Biochimie 194 (2022) 108–117

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Schistosome TRPML channels play a role in neuromuscular activity and tegumental integrity

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ARTICLE INFO

Article history:
Received 25 August 2021
Received in revised form
16 December 2021
Accepted 31 December 2021
Available online 3 January 2022

Keywords:
Schistosoma mansoni
TRPML
Lysosomes
Tegument

ABSTRACT

Schistosomiasis is a neglected tropical disease caused by parasitic flatworms of the genus *Schistosoma*. Mono-therapeutic treatment of this disease with the drug praziquantel, presents challenges such as inactivity against immature worms and inability to prevent reinfection. Importantly, ion channels are important targets for many current anthelmintics. Transient receptor potential (TRP) channels are important mediators of sensory signals with marked effects on cellular functions and signaling pathways. TRPML channels are a class of Ca^{2+}-permeable TRP channels expressed on endolysosomal membranes. They regulate lysosomal function and trafficking, among other functions. *Schistosoma mansoni* is predicted to have a single TRPML gene (SmTRPML) with two splice variants differing by 12 amino acids. This study focuses on exploring the physiological properties of SmTRPML channels to better understand their role in schistosomes. In mammalian cells expressing SmTRPML, TRPML activators elicit a rise in intracellular Ca^{2+}. In these cells, SmTRPML localizes both to lysosomes and the plasma membrane. These same TRPML activators elicit an increase in adult worm motility that is dependent on SmTRPML expression, indicating a role for these channels in parasite neuromuscular activity. Suppression of SmTRPML in adult worms, or exposure of adult worms to TRPML inhibitors, results in tegumental vacuolations, balloon-like surface exudates, and membrane blebbing, similar to that found following TRPML loss in other organisms. Together, these findings indicate that SmTRPML may regulate the function of the schistosome endolysosomal system. Further, the role of SmTRPML in neuromuscular activity and in parasite tegumental integrity establishes this channel as a candidate anti-schistosome drug target.

1. Introduction

Schistosomiasis is a debilitating parasitic disease of poverty. It is caused by infection by parasitic flatworms (blood flukes) from the genus *Schistosoma* [1]. Chronic morbidity resulting from infection impacts childhood development and causes as many as 200,000 deaths annually [2–4]. Currently there is no vaccine for schistosomiasis and the only therapeutic drug is praziquantel (PZQ) [4–7], which is effective against all species of human schistosomes. However, PZQ has significant limitations [8–10] including ineffectiveness against juvenile worms and an inability to prevent reinfection. Furthermore, with only a single drug available, the emergence of drug resistance would be dire [9,11,12]. Consequently, the need for new antischistosomals is urgent.

Ion channels underpins electrical excitability in cells. They are most notably central to the function of neuromuscular cells, but also play key roles in other cell types. Ion channels are also validated targets for a broad range of drugs, including many of the anthelmintics currently in use [6,13,14].

The transient receptor potential (TRP) ion channel superfamily has been the focus of intense research in recent years. Members of this family are non-selective cation channels that display a wide range of functions, most notably sensory signal transduction. TRP channels often exhibit polymodal activation, with different, seemingly unrelated signals capable of opening an individual channel. Because of their likely important functions in parasitic worms. TRP channels have begun to be intensively investigated as therapeutic targets to regulate infection. Indeed, a schistosome TRP channel ($SmTRPM_{PZQ}$) has recently been identified as a high-

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https://doi.org/10.1016/j.biochi.2021.12.018
0300-9084/

affinity, stereoselective receptor for PZQ [15].

Based on structural homology TRP channels are categorized into subfamilies (TRPC, TRPV, TRPA, TRPM, TRPP, TRPN, TRPML) [16–18]. In *S. mansoni* genome,15 predicted TRP channel genes representing 5 subfamilies (TRPC, TRPA, TRPM, TRPP, TRPML) are found. There are no predicted genes encoding TRPV channels in schistosomes, and we have shown that a schistosome TRPA1-like channel exhibits pharmacological properties that overlap with mammalian TRPV1 channels [19].

One group of schistosome TRP channels that has not been explored previously is the TRPML (Mucolipin) subfamily. TRPML channels are mainly intracellular channels, though they can be expressed on the plasma membrane as well [20,21]. Mammals have three TRPML subtypes, of which TRPML1 is the best studied, in part because mutations in the gene for this channel cause the childhood neurodegenerative lysosomal storage disease mucolipidosis type IV. TRPML channels are expressed on endolysosomal membranes and appear to regulate endolysosomal trafficking, lysosomal ion homeostasis, autophagy, nutrient acquisition, and other functions [22]. Genetic ablation of *Drosophila* TRPML recapitulates many of the cellular defects found in human TRPML1 mutations [23], suggesting a significant role for TRPML1 in regulating lysosome function in other metazoans in addition to mammals.

Analysis of the *S. mansoni* genome revealed a single TRPML gene (Smp_198800) with two splice variants (Smp_198800.1, Smp_198800.2) differing by a 12 amino acid insert found in Smp_198800.2. Here, we investigate the physiological and functional properties of *S. mansoni* TRPML (SmTRPML) channels. We find that the mammalian TRPML activators ML-SA1 and MK6-83 elicit currents in CHO-K1 cells expressing SmTRPML and that these activators also produce hyperactivity in adult schistosomes. These effects on worm activity are prevented by suppression of SmTRPML expression, indicating that the effects on motility are mediated through SmTRPML. SmTRPML expression co-localizes with lysosomal markers in the CHO-K1 cells but is also found on the plasma membrane. Interestingly, we find that both application of a TRPML inhibitor to schistosomes and suppression of SmTRPML expression in adult worms disrupts the worm tegument. These results suggest that TRPML is required for regulation of the parasite tegument and thereby represents a potential target for anti-schistosome therapeutics.

2. Materials and methods

2.1. Ethics statement

Studies were carried out in strict conformity with the recommendations in the guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health. Animal handling and experimental procedures were undertaken in compliance with the University of Pennsylvania's Institutional Animal Care and Use Committee (IACUC) guidelines (Animal Welfare Assurance Number A3079-01). The IACUC authorized these studies under protocol number 806056.

2.2. Reagents

ML-SA1, MK6-83, praziquantel, and serotonin were from Sigma-Aldrich (St. Louis MO). LysoTracker RED DND-99 was from Life Technologies, Inc. ML-SI3 was obtained from Enamine, code (s) EN 300–314172. Stock solutions were made in dimethyl sulfoxide (DMSO, ATCC, Manassas, VA) and subsequently diluted to an appropriate concentration in culture or recording media. All oligonucleotides were bought from Integrated DNA Technologies (IDT, Coralville, IA).

