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Mutations in the extracellular domains of glutamate-gated chloride channel α3 and β subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity

Annete I. Njue,* Jon Hayashi,† Lyle Kinne,† Xiao-Peng Feng* and Roger K. Prichard*

**Institute of Parasitology, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada*

†*FMC Corporation, Neurobiology, Princeton, New Jersey, USA*

Abstract

Two full-length glutamate-gated chloride channel (GluCl) cDNAs, encoding GluClα3 and GluClβ subunits, were cloned from ivermectin-susceptible (IVS) and -resistant (IVR) *Cooperia oncophora* adult worms. The IVS and IVR GluClα3 subunits differ at three amino acid positions, while the IVS and IVR GluClβ subunits differ at two amino acid positions. The aim of this study was to determine whether mutations in the IVR subunits affect agonist sensitivity. The subunits were expressed singly and in combination in *Xenopus laevis* oocytes. Electrophysiological whole-cell voltage-clamp recordings showed that mutations in the IVR GluClα3 caused a modest but significant threefold loss of sensitivity to glutamate, the natural ligand for GluCl receptors. As well, a significant decrease in sensitivity to the anthelmintics ivermectin and moxidectin was observed in the IVR GluClα3 receptor. Mutations in the IVR GluClβ subunit abolished glutamate sensitivity. Co-expressing the IVS GluClα3 and GluClβ subunits resulted in heteromeric channels that were more sensitive to glutamate than the respective homomeric channels, demonstrating co-assembly of the subunits. In contrast, the heteromeric IVR channels were less sensitive to glutamate than the homomeric IVR GluClα3 channels. The heteromeric IVS channels were significantly more sensitive to glutamate than the heteromeric IVR channels. Of the three amino acids distinguishing the IVS and IVR GluClα3 subunits, only one of them, L256F, accounted for the differences in response between the IVS and IVR GluClα3 homomeric channels.

Keywords: anthelmintic resistance, *Cooperia oncophora*, glutamate-gated chloride channel, ivermectin, moxidectin, two-electrode voltage clamp.

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Ivermectin (22,23-dihydroavermectin B_{1a}) and moxidectin are potent endectocides that are widely used to control parasite infections in domestic animals (Campbell 1989; McKellar and Benchaoui 1996). Ivermectin is believed to exert its antiparasitic effects by activating glutamate-gated chloride channels (GluCls; Cully *et al*. 1994, 1996; Dent *et al*. 1997, 2000; Vassilatis *et al*. 1997). These inhibitory receptors are found only in invertebrates (Cleland 1996), and belong to the superfamily of ligand-gated ion channels. The GluCls of *Caenorhabditis elegans* have been studied extensively. Four GluCl subunits, GluClα, GLC-3, avr-14 (GluClα3), and avr-15, form ivermectin-sensitive channels when expressed in *Xenopus* oocytes (Cully *et al*. 1994; Dent *et al*. 1997, 2000; Laughton *et al*. 1997a; Horoszok *et al*. 2001). A fifth subunit, GluClβ, forms glutamate-sensitive channels that do not respond to ivermectin (Cully *et al*. 1994). A GluClα subunit cloned from *Drosophila melanogaster* forms ivermectin-sensitive channels in *Xenopus* oocytes (Cully *et al*. 1996). A strong correlation between ivermectin's nematocidal effect and the potentiation and activation of an ivermectin-sensitive chloride current in *Xenopus* oocytes has been demonstrated (Arena *et al*. 1995). Ivermectin also acts on nematode γ-aminobutyric acid (GABA)-gated chloride channels, and this may also contribute to the antiparasitic effects of macrocyclic endectocides (Holden-Dye and Walker 1990; Feng *et al*. 2002).

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Address correspondence and reprint requests to Roger Prichard, Institute of Parasitology, McGill University, 21 111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9.
E-mail: roger.prichard@mcgill.ca

Abbreviations used: DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; GluCl, glutamate-gated chloride channel; IVR, ivermectin-resistant; IVS, ivermectin-sensitive; LGIC, lignad-gated ion channel; MUT, mutant.

Cooperia oncophora is a nematode that belongs to the superfamily Trichostrogyloidea, and is a common parasite of cattle in temperate climates (Parmentier *et al*. 1995). It is one of the dose-limiting species for ivermectin in cattle (Shoop *et al*. 1995). Ivermectin resistance involving *Cooperia* species has been reported (Vermunt *et al*. 1996; Coles *et al*. 1998, 2001; Anziani *et al*. 2001; Familton *et al*. 2001; Fiel *et al*. 2001), and highlights the need to understand ivermectin's mode of action and mechanisms of resistance. Milbemycins, a class of compounds structurally related to avermectins, also activate GluCls (Arena *et al*. 1995). While some reports suggest that moxidectin, a milbemycin, is effective against ivermectin-resistant nematodes (Craig *et al*. 1992; Pankavich *et al*. 1992), other reports suggest that ivermectin resistance leads to loss of sensitivity to moxidectin (Conder *et al*. 1993; Shoop *et al*. 1993). Moxidectin has been shown to be ineffective against ivermectin-resistant *C. oncophora* (Vermunt *et al*. 1996).

In an attempt to understand the role of GluCls in ivermectin resistance in *C. oncophora*, we have cloned two full-length cDNAs, encoding GluClα3 and β subunits (Njue & Prichard, submitted). These subunits show high homology to related sequences in *C. elegans*; at the amino acid level, *C. oncophora* GluClα3 shares 80% identity with the *C. elegans* avr-14B/GluClα3B, while *C. oncophora* GluClβ shares 76% identity with *C. elegans* GluClβ subunit. Genetic variation analysis of short fragments of the *C. oncophora* GluCl genes in ivermectin-susceptible (IVS) and -resistant (IVR) worms showed the *GluClα3* gene to be polymorphic, and the *GluClβ* gene less so (Njue & Prichard, submitted). Initial isolation of the IVR *C. oncophora* was reported by Coles *et al*. (1998). Here, we report the cloning of full-length GluClα3 and β subunit alleles from IVS and IVR worms. Three non-synonymous mutations were identified in the N-terminal extracellular domains of IVR GluClα3. As well, two mutations were identified in the N-terminal domain of IVR GluClβ. To determine whether mutations in the IVR GluCl subunits affect receptor function, the subunits were expressed in *Xenopus* oocytes and responses examined. We demonstrate that *C. oncophora* GluCls form functional homomeric and heteromeric channels when expressed in *Xenopus* oocytes, and show that mutations in the two GluCl subunits affect agonist sensitivity.

