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ORIGINAL PAPER

The actions of chloride channel blockers, barbiturates and a benzodiazepine on *Caenorhabditis elegans* glutamate- and ivermectin-gated chloride channel subunits expressed in *Xenopus oocytes*

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Abstract The pharmacology of *Caenorhabditis elegans* glutamate-gated chloride (GluCl) channels was determined by making intracellular voltage-clamp recordings from *Xenopus* oocytes expressing GluCl subunits. As previously reported (Cully et al. 1994), GluClα1β responded to glutamate (in a picrotoxin sensitive manner) and ivermectin, while GluClβ responded only to glutamate and GluClα1 only to ivermectin. This assay was used to further investigate the action of chloride channel compounds. The arylaminobenzoate, NPPB, reduced the action of glutamate on the heteromeric GluClα1β channel (IC_{50} 6.03 $\pm$ 0.81 μM). The disulphonate stilbene, DNDS, blocked the effect of both glutamate and ivermectin on GluClα1β channels, the action of glutamate on GluClβ subunits, and the effect of ivermectin on GluClα1 subunits (IC_{50}s 1.58–3.83 μM). Surprisingly, amobarbital and pentobarbital, otherwise known as positive allosteric modulators of ligand-gated chloride channels, acted as antagonists. Both compounds reduced the action of glutamate on the GluClα1β heteromer (IC_{50}s of 2.04 $\pm$ 0.5 and 17.56 $\pm$ 2.16 μM, respectively). Pentobarbital reduced the action of glutamate on the GluClβ homomeric subunit with an IC_{50} of 0.59 $\pm$ 0.09 μM, while reducing the responses to ivermectin on both GluClα1β and GluClα1 with IC_{50}s of 8.7 $\pm$ 0.5 and 12.9 $\pm$ 2.5 μM, respectively. For all the antagonists, the mechanism is apparently non-competitive. The benzodiazepine, flurazepam had no apparent effect on these glutamate- and ivermectin-gated chloride channel subunits. Thus, arylaminobenzoates, disulphonate stilbenes, and barbiturates are non-competitive antagonists of *C. elegans* GluCl channels.

Keywords *C. elegans* · Nematode · Ivermectin receptor · Barbiturates · Chloride channel

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Introduction

Chloride channels probably occur in all cell types and may be divided into those of neuronal origin, those present in smooth and striated muscle, and those found in various epithelia (Greger 1990). In neurones, there are chloride channels involved in the maintenance of the resting membrane potential and those that are ligand-gated, where the ligand can be one of almost any of the classical transmitters. However, GABA- and glycine-gated chloride channels are particularly widespread in vertebrates (Olsen and Tobin 1990; Betz 1991). In addition, there are a number of invertebrate phyla, viz, arthropods, molluscs, and nematodes, where there are also glutamate-gated chloride channels (Cleland and Selverston 1998; Cull-Candy 1976; Lea and Usherwood 1973a, b; Cully et al. 1994; Duan and Cooke 2000; Ikemoto and Akaike 1988; Ikemoto et al. 1988; Kehoe and Vulfius 2000). Certain of these channels have been cloned and are related structurally to GABA- and glycine-gated chloride channels (Cully et al. 1996). It has been proposed that these glutamate-gated chloride channels are the counterparts of the vertebrate glycine-gated channels (Vassilatis et al. 1997). Ligand-gated chloride channels have been described where glutamate and GABA share a receptor, e.g., on crayfish

striated muscle (Franke et al. 1986), or where separate glutamate and GABA receptors activate a common chloride channel, e.g., on *Aplysia* neurons (King and Carpenter 1987, 1989).

Glutamate- and ivermectin-gated chloride channel subunits have been identified in *Caenorhabditis elegans*, for example, GluClα1 (Cully et al. 1994), GluClα2 (Dent et al. 1997), GluClβ (Cully et al. 1994). Currently, a total of nine glutamate-gated chloride–related cDNAs have been isolated from *C. elegans* (http://www.wormbase.org 2004). The M2 region of these subunits share high homology with the bovine GABA-Aα1 subunit (Xu et al. 1995), the bovine GABA-Aβ1 subunit (Fisher et al. 1997), and the *Drosophila* Rdl (resistance to dieldrin) GABA-A receptor (ffrench-Constant et al. 1991). Studies by Cully et al. (1994) have shown that the *C. elegans* GluClα1 subunit contained the ivermectin-binding site while the GluClβ subunit bound glutamate. The GluClα1β heteromer was insensitive to GABA and glycine together with the mammalian glutamate agonists *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazol propionic acid (AMPA), and kainate (Cully et al. 1994). The GluClα1β heteromer was also insensitive to the NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) but was sensitive to the non-competitive GABA-A antagonist, picrotoxin. However, the sensitivity to picrotoxin varied, e.g., homomeric GluClα1 and the heteromeric GluClα1β receptors had similar sensitivities with an IC_{50} of around 50 μM, while homomeric GluClβ receptors were about 1,000 times more sensitive (Cully et al. 1996). This suggests that the subunit composition has an important role in the pharmacology of glutamate-gated chloride (GluCl) channels.

