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GLC-3: a novel fipronil and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans*

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1 We report the cloning and expression of a novel *Caenorhabditis elegans* polypeptide, GLC-3, with high sequence identity to previously cloned L-glutamate-gated chloride channel subunits from nematodes and insects.

2 Expression of *glc-3* cRNA in *Xenopus* oocytes resulted in the formation of homo-oligomeric L-glutamate-gated chloride channels with robust and rapidly desensitizing currents, an EC_{50} of 1.9 ± 0.03 mM and a Hill coefficient of 1.5 ± 0.1. GABA, glycine, histamine and NMDA all failed to activate the GLC-3 homo-oligomer at concentrations of 1 mM. The anthelminthic, ivermectin, directly and irreversibly activated the L-glutamate-gated channel with an EC_{50} of 0.4 ± 0.02 μM.

3 The GLC-3 channels were selective for chloride ions, as shown by the shift in the reversal potential for L-glutamate-gated currents after the reduction of external Cl^- from 107.6 to 62.5 mM.

4 Picrotoxinin failed to inhibit L-glutamate agonist responses at concentrations up to 1 mM. The polycyclic dinitrile, 3,3-bis-trifluoromethyl-bicyclo[2,2,1]heptane-2,2-dicarbonitrile (BIDN), completely blocked L-glutamate-induced chloride currents recorded from oocytes expressing GLC-3 with an IC_{50} of 0.2 ± 0.07 μM. The phenylpyrazole insecticide, fipronil, reversibly inhibited L-glutamate-gated currents recorded from the GLC-3 receptor with an IC_{50} of 11.5 ± 0.11 μM.

5 In this study, we detail the unusual antagonist pharmacology of a new GluCl subunit from *C. elegans*. Unlike all other native and recombinant nematode GluCl reported to date, the GLC-3 receptor is insensitive to picrotoxinin, but is sensitive to two other channel blockers, BIDN and fipronil. Further study of this receptor may provide insights into the molecular basis of non-competitive antagonism by these compounds.

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Abbreviations: BIDN, 3,3-bis-trifluoromethyl-bicyclo[2,2,1]heptane-2,2-dicarbonitrile; DMSO, dimethyl sulphoxide; EBOB, 1-(4-ethynylphenyl)-4-[2,3H_2]propyl-2,6,7-trioxabicyclo[2,2,2]octane; Fip, fipronil; GluCl, glutamate-gated chloride channel; HisCl, histamine-gated chloride channel; PKC, protein kinase C; PTX, picrotoxinin; SL, spliced leader; SOS, standard oocyte saline; TBPS, *t*-butylbicyclophosphorothionate

Introduction

The completion of the *Caenorhabditis elegans* genome sequence (*C. elegans* Sequencing Consortium, 1998) has permitted the first prediction of the total number of ionotropic receptor subunits present within an organism. The sequence predicts that the nematode genome encodes about 90 such subunits (Bargmann, 1998), including 37 ligand-gated anion channel subunits, most of which are of unknown function. The L-glutamate-gated chloride channels (GluCls) are an important group of ionotropic receptors, to date found only on invertebrate nerve and muscle cells (reviewed by Cleland, 1996). The GluCls are closely related to receptors for GABA and glycine that gate chloride channels and are presumed to possess a similar pentameric structure. The *C. elegans* GluCl subunits cloned to date share about 40% identity with glycine receptor subunits and 30–35% identity with mammalian $GABA_A$ and $GABA_C$ receptor subunits at the amino-acid level. There is strong evidence that the GluCls are targeted by the avermectin/milbemycin family of endectocides and insecticides (Arena *et al*., 1992; 1995; Cully *et al*., 1994; 1996; Dent *et al*., 2000), though it is not known whether all GluCls are sensitive to these compounds. The invertebrate GABA-gated chloride channels are also targeted by insecticides such as the phenylpyrazole, fipronil, providing further evidence of the importance of the ligand-gated anion channel superfamily in pest control.

Many recombinant GluCls described to date are blocked by the convulsant picrotoxinin (PTX), a plant derived toxin that blocks vertebrate and invertebrate native and recombinant GABA- and glycine-gated anion channels (Cull-Candy, 1976; Olsen & Tobin, 1990; Pribilla *et al*., 1992; Hosie *et al*., 1997). A number of convulsants also act at insect GABA-gated anion channels, including PTX, the picrodendrins (Hosie *et al*., 1996), the bicyclic dinitrile BIDN (Rauh *et al*., 1997; Hamon *et al*., 1998) as well as fipronil (Cole *et al*., 1993; Hosie *et al*., 1995). The antagonist activity of these

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compounds is thought to target the convulsant binding sites of the *Drosophila melanogaster* GABA-gated chloride channel subunit, RDL (Shirai *et al*., 1995; Hosie *et al*., 1997). Fipronil, PTX and BIDN antagonism of RDL are all affected by a mutation (A302S) in the channel-lining second transmembrane region, M2, (Hosie *et al*., 1995; Shirai *et al*., 1995) that makes the channel resistant to picrotoxinin and dieldrin (ffrench-Constant *et al*., 1993; Hosie *et al*., 1995). An equivalent mutation of the *C. elegans* GluClβ subunit (A279T) reduces the sensitivity of this receptor to PTX by 10,000 fold (Etter *et al*., 1999). BIDN and fipronil have not so far been tested on a nematode ligand-gated anion channel.

