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Native subunit composition of two insect nicotinic receptor subtypes with differing affinities for the insecticide imidacloprid

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ABSTRACT

Neonicotinoid insecticides, such as imidacloprid, are selective agonists of insect nicotinic acetylcholine receptors (nAChRs) and are used extensively to control a variety of insect pest species. The brown planthopper (*Nilaparvata lugens*), an insect pest of rice crops throughout Asia, is an important target species for control with neonicotinoid insecticides such as imidacloprid. Studies with nAChRs purified from *N. lugens* have identified two [^{3}H]imidacloprid binding sites with different affinities ($K_d = 3.5 \pm 0.6$ pM and 1.5 ± 0.2 nM). Co-immunoprecipitation studies with native preparations of *N. lugens* nAChRs, using subunit-selective antisera, have demonstrated the co-assembly of Nlα1, Nlα2 and Nlβ1 subunits into one receptor complex and of Nlα3, Nlα8 and Nlβ1 into another. Immunodepletion of Nlα1 or Nlα2 subunits resulted in the selective loss of the lower affinity imidacloprid binding site, whereas immunodepletion of Nlα3 or Nlα8 caused the selective loss of the high-affinity site. Immunodepletion of Nlβ1 resulted in a complete absence of specific imidacloprid binding. In contrast, immunodepletion with antibodies selective for other *N. lugens* nAChR subunits (Nlα4, Nlα6, Nlα7 and Nlβ2) had no significant effect on imidacloprid binding. Taken together, these data suggest that nAChRs containing Nlα1, Nlα2 and Nlβ1 constitute the lower affinity binding site, whereas nAChRs containing Nlα3, Nlα8 and Nlβ1 constitute the higher affinity binding site for imidacloprid in *N. lugens*.

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1. 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). The great abundance of nAChRs within the insect central nervous system (CNS) has led to the development of economically important insecticides targeting these receptors. Of particular significance has been the introduction of neonicotinoid insecticides such as imidacloprid in the early 1990s. Neonicotinoids are nAChR agonists that display significantly higher affinity binding for insect nAChRs than for mammalian nAChRs (Tomizawa et al., 2000).

The brown planthopper *Nilaparvata lugens* is a major insect pest of rice crops throughout Asia. Two binding sites for the neonicotinoid insecticide imidacloprid have been identified in nAChRs preparations purified from *N. lugens* (Liu et al., 2005), a feature that has been described in other insect species (Lind et al., 1998). However, despite the longstanding interest in identifying the precise subunit composition of neonicotinoid-sensitive nAChRs, this goal has not yet been achieved in any insect species.

In the model insect species *Drosophila melanogaster*, 10 nAChR subunits (Dα1–Dα7 and Dβ1–Dβ3) have been identified, and a similar number of nAChR subunits have been identified in other insect species (Jones et al., 2007; Millar and Denholm, 2007). In the case of insects such as *Drosophila*, all candidate nAChR subunits identified by genome sequencing have been cloned but, despite this, considerable problems remain in generating functional insect nAChRs in heterologous expression systems (Millar, 1999; Lansdell et al., 2008).

Previous studies have described the molecular cloning of five nAChR subunits from *N. lugens* (Nlα1-Nlα4 and Nlβ2) and also the identification of a point mutation (Y151S) in two different *N. lugens* nAChR subunits (Nlα1 and Nlα3) that is associated with resistance to neonicotinoid insecticides (Liu et al., 2005, 2006). Recently, additional nAChR subunits (Nlα8 and Nlβ1) have also been cloned from *N. lugens* (Yao et al., 2009; Yixi et al., 2009). Unfortunately, as

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with other insect species, difficulties remain in the efficient functional expression of recombinant receptors from nAChR subunits cloned from *N. lugens* (Millar, 1999; Millar and Lansdell, in press). The cloning and sequencing of *N. lugens* nAChR subunits has, however, facilitated the generation of subunit-selective antisera. In the present study we describe the use of antisera raised against bacterially-expressed fusion proteins to examine the subunit composition of imidacloprid binding sites in native nAChRs in *N. lugens*.

2. Materials and methods

2.1. Chemicals

[³H]Imidacloprid (32 Ci/mmol) was generously provided by Syngenta Limited, European Regional Centre, Guildford, United Kingdom. Imidacloprid was purchased from Sigma–Aldrich (USA).