Materials and methods

Generation of full-length cDNAs, and cRNA synthesis

Total RNA extracted from bulk IVS and IVR adult worms using Trizol Reagent™ was reverse-transcribed using an oligo-dT primer (Gibco-BRL, Gaithersburg, MD, USA). This was used as template for PCR amplification of the *C. oncophora* GluClα3 and GluClβ cDNAs. Subunit cDNAs generated were subcloned into the pT7TS vector, which contains 5′-and 3′ untranslated *Xenopus* β-globin regions (Dent *et al*. 1997). The clones were sequenced from both directions using the vector primers T7 and SP6.

The IVS and IVR GluClα3 subunits differ at three amino acid positions (Fig. 1). To determine the effect of each mutation on channel properties, site-directed mutagenesis was used to construct three mutants (MUT 1, 2, and 3), each bearing one of the amino acid substitutions that distinguish IVS and IVR GluClα3 (Table 1). All three mutations required a single base substitution. Site-directed mutagenesis was performed on pT7TS-IVS GluClα3 clones using the QuickChange mutagenesis kit (Stratagene), and the successful incorporation of mutations was confirmed by sequencing.

All cDNA/pT7TS plasmids were linearized using *Xba* 1 immediately downstream of the 3′ untranslated β-globin sequence, and cRNA was transcribed using the T7 mMessage mMachine Kit (Ambion, Austin, TX, USA). Quality and quantity of cRNA were checked by agarose gel electrophoresis and absorption spectroscopy. Samples were stored at − 80°C until use.

Oocyte preparation and injection

Ovarian tissue was isolated from mature female *X. laevis* by a simple surgical procedure. The frogs were anaesthetized by immersion in neutralized ethyl m-aminobenzoate solution (tricaine, methanesulfonate salt, 0.2% solution w/v; Sigma, St Louis, MO, USA). The isolated ovarian lobes were placed in a dish containing OR2 calcium-free buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH adjusted to 7.5 with NaOH) and dissected into smaller clumps of oocytes. These were rinsed several times, and gently shaken in OR2 containing 4 mg/mL collagenase type 1A (Sigma) for 1 h at 17°C. The oocytes were rinsed thoroughly in OR2 and manually defolliculated before being placed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH adjusted to 7.4 with NaOH) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. Then, 9.2 nL of cRNA (∼1 ng/subunit) in RNAse-free water were injected cytoplasmically. To express the heteromeric GluClα3/GluClβ receptors, the two subunit cRNAs were mixed at a ratio of 1 : 1 before injection into oocytes. Injected oocytes were maintained at 17°C in ND96 supplemented with penicillin and streptomycin, and electrophysiological recordings were performed at room temperature (22–25°C) 2–3 days after injection.

Electrophysiological recordings

Whole-cell currents were recorded from oocytes using the two-electrode voltage-clamp technique. Oocytes were voltage-clamped at a holding potential of − 80 mV using Axoclamp 2B voltage clamp amplifier (Axon Instruments, Union City, CA, USA). Recordings were made in ND96. Recording microelectrodes were filled with 3 M potassium acetate, and had tip resistances of 1–3 MΩ. Oocytes were held in a 0.13 mL bath, and continually superfused with ND96 at a rate of 0.8 mL/min. Drugs were applied by local microperfusion using an electronic digital pipette, and the duration of application was 4 s. For the dose–response experiments, recordings were made sequentially from the lowest to the highest drug concentration, with an interval of 2 min between applications. For the glutamate responses, a second application of the same concentration was applied every third response to ensure reproducibility. Glutamate was dissolved in ND96. Ivermectin and moxidectin were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and diluted in ND96.

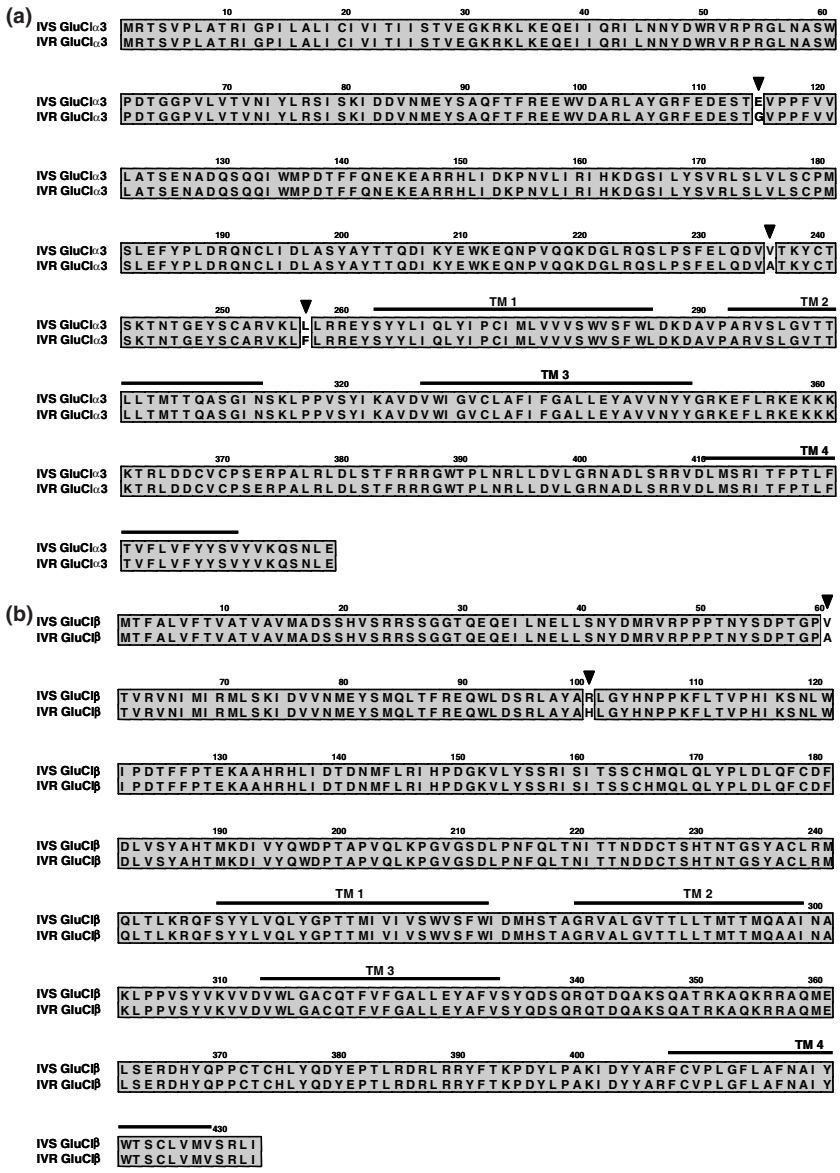

Fig. 1 Comparing the predicted amino acid sequences of the *C. oncophora* GluClα3 (a) and GluClβ (b) subunits from IVS and IVR worms. Conserved residues are shaded grey. The putative transmembrane domains, TMs 1–4, are overlined. (a) The IVS and IVR GluClα3 sequences differ at three amino acid positions (highlighted by ▼), all located in the N-terminal extracellular domain. (b) The IVS and IVR GluClβ sequences differ at two amino acid positions, both found in the N-terminal extracellular domain.