2.3. Isolation of schistosomes

The NIAID Schistosomiasis Resource Center through BEI Resources provided *Biomphalaria glabrata* snails infected with *S. mansoni* (NMRI strain, catalog #NR-21962) and Swiss-Webster mice infected with *S. mansoni* (NMRI strain, catalog #NR-21963), under NIH-NIAID Contract HHSN272201700014I. We also infected Swiss-Webster mice with cercariae obtained in our lab, as described [24,25]. Adults worms were perfused at 6–7 weeks post infection from mice as described [24,25] and were maintained in standard Schistosome Medium of RPMI (Thermo Fisher, Philadelphia, PA), plus 10% FBS (Gem Cell, Gemini Bio Products, West Sacramento, CA) and 100 U/ml penicillin/100 mg/ml streptomycin (Corning Life Sciences, Tewksbury, MA), at 37 °C and 5% CO_2.

2.4. Plasmids

Codon optimization, gene synthesis, and cloning (into pcDNA3.1Zeo$^{(+)}$) of the predicted SmTRPML sequences were done by Genscript, yielding the plasmid pcSmTRPML. This codon-optimized pcSmTRPML was fused with GCaMP6f in pcDNA3.1Zeo$^{(+)}$ at the predicted C-terminus using a strategy similar to that used elsewhere [26] for fusing GCaMP6f to the C-terminus of the Orai1 protein. Briefly, the pGP-CMV-GCaMP6f plasmid (Addgene) was used as a template for PCR of the full-length GCaMP6f sequence, using primers incorporating restriction sites (EcoR1/Not1) compatible with sites at the 3′ end of SmTRPML coding region of pcSmTRPML. Both pcSmTRPML and the amplified GCaMP6f coding sequence were cut with EcoR1 and Not1 and ligated to form the pcSmTRPML-GCaMP6f fusion plasmid. Due to a stop codon created in the SmTRPML-GCaMP6f linker by this process, we designed a primer for mutagenesis with the Q5 Mutagenesis Kit (NEB) that removed the stop codon and created an in-frame linker. The linker between SmTRPML and GCaMP6f contained the amino acid sequence KANSGLPCFATMVDSS. To create the SmTRPML2-GCaMP6f splice variant fusion plasmid, a primer inserting the 12 amino acid stretch found in SmTRPML2 but not in SmTRPML1 was designed and used for mutagenesis with the Q5 Mutagenesis kit. All sequences were verified by Sanger sequencing (Eurofins).

2.5. Effect of pharmacological compounds on motility of schistosomes

We analyzed the effects of TRPML activators (ML-SA1, MK6-83) and the TRPML inhibitor ML-SI3 [27] on motility of adult *S. mansoni*. Protocols were similar to those described previously [19]. Adult worms were tested for motility in Schistosome Medium at 37 °C on a Tokai Hit (Shizuoka, Japan) thermoplate. Briefly, single adult parasites were each placed in individual wells of a 24-well plate for 15 min at 37 °C to obtain a baseline level of activity using the Worm Motel imaging system and software described previously [19]. Test compounds were then added to the medium to appropriate final concentrations, and motility measured again over the course of another 15 min. Each worm thus served as its own control. As our vehicle control, we used 0.1% (v/v) DMSO. Serotonin (40 μM), which increases schistosome motility [28], and PZQ (500 nM), which paralyzes schistosomes, served as controls to confirm that the analysis system was measuring worm activity accurately.

2.6. CHO-K1 cells and transfection

CHO-K1 cells were obtained from ATCC. They were grown in Ham's F-12 Kaighn's medium (Thermo Fisher) supplemented with 10% fetal bovine serum (Gem Cell, Gemini Bio Products), and 100 U/

ml penicillin/100 mg/ml streptomycin (Corning Life Science) at 37 °C and 5% CO_2. Cells ranged between passages 5 to 11 were used in these experiments. For Ca^{2+} imaging, we plated 2.5 × 10^5 cells 24h prior to transfection onto a 35 mm glass bottom dish (14 mm microwell glass diameter; MatTek corporation, Ashland, MA). We transfected the CHO-K1 cells using Lipofectamine 2000 reagent (Thermo Fisher) with the pSmTRPML-GCaMP6f or, for controls, with both the empty pcDNA3.1/zeo$^{(+)}$ and the pGP-CMV-GCaMP6f plasmid [29]. Unless otherwise noted, we tested only one of the two splice variants (SmTRPML1, aka Smp_198800.1).

2.7. Calcium imaging

Ca^{2+} signals were measured in CHO-K1 cells with the genetically-encoded Ca^{2+} indicator GCaMP6f at 48h following transfection, using methods similar to those described by us and others [19,30]. Tested compounds (and concentrations) were ML-SA1 (20 μM); MK6-83 (20 μM); ML-SI3 (25 μM), DMSO (0.01%) and ionomycin (1 μM). The procedure for calcium imaging has been described in detail elsewhere [31].

2.8. LysoTracker Red staining

CHO-K1 cells transfected with the SmTRPML-GCaMP6f plasmid were incubated with 75 nM LysoTracker Red (Invitrogen) in HBSS (Hank's Balanced Salt Solution) including Ca^{2+} and Mg^{2+} for 1h at 37 °C. When labelling was complete the solution was replaced by fresh medium. Cells were observed and imaged under Spinning disk Confocal microscopy (20x, 63x oil) [32].

2.9. RNA extractions and RT-PCR

Direct-zol ™ RNA miniprep kit (ZYMO Research, Irvine, CA) was used to extract RNA from adult worms, juvenile worms and CHO-K1 cells. For RT-PCR, we used 500 ng RNA, in the Super Script ™ III One Step RT-PCR with Platinum ™*Taq* kit (Invitrogen) to amplify the sequences. Primers used for amplification of SmTRPML were SmTRPML1F(W) (5′-GCTTCAACATCATCACCAACAA-3′); SmTRPML1R(W)(5′-GAAACATGAGCAGCACGATAAA-3′) SmTRPML2F(W) (5′-GGTCCATATCGTTCATCAGATCC-3′); SmTRPML2R(W)(5′-TCAGCACCATCTTCTGTTGTT-3′); SmTRPML1F(C)(5′-CAGCCCATACCAGTCCTATTTC-3′); SmTRPML1R(C)(5′-TAGCTCACGGACAGCATAGA-3′); SmTRPML2F(C)(5′-CAGCCCATACCAGTCCTATTTC-3′); SmTRPML2R(C)(5′-TAGCTCACGGACAGCATAGA-3′). PCR runs were 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with number of cycles determined empirically.

