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RESEARCH PAPER

Mechanism of action of the insecticides, lindane and fipronil, on glycine receptor chloride channels

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BACKGROUND AND PURPOSE

Docking studies predict that the insecticides, lindane and fipronil, block $GABA_A$ receptors by binding to 6′ pore-lining residues. However, this has never been tested at any Cys-loop receptor. The neurotoxic effects of these insecticides are also thought to be mediated by $GABA_A$ receptors, although a recent morphological study suggested glycine receptors mediated fipronil toxicity in zebrafish. Here we investigated whether human α1, α1β, α2 and α3 glycine receptors were sufficiently sensitive to block by either compound as to represent possible neurotoxicity targets. We also investigated the mechanisms by which lindane and fipronil inhibit α1 glycine receptors.

EXPERIMENTAL APPROACH

Glycine receptors were recombinantly expressed in HEK293 cells and insecticide effects were studied using patch-clamp electrophysiology.

KEY RESULTS

Both compounds completely inhibited all tested glycine receptor subtypes with IC_{50} values ranging from 0.2–2 μM, similar to their potencies at vertebrate $GABA_A$ receptors. Consistent with molecular docking predictions, both lindane and fipronil interacted with 6′ threonine residues via hydrophobic interactions and hydrogen bonds. In contrast with predictions, we found no evidence for lindane interacting at the 2′ level. We present evidence for fipronil binding in a non-blocking mode in the anaesthetic binding pocket, and for lindane as an excellent pharmacological tool for identifying the presence of β subunits in αβ heteromeric glycine receptors.

CONCLUSIONS AND IMPLICATIONS

This study implicates glycine receptors as novel vertebrate toxicity targets for fipronil and lindane. Furthermore, lindane interacted with pore-lining 6′ threonine residues, whereas fipronil may have both pore and non-pore binding sites.

Abbreviations

I_{max}, saturating current magnitude; M2, second transmembrane domain; M3, third transmembrane domain; n_H, Hill coefficient; WT, wild type

Introduction

The phenylpyrazole derivative, fipronil, is successful as an insecticide because it inhibits the chloride channels of insect $GABA_A$ receptors at much lower concentrations than those of vertebrate $GABA_A$ receptors (Hainzl *et al.*, 1998; Ikeda *et al.*, 2001; Li and Akk, 2008; receptor nomenclature follows Alexander *et al.*, 2011). The neurotoxic effects of fipronil in vertebrates are mediated, at least partly, by $GABA_A$ receptors (Mohamed *et al.*, 2004). A role for the chloride channels in glycine receptors in the mediation of vertebrate fipronil toxicity has also been suggested on the basis of a behavioural and

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morphological study into fipronil-induced locomotor defects in zebrafish (Stehr *et al.*, 2006). However, the effects of fipronil on glycine receptors have never been quantitated.

The chlorinated cyclodiene, lindane (γ-hexachlorocyclohexane), is a first-generation insecticide that exhibits similar inhibitory potencies at insect and vertebrate $GABA_A$ receptors (Narahashi, 2002). Like fipronil, its neurotoxic effects in vertebrates are at least partly mediated by $GABA_A$ receptors (Sunol *et al.*, 1989; Hall, 1999). Lindane is widely used as a model compound for understanding insecticide inhibitory mechanisms at $GABA_A$ receptors, and, as discussed below, evidence to date suggests that it inhibits $GABA_A$ receptors by a mechanism similar to that of fipronil (Chen *et al.*, 2006). Although lindane has been shown to inhibit native glycine receptors in cultured cerebellar granule neurons (Vale *et al.*, 2003), its glycine receptor subunit specificity is not known.

Glycine receptors and $GABA_A$ receptors both belong to the pentameric, Cys-loop, ion channel receptor family. Individual subunits of these receptors consist of a large N-terminal ligand-binding domain and a transmembrane domain that comprises four transmembrane α-helices, termed M1–M4 (Miller and Smart, 2010). Each of the five subunits contributes an M2 domain to the lining of a central ion-selective pore. Several molecular docking studies have proposed that lindane and fipronil both inhibit ion flux through $GABA_A$ receptors, by binding to 2′ and 6′ pore-lining residues in the M2 domain (Chen *et al.*, 2006; Ci *et al.*, 2007; Law and Lightstone, 2008; Cheng *et al.*, 2009). One of these studies predicted that both compounds also bind in the intramembranous alcohol and anaesthetic binding pocket formed by the M1, M2 and M3 domains (Law and Lightstone, 2008). To date, however, there is only limited experimental evidence for lindane or fipronil binding in the pore, or any other region, of $GABA_A$ or glycine receptors. Although several studies have shown that naturally occurring mutations to 2′ residues in $GABA_A$ receptors can modulate the inhibitory potency of both lindane and fipronil (Cole *et al.*, 1995; Hosie *et al.*, 1995; Le Goff *et al.*, 2005; Hirata *et al.*, 2008), a systematic 2′ mutagenesis study has yet to be performed, and there is as yet no experimental evidence at all for a role of 6′ (or any other) residues in coordinating these compounds in any Cys-loop receptor.

Given all these considerations, we set out to investigate the mechanisms of action of both lindane and fipronil at recombinant human α1, α1β, α2 and α3 glycine receptors. Our first aim was to test whether any of these glycine receptor subtypes are sufficiently sensitive to either compound so as to represent possible targets for neurotoxicity. The second aim was to compare the inhibitory mechanisms of fipronil and lindane. This involved both a functional characterisation of their inhibitory effects and an investigation into the roles of 2′ and 6′ pore-lining residues in coordinating each molecule in the α1 glycine receptor pore. The final aim was to investigate the possibility of these molecules binding to a non-pore site involving the M3 domain. Our results showed that fipronil and lindane inhibited glycine receptor isoforms with sufficient potency so as to implicate them as possible toxicity targets. We also confirmed that both compounds bound in the pore, and we provided evidence that fipronil could also bind to a non-pore site.

Methods

Mutagenesis and expression of glycine receptor cDNAs

The human α1 glycine receptor cDNA was subcloned into the pCIS2 plasmid vector, the human α2 and α3 subunit cDNAs were subcloned into the pcDNA3.1 plasmid vector and the human β subunit cDNA was subcloned into pIRES2-EGFP plasmid vector. The empty pEGFP plasmid vector (Clontech, Mountain View, CA) was also used as a transfection marker. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), and the mutations were confirmed by DNA sequencing. HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $mg \cdot mL^{-1}$) and 10% serum supreme and maintained at 37°C in a humidified 5% incubator. Cells were co-transfected with glycine receptor and EGFP cDNAs in a ratio of 10:1 using a calcium phosphate precipitation protocol and incubated at 37°C in a humidified 3% incubator. The 1:20 ratio was also maintained when cells were co-transfected with glycine receptor α1 and β cDNAs. About 18 h post transfection, cells were washed twice with phosphate buffered saline and returned to the cultured medium. Cells were used for recording over the following 24–72 h.

Electrophysiology

Cells were viewed using an inverted fluorescent microscope, and currents were recorded using the whole-cell patch-clamp configuration. Cells expressing recombinant glycine receptors were identified by their green fluorescence. Cells were perfused by extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/NaOH and 10 glucose (pH 7.4 adjusted with NaOH). Patch pipettes were fabricated from borosilicate haematocrit tubing (Hirschmann Laborgerate, Eberstadt, Germany) and heat polished. Pipettes had a tip resistance of 1–2 MΩ when filled with the intracellular solution consisting of (mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 EGTA (pH 7.4 adjusted with CsOH). After establishment of the whole-cell recording configuration, cells were voltage clamped at –40 mV, and membrane currents were recorded using an Axopatch 1D and pClamp 10 software (Molecular Devices Inc., Sunnyvale, CA, USA). Currents were filtered at 500 Hz and digitized at 2 KHz.

Because α1 subunits can form functional receptors with or without β subunits, it was necessary to confirm that the expressed receptors incorporated β subunits. As the glycine receptor β subunit cDNA was cloned into the pIRES2-EGFP plasmid vector, we used green fluorescent protein fluorescence to identify cells transfected with the β subunit. The successful incorporation of β subunits into functional heteromeric glycine receptors was also confirmed pharmacologically by their characteristic reduction in picrotoxin sensitivity as described below.