Table 1 *C. oncophora* GluClα3 mutants tested

GluClα3 mutant	Nucleotide mutation	Amino acid change
MUT 1	GAG to GGG	E114G
MUT 2	GTT to GCT	V235A
MUT 3	CTT to TTT	L256F

To determine the effect of each amino acid substitution on channel properties, three mutants, each representing one of the amino acid substitutions, were constructed. The letter preceding the position number refers to the amino acid in the IVS GluClα3 subunit, and the letter following the number refers to the amino acid in the IVR GluClα3 subunit. For each mutant, the remainder of the sequence was identical to that of the IVS GluClα3 subunit.

Data acquisition and analysis were conducted using the pCLAMP suite of programs (Axon Instruments). The current–voltage relationship of the glutamate-induced response was determined over a voltage range of − 80 to +10 mV 250 μM glutamate was first applied at − 80 mV. This was followed by repeated glutamate application at holding potentials of − 40, − 20, 0, and 10 mV. The peak amplitude of the glutamate-induced currents at various potentials was normalized by assigning 100% to the value at − 80 mV. The glutamate dose–response relationships were measured by applying a series of glutamate concentrations to each oocyte. For each oocyte, measurements for the glutamate dose–response curves were normalized by assigning 100% to current activated by saturating concentrations of glutamate in the same oocyte. Because the effects of ivermectin and moxidectin were irreversible, the responses to successive drug administration were taken to be additive (Arena

et al. 1995). The ivermectin and moxidectin responses were normalized to the maximal response elicited with 5 μM ivermectin or moxidectin, respectively. Dose–response data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Dose–response curves were fitted to the equation:

$$I/I_{max} = 1/[1 + (EC_{50}/[D])^{h}],$$

where I_{max} is the maximal response, [D] is the drug concentration, EC_{50} is the concentration of agonist necessary to elicit half the maximum response, and h is the Hill coefficient. Data are given as means ± SE. Statistical analyses were performed using Student's unpaired two-tailed *t*-test, with a significance level of $p < 0.05$.

Drugs

Except for moxidectin, all drugs were purchased from Sigma. Moxidectin was a gift from Fort Dodge Animal Health.

Results

Full-length cDNAs encoding *C. oncophora* GluClα3 and GluClβ subunits were obtained from IVS and IVR worms. The predicted protein sequences were aligned using Clustal W and are shown in Fig. 1. Comparison of the IVS and IVR GluClα3 subunits showed three amino acid differences at positions 114, 235, and 256. The first two mutations (E114G and V235A) were a result of transitional substitutions at the second codon position (Table 1), while the position 256 mutation (L256F) was caused by a substitution at the first codon position. All three mutations were located in the N-terminal extracellular domain. Alignment of the IVS and IVR GluClβ subunits also revealed two mutations (V60A and R101H) in the N-terminal extracellular domain, both resulting from transitional substitutions at the second codon position (Fig. 1b).

cRNAs corresponding to the IVS and IVR GluClα3 and GluClβ subunits were expressed in *Xenopus* oocytes as homomers and heteromers. Initially, 5 ng of cRNA (in 26 nL) were injected into oocytes. This led to a dramatic increase in holding current, which may have been caused by spontaneous opening of channels in the absence of agonist. This was particularly so in oocytes expressing the IVS GluClα3–GluClβ heteromeric channels. As well, this 'leakiness' was more evident when recordings were done three or more days postinjection. As a result, the amount of RNA injected in ooctyes was reduced to ∼1 ng, and recordings were done from as early as 24 h post-injection and continued on day 2 post-injection.

The IVS and IVR GluClα3 subunits were expressed in *Xenopus* oocytes as homomers, and as heteromers with the IVS and IVR GluClβ subunits, respectively. Figure 2 illustrates the responses evoked in homomeric and heteromeric channels by increasing concentrations of glutamate, and the corresponding dose–response relationships. At a holding potential of − 80 mV, glutamate application elicited robust inward currents in oocytes expressing IVS and IVR GluClα3 subunits (Fig. 2a). The responses were dose-dependent, rapid in onset and completely reversible. The normalized dose–response curves for the IVS and IVR GluClα3 homomeric receptors are shown in Fig. 2(b). The presence of three mutations (E114G, V235A, and L246F) in the IVR GluClα3 subunit resulted in a shift of glutamate response curve of the receptor to the right. Glutamate EC_{50} values showed a significant threefold increase from 29.7 ± 4 ($n = 3$) in IVS GluClα3–96.1 ± 4.4 μM ($n = 3$) in IVR GluClα3 (Table 2a). Hill slopes for the two receptors were 2.4 ± 0.21 and 2.4 ± 0.45, respectively, suggesting that binding of more than one glutamate was required to gate the channel.

As with the GluClα3 subunits, the IVS GluClβ subunit also formed functional glutamate-gated homomeric channels (Figs 2a and b). Compared to the robust responses elicited by glutamate in the GluClα3 homomeric channels, the IVS GluClβ channel responses were smaller (Fig. 2a). The mean maximum current for the GluClα3 homomeric receptor was six times that for the GluClβ homomeric receptor. The EC_{50} value for glutamate for the GluClβ receptor was 185.6 ± 24.9 μM ($n = 3$) and the Hill value 2.2 ± 0.02. The IVR GluClβ subunit channel did not respond to any of the glutamate concentrations tested (10 nm to 1 mM).