2.10. RNA interference

We used siRNAs to knock down RNAs encoding both predicted splice variants of the single *S. mansoni* TRPML-like gene (Smp_198800) in adult worms [33,34]. As a control, we used luciferase siRNA (Silencer Firefly Luciferase, GL2+GL3, Life Technologies, Inc.), which has no significant resemblance to any sequences from the *S. mansoni* genome. siRNAs against SmTRPML were designed using the SciTools Software suite from IDT. The siRNA sequence was 5′-CACCUGUUGGUAAAUUUCAUGCCAA-3′ 3′-CUGUGGACAACCAUUUAAAGUACGGUU-5'. The procedure for electroporation has been described in detail [31]. These worms were then tested for sensitivity to TRPML modulators or maintained in culture over the course of several days to observe any changes in morphology, survival, or function.

2.11. qRT-PCR

We used qRT-PCR to measure levels of knockdown by RNAi. The worms were harvested 48h after RNAi for qPCR. Direct-Zol ™ RNA Mini Prep (ZYMO Research, Irvine, CA) was used to extract the total RNA from adult worms. qRT-PCR was performed using Brilliant II SYBR green qRT-PCR Master Kit (Agilent Technologies, Santa Clara, CA) on an Applied Biosystems 7500 instrument as described [35]. Primers used for the amplification of 18S ribosomal RNA have been described [35]. Primers used for amplification of SmTRPML were TRPML-FWDSET1 (5′GATGCGTCGTCGGTTGTATTA3′) and TRPML-REVSET1 (5′CCTTGAGGATCTGAACGAAGAG3′). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [36].

2.12. Data analysis

Analysis of data was done with GraphPad Prism or Microsoft Excel, expressed as arithmetic means ± SEM and tested for statistical significance using the statistical tests noted in the text and figure legends. Figures showing normalized change in fluorescence were analyzed and plotted using the R v.3.4.0 (ggplot2 package). In the drug response studies, each worm served as its own control, and we therefore compared means using paired t-tests (on the raw data, prior to normalization).

3. Results

3.1. TRPML activators elicit hyperactivity in adult schistosomes

We tested the effects of two activators of mammalian TRPML channels on schistosome motor activity. ML-SA1 is a agonist of mammalian TRPML1 channels, and it also exhibits activity against TRPML2 and TRPML3 channels [37]. ML-SA1 induces a conformational change in the channel selectivity filter which allows cations such as Ca^{2+} to flow through the channel [38].

ML-SA1 elicited a significant motility increase in adult *S. mansoni* worms. Male worms exhibited hyperactivity in 15 μM, 20 μM and 30 μM ML-SA1 while adult female worms showed significant increases in motility in the presence of 40 μM and 50 μM ML-SA1 (Fig. 1A and B). Higher concentrations of ML-SA1 elicited no hyperactivity in either males or females. We observed no significant effect of ML-SA1 on motility of juvenile (3–4-weeks post infection) worms (data not shown). We did not observe tegumental damage in adult or juvenile worms after exposure to ML-SA1.

We also studied the effects of another activator, MK6-83, on motility of adult worms. MK6-83 is a novel agonist of TRPML1 [39]. Like ML-SA1, MK6-83 significantly increased motility of male worms at 20 μM, 40 μM and 60 μM concentrations, with no effect at 80 μM; in females MK6-83 failed to show any measurable effect (Fig. 1C and D).

3.2. Knockdown of SmTRPML eliminates hyperactivity response of schistosomes to ML-SA1

We used siRNA predicted to target both predicted SmTRPML splice variants to suppress SmTRPML expression and to determine whether the effects of ML-SA1 on worm activity are dependent on expression of SmTRPML. qRT-PCR analysis revealed reduced expression of SmTRPML RNA by 55% in male worms and by 86% in female worms, (Fig. S1). Male and female worms with reduced SmTRPML expression no longer exhibited hyperactivity in response to ML-SA1 (males - 20 μM; Fig. 2A; females - 40 μM, Fig. 2B). Importantly, this effect was selective, as SmTRPML suppression had no effect on serotonin-elicited hyperactivity (Fig. S2).

3.3. Both the TRPML inhibitor ML-SI3 and suppression of TRPML1 expression disrupts tegumental integrity of adult schistosomes

ML-SI3 is a potent inhibitor of TRPML1 (IC_{50} value 1.6 μM) and TRPML2 (IC_{50} 2.3 μM); it is also a less effective inhibitor (IC_{50} = 12.5 μM) of TRPML3 [40,41]. We cultured male and female adult schistosomes in media containing ML-SI3 (25–75 μM) for 24h, followed by culture in media without drug. For five days worms exhibited progressive tegumental degradation (Fig. 3) whereas worms cultured with 0.1% DMSO exhibited no disruption of the tegument. Interestingly, we also observed a 63% reduction of SmTRPML expression in adult worms on the fifth day following 24h

