close agreement with previous reports that imidacloprid binds with high affinity to two sites (*K*d = 0.004 nM and 1.2 nM) in membrane preparations from the leafhopper *Nephotettix cincticeps* (Lind *et al*., 1998).

Competition of IPPA152002 and IPPA152004 against [³H]imidacloprid binding

IPPA152002 and IPPA152004, as well as clothianidin and imidacloprid itself, were tested for their ability to displace [^{3}H]imidacloprid binding to *N. lugens* membranes.

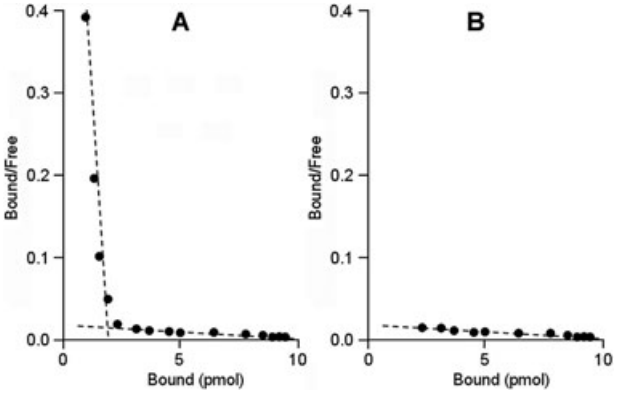

Figure 3. Scatchard plot of data presented in Fig. 2. (A) Without 0.01 μM IPPA152002; (B) With 0.01 μM IPPA152002. In all cases, representative plots are shown that is typical of at least three independent experiments. The Scatchard lines were indicated by dashed lines.

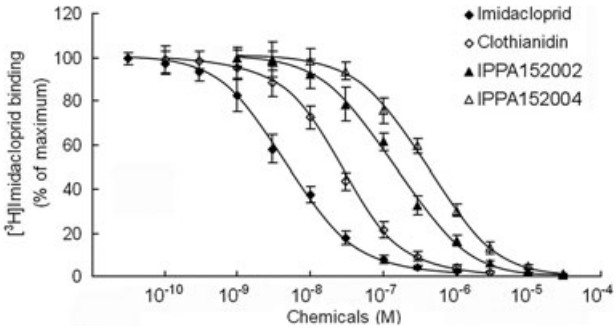

Figure 4. Competition binding with different neonicotinoids displacing [^{3}H]imidacloprid binding. Data are means of at least three independent experiments ± SEM. The concentration of [^{3}H]imidacloprid here is 1.0 nM.

Although IPPA152002 and IPPA152004 weakly displaced [^{3}H]imidacloprid binding, with *K*i values of 0.15 ± 0.03 μM and 0.42 ± 0.07 μM, imidacloprid itself (*K*i = 0.0045 ± 0.008 μM) and another commercial neonicotinoid clothianidin (0.026 ± 0.005 μM) were far more potent. These data indicated that IPPA152002 and IPPA152004 were weak displacers of [^{3}H]imidacloprid binding in *N. lugens* native nAChRs (Fig. 4).

Discrimination between high and low affinity binding sites in native nAChRs

Although IPPA152002 (as well as IPPA152004) showed poor ability to displace [^{3}H]imidacloprid binding in *N. lugens* membranes, IPPA152002 could completely displace [^{3}H]imidacloprid binding at its higher affinity site (*K*d = 0.0035 ± 0.0006 nM for imidacloprid) when 0.01 μM IPPA152002 was added (Fig. 2). Scatchard plot clearly showed only a single binding site for imidacloprid present when 0.01 μM IPPA152002 was included in saturation binding studies (Fig. 3B), compared with 2 sites in the absence of IPPA52002. The *K*d and *B*max values for this unique site were 1.41 ± 0.24 nM and 18.5 ± 3.1 fmol/mg, which were nearly identical to the values (1.47 ± 0.22 nM and 18.3 ± 2.3 fmol/mg) of the lower affinity site in saturation binding without IPPA152004. These results indicate that IPPA52002 acted preferentially at the very high affinity imidacloprid binding site in *N. lugens* membranes.

Potency of IPPA152002 and IPPA152004 on heterologously expressed hybrid nAChRs

The influence of the two *cis*-nitroemthylene neonicotinoids was investigated on hybrid nicotinic acetylcholine receptors composed of *N. lugens* Nlα1 and rat β2 subunits. The influence of the Y151S mutation, previously demonstrated to impair imidacloprid agonist efficacy in Nlα1-containing receptors, was also evaluated (Liu *et al*., 2005).

