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Structure–Activity Relationships of Acyclic Nicotinoids and Neonicotinoids for Insect Nicotinic Acetylcholine Receptor/Ion Channel Complex

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The insect nicotinic acetylcholine (ACh) receptor (nAChR) is a target site for the neonicotinoid insecticides such as imidacloprid and its acyclic derivative acetamiprid. The structure–activity relationships of acetamiprid homologues and 3-pyridylmethylamines (known as the essential structural requirement of nicotinoid) are compared in terms of the affinity to the [^{3}H]α- bungarotoxin (α-BGT) site (designated as ACh site) and the [^{3}H]phencyclidine (PCP) site [designated as noncompetitive blocker (NCB) site] of the insect nAChR from the honeybee heads. Increasing the chain length of alkyl substituents (from methyl to *n*-butyl) on an amino nitrogen atom of acetamiprid homologue and 3-pyridylmethylamine reduces the potency as inhibitors of [^{3}H]α-BGT binding, whereas it confers the enhanced potency as inhibitors of [^{3}H]PCP binding in the insect nAChR. Scatchard analysis reveals that homologues of acetamiprid and 3-pyridylmethylamine having *n*-butyl substituents interact with the high-affinity binding site for [^{3}H]PCP, which is considered to be the NCB site located in the ion channel of the insect nAChR. The interaction of acetamiprid homologues with the ACh or NCB site of nAChR is selective for insects, while that of the 3-pyridylmethylamines is effective for both insect and *Torpedo* [Tomizawa et al., J Pesticide Sci 21:412–418 (1996)]. The explorations in further structural modification of neonicotinoid compounds may facilitate development of new insecticides or probes for the ion channel of insect nAChR. Arch. Insect Biochem. Physiol. 37:17–23, 1998. © 1998 Wiley-Liss, Inc.

Key words: acetamiprid; neonicotinoids; nicotinic acetylcholine receptor; nicotinoids; noncompetitive blocker; 3-pyridylmethylamines

Abbreviations used: ACh = acetylcholine; α-BGT = α-bungarotoxin; B_{max} = maximal binding capacity; IC_{50} = concentration of test compound for the 50% inhibition of specific binding; K_D = dissociation constant; LD_{50} = median lethal dose; NCB = noncompetitive blocker; n_H = Hill coefficient; nAChR = nicotinic ACh receptor; PCP = phencyclidine; SAR = structure–activity relationship.

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INTRODUCTION

Nicotine, known as an insecticidal alkaloid in tobacco leaves, induces an excitatory action in insects through its interaction with the acetylcholine (ACh) site of the insect nicotinic ACh receptor (nAChR). Studies on structure–activity relationships (SAR) of insecticidal nicotine and its related compounds (designated as nicotinoids) reveal that the insecticidal nicotinoids must provide a 3-pyridylmethylamine moiety with a highly basic amino nitrogen atom as an essential structural requirement for the insecticidal activity and interaction with the ACh site of insect nAChR (Yamamoto et al., 1962; Tomizawa and Yamamoto, 1992; Tomizawa, 1994). The novel potent insecticides nitromethylene heterocycles (Kagabu et al., 1992) and imidacloprid (Moriya et al., 1992), as well as their acyclic series nitenpyram (Minamida et al., 1993) and acetamiprid (Takahashi et al., 1992), bind to the insect nAChR (Bai et al., 1991; Tomizawa and Yamamoto, 1992, 1993; Liu and Casida, 1993; Liu et al., 1993; Tomizawa, 1994; Tomizawa et al., 1995b). The SARs of imidacloprid congeners, in terms of their insecticidal activity and binding affinity to the ACh site of insect nAChR, resemble those of the nicotinoids, leading to their designation as "neonicotinoids" (Tomizawa and Yamamoto, 1993). These neonicotinoids are known to have highly selective toxicity, which is mostly attributable to their higher affinity for the insect than that for the vertebrate nAChRs (Liu and Casida, 1993; Zwart et al., 1994; Tomizawa et al., 1995a; Yamamoto et al., 1995).