Chloride channel blockers comprise a very heterogeneous group of compounds, and antagonists of neuronal chloride channels have nothing in common with blockers of the epithelial chloride channels (Greger 1990). The arylaminobenzoate, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and the disulphonate stilbene derivative, 4,4-dinitrostilbene-2,2-disulphonic acid (DNDS) are examples of non-specific chloride channel blockers which act on ion channels in neurones, muscle, and kidney epithelia (Greger 1990; Wangemann et al. 1986). The presence of a propyl group separating the phenyl ring and the amino group in NPPB is optimal while an anionic site is mandatory for chloride-blocking activity (Greger 1990). The blocking ability of NPPB and DNDS varies between preparations. For example, both NPPB and DNDS decrease a glutamate-gated chloride channel on salamander cone photoreceptors (Picaud et al. 1995). There is a chloride-sensitive channel from rough endoplasmic reticulum membranes of rat hepatocytes which is blocked by 300-μM NPPB while DNDS failed to inhibit this channel (Eliassi et al. 1997). Other chloride channels are resistant to both NPPB and DNDS, e.g., the cAMP-activated apical membrane chloride channels in *Necturus* gallbladder epithelium (Copello et al. 1993). Another arylaminobenzoate, flufenamic acid, 200 μM, reduced the response to 1 μM ivermectin on GluClα1β subunits expressed in oocytes by 60% (Cully et al. 1994) but had no effect when applied at 100 μM on the response to 50 μM glutamate on native glutamate-gated chloride channels in *C. elegans* pharynx (Pemberton et al. 2001).

Both barbiturates and benzodiazepines bind to specific modulatory sites on mammalian GABA-A-gated chloride channel receptors (Olsen 1982). Barbiturates enhance the action of GABA at these receptors (Birnir et al. 1997) while benzodiazepines also enhance the action of GABA-A receptors but can act through a range of sedative, hypnotic, anxiolytic, and muscle-relaxant actions (Costa et al. 1975). The benzodiazepine-binding site is located on the GABA-A α subunit but the presence of a γ subunit is also required (Sigel and Buhr 1997). In contrast, the barbiturate-binding site is located on the GABA-A β subunit and does not require the presence of a γ subunit for activation. Both receptor sites are distinct from the GABA-binding site (Leeb-Lundberg et al. 1980). The barbiturate-binding site is associated with that for picrotoxin (Smith and Olsen 1995; Thompson et al. 1996). Picrotoxin inhibits GABA responses in a non-competitive manner (Simmonds 1982) by acting deep within the chloride channel of the GABA-A receptor in the Val257-Thr261 region near the putative cytoplasmic end of M2 of the α1 subunit (Xu et al. 1995).

The present study sets out to determine the actions of NPPB, DNDS, amobarbital, pentobarbital, and flurazepam on *C. elegans* glutamate-gated GluClα1β and GluClβ subunits and *C. elegans* ivermectin-gated GluClα1β and GluClα1 subunits expressed in *Xenopus* oocytes in an attempt to further define the pharmacological profile of *C. elegans* glutamate- and ivermectin-gated chloride channels.

Materials and methods

In vitro transcription of cDNA

cDNA clones of the *C. elegans* glutamate-gated chloride channel α1 and β subunits were obtained from Merck (Rahway, New Jersey, USA). They were inserted into pBluescript™ S4 plasmids (Stratagene), in between the Not1 and EcoRV restriction enzyme cleavage sites. The plasmids were transformed into *E. coli* (XL1-BLUE, Stratagene) bacteria and incubated in LB medium. The plasmids were isolated from the cells using a QIAgen plasmid purification kit (QIAgen, Crawley, W. Sussex, UK). The clones were then excised from the plasmids using the restriction enzymes Not 1 and Sac 1 (Promega,

Southampton, UK) and purified with phenol to remove any contaminating proteins.

The transcription was performed using a RiboMAX™ large-scale RNA production system (Promega, UK). Linearized DNA was incubated with an RNA polymerase, a ribonuclease inhibitor, and a mixture of the four nucleotides. This mixture was incubated for 3 h at 30°C. At the end of that time, a DNase was added to remove the original DNA from the sample. Small samples were then taken from the mixture and treated with TCA for measurement of radiolabel incorporation. The transcripts were cleaned using an RNeasy™ spin column (QIAgen, Crawley, UK), a small sample was run on an RNA gel to check integrity, and the remainder was stored as an ethanolic precipitate until required for injection into oocytes.

Small samples of the transcripts were translated in the rabbit reticulocyte lysate cell-free translation system (Promega, UK) in order to ascertain that the translation products of the transcripts were of the correct size.

Oocyte preparation and injection

Mature female *Xenopus laevis* were purchased from Blades Biologicals (Edenbridge, Kent, UK) and kept in 30 cm deep filtered tap water at about 15°C. The water was left to stand for several days before the toads were placed in it to allow dispersal of any chlorine. To obtain oocytes, a toad was anaesthetized in a cold 1.5% w/v aqueous solution of ethyl-*m*-aminobenzoate (tricaine; Sigma, Poole, UK) for about 40 min. A small incision (about 1 cm) was made into the ventral surface of the abdominal cavity above the bladder to one side of the midline. Several lobes of the ovary were removed and placed in calcium-free standard oocyte saline (SOS) and the body wall and skin sutured separately. SOS had the following composition: NaCl 100; KCl 2; $CaCl_2$ 1.8; $MgCl_2$ 1.0; HEPES 5; all values in mM. The pH was adjusted to 7.6 before use. For calcium-free SOS, the $CaCl_2$ was simply omitted.

The oocytes were treated with a 2 mg ml^{-1} solution of collagenase for 15–20 min. The cells were then manually separated from each other and stored in SOS at 4°C until required for injection.

