A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper)

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Neonicotinoids, such as imidacloprid, are nicotinic acetylcholine receptor (nAChR) agonists with potent insecticidal activity. Since its introduction in the early 1990s, imidacloprid has become one of the most extensively used insecticides for both crop protection and animal health applications. As with other classes of insecticides, resistance to neonicotinoids is a significant threat and has been identified in several pest species, including the brown planthopper, *Nilaparvata lugens*, a major rice pest in many parts of Asia. In this study, radioligand binding experiments have been conducted with whole-body membranes prepared from imidacloprid-susceptible and imidacloprid-resistant strains of *N. lugens*. The results reveal a much higher level of [^{3}H]imidacloprid-specific binding to the susceptible strain than to the resistant strain (16.7 ± 1.0 and 0.34 ± 0.21 fmol/mg of protein, respectively). With the aim of understanding the molecular basis of imidacloprid resistance, five nAChR subunits (Nlα1–Nlα4 and Nlβ1) have been cloned from *N. lugens*. A comparison of nAChR subunit genes from imidacloprid-sensitive and imidacloprid-resistant populations has identified a single point mutation at a conserved position (Y151S) in two nAChR subunits, Nlα1 and Nlα3. A strong correlation between the frequency of the Y151S point mutation and the level of resistance to imidacloprid has been demonstrated by allele-specific PCR. By expression of hybrid nAChRs containing *N. lugens* α and rat β2 subunits, evidence was obtained that demonstrates that mutation Y151S is responsible for a substantial reduction in specific [^{3}H]imidacloprid binding. This study provides direct evidence for the occurrence of target-site resistance to a neonicotinoid insecticide.

crop protection | insecticide resistance | nicotinic receptor

Imidacloprid is a nicotinic acetylcholine receptor (nAChR) agonist with potent insecticidal activity. It was the first member of the neonicotinoid class of insecticides to be commercialized (in 1991) and is used extensively for both crop protection and animal health applications. Indeed, neonicotinoid insecticides are now estimated to have annual worldwide sales of ≈$1 billion (1). Although nAChRs are important excitatory, neurotransmitter-gated ion channels in both vertebrates and invertebrates, the selective toxicity of neonicotinoids for insects has been attributed, at least in part, to their high affinity for insect receptors (1–3). Nicotinic receptors are pentameric transmembrane complexes assembled from a diverse family of subunit subtypes (4, 5). In the model insect species *Drosophila melanogaster*, 10 nAChR subunits have been identified by molecular cloning (5). An important question, which is now being examined by studies of both native and recombinant nAChRs (5–7), is the contribution of particular nAChR subunits to the pharmacological and physiological properties of this diverse family of receptors.

Insecticide resistance is a major worldwide problem for the effective control of insect pests. Resistance has emerged in field populations of insects to all major insecticide classes, including organophosphates, carbamates, pyrethroids, and neonicotinoids (8–10). Since the introduction of imidacloprid, evidence of resistance has been slow to emerge but now involves a number of important insect pests (10). In the best-studied example of neonicotinoid resistance, involving the whitefly *Bemisia tabaci*, resistance is attributable to enhanced oxidative detoxification of neonicotinoids by overexpressed monooxygenases, rather than to target-site changes in nAChRs (11). For other major insecticide classes, both target-site modifications and enhanced detoxification have been identified as being important resistance mechanisms (12). Examples of target-site changes include a highly conserved mutation in the insect $GABA_A$ receptor subunit (rdl) that confers resistance to dieldrin and other cyclodienes in a range of insects (13, 14), mutations in the insect Na^+ channel that confer resistance to pyrethroid and diphenylethane (e.g., DDT) insecticides (9, 15, 16), and mutations in acetylcholinesterase, the target site of organophosphate and carbamate insecticides (17, 18). To date, however, there is no direct evidence of target-site resistance to neonicotinoids occurring in nAChR subunits.

Recently, evidence has been obtained for resistance to imidacloprid in the brown planthopper *Nilaparvata lugens*, a major rice pest (19). The lack of cross-resistance to other insecticide classes and the lack of marked synergism by inhibitors of detoxifying enzymes suggested that resistance might be due to a mutation within the target site of imidacloprid rather than due to enhanced detoxification (19). To examine this possibility, five nAChR subunits (Nlα1–Nlα4 and Nlβ1) were cloned from *N. lugens.* By comparison of nAChR subunit sequences from imidacloprid-susceptible and resistant *N. lugens* strains, a mutation was identified in two nAChR α subunits at a position close to the predicted agonist binding site. To examine the influence of the mutation on nicotinic agonist binding, cloned *N. lugens* nAChR subunit cDNAs were expressed in a cultured *Drosophila* cell line (as hybrid receptors with the rat nAChR β2 subunit). These studies have provided clear evidence for the resistance-associated mutation playing a direct role in neonicotinoid insecticide binding.