2.2. Membrane protein extraction and radioligand binding

Membranes were prepared from *N. lugens* as described previously (Liu et al., 2005). *N. lugens* (3-day-old female; 50 mg) 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 $1000 \times g$ for 30 min. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at $30,000 \times g$ 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 [³H]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, USA) on an LS6500 Liquid Scintillation Counter (Beckman Coulter, Fullerton, 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 [³H]imidacloprid concentrations used).

2.3. Antibodies and immunoprecipitation experiments

Polyclonal antisera (N3-I and N8-I), specific for the *N. lugens* α3 (Nlα3) and α8 (Nlα8), were prepared as described previously (Yixi et al., 2009). Polyclonal antisera (N1-I, N2-I, N4-I, N6-I, N7-I, Nb1-I and Nb2-I), specific for α1 (Nlα1), α2 (Nlα2), α4 (Nlα4), α6 (Nlα6), α7 (Nlα7), β1 (Nlβ1) and β2 (Nlβ2) subunits were raised against bacterially-expressed fusion proteins containing the large cytoplasmic loop of Nlα1 (Val[372]–Ala[430]), Nlα2 (Phe[424]–Tyr[485]), Nlα4 (Val[394]–Ala [450]), Nlα6 (Phe[392]–Phe [439]), Nlα7 (Phe[399]–Val [445]), Nlβ1 (Tyr[368]–His[428]) and Nlβ2 (Phe[355]–Ala[408]) subunits. Fusion proteins were purified as described previously (Schloß et al., 1988) and purified on protein G-Sepharose (GammaBind plus, Pharmacia, Germany). Detergent extracts of *N. lugens* membranes were prepared as described previously for *Drosophila* (Schloß et al., 1988) and immunoprecipitation performed by methods described previously (Yixi et al., 2009). GammaBind plus Sepharose was equilibrated in buffer A and 50 μl of a 1:1 GammaBind plus Sepharose/buffer A (10 mM Tris–HCl, pH 7.5, 280 mM sucrose,

10 mM NaCl, 100 μg/ml PMSF) suspension incubated for 2 h at 4 °C with 10 mg of antibody. Detergent extract was preabsorbed with GammaBind plus Sepharose to eliminate nonspecific binding and then the preabsorbed detergent extract (2.5 mg of protein) incubated overnight with antibody-coupled GammaBind plus Sepharose in a final volume of 1 ml of buffer A. In competition experiments, an ∼1000-fold molar excess of fusion protein (described above) was included in the incubation mixture. Immunoprecipitates were collected by centrifugation and pellets were washed twice with buffer A, twice with buffer B (buffer A containing 1 M NaCl), and once with buffer C (50 mM Tris–HCl, pH 6.8). Proteins were eluted from the GammaBind plus Sepharose, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted on to nitrocellulose and then detected using the ECL detection system, following the manufacturer's instructions (Millipore Corporation, Billerica, MA, USA).

2.4. Radioligand binding on detergent extracts

Detergent extracts of *N. lugens* membranes (2.0 mg) were incubated with 10 nM [³H]imidacloprid in the absence, or presence, of 10 μM unlabeled imidacloprid for 2 h at 4 °C. Aliquots (200 μl) were divided into two parts. Half of the sample was used for total radioligand binding determination as above description. The other half was preabsorbed with GammaBind plus Sepharose and then incubated overnight with antibody-coupled GammaBind plus Sepharose in a final volume of 200 μl of buffer A. Immunoprecipitates were collected by centrifugation as described above and the remaining supernatants were also collected. The radioactivity in the immunodepleted supernatants was determined as described above.

2.5. Data analysis

Radioligand binding data was analyzed by non-linear regression using Microsoft Excel solver macro to determine the dissociation constant (K_d) and maximal binding capacity (B_{max}) from double hyperbola plots in saturation data (Bowen and Jerman, 1995). Scatchard analysis was used to estimate the binding parameters (Ray et al., 1996).

