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RESEARCH PAPER

A molecular characterization of the agonist binding site of a nematode cys-loop GABA receptor

Mark D Kaji*, Ariel Kwaka, Micah K Callanan, Humza Nusrat, Jean-Paul Desaulniers and Sean G Forrester

Faculty of Science, University of Ontario Institute of Technology, Oshawa, ON, Canada

Correspondence
Sean Forrester, Faculty of Science, University of Ontario Institute of Technology, 2000 Simcoe street N, Oshawa, Ontario L1H7K4, Canada. E-mail: sean.forrester@uoit.ca

*Present address: Institute of Parasitology, Macdonald Campus, McGill University, 21111 Lakeshore Road, Ste Anne de Bellevue, H9X 3V9, Canada.

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BACKGROUND AND PURPOSE

Cys-loop GABA receptors represent important targets for human chemotherapeutics and insecticides and are potential targets for novel anthelmintics (nematicides). However, compared with insect and mammalian receptors, little is known regarding the pharmacological characteristics of nematode Cys-loop GABA receptors. Here we have investigated the agonist binding site of the Cys-loop GABA receptor UNC-49 (Hco-UNC-49) from the parasitic nematode *Haemonchus contortus*.

EXPERIMENTAL APPROACH

We used two-electrode voltage-clamp electrophysiology to measure channel activation by classical GABA receptor agonists on Hco-UNC-49 expressed in *Xenopus laevis* oocytes, along with site-directed mutagenesis and *in silico* homology modelling.

KEY RESULTS

The sulphonated molecules P4S and taurine had no effect on Hco-UNC-49. Other classical Cys-loop $GABA_A$ receptor agonists tested on the Hco-UNC-49B/C heteromeric channel had a rank order efficacy of GABA > trans-4-aminocrotonic acid > isoguvacine > imidazole-4-acetic acid (IMA) > (*R*)-(–)-4-amino-3-hydroxybutyric acid [*R*(–)-GABOB] > (*S*)-(+)-4-amino-3-hydroxybutyric acid [*S*(+)-GABOB] > guanidinoacetic acid > isonipecotic acid > 5-aminovaleric acid (DAVA) (partial agonist) > β-alanine (partial agonist). *In silico* ligand docking revealed some variation in binding between agonists. Mutagenesis of a key serine residue in binding loop C to threonine had minimal effects on GABA and IMA but significantly increased the maximal response to DAVA and decreased twofold the EC_{50} for *R*(–)- and *S*(+)-GABOB.

CONCLUSIONS AND IMPLICATIONS

The pharmacological profile of Hco-UNC-49 differed from that of vertebrate Cys-loop GABA receptors and insect resistance to dieldrin receptors, suggesting differences in the agonist binding pocket. These findings could be exploited to develop new drugs that specifically target GABA receptors of parasitic nematodes.

Abbreviations

3-APA, 3-aminopropylphosphonic acid; AHBA, 4-amino-2-hydroxybutyric acid; DAVA, 5-aminovaleric acid; GAA, guanidinoacetic acid; GPA, guanidinopropionic acid; IMA, imidazole-4-acetic acid; P4S, piperidine-4-sulphonic acid; *R*(–)-GABOB, (*R*)-(–)-4-amino-3-hydroxybutyric acid; RDL, resistance to dieldrin; *S*(+)-GABOB, (*S*)-(+)-4-amino-3-hydroxybutyric acid; TACA, trans-4-aminocrotonic acid; ZAPA, (Z)-3-[(Aminoiminomethyl)thio]prop-2-enoic acid sulphate

Tables of Links

TARGETS
Ligand-gated ion channels
$GABA_A$ receptors
$GABA_C$ receptors
Glutamate receptors
Glycine receptors

LIGANDS	
β-Alanine	Isonipecotic acid
Bicuculline	Muscimol
Glutamate	Picrotoxin
Glycine	P4S, piperidine-4-sulphonic acid
GPA, guanidinopropionic acid	Taurine
Isoguvacine	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Nematode Cys-loop GABA receptors have been shown to play key roles in muscle contraction involved in both locomotion and defecation (Schuske *et al.*, 2004). In addition to their unique biological functions, these receptors exhibit a unique pharmacology. One notable feature is the low efficacy of the classical GABA receptor antagonist bicuculline on the Cys-loop GABA receptors UNC-49 and EXP-1 from *Caenorhabditis elegans* (Bamber *et al.*, 2003; Beg and Jorgensen, 2003). This phenomenon has also been observed in the analysis of Cys-loop GABA receptors from somatic muscles of the parasitic nematode *Ascaris suum* (Wann, 1987; Holden-Dye *et al.*, 1988; Martin, 1993). From these observations, it has been suggested that the agonist binding site of nematode GABA receptors exhibit structural differences compared with mammalian $GABA_A$ receptors (Bamber *et al.*, 2003). Indeed, sequence and phylogenetic analysis of the UNC-49 receptor have suggested that they are unlike any subtype of mammalian receptors (Bamber *et al.*, 2003; Siddiqui *et al.*, 2010). Nematode GABA receptors have also been shown to be targets for nematicides such as piperazine (Martin, 1982; Brown *et al.*, 2012; Hernando and Bouzat, 2014). There are, therefore, good reasons for research on the development of new nematicides that target nematode Cys-loop GABA receptors which would have limited activity on GABA receptors from the mammalian host.

The *unc-49* gene encodes three alternatively spliced proteins of which UNC-49B is required for channel activity; UNC-49C has a modulatory role and UNC-49A does not participate in receptor formation (Bamber *et al.*, 1999; 2005). These channels are located at the neuromuscular junction and control body movement via muscle relaxation (Bamber *et al.*, 1999). The UNC-49 receptor has also been shown to bind the classical $GABA_A$ receptor agonist muscimol in both *in vitro* and *in vivo* studies with the latter resulting in *C. elegans* paralysis (McIntire *et al.*, 1993; Richmond and Jorgensen, 1999; Siddiqui *et al.*, 2010). Both the *unc-49b* and *unc-49c* subunit cDNAs have also been isolated and partially characterized from the sheep parasitic nematode *Haemonchus contortus* (Hco-UNC-49). The amino acid sequence of each subunit shares about 80% similarity when compared with the *C. elegans* UNC-49 orthologue subunits (Siddiqui *et al.*, 2010). The *unc-49* gene is also present in the genomes of all parasitic nematodes for which there is genome sequence available (Accardi *et al.*, 2012).

