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IPPA08 allosterically enhances the action of imidacloprid on nicotinic acetylcholine receptors

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ABSTRACT

Our previous study showed that IPPA08, a *cis*-configuration neonicotinoid compound with unique oxabridged substructure, acted as a specific synergist to neonicotinoid insecticides targeting nicotinic acetylcholine receptors (nAChRs). Heteropentamer nAChRs have diverse characteristics and can form canonical and noncanonical subunit interfaces. While canonical interfaces have been exploited as targets of many drugs, noncanonical interfaces have received less attention. In this study, the mechanism of IPPA08 synergism was evaluated on hybrid nAChRs consisting of three α1 subunits from the brown planthopper and two rat β1 subunits (Nlα1/rβ2) expressed in *Xenopus* oocytes. IPPA08 alone evoked inward currents, but only at very high concentrations, greater than 1 mM. However, at concentrations below 200 μM, IPPA08 slowed the decay of inward currents evoked by imidacloprid, but not by acetylcholine, and also increased the sensitivity of Nlα1/rβ2 to imidacloprid. Both modulations by IPPA08 were concentration-dependent in the same concentration range of 10–150 μM. Experimentally induced mutations in canonical (α+/β−) and noncanonical (β+/α−) interfaces of Nlα1/rβ2 receptors were also examined to evaluate the presence of possible binding sites for IPPA08 on the receptors. Our results showed that mutations in the canonical interfaces affected only the potency of IPPA08 as an agonist, while mutations in the noncanonical interfaces affected only the synergistic action of IPPA08. Based on these results, we propose that at low concentrations IPPA08 can act as a positive allosteric modulator of noncanonical interfaces, and likely slow the decay of currents through stabilizing the open-channel state caused by the action of imidacloprid on canonical interfaces.

1. Introduction

In the insect central nervous system (CNS), nicotinic acetylcholine receptors (nAChRs) are the most abundant neurotransmitters and therefore they serve as important targets for insecticides such as neonicotinoids (Breer and Sattelle, 1987; Millar and Denholm, 2007). nAChRs are cys-loop receptors and pentameric complexes assembled from identical subunits (homopentamer receptors) or different subunits (heteropentamer receptors) with the binding sites located at the interface between two adjacent subunits for important ligands such as the endogenous neurotransmitter, acetylcholine (ACh), and neonicotinoid insecticides (Brejc et al., 2001; Millar and Denholm, 2007; Smit et al., 2001). The ligand binding pocket at the interface of subunits consists of loops A, B, and C from one subunit (α subunit) and loops D, E, and F from the adjacent subunit (non-α subunit for heteropentamer receptors or α subunit for homopentamer receptors) (Brejc et al., 2001; Smit et al., 2001).

In insects, the binding site of neonicotinoids is at the interface between one α subunit and one β subunit in heteropentamer receptors consisting of two or more different subunits (Chamaon et al., 2002; Huang et al., 2000; Li et al., 2010; Liu et al., 2009; Schulz et al., 2000). Since nAChRs are complexes consisting of five subunits, there are five interfaces between two adjacent subunits in the heteropentamer receptor. Not all these interfaces have the binding pockets that are formed directly by loops A, B, and C

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from the α subunit (referred to as α+) and by loops D, E, and F from the β subunit (referred to β−). Thus far, studies on neonicotinoid binding sites have been confined to the α+/β− interfaces, with several reports confirming the importance of specific loops in α+ and β- subunits (Shimomura et al., 2004, 2006; Song et al., 2009). If α+/β− interfaces are considered as canonical interfaces, it is reasonable to presume that there should be some noncanonical interfaces, such as a β+/α−, α+/α− or β+/β− interface based on the subunit stoichiometry (Seo et al., 2009). Such revelation will help develop compounds binding to the putative pocket at the noncanonical interfaces and understand the relationship between compounds acting at the noncanonical and canonical interfaces.

Recently, some oxabridged compounds were synthesized from nitromethylene analogues of imidacloprid (Shao et al., 2010). Several of these compounds showed high insecticidal activities, and a compound with a 7-member oxabridge designated as cycloxaprid is being commercialized (Fig. S1). Compared to the high insecticidal activity of cycloxaprid, a compound with an 8-member oxabridge, IPPA08 (Fig. S1), showed extremely low toxicity against insects, but exhibited significant synergistic effects with other commercial neonicotinoids; i.e., IPPA08 increased their insecticidal activities against a range of insect species (Bao et al., 2016). In a previous study, we demonstrated that IPPA08 did not exert its synergistic effects by inhibiting the activities of detoxification enzymes, such as P450 monooxygenases (P450s), and that it could possess a distinct mode of action (Bao et al., 2016). In this study, we evaluated the possible action mode of IPPA08 and its relationship with neonicotinoids through target proteins.

2. Material and methods

2.1. Chemicals

Acetylcholine (ACh), dihydro-β-erythroidine (DHβE) and imidacloprid were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]imidacloprid (32 Ci/mmol) was generously provided by Syngenta Ltd., European Regional Centre, Guildford, UK. The representative oxabridged neonicotinoid, IPPA08, was synthesized and purified as previously described (Shao et al., 2010).

