Diethylcarbamazine, TRP channels and Ca$^{2+}$ signaling in cells of the Ascaris intestine

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Research Article

Keywords:

Posted Date: February 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1262219/v1

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Abstract

The nematode parasite intestine absorbs nutrients, is involved in innate immunity, can metabolize xenobiotics and as we show here, is also a site of action of the anthelmintic, diethylcarbamazine. Diethylcarbamazine (DEC) is used to treat lymphatic filariasis and activates TRP-2, GON-2 & CED-11 TRP channels in Brugia malayi muscle cells producing spastic paralysis. DEC also has effects on ascarid nematode parasites. Using PCR techniques, we detected, in A. suum intestine, message for: Asu-trp-2, Asu-gon-2, Asu-ced-11, Asu-ocr-1, Asu-osm-9 and Asu-trpa-1. Comparison of amino-acid sequences of the TRP channels of B. malayi, and A. suum revealed noteworthy similarity, suggesting that the intestine of Ascariis will also be sensitive to DEC. We used Fluo-3AM as a Ca$^{2+}$ indicator and observed characteristic unsteady time-dependent increases in the Ca$^{2+}$ signal in the intestine in response to DEC. Application of the TRP channel inhibitor, 2APB, prevented DEC mediated increases in intracellular Ca$^{2+}$. These observations are important because they emphasize that the nematode intestine, in addition of muscle, is a site of action of DEC as well as other anthelmintics. DEC may also enhance the Ca$^{2+}$ toxicity effects of other anthelmintics acting on the intestine or, increase the effects of other anthelmintics that are metabolized and excreted by the nematode intestine.

Introduction

Soil-transmitted helminths (STHs), including Ascaris, Trichuris, and hookworm, are a primary medical and public health concern in many developing countries. It is estimated that approximately 807 million to 1.2 billion people are infected with Ascaris lumbricoides $^1$. Although not usually fatal, parasitic infections have a detrimental effect on morbidity, reducing worker productivity by 6.3 million Disability Adjusted Life Years (DALYs) per year. Parasitic infections affect worker health, output, and the performance of children in school. In livestock, infestations lead to reduced food yields, impacting economic returns and exacerbating poverty $^2$.

Currently, there are no effective vaccines against STH infections, and treatment relies on the use of chemotherapeutics, usually using one of the three major classes of anthelmintics: benzimidazoles (albendazole/mebendazole), macrocyclic lactones (ivermectin), or nicotinic cholinergics (levamisole and pyrantel). There is a risk of resistance developing in humans associated with regular use of these compounds, as seen in animal parasites $^3$. With the limited number of anthelmintic drugs and the risk of resistance development, identifying the modes of action of existing anthelmintics and predicting rational anthelmintic combinations, which are more potent and effective, rather than relying on empirical combinations, is crucial.

The classic drug diethylcarbamazine (DEC) is used to treat lymphatic filariasis caused by filarial parasites including Brugia malayi and Wuchereria bancrofti $^4,5$, but DEC has also been used to treat STH infections, including ascariasis $^6$. Although DEC has been used for over seventy years, its mode of action is poorly understood. DEC’s recently discovered actions are to open nematode muscle Transient Receptor Potential (TRP) channels, including the TRPMs, GON-2 & CED-11, and the TRPC, TRP-2 $^7$. The opening of muscle cell TRPs causes entry of Ca$^{2+}$ and transient spastic paralysis. TRP channels in nematodes are not only found in muscle cells, but they are also found distributed throughout the different tissues of nematodes including the intestine $^8,5$. This suggests that DEC will also affect the cells of the intestine and affect its vital functions.

The nematode intestine is a long tube of single-layer columnar epithelial cells that connects the base of the pharynx to the anus. The intestine is vital for nematode survival. It is essential for digestion and transport ion solutes and nutrients $^{10-14}$. It is involved in defense against environmental toxins and microbial infection mediated by innate immunity and release of antimicrobial peptides $^{15,16}$. The intestine is a major site of metabolism of xenobiotic drugs by cytochrome P450 oxidative enzymes $^{17}$; it excretes xenobiotic compounds and their metabolites (including anthelmintic drugs) via its numerous ABC transporters and organic anion transporters $^{18}$. Toxic effects on the nematode intestine will produce a cascade of damage on other tissues including the neuromuscular and reproductive tissues of the parasite.

The intestine of parasitic nematodes is also one of the sites of action of anthelmintic drugs. The benzimidazoles, like mebendazole, disrupt the microtubules and block the transport of secretory granules and movement of subcellular organelles in the intestine of Ascaris suum $^{19}$. Levamisole, a cholinergic anthelmintic, targets nicotinic ion-channel receptors that are present in muscle $^{20}$ and intestinal cells of A. suum $^{21}$. Another potential anthelmintic acting on the intestine is Cry5B, a Cry protein recovered from Bacillus thuringiensis, which forms pores in the intestine membranes $^{22-26}$. Cry5B has potent toxic effects on a range of parasitic nematodes, including hookworm, Strongyloides and A. suum $^{26-28}$.

Here we study the effects of DEC on the intestine of the pig worm, Ascaris suum, which is genetically the same species as the human worm, Ascaris lumbricoides $^{29,30}$. We used Ca$^{2+}$ imaging to record simultaneously from groups of intestine cells in isolated open intestine flap preparations. This allowed us to separate and characterize the effects of anthelmintics on the intestine from the underlying muscle and hypodermis of the worm. We identified the message of different types of TRP channels in the intestine to compare it with the underlying muscle bags of the parasite and found different levels of tissue expression. The Ca$^{2+}$ imaging also showed that DEC produces an increase in intracellular Ca$^{2+}$ in the intestine by opening TRP channels that allow entry of extracellular Ca$^{2+}$ but DEC did not mediate an intracellular release of Ca$^{2+}$. The rise in the cytoplasmic Ca$^{2+}$ will be toxic and limit normal functions, including metabolism and excretion of xenobiotic drugs. By this mechanism, DEC will synergize and enhance the effects of other anthelmintics.

