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Activation and Modulation of Human α4β2 Nicotinic Acetylcholine Receptors by the Neonicotinoids Clothianidin and Imidacloprid

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Neonicotinoids are synthetic, nicotine-derived insecticides used for agricultural and household pest control. Though highly effective at activating insect nicotinic receptors, many neonicotinoids are also capable of directly activating and/or modulating the activation of vertebrate nicotinic receptors. In this study, we have investigated the actions of the neonicotinoids clothianidin (CTD) and imidacloprid (IMI) on human neuronal α4β2 nicotinic acetylcholine receptors. The data demonstrate that the compounds are weak agonists of the human receptors with relative peak currents of 1–4% of the response to 1 mM acetylcholine (ACh). Coapplication of IMI strongly inhibited currents elicited by ACh. From Schild plot analysis, we estimate that the affinity of IMI for the human α4β2 receptor is 18 μM. The application of low concentrations of CTD potentiated responses to low concentrations of ACh, suggesting that receptors occupied by one ACh and one CTD molecule have a higher gating efficacy than receptors with one ACh bound. Interestingly, subunit stoichiometry affected inhibition by CTD, with $(\alpha4)_2(\beta2)_3$ receptors significantly more strongly inhibited than the $(\alpha4)_3(\beta2)_2$ receptors.

Key words: nicotinic receptor; neonicotinoid; insecticide

Neonicotinoids are synthetic, nicotine-based compounds that have been used with great success as insecticides against agricultural and household pests. Neonicotinoids were first described in the 1970s and have grown to account for over 15% of the global insecticide market at present (Jeschke and Nauen, 2008). The compounds bind with high affinity to the transmitter binding site in the insect nicotinic receptor, leading to depolarization of the cell, eventually resulting in death of the insect (Salgado and Saar, 2004; Tan et al., 2007).

Although most neonicotinoids are highly selective for nicotinic receptors from insects, animal studies have shown that exposure to the neonicotinoid imidacloprid (IMI) affects the central nervous system similarly to nicotine, leading to tremors, impaired papillary function, and hypothermia (Sheets, 2001). Human exposure to the insecticide can result from petting dogs treated with imidacloprid-based insecticides to control fleas up to 4 weeks before (Craig et al., 2005), and clinical case reports have described, in extreme cases, fatal outcomes following poisoning with neonicotinoids (Proenca et al., 2005; Shadnia and Moghaddam, 2008). Overall, however, data on human toxicity are limited.

Many neonicotinoids can interact with high affinity and act as strong agonists on the native insect nicotinic receptor (for reviews see Matsuda et al., 2005; Tomizawa and Casida, 2005). The neonicotinoid clothianidin (CTD) is as efficacious, but a more potent, activator of nicotinic receptors in cholinergic neurons from *Drosophila* larvae or the American cockroach as the transmitter acetylcholine (ACh; Brown et al., 2006; Tan et al., 2007). In contrast, vertebrate receptors are typically only weakly sensitive to neonicotinoids. In clonal rat pheochromocytoma (PC12) cells, IMI weakly activates the nicotinic receptors but is capable of antagonizing currents elicited by carbachol (Nagata et al., 1998). This suggests that IMI has low gating efficacy on the types of nicotinic receptors expressed in PC12 cells. IMI is similarly ineffective on recombinant chick α7 and α4β2 receptors expressed in *Xenopus* oocytes, with the concentration–effect curves placed at concentrations exceeding that for ACh by up to two orders of magnitude (Matsuda et al., 1998; Ihara et al., 2003). A previous study showed that currents elicited by ACh from chick α4β2 receptors can be potentiated by CTD and IMI (Toshima et al., 2008).

Here we investigated the activation and modulation of human neuronal-type α4β2 nicotinic receptors by two widely used neonicotinoids, clothianidin and imida-

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cloprid. The α4β2 receptor forms most of the high-affinity nicotine binding sites in the brain. The motivation for the studies comes from the implication of involvement of nicotinic receptors in the clinical manifestation of neonicotinoid poisoning, and the lack of previous studies on neonicotinoid actions on human nicotinic receptors. The data indicate that CTD and IMI are weak agonists of human nicotinic receptors. At high concentrations, both compounds competitively inhibit receptor activation by nicotinic agonists.

MATERIALS AND METHODS

This work was conducted on human neuronal-type α4β2 nicotinic ACh receptors. A human embryonic kidney (HEK) 293 cell line stably expressing human α4β2 receptors (Zhang and Steinbach, 2003) was a gift from Dr. J.H. Steinbach (Washington University School of Medicine, St. Louis, MO). In some experiments, receptors containing the α4 and β2 subunits were transiently expressed in *Xenopus* oocytes. The cDNAs for the subunits were linearized by Xho I (Fermentas, Glen Burnie, MD) digestion, and the cRNA was produced using an mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with 7–14 ng total cRNA in a volume of 19 nl and incubated at 16°C for 2–3 days before recording. To control subunit stoichiometry, the ratio of cRNAs was held at 1:9 (α4:β2, resulting in a 2:3 ratio) or 9:1 (3:2 ratio).