Despite the important biological role these receptors play in a wide variety of both parasitic and free-living nematodes, there is still relatively little known about the characteristics of the agonist binding site. The work presented here describes the results of a comprehensive analysis of the agonist pharmacology of the UNC-49 receptor from *H. contortus* which was complemented by *in silico* homology modelling and site-directed mutagenesis. Based on the overall data, there appear to be several characteristics that are distinct from mammalian $GABA_A$ and insect Cys-loop GABA receptors, suggesting that the agonist binding site exhibits some structural differences.

Methods

Copy RNA (cRNA) synthesis

Hco-unc-49b (Genbank Accession #: EU939734.1) and *Hco-unc-49c* (Genbank Accession #: EU049602.1) cDNA were previously cloned into the expression vector PT7TS and stored in 50% w/v glycerol at –80°C (Dent *et al.*, 1997). Approximately, 0.4–1 μg of linearized plasmid was used for the T7 RNA polymerase mMESSAGE mMACHINE *in vitro* transcription kit from Ambion (Austin, TX, USA). cRNA was DNase treated, precipitated using lithium chloride and resuspended in nuclease-free water.

Site-directed mutagenesis

Amino acid changes to position 215 in Hco-UNC-49B subunits were generated using the QuikChange® site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). Nucleotide sequence verification was provided by Genome Quebec (Montreal, Quebec, Canada). To examine the effect of mutations at this position, all Hco-UNC-49B 215 mutants were co-expressed with Hco-UNC-49C wild-type subunits.

Xenopus laevis *oocyte expression*

All animal care and experimental procedures followed the University of Ontario, Institute of Technology Animal Care Committee and the Canadian Council on Animal Care guide-

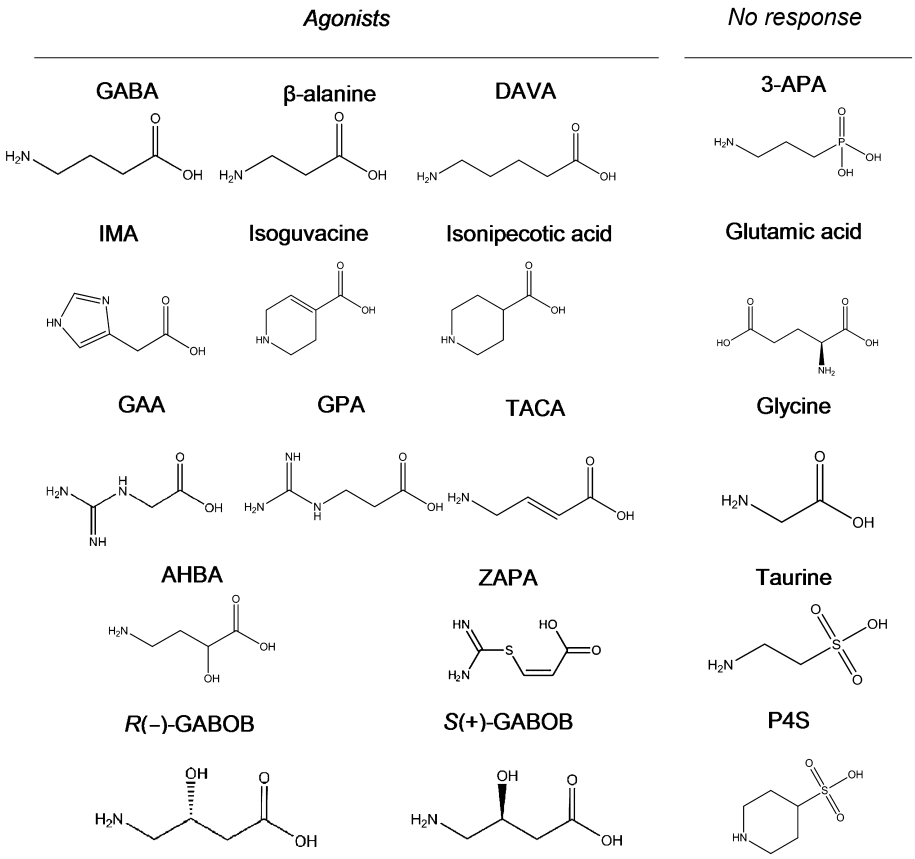

Figure 1

Chemical structures of the compounds assayed for agonist activity on the Hco-UNC-49B and BC receptor complexes using two-electrode voltage-clamp techniques. Compounds that activate Hco-UNC-49 are listed under Agonists, while those that elicited no current response are listed under No response.

lines. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Female *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI, USA). They were housed in a climate-controlled, light-cycled room with regular changes to the water. Frogs were anaesthetized with 0.15% 3-aminobenzoic acid ethyl ester methanesulphonate salt (MS-222) (Sigma-Aldrich, Oakville, ON, Canada), prior to surgical removal of a section of ovary. MS-222 was buffered with $NaHCO_3$ to pH 7 +/– 0.5. Ovarian lobe extraction was followed by a defollliculation treatment of 2 mg·mL^{-1} collagenase-II (Sigma-Aldrich) in calcium-free oocyte Ringer's solution [82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5 (Sigma-Aldrich)]. Defolliculation took place at room temperature under light shaking for 2 h. Collagenase was washed from the oocytes with ND96 solution (1.8 mM $CaCl_2$, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5) and allowed 1 h to recover at 18°C in ND96 supplemented with 275 µg·mL^{-1} pyruvic acid (Sigma-Aldrich) and 100 µg·mL^{-1} of the antibiotic gentamycin (Sigma-Aldrich). Stage V and VI oocytes were selected for cytoplasmic injection of cRNA. Injections were carried out using a Drummond Nanoject II (Drummond Scientific Company, Broomhall, PA, USA). Roughly, 25 ng of cRNA was injected into the selected oocytes. To form heteromeric channels, equal concentrations of *Hco-unc-49b* and *Hco-unc-49c* were mixed and injected into the oocytes. To examine the effect of changes at position 215, mutant *Hco-unc-49b* cRNA was co-injected with wild-type *Hco-unc-49c*. Injected oocytes were stored in supplemented ND96 and allowed to recover and express the cRNA for 48 h.

