Role in the Selectivity of Neonicotinoids of Insect-Specific Basic Residues in Loop D of the Nicotinic Acetylcholine Receptor Agonist Binding Site

Masaru Shimomura, Maiko Yokota, Makoto Ihara, Miki Akamatsu, David B. Sattelle, and Kazuhiko Matsuda

ABSTRACT

The insecticide imidacloprid and structurally related neonicotinoids act selectively on insect nicotinic acetylcholine receptors (nAChRs). To investigate the mechanism of neonicotinoid selectivity, we have examined the effects of mutations to basic amino acid residues in loop D of the nAChR acetylcholine (ACh) binding site on the interactions with imidacloprid. The receptors investigated are the recombinant chicken α4β2 nAChR and Drosophila melanogaster D2/chicken β2 hybrid nAChR expressed in Xenopus laevis oocytes. Modifications of Thr77 in loop D of the β2 subunit resulted in a barely detectable effect on the imidacloprid concentration-response curve for the α4β2 nAChR. T77R/E79V double mutations shifted the curve dramatically to higher affinity binding of imidacloprid. Likewise, T77K/E79R and T77N/E79R double mutations in the D2/β2 nAChR also resulted in a shift to a higher affinity for imidacloprid, which exceeded that observed for a single mutation of Thr77 to basic residues. By contrast, these double mutations scarcely influenced the ACh concentration-response curve, suggesting selective interactions with imidacloprid of the newly introduced basic residues. Computational, homology models of the agonist binding domain of the wild-type and mutant α4β2 and D2/β2 nAChRs with imidacloprid bound were generated based on the crystal structures of acetylcholine binding proteins of Lymnaea stagnalis and Aplysia californica. The models indicate that the nitro group of imidacloprid interacts directly with the introduced basic residues at position 77, whereas those at position 79 either prevent or permit such interactions depending on their electrostatic properties, thereby explaining the observed functional changes resulting from site-directed mutagenesis.

Nicotinic acetylcholine receptors (nAChRs) play a central role in rapid cholinergic synaptic transmission (Sattelle, 1980; Sattelle and Breer, 1990) and are important targets of insecticides (Gepner et al., 1978; Matsuda et al., 2001, 2005). Of the insecticides acting on insect nAChRs, imidacloprid and its analogs (Fig. 1), referred to as neonicotinoids, are used worldwide as agrochemicals (Matsuda et al., 2001, 2005; Tomizawa et al., 2003; Tomizawa and Casida, 2005). 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 et al., 2003; Tomizawa and Casida, 2005). Neonicotinoids possess either a nitro or a cyano group; these groups have been postulated to contribute directly to their selectivity (Matsuda et al., 2001, 2005; Tomizawa et al., 2003; Tomizawa and Casida, 2005). The molecular targets of neonicotinoids are nAChRs, which belong to the Cys-loop family of ligand-gated ion channels and usually consist of α and non-α subunits (Corringer et al., 2000; Karlin, 2002; Lindstrom, 2003). How-

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ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; PCR, polymerase chain reaction; SOS, standard oocyte saline; PDFAMS, Protein Discovery Full Automatic Modeling System; AChBP, acetylcholine binding protein; PDB, Protein Data Bank.
ever, α7, α8, and α9 subunits each form functional homomers when expressed in *Xenopus laevis* oocytes (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994), although α7 (Palma et al., 1999; Azam et al., 2003) and α9 (Elgoyhen et al., 2001) subunits can coassemble with other subunits, resulting in characteristics distinct from those of homomers.

The binding site for ACh and other agonists is formed by six loops, A–F, in the extracellular, N-terminal domain. Loops A–C are confined to α subunits, whereas loops D–F are present either in non-α subunits of α/non-α heteromers or in the α subunits of homomers (Corringer et al., 2000) and α/α heteromers (e.g., α9/α10) (Elgoyhen et al., 2001).