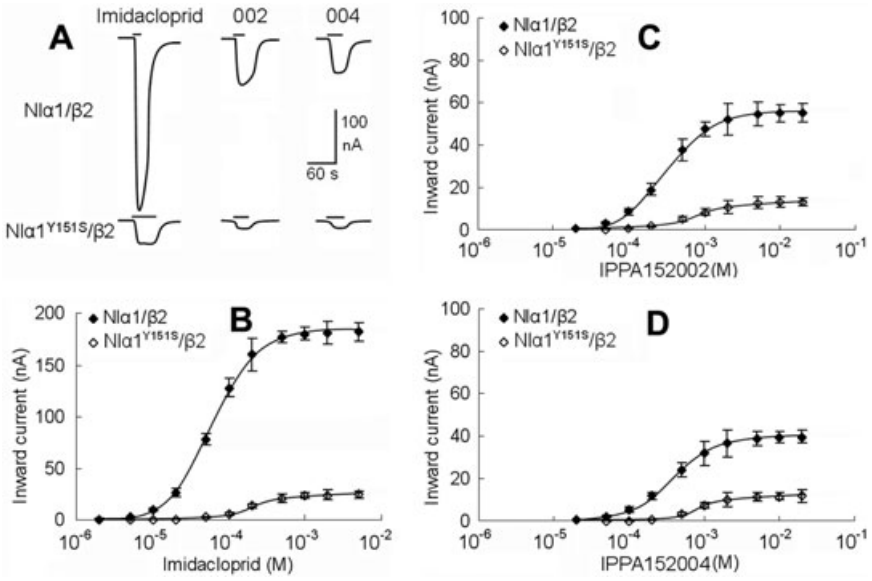

Figure 5. The representative whole-cell responses and agonist dose–response curves from hybrid nAChRs expressed in *Xenopus* oocytes, containing Nlα1/β2 or Nlα1^{Y151S}/β2. (A) The inward currents evoked by 1 mM imidacloprid, IPPA152002 and IPPA152004. (B) The agonist dose–response curves of imidacloprid in oocytes expressing Nlα1/β2 and Nlα1^{Y151M}/β2. (C) The agonist dose-response curves of IPPA152002. (D) The agonist dose–response curves of IPPA152004. Data are means of at least three independent experiments ± SEM.

In oocytes co-injected with cRNA encoding the Nlα1 and rat β2 subunits, obvious inward currents were detected in response to applications of IPPA152002 and IPPA152004, although these were significantly smaller than that of imidacloprid at the same concentration (1 mM, Fig. 5A). The inward currents evoked by IPPA152002 and IPPA152004, as well as imidacloprid, were completely inhibited by DHβE, a well-known antagonist of nAChRs (data not shown). In voltage-clamp electrophysiological studies, these chemicals evoked inward currents in a dose-dependent manner in *Xenopus* oocytes. As observed in the representative response, the maximal inward currents (*I*max) for IPPA152002 and IPPA152004 were only 30.0% and 21.9% of that for imidacloprid (Fig. 5). In addition, the calculated EC_{50} values for IPPA152002 and IPPA152004 were 5.2- and 6.3-fold of that for imidacloprid. Data for I_{max} and EC_{50} values are summarized in Table 2.

The Y151S mutation in Nlα1 dramatically reduced *I*max (12.8% of wildtype) and caused a rightward shift (3.1-fold) in the agonist dose-response curve for imidacloprid (Fig. 5B), but had little influence on acetylcholine potency, which is in close agreement with our previous report (Table 2) (Liu *et al.*, 2006). This mutation also showed a significant influence on IPPA152002 and IPPA152004 agonist activity, with a 75.8% and 70.6% reduction in *I*max, and a 2.4- and 2.1-fold increase in EC_{50} (Fig. 5C, D). These results showed the Y151S mutation affected the agonist potency of IPPA152002 and IPPA152004, although this effect was reduced when compared with imidacloprid.