Compounds tested and their preparations

(*R*)-(-)-4-amino-3-hydroxybutyric acid [*R*(–)-GABOB] was purchased from AstaTech Inc. (Bristol, PA, USA), (Z)-3-[(Aminoiminomethyl)thio]prop-2-enoic acid sulphate (ZAPA) and isoguvacine hydrochloride were purchased from Tocris Bioscience (Minneapolis, MN, USA). All other compounds were purchased from Sigma-Aldrich. These are GABA, β-alanine, 5-aminovaleric acid (DAVA), imidazole-4-acetic acid (IMA), guanidinoacetic acid (GAA), 4-amino-2-hydroxybutyric acid (AHBA), isonipecotic acid, trans-4-aminocrotonic acid (TACA), guanidinopropionic acid (GPA), (*S*)-(+)-4-amino-3-hydroxybutyric acid [*S*(+)-GABOB], 3-aminopropylphosphonic acid (3-APA), glutamic acid, glycine, piperidine-4-sulphonic acid (P4S) and taurine (see Figure 1). Initial millimolar stock concentrations were dissolved in ND96. Water insoluble compounds GPA and GAA

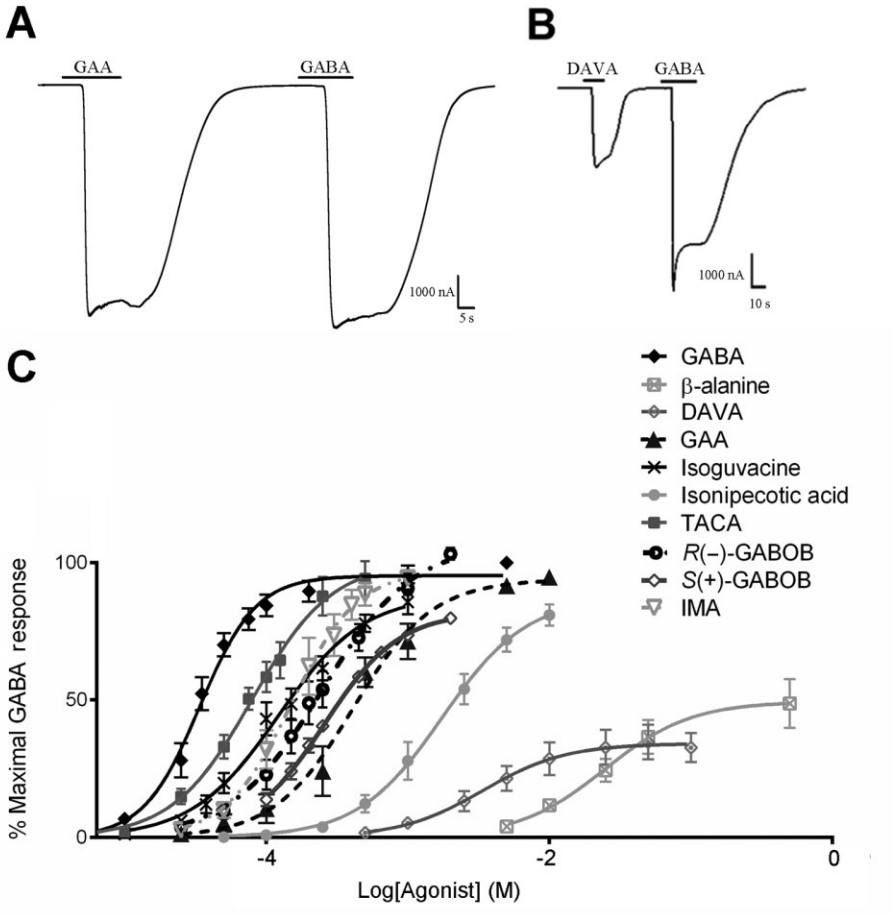

Figure 2

Comparative electrophysiological recordings of the maximal currents produced on the Hco-UNC-49BC receptor by 500 μM GABA with (A) 10 mM GAA and (B) 25 mM DAVA. (C) Concentration-response curves comparing agonist responses relative to maximal GABA; each data point is a mean ± SEM with $n > 3$.

were dissolved in 100% DMSO. For these solutions, 0.1% DMSO was added to ND96 wash solution and used in electrophysiological recordings.

Electrophysiological recordings

Channel activity was measured using the two-electrode voltage-clamp technique, utilizing an Axoclamp900A amplifier (Molecular Devices, Sunnyvale, CA, USA). Oocytes were clamped at –60 mV through microelectrodes filled with 3 M KCl (1–5 MΩ resistance), connected to Axon Instruments headstages (Molecular Devices) via Ag|AgCl wires. Borosilicate glass microelectrodes were created using a P-97 Flaming/Brown micropipette puller (Sutter Instruments Company, Novato, CA, USA). Oocytes were exposed to various compounds using a gravitational flow system into an RC-1Z perfusion chamber (Warner Instruments Inc., Holliston, MA, USA). ND96 was used to wash compounds from the oocytes once a maximal current response was achieved. Relative concentration-response curves were generated on single oocytes expressing the Hco-UNC-49 receptors by administering each compound at incremental increases in concentration (i.e. administered from low to high concentration) with an ND96 wash between each dose. The maximal response of each compound was determined relative to that of the maximal GABA response (Figure 2).

Data analysis

Concentration-response curves were produced using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Curves were generated using the equation from Prism's log[agonist] versus normalized response-variable slope setting:

$$I_{max} = \frac{1}{1+\left(\frac{EC_{50}}{[D]}\right)^{h}}$$

where I_{max} is the maximal current response of the agonist, EC_{50} is the concentration of agonist that produces half maximal response, $[D]$ is the concentration of agonist and h is the Hill slope.

