Actions of the anthelmintic ivermectin on the pharyngeal muscle of the parasitic nematode, *Ascaris suum*

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(*Received 15 February 1997; revised 29 April and 16 May 1997; accepted 16 May 1997*)

SUMMARY

The anthelmintic ivermectin has a number of effects on nematodes which result in changes in behaviour, particularly locomotion, including paralysis and an inhibition of feeding. This paper describes the application of an *in vitro* pharmacological approach to further delineate the action of ivermectin on feeding behaviour. Contraction of *Ascaris suum* pharyngeal muscle was monitored using a modified pressure transducer system which detects changes in intrapharyngeal pressure and therefore contraction of the radial muscle of the pharynx. The pharynx did not contract spontaneously. However, serotonin (5-HT, 100 μM) stimulated rhythmic contractions and relaxations (pumping) at a frequency of 0·5 Hz. γ-Aminobutyric acid (GABA) and glutamic acid inhibited the pumping elicited by 5-HT. The duration of inhibition was concentration dependent (1–1000 μM) with a threshold of 1 μM and 10 μM respectively ($n = 8$). Ivermectin also inhibited pharyngeal pumping (1–1000 nM). At lower concentrations, ivermectin (1–10 pM) potentiated the GABA and glutamate inhibition, so that inhibition occurred at concentrations which were below threshold in the absence of ivermectin. These data provide evidence that the pharynx is a site for the action of ivermectin. Thus interruption of pharyngeal processes such as, feeding, regulation of hydrostatic pressure and secretion may provide a new site of anthelmintic action.

Key words: *Ascaris suum*, nematode, pharynx, ivermectin, γ-aminobutyric acid (GABA), glutamate.

INTRODUCTION

Ivermectin, (22, 23 dihydroavermectin B_{1a}), a semi-synthetic avermectin analogue, is a potent anthelmintic and insecticide. It was introduced commercially in 1981 and has become the drug of choice for treating conditions caused by nematode and arthropod parasites (Campbell, 1989). In humans, ivermectin is effective in the treatment and prevention of Onchoceriasis (river blindness, Campbell, 1985), and in dogs, against the heartworm, *Dirofilaria immitis*.

Evidence to date suggests that avermectins modulate anion channels (Rohrer & Arena, 1995) and this is consistent with the inhibitory effect of the avermectins on a number of invertebrate muscle preparations, such as locust and crayfish muscle (Duce *et al*. 1995; Zufall, Franke & Hatt, 1989). Initial studies on nematodes have shown that ivermectin acts on somatic musculature resulting in a paralysis. Electrophysiological studies on the somatic musculature of *Ascaris* suggest that ivermectin acts via a GABA-gated chloride channel as a non-competitive antagonist (Holden-Dye & Walker, 1990). However, relatively high concentrations are required compared to the effective anthelmintic concentration.

Ivermectin also has potent effects on nematode pharyngeal muscle function. Previous studies in nematodes have shown that pharyngeal pumping rate can be reduced and even totally inhibited by nanomolar concentrations of the drug. For example, in the free-living nematode, *Caenorhabditis elegans* (Avery & Horvitz, 1990), ivermectin has an EC_{50} (half maximal response) of 5 nM, and in the parasitic nematode *Haemonchus contortus* (Geary *et al*. 1993) an EC_{50} of between 1 and 10 pM. *In vitro* studies on the hookworms, *Necator americanus* and *Ancylostoma ceylanicum* have shown ivermectin to inhibit pharyngeal pumping and thus ingestion (Richards, Behnke & Duce, 1995). A more recent study by Martin (1996), investigated the action of the avermectin analogue, milbemycin D, with results demonstrating that the pharyngeal muscle of *Ascaris* possesses glutamate receptors which are sensitive to milbemycin.

These observations suggest that ivermectin may have a site of action within the enteric nervous system that controls pharyngeal muscle activity. Innervation of the pharynx is complex, involving cholinergic, serotoninergic and peptidergic nerve elements within the enteric nervous system (ENS) (Brownlee *et al*. 1994, 1996). In the present study the effects of ivermectin, GABA and glutamate, and their resulting interactions on pharyngeal pumping behaviour in *Ascaris*, have been investigated.