3. Results

3.1. Co-immunoprecipitation of subunits from native nAChRs

Previous studies of recombinant nAChRs have provided evidence for the co-assembly of the *N. lugens* Nlα1 and Nlα2 subunits, together with a mammalian β subunit (rat β2), to form a functional hybrid (Nlα1/Nlα2/β2) receptor (Liu et al., 2009). In addition, evidence has been obtained for the co-assembly of Nlα3 and Nlα8, together with rat β2, into another functional hybrid (Nlα3/Nlα8/β2) receptor (Yixi et al., 2009). Evidence has also been obtained demonstrating the co-immunoprecipitation of Nlα3 and Nlα8 from preparations of native *N. lugens* nAChRs (Yixi et al., 2009). To examine whether Nlα1 and Nlα2 co-assemble in native nAChRs, immunoprecipitation studies were performed with subunit-specific antibodies. Polyclonal antisera (N1-I and N2-I) were generated against bacterial fusion proteins containing the main cytoplasmic loops of the Nlα1 and Nlα2 subunits. Detergent extracts of *N. lugens* head membranes were immunoprecipitated with either N1-I (Fig. 1A and C) or N2-I (Fig. 1E and G) and immunoprecipitated proteins were then analyzed by immunoblotting with either N1-I (Fig. 1A and G) or N2-I (Fig. 1C and E). In each case a single specific band was detected (indicated by a single asterisk; Fig. 1A, C, E and G) that could be blocked by the
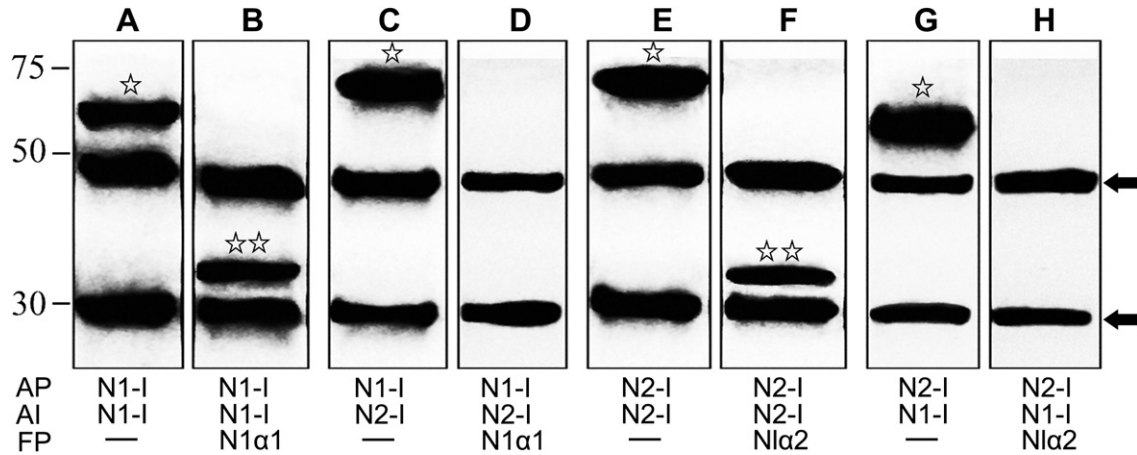
Fig. 1. Co-immunoprecipitation of Nlα1 and Nlα2 from native nAChRs. Detergent extracts of *N. lugens* head membranes were immunoprecipitated with either N1-I (A and C) or N2-I (E and G). Immunoprecipitated proteins were then analyzed by immunoblotting with N1-I (A and G) or N2-I (C and E). In each case a single specific band was detected (indicated by a single asterisk) that could be blocked by inclusion of the corresponding fusion protein (N1α1 in B and D; Nlα2 in F and H). When the appropriate antisera were used for immunoblotting, the location of the corresponding fusion protein is indicated by a double asterisk. In all cases, the immunoglobulin heavy and light chains (from the antisera used for immunoprecipitation) were also detected (indicated by arrow heads). The identity of the two immunoglobulin bands was further confirmed by immunoblotting with an anti-mouse IgG antibody (data not shown). Antibody used in precipitation (AP) and antibody in immunoblotting (AI) were indicated under each lane. Fusion protein (FP) used for competition was also indicated by subunit name.

corresponding fusion proteins of Nlα1 (Fig. 1B and D) or Nlα2 (Fig. 1F and H). The data demonstrates the absence of cross-reactivity of the antisera for the Nlα1 and Nlα1 subunits and provides evidence for co-immunoprecipitation of these two subunits in native nAChR preparations.