Glutamate also elicited responses in cells coexpressing IVS GluClα3 and IVS GluClβ subunits (Fig. 2). Relative to the respective homomeric receptors, the dose–response curve of the heteromeric channel shifted to the left. The EC_{50} value was estimated to be 13.4 ± 2.5 μM ($n = 3$), and the Hill slope 1.9 ± 0.28. In contrast, co-expressing the IVR GluClα3 and IVR GluClβ subunits shifted the concentration–response curve to the right, relative to the IVR GluClα3 homomeric receptor, indicating a reduction in glutamate sensitivity. The EC_{50} value obtained for this channel was 171.6 ± 20.7 μM ($n = 3$), manifesting a significant approximately 13-fold decrease in glutamate sensitivity relative to the heteromeric IVS receptor. Uninjected and water-injected oocytes did not show any response to glutamate.

The current–voltage relationship for the glutamate-sensitive current from the IVS GluClα3 homomeric channels showed a slightly outwardly rectifying voltage dependence (Fig. 3). The reversal potential was − 22 ± 1 mV ($n = 3$), close to the Nernst potential for chloride in *Xenopus* oocytes (− 24 mV, with 103.6 mM extracellular chloride and assuming 40 mM intracellular chloride; Dascal 1987).

The effects of ivermectin and moxidectin were also tested on the homomeric channels. Unlike the glutamate response, the responses to ivermectin and moxidectin were slow to activate and irreversible (Fig. 4). The current did not return to baseline even after washing for several minutes with drug-free ND96. Application of 250 μM glutamate and 5 μM moxidectin during maximal response to ivermectin did not elicit additional response. No response to ivermectin or

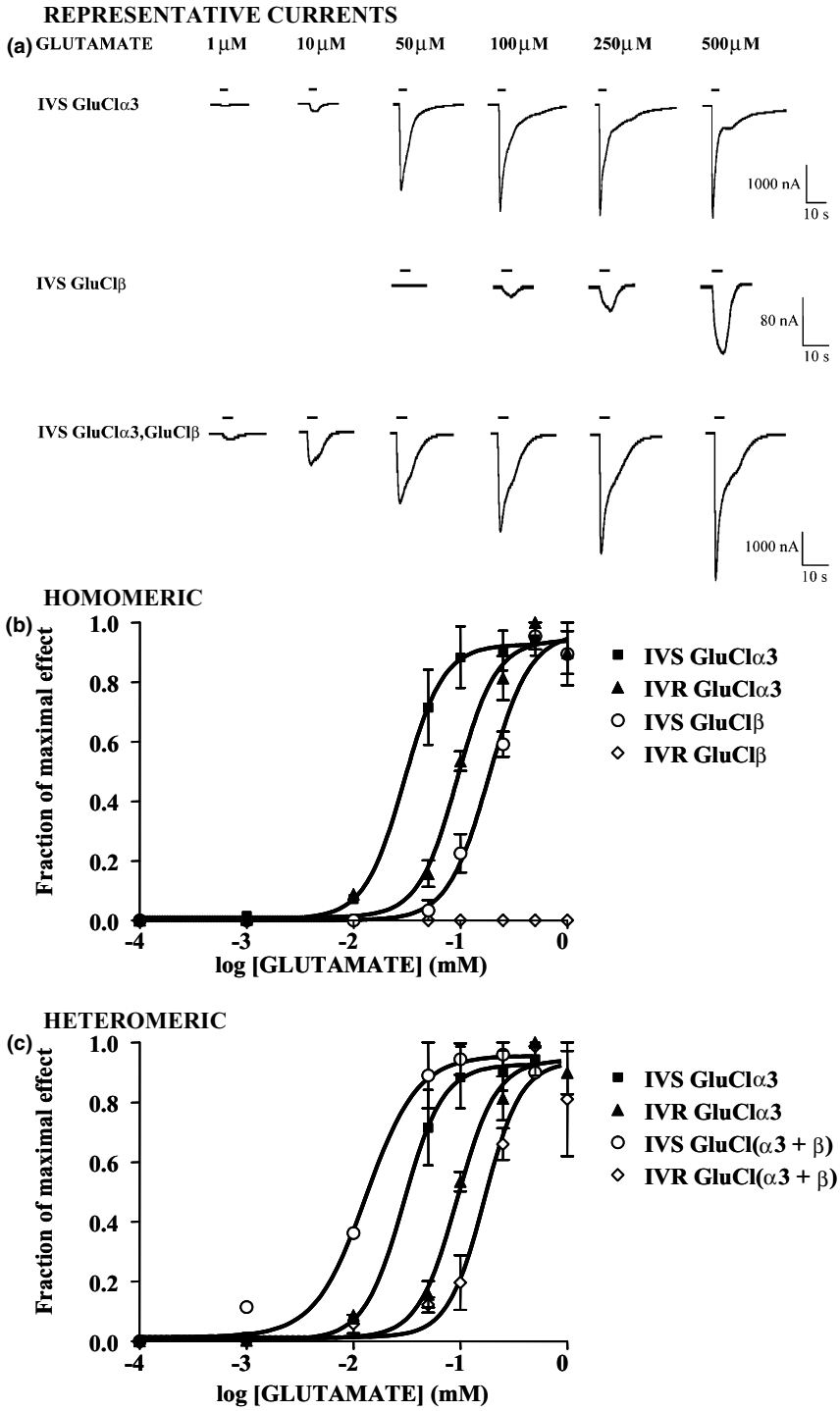

Fig. 2 Activation of IVS GluClα3 and GluClβ receptors by glutamate. IVS GluClα3 and GluClβ subunits form functional glutamate-activated homomeric and heteromeric channels when expressed in *Xenopus* oocytes (a, b). (a) Representative current traces from oocytes activated with glutamate at concentrations shown above each trace. Currents were measured using the whole-cell two-electrode voltage clamp technique, at $V_{hold} = -80$ mV. The GluClβ homomeric channel is less sensitive to glutamate than the GluClα3 homomeric and GluClα3/GluClβ heteromeric channels. (b) Normalized dose–response curves for glutamate-induced currents for oocytes expressing homomeric receptors (same oocytes as in (a) for IVS). EC_{50} values for IVS GluClα3 (■), IVR GluClα3 (▲), and IVS GluClβ (○) homomeric receptors = 29.7, 96.1, and 185.6 μM, respectively. IVR GluClβ (◇) receptor showed no response to glutamate. (c) Glutamate dose–response curves for ooctyes expressing homomeric GluClα3 (same as b) and heteromeric Co GluClα3/GluClβ channels. Compared to the IVS heteromeric channel (○), the IVR heteromeric channel (◇) showed a shift to the right, with an approximately 13-fold increase in EC_{50} value (13.4 μM and 171.6 μM for the IVS and IVR heteromeric channels, respectively).