Data analysis

Results are expressed as mean ± SEM of three or more independent experiments. The Hill equation was used to calculate the saturating current magnitude (I_{max}), half-maximal concentration (EC_{50}) and Hill coefficient (n_H) values for glycine

Table 1

EC_{50} and n_H values for glycine-dependent activation of all WT and mutant glycine receptors examinedin this study

Glycine receptor	EC_{50} (μM)	n_H	*n*
α1	28 ± 3	1.4 ± 0.2	10
α1 β	24 ± 2	1.9 ± 0.3	6
α2	120 ± 5	2.0 ± 0.1	6
α3	296 ± 20	2.0 ± 0.1	7
α1-G2′A	27 ± 2	1.8 ± 0.1	4
α1-G2′S	29 ± 3	2.1 ± 0.3	5
α1-G2′P	140 ± 7	2.3 ± 0.2	4
α1-T6′A	5.9 ± 1.6	1.2 ± 0.1	6
α1-T6′F	6.2 ± 0.5	0.8 ± 0.1	6
α1-T6′S	1.1 ± 0.2	1.1 ± 0.2	5
α1-T6′V	385 ± 25	1.9 ± 0.3	6
α1 β-F6′T	31 ± 2	1.5 ± 0.2	5
α1-A288F	280 ± 10	2.1 ± 0.2	7
α1-A288G	3.5 ± 0.9	1.7 ± 0.1	4
α1-A288S	38 ± 3	1.4 ± 0.3	5
α1-A288T	42 ± 4	1.9 ± 0.3	4

activation. A similar equation was also used to calculate the half-maximal concentrations for inhibition (IC_{50}) and n_H values of the inhibitors tested in this study. All curves were fitted using a nonlinear least squares algorithm (Sigmaplot 11.0; Jandel Scientific, San Rafael, CA). Statistical significance was determined by paired or unpaired Student's *t*-test, as appropriate, with $P < 0.05$ representing significance.

Materials

Glycine, picrotoxin, lindane and fipronil were obtained from Sigma (St Louis, MO). Picrotoxin was prepared as 100 mM stock in dimethylsulphoxide. Both lindane and fipronil were prepared as 30 mM stocks in dimethylsulphoxide and glycine was prepared as a 1 M stock in water. All stocks were frozen at –20°C. From these stocks, solutions for experiments were prepared on the day of recording. Solutions were applied to cells via gravity forced perfusion and parallel microtubules, and manual control of this system was achieved via a micromanipulator with a solution exchange time < 250 ms. Experiments were conducted at room temperature (19–22°C).

Results

Differential effects of lindane and fipronil at recombinant glycine receptors

All experiments were performed on recombinantly expressed human α1, α1β, α2 and α3 glycine receptors. Glycine dose–response relationships were determined for each receptor, with averaged EC_{50} and n_H values summarized in Table 1. These values are comparable with those previously determined in our laboratory (Hawthorne *et al.*, 2006; Yang *et al.*, 2007; Chen *et al.*, 2009). In all experiments described in this study, the glycine concentration required to produce a given EC value was determined empirically for each cell.

We first quantitated the glycine concentration dependence of the inhibition produced by 30 μM lindane and fipronil at each of the four receptors. When lindane was co-applied with EC_{50} glycine to the α1 glycine receptor, a slowly developing and slowly recovering inhibition was observed (Figure 1A, left). Fipronil produced a qualitatively similar inhibitory effect, although there was sometimes evidence for a rapid transient potentiation preceding the onset of inhibition (Figure 1A, centre). The magnitude of this potentiation was enhanced by reducing the glycine concentration from EC_{50} to EC_{20} (Figure 1A, right). The average magnitude of this potentiation, averaged from five cells at EC_{20} glycine, was 117 ± 4%. As this potentiation exhibited rundown with successive drug applications, its fipronil sensitivity was not quantitated. For both fipronil and lindane, steady-state inhibition was quantitated by measuring the percentage of glycine-activated current remaining after a 30 s closed state drug application, as shown in the examples in Figure 1B. We employed closed state drug application in most experiments for two reasons. First, we reasoned that it was more physiologically relevant than applying drugs in the open state, given that synaptically activated glycine receptors may open too briefly to allow slow-acting blockers such as these to equilibrate. The second reason was technical: as both drugs are very lipophilic, it proved to be much simpler to control for the effects of drug accumulation in the bath and perfusion system if a known glycine concentration were applied via a single perfusion tube in the absence of drug. We compared the relative magnitudes of fipronil and lindane inhibition at the α1, α1β, α2 and α3 glycine receptors, by applying each drug for 30 s in the closed state and then immediately applying EC_{20}, EC_{50} or EC_{100} glycine concentrations to determine the extent of inhibition. Sample recordings are presented in Figure 1B–D, and averaged data are presented in Figure 1E.

The incorporation of β subunits into glycine receptors results in a characteristic reduction in picrotoxin sensitivity (Pribilla *et al.*, 1992; Handford *et al.*, 1996). To confirm the successful incorporation of β subunits into functional glycine receptors, the effects of 20 μM picrotoxin on currents activated by an EC_{50} glycine concentration were compared at the α1 and α1β glycine receptors. Examples of the effects of picrotoxin at both receptors are shown in Figure 1C. Picrotoxin inhibited α1 and α1β glycine receptors to 15.8 ± 2.2% ($n = 15$ cells) and 87.6 ± 2.0% ($n = 14$ cells), respectively, of the original current magnitude. This difference, which was highly statistically significant ($P < 0.001$, by unpaired *t*-test), indicates that the majority of functional glycine receptors formed by co-expression of α1 and β subunits did indeed comprise α1β heteromers.

Averaged results from 7–10 cells for both lindane and fipronil at EC_{20}, EC_{50} and EC_{100} glycine concentrations are summarized in Figure 1E. Whereas 30 μM lindane potently inhibited α1, α2 and α3 homomeric glycine receptors, it had no effect at all at α1β glycine receptors. Indeed, even an extended (90 s) closed state application of 100 μM lindane had no significant inhibitory effect on these receptors