Searches of the *C. elegans* genome sequence revealed a predicted polypeptide, ZC317.3, with a high amino-acid identity to the cloned GluCl subunits. We report here the amplification and expression of the cDNA encoding this polypeptide together with an agonist and antagonist profile of the expressed receptor including the convulsant $GABA_A$ antagonists, BIDN and fipronil. Based on the sequence of the polypeptide and functional expression data we conclude that this gene, designated *glc-3*, encodes a further GluCl subunit from *C. elegans*.

Methods

Cloning of ZC317.3 cDNA

The N2 strain of *C. elegans* was maintained on the OP50 strain of *E. coli* in Petri dishes. RNA was extracted from mixed stage worms using the Trizole™ reagent (Life Technologies, Paisley, U.K.) and cDNA synthesized from total RNA using oligo-dT_{17} (in the initial experiments) or random hexamers (for full-length amplifications) as primers. Reagents used were from the Superscript system (Life Technologies). Amplification of the full-length ZC317.3 cDNA was carried out in a 50 μl reaction volume using specific oligonucleotide primers corresponding to the 5′ ($^{5'}$CTTGATGAGTCTCCGTTCACTTC$^{3'}$) and 3′ ($^{5'}$CAATTTCATTTGGCTTCCGGTGCG$^{3'}$) ends of the predicted gene and 2.6 units of Expand™ High Fidelity DNA polymerase (Boehringer, Lewes, U.K.). The PCR amplification consisted of a 2-min 'Hot Start' at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, an annealing temperature of 55°C for 30 s and an extension time of 30 s at 72°C with a final extension step at 72°C for 5 min. The cDNA was subcloned into pGEM®-T Easy (Promega, Southampton, U.K.) for cRNA synthesis. DNA sequencing reactions were carried out by DNASHEF, Edinburgh, U.K. and analysed using the GCG package mounted on a Silicon Graphics Unix workstation.

Preparation of synthetic cRNA for oocyte injection

The recombinant plasmid containing the full-length *glc-3* cDNA was linearised at the 3′ end of the insert. The linearised plasmid was diluted to a final concentration of 1 $\mu g\ \mu l^{-1}$ with RNAse free water. 15–35 μg of 7-methylguanosine capped cRNA was synthesized from 1 μg of DNA template using T7 RNA polymerase and a capped RNA transcription kit (mMessage mMachine, Ambion, Austin, TX, U.S.A.).

Oocyte preparation and injection

Ovarian tissue was surgically extracted from *Xenopus laevis* females anaesthetized using 0.2% (w v^{-1}) tricaine (3-aminobenzoic acid ethyl ester, methane sulphonate salt), (Sigma Chemical Co., St Louis, U.S.A.), and the isolated ovarian lobes dissected into small clumps each containing approximately 50 oocytes. The follicular layers were defolliculated manually from the oocytes following a 5–10 min incubation with collagenase (Sigma type1A, 2 mg ml^{-1}) in a calcium-free version of standard oocyte saline (SOS); normal SOS is (mM) NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.6). Reduced [Cl^-] SOS contained 54.9 mM NaCl and 45.1 mM sodium gluconate instead of 100 mM NaCl; all other constituents were the same as normal SOS. Oocytes were then transferred to SOS supplemented with 2.5 mM sodium pyruvate, streptomycin (100 $\mu g\ ml^{-1}$), penicillin (100 units ml^{-1}) and gentamycin (50 $\mu g\ ml^{-1}$). All antibiotics were from Sigma.

Each oocyte was injected cytoplasmically (Drummond 'Nanoject' microinjector, Broomhall, PA, U.S.A.) with 50 nl of capped cRNA (1 ng nl^{-1}). Injected oocytes were placed at 17°C and allowed to incubate for 1–6 days with replacement of saline at 24 h intervals. The data were obtained from oocytes incubated for 3–6 days after injection.

Electrophysiological recordings

Membrane current measurements were made using a two-electrode voltage-clamp amplifier OC-725C (Warner, Warner Instrument Corp, U.S.A.). Microelectrodes were pulled from borosilicate glass capillaries (Clark Electromedical, U.K.) on a microelectrode puller (Scientific and Research Instruments, Ltd., U.K.) to a resistance, when filled with 3 M KCl, of between 0.5 and 5 MΩ. Each oocyte was positioned in a Perspex-recording chamber and impaled with two microelectrodes. The bath was perfused with normal SOS, unless otherwise stated, supplied through a gravity-fed system at a rate of 5 ml min^{-1}. Oocytes were normally maintained at a holding potential of $E_h = -80$ mV and all membrane currents were recorded on a Gould BS-272 chart recorder.

