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Original Article

Responses to Neonicotinoids of Chicken α7 Nicotinic Acetylcholine Receptors: Effects of Mutations of Isoleucine 191 in Loop F to Aromatic Residues

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The effects on the responses to neonicotinoids and related nicotinic agonists of three site-directed mutations (I191W, I191F and I191Y) in loop F of the acetylcholine-binding site were studied using the chicken α7 nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus laevis* oocytes. Voltage-clamp electrophysiology was employed to show that, whereas the I191F mutation scarcely affected the concentration-response curves for neonicotinoids, the I191W mutation increased the maximum amplitude of responses to these ligands. By contrast, the I191Y mutation reduced the maximum amplitude of responses of the α7 nAChR to the insecticides.

Keywords: imidacloprid, neonicotinoid, desnitro-imidacloprid, nicotinic acetylcholine receptor, chicken α7 subunit, loop F.

INTRODUCTION

Insect nicotinic acetylcholine receptors (insect nAChRs) are targeted by several classes of neurotoxic insecticides.[1,2] Of these, imidacloprid and related insecticides (Fig. 1), collectively referred to as neonicotinoids, have enjoyed the greatest commercial success.[3] Bai *et al.* (1991) provided direct evidence for the agonist actions of imidacloprid on insect nAChRs.[4] Imidacloprid and its nitromethylene analog depolarized an identified cockroach motor neuron, which was blocked by α-bungarotoxin (α-BTX), a polypeptide nAChR antagonist. Also, it was found that the binding of [^{125}I]α-BTX to cockroach membranes was displaced by neonicotinoids.[4] Following these results, Tomizawa *et al.* showed in 1992 that [^{3}H]α-BTX-binding to honey bee, as well as housefly, head membranes was displaced by imidacloprid.[5]

The selectivity of neonicotinoids is an important feature that ensures they are safe to mammals. Even though this has been shown to result from the selective actions of neonicotinoids on insect nAChRs,[3,6,7] little is known about the underlying molecular mechanism. Since many neonicotinoids are able to activate nAChRs, they are postulated to interact with the binding site of ACh. Thus, it is conceivable that molecular features of insect nAChRs contributing to their selective interactions with neonicotinoids are present in one or more of the 6 loops (loops A–F) that make up the agonist-binding site.[3,8]

Using a combination of voltage-clamp electrophysiology and site-directed mutagenesis, we have shown that loops C, D and F play important roles in such interactions.[3,9–12] Mutations to acidic residues of G189 in loop F[9] and Q79 in loop D[10] of the chicken α7 nAChR were found to markedly reduce the responses of the nAChR to neonicotinoids, whereas such changes were not observed for the responses to desnitro-imidacloprid (DN-IMI, Fig. 1), an imidacloprid derivative lacking the nitro group. In addition, mutations of Q79 to basic residues enhanced the sensitivity of the α7 nAChR to neoni-

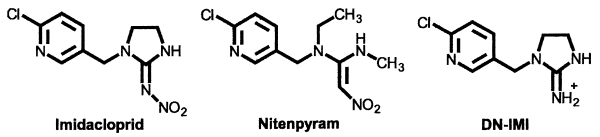

Fig. 1. Chemical structures of imidacloprid, nitenpyram and desnitro-imidacloprid.

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Table 1. Amino acid sequences in loop F

Subunits	Amino acid sequences										
						191					
Drosophila melanogaster ARD	D	L	S	D	Y	**W**	K	S	G	T	W
Drosophila melanogaster SBD	D	L	T	E	F	**Y**	L	S	V	E	W
Heliothis virescens β1	D	L	S	D	Y	**W**	K	S	G	T	W
Locusta migratoria β	D	L	S	D	Y	**W**	K	S	G	T	W
Manduca sexta β1	D	L	S	D	Y	**W**	K	S	G	T	W
Myzus persicae β1	D	L	S	D	Y	**W**	K	S	G	T	W
Chicken α7	D	I	S	G	Y	**I**	S	N	G	E	W
Chicken β2	S	L	D	D	F	**T**	P	S	G	E	W
Chicken β4	S	M	D	D	F	**T**	P	S	G	E	W
Human β2	S	L	D	D	F	**T**	P	S	G	E	W
Human β4	S	M	D	D	F	**T**	P	S	G	E	W