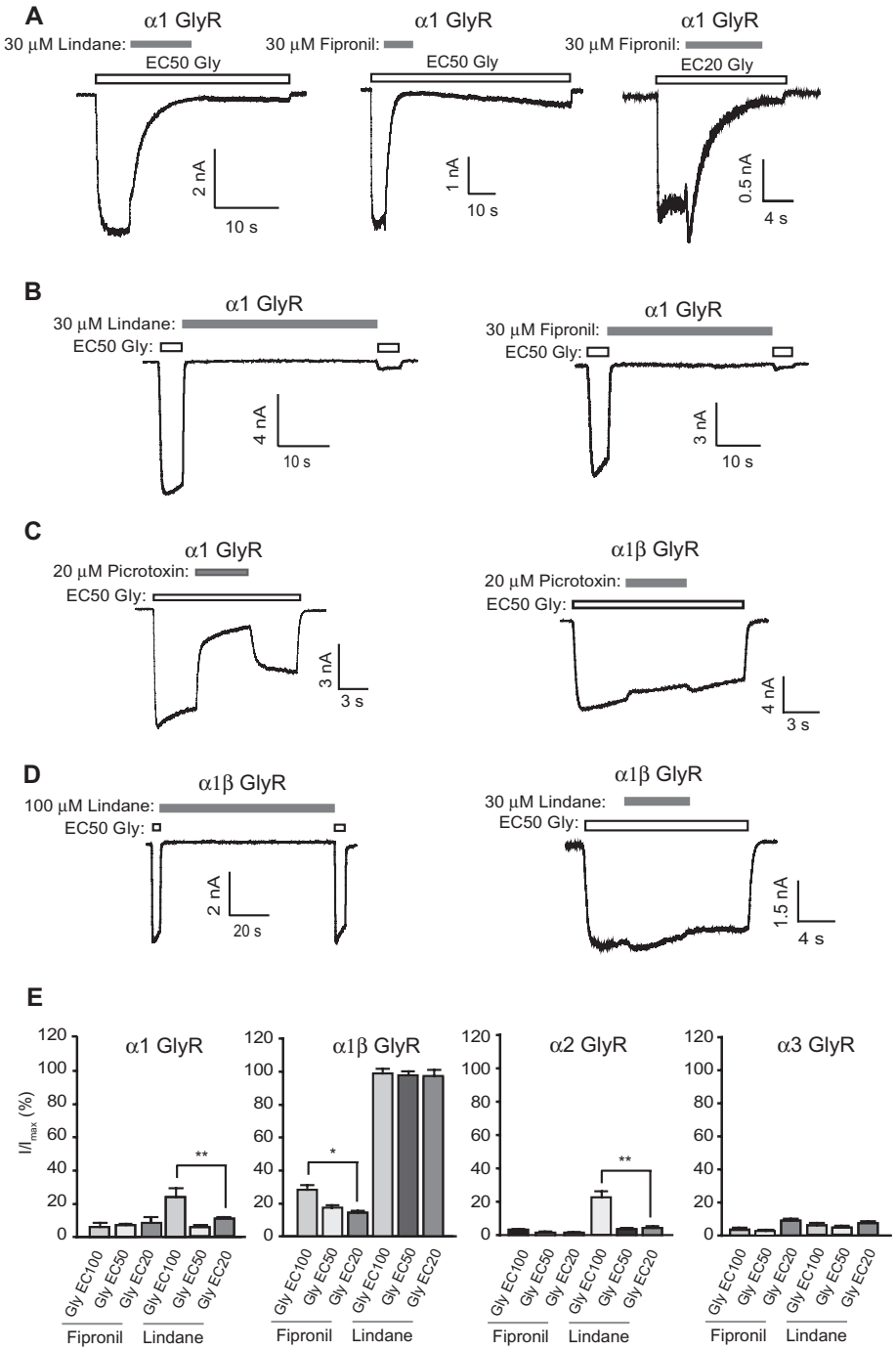

Figure 1

Effect of glycine concentration on steady-state inhibition by lindane and fipronil. (A) Left and centre panels show examples of the effects of 30 μM lindane and fipronil, respectively, applied to α1 glycine receptors (GlyR) in the presence of an EC_{50} glycine (Gly) concentration. Right panel shows transient potentiation of 30 μM fipronil when co-applied with EC_{20} glycine. In all experiments, the glycine concentration corresponding to the stated EC value was determined empirically for each cell. In this and all subsequent figures, the durations of glycine and drug applications are indicated by unfilled and filled bars respectively. (B) Examples of the effects of 30 μM lindane and fipronil at α1 glycine receptors in the closed state. (C) Examples of differential effects of 20 μM picrotoxin at α1 glycine receptors (left panel) and α1β glycine receptors (centre panel). Mean percentage inhibition by picrotoxin at both receptors is given in the text. (D) The left panel shows that an extended closed-state application of 100 μM lindane has no effect at α1β glycine receptors. The right panel shows that an open-state application of 30 μM lindane also has no inhibitory effect. (E) Percentage of maximum control current remaining (i.e. I/I_{max}) following a 30 s close-state exposure to fipronil and lindane followed immediately by applying the indicated glycine concentrations at α1, α1β, α2 and α3 glycine receptors. All values were averaged from at least five experiments. $^*P < 0.05$, $^{**}P < 0.01$, EC_{20} significantly different from corresponding EC_{100} value by unpaired *t*-test.

Table 2

IC_{50} and n_H values for lindane and fipronil inhibition of indicated glycine receptor subtypes

Glycine receptor	Lindane IC_{50} (μM)	n_H	*n*	Fipronil IC_{50} (μM)	n_H	*N*
α1	0.9 ± 0.1	1.0 ± 0.1	7	1.96 ± 0.03	1.6 ± 0.2	6
α1β	No block		7	2.14 ± 0.44	1.0 ± 0.1	5
α2	0.28 ± 0.03*	1.1 ± 0.1	7	0.52 ± 0.08*	1.3 ± 0.1	5
α3	0.25 ± 0.07*	0.8 ± 0.1	5	0.35 ± 0.01*	1.6 ± 0.1	5

*$P < 0.05$, significantly different from corresponding α1 glycine receptor value by unpaired *t*-test.

(Figure 1D, left, typical of 5 experiments). An open-state application of 30 μM lindane also had no inhibitory effect on these receptors (Figure 1D, right, typical of >10 experiments). The averaged results presented in Figure 1E also show that fipronil and lindane exhibited significantly reduced potencies at high glycine concentrations at the α1β, and the α1 and α2 glycine receptors respectively.

Full dose–response relationships for both compounds applied in the closed state at α1, α1β, α2 and α3 glycine receptors were quantitated at the respective EC_{50} glycine concentrations. Figure 2A shows sample dose–response relationships for lindane and fipronil at the α1, and for fipronil at the α1β glycine receptors. Averaged dose–response relationships at each of the four receptors are shown separately for lindane and fipronil in Figure 2B, with mean parameters of best fit to individual dose–response relationships summarized in Table 2. Both drugs exhibited significantly higher potencies at α2 and α3, relative to α1 glycine receptors. Although the mean fipronil IC_{50} was similar at α1 and α1β glycine receptors, its reduced potency at high concentrations at the α1β glycine receptor (see Figure 1D) was explained by its significantly lower n_H value (Table 2). The lindane and fipronil dose–response relationships were also quantitated in the open state (i.e. with drugs co-applied with an EC_{50} glycine concentration) at the α1 glycine receptor. Averaged results presented in Figure 2C indicate that their respective potencies were independent of whether they were applied in the closed or open states. Given the significant time required for the drugs to equilibrate with their binding sites at 30 μM (Figure 1), it is possible that at low (<1 μM) drug concentrations that the drug application times used here might not have been sufficient to approach equilibrium. Thus, drug IC_{50} values may be systematically overestimated in our study.

The time dependence of recovery from lindane and fipronil inhibition was quantitated at the EC_{50} glycine concentration. Following block by 30 μM of each drug, recovery was assayed by applying short pulses of glycine separated by 30 s washes in extracellular control solution. Sample experiments for lindane and fipronil at the α1 glycine receptor are shown in Figure 3A,B, with averaged recovery profiles for lindane and fipronil at all four glycine receptors summarized in Figure 3C,D. Following lindane inhibition, currents recovered to a maximum of around 70% of original current magnitude at the α1 and α2 glycine receptors, although currents appeared to stabilize at a much lower level at the α3 glycine receptor. Following fipronil inhibition, currents recovered completely at α1β glycine receptors, but at much slower rates at the three homomeric glycine receptors (Figure 3D). As with lindane, recovery from fipronil inhibition was slowest at the α3 glycine receptor.

When a similar recovery experiment was performed at EC_{100} glycine, recovery from lindane inhibition at α1 and α2 glycine receptors was complete by the second post-inhibition glycine application (e.g. Figure 4A). Averaged results, presented in Figure 4B, confirmed this trend. An example of the recovery profile from fipronil inhibition at EC_{100} glycine is shown in Figure 4C, with averaged recovery profiles for all four glycine receptors presented in Figure 4D. The higher glycine concentration enhanced fipronil recovery rates, leading to full recovery in α1, α2 and α1β glycine receptors, and to an improved but still incomplete level of recovery in the α3 glycine receptor. Figure 4E shows the results of similar experiments using fipronil, in place of lindane (as in Figure 4B). Together, these results show that the unbinding rates of both drugs (i.e., recovery) were enhanced by increasing glycine concentrations, with lindane showing the stronger dependence on glycine concentration.

Molecular determinants of lindane and fipronil inhibition

Lindane and fipronil are predicted to bind to the 2′ and 6′ pore-lining residues of the human β3 homomeric $GABA_A$ receptors (Chen *et al.*, 2006). Consistent with this, the sensitivity of *Drosophila melanogaster* RDL $GABA_A$ receptors to lindane and fipronil is dramatically reduced by naturally occurring A2′S and A2′G mutations (Cole *et al.*, 1995; Hosie *et al.*, 1995), whereas the sensitivity of an *Musca domestica* glutamate-gated chloride channel (GluCl) receptor is increased by the reverse S2′A mutation (Hirata *et al.*, 2008). There is, however, no information from any Cys-loop receptor concerning the possible role of 6′ residues as determinants of lindane and fipronil sensitivity. Here we investigated the roles of 2′ and 6′ α1 glycine receptor residues as putative binding sites for both molecules. Figure 5A shows a sequence alignment of the inner pore regions of all anionic Cys-loop receptors considered here. Figure 5B shows the structure of the M2 pore-lining region of the α1 glycine receptor based on the recently published crystal structure of the *Caenorhabditis elegans* α homomeric GluCl receptor (Hibbs and Gouaux, 2011). Pore-lining residues known to be involved in coordinating channel blockers are shown in colour.

