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Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors

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1 Imidacloprid is a new insecticide with selective toxicity for insects over vertebrates. Recombinant (α4β2) chicken neuronal nicotinic acetylcholine receptors (AChRs) and a hybrid nicotinic AChR formed by co-expression of a *Drosophila melanogaster* neuronal α subunit (SAD) with the chicken β2 subunit were heterologously expressed in *Xenopus* oocytes by nuclear injection of cDNAs. The agonist actions of imidacloprid and other nicotinic AChR ligands ((+)-epibatidine, (−)-nicotine and acetylcholine) were compared on both recombinant nicotinic AChRs by use of two-electrode, voltage-clamp electrophysiology.

2 Imidacloprid alone of the 4 agonists behaved as a partial agonist on the α4β2 receptor; (+)-epibatidine, (−)-nicotine and acetylcholine were all full, or near full, agonists. Imidacloprid was also a partial agonist of the hybrid *Drosophila* SAD chicken β2 receptor, as was (−)-nicotine, whereas (+)-epibatidine and acetylcholine were full agonists.

3 The EC_{50} of imidacloprid was decreased by replacing the chicken α4 subunit with the *Drosophila* SAD α subunit. This α subunit substitution also resulted in an increase in the EC_{50} for (+)-epibatidine, (−)-nicotine and acetylcholine. Thus, the *Drosophila* (SAD) α subunit contributes to the greater apparent affinity of imidacloprid for recombinant insect/vertebrate nicotinic AChRs.

4 Imidacloprid acted as a weak antagonist of ACh-mediated responses mediated by SADβ2 hybrid receptors and as a weak potentiator of ACh responses mediated by α4β2 receptors. This suggests that imidacloprid has complex effects upon these recombinant receptors, determined at least in part by the α subunit.

Keywords: Imidacloprid; neonicotinoid insecticide; epibatidine; chicken α4β2 nicotinic receptor; *Drosophila* α subunit (SAD)

Introduction

The nitroguanidine, imidacloprid, is a commercially-important member of neonicotinoid insecticides for which nicotinic acetylcholine receptors (AChRs) are the target molecules (for reviews see Elbert *et al*., 1991; Leicht, 1996). Soloway *et al*., (1979) first described insecticidal actions and selective toxicity to insects of heterocyclic compounds containing a nitromethylene moiety. Related neonicotinoids with insecticidal activity, the nitromethylenes, depolarized the postsynaptic membrane and blocked excitatory postsynaptic potentials at an identified nicotinic cholinergic synapse between mechanosensory afferents and giant interneurones in the cockroach, *Periplaneta americana* (Schroeder & Flattum, 1984; Sattelle *et al*., 1989). Further evidence for a nicotinic site of action was provided by the ability of many neonicotinoids to displace [^{125}I]-α-bungarotoxin binding to *Periplaneta* nervous system membranes (Buckingham *et al*., 1995). The nitromethylene, nithiazin, gated single channels in neurones of *Musca domestica* with properties similar to those gated by ACh (Leech *et al*., 1991) and showed a depolarizing action on a cloned locust (*Schistocerca gregaria*) homomer-forming α subunit, αL1 (Marshall *et al*., 1990).

Imidacloprid, 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine (Figure 1), was the result of further structural developments of the prototype nitromethylenes (Kagabu *et al*., 1992; Shiokawa *et al*., 1992; Moriya *et al*., 1993). It is a potent insecticide, active on a variety of insect species, is selectively toxic to insects over vertebrates (Moriya *et al*., 1993) and has a higher potency for insect over vertebrate nicotinic AChRs (Zwart *et al*., 1994). Bai *et al*. (1991) found that imidacloprid depolarizes an identified cockroach motor neurone and displaces [^{125}I]-α-bungarotoxin binding to cockroach nerve cord preparations, establishing its action on native, α-bungarotoxin-sensitive insect nicotinic AChRs. Binding of imidacloprid and related compounds to nicotinic AChRs has also been shown for other invertebrate (and vertebrate) membrane preparations (Tomizawa & Yamamoto, 1992; 1993; Liu & Casida, 1993).