Discussion

The commercial neonicotinoids have different action sites in different insect species, although these neonicotinoids

Table 2. Maximum current (I_{max}) and EC_{50} values for nAChR subunit combinations

Subunits combinations	Agonist	I_{max} (nA)	EC_{50} (μM)	*n*
Nlα1/β2	Acetylcholine	226.5 ± 19.6	36.4 ± 5.2	3
	Imidacloprid	181.7 ± 14.5	59.3 ± 4.4	4
	IPPA152002	54.6 ± 7.5	306.1 ± 43.2	6
	IPPA152004	39.8 ± 5.9	372.6 ± 59.7	5
Nlα2^{Y151S}/β2	Acetylcholine	229.2 ± 23.7	41.0 ± 5.8	3
	Imidacloprid	24.2 ± 1.7	182.2 ± 11.6	3
	IPPA152002	13.2 ± 2.1	734.6 ± 95.8	7
	IPPA152004	11.7 ± 2.5	786.2 ± 100.5	5

Data are means of at least three independent experiments ± SEM. *n*: the number of independent experiments.

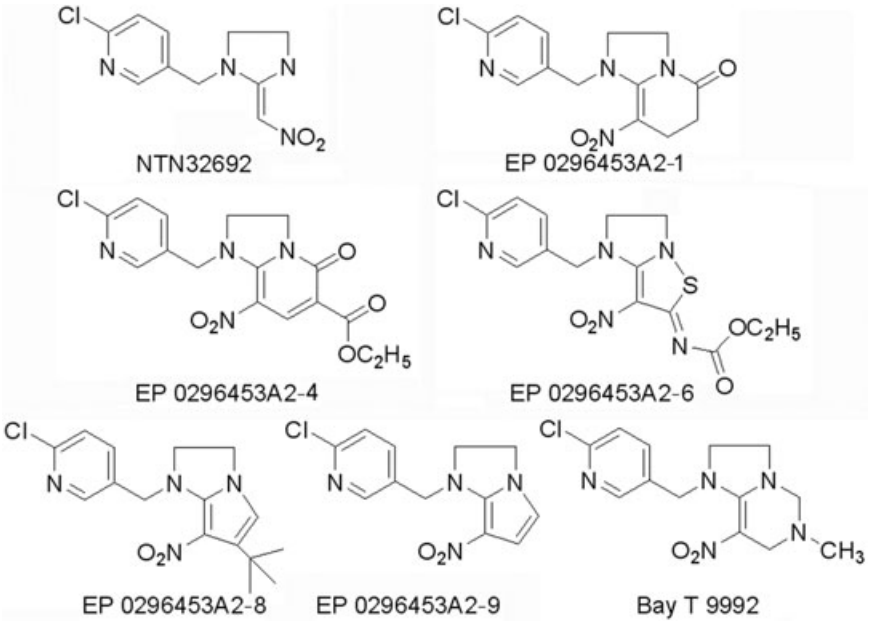

Figure 6. Dicyclic compounds with high bioactivity.

are well known to act on insect nAChRs. Two binding sites for imidacloprid were found in *Myzus persicae* (Hemiptera: Aphididae), *N. cincticeps* (Hemiptera: Cicadellidae) and *N. lugens* (Hemiptera: Delphacidae), but only one in *Periplaneta americana* (Dictyoptera: Blattidae), *Lucilia sericata* (Diptera: Calliphoridae), *Drosophila melanogaster* (Diptera: Drosophiloidae), *Manduca sexta* (Lepidoptera: Sphingidae), *Heliothis virescens* (Lepidoptera: Noctuidae), and *Ctenocephalides felis* (Siphonaptera: Pulicidae) (Lind *et al*., 1998; Liu *et al*., 2005; Tomizawa *et al*., 2005); and also this study). These studies demonstrate that only Hemipteran insects have very a high-affinity binding site for imidacloprid (with *K*d values close to or less than 0.1 nM), in addition to the lower affinity site that is also present in insects from other orders (with *K*d values more than 1 nM) (Lind *et al*., 1998; Liu *et al*., 2005). The presence of very high-affinity imidacloprid binding sites only in the hemipteran insects may explain why imidacloprid is particularly useful in controlling insect pests from Hemiptera, such as planthoppers, aphids and leafhoppers.

