Fipronil is a Potent Open Channel Blocker of Glutamate-Activated Chloride Channels in Cockroach Neurons

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ABBREVIATIONS: GABA, γ -aminobutyric acid; GluCl, glutamate receptor chloride channel;

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ABSTRACT

Fipronil, a phenylpyrazole insecticide, displays high insecticidal activity and reduced mammalian toxicity. To better elucidate the mechanism of its selective toxicity between insects and mammals and activity against dieldrin-resistant insects, we studied fipronil action on glutamate-gated chloride channels (GluCl_s), unique invertebrate ligand-gated chloride channels, in cockroach thoracic ganglion neurons, using the whole-cell patch clamp technique. Glutamate evoked two types of chloride currents, a desensitizing current and a non-desensitizing current. Fipronil differentially inhibited these two types of currents with different potencies and with different rates of reversibility. Fipronil inhibited the desensitizing and non-desensitizing GluCl_s with IC₅₀s of 801 nM and 10 nM, respectively. Kinetic analysis revealed that fipronil block required channel opening. Recovery of the desensitizing current from fipronil block required channel opening, whereas recovery of non-desensitizing current from block was independent of channel opening. The high potency of fipronil against the non-desensitizing current was due to a slow unblocking rate constant. In addition, when the non-desensitizing GluCl_s were occupied by picrotoxinin, the receptors became less sensitive to fipronil block. It is concluded that GluCl_s are a critical target for fipronil especially for the selective toxicity between mammals and insects, and that fipronil block of GluCl_s may play a role in the lack of the cross-resistance with dieldrin.

Fipronil, a phenylpyrazole insecticide introduced in 1992 (Colliot et al., 1992), is a potent blocker of GABA receptors which are believed to be its primary target site. Because of its high insecticidal activity against many insects and other arthropod pests and low toxicity to mammals (Tingle et al., 2003), fipronil has found many uses in agriculture, veterinary medicine and urban pest control. The oral LD₅₀ of fipronil in rats was estimated to be 91 mg/kg, and the LD₅₀s in insects were 0.07 mg/kg in corn rootworm (Scharf and Siegfried, 1999) and 0.13 mg/kg in housefly (Hainzl and Casida, 1996). Thus, the sensitivity of insects to fipronil is 700-1300-fold higher than that of rats. At the level of GABA receptors, fipronil also displays significant insect selectivity: the IC₅₀ against cockroach GABA receptors of fipronil was 59-fold lower than that of rat GABA_A receptors (Zhao et al., 2003). Therefore, the differences in GABA receptor sensitivity to fipronil can only partially explain the selective toxicity between mammals and insects. We propose that differences in the fipronil sensitivity of another target site may also play a role in the selective toxicity.

The cyclodiene insecticide dieldrin is also known to act at the GABA receptor (Nagata and Narahashi, 1994). A single gene conferring 4000-fold resistance to dieldrin (Rdl) was identified and found to code for a GABA-gated chloride channel subunit (French-Constant et al., 1993). Dieldrin resistance was associated with a point mutation of Ala to Ser at position 302 in the second transmembrane domain, which is thought to line the pore of the channel. Dieldrin resistance is often associated with much lower levels of fipronil resistance (Cole et al., 1995; Tingle et al., 2003). Furthermore, after several years of use in more than 60 countries, the field performance of fipronil has not been hampered by resistance. The Ala-Ser mutation of the *Drosophila* (DMRdl) GABA receptor is not associated with reduced fipronil sensitivity (Hosie et al., 1995) but equivalent mutation of *Heliothis* GABA receptor (HVRDL) showed reduced

sensitivity to fipronil (Wolff and Wingate, 1998). Thus, a target site other than Rdl GABA receptor might be involved in the effectiveness of fipronil on dieldrin-resistant insects.

Glutamate-gated chloride channels (GluCl_s) are also a potential insect-specific target of fipronil because of their unique presence in invertebrates but not in vertebrates (Cleland, 1996; Raymond and Sattelle, 2002). Recent studies showed that fipronil exhibited inhibitory actions on GluCl_s (Cully et al., 1994; Horoszok et al., 2001; Ikeda et al., 2003), but the results are not quantitative enough to compare with the effects on GABA receptors. At least two types of GluCl_s, one picrotoxin-insensitive and the other picrotoxin-sensitive, were found to co-exist in cockroach neurons (Raymond et al., 2000), and these were subsequently characterized as desensitizing and non-desensitizing, respectively (Ikeda et al., 2003). Fipronil only modestly inhibited the desensitizing GluCl with an IC₅₀ of 730 nM (Ikeda et al., 2003), but its action on the non-desensitizing GluCl has not yet been examined in detail.

The differential sensitivities of the desensitizing and non-desensitizing GluCl_s to fipronil were examined in cockroach thoracic ganglion neurons using the whole-cell patch clamp technique. Fipronil blocked non-desensitizing GluCl_s much more potently than it blocked the desensitizing GluCl_s. Furthermore, the block of non-desensitizing GluCl_s by fipronil was shown for the first time to occur primarily through an open-channel blocking mechanism. When co-applied with glutamate to non-desensitizing GluCl_s, fipronil blocked these receptors more potently than it blocked GABA receptors. It is concluded that the selective blocking action of fipronil on non-desensitizing GluCl_s is more significant than previously thought and is probably one of the potential mechanisms accounting for its selective toxicity between insects and mammals and its high activity against dieldrin-resistant insects.