moxidectin was observed in uninjected or water-injected oocytes. Oocytes expressing IVS GluClα3 homomeric channels displayed an ivermectin dose–response relation with an EC50 of 0.5 ± 0.12 μM ($n = 3$) and a Hill slope of 2.1 ± 0.5 (Table 2b). As with the glutamate response, the ivermectin response of the IVR GluClα3 receptor was shifted to the right relative to the IVS GluClα3 receptor, with a significant increase in the ivermectin EC_{50} of about threefold ($EC_{50} = 1.3$ μM ± 0.11, Hill slope 2.2 ± 0.18, $n = 3$). In contrast, the IVS GluClα3 moxidectin dose–response curve was shifted to the left relative to the IVS GluClα3 ivermectin curve (Fig. 4). The EC_{50} was estimated to be 0.2 ± 0.06 μM ($n = 3$), and the Hill slope 1.6 ± 0.36. A similar trend was seen when the IVR GluClα3 receptor dose–response curves

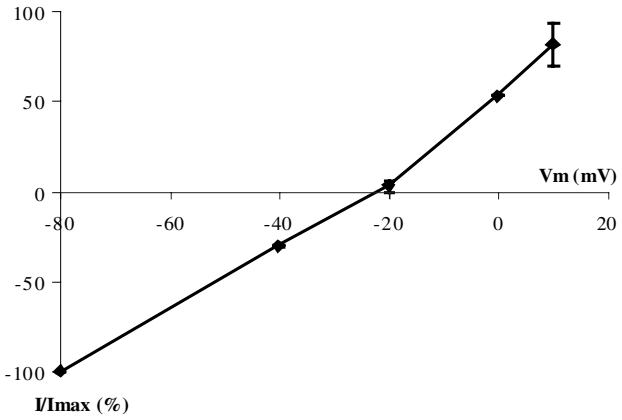

Fig. 3 Current–voltage (I/V) relationship for the glutamate-gated currents of the IVS GluClα3 receptor was tested at potentials ranging from −80 mV to +10 mV. Current amplitude were normalized by assigning 100% to the glutamate-induced current recorded at −80 mV. The reversal potential was −22 ± 1 mV ($n = 3$ ooctyes).

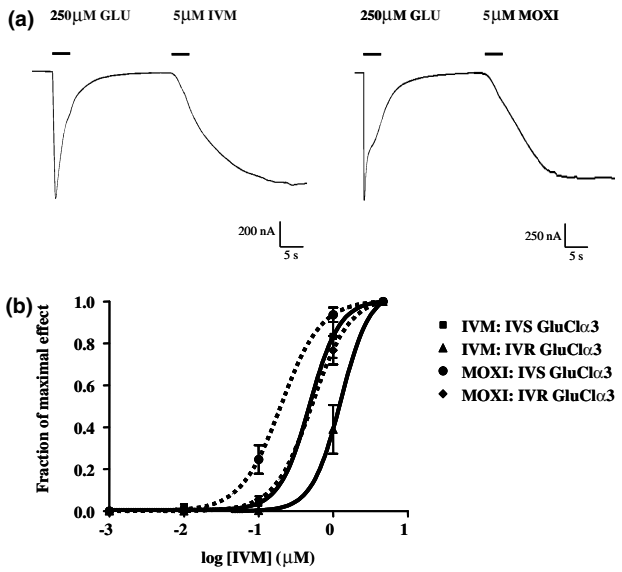

Fig. 4 Activation of IVS and IVR GluClα3 receptors by ivermectin (IVM) and moxidectin (MOXI; a, b). Evidence that ivermectin and moxidectin are agonists of *C. oncophora* GluClα3 receptors. (a) Currents induced by glutamate, ivermectin and moxidectin, applied at the concentrations indicated. While the glutamate response is rapid in activation of the membrane current and completely reversible, the ivermectin and moxidectin responses were slow in activation and irreversible. (b) Normalized dose–response curves for ivermectin and moxidectin. The EC_{50} values for ivermectin for IVS (■) and IVR (▲) GluClα3 receptors = 0.5 and 1.3 μM, respectively. The EC_{50} values for moxidectin for IVS (●) and IVR (◆) GluClα3 receptors = 0.2 and 0.5 μM, respectively.

for ivermectin and moxidectin were compared. The IVR GluClα3 moxidectin curve had an EC_{50} value similar to that of the IVS GluClα3 ivermectin curve (0.5 ± 0.05 μM, $n = 3$ and 0.5 ± 0.12 μM, $n = 3$, respectively). Relative to the IVS GluClα3 moxidectin curve, IVR GluClα3 also showed a significant decrease in moxidectin EC_{50} of approximately 2.6-fold. No ivermectin- or moxidectin-activated chloride currents were observed in oocytes expressing GluClβ homomeric channels.

Table 2 Summary of effects of (a) glutamate and (b) ivermectin (IVM) and moxidectin (MOXI) on IVS, IVR and mutant GluCl receptors

Receptor	EC_{50} (μM)	Hill slope	Ooctyes (*n*)
(a) Glutamate responses			
IVS GluClα3	29.7 ± 4	2.4 ± 0.21	3
IVR GluClα3	96.1 ± 4.4	2.4 ± 0.45	3
IVS GluClβ	185.6 ± 24.9	2.2 ± 0.02	3
IVR GluClβ	–	–	3
IVS GluCl(α3 + β)	13.4 ± 2.5	1.9 ± 0.28	3
IVR GluCl(α3 + β)	171.6 ± 20.7	2.5 ± 0.28	3
MUT 1 (E114G)	25.7 ± 5.2	1.8 ± 0.21	3
MUT 2 (V235A)	27.8 ± 2.6	2.2 ± 0.27	3
MUT 3 (L256F)	100.6 ± 27	1.5 ± 0.35	3
(b) IVM and MOXI responses			
IVM- IVS GluClα3	0.5 ± 0.12	2.1 ± 0.5	3
IVM- IVR GluClα3	1.3 ± 0.11	2.2 ± 0.18	3
MOXI- IVS GluClα3	0.2 ± 0.06	1.6 ± 0.36	3
MOXI- IVR GluClα3	0.5 ± 0.05	1.7 ± 0.22	3
IVM- MUT 1 (E114G)	0.6 ± 0.18	2.3 ± 0.3	2
IVM- MUT 2 (V235A)	0.4 ± 0.01	2.2 ± 0.33	3
IVM- MUT 3 (L256F)	1.2 ± 0.11	1.9 ± 0.16	2

EC_{50} and Hill slope values are expressed as means ± SEM. To express heteromeric channels, cRNAs for the GluClα3 and β subunits were injected in a 1 : 1 ratio.