Materials and Methods

Brown Planthopper (*N. lugens*). The susceptible (S) strain of *N. lugens* was a laboratory strain, obtained from Jiangsu Academy of Agricultural Sciences (Nanjing, China) in April 2000, which had been collected before imidacloprid application and reared in a greenhouse for >10 years. The imidacloprid-resistant (R) strains were laboratory strains selected from a field population originally collected from hybrid paddy rice in Jiangpu, Jiangsu, China, in August 2000. Selection of imidacloprid-resistant *N.*

Abbreviations: AChBP, acetylcholine binding protein; nAChR, nicotinic acetylcholine receptor; HA, hemagglutinin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY378698–AY378700, AY378702, and AY378703).

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lugens with a resistance ratio of 73-fold after 25 generations (T25) was described in ref. 19. After further selection, the resistance ratio after 35 generations (T35) had increased to 250-fold (20). All test insects were reared in the laboratory at 25 ± 1°C and 16-h light/8-h dark photoperiod.

Extraction of the Membrane Protein from Planthoppers. Membranes were prepared from *N. lugens* by using methods described in ref. 21. *N. lugens* (3-day-old female; 2 mg) were homogenized in 2 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 1,000 × *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 the 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.

Oligonucleotide Primers. Degenerate primers, BP1 (ATT/C/A ATG ACN ACN AAT/C GTN TGG), BP2 (ATG AAG/A TTC/T GGN TCN TGG AC), and BP3 (ACI GTG/A TAG/A AAN AG/AN GTC/T TT), were designed from the conserved N-terminal regions of insect nAChR subunits (22). Gene-specific primers were based on the nucleotide sequences of individual genes.

RT-PCR and Rapid Amplification of cDNA Ends (RACE). Total RNA was isolated from adult females by TRIzol kit (Invitrogen). Synthesis of first-strand cDNAs and RT-PCR were performed as described in ref. 16. RACE was carried out by using the Smart Race cDNA Amplification kit (Clontech) according to the manufacturer's instructions.

Cloning and Sequencing. PCR fragments were recovered from agarose gels by using the Wizard PCR Preps DNA gel extraction kit (Promega) and then cloned into the pGEM-T vector (Promega), and plasmid DNAs were prepared by using the Plasmid Purification kit (Promega). Plasmid DNAs (200–500 ng) and PCR fragments (30–90 ng) were used as templates for Taq Dye terminator cycle sequencing reactions (PE Biosystems, Foster City, CA). To verify the genotypes of individual susceptible and resistant planthoppers, genomic DNA was extracted from single insects and short (≈200-bp) fragments were amplified by PCR. Amplified fragments were cloned, and three to four independent cDNA clones were sequenced from each individual.

Allele-Specific PCR. The genotype of susceptible and resistant planthoppers was examined by using the bidirectional amplification of specific alleles method (23). Primers were designed to permit detection of the Y151S mutation in the gene encoding the Nlα1 subunit. PCR amplification was performed on genomic DNA from single insects with the following oligonucleotide primers: P (ACA CGT CCC CAG TGA GCA), Q (GTC GGT GGA ATG ATC TGT GC), A (ggg ggg ggc cGT TTG GAT CCT GTA CAT C), and B (ggg ggg ggc gCA TGA TTG CCG TCG T), by using methods described in ref. 23. PCR was performed for 35 cycles of 30 sec at 94°C, 30 sec at 58°C, and 60 sec at 72°C. Amplified bands were detected by agarose gel electrophoresis and comprised (*i*) a 900-bp "common" fragment from all individuals (primers P and Q), (*ii*) a 540-bp fragment amplified from individuals with the wild-type (Y151) allele (primers P and B), and (*iii*) a 370-bp fragment from individuals with the mutant (S151) allele (primers Q and A). The presence/absence of the 540- and 370-bp fragments allowed all insects to be genotyped rapidly as wild-type homozygote (540 bp), mutant homozygote (370 bp), or heterozygote (540 bp plus 370 bp) for this allele.

Epitope Tagging. To facilitate protein detection by Western blotting, a recombinant epitope tag was introduced into Nlα1 and Nlα1^{Y151S} cDNAs. In both cases, the nine amino acid influenza hemagglutinin (HA) epitope (YPYDVPDYA) was introduced within the predicted large (M3–M4) intracellular loop region to create plasmids pRmHa3-Nlα1^{HA} and pRmHa3-Nlα1$^{Y151S\text{-}HA}$. Mutagenesis was performed by using the QuikChange method (Stratagene), and all mutated cDNAs were verified by nucleotide sequencing.