We have shown previously that when the nitro group of imidacloprid interacts with basic residues, the nitrogen atoms in the imidazolidine ring of imidacloprid become positive, thereby strengthening cation-π interactions with tryptophan residues in loop B (Matsuda et al., 2001, 2005). This “induced-fit” mechanism can also account for the selective actions of other neonicotinoids such as thiacloprid and acetamiprid, both of which possess a cyano group. Consistent with this view, we have found that Q79R and Q79K mutations in loop D of chicken α7 homomer enhance the peak current amplitude of the currents recorded from the expressed receptor in response to imidacloprid and nitenpyram (Shimomura et al., 2002). Because most insect nAChR non-α subunits possess basic residues in loop D at the position corresponding to Gln79 (residue numbering is from the start methionine) of the α7 subunit (Table 1), it was postulated that such basic residues are likely to contribute to the selective neonicotinoid actions on insect nAChR. Nevertheless, although significant, the shift in the neonicotinoid concentration-response curves resulting from the Q79R and Q79K mutations in the α7 nAChR was small (Shimomura et al., 2002) and insufficient to account for neonicotinoid selectivity. In addition, it is possible that changes in the responses to neonicotinoids resulting from the addition of basic residues to loop D may be limited to the homomeric α7 nAChRs.

In the present study, two-electrode voltage-clamp electrophysiology has been employed to investigate the effects on the responses to imidacloprid of chicken α4β2 and *Drosophila melanogaster* Dco2/chicken β2 hybrid nAChRs of mutating Thr77 and Glu79 in the β2 subunit to basic amino acid residues. These residues correspond to Gln79 and Tyr81 of the α7 nAChR (see Table 1). To assist in interpreting the results from mutagenesis experiments, three-dimensional models for the agonist binding site of the nAChRs with imidacloprid docked have been constructed based on the crystal structures of acetylcholine binding proteins (AChBPs), with either nicotine (Celie et al., 2004) or epibatidine (Hansen et al., 2005) bound, both of which share the pyridine ring with imidacloprid. The models indicate that not only basic residues but also neighboring structural features are involved in the selectivity of nAChR-imidacloprid interactions.

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**TABLE 1**

<table>
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<th>Subunits</th>
<th>Amino Acid Number of Chicken β2 Subunit&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>73</td>
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<tr>
<td><em>Vertebrates</em></td>
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</tr>
<tr>
<td>Chicken α7</td>
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<tr>
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<tr>
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<td>N</td>
</tr>
<tr>
<td>Myzus persicae β1</td>
<td>N</td>
</tr>
<tr>
<td>Heliothis virescens β1</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Residue numbering is from the start methionine.
Materials and Methods

Preparation of DNAs Encoding Mutant β2 Subunits. The chicken nAChR β2 subunit cDNA in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) was used as a template for mutagenesis (Swick et al., 1992; Bertrand et al., 1994). Mutations were introduced by PCR as described previously (Matsuda et al., 2000; Shimomura et al., 2002). Oligonucleotides T77N sense and T77N antisense were prepared to generate the β2 T77N mutation in the β2 subunit. The T7 primer (5'-TAAATAGCAGCTACTAAGGGGACC3'-T) and β2 antisense primer (5'-CTTCATTGACAGCACATGC-3') were designed, respectively, on the basis of the sequence flanking the multiple cloning site of the pcDNA3.1 vector and the β2 cDNA approximately 1.3 kilobase pairs downstream of the start methionine codon. The first round PCR was carried out using 1 U of KOD-Plus polymerase (Toyobo, Shiga, Japan), 100 ng of wild-type pcDNA3.1-β2 of the first round PCR products and 0.3 X. laevis Mature/nAChR/H11032/1.3-kilobase pair BamHI fragment of pcDNA3.1-β2 were digested using BamHI (Takara, Shiga, Japan) and subcloned into rose gel (Promega, Madison, WI), the isolated PCR fragment was a single DNA band. After purification using a low melting-point agarose gel (Promega, Madison, WI), the isolated PCR fragment was digested using BamHI (Takara, Shiga, Japan) and subcloned into pcDNA3.1-β2. This plasmid was cut with BamHI and ligated with a 1.3-kilobase pair BamHI fragment of pcDNA3.1-β2 to complete the full-length mutant β2 subunits. Other DNA constructs encoding the T77N, T77R, E79R, E79V, T77K/E79R, T77N/E79R and T77R/E79V mutants were prepared in a similar manner.