Averaged EC_{50} values, along with their SEM were calculated from at least four replicate oocytes from two separate batches. However, the typical number of replicates was ≥ 5 and the overall number of replicates for each compound tested was based on the number of oocytes that survived

testing and were of good quality. Statistical analysis was performed using Student's *t*-test, where indicated, in order to determine significance ($P < 0.05$).

Homology modelling

The *C. elegans* glutamate-gated chloride channel crystal structure (PDB 3RIF) was used as a template in MODELLER 9.14 (Sali and Blundell, 1993) for the generation of Hco-UNC-49B extracellular domain homodimer. Previous research has suggested that the agonist binding site lies between two Hco-UNC-49B subunits (Accardi and Forrester, 2011). The most energetically favourable models were assessed by their DOPE score and with PROCHECK Ramachandran plot analysis. USCF Chimera 1.6.1 was used for model analysis and molecular distance measurements (Pettersen *et al.*, 2004). Sequence alignments were generated using ClustalW (Larkin *et al.*, 2007).

Computational agonist docking

Energetically reduced zwitterion ligands were obtained from the Zinc database, http://zinc.docking.org/ (Irwin *et al.*, 2012). AutoDock tools (Morris *et al.*, 2009) were used to prepare molecules for hypothetical docking performed by AutoDock Vina (Trott and Olson, 2010). The centre of the 30 × 30 × 30 Å docking search box was placed in the predicted aromatic box of the agonist binding site. A maximum of 50 binding models all within a range of 5 kcal·mol^{-1} from the best scoring pose were generated for each agonist.

Results

Pharmacological profile of Hco-UNC-49

Application of GABA to oocytes injected with *Hco-unc-49b* produced an EC_{50} of 76 ± 6 μM, whereas oocytes injected with a mixture of *Hco-unc-49b/c* produced an EC_{50} of 59 ± 8 μM. These current responses were of microampere amplitude, concentration-dependent and comparable with previous work in our laboratory (Siddiqui *et al.*, 2010; Accardi and Forrester, 2011). Oocytes injected with water elicited no current responses to any of the compounds tested in this study within the concentration ranges used.

Of all the compounds tested against Hco-UNC-49, those that displayed no current responses (at the maximum concentration indicated), were glycine (5 mM), taurine (5 mM), P4S (5 mM), 3-APA (500 μM) and glutamic acid (5 mM). Compounds that displayed initial agonist activity were analysed by means of concentration-response curves (Figure 2C). From these experiments, the rank order efficacy for Hco-UNC-49B/C was determined to be GABA > TACA > isoguvacine > IMA > *R*(−)-GABOB > *S*(+)-GABOB > GAA > isonipecotic acid > DAVA > β-alanine. GPA (5 mM) only achieved 3% of a maximal GABA current. ZAPA and AHBA also weakly activated the channel at maximal concentrations of 500 μM and 100 mM, achieving 11% and 46% of maximal GABA responses respectively (Table 1). DAVA and β-alanine were weak partial agonists with maximal responses of 33% and 49%, respectively, on the Hco-UNC-49B/C channel.

In addition to the UNC-49B/C heteromeric channels, the nematode UNC-49B subunit can readily form homomeric channels in *Xenopus* oocytes. However, because it has not been determined which receptor form (i.e. UNC-49B/C or UNC-49B) is present in *H. contortus*, the Hco-UNC-49B homomeric channel was also examined for most of the ligands. Both the homomeric and heteromeric channels shared a very similar trend in their rank order efficacy and partial agonism for all the compounds examined in this study. Moreover, consistent with other studies (Siddiqui *et al.*, 2010; Accardi and Forrester, 2011), the homomeric channel generally exhibited a lower sensitivity to agonists compared with the heteromeric channel (Table 1).

The role of serine 215

Previous research on $GABA_A$ and $GABA_C$ receptors found that a conserved threonine at positions 202 and 244, respectively, in binding loop C plays a key role in the activation of the channel by GABA where a change to a serine causes a significant change in GABA sensitivity (Amin and Weiss, 1993; 1994). Recently, a T244S mutation in the $GABA_C$ receptor was found to have a more dramatic effect on molecules such as IMA, *R*(−)-GABOB and *S*(+)-GABOB, nearly eliminating sensitivity or causing them to shift from agonist to antagonist (Yamamoto *et al.*, 2012a). Interestingly, in the analogous position (215) of the UNC-49B subunit, the naturally occurring residue is a serine (Figure 3A). Previous work from our laboratory found that mutating this serine to a threonine in Hco-UNC-49B had very little effect on GABA sensitivity compared with wild-type receptors (Accardi and Forrester, 2011). However, changing this residue to the chemically different alanine (S215A) caused a 250-fold reduction in the sensitivity of the receptor to GABA (data not shown). To further examine the role of this position, we analysed other agonist responses of the S215T receptor. Interestingly, like GABA, the S215T change had little effect of the sensitivity of the agonist IMA. In comparison, a more noticeable effect of this mutation was observed with GABOB where S215T exhibited a moderate twofold decrease in the EC_{50} of both *R*(−)-GABOB and *S*(+)-GABOB (Table 2). For the partial agonist DAVA, the introduction of a threonine at this position did not change the EC_{50} value but did significantly increase the maximal current response (Figure 3B).

