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Recording drug responses from adult *Dirofilaria immitis* pharyngeal and somatic muscle cells

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ABSTRACT

Despite being considered one of the most pathogenic helminth infections of companion animals, members of macrocyclic lactone class are the only drugs available for the prevention of heartworm disease caused by *Dirofilaria immitis*. Alarmingly, heartworm prevention is at risk; several studies confirm the existence of macrocyclic lactone resistance in *D. immitis* populations across the United States.

To safeguard the long term prevention and control of this disease, the identification and development of novel anthelmintics is urgently needed. To identify novel, resistance-breaking drugs, it is highly desirable to:

Unfortunately, none of the three above statements can be answered sufficiently for *D. immitis* and most of our hypotheses derive from surrogate species and/or *in vitro* studies. Therefore, the present study aims to improve our fundamental understanding of the neuromuscular system of the canine heartworm by establishing new methods allowing the investigation of body wall and pharyngeal muscle responses and their modulation by anthelmintics. We found that the pharynx of adult *D. immitis* responds to both ivermectin and moxidectin with EC_{50}s in the low micromolar range. We also demonstrate that the somatic muscle cells have robust responses to 30 μM acetylcholine, levamisole, pyrantel and nicotine. This is important preliminary data, demonstrating the feasibility of electrophysiological studies in this important parasite.

a) Understand the mode of action of the gold-standard
b) Understand the mechanism(s) of resistance against the gold-standard, and
c) Implement a highly predictive screening cascade.

1. Introduction

Dirofilaria immitis is the causative agent of heartworm disease and is widespread in warm and humid areas all over the world (McCall et al., 2008; Puttachary et al., 2013; Mani et al., 2016). Despite the introduction of the macrocyclic lactone anthelmintics, in a variety of safe, efficacious, and convenient heartworm preventatives starting from 1987 (Venco et al., 2004; McTier et al., 2019), a tremendous number of cases in both domestic cats and dogs is diagnosed every year (Wolstenholme et al., 2015).

In general, three factors namely (i) the vector, (ii) the parasite, and (iii) the host determine the spread of heartworm disease (Brown et al., 2012). Major reasons for the continuous severity of incidences are the following:

(i) The peri-urban prevalence of heartworm-positive natural reservoirs (wild canids like coyotes, foxes, jackals and wolves (Simon et al., 2012)), but also stray and neglected dogs (American Heartworm et al., 2004),
(ii) the spread of the parasite and its vector into regions where they have not been endemic before (e.g. due to climate change) (McCall et al., 2008), and
(iii) the lack of awareness (e.g. travel into endemic regions) and insufficient compliance of pet owners to treat their animals with preventatives according to the instructions (Brown et al., 2012).

As macrocyclic lactones are the only drug class available to prevent heartworm disease, the conscientious utilization and preservation of

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their effectiveness is obviously of major importance (Bowman and Mannella, 2011). There are also reports of resistance to the macrocyclic lactones developing in *D immitis* (Pulaski et al., 2014; Bourguinat et al., 2015; Blagburn et al., 2016). Therefore, there is an urgent need to identify and develop novel anthelmintics to prevent and/or control the disease.

To support the successful identification of novel, resistance-breaking drug candidates, it would be of significant benefit to understand how the gold-standard drug class exerts its anthelmintic effects against the pathogen of interest.

Unfortunately, the extremely high potency of the macrocyclic lactones against *D. immitis* larvae *in vivo* (*e.g.* C_{MAX} of 3 ng/ml for ivermectin) is not mirrored by their activity *in vitro* (where 17.5 μg/ml ivermectin fails to induce significant anthelmintic effects) (Evans et al., 2013; Berrafato et al., 2019). Furthermore, it is not understood why the macrocyclic lactones are most effective against third-stage and fourth-stage *D. immitis* larvae. In contrast, only at significantly increased drug concentrations anthelmintic effects are observed against adult worms and microfilariae, respectively (Bowman and Atkins, 2009; Bowman and Mannella, 2011).

Macrocyclic lactones are known to be potent activators/modulators of inhibitory ligand-gated ion channels - the glutamate-gated chloride channels (GluCls) - of nematodes and insects (Wolstenholme et al., 2015). In gastrointestinal nematodes, GluCls are widely expressed in the nervous system and in the pharynx (Wolstenholme, 2012). In *D. immitis* the expression pattern of GluCl subunits is not fully described, and more importantly, the contribution of GluCl-directed effects have never correlated to in vivo effects of this drug class (Wolstenholme et al., 2015). Many anthelmintic classes act on nematode ion channels (Abongwa et al., 2017), but to date there have not been direct assays of drug effects on the *D. immits* neuromuscular system.

Therefore, this study aims to improve our understanding of the neuromuscular system of the canine heartworm *D. immitis* by the establishment of preparations, allowing the measurement of responses in body wall and pharyngeal muscle of adult worms and their modulation by anthelmintics.