Preparation and Nuclear Injection of X. laevis Oocytes. Mature X. laevis female frogs were anesthetized by immersion in 1.5 g/l tricaine for 30 to 45 min, depending on body weight, before removal of a part of the ovary. We made as much effort as possible to minimize animal suffering and reduce the number of animals used. Oocytes at stage V or VI of development were separated from the follicle cell layer after treatment with 2 mg/ml collagenase (type IA; Sigma, St. Louis, MO). The nucleus of each defolliculated oocyte was removed of a part of the ovary. We made as much effort as possible to generate the response to ACh. For example, data from the wild-type chicken nAChR-imidacloprid complex were constructed based on the sequence flanking the multiple cloning site of the pcDNA3.1 vector and the β2 cDNA approximately 1.3 kilobase pairs downstream of the start methionine codon. The first round PCR was carried out using 1 U of KOD-Plus, 20 ng each primer and 0.3 mM dNTP mixture in a 50-μl solution for 30 cycles of 94°C for 15 s, 45°C for 30 s, and 68°C for 60 s. The second-round PCR was conducted using 1 U of KOD-Plus, 20 ng each of the first round PCR products and 0.3 μM primers (T7 primer and β2 antisense), and 0.2 mM dNTP mixture in a 50-μl solution for 30 cycles of 94°C for 15 s, 48°C for 30 s, and 68°C for 90 s, yielding a single DNA band. After purification using a low melting-point agarose gel (Promega, Madison, WI), the isolated PCR fragment was digested using BamHI (Takara, Shiga, Japan) and subcloned into pcDNA3.1-β2. This plasmid was cut with BamHI and ligated with a 1.3-kilobase pair BamHI fragment of pcDNA3.1-β2 to complete the full-length mutant β2 subunits. Other DNA constructs encoding the T77N, T77R, E79R, E79V, T77K/E79R, T77N/E79R and T77R/E79V mutants were prepared in a similar manner.

Bioinformatics. X. laevis oocytes or somatic cells were cultured in a recording chamber that was perfused with a saline solution with water molecules was minimized using a procedure similar to that outlined above. The model of the wild-type D. melanogaster D2/chicken β2 nAChR bound by imidacloprid was constructed in a manner similar to that used to study α4β2 nAChR-imidacloprid complex. In mutant receptor models, Thr77 and Gln79 of the β2 subunit were replaced by the mutated residues in the alignment.
Subsequent procedures were the same as employed for the wild-type proteins.

Results

In voltage-clamp electrophysiological studies, ACh and imidacloprid evoked inward currents in a dose-dependent manner in *X. laevis* oocytes expressing the wild-type and mutant αβ2 and Do2β2 nAChRs. The concentration-response curves for ACh and imidacloprid for the wild-type αβ2 and Do2β2 nAChRs, newly measured as control receptors in this study, resemble closely those previously reported (Shimomura et al., 2003, 2005). The $I_{\text{max}}$ (normalized maximum response) and pEC$_{50}$ [log (1/EC$_{50}$ (M))] values of ACh for the nonmutated αβ2 nAChR were 1.18 ± 0.04 and

Fig. 2. Concentration-response curves of acetylcholine (ACh) (A, C, E, and G) and imidacloprid (IMI) (B, D, F, and H) obtained for wild-type, T77K, T77N, and T77R mutants of the αβ2 (A and B) and Do2β2 (C and D), and wild-type, E79R, and E79V mutants of the αβ2 (E and F) and Do2β2 (G and H) nicotinic acetylcholine receptors expressed in *X. laevis* oocytes. Each point plotted represents mean ± S.E.M. of four to eight experiments.
5.14 ± 0.07 (n = 7), respectively, whereas the $I_{\text{max}}$ and $pEC_{50}$ values for the wild-type Do2β2 nAChR were 1.02 ± 0.02 and 4.76 ± 0.03 (n = 7), respectively (Fig. 2, Table 2). The responses to imidacloprid of the wild-type α4β2 nAChR were too small to obtain the $I_{\text{max}}$ and $pEC_{50}$ values, whereas imidacloprid activated the wild-type Do2β2 nAChR with $I_{\text{max}}$ and $pEC_{50}$ values of 0.55 ± 0.03 and 5.45 ± 0.08 (n = 4), respectively (Fig. 2, Table 2).