Drug application protocol

Fipronil (Chemserv, U.S.A.) and BIDN (donated by DuPont Agricultural Products) were dissolved in DMSO ($<1\%$ v v^{-1} final concentration in saline) and ivermectin (Sigma) was dissolved in ethanol ($<0.1\%$ v v^{-1} final concentration in saline); these solvent concentrations were shown to have no effect either on the responses of GluCl expressing or uninjected oocyte membranes when cells were clamped at $E_h = -80$ mV. A dose-dependent relationship for L-glutamate was generated by challenging the expressed receptor first with 3 mM applications of L-glutamate followed by increasing concentrations of agonist (10 μM to 100 mM) at 5 min intervals, as a result of which agonist responses of constant amplitude were consistently observed. The response to each concentration of agonist was expressed as a percentage of the peak response (%I_{max}). The response to each concentration of ivermectin was

obtained on individual oocytes and normalized to the current in response to 3 mM L-glutamate for that oocyte. For studies on fipronil and other antagonists, oocytes were incubated in different antagonist test concentrations for 2 min prior to application of 3 mM L-glutamate in the continued presence of the test concentration of the antagonist. The process was repeated with a higher concentration of the antagonist following a 3–5 min recovery period in normal SOS. The amplitude of the response to co-application of antagonist and L-glutamate was normalized to the values obtained in the presence of 3 mM L-glutamate in physiological saline.

Data were analysed using Graphpad prism (Graphpad Software) and presented as the mean ± the standard error of the mean. Hill coefficients were calculated using the following equation.

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

This equation was used to fit a sigmoid curve of variable slope to the normalized data. I_{max} and I_{min} represent the maximal and minimal currents induced by a particular agonist. The EC_{50} is the concentration of agonist necessary to elicit half the maximum response and n_H is the slope (Hill) coefficient.

Results

PCR amplification of the ZC317.3 cDNA

Our initial attempts to detect mRNA transcribed from the ZC317.3 gene used oligonucleotide primers designed to well-conserved portions of GluCl subunits in combination with generic 3′ (oligo-dT) and 5′ (SL1) sequences. These experiments suggested that the computer-predicted ends of the open reading frame were accurate, allowing us to synthesize oligonucleotides that included the predicted initiation and termination codons. Using these primers, PCR resulted in the amplification of an approximately 1400 bp cDNA. The cDNA was cloned into pGEM®-T Easy and sequenced: the sequence has been submitted to the EMBL database (Accession Number AJ243914). The ZC317.3 cDNA includes an open reading frame of 484 amino acids containing all the characteristics of invertebrate L-glutamate-gated chloride channel subunits (Figure 1). These features include a predicted signal peptide at the N-terminus and a long N-terminal extracellular domain that contains a characteristic 2nd pair of cysteine residues, in addition to the dicysteine loop (amino-acids 138 and 152 of the mature polypeptide; positions 218 and 232 on Figure 1), at amino-acids 199 and 210 of the predicted mature GLC-3

