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Nicotinic acetylcholine receptor β1 subunit from the brown planthopper, *Nilaparvata lugens*: A-to-I RNA editing and its possible roles in neonicotinoid sensitivity

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ABSTRACT

Nicotinic acetylcholine (ACh) receptors (nAChRs) are ligand-gated ion channels which mediate fast cholinergic synaptic transmission in insect and vertebrate nervous systems. The nAChR agonist-binding site is formed by loops A–C present in α subunits together with loops D–F present in either non-α subunits or homomer-forming α subunits. A new non-α subunit was cloned from *Nilaparvata lugens*, a major rice pest in many parts of Asia, showing very high amino acid identity to other insect β1 subunits, and was denoted as *N. lugens* β1 (Nlβ1). Six A-to-I RNA editing sites were found in Nlβ1 N-terminal domain, in which only one site was previously reported in *Drosophila melanogaster* Dβ1 and the other five were newly identified. Among the six editing sites, four caused amino acid changes, in which the site 2 (E2) and site 5 (E5) caused an N to D change in loop D (N73D) and loop E (N133D) respectively. E2 frequency was high in Sus (susceptible) strain and E5 frequency was high in Res (resistant) strain. By expressing in *Xenopus* oocytes, N73D editing was found to reduce the agonist potency of both ACh and imidacloprid, and the influence on ACh was more significant than on imidacloprid. By contrast, N133D editing only affected imidacloprid potency. These results indicated, although E2 and E5 editings both caused an N to D change in important loops, their roles in neonicotinoid insensitivity might be different.

1. Introduction

The nAChRs are ligand-gated ion channels mediating fast cholinergic synaptic transmission in insect and vertebrate nervous systems (Matsuda et al., 2001). Nicotinic receptors have long been recognized as potential targets for insecticidal compounds, and over the last 20 years 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 agricultural, veterinary and medical importance (Millar and Denholm, 2007). Neonicotinoid insecticides are insect-selective nicotinic acetylcholine receptor (nAChR) agonists that are used extensively in areas of crop protection and animal health (Matsuda et al., 2001; Tomizawa and Casida, 2005; Millar and Denholm, 2007). Neonicotinoids act selectively on insect nAChRs, accounting at least in part for the selective toxicity to insects over vertebrates (Matsuda et al., 2001, 2005; Tomizawa and Casida, 2005). A nitro or cyano group in neonicotinoids has been postulated to contribute directly to their selectivity (Matsuda et al., 2001; Tomizawa and Casida, 2005).

Numerous biochemical studies have provided extensive evidence for a structure model of the agonist site with contributing amino acids from three distinct regions of the α subunits (referred to as loops A–C) and from at least three regions of the non-α (β, γ or δ) subunits (loops D–F) (Prince and Sine, 1998; Arias, 2000; Corringer et al., 2000; Grutter and Changeux, 2001; Brejc et al., 2001). Loops A–F are present in insect nAChRs subunits, but it has proved to be difficult to study insect nAChRs agonist site due to difficulties which have been encountered in expressing recombinant insect nicotinic receptors (Tomizawa and Casida, 2001; Millar, 2003). Despite this, it has been possible to generate functional hybrid nicotinic receptors by the co-expression of insect α subunits with the vertebrate neuronal β subunits in heterologous expression systems, such as *Drosophila* S2 cells and *Xenopus* oocytes (Bertrand et al., 1994; Lansdell et al., 1997; Lansdell and Millar, 2000). However, this strategy is not suitable to express insect β subunit, because no functional pentamer consisting of insect β and either insect or vertebrate α subunits has been identified up to now (Lansdell and Millar, 2000; Yao et al., 2008). Another way to do the pharmacological studies on insect nAChRs, especially for insect

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β subunit, is to construct the artificial subunit chimeras (Shimomura et al., 2005; Yao et al., 2008).

In insects, β1 appears to be the most abundant non-α nAChR subunit. In brown planthopper (*Nilaparvata lugens*), a major rice pest in many parts of Asia, a β subunit was cloned and named as Nlβ1 based on its identity (54%) to *Drosophila melanogaster* Dβ1. However, this β subunit had distinct properties in its amino acid sequence, such as the presence of charged residues within its predicted fourth transmembrane (M4) domain, and showed some differences even in the conserved loops D–F compared with other insect β1 subunits, which indicates this β subunit might not be a real β1 subunit (Liu et al., 2005). For this reason, we tried to clone a β1 subunit from *N. lugens* based on the conserved regions specific in other insect β1 subunits. The single nucleotide polymorphisms (SNPs) and pharmacological properties of the new cloned gene were also included.

