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Depolarization of the tegument precedes morphological alterations in *Echinococcus granulosus* protoscoleces incubated with ivermectin

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Abstract The nematocidal activity of ivermectin (IVM) largely arises from its activity as a potent agonist of muscular and neuronal glutamate-gated chloride channels. A cestocidal effect has also been suggested following in vitro treatments, but the molecular basis of this activity is not clear. We studied the effect of IVM on the metacestode stage of the tapeworm *Echinococcus granulosus* by assessing the viability, ultrastructure, and tegumental membrane potential as a function of drug concentration and incubation time. Concentrations of 0.1 and 1.0 μg/ml of IVM had no effect on any of these three parameters for up to 6 days of treatment. A concentration of 10 μg/ml, however, elicited a sequence of alterations that started with a ~20-mV depolarization of the tegumental membrane, and was followed by rostellar disorganization, rigid paralysis and, eventually, loss of viability. It is likely that the IVM-induced depolarization of the tegument acts as the signal that initiates the cascade of degenerative processes that leads to the parasite's death. This would place the tegument as the primary target of action of IVM on cestodes. As an appropriate chemotherapy for the hydatid disease is still lacking, the cestocidal effect of IVM reported here is worth considering.

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Introduction

The nematocidal activity of ivermectin (IVM) largely arises from its activity as a potent agonist of muscular and neuronal glutamate-gated chloride channels (Cleland 1996; Martin et al. 1996, 1997, 1998; Martin 1997; Wolstenholme 1997; Dent et al. 2000). Upon binding, IVM irreversibly opens these chloride-selective ion channels (Cully et al. 1994; Dent et al. 1997), thus hyperpolarizing, and reducing the input resistance of, the cell. As a consequence, excitatory synaptic currents are less effective in depolarizing the cell to threshold and, therefore, action-potential firing in muscle and neurons is abolished; this is, eventually, followed by the parasite's death. The paralyzing effect of IVM on the pharyngeal musculature of *Caenorhabditis elegans*, which leads to starvation, is particularly well understood (Dent et al. 1997).

It is generally agreed that nematodes are the only helminths sensitive to IVM following oral treatments. Interestingly enough, however, Casado et al. (1989) reported the in vitro cestocidal effect of IVM on the metacestode stage of the tapeworm *Echinococcus granulosus* (''protoscoleces''), and a comparable result was found by Ochieng'-Mitula and Burt (1996) upon direct injection of IVM into hydatid cysts of infected animals.

As a step towards understanding the molecular basis of the protoscolicidal action of avermectins, we assessed the viability, tegumental morphology, and tegumental membrane potential of *E. granulosus* protoscoleces exposed (*in vitro*) to concentrations up to 10 μg/ml of IVM for up to 24 h. We found that depolarization of the tegument precedes, and perhaps initiates, the series of events that culminates in the metacestode's death. The involvement of glutamate-gated Cl^- channels in this process is, at present, not a foregone conlusion.

Materials and methods

E. granulosus protoscoleces were obtained from hepatic hydatid cysts of naturally infected sheep slaughtered at a municipal abattoir

