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Review

Insect nicotinic acetylcholine receptors (nAChRs): Important amino acid residues contributing to neonicotinoid insecticides selectivity and resistance

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels which mediate fast cholinergic synaptic transmission in insect and vertebrate nervous systems. The great abundance of nAChRs within the insect central nervous system has led to the development of insecticides targeting these receptors, such as neonicotinoid insecticides. Neonicotinoid insecticides act selectively on insect nAChRs, accounting at least in part for the selective toxicity to insects over vertebrates. Some important amino acid residues in insect nAChR α and β subunits contribute to neonicotinoid insecticides selectivity, including important residues in loop C, the region loop B to the N-terminus and loop B-C interval of insect α subunit, and important residues in loop D, E and F of insect β subunit. Important residues contributing to neonicotinoid insecticides selectivity may also contribute to the resistance to these insecticides, if they mutate to other residues identical or similar to the corresponding residues in vertebrate subunits. The first point mutation Y151S has been identified in insect α subunit loop B to be associated with neonicotinoid insecticides resistance, which decreased neonicotinoid insecticides affinity remarkably, but showed little effects on insect nAChRs normal function.

Key words: Nicotinic acetylcholine receptor, neonicotinoid insecticides, selectivity, resistance.

INTRODUCTION

Most commercially important insecticides are neurotoxins that act on ion channels, receptors or enzymes within the insect nervous system (Bloomquist, 1996; Narahashi, 1996; Casida and Quistad, 1998). Examples include pyrethroids which act on voltage-gated sodium channels (Vais et al., 2001; Soderlund and Knipple, 2003), organophosphates and carbamates which inhibit acetylcholinesterase (Casida and Quistad, 1998), and cyclodienes which act on insect GABA-gated ion channels (Buckingham et al., 2005). In recent years, one of the most promising areas in insecticide development is the identification of compounds acting on insect nicotinic acetylcholine receptors (nAChRs), referred as neonicotinoid insecticides (Casida and Quistad, 1998; Matsuda et al., 2001). Imidacloprid, the first of the neonicotinoid class of insecticides, was patented in 1985 by Bayer and was first marketed in 1991. Other neonicotinoid insecticides have subsequently been developed and brought to the market, including nitenpyram (in 1995 by Takeda), acetamiprid (in 1996 by Nippon Soda), thiamethoxam (in 1998 by Syngenta), thiacloprid (in 2000 by Bayer), clothianidin (in 2002 by Takeda and Bayer) and dinotefuran (in 2002 by Mitsui) (Millar and Denholm, 2007; Figure 1).

Neonicotinoid insecticides are insect-selective nAChRs agonist, and the great abundance of nAChRs within the insect central nervous system (CNS) has led to the quick development and extensive use of neonicotinoid insecticides. This paper provides a summary on important amino acid residues in insect n nAChRs contributing to neonicotinoid insecticides selectivity.

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NEONICOTINOID INSECTICIDES: SELECTIVITY AND RESISTANCE

Neonicotinoid insecticides show selective toxicity to insects over vertebrates, and are used extensively in areas of crop protection and animal health (Matsuda et al., 2001; Tomizawa and Casida, 2005; Millar and 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 and Casida, 2005). Neonicotinoid insecticides possess either a nitro or a cyano group, which have been postulated to contribute directly to their selectivity (Matsuda et al., 2001; Tomizawa and Casida, 2005).

Imidacloprid and other neonicotinoid insecticides, like other systemic insecticides, display prolonged persistence which is likely to generate high selection pressure for resistance (Taylor and Georghiou, 1982). Resistance to imidacloprid has been reported in a range of species, including *Nilaparvata lugens* (brown planthopper), a major rice pest in many parts of Asia (Nauen and Denholm, 2005; Liu et al., 2003; Liu and Han, 2006; Wang et al., 2008). However, because of its characteristics, including a novel mode of action (Devine et al., 1996; Bao et al., 2008), imidacloprid resistance in field population appears to develop slowly and the mechanism is not well understood. Although a point mutation has been identified to confer resistance to all neonicotinoid insecticides in brown planthopper, *N. lugens* (Liu et al., 2005, 2006), there has been no work to establish the prevalence of the mutation in field populations (Liu et al., 2006).

