Neuroscience Letters 589 (2015) 163–168

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Identification of key amino acid differences between *Cyrtorhinus lividipennis* and *Nilaparvata lugens* nAChR α8 subunits contributing to neonicotinoid sensitivity

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HIGHLIGHTS

- Neonicotinoids showed high toxicity to *Cyrtorhinus lividipennis*.
- A novel nAChR subunit Clα8 was cloned from *Cyrtorhinus lividipennis*.
- Key amino acid differences were found between Clα8 and *Nilaparvata lugens* α8.
- One different residue contributed to neonicotinoid sensitivity directly.
- One residue influenced sensitivity by enhancing direct effect of the other.

ARTICLE INFO

Article history:
Received 29 August 2014
Received in revised form
10 December 2014
Accepted 16 January 2015
Available online 20 January 2015

Keywords:
Cyrtorhinus lividipennis
Neonicotinoid selectivity
Nicotinic acetylcholine receptor
α8 subunit

ABSTRACT

High sensitivity to neonicotinoid insecticides have been reported in the miridbug *Cyrtorhinus lividipennis*, an important predatory enemy of rice planthoppers, such as *Nilaparvata lugens* (brown planthopper). In the present study, the sensitivity of neonicotinoid insecticides between *C. lividipennis* and *N. lugens* were detected and compared. The results showed that neonicotinoid insecticides were much more toxic to the miridbug than to the brown planthopper. A nicotinic acetylcholine receptor subunit was cloned from the miridbug and denoted as α8 subunit (Clα8) according to sequence similarities and important functional motifs. Key amino acid differences were found in specific loops from α8 subunits between *C. lividipennis* (Clα8) and *N. lugens* (Nlα8). In order to understand the roles of key amino acid differences in insecticide sensitivities, the different amino acid residues in specific loops of Nlα8 were introduced into the corresponding sites in Clα8 to construct several subunit mutants. Clα8 or subunit mutants were co-expressed with rat β2 to obtain the functional receptors in *Xenopus* oocytes. The single mutation N191F in loop B reduced imidacloprid sensitivity, with EC_{50} value in $Cl\alpha8^{N191F}/\beta2$ of 15.21 μM and 5.74 μM in Clα8/β2. Interestingly, although the single mutation E240T in loop C did not cause the significant change in imidacloprid sensitivity, it could enhance the effects of N191F and cause more decrease in imidacloprid sensitivity. The results indicated that E240T might contribute to neonicotinoid sensitivity in an indirect way.

1. Introduction

With the development of agriculture science and technology, a variety of pest control methods are available, such as chemical control, biological control, physical control and remote sensing [1]. Chemical and biological controls have been studied by many researchers, especially for two species predator–prey system or three-specie food chain system [2–4]. Chemical insecticides are considered useful because they can quickly wipe out a significant portion of pest population. However, the overuse of chemical insecticides causes many ecological and environmental problems and becomes a big health hazard to human being and natural enemies. Thus, in many cases, the biological and chemical control methods should be considered together for a better balance [5,6].

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http://dx.doi.org/10.1016/j.neulet.2015.01.046
0304-3940/

In previous researches, the brown planthopper outbreaks appeared to be prevented by predation of *Cyrtorhinus lividipennis* (Reuter) (Hemiptera: Miridae) [7,8], *Microvelia douglasi atrolineata* Bergroth (Hemiptera: Veliidae) [9] and spiders [10,11] in tropical rice without insecticides. Among several predators reported on rice hoppers, the green miridbug, *C. lividipennis*, is widely distributed in rice fields and plays important roles in biological controls against rice planthoppers. As the dominant predator in the irrigated rice, *C. lividipennis* mainly preyed on both eggs and young nymphs of rice planthoppers [11]. A predator nymph consumed an average of 7.5 eggs or 1.4 hoppers per day for a period of 14 days. Adults consumed about 10.2 eggs or 4.7 nymphs or 2.4 adults per day for a period of 10 days [12].

However, the irrational use of insecticides has led to the strong lethality to *C. lividipennis*. High sensitivity to neonicotinoid insecticides have been reported previously in *C. lividipennis*, in a laboratory study, and under field conditions [13,14]. Previous studies found that neonicotinoid insecticides like imidacloprid (selectivity ratio = 0.05) and clothianidin (selectivity ratio = 0.02) have no selectivity between *C. lividipennis* and *Nilaparvata lugens*, and *C. lividipennis* was more sensitive to these insecticides [15]. In addition, the sublethal dose of the neonicotinoid insecticides significantly impacted the egg hatchability, predacious number and fecundity of *C. lividipennis* [14,16].