Heterologous Expression in *Drosophila* S2 Cells. *N. lugens* nAChR subunit cDNAs were subcloned into plasmid expression vector pRmHa3, which contains an inducible metallothionein promoter (24). Because the Nlα1 and Nlα4 subunit cDNAs were incomplete at their 5′ ends, chimeras of these subunits were constructed in pRmHa3 in which the signal peptide and 16 amino acids at the N terminus of the predicted mature protein were replaced by the corresponding region of the *Drosophila* Dα2/SAD subunit. Schneider's *Drosophila* S2 cells were maintained and transfected by a modified calcium phosphate method, as described in refs. 25 and 26. Cells were transfected with plasmid pRmHa3 containing appropriate nAChR subunit cDNAs. Expression of nAChR subunit cDNAs from the metallothionein promoter of pRmHa3 was induced by the addition of $CuSO_4$ (0.6 mM) for 24 h.

Radioligand Binding. [^{3}H]Epibatidine [48 Ci (1 Ci = 37 GBq)/mmol)] was purchased from NEN. [^{3}H]Imidacloprid (32 Ci/mmol) was generously provided by Bayer HealthCare, Monheim, Germany. Radioligand binding to membrane preparations and transiently transfected S2 cells was described in ref. 27. Samples were assayed by filtration onto Whatman GF/B filters presoaked in 0.5% polyethylenimine, followed by rapid washing by using a cell harvester (Brandel, Bethesda, MD). Amounts of total protein were determined by a Bio-Rad DC protein assay using BSA standards.

Immunoblotting. To confirm the expression of Nlα1 and Nlα1^{Y151S} nAChR subunits, HA-tagged subunit cDNAs (Nlα1^{HA} and Nlα1$^{Y151S\text{-}HA}$) were expressed by transient transfection in *Drosophila* S2 cells. Immunoblotting was performed as described in refs. 28 and 29. Total cellular protein (200 μg) from transfected S2 cells was separated by SDS/PAGE and then electroblotted onto Hybond-C nitrocellulose membranes (Amersham Pharmacia). Membranes were blocked and then incubated with 1:1,200 dilution of mAbHA-7 (Sigma) for 1 h at room temperature. The nitrocellulose membrane was washed thoroughly, incubated with 1:1,200 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce), and processed by using the enhanced chemiluminescence detection system (Amersham Pharmacia).

Results

Radioligand Binding to nAChRs from Imidacloprid-Susceptible and Imidacloprid-Resistant *N. lugens*. High-affinity specific binding of [^{3}H]imidacloprid was detected in whole-body membrane preparations from an imidacloprid-susceptible strain of the brown planthopper *N. lugens* (Fig. 1*A*), as was reported for other insect species in refs. 21 and 30–33. Initial saturation radioligand-binding studies revealed two high-affinity binding sites for imidacloprid (K_d < 0.01 nM and 1.5 nM; Fig. 1) to native nAChRs from *N. lugens*. These findings are 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* (21). Further binding studies were performed with membranes isolated from imidacloprid-susceptible (S) and imidacloprid-resistant (R-T35) strains of *N. lugens* (20). Much higher levels of

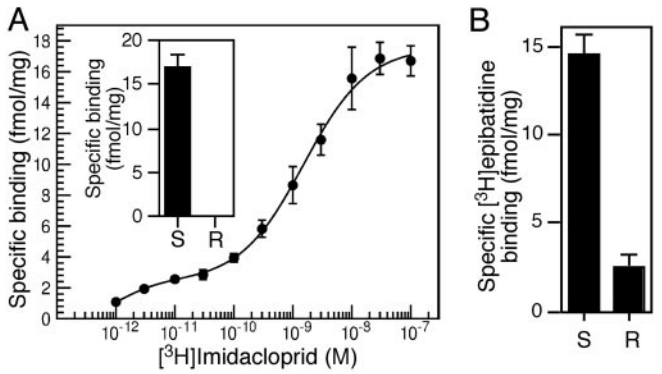

Fig. 1. Radioligand binding to native *N. lugens* nAChRs. (*A*) Equilibrium saturation binding was performed with [^{3}H]imidacloprid on whole-body membranes prepared from imidacloprid-susceptible *N. lugens*. Data points are means of triplicate samples. (*Inset*) Summary of binding data obtained with susceptible (S) and resistant (R) *N. lugens*. Specific binding of [^{3}H]imidacloprid was detected in membranes isolated from the susceptible strain. In contrast, only very low levels of [^{3}H]imidacloprid binding could be detected to the membranes from the resistant strain. (*B*) Binding of [^{3}H]epibatidine to imidacloprid-susceptible and imidacloprid-resistant *N. lugens*. Specific binding of [^{3}H]epibatidine was detected in the susceptible strain, but significantly lower levels were detected in resistant insects.