INSECT nAChRs: THE TARGET OF NEONICOTINOID INSECTICIDES

The nAChRs are ligand-gated ion channels mediating fast cholinergic synaptic transmission in insect and vertebrate nervous systems (Matsuda et al., 2001). In mammals and other vertebrates nAChRs are expressed both at the neuromuscular junction (the “muscle-type” nAChRs) and within the central and peripheral nervous system (“neuronal” nAChRs). In insects, although nAChRs are not expressed at the neuromuscular junction (where synaptic transmission is glutamatergic), acetylcholine is the major excitatory neurotransmitter in insect brain (Breer and Sattelle, 1987). The most extensively characterized nAChRs is that expressed within the electric organ of fish such as the marine ray *Torpedo* (Unwin, 1996). Affinity labeling, mutagenesis and structural 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 binding site segments A, B, and C) and from at least three regions of the non-α (β, γ or δ) –subunits (segments D, E, and F) (Prince and Sine, 1998; Arias, 2000; Corringer et al., 2000; Grutter and Changeux, 2001). Most features of the model are present and confirmed in the binding site identified within the solved structure of a molluscan, glial-derived soluble ACh binding protein (AChBP), a homopentameric structural and functional homolog of the N-terminal ligand binding domain of a nAChR α-subunit (Brejc et al., 2001; Smit et al., 2001).

The agonist site structure model of nAChRs was derived from few species up to the present and it remains unknown whether the structure is suitable for all animals because of the diversity in nAChRs. A total of 17 nAChR subunits (α1–α10, β1–β4, γ, δ and ε) have been identified in vertebrate species, which can co-assemble to form multiple functional homopentamers (α7, α8 and α9) or heteropentamers (Corringer et al., 2000). The genome sequencing projects of insects had revealed 10, 10, 11, 12 and 12 subunits in *Drosophila melanogaster* (Adams et al., 2000), *Anopheles 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 agonist site structure model derived from *Torpedo* nAChRs and molluscan AChBP might not be suitable for all nAChRs from different animal species, although most nAChR subunits possess the key residues included in the agonist site structure model. In insect, no functional nAChR pentamers were identified even when insect nAChR subunits were heterologously expressed together with different insect subunit combinations or all subunits from one insect species (Lansdell and Millar, 2000; Liu et al., 2005, 2006). The fact, that the key residues in the agonist structure model were included in insect nAChRs and no functional pentamers were identified in the expression of recombinant nAChRs from insect species in heterologous expression systems, gives some indications that the model is not generally suitable for some species, or other important residues in the regions outside these six loops also play essential roles in nAChRs function. Recently, the amino acid clusters between loop B and C of insect nAChR α subunit were identified essential to agonist binding (Liu et al., 2008).

IMPORTANT AMINO ACID RESIDUES IN INSECT nAChR α SUBUNIT

Although Loops A, B and C exist in insect α subunits and Loops D, E and F. In insect β subunits, the difficulties were encountered in expressing recombinant insect nicotinic receptors (Tomizawa and Casida, 2001; Millar, 2003; Millar and Denholm, 2007). Despite this, however, 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 the heterologous expression systems, such as *Drosophila* S2 cells and *Xenopus* oocytes (Bertrand et al., 1994; Lansdell et al.,

1997; Lansdell and Millar, 2000). A concern, however, is that these hybrid receptors may not faithfully reflect insect nAChRs (Tomizawa and Casida, 2001).

