The time-course of the response to the FMRFamide-related peptide PF4 in *Ascaris suum* muscle cells indicates direct gating of a chloride ion-channel

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SUMMARY

We investigated the effects of PF4 on *Ascaris suum* somatic muscle cells using a 2 electrode current-clamp technique. PF4 is a FaRP (FMRFamide-related peptide), originally isolated from the free-living nematode *Panagrellus redivivus*. PF4 caused hyperpolarization and an increase in chloride ion conductance when it was applied to the muscle cells of the *Ascaris* body wall. The delay between the application of the peptide and the appearance of the response was measured and compared with that of gamma-amino butyric acid (GABA), a compound that directly gates ion channels, and with PF1, a FaRP that acts via an intracellular signal transduction mechanism. The PF4 and GABA delay times were not significantly different; they were $1{\cdot}51 \pm 0{\cdot}11$ sec and $1{\cdot}22 \pm 0{\cdot}10$ sec respectively. The delay following application of PF1, $3{\cdot}75 \pm 0{\cdot}51$ sec, was significantly longer. The rapid response to PF4 is consistent with direct gating of a chloride ion channel, which has not been described elsewhere in the literature.

Key words: *Ascaris suum*, FMRFamide-related peptides, PF4, chloride channels, current clamp.

INTRODUCTION

Parasitic nematodes are a major cause of human morbidity and mortality (World Health Organization, 1998) and are economically important pathogens of livestock and crops. Drug-resistant strains of nematode have developed to all the classes of nematocidal drugs that are in common use (Waller, 1994), resulting in a need for new compounds that have novel pharmacological actions. The search for suitable drug targets in parasitic nematodes has focused on aspects of nematode biology that differ from their hosts. Receptors for the FMRFamide-related peptides, or FaRPs, represent a potential target for nematocide discovery (Geary *et al*. 1999). These neuroactive peptides are widespread in free-living and parasitic nematodes but are structurally distinct from their closest homologues in vertebrates. A total of 59 FaRPs are encoded on the *Caenorhabditis elegans* genome, of which 13 have been isolated biochemically from *C. elegans* (Li *et al*. 1999 for a review). FaRPs have also been extracted from other nematode species, including 19 from *Ascaris suum* (Davis & Stretton, 1996). Of the FaRPs tested to date most have marked effects on *A. suum* somatic muscle when tested on isolated tissue preparations (Brownlee & Fairweather, 1999). The FaRPs used in these experiments had been extracted from both free-living and parasitic nematodes, which indicates some level of conservation of FaRP receptor functionality between nematode species. Their distribution and physiological activity across several nematode species increases the appeal of the FaRPs as targets for anthelmintic discovery and development.

FaRPs have been localized to neurones by immunocytochemical studies (Brownlee, Fairweather & Johnston, 1993; Brownlee *et al*. 1993; Fellowes *et al*. 1999; Sithigorngul, Stretton & Cowden, 1990): it is clear that they have a neurotransmitter and/or a neuromodulatory function. Most of the FaRPs have either excitatory or inhibitory effects on *A. suum* muscle; however, a few show more complex biphasic effects. In general, the exact mechanisms through which the nematode neuropeptides act are not understood. One exception is PF1, the first FaRP to be extracted from the free-living nematode *Panagrellus redivivus*. PF1 is thought to act via intracellular nitric oxide release, which appears to activate a potassium channel in *A. suum* muscle membrane, thereby hyperpolarizing and relaxing the muscle (Bowman *et al*. 1995). There has been some investigation into the mechanism of action of FaRPs from other organisms. FMRFamide, the first FaRP to be isolated and characterized, has been shown to directly gate 2 sodium ion-channels in the snail, *Helix aspersa* (Green, Falconer & Cottrell, 1994). FMRFamide has also been shown to act via a G-protein coupled system in another snail, *Helisoma trivolvis*

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(Haydon *et al*. 1991). LyCEP, a FaRP isolated from *Lymnaea stagnalis*, also acts via a G-protein in *L. stagnalis* neurones (Tensen *et al*. 1998). Thus, there is precedent for a variety of modes of action among the FaRPs. If nematode neuropeptides (or their receptors) are to be considered as targets for anthelmintics, it would be useful to gain a better understanding of their biology. The aim of the present study was to elucidate the mechanisms of action of PF4, the fourth FaRP to be isolated from *P. redivivus*. It is known to have an inhibitory effect on *A. suum* somatic muscle, where its actions are highly dependent on the concentration of chloride ions in media bathing the muscle tissue (Holden-Dye, Brownlee & Walker, 1997; Maule *et al*. 1995). *A. suum* was selected as the experimental model in this study due to its large size, hence suitability for electrophysiological experiments, and because of the data available from previous studies showing that muscle cells from this parasite are highly sensitive to PF4.