Our recent study indicates that structural modification of the aforementioned 3-pyridylmethylamines in increasing the chain length of dialkyl substituents on an amino nitrogen atom alters their target from the ACh to noncompetitive blocker (NCB) sites of the nAChR/ion channel complexes from both *Torpedo* electric organ and honeybee head (Tomizawa et al., 1996a). Because acetamiprid provides a 3-pyridylmethylamine moiety and shares a consistent binding site with nicotinoids, it is considered to be an acyclic type of neonicotinoids. Thus, these prompt us to compare the SARs of 3-pyridylmethylamines and acetamiprid congeners (refered to as acyclic neonicotinoids) with various alkyl substituents on an amino nitrogen atom in terms of their affinities to the [^{3}H]α-bungarotoxin (α-BGT) site [designated as ACh site (Sattelle et al., 1983; Lummis and Sattelle, 1985)] and the [^{3}H]phencyclidine (PCP) site [designated as NCB site located in the ion channel (Eldefrawi et al., 1982; Sattelle et al., 1985; Tomizawa et al., 1995b)] of the insect nAChR.

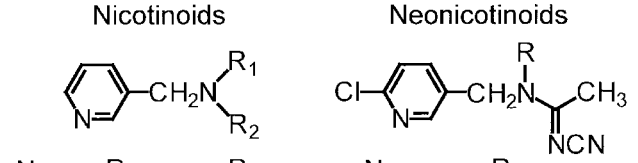
MATERIALS AND METHODS

Chemicals

N-[Propionyl-^{3}H]propionylated α-bungarotoxin ([^{3}H]α-BGT; 68.0 Ci/mmol) and [piperidyl-3,4-^{3}H(*N*)]phencyclidine ([^{3}H]PCP; 49.9 Ci/mmol) were purchased from Amersham Life Science (Arlington Heights, IL) and New England Nuclear Research Products (Boston, MA), respectively. Acetamiprid and its *N*-alkyl homologues (N^1[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-alkylacetamidine) were obtained from Nippon Soda Co., Ltd. (Odawara, Kanagawa, Japan) and Agro-Kanesho Co., Ltd. (Tokorozawa, Saitama, Japan). Other compounds employed in this study were available from our previous studies (Tomizawa 1994; Tomizawa et al., 1995b). Chemical structures of 3-pyridylmethylamines and acetamiprid homologues are drawn in Figure 1.

Binding Assays

The honeybee (*Apis mellifera*) head membranes were prepared according to the methods of Sherby et al. (1986) and Tomizawa et al. (1995b). The honeybee head membranes (~0.4 mg protein) were incubated with 2 nM [^{3}H]α-BGT for 60 min at 25°C in the absence and presence of test compounds, in a total volume of 0.25 ml of 20 mM sodium phosphate buffer, pH 7.4 containing 1 mM EDTA and 0.1 mM phenylmethanesulfonyl fluoride (Tomizawa and Yamamoto, 1993).



No.	R_1	R_2
1	H	CH_3
2	H	C_2H_5
3	H	n-C_3H_7
4	H	n-C_4H_9
5	CH_3	CH_3
6	C_2H_5	C_2H_5
7	n-C_3H_7	n-C_3H_7
8	n-C_4H_9	n-C_4H_9

No.	R
9*	CH_3
10	C_2H_5
11	n-C_3H_7
12	n-C_4H_9

* acetamiprid

Fig. 1. Chemical structures of 3-pyridylmethylamines and acetamiprid homologues.

Alternatively, 5nM [^{3}H]PCP was incubated for 40 s at 25°C with ~0.3 mg protein of honeybee head membranes in the absence and presence of test compounds, in 0.25 ml of 20 mM Tris buffer, pH 7.4 containing 0.1 mM diisopropylfluorophosphate and 0.02% sodium azide (Tomizawa et al., 1995b). The incubated mixtures were rapidly filtered on Whatman GF/B filters presoaked in 0.05% polyethylenimine. The filter was rinsed three times with 2.5 ml of ice-cold 0.9% sodium chloride. Radioactivity remaining on the filter was measured by liquid scintillation counting. Specific binding was defined as the difference in radioactivity in the absence or presence of 5 μM unlabeled α-BGT (for [^{3}H]α-BGT binding) or 10 μM unlabeled PCP (for [^{3}H]PCP binding).

The dissociation constant (K_D), maximal binding capacity (B_{max}) and Hill coefficient (n_H) of [^{3}H]PCP binding were determined by titration of unlabeled PCP (0–200 nM) on the binding of 5 nM [^{3}H]PCP.

Data Analysis

Data were analyzed by iterative nonlinear least-squares regression using Sigmaplot (Jandel Scientific Software, San Rafael, CA) and LIGAND (Biosoft, Cambridge, UK) programs. Statistically significant differences between the two groups were made by Student's *t*-test.