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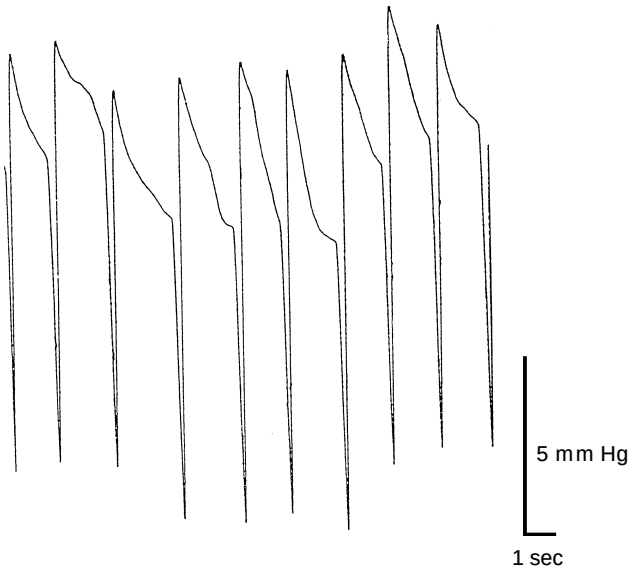

Fig. 1. Recording showing pharyngeal pumping behaviour in *Ascaris* induced by the presence of 100 μM 5-HT. The downward deflection indicates contraction of the pharyngeal muscle, the upward deflection indicates relaxation (power stroke).

MATERIALS AND METHODS

Mature adult specimens of *Ascaris suum* were obtained from pigs slaughtered at a local abattoir and maintained in the laboratory for up to 5 days in artificial perienteric fluid (APF, composition in mM: NaCl 67, NaAcetate 67, $CaCl_2$ 3, $MgCl_2$ 15·7, KCl 3, Tris 5, pH 7·6 with glacial acetic acid) containing 3 mM glucose at 37 °C.

Preparation

Preparation of the pharynx for measurement of intrapharyngeal pressure was via microdissection of the head region in normal APF. The anterior 2 cm of the worm was cut along the left lateral line and pinned cuticle side down in a Sylgard-lined Perspex chamber. The intestine (attached to the pharynx) was carefully teased from the surrounding ventral muscle field in order to ensure that the circumpharyngeal anterior nerve ring remained intact. The pharyngeal preparation was then transferred to a Sylgard-lined perfusion chamber containing modified APF (3 mM $MgCl_2$) at 37 °C. Subsequently, the head region was pinned cuticle side down and a piece of saline-filled Portex tubing (outer diameter, 1 mm) was inserted into the intestine and carefully moved anteriorly until a position adjacent to the pharyngeal-intestinal valve at the most distal end of the pharynx had been reached. It was secured in place by tightening a thread (Mersilk, gauge 4·0 braided silk, Ethicon, UK) around the tubing contained within the intestinal portion of the preparation, and connected to a Micron miniature pressure transducer (0–400 mmHg at full sensitivity, MP-15; Linton Instrumentation, Norfolk, UK). The entire system was filled with modified APF which had been boiled for 5 min to minimize the formation of air bubbles, thus enabling pressure changes associated with the pharynx to be transduced more efficiently. Drugs and the perfusate were directed at the pharynx, and drug solutions were separated from the perfusate by air bubbles. This method of application allows rapid and complete change of the composition of the perfusion stream over the pharynx. Studies with dye indicated that drugs would be evenly distributed in the bath within 1 min and cleared after a further 70 sec ($n = 8$); this being independent of concentration. The volume of the bath was 3·5 ml, perfusion rate of the experimental chamber was set at a constant 7 ml/min and the temperature was monitored throughout the experiment.

Drugs

Serotonin (5-hydroxytryptamine, (5-HT), creatine sulphate complex; Sigma Chemical Company Ltd, Poole, Dorset, UK), in the concentration range of 1–1000 μM was added to the perfusate in order to stimulate pharyngeal pumping. All drugs were applied to the preparation by addition to the perfusate. Acetylcholine (ACh), GABA, glutamate (L-glutamic acid, monoammonium salt) and picrotoxin were obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK). Each drug concentration was diluted in APF (containing 100 μM 5-HT) and applied for 1·5 min in order to test for bioactivity on pharyngeal pumping. Ivermectin was a gift from Merck Sharp & Dohme (Rahway, USA). The vehicle for ivermectin was 1% dimethyl sulfoxide and control experiments indicated that the vehicle had no direct effect on the pharynx or pharyngeal pumping behaviour. The peptide, KSAYMRFamide (AF8), was supplied by Alta Bioscience Ltd, Birmingham, UK.

Method

A hard copy of the results was obtained on a Polygraph 2000-506 series recording system (Linton Instrumentation, Norfolk, UK). Concentration–response relationships were determined for glutamate, GABA and ivermectin. Initially, a range of drug concentrations was added to enable the elucidation of subthreshold and threshold responses for glutamate, GABA and ACh. The effect of co-application of a subthreshold concentration of glutamate and ivermectin, GABA and ivermectin, ACh and ivermectin and the peptide KSAYMRFamide and ivermectin on pharyngeal pumping was monitored. For these experiments 1 pM ivermectin was applied to the preparation 15 min prior to addition of the subthreshold concentrations of glutamate, GABA, ACh and the peptide AF8.