Two-electrode current-clamp experiments were carried out to record the membrane potential and conductance response of *A. suum* somatic muscle cells to PF4. We compared the PF4 response with responses to gamma-amino butyric acid, a neurotransmitter that directly-gates chloride ion channels, and to PF1, a FaRP shown to act via intracellular transduction mechanisms. The primary objective of the work described here was to determine the relative speed of the response to PF4 in order to help determine the mechanism linking the PF4 receptor with its effectors.

MATERIALS AND METHODS

A. suum, obtained from slaughtered pigs, were maintained at 25 °C in Locke's solution that was changed twice daily (composition in mM: NaCl 154; $CaCl_2$ 2·1; KCl 5·6; $NaHCO_3$ 1·8; glucose 5·6). In order to apply compounds to the somatic muscle cells, the tissue was prepared as follows. A 1 cm long section was excised from the anterior third of the worm and was cut along one lateral line. The flap of tissue was pinned out, cuticle side down, in a Sylgard-lined recording chamber of volume 2 cm^3. The gut was removed revealing the bag regions of the somatic muscle cells.

The recording chamber was maintained at 32–34 °C and the flap preparation was constantly perfused with *Ascaris* Ringer solution at 5 ml/min (ARS, composition in mM: NaCl 135; KCl 3·4; $CaCl_2$ 3; $MgCl_2$ 15·7; glucose 3; Tris 5; pH was adjusted with maleic acid).

The target cell was impaled with 2 borosilicate glass (type GC120-15, Garner Glass, USA) micropipettes of resistance 20–40 MΩ, filled with 2 M Kacetate (Fig. 1). PF4 and PF1 (10 μM) and gamma-amino butyric acid (GABA, 100 μM) were prepared using ARS. Compounds were pressure-ejected onto the target cell through a fine-bore plastic tube positioned $\leqslant 50$ μm from the cell surface (Neurophore BH-2 system, Medical Systems Corporation, New York). Air pressure (2 kg/cm^2) was provided by a modified hand pump (Hozelock 4036, UK). In those experiments in which 2 compounds were compared by applying them to the same target cell, PF4 was applied before or after either GABA or PF1. To ensure $> 98\,\%$ wash-out of the compounds, 5 min were allowed to elapse between application of different compounds.

The pressure ejection was recorded on tape as a square wave along with the membrane potential responses. There was a measurable delay between the start of the square wave marking the pressure ejection and the expulsion of solution from the delivery tube. This 'Neurophore switching delay' was quantified by measuring the volume output into mineral oil for ejection pulses of varying duration, plotting volume against pulse duration and determining, by linear extrapolation, the x-intercept to obtain the lag time due to switching delay.

Intracellular recordings were made using the Axopatch-2A amplifier (Axon Instruments, Foster City, USA). Experiments were recorded on videotape (Betamax hifi, modified to record DC potentials from 0–10 KHz) and were filtered and digitized to facilitate curve fitting (Digidata 1200 series interface, Axon Instruments, USA).

PF4 and PF1 were gifts from Pharmacia Corporation, Kalamazoo, USA. All other chemicals were obtained from Sigma-Aldrich Company, Ltd, Poole, UK.

Results are expressed as mean ± standard error of the mean, unless stated otherwise, and significance was tested using analysis of variance (ANOVA). In order to quantify the hyperpolarization response to each of the compounds, first-order exponential curves were fitted to the experimental data using Origin 4.0 (Microcal Software Inc., Northampton, USA). Curve fitting provided a repeatable method of measuring the various parameters of the hyperpolarization: T_0, t_1, V_0, A_1 (Equation 1 and Fig. 2). Equation 1 was used to derive the start of the hyperpolarization, T. This proved to be a more accurate method of determining the beginning of the response than estimating it by eye, since pressure ejection artefacts sometimes occurred, obscuring the initial phase of the hyperpolarization. To ascertain the delay between the start of the pressure ejection of a compound and the start of the response, the time at which application began was subtracted from the calculated value T.