Insecticidal Activity

One microliter of a 3-pyridylmethylamine in acetone was topically applied on the notum of 3–4 days old female housefly (*Musca domestica*). Administration of acetamiprid (compound **9**) was carried out by intrathoracic injection of 0.22 μl in 25% dimethyl sulfoxide solution in water (Liu et al., 1993; Tomizawa et al., 1995b). Signs of intoxication were observed and mortalities were recorded after 24 h at 27 ± 2°C and 60% relative humidity.

RESULTS

Structure–Activity Relationship of 3-Pyridylmethylamines

The systematic structural modification of *N*-alkyl substituted 3-pyridylmethylamines in increasing the chain length of alkyl substituent from methyl to *n*-butyl (compounds 1–**4**, Fig. 1) enhanced the potency as inhibitors of [^{3}H]PCP binding, whereas it reduced the potency as inhibitors of [^{3}H]α-BGT binding (Table 1). This opposite relationship in the site selectivity was seen more clearly in the *N,N*-dialkyl series (compounds **5**–**8**).

TABLE 1. Structure–Activity Relationships of 3-Pyridylmethylamines and Acyclic Neonicotinoids for the [^{3}H]PCP and [^{3}H]α-BGT Bindings to the Honeybee Head Membranes

	IC_{50} (μM)[a]	
Compound No.	[^{3}H]PCP binding	[^{3}H]α-BGT binding
1	430 ± 30	340 ± 45
2	84 ± 3	360 ± 37
3	74 ± 1	>1000 (34%)
4	42 ± 4	>1000 (35%)
5	55 ± 5	130 ± 8
6	4.0 ± 0.2	190 ± 5
7	1.9 ± 0.1	260 ± 26
8	0.64 ± 0.06	>1000 (21%)
9	310 ± 18	8.0 ± 1.0
10	40 ± 9	47 ± 2
11	63 ± 6	>300 (32%)
12	27 ± 1	≥300 (49%)

[a]Mean ± S.D. of three experiments.
Numerical values in the parenthesis are percent inhibition at indicated concentrations of test compounds. Data for compounds **5** to **8** are taken from our previous study (Tomizawa et al., 1996a). Chemical structures of test compounds are illustrated in Figure 1.

Structure–Activity Relationship of Acyclic Neonicotinoids

An acyclic neonicotinoid with *N*-methyl substituent (compound **9**, acetamiprid) displayed a highest potency as inhibitor of the [^{3}H]α-BGT binding. When the *N*-methyl group of compound **9** was replaced with *N*-ethyl, *N*-*n*-propyl or *N*-*n*-butyl substituents (compounds **10**–**12**), the affinity to the [^{3}H]α-BGT binding site markedly decreased. However, this structural modification conferred the enhanced potency as inhibitors of [^{3}H]PCP binding (Table 1).

In addition, we also demonstrated that the 0.3 mM of compounds **10**, **11** and **12** inhibited 40, 29, and 42% (respectively) of the 2 nM [^{3}H]PCP binding stimulated by 0.1 mM carbachol (activated status of the receptor) and gave no effect on the 1 nM [^{3}H]α-BGT binding in the *Torpedo nobiliana* electric organ.

Influences of 3-Pyridylmethylamine and Acyclic Neonicotinoid on Kinetic Parameters of [^{3}H]PCP Binding

Scatchard analysis revealed that both compounds **8** and **12** competitively inhibited the [^{3}H]PCP binding in the honeybee. The K_D value in the presence of compound **8** or **12** was significantly different from control, while B_{max} value remained unchanged. Hill coefficient indicated a single site action in each case (Fig. 2).