Materials And Methods

Collection and maintenance of A. suum worms:

Adult female A. suum worms were collected from the JBS Swift and Co. pork processing plant at Marshalltown, Iowa. Worms were maintained in Ascaris Ringers Solution (ARS: 13 mM NaCl, 9 mM CaCl$_2$, 7 mM MgCl$_2$, 12 mM C$_6$H$_{12}$NO$_3$/ Tris, 99 mM NaC$_2$H$_3$O$_2$, 19 mM KCl and 5 mM glucose pH 7.8) at 32°C for
Measurement of Ca
spontaneous Ca
intestinal samples were left under blue light for a minimum of 3 minutes to promote settling and equilibration of the fluorescent signal and monitor for any

All solutions were delivered to the chamber under gravity feed through solenoid valves controlled using a VC-6 six-channel Valve Controller (Warner Instruments, USA). One microgram (1 µg) of total RNA from each tissue was used to generate cDNA by reverse transcription (RT) using SuperScript IV VILO™ Master Mix (Life Technologies, USA) following the manufacturer's protocol. PCR was conducted to detect the presence of Asu-gon-2, Asu-trp-2, Asu-ced-11, and Asu-ocr-1, Asu-osm-9, and Asu-trpa-1 using primers that were designed for targeting encoding regions of each gene (Table 1). Asu-gapdh was used as a reference gene. Negative controls included enzyme, water, and both forward and reverse primers for the target with no cDNA template. The cycling conditions for PCR were an initial denaturation for 2 min at 95°C, followed by 35 cycles of 95°C for 30 sec, 58°C for 35 sec, 72°C for 45 sec, and a final extension at 72°C for 10 min using GoTaq® G2 Hot Start Green Master Mix (Promega, USA). The PCR products of each gene were then separated on an individual 1% Agarose containing SYBR® Safe DNA Gel Stain (ThermoFisher Scientific), for 45mins at 100V, followed by visualization under UV light to confirm the presence of the genes. All photographs were acquired using Visionworks™ software (Analytik Jena) with an exposure setting of 3 seconds per 1 frame. Original gel pictures are presented in Supplementary Fig. 1.

Analysis of mRNA levels by Quantitative Real-time PCR

To quantify the relative mRNA transcript levels of each identified TRP channel in the intestine and muscle bags, cDNA was synthesized from 1 µg of RNA from each adult female A. suum as described above. Target genes with fragments ranging from 150 to 200 bp were amplified by qPCR from each cDNA sample in triplicate. The same procedure was used for the reference gene Asu-gapdh. All primers for qPCR are presented in Table 2. The quantitative PCR reaction mixture consisted of 1 µl of cDNA template, 1 µl of forward and reverse primer mix, and 10 µl of PowerUp™ SYBR® Green Master Mix (Applied Biosystems, ThermoFisher, USA), with the final volume made up to 20 µl with Nuclease-free water. The cycling conditions included an initial denaturation for 10 seconds at 95°C, 40 cycles of 95°C for 15 seconds and 58°C for 30 seconds followed by a final melting curve step. Cycling was performed using a QuantStudio™ 3-96 well 0.1 ml Block Real time PCR Detection system (ThermoFisher, USA), and transcript quantities were derived by the system software, using the generated standard curves. mRNA expression levels for each subunit (Asu-gon-2, Asu-trp-2, Asu-ced-11, Asu-ocr-1, Asu-osm-9, and Asu-trpa-1) were estimated relative to the reference gene (Asu-gapdh) using the Pfaffi Method. The qPCR experiments were repeated 3 times for each gene and for each DEC treatment (all subunit mRNA quantifications were performed in triplicate for worm's muscle bag sample and intestinal tissue sample: 15 biological replicates each with three technical replicates).

Dendrogram construction

All protein sequences used for the construction of the dendrogram were acquired either from Wormbase Parasite, The European Nucleotide Archive (ENA) or UNIPROT PROTOT repositories. Accession number links to sequences used for each gene of each organism can be found in Table 3. All protein alignments were done using MAFFT (http://mafft.cbrc.jp/alignment/software/) 49. Amino acid similarity and identity was analyzed using MAFFT alignments. Percent identity was the percent ratio of identical amino acids and the total number of residues in the longest sequence. Similarly, percent similarity was determined by dividing number of identical and similar amino acids by the total number of residues in the longest sequence (x100). Phylogenetic analysis was done using the software MEGA X software 50 using the maximum likelihood method based on Le and Gascuel model 51.

Preparation and loading Fluo-3AM

A 2 cm section of the intestine was removed from the body piece using fine forceps and cut open. The intestinal flap was placed onto a coverslip (24 x 50 mm), and pinned using a slice anchor (26 x 1mm x 1.5mm grid, Warner Instruments, Hamden, CT), immersed in Ascaris Perienteric Fluid APF (23 mM NaCl, 110 mM NaAc, 24 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 5 mM HEPES, 11 mM D-glucose) in a laminar ow chamber (Warner RC26G, Warner Instruments, Hamden, CT). Fluo-3AM loading was achieved by incubating the intestine in APF solution with no added CaCl₂ (<100 µM Ca²⁺) containing 5 µM Fluo-3AM and 10% Pluronic F-127 (10% v/v) for one hour with the chamber connected to a Dual Automatic Temperature Controller (Warner Instruments, Hamden, CT) maintained at 34-36°C. After incubation, the Fluo-3AM solution was discarded, and the sample was incubated in APF containing 1 mM CaCl₂ for an additional 15 minutes at 34-36°C to promote Ca²⁺ loading. Intestinal tissues were then continuously perfused with APF containing 1 mM CaCl₂ and exposed to either 10 µM diethylcarbamazine (DEC), 10 µM 2-APB, 10 µM DEC and 2-APB or 10 mM CaCl₂ which was used as a positive control. For no added Ca²⁺ experiments, 1 mM CaCl₂ APF was exchanged for no added CaCl₂ APF (measured<100 µM Ca²⁺) after 10 minutes incubation and the sample was incubated for an additional 5 minutes before the beginning of each experiment at 34-36°C. During recordings, preparations were continually washed in solutions with no added CaCl₂. For recordings with La³⁺, intestinal preparations were incubated in 1mM CaCl₂ APF containing 100 µM LaCl₃ to inhibit TRP channels for 10 minutes. Samples were the incubated in no added CaCl₂ and 100 µM LaCl₃ for additional 5 minutes at 34-36°C. For all experiments, samples were exposed to solutions containing no added CaCl₂ and 100 µM LaCl₃. All incubations were done in the absence of light to prevent degradation of the fluorescent dye.