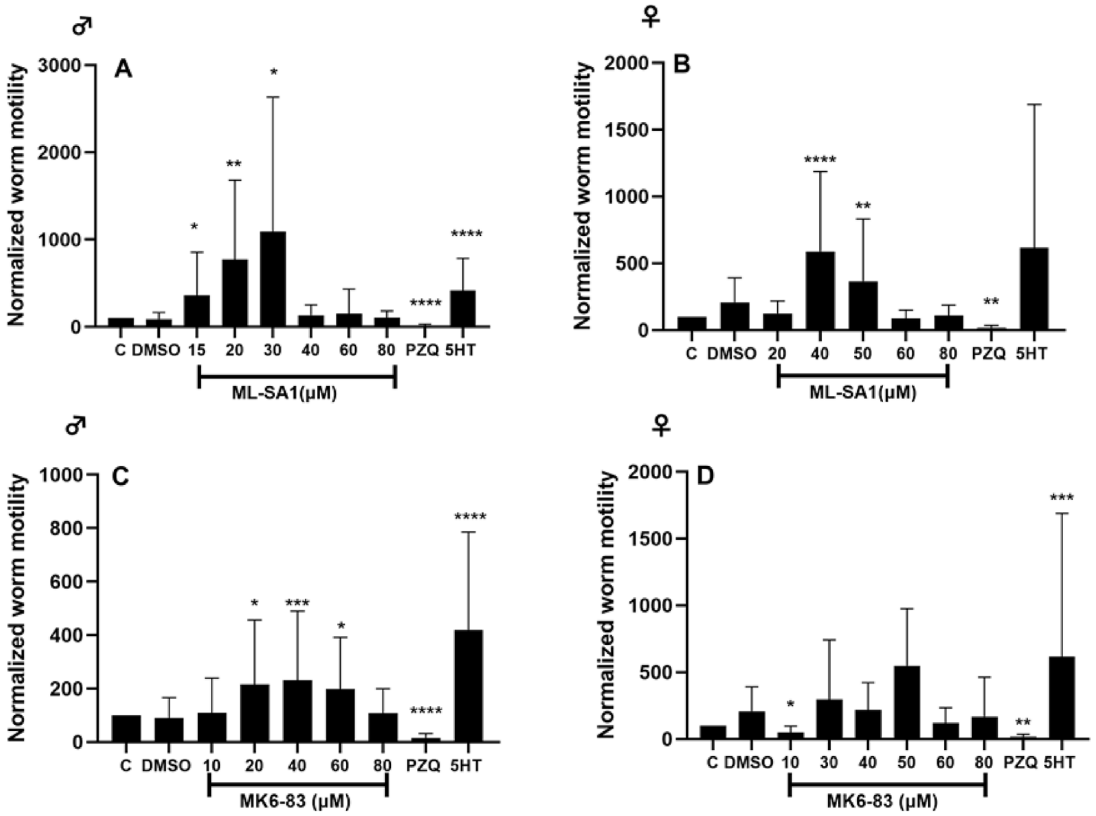

Fig. 1. TRPML activators elicit hyperactivity in *S. mansoni* adults. *S. mansoni* adults (approximately 7 weeks post infection) exhibit hyperactivity in response to the TRPML activators ML-SA1 (A, B) and MK6-83 (C, D). A, C show responses of males; B, D show responses of females. Motility was examined before and after addition of compound. Control (C) is media only and DMSO is 0.1% DMSO (the stock solvent) with no added activator. Each individually tested worm served as its own control and data were normalized to the control response for each worm. 5-HT was used as a positive control for hyperactivity (n = 43) and PZQ served as a positive control for paralysis (n = 48). A, n = 25; B, n = 24; C, n = 24; D, n = 16 for each concentration, with n = 24–48 for Controls. (C) Adult males exposed to MK6-83 exhibit a significant increase in motility of worms from 20 μM to 60 μM (n = 24) and a decrease at 80 μM as compared to control worms (n = 48). (D) Adult females exposed to MK6-83 did not exhibit any significant increase in motility compared to controls (n = 24).

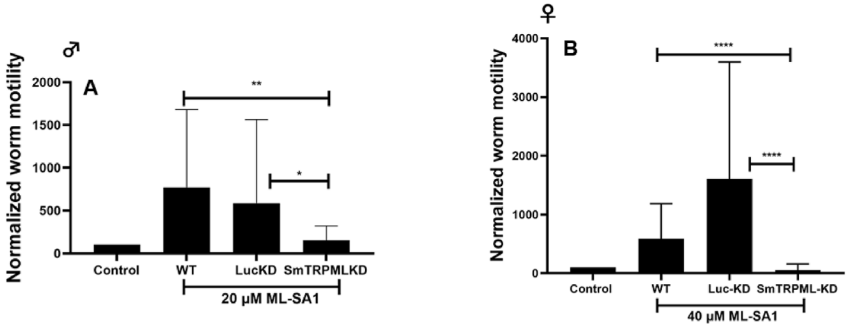

Fig. 2. Knockdown of SmTRPML expression eliminates effects of the TRPML activator ML-SA1 on adult schistosome motility. Comparison of ML-SA1-dependent motility between normal adult male (A) or female (B) *S. mansoni* (WT) and those electroporated with 5 μg siRNA targeted against SmTRPML (KD). Control worms (Control, no knock down, no drug) are also shown. To control for non-specific effects of electroporation with siRNA, worms were electroporated with 5 μg luciferase (Luc) siRNA were also exposed to ML-SA1. A) 20 μM ML-SA1; n = 24, all conditions. B) 40 μM ML-SA1; n = 37 (Luc-KD, Control), n = 24 (WT), n = 37 (SmTRPML-KD). *, **, ****,$P < 0.05$,$P < 0.01$,$P < 0.0001$ respectively, unpaired *t*-test vs. control worms for each ML-SA1 concentration.

exposure with 75 μM ML-SI3 (Fig. S 4). There is precedence for ion channel blockers reducing expression of genes for the targeted channel [42,43].

Adult male and female schistosomes in which SmTRPML expression was suppressed also exhibited significant blebbing on the tegumental surface as compared to control worms which remained fully viable indicating that the SmTRPML channel is playing some role in maintaining the tegumental integrity of schistosomes. (Fig. 4). All the worms exposed to drug showed tegumental damage, while 30% of the worms treated with RNAi showed this phenotype after 7–14 days.

3.4. Mammalian cells expressing SmTRPML respond to TRPML activators and inhibitors, with SmTRPML co-localizing lysosomal markers and to the plasma membrane

To further address whether the observed responses of schistosome to TRPML activators are mediated by SmTRPML channels, we expressed codon-optimized (mammalian) SmTRPML. (SmTRPML1) fused at the C-terminus to the genetically encoded Ca^{2+} indicator GCaMP6f [29] in CHO-K1 cells. Mammalian TRPML channels are expressed primarily on endolysosomal membranes, but also on plasma membranes in heterologous expression model [44]. To determine whether SmTRPML exhibits a similar expression pattern, we used confocal microscopy to examine the localization of SmTRPML-GCaMP6f and the lysosomal marker LysoTracker Red DND-99 in CHO-K1 cells as shown in Fig. 5. We observed that 7.0% of transfected cells expressed GFP fluorescence (indicative of SmTRPML expression) that co-localizes with LysoTracker Red, indicating expression on endolysosomes, GFP fluorescence also localizes to plasma membrane.

Using Ca^{2+} imaging protocols, we find that 20 μM ML-SA1 and 20 μM MK6-83 each elicit a significant increase in Ca^{2+} signals in CHO-K1 cells expressing SmTRPML1, compared to those expressing

