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The *Haemonchus contortus* UNC-49B subunit possesses the residues required for GABA sensitivity in homomeric and heteromeric channels

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ABSTRACT

Hco-UNC-49 is a GABA receptor from the parasitic nematode *Haemonchus contortus* that has a relatively low overall sequence similarity to vertebrate GABA receptors but is very similar to the UNC-49 receptor found in the free living nematode *Caenorhabditis elegans*. While the nematode receptors do share >80% sequence similarity they exhibit different sensitivities to GABA. In addition, the UNC-49C subunit appears to be a positive modulator of GABA sensitivity in the *H. contortus* heteromeric channel, but is a negative modulator in the *C. elegans* heteromeric channel. The cause(s) of these differences is currently unknown since the structural elements essential for GABA sensitivity in nematode receptors have been largely unexplored. Thus, the overall aim of this study was to investigate the residues that are important for UNC-49 receptor sensitivity through the use of homology modeling, site-directed mutagenesis, and two-electrode voltage clamp. This study revealed that Met^{170} in Loop B of the GABA binding-site may partially account for the observed differences in GABA receptor sensitivity between the nematode species. Residues in Loops A–D that have been reported to form the GABA binding pocket in mammalian receptors, including those forming the conserved 'aromatic box', also appear to play analogous roles in Hco-UNC-49. In addition, the two mutations that produced the most significant reduction in GABA sensitivity were R66S and Y166S. Homology modeling indicates that these two residues share a hydrogen bond and are positioned close to the carboxyl end of the GABA molecule. However, of residues examined in this study, only those on the Hco-UNC-49B subunit and not its subunit partner, Hco-UNC-49C, appear important for GABA sensitivity. Overall, results from this study suggest that the binding site of the UNC-49 heteromeric GABA receptor exhibits some differences compared to classical vertebrate $GABA_A$ receptors.

1. Introduction

Fast synaptic neurotransmission in both vertebrate and invertebrate systems is mediated, in part, by a large superfamily of ligand-gated ion channels (LGICs) known as Cys-loop receptors. In mammals, the Cys-loop superfamily can be broadly categorized as excitatory receptors [nicotinic acetylcholine receptors (nAChR), serotonin ($5\text{-}HT_3$) receptors] and inhibitory receptors (glycine or GABA-gated chloride channels) [1]. In the nematode *Caenorhabditis elegans*, inhibitory neurotransmission is mediated, in part, by ionotropic GABA receptors (UNC-49 receptors) which are concentrated at neuromuscular junctions where they play a key role in locomotion [2,3]. These nematode GABA receptors differ from mammalian $GABA_A$ receptors in sequence homology, pharmacology and overall function [4].

The *unc-49* gene encodes for three GABA receptor subunits (UNC-49A, UNC-49B and UNC-49C) which are involved in *C. elegans* locomotion [2]. However, only UNC-49B and UNC-49C are expressed at physiologically relevant levels and co-assemble to form the native receptor *in vivo* [2,5]. While UNC-49C does not appear to be essential for receptor function *in vivo*, its association with UNC-49B causes a decreased sensitivity to GABA as well as the channel blocker picrotoxin [2,4] and contributes directly to neurosteroid sensitivity [6]. The UNC-49 channel is also present in other nematodes such as the sheep parasite *Haemonchus contortus* [7]. However, the *H. contortus* UNC-49 channel possesses a 2.5-fold higher sensitivity to GABA compared to the *C. elegans* channel [7]. Another difference between the nematode channels is that UNC-49C appears to be a positive modulator of GABA sensitivity in the *H. contortus* heteromeric channel, but is a negative modulator in the *C. elegans* heteromeric channel [2,7]. These differ-

Abbreviations: $5\text{-}HT_3$, serotonin (5-hydroxytryptamine)-gated ion channel receptor; AChBP, acetylcholine binding protein; cRNA, copy RNA; LGIC, ligand-gated ion channels; MS-222, 3-aminobenzoic acid ethyl ester, methanesulphonate salt; nAChR, nicotinic acetylcholine receptor.

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ences may be attributed to differences within the agonist binding pocket of each nematode receptor. However, the essential elements involved in GABA sensitivity in nematode GABA receptors remain largely unknown. Consequently, it is not known whether UNC-49C directly contributes to GABA binding when associated in a channel complex with UNC-49B. A binding site involving both UNC-49B and UNC-49C would be analogous to mammalian $GABA_A$ receptors where the binding site lies on the interface of an α and β subunit [8].

Knowledge regarding the molecular elements required for mammalian $GABA_A$ receptor activation has stemmed from mutational analysis, photoaffinity labeling, radioligand-binding assays and *in silico* homology modeling [9–13]. From these studies it is generally accepted that the GABA binding site is comprised of the interactions between six discontinuous loops (Loops A–F) found within the extracellular domains of interacting subunits; Loops A–C of the principle subunit and Loops D–F of the adjacent subunit [8]. These loops can be readily observed in homology models using the related acetylcholine binding protein (AChBP) as a template [12,14,15]. Parallel mutagenesis and radioligand-binding assay studies have identified key residues within these loops that are essential for ligand binding and channel activation [9,10,12,16–19]. Of particular importance are the 'aromatic box' residues from Loops A–D which have been shown to be essential for GABA binding via direct interactions with the GABA molecule [12,15] or stablization of the binding pocket [8,15].