Material and Methods

Preparation of Cockroach Neurons. Adult American cockroaches, *Periplaneta americana*, were purchased from Carolina Biological Supply Company (Burlington, NC) and were maintained at 29°C with free access to water and food. Isolation of neurons from cockroaches was performed using enzymatic digestion and mechanical dissociation (Alix et al., 2002). Briefly, a cockroach was immobilized with pins dorsal side up and was dissected to access the ventral nerve cord. Three thoracic ganglia were isolated and immersed in the normal cockroach saline solution containing 200 mM NaCl, 3.1 mM KCl, 4 mM MgCl₂, 20 mM D-glucose, and 10 mM HEPES-acid (pH 7.3). The ganglia were then incubated for 30 min at room temperature (22~24°C) in cockroach saline solution supplemented with collagenase (type A, 0.5 mg/ml, Boehringer Mannheim, Germany) and hyaluronidase (type I-S, 1 mg/ml, Sigma, St. Louis, MO). The ganglia were then rinsed twice in normal saline solution supplemented with 5 mM CaCl₂ and fetal calf serum (5% by volume), and were mechanically dissociated by gentle repeated trituration through a fire-polished Pasteur pipette. The dissociated neurons, suspended in the supplemented normal saline solution, were allowed to settle on cover slips coated with poly-L-lysine hydrobromide (MW, >30,000, Sigma). The neurons were incubated at 24°C overnight before experiments. The resting potential of dissociated neurons was -75.2 ± 2.3 mV (n = 36). To prevent fipronil contamination each cell culture dish was used only once and the recording chamber was thoroughly washed with alcohol after each experiment.

Whole-Cell Current Recordings. Neurons were continuously perfused with cockroach external solution containing 167 mM NaCl, 3.1 mM KCl, 33 mM D-gluconic acid, 5 mM CaCl₂, 4 mM MgCl₂, and 10 mM HEPES-acid (pH 7.4). The osmolarity was 420 mOsm. Ionic currents were recorded using the whole-cell patch clamp technique at room temperature (23°C). Pipette

electrodes were made from 1.5 mm (o.d.) borosilicate glass capillary tubes, and had a resistance of 2-3 M Ω when filled with the standard internal solution containing: 15 mM NaCl, 170 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 10 mM EGTA, 10 mM phosphocreatine diTris, 20 mM HEPES-acid and 3 mM ATP-Mg²⁺. The pH was adjusted to 7.4 with KOH, and the osmolarity was 420 mOsm. The membrane potential was clamped at -60 mV unless otherwise stated. The recording of whole-cell currents began 10 min following membrane rupture so that the cell interior milieu was adequately equilibrated with the pipette solution. Currents through the electrode were recorded with an Axopatch-200A amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz, and stored by a PC-based data acquisition system that also provided preliminary data analysis. Data, when quantified, were expressed as the mean $\pm$ S.E.M.

Drug Application. A U-tube was used for rapid, controlled application of external solution containing glutamate alone or with blockers. The U-tube was a loop of fine tubing with a single hole that was placed near the cell. External solution containing glutamate alone or with blockers was fed through the tube by gravity from a container located above the bath. Closure of a computer-operated solenoid valve in the outlet side of the tube allowed the U-tube solution to flow out of the hole located near the cell. Another valve controlling a suction tube with an opening on the other side of the cell was also opened, allowing the test solution to be sucked away quickly. With this method, the external solution surrounding the cell could be completely exchanged with a test solution within 30 ms. Test compound was either co-applied with glutamate in the U-tube or added to the bath.

Chemicals. Monosodium glutamate (Sigma) was first dissolved in de-ionized water as the stock solution and then diluted with the cockroach external solution immediately before experiments. Fipronil (provided by Rhone-Poulenc Yuka Agro K.K., Akeno, Japan) was first

dissolved in dimethylsulfoxide to make stock solutions, and then diluted with the external solution shortly before experiments. The final concentrations of dimethylsulfoxide in test solutions were 0.1% (v/v) or less which had no effect on the glutamate-induced currents.

Analysis. Whole-cell currents were initially analyzed with the pClamp version 6.0.4 software to measure the current amplitudes and decay kinetics. The statistical analysis and the nonlinear regression analysis were carried out using the Sigmaplot 2001 software (SPSS). The dose-response relationship for glutamate to activate the glutamate receptor was evaluated by fitting the data to Hill equation: $I = I_{\max} C_{50}^h / (C^h + C_{50}^h)$, where I is the current amplitude relative to the control maximum current, $I_{\max}$, C is the concentration of chemical, C_{50} is the concentration of chemical inducing a half maximum response, and h is the Hill coefficient. The dose-response relationship for test chemicals to modulate the glutamate response was evaluated with a similar Hill equation to obtain EC_{50} or IC_{50} values.