All solutions were delivered to the chamber under gravity feed through solenoid valves controlled using a VC-6 six-channel Valve Controller (Warner Instruments, Hamden, CT) through an inline heater set at 37°C (Warner Instruments, Hamden, CT) at a rate of 1.5mL/min. At the start of all experiments, intestinal samples were left under blue light for a minimum of 3 minutes to promote settling and equilibration of the fluorescent signal and monitor for any spontaneous Ca²⁺ signaling before application of any compound.

Measurement of Ca²⁺ fluorescence
All recordings were performed on a Nikon Eclipse TE3000 microscope (20X/0.45 Nikon PlanFluor objective), fitted with a Photometrics Retiga R1 Camera (Surrey, BC, Canada). Light control was achieved using a Lambda 10-2 two-filter wheel system with a shutter controller (Lambda Instruments, Switzerland). Filter wheel one was set on a green filter between the microscope and camera. Filter wheel two was set on the blue filter between a Lambda LS Xenon bulb light box, which delivered light via a fiber optic cable to the microscope (Lambda Instruments, Switzerland). Blue light emission was controlled by using a shutter. Minimal illumination exposure was used to prevent photobleaching.

All Ca$^{2+}$ signal recordings were acquired and analyzed using MetaFluor 7.10.2 (MDS Analytical Technologies, Sunnyvale, CA) with exposure settings at 150 ms with 2x binning. Maximal percent Ca$^{2+}$ signal amplitudes ($\Delta F$) were calculated using the equation $F1-F0/F0 \times 100$, where $F1$ is the fluorescent value and $F0$ is the baseline value. All F0 values were determined as being the value when any stimulus was applied to the sample for all traces analyzed.

Details of numbers of regions and numbers of preparations used for measurements of the Ca$^{2+}$ signals

Calcium signals from each intestine were collected from 50 square 50 μm x 50 μm areas across the intestine covering a total area of 125,000 μm$^2$ that included 800-1000 individual enterocytes. The average fluorescence amplitude was calculated for each intestinal exposure of all 50 regions. For the DEC experiments, enterocytes were exposed to 10 µM DEC for 5 minutes followed by 10 mM CaCl$_2$ as the positive control. For the 2-APB experiments enterocytes were exposed to 10 µM 2-APB for 5 minutes followed by 10 mM CaCl$_2$. For the study of the effects of 2-APB on the DEC responses, preparations were exposed to 10 µM 2-APB and 10 µM DEC at the same time for 5 minutes before 2-APB was removed and DEC was left for an additional 5 minutes. 10 mM CaCl$_2$ was then used as a positive control. Finally, for the no added Ca$^{2+}$ and La$^{3+}$ experiments 10 µM DEC was applied for 5 minutes before subjected to 10 mM CaCl$_2$.

Statistical Analysis

Statistical analysis of all data was done using GraphPad Prism 5.0 (Graphpad Software, Inc., La Jolla, CA, USA). To ensure reproducibility, we repeated our experiments: the numbers of female worms, intestine preparations, the concentrations, and durations of applications of 2-APB, DEC, La$^{3+}$ are provided in the legends of the figures. Analysis of Ca$^{2+}$ amplitudes and time to peak were done using either unpaired or paired student t tests. $P < 0.05$ was used. The data are presented as relative expression levels in mean ± SEM for each TRP channel subunit relative to Asu-gapdh in muscle bags and intestinal tissue. Comparisons were made using Tukey’s post hoc test.

Chemicals

Source of chemicals: 2-APB was procured from Tocris; Sigma Aldrich supplied all other chemicals.

Results

**TRP channels in Ascaris**

In the filarial nematode, *Brugia malayi*, DEC stimulates spastic muscle paralysis by opening muscle TRP channels of the TRPM group (*Bma*-GON-2 and *Bma*-CED-11) and the TRPC group (*Bma*-TRP-2) [7]. We blasted these genes against the *A. suum* database (Wormbase) and found *Asu*-gon-2, *Asu*-ced-11, *Asu*-trp-2. We also found the TRPV genes, *Asu*-ocr-1 & *Asu*-osm-9, and the TRPA channel gene, *Asu*-trpa-1. We compared the sequences of these six TRP *A. suum* genes: *Asu*-gon-2, *Asu*-ced-11, *Asu*-trp-2, *Asu*-ocr-1, *Asu*-osm-9, and *Asu*-trpa-1 with the sequences of the same genes in: 1) the Clade III nematode parasites, *Parascaris equorum* and *Brugia malayi*; 2) the Clade V nematode, *C. elegans*; 3) the Clade I nematode parasite, *Trichuris muris*; 4) the schistosome, *Schistosoma mansoni* and, 5) the fly, *Drosophila melanogaster*. Accession number links to all sequences used can be found in Table 3. The dendrogram, Fig. 1, shows that the TRP channels of *A. suum* and *B. malayi* are more closely related than the TRP genes of *C. elegans*, *S. mansoni*, and *D. melanogaster*. The close relationship of the TRP channels of *A. suum* and *B. malayi* encouraged the hypothesis that TRP channels of *A. suum* and *B. malayi* may have a similar pharmacology and possible sensitivity to DEC.