in Alcalá de Henares, Spain. Protoscoleces were removed by aseptic techniques and tested for viability as previously described by Casado et al. (1986). Aliquots of ~1,500 protoscoleces were maintained in Leighton tubes in 10 ml of Medium 199 (ICN, Costa Mesa, Calif.) containing 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37 °C without change of medium or gassing. A 1 mg/ml stock solution of IVM [a mixture of 80% (w/w) 22,23-dihydroavermectin$_{1a}$ and 20% (w/w) 22,23-dihydroavermectin B_{1b}, a gift from Merck, Sharp & Dohme, Munich, Germany] was prepared in a 0.1% (v/v) mixture of dimethyl sulfoxide (DMSO) and water, and was appropriately diluted for experiments giving final concentrations of 0.1, 1.0, and 10 μg/ml of IVM. In both control and treated samples, viability was tested with the methylene-blue exclusion test, and tegumental morphology was assessed by electron microscopy. For both scanning (SEM) and transmission (TEM) electron microscopy, samples of protoscoleces were fixed with glutaraldehyde (5% in 0.1 M Milloning buffer), postfixed with osmium tetroxide (1% in 0.1 M Milloning buffer), and dehydrated in a graded acetone series as previously described by Pérez-Serrano et al. (1994). Briefly, after full dehydration, protoscoleces were embedded in Spurr's resin, and 0.5-μm-thick sections were cut and stained with toluidine blue in borax. Ultrathin sections were stained with uranyl acetate (1%) and Reynold's lead citrate, and examined with a Zeiss EM 10 transmission electron microscope at 80 kV. For SEM, protoscoleces were fixed and dehydrated as indicated above, dried in a Polaron Critical Point Dryer, mounted on stubs, coated with a Au-Pd alloy (500-Å thickness), and viewed in a Zeiss DMS Digital Scanning electron microscope at 15 kV. For membrane potential measurements, glass micropipettes were pulled from 1-mm (outer diameter) capillary tubing and filled with a 3-M KCl solution. Only micropipettes with resistances ranging from 10 to 40 MΩ were used. The electronics and optics of the experimental setup, as well as the criteria for acceptable tegumental impalements, were as described in Ferreira et al. (1992) and Ibarra and Reisin (1994). For impalements, protoscoleces were held on a nylon mesh (~ 200 μm) covering a 35-mm culture dish, and bathed in Medium 199. In Fig. 4, each data point represents the mean of 60 membrane-potential measurements (± SD) recorded on 60 different protoscoleces corresponding to three different batches of parasites (20 protoscoleces per batch).

Results

As a control, *E. granulosus* protoscoleces were incubated in Medium 199 containing 0.1% (v/v) DMSO and no IVM for up to 6 days at 37 °C. The parasite's viability remained intact (Fig. 1), the morphology was unaltered (Fig. 2a, b), and the potential difference across the outer tegumental membrane stayed close to –55 mV (Figs. 3a, 4). In contrast, when the protoscoleces were incubated in medium containing 10 μg/ml IVM (11.5 μM), a depolarization from –55 mV to –35 mV was recorded after 3 h of in vitro treatment (Fig. 3b, 4). Continued exposure to this agent further depolarized the tegument, the membrane potential reaching a value of –12 ± 5 mV after 24 h (Fig. 4). At this time, however, only 10% of protoscoleces had lost viability (Fig. 1), and no clear tegumental damage was detected with electron microscopy. The first signs of morphological alteration were first evident only after 3 days of continuous incubation with 10 μg/ml IVM: the soma region (Fig. 2c) and the distal cytoplasm (Fig. 2e) were markedly contracted, and the viability dropped to ~70% (Fig. 1). The microtriches were, however, still unaltered (Fig. 2d). Incubation with lower concentrations of IVM (0.1 and 1.0 μg/ml) for 6 days failed to elicit noticeable changes in viability (Fig. 1), ultrastructure (Fig. 2f) or tegumental membrane potential (Fig. 4).

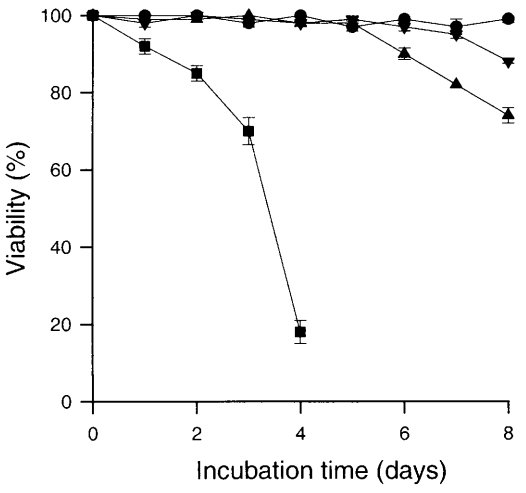

Fig. 1 Survival of *Echinococcus granulosus* protoscoleces after exposure to ivermectin (IVM). ● Control; ▼ 0.1 μg/ml IVM; ▲ 1 μg/ml IVM; ■ 10 μg/ml IVM. Viability was assessed by the methylene-blue exclusion test. Each data point represents the mean (± SD) of three measurements (one per batch of parasites)

Discussion

The in vitro cestocidal effect of IVM described here contrasts with its inefficacy following oral treatments. Most likely, this is due to the fact that the concentration of IVM normally attained in the hydatid-cyst fluid during treatment is well below 10 μg/ml, which is the lowest IVM concentration that exhibited cestocidal effect in our experiments. However, the observed effect suggests that a related avermectin might be successful as a non-invasive therapy for the hydatid disease. Surgery is, at present, the treatment of choice.