Results

Differential Actions of Fipronil on Two Types of GluCl_s. In isolated cockroach neurons, two types of GluCl currents were recorded in response to application of 100 μ M glutamate at a holding potential of -60 mV: the desensitizing current and the non-desensitizing current (Fig. 1). The desensitizing current (Fig. 1A) was characterized by a fast rising phase and a fast decay phase (observed in 18 out of 145 cells), and the non-desensitizing current (Fig. 1B) was characterized by a slower rising phase and a very slow decay phase (observed in 56 out of 145 cells). Most neurons exhibited a mixed-type current with a fast transient component followed by a slow component upon application of 100 μ M glutamate (Fig. 1C). The kinetic properties of the mixed-type current and its differential modulation by drugs suggested that the mixed-type current represented a composite of desensitizing and non-desensitizing currents. The EC_{50} s determined from the dose-response relationship were 37 μ M for the desensitizing current (Ikeda et al., 2003) and 116 ± 4 μ M ($n = 6$) for the non-desensitizing current.

The two types of GluCl currents responded differently to the blocking action of fipronil. Following co-applications of 1 μ M fipronil with 100 μ M glutamate via a U-tube, the peak amplitude of the desensitizing current was gradually decreased (Fig. 1A). The rising phase of the current was not changed but the decay phase was slightly accelerated. As a result, the time to the peak of the current was shortened. After three consecutive co-applications at an interval of 2 min, the fipronil-induced inhibition reached a steady-state level of 57% of the control and the decay time constant was reduced from 425 ms to 297 ms.

In another neuron that generated mainly the non-desensitizing current, co-application of 1 μ M fipronil with 100 μ M glutamate greatly accelerated the current decay without changing the rising phase (Fig. 1B). At the end of an 8-s application of fipronil, the current was inhibited

almost completely. In the second and third applications of fipronil, the glutamate-induced currents remained small. The steady-state currents evoked by 100 μ M glutamate were inhibited by $96.3 \pm 3.5\%$ ($n = 7$). The differential action of fipronil on the two types of glutamate-induced currents could also be seen in neurons generating the mixed-type current (Fig. 1C). Thus, a use-dependent inhibition is seen with fipronil in both the desensitizing and the non-desensitizing current, and the non-desensitizing current is much more sensitive than the fast transient current to fipronil block.

Blocking Action of Fipronil on Desensitizing GluCl_s. The potency and reversibility of the inhibitory action of fipronil on the desensitizing GluCl currents were analyzed in detail. As shown in Fig. 2, when 1 μ M fipronil was applied via bath perfusion and U-tube, the peak amplitudes of the desensitizing currents evoked by 100 μ M glutamate at an interval of 2 min were gradually inhibited to 26.2% of the control (b over a). The degree of inhibition was similar to that previously reported (Ikeda et al., 2003). After washing with fipronil-free external solutions for 10 min, only a slight recovery was seen (c vs. b traces) in the absence of repetitive glutamate stimulation. However, recovery occurred rapidly when the receptors were activated by glutamate. Most of the recovery must have occurred during the first application of glutamate to activate GluCl_s (c) because the current (d) in response to the 2nd application of glutamate reached near the maximum recovery level during repeated activation by glutamate. The recovery from the fipronil block was also discernible as a sustained inward current following the transient peak current in trace c. The results suggested that the fipronil molecule was trapped in the channel of the resting receptor and that unblock of the desensitizing GluCl_s required the activation or opening of GluCl_s. This implies that the fipronil-blocked receptors do not undergo fast desensitization.

Fipronil block of the desensitizing GluCl_s was concentration dependent. Desensitizing currents were evoked by a series of 30-s applications of 100 μ M glutamate at an interval of 2 min. After several stable control recordings were established, fipronil at different concentrations ranging from 10 nM to 10 μ M was co-applied with glutamate. The peak current amplitude was gradually decreased, reaching a steady-state level of block after four consecutive co-applications of each fipronil concentration. The concentration-response curve for steady-state block of desensitizing currents caused by fipronil is illustrated by closed circles in Fig. 3. The fitting to the data with a sigmoid curve gave an IC₅₀ of 801 ± 207 nM ($n = 3-5$), a value similar to that of the previous report (Ikeda et al., 2003).

Blocking Action of Fipronil on Non-Desensitizing GluCl_s. To obtain the concentration-response relationship for fipronil block of the non-desensitizing GluCl_s, two protocols were used. In the first protocol, the effect of bath-applied fipronil on the non-desensitizing current was monitored at a 30-s interval by a 2-s pulse of glutamate and fipronil co-application. The steady-state inhibition curve is plotted as open circles in Fig. 3. The fit to the data gave an IC₅₀ of 10 ± 0.9 nM ($n = 3-10$). Thus, fipronil is about 80 times more potent against the non-desensitizing current compared to the desensitizing current.

In the second protocol, fipronil was co-applied with 100 μ M glutamate for 20 s to observe the steady-state fipronil block of the activated receptors. The sustained currents were measured at the end of 20-s co-applications of glutamate and fipronil (Fig. 4A). While the currents shown in Fig. 4A may contain desensitizing GluCl currents, the non-desensitizing components can be obtained by subtraction: since the desensitizing current was minimally affected by 100 nM fipronil and also since the non-desensitizing current was almost completely inhibited, the non-desensitizing current can be obtained by subtracting the current after the 3rd application of fipronil from the

total current. The subtracted non-desensitizing currents are illustrated in Fig. 4B. The inhibition of the sustained current following the first, second, and third co-applications of fipronil with glutamate was calculated and is shown in Fig. 4C. The IC_{50} s were estimated to be 27.0 ± 5.5 , 17.3 ± 4.2 and 11.9 ± 3.5 nM fipronil for the first, second, and third co-applications, respectively ($n = 3-9$). The IC_{50} value of 11.9 nM after the third co-application is similar to the value obtained from combined bath and U-tube application (Fig. 3). Thus, fipronil blocked the non-desensitizing current in a use-dependent manner.

