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Original Article

Potentiating and blocking actions of neonicotinoids on the response to acetylcholine of the neuronal $\alpha4\beta2$ nicotinic acetylcholine receptor

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Effects of imidacloprid, clothianidin, thiacloprid and related compounds on the acetylcholine (ACh)-induced response of the recombinant, expressed chicken $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR) were investigated using voltage-clamp electrophysiology. Imidacloprid and clothianidin enhanced the amplitude of the response to ACh of $\alpha4\beta2$ nAChR. In complete contrast, thiacloprid attenuated the amplitude of the response to ACh of $\alpha4\beta2$ nAChR. Replacing the nitro group of imidacloprid by a cyano group abolished the potentiating action, whereas exchanging the cyano group of thiacloprid for a nitro group conferred the ability to potentiate the ACh response. All three neonicotinoids shifted the ACh concentration–response curve without influencing the peak current amplitude of the ACh response. ©Pesticide Science Society of Japan

Keywords: ion channels, imidacloprid, clothianidin, thiacloprid, neonicotinoids, $\alpha4\beta2$ nicotinic acetylcholine receptor.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of the cys-loop superfamily of ligand-gated ion channels that play a central role in fast cholinergic neurotransmission.[1)] So far, several classes of insecticides targeting nAChRs have been developed and used worldwide for pest control.[2–4)] Neonicotinoids acting on insect nAChRs are comparable with organophosphates and synthetic pyrethroids in terms of current market share. Targeting nAChRs raises concerns relating to mammalian toxicity, yet neonicotinoids show high selective toxicity to insects over vertebrates.[2,5–7)] Studies using radioligand binding assays as well as work deploying voltage-clamp electrophysiology have shown that neonicotinoid selectivity is based, at least in part, on their selective actions on the target molecules, insect nAChRs.[2,5–7)]

Many neonicotinoids are agonists of native and recombinant nAChRs, yet their agonist actions are dramatically affected by even small structural variations.[5)] In general, neonicotinoids containing nitroguanidine and chemically-related moieties exhibit higher agonist efficacy than those with a ring structure.[8–12)] A conspicuous example is clothianidin (Fig. 1) and its analog P-CH-CTD, containing a pyridine ring and a C=C double bond, both of which were found to evoke a larger amplitude response than even the neurotransmitter itself (ACh) when tested on recombinant *Drosophila* Dα2/chicken β2 hybrid nAChR expressed in *Xenopus laevis* oocytes[10)] and on native nAChRs of *Drosophila* cholinergic neurons.[13)] Single channel analysis disclosed that P-CH-CTD opens *Drosophila* nAChR channels in the highest conductance states more frequently than ACh, possibly contributing to super agonist action.[13)]

Not all neonicotinoids are nicotinic agonists. For example,

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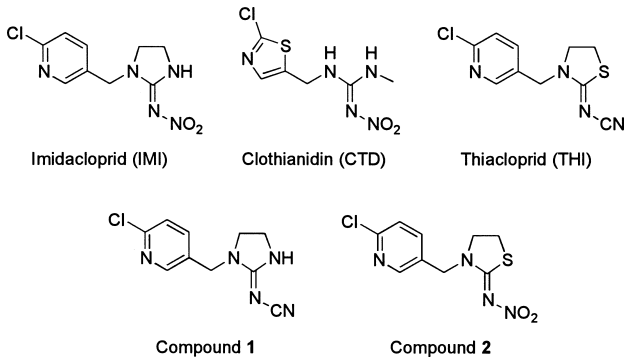

Fig. 1. Chemical structures of imidacloprid, clothianidin, thiacloprid and their analogs (Compounds **1** and **2**).

we have shown that alkylene-tethered bis-neonicotinoids, which possess two conventional neonicotinoid units joined by an alkyl linker, are antagonists of nAChRs in cell bodies of the terminal abdominal ganglion neurons of the American cockroach, *Periplaneta americana*.[14] Also, imidacloprid tested at 1 μM, a concentration not normally inducing currents in such cells, attenuated the responses to ACh when co-applied with the neurotransmitter.[11]

Neonicotinoids are ineffective in activating the vertebrate $\alpha4\beta2$ nAChR, yet imidacloprid has been found to potentiate the response of $\alpha4\beta2$ when co-applied with ACh.[9,15] Although this finding is of interest, the underlying mechanism remains to be elucidated.

