RESEARCH PAPER

On the mode of action of emodepside: slow effects on membrane potential and voltage-activated currents in *Ascaris suum*

SK Buxton¹, C Neveu², CL Charvet², AP Robertson¹ and RJ Martin¹

¹Department of Biomedical Sciences, Iowa State University, Ames, IA, USA, and ²INRA, UR1282 Infectiologie Animale et Santé Publique, Nouzilly, France

Correspondence

Richard Martin, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011-1250, USA. E-mail: rjmartin@iastate.edu

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BACKGROUND AND PURPOSE

Anthelmintics are required for treatment and prophylaxis of nematode parasites of humans and domestic animals. Emodepside, a cyclooctadepsipeptide, is a modern anthelmintic that has a novel mode of action involving a Ca-activated K channel (SLO-1) in *Caenorhabditis elegans*, sometimes mediated by a latrophilin (LAT) receptor. We examined mechanisms of action of emodepside in a parasitic nematode, *Ascaris suum*.

EXPERIMENTAL APPROACH

RT-PCR was used to investigate expression of *slo-1* and *lat-1* in *A. suum* muscle flaps, and two-micropipette current-clamp and voltage-clamp techniques were used to record electrophysiological effects of emodepside.

KEY RESULTS

Expression of *slo-1* and *lat-1* were detected. Emodepside produced a slow time-dependent (20 min), 4-aminopyridine sensitive, concentration-dependent hyperpolarization and increase in voltage-activated K currents. Sodium nitroprusside increased the hyperpolarizations and K currents. N-nitro-L-arginine inhibited the hyperpolarizations and K currents. Phorbol-12-myristate-13 acetate increased the K currents, while staurosporine inhibited the hyperpolarizations and K currents. Iberiotoxin reduced these emodepside K currents. The effect of emodepside was reduced in Ca-free solutions. Emodepside had no effect on voltage-activated Ca currents.

CONCLUSIONS AND IMPLICATIONS

Asu-slo-1 and *Asu-lat-1* are expressed in adult *A. suum* muscle flaps and emodepside produces slow activation of voltage-activated Ca-dependent SLO-1-like K channels. The effect of emodepside was enhanced by stimulation of protein kinase C and NO pathways. The data are consistent with a model in which NO, PKC and emodepside signalling pathways are separate and converge on the K channels, or in which emodepside activates NO and PKC signalling pathways to increase opening of the K channels.

Abbreviations

NNLA, N-nitro L-arginine; PKC, protein kinase C; SNP, sodium nitroprusside

Introduction

Parasitic nematode infections, classed as neglected tropical diseases, remain a problem in both human and veterinary

medicine. The estimated global prevalence of parasitic nematode infections in humans is over two billion and the global prevalence of ascariasis alone is over 800 million (Hotez *et al.*, 2007; 2008). These parasites are debilitating to humans,

causing lost work days, cognitive impairment and poor growth; they also depress agricultural production and food supply. Ascariasis is caused by *Ascaris lumbricoides* in humans: this parasite is nearly identical to the parasite, *Ascaris suum* of pigs, which is a good model of the human parasite, *A. lumbricoides*. In the absence of effective vaccines and adequate sanitation, anthelmintic drugs are used for treatment and prophylaxis. However, there have been reports of growing resistance to the classic anthelmintic drugs: benzimidazoles (albendazole), nicotinic agonists (levamisole/pyrantel) and macrocyclic lactones (ivermectin) (Prichard, 1990; 1994; Sangster and Gill, 1999; Sangster, 2001; Kaplan, 2002; Wolstenholme *et al.*, 2004; Martin and Robertson, 2007; James *et al.*, 2009). More recently, novel 'resistance-busting' anthelmintics (emodepside, a cyclooctadepsipeptide; monepantel, an amino-acetonitrile derivative, and derquantel, a paraherquamide derivative) have been released into the market. The need for the new anthelmintics and ways to combat resistance to the currently available anthelmintics cannot be overstated.

Sasaki *et al.* (1992) reported the isolation of the cyclooctadepsipeptide PF1022A from cultured *Mycelia sterilia*, a fungus found on the leaves of *Camellia japonica*. Emodepside is a semisynthetic analogue of PF1022A made by attaching two morpholine rings at the *para*-position of the two phenylactic acids (Harder *et al.*, 2005) in order to enhance pharmacokinetic properties. Both emodepside and PF1022A have been shown to be effective against benzimidazole-, levamisole- and ivermectin-resistant nematode parasite isolates (Samson-Himmelstjerna von *et al.*, 2005), and to be effective against a wide range of nematode parasites (Schurmann *et al.*, 2007).

Willson *et al.* (2003) reported that in *A. suum*, emodepside caused muscle relaxation and a 4-aminopyridine sensitive, Ca-dependent hyperpolarization of body wall muscle cells. The similarity of this response to that of the inhibitory neuropeptides PF1/PF2 led to the suggestion that emodepside may be acting at the neuromuscular junction to release an inhibitory neuropeptide with similar action to the neuropeptides PF1/PF2.

Two molecular effectors have been implicated in the mode of action of emodepside. The first, a latrophilin-like receptor, HC110-R, co-precipitated with PF1022A and was cloned from *Haemonchus contortus* (Saeger *et al.*, 2001). Here, it is interesting to note that FMRFamide-like neuropeptides like PF2 have been tested and found to act as ligands of HC110-R (Muhlfeld *et al.*, 2009). However, observations using latrophilin gene knockouts in *Caenorhabditis elegans*, found that emodepside still depressed locomotion but that, in the pharyngeal muscle, inhibitory effects were reduced (Willson *et al.*, 2003; Guest *et al.*, 2007). The observations suggest that a latrophilin receptor is involved in the response to emodepside in the pharyngeal feeding circuit, but that other molecular effectors are also involved in the inhibition of locomotion. The second emodepside effector was identified in mutagenesis screens in *C. elegans*. These delivered multiple alleles of the voltage-gated Ca-sensitive K channel gene, *slo-1*, such that *slo-1* loss-of-function mutants were discovered to be resistant to the pleiotropic effects of emodepside on reproduction, locomotion and feeding.

Effects of emodepside on the voltage-activated currents in a parasitic nematode have not been studied. Here, we find

evidence of the expression of SLO-1 K channels [Slo (BK) K channels, Alexander *et al.* 2009] and LAT-1 protein, and we have used current- and voltage-clamp electrophysiological techniques to investigate the mode of action of emodepside on membrane potential and voltage-activated currents in *A. suum*, as an example of a parasitic nematode. We find that emodepside activated K currents with SLO-1-like properties with effects being enhanced by protein kinase C (PKC) and NO signalling pathways.