The electrophysiological experiments in HEK cells were performed using whole-cell macroscopic and cell-attached single-channel patch clamp recordings. The bath solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 10 HEPES, pH 7.4. In macroscopic recordings, the pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.4. In single-channel recording, the pipette solution contained (in mM): 142 KCl, 5.4 NaCl, 1.8 $CaCl_2$, 1.7 $MgCl_2$, 10 HEPES, pH 7.4.

In single-channel recordings, the patch voltage was determined based on the combination of cell membrane potential and applied potential. The cell membrane potential was estimated from the reversal potential of nicotinic receptor currents, assuming that the currents reverse at 0 mV.

In macroscopic recordings, the drugs (ACh, neonicotinoids) were applied using the SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT). The cells were typically clamped at −60 mV. In single-channel recordings, the drugs were added to the pipette solution. Currents were amplified with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), digitized at 10 kHz (whole-cell recordings) or 50 kHz (single-channel recordings), and saved on a PC hard disk using a Digidata 1300 Series interface (Molecular Devices) for further analysis.

Standard two-electrode voltage clamp was used to record the currents from *Xenopus* oocytes. Both voltage and current electrodes were patch-clamp electrodes (OD = 1.2 mm, ID = 0.69 mm; Warner Instruments) filled with 3 M KCl and had resistances of 0.5–1.5 MΩ. The oocytes were clamped at −60 mV. The chamber was perfused continuously at approximately 5 ml/min, and bath solution was perfused between all test applications. Solutions were switched via pClamp using a VC-8T valve controller (Warner Instruments) and were applied from glass reservoirs via metal or Teflon tubing to reduce adsorption. The current responses were amplified with an Axoclamp 900A amplifier (Molecular Devices), digitized with a Digidata 1300 series digitizer (Molecular Devices) at a 100-Hz sampling rate, and stored using pClamp. All experiments were performed at room temperature.

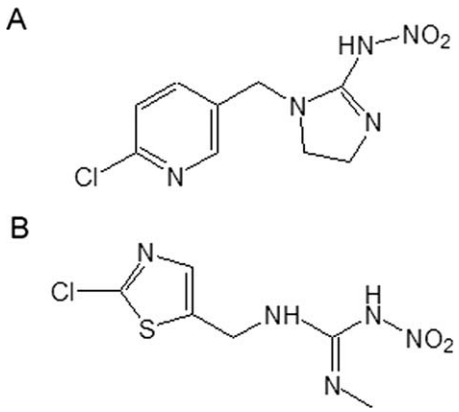

Fig. 1. Molecular structures of the neonicotinoids imidacloprid (**A**) and clothianidin (**B**).

Macroscopic recordings were obtained by exposing the cell to a drug (ACh, CTD, IMI) for 4–6 sec (HEK cells) or 15–25 sec (oocytes), followed by washout in bath to verify recovery. In the beginning of the experiment, two or three applications of ACh were made to obtain a stable control response. This was followed by an application of ACh + neonicotinoid or neonicotinoid alone. Finally, each cell was exposed again to ACh, to verify lack of rundown or nonrecovered desensitization. Successive drug applications were separated by 1–3 min washout periods in bath solution. In cases in which a cell was exposed to several concentrations of a neonicotinoid, each neonicotinoid application was preceded by exposure to ACh, to which the following response to neonicotinoid was normalized. The effects of the neonicotinoids were studied using coapplication of a nicotinic agonist and the neonicotinoid drug; no experiments employing preexposure were conducted.

The analysis of macroscopic currents was conducted using pClamp 9.0 (Molecular Devices) and was aimed at determining the peak amplitude. Single-channel currents from HEK cells expressing α4β2 receptors consisted of isolated openings that were analyzed with respect to single-channel amplitude to determine the single-channel conductance. The analysis was conducted using the QuB Suite (qub.buffalo.edu).

All reagents, including the neonicotinoids IMI and CTD (Fig. 1), were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (300 μM) of IMI and CTD were made in bath solution (for macroscopic experiments) or pipette solution (for single-channel experiments). Final dilutions were made as needed on the day of the experiment.