In the present study, using two-electrode voltage-clamp electrophysiology, we investigated the actions of several neonicotinoids (Fig. 1) on the ACh dose–response curve for chicken $\alpha4\beta2$ nAChR expressed in *Xenopus laevis* oocytes. We show that only neonicotinoids possessing a nitro group enhance the amplitude of the response to ACh recorded from $\alpha4\beta2$ nAChR, whereas thiacloprid with its cyano group attenuates the ACh response. A possible explanation is offered involving differences in ligand-$\alpha4\beta2$ nAChR interactions.

Materials and Methods

1. Expressing nicotinic AChRs in Xenopus oocytes by nuclear injection of cDNA

Oocytes at stage V or VI of development were removed under anesthetic (immersion in 1.5 g/l tricaine for 30 min) from adult female *Xenopus laevis* and separated from the follicle cell layer by treatment with 2 mg/ml collagenase (Type 1A; Sigma-Aldrich Japan, Tokyo, Japan) for 30 min at room temperature (20–25°C). Care was taken to reduce suffering and to minimize the number of animals used in accordance with the UK Animals (Scientific Procedures) Act, 1986. The nucleus of each defolliculated oocyte was injected with 2 ng of a 1 : 1 mixture of $\alpha4$ and $\beta2$ cDNAs cloned in the pcDNA3.1(+) expression vector. Then oocytes were incubated at 16°C in standard oocyte saline (SOS; 100 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.0 mM HEPES, pH 7.6), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamycin, and 2.5 mM sodium pyruvate. Electrophysiology was performed 2–5 days postinjection.

2. Electrophysiology

Xenopus laevis oocytes were secured in a recording chamber perfused continuously (7–10 ml/min) with SOS, containing 0.5 μM atropine to eliminate any response resulting from activation of endogenous muscarinic AChRs.[16] Membrane currents were recorded using 2.0 M KCl-filled electrodes (resistance, 0.5–5 MΩ) linked to a GENECLAMP 500B (Molecular Devices, Sunnyvale, CA, USA) amplifier at a membrane potential of -100 mV. Current signals were digitized prior to analysis on line.

Dimethyl sulfoxide (DMSO) stock solutions of neonicotinoids were diluted with SOS containing 0.5 μM atropine to prepare test solutions. DMSO at 1% or lower concentrations had no effect on the response of $\alpha4\beta2$ to ligands. Solutions of ACh in SOS were prepared by adding ACh chloride (Sigma-Aldrich Japan, Tokyo, Japan) directly to atropine-containing saline immediately prior to experiments. Oocytes were challenged with test compounds at intervals of 3 min to minimize the effects of desensitization and to ensure wash out of neonicotinoids. Clothianidin was a gift from Sumitomo Chemical Co., whereas imidacloprid, thiacloprid, compound **1** (a cyano version of imidacloprid) and compound **2** (a nitro version of thiacloprid) were generously provided by Bayer CropScience Co.

3. Data analysis

The peak amplitude of each response was normalized to the response to 100 μM ACh. Normalized ACh dose–response data were analyzed by non-linear regression analysis using GraphPad Prism software (GraphPad Software, CA, USA), as described previously.[16] The amplitude of each ACh response recorded in the presence of neonicotinoids was normalized to the control response to ACh measured before co-application with neonicotinoids. Concentration–response data for the agonist action of ACh and the inhibitory action of thiacloprid for the ACh-induced response were fitted to the Hill equation. Data on neonicotinoid-induced potentiation were processed by the spline method using GraphPad Prism software.