Methods

Collection and maintenance of worms

A. suum were collected weekly from Tyson Foods Inc. pork packing plant, Flindt Drive, Storm Lake, IA, USA and from JBS Swift and Co. pork processing plant, Marshalltown, IA, USA. The adult worms were kept in Locke's solution (NaCl 155 mM, KCl 5 mM, CaCl₂ 2 mM, NaHCO₃ 1.5 mM, glucose 5 mM) at 32°C. The solution was changed twice a day, and the worms were used within 4 days of collection.

Sequence and gene expression analysis

Database searches were performed with the BLAST Network Service (NCBI), using the tBLASTn algorithm (Altschul *et al.*, 1997). Signal peptide predictions were carried out using the Signal P 3.0 server (Bendtsen *et al.*, 2004). Conserved protein domains, as well as membrane-spanning regions were predicted using the SMART program (Schultz *et al.*, 1998). Total RNA was prepared from body muscle flaps (see below) from *A. suum* adult worms using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations.

RNA pellets were dissolved in 100 µL of RNase-free resuspension solution (Ambion, Austin, TX, USA) and were DNase treated with the TURBO DNA-free kit (Ambion). The RNA concentration was measured using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA synthesis was performed on 5 µg of total RNA using the oligo (dT) RACER primer (Invitrogen) and the superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reverse transcription-PCR experiments were carried out on first strand cDNA using two rounds of nested PCR, in a final volume of 20 µL, containing 200 ng of first-strand cDNA (or 1 µL of 1/100 dilution of amplification product from the first round of PCR), 1 U of GoTaq polymerase (Promega, Charbonnières, France), 0.25 mmol·L⁻¹ dNTPs each and 0.3 µmol·L⁻¹ of each primer. Primer sequences used for RT-PCR experiments are provided in Supporting Information Table S1. The reaction mixture was denatured by heating at 94°C for 5 min, followed by 33 cycles at 94°C for 45 s, 56°C for 45 s and 72°C for 45 s. A final extension step was performed at 72°C for 5 min. Amplification products were cloned in pGEM-T vector (Promega) and sequence-checked by GATC Biotech (Konstanz, Germany).

Somatic muscle preparation

The anterior portion of the worm about 4 cm away from the head was used in all recordings. About 1 cm of this part of the worm was cut-out and the resulting cylindrical worm piece

was cut open along a lateral line to form a muscle flap. The gut was removed to expose the muscle cells and the muscle flap was pinned onto a 35 × 10 mm Petri-dish lined with Sylgard and containing the perfusion solution. The preparation was placed in the experimental chamber and perfused with low-potassium *Ascaris* perienteric fluid (APF-Ringer) solution (in mM: NaCl 23, Na acetate 110, KCl 3, CaCl₂ 6, MgCl₂ 5, glucose 11, HEPES 5, pH adjusted to 7.6 with NaOH), unless otherwise stated, at a rate of 4 mL·min⁻¹ through a 20-gauge needle. The perfusion needle was placed directly above the muscle bag from which recordings were made. The preparation in the experimental chamber was kept at 34°C by using a Warner heating collar (DH 35) and heating the perfusate with a Warner SH-27B inline heater (Hamden, CT, USA).

Electrophysiology of somatic muscle

Two micropipette voltage- and current-clamp techniques were used in examining the electrophysiological effects of emodepside on the muscle bag of *A. suum* (Figure 1A). The micropipettes, made of borosilicate capillary glass with internal diameter 0.86 mm and external diameter 1.50 mm (Harvard Apparatus, Holliston, MA, USA) were pulled on a Flaming Brown micropipette puller (Sutter Instrument Co, Novato, CA, USA). The micropipettes were filled with 3 M potassium acetate to study the membrane potential and K current effects. The voltage-sensing micropipettes had resistances of 20–30 MΩ, but the tip of the current-injecting micropipettes for the current-clamp and voltage-clamp was broken to have a resistance of 3–6 MΩ. The muscle bag was impaled with both micropipettes for all recordings. An Axoclamp 2B amplifier, a 1320A Digidata interface and pCLAMP 8.2 software (Molecular Devices, Sunnyvale, CA, USA) were used in all recordings. In all current- and voltage-clamp recordings, the experiment sets were repeated on different worms to obtain the desired number of observations. In the current-clamp experiments, 40 nA hyperpolarizing pulses were injected for 500 ms at 0.30 Hz through the current-injecting micropipette and the change in membrane potential recorded with the voltage-sensing micropipette (Figure 1B).

In both current- and voltage-clamp experiments, cells close to the nerve cord were used. We kept the amplifier gain high (~100) and the phase lag low (~0.15–0.5 ms) to limit oscillations, but adjusted these in some recordings. For activation of the K currents, the muscle bags were held at –35 mV and stepped up to 0, 5, 10, 15, 20, 25 and 30 mV (Figure 1C); the currents lasted for 40 ms. For activation of the Ca currents, the muscle bags were stepped from the holding potential of –35 mV to –25 mV and then in steps of 5 mV up to 20 mV. The Ca currents were isolated from the K currents by using 4-aminopyridine (5 mM) in the perfusion solution and equimolar amounts (1.5 M) of potassium and cesium acetates in the recording micropipettes, and not making large depolarizations (Verma *et al.*, 2009). There was no need to block voltage-activated Na currents in isolating the Ca currents because nematodes lack voltage-activated Na currents. Calcium substitution experiments were performed by replacing calcium in the low-potassium APF-Ringer solution with equimolar cobalt.

The currents were leak-subtracted using pCLAMP 8.2 software. All drugs were initially applied in current clamp and the

Figure 1

Electrophysiological techniques for recording from *Ascaris suum*. (A) *A. suum* muscle bag showing the current (I) and voltage (V) micropipettes in the bag and the perfusion needle (P). (B) Current-clamp protocol used: bottom trace shows the 40 nA currents injected for 500 ms at 0.30 Hz; top trace shows the resting membrane potential (straight line) and the membrane response to the injected current (downward deflections). (C) Voltage-clamp protocol: bottom trace shows the voltage steps from the holding potential of –35 mV to 0 mV and in steps of 5 mV to 30 mV; top trace shows the K currents generated in response to the voltage steps.

effects on the voltage-activated currents investigated in voltage-clamp with the drugs still perfusing. Control measurements of membrane potential and K currents were made before drug applications, then at 10 min intervals. Unless otherwise stated, all drugs used to study the mechanisms of action of emodepside were applied for 10 min before co-application for 10 min with emodepside and effects on membrane potential and K current compared with the control at 0 min. The preparation was then washed in drug-free solutions and the wash (post-emodepside) effect measured following a 20 min time period. Our subsequent account is based on recordings from more than 130 muscle cells, each from a separate *A. suum* preparation. The membrane potentials of the selected cells recorded from were all

more negative than -25 mV with stable input conductances <4.0 μ S.