The $pEC_{50}$ value of ACh for the α4β2 nAChR was minimally shifted by T77K [5.08 ± 0.13 (n = 5)], T77N [5.47 ± 0.12 (n = 5)], T77R [4.83 ± 0.12 (n = 6)], E79R [4.86 ± 0.16 (n = 4)], and E79V [4.76 ± 0.05 (n = 4)] mutations (Fig. 2, A and E; Table 2). Likewise, imidacloprid failed to activate the α4β2 nAChR irrespective of the presence or absence of such single amino acid replacements in loop D (Fig. 2, B and F, Table 2).

When Thr77 in loop D of the β2 subunit was replaced by lysine in the Do2β2 nAChR, the ACh concentration-response curve was shifted to the right ($pEC_{50} = 4.55 ± 0.03 (n = 6)$) (Fig. 2C, Table 2). However, this was unique, and all other changes in the concentration-response curve of ACh for the Do2β2 nAChR after a single amino acid mutation at position 77 or 79 in loop D of the β2 subunit were minimal: Do2β2 (T77N) mutant, 4.88 ± 0.04 (n = 5); Do2β2 (T77R) mutant, 4.83 ± 0.04 (n = 5); Do2β2 (E79R) mutant, 4.70 ± 0.05 (n = 5); Do2β2 (E79V) mutant, 4.75 ± 0.04 (n = 6) (Fig. 2, C and G, Table 2). On the other hand, T77N or T77R mutation in loop D of the β2 subunit resulted in significant shifts of the imidacloprid concentration-response curve to the left [$pEC_{50}$ values of imidacloprid for the T77N and T77R mutants of Do2β2 nAChR were 6.10 ± 0.12 (n = 4) and 6.11 ± 0.12 (n = 7), respectively] (Fig. 2D, Table 2). In all other cases, the concentration-response curves for the Do2β2 nAChR of imidacloprid resulting from single amino acid replacements were similar to those observed for Do2β2 wild-type nAChR (Fig. 2, D and H, Table 2).

To help interpret these results, three models of the ligand binding site of the α4β2 and Do2β2 nAChRs bound by imidacloprid were constructed (Fig. 3). The wild-type models (Fig. 3, A and B) show Thr77 in proximity to the nitro group of imidacloprid. Comparing α4β2 and Do2β2 nAChR models indicates stronger contact with the nitro group of the insecticide in the case of the hybrid Do2β2 nAChR. In both cases, Glu79 seems to indirectly influence the interactions of Thr77. Thus, mutation of Thr77 and Glu79 is predicted to influence imidacloprid interactions with both nAChRs under investigation. Fig. 3, C and D, show that T77R;E79V double mutations in the α4β2 and Do2β2 nAChRs can assist in the electrostatic interactions of the basic residue with the nitro group of imidacloprid, thereby enhancing markedly agonist affinity. Therefore, we have also investigated the effects of combined mutations of Thr77 and Glu79 in loop D to residues observed in the insect non-α subunits on the responses to imidacloprid of the α4β2 and Do2β2 nAChRs.

Compared with the effects of the single amino acid mutations, the effects of double amino acid mutations at positions 77 and 79 were striking. The T77R;E79V double mutant of the α4β2 nAChR showed greatly enhanced responses to imidacloprid (Fig. 4, A and B). The $pEC_{50}$ and $I_{\text{max}}$ values of imidacloprid for the α4β2 (T77K;E79R) mutant were 4.50 ± 0.18 and 0.53 ± 0.06 (n = 6), respectively (Table 2).

