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A constitutively active G protein-coupled acetylcholine receptor regulates motility of larval *Schistosoma mansoni*

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ABSTRACT

The neuromuscular system of helminths controls a variety of essential biological processes and therefore represents a good source of novel drug targets. The neuroactive substance, acetylcholine controls movement of *Schistosoma mansoni* but the mode of action is poorly understood. Here, we present first evidence of a functional G protein-coupled acetylcholine receptor in *S. mansoni*, which we have named SmGAR. A bioinformatics analysis indicated that SmGAR belongs to a clade of invertebrate GAR-like receptors and is related to vertebrate muscarinic acetylcholine receptors. Functional expression studies in yeast showed that SmGAR is constitutively active but can be further activated by acetylcholine and, to a lesser extent, the cholinergic agonist, carbachol. Anti-cholinergic drugs, atropine and promethazine, were found to have inverse agonist activity towards SmGAR, causing a significant decrease in the receptor's basal activity. An RNAi phenotypic assay revealed that suppression of SmGAR activity in early-stage larval schistosomulae leads to a drastic reduction in larval motility. In sum, our results provide the first molecular evidence that cholinergic GAR-like receptors are present in schistosomes and are required for proper motor control in the larvae. The results further identify SmGAR as a possible candidate for antiparasitic drug targeting.

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1. Introduction

Schistosomiasis is a debilitating, chronic infection that affects over 200 million people in 74 endemic countries. Trematodes of the genus *Schistosoma* are the causative agents of the disease [1], with *S. mansoni* responsible for nearly half the infections. Currently, there is a single therapeutic option, praziquantel, and no vaccine is available. Reports of emerging resistance to praziquantel [reviewed in [2]], as well as its lack of efficacy against the migratory larval stages of the parasite [3] underpin the need to develop new therapeutic targets. One area that has been especially productive in the search for new drug targets is the parasite nervous system, exemplified by the success of ivermectin, pyrantel and the more recently discovered octadepsipeptides [4].

The schistosome nervous system is involved in a variety of processes that are essential to parasite survival including migration, attachment, feeding and reproduction [5]. It is hypothesized to play a role in signal transduction via synaptic and paracrine mechanisms, as schistosomes lack a circulatory system and thus the capability for classical endocrine signaling. The key interaction controlling neuronal signaling in schistosomes involves neuroactive compounds binding to their cognate receptors and eliciting effects directly or via second messenger cascades [reviewed in [6–8]]. These receptors fall into two broad classes: the Cys-loop ligand-gated ion channels and the metabotropic, heptahelical G protein-coupled receptors (GPCR). Sequencing of the *S. mansoni* genome [9,10] has provided a large complement of putative neuroreceptors from both classes. Several have been cloned and characterized, including receptors for dopamine, histamine, glutamate and serotonin [11–17]. Relatively less, however, is known about the cholinergic system of schistosomes.

Acetylcholine (ACh) is a quaternary amine neurotransmitter that elicits a variety of biological effects. In vertebrates, ACh acts primarily as an excitatory neurotransmitter and controls processes such as muscular contraction, glandular secretion and memory formation [18]. ACh plays a similar excitatory role among invertebrates and its role in nematode motor function is well characterized. A notable exception to the excitatory role of ACh occurs in schistosomes, where there is evidence of ACh acting as a major inhibitory neurotransmitter or modulator. Activation of ACh receptors in *S. mansoni* manifests as muscular relaxation resulting in

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flaccid paralysis [19,20]. Schistosomes have several putative ACh receptors that may be responsible for this phenomenon [reviewed in [7]]. The majority of these receptors are nicotinic ion channels, some of which have been cloned and characterized *in vitro* [21,22]. However, two muscarinic cholinergic receptors are also predicted. One of these appears to be truncated (Smp_152540) [10] but the other has all the structural features of a full-length GPCR and is worthy of further investigation.

Muscarinic acetylcholine receptors (mAChRs) are members of the heptahelical GPCR superfamily and are structurally related to rhodopsin (Family A GPCRs). They mediate their effects by interaction with heterotrimeric G proteins, causing changes in intracellular Ca^{2+} or cyclic adenosine monophosphate (cAMP). The term "muscarinic" is derived from these receptors' preferential binding and activation by the fungal toxin muscarine [23]. There are 5 subtypes of mAChRs in vertebrate organisms [reviewed in [24]]. Vertebrate mAChRs are located in both the central and peripheral nervous systems and are involved in a vast array of physiological processes such as memory, smooth muscle contraction and regulation of neurotransmitter release. Invertebrate mAChRs, also known as G protein-coupled acetylcholine receptors (GARs), share this functional diversity with their vertebrate homologs. Three GAR subtypes have been identified in parasitic and free-living nematodes [25–28]. Similar to vertebrate receptors, they may act in either an excitatory or inhibitory manner and are located on neurons contributing to several important nematode activities, such as muscular contraction, sensory perception and reproduction. Although structural similarity and broad expression patterns define the invertebrate GARs and vertebrate mAChRs as homologs, there are significant differences in their pharmacological profiles [28,29]. This unique pharmacology, combined with their functional importance, marks helminth GARs as promising targets for antiparasitics.