Time control experiments to test the stability of effects of NO modulators, PKC modulators and iberiotoxin

We conducted separate time-control experiments, without emodepside, to determine the time-course and stability of effects of the NO and PKC modulators on membrane potential and voltage-activated K currents. We found that sodium nitroprusside (SNP; 1 mM, $n = 4$), staurosporine (1 μ M, $n = 3$) and N-nitro L-arginine (NNLA; 100 μ M, $n = 6$) had no significant effect ($P > 0.05$) on membrane potentials or voltage-activated K currents when tested at 10, 20 and 30 min during their application. Phorbol 12-myristate 13-acetate (PMA) produced an increase in voltage-activated K currents and a small hyperpolarization within 5 min of application, and did not show further change. We also found that iberiotoxin (10 nM) had no effect at 10, 20 and 30 min on membrane potential, but iberiotoxin (by 10 min) reduced ($n = 3$) voltage-activated K currents and this effect was not significantly ($P > 0.05$) changed at 20 and 30 min.

Data analysis

All acquired data were displayed on a Pentium IV desktop computer. Graph Pad Prism software (version 5.0, San Diego, CA, USA) and Clampfit 9.2 (Molecular Devices) were used for the analysis. We observed the biggest effect of emodepside on the late phase K currents (L_K : we measured the average current during the period 30–40 ms after the step potential start) at a step potential of 0 mV. The reversal potential was calculated for voltage-activated K current experiments. To obtain the activation curves, we calculated the conductance changes from the outward K currents and the driving force, $E_{\text{rev}} - V$. The activation curves were then fitted by the Boltzmann equation: $G = G_{\text{max}}/[1 + \exp \{(V_{50} - V)/K_{\text{slope}}\}]$, where G = conductance, G_{max} = maximal conductance change, V_{50} = half-maximal voltage and K_{slope} = slope factor.

The current was expressed as percent control and plotted against voltage to determine how the currents changed with each voltage step. For experiments on the same preparation, one-tailed paired Student's t -tests were used to estimate statistical significance, set at $P < 0.05$. For comparison between effects on different preparations, an unpaired t -test was used. We show mean $\pm$ SEM values throughout the text.

To measure the emodepside-sensitive current as a proportion of the 4-aminopyridine sensitive current (at 0 mV) we first measured the control L_K current, then the L_K current at 10 min after application of emodepside (1 μ M): the difference was the current activated by emodepside. Next, we applied 4-aminopyridine (5 mM) for 5 min and again measured the L_K current: the difference between the L_K current in the presence of 4-aminopyridine and the L_K current at 10 min after emodepside was the 4-aminopyridine-sensitive current. The proportion was then determined and expressed as a %

Materials

Emodepside was generously provided by Achim Harder (Bayer HealthCare AG, Leverkusen, Germany). Emodepside stocks of 2 mM and 10 mM in 100% DMSO were prepared every 2

weeks. The working emodepside concentrations of 1 and 10 μ M were prepared so that the final DMSO concentration was 0.1%. PF1 (SDPNFLRFamide, EZBiolab, Westfield, IN, USA) stocks of 1 mM were prepared in distilled water every week. Ryanodine, staurosporine, iberiotoxin and PMA were obtained from Tocris Biosciences (Ellisville, MO, USA); 2 mM stock ryanodine was made every week. All stock concentrations were kept at -20°C , thawed and used once. Glucose, 4-aminopyridine, SNP and NNLA were obtained from Sigma-Aldrich (St. Louis, MO USA). HEPES was obtained from Calbiochem, EMD Biosciences Inc. (La Jolla, CA, USA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Results

Slo-1 and lat-1 homologous genes from A. suum are expressed at the adult stage of A. suum

Using *C. elegans* *slo-1* and *lat-1* deduced amino-acid sequences for query, databank searches revealed homologous *A. suum* sequences for both genes. The alignments of *C. elegans* SLO-1 and LAT-1 sequences with their respective *A. suum* counterparts are presented in Figure 2. *A. suum* *slo-1* cDNA sequence (GenBank accession no. ACC68842.1) encodes a putative protein of 1117 amino-acids, sharing 78% identity and 87% similarity with the Cel-SLO-1 sequence. SLO-1 sequences from both nematode species possess seven transmembrane-spanning domains (S0–S6), a P-loop, four hydrophobic intracellular segments (S7–S10) and a 'Ca²⁺ bowl' that typifies large conductance Ca-sensitive K channels (Wallner *et al.*, 1996; Wei *et al.*, 1996; Schreiber and Salkoff, 1997; Lim *et al.*, 1999; Ghatta *et al.*, 2006).

The cDNA sequence for *A. suum*-*lat-1* encodes a predicted protein of 986 amino-acids (Figure 2B), which has 38% identity and 54% similarity to the Cel-LAT-1 sequence. Despite this limited conservation at the amino-acid level, the LAT-1 sequences from both nematode species share critical characteristic features: seven transmembrane regions, a galactose-binding lectin domain (PFAM Gal_Lectine: PF02140), a hormone receptor domain (SMART HormR: SM 00008) and two G-protein coupled protein domains (SMART GPS: SM00303 and PFAM 7Tm_2: PF00002).

In order to provide further evidence for the expression of *slo-1*- and *lat-1*-like genes in the muscles of *A. suum* adult worms, we performed a set of RT-PCR experiments using gene-specific primers (Supporting Information Table S1) designed from *Asu-slo-1* and *Asu-lat-1* cDNA sequences respectively. For both candidates, a single amplification product at the expected size was obtained (Supporting Information Figure S1). Sequencing reactions showed that the amplicons corresponded to *Asu-slo-1* and *Asu-lat-1*, respectively, and confirmed their expression in the muscle flaps of adult *A. suum*. Evidence for the presence of these two genes encouraged electrophysiological investigation of effects of emodepside.

