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Glutamate-mediated signaling in *Schistosoma mansoni*: A novel glutamate receptor is expressed in neurons and the female reproductive tract

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ABSTRACT

L-Glutamate is a major neurotransmitter of both vertebrates and invertebrates. Earlier studies have shown that glutamate stimulates neuromuscular activity in the bloodfluke, *Schistosoma mansoni*, but its mode of action is unknown. Here we describe a novel glutamate receptor in *S. mansoni* (SmGluR), the first of its kind to be identified in a parasitic flatworm. SmGluR belongs to the G protein-coupled receptor (GPCR) superfamily and is distantly related to metabotropic glutamate receptors from other species. The full-length receptor cDNA was cloned, stably expressed in HEK-293 cells and shown to be activated by glutamate, whereas aspartate and the glutamate derivative, gamma-aminobutyric acid (GABA) had no significant effect. Among the classical (mammalian) agonists and antagonists tested, only LY341495 was able to interact with the schistosome receptor, suggesting that the pharmacological profile of SmGluR is substantially different from that of receptors in the host. The presence of SmGluR in the parasite was verified by immunoprecipitation and Western blot analyses, using a specific peptide antibody. Confocal immunolocalization studies revealed that SmGluR is strongly expressed in the nervous system of adult worms and larvae. In the adults, the receptor was detected in the longitudinal nerve cords and cerebral commissures, as well as the peripheral nerve fibers and plexuses innervating the acetabulum and the somatic musculature. Outside the nervous system, SmGluR was detected along the length of the female reproductive system, including the oviduct, ootype and the uterus. A comparative expression analysis at the RNA level revealed that SmGluR is expressed at about the same level in cercaria and adult stages, as determined by quantitative reverse-transcription PCR. The results identify SmGluR as an important neuronal receptor and provide the first molecular evidence for a glutamate signaling system in schistosomes.

1. Introduction

The amino acid L-glutamate is an important neurotransmitter of vertebrates and many invertebrate phyla. Glutamate mediates its effects by interacting with different types of receptors, both ionotropic-gated channels and metabotropic glutamate receptors (mGluRs). The latter belong to the G protein-coupled receptor (GPCR) superfamily (Class C) and are structurally related to metabotropic gamma-aminobutyric acid (GABA) and Ca^{2+}-sensing receptors. mGluRs have a distinctive modular structure, consisting of a large N-terminal extracellular domain, followed by a seven transmembrane (7-TM) anchoring segment and an intracellular C-terminal tail of variable length. The N-terminal extracellular domain carries a conserved Venus Flytrap (VFT) module, which contains the glutamate-binding site, and is separated from the 7-TM region by a short cysteine-rich linker [1]. mGluRs are further subdivided into three major groups based on sequence similarity, pharmacological properties and signal transduction pathways [2]. Group I includes mGluR1 and mGluR5 along with their splice variants; they are typically coupled to Gq/11 proteins and signal through changes in intracellular calcium and the inositol phospholipid pathway. In contrast, Group II (mGluR2 and mGluR3) and Group III receptors (mGluR4, mGluR6, mGluR7 and mGluR8) bind to Gi/o proteins and signal primarily through inhibition of adenylate cyclase and a decrease in cellular cAMP [3].

The bloodfluke *Schistosoma mansoni* is the major causative agent of schistosomiasis, a public health problem that affects more than 210 million people worldwide. *S. mansoni* is responsible for the majority of cases in sub-Saharan Africa, the Middle East, the Caribbean and South America [4]. In the absence of a vaccine, which is still lacking for all helminth infections, chemotherapy is the only way to combat this disease. Currently the control of schistosomiasis rests with a single drug, praziquantel, which is effective against adult worms but not the larval stages. Recent reports of sporadic resistance to praziquantel in different parts of the world [5,6] underscore the need for alternative therapies. The schisto-

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some nervous system is an attractive target for development of new therapeutic drugs. Being an acoelomate, *S. mansoni* lacks the capacity for endocrine signaling and must rely entirely on the nervous system to control vital functions, particularly those that involve the musculature such as movement and attachment to the host. Disruption of neuronal signaling with a specific drug would be expected to interfere with these essential activities and ultimately cause the worm to be eliminated from the host.

Neurotransmitter receptors are considered to be strong targets for drug discovery and schistosomes have a rich diversity of neuroactive substances and receptors that could be targeted in this manner [7,8]. Some of these receptors have been cloned and characterized [9–14] but there are many more encoded in the genome that have not been investigated. Glutamate receptors are particularly promising targets because of their involvement in fast excitatory transmission [15]. Many of the neuroactive drugs under development for treatment of human diseases work by targeting glutamate receptors [16–18] and the mainstay of nematode control, ivermectin, also works through a glutamate receptor. The *S. mansoni* genome encodes several putative glutamate-gated channels, mGluRs and one unusual glutamate binding protein that may be unique to flatworms ([8], Taman and Ribeiro; unpublished results). These receptors could prove to be important drug targets and are worthy of further investigation.