2. Methods

2.1. Parasites

The *D. immitis* strain, Georgia-2 (GA-2), GA, USA, was used in all experiments. This strain originated from a blood collection of a dog from Vidalia, GA, USA, in April 2013. Following an initial passage through the mosquito vector (*Aedes aegypti*; black-eyed Liverpool strain), it has been maintained in laboratory beagle dogs at TRS Labs Inc., Athens, GA, USA (Berrafato et al., 2019).

All animal procedures were approved by the local animal care and use committee and by governmental authorities. In total, 5 consecutive experimental blocks were conducted. Two dogs were infected per experimental block.

In total, 10 beagle dogs were inoculated with 200 motile, viable third-stage larvae of *D. immitis* by subcutaneous injection in the inguinal region. Euthanasia, necropsy and parasite recovery were performed 160±10 (153, 155, 159, 166 and 169) days post infection.

2.2. Parasite maintenance

Freshly recovered *D. immitis* adult females (Fig. 1A) were transported at room temperature in Roswell Park Memorial Institute 1640 medium (RPMI, Invitrogen, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (100X; Fisher Scientific, USA). After arrival worms were maintained at 20 °C using the same media, which was changed daily. On the day of the experiments, adult female worms were warmed to 37 °C in a 5% CO_2 incubator.

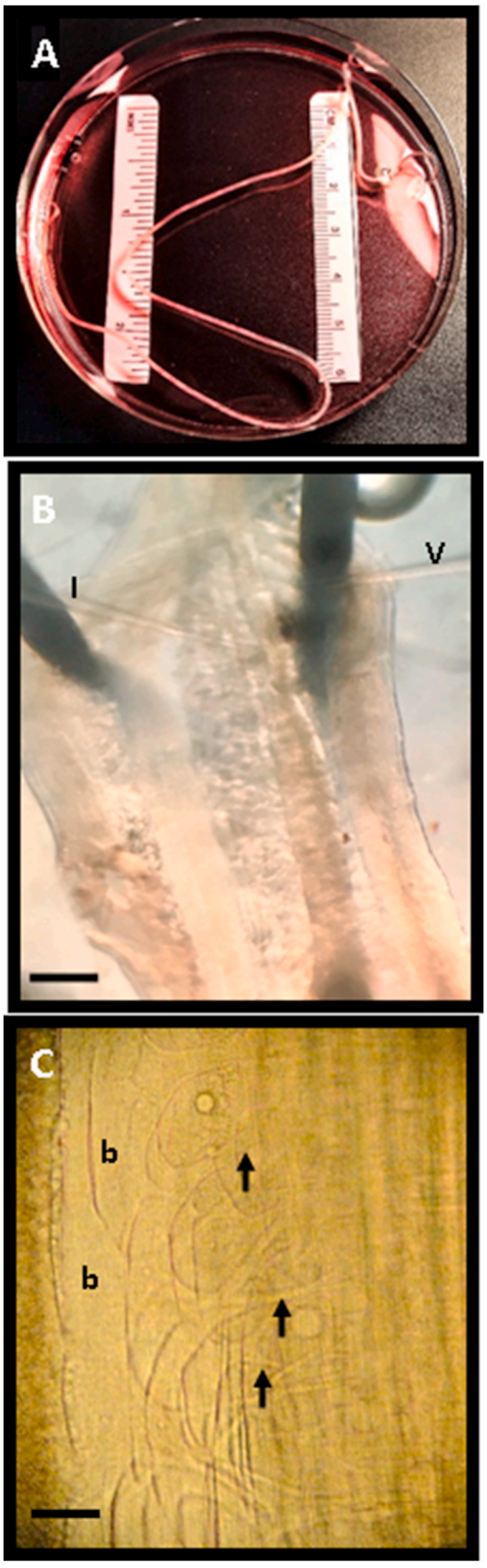

Fig. 1. Preparations for electrophysiological recording. A. Adult female *D. immitis*. **B.** Dissected pharyngeal preparation with current injecting (I) and voltage sensing (V) glass electrodes. **C.** Somatic muscle preparation, b = muscle bags, = muscle 'arm projections' on to nerve cord (Scale bar = 200 μm).

2.3. Drugs

Nicotine, L-glutamate, acetylcholine, levamisole, pyrantel, ivermectin and moxidectin were obtained from Sigma-Aldrich (MO, USA). Stocks of ivermectin and moxidectin were prepared in DMSO and diluted 1:1000 to give final working concentrations (0.1% DMSO). Nicotine, acetylcholine, L-glutamate, pyrantel and levamisole were dissolved in calcium- or calcium-free Ringer (see details for exact composition in each section).