2. Materials and methods

2.1. Experimental insects

The susceptible strain (Sus) of *N. lugens* was a laboratory strain obtained from China National Rice Research Institute in September 2001. The resistant strain (Res) was a laboratory selected strain originally collected from a field of hybrid paddy rice in Hangzhou (Zhejiang, China) in August 2006, with the resistance ratio 127-fold to imidacloprid. Insects were kept indoors at 25 (±1) °C, humidity 70–80% and 16/8 h light/dark.

2.2. Amplification of cDNA and corresponding genomic DNA

Total RNA and genomic DNA was isolated from a single individual of the 5th instar female from Sus strain using TRIzol® reagent (Invitrogen). Synthesis of first-strand cDNAs was carried out according to the reverse transcriptase XL (AMV) (TaKaRa) protocol with oligo dT_{18}. The first-strand cDNA (1 μL) was used as a template for PCR. Degenerate primers, BF1 (GCN TTY GTN CAR YTN ATH AAY GT), BF2 (TGG ACN TTY AAY GGN GAY CAR GT) and BR1 (CAT NCC NGG CAT YTC CAT CAT CCA), were designed from the conserved regions of insect nAChR β1 subunits (Fig. 1). The components of PCR were PCR buffer containing 0.1 mM dNTP, 5 μM each primer, and 1.0 U of Go-Taq DNA polymerase (Promega) in a total volume of 20 μL. Thermal cycling conditions were 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min. The last cycle was followed by final extension at 72 °C for 10 min. The amplified product was separated onto agarose gel and purified using the Wizard PCR Preps DNA Purification System (Promega). Purified DNA was ligated into the pGEM-T easy vector (Promega) and several independent subclones were sequenced from both directions. The full-length cDNA was obtained by the rapid amplification of cDNA ends (RACE) according to the Smart Race kit (Clontech) protocol with gene-specific primers (GSPs) for 5′-RACE (5′-GSP1: GAT GAT GAT GTA GAA GGT GAT GTC; 5′-GSP2: CTT CCA ATA GTC GGA CAG GTC GAC) and 3′-RACE (3′-GSP1: TAC CTG CTC TTC ACC TTC ATC ATG; 3′-GSP2: TTC CTC TAC TAC CTG CCG ATC ATC). The RACE products were treated as described above and several independent subclones were sequenced from both directions.

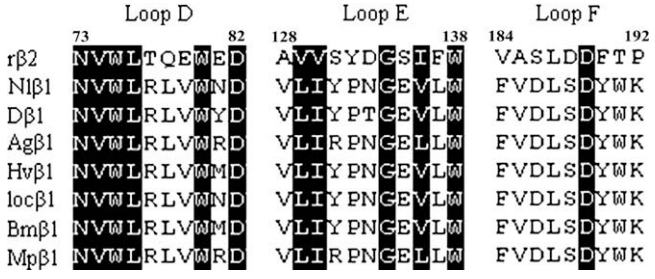

Fig. 1. Alignment of insect β1 and rat β2 subunits in loops D–F. rβ2 (*Rattus norvegicus*, L31622), Nlβ1 (*Nilaparvata lugens*, FJ358493), Dβ1 (*Drosophila melanogaster*, X07956), Agβ1 (*Aphis gossypii*, AF527785), Hvβ1 (*Heliothis virescens*, AF096880), locβ1 (*Locusta migratoria*, AJ000393), Bmβ1 (*Bombyx mori*, EU082071) and Mpβ1 (*Myzus persicae*, AJ251838) are used in the alignment.

Intron sites were identified based on the Nlβ1 orthologue from *D. melanogaster*, Dβ1, and each intron was verified by genomic DNA RT-PCR with specific primers in exons on both sides of the corresponding intron. For each intron, each sequencing reaction was carried out from both ends and the full sequence was not complete for some introns over 1300 bp length. Then the exons were verified by genomic DNA RT-PCR with specific primers in introns on both sides of the corresponding exon, with the exception of the first and last exons. For the first exon, the forward primer was designed on the start sequence of Nlβ1 cDNA and the backward primer on the sequence of the first intron. For the last exon, the backward primer was designed on the sequence before poly A tail and the forward primer on the last intron. The Nlβ1 cDNA corresponding sequence in genomic DNA was combined, as shown in Fig. 1, as the final Nlβ1 cDNA full sequence.

2.3. RNA editing in different populations

Total RNA and genomic DNA was isolated from a single individual of the 5th instar female from Sus strain using TRIzol® reagent (Invitrogen). DNA contamination in the total RNA was eliminated by treating the samples with TURBO DNA-*free*™ (Ambion, Austin, TX, USA).

