

Actions of imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors of American cockroach neurons and their relationships with insecticidal potency

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The actions of neonicotinoid insecticides on nicotinic acetylcholine receptors (nAChRs) in the terminal abdominal ganglion neurons of the American cockroach were investigated using whole-cell patch-clamp electrophysiology. Neonicotinoids possessing a nitromethylene group showed higher agonist affinity than the corresponding nitroimine analogues, whereas compounds with an acyclic guanidine moiety showed greater agonist efficacy than the corresponding cyclic compounds. Imidacloprid showed the lowest agonist efficacy of all neonicotinoids and low concentrations of imidacloprid attenuated acetylcholine-induced currents. However, such blocking actions were minimal with other neonicotinoids. The diverse actions of neonicotinoids on nAChRs, combined with target accessibility based on hydrophobicity, appears to account for their insecticidal potency on cockroaches measured in the presence of metabolic inhibitors. © Pesticide Science Society of Japan

Keywords: imidacloprid, clothianidin, neonicotinoids, nicotinic acetylcholine receptors, whole-cell patch-clamp electrophysiology, insecticidal activity.

Introduction

Neonicotinoids are a class of insecticides acting selectively on insect nicotinic acetylcholine receptors (nAChRs). These compounds display strong insecticidal activities against various insect pests (including those showing resistance to other classes of insecticides) and good levels of safety in vertebrates, which has led to neonicotinoids showing the fastest growing sales of insecticides worldwide.¹⁾

The targets of neonicotinoids, nAChRs, belong to the cysteine-loop superfamily of ligand-gated ion channels that mediate fast synaptic transmission in both insects and mammals.²⁾ The safety of neonicotinoid insecticides has been shown to stem mainly from their excellent selectivity for insect nAChRs.^{3,4)} The insecticidal potency is influenced not only by the intrinsic actions on the targets, but also by accessibility to nAChRs and metabolism in insects.⁵⁾ To date, the nicotinic potencies of

many neonicotinoids have been characterized using binding assays to examine the correlation between binding potency and insecticidal potency as well as to clarify the structure–activity relationship.^{5–11)} However, relatively few electrophysiological studies have been conducted for such purposes.^{3,12–15)}

We have previously investigated the agonist actions of clothianidin and related neonicotinoids on a recombinant fruit fly *Dα2* (SAD)/chicken $\beta 2$ hybrid nAChR expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp electrophysiology.¹⁶⁾ Neonicotinoids with a cyclic-guanidine (or amidine) group show lower agonist efficacy than those with corresponding acyclic moieties, and the nitromethylene compounds are more potent in terms of the EC_{50} in activating the nAChR than the corresponding nitroimine compounds.¹⁵⁾ However, it is not clear whether such structure–activity relationships exist in native insect nAChRs. Therefore, we have investigated the actions of neonicotinoids on nAChRs on the terminal abdominal ganglion (TAG) neurons of American cockroaches using whole-cell patch-clamp electrophysiology. Here we report that the structure–agonist activity relationship observed for the TAG nAChRs resembles that observed for

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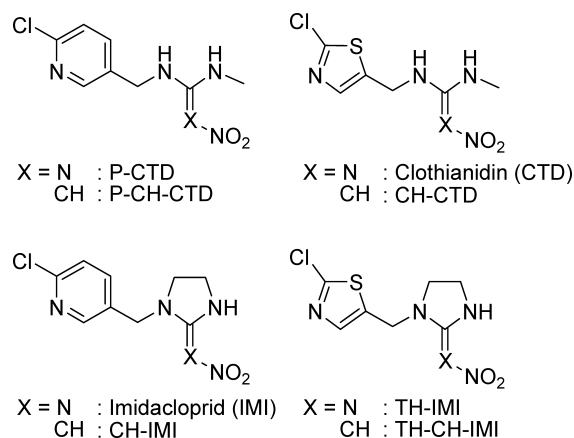


Fig. 1. Chemical structures of tested compounds and their abbreviated names.

the $D\alpha 2\beta 2$ hybrid nAChR.¹⁶ Also, we show that the diverse actions of neonicotinoids on the nAChRs contribute in a complex manner to their insecticidal potency toward the American cockroaches.

Materials and Methods

1. Insects

American cockroaches *Periplaneta americana*, provided by Earth Chemical Co. Ltd. (Hyogo, Japan), were reared in a room controlled at 25–28°C with about 60% humidity.