Equation 1: exponential decay arranged in terms of the start of the hyperpolarization response.

$$T = T_0 - t_1 \cdot \ln\left[\frac{V - V_0}{A_1}\right],$$

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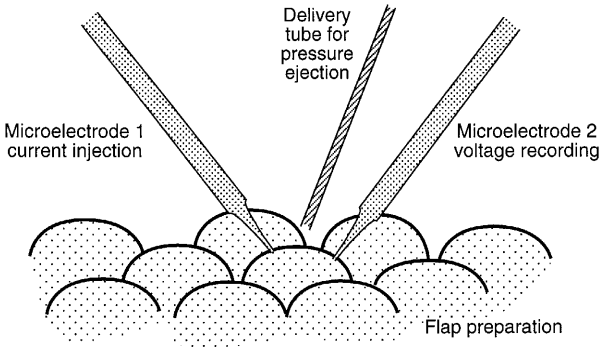

Fig. 1. *Ascaris suum* muscle flap preparation placed cuticle-side down in the recording chamber. The narrow plastic delivery tube is positioned above the target cell, 50 μm from the cell surface.

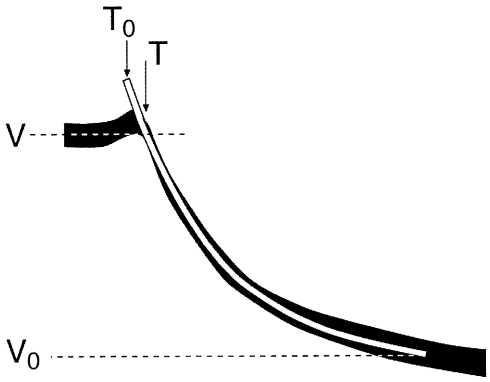

Fig. 2. An illustration of the terms used in Equation 1. The black line represents an experimental trace showing membrane hyperpolarization, the superimposed white line is the fitted exponential curve.

where T is the start of the response in sec, T_0 is the x offset (the minimum value for x) in sec, t_1 is the decay constant of the curve in sec, V is the resting membrane potential in volts, V_0 is the y offset (the most negative value of y) in volts, A_1 is the amplitude in volts. The terms of Equation 1 are illustrated in Fig. 2.

To further investigate the delay between ejection of the compounds and the response, their diffusion rates were also investigated. Diffusion calculations (Equation 2) were carried out using a computer spreadsheet (Microsoft Excel 97, Microsoft Corp., USA).

RESULTS

Conductance

A representative trace from a current-clamped muscle cell is shown in Fig. 3A. The resting membrane potential of the *A. suum* somatic muscle cells was found to be $-31{\cdot}9 \pm 0{\cdot}9$ mV ($n = 72$), which is in line with the findings of other workers (Martin, 1980: -31 ± 1 mV; Holden-Dye *et al.* 1997: $-31{\cdot}4 \pm 0{\cdot}4$ mV). We determined that the input conductance of resting muscle cells was $2{\cdot}27 \pm 0{\cdot}05$ μS ($n = 102$). Previous studies obtained similar values for the input conductance (Martin, 1980: $2{\cdot}4 \pm 0{\cdot}2$ μS; Holden-Dye *et al.* 1997: $1{\cdot}79 \pm 0{\cdot}06$ μS).

