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Comparative pharmacology of flatworm and roundworm glutamate-gated chloride channels: Implications for potential anthelmintics

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ABSTRACT

Pharmacological targeting of glutamate-gated chloride channels (GluCls) is a potent anthelmintic strategy, evidenced by macrocyclic lactones that eliminate numerous roundworm infections by activating roundworm GluCls. Given the recent identification of flatworm GluCls and the urgent need for drugs against schistosomiasis, flatworm GluCls should be evaluated as potential anthelmintic targets. This study sought to identify agonists or modulators of one such GluCl, SmGluCl-2 from the parasitic flatworm *Schistosoma mansoni*. The effects of nine glutamate-like compounds and three monoterpenoid ion channel modulators were measured by electrophysiology at SmGluCl-2 recombinantly expressed in *Xenopus laevis* oocytes. For comparison with an established anthelmintic target, experiments were also performed on the AVR-14B GluCl from the parasitic roundworm *Haemonchus contortus*. L-Glutamate was the most potent agonist at both GluCls, but L-2-aminoadipate, D-glutamate and D-2-aminoadipate activated SmGluCl-2 (EC_{50} 1.0 ± 0.1 mM, 2.4 ± 0.4 mM, 3.6 ± 0.7 mM, respectively) more potently than AVR-14B. Quisqualate activated only SmGluCl-2 whereas L-aspartate activated only AVR-14B GluCls. Regarding the monoterpenoids, both GluCls were inhibited by propofol, thymol and menthol, SmGluCl-2 most potently by thymol (IC_{50} 484 ± 85 μM) and least potently by menthol (IC_{50} > 3 mM). Computational docking suggested that agonist and inhibitor potency is attributable to particular interactions with extracellular or membrane-spanning amino acid residues. These results reveal that flatworm GluCls are pharmacologically susceptible to numerous agonists and modulators and indicate that changes to the glutamate γ-carboxyl or to the propofol 6-isopropyl group can alter the differential pharmacology at flatworm and roundworm GluCls. This should inform the development of more potent compounds and in turn lead to novel anthelmintics.

1. Introduction

Flatworm parasites are responsible for an astounding disease burden in the developing world. This is exemplified by blood flukes that currently inflict schistosomiasis on hundreds of millions of people (Gryseels et al., 2006; King, 2010). The disease can be deadly but in most cases causes a prolonged morbidity (van der Werf et al., 2003; King et al., 2005) that is clearly associated with oppressive poverty in the affected societies (King, 2010). Treatment of schistosomiasis relies largely on the drug praziquantel, which has been very successful (Doenhoff et al., 2008). A refractory period in juvenile flukes (Pica-Mattoccia and Cioli, 2004; Botros et al., 2005), however, likely renders praziquantel ineffective in areas of high transmission (Gryseels et al., 2001; Doenhoff et al., 2008), and perhaps more alarmingly, very few drugs are available as alternative treatments (Thetiot-Laurent et al., 2013). There is thus a great need for new anthelmintics for schistosomiasis (Caffrey and Secor, 2011; Thetiot-Laurent et al., 2013).

Abbreviations: ECD, extracellular domain; *cis*-ACBD, *cis*-1-aminocyclobutane-1,3-dicarboxylate; GABA, γ-aminobutyric acid; $GABA_AR$, type A γ-aminobutyric acid receptor; GluCl, glutamate-gated chloride channel; GlyR, glycine receptor; iGluR, (tetrameric) ionotropic glutamate receptor; pLGIC, pentameric ligand-gated ion channel (or Cys-loop receptor); TMD, transmembrane domain.

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One of the most effective anthelmintic targets thus far is the glutamate-gated chloride channel (GluCl) of roundworms, a membrane-bound receptor-channel in neuronal and muscle cells, where it mediates an inhibitory chloride current in response to neurotransmitter binding (Wolstenholme, 2012). It is a uniquely invertebrate member of the pentameric ligand-gated ion channel (pLGIC) family, also known as Cys-loop receptors. GluCls are closely related to mammalian glycine receptor (GlyR) and Type A GABA receptor ($GABA_AR$) pLGICs (Dent, 2006). The potency of the GluCl as an anthelmintic target is demonstrated by the widely used macrocyclic lactone, ivermectin, which binds with high affinity to the membrane-spanning domain of roundworm GluCls, irreversibly activating a chloride current (Dent et al., 2000; Lynagh and Lynch, 2012b). In the parasitic nematode, this depresses motor, sensory and secretory systems (Kass et al., 1980; Perry, 2001; Moreno et al., 2010), which serves to eliminate nematode infections from millions of humans suffering from diseases such as river blindness and lymphatic filariasis (Omura, 2008).