The depolarizing action of IVM on the tegument of *E. granulosus* contrasts with its hyperpolarizing effect on neurons and muscle of nematodes and other invertebrates. While the latter is explained by an increase in Cl^- permeability (due to the activation of glutamate/IVM-gated Cl^- channels), the former could be accounted for by an increase in the tegumental permeability to Na^+, a decrease in the permeability to K^+, and/or Cl^-, or an increase in the permeability to cations in general. Some of the ion channels present in the outer tegumental membrane of *E. granulosus* protoscoleces have already been characterized at the single-channel level. Grosman and Reisin (1995, 1997) described the presence of a Ca^{2+}-activated voltage-sensitive K^+ channel, and a non-selective cation channel. Chloride-selective channels were not found. Whether the tegumental non-selective cation channels described by Grosman and Reisin (1995, 1997) can be gated by IVM is currently under study.

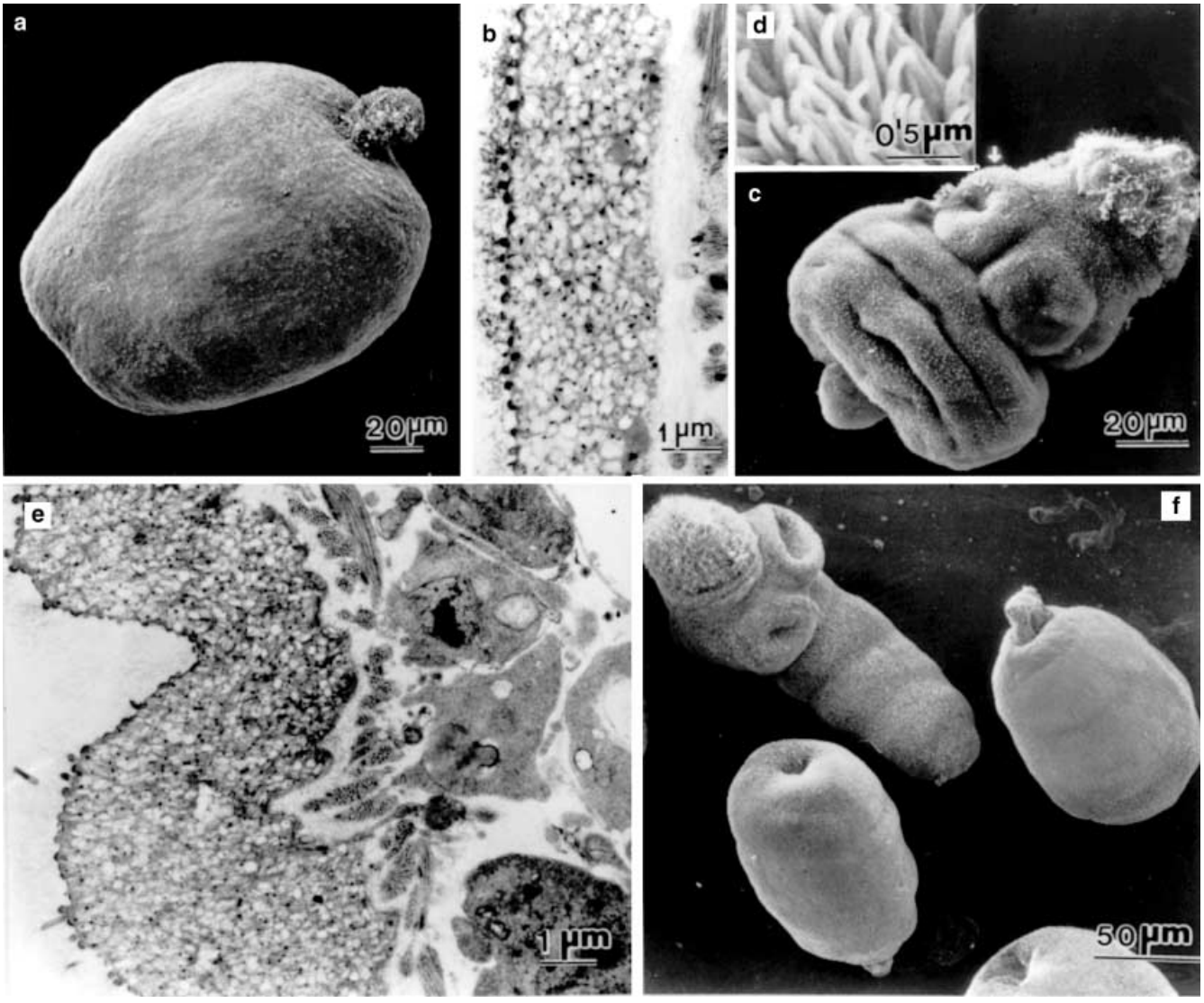

Fig. 2a–f Scanning (SEM) and transmission (TEM) electron micrographs of control and treated protoscoleces. **a** SEM of an untreated protoscolex. **b** TEM of the distal cytoplasm of an untreated protoscolex. **c** SEM after 3 days of incubation with 10 µg/ml IVM. The soma (i.e., the posterior region) appears contracted. **d** SEM after 3 days of incubation with 10 µg/ml IVM. Microtriches appear unaltered. **e** TEM after 3 days of incubation with 10 µg/ml IVM. The distal cytoplasm appears contracted. **f** SEM after 6 days of incubation with 0.1 µg/ml IVM. None of the protoscoleces (two invaginated and one evaginated) show signs of ultrastructural changes