In 1988, Nihon Tokushu Noyaku Seizo Company synthesized series of *cis*-configuration compounds based on compound NTN32692 (Fig. 6). It was found that several compounds (Fig. 6) exhibited high insecticidal activities against green leafhopper *N. virescens*, and the mortality is 100% at 200 ppm (Shiokawa *et al*., 1988). The dicyclic neonicotinoid analogue containing a tetrahydropyrimidine ring was reported by Bayer in 1992, in which the nitro group is *cis* to the chloropyridinylmethyl moiety and showed obvious biological activity. Casida and co-workers reported that this compound exhibited high affinity on nAChR of *Musca* or *Drosophila* head membranes (Latli *et al*., 1997). For example, the compound Bay T 9992 (Fig. 6) could displace [^{3}H]imidacloprid binding with IC_{50} values of 0.73 $\pm$ 0.37 nM. These results suggest that neonicotinoids with nitro group in *cis*-configuration also possess insecticidal activity. In the present study, compounds IPPA152002 and IPPA152004 are nitromethylene neonicotinoids, containing a tetrahydropyridine ring with exo-ring ether modifications which makes the nitro group in *cis*-configuration. Previous studies showed that they exhibited good insecticidal activities against pea aphid (*Aphis craccivora*), army worm (*Mythimna separata*) and brown planthopper (*N. lugens*) (Tian *et al*., 2007; Shao *et al*., 2008). Structural property and insecticidal activity of *cis*-nitromethylene neonicotinoids on target insects of commercial neonicotinoids, such as imidacloprid, make it interesting to find out the action mode of these potential neonicotinoids. When 0.01 μM of IPPA152002 was included, there was a complete absence of the very high-affinity binding site for imidacloprid. Because the used concentration (0.01 μM) was much lower than the *K*i value (0.15 μM) in competition binding studies, its complete displacement of [^{3}H]imidacloprid binding at the very high-affinity binding site indicated this *cis*-nitromethylene neonicotinoid preferentially acted at the high affinity [^{3}H]imidacloprid binding site. In contrast to the preferential acting at the high affinity site, both IPPA152002 and IPPA152004 were weak displacers of [^{3}H]imidacloprid binding in *N. lugens* native nAChRs, which indicated these two *cis*-neonicotinoids interacted weakly at the lower

affinity imidacloprid binding site. Of course, *cis*-nitromethylene neonicotinoids may have other acting sites besides that found here by displacement of [^{3}H]imidacloprid binding in native *N. lugens* nAChRs, which need further investigation.

The Y151S mutation was identified in α subunits from a resistant population of *N. lugens* which was selected from a field population by laboratory selection over 30 generations (Liu *et al*., 2005). The mutation Y151S is located within loop B of the Nlα1 subunit, a region which lies close to the nAChR agonist binding site (Grutter & Changeux, 2001). We have shown previously, from comparisons of Nlα1/β2 and Nlα1^{Y151S}/β2 nAChRs, that the Y151S mutation dramatically reduces sensitivity to neonicotinoid insecticides, such as imidacloprid (Liu *et al*., 2005, 2006). In the present study, the Y151S mutation was also found to show great effect on the agonist potency of IPPA152002 and IPPA152004, reflected in a reduction of *I*max and an increase in EC_{50}. These results suggest that IPPA152002 and IPPA152004 were agonists on hybrid Nlα1/β2 nAChRs. However it cannot be concluded that Nlα1 forms part of the high affinity site for imidacloprid, IPPA152002 and IPPA152004 in the whole insect because subunit stoichiometry would be expected to give rise to multiple nAChRs and as such the nature of the binding sites is unknown. For example, although epibatidine only has one binding site in *D. melanogaster* native nAChRs, it shows agonist potency on more than one hybrid receptors containing α subunits from *D. melanogaster* (Lansdell & Millar, 2000). As well as IPPA152002 and IPPA152004, although they showed agonist potency on Nlα1/β2 receptors, more direct evidences are needed to show that they actually act on Nlα1 containing native receptors in *N. lugens*.

Overall this data further demonstrates that hemipteran insects display a very high affinity binding site (*K*d < 0.2 nM) in addition to a second lower affinity binding site, also found in other insect species (*K*d > 1.0 nM). The *cis*-neonicotinoids used in this study interact at similar sites to imidacloprid and clearly discriminate between the two binding sites, potently interacting at the very high affinity imidacloprid binding site but only weakly at the lower affinity imidacloprid binding site. Taken together, the biology and the pharmacology data suggest that for effective hemipteran control, neonicotinoids must act with high affinity to both binding sites. The *cis*-neonicotinoids will be useful compounds to discriminate between the two imidacloprid binding sites.