Pipettes for injecting RNA were pulled from thin-walled capillary tubes on a two-stage vertical electrode puller. The tip of the micropipette was broken under a microscope to a diameter of 10–15 μm and filled with mineral oil to maintain a constant interface between the RNA solution and the pressure port. The micropipette was attached to a microelectrode holder with a pressure port in a hydraulic microdrive. The concentration of the RNA solution was 1 μg ml^{-1} in each case. In the case of the GluClα1/GluClβ mixture, the ratio of the two subunits was 1:1. A drop of RNA solution was placed on a piece of sterile paraffin film and drawn up into the pipette. The oocytes to be injected were placed in a drop of SOS medium to prevent movement of the cell. Each cell was injected with 50 nl of the RNA solution and then kept in sterile wells of SOS at 18–20°C until required.

Electrophysiological recordings from oocytes

A standard two-electrode voltage-clamp was used for electrophysiological recording. The intracellular electrodes were filled with 3 M potassium chloride (resistance, 1–2 MΩ). Silver/silver chloride wires connected the electrodes to the headstage of an Axoclamp 2A current/voltage-clamp (Axon Instruments, California, USA). Signals were monitored on a chart recorder. The oocyte was placed in a perfusion chamber and perfused with SOS at a rate of approximately 8 ml min^{-1}. After impaling with two electrodes, the oocyte was allowed to recover before voltage clamping at −80 mV. Drugs were dissolved in SOS, or in the case of ivermectin (22,23,dihydroavermectin B1A), dissolved in DMSO and diluted in SOS to a final concentration of DMSO <0.5%. The sodium salt of L-glutamate was used to avoid possible pH effects. Drug application was by local perfusion at a rate of about 8 ml min^{-1}. A bubble was introduced between the SOS and drug so that the bubble escaped through a small hole immediately prior to the bath to minimize agitation in the bath and allow rapid on/off responses to be seen. Application of agonist was 10 s in the case of glutamate and 30 s in the case of ivermectin. Antagonists were applied for 3–5 min prior to agonist application.

Data analysis

In each case, the experiments were repeated at least four times. All values are quoted as mean ± standard error of the mean. Error bars on graphs also show the standard error of the mean. Concentration–response curves were fitted using non-linear regression in GraphPad Prism (version 2, San Diego, USA). Data on graphs are shown as a percentage of the maximum response. Unpaired Student's *t* tests were carried out where appropriate, and $P < 0.05$ was taken as significant.

Drugs

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), amobarbital, pentobarbital, flurazepam, ivermectin, glutamate (sodium salt), and picrotoxin were obtained from

Sigma–Aldrich. 4,4-Dinitrostilbene-2,2-disulphonic acid (DNDS) was obtained from Fluorochem, UK.

Results

Effect of glutamate and ivermectin on glutamate- and ivermectin-gated chloride channels

No responses to either glutamate or ivermectin were obtained from un-injected or water injected control oocytes (data not shown). DMSO (0.5%) (used as vehicle) neither elicited a response nor reduced the amplitude of glutamate responses in injected oocytes. The maximum response seen to glutamate in oocytes injected with GluClα1 and GluClβ in a 1:1 ratio to form a GluClα1β heteromer was 4.2 μA, and the EC_{50} for glutamate on this heteromer was 1.8 ± 0.3 mM ($n = 13$), Table 1. The EC_{50} for glutamate on the homomer, GluClβ, was 0.8 ± 0.08 mM ($n = 9$) which was significantly different from the EC_{50} for the heteromer, $P < 0.001$. The maximum response seen to ivermectin in oocytes injected with the heteromer was 3.4 μA with an EC_{50} of 1.02 ± 0.06 μM ($n = 10$), Table 1. The EC_{50} for ivermectin on the homomer, GluClα1, was 0.97 ± 0.05 μM ($n = 10$). However, there was no significant difference between the EC_{50} values on the heteromer and GluClα1 homomer. The Hill coefficients of the concentration–response curves for glutamate were similar for the heteromer, 1.3 ± 0.2, and the β homomer, 1.8 ± 0.4, Table 1. However, the Hill coefficients for the concentration–response curves for ivermectin were significantly different with values of 2.1 ± 0.2 and 1.4 ± 0.1, for the heteromer and the GluClα1 homomer, respectively, $P < 0.05$. A Hill coefficient of greater than one implies that more than one agonist molecule is required to gate the channel. The glutamate responses of GluClα1β reversed within a few seconds while those of ivermectin took much longer to reverse, i.e., around 80% reversal over 45–60 min. The reversal potential of the response of the GluClα1β heteromer to glutamate was −21 mV, a value equal to the reversal potential for chloride in the system.