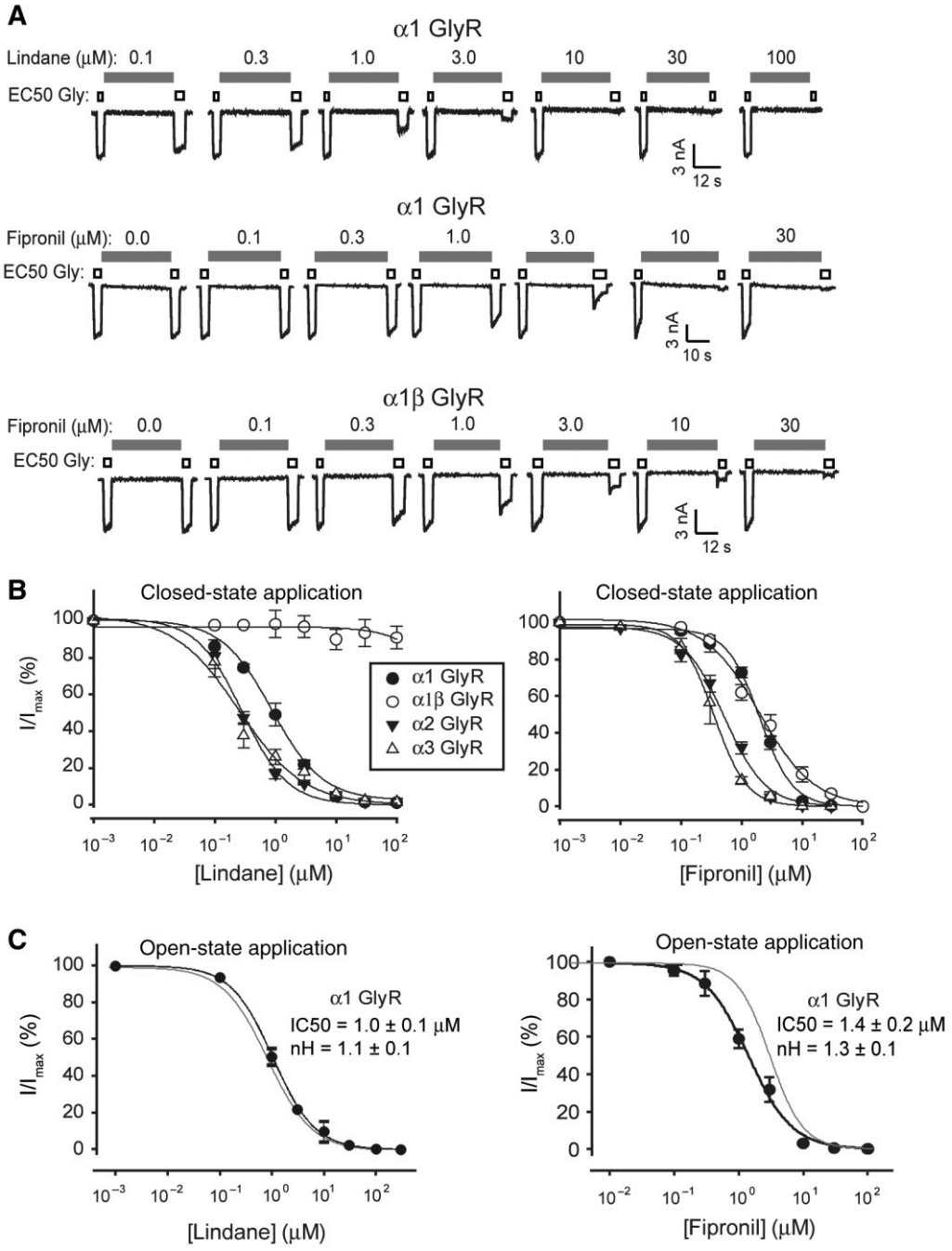

Figure 2

Dose–response relationships for lindane and fipronil at α1, α1β, α2 and α3 glycine receptors (GlyR) at EC_{50} glycine. (A) Examples of the effects of indicated concentrations of lindane (top) or fipronil (centre) at the α1 glycine receptor and (bottom) fipronil at the α1β glycine receptor. All traces in a given row were recorded from the same cell. (B) Averaged dose–response curves for lindane (left panel) and fipronil (right panel) at the indicated receptors, when applied in the closed state. At least five full concentration–response relationships were averaged for each compound at each receptor. Mean IC_{50} and n_H values of best fit to individual dose–response curves are summarized in Table 2. (C) Averaged dose–response relationships for lindane (left panel) and fipronil (right panel) when co-applied EC_{50} glycine at α1 glycine receptors. The mean IC_{50} and n_H values are indicated ($n = 5$ cells each). The corresponding α1 glycine receptor dose–response curves from B are included as grey lines. It is important to note that at low (<1 μM) drug concentrations that the drug application times might not have been sufficient to approach equilibrium. Thus, drug IC_{50} values may be systematically overestimated in these experiments.

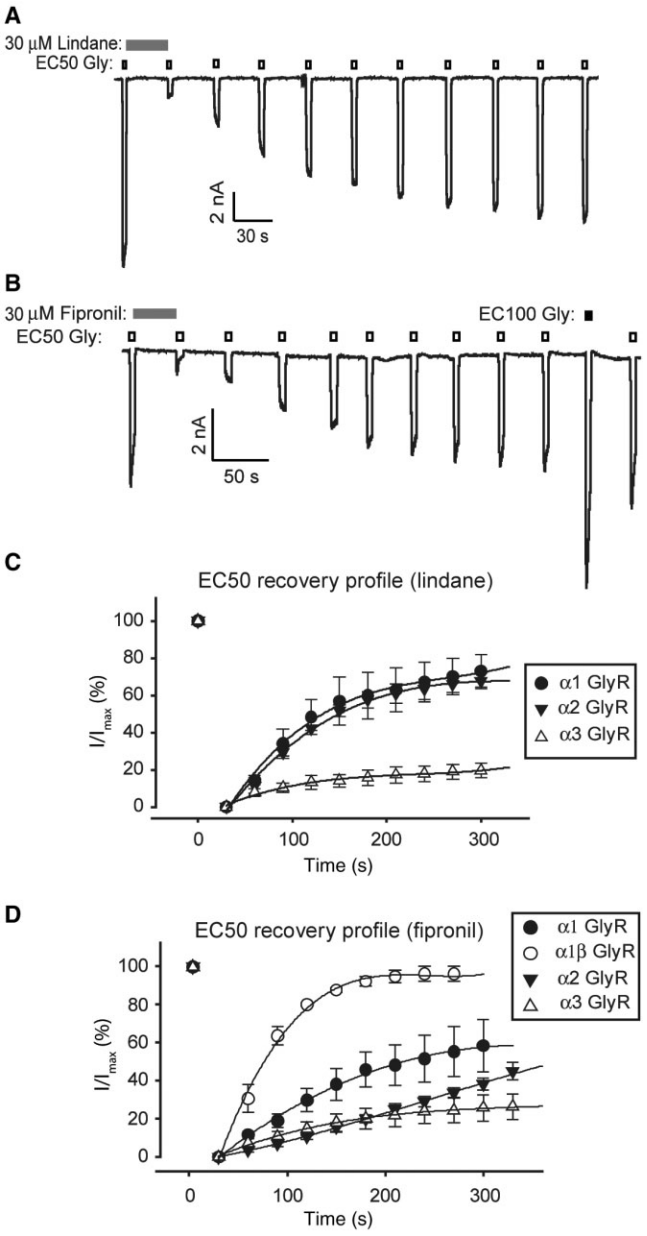

Figure 3

Time dependence of recovery from lindane- and fipronil-induced inhibition at EC_{50} glycine (Gly). (A) Sample recovery profile following inhibition by 30 μM lindane at the α1 glycine receptor (GlyR). Recovery was monitored by applying brief glycine pulses at 30 s intervals. (B) Similar experiment for fipronil. (C) Averaged recovery profiles from lindane inhibition at α1, α2 and α3 glycine receptors. (D) Averaged recovery profiles from fipronil inhibition at α1, α1β, α2 and α3 glycine receptors. In this and the subsequent figure, all averaged recovery profiles were averaged from at least five cells.