The use-dependent block of non-desensitizing GluCl_s by fipronil was further examined. Non-desensitizing currents were evoked by 2-s applications of 100 μ M glutamate. Fipronil at a concentration of 100 nM was bath-perfused for 10 min, during which a series of repetitive co-applications of fipronil and glutamate were performed at an interval of 30 s (Fig. 5A). Currents were suppressed gradually during repeated co-applications and eventually reached a very low levels at the end of 10-min fipronil perfusion. In another protocol shown in Fig. 5B, non-desensitizing currents were also evoked by 2-s applications of 100 μ M glutamate, but, during bath perfusion of 100 nM fipronil, no glutamate was applied to the neuron until at the end of 10-min fipronil perfusion. The results showed that the non-desensitizing steady-state currents were inhibited to $10.4 \pm 1.4\%$ ($n = 7$) of the control by fipronil when GluCl receptors were repeatedly activated by glutamate, whereas the steady-state currents were decreased only to $90.6 \pm 2.0\%$ ($n = 6$) of the control by fipronil when GluCl receptors were kept in a resting state (Fig. 5C). The activation of the receptors significantly enhances the blocking action of fipronil on non-desensitizing GluCl_s ($p < 0.01$).

Kinetic Analysis of Fipronil Block of Non-Desensitizing GluCl_s. To estimate the rate of fipronil block of the activated glutamate receptor, fipronil at various concentrations was co-

applied with 100 μ M glutamate for 20 s via a U-tube perfusion system. In the absence of fipronil, the current evoked by 20-s application of glutamate decayed slowly. The time constant for decay of the non-desensitizing current could be determined from neurons generating the mixed current 3 s after its onset, as the desensitizing current, which decayed with a time constant of 400 ms, was essentially over by then. The glutamate current decayed faster as the fipronil concentration was increased from 10 to 1000 nM (Fig. 6). The time constants thus determined decreased from a control of 29.4 s to 23.6, 15.8, 9.7, 5.1 and 2.5 s, respectively, by 10, 30, 100, 500 and 1000 nM fipronil. The reciprocal of these time constants is plotted as a function of fipronil concentration in Fig. 6. The slope of the linear relationship gave the binding rate constant of $3.04 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and the unbinding rate constant of $5.77 \times 10^{-2} \text{ s}^{-1}$ ($n = 3-7$). These values are of the same order of magnitude as those for blocking the cockroach GABA-activated chloride channel (Zhao et al., 2003).

Fipronil Preferentially Blocks Non-Desensitizing GluCl_s in the Activated State. To assess the activated receptor block, a 30-s co-application of fipronil with glutamate was used (Fig. 7A). To assess the block when receptors are repetitively activated, brief 2-s pulses of glutamate and fipronil were co-applied at 30-s intervals while fipronil was perfused in the bath (Fig. 7B). The block of the activated receptor developed with a time constant of $18.0 \pm 3.1 \text{ s}$ ($n = 4$) (Fig. 7C), whereas the block during repetitive receptor activations occurred much more slowly, with a time constant of $83.1 \pm 12.4 \text{ s}$ ($n = 4$) (Fig. 7B). However, if the time courses of fipronil block using the two protocols was expressed in terms of the duration during which the receptors were activated, the fipronil block induced by brief repetitive 2-s co-applications of glutamate and fipronil became faster, with a time constant of $5.1 \pm 1.2 \text{ s}$ ($n = 4$) (Fig. 7C). Thus, repetitive openings of the glutamate receptor are more effective than a single long opening for

fipronil block. This result together with that shown in Fig. 5B suggests that the activated receptors are more sensitive to the blocking action of fipronil than desensitized receptors.

Recovery of Desensitizing and Non-Desensitizing GluCl_s from Fipronil Block. In order to compare the recovery of desensitizing and non-desensitizing currents from fipronil block, experiments depicted in Fig. 8 were performed. During a 30-s co-application of 100 μ M glutamate and 1 μ M fipronil, the peak was reduced to about 40% of the control while the steady-state current was decreased to $5.8 \pm 2.4\%$ ($n = 5$) of the control (Fig. 8A and D). After washing the neuron with fipronil-free external solution for 10 min, the peak current remained decreased in response to the application of 100 μ M glutamate, while the steady-state current recovered to $24.9 \pm 4.9\%$ ($n = 5$) of the control (Fig. 8B and D). A second application of glutamate 30 s later revealed that the peak current completely returned to the control level, while the steady-state current increased slightly (Fig. 8B, washout-2). The rapid recovery of the peak current during the 2nd test pulse after washout was similar to that described in Fig. 2Bd.