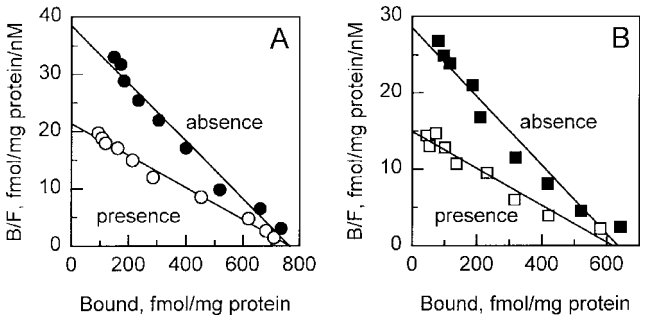


Fig. 2. Scatchard plots of 5 nM [^{3}H]PCP binding to the membranes from the honeybee heads. The binding reactions were performed (**A**) in the absence (●) and presence (○) of 0.6 μM 3-pyridylmethyl-*N,N*-di-*n*-butylamine (compound **8**) (data from Tomizawa et al., 1996a); (**B**) in the absence (■) and presence (□) of 30 μM *N*-*n*-butylacetamiprid (compound **12**). Binding parameters are indicated as follows: (A) K_D 20 ± 1 nM, B_{max} 760 ± 12 fmol/mg protein and n_H 1.06 ± 0.03 in the absence of compound **8**, whereas K_D 40 ± 2 nM,** B_{max} 760 ± 25 fmol/mg protein, and n_H 1.03 ± 0.05 in the presence of compound **8**; (**B**) K_D 21 ± 1 nM, B_{max} 630 ± 20 fmol/mg protein, and n_H 1.04 ± 0.03 in the absence of compound **12**, while K_D 41 ± 1 nM,** B_{max} 620 ± 14 fmol/mg protein, and n_H 1.02 ± 0.03 in the presence of compound **12**. The values are mean ± S.D. of three experiments. **Significant difference between control and treatment ($P < 0.01$).

Insecticidal Activities and Signs of Intoxication of 3-Pyridylmethylamines and Acyclic Neonicotinoid

The 3-pyridylmethylamines (compounds **4**, **5**, and **8**) showed a certain level of insecticidal activity against houseflies. The signs of intoxication of the compound **5** was quite different from those of compounds **4** and **8** (Table 2). The compound **5** with dimethyl substituents induced the excitatory effects such as convulsions, leg tremors, and motion of wings, resembling the signs induced by nicotine (Schmeltz, 1971; Tomizawa et al., 1995b). However, treatments with compounds **4** and **8** having *n*-butyl substituents resulted in anesthetic effects similar to those of ether and hallucinogen PCP (Tomizawa et al., 1995b), and houseflies awoke within 5–6 h at low doses. The acetamiprid having an *N*-methyl group (compound **9**) displayed a similar insecticidal activity to imidacloprid (Tomizawa et al., 1995b), inducing the excitatory symptoms such as immediate convulsions, leg tremors, and motion of wings as those induced by nicotine and imidacloprid (Schmeltz, 1971; Sone et al., 1994; Tomizawa et al., 1995b).

DISCUSSION

We recently reported that increasing the chain length of dialkyl substituents on an amino nitrogen atom of 3-pyridylmethylamine, an essential structural moiety of nicotinoid, leads to the diminished affinity to the ACh site and the enhanced affinity to the NCB site in both *Torpedo* and honeybee nAChR/ion channel complexes (Tomizawa et al., 1996a). The present study finds that structural modification of the acyclic neonicotinoids (acetamiprid congeners) in elongation of the *N*-alkyl chain can also enhance the affinity to the [^{3}H]PCP binding site and reduce the affinity to the ACh site, as indicated by the inhibition of [^{3}H]α-BGT binding in the insect nAChR. This SAR is consistent with that of the monoalkyl and dialkyl substituted 3-pyridylmethylamines. A similar SAR is also seen in the symmetrically substituted tetraalkylammonium compounds (Bakry et al., 1982; Tomizawa et al., 1996a). Scatchard analysis of the [^{3}H]PCP binding reveals that the *N*-*n*-butylacetamiprid (compound **12**) as well as the 3-pyridylmethyl-*N,N*-di-*n*-butylamine (compound **8**) interacts with the high-affinity site for PCP, which is considered to be an NCB site located in the ion channel of the insect nAChR (Eldefrawi et al., 1982; Sattelle et al., 1985;

TABLE 2. Insecticidal Activities and Signs of Intoxication of 3-Pyridylmethylamines and Acyclic Neonicotinoid Against Adult Houseflies, *Musca domestica*

Compound No.	LD_{50} (μg/female)[a]	Signs of intoxication
4	59 ± 5[b]	anesthetic effects resembling that induced by ether and PCP
5	37 ± 2[b]	excitatory effects such as convulsions, leg tremor, and motion of wings
8	50 ± 1[b]	anesthetic effects resembling that induced by ether and PCP
9	0.42 ± 0.02[c]	excitatory effects such as immediate convulsions, leg tremor, and motion of wings, followed by paralysis

[a]Mean ± S.D. of three experiments.
[b]By topical application
[c]By intrathoracic injection.
Due to a solubility problem of compound **12**, it allowed to test at 0.5–1.0 ng/female with no significant intoxication signs and mortality. Chemical structures of test compounds are listed in Figure 1.