Previous immunoprecipitation studies provide evidence that the insect Mpβ1 subunit (from the aphid *Myzus persicae*) can co-assemble with both the Mpα1 and Mpα2 subunits, but not with other *M. persicae* α subunits (Huang et al., 2000). In addition, *Drosophila* nAChR Dα1, Dα2 and Dβ1 subunits have been shown to share a similar distribution in the nervous system (Jonas et al., 1994). In order to examine the ability of the *N. lugens* Nlβ1 subunit to co-assemble with other nAChR subunits, polyclonal antisera (Nb1-I) was generated against bacterial fusion proteins containing

the main cytoplasmic loop of Nlβ1 subunit. Detergent extracts of *N. lugens* head membranes were immunoprecipitated with Nb1-I and immunoprecipitated proteins were then analyzed by immunoblotting with Nb1-I (Fig. 2A), N1-I (Fig. 2C), N2-I (Fig. 2E), N3-I (Fig. 2G) or N8-I (Fig. 2I). In each case, a single specific band was detected (indicated by a single asterisk) that could be blocked by the fusion protein of Nlβ1 (Fig. 2 B, D, F, H and J). In addition, in experiments in which native nAChRs were immunoprecipitated with N1-I, N2-I, N3-I or N8-I, the Nb1-I antisera detected a single band corresponding to Nlβ1 (data not shown), demonstrating the absence of cross-reactivity of this antisera. This data concerning co-immunoprecipitation of Nlβ1, taken together with data demonstrating co-immunoprecipitation of Nlα1/Nlα2 (Fig. 1) and Nlα3/Nlα8 (Yixi et al., 2009), provides evidence for the co-assembly of
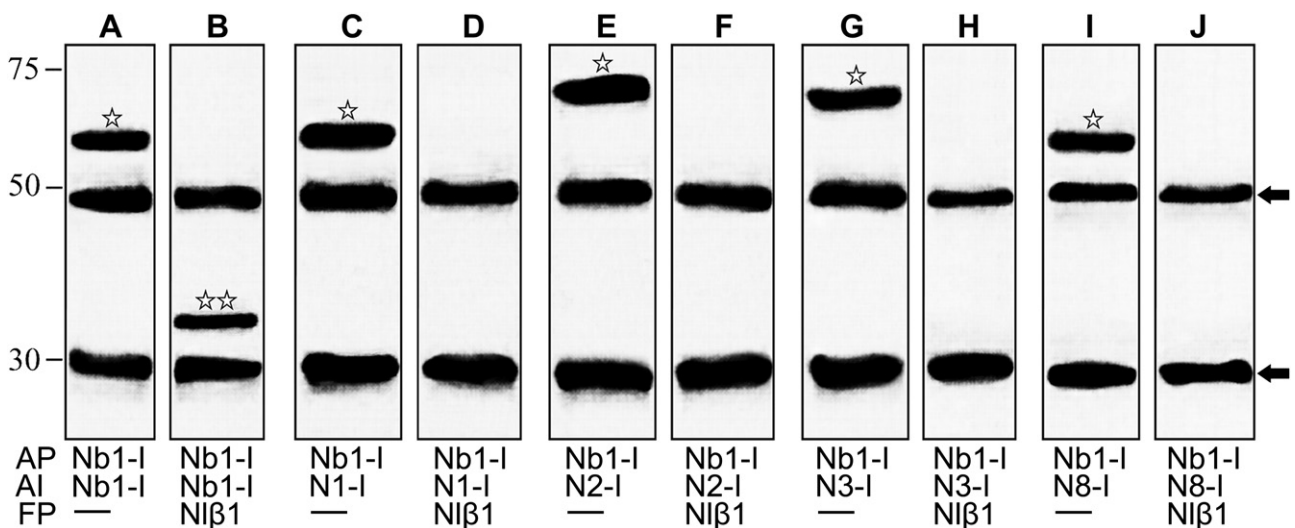