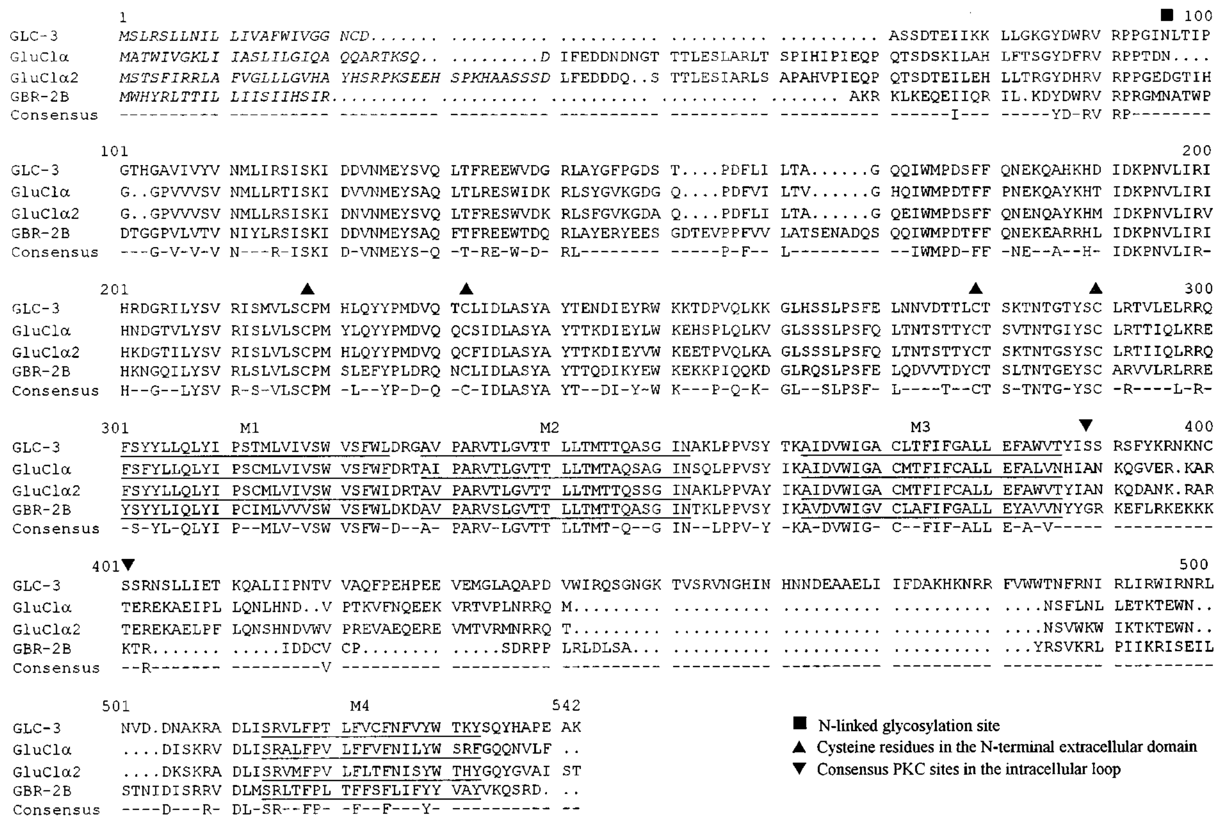

Figure 1 Alignment of the amino acid sequence (single-letter code) of the *C. elegans* GLC-3 subunit with the GluClα (Cully *et al*., 1994), GluClα2 (Dent *et al*., 1997; Vassilatis *et al*., 1997) and GBR-2B (Laughton *et al*., 1997) receptor subunits. The amino acid sequences were aligned using the computer program PILEUP. Where the amino acids are identical in all four sequences a consensus is shown along the bottom of the alignment; an absence of identity is indicated by a dash. The putative signal peptides are shown in italics and the transmembrane regions (M1–4) are underlined. For the GLC-3 sequence only the N-linked glycosylation site, the four cysteine residues in the extracellular domain and the consensus sites for phosphorylation by PKC in the intracellular loop are shown. Dots indicate gaps in the alignment.

polypeptide (positions 279 and 290 on the alignment shown in Figure 1), that distinguishes the L-glutamate- and glycine-gated chloride channels from the ionotropic GABA receptors. A candidate site for N-linked glycosylation is located in the extracellular domain at residue 26 of the mature polypeptide. The extracellular domain is followed by four hydrophobic regions (M1 – M4) that are predicted to cross the plasma membrane. Comparison of the cDNA sequence with the genomic sequence and with the amino-acid sequence predicted by Genefinder revealed that one of the intron/exon boundaries was not as predicted, resulting in a longer intracellular loop between M3 and M4. This loop contains two consensus sites for phosphorylation by protein kinase C.

When used to search the protein sequence databases, ZC317.3 exhibited greater than 50% amino-acid sequence identity to several invertebrate GluCl subunits (Table 1). A tree illustrating the relationship between the *C. elegans* and *Drosophila* GluCl subunits is shown in Figure 2 and an alignment of the GluClα-like subunits is shown in Figure 1. The *C. elegans* ZC317.3 gene is therefore designated *glc-3* and the subunit it encodes GLC-3. GLC-3 appears to be most closely related to the previously characterized GluClα and -α2 subunits. It is noteworthy that neither the N-linked glycosylation site nor the protein kinase C sites are conserved between GLC-3 and the other *C. elegans* α subunits, though a glycosylation site is present at a homologous position on the GBR-2 (GluClα3) subunits (Laughton *et al*., 1997). Compared to GluClα and α2, GLC-3 is truncated by about 30 amino acids at the N-terminus, producing an extracellular domain similar in size to GBR-2, and has a slightly longer intracellular loop than any of the other GluCl subunits.

Table 1 Amino acid identities of GLC-3 with other *C. elegans* GluCl subunits. Accession numbers and bibliographic data are given in Figures 1 and 2

Subunit	*% identity*
GluClα	60
GluClα2S	66
GBR-2B	58
GluClβ	50
C27H5.8	40
*Dm*GluCl	50

A functional homomeric GluCl is expressed in Xenopus *oocytes injected with glc-3 cRNA*

Xenopus oocytes injected with *in vitro*-transcribed cRNA encoding the GLC-3 polypeptide responded to L-glutamate, yielding robust, dose-dependent inward currents (at a holding potential of $E_h = -80$ mV) (Figure 3a, b). GABA, histamine, glycine and the excitatory L-glutamate-gated channel agonist, NMDA, at concentrations of 1 mM ($n=5$ tests each on a different oocyte for each ligand) all failed to activate the expressed receptor (data not shown). Control oocytes injected with the same volume of deionized water and non-injected oocytes failed to respond to L-glutamate when it was applied at concentrations from 3 μM to 10 mM ($n=6$). The conformationally constrained L-glutamate analogue, ibotenic acid, activated the GLC-3 receptor at 1 mM, but produced non-desensitizing currents of a smaller amplitude and slower onset than L-glutamate (Figure 3a). The EC_{50} and Hill coefficients for L-glutamate were 1.90 ± 0.03 mM and 1.5 ± 0.10, ($n=6$) respectively (Figure 3b). L-glutamate evoked-currents (I_{glu}) showed a rapid onset and desensitized rapidly in the presence of the neurotransmitter. Reduction of the external NaCl concentration to 54.9 mM and its partial replacement by 45.1 mM monosodium gluconate shifted the reversal potential (E_{rev}) for L-glutamate-sensitive currents from -16.6 to -7.3 mV, which is in the appropriate direction predicted by the Nernst equation (Figure 3c, $n=4$).