**TRP channels expression in Ascaris intestine**

To determine if these TRP channel genes are expressed in the intestine and muscle bags of *Ascaris*, we generated screening primers targeting the coding region of these six genes (Table 1). We compared the expression found in the intestine and expression found in the muscle bag regions of the same animal. We found that all six genes were expressed in either the intestine or the muscle bag regions, or both (Fig. 2; for original uncropped gel pictures see Supplementary Fig. 1). Expression of the TRPM channels, *Asu*-gon-2, and *Asu*-ced-11, were found in both tissues, with *Asu*-gon-2 appearing to show a similar band intensity in both the intestine and muscle bags, whereas *Asu*-ced-11 showed brighter bands in the muscle bags (Fig. 2A & B). *Asu*-trp-2 expression appeared to have similar levels of band intensity in both tissues (Fig. 2C). We also observed expression for the TRPV channels, with *Asu*-ocr-1 appearing having stronger band intensity in the intestine (Fig. 2D) while *osm*-9 had stronger band intensity in the muscle bags (Fig. 2E). Lastly, *Asu*-trpa-1 was expressed in both the intestine and the muscle bag regions (Fig. 2F). Thus, the TRP channels (*Asu*-GON-2, *Asu*-CED-11 & *Asu*-TRP-2) that are associated with mediating DEC effects on the muscle of the filarial parasite, *Brugia malayi*, are present in the intestine of *A. suum*.

**TRP channels have tissue-specific expression levels in Ascaris**

Variations in band intensity for the different TRP channels between the intestine and muscle bag regions suggested differences in the relative mRNA expression levels for each channel. We used qPCR to measure the mRNA expression levels for each of the six channels in the intestine relative to the reference gene, *Asu*-gapdh. We compared the TRP channel expression levels to their paired muscle bag counterparts.

Our analysis showed that *Asu*-gon-2 had a higher expression level (4.5-fold) in the intestine compared to the muscle bag (Fig. 3, black bar). We observed that *Asu*-trp-2 had similar levels of expression in both the intestine and muscle bag (Fig. 3, blue bar). For *Asu*-ced-11, we observed a reduced expression in the
intestine compared to muscle, suggesting that the CED-11 function in the intestine is less significant, Fig. 3. For the TRPV channels, OCR-1, and OSM-9, we observed that OCR-1 has a higher expression level in the intestine, 20-fold (Fig. 3, red bar), and that OSM-9 expression is below a detectable level in the intestine (Fig. 3). Lastly, for TRPA-1, although we observed similar levels of band intensity on the gels, qPCR showed expression at a higher level in the intestine (3.5-fold) compared to the muscle (Fig. 3, green bar). High levels of diverse TRP channel transcripts could suggest a functional role for these receptors in A. suum intestine.

In B. malayi, DEC activates TRP channels that involve TRP-2, GON-2, and/or CED-11-subunits. The presence of clear and significant expression levels of Asu-gon-2 and similar expression of Asu-trp-2 but not Asu-ced-11 suggests that the GON-2 and TRP-2 channels may be activated by DEC. We do not exclude the possibility that DEC may also interact with the TRPV channel, OCR-1, and the TRPA channel, TRPA-1, in the intestine of A. suum based on their high levels of expression in the intestine.

DEC stimulates a prolonged Ca\textsuperscript{2+} signal in the Ascaris intestine via TRP channels.

We loaded the cells of the intestines of A. suum with the Ca\textsuperscript{2+} reporter Fluo-3-AM (Methods). Increasing the Ca\textsuperscript{2+} concentration in the APF bathing solution from 1 mM to 10 mM provides a valuable control test for the condition of the intestine preparation. In fresh preparations, it produced a rapid and smooth increase in the Fluo-3 fluorescence starting within 20 seconds of application. This Ca\textsuperscript{2+} induced rise in cytosolic calcium declined immediately after the CaCl\textsubscript{2} concentration was returned to 1 mM. The time for the CaCl\textsubscript{2} signal to reach the peak was relatively fast, taking a mean of 3 minutes, Fig. 4A & D.

Previous work on the muscle cells of the filarial nematode, B. malayi, showed that DEC opens their Transient Receptor Potential channels (TRPs), causing spastic paralysis \textsuperscript{7}. We anticipated that DEC would also open TRP channels in the intestines of Ascaris. We bathed the intestines in 10 \textmu M DEC for 5 minutes and observed that DEC produced a slow but uneven 'sometimes step-like' Ca\textsuperscript{2+} signal in the intestine as opposed to the smoother rise caused by the CaCl\textsubscript{2} and was initiated within 30 seconds of the DEC application (Fig. 4B). The increase continued to rise after removal and washout of DEC before eventually declining; the Ca\textsuperscript{2+} concentration reached its peak at a mean of 14.5 minutes after DEC exposure (Fig. 4D). The peak amplitude of the DEC signal was significantly smaller (10\%) and slower than that produced by the application of 10 mM CaCl\textsubscript{2} (31\%), Fig. 4C. Based on our observations, DEC can generate a detectable Ca\textsuperscript{2+} signal with unique characteristics.

To determine if the observed DEC mediated responses were due to effects on TRP channels, we used the broad-spectrum TRP channel antagonist, 2-APB (2-aminoethoxydiphenyl borate). Application of 10 \textmu M 2-APB alone had no effect on the Ca\textsuperscript{2+} signal (Fig. 5C; black bar). We found that 10 \textmu M 2-APB did not inhibit the effect of 10 \textmu M DEC if applied after the DEC: the Ca\textsuperscript{2+} response continued in the presence of 2-APB and after removal of DEC, Fig. 5A. However, if 2-APB was applied at the same time as DEC, it prevented any increase in the intracellular Ca\textsuperscript{2+} fluorescence (Fig. 5B & 5C; white bar) until the 2-APB was removed, which generated an increase in fluorescence that continued after DEC was removed (Fig. 5B & 5C, grey bar).