Fig. 2. Co-immunoprecipitation of Nlβ1 and four α subunits from native nAChRs. Detergent extracts of *N. lugens* head membranes were immunoprecipitated with Nb1-I and immunoprecipitated proteins were then analyzed by immunoblotting with Nb1-I (A), N1-I (C), N2-I (E), N3-I (G) or N8-I (I). In each case, a single specific band was detected (indicated by a single asterisk) that could be blocked by the fusion protein of Nlβ1 (B, D, F, H and J). A band corresponding to the fusion protein of Nlβ1 was detected, as expected (indicated by double asterisks in B). In all cases, the immunoglobulin heavy and light chains (from the antisera used for immunoprecipitation) were also detected (indicated by arrow heads). Antibody used in precipitation (AP) and antibody in immunoblotting (AI) were indicated under each lane. In each lane, fusion protein (FP) of Nlβ1 was used for competition (also indicated under each lane).
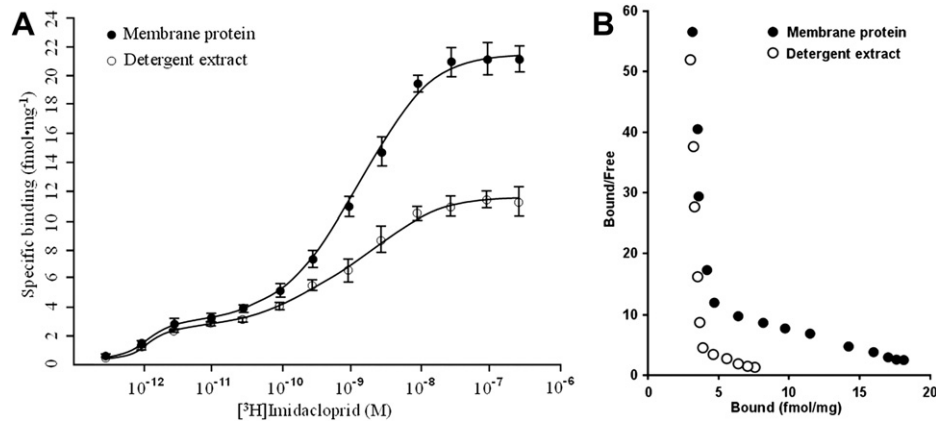
Fig. 3. Radioligand binding on *N. lugens* native nAChRs. (A) Equilibrium saturation binding of [3H]imidacloprid with membrane preparations and with detergent extracts from *N. lugens*. Data points are means of at least three independent experiments. (B) Scatchard plot of data presented in A. In all cases, representative plots are shown that are typical of at least three independent experiments.

Nlα1, Nlα2 and Nlβ1 in one native receptor and of Nlα3, Nlα8 and Nlβ1 in another native nAChR.

3.2. Radioligand binding to native nAChRs in N. lugens

[3H]imidacloprid binding was performed with both native membrane preparations and with detergent extracts of *N. lugens*. High-affinity specific binding of [3H]imidacloprid was detected in *N. lugens* membrane preparations (Fig. 3). Saturation radioligand binding studies revealed two high-affinity binding sites for imidacloprid ($K_d = 3.5 \pm 0.6$ pM and 1.5 ± 0.2 nM; Fig. 3A). The presence of two distinct binding sites is well illustrated by the non-linear nature of the data when analyzed by a Scatchard plot (Fig. 3B). These results are in agreement to our previous findings (Liu et al., 2005), and with previous reports demonstrating that imidacloprid binds with relatively high affinity to two sites ($K_d = 4.0$ pM and 1.2 nM) in membrane preparations from the leafhopper *Nephotettix cincticeps* (Lind et al., 1998). In detergent extracts of *N. lugens* head membranes, two imidacloprid binding sites with different affinities were also observed (Fig. 3A), as illustrated by a Scatchard plot (Fig. 3B). The K_d values for the two imidacloprid binding sites estimated from membrane preparations and from detergent extracts were not significantly different (Table 1). However, a decrease in B_{max} for the lower affinity site was observed when comparing detergent extracts to membrane preparations (7.8 $\pm$ 1.5 fmol/mg and 18.3 $\pm$ 2.3 fmol/mg protein, respectively). It is possible that this may reflect either a reduced stability of the receptor when solubilised in detergent or reduced retention during sample harvesting onto glass fiber filters. However, the similarity in binding affinities detected in the presence and absence of detergent suggests that detergent solubilisation is not causing significant alteration of the imidacloprid binding site.