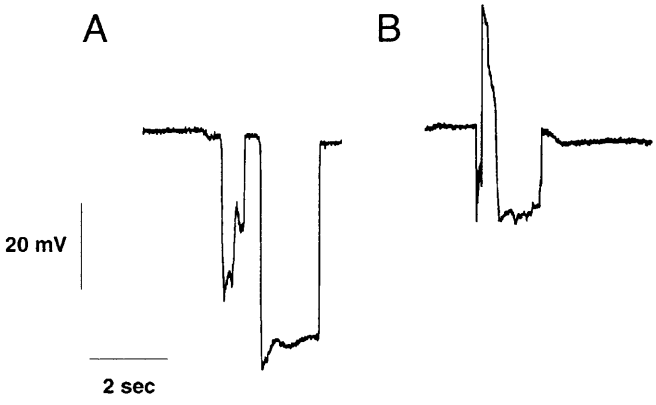

Fig. 3a, b Example recordings of tegument impalements. **a** Control protoscolex (i.e., no IVM); **b** protoscolex incubated with 10 µg/ml IVM for 16 h

Alternatively, it seems reasonable to assume that, due to its high solubility in lipids, IVM can form non-selective cation channels by itself like many antibiotics do. The formation of non-selective cation channels by IVM itself was observed when this compound was applied to the internal side of excised membrane patches of *Ascaris suum* muscle membranes (Martin and Pennington 1989).

Our results highlight the need for further studies on the effect of avermectin-related drugs on tapeworms. Measurement of the tegumental membrane potential proved to be a simple, yet highly sensitive probe to assess the early effects of these compounds in *E. granulosus*, and promises to be an invaluable tool in the search for an adequate anthelmintic pharmacotherapy.

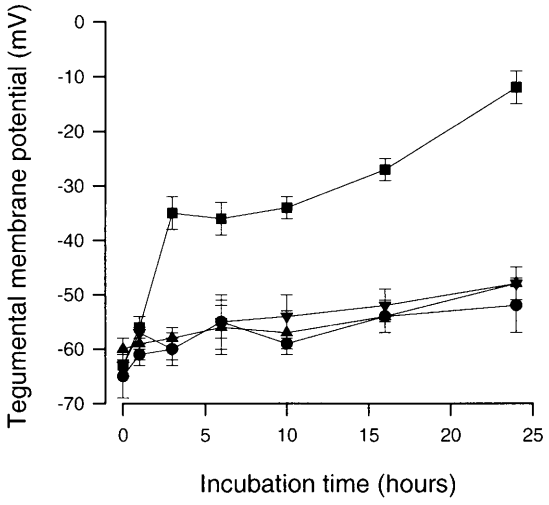

Fig. 4 Time course of the tegumental membrane potential of protoscoleces incubated in Medium 199 containing: ● no IVM; ▼ 0.1 μg/ml IVM; ▲ 1 μg/ml IVM; ■ 10 μg/ml IVM. Each data point represents the mean (±SD) of 60 membrane-potential measurements recorded on 60 different protoscoleces corresponding to three different batches of parasites

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