Table 2. PCR primers used for the preparation of mutant cDNAs

Mutants	Directions	Nucleotide sequences
I191F	sense	GCAGATATATCCGGCTATTTTTCAAATGGAGAG
	antisense	CTCTCCATTTGAAAAATAGCCGGATATATCTGC
I191Y	sense	GATATATCCGGCTATTATTCAAATGGAGAG
	antisense	CTCTCCATTTGAATAATAGCCGGATATATC
I191W	sense	GATATATCCGGCTATTGGTCAAATGGAGAG
	antisense	CTCTCCATTTGACCAATAGCCGGATATATC

cotinoids,[10] suggesting that the changes in the responses of the nAChR to neonicotinoids resulting from site-directed mutagenesis are due to electronic interactions of the nitro group of the insecticides with the amino acids newly added to loops D and F. Based on these results, together with findings obtained using a three-dimensional model constructed for a complex of imidacloprid with the ligand-binding site of the α7 nAChR,[11] it is postulated that loops D and F are likely to be located in proximity to the nitro group of neonicotinoids tested when the insecticides bind to the nAChR. On the other hand, we have also found that replacement of the chicken α4 subunit with *Drosophila* ALS and SAD subunits of the chicken $\alpha4\beta2$ nAChR expressed in *Xenopus* oocytes resulted in shifts of the dose-response curves for neonicotinoids to lower concentrations.[13,14] Therefore, structural features of the SAD subunit contributing to the sensitivity of the hybrid SADβ2 nAChR to neonicotinoids were explored. As a result, an amino acid in loop C, combined with a loop B–C interval region, was found to play a key role in defining aromatic residues involved in nAChR-imidacloprid interactions.[12] Nevertheless, it is likely that there will be other regions involved in the selective recognition of neonicotinoids.

In loop F of insect non-α subunits, aromatic residues are present at the position corresponding to I191 of the chicken α7 subunit and tryptophan residues are most frequently observed (Table 1). Thus, in the present study we have used site-directed mutagenesis to investigate the role of this tryptophan residue in its interactions with neonicotinoids. We have generated I191F, I191W and I191Y mutations of the chicken α7 subunit and studied their effects on receptor pharmacology when expressed in *Xenopus* oocytes. These mutations were designed to test whether tryptophan only, or aromatic residues in general, can strengthen nAChR-neonicotinoid interactions.

MATERIALS AND METHODS

1. Preparation of DNAs Encoding Mutant α7 Subunits

The chicken nAChR α7 subunit cDNA[15] in the pMT3 vector[16] was used as a template for mutagenesis. A series of mutations were introduced by PCR as described earlier.[9–11] Oligonucleotides I191F sense and I191F antisense (Table 2) were prepared to generate the α7 I191F mutation on the α7 subunit containing the I191F mutation, while KM001 (5′-TGTCCACTCCCAGGTCCAACTG-3′) and KM012 (5′-CTCCATGCTTGACAGGCTGCATC-3′) were designed, respectively, on the basis of the sequence flanking the multiple

cloning site of the pMT3 vector and the α7 cDNA about 1.1 kbp downstream of the start methionine codon. The first round PCRs were carried out using 2.5 U of LA-Taq (Takara, Otsu, Shiga, Japan), 100 ng of the wild-type pMT3-α7 as template, 0.3 μM primers (KM001 and I191F antisense; KM012 and I191F sense) and 0.4 mM dNTP mixture in a 50 μl solution for 30 cycles of 98°C 30 sec, 48°C 30 sec, 72°C 60 sec. The second round PCR was conducted using 1.25 U of LA-Taq, 20 ng each of the first round PCR products and 0.3 μM primers (KM001 and KM012) in a 50 μl solution for 30 cycles of 98°C 30 sec, 60°C 30 sec, 72°C 90 sec, yielding a single DNA band. After purification using a low melting-point agarose gel (Promega, Madison, WI, USA), the isolated PCR fragment was digested using *Pst*I and *Eco*RI (Takara, Otsu, Shiga, Japan) and subcloned into pMT3-α7. This plasmid was cut with *Eco*RI and ligated with a 1.2 kbp *Eco*RI fragment of pMT3-α7 to complete the full-length mutant α7. Other DNA constructs encoding the I191W and I191Y mutants were prepared in the same manner and the entire sequences of all mutants were confirmed by automated DNA sequencing. Oligonucleotides used as primers for the mutagenesis are listed in Table 2.