Homology modelling and docking

The *C. elegans* glutamate-gated chloride channel crystal structure (PDB 3RIF) was used as a template for homology modelling of the Hco-UNC-49B extracellular domain (Hibbs and Gouaux, 2011). Figure 4A depicts the resulting homodimer with GABA docked into the binding pocket between the principal and complementary subunits. GABA docked into the defined binding pocket of Hco-UNC-49 in an elongated conformation of the alkyl backbone (Figure 4B). The docked GABA molecule aligned its carboxyl group with Arg66 of loop D. In addition, the GABA amine-nitrogen atom docks 4.6 Å from Tyr166 in loop B and 3.6 Å from Tyr218 of loop C, implying involvement of either or both residues in π-cation interactions. All other full agonists docked in a similar pose with their amine-nitrogen atom docking in similar distances from Tyr166 and Tyr218 and their carboxyl group in proximity to Arg66. However, the partial agonist β-alanine docked with its amine-nitrogen atom farther from both aromatic residues. In addition, compared with the other agonists, the

Table 1

EC_{50}, Hill slope and maximal current responses of agonists at the heteromeric and homomeric Hco-UNC-49 channels

Compounds	Hco-UNC-49BC EC_{50} ± SEM (μM) (*Hill slope ± SEM*)	% Maximal GABA	*n*	Hco-UNC-49B EC_{50} ± SEM (μM) (*Hill slope ± SEM*)	% Maximal GABA	*n*
GABA	59 ± 8 *(2.5 ± 0.42)*	100	9	76 ± 6 *(2.62 ± 0.12)*	100	7
TACA	78 ± 5 *(2.25 ± 0.33)*	103	11	125 ± 18 *(2.2 ± 0.03)*	99	4
Isoguvacine	99 ± 12 *(1.95 ± 0.3)*	86	14	119 ± 20 *(1.66 ± 0.19)*	61	4
IMA	175 ± 21 *(1.93 ± 0.17)*	94	11	235 ± 19 *(2.19 ± 0.19)*	94	13
R(−)-GABOB	234 ± 43 *(1.67 ± 0.11)*	103	6	–	–	–
S(+)-GABOB	382 ± 22 *(1.11 ± 0.07)*	80	6	–	–	–
GAA	482 ± 106 *(1.39 ± 0.15)*	95	5	432 ± 33 *(1.98 ± 0.27)*	98	6
Isonipecotic acid	1725 ± 362 *(1.78 ± 0.23)*	81	4	5343 ± 1523 (1.12 ± 0.1)	70	6
DAVA[a]	3914 ± 520 *(1.47 ± 0.18)*	31	7	4350 ± 290 *(1.47 ± 0.12)*	8	7
β-alanine[a]	25721 ± 2806 *(1.49 ± 0.19)*	49	7	40201 ± 6166 *(1.5 ± 0.1)*	15	6
GPA	5 mM activates		6	5 mM activates		6
	2.54 ± 0.18%[b]			0.23 ± 0.04%[b]		
AHBA	100 mM activates		3	100 mM activates		3
	46.3 ± 9.8%[b]			29.6 ± 0.3%[b]		
ZAPA	500 μM activates		6	–	–	–
	10.5 ± 1.8%[b]					

Maximal responses are reported as a percentage of a saturating GABA response. Corresponding replicate numbers, *n*, are included. Rank order efficacy for the Hco-UNC-49BC channel is presented in descending order. Italics in parentheses represent Hill slope data. AHBA, 4-amino-2-hydroxybutyric acid; DAVA, 5-aminovaleric acid; GAA, guanidinoacetic acid; GPA, guanidinopropionic acid; IMA, imidazole-4-acetic acid; *R*(−)-GABOB, (*R*)-(−)-4-amino-3-hydroxybutyric acid; *S*(+)-GABOB, *S*)-(+)-4-amino-3-hydroxybutyric acid; TACA, trans-4-aminocrotonic acid; ZAPA, *Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid sulphate.

[a]Partial agonist.

[b]Percentage activation compared with a maximal GABA response (500 μM).

β-alanine molecule fails to extend as far across the binding cleft and as such its amine group is the furthest away from Glu164 (Figure 5).

R(−)-GABOB and *S*(+)-GABOB, bound with similar positioning of their carboxyl groups, oriented closer to Arg66 than that of GABA. The amine group of *R*(−)-GABOB positions slightly closer to Tyr166 compared with *S*(+)-GABOB. The 3-hydroxy groups of both stereoisomers were placed close (~3 Å) to Ser215 with the more efficacious *R*(−)-GABOB directed closer to this residue.

Discussion

The nematode UNC-49 receptor has been previously investigated regarding its sensitivity to allosteric modulators and anthelmintics such as ivermectin and piperazine (Bamber *et al.*, 2003; Brown *et al.*, 2012; Hernando and Bouzat, 2014). However, little is known regarding the characteristics of its agonist binding site or its sensitivity to a range of classical GABA receptor agonists. To address this, we describe a pharmacological profile of a range of classical GABAeric compounds on the nematode Cys-loop GABA receptor UNC-49.

Comparison of the pharmacological profile with in silico *modelling*

Based on the pharmacological analysis, it appears that there is a size restriction that dictates partial and full agonism in addition to efficacy (Table 1). This length-efficacy link is highlighted by the molecules glycine, β-alanine, GABA and DAVA. An increase or decrease of a single carbon in the backbone of GABA prevents maximal channel activation and reduces efficacy by an order of magnitude. Glycine does not activate the UNC-49 receptor while β-alanine and DAVA are partial agonists with low efficacy. Ligand-docking results have also revealed certain trends in the activation of the UNC-49 receptor by agonists. In all cases, the amine-nitrogen atom of all agonists docked between Tyr166 and 218 with its position closer to Tyr218. A recent molecular dynamics simulation of GABA docking to the insect resistance to dieldrin (RDL) GABA receptor also found that the amine-nitrogen of GABA docked between the analogous residues Phe206 and Tyr254 at a distance of 5.87 and 3.93 Å respectively. Both of these residues have been shown to be involved in π-cation interactions which are essential for ligand binding (Ashby *et al.*, 2012). Our docking results have also indicated that, compared with the full agonists, β-alanine docks with its amine

group farthest from both Tyr166 and Tyr218 and DAVA with its amine group closest to Tyr218.