The recovery of the non-desensitizing current from the block caused by 1 μ M fipronil was incomplete (Fig. 8B). However, since the non-desensitizing current was 80 times more sensitive to fipronil block than the desensitizing current (Fig. 3), it was necessary to compare the recovery after washing using the equivalent concentrations of fipronil that cause the same degree of block. Thus, additional experiments were performed for the recovery of the non-desensitizing current using 10 nM fipronil (Fig. 8C). The steady-state current was decreased to $71.6 \pm 5.2\%$ ($n = 5$) of the control during co-application of 100 μ M glutamate and 10 nM fipronil. After washing the neuron with fipronil-free external solutions for 10 min, during which no glutamate was applied, the steady-state currents recovered to $93.0 \pm 3.1\%$ ($n = 5$) of the control (Fig. 8D). It was

concluded that non-desensitizing GluCl_s, when blocked by a moderate concentration of fipronil, could fully recover in the absence of receptor activation.

Interaction of Fipronil and Picrotoxinin in Blocking the Non-Desensitizing Current.

Picrotoxinin and fipronil block rat GABA_A receptors by binding to different receptor sites (Ikeda et al., 2001). Although picrotoxinin had little or no effect on the desensitizing GluCl_s, it blocked the non-desensitizing GluCl_s potently, with an IC₅₀ on the order of 4 μM. Thus, a question arose whether picrotoxinin and fipronil interacted with each other in blocking the non-desensitizing GluCl_s. The time-dependent block of the non-desensitizing current by 100 μM picrotoxinin co-applied with 100 μM glutamate is illustrated in Fig. 9A. The block of non-desensitizing current by picrotoxinin was nearly complete at the end of the 1st 5-s glutamate/picrotoxinin co-application (Fig. 9Ab) as evidenced by the complete block by the 2nd co-application of picrotoxinin and glutamate (Fig. 9Ac). The recovery from picrotoxinin block is illustrated in Fig. 9Ba and 9Bb. After washing the neuron with picrotoxinin-free external solution for 2 min, the first glutamate pulse induced a current with a slow rising phase (Fig. 9Ba). The recovery was essentially complete at the end of a single 5-s glutamate pulse, as indicated by the near normal current evoked by the 2nd application of glutamate applied 1 min later (Fig. 9Bb). These results suggest that the picrotoxinin molecule is trapped in the closed channel when the receptor is at rest and that the picrotoxinin molecule egresses the channel only when it is open. Thus, picrotoxinin is an open channel blocker.

If fipronil and picrotoxinin were mutually exclusive in blocking the non-desensitizing GluCl_s, one would expect that picrotoxinin would alter the time course and degree of fipronil block, as fipronil could not affect the picrotoxinin-blocked channel until the channels were free of picrotoxinin. This was indeed the case as shown in Fig. 9C. After picrotoxinin block had been

established, the neuron was washed with picrotoxinin-free external solution for 2 min and subsequently exposed to 1 μ M fipronil co-applied with glutamate. The glutamate-induced current had a slow rising phase, reaching a peak near 3 s and then began to show slow decay. These changes in current kinetics are consistent with the hypothesis that fipronil cannot affect the channel until it is free of picrotoxinin. In the absence of picrotoxinin, fipronil blocked the non-desensitizing GluCl in a time-dependent manner without affecting the rising phase (Fig. 9D). As a consequence of their mutual exclusion, the fipronil block measured at the 5-s time point was less in the channel previously blocked by picrotoxinin than in the control channel. On average, the fipronil blocks were $59.6 \pm 2.2\%$ ($n = 10$) and $80.0 \pm 2.7\%$ ($n = 6$), respectively, for the picrotoxinin-blocked channel and the control channel ($p < 0.01$) (Fig. 9E).

Discussion

The present study has demonstrated that the desensitizing and non-desensitizing currents produced by glutamate in acutely dissociated cockroach neurons differ not only in their activation and decay phase but also in their sensitivity to picrotoxinin and fipronil. Fipronil was found to block the desensitizing current with an IC_{50} of 801 nM, whereas it more potently blocked the non-desensitizing current, with an IC_{50} of 10 nM.

Two components of glutamate-gated chloride currents have previously been reported in cockroach dorsal unpaired median neurons (Raymond et al., 2000). These two types of currents exhibited different pharmacological profiles in terms of ibotenate and picrotoxin sensitivity. One component was sensitive to picrotoxin and fipronil, but insensitive to ibotenate, while the other was insensitive to 100 μ M picrotoxin, but sensitive to ibotenate. We identified two ibotenate-sensitive GluCl_s in cockroach neurons, only one of which was sensitive to picrotoxin (unpublished observation). The picrotoxin-insensitive current undergoes rapid desensitization while the picrotoxin-sensitive current is non-desensitizing. In the present study, we have further characterized the mechanism of the blocking action of fipronil on both desensitizing and non-desensitizing currents.

Fipronil blocked the desensitizing current in a use-dependent manner. One of the requirements for use-dependent block is a state-dependent change in affinity for the blocker or its access to the binding site. When fipronil was co-applied with glutamate, the rising phase of the current was not altered, but the decaying phase was accelerated, indicative of an open channel blocking action. Once fipronil block had been established, recovery from block did not occur until the channel was opened by activation of the receptor. This result suggests that the fipronil

molecule is trapped in the closed channel. Both open channel block and blocker trapping phenomena are necessary and sufficient to account for the use-dependent block.