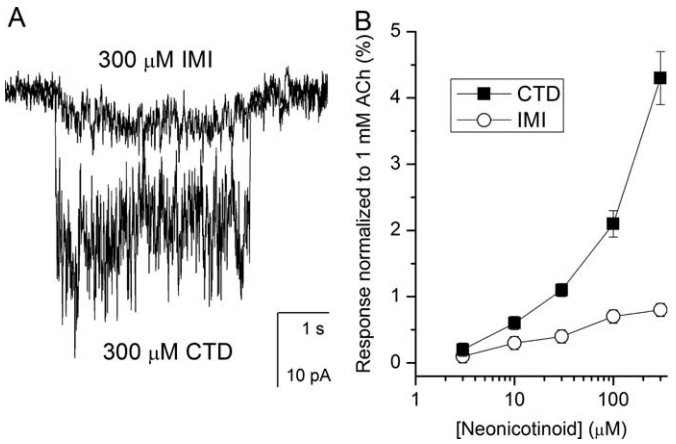

Fig. 2. Direct activation of human α4β2 receptors by the neonicotinoids CTD and IMI. **A:** An HEK cell stably expressing α4β2 receptors was exposed to 300 μM CTD or 300 μM IMI. For comparison, in the same cell the peak response to 1 mM ACh was 625 pA. **B:** The concentration–response relationship for CTD and IMI. Responses are normalized to peak currents elicited by 1 mM ACh. The data show mean ± sem from four cells for each compound.

RESULTS

The Neonicotinoids CTD and IMI Directly Activate the Human α4β2 Nicotinic Receptor

Exposure of HEK cells stably expressing human α4β2 receptors to the neonicotinoids CTD or IMI resulted in inward current. The peak amplitudes were small compared with those observed in the presence of saturating concentrations of ACh. Responses to 300 μM CTD had a peak amplitude of 4% ± 1% (mean ± sem; n = 10 cells) of that observed in response to 1 mM ACh. IMI was even less effective at eliciting channel activity. The peak response in the presence of 300 μM IMI was only 0.6% ± 0.3% (n = 10 cells) of the response observed in the presence of 1 mM ACh. Sample current responses in the presence of CTD or IMI are shown in Figure 2A.

The concentration–response measurements conducted in the presence of 3–300 μM CTD showed no saturation of peak amplitude levels (Fig. 2B). This indicates that CTD is a low-affinity, but, potentially, a high-efficacy agonist of the human α4β2 receptor. Estimates of the IMI concentration–response relationship were complicated by the small current responses. However, there were no significant differences in peak amplitudes in the presence of 100 and 300 μM IMI. This suggests that IMI is a low-efficacy, but, potentially, a high-affinity, agonist of the human α4β2 receptor. The neonicotinoid concentrations were limited by solubility limits in aqueous solutions.

IMI Strongly Inhibits α4β2 Receptors Activated by Nicotinic Agonists

We next examined the modulatory effect of CTD and IMI on receptors activated by ACh. The experiments were conducted under three conditions: in the presence of 1 mM ACh (a saturating concentration), 100 μM ACh (~EC50), and 5 μM ACh (~EC10).

IMI had a strong effect on currents elicited by ACh. Coapplication of 300 μM IMI with 1 mM ACh reduced the peak response to 41% ± 4% of control (n = 5 cells). The peak current was reduced to 10% ± 11% (n = 5 cells) in the presence of 100 μM ACh and to 4% ± 3% (n = 5 cells) in the presence of 5 μM ACh. Sample traces are shown in Figure 3A. Assuming that activity from IMI-occupied receptors is insignificant, i.e., IMI acts as a competitive antagonist, we employed Schild plot analysis to estimate the affinity of IMI to the human α4β2 receptor. By using the data obtained at 3–300 μM IMI, we estimate that the K_D for IMI is 18.2 ± 0.9 μM (Fig. 4).

The presence of up to 300 μM CTD was without inhibitory effect on currents elicited by ACh (Fig. 3B). Furthermore, under some conditions, the application of CTD led to enhancement of the response elicited by ACh. When coapplied with 5 μM ACh (EC10), the addition of 10–100 μM CTD resulted in a significant increase in peak response. The effect was the greatest in the presence of 30 μM CTD (164% ± 26% of control, n = 5 cells; $P < 0.001$), diminishing at higher concentrations of the neonicotinoid. The potentiating effect of CTD was absent when higher concentrations of ACh were used to activate the receptor. The findings are summarized in Figure 3C.

The Neonicotinoids Do Not Affect Single-Channel Conductance of α4β2 Receptors

To exclude the possibility that reduced single-channel conductance contributes to the small macroscopic responses in the presence of CTD or IMI, we conducted single-channel patch-clamp experiments on HEK cells stably expressing α4β2 receptors. Receptors in cell-attached patches were activated by 100 μM ACh, 300 μM CTD, or 300 μM IMI, and single-channel activity recorded at applied potentials of 0 to +100 mV (roughly, V_M of −30 to −130 mV). The single-channel conductance of the dominant (>80% of all openings) type of activity was estimated as slope conductance from the amplitude–voltage plot. The data indicate that the single-channel conductance is similar in the presence of ACh, CTD, or IMI. We estimate that the single-channel conductance is 45 ± 4 pS (n = 3 patches), 50 ± 14 pS (n = 3 patches), or 53 ± 10 pS (n = 3 patches) in the presence of ACh, CTD, or IMI, respectively. These values are in general agreement with previous estimates of single-channel conductance of α4β2 receptors (Buisson et al., 1996; Nelson et al., 2003) and indicate that the smaller macroscopic responses to neonicotinoids are not due to reduced single-channel conductance.