2.2. Expression and electrophysiological recordings in Xenopus oocytes

The nAChR subunit, Nlα1, from *Nilaparvata lugens*, the rβ2 subunit from *Rattus norvegicus* and Cα7 subunits from chicken were subcloned into the expression vector pGH19 as previously described (Liu et al., 2006). Subunit cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, Austin, TX, USA) according to the manufacturer's protocol. *Xenopus* oocyte preparation, cRNA injection and electrophysiological recordings were performed as previously described (Liu et al., 2006). In the antagonist test, equilibrium was first achieved by applying the antagonist 5 min prior to applying the agonist. IPPA08 and neonicotinoids were applied together to test the effects of IPPA08 on the potential agonist actions of neonicotinoids.

2.3. Constructing mutant Nlα1 and rβ2 subunits

Alignment of Nlα1 and Rα3 (Rat nAChR α3 subunit) protein sequences revealed differences in amino acids in loops A, B, and C from the canonical interface and also in loops D, E, and F from the putative noncanonical interface (Fig. 1A). Similarly, an alignment of rβ2 and Nlβ1 subunits revealed amino acid differences in loops D, E, and F from the canonical interface and also in loops A, B, and C from the putative noncanonical interface (Fig. 1B). Based on these amino acid differences mutations were created in Nlα1 and rβ2 by site-directed mutagenesis using the QuickChange method (Stratagene). Because only one amino acid residue is different between Nlα1 and Rα3 in loop A, N84 (amino acid with uncharged polar side

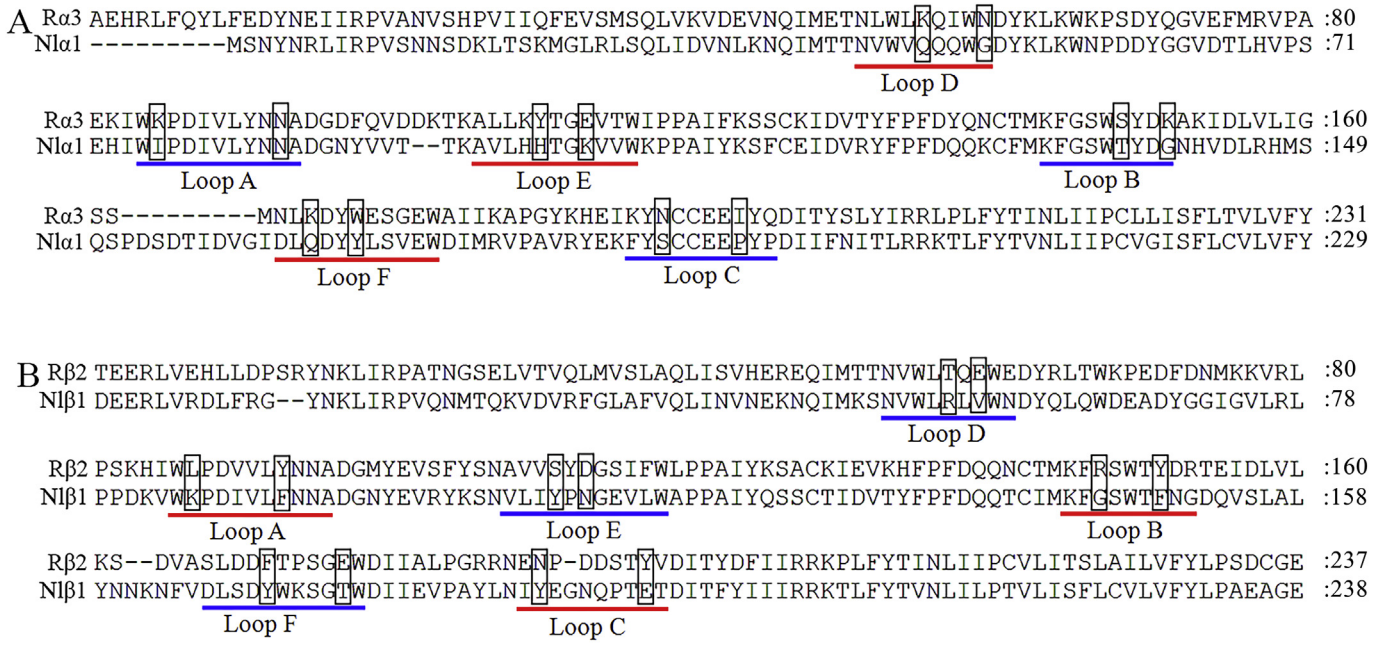

Fig. 1. Alignment of amino acid sequences of nAChR subunits. Blue underlines indicate loops constituting the canonical interfaces. Red underlines indicate loops constituting the noncanonical interfaces. The key sites for mutation are shown in boxes. (A) At each boxed site, the residue in Nlα1 was experimentally mutated to the corresponding residue in Rα3 to construct the Nlα1 mutant except residue N84. Because only one amino acid residue is different between Nlα1 and Rα3 in loop A, the residue N84 (amino acid with uncharged polar side chain) was mutated into residue K (amino acid with positive charged side chain) to study the influence of residues in loop A. The mutated residues are numbered based on the Nlα1 sequence. (B) At each boxed site, the residue in Rβ2 was experimentally mutated into the corresponding residue in Nlβ1 to construct the Rβ2 mutant. The mutated residues are numbered based on the Rβ2 sequence. Nlα1, *Nilaparvata lugens* α1 subunit, Genbank AAQ75737.1; Nlβ1, *N. lugens* β1 subunit, ACJ07013.1; Rα3, *Rattus norvegicus* α3 subunit, AAA41673.1; Rβ2, *R. norvegicus* β2 subunit, AAC78724.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)