We tested the inhibitory potency of 30 μM of lindane and fipronil on currents activated by EC_{50} glycine at a variety of mutant α1 glycine receptors. The glycine EC_{50} and n_H values for all mutant receptors employed in this study are summarized in Table 1. At the 2′ position, we initially investigated the roles of the G2′A and G2′P mutations. The G2′A mutation is a conservative substitution that has dramatic effects on glycine receptor sensitivity to other pore-binding molecules including cyanotriphenylborate, nifedipine and picrotoxinin (Rundstrom *et al.*, 1994; Yang *et al.*, 2007; Chen *et al.*, 2009). The α2 and α3 glycine receptor subunits both contain endogenous 2′ alanine residues (Figure 5A). The G2′P mutation is highly non-conserved but was tested because the glycine receptor β subunit contains an endogenous 2′ proline (Figure 5A). Four 6′ mutants were chosen for analysis: T6′S, T6′A, T6′V and T6′F. The small β-branched side chain of the endogenous threonine exposes a polar hydroxyl group and a non-polar methyl group to the pore, giving it the ability to participate in both hydrogen bonds and non-specific hydrophobic interactions respectively. Serine, generally considered a conservative substitution, retains the reactive hydroxyl group, although the hydrophobic methyl group is lost. This increases the accessibility of the hydroxyl group, possibly offering more opportunities for hydrogen bond formation. Valine retains the β-branched side chain structure but replaces the threonine hydroxyl with a second hydrophobic methyl. Both the hydroxyl and methyl groups are absent in alanine, which has a short, non-reactive side chain. We also screened the non-conservative T6′F mutation as the β subunit contains an endogenous phenylalanine at this position (Figure 5A). Sample recordings for lindane and fipronil, applied in the closed state, at the G2′P, G2′A and T6′F mutant glycine receptors are shown in Figure 6A,B, respectively, with averaged results for all mutant receptors summarized in Figure 6C,D respectively. A striking feature was that the G2′P mutation eliminated fipronil sensitivity but had no effect on lindane sensitivity. This result strongly suggests that lindane does not interact at the 2′ level of the pore. Because G2′P eliminated fipronil sensitivity and G2′A produced a weak (but not statistically significant) reduction in fipronil sensitivity (Figure 6D), it remained possible that fipronil may interact at the 2′ level of the pore. As stronger evidence was needed for such an interaction, we investigated the effect of the G2′S mutation. As shown in Figure 6D, this relatively conservative mutation produced a dramatic reduction in fipronil sensitivity, strengthening the case for fipronil interacting with molecular groups at the 2′ pore-lining level. All four T6′ mutations dramatically reduced the inhibitory effects of both drugs.

As drugs were applied in the closed state, it was possible that T6′ mutations may have induced a closed conformation that prevented access of drugs to their binding site. We also investigated the effect of drugs applied in the open state to the T6′S mutant glycine receptor, as this was the most conservative T6′ mutant we investigated. As shown in the sample recordings in Figure 6E,F, 30 μM concentrations of lindane and fipronil had no effect on T6′S mutant glycine receptor currents when co-applied with EC_{50} glycine. Similar results were recorded from three cells for each drug.

From these results, we hypothesized that that the β subunit 6′ phenylalanine residue was responsible for the loss of lindane sensitivity at α1β glycine receptors. To test this, we investigated the lindane sensitivity of receptors formed by co-expression of unmutated α1 subunits and F6′T mutant β subunits. As shown in Figures 6C and 7A, these receptors were indeed highly sensitive to lindane inhibition. However, because it is possible the mutant β subunit may not have

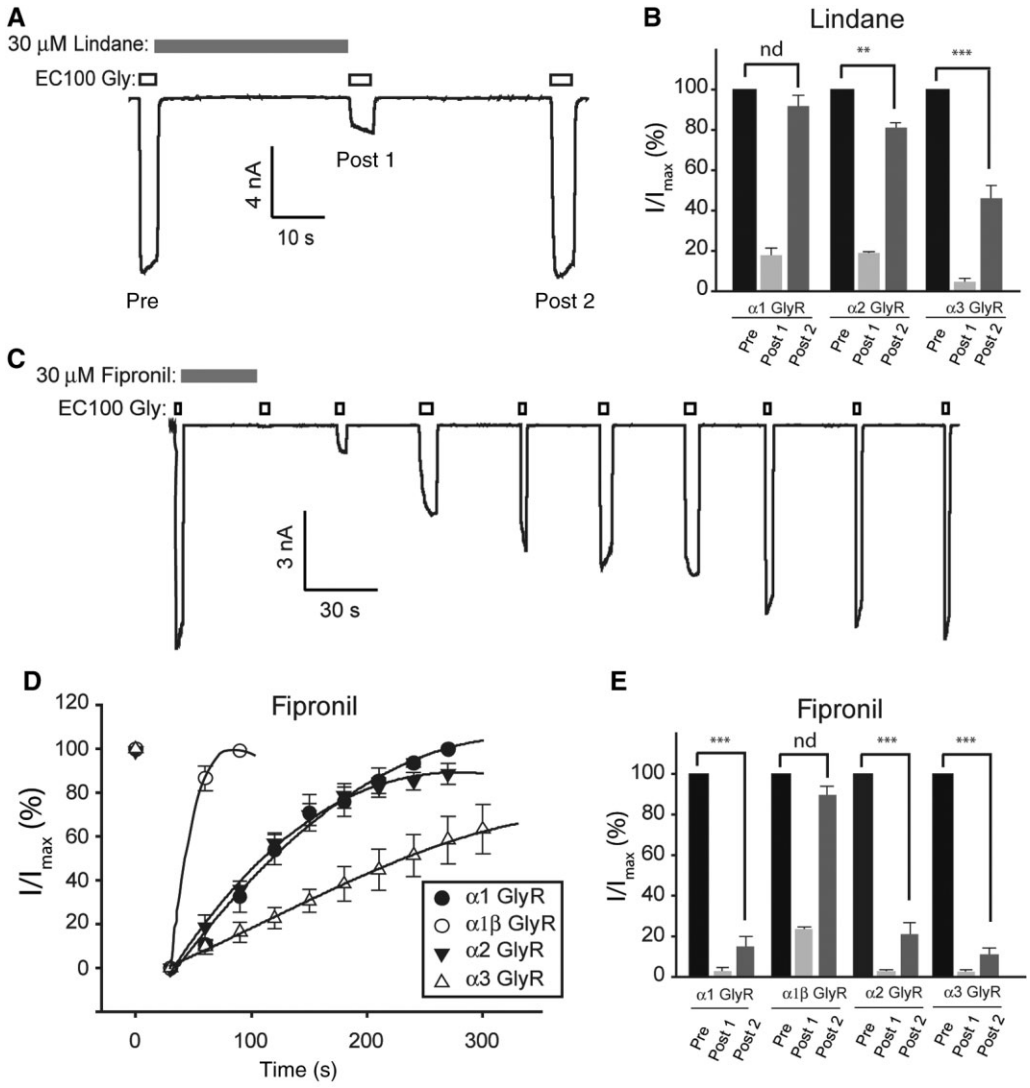

Figure 4

Time dependence of recovery from lindane- and fipronil-induced inhibition at EC_{100} glycine (Gly). (A,B) Sample recovery profile following inhibition by 30 μM lindane at the α1 glycine receptor (GlyR). Recovery was monitored by applying brief glycine pulses immediately after lindane removal (post1) and 30 s later (post2). The post1 and post2 currents were expressed as a percentage of control (pre) current magnitude. These parameters were averaged for α1, α2 and α3 glycine receptors in panel B. (C) Sample recovery profile following inhibition by 30 μM fipronil at the α1 glycine receptor. (D) Averaged recovery profiles from fipronil inhibition at α1, α1β, α2 and α3 glycine receptors. (E) Percentage recovery from fipronil inhibition at post1 and post2 time points. These experiments are directly comparable with those summarized in B. nd (no difference) $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from WT glycine receptor; unpaired *t*-test.

expressed in these experiments, it was necessary to demonstrate functional expression of the mutant β subunits. Picrotoxin sensitivity is not informative in this case as the mutant heteromers and unmutated α1 homomers exhibit similarly high picrotoxin sensitivities (Shan *et al.*, 2001). However, we found that the rate of current recovery from lindane inhibition at the mutant heteromeric receptors was dramatically faster than that observed at unmutated α1 glycine receptors (e.g. compare Figure 7A with Figure 1A). The averaged recovery rate data for the two receptors, quantitated in Figure 7B, confirm this trend and thus provide clear evidence for the strong functional expression of F6′T mutant β subunits. This result supports the hypothesis that β subunit 6′ phenylalanine residues are solely responsible for the lack of lindane sensitivity in α1β glycine receptors. This mechanism does not apply to fipronil, because even unmutated α1β heteromeric glycine receptors exhibited high fipronil sensitivity (Figure 1D). However, as with lindane, β subunit incorporation dramatically enhances the fipronil recovery rate (Figure 3D). The implications of all these results for lindane and fipronil binding mechanisms are considered below.