The α4β2 Receptor Stoichiometry Affects Sensitivity to CTD

Receptors consisting of α4 and β2 subunits can have a stoichiometry of 2 α subunits:3 β subunits or 3 α

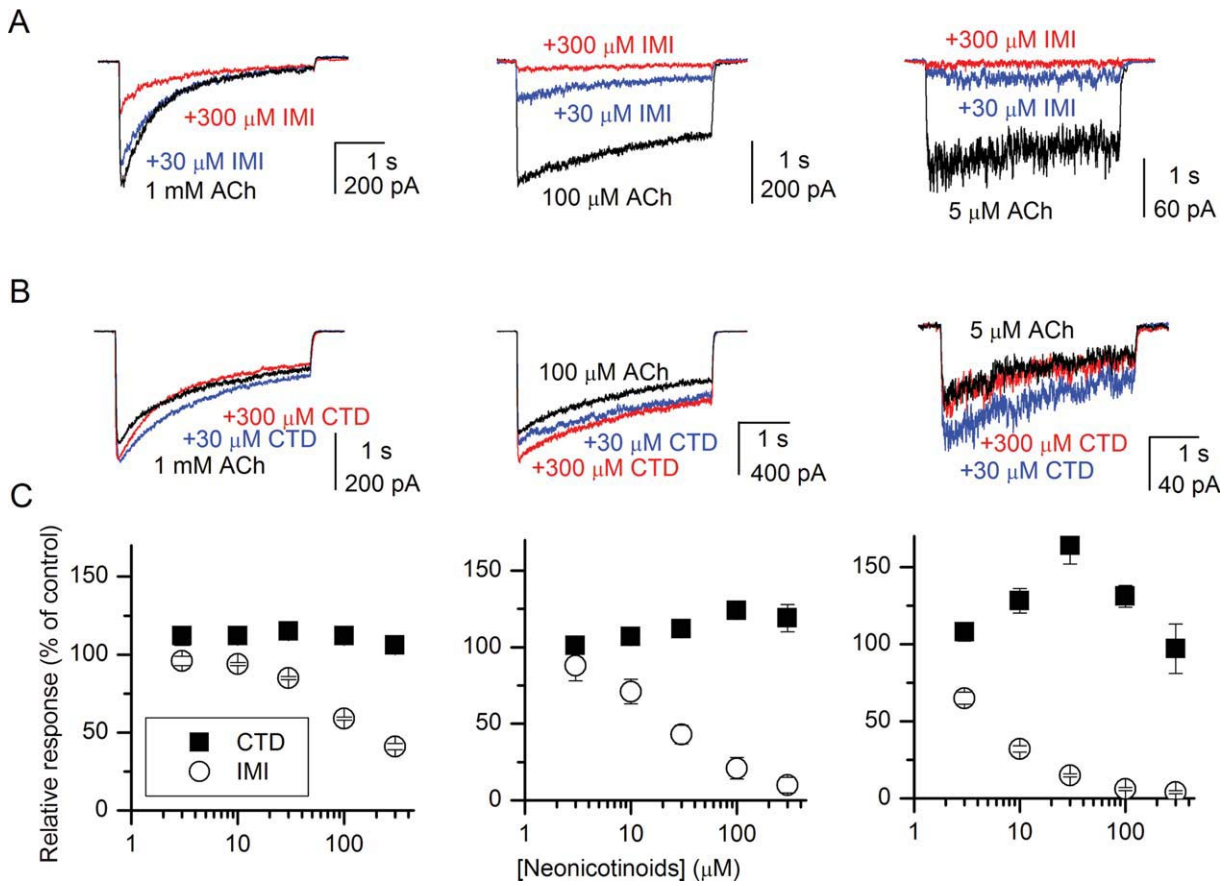

Fig. 3. Modulation of human α4β2 receptors by the neonicotinoids CTD and IMI. **A:** Current traces from HEK cells stably expressing α4β2 receptors. The receptors were activated by 1 mM ACh (left), 100 μM ACh (middle), or 5 μM ACh (right) in the absence and presence of 30 or 300 μM IMI. The presence of IMI results in reduction of current amplitude. **B:** Current traces from HEK cells stably expressing α4β2 receptors. The receptors were activated by 1 mM ACh (left), 100 μM ACh (middle), or 5 μM ACh (right) in the absence and presence of 30 or 300 μM CTD. The presence of CTD has no effect on currents elicited by 1 mM ACh. Currents elicited by 100 μM or 5 μM ACh are potentiated by CTD. **C:** Summary of the effects of CTD and IMI on currents elicited by 1 mM ACh (left), 100 μM ACh (middle), or 5 μM ACh (right).

subunits:2 β subunits. The stoichiometry of α4β2 receptors in the HEK cell line used in the studies described above is 3 α subunits:2 β subunits (Li and Steinbach, 2010). The switch from one form to another can take place in response to a long-term exposure to a nicotinic agonist or by culturing the cells at lower temperatures but is typically achieved by overexpression of the α or β subunit (Nelson et al., 2003).