2.4. Pharyngeal electrophysiology

For *D. immitis* we adapted the methods used previously to record drug responses from the *Ascaris suum* pharynx (Choudhary et al., 2020). Briefly, we made ~0.5 cm tissue flaps from the anterior of the worm containing the pharynx and pinned them onto a Sylgard™-lined bath chamber maintained at 28 °C by inner circulation of warm water. The preparation was continuously perfused with calcium-free Ringer (Ca-free Ringer) [composition (mM): NaCl 23, Na-acetate 110, KCl 24, $MgCl_2$ 11, glucose 11, and HEPES 5; adjusted the pH to 7.6 with NaOH]. The pharyngeal recordings are conducted in calcium-free media to increase the stability of the preparation as calcium causes pharyngeal contraction. The rate of perfusion was 3.5–4 ml/min through a 20-gauge needle placed directly above the region of the pharynx to be recorded from. Recordings were made by impaling the posterior region of pharynx with two microelectrodes, namely current-injecting (I) and voltage-recording electrodes (V) (Fig. 1B). The injected step current was −300 nA for 500 ms at 0.3 Hz. All preparations used for recording had resting membrane potentials more negative than −20 mV (−23.6 ± 1.4 mV for *D. immitis* and −21.5 ± 0.8 mV for *A. suum*) and a resting conductance less than 250 μS. For each series of recordings for each drug, ivermectin and moxidectin were applied in increasing concentrations (0.1, 1, 3, 10 and 30 μM) for 2 min consecutively.To assess the effects of compounds we measured the maximum change in membrane potential (ΔV_{max}) in mV and the maximum change in conductance (ΔG_{max}) in μS for each drug application. Conductance was calculated using Ohm's law, where G = I/V. In brief, if the thickness of the recording trace decreased in response to drugs, representing an increase in conductance and therefore the opening of ionic channels in the membrane. An increase in thickness of trace represents the opposite - decrease in conductance and closing of ionic channels. For comparative purposes, we recorded pharyngeal responses in *Ascaris suum* as described previously (see Puttachary et al., 2013 for details). In brief, female *A. suum* worms were obtained from the JBS Swift and Co. pork processing plant at Marshalltown, Iowa and maintained in Ringer (13 mM NaCl, 9 mM CaCl2, 7 mM MgCl2, 12 mM C4H11NO3/Tris, 99 mM NaC2H3O2, 19 mM KCl and 5 mM glucose pH 7.8) at 32 °C for 24 h to allow for acclimatization prior any experiments.The drugs were perfused in increasing concentration (0.1, 1, 3, 10 and 30 μM) for 2 min consecutively without wash as it is not possible to remove ivermectin or moxidectin from the preparation once exposed.

2.5. Somatic muscle electrophysiology

For the *D. immitis* muscle preparation and recording we adapted the protocols developed for *Brugia malayi* (Robertson et al., 2011, 2013; Verma et al., 2017). Briefly, ~0.5 cm long body tubes were cut from the anterior region and placed in a Sylgard™-lined recording chamber filled with Ringer [23 mM NaCl, 110 mM Na acetate, 5 mM KCl, 6 mM CaCl2, 4 mM MgCl2, 5 mM Hepes, 10 mM D-glucose, and 11 mM sucrose, pH adjusted to 7.2 with NaOH and ~320 mOsmol]. The muscle tube was first immobilized in the chamber along one side with Glushield cyanoacrylate glue (Glustitch Inc., BC, CA) and was then cut open longitudinally by using a fine spring scissor to expose the muscle cells (Fig. 1C). Muscle flaps were incubated for 15–30 s in 1 mg/ml collagenase (type 1A, Sigma-Aldrich, St. Louis, USA) followed by five washes with bath solution.

The patch-clamp technique was used to record whole-cell currents from individual muscle cells. We filled patch pipettes with a solution containing [120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM Tris, 0.25 mM $CaCl_2$, 4 mM NaATP, 5 mM EGTA, and 36 mM sucrose (pH 7.2 with KOH), ~315–330 mOsmol]. Pipettes with resistances of 3–5 MΩ were used. The preparation was continuously perfused with ringer at 2 ml/min.

Muscle cells were held at −40 mV, and drugs (30 μM) were perfused for 30 s sequentially with a minimum of 2 min wash period between drug applications. The peak current responses from whole cell recordings were used for analysis. We measured inward peak current responses (I_{peak}) to different agonists for analysis. All preparations used for recording had resting membrane potentials more negative than −20 mV (−29.2 ± 1.0 mV).

2.6. Statistical analysis

All statistical analyses were carried out using GraphPad Prizm v6 software (San Diego, Ca). In Figs. 2–5 we plotted the maximum change in membrane potential (ΔV_{max}) and the maximum change in conductance (ΔG_{max}) against log drug concentrations from 5 experiments. We used the log agonist vs. response equation (*nH* constrained to 1) to generate concentration-response curves to calculate EC_{50} values for ivermectin and moxidectin.