Emodepside has an inhibitory effect on spiking

Initially, we examined the effects of emodepside on muscle spiking under current-clamp as a way of detecting inhibitory

A

Figure 2

Conservation of SLO-1 and LAT-1 sequences in *C. elegans* and *A. suum*. Alignments of deduced amino-acid sequences were constructed using the MUSCLE algorithm (Edgar, 2004) and further processed using GeneDoc program (<http://www.nrbsc.org/gfx/genedoc/index.html>). Identical amino-acids between *C. elegans* and *A. suum* sequences are shaded in dark blue and distinct amino-acids sharing similar physico-chemical properties are shaded in light blue. Predicted signal peptide sequences are shaded in grey. The transmembrane domains (TM) are noted below the sequences. (A) Comparison of Asu-SLO-1 (GenBank accession n° ACC68842.1) and Cel-SLO-1 (GenBank accession n° NP_001024259.1) showing the 7 TM domains (S0–S6), P-loop, S7–S10 intracellular domains and 'Ca²⁺ bowl'. Domain annotation corresponds to *A. suum* SLO-1. (B) Comparison of Asu-LAT-1 (GenBank accession n° ADY40714.1) and Cel-LAT-1 (GenBank accession n° NP_495894.1). Domain annotations correspond to *A. suum* LAT-1.

B

Figure 2

Continued.

Figure 3

Effect of emodepside (1 μM) on membrane spiking. (A) Representative current-clamp trace showing the spiking induced by ryanodine (1 μM) and the inhibitory effect of emodepside on the spikes. (B) Bar chart (mean ± SEM) of the effects of ryanodine on the frequency of spikes. Ryanodine caused a time-dependent increase in the spike frequency; comparison was made between 20 min and 35 min of ryanodine application ($P < 0.05$, $n = 7$, paired *t*-test). (C) Bar chart (mean ± SEM) of the effects of emodepside on the frequency of ryanodine-induced spikes. Emodepside abolished the increase in spike frequency; comparison was made between 20 min of ryanodine application and 15 min (35 min) of emodepside + ryanodine application ($P < 0.05$, $n = 5$, paired *t*-test). (D) Bar chart (mean ± SEM) of the effects of PF1 (1 μM) on the ryanodine-induced spike frequency. After just 10 min application, PF1 caused a decrease in spike frequency ($P < 0.01$, $n = 5$, paired *t*-test).

actions of emodepside. We activated spiking by applying 1 μM ryanodine (Puttachary *et al.*, 2010) and compared effects of emodepside with the neuropeptide PF1 because emodepside has been suggested to act by stimulating release of inhibitory neuropeptides like PF1.

Ryanodine (1 μM) produced a slow time-dependent increase in the spike frequency and comparison between the 20 and 35 min period (Figure 3B) shows a significant increase of 15 spikes per minute ($P < 0.05$, $n = 7$) in spike frequency.

Figure 3A shows a representative trace of the inhibitory effect of emodepside on the ryanodine-induced spiking. Emodepside significantly inhibited the ryanodine increase in spike frequency between the 20 and 35 min period (Figure 3C) by 9.8 spikes·min⁻¹ ($P < 0.05$, $n = 5$). We also tested the effect of PF1 (1 μM) on ryanodine spiking and found that it was more potent and faster acting than emodepside as PF1 decreased the ryanodine spike frequency by 46 spikes per minute in a shorter time of 10 min ($P < 0.01$, $n = 5$, Figure 3D).

Figure 4

Effect of emodepside (1 μ M) on membrane potential. (A) Representative current-clamp trace showing the membrane potential before, during and after 10 min application of 1 μ M emodepside. (B) Bar chart (mean $\pm$ SEM) of the effects of emodepside on the membrane potential. Comparison was made between membrane potential before and during emodepside application. Emodepside caused a significant membrane hyperpolarization ($P < 0.001$, $n = 10$, *paired t-test*) which was reduced in the presence of 4-aminopyridine (5 mM) ($P < 0.01$, $n = 8$, *unpaired t-test*). Emodepside (10 μ M) caused an increased hyperpolarization in comparison to 1 μ M emodepside ($P < 0.05$, *unpaired t-test*). (C) Bar chart (mean $\pm$ SEM) of the effects of NO on emodepside-induced hyperpolarization. In the presence of 1 mM SNP, emodepside (1 μ M) caused an increased hyperpolarization ($P < 0.01$, $n = 4$, *paired t-test*). NNLA (100 μ M) decreased the hyperpolarization caused by emodepside ($P < 0.01$, *unpaired t-test*). (D) Bar chart (mean $\pm$ SEM) of the effects of protein kinase modulators on emodepside-induced hyperpolarization. PMA (10 μ M) had no significant effects on the hyperpolarization caused by emodepside. However, staurosporine (1 μ M) decreased the hyperpolarization caused by emodepside ($P < 0.05$, *unpaired t-test*).

Effect of emodepside on membrane potential and input conductance

Figure 4A shows a representative recording of the membrane potential before, during and after application of emodepside (1 μ M). Perfusion of emodepside for 10 min produced a slowly developing -5.2 mV hyperpolarization, which continued to increase during drug application; the resting input

conductance was 3.5 μ S before drug application and was not changed significantly by emodepside. We observed that the onset of membrane hyperpolarization produced by emodepside varied between 30 s to 3 min and the mean hyperpolarization at 10 min is shown in Figure 4B. The hyperpolarization was not reversible but continued to increase gradually despite washing. At a higher concentration of

10 μ M emodepside at 10 min, the hyperpolarization was greater ($P < 0.001$, $n = 6$, Figure 4B), but there was still no significant change in input conductance. Comparison of the hyperpolarization caused by 1 and 10 μ M emodepside showed that the hyperpolarization was concentration-dependent; the difference, -2.6 ± 1.3 mV, was significant ($P < 0.05$, $n = 16$). The membrane potentials of some of the muscle cells showed spontaneous small depolarizing potentials which were reduced or completely abolished by the emodepside.

To test if the membrane hyperpolarization caused by emodepside was mediated by opening of K channels, we used the K channel blocker, 4-aminopyridine (5 mM), in the perfusion solution throughout the experiment. In the presence of 4-aminopyridine, the mean resting membrane potential was unchanged but the hyperpolarizing effect of emodepside at 10 min was significantly reduced (Figure 4B).

Emodepside effect on membrane potential: role of NO and PKC

The slow hyperpolarizing effect of emodepside and sensitivity to 4-aminopyridine has similarities to the effect of the neuropeptide PF1 in *A. suum* and the effects of PF1 are mediated by NO synthase (Bowman *et al.*, 1995; 2002; Verma *et al.*, 2009). We therefore tested the effects of NNLA, an inhibitor of inducible NO synthase (iNOS) and SNP, an NO-donor on the actions of emodepside.