3.3. Radioligand binding to detergent extracts after immunodepletion

Previous studies of recombinant nAChRs expressed in *Xenopus* oocytes (in which functional expression has been achieved only by co-expression of a mammalian β subunit) have identified two triplet nAChRs with distinct subunit compositions: Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 (Liu et al., 2009; Yixi et al., 2009). In order to investigate whether Nlβ1, together with these four *N. lugens* α subunits, constitute native imidacloprid binding sites in *N. lugens*, [3H]imidacloprid binding was performed with detergent extracts after immunodepletion by subunit-specific antisera.

Receptor depletion by immunoprecipitation with subunit-specific antisera caused a dose-dependent reduction in [3H]imidacloprid binding (Fig. 4). Immunodepletion by antisera specific for either Nlα1 or Nlα2 (N1-I or N2-I) reduced specific [3H]imidacloprid

Table 1
Summary of [3H]imidacloprid binding on *N. lugens* membranes and detergent extracts.

	Site 1		Site 2	
	K_d (pM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
Whole body membrane	3.5 ± 0.6	4.1 ± 0.6	1.5 ± 0.2	18.3 ± 2.3
Detergent extract	3.3 ± 0.8	3.8 ± 0.5	1.4 ± 0.3	7.8 ± 1.5
Supernatant after N1-I immunodepletion	3.4 ± 0.7	2.1 ± 0.4	–	–
Supernatant after N2-I immunodepletion	3.2 ± 0.9	2.4 ± 0.3	–	–
Supernatant after N3-I immunodepletion	–	–	1.5 ± 0.4	5.1 ± 1.1
Supernatant after N8-I immunodepletion	–	–	1.4 ± 0.4	4.4 ± 0.7
Supernatant after Nb1-I immunodepletion	–	–	–	–

The amounts of N1-I, N2-I, N3-I and N8-I are 6 mg and the amount of Nb1-I is 7 mg (see Fig. 4).
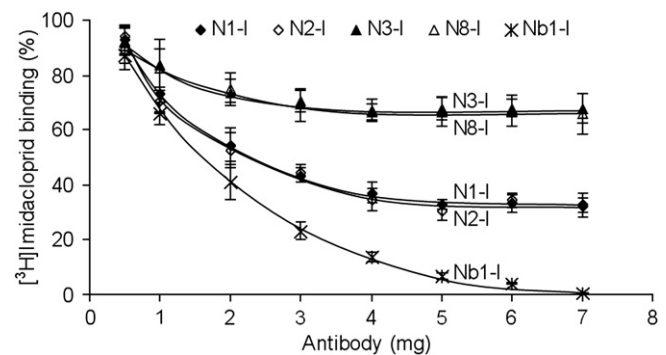

Fig. 4. Immunoprecipitation of [3H]imidacloprid binding sites by subunits specific antisera. [3H]imidacloprid binding on detergent extracts of *N. lugens* membrane proteins was performed after immunodepletion by increasing amounts of subunit-specific antisera. Data points are means of at least three independent experiments.
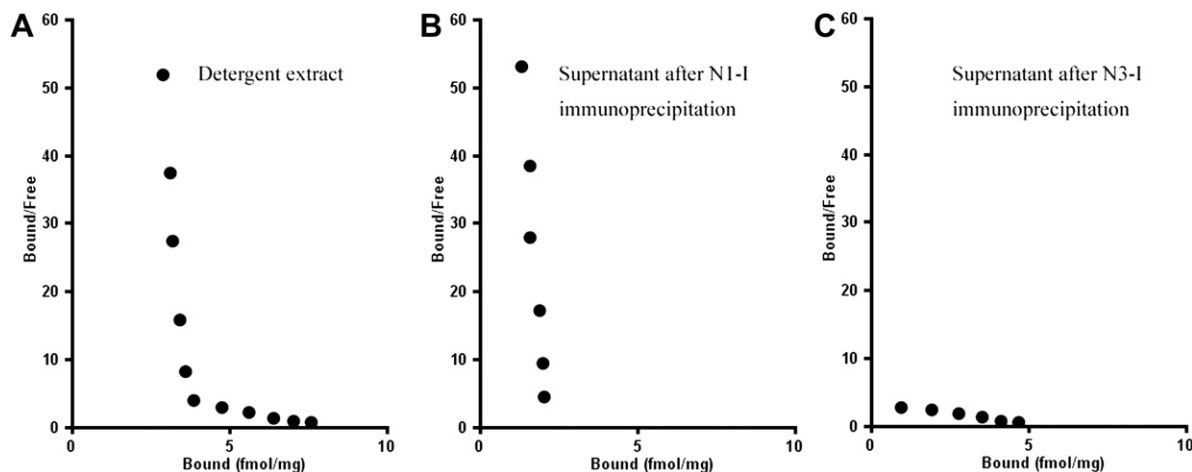
Fig. 5. Scatchard plot of equilibrium saturation [³H]imidacloprid binding data on detergent extracts. (A) Binding on detergent extracts without prior immunodepletion. (B) Binding on detergent extracts after immunodepletion by Nlα1-specific antisera N1-I (corresponding to 6 mg in Fig. 4). (C) Binding on detergent extracts after immunodepletion by Nlα3-specific antisera N3-I (corresponding to 6 mg in Fig. 4). In all cases, representative plots are shown that are typical of at least three independent experiments.