Here we tested whether receptor sensitivity to neonicotinoids depends on subunit stoichiometry. Because the receptors containing 2 α subunits per receptor typically show relatively small maximal currents (Moroni et al., 2006; X. Jin and J.H. Steinbach, unpublished data), we conducted these experiments on receptors expressed in *Xenopus* oocytes. The switch in subunit stoichiometry was achieved by altering the ratio of cRNAs injected into the oocytes and confirmed by testing the oocytes for sensitivity to ACh. Oocytes injected with α4 and β2 subunit cRNAs in a 9:1 ratio had an ACh EC50 of 47 μM, whereas those injected in a 1:9 ratio had an EC50 of 0.9 μM (Fig. 5A). Previous work has shown that differences in subunit stoichiometry, resulting from changes in subunit availability when different amounts of cRNA are used for injections, underlie differential sensitivity to ACh (Moroni et al., 2006; Carbone et al., 2009).

Coapplication of 300 μM CTD or IMI with 1 mM ACh (a saturating concentration) reduced the peak response. The application of IMI reduced the peak response to 46% ± 8% (n = 10 cells) or 51% ± 11% of control (n = 15 cells) in $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ receptors, respectively. The effects are not significantly different from each other ($P > 0.27$) or from the values obtained for HEK cells stably expressing the $(\alpha4)_3(\beta2)_2$ receptors [$P > 0.07$ for $(\alpha4)_2(\beta2)_3$; $P > 0.18$ for $(\alpha4)_3(\beta2)_2$]. When CTD was coapplied with ACh, the peak response was reduced to 83% ± 6% (n = 10 cells) of control in oocytes expressing $(\alpha4)_3(\beta2)_2$ receptors and to 73% ± 9% (n = 15 cells) in oocytes expressing $(\alpha4)_2(\beta2)_3$ receptors. The effects of CTD on $(\alpha4)_2(\beta2)_3$

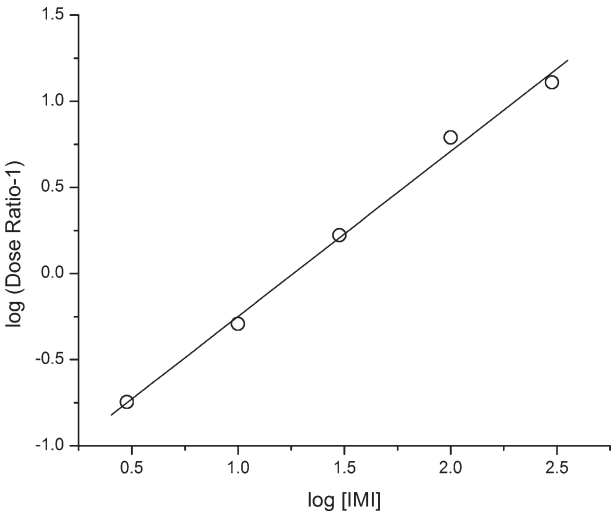

Fig. 4. Schild plot analysis of antagonism by IMI. The linear fit of the relationship between log (dose ratio − 1) and log [IMI] gave a Y-intercept of −1.21 ± 0.07 and a slope of 0.96 ± 0.04. The slope value of nearly 1 indicates that IMI acts as a competitive antagonist. Dose ratio was calculated as the ratio of the half-maximal ACh concentration in the presence of various concentrations of IMI over control. We estimate that the K_D for IMI is 18.2 μM.