To amplify full cDNA sequence, the specific primers were designed on the start sequence of Nlβ1 cDNA and the sequence before poly A tail (cDNA-F: CGA ACC AGT CAG TAG TTG CTC GCG CCA C; cDNA-R: TTA AAC AGT TAA CAT TTA TTG TTA TCA TTG ACA TT). PCR fragments were amplified from RNA using AccessQuick RT-PCR system (Promega) with 2 μL RNA in a total volume of 25 μL and the following program: 48 °C for 30 min followed by 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR products were purified, cloned and sequenced as described above. Eight insects were used for each population and 10 clones from each insect were sequenced. Sequences were analyzed using Vector NTI Advance 10 (Invitrogen) and were also inspected manually.

To amplify each exon, the specific primers were designed as described above. The exon genomic fragments were amplified using Go-taq green master mix (Promega) following cycling regime: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 1.5 min and a final extension at 72 °C for 5 min. The PCR products were purified as described above and sequenced directly from both directions.

2.4. Construction of expression plasmid

Nlβ1 full-length cDNA was PCR amplified from pGEM-T-subunit plasmid using the gene-specific primers including EcoRI and XbaI specific regions (EcoRI-F: CCG AAT TCT GCC TGA CAA TGG ACG TGT CCC ACC; XbaI-R: GGT CTA GAG GTA CAA TAT CAC GAT AAC AAA TGG GAA AC). PCR product and the expression vector pGH19 were treated by EcoRI and XbaI, and purified by Wizard PCR Preps DNA Purification System (Promega, USA). Then the treated and purified PCR product was subcloned into vector pGH19. *N. lugens* nAChR Nlα1 subunits (database accession number AY378698) and *Rattus*