Across from these putative π-cation contributing residues is Arg66 that may bind the negatively charged carboxyl group of a ligand. The majority of agonists dock with their carboxyl group roughly 3 Å from this residue. An R66S mutation of Hco-UNC-49B greatly reduces GABA activation of the Hco-UNC-49 channel (Accardi and Forrester, 2011). This Arg-carboxyl association has been previously described for $GABA_A$ (Wagner *et al.*, 2004), $GABA_C$ (Harrison and Lummis, 2006), RDL (McGonigle and Lummis, 2010) and glycine receptors (Pless *et al.*, 2011), suggesting a conserved role for this residue. The carboxyl group of β-alanine is positioned close to Arg66 but its amino group, when compared with the other agonists, is further from Glu164, a residue which has been shown in the RDL receptor to form an essential ionic or hydrogen bond with GABA (Ashby *et al.*, 2012). Interestingly, at the RDL receptor, β-alanine is a full agonist and has about a 25–30-fold higher efficacy compared with its efficacy at Hco-UNC-49 (Hosie and Sattelle, 1996; McGonigle and Lummis, 2010) demonstrating some differences in the binding site and the binding of β-alanine between nematode and insect Cys-loop GABA receptors.

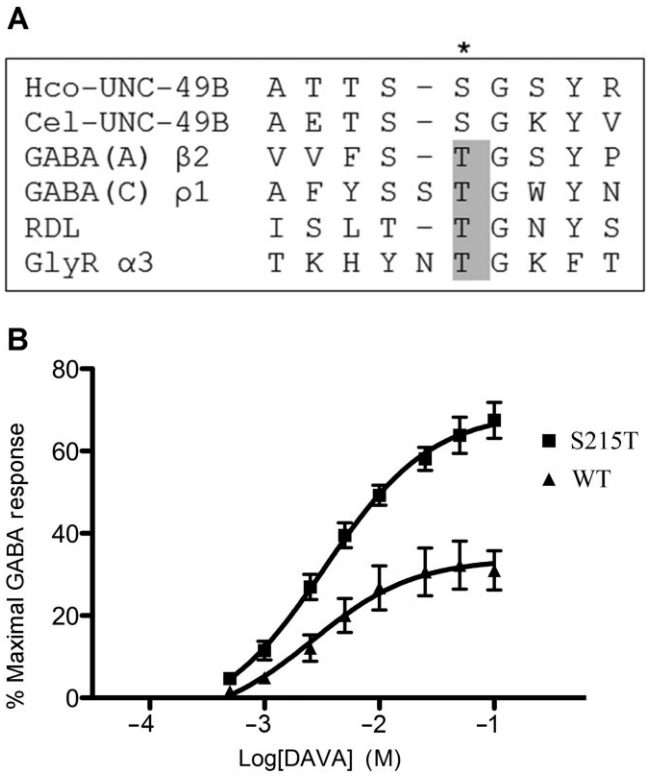

Figure 3

(A) Sequence alignment of Hco-UNC-49B loop C residues with other Cys-loop receptors (*Caenorhabditis elegans* UNC-49, human $GABA_A$ β2, human $GABA_C$ ρ1, *Drosophila melanogaster* RDL and human GlyR α3). The analogous positioning of the S215 residue mutated in this study is denoted by *. (B) DAVA concentration-response curves comparing wild-type Hco-UNC-49BC and S215T receptors. Each data point is a mean ± SEM with $n > 5$.

The cyclic compounds isoguvacine and isonipecotic acid contain a bulky piperidine in place of an amine group. The lower efficacy of isonipecotic acid compared with isoguvacine has been previously observed for both vertebrate (Kusama *et al.*, 1993) and invertebrate (Hosie and Sattelle, 1996) GABA receptors and may be a result of the reduced planarness of isonipecotic acid (Woodward *et al.*, 1993) caused by the lack of a double bond in the ring structure.

Previous research has shown that nematode Cys-loop GABA receptors including Hco-UNC-49 are also targets for nematicides such as piperazine (Martin, 1982; Brown *et al.*, 2012; Hernando and Bouzat, 2014). Unlike zwitterions such as GABA, piperazine lacks a carboxyl group which is likely to cause it to bind differently to GABA receptors and possibly interact with different residues. Indeed, there is relatively little known about the interaction of piperazine with key binding site residues on Cys-loop GABA receptors. Because of this lack of knowledge, we did not include a model of piperazine for this study, as a more extensive examination using both *in silico* modelling and mutagenesis would be required to reveal the characteristics of its binding site and mechanism of activation.

The role of S215 in the Hco-UNC-49 receptor

Previous research has shown that a threonine residue in loop C is important for the ability of several agonists to activate

Table 2

EC_{50} and maximal current responses of IMA, DAVA and the enantiomers of GABOB at wild-type Hco-UNC-49BC and S215T receptor

	Wild type		S215T	
	EC_{50} ± SEM (μM)	% Maximal GABA	EC_{50} ± SEM (μM)	% Maximal GABA
R(–)-GABOB	234 ± 43	103 ± 2	129 ± 20	101 ± 2
S(+)-GABOB	382 ± 22	80 ± 2	194 ± 31*	90 ± 2
IMA	175 ± 20	94 ± 3	163 ± 56	94 ± 3
DAVA	3914 ± 520	33 ± 5	3900 ± 227	67 ± 4*

Maximal responses are reported as a percentage of a saturating GABA response. $n \geq 5$ for all experiments. DAVA, 5-aminovaleric acid; IMA, imidazole-4-acetic acid; *R*(–)-GABOB, (*R*)-(–)-4-amino-3-hydroxybutyric acid; *S*(+)-GABOB, *S*)-(+)-4-amino-3-hydroxybutyric acid.
*Significantly different from wild type ($P < 0.001$).