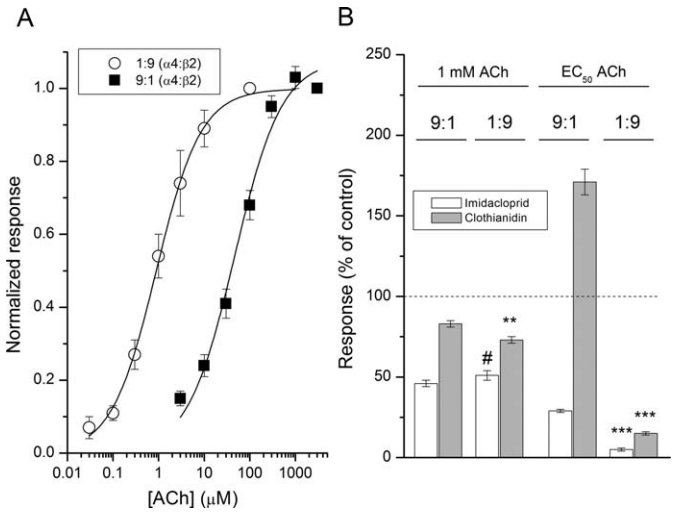

Fig. 5. Effect of subunit stoichiometry of human α4β2 receptors expressed in *Xenopus laevis* oocytes on activation by ACh and modulation by the neonicotinoids CTD and IMI. **A:** The relationships between peak response and ACh concentration are given for α4β2 receptors from oocytes injected with 1:9 (circles) and 9:1 (squares) cRNA ratios. The curves were fitted to the Hill equations. The best-fit parameters for 1:9 ratio are: maximal response = 1 ± 0.2, EC50 = 0.89 ± 0.06 μM, and n_H = 0.9 ± 0.1. The best-fit parameters for 9:1 ratio are: maximal response = 1.1 ± 0.1, EC50 = 47 ± 1 μM, and n_H = 0.8 ± 0.1. **B:** Summary of the modulation data for 300 μM CTD or 300 μM IMI on currents elicited by a saturating concentration (1 mM) of ACh or an EC50 concentration (1 μM for 1:9 ratio, and 50 μM for 9:1 ratio). The data show mean ± sem. Statistical analyses (*t*-test) were conducted to compare the effects of the neonicotinoids on oocytes injected with 1:9 vs. 9:1 ratio. ★★$P < 0.01$, ★★★$P < 0.001$, #not significant.

receptors is statistically different from the effect on $(\alpha4)_3(\beta2)_2$ receptors ($P < 0.01$). The data are summarized in Figure 5B.

We also tested neonicotinoid modulation of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ receptors activated by an EC50 concentration of ACh (1 and 50 μM for the two forms, respectively). IMI had an inhibitory effect on ACh-elicited currents. The peak responses from the $(\alpha4)_3(\beta2)_2$ receptors were reduced to 29% ± 2% of control (n = 6 cells) when 300 μM IMI was coapplied with ACh. In oocytes expressing $(\alpha4)_2(\beta2)_3$ receptors, the peak response was reduced to 5% ± 2% of control (n = 4 cells). The data are summarized in Figure 5B.

The effect of CTD was dependent on subunit stoichiometry. The $(\alpha4)_3(\beta2)_2$ receptors were potentiated by CTD. When 300 μM CTD was coapplied with 50 μM ACh (an EC50 concentration), the peak response was enhanced to 171% ± 19% of control. This effect is similar to CTD-elicited potentiation seen in HEK cells stably expressing the $(\alpha4)_3(\beta2)_2$ variant (Fig. 3B,C). In contrast, in oocytes expressing $(\alpha4)_2(\beta2)_3$ receptors, coapplication of 300 μM CTD with ACh inhibited the peak response to 15% ± 2% of control (n = 4 cells).

We also tested direct activation by CTD and IMI of α4β2 receptors expressed in *Xenopus* oocytes. The $(\alpha4)_3(\beta2)_2$ variant gave small current responses to applications of 300 μM CTD or IMI. We estimate that the peak current in the presence of CTD is 4% ± 1% (n = 3 cells) of that in the presence of saturating ACh. Direct activation by IMI was <1% of that observed for ACh. Both values are similar to those in HEK cells stably expressing the $(\alpha4)_3(\beta2)_2$ variant. Currents elicited by CTD and IMI from oocytes expressing $(\alpha4)_2(\beta2)_3$ receptors were too small to quantify reliably.

DISCUSSION

Neonicotinoids are synthetic, nicotine-derived compounds that are widely used for agricultural and household pest control. Although neonicotinoids act with high selectivity on insect nicotinic receptors, several studies have demonstrated that the compounds can activate and/or modulate nicotinic receptors from vertebrates. Here, we have investigated the activation and modulation of human α4β2 nicotinic receptors by the neonicotinoids CTD and IMI. The data show that CTD and IMI directly activate the human nicotinic receptors, although the relative responses, at concentrations to which we were limited by solubility, were much lower than in the presence of the transmitter ACh. Despite the low peak amplitudes, responses to CTD and IMI were reliably observed in cells that were responsive to ACh. In addition, the application of IMI reduced responses to ACh.