The T77K;E79R, T77N;E79R, and T77R;E79V mutations of the Do2β2 nAChR shifted the imidacloprid concentration-response curve dramatically to the left (Figs. 4, C and D, and 5D). The $pEC_{50}$ values of imidacloprid for the T77K;E79R, T77N;E79R, and T77R;E79V mutants were 5.87 ± 0.07, 6.58 ± 0.06, and 6.19 ± 0.12, respectively (Table 2). The $I_{\text{max}}$ values obtained from the dose-response curve of imidacloprid for the T77K;E79R, T77N;E79R, and T77R;E79V mutants were 0.63 ± 0.03 (n = 5), 0.65 ± 0.02 (n = 6), and 0.44 ± 0.03 (n = 8), respectively (Table 2). By contrast, for both receptor

### Table 2

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<th>Acetylcholine</th>
<th>Imidacloprid</th>
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<tr>
<td>$I_{\text{max}}$</td>
<td>$pEC_{50}$</td>
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<tr>
<td>α4β2 Wild-type</td>
<td>1.18 ± 0.04</td>
</tr>
<tr>
<td>α4β2 T77K</td>
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<td>α4β2 T77N</td>
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<tr>
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<tr>
<td>α4β2 E79R</td>
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<td>α4β2 E79V</td>
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</tr>
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<td>α4β2 T77R;E79V</td>
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</tr>
<tr>
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</tr>
<tr>
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N.D., not determined because the concentration-response curve did not plateau even at 1 mM.

* $P < 0.05$; ** $P < 0.01$.

Data are from Shimomura et al. (2005).
types, the dose-response curves for ACh were much less affected by the double mutations in loop D of the β2 subunit (Fig. 5, A and C, Table 2).

Discussion

In this study, we have shown that amino acid substitutions in loop D designed to mimic insect non-α subunits greatly increase the affinity of imidacloprid in terms of the pEC\textsubscript{50} value for the α4β2 and α2β2 nAChRs, whereas the impact on ACh concentration-response curve is much smaller. This points to an important role for loop D in the selectivity of imidacloprid for the recombinant nAChRs investigated.

To enhance significantly the imidacloprid-sensitivity of the α4β2 nAChR in terms of the shift of pEC\textsubscript{50} value, it was necessary to replace not only Thr77 by a basic residue, but also Glu79 by the neutral residue valine. An interpretation of this result is that the electrostatic force of Arg77 enhancing the affinity of Glu79 only when the T77R mutation was added to loop D. However, this “lock” is removed by the E79V mutation, resulting in an enhancement of the current amplitudes in response to imidacloprid. The shift of the imidacloprid concentration-response curve for the α4β2 and α2β2 nAChRs by the T77R;E79V double mutation was smaller than that induced by the T77R;E79V double mutation. In view of the result that the ACh concentration-response curves were shifted by the T77R;E79V mutation to higher concentrations irrespective of the kinds of nAChRs tested (Table 2), this mutation might alter the conformation of the agonist binding site to reduce the affinity of all agonists, thereby counteracting the enhancement of the affinity for imidacloprid by the basic residues.

We have generated three-dimensional models of wild-type and mutant α4β2 and α2β2 nAChRs bound by imidacloprid to help understand the mechanism underlying the results of the site-directed mutagenesis (Fig. 3). The locations of key amino acid residues [Tyr121 (loop A), Trp177 (loop B), Tyr218 (loop C), Cys220 (loop C), Cys221 (loop C), and Tyr225 (loop C) of the α4 subunit; and Trp75 (loop D) and Phe137 (loop E) of the β2 subunit; residue numbering is from the start methionine] in the models resemble those located in the model reported previously by Le Novère et al. (2002). The nitro group seems to be located apart from Thr77 in the wild-type α4β2 nAChR model (Fig. 3A), whereas the nitro group oxygens make contact with Arg77 in the T77R in the T77R;E79V mutant model (Fig. 3C), consistent with the shift to lower concentrations of the imidacloprid concentration-response curve after the T77R;E79V double mutation (Figs. 4 and 5). Likewise, the T77R;E79V double mutation, which resulted in a significant decrease of the EC\textsubscript{50} value of imidacloprid for the α2β2 hybrid nAChR (Figs. 4 and 5), placed the nitro group in contact with Arg77 in the model (Fig. 3D). Thus, the enhancement of the recombinant AChRs after the double site-directed mutagenesis is probably due to direct electrostatic interactions of the nitro group of imidacloprid with the basic residues in loop D.