Imidacloprid actions on recombinant nicotinic AChRs comprising insect and vertebrate subunits may provide insights into its mechanism of action. Heterologous expression with *Xenopus* oocytes permits the study of ligand interactions with receptors of known subunit composition. Five cDNAs encoding putative insect nicotinic AChR subunits have been cloned: αL1 (α) from *Schistocerca gregaria* (Marshall *et al*., 1990), and ALS (α) (Bossy *et al*., 1988), SAD (α) (Bertrand *et al*., 1994), ARD (non-α) (Hermans-Borgmeyer *et al*., 1986) and SBD (non-α) (Sawruk *et al*., 1990b) from *Drosophila melanogaster*. No combination of *Drosophila* α subunits with either, or both, of the 2 *Drosophila* non-α-subunits so far cloned has been shown to result in robust functional nicotinic AChRs. However, the *Drosophila* nicotinic AChR subunits are all expressed in the nervous system, (Gundelfinger, 1992) and the α subunits can form functional receptors when co-expressed with a chicken neuronal β2 subunit (Bertrand *et al*., 1994).

To examine the role of α-subunits in determining the actions of imidacloprid we used heterologous nicotinic AChR

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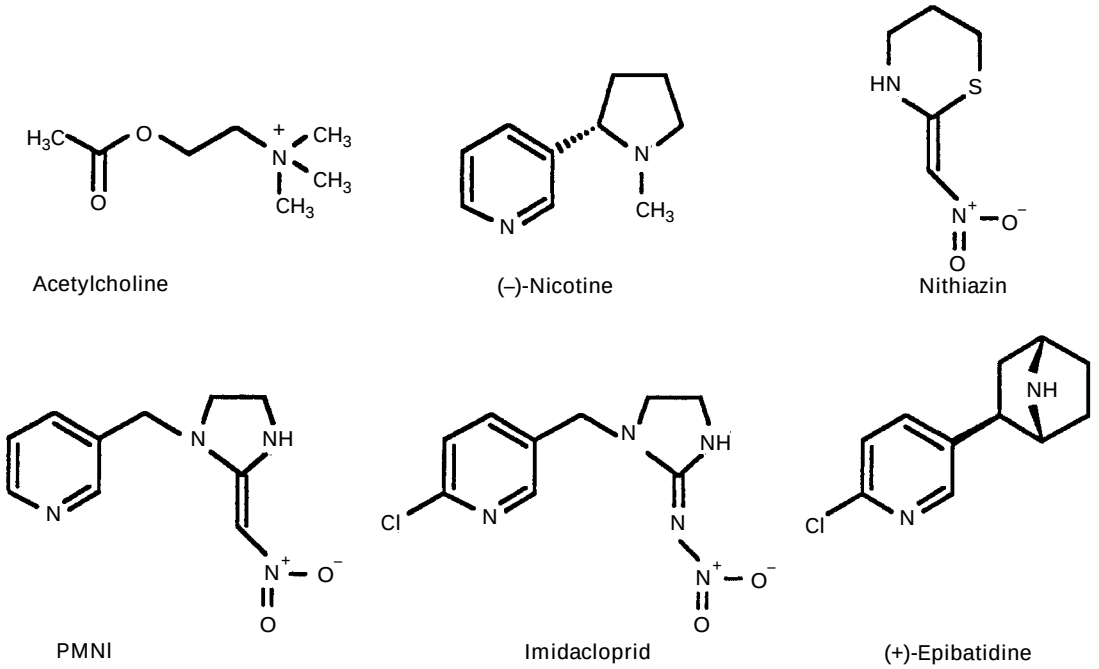

Figure 1 Chemical structures of nicotinic cholinoceptor ligands used in this study and/or referred to in the text including the nitroguanidine imidacloprid and related nitromethylene heterocycles.

expression in *Xenopus* oocytes. The effects of the compound on the expressed chicken neuronal $\alpha4\beta2$ nicotinic AChR were tested. This is the most abundant neuronal nicotinic AChR *in vivo* accounting for most of the high-affinity [^{3}H]-nicotine binding sites in chicken brain (Schoepfer *et al*., 1988; Whiting *et al*., 1987a,b; 1991; Morris *et al*., 1990). The findings were compared with data from the co-expression of the *Drosophila* SAD and chicken $\beta2$ subunits. We show that imidacloprid activates both recombinant receptors as a partial agonist, with higher apparent affinity for the *Drosophila*/chicken hybrid receptor.