The nAChR subunit composition has been identified to influence neonicotinoid insecticides selectivity, such as the replacement of the α4 subunit of the chicken α4β2 nAChRs by *Drosophila* Dα2 subunit resulting in a marked increase of imidacloprid selectivity and demonstrating that insect α subunit has structural features favorable for interactions with neonicotinoids (Matsuda et al., 1998; Lansdell and Millar, 2000; Ihara et al., 2003). In chicken α4β2 nAChRs, the combination of replacing the region loop B to the N-terminus of α4 subunit by the corresponding region of Dα2 subunit and E219P mutation in the YECC motif in loop C resulted in a marked increase of imidacloprid selectivity, which showed that the region loop B to the N-terminus in the Dα2 subunit contributes to the high imidacloprid sensitivity of the hybrid Dα2β2 nAChRs (Shimomura et al., 2005). Using the similar techniques, loop B–C interval region and proline residue in the YPCC motif in loop C from the Dα2 were also found to play important roles in its selective interactions with imidacloprid (Shimomura et al., 2004). Because mutation of proline residue in the YPCC motif in Dα2 loop C only showed minimal effects on ACh potency, such mutation may lead to a neonicotinoid resistant phenotype in insects.

In a laboratory strain of *N. lugens* with high resistance to imidacloprid, the point mutation Y151S mutation in nAChR α subunit has been identified to contribute to neonicotinoid insecticides resistance (Liu et al., 2005, 2006). Radioligand binding studies, performed with both native nAChR preparations and heterologously –expressed recombinant nAChRs in *Drosophila* S2 cells, demonstrated that the Y151S resistance-associated point mutation is responsible for a dramatically reduced level of specific [^{3}H]imidacloprid binding (Liu et al., 2005). By expression of recombinant nAChRs in *Xenopus* oocytes, this Y151S mutation was found to show remarkable effects on all neonicotinoid insecticides potencies, but little on ACh (Liu et al., 2006). Despite the evidence that the potency of all neonicotinoid compounds is reduced by the mutation, an interesting finding is that this effect is less pronounced for the tertrahydrofuryl compound, dinotefuran. In contrast to dinotefuran, all of the other neonicotinoid insecticides examined contain a chlorinated heterocyclic (chloropyridyl or chlorothiazolyl) group. Because Y151 is an important residue for ligand binding and conserved in most vertebrate and insect α subunits, it is not possible for the Y151S mutation involved in neonicotinoid insecticides selectivity.

Although all residues contributing to the six loops were thought to be important for agonist binding, some of them play the roles in a weak manner, including Y151 in loop B (Corringer et al., 2000). Various residues at the 151-site are found among invertebrate nAChR α subunits, including M151 (Methionine) in *Caenorhabditis elegans* α subunit acr18, belonging to DEG-3 group (Brown et al., 2006). When Y151M mutation was introduced into insect α subunit, imidacloprid was found to act as an antagonist on insect nicotinic acetylcholine receptor containing the Y151M mutation (Zhang et al., 2008). Although the Y151M mutation resulted in the complete loss of agonist action of imidacloprid on insect Nlα1/β2, imidacloprid interferes with the normal biological function of nAChRs Nlα1^{Y151M}/β2 by inhibiting the response to acetylcholine and should maintain activity against insect nAChRs containing the Y151M mutation. Consequently, despite the effects of the Y151M mutation upon imidacloprid action, the mutation may not lead to an imidacloprid resistant phenotype.

IMPORTANT AMINO ACID RESIDUES IN INSECT nAChR β SUBUNIT

The recombinant insect/vertebrate α/β nAChRs in the heterologous expression systems are the best available model at present, 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 till now (Bertrand et al., 1994; Lansdell et al., 1997; Lansdell and Millar, 2000; Liu et al., 2006).

Another way to do the pharmacological studies on insect nAChRs, especially for insect β subunit, is to construct the artificial subunit chimeras, although it also can not reveal the complete features of its wild type (Lansdell and Millar, 2004; Shimomura et al., 2005).