binding by approximately 67% (66.3 ± 7.3% for N1-I and 67.6 ± 5.9% for N2-I). Immunodepletion with antisera specific for either Nlα3 or Nlα8 (N3-I or N8-I) reduced specific [³H]imidacloprid binding by approximately 33% (32.5 ± 4.1% for N3-I and 33.3 ± 3.6% for N8-I). Data presented in a Scatchard plot (Fig. 5) illustrates that only the higher affinity imidacloprid binding site (3.4 ± 0.7 pM) remains in detergent extracts immunodepleted by N1-I (Fig. 5B), and only the lower affinity binding site (1.5 ± 0.4 nM) remains in detergent extracts immunodepleted by N3-I (Fig. 5C). In detergent extracts of *N. lugens* head membranes, immunodepletion by antisera selective for Nlβ1 (Nb1-I) completely abolished specific [³H]imidacloprid binding (Fig. 4). Immunodepletion with antisera specific for Nlα4, Nlα6, Nlα7 or Nlβ2 (N4-I, N6-I, N7-I or Nb2-I) had no significant effect on specific [³H]imidacloprid binding.

4. Discussion

Neonicotinoid insecticides, such as imidacloprid, are selective agonists of insect nicotinic acetylcholine receptors (nAChRs) and are used extensively in crop protection and animal health to control a variety of insect pest species. Since the introduction of imidacloprid as a commercial insecticide in 1990, six other neonicotinoid compounds have been approved for use as insecticides. These include nitenpyram (in 1995), acetamiprid (in 1996), thiamethoxam (in 1998), thiacloprid (in 2000), clothianidin (in 2002) and dinotefuran (in 2002).

Two binding sites for imidacloprid have been identified in insects such as the aphid *M. persicae*, the leafhopper *N. cincticeps* and the planthopper *N. lugens*. In contrast, only a single imidacloprid binding site has been identified in several other insect species, including the cockroach *Periplaneta americana*, the green bottle fly *Lucilia sericata*, the furit fly *D. melanogaster*, the tobacco hornworm *Manduca sexta*, the tobacco budworm *Heliothis virescens*, and the cat flea *Ctenocephalides felis* (Lind et al., 1998; Tomizawa et al., 2005; Liu et al., 2005). These studies demonstrate that, whereas all insect species examined have a relatively high-affinity binding site for imidacloprid (with a K_d value close to 1 nM), only hemipteran insects have a very high-affinity binding site for imidacloprid (with a K_d values close to or less than 0.1 nM) (Lind et al., 1998; Liu et al., 2005). The presence of very high-affinity imidacloprid binding sites in hemipteran insects may explain why imidacloprid is particularly useful in controlling insect pests from the insect order Hemiptera, such as planthoppers, aphids and leafhoppers.