Methods

Construction of nuclear injection vectors containing nicotinic AChR subunit clones

The *Drosophila* SAD cDNA (Sawruk *et al*., 1990a) was subcloned into the nuclear expression vector pMT3 (Swick *et al*., 1992). Oligonucleotides were designed to enable amplification of the protein-coding region of a cDNA clone in pBluescript which incorporated restriction sites for subsequent subcloning. These were: mso136 (forward primer, *Sal*I site) 5′GCCGTGTCGACACCATGGCTCCTGG mso137 (reverse primer, *Kpn*I site) 5′CGTTCGGTACCATTTAATTCTTCTT. The SAD cDNA was amplified with LA-PCR (Barnes, 1994). Reactions were performed in 50 mM KCl, 10 mM Tris HCl pH 9.0 and 0.1% Triton X100 (Promega) with the oligonucleotides at 0.2 μM and dNTPs at 0.2 mM; 0.7 μl of a 12 : 1 mixture of *Taq* DNA polymerase (Promega 5 units μl^{-1}) and *Pfu* polymerase (Stratagene 2.5 units μl^{-1}) were added during a 94°C 5 min hot start, followed by 30 cycles of 94°C, 45 s; 55°C, 45 s; 68°C, 3 min and one cycle of 68°C, 10 min. A single band was produced. The band was gel purified and subcloned into *Sal*I, *Kpn*I cut pMT3. The structure of the resulting plasmid was confirmed by DNA sequencing.

cDNAs encoding the chicken neuronal $\alpha4$ nicotinic AChR subunit in the pMT3 vector and the $\beta2$ subunit in the pCDM8 vector (Invitrogen, U.K.) were kindly provided by Dr M. Ballivet and Dr J. Patrick respectively.

Xenopus *oocytes and nuclear injection*

Stage IV–V oocytes were dissected from mature female *Xenopus laevis* and defolliculated manually after a 30 min incubation with collagenase (Sigma type IA, 2 mg ml^{-1}) in a calcium-free version of oocyte saline. Normal oocyte saline composition was as follows (mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5; pH 7.5. The nucleus of each oocyte was injected with 1 ng of each cDNA in 20 nl distilled water and incubated at 17–18°C in normal saline supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamicin (50 μg ml^{-1}) and 2.5 mM sodium pyruvate. Electrophysiology was performed 2–4 days after nuclear injection.

Electrophysiology of Xenopus *oocytes*

Oocytes were secured in a Perspex recording chamber (80 μl volume) and perfused continuously with normal oocyte saline (5–7 ml min^{-1}) by use of a gravity-fed system (Buckingham *et al*., 1994). Atropine 0.5 μM was included in the saline to suppress any responses resulting from activation of endogenous muscarinic acetylcholine receptors (Lupu-Meiri *et al*., 1990; Blake *et al*., 1993). Membrane currents were measured by the two-electrode voltage-clamp method, with 2 M KCl-filled electrodes (resistance = 0.5–5 MΩ) and a GENECLAMP 500 amplifier (Axon Instruments, U.S.A.). Unless stated otherwise, the oocyte membrane potential was clamped at −100 mV. Signals digitized by a TL-1 interface (Axon Instruments, U.S.A.) were stored on an IBM computer with Axotape software (version 1.2.01, Axon Instruments) and subsequently analysed off-line.

Test solutions were prepared by adding stock solutions of each compound to the standard oocyte saline containing atropine. Stock solutions of acetylcholine, (−)-nicotine and (+)-epibatidine in distilled water and imidacloprid in dimethylsulphoxide (DMSO) were stored in a freezer at

−20°C, whereas stock solutions of ACh were freshly prepared immediately before experiments. In early experiments, DMSO was used as a solvent for imidacloprid stocks to ensure full solvation. However, the limit of solubility of imidacloprid in saline was subsequently found to exceed 0.1 mM. Therefore, when imidacloprid was tested at concentrations higher than 100 μM it was dissolved directly into the saline to avoid any possible non-specific effects of the solvent. DMSO at concentrations lower than 0.2% (v/v) did not affect the amplitude or shape of responses ($n=6$ for SADβ2 and 2 for $\alpha4\beta2$).

Oocytes were challenged with compounds at intervals of 3–5 min to minimize the possible occurrence of desensitization. Only oocytes which gave stable responses to at least 2 successive applications of 10 μM ACh were used. Dose-response data were obtained by challenging oocytes with increasing concentrations of each agonist and the amplitude of the current recorded in response to each challenge was normalized to the maximum amplitude of the current response to 10 μM ACh. Data were not normalized to higher concentrations of ACh because such applications often desensitized the receptors so strongly that similar current responses could not be observed repeatedly, even with a 10 min interval between each challenge.