Despite their successful treatment of numerous roundworm infections, macrocyclic lactones are largely ineffective against flatworms, with practically no activity in flukes (Shoop et al., 1995) and relatively low potency at tapeworms (Campbell et al., 1983; Perez-Serrano et al., 2001). Consequently, flatworm GluCls did not appear relevant as anthelmintic targets. Indeed, it was unclear if GluCls were even present in flatworms until very recently, when four GluCl subunits, SmGluCl1-4, were isolated from *Schistosoma mansoni* (Dufour et al., 2013). SmGluCl-2 and -3 form homomeric channels that are not activated by macrocyclic lactones, but their robust responses to L-glutamate, together with the conservation of similar transcripts in other flatworms (Dufour et al., 2013), suggest that their pharmacological modulation could constitute a novel treatment for a wide range of flatworm parasites. Furthermore, these channels reveal similarities with roundworm GluCls, suggesting that it may be possible for certain compounds to target both roundworm and flatworm GluCls, potentially leading to anthelmintics of an unprecedented broad spectrum. A recent high-resolution structure of a homomeric roundworm GluCl from *Caenorhabditis elegans* has outlined general pLGIC architecture and precisely defined the binding sites for glutamate and ivermectin (Hibbs and Gouaux, 2011). Glutamate binds in the extracellular domain (ECD), between principal Loops A, B and C of one subunit and complementary Loops D, E, F and G of an adjacent subunit. Ivermectin occupies a cavity between adjacent subunits in the transmembrane domain (TMD), which in mammalian pLGICs contains binding sites for various modulators of agonist-induced activation.

In the present work, a flatworm GluCl was examined as a pharmacological target in comparison to a roundworm GluCl that is already established as a useful anthelmintic target. To this end, the SmGluCl-2.1 from *S. mansoni* and the AVR-14B GluCl from *Haemonchus contortus* were recombinantly expressed in *Xenopus laevis* ooctyes, and both channels were tested for activation or modulation by several compounds. These GluCls were selected according to their qualities representative of other GluCls from the respective phyla: SmGluCl-2.1 shows robust responses to glutamate and is phylogenetically similar to numerous other flatworm GluCls, both trematode and cestode (Dufour et al., 2013); AVR-14B is highly conserved in parasitic roundworms (Beech et al., 2010), has typical roundworm GluCl ivermectin sensitivity (McCavera et al., 2009) and is a verified nematicidal target (Glendinning et al., 2011). Compounds were selected due to their analogy with known agonists that bind to the ECD or modulators that bind to the TMD of other pLGICs. Several compounds acted as moderate-to-low affinity agonists or inhibitors, suggesting sites for potential anthelmintic compounds are possessed by flatworm and roundworm GluCls alike.

2. Materials and methods

2.1. Drugs, chemicals, reagents

S. mansoni SmGluCl-2.1 (hereafter referred to as SmGluCl-2; (Dufour et al., 2013); in the pT7TS vector) and *H. contortus* AVR-14B (in pT7TS) cDNAs were kind donations from Professor Timothy Geary (Institute of Parasitology, McGill University, Montréal, Canada) and Professor Adrian Wolstenholme (Department of Infectious Diseases, University of Georgia, Athens, GA, USA), respectively. The AVR-14B Arg95Ala mutant cDNA was constructed using mutagenesis primers synthesized by Eurofins MWG Operon (Ebersberg, Germany) and the Quikchange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Böblingen, Germany), and it was confirmed by DNA sequencing (Eurofins MWG Operon). XbaI was purchased from Fisher Scientific Germany GmbH (Schwerte, Germany). The mMESSAGE mMACHINE T7 Kit for transcription was purchased from Life Technologies GmbH (Darmstadt, Germany). Chemicals and drugs were purchased from AppliChem GmbH (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Sigma–Aldrich (Munich, Germany) or Tocris Bioscience (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany).

2.2. Electrophysiological experiments

X. laevis oocytes were obtained, defolliculated and stored as previously described (Lynagh et al., 2013). After cDNA linearization with XbaI and cRNA synthesis with the mMESSAGE mMACHINE T7 kit, 4 ng cRNA was injected into defolliculated oocytes, and oocytes were stored in frog Ringer's solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4 with NaOH; 50 μg/mL gentamycin). 2–5 days later, oocytes were transferred to a recording chamber and constantly perfused with bath solution (115 mM NaCl, 1 mM KCl, 1.8 mM CaCl2, 10 mM HEPES; pH 7.4 with NaOH). Oocytes were two electrode voltage-clamped at −70 mV with micropipettes filled with 3 M KCl. Currents were filtered at 200 Hz and sampled at 1000 Hz with a Geneclamp 500B amplifier, Digidata 1322A interface and Clampex software (Molecular Devices, Sunnyvale, CA, USA). Currents were measured in response to increasing concentrations of L-glutamate or other agonists, each dissolved in bath solution. Modulation of L-glutamate-induced currents was tested by co-applying increasing concentrations of the compound in question with the half maximal effective concentration (EC_{50}) of L-glutamate.

2.3. Amino acid sequence alignments, homology modeling and dockings

Amino acid alignments were performed with ClustalX2 (Larkin et al., 2007). To estimate the binding sites for the compounds tested, comparative models of SmGluCl-2 and AVR-14B were built on the template crystal structure of the *C. elegans* GLC-1 GluCl (PDB entry 3RIF; (Hibbs and Gouaux, 2011)) using Modeller (Eswar et al., 2006). Computational docking was performed with AutoDock Vina including flexible side chains (Trott and Olson, 2010). Glutamate and related compounds were docked to each model within a cube of sides 20 Å encompassing the L-glutamate binding site identified in the *C. elegans* GLC-1 GluCl (Hibbs and Gouaux, 2011). Modulators were docked to a $28 \times 28 \times 25$ Å volume in the extracellular half of the TMD, including intrasubunit cavities of two adjacent