Tomizawa et al., 1995b). Compounds **4** and **8** selectively interacting with the NCB site produce the anesthetic effects in insect. The compound **9** (acetamiprid), which prefers the ACh site, induces excitatory effects, while the compound **5** is not clear in the relationship between the affinity on binding assays and the intoxication signs. It is interesting that the intoxication signs induced by the administration of test compounds may be corresponding to what is predicted from the radioligand binding assays.

The acyclic neonicotinoids show an important feature that is distinctly different from that of the 3-pyridylmethylamines. Acetamiprid with *N*-methyl group (compound **9**) displays much lower agonistic potency compared with a 3-pyridylmethylamine, owning dimethyl substituents (compound **5**) and nicotine in the *Torpedo* nAChR (Tomizawa et al., 1995a, 1996a). Furthermore, *N*-*n*-butylacetamiprid (compound **12**) shows little inhibition of the [^{3}H]PCP binding in the opened status of the *Torpedo* receptor, whereas a 3-pyridyl–methylamine with di-*n*-butyl substituents (compound **8**) is suitable as an open-channel blocker of the *Torpedo* nAChR (Tomizawa et al., 1996a). These findings suggest that the acetamiprid and its *N*-alkyl homologues prefer the insect over vertebrate receptor.

The 3-pyridylmethylamines with a highly basic amino nitrogen atom mostly ionize by protonation at physiological pH (Yamamoto et al, 1962; Tomizawa and Yamamoto, 1992). On the other hand, the neonicotinoids provide the partial positively charged nitrogen atom by the strong electron-withdrawing effect of the neighboring substituents, the partially positive nitrogen atom corresponds to the ionized amino nitrogen atom of the 3-pyridylmethylamine (Yamamoto et al., 1995; Kagabu and Matsuno, 1997). The ACh site of the insect nAChR accepts either ionized or partially positive nitrogen atoms, while the ACh site of vertebrate nAChR prefers the ionized one (Yamamoto et al., 1995). The present study indicates that the 3-pyridylmethylamines and acyclic neonicotinoids with longer alkyl chains act on the NCB site of insect nAChR. The nAChR subunit has four transmembrane segments (referred to as M1, M2, M3, and M4) based on the hydrophobicity profile of the primary amino acid sequence. Affinity labeling and site-directed mutagenesis experiments reveal that the hydrophobic M2 segment is a component of the ion channel (reviewed in Galzi et al., 1991; Gundelfinger and Hess, 1992; Karlin and Akabas, 1995; Hucho et al., 1996). The homologous amino acid residues from the M2 segment of each subunit form several rings in the lumen of the channel. Most importantly, the Serine and Threonine rings contribute to the cation permeation and target for NCBs (reviewed in Galzi et al., 1991; Karlin and Akabas, 1995; Hucho et al., 1996). The hydroxy oxygens in the Serine and Threonine rings may serve as the binding site for the ionized or partially positive nitrogen atom of the 3-pyridylmethylamine or acyclic neonicotinoid, as described in the interaction of bis-ammonium blockers with the ion channel of nAChR (Brovtsyna et al., 1996). Furthermore, the hydrophobicity and steric effect by introduction of the longer alkyl chains influence the interaction of ligand with the α-helices of M2 domains of ion channel.

The neonicotinoid compounds provide many possibilities for further structural modification as stated by Yamamoto and Tomizawa (1993) and Kagabu (1996). The knowledge of neonicotinoid SAR leads the successful way to isolating the nAChRs from *Drosophila* and *Musca* heads (Tomizawa et al., 1996b). Our investigation suggests that homologous structural modifications of the acyclic neonicotinoids and the 3-pyridylmethylamines alter their target from the ACh to NCB sites in the insect nAChR. The interaction of acetamiprid and its *N*-alkyl congeners with the ACh or NCB site of nAChR is selective for insects, while that of the 3-pyridylmethylamines is effective for both insects and vertebrate. The results obtained from the present study may help expedite the development of new insecticides or probes for the ion channel of insect nAChR.

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