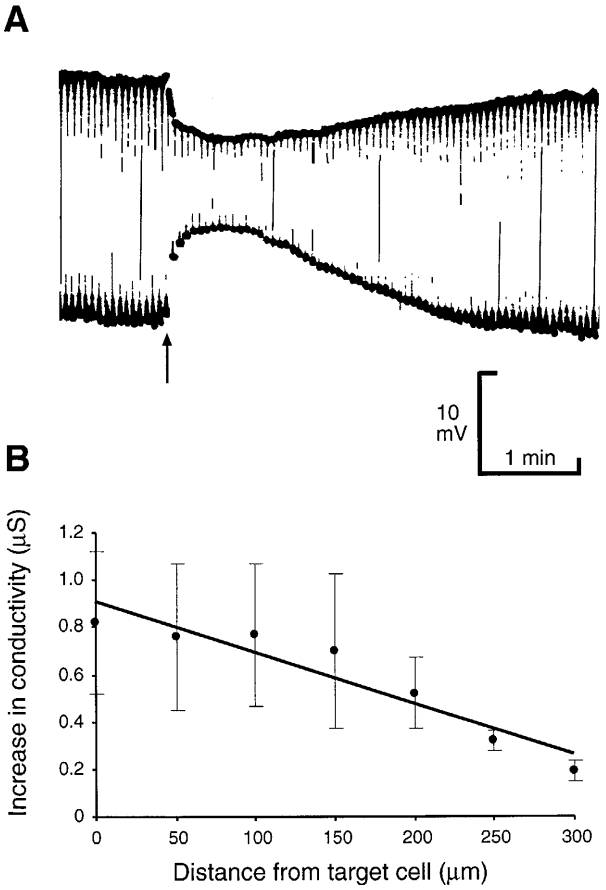

Fig. 3. (A) Recording of the membrane potential and input conductance of an *Ascaris* muscle cell. The upper edge of the trace is the membrane potential of the cell, the downward deflections (initially 25 mV) are due to injections of current into the cell which cause corresponding hyperpolarization. The width of the trace is inversely proportional to the electrical conductance of the membrane. Prior to application of PF4 (arrow) the resting potential was -27 mV and the conductance was $1{\cdot}6$ μS. The effect of PF4 was to hyperpolarize the cell to -33 mV and to increase the conductance to $4{\cdot}7$ μS. (B) Response of the target cell (~ 150 μm in diameter) plotted against the horizontal distance between the delivery tube and the cell. Mean responses are shown and the error bars indicate the standard error of the mean ($n = 5$). The line was fitted by linear regression.

Input conductance increased following a 20 sec application of 10 μM PF4 to $3{\cdot}45 \pm 0{\cdot}68$ μS, which compares with published data (Holden-Dye *et al.* 1997: 5 ± 1 μS). As the delivery tube was moved laterally from the target cell, the change in conductance decreased (Fig. 3B), indicating that the receptors responding to PF4 were present on the bag region of the muscle cell. The response fell by 50 %

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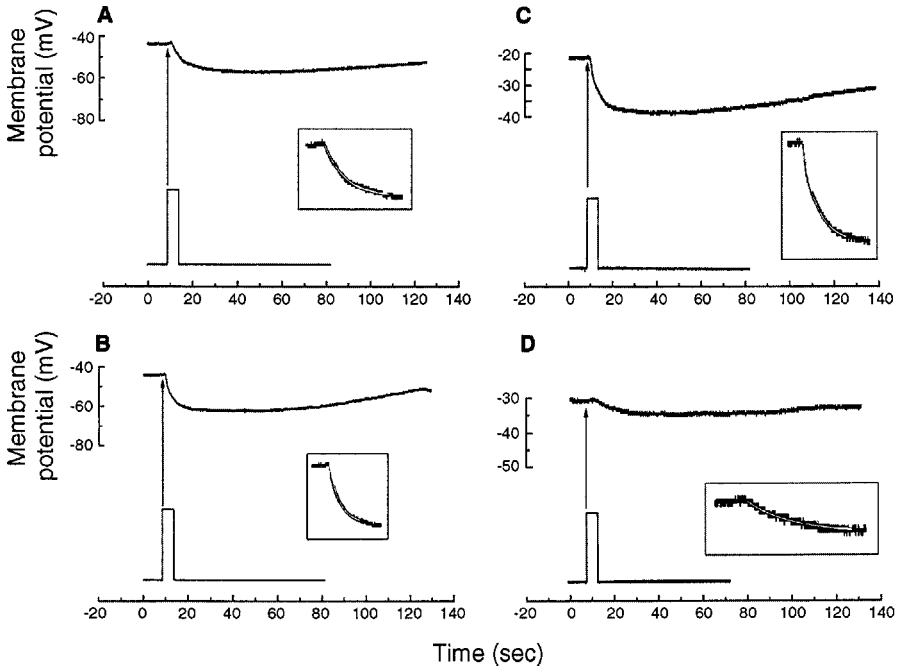

Fig. 4. Cell membrane potential plotted against time in each of 4 representative experiments. In each case, the rectangular pulse indicates the time-course of the pressure pulse for compound application and the arrow shows the start of application. The single exponential curve fitted to each hyperpolarization response is shown as a white line superimposed on the black trace in the inset in each graph. (A) Target cell responding to PF4; (B) GABA application to the same cell; (C) another cell responding to PF4; (D) PF1 application to the same cell.

at a distance of 225 μm from the centre of the bag region of the target cell.