2. Preparation and Nuclear Injection of Xenopus oocytes

Mature *Xenopus laevis* females were anesthetized by immersion in 1.5 g/l tricaine for 30–45 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 (Sigma, type IA). The nucleus of each defolliculated oocyte was injected with 20 nl of cDNA in distilled water (0.1 ng/nl) and incubated at 18°C in standard oocyte saline (SOS) of the following composition (in mM); NaCl 100, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and HEPES 5.0, pH 7.6, supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml) and 2.5 mM sodium pyruvate. Electrophysiology was performed 3–6 days after nuclear injection.

3. Electrophysiology

Xenopus oocytes were secured in a recording chamber and perfused continuously with SOS (7–10 ml/min) containing 0.5 μM atropine using a gravity-fed delivery system described previously.[10] Membrane currents were recorded using 2.0 M KCl-filled electrodes (resistances 0.5–5.0 MΩ) and a GeneClamp 500B (Axon Instruments, Union, CA, USA) amplifier. The oocyte membrane was clamped at -100 mV. Currents were displayed using a pen recorder.

To prepare test solutions, stock solutions of ligands were diluted with SOS containing 0.5 μM atropine. Stock solutions of nitenpyram (100 mM) and DN-IMI (10 mM) in SOS were stored at 4°C, whereas solutions of ACh in SOS were always prepared immediately prior to experiments. Stock solutions of imidacloprid (300 mM) were prepared in DMSO, and diluted with SOS prior to tests. DMSO at concentrations lower than 1% (v/v) had no effect on the responses. Compounds were delivered to the experimental chamber at intervals of 3–5 min. Concentration-response data were obtained by challenging oocytes with increasing concentrations of an agonist and the peak amplitude of the current recorded in response to each challenge was normalized to the maximum amplitude of the response to ACh. Data from wild-type, I191F mutant and I191W mutant receptors were normalized to the response to 1 mM ACh, while those from the I191Y mutant were normalized to the response to 100 mM ACh.

Using GraphPad 'Prism' (GraphPad Software, San Diego, CA, USA), a non-linear regression analysis was applied to normalized data to obtain I_{max}, the maximum normalized response, EC_{50}, the concentration (in M) giving half the maximum normalized response and n_H, the Hill coefficient, as described earlier.[10] Experiments were performed at room temperature (19–25°C). Imidacloprid and DN-IMI were synthesized as described respectively by Moriya *et al.*[17] and Latli *et al.*,[18] whereas nitenpyram was a gift from Sumitomo Chemical Takeda Agro Co. ACh (chloride salt) and atropine (sulfate salt) were purchased from Sigma Aldrich Japan (Tokyo, Japan). The structures of neonicotinoids and DN-IMI tested in this study are illustrated in Fig. 1.

RESULTS AND DISCUSSION

Previous studies showed that site-directed mutagenesis in loop F markedly influenced the maximum responses of the α7 nAChR to neonicotinoids, suggesting a role for this loop in nAChR-imidacloprid interactions.[9] However, prior to this study, it was not clear whether or not loop F of insect nAChRs possesses any structural feature(s) favorable for selective interactions with neonicotinoids. In two-electrode voltage-clamp studies, ACh, imidacloprid, nitenpyram and DN-IMI evoked inward currents in a dose-dependent manner in *Xenopus* oocytes expressing the wild-type and mutant chicken α7 nicotinic AChRs (Figs. 2 and 3). For the wild-type receptor, the I_{max} (the maximum response normalized to that observed in response to 1 mM ACh) and pEC_{50} ($=-\log EC_{50}$; unit of EC_{50}: M) values of ACh obtained from the concentration-response curve were 1.00 ± 0.03 and 3.94 ± 0.04 (n=4), while the I_{max} and pEC_{50} values of imidacloprid were 0.54 ± 0.04 and 3.45 ± 0.08 (n=4), respectively (Fig. 3 and Table 3). These baseline data, newly measured for this study, resembled closely those reported earlier.[9,10]

ACh and imidacloprid actions on the I191F mutant receptor were similar to those recorded for the wild-type receptor (Figs. 2 and 3, Table 3). However, when I191 was replaced by tryptophan, the I_{max} value of imidacloprid was significantly increased ($I_{max}=1.01\pm0.06$ (n=7)). Furthermore, the concentration-response curve of ACh was significantly shifted to lower concentrations by the I191W mutation ($pEC_{50}=4.39\pm0.08$ (n=12)).