Normalized data were fitted by use of GraphPad 'Prism' (GraphPad Software, U.K.) to the following equation:

$$\varphi = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{(\log EC_{50} - [A])n_H}}$$

where φ is the normalized response to a compound applied at concentration [A], I_{max} and I_{min} are the maximum and the minimum normalized responses, respectively, EC_{50} is the concentration giving half the maximum normalized response and n_H is the Hill coefficient. Experiments were performed at room temperature (25±5°C).

Imidacloprid was synthesized *de novo* and acetylcholine, (−)-nicotine (free base) and (+)-epibatidine (HCl salt) were obtained from Sigma-Aldrich Co. (Dorset, U.K.).

Results

Imidacloprid, ACh, (−)-nicotine and (+)-epibatidine evoked dose-dependent, inward currents in *Xenopus* oocytes expressing chicken neuronal $\alpha4\beta2$ nicotinic AChRs recorded under two-electrode voltage-clamp (Figure 2a). Figure 3a shows the dose-response curves and Table 1 summarizes the pEC_{50} ($=-\log_{10} EC_{50}$), Hill coefficient and normalized maximum response for each compound. Imidacloprid was found to have the lowest pEC_{50} (<4.10), whereas (+)-epibatidine, which shares the 6-chloro-3-pyridyl moiety with imidacloprid (Spande *et al*., 1992), showed the highest pEC_{50} (8.88±0.03) of the ligands tested in this study on the recombinant vertebrate receptor. ACh and (−)-nicotine activated the $\alpha4\beta2$ receptor with similar apparent affinities (ACh, $pEC_{50}=6.30$; (−)-nicotine, $pEC_{50}=6.39$). The maximum amplitude of responses to (−)-nicotine, (+)-epibatidine and ACh were similar, indicating that they were full agonists of the receptor. Although the maximum amplitudes of responses to epibatidine were significantly smaller than those to ACh ($P<0.001$, t test), epibatidine-evoked responses desensitized rapidly, suggesting that the maximum current is likely to have been underestimated. However, imidacloprid did not evoke maximum current amplitudes at saturating concentrations and was therefore a partial agonist (Table 1).

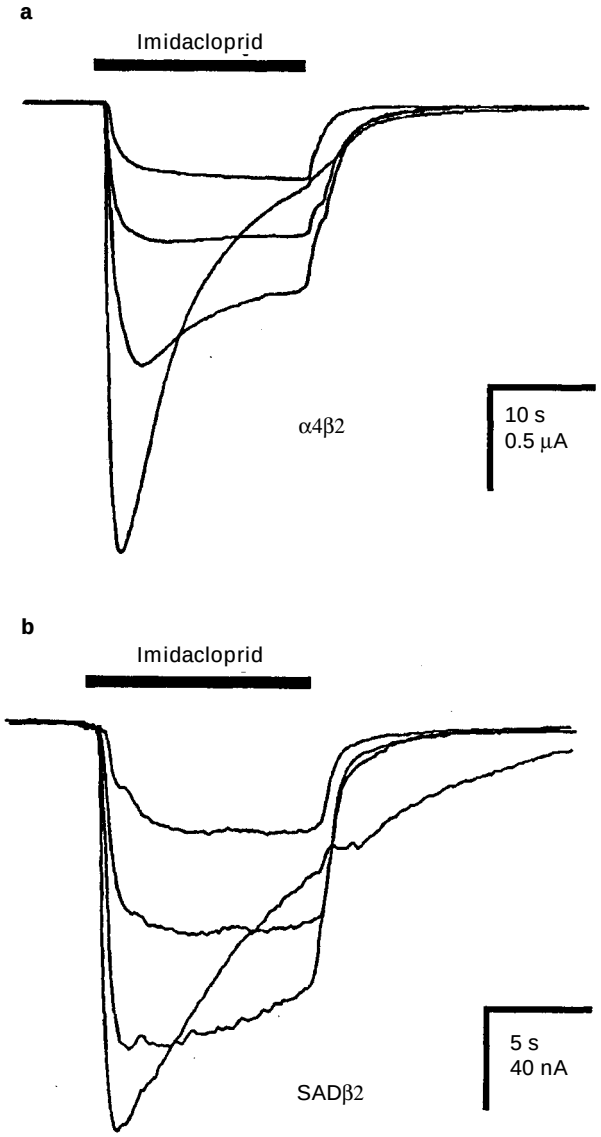

Figure 2 Activation by imidacloprid of chicken $\alpha4\beta2$ and the hybrid nicotinic acetylcholine receptor formed by co-expressing the chicken $\beta2$ subunit with *Drosophila* SAD. (a) Inward current responses of chicken $\alpha4\beta2$ to (from top to bottom) 10, 30 100 and 300 μM imidacloprid; (b) inward current responses of SAD $\beta2$ to (from top to bottom) 1, 2, 4 and 20 μM imidacloprid.