Delay time

The target cells responded to PF4 in 1·5 ± 0·3 sec, a delay that was not significantly different from the response to GABA (1·2 ± 0·1 sec, ANOVA, $P > 0·1$). In contrast, there was a significantly longer delay period between the application of PF1 and the response of the target cell (3·8 ± 0·5 sec, ANOVA, $P < 0·01$). See Table 1.

Responses of typical target cells to PF4, GABA and PF1 are shown in Fig. 4. The time-course of the hyperpolarization response was quantified by fitting a first-order exponential decay curve to the record of the membrane potential. The inset boxes in Fig. 4 show the exponential curves superimposed on the hyperpolarizations. The fitted curves described the rate of hyperpolarization and allowed the onset of the response (T) to be calculated using Equation 1. By subtracting the start of the pressure ejection from T, the delay between application and response was calculated.

The hyperpolarization response to PF4 (Fig. 4A) developed rapidly; the time-constant of the curve fitted to the response was 4·3 ± 0·3 sec. Although significantly different from the time-constant of the GABA response of 3·2 ± 0·2 sec (ANOVA, $P < 0·01$), the PF4 response was similar in the swift increase in membrane conductance leading to membrane hyperpolarization. On the other hand, the response to PF1 developed slowly; the time-constant of the fitted curve was 12·0 ± 1·8 sec. Coupled to the small/negligible change in membrane conductance elicited by PF1, the lengthy time constant underlines the difference in receptor-ion channel interactions between PF1 and PF4 responses.

Table 1. Delay time and time-constant of response of *Ascaris suum* to GABA, PF4 and PF1

(The mean time ± standard error of the mean is shown for each compound. The values were derived from single exponential curves fitted to the hyperpolarization responses to each compound. The delay of action was obtained by measuring the time between application of the compound and the start of the response. $n = 57$ for GABA, 62 for PF4 and 14 for PF1.)

	GABA	PF4	PF1
Delay of action (s)	1·22 ± 0·10	1·51 ± 0·11	3·75 ± 0·51
Time-constant (t1) (s)	3·20 ± 0·23	4·31 ± 0·28	12·03 ± 1·83

Bowman *et al*. (1995) described PF1 receptors on both the muscle bag and hypodermis of *Ascaris*. The similar size of PF1 and PF4 means they have very similar diffusion coefficients. Since the position of the delivery tube was always closest to cells nearest the nerve cord and therefore a substantial distance away from the lateral lines the minimum distance for the drug to travel to reach the hypodermis is the

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thickness of the muscle cell layer (approx. 750 μm). Simple extrapolation of the line in Fig. 3B indicates that at such a distance the effect of PF1 would be negligible if any. Taken together with the compelling evidence that PF1 has a direct effect on isolated muscle bags (Martin & Valkanov, 1996) the possibility that any delay of PF1 action is due to the compound acting on the hypodermis, rather than directly on muscle, would seem extremely unlikely.

From Table 1 and Fig. 4 it can be seen that the voltage responses to PF4 and GABA have similar onsets and time-courses, consistent with the interpretation that PF4, like GABA, is a directly-gating ligand. In comparison, the voltage response to PF1, a ligand that acts via intracellular signal transduction, exhibits slower onset kinetics and progresses at a slower rate. The delay of the response and its significance is considered in the Discussion section.

DISCUSSION

Previous studies have shown that the *in vitro* effects of PF4 on *A. suum* muscle are highly dependent on the concentration of chloride ions in the medium (Holden-Dye *et al.* 1997; Maule *et al.* 1995). Although similar in appearance, the response to PF4 is distinct from that of GABA (Holden-Dye *et al.* 1997).

The present study compared the delay in ionic conductances recorded in *A. suum* muscle cells following application of PF4, GABA and PF1. Based on previous studies, each of these compounds act on receptors present on the somatic muscle cell surface in *A. suum*. As shown, PF4 acts locally. GABA (Martin, 1985) and PF1 (Bowman *et al.* 1995; Martin & Valkanov, 1996) also act on receptors in somatic muscle cell membranes. The common anatomical site of action for these compounds allowed comparison of the respective delays of action.