In the presence of 100 μ M NNLA, the emodepside hyperpolarization was significantly reduced to -1.3 ± 0.44 mV ($P < 0.01$, $n = 6$, Figure 4C). In contrast, in the presence of 1 mM SNP, emodepside caused a significantly increased hyperpolarization of -9.5 ± 1.6 mV ($P < 0.01$, $n = 4$, Figure 4C). Both of these observations suggested a role for NO in the mediation of the emodepside effects. In time control experiments, NNLA and SNP had no significant effect by themselves on the membrane potential.

We further investigated the role of PKC in mediating the effects of emodepside as emodepside has been suggested to bind to latrophilin-like receptors in *C. elegans* which can signal through G_q proteins and PKC in other preparations. Staurosporine (1 μ M), a broad-spectrum protein kinase inhibitor, significantly reduced the hyperpolarization produced by emodepside (1 μ M) to -2.1 ± 0.48 mV ($P < 0.05$, $n = 4$, Figure 4D). Staurosporine, by itself, had no significant effects on the membrane potential. The PKC activator, PMA (10 μ M), produced a small but significant hyperpolarization (-2.1 ± 0.3 mV, $P < 0.05$, $n = 5$). However, in the presence of the 10 μ M PMA, 1 μ M emodepside produced an additional membrane hyperpolarization of -4.9 ± 1.0 mV ($P < 0.01$, $n = 5$, Figure 4D). The effects of PMA and staurosporine are consistent with a role for PKC in the emodepside signalling pathway.

Effect of emodepside (1 μ M) on voltage-activated K currents

We investigated the effects of emodepside (1 μ M) on the voltage-activated K currents. Given the slow onset of emodepside effects on spiking and membrane potential, we investigated the time-dependent effects of emodepside on the K currents (Figure 5). In control recordings, we tested and

observed no significant change in the voltage-activated K currents at 10, 20, 30 and 40 min. We applied emodepside for 30 min and examined the effect on the K currents at 10 min intervals. Figure 5A shows representative K current traces of the control and the effects of emodepside at 10, 20 and 30 min, where it produced time-dependent increases in the currents. The maximum increase in the late phase K currents (L_K) was observed at the 0 mV voltage step. At the higher voltage steps, emodepside produced smaller changes with no effect at +30 mV. In the sample plot of Figure 5B, emodepside increased L_K at 0 mV to 130%, 146% and 167% at 10 min, 20 min and 30 min respectively. A similar trend was observed in all recordings (0 mV, Figure 5C, $n = 4$), where emodepside significantly increased the currents at 10 min, 20 min and 30 min. We tested the effects of emodepside on the K current activation curves and were only able to see small changes in the kinetic parameters V_{50} and G_{max} throughout the application of emodepside.

When we applied emodepside for 10 min and then washed for 20 min (post-emodepside period), we observed a continued increase in the K current following the onset of washing (Figure 5D–F). In the sample plot in Figure 5E, L_K increased to 133% after 10 min exposure to emodepside and to 186% following a further period of 20 min washing. We observed a similar effect in all 9 recordings, as shown in Figure 5F.

Effect of emodepside (10 μ M) on voltage-activated K currents

We increased the concentration of emodepside to 10 μ M in order to resolve effects on the kinetic parameters of the K current activation curves. Figure 6A shows representative traces and the average effect from 6 recordings, of emodepside (10 μ M) on the L_K is shown in Figure 6B. The activation curve, Figure 6C, shows that 10 μ M emodepside shifted V_{50} by 2.7 mV in the hyperpolarizing direction and by 6.4 mV during the post-emodepside period; there was no change in the G_{max} . Similar hyperpolarizing shifts without changes in G_{max} were observed in all 6 experiments: 2.9 ± 0.3 mV ($P < 0.05$) in 10 μ M emodepside and 6.6 ± 1.0 mV ($P < 0.01$) in the post-emodepside period. In control experiments we found that the conductance-voltage plots showed little or no change in V_{50} throughout a 40 min recording period: specifically, at 20 min it was 0.28 ± 0.10 mV, $n = 6$; at 30 min it was 0.50 ± 0.20 mV, $n = 6$. The shape of the conductance activation curve with an increase in V_{50} , can explain why the emodepside K current is greater at 0 mV with little change near +30 mV: emodepside is not altering the maximum opening of channels but their voltage-sensitivity.

Ca is required for effects of emodepside on K currents

We replaced extracellular Ca with equimolar Co to determine the Ca requirement for the action of emodepside on K currents. In the absence of Ca, the effect of emodepside (1 μ M) was reduced and we observed only a $12.0 \pm 5.0\%$ increase in L_K at 10 min and a $33.0 \pm 17.0\%$ increase post-emodepside. The increases were not significantly different from the control L_K ($P > 0.05$, $n = 10$). These observations show that Ca was required for effects of emodepside on the L_K current and

Figure 5

Effect of emodepside (1 μM) on voltage-activated L_K currents. (A) Representative voltage-clamp traces of control K current and the time-dependent effects of emodepside on the K currents, all at 0 mV. (B) Sample plot of current (% control) against voltage showing the voltage-dependence of the K currents increased by emodepside at different time points. (C) Bar chart (mean ± SEM) of the effects of emodepside on L_K currents. Comparison was made between the control 0 mV step current at 30–40 ms and the corresponding current increased by emodepside at 10, 20 and 30 min. Emodepside increased L_K currents at 10 min ($P < 0.01$, $n = 4$, paired t -test), 20 min ($P < 0.01$, $n = 4$, paired t -test) and 30 min ($P < 0.05$, $n = 4$, paired t -test). (D) Representative voltage-clamp traces of control L_K current, the effects of emodepside on the L_K current and the L_K currents after 20 min wash (post-emodepside). (E) Sample current (% control) versus voltage plot showing how the L_K currents change with voltage. Emodepside increased the current after 10 min and the increase was sustained at the post-emodepside period. (F) Bar chart (mean ± SEM) of the effects of emodepside on the L_K currents. After 10 min perfusion, emodepside increased the L_K currents ($P < 0.001$, $n = 9$) and the increase was sustained during the post-emodepside period ($P < 0.01$, $n = 9$).

Figure 6

Effect of emodepside (10 μ M) on the voltage-activated L_K currents. (A) Representative traces of the control L_K current, the effects of emodepside on the current and the post-emodepside L_K current all at 0 mV. (B) Bar chart (mean $\pm$ SEM) of % increase in L_K currents produced by emodepside ($P < 0.05$, $n = 6$, paired t -test) and the continued increase at the post-emodepside period ($P < 0.05$, $n = 6$, paired t -test). (C) Sample activation curve of the L_K currents; conductance-voltage plots were fitted to the Boltzmann equation. Emodepside shifted the V_{50} in the hyperpolarization direction and the shift was sustained during the post-emodepside period. Essentially no change in $G_{\max}$ was observed.

because I_{a-} and I_{K-} -like K currents are present in *A. suum* under Ca-free conditions (Martin *et al.*, 1992) the experiments suggest that emodepside does not act directly on I_{a-} or I_{K-} -like K currents.