Investigation of a 'non-blocking' fipronil binding site

The experiments described above were performed using drug applications that were long enough to produce a steady-state

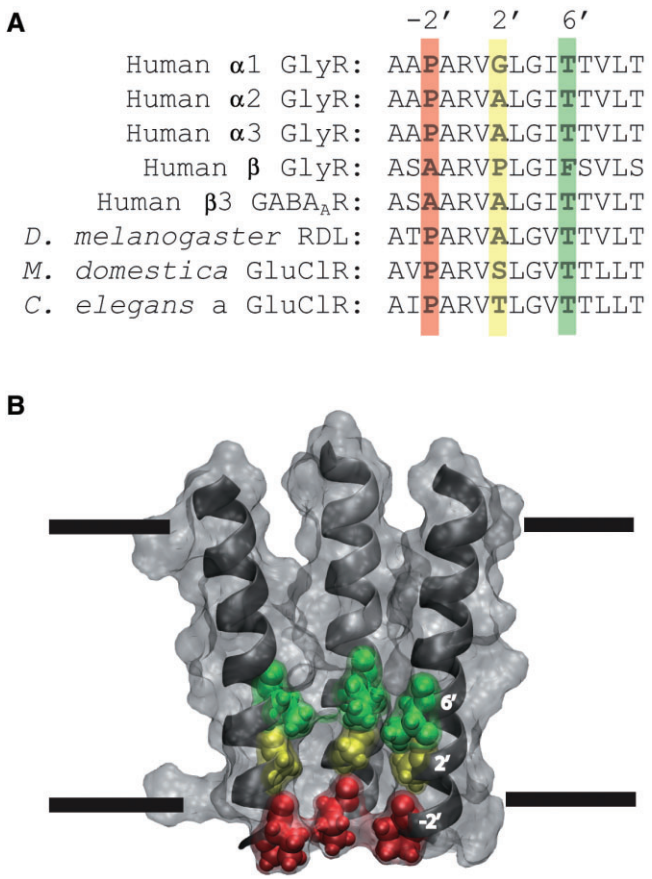

Figure 5

Sequence alignment of anionic Cys-loop receptor subunits and a model structure of the inner part of the α1 glycine receptor (GlyR) pore. (A) Sequence alignment of indicated subunits with pore-lining residues at the –2′, 2′ and 6′ positions highlighted in red, yellow and green respectively. (B) A model structure of the M2 domains of three contiguous α1 glycine receptor subunits based on the structure of the *C. elegans* α GluCl receptor (GluClR; PDB code: 3RHW). Residues are colour coded and labelled as in panel A. Intracellular (bottom) and extracellular (top) membrane boundaries are indicated by horizontal black bands.

level of inhibition. However, a shorter application of each drug revealed a distinct difference in their modes of action. As shown in the example in Figure 8A (left), a brief (5 s) application of 30 μM lindane simply produced partial block, as expected for a slowly-equilibrating pore blocker. In contrast, a 5 s application of 30 μM fipronil produced no instantaneous block, as shown by the undiminished initial magnitude of the subsequent control glycine application (Figure 8A, right). However, fipronil-dependent inhibition then developed rapidly despite the absence of fipronil in free solution. We have previously reported a similar effect for dihydropyridines (Chen *et al.*, 2009), which we considered was most likely to be explained by these molecules binding in crevices between or behind M2 domains in the closed state and being released into the pore in the open state. It is possible that one possible location of this M2 crevice site might be the alcohol and anaesthetic binding site formed by A288 in the M3 domain of the glycine receptor (Lobo and Harris, 2005; Lobo *et al.*, 2008). The plausibility of fipronil binding in this location is supported by a recent molecular docking study (Law and Lightstone, 2008).

To determine whether A288 might form a non-blocking fipronil site, we investigated the effects of the conservative mutations, A288S and A288T, and the non-conservative large substitution, A288F, on receptor sensitivity to both lindane and fipronil. As none of these mutations had a significant effect on receptor sensitivity to lindane (Figure 8E), we conclude that they were unlikely to have excessively disrupted pore structure around the 6′ region. Although the A288S and A288T mutations had no effect on fipronil sensitivity (Figure 8F), the A288F mutation completely eliminated fipronil inhibitory potency regardless of whether it was applied in the closed or open states (Figure 8C,D). However, the transient fipronil-mediated potentiation remained intact (e.g. Figure 8C, representative of five cells). Because the A288F mutation selectively eliminates fipronil inhibition, we propose that fipronil (but not lindane) accesses its pore-binding site via a lateral portal in the vicinity of A288, and not by traversing along the axis of symmetry of the pore from the extracellular bulk solution. S15′ in the M2 domain is also proposed to contribute to this putative fipronil binding site (Law and Lightstone, 2008). However, we found the S15′I mutation had no significant effect on the sensitivity of α1 glycine receptors to either lindane or fipronil (not shown).

Discussion

Glycine receptors as potential targets of lindane and fipronil toxicity in vertebrates

One original motivation for this study was to test whether glycine receptors were likely targets for fipronil or lindane toxicity in humans. We found that fipronil inhibited human α1, α1β, α2 and α3 glycine receptor isoforms with IC_{50} values ranging from 0.4 to 2 μM (Table 2). This is certainly comparable with its inhibitory potency range (1–2 μM) at major mammalian $GABA_A$ receptor isoforms (Ikeda *et al.*, 2001; Li and Akk, 2008), suggesting that all human glycine receptor isoforms could potentially be fipronil toxicity targets. This conclusion is supported by a recent behavioural and morphological study, which inferred that glycine receptor inhibition was responsible for fipronil-mediated locomotor defects in zebrafish (Stehr *et al.*, 2006). Lindane has previously been shown to be a partial (~50%) inhibitor of a variety of recombinant $GABA_A$ receptor isoforms, also exhibiting IC_{50} values in the 1–2 μM range (Aspinwall *et al.*, 1997; Maskell *et al.*, 2001). Lindane inhibited totally the native glycine receptors in cultured cerebellar granule cells with an IC_{50} near 5 μM (Vale *et al.*, 2003). Here we found that lindane completely inhibited the four tested glycine receptor subtypes with IC_{50} values ranging from 0.2 to 1 μM, with the α2 homomeric glycine receptor exhibiting the highest sensitivity (Table 2). This implies that lindane toxicity might also be mediated by glycine receptors, particularly in mammalian embryos, where α2 homomers are the dominant glycine receptor isoform (Becker *et al.*, 1988). In summary, therefore, the present study supports the conclusion that toxic doses of fipronil and