For statistical analysis of EC_{50} values (Fig. 6) we determined concentration-response relationships for each individual pharyngeal preparation (n = 5) and the maximum change in membrane potential (ΔV_{max}) and the maximum change in conductance (ΔG_{max}) were plotted against log drug concentrations for each experiment. The log agonist vs. response equation (bottom constrained to 0 and *nH* constrained to 1)

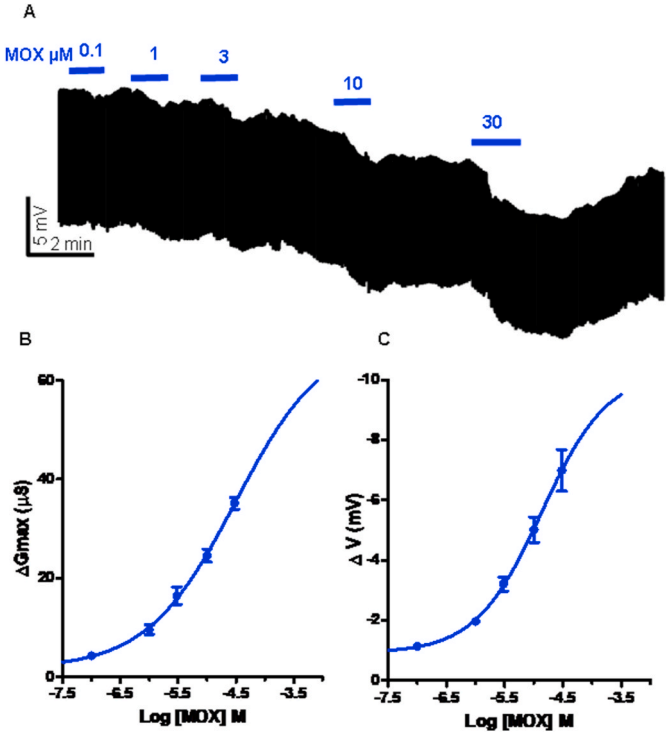

Fig. 2. Effect of ivermectin on the *D immitis* pharynx. A. Representative two-electrode pharyngeal recording demonstrating the effect of ivermectin (IVM) on *D. immitis* pharynx. **B.** Concentration response obtained from calculating average in peak conductance change after ivermectin applications. **C.** Concentration response curve obtained from calculating average change in membrane potential after ivermectin applications. Five recordings from five different worms.

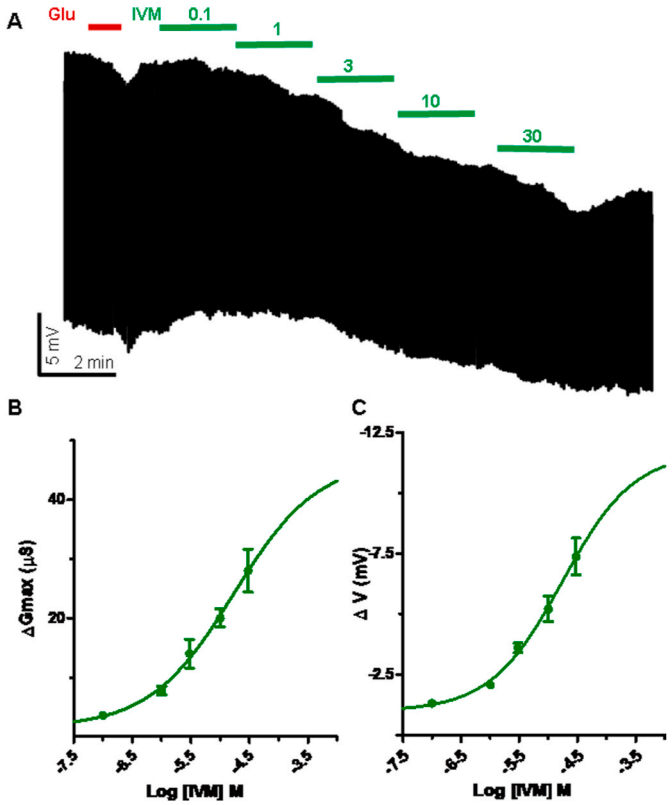

Fig. 3. Effect of moxidectin on the *D immitis* pharynx. A. Representative two-electrode pharyngeal recording demonstrating the effect of moxidectin (MOX) on *D. immitis* pharynx. **B.** Concentration response obtained from calculating average in average peak conductance after moxidectin applications. **C.** Concentration response curve obtained from calculating average change in membrane potential after moxidectin applications. Five recordings from five different worms.