We checked the response to 10 mM CaCl\textsubscript{2} after 2-APB and DEC applications to ensure that the preparation was still responding normally and saw the usual ~30\% increase in the fluorescent signal (Fig. 5C dark grey bar). Thus, 2-APB does not irreversibly inhibit the Ca\textsuperscript{2+} signaling machinery. The histogram shown in Fig. 5C summarizes the mean % changes in Ca\textsuperscript{2+} fluorescence changes from 6 similar experiments: co-application of 2-APB and DEC prevents the increase in the DEC signal (Fig. 5C; compare white bar and light grey bar), but when 2-APB was removed in the presence of DEC, we observed the increased Ca\textsuperscript{2+} signal with its characteristic phenotype and similar peak (Fig. 5B & 5C; light grey bar). Our results suggest that DEC allows the entry of calcium into the intestine by the opening of TRP channels. The continued increase in Ca\textsuperscript{2+} fluorescence after removal of DEC may relate to a slow wash-off effect of DEC or downstream secondary Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release from intracellular stores \textsuperscript{31}.

Prolonged DEC exposure does not change TRP mRNA levels in the intestine or muscle

Maintained applications of diethylcarbamazine to adult B. malayi produces a temporary spastic paralysis that lasts for less than 4 hours that is followed by recovery \textsuperscript{7}. We were interested in determining if there was an accommodation to DEC in A. suum mediated by reducing TRP channel message during a 4-hour exposure. We treated isolated intestine flaps and muscle flaps with 10 and 100 \textmu M DEC for 4 hours. Again, we used qPCR to measure relative mRNA levels of the six TRP channels in the treated flaps and compared them to untreated controls that were bathed in RPMI. We observed no significant changes in message levels for any TRP channels in either the intestines or muscle cells samples treated with either 10 \textmu M or 100 \textmu M DEC (Fig. 6A & B). These observations suggest that TRP channel expression in intestines or muscle cells of A. suum is not sensitive to DEC over this time-scale.

DEC mediated signals are dependent on extracellular Ca\textsuperscript{2+}

In the C. elegans intestine, the TRPM6 and TRPM7 channel homologs, GON-2 and GTL-1, have nearly identical pore domains \textsuperscript{32–34} and exhibit greater than 60-fold selectivity for Ca\textsuperscript{2+} over Na\textsuperscript{+} \textsuperscript{35} and have the highest Ca\textsuperscript{2+} selectivity of the TRP channels. These channels have been demonstrated to regulate IP\textsubscript{3}-dependent oscillatory Ca\textsuperscript{2+} signals and regulate the nematode’s defecation cycle \textsuperscript{31}. GON-2/GTL-1 also promotes Mg\textsuperscript{2+} entry and homeostasis in the C. elegans intestine regulating electrolyte absorption and supply \textsuperscript{36}.

To determine if our DEC activated TRP channels produced entry of extracellular Ca\textsuperscript{2+} to deliver the increased cytosolic Ca\textsuperscript{2+} rather than via an intracellular release mechanism, we removed Ca\textsuperscript{2+} from the extracellular environment by washing and exposing intestines to DEC in APF buffer with no added CaCl\textsubscript{2} (<100 Ca\textsuperscript{2+} \textmu M). Fig. 7A shows an intestine treated with 10 \textmu M DEC bathed in the no added Ca\textsuperscript{2+} APF. No increase in fluorescence during or after the application is seen. But interestingly, there was a reduction (~13\%) in the calcium signal suggesting that the cytosolic Ca\textsuperscript{2+} was being leached from the preparation (Fig. 7A & C, white bar). DEC opening TRP channels in the plasma membrane that are permeable to Ca\textsuperscript{2+} may explain the effect. The intracellular concentration is leached out because it is initially higher than the no added Ca\textsuperscript{2+} extracellular levels. To show that the intestinal sample was still functioning physiologically
The intestine of parasitic nematodes is increasingly being recognized as a major site of action of a number of anthelmintic drugs in addition to the channels (GON-2) and an elaborate network of microtubules that includes a terminal web under the apical microvilli facing the somatic body muscle cells. The digestion of food involves the secretion of proteases and lipases and the absorption of nutrients including glucose into the intestine cells. Columnar cells, electrically coupled to their neighbors, are polarized with the apical region bearing microvilli and the basolateral region facing the somatic body muscle cells. Damage to the cells of the intestine will adversely affect these vital functions.

UDP transferases and V-type ATPases are crucial for nutrient absorption and homeostasis. The nematode intestine carries out essential functions for survival, including: 1) digestion and nutrient absorption, 2) pH regulation via apical membranes, 3) storage of lipids, 4) innate immunity, 5) secretion of bactericidal peptides, 6) drug metabolism with P450 cytochromes and UDP transferases, and 7) excretion by organic-anion-transporter (OAT-1), P-glycoprotein- and MDR-transporters into the lumen of the intestine. Damage to the cells of the intestine will adversely affect these vital functions.

The intestine cells are columnar cells, electrically coupled to their neighbors, and polarized with the apical region bearing microvilli and the basolateral region facing the somatic body muscle cells. The digestion of food involves the secretion of proteases and lipases and the absorption of nutrients including glucose and lipids. It is facilitated by a range of selective transporters. The processes of absorption of nutrients and secretion of proteases also require significant endocytosis and exocytosis utilizing different types of vacuoles and vesicles that are present in the intestinal cells; these vesicles are moved within the cell on an elaborate network of microtubules that includes a terminal web under the apical microvilli. Absorption of Ca²⁺ and Mg²⁺ is mediated by TRPM-like ion-channels (GON-2).