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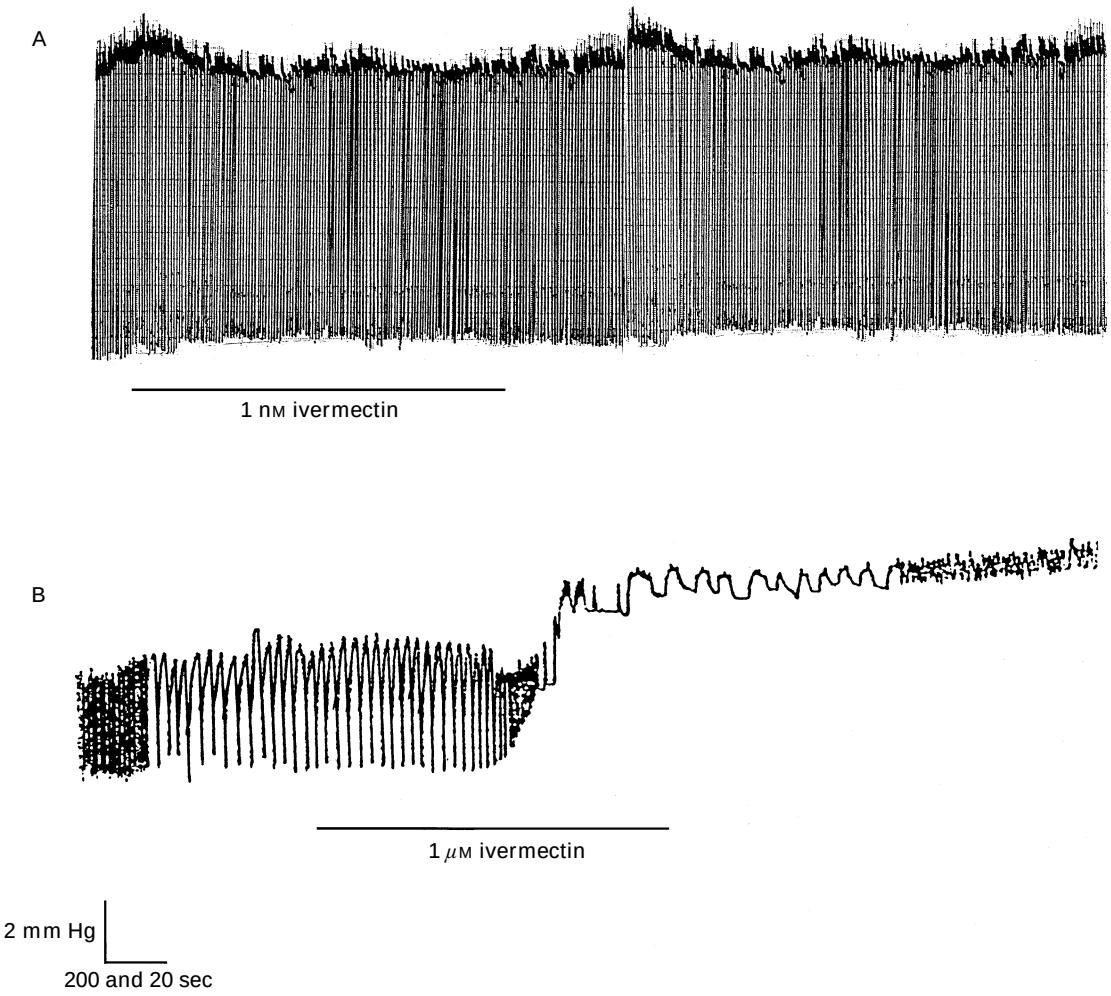

Fig. 2. Recording showing the effect of 1 nM (A) and 1 μM (B) ivermectin on pharyngeal pumping behaviour in *Ascaris* (different preparations). Horizontal line indicates duration of ivermectin application.

Perfusion of picrotoxin (100 μM–1 mM), an antagonist of $GABA_A$ receptors, for 10 min prior to addition of GABA and glutamate was also carried out to investigate the receptor profile. For a detailed discussion on the pharyngeal preparation technique used to study the pumping activity of *Ascaris*, see Brownlee *et al*. (1995).

Analysis

Pharyngeal pumping behaviour is complex and consists of a number of components, for example, frequency, amplitude, rate and change of average pressure, etc. In the present study it was decided to investigate the inhibition of pharyngeal function, whereby inhibition was classified as the complete cessation or near-complete cessation (small amplitude changes; 10% of total amplitude) of pharyngeal pumping. This cessation in pumping can be anywhere in the range between relaxed to contracted state of the pharyngeal muscle. It should be noted that to date no drug has been found to affect one of the variables of pharyngeal pumping and not eventually (at higher concentrations) inhibit pharyngeal pumping as defined above. Duration of inhibition was measured from the onset of inhibition until recovery of pharyngeal pumping.