Table 1 A summary of the effect of glutamate and ivermectin on GluCl channels

Agonist	Channel	EC_{50}	Hill coefficient	n
Glutamate	GluClα1β	1.8 ± 0.3 mM	1.3 ± 0.2	13
Glutamate	GluClβ	0.8 ± 0.08 mM	1.8 ± 0.4	9
Ivermectin	GluClα1β	1.02 ± 0.06 μM	2.1 ± 0.2	10
Ivermectin	GluClα1	0.97 ± 0.05 μM	1.4 ± 0.1	10

The values are given for the EC_{50}s of glutamate and ivermectin against either the heteromeric or homomeric channels as indicated

The effect of picrotoxin and non-selective chloride channel blockers on glutamate- and ivermectin-gated chloride channels

Three compounds were tested on the GluCl channels, viz, picrotoxin, NPPB, and DNDS. The IC_{50} values are summarized in Table 2. Picrotoxin (100 μM) blocked the response of GluClα1β to glutamate in a non-competitive manner (Fig. 1a; $n = 6$). In a further series of experiments, the effect of a range of concentrations of picrotoxin at blocking the response to 1 mM glutamate was tested. Picrotoxin blocked the response with an IC_{50} of 18.52 ± 1.02 μM ($n = 5$; Fig. 1b; Table 2). The non-selective chloride channel blocker NPPB (100 μM) also reduced the maximum response of oocytes expressing GluClα1β to glutamate (Fig. 1c; $n = 4$) i.e., acting in a non-competitive manner. Experiments to determine the IC_{50} against 1 mM glutamate showed that this compound was significantly more potent than picrotoxin (IC_{50} of 6.03 ± 0.81 μM, $n = 4$; $P < 0.05$; Fig. 1d; Table 2). Another non-selective chloride channel blocker, DNDS, 100 μM, also reduced the glutamate response of the GluClα1β heteromer in a similar non-competitive manner to NPPB (Fig. 1e; $n = 5$). Further experiments testing this compound against the response to 1 mM glutamate yielded an IC_{50} of 1.58 ± 0.34 μM, (Fig. 1f; $n = 5$; Table 2). Therefore, this compound was significantly more potent than either picrotoxin or NPPB, $P < 0.05$. The effect of DNDS on the glutamate response in oocytes expressing the GluClβ homomer was similar with an IC_{50} of 2.38 ± 0.26 μM ($n = 4$; Table 2). DNDS, 100 μM, also reduced the response to ivermectin in oocytes expressing GluClα1β in a non-competitive manner (Fig. 2a, c; $n = 5$). Experiments to test the ability of DNDS to block the response to 500 nM ivermectin yielded an IC_{50} of 3.52 ± 0.30 ($n = 4$; Table 2). DNDS also exhibited a similar non-competitive block of the response to ivermectin for GluClα1 (100 μM DNDS, Fig. 2b, $n = 5$). The IC_{50} against 500 nM ivermectin was 3.83 ± 0.30 μM ($n = 4$; Table 2).

Table 2 A summary of the effects of antagonists (100 μM) on GluCl channels

Antagonist	Agonist	Channel	IC_{50} μM	n
Picrotoxin	Glutamate	GluClα1β	18.52 ± 1.02	5
NPPB	Glutamate	GluClα1β	6.03 ± 0.81	4
DNDS	Glutamate	GluClα1β	1.58 ± 0.34	5
DNDS	Glutamate	GluClβ	2.38 ± 0.26	4
DNDS	Ivermectin	GluClα1β	3.52 ± 0.30	4
DNDS	Ivermectin	GluClα1	3.83 ± 0.30	4

The values are given for the IC_{50}s against glutamate (1 mM) and ivermectin (500 nM) for either the heteromeric or homomeric channels as indicated