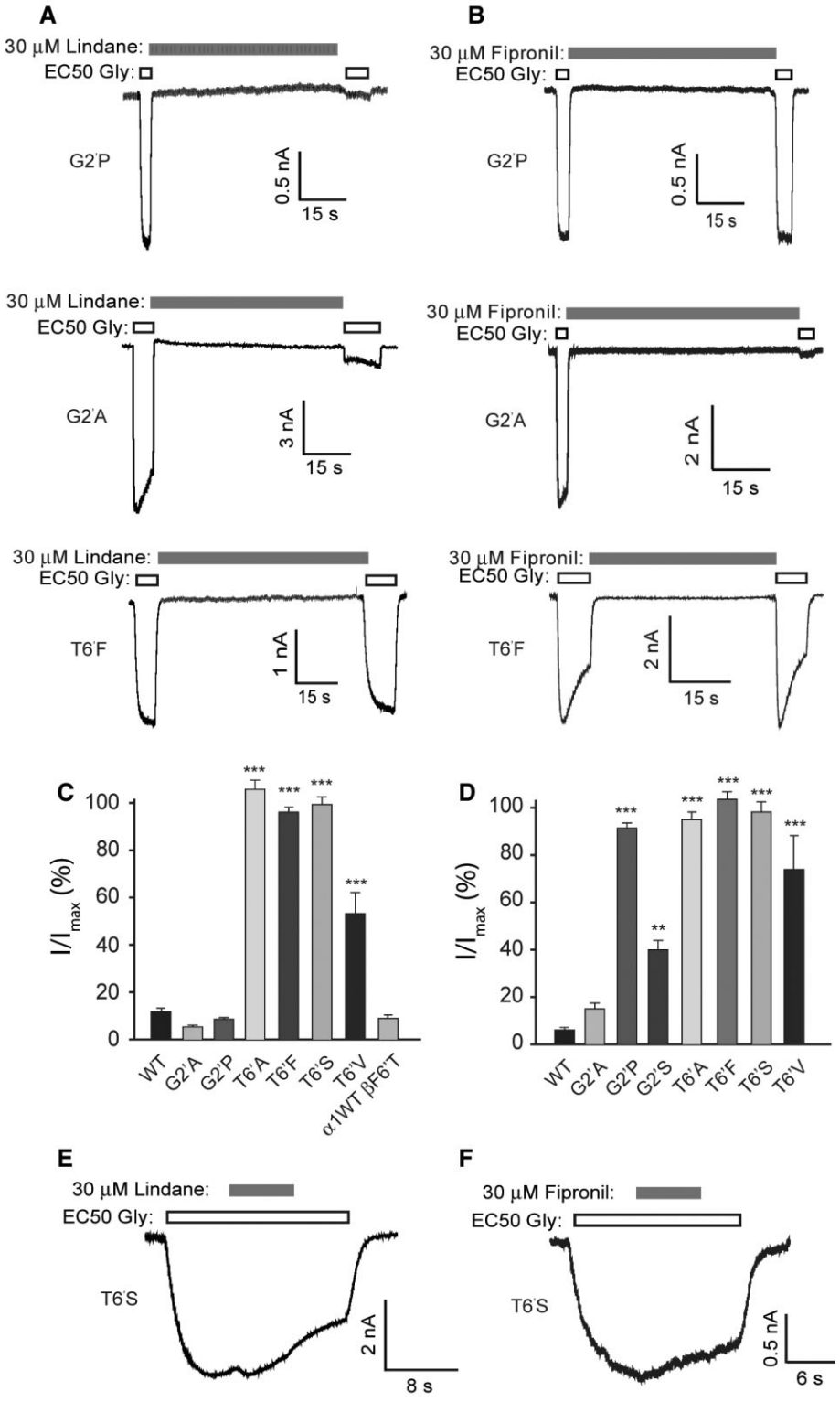

Figure 6

Effects of 2′ and 6′ mutations on α1 glycine receptor (GlyR) sensitivity to lindane and fipronil. (A) Examples of the effects of 30 μM lindane on EC_{50} glycine (Gly) currents at indicated mutant α1 glycine receptors. (B) Results of similar experiments for fipronil. (C) Percentage of maximum control current remaining (i.e. I/I_{max}) following a 30 s exposure of 30 μM lindane at α1 and α1β glycine receptors incorporating the indicated mutations. (D) Similar plot for fipronil. All results in C and D were averaged from at least five experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from WT glycine receptor; unpaired *t*-test. (E) Sample recording revealing an absence of inhibition by 30 μM lindane of the T6′S mutant α1 glycine receptor when co-applied with EC_{50} glycine. (F) Similar result for 30 μM fipronil at the same mutant glycine receptor.

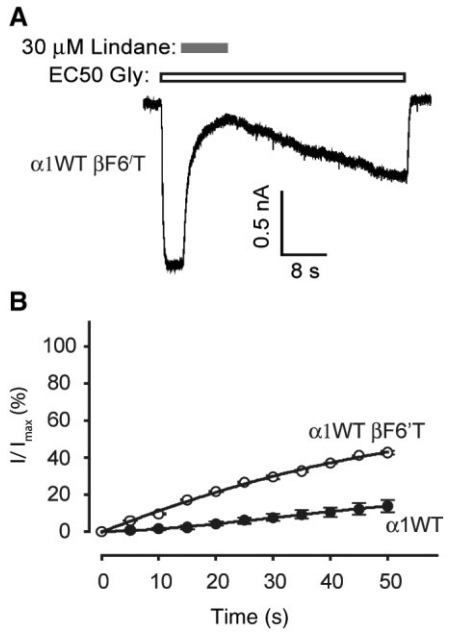

Figure 7

Evidence for the functional expression of F6′T mutant β subunits in heteromeric glycine receptors (GlyR). (A) Sample recording showing relatively rapid recovery from lindane inhibition. (B) Averaged recovery profiles from lindane inhibition at the indicated glycine receptors.

lindane are likely to produce neuronal hyperexcitability in humans and other vertebrates by inhibiting both glycine and $GABA_A$ receptors.

Given that both drugs exhibit a slow unbinding rate (Figure 3), it is possible that extremely low (nM) concentrations of drug could accumulate at binding sites and produce significant inhibitory effects. However, it is difficult to convincingly quantitate slow (i.e. minutes to hours) blocking rates with electrophysiological techniques, using lipophilic compounds whose effects are poorly reversible. Our approach was to pre-incubate HEK293 cells expressing α1 glycine receptors with 0.1–1 μM of each compound for 1 h and use a yellow fluorescent protein-based anion-influx fluorescence assay, as previously described (Gilbert *et al.*, 2009), to probe for reductions in the anion influx rate, relative to non-drug-treated cells. With this assay, we observed no significant reduction in anion flux rates at lindane and fipronil concentrations less than 1 μM (not shown).

Inhibitory sites of action of lindane and fipronil

Two lines of evidence strongly suggest that both lindane and fipronil bind in the glycine receptor channel pore. First, functional data indicate that both compounds are non-competitive antagonists (Figure 1E), and that recovery from inhibition is strongly glycine concentration-dependent (Figures 3 and 4). These are both classic properties of open channel blockers. Second, site-directed mutagenesis evidence strongly supports a pore-binding site for both compounds. The molecular evidence for a pore-binding site is now considered in detail.

A computational docking study concluded that lindane binds to homomeric β3 $GABA_A$ receptors via hydrogen bonds with T6′ and hydrophobic interactions with A2′ (Chen *et al.*, 2006). Experimental support for a 2′ binding interaction is provided by the finding that, in the *Drosophila* resistant to dieldrin (RDL) $GABA_A$ receptor, A2′S and A2′G mutations reduced lindane sensitivity (Cole *et al.*, 1995), whereas the reverse S2′A mutation in an *M. domestica* GluClR enhanced lindane sensitivity (Hirata *et al.*, 2008). There is, however, no experimental evidence to date that lindane interacts with 6′ residues in any Cys-loop receptor. Here we found that lindane inhibitory potency at the α1 glycine receptor was not affected by the extremely non-conserved G2′P mutation. This is strongly suggests that lindane does not interact with 2′ pore-lining groups in the α1 glycine receptor. In contrast, we found the conservative T6′A, T6′S and T6′V mutations dramatically reduced lindane inhibitory potency. Threonine residues can contribute to both hydrogen bonds and hydrophobic interactions. However, selective elimination of their hydrophobic interaction capability by the T6′S mutation, of hydrogen bonding by the T6′V mutation and of both binding capabilities by the T6′A mutation, imply that lindane interacts with 6′ threonine, via both types of bonds. The loss of lindane sensitivity produced by the T6′F mutation was not surprising given the bulk and hydrophobic nature of this amino acid (Sedelnikova *et al.*, 2006). Indeed, because heteromeric α1β glycine receptors incorporating the reverse β subunit F6′T mutation exhibited a high sensitivity to lindane, typical of α1 homomeric glycine receptors, we conclude that the β subunit F6′ side chains alone are responsible for the loss of lindane sensitivity in α1β glycine receptors. Picrotoxin exhibited an identical dependence on 6′ side chain identity in α1β glycine receptors (Shan *et al.*, 2001).

Molecular docking studies suggest that fipronil binds to homomeric β3 $GABA_A$ receptors via hydrogen bonds with the hydroxyl group of T6′ and hydrophobic interactions with A2′ and T6′ (Chen *et al.*, 2006; Ci *et al.*, 2007; Cheng *et al.*, 2009). Consistent with this, naturally occurring A2′S and A2′G mutations in the *Drosophila* RDL $GABA_A$ receptor dramatically reduced fipronil sensitivity (Cole *et al.*, 1995; Hosie *et al.*, 1995; Le Goff *et al.*, 2005), whereas the reverse S2′A mutation enhanced fipronil sensitivity (Hirata *et al.*, 2008). At the α1 glycine receptor, we found that the inhibitory potency of fipronil was modestly reduced by the G2′A mutation (although this was not significant) and was more dramatically reduced by the G2′S mutation. A parsimonious explanation for these results is that a progressively increasing side chain volume at the 2′ level displaces fipronil from its site by simple steric interference. The elimination of fipronil sensitivity by the G2′P mutation is difficult to interpret due to the non-conservative nature of this mutation but is also consistent with a steric displacement model. Because fipronil sensitivity was reduced by the T6′S and T6′V mutations and eliminated by the T6′A mutation, we conclude that hydrogen bond and hydrophobic interactions are both important in binding fipronil to T6′. This fits with the results of the $GABA_A$ receptors molecular docking simulations. Although both G2′P and T6′F mutations individually eliminated fipronil sensitivity, the α1β glycine receptor (which contains endogenous β subunit P2′ and F6′ residues) showed a fipronil sensitivity similar to that of the α1 glycine receptor. The β subunit