Results are expressed as the mean ± S.E. mean. Concentration–response curves were fitted to the logistic equation using non-linear regression analysis (Graph Pad Prism, version 2, GraphPad Software Inc. San Diego, CA 92121, USA) and EC_{50} values are given with 95% confidence limits. Statistical significance was tested using the unpaired two-tailed Student's *t*-test.

Controls

Controls in which APF containing 1% dimethyl sulfoxide when added to the preparation had no effect on pharyngeal pumping behaviour.

RESULTS

The pharyngeal muscle of *Ascaris* did not contract spontaneously, but 5-HT (10–1000 μM) stimulated rhythmic contractions of the pharynx (Brownlee *et al*. 1995). In all experiments, 5-HT was added to the

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perfusate at a concentration of 100 μM so as to maintain pharyngeal pumping. The pumping behaviour consisted of a series of regular rhythmic contractions, which were characterized by a general increase in muscle tone followed by a spontaneous contraction and relaxation (Fig. 1). This relaxation is the 'power stroke', forcing the contents from the pharyngeal lumen via the pharyngeal–intestinal valve into the intestine. The contractions had a frequency of $0{\cdot}5 \pm 0{\cdot}03$ Hz ($n = 6$) and an amplitude in the range 5–15 mmHg.

Ivermectin effects

Ivermectin alone did not inhibit pharyngeal pumping behaviour at low concentrations, 1 pM–1 nM (see Fig. 2A) over a 30–45 min period. However, it should be noted that 1 pM ivermectin on occasion did reduce amplitude of the pumping behaviour over the time-course of the experiment, but this concentration never induced inhibition. At higher concentrations (1–500 nM), pharyngeal pumping was inhibited for periods of up to 60 min. This inhibition was characterized by the pharynx exhibiting irregular, small amplitude pumping in a hyper-relaxed state. At higher concentrations (1 μM) of the drug, this small amplitude pumping was abolished in the hyper-relaxed state (see Fig. 2B). The onset of ivermectin effects (inhibition) are concentration dependent ($n = 12$), that is, the higher the concentration the sooner the response from the pharynx is observed (for example; at 1 μM = 60 sec; 10 μM = 30 sec). These effects are irreversible and remain for the time-course of the experiment (3–4 h) unless lower concentrations of ivermectin are used (1–10 pM). It should be noted that not all preparations responded by inhibition of pharyngeal pumping to 500 nM ivermectin. For example, of the 53 different preparations used in this study, 9 of them (approximately 17 %) did not exhibit any effect on pharyngeal behaviour at this concentration of ivermectin.

GABA and glutamate effects

GABA caused an inhibition of pharyngeal pumping in the concentration range 1–1000 μM. The duration of inhibition was concentration dependent ($n = 7$, Fig. 3) and up to periods of 6 min. This reversible inhibition of pharyngeal pumping behaviour was associated with the pharyngeal muscle remaining in a contracted state. The estimated EC_{50} was 23 μM (95 % confidence limits, 9·8–55·3 μM, $n = 6$).

The effect of glutamate was not as potent as that of GABA. Both the GABA and glutamate concentration–response curves (or individual results for each concentration (10–100 μM) were significantly different ($P < 0{\cdot}0001$, $n = 6$; unpaired Student's *t*-test). Inhibition of pharyngeal pumping by glutamate was in the 10–1000 μM range ($n = 6$, Fig. 3), with an estimated EC_{50} of 491 μM (95 % confidence limits, 59·3–4063·0 μM, $n = 6$). The duration of this inhibition (in the contracted state) was not as long as that induced by GABA on the pharynx which caused the muscle to remain in a contracted state for periods up to 3 min. The longer term effect of glutamate caused irregularity of pumping behaviour in terms of both frequency and amplitude over the time-course of the experiment.

Interaction of GABA and glutamate with ivermectin

When subthreshold concentrations of GABA (0·1 or 1 μM) (Fig. 4A–C) and glutamate (1 μM) (Fig. 4D) were added to the preparation there was no inhibition, though a slight reduction in amplitude of pharyngeal pumping was apparent. However, following perfusion with 1 pM ivermectin and then addition of the same subthreshold concentration of amino acid, a clear inhibitory response to GABA (0·1 μM, $n = 6$; Fig. 4A–C); and glutamate (1 μM, $n = 6$; Fig. 4D) was obtained. This inhibition was irreversible, with the inhibition remaining throughout the time-course of the experiment. However, it should be noted that the potentiation of the GABA inhibitory effect was occasionally reversible upon the first application of GABA following the onset of perfusion with 1 pM ivermectin (see Fig. 4C). Even in the case of Fig. 4C, the second application of GABA caused irreversible inhibition of pharyngeal pumping. This indicates that there is a time-dependent effect on potentiation of the irreversible GABA inhibition. Ivermectin had no observable effect on the ACh or peptide (KSAYMRFamide) responses associated with the pharynx (not shown).