To rule-out the possibility that the time-constant of the cell membrane was long enough to delay the hyperpolarization response, the time-constant was measured at the resting membrane potential while depolarizing current pulses were injected into the cell. Measurement was carried out by fitting a first-order exponential curve to the trace at the point where the membrane was charging to the hyperpolarized potential. The intrinsic time-constant of the cell membrane, which describes the rate of change of the membrane potential, was 10 ms ($n = 5$). This was 2 orders of magnitude less than the delay in the response to PF4, GABA or PF1. Therefore, the hyperpolarization response to applied ligands was not significantly delayed by the intrinsic time-constant of the membrane.

Our results show that PF4 resembles GABA in terms of the speed of the membrane response to the compounds. Both PF4 and GABA elicit a similar rapid hyperpolarization. Given that GABA is a directly-gating ligand of chloride ion channels, this observation is consistent with PF4 gating an ion-channel directly. Comparison with PF1 showed that this second messenger-mediated FaRP response has a significantly longer delay of action, concordant with signal transduction taking place within the somatic muscle cells. Directly-gated ion channels open rapidly in response to the appropriate ligand. An extensively studied example from invertebrates is the glutamate-gated chloride channel in crayfish muscle. Using an electronically controlled switch, the solution bathing the crayfish muscle cell membrane was changed in < 1 ms (Franke, Hatt & Dudel, 1987). In the experiments carried out by Franke *et al.* (1987) the delay between application of glutamate and appearance of a response was shown to be 0·4–0·6 ms. The delay seen in the *Ascaris* somatic muscle cell response to GABA and PF4

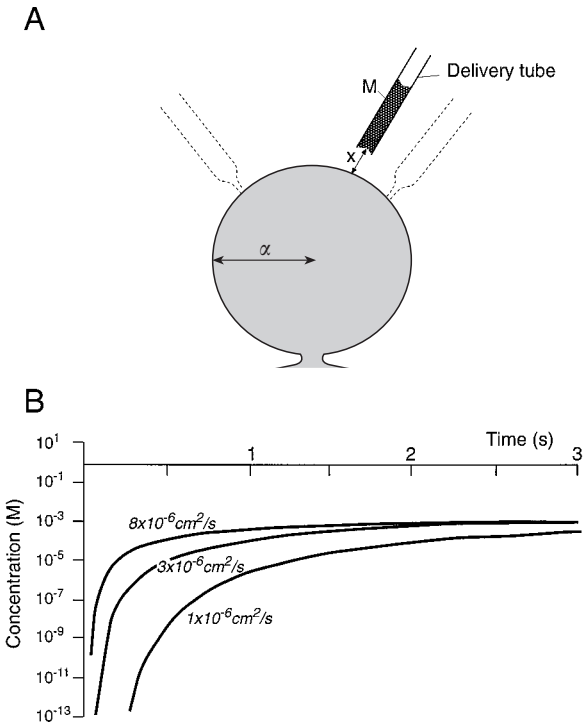

Fig. 5. (A) Terms used in Equation 2. The bag region of the somatic muscle cell (lightly shaded) is impaled by 2 micropipettes with the delivery tube positioned above the cell. The radius of the cell is α and the perpendicular distance between the end of the delivery tube and the bag surface is x. The delivery tube contains M moles of solution (densely shaded). (B) Effect of the diffusion coefficient on ligand concentration at the receptors. Cumulative concentration is plotted against time according to Equation 2. The cell is assumed to be 150 μm in diameter, the tube is positioned 50 μm above the cell, the amount of ligand delivered is 1 pmole. The lines are labelled with the relevant diffusion coefficient (D). Maximum concentration is achieved very rapidly when the value of D is low, much more slowly when it is high.