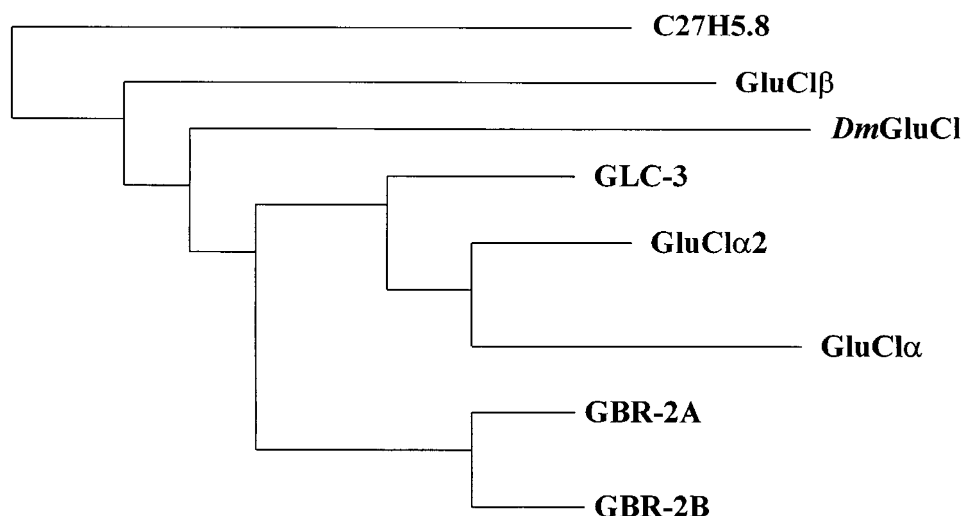

Figure 2 Unrooted neighbour-joining tree of the amino-acid sequences of invertebrate Glu-Cl subunits. Sequences were aligned using Pileup and the tree calculated using Phylip and 100 bootstrap replicates. All the sequences shown are from *C. elegans* except *Dm*GluCl, which is the *Drosophila* GluCl described by Cully *et al*. (1996). Accession numbers: GluClα U14524, GluClα2 AJ000537, GluClβ U14525, GBR-2A U40573, GBR-2B U41113, *Dm*GluCl U58776, C27H5.8 U14635.

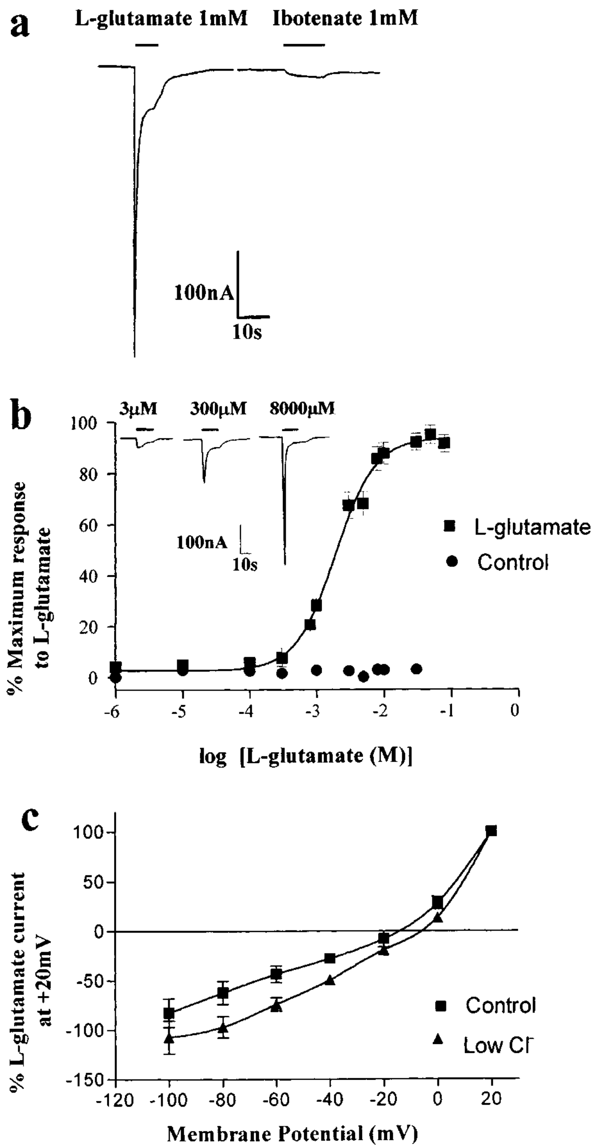

Figure 3 Responses to L-glutamate and other candidate ligands of *Xenopus* oocytes expressing GLC-3. Two-electrode voltage clamp electrophysiology was used to record L-glutamate activated inward currents in oocytes injected with *in vitro*-synthesized *glc-3* RNA. (a) Inward currents recorded in the presence of 1 mM L-glutamate or 1 mM ibotenic acid, from oocytes voltage-clamped at -80 mV. Horizontal bars indicate the duration of agonist application and the downward deflection denotes an inward current. (b) L-glutamate concentration-response curves of oocytes injected with *in vitro* cRNA encoding the GLC-3 subunit and water-injected control oocytes clamped at -80 mV. Data were normalized to the maximum L-glutamate response seen with each oocyte and are shown as the standard error of the mean (s.e.mean) of $\geqslant$ six oocytes taken from a minimum of two animals. The inset shows typical responses observed following the application of 3 μM, 300 μM and 8 mM L-glutamate. (**c**) Current-voltage relationship of the L-glutamate-induced currents (3 mM) in *Xenopus* oocytes pre-injected with cRNA encoding GLC-3 subunits in normal saline and reduced [Cl^-] saline. Data were normalized to the response observed at a voltage clamp of $+20$ mV and each point is the mean of the responses seen in $\geqslant$ 3 oocytes$\pm$the standard error of mean.