Perfusion of 100 μM picrotoxin had no effect (potentiation or reduction) on either the GABA (10 μM) or glutamate (100 μM) response (not shown). However, 500 μM picrotoxin partially blocked the response to glutamate (reduced the amplitude of pumping) (Fig. 5A; $n = 6$) and reduced the GABA induced effect (duration of inhibition). Application of 1 mM picrotoxin blocked the GABA response (Fig. 5B; $n = 6$); but had no additional effect on the glutamate induced response (reduction in amplitude).

DISCUSSION

In this study, changes in intrapharyngeal pressure in *Ascaris* were used to monitor the frequency and amplitude of pharyngeal pumping. The frequency and form of these pressure changes closely match the pattern and frequency of the *Ascaris* pharyngeal muscle action potentials as recorded by Del Castillo *et al.* (1964*a*). The fast relaxation shown in Fig. 1 is

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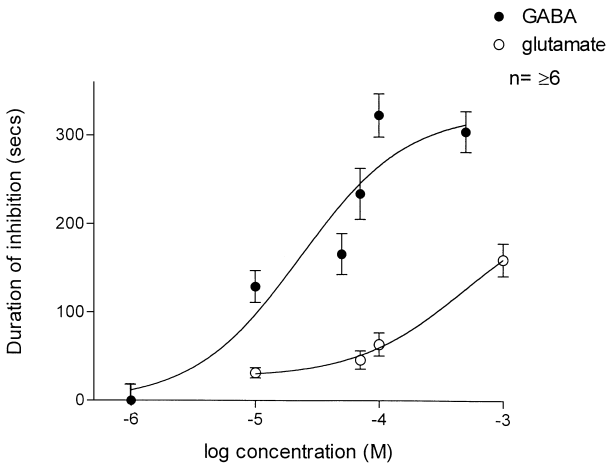

Fig. 3. Dose–response curve showing the effect of increasing GABA and glutamate concentration (μM) on the duration of pharyngeal pumping inhibition in *Ascaris* ($n = 6$). EC_{50} GABA is 23 μM (95% confidence limits, 9·8–55·3 μM). EC_{50} glutamate is 491 μM (95% confidence limits, 59·3–4063·0 μM).

likely to be the functional consequence of the fast negative potential recorded by Del Castillo & Morales (1967), and as such would be caused by the rapidly activating and inactivating potassium channel described by Byerly & Masuda (1979). The transducer measures pharyngeal pressure changes and not somatic muscle responses to either 5-HT, GABA, glutamate, ivermectin or any of the drugs. The ability to monitor intrapharyngeal pressure changes, and thus pumping behaviour, has enabled the initiation of studies to determine the role of ivermectin and other anthelmintics in modifying this nematode behaviour. For a discussion of the role of serotonin (5-HT) in pharyngeal function of nematodes see Brownlee *et al*. (1995).

Studies to date have suggested that GABA is an inhibitory neurotransmitter in nematodes (Del Castillo, De Mello & Morales, 1964*b*, *c*; Holden-Dye & Walker, 1990). Immunocytochemical localization studies have shown GABA-like immunoreactivity (IR) to be present within inhibitory motoneurons of *Ascaris* and *C. elegans* (Johnston & Stretton, 1987; McIntire *et al*. 1993). Physiological studies have shown that nematode somatic muscle relaxes when GABA is applied and this is associated with an increase in chloride conductance of muscle cells (Holden-Dye & Walker, 1990). The evidence for glutamate acting as a neurotransmitter in nematodes is poor. However, biochemical studies have shown that there are membrane-associated binding sites for glutamate in *C. elegans* and *H. contortus* (Schaeffer & Haines, 1989; Rohrer, Evans & Bergstrom, 1990; Schaeffer *et al*. 1990; Cully & Paress, 1991; Rohrer *et al*. 1994) whereas electrophysiological studies in *Ascaris* reveal a glutamate-gated chloride channel (Martin, 1996). Further evidence for a glutamatergic system comes from the isolation of 2 cDNAs from *C. elegans* which encode a glutamate-gated chloride channel which is sensitive to ivermectin (Cully *et al*. 1994).

