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Specific loops D, E and F of nicotinic acetylcholine receptor β1 subunit may confer imidacloprid selectivity between *Myzus persicae* and its predatory enemy *Pardosa pseudoannulata*

Feng Song [a], Zhiqi You [b], Xiangmei Yao [a], Jiagao Cheng [b], Zewen Liu [a,*], Kejian Lin [c,**]

[a] Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China
[b] Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China
[c] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

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ABSTRACT

One nicotinic acetylcholine receptor non-α subunit was cloned from the pond wolf spider, *Pardosa pseudoannulata*, an important predatory enemy of some insect pests with agricultural importance, such as the green peach aphid *Myzus persicae*. The subunit shows high amino acid identities to insect β1 subunits (74–78%), and was denoted as Ppβ1. Although high identities are found between Ppβ1 and insect β1 subunits, amino acid differences are found within loops D, E and F, important segments contributing to ligand binding. The effects of amino acid differences within these loops were evaluated by introducing loops of insect or spider β1 subunits into rat β2 subunit and co-expressing with insect α subunit. The corresponding regions of rat β2 chimera $\beta2^{Mp\beta1}$ (β2 with loops D, E and F from *M. persicae* β1 subunit Mpβ1) were replaced by loops D, E and F of Ppβ1 singly or together to construct different chimeras. When these chimeras were co-expressed with insect Nlα1, it was found that the replacement of loops D, E and F of $\beta2^{Mp\beta1}$ by that of Ppβ1 resulted in a right-ward shift of the imidacloprid dose–response curves, reflecting increases in EC_{50}, compared to Nlα1/$\beta2^{Mp\beta1}$. By contrast, the influences on ACh potency were minimal. The further study showed that R81Q, N137G and F190W differences, within loops D, E and F respectively, contributed mainly to these sensitivity changes. This study contributes to our understanding of the molecular mechanism underlying selectivity of neonicotinoids against insects over spiders.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast cholinergic synaptic transmission in invertebrate and vertebrate nervous systems (Matsuda et al., 2001). In vertebrates, nAChRs are expressed at both the neuromuscular junction and within the central and peripheral nervous system. In insects, although nAChRs are not expressed at the neuromuscular junction, the great abundance of nAChRs is within the central nervous system (CNS), which has led to the development of insecticides targeting these receptors (Breer and Sattelle, 1987). 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).

In nAChRs, the ligand-binding site is located at the interface between two subunits (Arias, 2000; Corringer et al., 2000). The most extensively characterized nAChR 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 structural model of the agonist binding pocket with amino acid residues contributed by three distinct loops of the α-subunits (referred to as binding site loops A, B, and C) and from at least three loops of the non-α (β, γ or δ) -subunits (loops D, E, and F) (Prince and Sine, 1998; Arias, 2000; Corringer et al., 2000; Grutter and Changeux, 2001; Brejc et al., 2001; Smit et al., 2001). These key residues interact with the agonists directly, and the other residues in these loops may interact with the agonists in direct or in indirect mode (for a model diagram, see 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).

* Corresponding author. Tel./fax: +86 25 84399051.
** Corresponding author. Tel./fax: +86 10 62815929.
E-mail addresses: jemunson@njau.edu.cn (Z. Liu), kjlin@ippcaas.cn (K. Lin).

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A large number of nAChRs subunits have been found in different insect species, and functional studies have been performed extensively *in vivo* and in vitro. In the fruit fly *Drosophila melanogaster*, an extensively studied model insect species, ten nAChR subunits (Dα1–Dα7 and Dβ1–Dβ3) have been identified and characterized by molecular cloning (Millar, 2003; Jones et al., 2007). Genome sequencing has revealed a similar level of nAChR subunit diversity in other insect species (Jones et al., 2007; Millar and Denholm, 2007), including the malaria mosquito *Anopheles gambiae* (Jones et al., 2005), the honeybee *Apis mellifera* (Jones et al., 2006), the silkworm *Bombyx mori* (Shao et al., 2007) and the flour beetle *Tribolium castaneum* (Jones and Sattelle, 2007). Despite the considerable difficulties that have been encountered in the heterologous expression of insect nAChRs (Millar, 1999; Millar and Lansdell, in press), expression of functional recombinant nAChRs has been reported for several insect nAChR α subunits when co-expressed with vertebrate β subunits (Bertrand et al., 1994; Millar and Lansdell, in press). For insect β subunits, the only available way now is to construct artificial subunit chimeras, although it might not reveal the complete features of a native receptor (Lansdell and Millar, 2004; Shimomura et al., 2006). Advances in characterization of insect nAChRs improve our understanding of neonicotinoid action mode and selectivity mechanisms. 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). 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). Neonicotinoids, such as imidacloprid, also show low toxicity to some spider species, such as the pond wolf spider, *Pardosa pseudoannulata*, an important predatory enemy of some insect pests with agricultural importance (Kunkel et al., 1999; Tanaka et al., 2000). The selectivity of neonicotinoids against insects versus spiders might be also from the difference in their nAChRs. However, there is no available information about spider nAChRs at present.