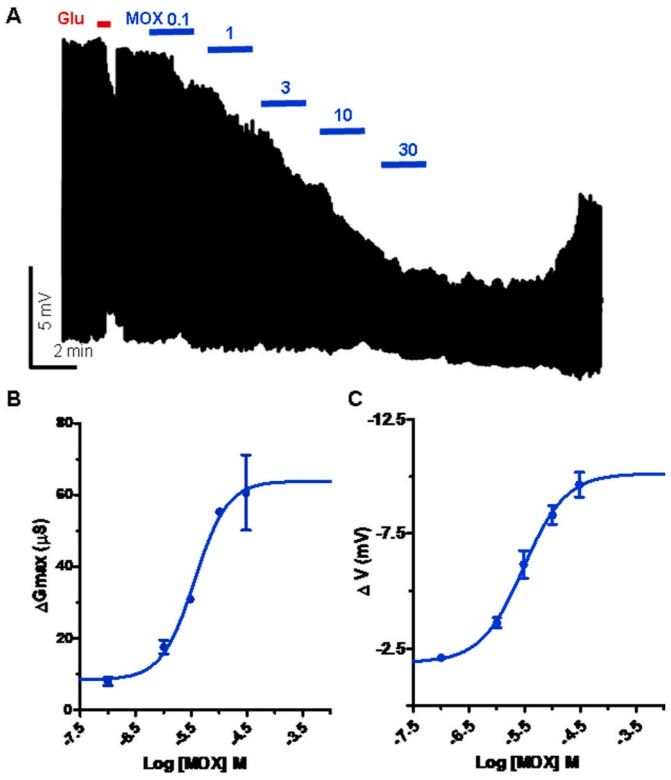

Fig. 4. Effect of ivermectin on the *A. suum* pharynx A. Representative two-electrode pharyngeal recording demonstrating effect of ivermectin (IVM) on *A. suum* pharynx. **B.** Concentration response obtained from calculating average in average peak conductance after ivermectin applications. **C.** Concentration response curve obtained from calculating average change in membrane potential after ivermectin applications. Five recordings from five different worms.

was used to generate concentration-response curves and obtain EC_{50} values for ivermectin and moxidectin. All responses were plotted as mean ± SEM. Unpaired *t* tests (two-tailed test) were used to determine whether there were significant differences between different treatments in the same or different preparations (*A. suum* and *D. immitis*).

For responses from somatic muscle cells the peak current responses (I_{peak}) from whole-cell recordings were plotted.

3. Results

3.1. Pharyngeal recordings

Figs. 2A and 3A show representative traces of two-electrode current clamp recordings from the pharynx of adult *D. immitis*. Glutamate (100 μM) was applied as control for both sets of recordings (n = 5 each). We tested two drugs that act on GluCls, ivermectin and moxidectin.

Fig. 2A is a representative trace demonstrating change in membrane potential (ΔV) and input conductance (ΔG) with application of increasing concentrations of ivermectin (0.1, 1, 3, 10 and 30 μM) on the pharynx. The EC_{50} values were 3.4 ± 0.6 μM for maximum change in conductance (Figs. 2B) and 5.2 ± 1.1 μM for maximum change in membrane potential (Fig. 2C) and were not significantly different ($p > 0.05$).

Fig. 3A is a representative trace demonstrating the concentration-dependent effect of moxidectin on membrane potential and input conductance when applied in increasing concentrations (0.1, 1, 3, 10 and 30 μM). The EC_{50} values were 2.0 ± 0.2 μM for maximum change in conductance (Figs. 2B) and 1.7 ± 0.1 μM for maximum change in membrane potential (Fig. 2C) and were not significantly different ($p > 0.05$).

Figs. 4 and 5 show representative traces of two-electrode current clamp recordings from the *A. suum* pharynx (n = 5 for each drug). As with *D. immitis* we tested ivermectin and moxidectin. Fig. 4A is a representative trace showing the change in membrane potential and conductance with application of increasing concentrations of ivermectin (0.1, 1, 3, 10 and 30 μM). As above, we calculated EC_{50} values, using both change in conductance (2.5 ± 0.2 μM, Fig. 4B) and in membrane potential (3.0 ± 0.6 μM, Fig. 4C). There was no significant difference in EC_{50} when we compared membrane potential and conductance changes ($p > 0.05$).

Moxidectin in the *A. suum* pharyngeal preparation on the other hand seems to be less potent when compared with *D. immitis* pharynx. Fig. 5A is a representative trace showing concentration-dependent effects of moxidectin on membrane potential and conductance when applied in increasing concentrations (0.1, 1, 3, 10 and 30 μM). As previously, we calculated EC_{50} using both change in conductance (2.2 ± 1.2 μM, Fig. 5B) and in membrane potential (1.4 ± 0.7 μM, Fig. 5C). As expected, the EC_{50} calculated using either change in conductance or change in membrane potential were not significantly different.

Fig. 6 shows bar graphs of EC_{50} for change in membrane potential (ΔV, Fig. 6a) and change in conductance (ΔG, Fig. 6b). We found there was no difference in sensitivity between the responses to ivermectin comparing the two species (*A. suum* and *D. immitis*). However, *D. immitis* was significantly more sensitive to moxidectin than *A. suum* (ΔV, $p < 0.005$; ΔG $p < 0.05$). Furthermore, in *D. immitis* moxidectin was significantly more potent than ivermectin (ΔV, $p < 0.05$; ΔG $p < 0.005$) whereas the reverse was true in *A. suum,* where ivermectin was significantly more potent than moxidectin (ΔV, $p < 0.05$).