2. Chemicals

The neonicotinoids tested are illustrated in Fig. 1. Imidacloprid, the nitromethylene analogue of imidacloprid (CH-IMI), the chlorothiazole analogue of IMI (TH-IMI) and the nitromethylene analogue of TH-IMI (TH-CH-IMI) were synthesized *de novo* as reported elsewhere.¹⁷ The others were provided by Sumitomo Chemical Takeda Agro Company (Hyogo, Japan). NIA16388 (propagyl propyl phenylphosphonate) was a gift from Dr. Keiichiro Nishimura, Emeritus Professor of Osaka Prefecture University. The other chemicals used in this study were purchased, either from Wako Pure Chemical Industries (Osaka, Japan), or from Sigma Aldrich Japan (Tokyo, Japan), and used without further purification. The stock solutions of the neonicotinoids were prepared using dimethyl sulfoxide (DMSO) and stored at –20°C prior to use.

3. Culture of cockroach neurons

Cockroach neurons were cultured as described earlier.¹⁸ The abdominal nerve cord from the terminal abdominal ganglion (TAG) to the 4th ganglion was removed from male adult American cockroaches and transferred carefully into a cockroach physiological saline with the following composition (in mM): NaCl 200, KCl 3.1, MgCl₂ 4, HEPES 10 and glucose 10 supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin. The TAG was then carefully desheathed using fine forceps, and subsequently treated with collagenase

(Sigma Type IA, 1 mg/ml) for 30 min. After the collagenase had been washed off, TAGs were transferred into the cockroach saline supplemented with 5 mM CaCl₂ and 10% of fetal bovine serum (FBS), and triturated using a disposable pipette tip to dissociate the neurons. TAG neurons were then placed onto poly-D-lysine-coated coverslips in 35-mm culture dishes, and the neurons were allowed to settle on the surface of the coverslips. About 30 min later, the culture dishes were filled with the CaCl₂/FBS-supplemented cockroach saline, and the neurons were incubated in a humidified incubator at 25°C until electrophysiological recordings were made.

4. Electrophysiology

The actions of neonicotinoids were recorded electrophysiologically using the conventional tight-seal whole-cell patch-clamp method.¹⁹ Briefly, dissociated TAG neurons on coverslips were placed in a recording chamber RC-16 (Warner Instruments, USA), which was placed on the stage of an inverted microscope equipped with phase contrast optics at ×400 magnification. The recording chamber was continuously superfused at a flow rate of 5 ml/min with a cockroach recording saline having the following composition (in mM): NaCl 200, KCl 3.1, MgCl₂ 4, CaCl₂ 5, HEPES 10 and glucose 10. Test solutions were prepared by diluting the DMSO stock solutions with the cockroach recording saline. Unless otherwise noted neonicotinoids were applied to the cockroach neurons using a U-tube system¹⁸ capable of switching the saline around the neuron within 100 ms. Neonicotinoids were bath-applied for 1 min prior to co-application with ACh when their antagonist actions were evaluated. Patch-pipettes were prepared with glass capillaries PG150T-10 (Harvard Apparatus, MA, USA) using a puller PP-83 (Narishige, Tokyo, Japan), and were filled with an internal saline of the following composition (in mM): KCl 170, Na pyruvate 20, MgCl₂ 1, CaCl₂ 0.5, EGTA 10 and HEPES 20 with pH 7.4. The osmolality of each saline was 420–430 mmol/kg.

The maximum amplitude of the inward current responses to neonicotinoids was normalized to those evoked by 100 µM ACh, and analyzed using GraphPad Prism Software version 4.03 (San Diego, CA, USA) according to the Hill equation (1)

$$Y = I_{\max} / (1 + 10^{(\log EC_{50} - [M]) \cdot n_H}) \quad (1)$$

where Y is the normalized response, [M] is the logarithm of the concentration of ligand, I_{\max} is the normalized maximum response and n_H is the Hill coefficient. Each point on the curve was derived from at least 4 separate electrophysiological recordings.