Actions of ivermectin on the expressed homomeric GLC-3 receptor

Oocytes expressing the GLC-3 protein exhibited slowly activating, irreversible, inward IVM-sensitive currents which were dose-dependent (Figure 4a). No such responses were observed in either uninjected oocytes or in oocytes injected with distilled water. Large, non-desensitizing, IVM-sensitive inward currents were observed following the application of 0.1 and 1 μM IVM, whereas 0.01 μM IVM failed to gate the channel ($n=3$). Dose-dependent currents elicited by ivermectin (Figure 4b; $n=6$) generated an EC_{50} value of 0.4 ± 0.02 μM and a Hill coefficient of 4.9 ± 0.84. We observed no potentiation of sub-maximal L-glutamate-gated currents in the presence of low (0.01 μM) concentrations of ivermectin (data not shown).

Actions of convulsant antagonists on GLC-3 receptors

Picrotoxinin (PTX) is a potent antagonist of many native and recombinant vertebrate and invertebrate GABA-gated chloride channels (Cull-Candy, 1976; Olsen & Tobin, 1990; Becker, 1992; Pribilla *et al.*, 1992), and of some native and

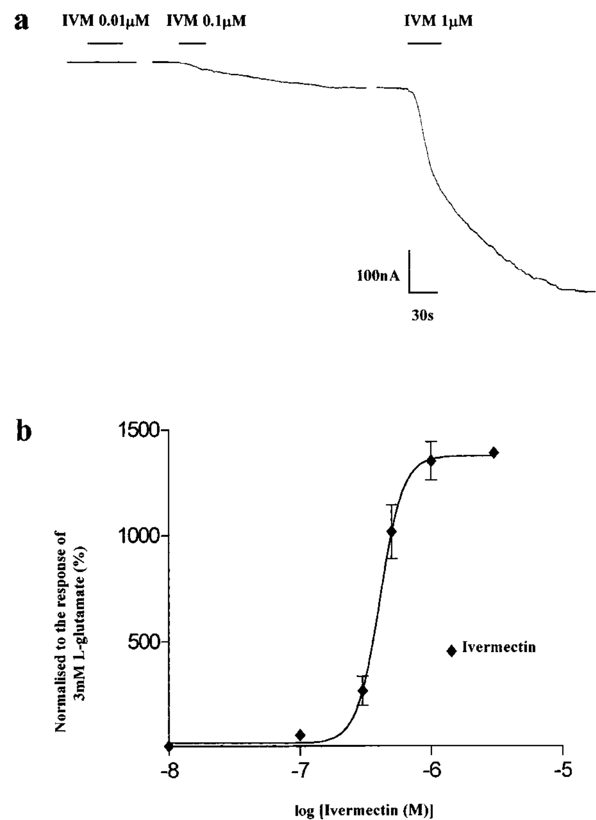

Figure 4 Ivermectin-induced dose-dependent inward currents. (a) Robust inward currents recorded in a single *Xenopus* oocyte expressing GLC-3 protein, voltage clamped at -80 mV. Horizontal bars above the trace indicate consecutive 30 s applications of IVM at increasing concentrations. (b) Dose-dependent ivermectin response curve. Each data point represents the ivermectin response from a minimum of six individual oocytes. The response is shown as the mean from at least six oocytes$\pm$standard error of the mean (s.e.mean), normalized to the response to 3 mM L-glutamate in each oocyte.

recombinant GluCls (Arena *et al*., 1992; Cully *et al*., 1994; Cleland, 1996; Vassilatis *et al*., 1997; Etter *et al*., 1999). However, at concentrations of 0.01 to 1000 μM, it failed to inhibit the robust L-glutamate (3 mM) agonist response in oocytes expressing the GLC-3 receptor (Figure 5, $n=6$). The bicyclic dinitrile convulsant, BIDN and the insecticide, fipronil (10 μM), had no direct effect on either uninjected oocytes or oocytes injected with GLC-3 cRNA, all of which were clamped at $E_h = -80$ mV. Both BIDN and fipronil resulted in a dose-dependent suppression of the amplitude of the L-glutamate-gated chloride currents ($n=5$ for both antagonists) (Figure 5b). The amplitude of the L-glutamate responses was restored after a 10 min wash in normal saline. For antagonist concentrations of 10 μM and above recovery was slower and often incomplete. The estimated IC_{50} for the co-application of BIDN and 3 mM L-glutamate was 0.2 ± 0.07 μM and for fipronil the IC_{50} was 11.5 ± 0.11 μM (Figure 5b).

Discussion

Analysis of the genomic sequence of *C. elegans* strongly suggested that the ZC317.3 gene encoded a GluCl subunit. Our ability to amplify a cDNA derived from this gene by RT–PCR methods shows that the gene is transcribed and thus it is very likely that the subunit polypeptide is expressed in the nematode. The amplified sequence indicates that the gene differs in its intron:exon structure from that predicted, but the 5′ and 3′ ends of the transcript appear to have been correctly predicted by the Genefinder programme. The polypeptide encoded by this clone exhibits the characteristic features of the 'cys-loop' receptor subunit family, with a long hydrophilic N-terminal region containing a 15 amino-acid dicysteine loop and four predicted membrane-spanning regions (M1–M4). The polypeptide also contains the second pair of cysteine residues in the extracellular domain that are typical of GluCl and glycine receptor subunits, as well as consensus sequences for one N-linked glycosylation and two phosphorylation sites. The sequence most closely resembles that of the *C. elegans* GluClα, GluClα2 and GBR-2 (GluClα3) subunits, though the level of identity between GLC-3 and the GluClα subunits is rather lower (60–66%) than that between GluClα and GluClα2 (76%) as illustrated in Figure 2. The overall significance of this may become clear once *in vivo* functions and subunit composition of the various GluCls have been established. The consensus PKC phosphorylation sites found in the long intracellular loop of ZC317.3 are not conserved in any of the other GluCl subunits and the lack of additional basic residues close to the consensus

