MOLECULAR & BIOCHEMICAL PARASITOLOGY

Levamisole and ryanodine receptors (II): An electrophysiological study in *Ascaris suum*

Sreekanth Puttachary, Alan P. Robertson, Cheryl L. Clark, Richard J. Martin*

Department of Biomedical Sciences, Iowa State University, Ames, IA 50011-1250, USA

ABSTRACT

Resistance to antinematodal drugs like levamisole has increased and there is a need to understand what factors affect the responses to these anthelmintics. In our previous study, we examined the role of ryanodine receptors in muscle contraction pathways. Here we have examined interactions of levamisole receptors, ryanodine receptors (RyRs), the excitatory neuropeptide AF2, and coupling to electrophysiological responses. We examined the effects of a brief application of levamisole on *Ascaris suum* body muscle under current-clamp. The levamisole responses were characterized as an initial primary depolarization, followed by a slow secondary depolarizing response. We examined the effects of AF2 (KHEYLRFamide), $1\,\mu M$ applied for $2\,min$. We found that AF2 potentiated the secondary response to levamisole and had no significant effect on the primary depolarization [1]. Further, the reversal potentials observed during the secondary response suggested that more than one ion was involved in producing this potential. AF2 potentiated the secondary response in the presence of $30\,\mu M$ mecamylamine suggesting the effect was independent of levamisole sensitive acetylcholine receptors. The secondary response, potentiated by AF2, appeared to be dependent on cytoplasmic events triggered by the primary depolarization. Ion-substitution experiments showed that the AF2 potentiated secondary response was dependent on extracellular calcium and chloride suggesting a role for the calcium-activated anion channel. Caffeine mimicked the AF2 potentiated secondary response and $0.1\,\mu M$ ryanodine inhibited it. $1.0\,\mu M$ ryanodine increased spiking showing that it affected membrane excitability. A model is proposed showing ryanodine receptors mediating effects of AF2 on levamisole responses.

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1. Introduction

Nematode parasites are a severe burden on the productive lives of humans and animals [2–4]. Treatment of these conditions with anthelmintics is limited to three main classes of drugs, and drug resistance has emerged in humans [2,3] as well as in animals [4,5] against each of the three classes of anthelmintic. The appearance of multidrug resistance in nematode parasites [6] is a worrying development. These concerns emphasize the requirement for understanding the mode of action of these compounds and mechanisms of resistance.

Our laboratory has studied levamisole, pyrantel, oxantel and morantel which belong to an important group of nicotinic anthelmintic drugs [7–12] that are used for treatment of ascariasis, *Trichuris* sp. and hookworm infections [13]. The target sites of these drugs include the pharmacologically distinctive ion channels that are nicotinic acetylcholine receptors (nAChRs) found on the body muscles of nematodes [14–16]. These drugs produce spastic paralysis of the parasitic nematode and have an advantage of acting rapidly on the parasite, effecting cures within 4 h.

We have seen in the previous paper that ryanodine receptors (RyRs) of *Ascaris suum* modulate the amplitude of the levamisole contraction by affecting g_{max} but not EC_{50}. The cellular mechanisms that modulate responses to anthelmintics are important to recognize and describe because they may be modified in anthelmintic resistance. In this paper we extend our previous observations on the role of RyRs in muscle contraction in *A. suum*, using the current-clamp technique. We demonstrate that RyRs and the entry of calcium play a role in modulating the secondary responses to levamisole and its potentiation by AF2. These observations demonstrate the role of RyRs in modulating the electrophysiological response, and hence, affect the contractile response to levamisole. It is possible that RyRs are modified with the development of resistance to levamisole in parasitic nematodes.

2. Materials and methods

2.1. Muscle flap preparation for electrophysiology

Adult *A. suum* were collected weekly from the Tyson's pork packing plant at Storm Lake, Iowa. Worms were maintained in Locke's

* Corresponding author. Tel.: +1 5152942470; fax: +1 5152942315.
E-mail address: rjmartin@iastate.edu (R.J. Martin).

solution [composition (mM): NaCl 155, KCl 5, CaCl$_2$ 2, NaHCO$_3$ 1.5 and glucose 5] at a temperature of 32 °C. The Locke's solution was changed daily and each batch of worms was used within 4 days of collection. We prepared 1 cm muscle tissue flaps by dissecting the anterior part of the worm, 2–3 cm caudal to the head. A body muscle flap preparation was then pinned onto a Sylgard^{TM}-lined double jacketed bath chamber maintained at 35 °C by inner circulation of warm water (Fisher scientific Isotemp 3016H, PA, USA). The intestines were removed to expose the muscle cells [1]. The preparation was continuously perfused, unless otherwise stated, with *Ascaris* Perienteric Fluid-Ringer (APF-Ringer) composition (mM): NaCl 23, Na-acetate 110, KCl 24, CaCl$_2$ 6, MgCl$_2$ 5, glucose 11, and HEPES 5; NaOH or acetic acid was used to adjust the pH to 7.6. The incoming perfusate was pre-warmed to 35 °C with an in-line heating system (SH 27B Warner instruments, CT, USA) before application. The rate of perfusion was 3.5–4 ml min^{-1} through a 20 gauge needle placed directly above the muscle bag recorded from. The calcium substitution experiments were done using cobalt APF-Ringer, composition (mM): NaCl 23, Na-acetate 110, KCl 24, CoCl$_2$ 6, MgCl$_2$ 5, glucose 11, and HEPES 5 mM; pH 7.6. Chloride was substituted by acetate in chloride free APF-Ringer. The experimental compounds were dissolved in APF-Ringer, cobalt APF-Ringer or chloride free APF-Ringer as described in the results. 1 μM levamisole was applied for a period of 10–20 s as described in the results. AF2 (1 μM) was applied for 2 min and followed by a minute wash prior to applications of levamisole.