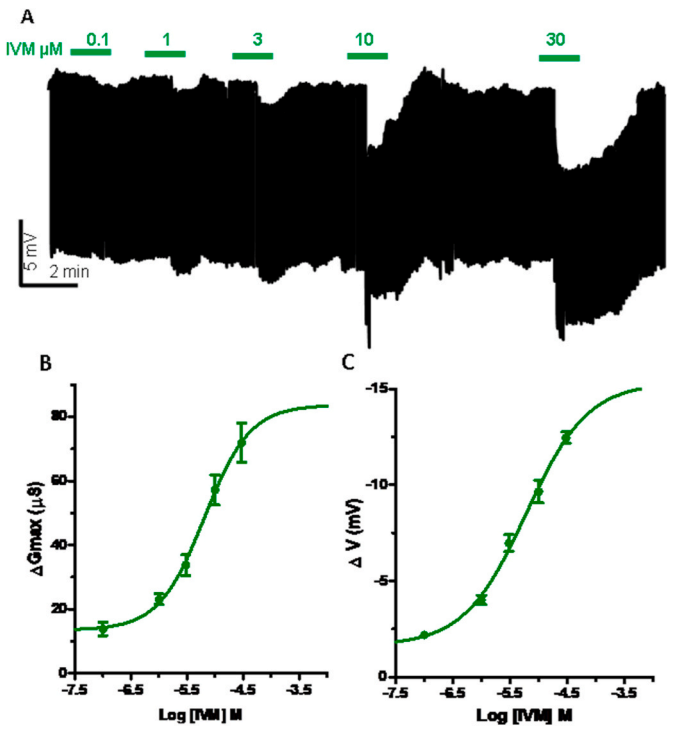

Fig. 5. Effect of moxidectin on the *A. suum* pharynx. A. Representative two-electrode pharyngeal recording demonstrating effect of moxidectin (MOX) on *A. suum* pharynx. **B.** Concentration response obtained from calculating average in average peak conductance after moxidectin applications. **C.** Concentration response curve obtained from calculating average change in membrane potential after moxidectin applications. N = 5.

3.2. Whole-cell somatic muscle recordings

Fig. 7A is a representative recording showing inward currents induced by different cholinergic agonists applied to somatic muscle cells during whole-cell voltage-clamped at −40 mV. We applied various cholinergic agonists to test for the presence of functional nAChR's on *D. immitis* muscle cells. Fig. 7A shows inward currents induced by acetylcholine, nicotine, levamisole and pyrantel. Fig. 7B, shows bar charts of the average peak currents (mean ± SE) produced by the different agonists (30 µM) (n = 6 muscles from 5 worms). Levamisole induced inward currents were the largest (−1278 ± 187 pA), followed by ACh (−893 ± 91.8 pA), pyrantel (−709 ± 92.2 pA), and nicotine (−63.3 ± 18.5 pA).

4. Discussion

The majority of antinematodal drugs act on the neuromuscular system of the parasite (Sangster et al., 2005; Holden-Dye et al., 2012; Abongwa et al., 2017; Verma et al., 2017). The macrocyclic lactones (avermectins and milbemycins) interact with glutamate gated chloride channels (Pemberton et al., 2001; Wolstenholme and Rogers, 2005; Geary and Moreno, 2012), while the cholinomimetics, including levamisole and pyrantel act on nicotinic acetylcholine receptors (Pinnock et al., 1988; Robertson and Martin, 1993) and emodepside acts on voltage-gated potassium channels (Guest et al., 2007; Kulke et al., 2014; Crisford et al., 2015; Kashyap et al., 2019). Other relatively new compounds such as derquantel (Ruiz-Lancheros et al., 2011; Epe and Kaminsky, 2013), monepantel (Kaminsky et al., 2011; Abongwa et al., 2018) and tribendimidine (Keiser et al., 2007; Hu et al., 2009; Robertson et al., 2015) also act on nematode nicotinic acetylcholine receptors (Bartos et al., 2006; Holden-Dye et al., 2013; Martin et al., 2015).