Emodepside effects on K currents: role of NO and PKC

We investigated the role of NO on the effects of emodepside (1 μ M) on the K currents using SNP as an NO donor and NNLA as an iNOS inhibitor. SNP (1 mM) by itself did not cause a significant change in the L_K currents even after 30 min ($P > 0.05$, $n = 4$). Figure 7A shows representative K current traces with control, effects of SNP and emodepside + SNP and post-emodepside L_K currents. In six preparations, emodepside, in the presence of SNP, increased the L_K currents at 10 min ($P < 0.05$, $n = 5$, Figure 7D) and a further increase during the post-emodepside period. The activation curve, Figure 7C, shows that in the presence of SNP, emodepside shifted the V_{50} in the hyperpolarization direction by 2.9 mV and during the post-emodepside period, the shift was

increased to 4.6 mV. The mean shift in the V_{50} at 10 min was 2.4 mV ($P < 0.05$, $n = 6$) and 6.2 mV ($P < 0.01$, $n = 6$) post-emodepside.

NNLA (100 μ M) had no significant effect on K currents or membrane potential in control experiments ($P > 0.05$, $n = 3$) but this compound did inhibit the effect of emodepside (1 μ M) on K currents. In the presence of NNLA, there was little effect ($1.9 \pm 1.0\%$ increase, $P > 0.05$, $n = 6$) of emodepside on the L_K currents at 10 min and the effect at the 20 min post-emodepside period was reduced to $26.0 \pm 11.0\%$ ($P < 0.05$, $n = 5$). Thus NNLA reduced the effects of emodepside on the K currents. The effects of both SNP and NNLA together indicated a role for NO during the action of emodepside.

We investigated the role of PKC in emodepside effects on the K currents using PMA as a PKC activator and staurosporine as a kinase inhibitor. PMA (10 μ M), by itself produced a small stable but significant increase in the K currents and a small hyperpolarization. Figure 8A shows representative traces of the control L_K current, the effects of PMA alone, of emodepside + PMA and of the post-emodepside period on the

Figure 7

Effect of emodepside (1 μ M) and SNP (1 mM) on the voltage-activated L_K currents. (A) Representative traces of control L_K current, the effects of emodepside + SNP on the L_K current and the post-emodepside L_K current, all at 0 mV. (B) Bar chart (mean $\pm$ SEM) of % increase in L_K current produced by emodepside in the presence of SNP ($P < 0.05$, $n = 5$, paired t -test) and the continued increase in the post-emodepside period ($P < 0.05$, $n = 4$, paired t -test). (C) Sample activation curve of the L_K currents; conductance-voltage plots were fitted to the Boltzmann equation. In the presence of SNP, emodepside shifted the V_{50} in the hyperpolarization direction. Essentially no change in G_{max} was observed.

L_K current. The mean data from all 5 recordings is summarised in Figure 8B. In the representative activation curve in Figure 8C, PMA shifted the V_{50} in the hyperpolarization direction, whilst emodepside + PMA caused a further hyperpolarizing shift in the V_{50} and during the post-emodepside period, there was a further additional hyperpolarizing shift. On average, PMA shifted V_{50} by 1.9 mV ($P < 0.05$, $n = 5$) and emodepside + PMA shifted it by 2.8 mV ($P < 0.01$, $n = 5$). There was no change in G_{max} . From these observations, it is clear that PMA reproduces some of the effects of emodepside.

The PKC inhibitor staurosporine (1 μ M) by itself had no significant effect on the K currents (at 30 min, mean decrease $4.4 \pm 4.0\%$, $P > 0.05$, $n = 3$). However, staurosporine inhibited the action of emodepside (1 μ M). Figure 8 shows effects on representative K currents and Figure 8E shows summary bar-charts. Staurosporine, at 10 min, had no effect on the L_K current. In the presence of staurosporine, emodepside (1 μ M) was no longer activating, but was inhibitory, with a mean decrease of 10% ($P < 0.05$, $n = 4$, Figure 8E). The effects of PMA mimicking emodepside, and the loss of emodepside activation when pretreated with staurosporine, together indicated a

role for PKC during the action of emodepside. The inhibitory action of emodepside following staurosporine suggests additional actions of emodepside but these were not explored further.

The 4-aminopyridine-sensitive K current includes I_a as well as the emodepside-activated current

In the presence of 4-aminopyridine (5 mM), emodepside (1 μ M) had no detectable effect on K currents ($P > 0.05$, $n = 8$). When we applied 4-aminopyridine after emodepside, and measured the emodepside-activated K current as a proportion of the total 4-aminopyridine-sensitive K current, it was only $50 \pm 16\%$ ($n = 6$). Martin *et al.* (1992) have shown that 5 mM 4-aminopyridine selectively inhibits I_a -like K currents but not I_k -like K currents in *A. suum* under Ca free conditions. The sensitivity of the emodepside K currents to 4-aminopyridine showed that emodepside did not activate I_k currents. The presence of Ca here, allows K currents to be activated by emodepside. The total 4-aminopyridine-sensitive current was bigger than the emodepside-activated current, indicating that

Figure 8

Effects of emodepside (1 μ M) plus PKC modulators on the voltage-activated L_K currents. (A) Representative traces of control L_K current, the effects of PMA (10 μ M) alone, emodepside + PMA and the post-emodepside period on the L_K current. (B) Bar chart (mean $\pm$ SEM) of the % increases in L_K currents caused by PMA ($P < 0.05$, $n = 5$, paired t -test), emodepside + PMA ($P < 0.01$, $n = 5$, paired t -test) and post-emodepside ($P < 0.05$, $n = 3$, paired t -test). Comparison was made with the % control L_K current. (C) Conductance-voltage plots were fitted to the Boltzmann equation. In the activation curves shown, PMA and emodepside + PMA shifted the V_{50} in the hyperpolarization direction. Essentially no change in G_{max} was observed. (D) Representative traces showing the effects of emodepside (1 μ M) + staurosporine (1 μ M) on the L_K current. (E) Bar chart (mean $\pm$ SEM) of % change in currents caused by emodepside + staurosporine. Comparison was made with the % control L_K current. In the presence of staurosporine, emodepside decreased the L_K ($P < 0.05$, $n = 4$, paired t -test) and the decrease was sustained during the post-emodepside period. (C) In the presence of staurosporine, emodepside had no significant effects on the V_{50} but significantly reduced the G_{max} ($P < 0.05$, $n = 4$, paired t -test).