In an attempt to determine whether one, two or all three mutations in the IVR GluClα3 subunit contributed to altered response to glutamate and ivermectin, the mutants, each representing one mutation, were expressed individually in *Xenopus* oocytes. Figure 5(a) compares the glutamate dose–responses of the mutants with those of the IVS and IVR GluClα3 homomeric channels. Mut 1 (E114G) and Mut 2 (V235A) both showed dose–response curves very similar to those of the IVS GluClα3 receptor, with EC_{50} values of 25.7 ± 5.2 ($n = 2$) and 27.8 ± 2.6 ($n = 3$) μM for Mut 1 and Mut 2, respectively. In contrast, the glutamate dose–response curve and EC_{50} value (100.6 μM ± 27, $n = 2$) of the Mut 3 (L256F) receptor were very similar to those of the IVR GluClα3 receptor. A similar trend was observed with the ivermectin dose–response curves (Fig. 5b). EC_{50} values for the Mut 1, Mut 2, and Mut 3 ivermectin curves were 0.6 ± 0.18, 0.4 ± 0.01, and 1.2 ± 0.11 μM, respectively.

The pharmacological properties of the homomeric channels were also examined. Ibotenate, a structural analog of glutamate known to activate GluCls (Lea and Usherwood 1973; Arena *et al*. 1992; Cully *et al*. 1994), elicited responses in IVS GluClα3, and IVS GluClβ homomeric channels (Fig. 6,

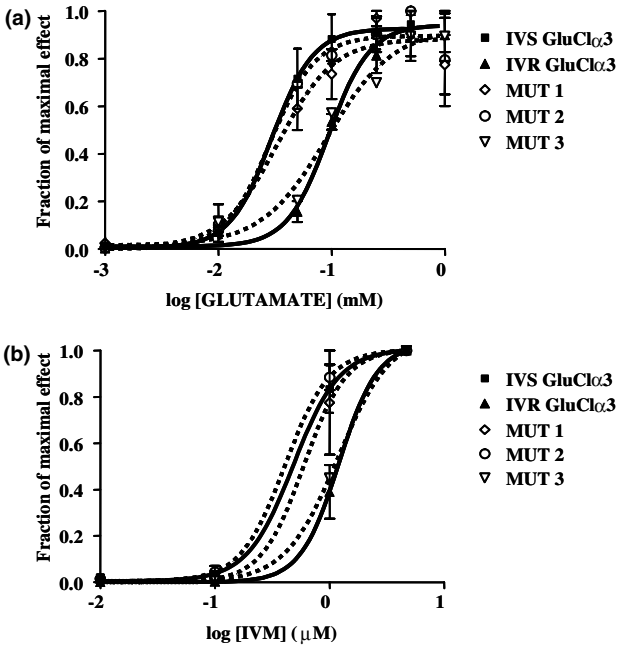

Fig. 5 Normalized dose–response curves of mutant GluClα3 receptors for glutamate and ivermectin (a, b). (a) The EC_{50} values for glutamate for Mut 1 (E114G, ◇), Mut 2 (V235A, ○), and Mut 3 (L256F, ▽) GluClα3 receptors = 25.7, 27.8, and 100.6 μM, respectively. (b) The EC_{50} values for ivermectin for Mut 1 (◇), Mut 2 (○), and Mut 3 (▽) receptors = 0.6, 0.4, and 1.2 μM, respectively.

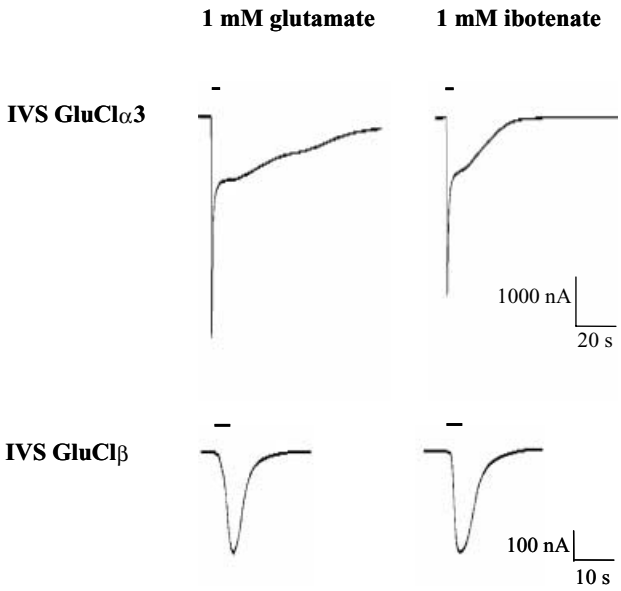

Fig. 6 Ibotenate activated IVS GluClα3 and IVS GluClβ homomeric channels. Currents activated by glutamate and ibotenate at concentrations shown above each current plot. The kinetics of the glutamate and ibotenate responses are similar in both channels.

Table 3 Pharmacology of Co GluClα3 and GluClβ homomeric channels

Agonist (ligand)	IVS GluClα3 channel response (nA)	IVS GluClβ channel response (nA)
250 μM Glutamate	− 5936 ± 409	− 311 ± 11
1 mM Ibotenate	− 4785 ± 362	− 310 ± 9
1 mM Aspartate	0	0
1 mM D-Glutamate	0	0
500 μM Quisqualate	0	0
500 μM Kainate	0	0
1 mM Glycine	0	0
1 mM GABA	0	0
1 mM NMDA	0	0

For each ligand, at least three oocytes were tested.

Table 3). For both channels, the kinetics of the glutamate and ibotenate responses were similar. While 1 mM ibotenate maximally activated the IVS GluClβ channel, the response of the IVS GluClα3 channel was 80 ± 6.1% of the response to 1 mM glutamate. Apart from ibotenate, no other agonist activated the IVS GluClα3 and IVS GluClβ homomeric channels (Table 2). The glutamate analogs D-glutamate, quisqualate, kainate and NMDA were inactive. As well, 1 mM concentrations of glycine and GABA failed to activate current.