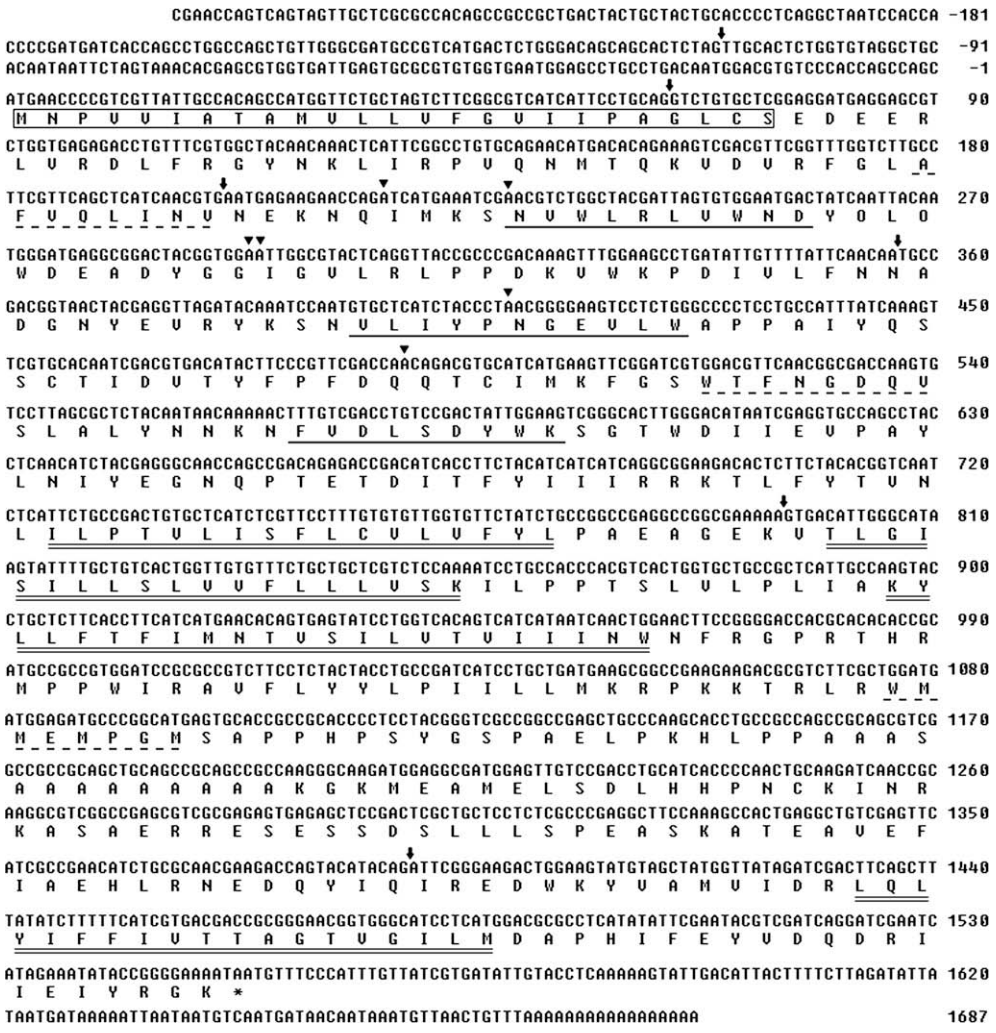

Fig. 2. Nucleotide and deduced amino acid sequence of Nlβ1. The putative signal peptide is boxed. The intron positions are indicated by vertical arrows. A-to-I RNA editing sites are marked with triangles. The degenerate primers used to amplify insect β1 specific fragment are underlined by dashed lines. Loops D–F, important to agonist-binding site in nAChR β subunit, are marked by single lines. Transmembrane domains (TM1–4) are double underlined. The stop codon, TAA, is indicated by a star.

norvegicus β subunit rat β2 (L31622) were subcloned into the expression vector pGH19 as described previously (Liu et al., 2006). In the rat β2 subunit, three important loops contributing to agonist binding were replaced by Nlβ1 (loops D and E) to construct chimeras $\beta2^{LoopD}$ and $\beta2^{LoopE}$. To construct the mutants, single insect-specific residue introduction into rat β2 or rat β2 chimeras was carried by site-directed mutagenesis using the QuikChange method (Stratagene). All plasmid, chimera and mutant constructs were verified by nucleotide sequencing.

2.5. Expression and electrophysiological recording in Xenopus oocytes

Subunit cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, USA). *Xenopus* oocyte preparation and cRNA injection were preformed as described previously (Liu et al., 2006; Yao et al., 2008). Electrophysiological recordings were made using a two-electrode voltage clamp (Multiclamp 700B Amplifier, Axon Instruments, USA) as previously described (Liu et al., 2006; Yao et al., 2008). Dose–response curves were fitted with the Hill equation:

$$I = I_{max}/\left[1 + (EC_{50}/x)^{nH}\right]$$

where I = the response, I_{max} = the maximum response, EC_{50} = half-maximal activation concentration, x = agonist concentration, and n_H = Hill coefficient.

3. Results

3.1. Isolation of Nlβ1 cDNA

RT-PCR and RACE techniques were used to clone the full-size *N. lugens* Nlβ1 cDNA. Fig. 1 shows the full-length cDNA sequence together with the deduced amino acid sequence (GenBank accession number, FJ358493). The sequence has an open reading frame (ORF) of 1554 bp and 517 deduced amino acids (Fig. 2). The deduced protein sequence of Nlβ1 shows high identity to insect nAChR β1 subunits, such as 94% to *Locusta migratoria* locβ1, 91% to *Anopheles gambiae* Agaβ1, 89% to *D. melanogaster* Dβ1 and 88% to *Myzus persicae* Mpβ1. The protein sequence lacks the vicinal cysteines characteristic of α subunits and thus has been denoted as a β subunit.