Both GABA and glutamate cause an inhibition of 5-HT stimulated pharyngeal pumping. The duration of this inhibition was concentration dependent with GABA more potent than glutamate. Ivermectin potentiated both the GABA and glutamate responses suggesting that ivermectin is very likely to be acting via a ligand-gated channel. This is similar to the mode of action in other invertebrates where it has been shown to act through GABA-, glutamate- or glycine-gated channels (Rohrer & Arena, 1995). However, in the nematode *C. elegans*, it has been reported that the site of action for ivermectin is through a glutamate-gated chloride channel, and evidence for this comes from expression studies in oocytes (Cully *et al*. 1994). It has been suggested that the avermectins may act to modify more than 1 type of ion channel, e.g. both chloride and calcium channels. To date there is relatively little evidence for a role for glutamate in the *Ascaris* pharynx and little evidence elsewhere in nematodes for glutamate as a neurotransmitter (see above). Electrophysiological studies have revealed a chloride-dependent glutamate response in *Ascaris* pharynx (Martin, 1996), whereas the current study shows that glutamate has an inhibitory effect on the pharynx which is potentiated by ivermectin.

In the somatic musculature of *Ascaris*, ivermectin inhibits GABA responses with micromolar potency (Holden-Dye & Walker, 1990) thus suggesting a mode of action through a GABA-gated chloride channel. The pharyngeal muscle of nematodes appears far more sensitive to the effects of ivermectin than the somatic musculature. In this study, picomolar concentrations of ivermectin, have effects on pharyngeal pumping, not directly, but via GABA and glutamate responses. In the parasitic nematode *Haemonchus contortus* (Geary *et al*. 1993) and the free-living nematode *C. elegans* (Avery & Horvitz, 1990) concentrations of ivermectin in the nanomolar range were effective at inhibiting pharyngeal pumping. Uptake of ivermectin may be through the mouth and pharynx during feeding behaviour, but a recent study provides evidence for uptake of the drug also across the cuticle (Smith & Campbell, 1996).

It is also interesting that, in this study, ivermectin had different apparent effects depending on the concentration used. At low concentrations (pM), there was no observable direct effect (indirect effect mediated by the potentiation of the GABA and glutamate responses) on the pharyngeal muscle and pumping behaviour, whereas at concentrations of 100–1000 mM, direct inhibitory effects which lasted the duration of the experiment were evident. In *C. elegans*, low concentrations of ivermectin cause potentiation of the glutamate response in the

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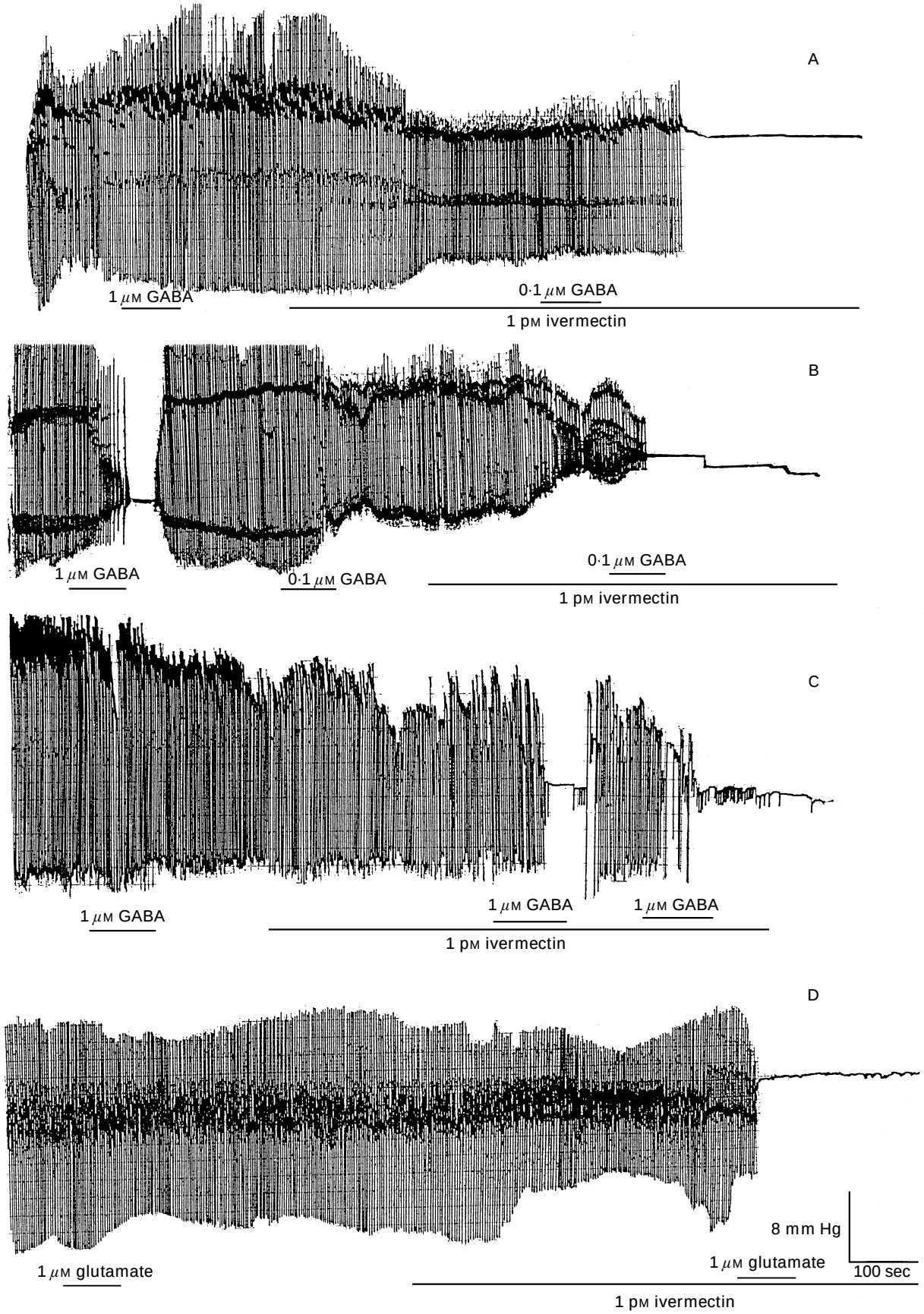