Despite considerable interest in this topic, little is known about the subunit composition of neonicotinoid-selective nAChRs. Immunoprecipitation studies conducted with native nAChRs have provided some information concerning the ability of different insect subunits to co-assemble with one another. This has included information concerning native nAChRs expressed in the fruit fly *D. melanogaster* (Chamaon et al., 2000, 2002; Schulz et al., 2000) and in *N. lugens* (Yixi et al., 2009), but has not provided information concerning the selectivity of such complexes for neonicotinoid insecticides.

In the extensively studied model insect species *D. melanogaster*, for which the complete genome sequence is known, all candidate nAChR subunits have been identified and cloned. However, despite this, the subunit composition of pharmacologically distinct nAChRs has not yet been completely established. In part, this is a result of difficulties in generating functional insect nAChRs in heterologous expression systems (Millar, 1999; Lansdell et al., 2008). Despite these problems, expression of functional recombinant nAChRs has been reported for several insect nAChR subunits when co-expressed with vertebrate nAChR subunits such as the rat β2 subunit (Millar and Lansdell, in press). This has permitted the functional characterization of hybrid nAChRs such as Nlα1/β2 (Liu et al., 2006) and the triplet subunit combination Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 (Liu et al., 2009; Yixi et al., 2009). By expression of hybrid nAChRs Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 in *Xenopus* oocytes (Liu et al., 2009; Yixi et al., 2009), it was found that imidacloprid has significantly different apparent affinities on hybrid Nlα3/Nlα8/β2 and on Nlα1/Nlα2/β2 nAChRs (EC₅₀ = 3.2 ± 0.5 and 350.4 ± 16.8 nM, respectively). Interestingly, the difference in these

Table 2
The co-immunoprecipitation among subunits in native *N. lugens* nAChRs.

	Nlα1	Nlα2	Nlα3	Nlα4	Nlα6	Nlα7	Nlα8	Nlβ1	Nlβ2
Nlα1		+	−	−	−	−	−	+	−
Nlα2	+		−	−	−	−	−	+	−
Nlα3	−	−		−	−	−	+	+	−
Nlα4	−	−	−		−	−	−	−	−
Nlα6	−	−	−	−		+	−	−	−
Nlα7	−	−	−	−	+		−	−	−
Nlα8	−	−	+	−	−	−		+	−
Nlβ1	+	+	+	−	−	−	+		−
Nlβ2	−	−	−	−	−	−	−	−	

+, Co-immunoprecipitation; −, no co-immunoprecipitation.

EC$_{50}$ values (109-fold) is broadly similar to the difference in binding affinity of imidacloprid to the two sites in native *N. lugens* nAChRs (420-fold). In the present study, we have attempted to circumvent the problems associated with heterologous expression of insect nAChRs by examining native nAChRs from *N. lugens*. Immunoprecipitation studies using subunit-selective antibodies have provided evidence that all imidacloprid binding sites in *N. lugens* contain Nlβ1. In addition, evidence has been obtained indicating that four *N. lugens* nAChR α subunits (Nlα1, Nlα2, Nlα3 and Nlα8) also contribute to the formation of imidacloprid binding sites. Based on co-immunoprecipitation studies (as summarized in Table 2), we have concluded that Nlα1, Nlα2 and Nlβ1 co-assemble to form one nAChR subtype and Nlα3, Nlα8 and Nlβ1 co-assemble to form a second nAChR subtype in *N. lugens*. In addition, the combination of radioligand binding and immunoprecipitation indicated that the Nlα1/Nlα2/Nlβ1 and Nlα3/Nlα8/Nlβ1 subunit combinations correspond to the lower and higher affinity imidacloprid binding sites, respectively. Antibody-mediated receptor depletion experiments suggest that the lower affinity imidacloprid binding site (Nlα1/Nlα2/Nlβ1) represents about 67% of total binding sites, whereas the higher affinity site (Nlα3/Nlα8/Nlβ1) represents about 33%. These findings argue against there being a third population of nAChRs making a major contribution to high-affinity imidacloprid binding sites in *N. lugens*.

In conclusion, we have obtained evidence for the co-assembly of *N. lugens* nAChR subunits Nlα1, Nlα2 and Nlβ1 in one receptor complex, and subunits Nlα3, Nlα8 and Nlβ1 in another receptor complex. Furthermore, we conclude that these correspond to the lower and higher affinity imidacloprid binding sites in the economically important insect pest *N. lugens*.

Acknowledgements

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