Discussion

GluCls are members of the ligand-gated ion channel (LGIC) superfamily which includes the cationic channels activated by acetylcholine and serotonin, as well as anionic channels activated by GABA, glycine, histamine, and serotonin (Ortells and Lunt 1995; Ranganathan *et al*. 2000; Zheng *et al*. 2002). GluCls are found only in invertebrates (Cleland 1996), and are therefore ideal drug targets. Ivermectin is a widely used anthelmintic which is known to interact with GluCls (Cully *et al*. 1994, 1996; Arena *et al*. 1995; Dent *et al*. 1997, 2000; Vassilatis *et al*. 1997). This interaction is thought to mediate at least part of its anthelmintic activity, since the ability of ivermectin to activate GluCls expressed in *Xenopus* oocytes correlates well with its biological activity (Arena *et al*. 1995).

In this study, we describe the function of two GluCl subunits, GluClα3 and GluClβ, from *C. oncophora*. *C. oncophora* GluClα3 subunit is a homolog of *C. elegans* avr-14B/GluClα3B (Dent *et al*. 2000) and *H. contortus* gbr-2B/GluClα3B (Jagannathan *et al*. 1999). *C. elegans* and *H. contortus GluClα3* genes are alternatively spliced to yield two subunits, GluClα3A and -3B (Jagannathan *et al*. 1999; Dent *et al*. 2000). While we did not clone a GluClα3A homolog from *C. oncophora*, it is likely that, given the close phylogenetic relationship among these three nematodes, the *C. oncophora GluClα3* gene may also be alternatively spliced. Initial amplification of the full-length *C. oncophora*

GluClα3 cDNA yielded two products (data not shown), and the second less abundant product, which most likely represents the GluClα3A, was not cloned. While *H. contortus* GluClα3B subunit binds ivermectin with high affinity, the 3A subunit does not (Cheeseman *et al*. 2001). The *C. elegans* avr14A/GluClα3A subunit, which is homologous to *H. contortus* GluClα3A, does not respond to glutamate or ivermectin when expressed in *Xenopus* oocytes (Dent *et al*. 2000). Like *C. elegans* GluClα3B, *C. oncophora* GluClα3 forms ivermectin-sensitive glutamate-gated channels when expressed in *Xenopus* oocytes. Other *C. elegans* GluCls that are activated by glutamate and ivermectin include glc-3 (Horoszok *et al*. 2001), GluClα2B, and GluClα2B (Dent *et al*. 1997). As well, a GluClα subunit also exists in *C. elegans* that forms a homomeric channel gated by ivermectin but not glutamate (Cully *et al*. 1994). The second *C. oncophora* subunit, GluClβ, is homologous to the *C. elegans* (Cully *et al*. 1994) and *H. contortus* (Delany *et al*. 1998) GluClβ subunits. Unlike *C. elegans* avr-14B/GluClα3B and *C. oncophora* GluClα3 receptors, homomeric channels formed by *C. oncophora* IVS GluClβ and *C. elegans* GluClβ are activated by glutamate, but not ivermectin.

C. oncophora GluClα3 and GluClβ subunits cloned from IVS and IVR worms show amino acid differences. Mutations in both subunits were found in the N-terminal extracellular domain, which carries the ligand binding site (Etter *et al*. 1996). When expressed in *Xenopus* oocytes, IVS and IVR GluClα3 subunits both formed ivermectin- and moxidectin-sensitive glutamate-gated channels. Mutations in the IVR GluClα3 subunit caused a modest but significant threefold decrease in sensitivity to glutamate, and significant ~ 2.5-fold reductions in sensitivity to ivermectin and moxidectin. Mutations in the IVR GluClβ subunit abolished responsiveness to glutamate. Mutations in genes encoding LGIC receptor subunits have been shown to confer drug resistance. A single A302S mutation in the *Drosophila melanogaster Rdl* gene, which encodes a GABA receptor subunit, confers resistance to cyclodiene insecticides (Ffrench-Constant *et al*. 1998). As well, a proline to serine mutation in the gene coding for the *D. melanogaster* GluClα subunit causes a significant reduction in sensitivity to ivermectin and nodulisporic acid (Kane *et al*. 2000). In *C. elegans*, mutations of *lev-1,* a gene which encodes a non-α nicotinic acetylcholine receptor (nAchR) subunit, causes a reduction in sensitivity to levamisole, even though this subunit only forms a levamisole-sensitive channel in the presence of other subunits (Fleming *et al*. 1997). Also in *C. elegans*, mutations in two of three GluCl genes, *avr-14*, *glc-1* or *avr-15*, confer modest or no resistance to ivermectin, while mutations in all three genes confer high-level (4000-fold) resistance to ivermectin (Dent *et al*. 2000).

To determine whether all three mutations in the IVR GluClα3 contribute to loss of glutamate and ivermectin sensitivity, each mutation was introduced singly to the IVS GluClα3 using site-directed mutagenesis. The responses of Mut 1 (E114G) and Mut 2 (V235A) receptors were similar to those of the IVS GluClα3 receptor, suggesting that the mutations at these positions had no influence on ligand binding. In contrast, Mut 3 glutamate and ivermectin dose–response curves were similar to the IVR GluClα3 receptor responses, suggesting that the L256F mutation accounted for the difference between IVS and IVR GluClα3 channels in response to these two ligands. Whether this amino acid contributes directly to ligand binding, or whether it causes conformational change that influences ligand binding, is not known, and requires further characterization. The L256F mutation does not map onto any of the six domains of the nAchR subunits that form the acetylcholine binding site (Corringer *et al*. 1995, 2000; Brejc *et al*. 2001). That this mutation is not found in critical regions may explain the modest loss of agonist sensitivity observed. In *D. melanogaster*, a single mutation in the TM2 region of the *rdl* gene that codes for a GABA-gated channel confers high-level resistance to the insecticide dieldrin (Ffrench-Constant *et al*. 1993). Also in *D. melanogaster*, a single mutation in the M2-M3 linker region of DmGluClα gene confers over 20-fold resistance to the novel insecticide and acaricide, nodulisporic acid (Kane *et al*. 2000).