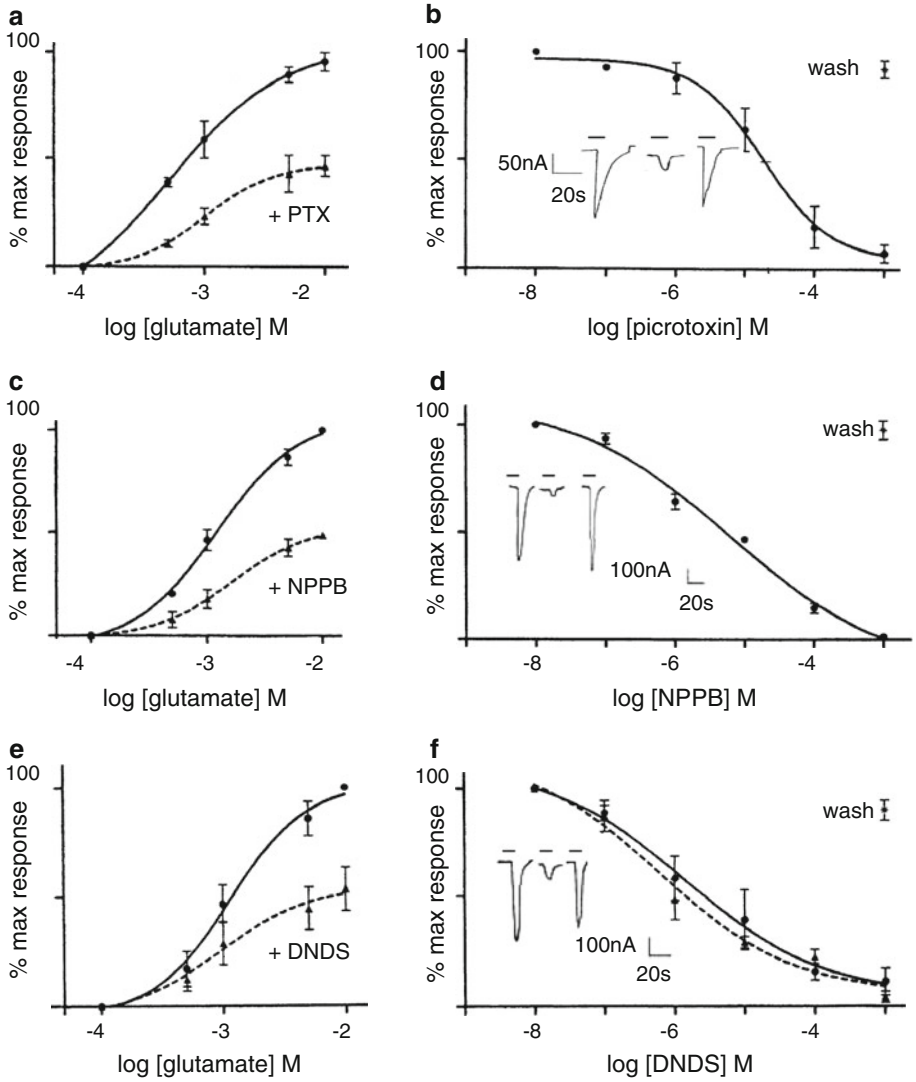

Fig. 1 The effect of chloride channel compounds on the glutamate response of oocytes expressing GluCl channels. **a** GluCl$\alpha 1\beta$. Concentration–response curves for glutamate in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM picrotoxin (PTX) ($n = 6$). **b** GluCl$\alpha 1\beta$. Effect of varying the concentration of picrotoxin on the response to 1 mM glutamate. The results are presented as the percentage of the maximum response obtained to 1 mM glutamate before the addition of antagonist ($n = 5$). The response after a wash (>15 min) is as indicated. The *inset* shows example traces to 1 mM glutamate before PTX (*left*), in the presence of 1 mM PTX (*middle*) and after a wash (>15 min, *right*). **c** GluCl$\alpha 1\beta$. Concentration–response curves for glutamate in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM NPPB ($n = 4$). **d** GluCl$\alpha 1\beta$. Effect of varying the concentration of NPPB on the response to 1 mM glutamate. The results are presented as the percentage of the maximum response obtained to 1 mM glutamate before the addition of antagonist ($n = 4$). The *inset* shows example traces to 1 mM glutamate before NPPB (*left*), in the presence of 100 μM NPPB (*middle*) and after a wash (>15 min, *right*). **e** GluCl$\alpha 1\beta$. Concentration–response curves for glutamate in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM DNDS ($n = 5$). **f** Effect of varying the concentration of DNDS on the response to 1 mM glutamate for GluCl$\alpha 1\beta$ ($n = 5$; *filled upright triangle*, *dashed line*) and GluClβ ($n = 4$; *filled circle*, *solid line*). The results are presented as the percentage of the maximum response obtained to 1 mM glutamate before the addition of antagonist. The *inset* shows example traces to 1 mM glutamate for GluCl$\alpha 1\beta$ before DNDS (*left*), in the presence of 1 mM DNDS (*middle*) and after a wash (>15 min, *right*)

Effects of barbiturates on glutamate- and ivermectin-gated chloride channels

The actions of two barbiturates, viz, amobarbital and pentobarbital, were examined for their effects on the glutamate and ivermectin responses on oocytes expressing heteromeric and homomeric glutamate- and ivermectin-gated chloride channels. The IC_{50} values are summarized in Table 3. Amobarbital, 100 μM, reduced the maximum glutamate response of oocytes expressing GluCl$\alpha 1\beta$ ($n = 4$; Fig. 3a). The IC_{50} for this compound against 1 mM glutamate was 2.04 ± 0.50 μM, ($n = 5$; Fig. 3c).

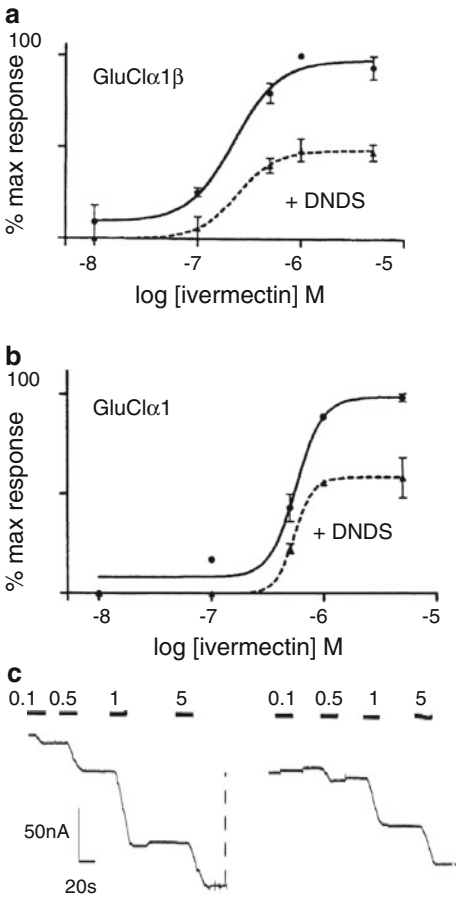

Fig. 2 The effect of DNDS on the response to ivermectin. **a** GluCl$\alpha 1\beta$. Concentration–response curves for ivermectin in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM DNDS ($n = 4$). **b** GluCl$\alpha 1$. Concentration–response curves for ivermectin in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM DNDS ($n = 5$). **c** Example traces of cumulative responses to ivermectin (0.1–5 μM, as indicated) for GluCl$\alpha 1\beta$ before DNDS (*left*) and in the presence of 100 μM DNDS (*right*)