In the present study, we report the cloning a nAChR non-α subunit from the pond wolf spider, *P. pseudoannulata*. Based on the key amino acid differences found within important loops D, E and F, the neonicotinoid selectivity between insects and spiders was evaluated by expressing the hybrid nAChRs in *Xenopus* oocytes and analysis by two-electrode voltage-clamp recording.

2. Materials and methods

2.1. Materials

Acetylcholine and imidacloprid were purchased from Sigma–Aldrich (USA). *P. pseudoannulata* was colleted from a field of hybrid paddy rice in Nanjing (Jiangsu, China) in August 2008.

2.2. Amplification of cDNA

Total RNA was isolated 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 (AAY GTN AAY GAR AAR AAY CAR ATH ATG AA), BR1 (ATN ACC ATN GCN ACR TAY TTC CAR TCY TC) and BR2 (ATR CAN GTY TGY TGR TCR AAN GGR AAR TA), were designed from the conserved regions of insect nAChR β1 subunits (Fig. 1). The components of PCR were PCR reaction buffer containing 0.1 mM dNTP, 5 μM each primer, and 1.0 unit 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: CAT CGG CAT TGT TGA ATA GCA C; 5′-GSP2: GTC GTT CCA CAT CAA CTG TAA CC) and 3′-RACE (3′-GSP1: AGT CTG GAA ACC TGA CAT TGT G; 3′-GSP2: GCG TTA CGA GTC GAA CGT GCT G). The RACE products were treated as described above and several independent subclones were sequenced from both directions.

2.3. Plasmids and mutagenesis

Nilaparvata lugens nAChR α subunit Nlα1 (AY378698) and *Rattus 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 loops (D, E and F) contributing to agonist binding were replaced by that of *Myzus persicae* Mpβ1 to construct the chimera $\beta2^{Mp\beta1}$ as previously described (Yao et al., 2008). Then loops D, E and F were replaced by that of Ppβ1 singly or together to construct chimeras $\beta2^{Mp\beta1\text{-}LoopD}$, $\beta2^{Mp\beta1\text{-}LoopE}$, $\beta2^{Mp\beta1\text{-}LoopF}$ and $\beta2^{Mp\beta1\text{-}LoopDEF}$. To construct the mutants, single insect-specific residue introduction into $\beta2^{Mp\beta1}$ was carried by site-directed mutagenesis using the QuikChange method (Stratagene). All plasmid, chimera and mutant constructs were verified by nucleotide sequencing.

2.4. In vitro transcription

Plasmids were linearized with NotI or NdeI. In vitro transcription, to generate cRNA, was performed using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, USA). Reactions were carried out according to the manufacturer's protocol. Transcripts were recovered by precipitation with propan-2-ol, dissolved in nuclease free water at a final concentration of 0.5 mg/mL and stored at −80 °C prior to use.

2.5. Oocyte preparation

Ovarian lobes were isolated from female *Xenopus laevis* frogs using standard procedures, as described previously (Yao et al., 2008). Clumps of stage V–VI oocytes were dissected in a sterile modified Barth's solution (NaCl 88 mM; KCl 1 mM; $MgCl_2$ 0.82 mM; $CaCl_2$ 0.77 mM; $NaHCO_3$ 2.4 mM; Tris–HCl 15 mM; with 50 U/mL penicillin and 50 μg/mL streptomycin; pH 7.4 adjusted with NaOH). The dissected oocytes were treated with collagenase (type IA, Sigma, USA; 65 min at 18 °C, 245 collagen digestion units/mL in Barth's solution, 10–12 oocytes/ml), rinsed, stored at 4 °C overnight, and manually defolliculated the following day before injection with cRNA (50 nL/oocyte). Nlα1 and rat β2 cRNAs were injected at a ratio of 1:1. The oocytes were incubated for approximately 60 h at 18 °C in Barth's solution containing 5% heat-inactivated horse serum (Gibco/Invitrogen, USA) and then stored at 4 °C. Experiments were carried out at a room temperature of 18–20 °C between 2 and 10 days after injection.

2.6. Electrophysiology

Oocytes, held in a 0.25 mL bath, were perfused at 4.5 mL/min with modified Ringer's solution (NaCl 150 mM, KCl 2.8 mM, HEPES 10 mM, $MgCl_2$ 2 mM, atropine sulfate 0.5 μM; pH 7.2, adjusted with NaOH) and voltage clamped at −70 mV using the two-electrode