Replacing the chicken α4 subunit with the *Drosophila* SAD (α) subunit increased the pEC_{50} of imidacloprid by about 37 fold ($pEC_{50}=5.65$), whereas the pEC_{50}s of other ligands were decreased by 5–40 fold (9 fold, (+)-epibatidine; 5.6 fold, (−)-nicotine; and 40 fold ACh). A series of imidacloprid-induced currents mediated by the SAD $\beta2$ receptors is shown in Figure 2b. The full-agonist effect of (+)-epibatidine was preserved, but (−)-nicotine and imidacloprid both behaved as partial agonists on this recombinant receptor (Figure 3b).

We examined the contributions of the Ca^{2+}-activated chloride current component to the imidacloprid dose-response curve by exchanging the Ca^{2+} ions in the saline for Ba^{2+} ions (dose-response curves not shown). The pEC_{50} values of imidacloprid were not significantly affected by elimination of Ca^{2+} ions from the saline (pEC_{50}s in barium saline were 4.30±0.04, $n=4$ and 5.82±0.06, $n=4$ for $\alpha4\beta2$ and SAD $\beta2$, respectively, $P<0.01$, t test). Thus, we considered that the dose-response curves of the compounds in the presence of

external Ca^{2+} ions faithfully reflected the dose-dependent increase in the cation influx induced by the compounds.

Current-voltage relationships of the imidacloprid-induced current were examined for both $\alpha4\beta2$ and SAD$\beta2$ receptors. In both cases, strong inward rectification was observed at potentials higher than −20 mV (Figure 4a and b). Although reversal potentials for the two nicotinic AChRs were not determined precisely due to the rectification, both *I*-V curves appeared to cross the potential-axis between −20 and −10 mV.

We tested for possible effects of imidacloprid, at concentrations just below threshold for its agonist action, on the amplitude of ACh responses. Following a 1 min perfusion in imidacloprid, oocytes were challenged with approximate EC_{50} concentrations of ACh co-applied with imidacloprid. In the case of SAD$\beta2$, 100 nM imidacloprid reversibly reduced the amplitude of the ACh (10 μM) responses to 81.1±1.9% ($n=6$) of control values. However, $\alpha4\beta2$-mediated responses to an approximate EC_{50} concentration of ACh (1 μM) were enhanced to 128.5±5.8% ($n=8$) of control values by 300 nM imidacloprid, when co-applied with ACh after pretreatment for 1 min (Figure 5).

Discussion

In this study, we demonstrated for two recombinant nicotinic AChRs that (a) imidacloprid is a partial agonist and (b) the pEC_{50} of imidacloprid, as well as that of other cholinoceptor ligands, is affected by the α subunit present in the receptor. A concentration of imidacloprid just below the threshold for

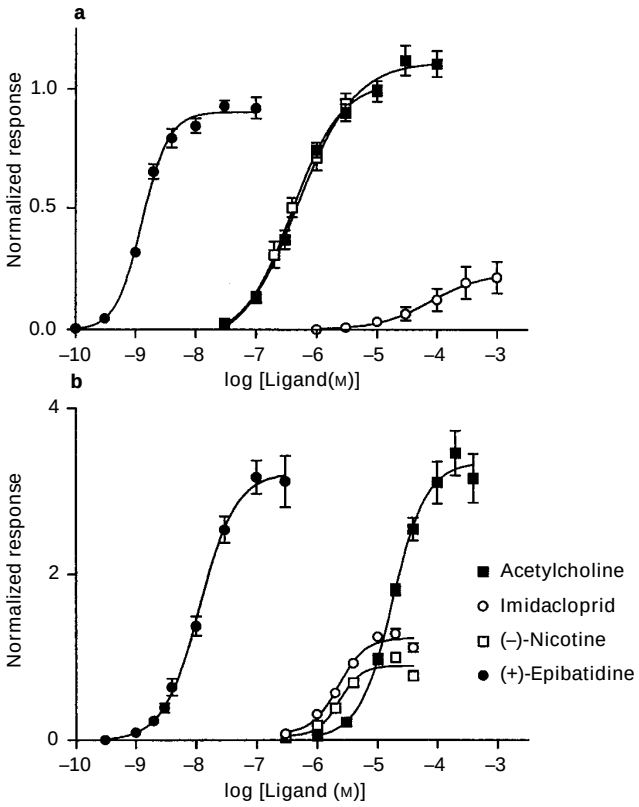

Figure 3 Dose-response relationships for imidacloprid, acetylcholine, (−)-nicotine and (+)-epibatidine obtained for recombinant $\alpha4\beta2$ (a) and SAD$\beta2$ (b) receptors. Data were normalized to the maximum response to 10 μM ACh being 1.0. Each data point represents the mean of 3–7 experiments; vertical lines show s.e.mean.