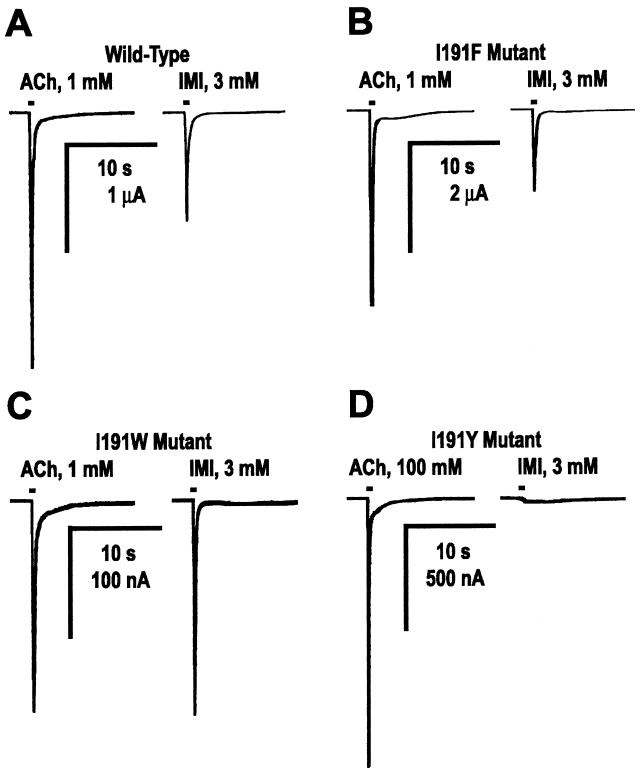

Fig. 2. Agonist actions of acetylcholine (ACh) and imidacloprid (IMI) on the wild-type (A) and mutant (I191F (B), I191W (C) and I191Y (D)) α7 nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus laevis* oocytes. The inward membrane currents induced by ACh and IMI at the maximum or nearly maximum concentrations are shown along with calibrations.

In contrast to the findings for the I191W mutation, the I191Y mutation significantly shifted the concentration curve of ACh to higher concentrations ($pEC_{50}=2.77\pm0.06$ (n=7)) and reduced the I_{max} value of imidacloprid ($I_{max}=0.03\pm0.01$ (n=8)). Similar results were obtained using the I191F, I191W and I191Y mutations when the agonist actions of nitenpyram were examined. Thus, the effect of the I191F mutation on the nitenpyram concentration-response curve was minimal, whereas the I191W and I191Y mutations increased and reduced, respectively, the maximum response of the α7 nAChR to nitenpyram. The data obtained for imidacloprid and nitenpyram on employing site-directed mutagenesis suggest that the effect of the tryptophan residue is not simply due to its aromatic nature.

DN-IMI, a denitrated metabolite of imidacloprid, was the most potent agonist tested on the α7 nAChR in this study, based on the pEC_{50} values. DN-IMI was a full agonist of the three mutant receptors tested as well as the wild-type receptor (Fig. 3), if ACh is defined as a full agonist. The pEC_{50} value of DN-IMI was scarcely influenced by the I191W mutation (Fig. 3, Table 3). Thus, the presence of the tryptophan residue in loop F appears to contribute to enhancing the interactions of the nitro group of neonicotinoids with the α7 nAChR. However, the rank order of agonist potency of the ligands tested (DN-IMI>ACh>imidacloprid and nitenpyram) was unaffected by either the I191F and I191W mutations, or the I191Y mutation in loop F. Thus, although the presence of the tryptophan residue at the position corresponding to I191 of the α7 subunit can play an important role in strengthening neonicotinoid-nAChR interactions, this residue cannot fully account for the selectivity of neonicotinoid actions.