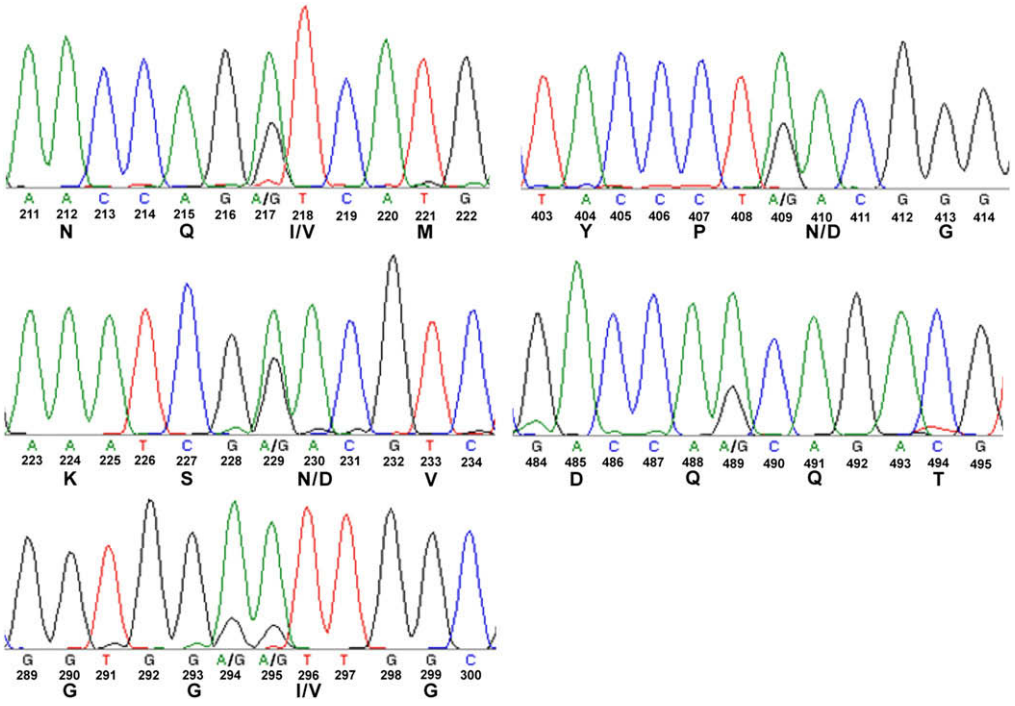

Fig. 3. A-to-I RNA editings in Nlβ1 N-terminal domain. The editing sites are indicated by A/G. The edited position refers to the nucleotide position of Nlβ1 ORF (open reading frame). The amino acid changes caused by A-to-I RNA editings are also shown under the nucleotide sequence.

Nlβ1 protein sequence possesses features common to insect nAChR β subunit, such as an extracellular N-terminal region with conserved residues within loops D–F which are involved in ligand binding, the cys-loop consisting of two disulphide bond-forming cysteines separated by 13 amino acid residues, four well-conserved transmembrane regions (TM1–4) and a variable intracellular region between TM3 and TM4. Loops D–F, contributing to ligand binding in nAChR β subunit, of Nlβ1 are identical to most other insect β1 subunits (Fig. 1).

3.2. A-to-I RNA editing

There were decades of synonymous or non-synonymous single nucleotide polymorphisms (SNPs) in the ORF of Nlβ1 in Sus and Res strains. Comparison of cDNA sequences with corresponding genomic sequences suggests that six SNPs detected in the N-terminal domain are the result of A-to-I editing (Fig. 3). Among these six A-to-I RNA editing sites, four resulted in non-synonymous mutations and other two were synonymous SNPs.

The editing sites in Nlβ1 were investigated in this study by examining the PCR-amplified genomic sequence and 10 cDNA sequences (clones) from the same individuals ($n = 8$ individuals and 80 clones) from different populations. Site 1 (E1), site 2 (E2), site 4 (E4) and site 5 (E5) were non-synonymous SNPs, and site 3 (E3) and site 6 (E6) were synonymous SNPs (Fig. 3). Among these 6 sites, only E2 and E5, resulting in an N to D substitution in loop D and loop E respectively, happened at different frequencies between strains (Fig. 4). The highest E2 frequency was in Sus strain and the highest E5 frequency was in Res strain.

3.3. Expression in Xenopus oocytes

Because E2 and E5 RNA editings resulted in an N to D substitution in loop D (N73D) and loop E (N133D) and happened at different frequencies among strains, it is interesting to evaluate the influence of the N to D substitution in loop D and loop F on the agonist potency. To evaluate the influence of N73D in loop D and N133D in loop E, loop D or loop E of Nlβ1 was introduced into rat β2 of Nlα1/β2 nAChRs to construct the hybrid Nlα1/$\beta2^{LoopD}$ or Nlα1/$\beta2^{LoopE}$ nAChRs, and then N73D or N133D mutation was introduced into $\beta2^{LoopD}$ or $\beta2^{LoopE}$ to construct mutant Nlα1/$\beta2^{LoopD\text{-}N73D}$ or Nlα1/$\beta2^{LoopE\text{-}N133D}$ (Fig. 1).

In oocytes co-injected with cRNA encoding the Nlα1 and rat β2 subunits, large inward currents were detected in response to applications of acetylcholine or imidacloprid (Fig. 5). In voltage-clamp electrophysiological studies, ACh and imidacloprid evoked inward currents in a dose-dependent manner in *Xenopus* oocytes. The inward current in response to 1 mM ACh in oocytes expressing Nlα1-β2 was 261.05 ± 15.32 nA (mean ± SEM), to which the peak amplitude of the current recorded in response to each challenge

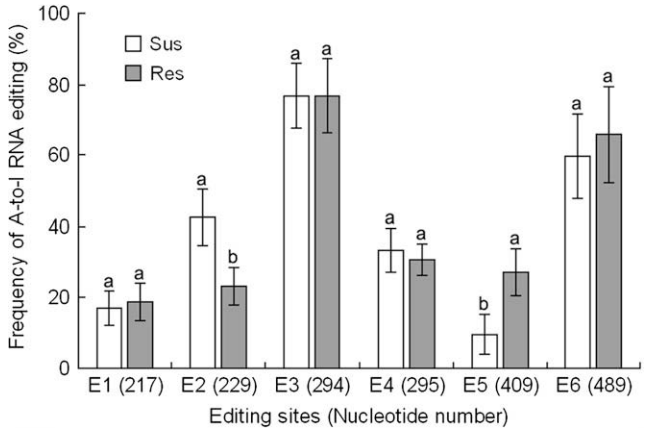

Fig. 4. Frequency of A-to-I RNA editings in different *N. lugens* strains. Eight individual insects were used for each population. Ten clones were analyzed per insect. Values represent the mean ± SD ($n = 8$). The edited position, referred to the nucleotide position of Nlβ1 ORF, is given in parentheses after each editing site. Different lowercase letters at each editing site indicate significant differences at $P < 0.05$ level between strains (*t* test).