Results

1. Potentiation of ACh-induced responses by imidacloprid and clothianidin

ACh evoked membrane currents in *Xenopus* oocytes expressing chicken $\alpha4\beta2$ nAChR were concentration-dependent with a pEC_{50} ($=-\log EC_{50}$) of 5.44 ± 0.11 ($n=9$), whereas imidacloprid barely activated vertebrate nAChR. Imidacloprid did not appear to evoke any response at concentrations lower than 10 μM (Fig. 2A); however, it enhanced the ACh-induced response when co-applied with 1 μM ACh (Fig. 2A, $n=8$), as reported earlier.[9] Here we have shown that the optimum con-

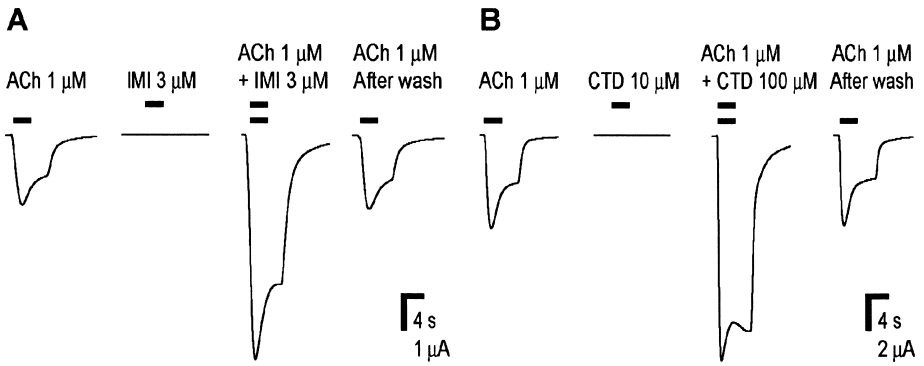

Fig. 2. Actions of imidacloprid (**A**) and clothianidin (**B**) on the response to acetylcholine (ACh) of chicken $\alpha4\beta2$ nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes recorded using two-electrode voltage-clamp electrophysiology. The neonicotinoids were co-applied with 1 μM ACh. Potentiation by neonicotinoids of the ACh-induced response was removed completely by washing with standard oocyte saline for 5 min. Abbreviations: IMI, imidacloprid; CTD, clothianidin.

centration for this action of imidacloprid is approximately 3 μM (Fig. 3A). Clothianidin also enhanced the response of $\alpha4\beta2$ nAChR to 1 μM ACh (n=5); however, in this case, the optimum concentration was higher than that observed for imidacloprid-induced potentiation (Fig. 3B). For both imidacloprid and clothianidin, the enhanced response to ACh was attenuated by increasing the ACh concentration from 1 μM to 100 μM (Figs. 3A and 3B, n=4).

To address the mechanism of potentiation, the effects of co-application of imidacloprid and clothianidin on the concentra-