5. Insecticidal assays

The insecticidal tests using male adult American cockroaches were conducted as previously reported.^{20,21} One microliter of a methanol solution containing 50 g/l of NIA16388 and 50 g/l of piperonyl butoxide (PB) was injected into cockroaches 1 hr before the injection of neonicotinoids to suppress metabolic

Table 1. Electrophysiological actions, insecticidal actions and the hydrophobicity parameter $\log k$ of neonicotinoids

Name	Structure		Electrophysiological action ^{a)}			Insecticidal action	Hydrophobicity ^{b)}	
	Ar	X	pEC ₅₀	I _{max}	n _H			
Acetylcholine			4.94±0.09	1.02±0.06	1.1±0.2	— ^{c)}	— ^{d)}	
Cyclic guanidine type								
Imidacloprid		N		5.88±0.40	0.11±0.02	0.9±0.3	10.4	0.23
CH-IMI		CH		7.14±0.14	0.24±0.02	1.7±0.8	10.1	-0.05
TH-IMI		N		5.80±0.17	0.30±0.03	0.8±0.2	9.07	0.28
TH-CH-IMI		CH		7.80±0.24	0.30±0.03	1.0±0.6	10.2	-0.03
Acyclic guanidine type								
P-CTD		N		5.73±0.24	0.31±0.04	1.0±0.5	9.52	0.11
P-CH-CTD		CH		7.41±0.32	0.44±0.06	1.2±0.9	9.22	-0.16
Clothianidin		N		5.80±0.15	0.68±0.06	0.8±0.2	10.2	0.17
CH-CTD		CH		7.71±0.18	0.47±0.03	2.4±1.5	10.2	-0.14

^{a)} The values shown (mean±SEM, $n=4$) were calculated using the concentration–response data illustrated in Fig. 1C.

^{b)} The values shown are the mean of data obtained from triplicate experiments. SEMs are not shown because they are small (<0.01).

^{c)} Could not be determined because of a lack of insecticidal activity.

^{d)} Not determined.

degradation in the insects. Then, various volumes (1–10 μl) of the methanol solution of each neonicotinoid, which contains DMSO at concentrations less than 1.0% (v/v), were injected into the ventral side of the abdomen. Three insects were used for each dose. The insects were kept at 25–28°C for 24 hr after the injection of test compounds, and then their symptoms were observed. The initial dose was set appropriately, and then the dose was either increased or decreased in intervals of 0.1 log units depending on the symptoms to determine the minimum lethal dose (MLD, mol/insect) at which two of three insects either died or were paralyzed. Experiments were repeated at least twice to obtain a converged MLD value. Neither the solvents nor the metabolic inhibitors alone had an effect on the motility of the cockroaches. The pMLD ($=\log(1/\text{MLD})$) values listed in Table 1 were used as indices for the insecticidal activity.

6. Hydrophobicity of compounds

Hydrophobicity is one important factor influencing the penetration of membrane barriers and subsequent transport of compounds to their targets. In this study, $\log k$, the logarithm of a retention factor (k), which can be measured, using high-performance liquid chromatography (HPLC), was determined to evaluate the hydrophobicity of compounds. The k value was defined as follows:

$$k = (t_R - t_0) / t_0 \quad (2)$$

where t_R and t_0 are the retention times of the compound and the unretained reference, potassium iodide, respectively. HPLC was carried out using a Shimadzu ClassVP system (Kyoto, Japan) with a Cadenza CD18 ODS column (100 mm×4.6 mm, Imtakt, Kyoto, Japan). The neonicotinoids tested were detected at 270 nm with an aqueous solvent containing 30% acetonitrile at a flow rate of 1 ml/min. The $\log k$ values of neonicotinoids, which were determined in triplicate experiments, are listed in Table 1.

Results

1. Electrophysiological actions of neonicotinoids

Inward currents were evoked in the dissociated TAG neurons in response to the challenge of ACh and neonicotinoids, with current amplitudes being reproducible after washing with saline for 1 min λ , excluding the treatment with neonicotinoids at concentrations higher than 100 μM . An example of the responses to ACh, imidacloprid and clothianidin is shown in Fig. 2A. These responses were blocked reversibly by a nicotinic receptor antagonist, mecamylamine (Fig. 2B). The responses of TAG neurons to ACh and neonicotinoids increased in a concentration-dependent manner (Fig. 3). The EC₅₀, I_{max} and n_H values for these compounds were determined according to Equation (1). The maximum responses to all neonicotinoids were smaller than the maximum response