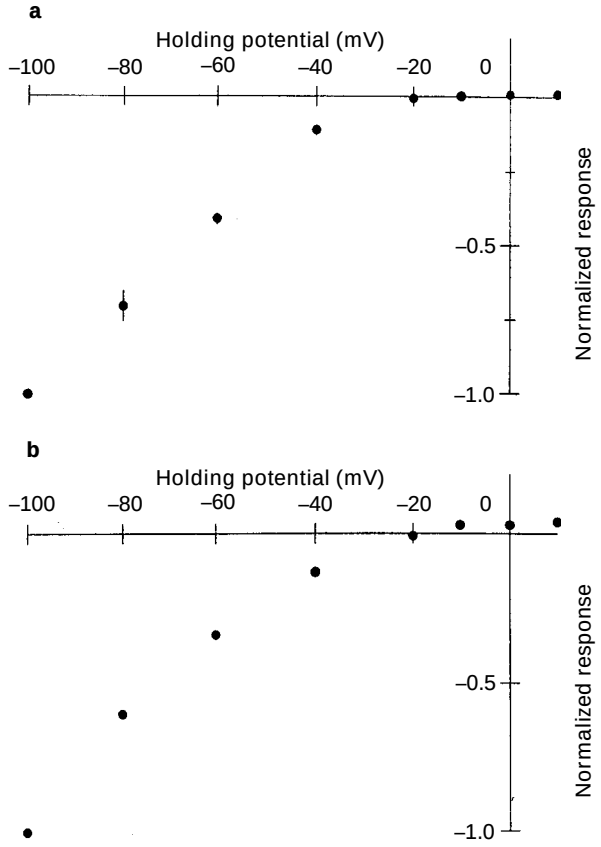

Figure 4 Current-holding potential relationships of the imidacloprid-induced current responses of the $\alpha4\beta2$ (a) and SAD $\beta2$ (b) nicotinic cholinoceptors. Test concentrations of imidacloprid were 10 μM for $\alpha4\beta2$ and 2 μM for SAD $\beta2$. The maximum response at each potential was normalized to that measured at −100 mV. The data are shown as mean ($n=4-7$ in (a) and $n=4-8$ in (b)); vertical lines show s.e.mean.

Table 1 pEC_{50}, n_H and I_{max} values derived from dose-response curves for the actions of candidate nicotinic cholinoceptor agonists

		α4β2			*SAD β2*	
Compounds	pEC_{50}	n_H	I_{max}†	pEC_{50}	n_H	I_{max}†
Imidacloprid	<4.10±0.06*	1.0±0.1	0.24±0.01	5.65±0.08	2.0±0.7	1.24±0.07
(+)-Epibatidine	8.88±0.03	2.0±0.3	0.91±0.02	7.94±0.03	1.4±0.1	3.22±0.06
(−)-Nicotine	6.39±0.05	1.1±0.1	1.03±0.03	5.64±0.11	2.4±1.4	0.90±0.07
Acetylcholine	6.30±0.08	0.9±0.1	1.11±0.03	4.74±0.04	1.5±0.3	3.35±0.11

The values shown are the result of a fit of the data (mean±s.e.mean, $n=3-9$), illustrated in Figure 3, to the Hill function. *A maximum could not be obtained for this curve. †The maximum current response expressed as a fraction of the response in the same oocyte to 10 μM acetylcholine. All values of I_{max}, except that for (−)-nicotine on $\alpha4\beta2$, differ significantly from that for acetylcholine ($P<0.001$, *t* test).