Replacement of Loop D, E and/or F of vertebrate β2 subunit by the corresponding regions of insect β1 subunit increased the neonicotinoid selectivity (Yao et al., 2008). In studies of single amino acid changes, the introduction of insect-specific loop D amino acid residues T77R/K/N and E79V/R into the chicken β2 subunit of Dα2/β2 hybrid nAChRs significantly increased the neonicotinoid selectivity (Shimomura et al., 2006). S131Y/R and D133N in loop E and T191W and P192K in loop F were also found to contribute to the neonicotinoid selectivity of Dα2/β2 (rat) hybrid nAChRs (Yao et al., 2008). Neonicotinoids possess either a nitro or a cyano group, which have been postulated to contribute directly to their selectivity (Matsuda et al., 2001; Tomizawa and Casida, 2005). T77R/K, S131R and P192K, mutations from a neutral residue to a basic residue, and E79V/R and D133N, mutation from an acidic residue to a neutral or basic residue, should change the electrostatic properties of the Nlα1-β2 nAChR agonist binding pocket, which could explain their roles in influencing neonicotinoid selectivity (Shimomura et al., 2006).

These studies show insect-specific residues or regions, in or out the important loops (Loop A-F), could enhance neonicotinoids selectivity, depending on residues' electrostatic properties (Shimomura et al., 2006). Because these

mutations in vertebrate β2 Loop D, E and F only showed minimal effects on ACh potency, such mutation may also lead to a neonicotinoid resistant phenotype in insects.

PROBLEMS ENCOUNTERED IN INSECT nAChRs STUDY

Although many insect nAChR subunits have been cloned, and genome sequencing of several insects also gives more information about insect nAChRs, some problems were encountered in the detailed functional and pharmacological characterisation of insect nAChRs.

1) The subunit composition of insect native and recombinant nAChRs is unknown. Until now, no functional nAChRs consisting of only insect subunits were identified. Although recombinant nAChRs of insect α subunit and vertebrate β subunit in the heterologous expression systems and the construction of artificial subunit chimeras are thought to be useful strategies, they may not faithfully reveal all features of insect nAChRs (Tomizawa and Casida, 2001; Lansdell and Millar, 2004; Shimomura et al., 2005).

2) The number of nAChR subunits is different among insect species. In *D. melanogaster*, an extensively studied model insect species, ten nAChR subunits (α1-α7 and β1-3) have been identified by molecular cloning (Tomizawa and Casida, 2001; Millar, 2003). The proliferation of insect genome sequencing projects is now starting to reveal a similar level of nAChR subunit diversity in other species, such as nine α (Agamα1-9) and one β (Agamβ1) in *A. gambiae* (Jones et al., 2005), nine α (Amelα1-9) and two β (Amelβ1-2) in *A. mellifera* (Jones et al., 2006), nine α (Bmα1-9) and three β (Bmβ1-3) in *B. mori* (Shao et al., 2007), eleven α (Tcasα1-11) and one β (Tcasβ1) in *T. castaneum* (Jones and Sattelle, 2007). It is found that at least two α subunits are missing from *D. melanogaster*, which shows this model insect species is not a suitable model for insect nAChRs study. Furthermore, the function and the roles in insecticide selectivity of these missing α subunits are unknown until now.

3) Insect nAChRs agonist site structure remains unknown. The agonist site structure model of nAChRs was derived from few species up to the present and it remains unknown whether the structure is suitable for all animals because of the diversity in nAChRs. Recently, the amino acid residues or residue clusters outside the six loops were found to play essential roles in agonist binding, especially for the amino acid clusters between loop B and C (Liu et al., 2008). This result indicated that the residues in the six loops could be necessary, but not enough for the activity of agonist binding.

4) The target subunit of different insecticides remains unknown. 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). Insecticides acting on insect nAChRs mainly include plant alkaloids (including nicotine), spinosyns, nereistoxin analogues and neonicotinoid insecticides. Although these insecticides all act on insect nAChRs, their target subunits are different. The available example is that *Drosophila* Dα6 subunit has been identified as a target site for spinosad (Chouinard et al., 2006; Orr et al., 2006), but not neonicotinoid insecticides such as imidacloprid (Lansdell and Millar, 2004), a finding which is consistent with the evidence that spinosad and neonicotinoids act upon different populations of nAChRs (Salgado and Saar, 2004).

Nicotinic receptors are a diverse family of neurotransmitter-gated ion channels, expressed in both vertebrate and invertebrate species. Although some progresses have been achieved in insect nAChRs and the selective insecticides acting on insect nAChRs have been developed well, the resolve of some important problems is in urgent need.

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