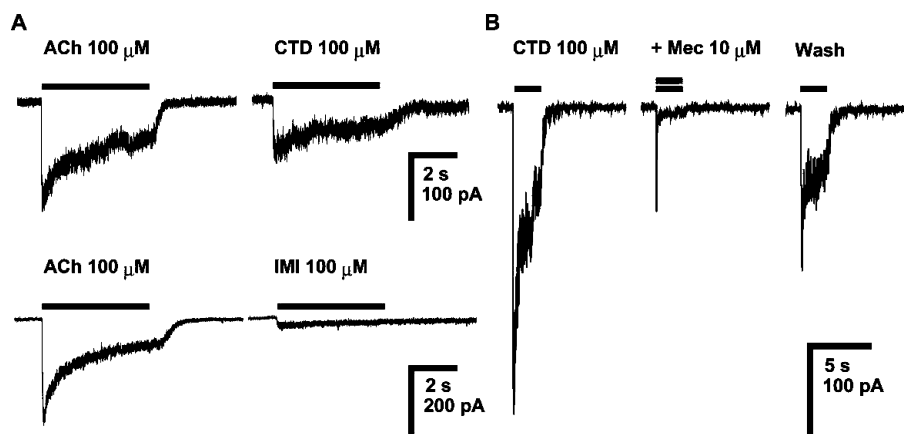


Fig. 2. Responses from dissociated neurons of American cockroaches, recorded using whole-cell patch-clamp electrophysiology. A, Responses to 100 μM clothianidin (CTD) and 100 μM imidacloprid (IMI). B, Blocking action of mecamylamine (Mec) on the CTD-evoked response.

to ACh.

The affinity of neonicotinoids for nAChRs in terms of the pEC_{50} [$=\log(1/\text{EC}_{50})$] values ranged from 5.74 to 7.72 (Table 1), which was higher than that of ACh (4.94 ± 0.09). The affinity of nitromethylene-type neonicotinoids was higher than of nitroimine-type compounds (*e.g.* CH-IMI *vs.* imidacloprid, CH-CTD *vs.* clothianidin, Table 1). Additionally, the I_{max} values suggest the agonist efficacy of compounds with a cyclic guanidine moiety was smaller than of compounds with the corresponding acyclic moiety (*e.g.* imidacloprid *vs.* P-CTD, CH-IMI *vs.* P-CH-CTD, Table 1). The n_{H} values estimated from the concentration–response curves for neonicotinoids were 0.8–2.4 (See Table 1), which were not significantly different from the value of ACh ($P > 0.05$, One-way ANOVA, Tukey's analysis).

To elucidate further the unidentified mechanism underlying the insecticidal potency, we examined the effects on the ACh-induced response of neonicotinoids at concentrations at which they did not evoke any inward current in the cockroach neurons. It was found that 10 nM imidacloprid blocked the response induced by 30 μM ACh by 63%, which was significantly greater ($P < 0.01$, one way ANOVA, Tukey's analysis)

than the blocking actions of TH-IMI, clothianidin and P-CTD tested at the same concentration (Fig. 4).

2. Log k values

It has been established that log k values correlate closely with the hydrophobicity parameter log P , where P is the *n*-octanol/water partition coefficient.²²⁾ The log k values obtained from the retention time of compounds in HPLC are shown in Table 1. TH-IMI showed the highest log k value (0.28), whereas P-CH-CTD showed the lowest (-0.16). The neonicotinoids with the nitroimine group were more hydrophobic in terms of log k than with corresponding nitromethylene group. Also, the compounds with the cyclic nitroguanidine moiety showed higher log k values than those with the corresponding acyclic moiety, while the compounds with the thiazole ring showed higher log k values than the corresponding compounds with the pyridine ring.