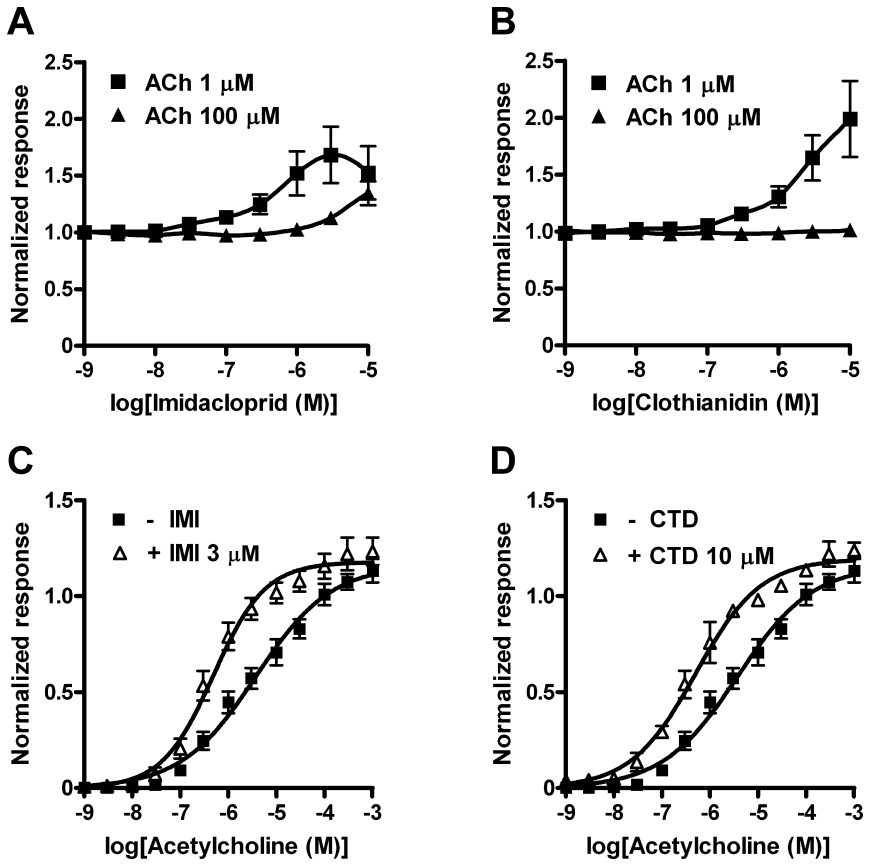

Fig. 3. Potentiation by imidacloprid (IMI) and clothianidin (CTD) of the response to acetylcholine (ACh) of chicken $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus laevis* oocytes. (**A**, **B**) Concentration-dependent actions of IMI (**A**) and CTD (**B**) on the response to 1 μM ACh of the $\alpha4\beta2$ nAChR. (**C**, **D**) Effects of co-application with IMI (**C**) and CTD (**D**) on the concentration–response curve of ACh for the nicotinic receptor. Each point plotted represents the mean±SEM of 4 to 8 experiments using at least two batches of oocytes.

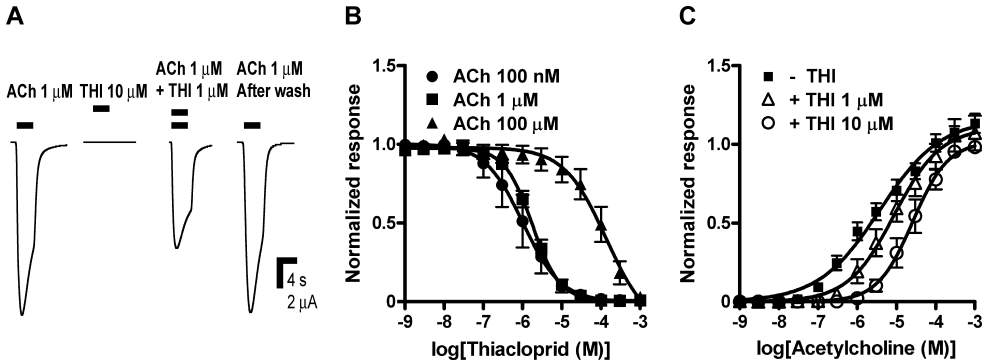

Fig. 4. Blocking actions of thiacloprid (THI) on the response to acetylcholine (ACh) of chicken $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus laevis* oocytes. (**A**) Responses of oocytes expressing the $\alpha4\beta2$ nAChR to 1 μM ACh, 10 μM THI and 1 μM ACh co-applied with various concentrations of THI. Blocking actions were removed completely by washing with standard oocyte saline for 5 min. (**B**) Effects of co-application with thiacloprid on the maximum current amplitude of the response to 1 μM ACh of $\alpha4\beta2$ nAChR. (**C**) Shifts of the ACh concentration–response curve by co-application with either 1 μM or 10 μM THI. In B–C, each point plotted represents the mean±SEM of 4 to 8 experiments using at least two batches of oocytes.

