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Association of Neonicotinoid Insensitivity with a Conserved Residue in the Loop D Binding Region of the Tick Nicotinic Acetylcholine Receptor

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Supporting Information

ABSTRACT: Neonicotinoid insecticides target nicotinic acetylcholine receptors (nAChR) in the nervous system of insects but are largely ineffective against ticks. This study aimed to identify the molecular basis for this insensitivity. A homology model of the nAChR binding domain was generated on the basis of the crystal structure of an acetylcholine-binding protein with the insecticide imidacloprid bound. We hypothesized that tick β-subunits would differ at a critical residue (Arg81) in their D loops. To test this, we sequenced nAChR genes from five tick species and found that instead of the conserved arginine found in insects, a glutamine was present in all the tick sequences.

The neonicotinoids have emerged as the most important class of synthetic insecticides of the past 20 years.[1,2] Imidacloprid was the commercial forerunner in this class, members of which function as agonists of insect nicotinic acetylcholine receptors (nAChRs). nAChRs make up a family of neurotransmitter-gated ion channels that mediate fast signaling at postsynaptic membranes of vertebrates and invertebrates. In insects, the nAChRs are restricted to the nervous system, where they are present at high densities.[3,4] Previous studies have suggested that differences in nAChR binding affinities underlie the selective toxicity of neonicotinoids for insects relative to vertebrates.[5−7] However, to date, the molecular basis of why these insecticides are also less effective against the closely related arachnid species such as ticks[8] has yet to be explored.

nAChRs exist as hetero- or homopentamers comprised of "α" and "non-α" type subunits; the classification of subunit types is based on the presence (α) or absence (non-α) of vicinal cysteine residues in their C loop region. Studies of mammalian nAChRs demonstrate that different subunit combinations can produce nAChRs with diverse pharmacological profiles.[9] Although insect nAChRs are as yet less well characterized than their vertebrate counterparts, there has been considerable progress in identifying gene families that encode individual subunits that constitute mature insect receptors. For example, the *Drosophila melanogaster* genome encodes 10 subunits; seven are α and three are non-α subtypes.[10]

The acetylcholine (like the nicotinic ligand) binding site is located within the extracellular domain of the receptor at the interface between subunits, where conserved sequences from six different regions (loops A−C and D−F of adjacent subunits) have been proposed to comprise the ligand binding domain (LBD).[11] High-resolution crystal structures of nAChRs have yet to be obtained; however, structures of closely related molluscan acetylcholine binding proteins (AChBPs), with sequences that are 25−30% identical to those of insect subunits, are useful modeling templates for the extracellular domains of nAChRs including the LBD.[12] More recently, AChBPs have been cocrystallized with bound nicotinic ligands and neonicotinoids, providing detailed structural information and identifying key residues involved in binding.[13,14]

The common feature of neonicotinoids crucial for insect selectivity is the presence of an electronegative group, either the N-nitro group present in imidacloprid or an N-cyano group in other members of the class.[3,15] Removal of this group has a dramatic effect on selectivity for different organisms. For example, desnitroimidacloprid exhibits similar levels of activity against both insect and vertebrate nAChRs.[16] Sequence analysis has identified a positively charged residue conserved in loop D of insect β-subunits that is replaced with uncharged or negatively charged residues in vertebrate species.[5] The side chain of this residue was proposed to electrostatically interact favorably (insect nAChRs) or repulsively or passively (vertebrate nAChRs) with the neonicotinoid electronegative pharmacophore. Substituting arginine at this position in a chick receptor (Q79R) produced an increase in sensitivity to imidacloprid, supporting the hypothesis that this residue is a determinant of neonicotinoid selectivity.[5] In addition, the first report of field-evolved resistance to a neonicotinoid associated with target site modification in the aphid, *Myzus persicae* (Figure 1), involves an arginine-to-threonine substitution at this key position in subunit β1.[17]

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Figure 1. Sequence alignment of the loop D regions of insect β-subunits with those of the *Ixodes scapularis* 8451 tick and *Pardosa pseudoannulata* (wolf spider). The arachnid species names are in bold. Residues that are identical in all species are highlighted in blue. The loop D residue of interest is highlighted in red (glutamine in arachnid) or green (arginine in insect). Figure S1 of the Supporting Information includes the full-length sequences of these and other insect β-subunits.

In this study, we used a combination of homology modeling and sequence determination to explore the structural basis for the insensitivity of tick species to neonicotinoids.

The only full sequence of an arachnid nAChR β-type subunit reported to date is that from the wolf spider.[18] We identified another arachnid nAChR β-subunit sequence (ISCW008451) in the preliminary genome of the deer tick (*Ixodes scapularis*) available at VectorBase.[19] Comparison of these sequences with those of insect β-subunits (Figure 1 and Figure S1 of the Supporting Information) shows that the positively charged arginine residue, conserved in the D loops of insect β-subunits, is replaced with an uncharged glutamine (Q81) in both arachnid sequences, suggesting this may be the site that is critical for the neonicotinoid selectivity of insects but not ticks. We developed a homology model of the tick receptor (Figure 2,