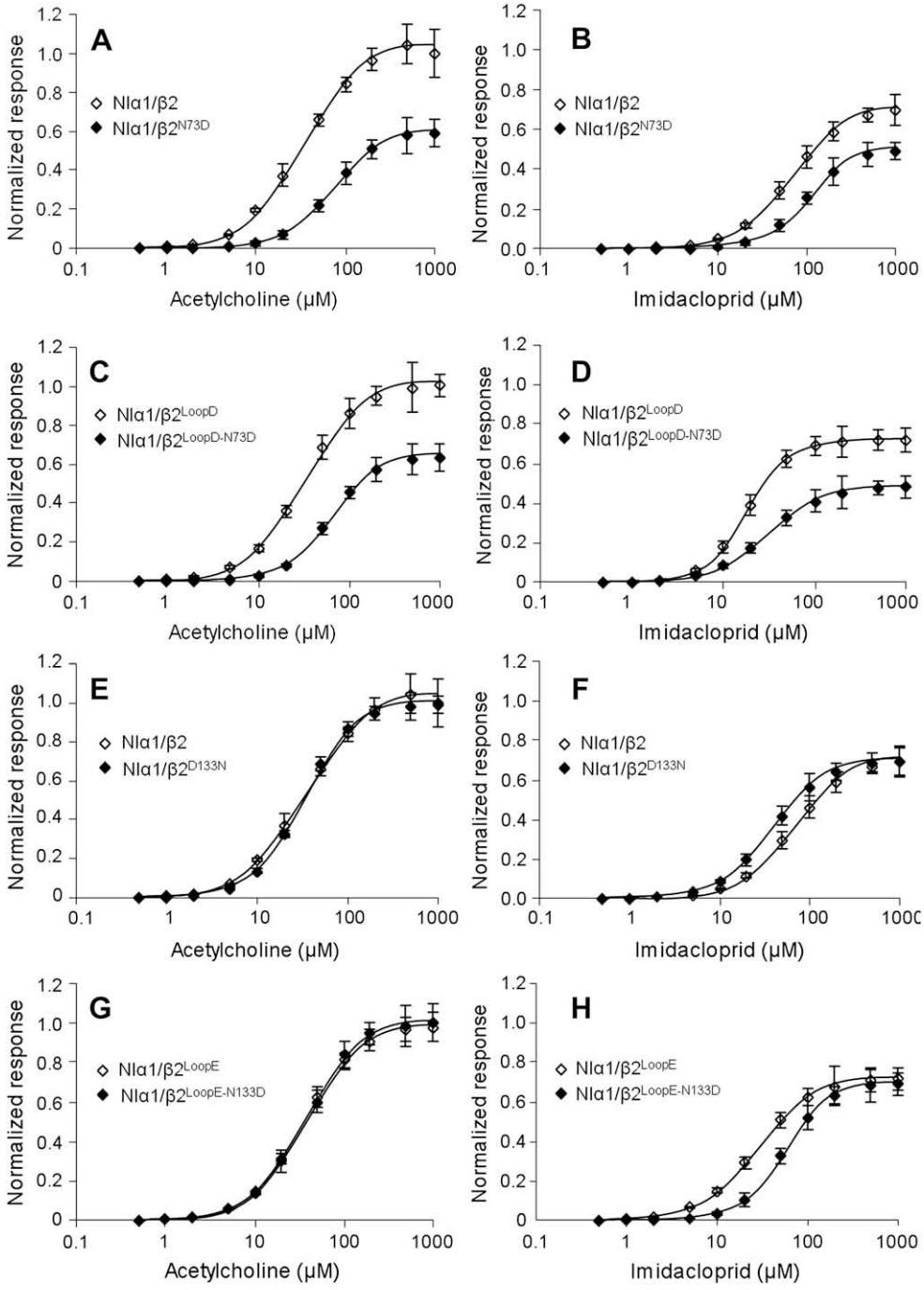

Fig. 5. Agonist dose–response curves from hybrid nAChRs expressed in *Xenopus* oocytes. A, C, E and G show ACh dose–response curves from different nAChRs containing Nlα1 and β2 or β2 mutant as indicated. B, D, F and H show imidacloprid dose–response curves from different nAChRs containing Nlα1 and β2 or β2 mutant as indicated. Data are means of at least four independent experiments ± SEM.