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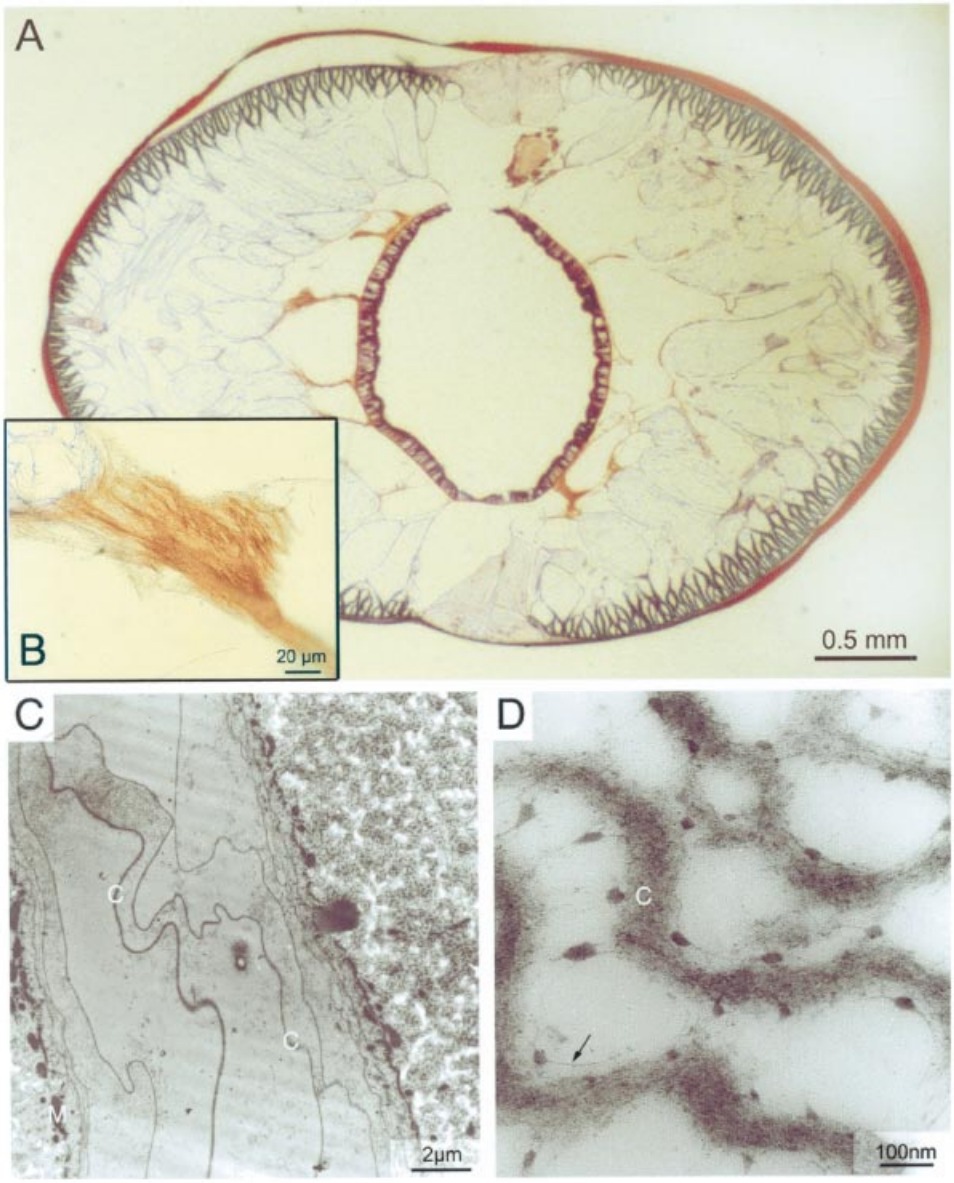

Fig. 6. Transverse section of *Ascaris suum* stained with Mallory's phosphotungstic acid-haemotoxylin to demonstrate collagen. Collagen is stained red, muscle fibres are blue (Everson Pearse, 1985). (A) Cross-section of the whole worm, showing that the cuticle and the interstitial spaces contain collagen. (B) Fibrous structure of the collagen layer covering the bag region of the muscle cells (boxed area of A). (C) Transmission electron micrograph of the interstitial space between the bag regions of adjacent somatic muscle cells. The edges of the muscle bags are demarcated by the row of mitochondria (M) found at the periphery of the bag. Intracellular glycogen granules give the bag a mottled appearance. The interstitial space contains collagen fibres (C). (D) Higher magnification view of the interstitial collagen network. The collagen fibres (C) have the amorphous appearance of vertebrate basal lamina, a collagenous component of basement membranes (Stevens & Lowe, 1992; Toner & Carr, 1971). Fine strands appear to connect neighbouring fibres (arrow), consistent with a mesh-like structure.

(assuming that the latter is also a directly-gating ligand) would be expected to be of a similar order to that shown by Franke *et al*. (1987). However, the delay of 1·2 and 1·5 sec for GABA and PF4 respectively indicate that other processes occur that increase the delay time, namely the neurophore switching delay and the delay due to diffusion.