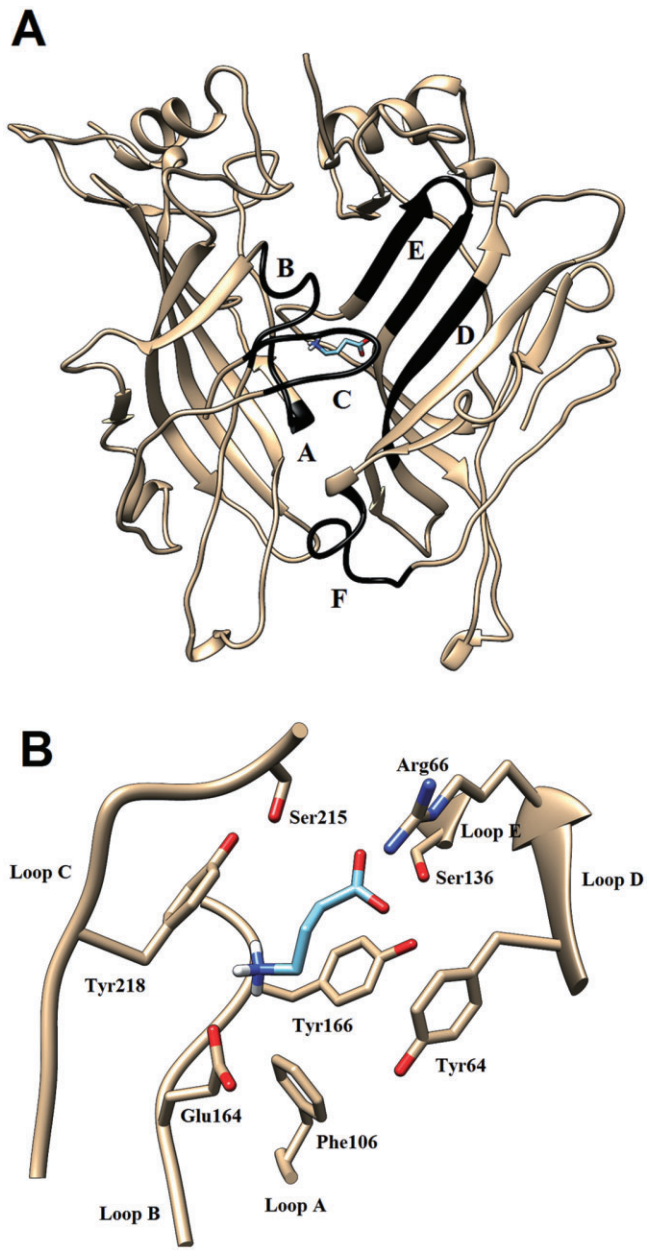

Figure 4

(A) Homology model of the Hco-UNC-49B extracellular domain homodimer. The discontinuous binding loops of the principal (A–C) and complementary (D–F) subunits are labelled and highlighted in black with a GABA molecule situated in the putative binding pocket. (B) A teal GABA molecule docked in the aromatic box. Relevant side chains of the residues (beige) that make up the binding pocket are labelled. Nitrogen and oxygen are coloured blue and red respectively.

the $GABA_C$ receptor (Yamamoto *et al.*, 2012a). T244S mutagenesis yielded a functional receptor but with a significant increase in the EC_{50} of GABA compared with wild type receptors. *R*(−)-GABOB failed to achieve maximal current gating at 1 mM and *S*(+)-GABOB displayed antagonist properties (Yamamoto *et al.*, 2012a). Interestingly, Hco-UNC-49 has a serine in the analogous position (S215). Like other Cys-loop GABA receptors, this position in Hco-UNC-49 appears to play an essential role in the binding site as its mutation to alanine (S215A) dramatically affects GABA activation. However, all other Cys-loop GABA receptors have the chemically similar threonine in the analogous position and the reverse mutation (S215T) introduced into Hco-UNC-49 did not significantly alter the EC_{50} of GABA (Accardi and Forrester, 2011), nor that of IMA (current study). However, this mutation did result in a moderate twofold increase in the efficacy of both enantiomers of GABOB and significantly enhanced the maximal response of the partial agonist DAVA. These results are consistent with other studies suggesting that this position is important for channel gating (Amin and Weiss, 1994; Yamamoto *et al.*, 2012a). It is possible that in Hco-UNC-49, the replacement of serine to a threonine at position 215 is somehow changing the position of DAVA allowing a more favourable interaction with key residues. Overall, these observations indicate that the naturally occurring serine seems to play, to a certain extent, a negative role in the activity of several agonists and may partially explain the distinct pharmacology of the nematode UNC-49 receptor.

The pharmacological profile of Hco-UNC-49 in comparison with vertebrate Cys-loop GABA receptors

There is now a sizable body of evidence suggesting that there is a diversity of invertebrate and bacterial GABA-gated ion channels that do not fall under the umbrella categorization of any vertebrate Cys-loop GABA receptor (Martin, 1993; Hosie and Sattelle, 1996; Bamber *et al.*, 2003; Thompson *et al.*, 2012). In this study, there were several noticeable differences between the profiles of the nematode in comparison with vertebrate GABA receptors. IMA was a full agonist for the Hco-UNC-49 and RDL receptors (Hosie and Sattelle, 1996), but a partial agonist for vertebrate GABA receptors (Kusama *et al.*, 1993). In addition, the nematode GABA receptor was insensitive to sulphonated compounds such as taurine and P4S which have been shown to exhibit efficacy at the $GABA_A$ receptor (Kusama *et al.*, 1993; Woodward *et al.*, 1993; del Olmo *et al.*, 2000). However, overall, the pharmacological profile appears to match more closely to vertebrate $GABA_A$ receptors than $GABA_C$ receptors. For example, GAA and DAVA both show antagonist properties at $GABA_C$ but are agonists of Hco-UNC-49 and $GABA_A$ receptors (Woodward *et al.*, 1993; Neu *et al.*, 2002; Chebib *et al.*, 2009; Yamamoto *et al.*, 2012b). One exception is the sensitivity of the Hco-UNC-49 receptor to the enantiomers of GABOB. Here, both the wild-type and S215T UNC-49 receptors were more sensitive to *R*(−)-GABOB than *S*(+)-GABOB, which is more consistent with $GABA_C$ receptors (Roberts *et al.*, 1981; Hinton *et al.*, 2008). However, taken together, Hco-UNC-49 channels exhibit a pharmacological profile, distinct from those of their vertebrate counterparts.