specific [^{3}H]imidacloprid binding were detected in membranes isolated from the imidacloprid-susceptible strain (16.7 ± 1.0 fmol/mg of protein; $n = 3$; Fig. 1*A*) than in membranes isolated from the resistant strain (0.34 ± 0.21 fmol/mg of protein, $n = 3$; Fig. 1*A*). The low level of specific binding observed with the resistant strain precluded an accurate estimate of binding affinity. These observations provide strong evidence to suggest that resistance to imidacloprid in *N. lugens* is a consequence of a modification to the target site. To examine the ability of susceptible and resistant insects to bind other nicotinic agonists, radioligand binding was performed with [^{3}H]epibatidine. As had been observed with [^{3}H]imidacloprid, higher levels of specific epibatidine binding were detected in the imidacloprid-susceptible strain (14.3 ± 1.2 fmol/mg of protein; $n = 3$; Fig. 1*B*) than in resistant insects (2.6 ± 0.6 fmol/mg of protein; $n = 3$; Fig. 1*B*).

Molecular Cloning of nAChR Subunits from *N. lugens*. Five different partial cDNA fragments were cloned from *N. lugens* by RT-PCR using degenerate primers designed to previously cloned insect nAChR subunit genes. These fragments were extended 5′ and 3′ by using RACE techniques to yield three full-length cDNAs and two almost-full-length cDNAs that lacked signal peptides and a short sequence of the N-terminal coding region. An alignment of the deduced protein sequences of these genes is shown in Fig. 2.

The cloned genes contained a single long ORF, the amino acid sequence of which showed clear sequence similarity to previously cloned nAChR subunits (Fig. 2) and features characteristic of the nAChR gene family (34). All sequences contained four hydrophobic putative transmembrane domains and a pair of cysteine residues [at positions 128 and 142; numbered according to the *Torpedo* α1 subunit (35)], believed to form a 15-aa disulfide-linked loop characteristic of ligand-gated ion channels. Based on their relative sequence similarity to nAChR subunits from *Drosophila*, the putative planthopper nAChR subunits were named as Nlα1 (73% similarity to Dα1/ALS), Nlα2 (63% similarity to Dα2/SAD), Nlα3 (56% similarity to Dα3), Nlα4 (32% similarity to Dα4), and Nlβ1 (63% similarity to Dβ1/ARD). Nlα4 has low sequence similarity to all known *Drosophila* subunits and may not, therefore, be the true orthologue of Dα4. An unusual feature of the Nlβ1 subunit is the presence of charged residues within its predicted fourth transmembrane (M4) domain (Fig. 2). It is possible that this unusual M4 domain is a consequence of alternative splicing; however, two independently isolated cDNA clones, which differed in the presence and absence of an N-terminal exon (accession nos. AY378703 and AY378704), had identical sequences in their M4 domain.

Identification of Potential Resistance-Related Mutations. Comparison of cDNA sequences of the four α subunits cloned from susceptible (S) and resistant (R) strains revealed several amino acid polymorphisms. However, only one of these polymorphisms (in both Nlα1 and Nlα3) was found to be associated with imidacloprid resistance. This polymorphism corresponds to the replacement of a tyrosine (Y) residue with a serine (S) residue at a position equivalent to Y151 in mature *Torpedo* nAChR α1 subunit (see Fig. 2). Although the amino acid numbering of Nlα1 and Nlα3 differs slightly from that of the *Torpedo* α1 subunit, this residue is referred here to Y151, to assist in comparisons with studies of other nAChR subunits. To examine the correlation between these mutations and resistance, the genomic sequence encoding this region of the Nlα1 and Nlα3 subunits was amplified by PCR. Twenty-two imidacloprid-susceptible (S) and 27 imidacloprid-resistant (R-T35) planthoppers were examined (see *Materials and Methods*). All susceptible individuals had tyrosine at this site in both Nlα1 and Nlα3, whereas all resistant individuals contained a serine at this position in both Nlα1 and Nlα3. The bidirectional amplification of specific alleles method (23) was used to further characterize the genotype of susceptible and resistant planthoppers at position Y151 in the gene encoding Nlα1 (Table 1). At generation 25, when resistance to imidacloprid was 73-fold (19), 59 of 70 individuals tested (84%) were heterozygous for Y151S, and the remaining 11 (16%) were homozygous for the mutation. By generation 35, when resistance had reached 250-fold (20), all insects tested were homozygous for the mutation.