Given that parasitic nematodes are not readily amenable to genetic

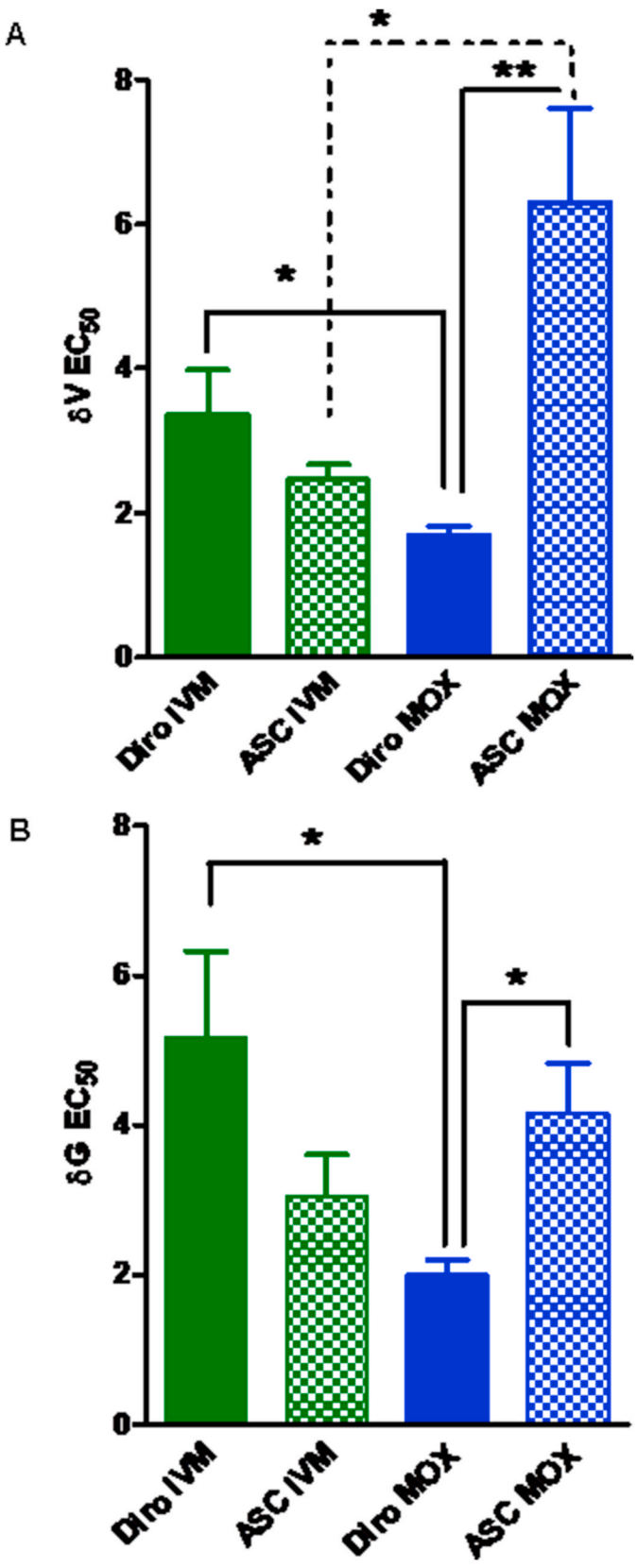

Fig. 6. Comparison of ivermectin and moxidectin effects on *D. immitis* and *A. suum* pharynx. Bar chart representing mean EC_{50} values for **A.** change in membrane potential and **B.** conductance for ivermectin (IVM) and moxidectin (MOX) in *D. immitis (Diro)* and *A. suum* (ASC). T-tests were used to compare pairs of experimental groups as indicated by connecting lines, *p < 0.05, **p < 0.01.