Nicotinic acetylcholine receptors (nAChRs), the targets of neonicotinoid insecticides [17,18], are receptors of acetylcholine, the neurotransmitter of synaptic transmission in the nervous systems of insects [19]. The binding site for neurotransmitter or other agonist ligand exists on the interface of the *N*-terminal domains of two adjacent subunits [18,20]. Amino acid mutations located in 6 loops (loop A–C from α subunit and loop D–F from β subunit) which constitute the binding pocket have been reported to associate with resistance to neonicotinoids in various insect species [21,22]. For example, mutations in these loops of insect α subunits and β subunits were reported to be involved in neonicotinoid resistance insecticides in *Myzus persicae*, Aphis gossypii, *N. lugens* and *Drosophila melanogaster* [23–28]. Thus, researches on these domains of nAChR subunits are significantly important for the insecticide sensitivity and target resistance in insects.

High sensitivity to neonicotinoid insecticides of *C. lividipennis* was well-documented, but there is little information and knowledge about the possible molecular mechanism. In this study, the toxicity of neonicotinoid insecticides to *C. lividipennis* and *N. lugens* was compared. The trials to understand the different toxicity between two insect species were performed through the comparison of insecticide sensitivities on nAChRs from two species.

2. Materials and methods

2.1. Insects

The field populations of the brown planthopper (BPH) and the green miridbug were collected from the same rice field in Nanning (Guangxi) in July 2013. The susceptible strain of BPH was original collected from Taizhou (Zhejiang) in August 2003 and reared in the laboratory without the contact of any insecticides. The susceptible strain of the green miridbug was originally collected from Taizhou (Zhejiang) in July 2007 and reared in the laboratory without the contact of any insecticides. Uniform sized BPH were selected and reared on rice plants kept in the illumination incubator. Rice plants pre-oviposited by BPH were used to rear miridbugs. Adult green miridbug were inoculated to these plants and confined for 2–3 days for the oviposition to obtain nymphs with specific ages. All plants and insects were maintained at 26 ± 1 °C, 75 ± 5% RH in the illumination incubator.

2.2. Chemicals

Acetylcholine, Imidacloprid and Clothianidin were purchased from Sigma–Aldrich (St. Louis, MO, USA)

2.3. Contact acute toxicity (LC_{50}) and selectivity assessment

The contact acute toxicity was tested according to the method described by Preetha et al. [15]. Glass scintillation vials (15 ml) were coated with 0.5 ml of tested insecticide and rolled until no drops were seen on the glass wall to insure the uniformity of insecticide membrane. The vials were air dried for 1 h to allow all the acetone to evaporate before introducing the tested insects. A pre-experiment was performed to estimate the range of insecticide concentrations. Then five proper concentrations were selected with 10–90% mortalities. Acetone alone was used as the control. Approximately 30 tritonymphs of uniform size insects (miridbug/BPH) were introduced into the treated vials and the mouth was covered with double gauze. After 3 h exposure, the tested nymphs were transferred into a plastic cup containing fresh rice plants pre-oviposited by BPH for miridbugs and rice plants only for BPH. The mortalities were recorded in 48 h after treatments and the mortality in each treatment was corrected by Abbott formula. The data obtained was analyzed using software Polo. Selectivity ratio was calculated as described previously [13] using the following formula:

$$\text{Selectivityratio} = LC_{50}\text{ofbeneficialspecies}/LC_{50}\text{ofpestspecies}$$

The insecticide is selective when the value is >1 and non-selective when the value is ≤1.

2.4. Molecular cloning of Clα8

Total RNA was isolated from approximately 10 *C. lividipennis* using TRIzol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). Specific primers (5′ GSP: GTAATTGGACAGCAGATCGTCGTACAGCC; 3′ GSP: GGAGTCTGGGT ACGTCAATGATGGG) were designed according to the unigene (CL1597.Contig1) from a transcriptome to obtain the full length by RACE technique using GeneRacer™ (Invitrogen). The amplified product was separated by the agarose gel electrophoresis and purified using Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Purified DNA was ligated into the pGEM-T Easy vector (Promega) and several independent sub-clones were sequenced from both directions. Sequence homology was determined using the NCBI BLAST online services at http://www.ncbi.nlm.nih.gov/BLAST. Protein alignments were performed using the ClustalW program (http://www.ebi.ac.uk/services/).

2.5. Expression and electrophysiological recording in Xenopus oocyte

The *N. lugens* Nlα8 (FJ481979) and *Rattus norvegicus* subunit rat β2 (L31622) were subcloned into the expression vector pGH19 as described previously [29,30]. Clα8 was subcloned into vector pGH19 at EcoRI and XbaI sites. Clα8 mutants ($Cl\alpha8^{N191F}$, $Cl\alpha8^{E240T}$ and $Cl\alpha^{N191F/E240T}$) were constructed by the overlap extension PCR using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Subunit cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, Foster, CA, USA). Xenopus oocyte preparation and cRNA injection were performed as described previously [29]. Electrophysiological recordings were made using a two-electrode voltage clamp (Multiclamp 700B Amplifier; Axon Instruments, Foster, CA, USA) as previously described [29].