Small direct activation responses can be a result of low affinity or low efficacy. Our data suggest that CTD is a low-affinity agonist of the human α4β2 receptor

because the concentration–effect relationship showed no indication of saturation of response at the highest concentration tested (300 μM). Furthermore, CTD was ineffective at inhibiting responses elicited by ACh, whereas responses to low concentrations of ACh were potentiated by CTD. This action is reminiscent of heteroligation previously described for other weak agonists such as choline and *d*-tubocurarine (Steinbach and Chen, 1995; Zwart and Vijverberg, 2000; Akk et al., 2005). In heteroliganded receptors, one transmitter binding site is occupied with ACh, the other with the secondary agonist (in the present study, CTD). If the gating efficacy of such receptors is greater than the efficacy of receptors singly liganded with ACh, then the application of the secondary drug results in potentiation of currents. We speculate that the increase in peak current observed in the presence of low ACh and CTD (Figs. 3B,C, 5B) arises from receptors in which one transmitter binding site is occupied by ACh and the other by CTD. In future studies, it will be interesting to determine whether the open probability of the α4β2 channel is increased when CTD is coapplied with low concentrations of ACh.

Our data suggest that IMI is a low-efficacy but high-affinity agonist of the human α4β2 receptor. The evidence for this comes from experiments in which IMI was coapplied with ACh. A statistically significant effect ($P < 0.001$) was observed at 10 μM IMI coapplied with 1 mM ACh (10 times EC50 concentration). From the Schild plot analysis, we estimate that the affinity of IMI for the human α4β2 receptor expressed in HEK cells is 18 μM.

It is well known that the α4β2 receptor can assemble in the stoichiometry of 2 α subunits:3 β subunits [$(\alpha4)_2(\beta2)_3$ receptors] and 3 α subunits:2 β subunits [$(\alpha4)_3(\beta2)_2$]. The switch, which can take place as a result of long-term exposure to nicotine (e.g., in smokers) or other agonists, is known to affect receptor sensitivity to ACh as well as other pharmacological and biophysical properties (Nelson et al., 2003; Moroni et al., 2006). We tested whether subunit stoichiometry affects sensitivity to CTD and IMI. To enhance the current levels, we conducted these experiments on receptors expressed in *Xenopus* oocytes. The data indicate that CTD is a stronger inhibitor of the $(\alpha4)_2(\beta2)_3$ than of the $(\alpha4)_3(\beta2)_2$ receptor. The effect is most striking when low concentrations of ACh are used to activate the receptors. In the presence of an EC50 concentration of ACh, the application of 300 μM CTD has a potentiating effect on the $(\alpha4)_3(\beta2)_2$ receptor. In contrast, the $(\alpha4)_2(\beta2)_3$ receptor is strongly inhibited by CTD. In future work, it will be interesting to determine whether the presence of an α subunit in the fifth position creates an additional binding site for CTD, thus enhancing its ability to activate the receptor. Single-channel experiments on HEK cells expressing α4β2 receptors indicate that the single-channel conductance is indistinguishable for ACh, CTD, and IMI.

Both the affinity and efficacy components contribute to the insecticidal activity of neonicotinoids (Nishiwaki et al., 2003; Ihara et al., 2004). In cholinergic neurons from *Drosophila* larvae, CTD is a significantly more efficacious agonist than IMI or the transmitter ACh (Brown et al., 2006). CTD and IMI are equally potent and efficacious on recombinant nicotinic receptors from the brown planthopper, *Nilaparvata lugens*, expressed in *Xenopus* oocytes (Liu et al., 2006). IMI is significantly weaker, compared with ACh, at activating chicken α7 receptors and is essentially inactive at chicken α4β2 receptors (Shimomura et al., 2002; Toshima et al., 2009). To the best of our knowledge, no previous studies have been conducted on human nicotinic receptors.

In sum, we have examined direct activation and modulation of human α4β2 nicotinic receptors by the neonicotinoids CTD and IMI. CTD potentiated responses to low concentrations of ACh but was largely without effect on currents elicited by saturating concentrations of the transmitter. In contrast, IMI inhibited currents from α4β2 receptors with high potency. The inhibitory effect was especially noticeable at lower transmitter concentrations, i.e., conditions under which the α4β2 receptors operate in vivo. The data thus suggest that IMI-containing insecticides may have stronger side effects on humans. The data also indicate that subunit stoichiometry can affect receptor modulation by neonicotinoids.

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REFERENCES

Akk G, Milescu LS, Heckmann M. 2005. Activation of heteroliganded mouse muscle nicotinic receptors. J Physiol 564:359–376.

Brown LA, Ihara M, Buckingham SD, Matsuda K, Sattelle DB. 2006. Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J Neurochem 99:608–615.

Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, Bertrand D. 1996. Human α4β2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: a patch-clamp study. J Neurosci 16:7880–7891.

Carbone AL, Moroni M, Groot-Kormelink PJ, Bermudez I. 2009. Pentameric concatenated (α4)(2)(β2)(3) and (α4)(3)(β2)(2) nicotinic acetylcholine receptors: subunit arrangement determines functional expression. Br J Pharmacol 156:970–981.

Craig MS, Gupta RC, Candery TD, Britton DA. 2005. Human exposure to imidacloprid from dogs treated with Advantage®. Toxicol Mech Methods 15:287–291.