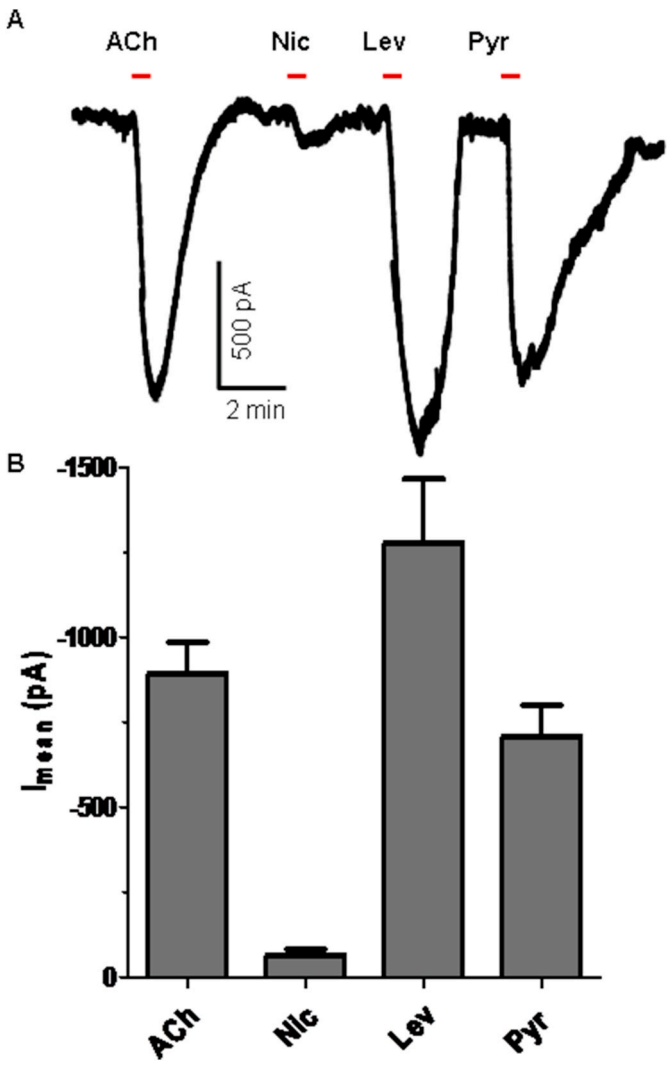

Fig. 7. Cholinomimetic effects on *D. immitis* somatic muscle. A. Representative single electrode patch-clamp recording from *D. immitis* muscles demonstrating effects of different nicotinic agonists. **B.** Bar chart representing peak inward current produced by single concentration of (30 μM) (n = 6 cells from 5 parasites).

manipulation, the study of the mode of action of these compounds has been heavily reliant on electrophysiological techniques (Martin et al., 1998; Wolstenholme and Rogers, 2005; Cook et al., 2006). Successful electrophysiology has been reported for a range of nematode parasite species including *Ascaris suum* (Walker et al., 1992), *Ascaridia galli* (Holden-Dye and Walker, 2006), *Oesophagostomum dentatum* (Martin et al., 1997), *Ancylostoma* sp. (Weeks et al., 2016) and *Brugia malayi* (Robertson et al., 2013; Verma et al., 2017); however, the species range is not extensive, and to date there have been no reports of electrophysiological recordings from *D. immitis*.

Despite *D. immitis* being considered as one of the most pathogenic helminths infecting companion animals, macrocyclic lactones are the only drug class currently approved for the prevention of heartworm disease (Maclean et al., 2017) while pharmacological intervention to kill adult parasites are suboptimal (Savadelis et al., 2018). As several studies confirm the existence of macrocyclic lactone resistance in heartworm populations across the United States (Pulaski et al., 2014; Bourguinat et al., 2015; Blagburn et al., 2016), there is a compelling need for new therapeutic agents for dirofilariasis prevention. However, our understanding of the pathogen itself as well as of how the macrocyclic lactones prevent the migration of *D. immitis* within their canine host are very limited. Therefore, this study aimed to contribute to the improvement of our understanding of the neuromuscular system of *D. immitis* and its response to anthelmintics. We were able to show that adult heartworm are amenable to electrophysiological recording techniques. More precisely, we have demonstrated that adult *D. immitis* can be dissected to expose muscle cells and the pharynx for single electrode patch-clamp or two-electrode current-clamp recordings. Previous studies have found that moxidectin is more potent than ivermectin (Awadzi et al., 2014; Barda et al., 2017; Boussinesq, 2018; Opoku et al., 2018) and our results conform with these findings. Also, it conforms with our observations of the relatively high EC_{50} values for effects on adult heartworm of these drugs. In addition, we have found differences in the responses when we compare *D. immitis* with *A. suum*. These observations also demonstrate that there are differences in GluCl pharmacology between worms from the same clade. Interestingly, we found the responses to ivermectin on the *A. suum* pharynx were reversible on washing, in contradiction to other studies that found ivermectin responses to be irreversible (Brownlee et al., 1997), we attribute our washable effect to the extremely vigorous focal perfusion provided by our recording system.

We have demonstrated through whole-cell patch-clamp recordings that nAChRs on adult *B. malayi* muscle cells are activated by the cholinergic anthelmintics acetylcholine, nicotine, levamisole, and pyrantel. Levamisole was the most potent nicotinic agonist under our observations (Verma et al., 2017). Although cholinomimetic drugs are not suitable for treatment of adult heartworms, the robust whole-cell responses to pyrantel and levamisole suggest the parasites possess nAChRs that are potential targets for therapeutic intervention with novel compounds (Martin et al., 1997; Abongwa et al., 2017). Our findings on ivermectin and moxidectin responses are also significant. The adult *D. immitis* pharynx is less sensitive to ivermectin than the large porcine nematode *A. suum.* However, the presence of robust responses to glutamate and high doses of ivermectin & moxidectin demonstrate the presence of a significant number of glutamate gated chloride channels on the pharynx that are potential targets for more selective new compounds. We believe that these observations and techniques can be employed to further investigate and characterize the *D. immitis* GluCls, nicotinic receptors and other ionic channels in this significant parasite. Future studies will focus on two fronts: further characterization of channels and drug responses in adult *D. immitis* and further development of our recording techniques to include the larval stages of this important parasite.

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Declaration of competing interest

D.K. was an employee of Bayer Animal Health GmbH when the experiments were conducted. With the exception of D.K., Bayer Animal Health GmbH had no role in the design or conduct of the experiments, analysis or interpretation of the data. All other authors declare no conflict of interest.

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