Pentobarbital, 100 μM, reduced the glutamate response of the heteromer ($n = 6$, Fig. 3b). The IC_{50} for this compound against 1 mM glutamate was 17.56 ± 2.16 μM ($n = 6$; Fig. 3c). For the GluClβ homomer, the IC_{50} was 0.59 ± 0.09 μM ($n = 4$; Table 3). The response of the GluCl$\alpha 1\beta$ heteromer and the GluCl$\alpha 1$ homer to ivermectin was also inhibited in a non-competitive manner by pentobarbital (100 μM; $n = 5$ and $n = 4$, respectively; Fig. 4a, b, d). The IC_{50}s for this compound against 500 nM ivermectin was 8.7 ± 0.50 μM ($n = 6$) and 12.9 ± 2.5 μM for the GluCl$\alpha 1\beta$ heteromer and the GluCl$\alpha 1$ homer, respectively ($n = 5$; Fig. 4c; Table 3). The benzodiazepine, flurazepam, was tested for a modulatory effect on a 1 mM glutamate response of the heteromer but found to have no effect when applied at concentrations from 10 nM to 1 mM ($n = 4$; data not shown).

Discussion

The GluCl subunits studied here, GluCl$\alpha 1$ and GluClβ, are encoded by *glc-1* and *glc-2*, respectively. The subunits have 45% amino acid identity and 63% amino acid similarity with each other, and around 21% of the subunit amino acids are conserved in at least 75% of GABA-A and glycine subunits with which they were compared (Cully et al. 1996). This conservation is strongest in the membrane-spanning domains and the N-terminal domain. However, these $\alpha 1$ and β subunits are insensitive to both GABA and glycine and to bicuculline and strychnine (Cully et al. 1994). Cully et al. found that the $\alpha 1$ subunit homomer is activated by ivermectin while the β subunit homomer responded to glutamate, an observation confirmed in the present study. The EC_{50} values obtained for glutamate in the present study were similar to those obtained by Cully et al., with the homomer being more sensitive to glutamate. However, the EC_{50} values for ivermectin were higher in the present study compared with those obtained by Cully et al. The Hill coefficients obtained for both glutamate and ivermectin on the heteromer and both homomers were again similar between the study of Cully et al. and the current study, suggesting that more than one agonist molecule is required to gate the channels. In contrast to the situation in *C. elegans*, the *Drosophila* GluClα subunit is gated by both ivermectin and glutamate (Cully et al. 1996). This subunit has 48% amino acid identity and 60% nucleotide identity with the *C. elegans* subunits. Its EC_{50} values for ivermectin and glutamate were 41 nM and 23 μM, respectively, with Hill coefficients greater than one.

Picrotoxin blocked the action of glutamate on the GluCl$\alpha 1\beta$ heteromer in the current study with an IC_{50} of 18.52 μM which is slightly lower than the value of 42 μM obtained by Etter et al. (1999). While these authors found a similar value for picrotoxin against ivermectin on the heteromer, there was a large difference in the EC_{50} for picrotoxin block of the homomers. In the case of GluCl$\alpha 1$, the value was 59 μM while for GluClβ the value was

Table 3 A summary of the effects of barbiturates (100 μM) on GluCl channels

Antagonist	Agonist	Channel	IC_{50} μM	*n*
Amobarbital	Glutamate	GluCl$\alpha 1\beta$	2.04 ± 0.5	5
Pentobarbital	Glutamate	GluCl$\alpha 1\beta$	17.56 ± 2.16	6
Pentobarbital	Glutamate	GluClβ	0.59 ± 0.09	4
Pentobarbital	Ivermectin	GluCl$\alpha 1\beta$	8.7 ± 0.50	6
Pentobarbital	Ivermectin	GluCl$\alpha 1$	12.9 ± 2.5	5

The values are given for the IC_{50}s against glutamate (1 mM) and ivermectin (500 nM) for either the heteromeric or homomeric channels as indicated