4-aminopyridine was not selective and inhibits the emodepside K current as well as the I_a -like K current.

Effects of iberiotoxin

We examined the effects of iberiotoxin; a selective inhibitor of high conductance Ca-activated K (SLO-1) channels with

IC_{50} values often in the sub-nanomolar ranges (Galvez *et al.*, 1990; Candia *et al.*, 1992; Gruhn *et al.*, 2002). Iberiotoxin (10 nM) was used to minimize effects on other channels (Sones *et al.*, 2009) and this inhibitor produced a significant decrease (-23.7% , $P < 0.05$, $n = 4$) in the control L_K currents (Figure 9), without a detectable change in membrane poten-

Figure 9

Effects of emodepside (1 μ M) and iberiotoxin (10 nM) on the voltage-activated L_K currents. (A) Representative traces of control L_K current, 10 nM iberiotoxin effect on the L_K currents, emodepside + iberiotoxin effect on the L_K currents and the post-emodepside L_K currents. (B) Current, expressed as percent control, at 0 mV showing the inhibitory effect of iberiotoxin and emodepside on the L_K currents.

tial, indicating the presence of iberiotoxin-sensitive SLO-1-like K channels in *A. suum*. The application of emodepside (1 μ M) was able to counter some of the effects of iberiotoxin, producing hyperpolarization (-4.1 ± 0.2 mV, $P < 0.05$, $n = 4$) and a return of the L_K currents towards control levels, Figure 9, but emodepside no longer increased the K current above the control ($P > 0.05$). Thus iberiotoxin reduced (but did not entirely block) effects of emodepside indicating that iberiotoxin-sensitive SLO-1-like channels are involved.

Emodepside effects on voltage-activated Ca currents

We investigated the effects of emodepside (1 μ M) on voltage-activated Ca currents. The inhibitory neuropeptide PF1, which emodepside may mimic, has been shown to significantly inhibit the voltage-activated Ca currents in *A. suum* (Verma *et al.*, 2009). In all nine recordings, we observed no significant effect of emodepside on the Ca currents (Figure 10).

Discussion

Different mode of action

Emodepside is a cyclooctadepsipeptide anthelmintic that selectively inhibits muscle contraction of nematodes

(Willson *et al.*, 2003). It is effective against nematode isolates that have developed resistance to drugs from the other major classes of anthelmintic (Samson-Himmelstjerna von *et al.*, 2005): ivermectin (an allosteric modulator of glutamate-activated Cl channels; Pemberton *et al.*, 2001), levamisole (a nematode selective nAChR agonist; Qian *et al.*, 2006; 2008) and febantel (a selective ligand for nematode β -tubulin; Miro *et al.*, 2006). Because emodepside retains its efficacy against these resistant isolates, it implies that emodepside has a different mode of action. In this paper we have examined effects of emodepside on the electrophysiology of *A. suum* muscle using two micropipette current- and voltage-clamp techniques with a view to determining its mechanism of action.

Emodepside is not a GABA receptor agonist

The earliest studies on the mechanisms of action of the cyclooctadepsipeptides used PF1022A. PF1022A seemed to exert its anthelmintic action on the nerves or muscle rather than affecting energy metabolism because low concentrations of PF1022A (<1 μ M) depressed the motility of the nematode parasite, *Angiostrongylus cantonensis* (Terada, 1992). Chen *et al.* (1996) reported that PF1022A bound to GABA receptors of *Ascaris suum* muscle suggesting a direct effect on nematode GABA receptors. However, direct electrophysiological recording from *Ascaris suum* muscle found that PF1022A did not act like GABA, nor did it act as a cholinergic antagonist (Martin *et al.*, 1996). Emodepside does not produce an increase in the

Figure 10

Effects of emodepside (1 μM) on the voltage-activated Ca currents. (A) Representative inward Ca current traces of control, 1 μM emodepside and the post-emodepside currents. (B) Bar chart (mean $\pm$ SEM) of the effects of emodepside on the inward Ca currents (green: 20 min post emodepside). Emodepside had essentially no effect on the voltage-activated inward Ca currents ($P > 0.05$, $n = 9$, paired t -test). (C) Current-voltage plot showing no effect of emodepside on the inward Ca^{2+} currents at the different voltage steps.

muscle membrane conductance like GABA or piperazine (Martin, 1982) again showing that these compounds do not act as GABA agonists.

GeBner *et al.* (1996) reported that the depsipeptides PF1022A, PF1022-001 (antipode of PF1022A), valinomycin, enniatin A₁ and beauvericin have ionophore activities, increasing the bilayer conductivity to monovalent ions like Na^+ , Li^+ , K^+ and Cs^+ . However, they reported that only PF1022A exerts high paralytic effect on *A. suum*, suggesting that the anthelmintic effects of the cyclooctadepsipeptides are not attributable to ion-carrier activity.

K-dependent hyperpolarization by releasing inhibitory neuropeptides (PF1/PF2)

Willson *et al.* (2003) tested the effects of emodepside on *Ascaris suum* muscle contraction and electrophysiology. They observed that 10 μM emodepside had a much slower inhibitory action on muscle contraction than GABA and produced a slow hyperpolarization with no detectable change in input conductance. The inhibitory neuropeptide, PF2, was also reported to cause slow inhibition of contraction of *A. suum* muscle, similar to emodepside (Fellowes *et al.*, 2000; Willson *et al.*, 2003). In this study we have observed similar, but concentration-dependent, effects with emodepside. Willson *et al.* (2003) showed that the K channel blocker 4-aminopyridine inhibited the effect of emodepside on membrane potential and suggested that emodepside may

stimulate the release of inhibitory neuropeptides, such as PF1 or PF2, to produce its effects. PF1 causes slow, non-reversible, concentration-dependent membrane hyperpolarization that is significantly blocked by 4-aminopyridine (Franks *et al.*, 1994; Verma *et al.*, 2009). Here, we have observed similar effects of emodepside on the membrane potential. Despite the similarity in the effects of PF1 and emodepside on the membrane potential, we have demonstrated clear differences in their mode of action. Unlike PF1, which inhibits voltage-activated Ca currents (Verma *et al.*, 2009), emodepside had no effects on voltage-activated Ca currents. We also observed that emodepside inhibited the ryanodine-induced spiking more slowly than PF1, probably because of the lack of emodepside effect on the voltage-activated Ca currents. Though there are similarities in the effects of emodepside and the inhibitory neuropeptides PF1 and PF2, our body of evidence would not support the proposal that emodepside stimulated the release of a PF1-like neuropeptide.