In oocytes expressing IVR GluClβ, no glutamate-activated currents were detected. This could be due to failure of the receptor to assemble on the cell surface. However, this seems unlikely, as co-expression of this subunit with the IVR GluClα3 resulted in the formation of receptors that were less sensitive to glutamate than the homomeric IVR GluClα3 receptors, suggesting coassembly of the two subunits. It is possible that the mutations in IVR GluClβ may have altered the binding site, preventing glutamate from binding. Heteromeric channels formed by coexpressing IVS GluClβ with IVS GluClα3 (Fig. 2c) or IVR GluClα3 (data not shown) were more sensitive to glutamate than the homomeric IVR GluClα3 channels. This reflects the contribution of a functional non-mutant GluClβ subunit to the glutamate sensitivity of the heteromeric channel. It is likely that, *in vivo*, the function of the IVR GluClβ subunit may be replaced by other non-mutant subunits. In *C. elegans*, avr-14/GluClα3, which is expressed in extrapharyngeal neurons, can functionally replace avr-15 and inhibit pharyngeal pumping in *avr-15* mutants (Dent *et al*. 2000).

Of the two amino acid positions substituted in IVR GluClβ, the position 60 residue is highly conserved, being occupied by a Val in all GluCls, except for *D. melanogaster.* While the residue at position 100 is Arg in most GluCls, this position is more variable. An attempt to rescue the function of IVR GluClβ by mutating A60V was unsuccessful. It would appear therefore that the R100H mutation may be important in determining the function of the GluClβ subunit. Whether the R100H mutation alone can abolish agonist sensitivity, or whether both mutations are required is not known, and requires further characterization. Interestingly, in

H. contortus GluClβ, the residue at position 100, Arg, corresponds to the residue identified in our mutant non-functional subunit. Whether the *H. contortus* GluClβ subunit forms a functional receptor in *Xenopus* oocytes is not known.

Heteromeric channels formed after coinjection of IVS GluClα3 and GluClβ cRNA showed a higher sensitivity to glutamate than either of the homomeric channels, demonstrating the co-assembly of the two subunits. Interestingly, the IVR GluClα3/IVR GluClβ heteromeric channels were approximately 13-fold less sensitive to glutamate than the IVS GluClα3/IVS GluClβ heteromeric channels. Whether the GluClα3 and GluClβ subunits assemble to form native receptors in the nematode is not known. LGIC receptors exist in their native form as heteromeric pentamers composed of two to four different yet closely related subunit types (Unwin 1993; Bechade *et al*. 1994; Macdonald and Olsen 1994; Chang *et al*. 1996). As members of the LGIC superfamily, the native GluCls are thought to exhibit the same heteropentameric structure. However, the exact composition of native GluCl receptors is not known. In *C. elegans*, the GluClβ subunit is expressed solely in the pharynx (Laughton *et al*. 1997b), while avr-14/GluClα3 is expressed in extrapharyngeal neurons (Dent *et al*. 2000), and it is therefore unlikely that these two subunits co-assemble to form a receptor. avr-15/GluClα2 is expressed in the pharynx, and the native pharyngeal receptor may be composed of avr-15 and GluClβ subunits (Dent *et al*. 1997; Pemberton *et al*. 2001). As well, other glutamate-sensitive, ivermectin-insensitive GluCl subunits may contribute to formation of the pharyngeal receptor complex (Pemberton *et al*. 2001). In *H. contortus*, GluClα and β co-localize in motor neuron commisures, and most likely contribute to formation of the GluCl receptor at this site (Delany *et al*. 1998; Portillo *et al*. 2003). The splice variants of the *H. contortus gbr-2/GluClα3* gene, GluClα3A and 3B, are also expressed in motor neurons (Jagannathan *et al*. 1999; Portillo *et al*. 2003). Whether or not these two splice variants co-localize with GluClα and -β subunits has not been confirmed (Portillo *et al*. 2003). *H. contortus* GluClα3B subunit, which is homologous to *C. oncophora* GluClα3, appears to be expressed in pharyngeal neurons (Portillo *et al*. 2003), and may therefore mediate ivermectin's inhibitory action on pharyngeal pumping (Geary *et al*. 1993). The complexity of native receptors is highlighted by a recent report that suggests co-assembly of a GluClα subunit and the GABA-gated chloride channel Rdl subunit in some receptors in *Drosophila* head membranes (Ludmerer *et al*. 2002).

Both IVS and IVR GluClα3 homomeric receptors were sensitive to ivermectin and moxidectin. Application of saturating concentrations of glutamate and moxidectin during maximal response to ivermectin caused no further change in membrane potential. Similarly, glutamate and ivermectin did not elicit any additional current during maximal response to moxidectin, suggesting that glutamate, ivermectin, and moxidectin activate the same channel. Heteromeric channels formed by IVS GluClα3 and β subunits gave a dose–response curve to ivermectin with an EC_{50} of 0.5 μM (data not shown), suggesting that the presence of the β subunit does not affect ivermectin sensitivity. Similar results have been demonstrated with *C. elegans* GluCl subunits, where expression of *C. elegans* GluClα2 together with *C. elegans* GluClβ had no apparent effect on the ivermectin dose–response curve (Vassilatis *et al*. 1997).

Our results show that the moxidectin dose–response curves are left-shifted relative to the ivermectin dose–response curve, indicating a higher sensitivity to moxidectin than ivermectin. However, as for glutamate, ivermectin and moxidectin dose–response curves for the IVR GluClα3 homomeric channel are right-shifted relative to the respective IVS GluClα3 channel curves, with significant increases in EC_{50} values. Interestingly, the moxidectin dose–response curve of the IVR GluClα3 channel was comparable to that of the IVS GluClα3 channel response to ivermectin. These findings suggest that while loss of sensitivity to ivermectin also results in a loss of moxidectin sensitivity, the efficacy of moxidectin in IVR is still comparable to the efficacy of ivermectin in IVS. In a dose-titration study, Ranjan *et al*. (2002) showed that in *H. contortus*, resistance to ivermectin results in resistance to moxidectin, and *vice-versa*, though moxidectin resistance develops more slowly. Consequently, ivermectin-resistant parasites may demonstrate sensitivity to the use-level of moxidectin.

In conclusion, we have shown that *C. oncophora* GluCl subunits form functional homomeric and heteromeric receptors when expressed in *Xenopus* oocytes. Mutation in GluClα3 subunit causes a modest loss of agonist sensitivity, while mutation in the GluClβ abolishes agonist sensitivity. The IVR worms used in this study are known to be resistant to ivermectin (Coles *et al*. 1998). In *C. elegans*, simultaneous mutations in three GluCl genes are required for high level resistance to manifest (Dent *et al*. 2000). It remains to be seen what changes occur in other *C. oncophora* GluCls, as well as other ivermectin targets such as the GABA receptor, that may enhance the effect of the L256F mutation, and help explain the mechanisms of ivermectin resistance.

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