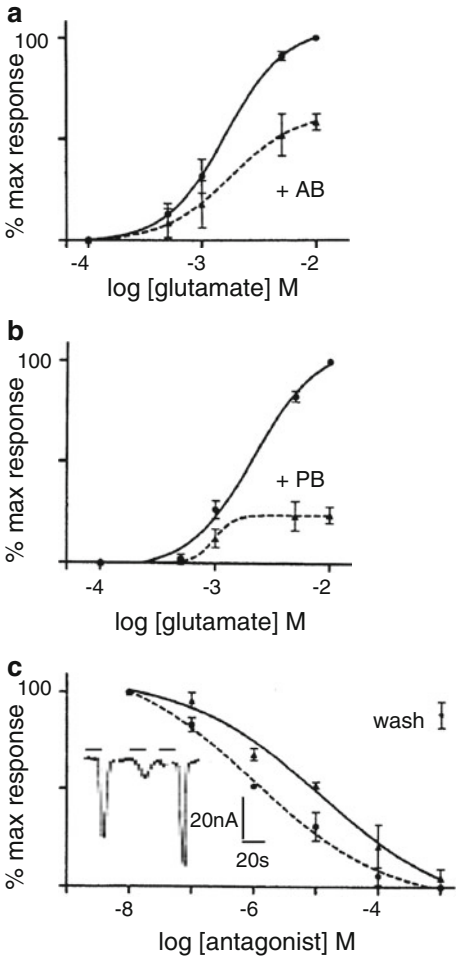

Fig. 3 The effect of barbiturates on glutamate responses in oocytes expressing GluCl$\alpha 1\beta$ heteromeric channels. **a** Concentration–response curves for glutamate in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM amobarbital (*AB*) ($n = 4$). **b** Concentration–response curves for glutamate in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM pentobarbital (*PB*) ($n = 6$). **c** Effect of varying the concentration of pentobarbital (*filled upright triangle*, *solid line*, $n = 5$) or amobarbital (*filled circle*, *dashed line*, $n = 6$) on the response to 1 mM glutamate. The results are presented as the percentage of the maximum response obtained to 1 mM glutamate before the addition of antagonist. The response after a wash (>15 min) is as indicated. The *inset* shows example traces to 1 mM glutamate for GluCl$\alpha 1\beta$ before amobarbital (*left*), in the presence of 100 μM amobarbital (*middle*) and after a wash (>15 min, *right*)

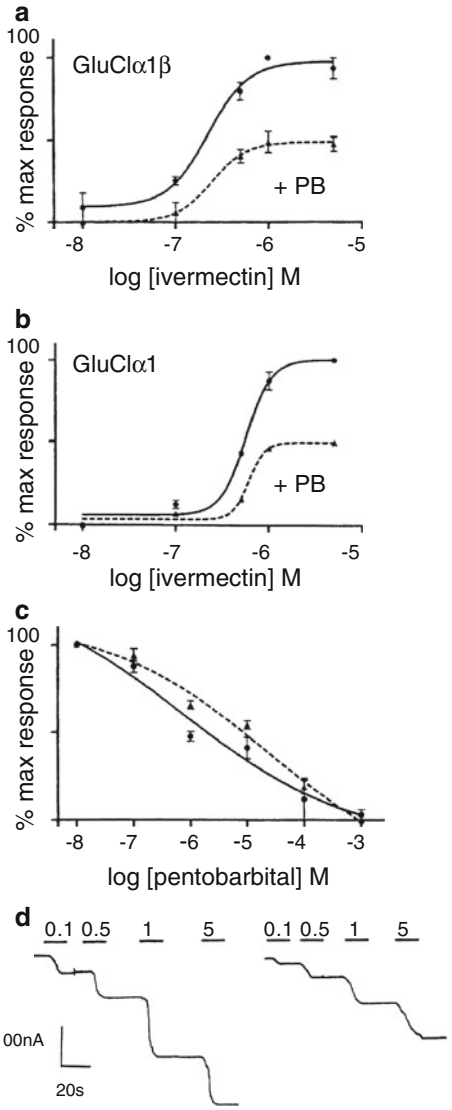

Fig. 4 The effect of pentobarbital on the response of GluCl channels to ivermectin. **a** GluCl$\alpha 1\beta$. Concentration–response curves for ivermectin in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM pentobarbital ($n = 5$). **b** GluClα1. Concentration–response curves for ivermectin in the absence (*filled circle*, *solid line*) and presence (*filled upright triangle*, *dashed line*) of 100 μM pentobarbital ($n = 4$). **c** Effect of varying the concentration of pentobarbital on the response to 500 nM ivermectin for GluCl$\alpha 1\beta$ (*filled circle*, *solid line*, $n = 6$) and for GluClα1 (*filled upright triangle*, *dashed line*, $n = 5$). The results are presented as the percentage of the maximum response obtained to 500 nM ivermectin before the addition of pentobarbital. **d** Example traces of cumulative responses to ivermectin (0.1–5 μM, as indicated) for GluCl$\alpha 1\beta$ before pentobarbital (*left*) and in the presence of 100 μM pentobarbital (*right*)

77 nM. Etter et al. concluded that picrotoxin blocked GluCl channels by binding to a site accessible when the channel was open. Picrotoxin weakly antagonised the action of glutamate on the native receptor in the pharynx of *C. elegans*, for example, 100 μM picrotoxin only reduced the response to 50 μM glutamate by 25% (Pemberton et al. 2001). Using the heteromer expressed in *Xenopus* oocytes, 50 μM picrotoxin reduced the response to 300 μM glutamate by 60% (Etter et al. 1999). Picrotoxin blocks other invertebrate glutamate-gated chloride channels, including the glutamate-gated chloride channels found on locust striated muscle (Cull-Candy 1976; Lea and Usherwood 1973a, b) and glutamate-gated chloride channels of lobster gastric mill muscle where high μM is required (Lingle and Marder 1981). Interestingly, low μM picrotoxinin, the

active component of picrotoxin, partially blocked glutamate-gated chloride channels of *Limulus polyphemus* neurones when glutamate was bath applied (Roberts and Walker 1982). Picrotoxin is potent as an antagonist of many invertebrate and vertebrate GABA-A receptors (Nistri and Constanti 1979). Resistance to picrotoxin can be achieved by mutating the 279 alanine of *C. elegans* GluClβ to threonine which is located in the second membrane-spanning domain (Etter et al. 1999). A similar situation occurs in insect GABA-A receptors, where a point mutation at 279 alanine to serine provides resistance to dieldrin and picrotoxin (ffrench-Constant et al. 1993). This demonstrates the similarity between the glutamate- and GABA-gated chloride channels for the picrotoxin site. Using a chimera receptor composed of the N-terminal of GluClα1 and the C-terminal of GluClβ, Etter et al. (1999) also showed that the C-terminal contained a high affinity-binding site for picrotoxin. In terms of their phylogenetic relationship to other ligand-gated ion channels, it has been proposed that the GluCl receptors of *C. elegans* may be orthologous to the vertebrate glycine receptor (Vassilatis et al. 1997) which itself may be derived from the GABA-A receptor family (Ortells and Lunt 1995).