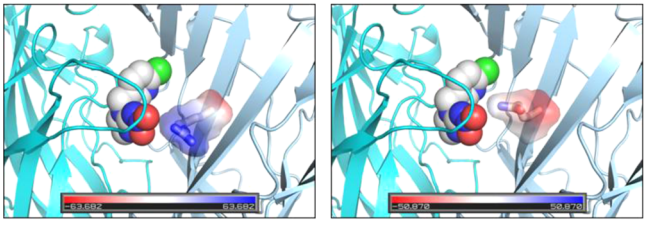

Figure 2. Modeled structures of nAChRs (ribbons) complexed with the neonicotinoid imidacloprid (space filling), with the loop D residue of the β-subunit shown as sticks overlaid with an electrostatic plot of that residue: (left) arginine side chain found in insects and (right) glutamine side chain found in arachnids. The model was created using the *Aplysia californica* binding protein structure (PDB entry 3C79) with the *I. scapularis* ISCW003334 α-subunit and ISCW008451 β-subunit sequences. These models show that the arginine found in insects could optimally interact electrostatically with the negative region of the insecticide, whereas the glutamine of the arachnids would not.

right) based upon an AChBP structure [Protein Data Bank (PDB) entry 3C79] and also introduced the insect-specific arginine replacement (Figure 2, left) at this site to investigate the differences in potential interactions of these residues with imidacloprid. These models suggest that while the insecticide would fit sterically in a binding site containing either arginine or glutamine, given the orientation of the ligand in the binding pocket, the electrostatic interactions would be much less favorable with glutamine than with arginine. Hence, we hypothesized that the lack of positive charge at this residue position was critical for the decreased activity of neonicotinoids toward arachnids.

To test the hypothesis, we determined the binding loop sequences of β-subunit nAChRs from five tick species of agricultural or medical interest: brown dog tick (*Rhipicephalus sanguineus*), cattle tick [*Rhipicephalus* (*Boophilus*) *microplus*], ornate cow tick (*Dermacentor reticulatus*), deer tick (*Ixodes scapularis*), and castor bean tick (*Ixodes ricinus*) (see the Supporting Information for experimental procedures). Figure 3

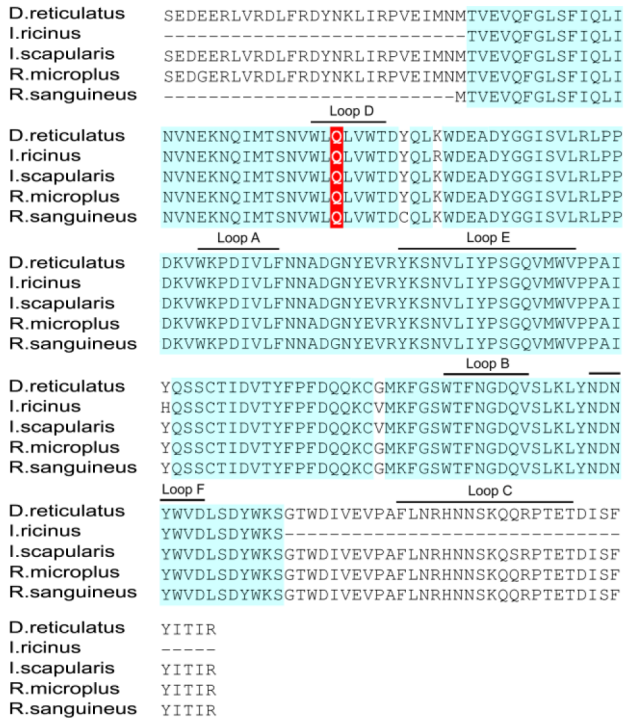

Figure 3. Sequence alignment of the binding site regions of β-subunits from the five tick species (*R. sanguineus*, *R.* (*Boophilus*) *microplus*, *D. reticulatus*, *I. scapularis*, and *I. ricinus*) determined in this study. Residues that are identical in all species are highlighted in blue. The conserved glutamine in loop D that is present in all these arachnids is highlighted in red. This residue is a highly conserved arginine in insect β-subunits. A larger version of this figure is included as Figure S2 of the Supporting Information.

shows that all five tick species have a glutamine residues at position 81 in loop D (highlighted in red) and not the highly conserved arginine present in insect β-subunits, consistent with our hypothesis.

A number of other residues within loops A–F comprising the binding domain have been shown to be important for neonicotinoid binding, so we considered whether they might also be involved in selectivity. These include residues within loop B (tryptophan 143), loop C (tyrosine 185 and proline 186), and residues within loop E (methionine 116 and isoleucine 118).[16,20] However, closer analysis of the residues at these positions in our tick LBD model did not reveal any significant interactions with the ligand, further implicating the loop D glutamine 81 replacement as the most likely cause of the neonicotinoid insensitivity.

Thus, we propose that the lack of efficacy and specificity of neonicotinoids for ticks (and other arachnids) is due to the absence of a positively charged residue at a critical position in the loop D binding region of their β-subunits. Previous studies

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proposed that this loop D arginine is a key physiochemical determinant for binding to the neonicotinoids in insects, and this study extends this to suggest a role for it in invertebrate species selectivity, although future functional studies will be needed to confirm this hypothesis.

Consequently, the identification of the role of this residue in insect versus tick selectivity should ultimately lead to the design of more specific nicotinic ligands and will form the basis for the development of tick-specific cell-based assays for identifying novel nicotinic agonists and antagonists suitable for the control of acari.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest: A.T. is employed by Bayer Animal Health.

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