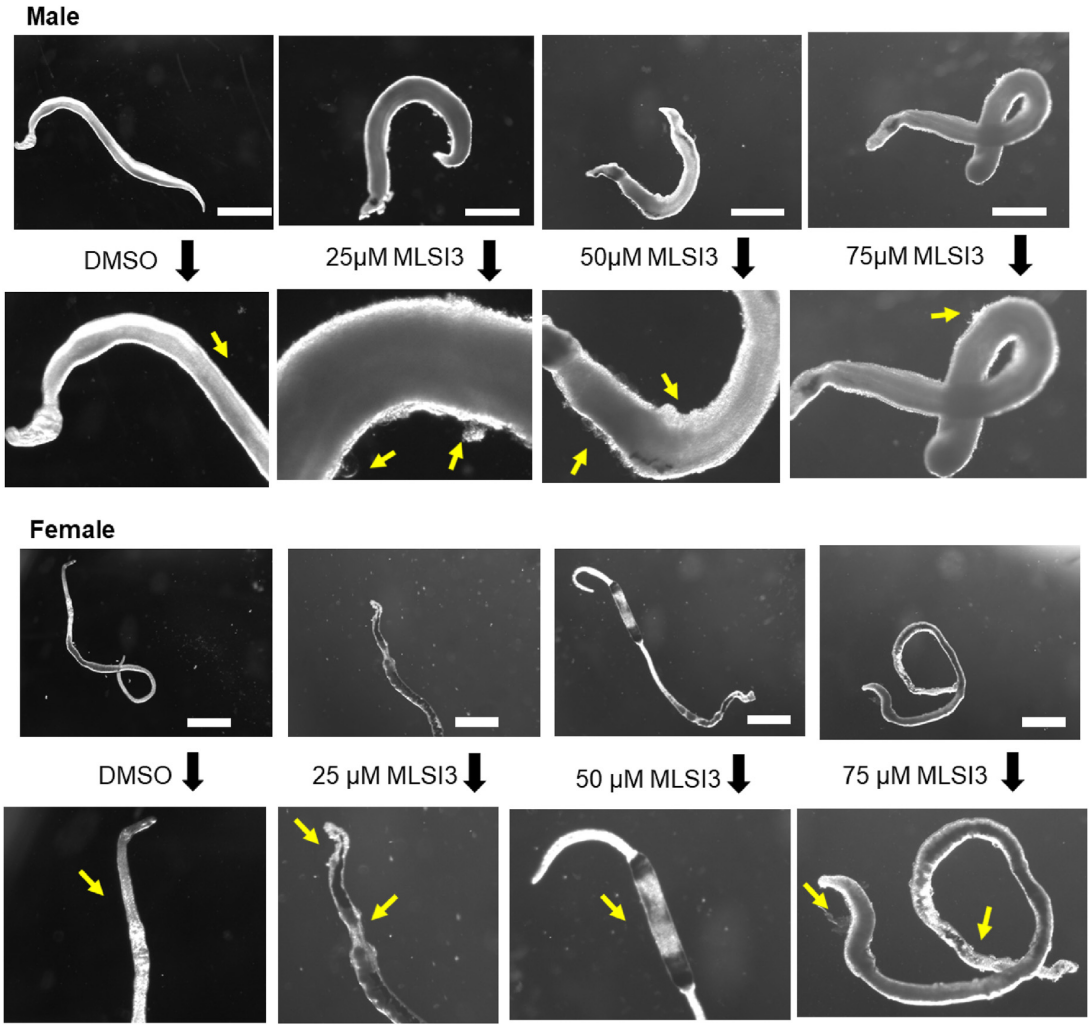

Fig. 3. Exposure of adult males and females to the mammalian TRPML inhibitor ML-SI3 at 25 μM, 50 μM and 75 μM results in "shredding" of the tegument. Worms were cultured in media plus drug (or 0.1% DMSO) for 24 h and then media alone for 5 days. Arrows indicate regions of tegumental disruption. Scale bar = 1 mm.

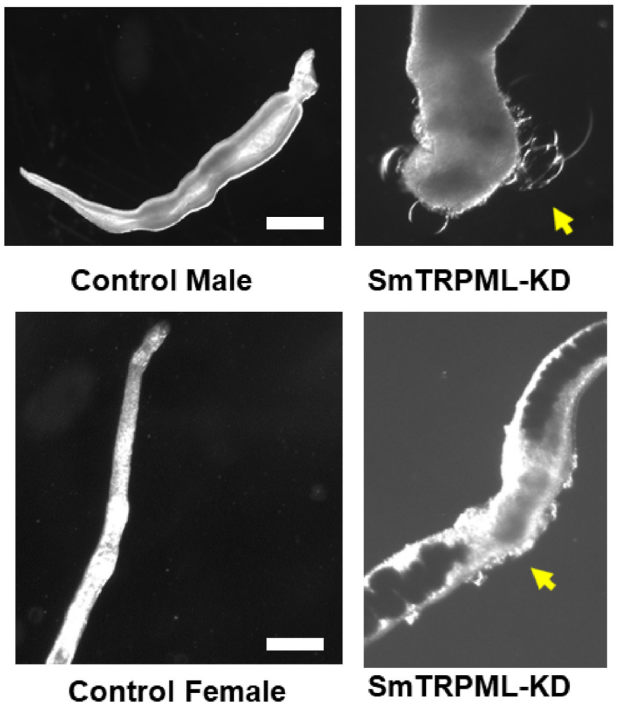

Fig. 4. Blebbing on the tegument of adult male and female worms when electroporated with SmTRPML siRNA compared to control. Yellow arrows indicate areas of tegumental blebbing. Scale bar = 1 mm.

only vector controls (Fig. 6). Additionally, we observed oscillation in the signal when cells were exposed to 20 μM MK6-83. Interestingly, the cells expressing vector controls also exhibited robust Ca^{2+} signals in response to these compounds (Fig. S3), likely indicating a high level of endogenous activator responsive TRPML channels in the CHO cells. However, statistical analysis revealed a significant difference ($P < 0.0001$, two-way ANOVA) in the response of control vs SmTRPML-transfected cells to both activators, Thus, a significant component of the Ca^{2+}signal elicited by these compounds depends on the expression of SmTRPML in these cells.

The response of SmTRPML1 to both activators is largely dependent on the presence of extracellular Ca^{2+}, through a component of the signal persists in the absence of extracellular Ca^{2+}, suggesting Ca^{2+} release of intracellular stores (Fig. 7).

Cells expressing SmTRPML2, the splice variant with the 12 amino acids inserts, did not respond to these activators with an increase in Ca^{2+} fluorescence. We are exploring whether the 12 amino acids inserts change the structure and pharmacological sensitivity of the channel protein, and whether this predicted variant is actually expressed in schistosomes.

To examine if mammalian TRPML antagonists inhibit SmTRPML, we tested ML-SI3 which acts as an antagonist (channel blocker) of the TRPML family with greatest activity against the human TRPML1 channel, (although it also blocks TRPML2 and TRPML3 with lower affinity [40]. We treated the CHO cells expressing SmTRPML with 25 μM ML-SI3 for 2h and then exposed them to 20 μM ML-SA1 following removal of inhibitor. No increase in intracellular Ca^{2+} was observed suggesting that this mammalian TRPML antagonist is also inhibiting SmTRPML (Fig. 8). Indeed, baseline Ca^{2+} levels appear to decrease significantly below those seen in cells not expressing the channel (Vector in Fig. 8), perhaps indicating inhibition of basal SmTRPML activity by ML-SI3.