The two chloride channel blockers used in this study, viz, NPPB and DNDS, are examples of arylaminobenzoates and disulphonate stilbenes, respectively, and have been tested for their blocking actions on a range of chloride channels. There have been relatively few studies where NPPB and DNDS have been tested against ligand-gated chloride channel responses. For example, DNDS, DIDS, and furosemide, 100 μM, reversibly reduced the response of a peptide-gated chloride inhibitory response of *Helix aspersa* neurons while NPPB had no blocking action (Pedder et al. 1998). *H. aspersa* acetylcholine-gated chloride channels are also reversibly blocked by 180 μM SITS, and this effect is non-competitive (Wright and Walker 1993). SITS, 100–500 μM, also reduced the GABA response on dorsal root ganglion neurones in a reversible and non-competitive manner without altering the GABA reversal potential (Gallagher et al. 1983). In contrast, both NPPB and DNDS have been widely studied in terms of their mode of action as blockers of other types of chloride channel. For example, disulphonate stilbenes such as DIDS bind preferentially to the outward-facing state of the anion transporter, AE1, of red blood cells (Jennings et al. 1998). It is likely that they bind to exposed lysine residues producing steric block of the ion transport mechanism (Abuladze et al. 2005). In general, chloride currents in the heart which are regulated by adenylyl cyclase-cAMP-PKA (ICl.PKA) are relatively insensitive to disulphonate stilbenes but are blocked by arylaminobenzoates (Hume et al. 2000). Arylaminobenzoates block the cystic fibrosis transmembrane conductance regulator (CFTR) when added to either side of the membrane while disulphonate stilbenes only block when added to the intracellular side of the membrane (Sheppard and Welsh 1999). The disulphonate stilbenes also inhibit single chloride channels prepared from *Torpedo californica* electroplax and incorporated in planar phospholipids bilayer membranes (Miller and White 1984).

In contrast to the situation with vertebrate GABA-A receptors, barbiturates inhibited both glutamate- and ivermectin-gated responses of *C. elegans* GluCl channels with IC_{50}s in the low μM range. Amobarbital which was potent against glutamate on the heteromer in this study was inactive against the native glutamate-gated chloride channel in the pharynx of *C. elegans* (Pemberton et al. 2001), perhaps reflecting a different subunit stoichiometry in this tissue e.g., the presence of an *avr-15* subunit rather than *glc-1*. In contrast, pentobarbital, 100 μM, enhanced both the amplitude and duration of the GABA responses of cultured locust neurones by up to 70% (Lees et al. 1987). At concentrations above 5 μM, pentobarbital also enhanced ionophoretically applied GABA on locust-dissociated neurones (von Keyserlingk and Willis 1992). However, 300 μM thiopentone reversibly depressed the amplitude of fast chloride-dependent glutamate inhibitory events on snail neurones (Judge et al. 1979). This depression was followed by an increase in duration of the response. These results demonstrate that invertebrate ligand-gated chloride channels can be modulated by barbiturates although the direction of the modulation can vary. It would be of interest to investigate the action of barbiturates on other invertebrate ligand-gated anion channels.

The *Drosophila* GABA-A subunit, RDL, is sensitive to the benzodiazepine, 4-chlorodiazepam but not to flunitrazepam (Hosie and Sattelle 1996) while flunitrazepam, but not diazepam, modulates the action of GABA on locust neurones (von Keyserlingk and Willis 1992). Flunitrazepam-binding sites have been identified in the central nervous system of insects but interestingly the most potent ligand at displacing this binding, Ro5-4864, is selective for the vertebrate peripheral benzodiazepine-binding site (Lummis 1990). However, in the present study, the benzodiazepine, flurazepam, failed to modulate *C. elegans* GluClα1β subunits expressed in oocytes or the native GluCl receptors in the pharynx (Pemberton et al. 2001). It may be that the glutamate-gated chloride channels in nematodes do not possess benzodiazepine modulatory binding sites. Since there are nine glutamate-gated chloride channels in *C. elegans,* it is possible that some of these may be related to mammalian GABA-A γ or δ subunits and may be necessary for benzodiazepine modulation. A third possibility, as demonstrated in insects, is that only certain benzodiazepines may be active on nematode glutamate-gated chloride channels (Nistri and Constanti 1978).

In summary, the current study has provided further information on the sensitivity of *C. elegans* GluCl channels to chloride channel compounds. It confirmed the effect of picrotoxin but failed to find evidence for a benzodiazepine-binding site. However, it has demonstrated that both aryl-aminobenzoates and disulphonate stilbenes act as non-competitive antagonists at these channels. In terms of the barbiturates, we can conclude that *C. elegans* GluCl channels, in common with vertebrate GABA-A-gated chloride channels, have a binding site. Intriguingly, evidence from GABA receptors on *Ascaris* body wall muscle suggests that these channels are insensitive to barbiturates (Holden-Dye et al. 1989). Thus, a nematode glutamate-gated chloride channel, but not a nematode GABA-gated chloride channel, is sensitive to barbiturates. Furthermore, in contrast to the vertebrate GABA-A receptor, barbiturates act as negative, not positive, allosteric modulators of the channels.

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