Glutamate is a well established neurotransmitter in helminths, both nematodes and flatworms. The most compelling evidence for a glutamatergic neuronal system comes from studies of *Caenorhabditis elegans*. In *C. elegans*, glutamate plays a major role in the control of locomotion and mediates a variety of locomotory behaviors associated with foraging, feeding, mechanosensory and avoidance responses [19]. The effects on locomotion are exerted indirectly, through receptors located in the central nervous system, rather than through direct effects on the musculature. Flatworms also employ glutamate within their nervous system but the mode of action is less clear. Glutamate-containing neurons have been identified in *Hymenolepis diminuta* [20] and *Fasciola hepatica* [21]. In *S. mansoni*, glutamate-like immunoreactivity was detected throughout the nervous system, including the cerebral ganglia, commissures and longitudinal nerve cords. Earlier studies reported that glutamate induced dose-dependent muscle contraction in isolated muscle fibers of adult *S. mansoni* [22]. More recently, researchers described a putative glutamate binding site in extracts of *S. mansoni* [23] and showed that worms treated with a glutamate agonist (kainate) exhibit powerful body wall contractions and hyperkinesias [23]. These observations suggest that glutamate has neuromuscular activity in schistosomes and highlight the potential of glutamate receptors for drug targeting.

Here we describe a novel metabotropic glutamate receptor in *S. mansoni*, the first to be identified in any of the parasitic helminths. The receptor responds to glutamate when expressed heterologously in mammalian cells but differs from mammalian mGluRs with respect to mechanism of signaling and pharmacological profile, consistent with a receptor that diverged early in evolution. An extensive *in situ* immunolocalization analysis reveals that SmGluR is widely distributed in the nervous system and it is also associated with the female reproductive tract.

2. Materials and methods

2.1. Neurochemicals

L-Glutamate, aspartate and γ-aminobutyric acid (GABA) were purchased from Sigma. (S)-3,5-DHPG ((S)-3,5-dihydroxyphenylglycine), DCG IV (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine), L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid), (*S*)-MCPG ((*S*)-a-methyl-4-carboxyphenylglycine), LY 341495 ((2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) from Tocris Bioscience, USA.

2.2. Parasites

A Puerto Rican strain of *S. mansoni* was used in all the experiments in this study. Sporocyst-infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute (Bethesda, MD, USA). Cercarial shedding was performed as previously described [13]. Adult *S. mansoni* worms were obtained by portal perfusion of CD1 female mice infected 6–8 weeks previously with approximately 150 cercaria/mouse.

2.3. Cloning of SmGluR

A BLAST analysis of an early version of the *Schistosoma mansoni* genome database (v3.1) identified a predicted coding sequence (Smp_012620) that has some characteristics of family C GPCRs and shares homology with metabotropic glutamate receptors from other species. This sequence was named SmGluR and it was cloned from reverse-transcribed *S. mansoni* cDNA by PCR. In brief, RNA was isolated from adult *S. mansoni* using TRIZOL® (Invitrogen) and reverse transcription (RT) was performed with M-MLV reverse transcriptase (Invitrogen) and oligo$(dT)_{12-18}$ primer, according to standard protocols. The resulting cDNA was used to amplify the open reading frame (ORF) with sequence-specific primers targeting the beginning and the end of the predicted coding sequence. The primer sequences were as follows: sense primer (5′-ATGGATATTGACCGTTTTATTTC-3′); antisense (5′-CTAAAGTATTCGGTTTTTATAGTTTACGTGATT-3′). The amplified product was cloned in Topo2.1 vector (Invitrogen) and the sequence was confirmed for at least two different clones.

2.4. Stable expression of SmGluR in mammalian cells

The full-length SmGluR cDNA was modified by PCR to introduce a Kozak sequence for optimal translational efficiency in mammalian cells [24] followed by a FLAG fusion tag (DYKDDDDK) at the receptor's N-terminal end. The modified cDNA was cloned between the SalI and NotI restriction sites of expression vector, pCI-neo (Promega) and the resulting construct (pCIneo-SmGluR) was verified by DNA sequencing. HEK-293 cells were transfected with expression plasmid pCIneo-SmGluR using Fugene6 (Roche), according to the manufacturer's recommendations. 48 h after transfection, the medium was replaced with fresh Dulbecco's modified eagles medium (DMEM) containing 5% fetal bovine serum (FBS) and 20 mM 4,2-hydroxyethyl-1 piperazineethane sulfonic acid (HEPES) containing G418 (Invitrogen) at 750 μg/ml final concentration and the cells were cultured at 37 °C in a 5% CO_2 incubator. Cells were passaged and the media changed every 2–3 days until cell colonies appeared approximately three weeks after transfection. Individual clones were transferred to 24-well plates for amplification and were maintained under selection with G418 at a concentration of 600 μg/ml. Individual clones expressing SmGluR were identified by immunofluorescence analysis (IFA) using anti-FLAG M2 antibody (10 μg/ml; Sigma).

2.5. Functional analysis of SmGluR

Stable transfectants were tested for receptor activity by measuring changes in intracellular cAMP following agonist stimulation. Briefly, HEK-293 cells expressing SmGluR were seeded in a standard 96-well plate at a density of 100,000 cells/well. The plate was