3. Insecticidal activity

It has been reported that the metabolic inhibitors PB and NIA16388 increased the insecticidal activity of neonicotinoids against American cockroaches.^{20,23,24)} Hence, we meas-

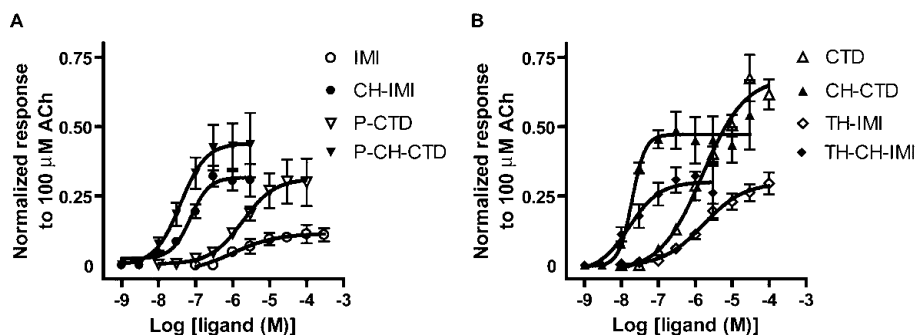


Fig. 3. Concentration–response relationships of neonicotinoids for cockroach nAChRs. Each plot is shown as the mean \pm standard error of the mean (SEM, $n=4$). Data were fitted according to the Hill equation described in Materials and Methods.

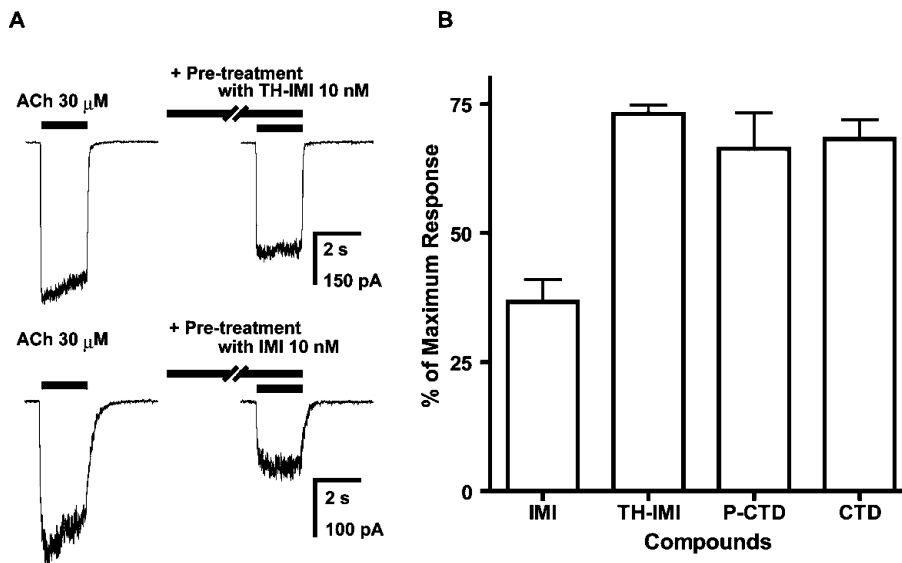


Fig. 4. Actions of low concentrations of neonicotinoids on the acetylcholine-induced response of cockroach terminal ganglion neurons. A, The blocking actions on the 30 μM acetylcholine-induced response of imidacloprid (IMI) and its thiazolyl analog TH-IMI at 10 nM. Neonicotinoids were bath-applied for 1 min prior to co-application with ACh for 2 s. B, Comparison of the blocking actions of 10 nM neonicotinoids. Each bar is shown as the mean ± standard error of the mean (SEM, $n=4$).

ured the MLD values for neonicotinoids in the presence of both synergists. The rank order of insecticidal potency in terms of pMLD values was imidacloprid > TH-CH-IMI, clothianidin, CH-CTD > CH-IMI > P-CTD > P-CH-CTD > TH-IMI.

Discussion

The inward currents evoked by ACh and neonicotinoids in the cockroach TAG neurons were blocked by 10 μM mecamylamine (Fig. 2B). Hence the observed responses are mediated by nAChRs in the TAG neurons. The maximal currents induced by all neonicotinoids were smaller than those induced by ACh, indicating that the insecticides are partial agonists of native nAChRs. In an earlier study, we found using voltage-clamp electrophysiology that clothianidin and related analogs showed higher agonist efficacy in terms of I_{\max} values than ACh for *Drosophila* Dα2/chicken β2 hybrid nAChRs expressed in *Xenopus* oocytes.¹⁶ The discrepancy between the maximum responses to neonicotinoids of native and recombinant nicotinic receptors is probably due to the difference in the subunits composing the nAChRs tested. If this is the case, then it is of importance to examine the actions of neonicotinoids on *Drosophila* native neurons expressing the Dα2 subunit.