Recombinant nAChRs Expressed in *Drosophila* S2 Cells. To facilitate heterologous expression in *Drosophila* S2 cells, *N. lugens* nAChR subunit cDNAs were subcloned into the *Drosophila* expression vector pRmHa3. As previously described in refs. 22 and 26, initial characterization of recombinant nAChRs containing insect and vertebrate (rat) nAChR subunits was conducted with [^{3}H]epibatidine. As has been found with nAChR subunits cloned from other insect species (22, 26, 36), no specific radioligand binding could be detected with any combination of *N. lugens* nAChR subunits. Despite difficulties in heterologous expression of insect nAChR subunits, a strategy that has proved successful with several insect species is to coexpress insect nAChR α subunits with a vertebrate non-α subunit such as rat β2 (26, 27, 37). Each of the four *N. lugens* α subunits (Nlα1–Nlα4) was coexpressed with the rat β2 subunit by transient transfection in *Drosophila* S2 cells. No specific binding of [^{3}H]epibatidine or of other nicotinic radioligands (such as [^{3}H]methyllycaconitine) could be detected in cells transfected with Nlα3 plus β2 or Nlα4 plus β2, but specific binding of [^{3}H]epibatidine was detected in cells transfected with Nlα1 plus β2 and Nlα2 plus β2.

To examine the influence of the Y151S mutation upon nicotinic radioligand binding, *Drosophila* S2 cells were cotransfected with Nlα1 plus β2 or Nlα1^{Y151S} plus β2 subunit combinations. Equilibrium saturation binding experiments were performed to determine B_{max} and K_d values for binding of [^{3}H]epibatidine. Specific binding of [^{3}H]epibatidine was detected in cells transfected with Nlα1 plus β2 ($B_{max} = 64.5 \pm 0.8$ fmol/mg of protein; $n = 3$; Fig. 3) and was of high affinity ($K_d = 0.32 \pm 0.04$ nM, $n = 3$). Specific binding of [^{3}H]epibatidine also was detected in cells transfected with Nlα1^{Y151S} plus β2, but at significantly lower levels ($B_{max} = 2.0 \pm 0.04$ fmol/mg of protein,