Experimental procedures

Chemicals

Acetylcholine (ACh), imidacloprid, clothianidin and dihydro-β-erythroidine (DHβE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]Imidacloprid (32 Ci/mmol) was generously provided by Syngenta Ltd, European Regional Centre, Guildford, UK. *cis*-nitromethylene neonicotinoids, IPPA152002 and IPPA152004, were synthesized and purified as previously reported (Tian *et al*., 2007; Shao *et al*., 2008).

Insects and bioassay

The susceptible strain (Sus) of *N. lugens* was a laboratory strain obtained from the China National Rice Research Institute in September 2001. The resistant strain (Res) was originally collected from a field of hybrid paddy rice in Anqing (Anhui, China) in September 2006 and continuously selected with imidacloprid in a laboratory for 16 generations. Insects were kept indoors at 25 (±1) °C, humidity 70–80% and 16/8 h light/dark.

The bioassay followed the microtopical application previously reported (Liu *et al*., 2003). A droplet (0.08 μl) of chemicals in acetone was applied topically to the prothorax notum of 2- to 3-day old macropterous adult females (unmated), with a hand microapplicator (Burkard Manufacturing Co. Ltd, Rickmansworth, UK). About 30insects were treated at each concentration, and every treatment was repeated three times. The controls used acetone instead of insecticide solution. The results were checked after 48 h.

Membrane protein extraction and radioligand binding

Membranes were prepared from *N. lugens* as previously reported (Liu *et al*., 2005). *N. lugens* (3-day-old female; 50 mg) from Sus strain were homogenized in 50 ml of extraction buffer [pH 7.2, 0.32 mM sucrose, 100 μM EDTA, 1% proteinase inhibitor mixture I (Sigma)]. The homogenate was centrifuged at 1000 × ***g*** for 30 min. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 30 000 × ***g*** for 60 min. The pellet was resuspended in incubation buffer (pH 7.4, 0.05 mM Tris, 0.12 mM NaCl, 100 μM EDTA). Protein content was determined by a Bio-Rad DC protein assay using BSA as standard (Bradford, 1976).

In a total volume of 300 μl incubation buffer, the receptor preparation (0.2–0.3 mg protein per assay) was incubated for 120 min at 4 °C with appropriate concentration of [^{3}H]imidacloprid. Samples were assayed by filtration onto Whatman GF/B filters presoaked in 0.5% polyethylenimine, followed by rapid washing with ice-cold saline buffer (pH 7.4, 20 mM Na_2HPO4, 0.15 M NaCl, 0.2% bovine serum albumin). The filters were transferred into the scintillation vials and the radioactivity remaining on the filter was assayed after overnight incubation in 3 ml scintillation cocktail (OptiPhase Supermix, PerkinElmer, MA, USA) on an LS6500 Liquid Scintillation Counter (Beckman Coulter, CA, USA). Specific binding was defined as the difference in radioactivity in the absence and the presence of unlabeled imidacloprid with an 1000-fold molar excess (compared to [^{3}H]imidacloprid concentrations used).

Competition binding was performed by including different concentrations of chemicals (imidacloprid, clothianidin, IPPA152002 and IPPA152004) in the incubation mixture containing 1.0 nM [^{3}H]imidacloprid and membrane protein (0.2–0.3 mg per assay).

Expression and electrophysiological recording in Xenopus oocytes

N. lugens nAChR subunits Nlα1 (*AY378698*, wildtype and mutant containing Y151S mutation) and *Rattus norvegicus* β2 subunit

(*L31622*) were subcloned into the expression vector pGH19 as described previously (Liu *et al*., 2006). Subunit cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, Austin, TX, USA). *Xenopus* oocyte preparation, cRNA injection and electrophysiological recordings were performed as described previously (Liu *et al*., 2006).

Data analysis

In Bioassay, LD_{50} values were determined on the basis of standard probit analysis (Bliss, 1935). In radioligand binding, nonlinear regression analysis with Microsoft Excel's solver macro was used to determine the dissociation constant (*K*d) and maximal binding capacity (*B*max) from double hyperbola plots in saturation data, and the *K*i and Hill coefficient values in displacement binding (Bowen & Jerman, 1995). Dose–response curves in electrophysiological recordings were fitted with the Hill equation as described previously (Liu *et al*., 2006).

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