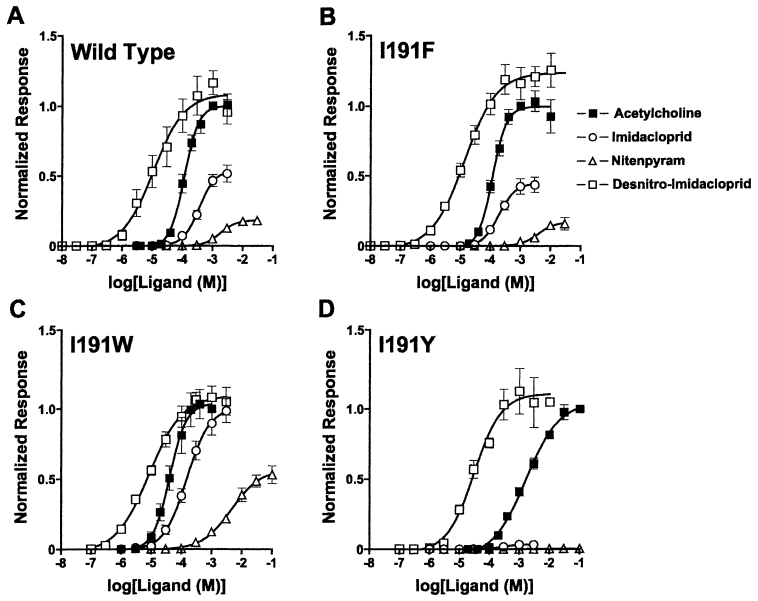

Fig. 3. Concentration-response curves for acetylcholine (ACh), imidacloprid, nitenpyram and desnitro-imidacloprid for wild-type (A) and mutant (I191F (B), I191W (C) and I191Y (D)) α7 nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Each point plotted represents the mean±standard error of the mean obtained from 4–12 experiments.

Table 3. I_{max} and pEC_{50} values[a] of neonicotinoid insecticides and related ligands for wild-type and mutant α7 receptors

Ligands	Wild-Type		I191F mutant		I191W mutant		I191Y mutant	
	I_{max}	pEC_{50}	I_{max}	pEC_{50}	I_{max}	pEC_{50}	I_{max}	pEC_{50}
ACh	1.00±0.03	3.94±0.04	1.00±0.03	3.93±0.05	1.04±0.06	4.39±0.08**	1.06±0.03	2.77±0.06**[,b]
Imidacloprid	0.54±0.04	3.45±0.08	0.45±0.03	3.70±0.08	1.01±0.06**	3.82±0.09	0.03±0.01**	ND[c]
Nitenpyram	0.18±0.01	2.74±0.05	0.17±0.03	2.41±0.17	0.57±0.05*	2.35±0.13	ND[c]	ND[c]
DN-IMI	1.08±0.06	4.97±0.14	1.24±0.04	4.87±0.09	1.10±0.04	5.07±0.09	1.11±0 .07	4.51±0.12*

a. The values shown are the result of non-linear regression analyses of the concentration-response data (mean±s.e.mean, n=4–12) illustrated in Fig. 3. The maximum currents are shown as a fraction of the response in the same oocyte to saturating concentrations of ACh (wild-type, I191F and I191W mutants: 1 mM; I191Y mutant: 100 mM). The Hill coefficients (not shown) are 1.9–0.7. b. Asterisks show significant differences (*, $P<0.05$; **, $P<0.01$) from the wild-type data with the one-way ANOVA, Dunnett's multiple comparison tests. c. Could not be determined because the responses were of very small amplitude even at the maximum concentrations tested.

When I191 is replaced by tyrosine, the hydroxyl group of Y191 may interact, by hydrogen bond formation, with other loops rather than the ligands tested. If this is the case, the agonist binding and subsequent changes of subunit conformation[19] could be interrupted by such an interaction, accounting for the marked reduction of the maximum responses to neonicotinoids seen with the I191Y mutation. In this context, the presence of a tyrosine residue in the *Drosophila* non-α subunit SBD (Table 1) at the position corresponding to I191 seems to present a contradiction, provided that insect nAChR subunits, whether they are α or non-α subunits, have structural features favorable for interactions with neonicotinoids. It is, however, difficult to express robust nAChRs containing the SBD subunit in *Xenopus* oocytes. Therefore, it is not clear how the presence of a tyrosine residue in loop F of this subunit influences the responses of nAChRs to neonicotinoids.

In conclusion, based on mutagenesis studies on the α7 receptor, we propose for the first time that a tryptophan residue in loop F, which is found in many insect non-α nAChR subunits, may contribute to strengthening neonicotinoid-insect nAChR interactions. Although the physicochemical factors involved in such a contribution remain to be resolved, the result obtained in this study enhances our understanding of the molecular basis of the selectivity of neonicotinoids for insect over vertebrate nAChRs.

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