The intestine of parasitic nematodes is increasingly being recognized as a major site of action of a number of anthelmintic drugs in addition to the neuromuscular system. These anthelmintics now include:

1. **Diethylcarbamazine (DEC):** The disturbance in the Ca²⁺ homeostasis of the intestine by an action of DEC on TRP channels can disrupt vital functions of the enterocytes and will contribute to the anthelmintic action of DEC against ascariasis. DEC produces a fall in calcium, suggesting that cytosolic calcium is leached through the DEC TRP channels. The application of the anthelmintic levamisole opens these nicotinic acetylcholine channels that produce a clear and characteristic disturbance in the Ca²⁺ homeostasis of the intestine.

2. **Benzimidazoles:** The intestine of nematodes is sensitive to the actions of the benzimidazole anthelmintics, mebendazole, and albendazole. These drugs bind selectively to the β-tubulin of nematodes, disrupt microtubules, and the movement of digestive vacuoles and lysosomes, leading to autolysis of the intestinal cells.

3. **Levamisole:** We have reported that the intestine expresses message for different acetylcholine gated ion channels subunits, including UNC-38, UNC-29, UNC-63, and ACR-8. The application of the anthelmintic levamisole opens these nicotinic acetylcholine channels that produce a clear and characteristic calcium signal. The function of these channels in the intestine cells may be paracrine, to communicate locally with adjacent intestine cells and underlying muscle cells, because the cells are not electrophoretically excitable like muscle cells or nerve cells.

4. **The Bacillus thuringiensis toxin:** Cry5B is a potent pore-forming toxin that can target multiple different species of gastrointestinal parasites including *Ancylostoma ceylanicum*. Cry5B appears to have two modes of action: 1) binding selectively to the CDH-8 cadherin present on the surface of the cell membrane, leading to oligomerization of the protein by crystallinization, 2) and 2) interaction with glycolipids produced by BRE-5, a glycosyltransferase that catalyzes the addition of monosaccharides onto glycolipids, leading to Cry5B insertion into the membrane forming the pore. Interestingly, *C. elegans* that are resistant to the anthelmintic levamisole, show increased hyper-susceptibility to Cry5B and vice versa with Cry5B resistant strains showing increased sensitivity to levamisole, resulting in a synergistic relationship between two anthelmintics. Here, we have observed that DEC has a direct effect on the intestine, in addition to the known effects of levamisole. In addition to levamisole, the two other cholinergic agonist anthelmintics, pyrantel and tribendimidine also have strong synergistic effects with Cry5B in *C. elegans* that is also seen in the hookworm, *Ancylostoma ceylanicum*. The observations that we describe here, suggest a mechanism by which DEC will add to, and potentiate, the effects of albendazole, levamisole, ivermectin, and Cry5B in combinations treatments for STH infections.
We have pointed out above that the nematode parasite intestine is a site of action of DEC, the benzimidazoles, cholinergic anthelmintics like levamisole, and Cry5B. These anthelmintic drugs disrupt the normal Ca\(^{2+}\) homeostasis and vital functions and suggest that combinations of these anthelmintics will have additive or synergistic effects. Adverse effects on the intestine will potentiate the effects of other anthelmintics that are metabolized or excreted by the intestine. One example is the macrocyclic lactone, ivermectin, which acts as positive allosteric modulators of the Glutamate-gated Chloride channel. P-glycoproteins have been suggested as candidate genes for ivermectin resistance by acting as efflux pumps. The P-glycoprotein gene pgp-9 has been repeatedly associated with macrocyclic lactone resistance in different nematode parasites. \(^ {45,46}\) Although P-glycoproteins are found in other nematode tissues, they are most strongly expressed in the intestine of the parasites. \(^ {39,47}\) The combination of albendazole with ivermectin or diethylcarbamazine is anticipated to enhance the treatment effect of STHs with albendazole alone, ivermectin alone, or diethylcarbamazine alone. This has, in fact, been seen in the study of the effects of albendazole, ivermectin, and diethylcarbamazine alone and combinations against *Ascariasis* and *Trichuriasis*. \(^ {48}\) The parasite intestine offers a common target where anthelmintics can have synergistic effects.

**Declarations**

**Data availability statements:**

The datasets analyzed during the current study are available in Wormbase Parasite, The European Nucleotide Archive (ENA) and UNIPROT repositories, https://parasite.wormbase.org/index.html, www.elixir-europe.org/platforms/data/core-data-resources, www.uniprot.org.

**Acknowledgements:**

We acknowledge the NIH National Institute of Allergy and Infectious Diseases grants R01AI047194 and R01AI155413 to RJM, the E. A. Benbrook Foundation for Pathology and Parasitology for support to RJM. The funding agencies had no role in the design, execution or publication of this study. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases.

**Author Contributions:**

Paul Williams and Richard Martin wrote the main manuscript text. Paul Williams, Richard Martin, and Alan P. Robertson provided interpretations, conceptions, and designs of experimental procedures. Paul Williams acquired and analyzed experimental data. Sudhanva Kashyap designed Fig. 1 and wrote the corresponding methods on production of the dendrogram. Mark McHugh was involved in generation and interpretation of data for Fig. 2. All authors have reviewed the manuscript.

**Competing interests:**

The authors declare no competing interests.