was normalized. Similar to our previous report (Yao et al., 2008), introduction of Nlβ1 loop D or loop E into β2 showed significant influences on imidacloprid potency, but influences on ACh potency were minimal. In Nlα1/β2^{LoopD}, the introduction of N73D mutation reduced both ACh and imidacloprid potency significantly, with remarked changes both in I_{max} and EC_{50} (Table 1). And the influence of N73D mutation on ACh was more significant than on imidacloprid (Fig. 5A–D). The introduction of N133D mutation in Nlα1/β2^{LoopE} didn't cause significant changes in I_{max} to both ACh and imidacloprid, and also didn't cause a significant right-shift in EC_{50} to ACh (Fig. 5G). Despite this, the big shift in EC_{50} to imidacloprid was observed in Nlα1/β2$^{LoopE\text{-}N133D}$ (pEC_{50} = 4.31 ± 0.07, n = 5), when compared with Nlα1/β2^{LoopE} (pEC_{50} = 4.58 ± 0.03, n = 5; Fig. 5H).

Because rat β2 also has N73 at the corresponding site, N73D mutation was also carried out in β2 of Nlα1/β2 to construct mutant Nlα1/β2^{N73D}. D133 is found in β2 at the corresponding site of N133 in Nlβ1, so the D133N reverse mutation was carried out in β2 of Nlα1/β2 to construct mutant Nlα1/β2^{D133N} (Fig. 1). Similar to the introduction of N73D mutation into Nlα1/β2^{LoopD}, the introduction of N73D mutation into Nlα1/β2 reduced both ACh and imidacloprid potency significantly. The introduction of D133N reverse mutation

Table 1
I_{max} and EC_{50} values of acetylcholine and imidacloprid for nAChRs containing Nlα1-β2 or -β2 chimeras/mutants expressed in *Xenopus* oocytes. Values shown are the result of a fit of the concentration–response data (mean ± SEM, $n = 5–7$). Different lowercase letters in each column indicate significant differences at $P < 0.05$ level (*t* test).

Oocytes	Acetylcholine		Imidacloprid	
	I_{max}	pEC_{50}	I_{max}	pEC_{50}
Nlα1/β2	1.01 ± 0.06 a	4.49 ± 0.04 a	0.71 ± 0.04 a	4.20 ± 0.05 d
Nlα1/β2^{N73D}	0.60 ± 0.08 b	4.16 ± 0.07 b	0.50 ± 0.06 b	4.01 ± 0.06 e
Nlα1/β2^{D}	1.01 ± 0.07 a	4.53 ± 0.09 a	0.72 ± 0.08 a	4.75 ± 0.09 a
Nlα1/β2^{D-N73D}	0.64 ± 0.09 b	4.23 ± 0.09 b	0.48 ± 0.07 b	4.53 ± 0.12 bc
Nlα1/β2^{D133N}	0.99 ± 0.09 a	4.52 ± 0.10 a	0.70 ± 0.06 a	4.43 ± 0.07 c
Nlα1/β2^{E}	0.98 ± 0.06 a	4.47 ± 0.05 a	0.72 ± 0.05 a	4.58 ± 0.03 b
Nlα1/β2$^{E-N133D}$	1.01 ± 0.11 a	4.46 ± 0.09 a	0.69 ± 0.07 a	4.31 ± 0.07 cd

into Nlα1/β2 caused a significant left-shift in EC_{50} to imidacloprid ($pEC_{50} = 4.20 \pm 0.05$ for Nlα1/β2, $n = 5$; and $pEC_{50} = 4.43 \pm 0.07$ for Nlα1/β2^{D133N}, $n = 7$; Fig. 5F), but showed little influence on ACh (Fig. 5E).

4. Discussion

The superfamily of pentameric ligand-gated ion channels (LGICs), including nAChR, 5-HT3, $GABA_A$ and $GABA_C$, and glycine receptors, mediates chemical synaptic transmission. The genome sequencing projects of insects had revealed 10, 10, 11, 12 and 12 subunits in *D. melanogaster* (Adams et al., 2000), *A. gambiae* (Jones et al., 2005), *Apis mellifera* (Jones et al., 2006), *Bombyx mori* (Shao et al., 2007) and *Tribolium castaneum* (Jones and Sattelle, 2007) respectively. The combinatorial assembly of these subunits produces a wide structural diversity of receptor oligomers, targeted to different subcellular compartments, which exhibit variable electrical properties (conductance, ion selectivity, rectification), pharmacologic characteristics (affinities for agonists, competitive antagonists and allosteric effectors, potency orders) and kinetics of activation and desensitization (Le Novere et al., 2002).