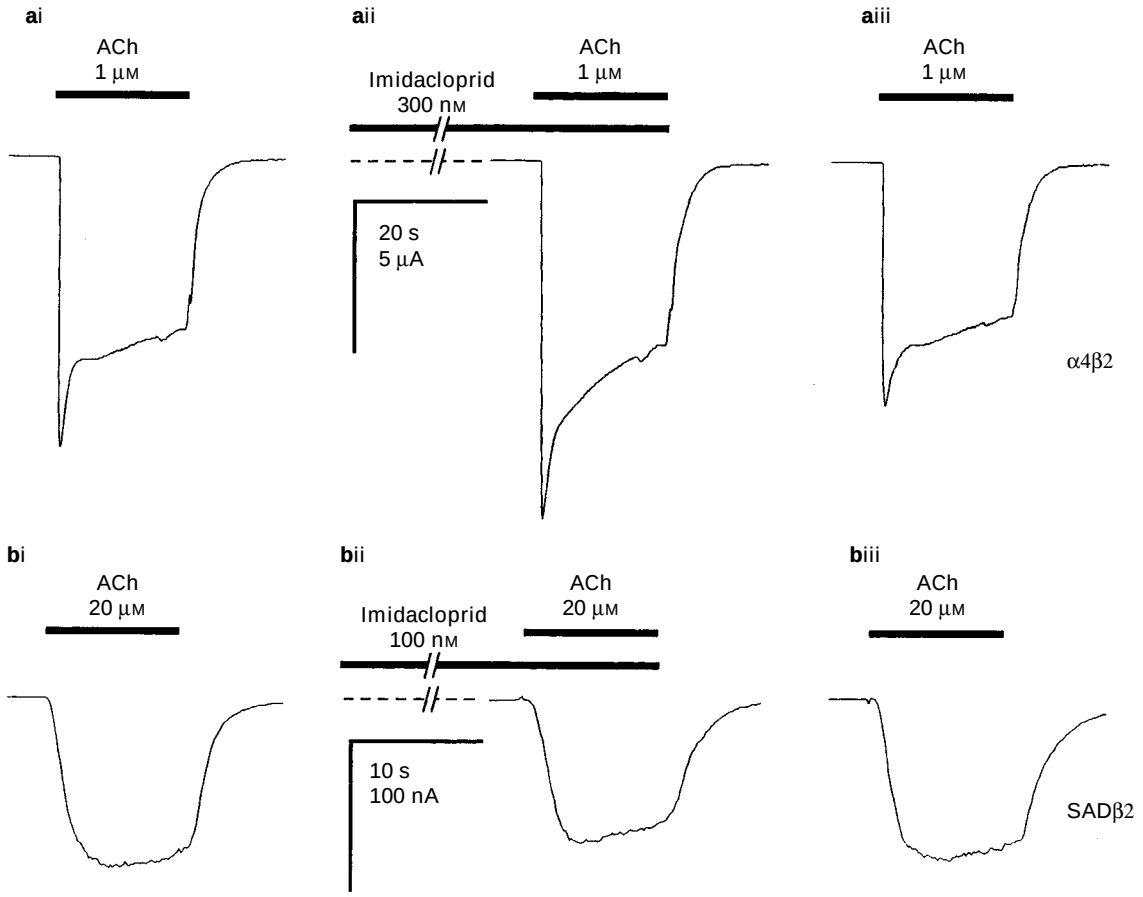

Figure 5 Effects of imidacloprid on inward current responses to ACh mediated by recombinant $\alpha4\beta2$ receptors (a,i–iii) and SAD$\beta2$ receptors (b, i–iii). ACh was applied at approximately EC_{50} concentrations (ai, bi). After at least 3 min had been allowed for recovery from possible desensitization, the oocytes were then perfused in saline containing imidacloprid at a concentration just below the threshold for agonist action, followed immediately by co-application of imidacloprid with ACh (aii, bii). (aiii and biii) Responses to the application of ACh after 5 min wash in normal saline.

agonist action acted as a weak antagonist of SAD$\beta2$ hybrid receptors. By comparison, PMNI applied to locust neurones at 0.1 μM, a concentration that evokes currents of about 28% of the maximum ACh response, blocks ACh responses by 92% (Zwart *et al*., 1992). Imidacloprid, therefore, does not exert a pronounced dual agonist/antagonist action on either recombinant receptor type under the conditions used in this study, a finding in accord with earlier studies on native insect nicotinic AChRs (Bai *et al*., 1991). That imidacloprid should act as a weak antagonist of SAD$\beta2$ receptors and as a weak augmenter of ACh responses on $\alpha4\beta2$ receptors suggests that the effect of the subunit exchange upon the interaction of this ligand with recombinant receptors is complex.