Latrophilin receptors

In 2004, Willson *et al.* described observations on the *C. elegans* pharynx with emodepside (100 nM) acting as an agonist to stimulate exocytosis and elicit pharyngeal paralysis. The paralysis of the pharynx produced by emodepside depended on the presence of LAT-1 with emodepside resistance appearing in *lat-1* null mutants. We observed evidence

of expression of *Asu-lat-1* in adult muscle flaps and PKC and NO signalling pathways suggesting activation of G-protein receptor(s) by emodepside. Muhlfeld *et al.* (2009) used surface plasmon resonance to show that the neuropeptides AF1, AF10 and PF2 bind, albeit with low affinities, to HC110-R, implying that these neuropeptides may be putative natural ligands of the latrophilin-like receptor. They observed no reasonable binding characteristics with PF1 and other neuropeptides. Our observations, however, do not rule out a possible direct effect of emodepside on K channels.

SLO-1 as a target for emodepside in C. elegans

Guest *et al.* (2007) described use of a mutagenesis screen to generate alleles of *slo-1* that encodes for a Ca-activated K channel in *C. elegans*. They observed that *slo-1*, but not *slo-2*, null mutants were more resistant to the inhibitory effects of emodepside than *lat-1* and *lat-2* (latrophilin receptor) double mutants. Guest *et al.* (2007) proposed that emodepside either directly or indirectly activates SLO-1 that is present in body wall muscle and motor neurons to produce its inhibitory effects in nematodes. Interestingly when *slo-1* was selectively expressed in the pharyngeal muscle of *slo-1* null mutants, there was no effect of emodepside on the frequency of pharyngeal pumping contrary to what might be expected if emodepside exerted a direct effect on SLO-1. However, *slo-1* may regulate other features of the pharyngeal activity such as pump duration and pump interval and the effect of emodepside on these parameters remains to be investigated.

SLO-1 as a target for emodepside in A. suum

We have observed expression of *Asu-slo-1*, an evolutionarily conserved homologue of the *slo-1* gene in adult *A. suum* body muscle flaps; and have also observed a high-conductance 250 pS K channel present in the *A. suum* bag membrane (unpublished single-channel observations). There is a Ca-dependent and voltage-activated K channel present in *A. suum* muscle which is affected by the inhibitory neuropeptide PF1 (Verma *et al.*, 2009). Our studies here indicate that the target of emodepside includes SLO-1-like K channels. Evidence supporting this possibility includes the following findings: a Ca-dependent and voltage-activated K current activated by emodepside; the inhibitory effects of 4-aminopyridine (5 mM) on this current (which also blocks Ca-independent I_{A-} but not Ca-independent I_{K-} like K currents in *A. suum*, Martin *et al.*, 1992); inhibitory effects of 10 nM iberiotoxin (which has selective effects on SLO-1 K currents, Galvez *et al.*, 1990; Candia *et al.*, 1992; Gruhn *et al.*, 2002). Not all of the emodepside K current was blocked by iberiotoxin which may be due to different types of β -subunits present with the SLO-1 α -subunits that mediate iberiotoxin sensitivity (Meera *et al.*, 2000) or effects on K channels other than SLO-1, I_A or I_K .

We observed that the effect of emodepside was very slow in onset and increased over a period of more than 10 min. For example, in our ryanodine-induced spiking experiments, we observed the speed of onset of the inhibitory action of emodepside was slower than the onset of action of PF1. The slowly developing effect of emodepside may be

due to the highly lipophilic nature of this compound and a membrane partitioning effect. Indeed, the potency of a similarly lipophilic anthelmintic, ivermectin also follows a slow time-course in electrophysiological experiments in *A. suum* (Kass *et al.*, 1980). Alternatively the slow time-course and persistence may be because emodepside does not directly activate the K channels but acts through a signalling cascade. We found that the emodepside effect was potentiated by a NO donor and a PKC activator, while antagonism of iNOS and inhibition of PKC with staurosporine inhibited the effects of emodepside. Interestingly, these signalling molecules are known activators of SLO-1 in other cells (Bolotina *et al.*, 1994; Mistry and Garland, 1998; Wang *et al.*, 1999; Holden-Dye *et al.*, 2007) and therefore emodepside may act through either or both of these signalling cascades and the signalling cascades may be in series or parallel. A number of studies on the mammalian orthologues of SLO-1 show that they are directly and alternately regulated by complex, multiple signalling cascades, involving NO and diacylglycerol or Ca-dependent PKC activation (Ghatta *et al.*, 2006; Salkoff *et al.*, 2006). Although emodepside is lipophilic, and this is consistent with the observed lack of a washout effect (Willson *et al.*, 2003; Schurmann *et al.*, 2007), we observed that the effect of emodepside on voltage-activated K currents continued to increase following washout. This is similar to PF1 (Verma *et al.*, 2009) and, therefore, an additional or alternative explanation may be the involvement of a second messenger(s) signalling cascade.

To conclude, we found that *Asu-slo-1* and *Asu-lat-1* genes were expressed in body muscle flaps of the nematode parasite *A. suum*. Emodepside induced a 4-aminopyridine-sensitive hyperpolarization of the muscle membrane potential and potentiated voltage- and Ca-dependent, 4-aminopyridine- and iberiotoxin-sensitive, SLO-1-like currents that were affected by modulators of NO and PKC signalling pathways. Emodepside had no effect on voltage-activated Ca currents. The effects are consistent with a model in which NO, PKC and emodepside signalling pathways converge on the K channels, and/or a model in which emodepside activates the K channels through an NO or a PKC signalling cascade. Further experiments on heterologous expression systems are required to distinguish between these modes of action.

Note added in Proof

The following two manuscripts have appeared recently describing further effects of emodepside and the role of SLO-1.

- 1) Crisford, A. *et al.* (2011) Selective toxicity of the anthelmintic emodepside revealed by heterologous expression of human KCNMA1 in *Caenorhabditis elegans*. *Molecular Pharmacology*. 79, 1031–1043.
- 2) Welz, C., *et al.* (2011). SLO-1-channels of Parasitic Nematodes Reconstitute locomotor Behaviour and Emodepside Sensitivity in *Caenorhabditis elegans slo-1* Loss of Function Mutants. *PloS Pathog.* April; 7(4): e100133.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Ethidium bromide stained agarose gel showing patterns revealed by RT-PCR for transcription of *Asu-slo-1* and *Asu-lat-1* from adult stage muscle flap. M: Molecular marker.

Table S1 Primer sequences used for *Asu-slo-1* and *Asu-lat-1* RT-PCR experiments

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