Fig. 4. Effect of ivermectin (1 pM) on the potentiation of GABA and glutamate-mediated responses on inhibition of pharyngeal pumping behaviour in *Ascaris*. (A–C) GABA; (D) glutamate. (A) Addition of 1 μM GABA prior to perfusion with ivermectin, and then addition of 0·1 μM GABA. (B) Addition of 0·1 and 1 μM GABA prior to perfusion with ivermectin, and then addition of 0·1 μM GABA. (C) Addition of 1 μM GABA prior to perfusion with ivermectin,

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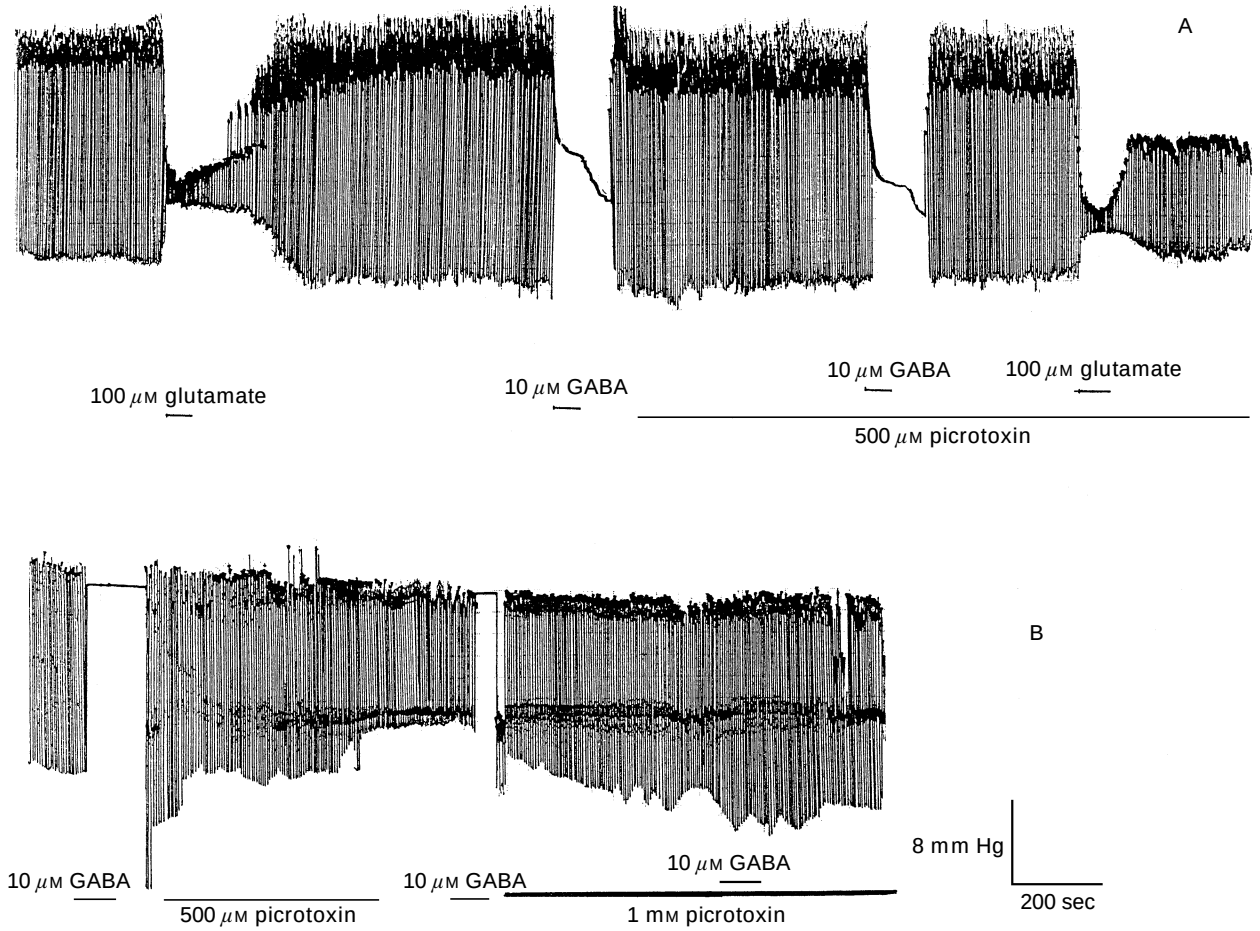