A large number of nicotinic AChR isoforms are expressed in the nervous systems of vertebrates (McGehee & Role, 1995) and several in insects (Gundelfinger, 1992). The diversity of nicotinic AChR properties in the nervous system of vertebrates with respect to cation selectivity and pharmacology is linked to differences in subunit composition (Karlin & Akabas, 1995). Our discovery that the presence of the *Drosophila* SAD subunit results in a significant increase in the apparent affinity of the recombinant receptor for imidacloprid, with a contrasting decrease of apparent affinities of other ligands, strongly suggests that the SAD subunit contributes to the high apparent affinity of the receptor for imidacloprid. It is of particular interest that (+)-epibatidine shares the 6-chloro-3-pyridyl moiety with imidacloprid (Figure 1), yet its pEC_{50} is decreased by the α subunit exchange, suggesting that a part of the 2-nitroimino-imidazolidine moiety of imidacloprid, at least, binds to the α subunit and the SAD subunit can recognize structural differences between the 2-nitroimino-imidazolidine moiety of imidacloprid and the azabicycloheptane moiety of (+)-epibatidine. The selectivity of imidacloprid for the insect/vertebrate hybrid (compared to the recombinant $\alpha4\beta2$ nicotinic AChR) seen in these experiments is less marked than the selectivity for *in situ* insect nicotinic AChRs over *in situ* vertebrate nicotinic AChRs (Zwart *et al*., 1994), suggesting that other factors may also contribute to the selectivity of native insect nicotinic AChRs to imidacloprid.

A model of agonist binding sites in the *Torpedo* receptor in which negative subsites in the γ and δ subunits recognize the quaternary nitrogen atom of acetylcholine has been proposed (Karlin & Akabas, 1995). On the basis of ligand structural comparisons and the electron deficient property deduced from its ^{15}N-NMR chemical shift, Yamamoto *et al*. (1995) predicted that the nitrogen atom in the imidazolidine moiety linking it to the 6-chloro-3-pyridylmethyl moiety of imidacloprid corresponds to the positively-charged nitrogen of the protonated form of nicotine. If this is so, then the Karlin and Akabas (1995) model of ligand/nicotinic AChR interaction would predict an interaction of this positively charged nitrogen atom with the negative subsite in the non-α subunit: that is, the $\beta2$ subunit of the recombinant receptors examined in the present study. If the $\beta2$ subunit were to be

exchanged for an insect non-α subunit, the pEC_{50} of imidacloprid may be further increased. However, as functional *Drosophila* subunit partner(s) of SAD remain to be identified, it is not yet possible to assess the role of insect non-α subunits in the action of imidacloprid by the methods adopted in this study.

Imidacloprid is a partial agonist of α4β2 and SADβ2 recombinant nicotinic AChRs, whereas (+)-epibatidine is a full agonist, suggesting that the imidazolidine moiety of imidacloprid also contributes to the partial-agonist action. It is not known whether this effect is also seen *in vivo*, where perhaps such an action might serve to suppress nicotinic cholinergic transmission by competition of imidacloprid with ACh for the nicotinic AChR. Imidacloprid has been shown to increase the activity of a particular subconductance state of the nicotinic AChR channels, reducing the activity of the main conductance in phaeochromocytoma (PC12) cells, thereby offering a possible explanation for the imidacloprid suppression of acetylcholine responses in these cells (Nagata *et al*., 1996). Heterologous expression of cloned subunits offers the advantage that the nicotinic AChR subunit composition is known, whereas PC12 cells, for example, are known to express α3, α5, α7, β2 and β3 nicotinic AChR subunits (Rogers *et al*., 1992; Henderson *et al*., 1994).

The current-voltage relationships of imidacloprid-induced responses in the α4β2 and the SAD β2 receptors are similar to those obtained for ACh (Bertrand *et al*., 1994), suggesting that both ACh and imidacloprid act on the same nicotinic AChR/channel molecules. The reversal potentials between −20 and −10 mV imply that the imidacloprid-induced responses are not the result of purely cationic currents, but probably include the chloride currents activated by Ca^{2+} influx (Miledi & Parker, 1984; Boton *et al*., 1989).

In conclusion, we have for the first time demonstrated that the α-subunit can contribute to selectivity for selected insect/vertebrate recombinant receptors and that imidacloprid acts as a partial agonist of recombinant insect/vertebrate hybrid receptors. The selectivity for the insect α subunit-containing receptors can be explained by assuming that the 2-nitroimino-imidazolidine moiety is, at least in part, recognized by the α-subunit, SAD. The partial agonist action of imidacloprid may contribute to whole-organism toxicity of these insecticides by competition with ACh for the receptor.

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