tion–response curve of ACh were examined (Figs. 3C and 3D). In the presence of 3 μM imidacloprid, the ACh concentration–response curve for $\alpha4\beta2$ was shifted to the left (Fig. 3C). The pEC_{50} value (6.31±0.07 (n=7)) obtained in the presence of imidacloprid differed significantly from the value (5.44±0.11) observed in the absence of imidacloprid (P<0.01, one-way ANOVA, Dunnet's test). In contrast to the effects on the EC_{50} value, the normalized maximum response to ACh was barely influenced by co-application with 3 μM imidacloprid (Fig. 3C). Similarly, 10 μM clothianidin also shifted the ACh concentration–response curve to the left (Fig. 3D), resulting in a pEC_{50} value of 6.29±0.08 (n=4) which differs significantly (P<0.01, one-way ANOVA, Dunnet's test) from that obtained in the absence of clothianidin.

2. Block of ACh-induced responses by thiacloprid

Unlike imidacloprid and clothianidin, thiacloprid attenuated the ACh-evoked response in a concentration-dependent manner when co-applied with ACh (Figs. 4A and 4B). Thus, an IC_{50} (M) was determined for the capacity of thiacloprid to reduce the response to 1 μM ACh to half its maximum amplitude. The pIC_{50} ($=-\log IC_{50}$) values for the responses to 100 nM, 1 μM and 100 μM ACh were 5.59±0.11 (n=4), 5.75±0.09 (n=4) and 3.87±0.20 (n=4), respectively.

When co-applied with 1 and 10 μM thiacloprid, the ACh concentration–response curve was shifted to the right. The difference between pEC_{50} values in the presence (4.58±0.08 (n=4)) and absence (5.44±0.11 (n=9)) of 10 μM thiacloprid was significant (P<0.01, one-way ANOVA, Dunnet's test), whereas the maximum response to ACh was unaffected (Fig. 4C).

3. Effects of replacing the nitro and cyano groups of neonicotinoids on ACh-induced responses

To explore further the potentiating action of imidacloprid and clothianidin on the ACh-induced response of $\alpha4\beta2$ nAChR, compound **1** containing a cyano group instead of the nitro group of imidacloprid, and compound **2** containing a nitro group instead of the cyano group of thiacloprid were investigated. Compound **1** showed no potentiating action regardless of whether the ACh concentration was 100 nM or 1 μM (Fig. 5A). On the other hand, compound **2**, although it only attenuated the current amplitude of the ACh-induced response of $\alpha4\beta2$ nAChR when co-applied with 1 μM ACh, potentiated the response to 100 nM ACh at concentrations of 1, 10 and 100 nM (Fig. 5B).

Discussion

In this study, we investigated the effects of imidacloprid, clothianidin and thiacloprid on the response to ACh of $\alpha4\beta2$ nAChR. Potentiation of the response was not limited to imidacloprid, but was also seen for clothianidin. Similar actions have also been observed for *d*-tubocurarine,[17] atropine[18] and choline,[19] and these observations have been interpreted in terms of an equilibrium two-site receptor occupation model.[19,20] According to this model, sequential occupation of the two agonist binding sites by two different agonists greatly enhances the probability of opening the nAChR channel. Potentiation by imidacloprid and clothianidin of the ACh-induced response may also be accounted for in a similar fashion, because the action was reduced by increasing the ACh concentration (Figs. 3A and 3B). Although imidacloprid and clothianidin are able to activate $\alpha4\beta2$ nAChR at high concentrations, potentiation of the ACh-induced response is unlikely to result from this property, because antagonists *d*-tubocurarine and atropine were also found to induce similar actions.[17,18]

Unlike imidacloprid and clothianidin, thiacloprid blocked the ACh-induced response. Interestingly, whether they were blocking or potentiating the $\alpha4\beta2$ ACh response, the actions