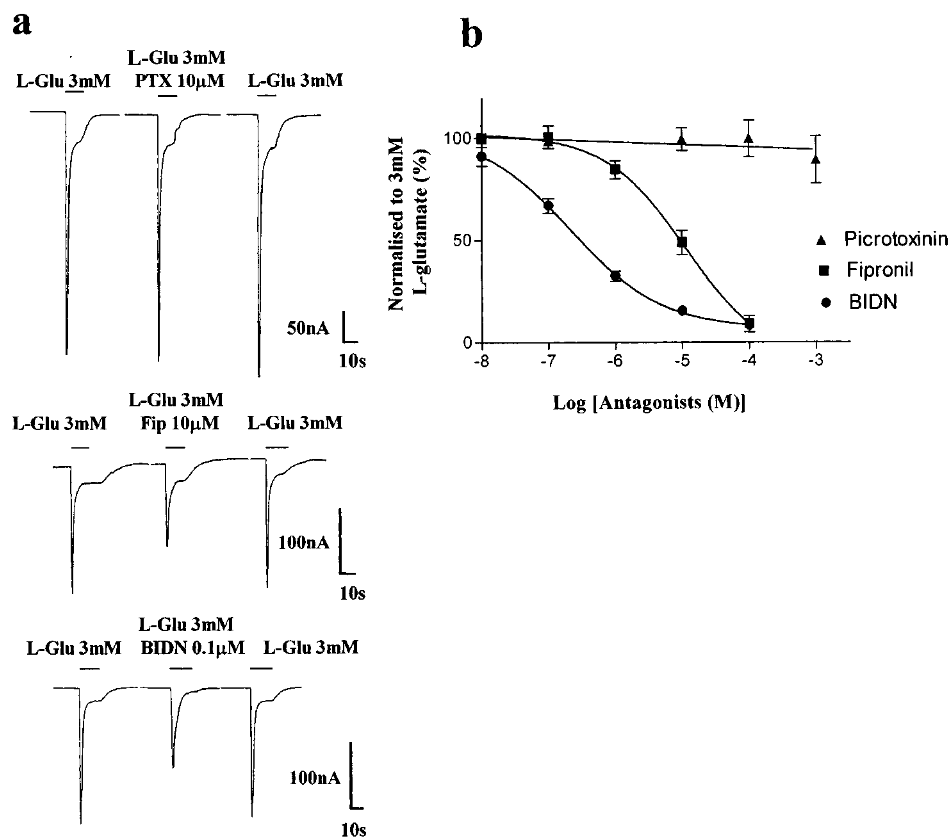

Figure 5 The GABA channel antagonists, picrotoxinin (PTX), fipronil (Fip) and BIDN were tested on 3 mM L-glutamate-elicited responses recorded from GLC-3 homo-oligomers. (a) For each antagonist, three responses are shown (Control) 3 mM L-glutamate, 3 mM L-glutamate in the presence of test antagonist, 3 mM L-glutamate recovery response after 3–5 min wash in normal saline. (b) Dose-response curves for the actions of fipronil, picrotoxinin and BIDN on the L-glutamate response of the GLC-3 receptor. Oocytes were incubated for 2 min in the candidate antagonist prior to co-application of L-glutamate and the test antagonist. The response 15 s after the co-application of L-glutamate plus antagonist was normalized to the response elicited from each oocyte after the application of 3 mM L-glutamate alone. Each point is the mean observation from a minimum of four oocytes.

phosphorylation sites may argue against any functional role for them *in vivo*.

The high level of sequence identity of the GLC-3 with the GluClα and GluClα2 subunits suggests that the novel polypeptide might have a similar function. When expressed in *Xenopus* oocytes, the functional properties of the receptor formed by this clone are indeed very similar to those described for the two previously cloned GluClα subunits in that they are activated by both L-glutamate and ivermectin. The EC_{50} for L-glutamate of 1.9 ± 0.03 mM for the GLC-3 receptor was close to the reported values of 1.36 ± 0.05 mM for the GluClα+β receptor (Cully *et al*., 1994) and 2.0 ± 0.3 mM for the GluClα2 receptor (Dent *et al*., 1997). Ibotenate was a weak agonist on the homomeric GLC-3 receptor, as reported for the GluClα+β receptor (Cully *et al*., 1994). The expressed channels appeared to be selective for Cl^- ions, as indicated by the shift in reversal potential for L-glutamate-gated current when the external sodium chloride was partially replaced by monosodium gluconate. The estimated Hill coefficient for L-glutamate was greater than 1, in agreement with all other expressed *C. elegans* GluCls (Cully *et al*., 1994; Dent *et al*., 1997; Vassilatis *et al*., 1997). An ability to irreversibly bind ivermectin, resulting in channel opening, may be diagnostic for α-like subunits of the GluCl, as the GluClβ subunit is not activated by these compounds (Cully *et al*., 1994), though Dent *et al*. (2000) have reported that one of the alternatively-spliced GluClα3 subunits does not form ivermectin- or glutamate-gated channels. Homomeric GLC-3 receptors are slowly and irreversibly activated by ivermectin in a dose-dependent manner similar to that described for GluClα2. Currents elicited by ivermectin (Figure 4b) generated an EC_{50} value of 0.4 ± 0.02 μM and a Hill coefficient of 4.91 ± 0.845. The EC_{50} for ivermectin at the GLC-3 receptor is similar to those reported for ivermectin phosphate at the α1 and α2 subunits (0.14 and 0.11 μM respectively) (Cully *et al*., 1994; Vassilatis *et al*., 1997).