4. Discussion

TRPML channels serve a variety of vital functions that include endolysosomal trafficking, ion homeostasis, amino acid utilization, and nutrient acquisition. TRPML channels also regulate neuromuscular development and activity, evident by the neurodegeneration exhibited in humans, other mammals, and *Drosophila* [23] carrying TRPML channel mutations. Given the conserved role of TRPML channels, we hypothesized they may also be critical to normal schistosome physiology and if so, could represent targets for therapeutics that alter worm function and viability.

In this report, we show that activators of mammalian TRPML channels elicit significant increases in neuromuscular activity and motility of adult *S. mansoni*. RNAi analysis shows that the effects of the TRPML activators on schistosome motor activity are dependent upon SmTRPML expression. Interestingly, there are precedents for links between Ca^{2+} release from endolysosomes via TRPML and cellular contraction. Thus, TRPML regulates actomyosin contractility and couples migration to phagocytosis in *Drosophila* macrophage-like hemocytes [44]. Similarly, TRPML-dependent release of intracellular Ca^{2+} regulates smooth muscle contractility in the lower urinary tract of mice [45]. Thus, dissecting the role of TRPML in regulating schistosome neuromuscular activity could lead to insights into the physiology of these organisms, and perhaps new therapeutic targets.

To further address whether the effects of TRPML activators on activity of adult worms is being mediated through SmTRPML1, we expressed SmTRPML in a heterologous expression model. SmTRPML responded to TRPML activators with a significant increase in Ca^{2+} signaling, an effect that is blocked by a TRPML inhibitor. CHO cells transfected with vector controls show a robust response to activators as well, likely indicating action on endogenous TRPML channels. These activators are targeted against mammalian TRPML channels and may exhibit lower potency against the distantly related SmTRPML channels, which may explain the larger response apparently due to the endogenous channels.

Mammalian TRPML channels are localized primarily in endolysosomes, but can also be detected in the plasma membrane, in part upon lysosomal exocytosis, and more dramatically in overexpression systems such as the one we used. SmTRPML localizes similarly to mammalian TRPML in our heterologous system co-localizing with a lysosomal marker and also appears to be expressed on the plasma membrane, consistent with other reports [20,46] as well as our results showing contributions of both extracellular and intracellular contributions to SmTRPML-mediated increases in Ca^{2+} levels (Fig. 7). Predictive software such as LOCTree2 (https://www.rostlab.org/services/locdb/) and LocSigDB (Negi, S. 2015, Database) revealed consensus lysosomal targeting motifs in SmTRPML like those used to effect endolysosomal localization of mammalian TRPMLs. The 12 amino acid difference between SmTRPML1 and SmTRPML2 lies in the large loop between transmembrane region S1 and S2, predicted to reside within the lumen of the endolysosome.

Unlike SmTRPML1, the predicted SmTRPML2 variant does not respond to TRPML activators in the heterologous system. To date we have detected only SmTRPML1 expression in adult worms and juvenile worms, perhaps indicating that predicted SmTRPML2 variant

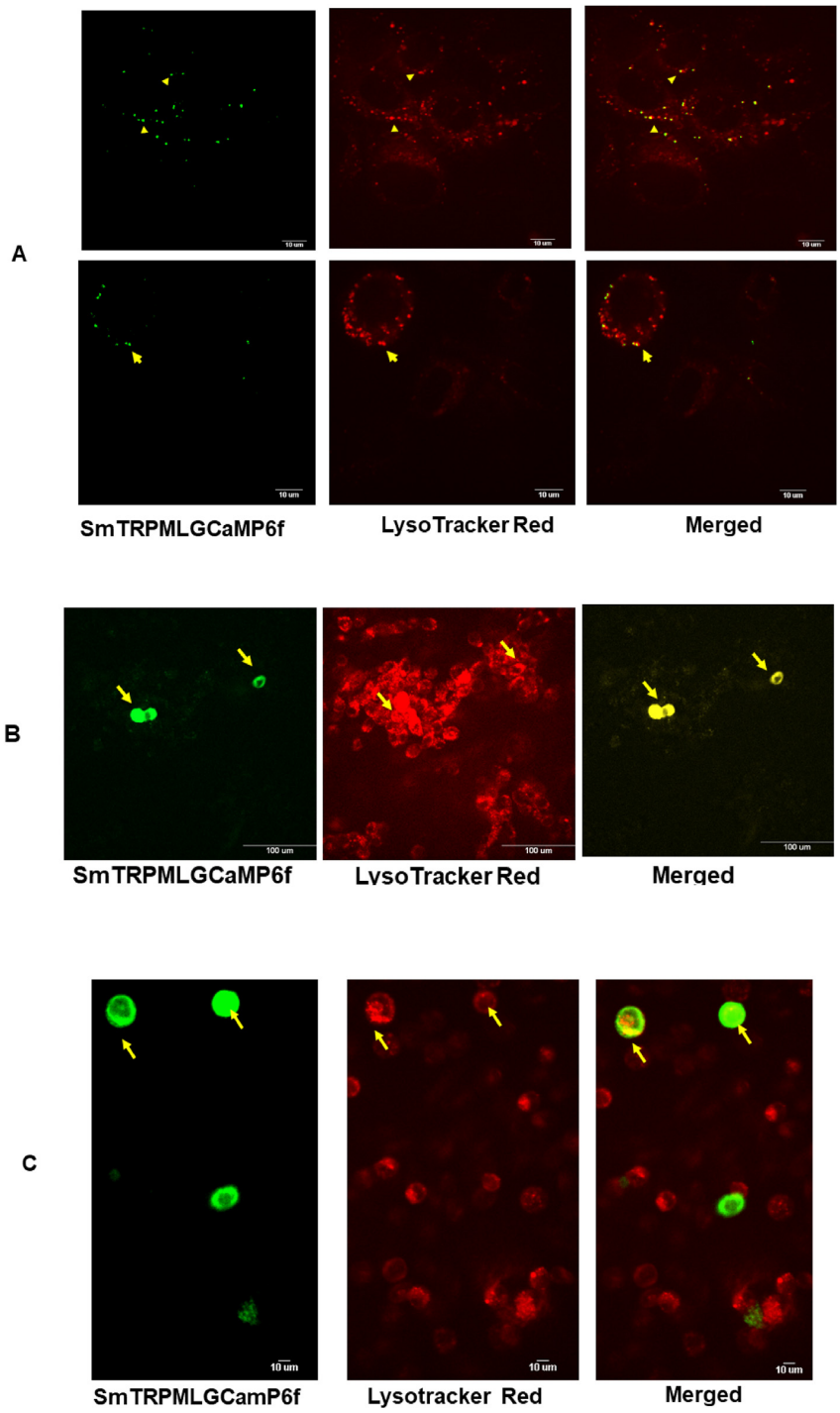

Fig. 5. SmTRPML expressed in CHO cells co-localizes with LysoTracker Red and to the plasma membrane. (A) and (B) SmTRPML co-localizes with the LysoTracker Red in CHO cells (scale bar = 10 μm) and (scale bar = 100 μm) respectively. Note the abundance of yellow intracellular vesicles in the merged image (yellow arrowheads). (C) SmTRPML also appears to be expressed on the plasma membrane in this heterologous overexpression system (yellow arrows) (Scale bar = 10 μm).