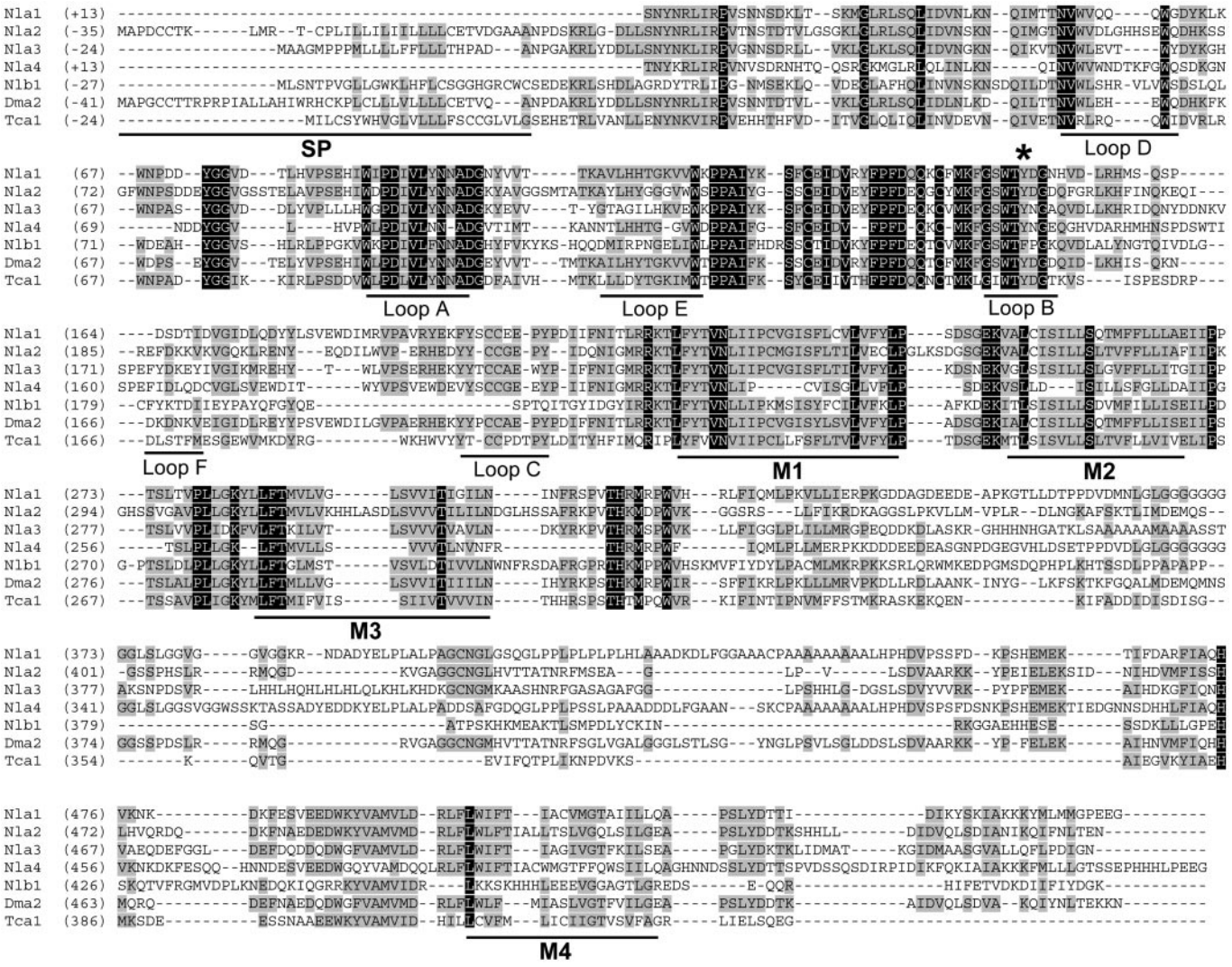

Fig. 2. Amino acid sequence alignments of *N. lugens* nAChR subunits. Also shown are the sequences of the *Drosophila* Dα2/SAD subunit and the *Torpedo* α1 subunit. Predicted signal peptide (SP) and transmembrane domains (M1–M4) are underlined. The location of six domains (loops A–F) believed to be important in forming the agonist/antagonist binding site also are indicated. Of these, three (loops A–C) are contributed by the α subunit interface and three (loops D–F) by the non-α interface in a heteromeric nAChR. The location of the mutation identified in this study (equivalent to Y151 of the *Torpedo* α1 subunit) is indicated by an asterisk.

$n = 3$; Fig. 3). This decrease corresponds to about a 32-fold lower level of specific binding than was detected with Nlα1 plus β2. Due to the low levels of specific binding detected with the Nl$\alpha1^{Y151S}$ plus β2 subunit combination, it was difficult to determine the affinity of binding accurately, which nonetheless did not appear to be significantly different from that observed with Nlα1 plus β2.

Table 1. Nl$\alpha1^{Y151}$ genotype frequency in *N. lugens*

Population	*n*	% homozygous wild type	% heterozygous	% homozygous mutant
S	60	100	0	0
R (T25)	70	0	84	16
R (T35)	79	0	0	100

Nl$\alpha1^{Y151}$ genotype frequency was examined by allele-specific PCR in three populations of *N. lugens*: the imidacloprid-susceptible population (S) and two resistant (R) populations. The resistant population isolated after 25 generations of selection with imidacloprid (T25) had a resistance ratio of 73 (19), whereas the population isolated after 35 generations (T35) had a resistance ratio of 250 (20).

Competition binding experiments were carried out to examine the influence of the Y151S mutation upon imidacloprid binding (Fig. 3). In cells transfected with Nlα1 plus β2, imidacloprid displaced [^{3}H]epibatidine binding with high affinity ($K_i = 24.3 \pm 2.3$ nM, $n = 3$; Fig. 3). Imidacloprid did not cause significant displacement of the low level of [^{3}H]epibatidine binding detected in cells transfected with Nl$\alpha1^{Y151S}$ plus β2, even at concentrations up to 10 μM (data not shown). To examine more directly the influence of the Y151S mutation upon imidacloprid binding, the studies were performed with [^{3}H]imidacloprid. In cells transfected with Nlα1 plus β2, specific binding of [^{3}H]imidacloprid was detected ($B_{max} = 39.9 \pm 8.4$ fmol/mg of protein; $n = 3$; Fig. 4*A*). In contrast, no significant specific binding of [^{3}H]imidacloprid was detected in cells transfected with Nl$\alpha1^{Y151S}$ plus β2 ($B_{max} = 0.4 \pm 1.2$ fmol/mg of protein; $n = 3$; Fig. 4*A*).