When the structure of imidacloprid is compared with that of clothianidin, two differences can be noted: (1) imidacloprid has a cyclic guanidine moiety, while clothianidin has an acyclic guanidine moiety; (2) imidacloprid has a 6-chloro-3-pyridyl group, while clothianidin has a 2-chloro-1,3-thiazol-5-yl group as the aryl moiety (Fig. 1). These structural differences can also be used to group the other compounds in the study. In general, the compounds with an acyclic guanidine

moiety evoked a greater maximum response than those with a cyclic guanidine moiety (Table 1, e.g. P-CTD vs. imidacloprid). On the other hand, when compared in terms of the pEC₅₀, the nitromethylene compounds were more potent than the corresponding nitroimine compounds (e.g. imidacloprid vs. CH-IMI). However, no clear correlation appears to be present between the pEC₅₀ values with the aryl ring structures. The Hill coefficients of neonicotinoids and ACh were within the margin of error, indicating similarity in the interactions with the cockroach nAChRs. Despite the variations in the maximal current amplitudes observed in the present study, the order of agonist affinity in terms of the pEC₅₀ and efficacy in terms of the I_{\max} for the cockroach AChRs resembled those observed in the hybrid Dα2β2 nAChR, suggesting that the mechanisms underlying the nAChR-neonicotinoid interactions might also be observed in other insect nAChRs.

It is conceivable that the insecticidal potency of neonicotinoids against American cockroaches is determined by a combination of their intrinsic activity at nAChRs, accessibility to the targets and metabolic degradation of compounds in insects. Hence, by suppressing metabolism, the insecticidal potency of neonicotinoids should be correlated with their actions on nAChRs if their accessibility to targets were taken into consideration on the basis of hydrophobicity. The insecticidal potency of compounds appears to have no clear correlation with the agonist potency in terms of the pEC₅₀ for the nAChRs. For example, the pEC₅₀ value of CH-IMI was higher than that of imidacloprid, but the insecticidal potency of CH-IMI (pMLD=10.1) was almost equivalent to that of imidacloprid (pMLD=10.4). This may be explained by the difference in hydrophobicity between the two compounds. CH-IMI was

more potent than imidacloprid in activating the cockroach nAChRs, but less hydrophobic in terms of $\log k$ than imidacloprid, and thereby inferior to imidacloprid in its ability to access the targets which will negatively impact on the insecticidal potency.

Additionally, the finding that 10 nM imidacloprid attenuated currents induced by ACh suggests that the complex actions of imidacloprid may account for increased insecticidal potency over similar compounds such as TH-IMI which showed a weaker blocking action of the ACh-induced response and a lower insecticidal activity than imidacloprid. However, it is still difficult to explain the insecticidal potency of clothianidin based on such an antagonist action.

The pEC₅₀ and $\log k$ values as well as the antagonist action on the ACh-induced response of clothianidin and P-CTD were similar, but the agonist efficacy of clothianidin was greater than of P-CTD. This may explain why the insecticidal potency of clothianidin was greater. Also, the high agonist efficacy of clothianidin may also explain why its insecticidal potency is comparable to that of imidacloprid, because both clothianidin and imidacloprid showed similar affinity for the cockroach nAChRs but the hydrophobicity and antagonist actions of clothianidin were inferior to those of imidacloprid.

In conclusion, we have evaluated the insecticidal actions of neonicotinoids on American cockroaches as well as their electrophysiological actions on the cockroach TAG neurons to clarify the structural and physicochemical factors contributing to these actions. Clothianidin and related acyclic guanidine compounds were found to induce greater maximum responses of the cockroach nAChRs than imidacloprid and related cyclic guanidine compounds. On the other hand, neonicotinoids with the nitromethylene moiety were found to activate the nAChRs at lower concentrations than those with the nitroimine moiety. Comparisons of the electrophysiological actions of neonicotinoids with their insecticidal activity suggested that hydrophobicity as well as affinity and efficacy in activating nAChRs play important roles in determining the insecticidal potency. It has been shown that clothianidin is superior to imidacloprid in controlling lepidopteran insects.²⁵⁾ Whether this is due to the higher agonist efficacy of clothianidin for nAChRs compared to that of imidacloprid remains to be studied. In addition, it is necessary to examine whether neonicotinoids with higher agonist efficacy generally show higher insecticidal activity using other insect species.

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