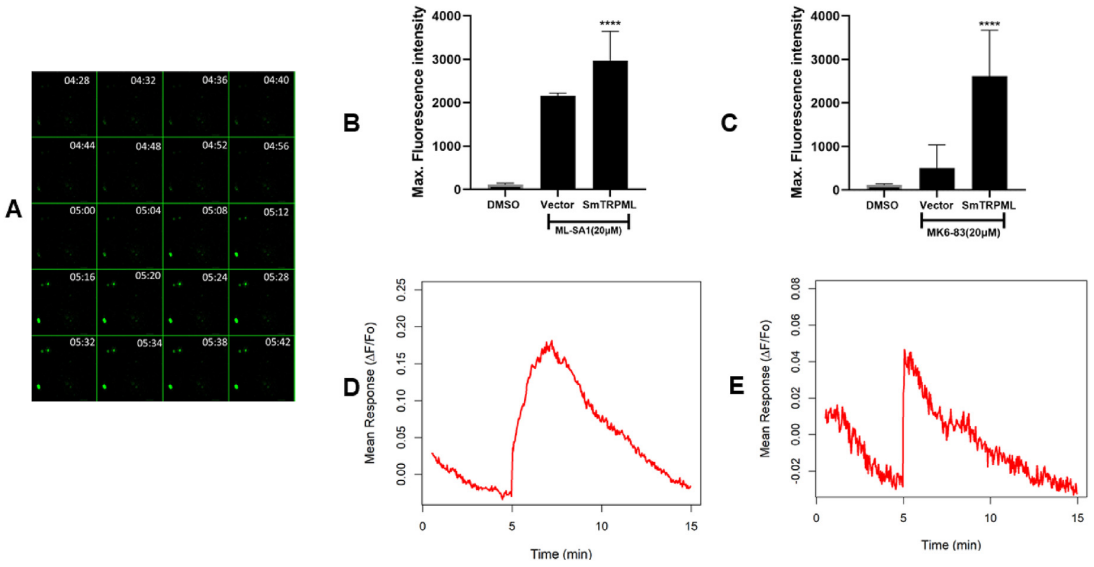

Fig. 6. TRPML activators elicit a significant increase in Ca^{2+} signals in CHO-K1 cells expressing SmTRPML. (A) Montage showing the increase in GCaMP6f fluorescence in CHO cells transfected with SmTRPMLG-GCaMP6f following exposure to 20 μM ML-SA1. Note increase in green fluorescence in these cells, indicating an increase in Ca^{2+} levels. Scale bar = 30 μm. (B, C) Normalized maximal GCaMP6f fluorescence intensity in response to 20 μM ML-SA1 (B) or MK6-83 (C) in cells transfected with either SmTRPML-pGP-CMV-GCaMP6f or a combination of empty vector (pcDNA3.1/zeo$^{(+)}$) and pGP-CMV-GCaMP6f plasmids (pc, n = 24, 3 independent transfections); SmTRPML-GCaMP6f, ML-SA1 (n = 24, 3 independent transfections); SmTRPML1GCaMP6f, MK6-83 (n = 12, 3 independent transfections). (D) and (E) Averaged GCaMP6f fluorescence intensity change vs. time in cells transfected with SmTRPML-GCaMP6f exposed and to 20 μM ML-SA1 (D) or 20 μM MK6-83.

is not in fact expressed in schistosomes. Nonetheless, we are currently investigating whether this absence of activation represents a key biological difference, perhaps related to the 12 amino acid insert found in SmTRPML2. Interestingly, ML-SA1 fails to activate *Drosophila* TRPML unless exogenous phosphatidylinositol 3, 5-biphosphate (PI (3,5) P_2) is applied. In contrast, mouse TRPML is readily activated by ML-SA1 independent of (PI (3,5) P_2) [37], and our results suggest that, at least for SmTRPML1, activation by ML-SA1 resembles the mammalian channel more than the *Drosophila* channel.

We also observe that knockdown of SmTRPML or exposure of adult worms to the TRPML inhibitor ML-SI3 causes tegumental disruption, suggesting a link between schistosome lysosomal function and tegumental pathology. Interestingly, previous work exposing worms to 1,7-bis(p-aminophenoxy) heptane (153C51), a compound with antischistosomal activity, also suggested this type of a connection [47]. The observation that ML-SI3 decreases SmTRPML RNA expression in worms opens the possibility that the effects on expression may contribute to the tegumental phenotype.

SmTRPML might also be playing a role in tegument repair, as knockdown of SmTRPML results in tegumental blebbing. Blebbing shows plasmalemmal protrusions and it is a feature of injured cells. Blebs serve as a messenger for injury -induced intracellular compartments that encapsulate damaged segments of the plasma membrane. In injured cells, an inrush of extracellular Ca^{2+} sets off a process of plasma membrane blebbing which anticipates cell death [48–54]. TRPML plays a role in cell membrane repair, presumably through lysosomal exocytosis and release of intracellular Ca^{2+} through the channel [55]. The tegument of adult schistosomes is a metabolically active cell membrane-like structure with a heptalaminate (double lipid bilayer) arrangement, and likely relies on such repair mechanisms for maintaining this critical parasite-host interface.

The tegument of schistosomes is a critical, active surface involved in nutrient acquisition, male-female signaling, immune evasion, and host-parasite interactions. Tegumental disruption is one of the hallmarks of PZQ activity, resulting in exposure of parasite antigens that can evoke host responses. Our finding that disruption of SmTRPML function or expression compromises the tegument lends credence to the potential for targeting of these channels therapeutically. Further structural investigations and electrophysiological characterizations will help facilitate the understanding of SmTRPML function and regulation in these parasites.

Funding statement

This work was funded by National Institutes of Health (https://www.nih.gov/) grants NIH R01-AI123173, NIH R21-AI132912 to RMG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure

All authors have approved the final article.