While there is ample knowledge regarding the structures important for mammalian GABA receptor activation, investigations into the elements involved in invertebrate GABA receptor sensitivity have been limited. Nonetheless, a recent study has identified several aromatic box residues within the *Drosophila melanogaster* RDL GABA receptor that may contribute to the binding site [20]. Indeed, the extensive information that is available regarding the structure/function of mammalian GABA receptors has now provided a good opportunity to begin to translate this knowledge to GABA receptors from invertebrate organisms. This is particularly important for our understanding of the UNC-49 group of subunits which appear to be unique to nematodes [21] and have been shown to exhibit differences in their sensitivity to various agonists and antagonists compared to mammalian $GABA_A$ receptors [4,7]. It is also important to note that the residues important for GABA sensitivity are not entirely equivalent between different GABA receptors [20]. Therefore, determining how the *H. contortus* UNC-49 agonist binding site compares to that of mammalian and other invertebrate $GABA_A$ receptors should contribute to our overall understanding of the structure and function of GABA receptors and possibly to the discovery of novel pesticides and anthelmintics.

The aim of this study was to evaluate the residues important for UNC-49 channel activation and at the same time investigate the subunit determinants required for GABA sensitivity. Through site-directed mutagenesis and homology modeling, this study has uncovered several amino acid residues in Loops A–D in the UNC-49 channel that are important for GABA sensitivity. However, in all loops examined, only mutations introduced in UNC-49B, and not UNC-49C, affected GABA sensitivity. Additionally, there is at least one amino acid in Loop B that may partially account for the observed differences in GABA sensitivity between *H. contortus* and *C. elegans* UNC-49 channels.

2. Materials and methods

2.1. Homology modeling and ligand docking

The protein coding sequence of Hco-UNC-49B (GenBank accession number ACL14329) and Hco-UNC-49C (ABW22635) were aligned with the coding sequence of the AChBP (P58154) using the align2d command in MODELLER 9v7 [22]. The crystal structure of the AChBP in the HEPES-bound state at 2.7 Å resolution (Protein Data Bank ID 1I9B) was selected as the template for homology modeling. A three-dimensional model of the extracellular region of the Hco-UNC-49 receptor was constructed using the default parameters in MODELLER through multi-subunit modeling in which one to five subunits were modeled in a single run by repeatedly aligning the template sequence to the desired number of Hco-UNC-49 subunit sequences. A total of 50 models were generated to compensate for low sequence identity. The most energetically favorable models were evaluated for violations in stereochemical, volume, and surface properties using PROCHECK [23] and Ramachandran plot analysis (RAMPAGE, http://mordred.bioc.cam.ac.uk/~rapper/rampage.php; [24]. All models were viewed and images generated using UCSF Chimera v 1.4.1 [25]. Protonated structural models of GABA were generated using ChemBio3D Ultra 12.0 (CambridgeSoft, Cambridge, UK) and were energy minimized using the MM2 force field. Docking of GABA into the Hco-UNC-49 receptor homology model was conducted using DOCK v 6.4. The predicted aromatic box residues Tyr^{64}, Phe^{106}, Tyr^{166} and Tyr^{218} were used to define the binding site with a radius of 10 Å.

2.2. Mutation introduction and in vitro transcription of Hco-UNC-49

The coding sequences of Hco-UNC-49B and Hco-UNC-49C were sub-cloned into a pT7TS transcription vector [26]. Introduction of the mutations in the Hco-UNC-49 coding sequence was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified using DNA sequencing (Genome Quebec). Linearized versions of the constructs (100–600 ng) were then used as a template in the mMessage mMachine *in vitro* transcription reaction using T7 RNA polymerase provided in a capped RNA transcription kit (Ambion, Austin, TX, USA). Capped *Hco-unc-49* copy RNA (cRNA) was precipitated using lithium chloride and subsequently resuspended in H_2O at a final concentration of 0.5 ng/nl. Approximately 10–25 μg of cRNA was generated per *in vitro* transcription reaction.

2.3. Expression of Hco-UNC-49 in Xenopus laevis oocytes

Oocytes were surgically removed from *X. laevis* females (Nasco) while anesthetised using 0.15% (w/v) 3-aminobenzoic acid ethyl ester, methanesulphonate salt (MS-222, pH 7; Sigma, Oakville, ON, CA), buffered using sodium bicarbonate at pH 7. Harvested oocytes were partitioned into small clusters of less than 20 oocytes and defolliculated using type II collagenase solution (2 mg/mL; Sigma) in OR2 buffer (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes pH 7.5) for 2 h under light shaking at room temperature. Oocytes were then transferred into ND96 frog saline (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES) supplemented with 275 μg/ml pyruvate (a carbon source), and gentamycin (100 μg/ml; Sigma). Oocytes were cytoplasmically injected with 50 nl of capped *Hco-unc-49* cRNA (0.5 ng/nl; wild-type and mutants) using the Drummond Nanoject microinjector (Broomhall, PA, USA). Incubation and maintenance of the injected oocytes occurred over a 1–4 day period at 20 °C while submerged in supplemented ND96. Continual replacement of supplemented ND96 occurred approximately every 12 h. Electrophysiological recordings were performed 2–5 days, post cRNA injection.