The unblocking rate constant of fipronil for the desensitizing current can be estimated from the slow time constant of recovery from fipronil block (Fig. 2Ac). The slow time constant of 5.7 s corresponds to an unblocking rate constant of $1.7 \times 10^{-1} \text{ s}^{-1}$, which, in combination with a K_d of 801 nM, would lead to the blocking rate constant of $2.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. This value for fipronil block of the desensitizing current is similar to that for fipronil block of the non-desensitizing current ($3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$).

Fipronil also blocked the non-desensitizing current in a time- and use-dependent manner. While having no effect on the rising phase of the current, fipronil induced a prominent decay and caused a profound reduction in the glutamate-induced current at the end of a 30-s pulse. The current remained small upon subsequent co-application of fipronil with glutamate, since the blocked channels did not recover during a 2-min interval between fipronil applications.

Although activation profoundly enhanced fipronil block of non-desensitizing GluCl_s with little recovery during a 30 s interpulse interval (Fig. 5), there was substantial recovery from block in the absence of channel opening after 10 min washing (Fig. 8). This observation suggests that the binding site of fipronil in non-desensitizing receptors is not as strongly protected by the activation gate as it is in the desensitizing receptors. Fipronil block of activated non-desensitizing GluCl_s can be estimated from the time-dependent block. The blocking rate constant estimated from the concentration dependence of time constants of fipronil block was $3.04 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and the unblocking rate constant was $5.77 \times 10^{-2} \text{ s}^{-1}$ (Fig. 6). After washing out fipronil, the unblocking rate constant would predict a substantial time-dependent increase in the current during a 30-s activation of the receptor by glutamate, but no such time-dependent recovery was

seen. Thus, the fipronil molecule escapes from the blocked receptor at a rate much slower than predicted from the unblocking rate constant (Fig. 6B). This result suggests that there exists more than one blocked state, and that Y-intercept of the concentration dependence of the on-rate is not simply reflecting the unblocking rate. That is, the affinity of the second blocked state of the channel for fipronil is much higher than that of the first blocked state of the channel. This would also explain the discrepancy between the IC_{50} of 10 nM determined at the steady-state block and the K_d value of 190 nM calculated from the blocking and unblocking rate constants for block of the open channel. However, if one were to use the apparent K_d of 10 nM and the blocking rate constant of $3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ to calculate the unblocking rate constant, the unblocking rate constant would become $3 \times 10^{-3} \text{ s}^{-1}$, which is small enough to explain the negligible increase in the current during a 30-s application of glutamate to activate the receptor after washout of fipronil. In contrast, there was a sizable recovery of the desensitizing current after the application of glutamate (Fig. 2).

It is interesting to note that the non-desensitizing current is more sensitive than the desensitizing current to the blocking action of fipronil and picrotoxinin. This situation is opposite to what has been reported for $GABA_A$ receptors where the desensitized state favors picrotoxin block (Newland and Cull-Candy, 1992; Pribilla et al., 1992; Zhang et al., 1994). An allosteric mechanism was proposed in which picrotoxin binds to the channel lumen inducing a rapid conformational change to the desensitized state (Zhang et al., 1994). According to this allosteric mechanism, the lower sensitivity of the desensitizing GluCl_s to fipronil block can be explained by two mechanisms: First, the channel opens transiently; and second, the blocked channel appears not to undergo desensitization (Fig. 2Ac).

The open channel block by fipronil is also inferred from the experiment in which the interaction of fipronil with picrotoxinin in blocking the non-desensitizing current was analyzed. Picrotoxinin blocked and unblocked the non-desensitizing current when the receptors were activated. The analysis of the kinetics and steady-state block of the picrotoxinin-blocked channel by fipronil suggests that once the channel was blocked by picrotoxinin, fipronil could not bind to the channel until the channel was free of picrotoxinin (Fig. 9C). This interaction could arise from direct competition for the same binding site or from allosteric interactions.

This is the first study in which the potency of fipronil against the non-desensitizing GluCl has been measured and analyzed. The IC_{50} of 10 nM indicates that this target is at least as sensitive as the GABA receptors of the same species (Zhao et al., 2003). In that study, an IC_{50} of 28 nM for fipronil block of GABA receptors was measured, but might be considered an underestimate of its potency because, due to the extremely slow action of fipronil on GABA receptors, the measurements were not made at equilibrium. Thus, GABA receptors and GluCls represent two high-affinity target sites for fipronil in insects.

The existence of two target sites for fipronil could be an important factor limiting the development of resistance. Dieldrin blocks only the GABA receptors in insects (Ikeda et al., 2003; Zhao et al., 2003; Zhao et al., 2004), and is subject to very high levels of target-site resistance, conferred by a point mutation of Ala to Ser in transmembrane domain II of the Rdl ligand-gated chloride channel subunit (French-Constant et al., 1993). Native GABA receptors from insects containing this mutation are highly resistant to dieldrin and picrotoxinin (Zhang, et al., 1994; Cole et al., 1995), as are heterologously-expressed Rdl homomultimers, which are activated by GABA (Buckingham et al., 1996). On the other hand, Rdl is also a component of GluCls in insects (Ludmerer et al., 2002), and although this may be unimportant in terms of

dieldrin resistance, it could play a role in the development of resistance of fipronil, which also potentially blocks GluCl_s. Heterologously-expressed Rdl homomultimers containing the resistance mutation may display reduced sensitivity to fipronil in some cases (Wolff and Wingate, 1998), which, although much less than the resistance to dieldrin, could lead to moderate levels of resistance to fipronil in some species, which is in fact seen (Cole et al., 1995). Studies of the action of fipronil against native GABA and non-desensitizing GluCl_s in insects with the Rdl resistance mechanism are needed to fully understand the moderate resistance to fipronil.