Ihara M, Matsuda K, Otake M, Kuwamura M, Shimomura M, Komai K, Akamatsu M, Raymond V, Sattelle DB. 2003. Diverse actions of neonicotinoids on chicken α7, α4β2 and *Drosophila*-chicken SADβ2 and ALSβ2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Neuropharmacology 45:33–144.

Ihara M, Matsuda K, Shimomura M, Sattelle DB, Komai K. 2004. Super agonist actions of clothianidin and related compounds on the SAD β2 nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. Biosci Biotechnol Biochem 68:761–763.

Jeschke P, Nauen R. 2008. Neonicotinoids-from zero to hero in insecticide chemistry. Pest Manag Sci 64:1084–1098.

Li P, Steinbach JH. 2010. The neuronal nicotinic α4β2 receptor has a high probability of being open. Br J Pharmacol 160:1906–1915.

Liu Z, Williamson MS, Lansdell SJ, Han Z, Denholm I, Millar NS. 2006. A nicotinic acetylcholine receptor mutation (Y151S) causes reduced agonist potency to a range of neonicotinoid insecticides. J Neurochem 99:1273–1281.

Matsuda K, Buckingham SD, Freeman JC, Squire MD, Baylis HA, Sattelle DB. 1998. Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. Br J Pharmacol 123:518–524.

Matsuda K, Shimomura M, Ihara M, Akamatsu M, Sattelle DB. 2005. Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular biology, and receptor modeling studies. Biosci Biotechnol Biochem 69:1442–1452.

Moroni M, Zwart R, Sher E, Cassels BK, Bermudez I. 2006. α4β2 Nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. Mol Pharmacol 70:755–768.

Nagata K, Song JH, Shono T, Narahashi T. 1998. Modulation of the neuronal nicotinic acetylcholine receptor-channel by the nitromethylene heterocycle imidacloprid. J Pharmacol Exp Ther 285:731–738.

Nelson ME, Kuryatov A, Choi CH, Zhou Y, Lindstrom J. 2003. Alternate stoichiometries of α4β2 nicotinic acetylcholine receptors. Mol Pharmacol 63:332–341.

Nishiwaki H, Nakagawa Y, Kuwamura M, Sato K, Akamatsu M, Matsuda K, Komai K, Miyagawa H. 2003. Correlations of the electrophysiological activity of neonicotinoids with their binding and insecticidal activities. Pest Manag Sci 59:1023–1030.

Proenca P, Teixeira H, Castanheira F, Pinheiro J, Monsanto PV, Marques EP, Vieira DN. 2005. Two fatal intoxication cases with imidacloprid: LC/MS analysis. Forensic Sci Int 153:75–80.

Salgado VL, Saar R. 2004. Desensitizing and non-desensitizing subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. J Insect Physiol 50:867–879.

Shadnia S, Moghaddam HH. 2008. Fatal intoxication with imidacloprid insecticide. Am J Emerg Med 26:631–634.

Sheets LP. 2001. Imidacloprid: a neonicotinoid insecticide. In: Kreiger R, editor. Handbook of pesticide toxicology,2nd ed. New York: Academic Press. p1123–1130.

Shimomura M, Okuda H, Matsuda K, Komai K, Akamatsu M, Sattelle DB. 2002. Effects of mutations of a glutamine residue in loop D of the α7 nicotinic acetylcholine receptor on agonist profiles for neonicotinoid insecticides and related ligands. Br J Pharmacol 137:162–169.

Steinbach JH, Chen Q. 1995. Antagonist and partial agonist actions of d-tubocurarine at mammalian muscle acetylcholine receptors. J Neurosci 15:230–240.

Tan J, Galligan JJ, Hollingworth RM. 2007. Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. Neurotoxicology 28:829–842.

Tomizawa M, Casida JE. 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu Rev Pharmacol Toxicol 45:247–268.

Toshima K, Ihara M, Kanaoka S, Tarumoto K, Yamada A, Sattelle DB, Matsuda K. 2008. Potentiating and blocking actions of neonicotinoids on the response to acetylcholine of the neuronal α4β2 nicotinic acetylcholine receptor. J Pestic Sci 33:146–151.

Toshima K, Kanaoka S, Yamada A, Tarumoto K, Akamatsu M, Sattelle DB, Matsuda K. 2009. Combined roles of loops C and D in the interactions of a neonicotinoid insecticide imidacloprid with the α4β2 nicotinic acetylcholine receptor. Neuropharmacology 56:264–272.

Zhang J, Steinbach JH. 2003. Cytisine binds with similar affinity to nicotinic α4β2 receptors on the cell surface and in homogenates. Brain Res 959:98–102.

Zwart R, Vijverberg HP. 2000. Potentiation and inhibition of neuronal α4β4 nicotinic acetylcholine receptors by choline. Eur J Pharmacol 393:209–214.