The α2β2 hybrid nAChR was more sensitive to imidacloprid than the α4β2 nAChR in terms of the shift of the imidacloprid concentration-response curve, suggesting that the α2 subunit possesses structural features favoring interactions with imidacloprid. This agrees well with earlier findings that the region upstream of loop B and an amino acid in loop C also play a role in determining selectivity (Shimomura et al., 2004a, 2005). However, the contribution of the non-α subunit β2 to the interactions with imidacloprid should not be undervalued, as demonstrated earlier by Lansdell and Millar (2000). The molecular modeling (Fig. 3B) showed closer proximity of the nitro group to Thr77 in the α4β2 nAChR compared with its position in the α4β2 nAChR (Fig. 3A), thereby facilitating hydrogen bond formation between the ligand and the hybrid nAChR. Consistent with the models, not only T77N;E79R and T77R;E79V double mutations but also T77N and T77R point mutations significantly increased the pEC\textsubscript{50} value of imidacloprid in the α2β2 nAChR, which was not observed for the α4β2 nAChR. All these results seem to suggest that the amino acids newly introduced to position 77 in loop D are able to interact more strongly with the nitro group of imidacloprid in the α2β2 nAChR than in the α4β2 nAChR, thereby resulting in the

![Fig. 3. Homology models of the agonist binding domain of the wild-type α4β2 (A) and α2β2 (B) nAChRs and their T77R;E79V mutants (C, α4β2 nAChR; D, α2β2 nAChRs) bound by imidacloprid constructed using the crystal structures of the AChBP from L. stragnalis (PDB code 1UW6) and A. californica (PDB code 2BYQ). The figures were made using Sybyl version 6.91 and PDBFAMS (see Materials and Methods for details). In the wild-type nAChR models, only imidacloprid, Arg77, and V79 are shown (main chain of the α4 and α2 subunits, orange; main chain of the β2 subunit, green; carbon, white; hydrogen, cyan; nitrogen, blue; oxygen, red; chloride, green blue). Enlarged views are shown under respective whole views.](https://molpharm.aspetjournals.org/patch/10.1124/mol.107.095127)
Fig. 4. Inward currents recorded using two electrode voltage-clamp electrophysiology in response to bath-applied acetylcholine (ACh) and imidacloprid (IMI) of the wild-type α4β2 nicotinic acetylcholine receptor (nAChR) (A) and its T77R;E79V mutant (B) and the wild-type Do2β2 nAChR (C) and its T77N;E79R mutant (D) expressed in X. laevis oocytes.

Fig. 5. Concentration-response curves of acetylcholine (ACh) (A and C) and imidacloprid (IMI) (B and D) obtained for wild-type, T77K;E79R, T77N;E79R, and T77R;E79V mutants of the α4β2 (A and B) and Do2β2 (C and D) nicotinic acetylcholine receptors expressed in X. laevis oocytes. Each point plotted represents mean ± S.E.M. of four to eight experiments.
significant shifts of the imidacloprid concentration-response curve to lower concentrations (Table 2). However, the high imidacloprid sensitivity of the Do2β2 nAChR may stem partly from the interactions of one of the two nitro group oxygens with other regions than loop D. Thus, further studies are necessary to fully understand the mechanism for the selectivity of neonicotinoids.

The EC50 value of imidacloprid in the T77N;E79R mutant of the Do2β2 nAChR is lower than 1 μM, which is close to the value determined for native nAChRs on the cockroach (Periplaneta americana) native neurons (Ihara et al., 2006). Therefore, the overall difference in imidacloprid sensitivity between insect and mammalian nAChRs in vivo can be accounted for by the interactions with loop D in the α-subunit combined with those with loop C and the region upstream of loop B in the α-subunit of insect nAChRs (Shimomura et al., 2004a, 2005).

In conclusion, we have shown that the basic residues observed only in insect nAChR loop D play a key role in the selective interactions of heteromeric nAChRs with neonicotinoids. It is conceivable that the double mutations in loop D enhance markedly the imidacloprid sensitivity of the D2 nAChR is lower than 1 μM. It is conceivable that the double mutations in loop D contribute to our understanding of the molecular mechanism for the effects of such structural changes on the ACh concentration-response curve are minimal. The present study contributes to our understanding of the molecular mechanism underlying selectivity of neonicotinoids and suggests a possible target region on which to focus the design of new insecticides.

References


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