Similarity to the Ascaris *GABA receptor*

The pharmacological profile of the UNC-49 receptor shares resemblance to that observed for the well-characterized *Ascaris* muscle receptor. First, most of the agonists analysed for activity at the Hco-UNC-49 receptor including muscimol

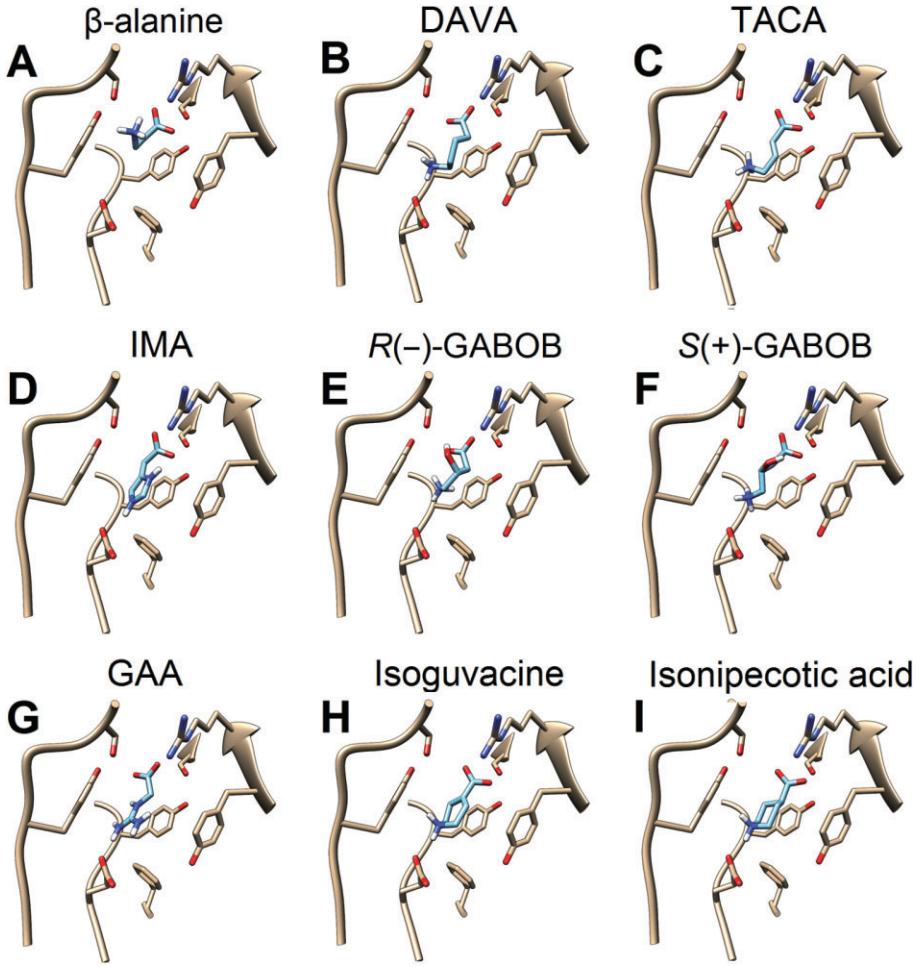

Figure 5

Comparative docking of agonists into the extracellular orthosteric binding site of Hco-UNC-49B. Shown are the residues (beige) of the defined aromatic box, with oxygen labelled red and nitrogen blue. Teal molecules in panels are: (A) β-alanine, (B) DAVA, (C) TACA, (D) IMA, (E) *R*(−)-GABOB, (F) *S*(+)-GABOB, (G) GAA, (H) isoguvacine and (I) isonipecotic acid.

are found to be less potent than GABA with similar trends in their rank order efficacy to those from *Ascaris* muscle receptors (Holden-Dye *et al.*, 1988; 1989; Siddiqui *et al.*, 2010). In addition, the different sensitivities of Hco-UNC-49 to the enantiomers of GABOB (i.e. R > S) are the same as observed for the *Ascaris* receptor (Holden-Dye *et al.*, 1989). Furthermore, the UNC-49 and the *Ascaris* GABA receptors are also unresponsive to sulphonated agonists of $GABA_A$ receptors (Holden-Dye *et al.*, 1989), relatively resistant to known vertebrate GABA receptor blockers such as picrotoxin (Holden-Dye *et al.*, 1988; Bamber *et al.*, 2003; Brown *et al.*, 2012) and competitive antagonists such as bicuculline (Holden-Dye *et al.*, 1988; Bamber *et al.*, 2003) and are not potentiated by benzodiazepines (Holden-Dye *et al.*, 1989; Bamber *et al.*, 2003). Finally, the UNC-49 and the previously characterized *Ascaris* GABA receptors are both located in muscle tissue (Holden-Dye *et al.*, 1989; Bamber *et al.*, 2005; Hernando and Bouzat, 2014). However, one difference between the UNC-49 and the *Ascaris* receptor is the efficacy of ZAPA which is very low at Hco-UNC-49 but is equal to that of GABA at the *Ascaris* receptor (Holden-Dye and Walker, 1988). This difference could possibly be attributed to differences in the receptor agonist binding site between species and/or how the receptors were examined (overexpression in oocytes verses *in situ*). Nevertheless, it appears that the UNC-49 receptor could be an attractive target for novel nematicides as it not only appears to exhibit a unique pharmacology but is also found in a wide range of parasitic nematodes and plays an important role in the function of the neuromuscular junction which is essential for locomotion and, probably, parasite-specific movement within the host (Accardi *et al.*, 2012).

In conclusion, we have elucidated the agonist pharmacology of the nematode Cys-loop GABA receptor UNC-49. Overall, the agonist profile is closer to that of $GABA_A$ than $GABA_C$ receptors, but the closest match is the *Ascaris* muscle receptor. Results from this study also provide further evidence that the agonist binding site of the UNC-49 receptor has a unique structure and sensitivity to various agonists and thus is a good candidate for both drug screening and rational drug design. Furthermore, as the overall sequence of the UNC-49 receptor differs significantly from vertebrate Cys-loop GABA

receptors, other possible drug binding sites could be investigated and used as a tool for nematocidal discovery.

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Author contributions

M. D. K., M. K. C., A. K. and H. N. performed the research. S. G. F. and M. D. K. designed the research study. J.-P. D. provided intellectual contributions, reagents and contributed to manuscript preparation. M. D. K. and A. K. analysed the data. M. D. K. and S. G. F. wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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