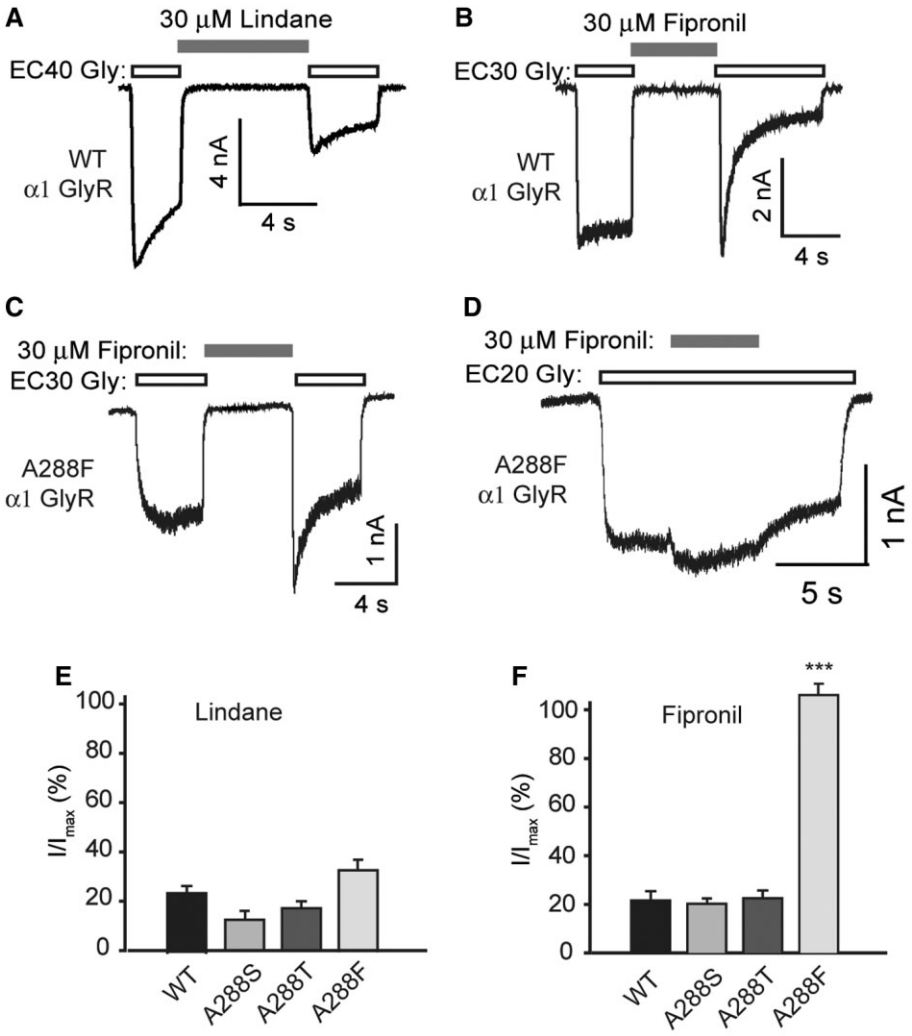

Figure 8

Effects of the A288F mutation on α1 glycine receptor (GlyR) sensitivity to lindane and fipronil. (A,B) Examples of the effects of brief (~5 s) closed-state applications of 30 μM lindane and fipronil on EC_{30-40} glycine currents at WT α1 glycine receptors. (C) Sample experiment showing that 30 μM fipronil applied in the closed state potentiates but does not inhibit A288F mutant α1 glycine receptors. (D) Sample experiment showing that 30 μM fipronil, when co-applied with EC_{20} glycine, also potentiates but does not inhibit A288F mutant α1 glycine receptors. (E) Percentage of maximum control current remaining (i.e. I/I_{max}) following a 5 s closed-state exposure of 30 μM lindane at α1 glycine receptors incorporating the indicated mutations. (F) Results of similar experiments for fipronil. All results in E and F were averaged from at least five experiments. ***$P < 0.001$, significantly different from WT glycine receptor; unpaired *t*-test.

contains many M2 residues that differ from their α1 subunit counterparts, and it is likely that some combination of these may have compensated for the deleterious effects (if any) of the endogenous β subunit P2′ and F6′ residues.

In summary, in agreement with molecular docking predictions (Chen *et al.*, 2006; Ci *et al.*, 2007; Cheng *et al.*, 2009) and functional studies on the $GABA_A$ receptor (Cole *et al.*, 1995; Hosie *et al.*, 1995; Le Goff *et al.*, 2005) and GluClR (Hirata *et al.*, 2008), the results of this study support the idea of fipronil and lindane binding in the α1 glycine receptor pore. We conclude that both molecules interact with T6′ residues via both hydrophobic interactions and hydrogen bonds. In contrast with modelling predictions, we found no evidence for lindane binding at the 2′ level. Fipronil, on the other hand, may simply require the space afforded by the endogenous 2′ glycine residues in order to adopt its optimal binding configuration with 6′ threonine residues. Of course, both drugs may also interact with other pore-lining residues not considered here.

A 'non-blocking' fipronil binding site

A brief fipronil application produced no instantaneous block, shown by the undiminished magnitude of the subsequent glycine application (Figure 7A, right). However, when glycine was applied immediately after fipronil was removed, fipronil-dependent inhibition developed rapidly despite the absence of fipronil in free solution. This implies the existence of both a non-blocking fipronil binding site that is readily accessed by fipronil from free solution in the closed state and a blocking fipronil site that is accessed more readily in the open state.

Fipronil is evidently able to migrate from the non-blocking site to the blocking site upon channel opening. We recently showed that dihydropyridines exhibit a similar phenomenon at this receptor (Chen *et al.*, 2009). A recent $GABA_A$ receptor molecular docking study (Law and Lightstone, 2008) predicted that fipronil and lindane can also bind at or near the intramembranous alcohol and anaesthetic binding site that is formed, at least partly, by A288 in the α1 glycine receptor M3 domain (Lobo and Harris, 2005). Hence, we investigated whether this site may form the non-blocking fipronil site. As the A288F mutation abolished fipronil sensitivity without affecting lindane sensitivity, it seems unlikely that this mutation would have severely affected the geometry of the pore blocking T6′ site. On the other hand, however, the A288F mutation is known to dramatically affect α1 glycine receptor sensitivity to anaesthetics and ivermectin (Yamakura *et al.*, 1999; Lynagh and Lynch, 2010), both of which are thought to bind at this site (Lobo and Harris, 2005; Collins and Millar, 2010). Thus, we conclude that A288F had a specific effect at the non-blocking site, either by preventing fipronil from binding to this site or from leaving this site to migrate to the pore in the open state. Our observation that fipronil still produced a transient potentiation in the A288F mutant glycine receptor suggests it may still have been able to bind at this site. Indeed, other molecules (e.g. alcohols, anaesthetics, avermectins, neurosteroids) thought to bind in the same pocket all produce potentiation of Cys-loop receptors (Hosie *et al.*, 2006; Li *et al.*, 2006; Li and Akk, 2008; Collins and Millar, 2010; Nury *et al.*, 2011).

Conclusion

As the glycine receptor isoforms investigated here are comparable with the major $GABA_A$ receptor isoforms in terms of their sensitivity to lindane and fipronil, we conclude that glycine receptors are likely to represent a novel target for fipronil and lindane toxicity in vertebrates. Our results also provide strong experimental support for molecular docking studies that had previously predicted that lindane and fipronil bind to anionic Cys-loop receptor 6′ threonine pore-lining residues via a mixture of hydrophobic interactions and hydrogen bonds. However, our results do not support the predicted interaction between lindane and 2′ pore-lining groups in α1 glycine receptors. In contrast, fipronil sensitivity was modulated by 2′ mutations in a manner that suggests that it requires the space afforded by the endogenous 2′ glycine residues in order to adopt its optimal conformation for binding with 6′ threonine residues. We also found that glycine receptor sensitivity to lindane (but not fipronil) was eliminated upon β subunit incorporation. This loss of sensitivity was found to be mediated solely by β subunit 6′ phenylalanine residues. Indeed, this loss of sensitivity was so profound that it renders lindane a more effective pharmacological tool than the existing standard, picrotoxin, for reporting the presence of β subunits in heteromeric αβ glycine receptors. Finally, we present evidence that fipronil (but not lindane) bound to the alcohol and anaesthetic binding pocket, without producing block and was able to migrate from there to the pore blocking site upon channel activation. Together, these findings provide new insights into insecticide binding and toxicity mechanisms and may contribute towards the rational design of novel insecticide-related compounds.

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Conflict of interest

None.

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