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Inhibition of *Schistosoma mansoni* ether-a-go-go related gene-encoded potassium channels leads to hypermotility and impaired egg production

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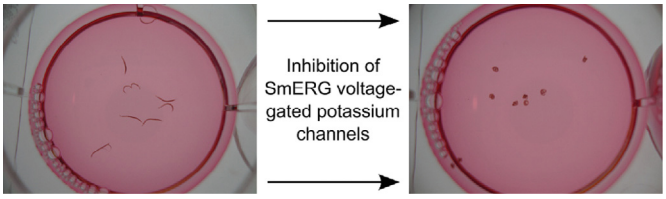
HIGHLIGHTS

- Schistosomes possess ether-a-go-go related genes.
- *Schistosoma mansoni* ERGS can be effectively inhibited by dofetilide.
- Inhibition of *S. mansoni* ERGs leads to hypermotility and production of abnormal eggs.

GRAPHICAL ABSTRACT



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ABSTRACT

The purpose of this work was to investigate the effect of ether-a-go-go related gene (ERG) potassium channel inhibition on *Schistosoma mansoni*. Use of dofetilide to block the schistosome ERGs resulted in a striking 'corkscrew' effect. The worms were unable to control their motility; they were hypermotile. The treated worms produced abnormal eggs, some of which consisted of little more than a spine. One of the *S. mansoni* ERGs (SmERGs), Smp_161140, was chosen for further study by RNAi. The transcript was knocked down to 50% compared to the controls. These RNAi-treated worms demonstrated seizure-like movements. In *S. mansoni*, as in other organisms, ERG channels seem to play a role in regulating muscle excitability. This work shows that egg production can be greatly reduced by effectively targeting muscle coordination in these important parasites.

1. Introduction

Schistosomiasis is the second most devastating parasitic disease worldwide (Olveda et al., 2014). The eggs laid by paired adult female schistosomes in the blood vessels of the definitive hosts are responsible for both the continuation of the lifecycle and for the pathology associated with infection. There are at least 240 million people infected with schistosomes, and 130,000 deaths are attributable to *Schistosoma mansoni* annually in Sub-Saharan Africa alone (van der Werf et al., 2003). Presently, treatment of schistosomiasis relies on one drug: Praziquantel. There are reports of reduced efficacy, and resistant parasites can be bred in the laboratory (Mwangi et al., 2014). There is a need, therefore, for both more basic research into the biology of schistosomes, as well as for focussed work towards potential new interventions. In this context, targeting the neuromuscular function of schistosomes could present a viable option for control (Atkinson et al., 2010; MacDonald et al., 2014; Salvador-Recatalà and Greenberg, 2012). Praziquantel targets Ca^{2+} channels (Greenberg, 2005, Kohn et al., 2003). Other anthelminthics affect the nervous systems of their respective target species (Rufener et al., 2010).

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Potassium channels of the ether-a-go-go (EAG) family are named for a phenotype in *Drosophila*; when the channels are mutated, these flies exhibit leg shaking activity upon exposure to ether (Kaplan and Trout, 1969). The subtype ether-a-go-go related genes (ERG) encode voltage-gated potassium channels, which are inwardly rectifying. These channels are made up of tetramers (Gong et al., 2004) and contribute to the repolarisation of the cell after an action potential, leading to the re-establishment of the resting membrane potential. The *Drosophila* ERG mutant is called *sei* for seizure (Titus et al., 1997; Wang et al., 1997). Flies with mutations at the *sei* locus have convulsive seizures. In particular, the flight motor pathway is affected. In *Caenorhabditis elegans*, channels encoded by ERG orthologue UNC-103 regulate the excitability of vulval muscles, and hence egg laying behaviour (Collins and Koelle, 2013). UNC-103 null animals lay more eggs, and lay underdeveloped eggs (Collins and Koelle, 2013). In humans, hERG channels are highly expressed in cardiac muscle and mutations in hERG cause long QT syndrome, which is a potentially fatal arrhythmia (Curran et al., 1995; Mitcheson et al., 2000). Dofetilide is an inhibitor of hERG (Rasmussen et al., 1992) and the residues in hERG which are necessary for dofetilide function have been identified (Kamiya et al., 2006). The dramatic effects of ERG channel inhibition in other organisms, in particular regarding egg production in *C. elegans*, have led us to investigate these channels in *S. mansoni*. The objective of the current study was to characterise the effect of ERG channel inhibition in schistosomes. Here we describe the effect on the worms of inhibiting the channels using dofetilide and one of the channels using RNAi. To our knowledge this is the first study of ERGs in schistosomes.

2. Materials and methods

2.1. Ethics statement

All animal experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No 123; revised Appendix A). Ethical approval was granted by the Regional Council (Regierungspraesidium) Giessen (V54-19 c 20/15 c GI 18/10).