Fig. 5. Trace showing the effect of 500 μM and 1 mM picrotoxin on GABA and glutamate induced effects on pharyngeal pumping in *Ascaris*. (A) Addition of 100 μM glutamate and 10 μM GABA before and during application of 500 μM picrotoxin. (B) Addition of 10 μM GABA before and after application of 500 μM and 1 mM picrotoxin. Long horizontal bar indicates duration of picrotoxin application. Short horizontal bar indicates duration of GABA/glutamate application.

glutamate-gated chloride channel (EC_{50}, 90 nM), whereas at higher concentrations ivermectin acts by irreversibly opening this channel (Arena *et al*. 1992; Cully *et al*. 1994). In the present study at higher concentrations of ivermectin, the pharynx remains in a hyper-relaxed state, whilst at lower concentrations of ivermectin there is potentiation of the glutamate and GABA responses. This suggests that ivermectin may inhibit *Ascaris* pharyngeal muscle via either a glutamate or GABA chloride channel in the pharynx. The location of these receptors within the enteric nervous system and the pharyngeal muscle remains to be established.

There are 2 possible explanations for the observation that ivermectin potentiates the inhibitory action of both GABA and glutamate. The first assumes that the molecular target for ivermectin is the α subunit of a nematode glutamate-gated chloride channel (as identified in *C. elegans*) and that this receptor for ivermectin is expressed on *Ascaris* pharyngeal muscle. Ivermectin would therefore potentiate the inhibitory action of glutamate on pharyngeal pumping. The effect of ivermectin on the GABA response may also be explained by an interaction at the glutamate-gated chloride channel. This would be the case if the GABA receptor is located presynaptically to the glutamate receptor in the neural circuit controlling pharyngeal pumping. An alternative explanation would be that ivermectin is able to interact, at a molecular level, with both glutamate and GABA receptors that control pharyngeal pumping.

The current evidence shows that the pharynx is extremely sensitive to the anthelmintic ivermectin, much more so than the somatic musculature. The pharyngeal muscle exhibits responses to picomolar concentrations of ivermectin by potentiation of both GABA and glutamate responses. The greater sen-

and then addition of 1 μM GABA after perfusion with ivermectin. (D) Addition of 1 μM glutamate prior to perfusion with ivermectin, and then addition of 1 μM glutamate. Long horizontal bar indicates duration of ivermectin application. Short horizontal bar indicates duration of GABA/glutamate application.

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sitivity of this muscle to ivermectin and a range of transmitters, including endogenous FMRFamide-related peptides (FaRPs) (Brownlee *et al*. 1995) suggests that it may be a novel target for chemotherapeutic control strategies. The irreversible nature of pharyngeal inhibition is consistent with the slow rate of dissociation for ivermectin observed in binding studies (Schaeffer, 1989; Cully, 1991).

In summary, this paper describes a method, originally used to investigate the effect of serotonin and nematode FaRPs (Brownlee *et al*. 1995), to investigate the role of ivermectin in the pharmacology of the pharyngeal muscle of *Ascaris in vitro*. Such studies will yield information on the role of neurotransmitters in the nematode ENS. The pharynx is particularly suitable for these studies as the perfused neurotransmitters have direct access to the pharyngeal muscle basal membrane. Studies have shown that ivermectin has a potent effect on pharyngeal pumping and potentiates both GABA and glutamate effects on the pharynx. This study also indicates that the pharynx of *Ascaris* and the modulation of pharyngeal pumping behaviour by neurotransmitters and drugs (ivermectin) can be quantified using this approach (Brownlee *et al*. 1995). The method described in this study is a suitable preparation for the exploitation of the nematode pharynx as an assay for the effect of drugs in nematodes. The pharmacology of the nematode enteric nervous system has been a much neglected area, and it is important that this is now addressed as the control of pharyngeal function is vital for nematode survival.

This investigation was supported by a Medical Research Council (MRC) Training Fellowship to David J. A. Brownlee. The financial support of the Wessex Medical Trust is also acknowledged.

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