Fipronil block of the GluCl_s of cockroach neurons is of paramount importance from the toxicological point of view. Because GluCl_s are not found in mammals (Cleland, 1996; Raymond and Sattelle, 2002), these receptors are an excellent target for developing insecticides with a high selective toxicity for insects over mammals.

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Notes

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Figure Legends

Fig. 1. Differential actions of fipronil on two types of GluCl currents. Currents in A, B and C were from separate neurons. Glutamate (100 μ M) and fipronil (1 μ M) were co-applied via a U-tube for 8 s, and currents during co-applications and control currents induced by glutamate alone were recorded at 2-min intervals from a holding potential of -60 mV. A, the desensitizing currents was inhibited by 41.3% after the third co-application of fipronil. B, fipronil induced a time-dependent block of the non-desensitizing current, reaching 93.9% block after the third co-application of fipronil. C, the desensitizing and non-desensitizing components in the mixed type current exhibited differential sensitivities to the blocking action of fipronil. The fast component was inhibited by 30.2%, while the slow component was inhibited by 90.5%.

Fig. 2. Reversible blocking action of fipronil on the desensitizing GluCl. A, a representative experiment showing the desensitizing GluCl currents recorded before, during and after bath and U-tube applications of fipronil. B, time course of, and recovery from, fipronil block of desensitizing GluCls. Currents were induced by a 30-s application of 100 μ M glutamate at an interval of 2 min and a holding potential of -60 mV. The current traces labeled a, b, c, and d in Fig. 2A correspond to those labeled in Fig. 2B. In a 12 min period between a and b, fipronil at 1000 nM was applied via a U-tube and bath perfusion after several stable control recordings. In a 10 min period between b and c, little or no recovery was seen when glutamate pulses were not applied even after washing the neuron with drug-free external solutions. A large current was seen in d when a second glutamate pulse was applied. Note that there is an inward slow current

following the peak during the first washout record c. This slow inward current developed with a time constant of 5.7 s.

Fig. 3. Dose-response relationships of fipronil block of the desensitizing and the non-desensitizing GluCl_s. The currents were evoked by 20-s U-tube and bath applications of 100 μ M glutamate and fipronil at a holding potential of -60 mV. The maximal peak current was measured for the desensitizing current and the steady-state current was measured for the non-desensitizing current. The currents were normalized to the respective control currents to calculate the blocking percentages. Each data point was the average value obtained from separate cells. The IC₅₀ of fipronil block of desensitizing currents was 801 ± 207 nM ($n = 3-5$), with a Hill coefficient of 0.89. The IC₅₀ of fipronil block of non-desensitizing currents was 10.0 ± 0.9 nM ($n = 3-10$), with a Hill coefficient of 1.32.

Fig. 4. Use-dependent fipronil block of the non-desensitizing GluCl_s in the activated state. A, a series of mixed-type currents were evoked by 20-s co-applications of 100 μ M glutamate without or with 100 nM fipronil via a U-tube at an interval of 2 min, at a holding potential of -60 mV. B, the slow component currents were derived from subtracting the fast component from the total currents in A. The dotted lines indicate the currents remaining at the end of the control current and of the current induced by the 1st co-application. C, dose-response relationship for fipronil block of the activated non-desensitizing GluCl_s. The current amplitudes were measured at the end of 20 s co-application during the 1st, 2nd, and 3rd pulses of fipronil and glutamate. The fit to the dose-response data gave IC₅₀ values of 27.0 ± 5.5 nM after the first (circles), 17.3 ± 4.2 nM after the second (triangles), and 11.9 ± 3.5 nM after the third (squares) applications ($n = 3-9$).

Fig. 5. Use-dependent block of non-desensitizing GluCl_s by fipronil. Two recording protocols were used: A, non-desensitizing GluCl currents were induced by 2-s applications of 100 μ M glutamate at an interval of 30 s. Fipronil at 100 nM was bath-perfused and co-applied with glutamate for 10 min after several stable control recordings. B, similar protocol to A but no glutamate was applied during 10-min bath perfusion of 100 nM fipronil. A test pulse of glutamate was applied to determine the unblocked GluCl_s. C, after 10-min bath perfusion of 100 nM fipronil, the steady-state currents from protocol A were suppressed to $10.4 \pm 1.4\%$ ($n = 7$) of the control, whereas those from protocol B were $90.6 \pm 2.0\%$ ($n = 6$) of the corresponding control, indicating that activation of the GluCl receptors significantly increases the blocking action of fipronil on non-desensitizing GluCl_s ($p < 0.01$).