CRediT authorship contribution statement

Swarna Bais: Conception of the work, Collection of data, Analysis of data, Writing of manuscript. **Abigail Norwillo:** Collection of data, Writing of manuscript. **Gordon Ruthel:** Analysis of data, Writing of manuscript. **De'Broski R. Herbert:** Writing of

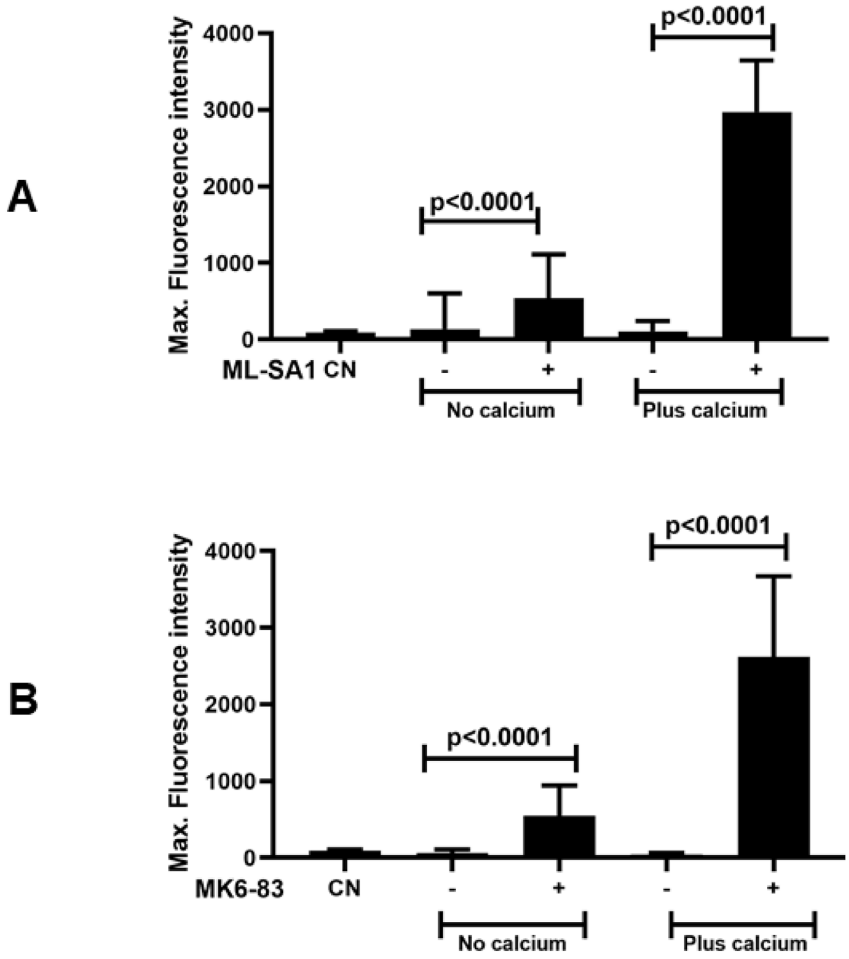

Fig. 7. Both extracellular Ca^{2+} and intracellular Ca^{2+} stores contribute to the SmTRPML-mediated increase in Ca^{2+}. Response of CHO cells expressing SmTRPML to 20 μM ML-SA1 (A; n = 65, 3 independent transfections) or 20 μM MK6-83 (B; n = 71, 3 independent transfections) in the presence or absence of extracellular Ca^{2+}. Note that SmTRPML-mediated increases compared to baseline occur in both conditions, indicating contributions from both influx of extracellular Ca^{2+} and release of intracellular stores. CN represents untransfected cells.

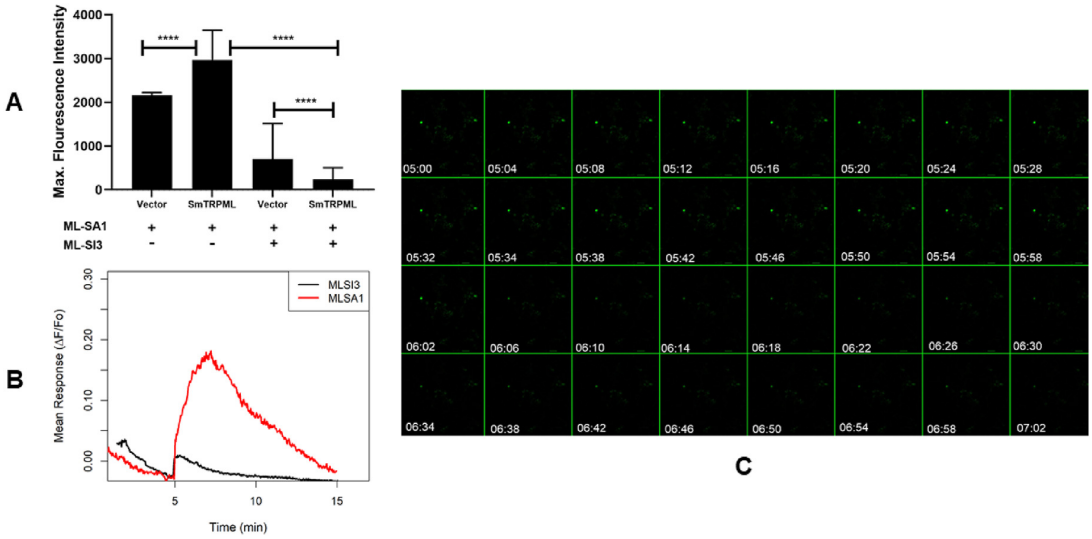

Fig. 8. The mammalian TRPML inhibitor ML-SI3 inhibits activation of SmTRPML expressed in CHO-K1 cells. (A) Cells were pre-incubated with 25 μM ML-SI3 for 2 h, and then tested for response to 20 μM ML-SA1 or exposed to ML-SA1 without ML-SI3 pre-incubation. Vector = cells transfected with pcNA3.1zeo^{+} plus p-GP-CMV-GCaMP6f (n = 33, 4 independent transfections); SmTRPML = cells transfected with SmTRPML-GCaMP6f and exposed to 20 μM ML-SA1 without (n = 24, 4 independent transfections) or with pre-incubation with 25 μM ML-SI3 (n = 138, 4 independent transfections) (B) Averaged GCaMP6f fluorescence intensity change vs. time in cells transfected with SmTRPML-GCaMP6f, treated with 25 μM MLSI3, and then exposed to 20 μM ML-SA1. C). **Montage image of fluorescence in CHO cells transfected with SmTRPML-GCaMP6f and exposed to ML-SA1 (20 μM) following pre-incubation with ML-SI3 (25 μM) for 2 h.**

manuscript. **Bruce D. Freedman:** Conception of the work, Writing of manuscript. **Robert M. Greenberg:** Conception of the work, Collection of data, Writing of manuscript, Analysis of data.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank Yue Ning for excellent technical support. We also thank the Penn Vet Imaging Core facility for their expertise and the use of imaging equipment and software. We thank the Schistosomiasis Resource Center for providing schistosome-infected snails and mice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2021.12.018.

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