The performance of the pressure ejection equipment was uniform throughout the experiments, and the mean neurophore switching delay was found to be $0{\cdot}77 \pm 0{\cdot}31$ (mean $\pm$ S.D., $n = 3$). Therefore, the mean switching delay was a significant proportion of the delay period recorded. Subtraction of the mean switching delay from the total delay of action gives the delay due to diffusion plus the physiological delay of signal transduction between receptor and effector. Thus, the delay due to diffusion plus the physiological delay was 0·45 sec for GABA and 0·74 sec for PF4. The delay due to diffusion was investigated further using Equation 2 (Blackman, Ginsborg & House, 1979, based on Purves, 1977). The terms of Equation 2 are illustrated in Fig. 5A.

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Equation 2: diffusion equation

$$C = \frac{M}{4\pi\alpha^2(\alpha+x)}\left[\frac{\alpha}{\sqrt{(\pi Dt)}}\exp\left(\frac{-x^2}{4Dt}\right) - \exp\left(\frac{Dt}{\alpha^2}+\frac{x}{\alpha}\right) erfc\left(\frac{\sqrt{(Dt)}}{\alpha}+\frac{x}{2\sqrt{(Dt)}}\right)\right],$$

where C is the concentration of ligand at time t, in sec, M is the quantity of ligand released from the delivery tube, in moles, x is the distance from the cell surface, in cm, D is the diffusion coefficient of the compound, in cm^2/sec, t is the time, in sec, α is the radius of the cell, in cm, exp is exponential function, *erfc* is error function.

According to Equation 2, the concentration of ligand, C, is directly proportional to the amount of ligand ejected onto the cell and is dependent upon α, x and D. Since the receptors are on the cell surface, only those ligand molecules in close proximity to the membrane will bind to receptors. The concentration of molecules around the target cell increases with time after ejection, according to Equation 2. The diffusion coefficient, D, affects the rate of diffusion, as illustrated in Fig. 5B; the smaller the value of D, the slower the diffusion of the ligand. The value of D reflects the ambient temperature, the size of the diffusing molecules and the viscosity of the surrounding medium. The diffusion coefficients for the compounds used in this study were not previously reported so were estimated from the D values of molecules with similar biophysical properties (e.g. molecular weight, charge, lipophilicity). The diffusion coefficient of GABA in aqueous solution is $\sim 8\times10^{-6}$ cm^2/sec (the D value of acetylcholine, Florence & Attwood, 1998). The diffusion coefficient of PF4 in an aqueous medium was estimated to be $\sim 3\times10^{-6}$ cm^2/sec, the experimentally obtained value for a structurally related nematode FaRP, AF2 (Sheehy *et al*. 2000). Hence the D values illustrated in Fig. 5B are 8×10^{-6}, 3×10^{-6}, and 1×10^{-6} cm^2/sec. From Fig. 5B it can be seen that the time for ligand concentration to reach 1 nM at the target cell varies from 0·1 sec when $D = 8\times10^{-6}$ cm^2/sec to 0·7 sec when $D = 1\times10^{-6}$ cm^2/sec. It is apparent that a low value of diffusion coefficient can produce a significant delay in the response, such as that recorded in our experiments.

In the presence of a diffusion barrier, molecules will diffuse more slowly than when in simple aqueous solution. The collagen matrix that surrounds *Ascaris* muscle cells ('interstitial ground substance' according to Rosenbluth, 1965) is highly likely to act as a diffusion barrier, and as such will reduce the diffusion coefficients of solutes, including peptides and small molecule neurotransmitters. Light and electron micrographs of the *Ascaris* extracellular collagen matrix (Fig. 6) show this layer to be 2–5 μm thick. The radii of the aqueous pores that traverse this layer were not verifiable from the micrographs. The delay due to diffusion through this collagen matrix would likely constitute a substantial part of the overall delay time recorded for all three of the compounds used in this study.

In summary, the archetypal FaRP, FMRFamide has been shown to directly-gate sodium channels in the snail, *Helix aspersa* (Green *et al*. 1994; Lingueglia *et al*. 1995). Our study compares the delay of action of PF4 with that of GABA and shows that the small difference between these two molecules is attributable to differences in their rates of diffusion and not to their mechanisms of action. PF4 is remarkable among peptides in that its rapid mechanism of action is consistent with direct gating of a chloride channel. To date, there are no reports of other chloride channels that open in direct response to FaRPs or any other peptides. Since other directly ligand-gated ion channels have already been shown to be the molecular targets for successful nematocides, including chemical classes typified by ivermectin and levamisole, this characteristic of the PF4 receptor makes it an attractive target for anthelmintic discovery.

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