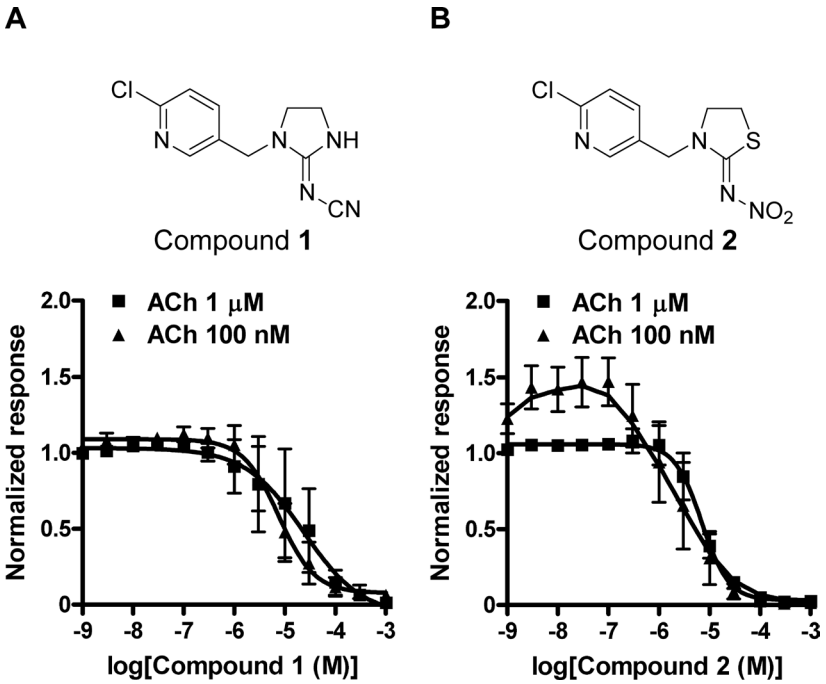

Fig. 5. Effects of replacing the functional group of imidacloprid and thiacloprid on the response to acetylcholine (ACh) of chicken $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus laevis* oocytes. (**A**) Effects of co-application with compound **1** on the maximum current amplitude of the response to 100 nM and 1 μM ACh of the $\alpha4\beta2$ nAChR. (**B**) Effects of co-application with compound **2** on the maximum current amplitude of the response to 100 nM and 1 μM ACh of the $\alpha4\beta2$ nAChR. Each pinot plotted represents the mean±SEM of 4 experiments using at least two batches of oocytes.

of these three neonicotinoids were all reversed by increasing the concentration of ACh. It is therefore conceivable that the differential action of thiacloprid is due either to its unique interaction at a particular site in the nAChR or to a lack of interaction of the kind seen for imidacloprid and clothianidin. Thiacloprid lacks a nitro group but instead possesses a cyano group, suggesting a possible role for the nitro group in potentiating the ACh-induced response of $\alpha4\beta2$ nAChR. In support of this, replacing the nitro group of imidacloprid by a cyano group abolished such an action, whereas replacing the cyano group of thiacloprid by a nitro group conferred the capacity to potentiate the ACh response (Fig. 5).

Our earlier findings[21] and recent photoaffinity labeling experiments and homology modeling[22] indicated that the nitro group of neonicotinoids may interact with the X residue in the YXCC motif of loop C in the N terminal agonist binding domain of the α subunit. The $\alpha4$ subunit has a glutamate residue as the X residue in loop C, which is predicted to result in electrostatic repulsion if it interacts directly with the nitro group. Thus, even if the nitro group is implicated in potentiation of the ACh-evoked response, it will interact with another position.

In conclusion, we investigated the actions of three commercial neonicotinoids and their analogs on the ACh-induced response of $\alpha4\beta2$ nAChR expressed in *Xenopus* oocytes using two-electrode voltage-clamp electrophysiology. An important finding of this study is that imidacloprid and clothianidin enhanced the response, whereas thiacloprid blocked it, suggesting a role for the nitro group in potentiation, which was supported by experiments using cyano and nitro analogs of imidacloprid and thiacloprid, respectively. The findings obtained in this study contribute to enhancing our understanding of the basis for the diverse actions of neonicotinoids on their targets and may assist in the future design of safer insecticides acting with improved selectivity for insect nAChRs.

Acknowledgments

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