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### Tables

**Table 1. Primer sequences used for RT-PCR.** Forward and reverse primer sequences for the TRP channel genes gon-2, trp-2, ced-11, ocr-1, osm-9 and trpa-1 and the reference gene gapdh in Ascaris suum.

| Gene          | Forward Primer | Reverse Primer |
|---------------|----------------|----------------|
| Asu-gon-2     | GTCGACGCTGCGAGTTACAG | ACGATCGCCAAATGATCA |
| Asu-trp-2     | GTGATGGGATGCTGGGAT | AGGAATAGAGAAAGGCA |
| Asu-ced-11    | ATTTCTACCAAGTCGTGAGT | TCATCGCTGAGCAACATCG |
| Asu-ocr-1     | AAAAGCTCAAGTTGCTGGA | TAGCAGTGTGATTTGCACA |
| Asu-osm-9     | CCGGATATGGAGATGCA | GACGATGGTATCCAGCAG |
| Asu-trpa-1    | ATGCTACTGCCGAGTGAGC | TTACGATCTCATATGGAGC |
| Asu-gapdh    | AGCAAGGCCCTCCTGAGAT | TCTCCAGTCGGGCTGAGA |

**Table 2. Primer sequences targeting middle of each gene for quantitative PCR.** Forward and reverse primers used for qPCR experiments for the TRP channel genes gon-2, trp-2, ced-11, ocr-1, osm-9 and trpa-1 and the reference gene gapdh in Ascaris suum.

| Gene          | Forward Primer | Reverse Primer |
|---------------|----------------|----------------|
| Asu-gon-2     | GTCGACGCTGCGAGTTACAG | ACGATCGCCAAATGATCA |
| Asu-trp-2     | GTGATGGGATGCTGGGAT | AGGAATAGAGAAAGGCA |
| Asu-ced-11    | ATTTCTACCAAGTCGTGAGT | TCATCGCTGAGCAACATCG |
| Asu-ocr-1     | AAAAGCTCAAGTTGCTGGA | TAGCAGTGTGATTTGCACA |
| Asu-osm-9     | CCGGATATGGAGATGCA | GACGATGGTATCCAGCAG |
| Asu-trpa-1    | ATGCTACTGCCGAGTGAGC | TTACGATCTCATATGGAGC |
| Asu-gapdh    | AGCAAGGCCCTCCTGAGAT | TCTCCAGTCGGGCTGAGA |

**Table 3. Accession numbers for genes analyzed for all species.** Hyperlinks to all gene and protein sequences for each organism used for the generation of the dendrogram in Fig. 1. N/A = Not Applicable as a suitable gene could not be found in the organisms’ genome. Note the two genes listed for A. suum OCR-1. Combination of the two genes led to better fit for the tree suggesting that the two genes may be one single gene.

**Figures**
| Species                  | GON-2   | CED-11   | TRP-2   | OCR-1   | OSM-9   |
|-------------------------|---------|----------|---------|---------|---------|
| Ascaris suum            | AgR007_g147 | AgB05_g111 | AgR004_g234 | AgR018X_g126 / AgR018X_g125 | AgR022_g181 |
|                         | AEUI03000013.1 | AEUI03000007.1 | AEUI03000009.1 | AEUI03000026.1 / AEUI03000026.1 | AEUI03000026.1 |
| Parascaris equorum      | PEQ_0000273201_LM470319.1 | PEQ_0001179501_LM463558.1 | PEQ_0001228701_LM481478.1 | PEQ_0001206301_LM464818.1 | PEQ_0001023201_LM463237.1 |
| Caenorhabditis elegans  | WBGene00001651 | WBGene00006615 | WBGene00006615 | WBGene00003838 | WBGene00003899 |
|                         | BX284601.5 | BX284603.4 | BX284603.4 | BX284605.5 | BX284604.4 |
| Brugia malayi           | WBGene00228694 | WBGene00227742 | WBGene00225507 | WBGene00225952 | WBGene00287459 |
|                         | CAAKNF010000194.1 | CAAKNF010000192.1 | CAAKNF010000192.1 | CAAKNF010000195.1 | CAAKNF010000196.1 |
| Trichuris muris         | WBGene00291806 | WBGene00291719 | WBGene00291415 | WBGene00287459 | WBGene00290215 |
| Schistosoma mansoni     | Smp_147140 | Smp_246790 | Smp_336170 | N/A | N/A |
|                         | LR214935.1 | LR214931.1 | LR214933.1 | LR214933.1 | LR214933.1 |
| Drosophila melanogaster | A8DYE2   | Q9VJJ7   | A0A0B4LGJ2 | M9P1S7 | Q9W3W0 |

Figure 1

*Phylogenetic of TRP channels protein sequences conservation across multiple parasitic species:* Phylogenetic tree showing conservation of the TRPM channels GON-2 and CED-11, TRPC channel TRP-2, TRPV channels OCR-1 and OSM-9 and TRPA channel TRPA-1 in multiple species; Clade III *Ascaris suum*, *Parascaris equorum* and *Brugia malayi*, Clade V *C. elegans*, Clade I *Trichuris muris*, schistosome, *Schistosoma mansoni* and the fly *Drosophila melanogaster*. Tree was constructed using MEGA X software by maximum likelihood method based on the Le and Gascuel model.
Figure 2

Localization of TRP channels in the intestine and muscle bag region. RT-PCR analysis of intestine (1i, 2i, 3i, 4i, 5i) and muscle bag (1b, 2b, 3b, 4b, 5b) of five separate female *A. suum* worms. Each lane represents the intestine or muscle bag of an individual worm. *Asu-gapdh* from the intestine (Ci) or muscle bag (Cb) was used as a positive control. N.C. = negative control, no cDNA template present. M = Fast Ruler Middle Range DNA Ladder (ThermoFisher Scientific). A) *Asu-gon-2*, B) *Asu-ced-11* C) *Asu-trp-2*, D) *Asu-ocr-1* E) *Asu-osm-9*, F) *Asu-trpa-1*. Note the reduced intensity of the bands (white circles) with *Asu-ced-11* from the intestine. All images are cropped. Images were taken under UV light with an exposure setting of 3 seconds per 1 frame. Original gel images are presented in Supplementary Fig. 1.