Fig. 6. Kinetic analysis of open-channel block of non-desensitizing GluCl receptors by fipronil. A, glutamate (100 μ M) was applied for 20 s to induce currents (control), with co-application of various concentrations of fipronil to assess the kinetics of block of the activated channel. After the initial rapid decay, the slow decay phase of the currents was fitted with a single exponential function to obtain the time constant (τ) of fipronil block of the non-desensitizing current. Each trace was obtained from a different cell. B, the reciprocal of the time constant ($1/\tau$) is plotted as a function of fipronil concentration to calculate the association and dissociation rate constants for fipronil interaction with the activated receptor. Data points are best fitted to the solid line according to the equation $1/\tau = k_{+1}[\text{Fipronil}] + k_{-1}$, where k_{+1} and k_{-1} are the association and dissociation rate constants, respectively, and [Fipronil] is the fipronil concentration. The

correlation coefficient of 0.996 gives a significance level of $p < 0.05$. $k_{+1} = 3.04 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{-1} = 5.77 \times 10^{-2} \text{ s}^{-1}$; the calculated $K_d = 190 \text{ nM}$ ($n = 3-7$).

Fig. 7. Comparison of fipronil block of the non-desensitizing GluCl currents as induced by long co-application of fipronil and glutamate with that induced by repetitive short co-applications. A, mixed-type GluCl currents evoked by 30-s applications of glutamate before (a) and during (b) co-application of 100 nM fipronil. B, GluCl currents evoked by repetitive 2-s applications of 100 μM glutamate at an interval of 30 s in the presence of 100 nM fipronil in both bath and U-tube solutions. The time course of block (expressed as a decrease in a value relative to the control) was plotted in terms of the total time exposed to fipronil and fitted to a single exponential function with a time constant of $83.1 \pm 12.4 \text{ s}$ ($n = 4$). C, comparison of the GluCl blocking actions of fipronil in terms of the duration of receptor activation with the protocols from panels A and B. The fipronil-induced current decay was normalized to the control (trace Ab divided by trace Aa) and is plotted as open circles, which were fitted to a single exponential function with a time constant of $18.0 \pm 3.1 \text{ s}$ ($n = 4$). The filled circles show the summation of 15 of 2-s activations of GluCl in the presence of fipronil (from panel B) and are fitted to a single exponential function with a time constant of $5.1 \pm 1.2 \text{ s}$ ($n = 4$).

Fig. 8. Block and unblock of GluCl by fipronil. Currents were evoked by applications of 100 μM glutamate for 30 s at an interval of 2 min at -60 mV holding potential. A, the control current, and the current remaining after the 3rd co-application of 1 μM fipronil with glutamate. B, the currents recorded after 10-min and 12-min washout of fipronil in the bath with no glutamate stimulations during the first 10-min washout. The fast component fully recovered from fipronil

block 2 min after glutamate stimulation while the slow component recovered only slightly. C, the blocking and unblocking of GluCl_s by co-application of 10 nM fipronil, using the same protocol as A and B. Both fast and slow components recovered after washing with fipronil-free external solutions for 10 min. D, the steady-state non-desensitizing GluCl currents were inhibited to $71.6 \pm 5.2\%$ ($n = 5$) and $5.8 \pm 2.4\%$ ($n = 5$) of the controls during 3rd co-applications of glutamate with 10 nM or 1000 nM fipronil, respectively, and recovered to $93.0 \pm 3.1\%$ ($n = 5$) and $24.9 \pm 4.9\%$ ($n = 5$) of the corresponding controls, respectively, after 10-min washout.

Fig. 9. Interaction of fipronil and picrotoxinin in blocking GluCl currents. The non-desensitizing GluCl currents were evoked by 5 s, 100 μ M glutamate pulses at a holding potential of -60 mV. Aa, a non-desensitizing control GluCl current. Ab and Ac, the currents recorded after the 1st and 2nd U-tube applications of 100 μ M picrotoxinin, respectively, at an interval of 1 min. Ba and Bb, the 1st and 2nd currents recorded 2 min after washing picrotoxinin in bath solution. Ba shows a slow rising phase but reaches the control level at the end of a single 5-s pulse. The 2nd current (Bb) appears normal as compared with Aa. Ca, 100 μ M picrotoxinin was applied again to induce the block. Cb, the current recorded by co-application of 1 μ M fipronil and 100 μ M glutamate 2 min after washing with picrotoxinin-free bath solution. An initial slow rising phase is followed by a slow decay phase. D, the currents evoked by glutamate without (Da) or with (Db) co-application of 1 μ M fipronil without pretreatment of picrotoxinin. Fipronil induced a pronounced decay phase while having no effect on the rising phase of the current. E, comparison of the fipronil block measured at the 5-s time point from the beginning of the non-desensitizing currents in GluCl_s pre-blocked by picrotoxinin (white column) and GluCl_s of the control (black column). The currents were blocked by $80.0 \pm 2.7\%$ ($n = 6$) and $59.6 \pm 2.2\%$ ($n = 10$) by co-

applications of 1 μ M fipronil in the absence or presence of picrotoxinin pretreatment, respectively ($p < 0.01$).

Fig. 1. Zhao et al. (2004)

Fig. 2. Zhao et al. (2004)

Fig. 3. Zhao et al. (2004)

Fig. 4. Zhao et al. (2004)

Fig. 5. Zhao et al. (2004)

Fig. 6. Zhao et al. (2004)

A. Activated receptor block

B. Resting receptor block

C. Time course of block

Fig. 7. Zhao et al. (2004)

Fig. 8. Zhao et al. (2004)

Fig. 9. Zhao et al. (2004)