2.2. Electrophysiology

A two-microelectrode current-clamp technique was employed to examine the electrophysiological effects in the bag region of *A. suum* muscle (Fig. 1A). Borosilicate capillary glass (Harvard Apparatus, Holliston, MA, USA, ID-0.86 mm, OD-1.5 mm) microelectrodes were pulled on a P-97 Flaming Brown Micropipette puller (Sutter Instrument Co., CA, USA). We used 3 M potassium acetate in the micropipettes which had resistances of 20–30 MΩ. The recordings were obtained by impaling the bag region of *A. suum* muscle with two microelectrodes, namely current-injecting (I) and voltage-recording electrodes (V). All experiments were performed using an Axoclamp 2A amplifier, a 1320A Digidata interface and Clampex 9 software (Molecular Devices, CA, USA). All data were displayed and analyzed on a PC based desktop computer.
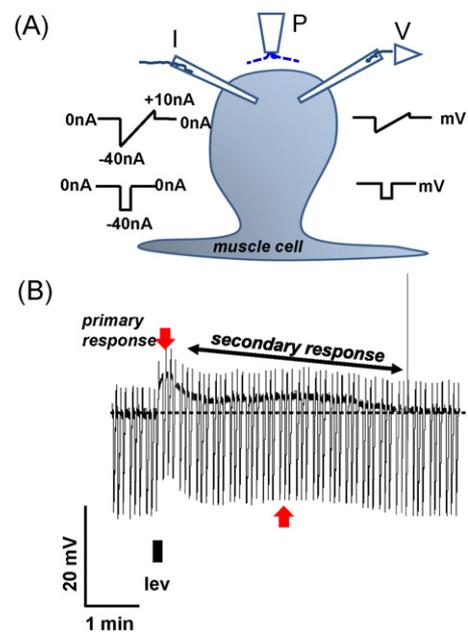
The current-injecting electrode injected hyperpolarizing ramp or step currents, while the voltage-recording electrode recorded the change in membrane potential in response to the injected currents. Our ramp current was a hyperpolarizing step of −40 nA changing linearly with time to a depolarizing current of 10 nA over a duration of 3 s at 0.2 Hz. The step current was −40 nA for 500 ms at 0.3 Hz. Each set of experiments were repeated on preparations from separate batches of worms. Cells with constant membrane potentials more negative than −20 mV for 20 min and a stable input conductance of <3.5 μS were selected for the recordings.

2.3. Drugs

AF2 (H-Lys-His-Glu-Tyr-Leu-Arg-Phe-NH2) [Sigma-Genosys, The Woodlands, TX, USA] 1 mM stock solutions were prepared in double distilled water every week and kept in aliquots at −20 °C. AF2, stock solutions were thawed just before use. All other chemicals were obtained from Sigma-Aldrich (MO, USA) and Acros-Organics (NJ, USA).

2.4. Analysis

The peak change in membrane potential (δV) and conductance (δG) were determined in response to drug applications. The dura-

Fig. 1. (A) Diagram showing the placement of the two micropipettes used for current-clamp and position of the micro perfusion system for continuous perfusion and application of drugs. P: microperfusion pipette. I: current-injecting electrode, injects ramp currents or step currents. V: voltage-recording electrode. (B) Representative trace showing the levamisole response and its two components in APF-Ringer namely, a primary depolarization and a secondary depolarizing response (secondary response). The darkest line of the recording is the membrane potential and the downward transients are the responses to injected current. The rapid primary depolarization (downward red arrow) is followed by a slow secondary response (upward red arrow). 1 μM levamisole was applied for 10 s as indicated by the filled rectangle below the trace. The discontinuous horizontal line indicates the original position of the resting membrane potential. The width of the trace is a reflection of membrane conductance; it gets narrower as membrane-ion channels open. The duration of the secondary response (T_{80}) was measured as the time taken (min) for the peak primary depolarization to decline by 80%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tion of the secondary depolarizing response (secondary response) to levamisole was measured as the time taken (min) for the peak primary depolarization to decline by 80% (T_{80}). We estimated reversal potentials by extrapolating from the membrane I–V plots using linear regression. We used the intracellular ionic concentration values of *Ascaris* estimated by Brading and Caldwell [17] to calculate ion-reversal potentials. For a single ion species, the reversal potential was calculated from the Nernst equation using estimates of the intracellular and extracellular concentrations of that ion. When ion channels selectively permeable to one species of ion open, then the membrane potential will move towards the reversal potential for that ion. We estimated the reversal potential from linear regression of the relationships between injected current and the membrane potential responses (the I–V plots) before and during conductance changes. The potential at which these plots cross, is the reversal potential of the ion channel that has opened and is determined by ions that flow through the ion channel. For example, a channel conducting only chloride ions, will have a measured reversal potential that matches the chloride Nernst potential. If the measured reversal potential does not match the Nernst potential of a single ion, it implies that more than one ion is involved in generating the potential.

We defined spikes in *A. suum* as brief repeating action potentials with amplitude greater than 5 mV appearing as a single spike for a duration up to 500 ms. We measured the spike frequency (min^{-1}), amplitude (mV) and spike gradient (mV s^{-1}). We tested the effects of ryanodine on spike parameters before levamisole application and during the rising phase of the levamisole depolarization. The spike