In insects, β1 appears to be the most abundant non-α nAChR subunits, which have been identified in a range of insects (Yao et al., 2008). In *N. lugens*, a non-α subunit had been cloned and labeled as β1 subunit based on its identity (54%) to *D. melanogaster* Dβ1. However, this β subunit had distinct properties in its amino acid sequence, such as the presence of charged residues within its predicted fourth transmembrane (M4) domain, and showed some differences even in the conserved loops and transmembrane domains compared with other insect β1 subunits (Liu et al., 2005). Because our new cloned non-α subunit shows very high identity (87–94%) at amino acid level to other insect β1 subunits and possesses features common to insect nAChR β1 subunits, it is thought as a real β1 subunit and denoted as Nlβ1. Although the originally labeled *N. lugens* β1 shows the highest identity (54%) to Dβ1 among three β subunits (Dβ1–3) from *D. melanogaster*, it also shows 43% identity to Dβ2. This identity is similar to 44% identity between Dβ1 and Dβ2, which suggests the originally named β1 might be a *N. lugens* β2 subunit. This information has been updated in GenBank (AY378703) and the originally labeled β1 is denoted as Nlβ2.

RNA editing is a process that results in the synthesis of proteins that are not directly encoded in the genome. One type of RNA editing involves the modification of individual adenosine bases to inosine in RNA by ADAR enzymes (adenosine deaminases acting on RNA) (Bass, 2002; Maas et al., 2003). Because inosine acts as guanosine during translation, A-to-I conversion in coding sequences often leads to amino acid changes and entails changes in protein function (Maas et al., 2003; Seeburg et al., 1998; Schmauss and Howe, 2002). Moreover, RNA editing may influence alternative splicing decisions, and RNA and alternative splicing are important factors contributing to diversity of nAChRs (Rueter et al., 1999; Millar, 2003; Jin et al., 2007). For insect nAChR genes, an extraordinary feature is that they can potentially create many different mRNAs by RNA editing and alternative splicing. Fox example, more than 30,000 insect nAChR α6 isoforms are theoretically possible through RNA editing and alternative splicing, without considering any linkage between these events (Grauso et al., 2002). In *D. melanogaster* Dβ1, there are four editing sites in the N-terminal domain, in which two lead to amino acid changes (Hoopengardner et al., 2003; Sattelle et al., 2005). Here, six editing sites were found in *N. lugens* Nlβ1 N-terminal domain, but only one site (E1) in Nlβ1 is identical to that (site 4) of *D. melanogaster* Dβ1. In the same nAChR subunit from different insect species, some A-to-I editing sites are a genomically encoded G in other species, which might provide the possible explanation for the loss of three editing sites and addition of five new editing sites in *N. lugens* Nlβ1, compared to *D. melanogaster* Dβ1 (Jin et al., 2007). For example, a genomically encoded G (position 216, Fig. 3) was found in Nlβ1, corresponding to the A-to-I editing site 3 in Dβ1 (Hoopengardner et al., 2003).

A-to-I conversion in coding sequences often leads to amino acid changes and entails changes in protein function (Seeburg et al., 1998; Schmauss and Howe, 2002). Among six editing sites found in Nlβ1, four were found to lead to amino acid changes, in which two (E2 and E5) caused an N to D change in loop D (N73D) and loop E (N133D) respectively. Interestingly, only E2 and E5 happened at different frequencies between the susceptible (Sus) and resistant (Res) strains. Although both caused an N to D change, E2 and E5 happened at high frequency in Sus and Res strains respectively. These results indicated, although E2 and E5 both caused an N to D change in important loops, their roles in imidacloprid sensitivity might be different.

The influence of two mutations (N37D and N133D) in loops D and E on nAChRs biological function and pharmacological property was evaluated by expressing in *Xenopus* oocytes. N73D mutation significantly reduced the agonist potency of both ACh and imidacloprid, and the influence on ACh was more significant than on imidacloprid. This result indicated N73D mutation should have the effects on insect nAChRs normal function, although it might also cause imidacloprid insensitivity. N73D mutation might make insect nAChRs resistless to the condition changes, including insecticide treatment, which might give the explanation for the lower E2 frequency in the laboratory selected Res strain. In contrast, N133D mutation in loop E only showed significant effects on imidacloprid potency, and the influences on ACh potency were minimal or negligible, which is similar to our previous study (Yao et al., 2008). This result showed that N133D mutation resulted in imidacloprid insensitivity on the heterologously expressed receptors, but not had influence on receptor normal function. And high E5 frequency in Res strain might be associated with imidacloprid insensitivity in this strain.

Acknowledgements

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