Detection of Nl$\alpha1^{HA}$ and Nl$\alpha1^{Y151S\text{-}HA}$ by Immunoblotting. Levels of expressed Nlα1 and Nl$\alpha1^{Y151S}$ subunit protein in transfected

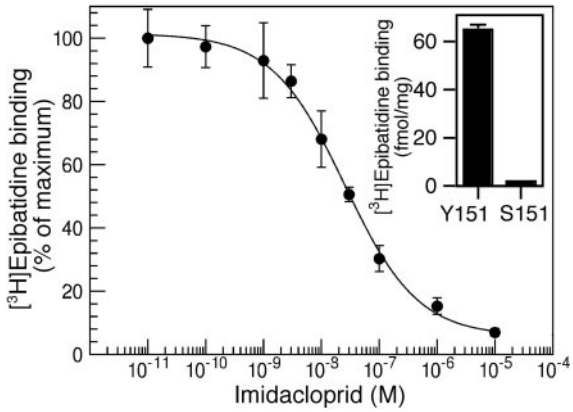

Fig. 3. Characterization of hybrid (Nlα1 plus rat β2 subunit) nAChRs expressed in *Drosophila* S2 cells. Specific binding of [^{3}H]epibatidine was detected in cells transfected with Nlα1 plus β2 subunits but at significantly lower levels in cells transfected with Nl$\alpha1^{Y151S}$ plus β2 (*Inset*). Competition binding with imidacloprid in cells transfected with Nlα1 and rat β2 subunits. Imidacloprid displaced binding of [^{3}H]epibatidine with high affinity (K_i = 24.3 ± 2.3 nM). Data points are means of triplicate samples from a single experiment and are typical of results obtain from three independent experiments.

Drosophila S2 cells were examined by immunoblotting with HA-tagged subunit cDNA constructs. The rationale was to determine whether the low level of radioligand binding could be attributed simply to low levels of expression of the Nl$\alpha1^{Y151S}$ subunit. HA-tagged subunit cDNAs (Nl$\alpha1^{HA}$ and Nl$\alpha1^{Y151S\text{-}HA}$) were transfected into *Drosophila* S2 cells, and total cellular proteins were separated by SDS/PAGE. Samples were transferred to nitrocellulose filters and examined by immunoblotting with a monoclonal antibody that recognizes the HA epitope (mAbHA-7). A band of the expected molecular mass (≈66 kDa) was detected in cells transfected with Nl$\alpha1^{HA}$ and Nl$\alpha1^{Y151S\text{-}HA}$ but was absent from mock-transfected S2 cells (Fig. 4*B*). The bands detected in cells transfected with Nl$\alpha1^{HA}$ and Nl$\alpha1^{Y151S\text{-}HA}$ were of similar intensity, suggesting that the Y151S mutation does not significantly alter levels of total subunit protein.

Discussion

Imidacloprid, like other systemic insecticides, displays marked residual persistence, which can generate prolonged selection pressure for resistance (38). Resistance to imidacloprid has been reported in a range of species including western flower thrips, Colorado potato beetle, German cockroach, and house fly (39, 40). In silverleaf whitefly and green peach aphid, high resistance was obtained by continuous selection in the greenhouse (41, 42). Previous studies from this laboratory also have shown that the brown planthopper (*N. lugens*) developed high resistance to imidacloprid after continuous selection (19, 20). In some species with high resistance to imidacloprid, piperonyl butoxide had very strong synergistic effects on imidacloprid, suggesting that P450 monooxygenases may play important roles in imidacloprid resistance (41, 42). Our previous studies of resistance to imidacloprid in *N. lugens* (19) revealed significant cross-resistance only to the insecticides that target nAChRs, suggesting that target insensitivity might play an important role in imidacloprid resistance.

A point mutation (Y151S), associated with resistance to imidacloprid in *N. lugens*, has been identified in two nAChR subunits Nlα1 and Nlα3. These subunits exhibit high sequence similarity to nAChR subunits from *Drosophila* (Dα1/ALS and Dα3), which have previously been shown to form a high-affinity binding site for imidacloprid (27). Heterologous expression studies with Nlα1 or Nl$\alpha1^{Y151S}$ have revealed that the Y151S mutation in Nlα1 is responsible for a loss of specific imidacloprid binding. Despite the absence of specific [^{3}H]imidacloprid binding in transfected *Drosophila* S2 cells, immunoblotting data confirm that the Y151S mutation has no significant effect on levels of total Nlα1 subunit protein expressed. Although the role of such mutations in conferring target-site resistance to insecticides is well documented, none of these reports relate to neonicotinoids. The association between Y151S and imidacloprid resistance shown by direct sequencing is supported further by work using allele-specific PCR to measure the frequency of Nlα1 genotypes in insects collected at two time periods during selection of the resistant strain. At generation 25, when resistance to imidacloprid was 73-fold, 84% were heterozygous for Y151S, and the remaining 16% were homozygous for the mutation. By generation 35, when resistance had reached 250-fold, all insects tested were homozygous for the mutation. This finding is consistent with the concentrations of imidacloprid used for selection, which were gradually increased as the experiment progressed (19, 20).