2.2. Biological material

The *Schistosoma mansoni* lifecycle was maintained in Syrian golden hamsters (*Mesocricetus auratus*) and *Biomphalaria glabrata* snails (Grevelding, 1995). Adult worms were obtained by hepatoportal perfusion of hamsters 42 days after infection.

2.3. Organ PCR

Reproductive organs were isolated from adult schistosomes, total RNA was extracted and 500 ng was used for cDNA synthesised by a previously described method (Hahnel et al., 2013). RT-PCR was carried out on cDNA from ovaries, testes, and whole schistosome couples, including a no template negative control. Purity of the cDNA was tested by PCR to amplify SmNPP-5 (negative control – expressed in tegument, but not in gonads) and SmFKBP12 (positive control known to be expressed in testes and ovaries) (Hahnel et al., 2013). To detect SmERG, the following primers were used: Smp_161140 core 1F AGCAGTTTGGGATTGGCTTGTTCTTC and Smp_161140 core 4R ATACCAAATACATGCAAGCCAATGAGC, resulting in a 478 bp product. Firepol DNA polymerase (Solis Biodyne) was used and products were visualised by agarose gel electrophoresis using GelRed (GeneOn) to stain the DNA.

2.4. Sequence alignment

The human ERG sequence given as a target of dofetilide by DrugBank (http://www.drugbank.ca/drugs/DB00204, ("DrugBank: a knowledgebase for drugs, drug actions and drug targets.," 2008)) was blasted against *Schistosoma mansoni* proteins at GeneDB.org (Logan-Klumpler et al., 2012). The four resulting proteins were aligned with the human channel sequence at multalin (http://multalin.toulouse.inra.fr) (Corpet, 1988) using the default settings. The alignment was repeated using the whole human sequence, and partial sequences of the SmERGs, such that the residue numbers refer to the human sequence. The sections used were as follows: Smp_161140 amino acids 519–609; Smp_152350, 495–585; Smp_196370, 358–448; Smp_151810, 540–630.

2.5. Dofetilide treatment

Adult worms were cultured in 3 ml M199 medium with 10% NCS (Sigma), 1% 1M HEPES (Roth), and 1% antibiotic-antimycotic (Sigma) at 37 °C with 5% CO_2 in six well plates (8 couples per treatment) as described before (Beckmann and Grevelding, 2010). Dofetilide (Sigma) was dissolved in DMSO, and added to the culture wells at final concentrations of 0 μM, 30 μM, 150 μM or 300 μM. All of the wells including the negative control contained 0.5% v/v of DMSO. Worms were transferred into a new plate each day with the same concentrations of drug. Eggs produced during the preceding 24 h were counted and observed. Treatment was stopped after three days.

2.6. RNAi

The 3′ end of Smp_161140 was chosen as it has no homology either to the other SmERGs, or to other *S. mansoni* sequences. DNA template for double-stranded RNA synthesis was produced by PCR with primers which both have the T7 promoter sequence at their 5′ ends: Smp_161140 1F T7, 5′-TAATACGACTCACTATAGGGAGAATGCCAATCAAAAGGGGAC-3′ and Smp_161140_W R, 5′-TAATACGACTCACTATAGGGAGACACATCTAGAAATTGGTTGCTCA-3′. The PCR product was checked on an agarose gel and then column-purified (Machery-Nagel) before use. Double–stranded RNA was synthesised using the Megascript kit (Ambion), according to the manufacturer's instructions. Paired adult schistosomes (8 couples per cuvette, 16 couples per condition) were placed in 20 μl of electroporation buffer (120 mM trehalose, 20 mM HEPES, 1 mM myo-inositol, 1 mM KCl, 1 mM $MgCl_2$, 1 mM K_2HPO_4, 0.4 mM KH_2PO_4, 1 mM glutathione, pH 6.9, conductivity <1.3 mS, modified from (Mizukami et al., 2010)) with the addition of 25 μg dsRNA (not included in negative controls) in electroporation cuvettes. The worms were electroporated using a square wave pulse of 125 V for 20 ms. Immediately following this step, the worms were transferred to pre-warmed M199 with supplements, and cultured at 37 °C with 5% CO_2 for five days. Every day, the medium was changed, the worms were observed, and the eggs were counted. At the end of the experiment, half of the worms were snap frozen in TriFast (Peqlab) for RNA extraction, and the other half were fixed and stored in AFA for carmine red staining. The experiment was carried out on three separate occasions, with 16 couples per treatment.

2.7. Staining and confocal microscopy

After the experiment, worms were fixed in AFA (2% acetic acid, 3% formaldehyde, 66.5% EtOH) and stored at room temperature until use. In preparation for viewing by confocal laser scanning microscopy (CLSM), specimens were stained in Carmine Red (Merck) as described previously (Beckmann and Grevelding, 2010). The parasites were then viewed using a Leica TCS SP2 confocal microscope with excitation at 488 nm and a 470 nm long pass filter.