Experiments with non-competitive antagonists of ionotropic receptors containing chloride channels revealed some novel features of the homomeric GLC-3 receptor. The GLC-3 channels are insensitive to picrotoxinin at 1 mM, but are inhibited by BIDN and fipronil with IC_{50}s of 0.22 and 11 μM respectively. This is the first report that any recombinant GluCl is sensitive to the actions of BIDN or a phenylpyrazole. These compounds are known to target GABA-gated chloride channels (Deng *et al*., 1993; Hosie *et al*., 1995; Rauh *et al*., 1997) and the new findings presented here indicate that GluCls and ionotropic GABA receptors show some similarities in their binding sites for certain convulsants. A naturally occurring mutation in the second membrane-spanning region (M2) of the *Drosophila* subunit RDL (A302S) confers both picrotoxinin and fipronil resistance (Pribilla *et al*., 1992; ffrench-Constant *et al*., 1993; Zhang *et al*., 1994; 1995; Gurley *et al*., 1995; Hosie *et al*., 1995; Wang *et al*., 1995). This mutation is predicted to lie deep in the GABA receptor pore (Olsen & Tobin, 1990; Smith & Olsen, 1995; Xu *et al*., 1995). However, the sequences of the M2 regions of the *C. elegans* GluCl subunits (Figure 1) are almost identical to each other, in particular the residue in M2 equivalent to A302 of RDL is a serine or threonine in all these subunits. This does not suggest any obvious reason for the observed differences in antagonist sensitivity between GLC-3 and the other GluCls. It is possible that residues outside the ion channel affect the antagonist sensitivity and specificity of the GluCls. Lynch *et al*. (1995) showed that mutations at the extracellular end of the M2 of the mammalian glycine receptor α subunit, notably alterations in lysine-274, transformed picrotoxin from an allosteric competitive antagonist to an allosteric potentiator at low (0.01–3 μM) concentrations and to a non-competitive antagonist at higher (>3 μM) concentrations. Again, the sequence of this region of GLC-3, in particular the basic residue equivalent to K274, is conserved. [^{3}H]-BIDN binding to insect membranes is displaced competitively by dieldrin but not by fipronil or PTX (Rauh *et al*., 1997). Fipronil is a non-competitive antagonist of [^{35}S]-TBPS and [^{3}H]-EBOB binding to insect GABA receptors (Cole *et al*., 1993; Deng *et al*., 1993), unlike PTX (Bloomquist, 1993; Casida, 1993). Therefore it is likely that BIDN and fipronil bind to distinct sites from dieldrin, PTX and each other, even though the activity of all four compounds is affected by the same mutation (A302S) in the RDL channel. The interesting pharmacology observed for GLC-3 indicates that this and other *C. elegans* GluCls may prove to be valuable tools for studying the interactions of non-competitive antagonists with ligand-gated chloride channels. In particular, it will be interesting to determine whether or not other native and other recombinant GluCls are sensitive to fipronil, BIDN and related compounds.

Very recently three novel ligand-gated chloride ion channels have been cloned from invertebrates; two histamine-gated chloride channel (HisCl) subunits from *Drosophila* and a 5HT-gated channel (MOD-1) from *C. elegans* (Zheng *et al*., 2000; Ranganathan *et al*., 2000). Though the histamine-gated channel subunits show close sequence similarity with the GluCls, the absence of any response to histamine by the GLC-3 receptor shows that this subunit is very unlikely to form part of a nematode HisCl. The sequence of MOD-1 is quite distinct from that of GLC-3 and the channel does not respond to L-glutamate or ivermectin (Ranganathan *et al*., 2000) so GLC-3 is also unlikely to form part of a 5HT-gated channel, based on sequence and pharmacological differences.

It might be that GLC-3 is only a minor component of the ivermectin receptors found in *C. elegans*, since Vassilatis *et al*. (1997) concluded that the majority of [^{3}H]-ivermectin binding sites present in membrane preparations could be accounted for by the GluClα and GluClα2 subunits. Dent *et al*. (2000) showed that mutations in three genes (*glc-1*, *avr-14* and *avr-15*) resulted in a 4000-fold resistance to ivermectin and almost complete abolition (>97%) of high-affinity (<3 nM) ivermectin binding. Alternatively, GLC-3 might co-assemble with one or more of the other GluCl subunits to form a component of the ivermectin-binding sites. Detailed investigation of this latest member of the GluCl gene family using the advanced molecular genetic techniques available for *C. elegans* will enhance our overall understanding of chemical neurotransmission in the nematode nervous system.

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