An interesting feature of this work is the discovery of the same mutation at homologous residues in two distinct nAChR α subunits. This discovery raises the intriguing question of whether both mutations are essential to generate a resistance phenotype, or whether a single mutated subunit is sufficient to confer at least some protection against imidacloprid. The configuration of subunits in native receptors is unclear at present and requires further investigation. Two possibilities are that either the subunits contribute to different populations of receptors, rendering each of these resistant, or they occur together in a heteromeric receptor. Interestingly, evidence for the existence of heteromeric nAChRs containing two different α-subunit subtypes has been reported in *Drosophila* (43). The need for insects to contain two mutated subunits to express resistance, if confirmed, has important evolutionary implications in terms of the expected frequency of resistant individuals in untreated populations and may help to explain the apparent scarcity of target-site resistance to these compounds, compared with enhanced detoxification (10).

Extensive photoaffinity labeling studies have been conducted with nAChRs purified from the electric organ of the marine ray *Torpedo*. This work, together with data derived from site-directed mutagenesis and structural studies, has led to a model in which the nAChR binding site lies at the interface of two adjacent subunits. This model proposes that three amino acids loops (loops A–C) are contributed by the "principal" subunit and three (loops D–F) by the "complementary" subunit (44), see

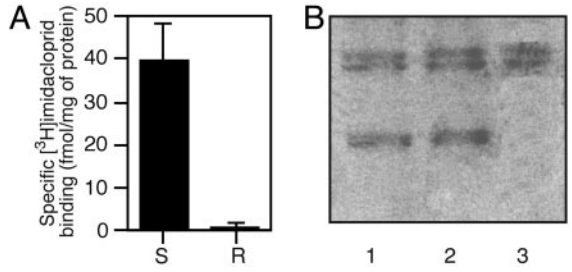

Fig. 4. Characterization of Nlα1 and Nl$\alpha1^{Y151S}$ subunits by radioligand binding and immunoblotting. (*A*) A saturating concentration of [^{3}H]imidacloprid (100 nM) was used to examine binding to transfected *Drosophila* S2 cells. In cells transfected with Nlα1 plus β2, specific binding of [^{3}H]imidacloprid was detected. In contrast, no significant specific binding of [^{3}H]imidacloprid was detected in cells transfected with Nl$\alpha1^{Y151S}$ plus β2. (*B*) Total cellular proteins (200 μg) from *Drosophila* S2 cells transfected with Nl$\alpha1^{Y151S\text{-}HA}$ (lane 1), Nl$\alpha1^{HA}$ (lane 2) or mock-transfected (lane 3) were separated by SDS/PAGE. After transfer to nitrocellulose, proteins were detected by immunoblotting with mAbHA-7. Specific immunoreactive bands of apparent molecular mass ≈66 kDa were detected corresponding to Nl$\alpha1^{Y151S\text{-}HA}$ and Nl$\alpha1^{HA}$ (lanes 1 and 2). Nonspecific bands of higher molecular mass are visible in all lanes.

Fig. 2. Photoaffinity labeling studies of *Torpedo* nAChRs have identified several aromatic residues believed to contribute to the nAChR binding site, including Y151 within loop B (45). Comparison of nAChR subunit sequences with that of the acetylcholine binding protein (AChBP) from the mollusc *Lymnaea stagnalis* (46) reveals significant sequence similarity. Residue Y151 in nAChR α subunits is at a position analogous to a histidine residue (H145) in the AChBP (46). X-ray diffraction studies of the *Lymnaea* AChBP reveal that H145 forms part of the AChBP agonist binding site (46). This finding, together with the photoaffinity labeling studies conducted with *Torpedo* nAChRs discussed above, supports the conclusion that Y151 is located at or close to the agonist binding site in nAChR subunits.

The data presented provide evidence that Y151 plays an important role in the binding of the neonicotinoid agonist imidacloprid to insect nAChRs. However, because Y151 is highly conserved between vertebrate and invertebrate nAChR subunits, this residue cannot be responsible for the excellent selectivity of neonicotinoids such as imidacloprid for insect nAChRs (1, 3). Whereas the binding of classical nicotinic agonists such as nicotine is thought to require cation–π interactions between the ammonium nitrogen and aromatic residues within the nAChR binding site (47), it has been proposed that the selectivity of neonicotinoids for insect nAChRs is due to interactions between an electronegative pharmacophore and a cationic subsite in the receptor (48, 49). Given the likely close proximity of Y151 to the nAChR agonist binding site (which, as discussed earlier, is supported by the atomic resolution structure of the *Lymnaea* AChBP), it seems plausible that the Y151S mutation might cause an induced conformational change within the nAChR binding site region that involves other amino acids, including those that are essential for the selective binding of the electronegative pharmacophore of neonicotinoid compounds.

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