In the present work, we describe the first functional analysis of a schistosome GAR (SmGAR), possibly the only full-length G protein-coupled acetylcholine receptor in *S. mansoni*. SmGAR is distantly related to nematode GARs and its expression is predicted to be highly up regulated during the early larval stages of the parasite [10]. Functional analysis in a heterologous system determined that SmGAR has high basal activity, consistent with a constitutively active receptor, but it is further activated by cholinergic agonists. Furthermore, RNAi phenotypic assays revealed that silencing of SmGAR causes significant disruption of larval motility, suggesting a potentially important role in early parasite migration.

2. Materials and methods

2.1. Parasites

Biomphalaria glabrata snails infected with a Puerto Rican strain of *S. mansoni* were generously provided by the Biomedical Research Institute and BEI Resources, MD, USA. Cercariae were obtained by exposing 6–8 week-old snails to bright light [30] for 2 h. Cercariae were then transformed into larval schistosomulae *in vitro* by mechanical shearing [30]. Schistosomulae were washed with Opti-MEM containing antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin and Fungizone 0.25 μg/ml) and cultured for 1–3 days in Opti-MEM (no antibiotics) supplemented with 6% fetal bovine serum at 37 °C/5% CO_2. Adult worms were recovered by portal perfusion [30] from adult female CD1 mice 7 weeks post-infection with 250 freshly shed cercariae/mouse.

2.2. Cloning of SmGAR

Total RNA was extracted from either pooled adult worms or 24-h-old schistosomulae, using Trizol (Invitrogen) or the RNeasy Micro Kit (Qiagen), according to manufacturers' instructions. RNA was reverse-transcribed (RT) using MML-V and Oligo-dT primer (Invitrogen). A negative control reaction lacking MML-V reverse transcriptase (-RT), was used to rule out the possibility of contamination of cDNA with genomic DNA. Primers to amplify the full length, predicted coding sequence of Smp_145540 (SmGAR) were designed using Oligo 6.2 [31]. Primer sequences were as follows: Forward 5′-ATGAATCTATTATTTTGTTTTC-3′ and Reverse 5′-TTATAATCTTCTAAAATCACC-3′. A proofreading Phusion High Fidelity Polymerase (New England Biolabs) was used for PCR amplification according to standard protocols. Cycling conditions were as follows: 98 °C/30 s, 30 cycles of 98 °C/10 s, 54 °C/60 s, 72 °C/60 s and a final extension of 72 °C/5 min. All PCR products were ligated to the pJet1.2 Blunt cloning vector (Thermo Scientific) and verified by DNA sequencing of at least two independent clones.

2.3. Bioinformatics

The predicted protein sequence of SmGAR (Smp_145540) was used as a query for a BLASTp search of the NCBI non-redundant protein dataset. Homologs were aligned with SmGAR using PROMALS3D [32] and the resulting multiple sequence alignment was then inspected manually to ensure the correct alignment of highly conserved Family A GPCR transmembrane (TM) motifs. Residues of interest are described both according to their numerical position in the primary SmGAR sequence and the Ballesteros and Weinstein numbering system for Family A GPCRs [33], which is shown as a superscript. The Ballesteros and Weinstein designator describes the TM helix where the residue is located (TM 1–7) and its position within the helix relative to a reference residue. The reference is an invariant amino acid of each TM helix, which is arbitrarily given the number 50. Thus, for example, the invariant reference for TM 3 in the schistosome receptor is $Arg248^{3.50}$ (position 3.50) and $Asp230^{3.32}$ is a TM 3 aspartate located eighteen residues upstream from the conserved reference (position 3.32). This system is used throughout the study to compare equivalent TM residues from different receptors. Identification of TM regions was performed by TMHMMv2.0 [34] and comparison of SmGAR with crystal structures of vertebrate GPCRs available in the general Protein Database (PDB), including the human β2-adrenergic receptor (PDB Accession# 2rh1) and the rat M3 muscarinic receptor (4daj). A neighbor-joining phylogenetic tree with 1000 bootstrap replicates was built from the multiple sequence alignment and visualized with FigTree 3.0 (http://tree.bio.ed.ac.uk/software/figtree/). Accession numbers of the sequences used in the alignment can be found in Table S1.

2.4. Yeast expression

Full-length SmGAR was ligated into a previously described yeast expression vector, Cp4258 [28,35]. The resulting construct (Cp4258–SmGAR) was confirmed by DNA sequencing and used to transform *Saccharomyces cerevesiae* strain Cy13393 (*MATαP*$_{FUS1}$*-HIS3 GPA1-Gαi2(5) can1 far1Δ1442 his3 leu2 lys2 sst2Δ2 ste14::trp1::LYS2 ste18γ6-3841 ste3Δ1156 tbt1-1 trp1 ura3*); kindly provided by J. Broach, Penn State University. This strain expresses the *HIS3* gene under the control of the *FUS1* promoter [35] and also includes an integrated copy of a chimeric Gα gene in which the first 31 and last 5 codons of the native yeast Gα (GPA1) were replaced with those of human $G\alpha_{i2}$ subunit. Strains containing $G\alpha_q$ and $G\alpha_s$ were also tested but found to yield no receptor activity when compared to Cy13393. Yeast was cultured according to a previously established protocol [28] until mid-log phase. Yeast (200 μl) were then transformed by the lithium acetate method using 200 μg of carrier DNA and 1 μg of Cp4258–SmGAR or empty plasmid as a negative control and positive transformants were selected on

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