![Figure 2](image)

Figure 3

Differential expression of TRP channels between intestine and muscle bag. Bar charts (expressed as mean ± SEM) demonstrating transcript level analysis for TRP channels from intestinal samples for GON-2 (red bar), TRP-2 (blue bar), CED-11, OCR-1 (red bar), OSM-9 and TRPA-1 (green bar) when compared to paired muscle samples. n = 15 individual worms over three biological replicates.

![Figure 3](image)

Figure 4

DEC stimulates Ca$^{2+}$ signals in *Ascaris suum* intestines: A) Representative Ca$^{2+}$ signal in response to 10 mM CaCl$_2$. Grey box indicates stimulus application. B) Representative Ca$^{2+}$ response to 10 µM DEC. Grey box represents stimulus application. C) Total amplitudes of Ca$^{2+}$ induced Fluo-3 fluorescence in response to 10 mM CaCl$_2$ and 10 µM DEC. * significantly different to CaCl$_2$ (CaCl$_2$ vs DEC *P* < 0.0255, *t* = 2.827, *df* = 7, paired *t*-test) n = 8 intestines from 7 female worms. D) Quantification of time for Ca$^{2+}$ to reach maximum in response to 10 mM CaCl$_2$ and 10 µM DEC. ** significantly different to CaCl$_2$ (CaCl$_2$ vs DEC *P* < 0.0014, *t* = 5.122, *df* = 7 paired *t*-test) n = 8 intestines from 7 female worms. All values represented as means ± SEM.

![Figure 4](image)

Figure 5

2-APB inhibits DEC mediated Ca$^{2+}$ signals A) Representative trace in response to 10 µM 2-APB being applied after 10 µM DEC initiates a Ca$^{2+}$ signal. Grey box represents DEC 10 µM application, bar represents 10 µM 2-APB. B) Representative trace in response to 10 µM 2-APB being applied at the same time as 10 µM DEC. Grey box represents DEC 10 µM application, bar represents 10 µM 2-APB. C) Total amplitudes of Fluo-3 fluorescence in response to 10 µM 2-APB (black
bar), 10 µM 2-APB & 10 µM DEC (white bar), 10 µM DEC (light grey bar) and 10 mM CaCl$_2$ (dark grey bar). N.S. not significantly different to 2-APB (2-APB vs 2-APB + DEC P = 0.6519, t = 0.4650, df = 10, unpaired t-test). 2-APB n = 6 intestines from 3 females; 2-APB + DEC n = 6 intestines from 4 females. * Significantly different to 2-APB + DEC (2-APB + DEC vs DEC P < 0.026 t = 3.102 df = 5, paired t-test). n = 6 intestines from 4 females. † Significantly different to DEC (DEC vs CaCl$_2$ P < 0.0326, t = 2.931, df = 5, paired t-test). n = 6 intestines from 4 females. All values represented as means ± SEM.

Figure 6

**Long-term DEC exposure does not change TRP channel expression:** A) Transcript level analysis of TRP channels in intestines for GON-2, TRP-2, CED-11, OCR-1, OSM-9, and TRPA-1 after 4-hour treatment with 10 µM DEC (Solid) or 100 µM (-lined) when compared to paired untreated intestinal samples. B) Transcript level analysis of TRP channels in muscles bags for GON-2, TRP-2, CED-11, OCR-1, OSM-9, and TRPA-1 after 4-hour treatment with 10 µM DEC (Solid) or 100 µM (lined) when compared to paired untreated muscle samples. One-way analysis of variance (ANOVA) showed no significant difference in transcript levels between 10 µM and 100 µM DEC treated samples in the intestine or muscle. All values represented as means ± SEM.

Figure 7

**DEC responses are initiated by Ca$^{2+}$ entry through TRP channels:** A) Representative trace of a 10 µM DEC response in the presence of no added Ca$^{2+}$ APF solution. Grey box indicates stimulus application. B) Representative trace of a 10 µM DEC response with 100 µM LaCl$_3$ and no added Ca$^{2+}$. Grey box indicates stimulus application. C) Comparison of total amplitudes of Fluo-3 fluorescence in samples with no added CaCl$_2$ in the bath solution in response to 10 mM CaCl$_2$ (black bar) and 10 µM DEC (white bar), (n = 8 intestinal preparations from 6 individual female *Ascaris*), and samples treated with 100 µM LaCl$_3$ and no added Ca$^{2+}$ in response to 10 mM CaCl$_2$ (grey bar) and DEC (lined bar), n = 10 intestinal preparations from 6 individual female worms. *** Significantly different to 10 mM CaCl$_2$ no added CaCl$_2$ (10 mM CaCl$_2$ no added CaCl$_2$ vs 10 mM CaCl$_2$ 100 µM LaCl$_3$ P < 0.0001, t = 5.802 df = 16, unpaired t-test). * Significantly different to DEC no added CaCl$_2$ (No added CaCl$_2$ DEC vs 100 µM LaCl$_3$ DEC P < 0.0124 t = 2.186, df = 16, unpaired t-test). All values represented as means ± SEM.

Figure 8

**Summary diagram of proposed DEC stimulation of Ca$^{2+}$ entry into intestinal cells via TRP channels:** Diagram representing the mode of action for DEC in the intestine of *Ascaris suum*. DEC activates the TRPM channel GON-2 (red) and TRPC channel TRP-2 (blue), to promote extracellular Ca$^{2+}$ entry. DEC may also interact with the TRPV channel, OCR-1 (pink), and the TRPA channel, TRPA-1 (orange), to promote Ca$^{2+}$ entry. Cation entry can lead to two possible pathways: 1) Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) leading to intracellular storage release via RYR receptors (green) or 2) activation of Ca$^{2+}$ channels (yellow) on the membrane by changes in membrane potential (ΔV) increasing extracellular Ca$^{2+}$ entry. 2-APB and La$^{3+}$ can inhibit TRP channels preventing Ca$^{2+}$ entry and subsequent Ca$^{2+}$ increases.

**Supplementary Files**

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