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The effects of the peptide KPNFIRFamide (PF4) on the somatic muscle cells of the parasitic nematode *Ascaris suum*

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1 Commonly used anthelmintic agents act on the muscle cells of parasitic nematodes to cause paralysis of the parasite and its expulsion from the host.

2 The motonervous system of nematodes contains neuropeptides, many of which are myoactive and elicit prolonged worm paralysis. Here we describe the actions of a novel peptide, KPNFIRFamide (Lys-Pro-Asn-Phe-Ileu-Arg-Phe-amide; PF4), which mediates relaxation of the somatic muscle of the parasitic nematode *Ascaris suum*. Its mechanism of action is compared to that of the inhibitory neuromuscular junction transmitter, γ-aminobutyric acid (GABA), which gates a chloride channel on *Ascaris* muscle.

3 Both PF4 and GABA hyperpolarized the muscle cells (EC_{50} values 98 nM and 59 μM, respectively; $n=6$) and this was accompanied by an increase in input conductance.

4 The increase in input conductance elicited by PF4 and a supramaximal concentration of GABA were additive (10 μM PF4, 7.78 ± 1.88 μS; 10 mM GABA, 4.68 ± 1.39 μS; 10 mM GABA and 10 μM PF4 12.05 ± 2.6 μS, $n=6$, $P<0.02$ with respect to PF4 alone; $P<0.01$ with respect to GABA alone).

5 The membrane potential response to 10 μM PF4 initially consisted of a fast hyperpolarization that occurred within 1 min of PF4 application. The reversal potential for this early response to PF4 (PF4-early) was determined at different extracellular chloride concentrations. Linear regression analysis of the natural logarithm of the extracellular chloride concentration against the reversal potential for PF4-early yielded a straight line with a slope of -29.6 ± 2.4 (-34.4 to -24.9, 95% confidence limits; $r^2=0.82$). This is close to the slope of -26.5 for a chloride-dependent event, as predicted by the Nernst equation. There was a significant correlation between the reversal potential for this event and the reversal potential for GABA ($r=0.94$; $P<0.001$; $n=12$).

6 The late response to PF4 (PF4-late) appeared after 1 min and consisted of a slow reduction in the hyperpolarization to a plateau level, before the return of the membrane potential to the resting value. PF4-late is not likely to be a chloride-dependent event as during the hyperpolarization caused by a supramaximal concentration of GABA the muscle cells depolarized when a supramaximal concentration of PF4 was added to the perfusate. The membrane potential in the presence of 1 mM GABA was -61.8 ± 4.8 mV and in the presence of 1 mM GABA with 10 μM PF4 was -47.5 ± 1.5 mV ($P<0.02$; $n=6$).

7 The conductance increase elicited by 30 μM GABA was blocked by 10 μM ivermectin (before ivermectin 0.97 ± 0.2 μS, after ivermectin 0.33 ± 0.12 μS; $n=5$; $P<0.05$; Student's paired t test) but the conductance increase elicited by 1 μM PF4 was not (before ivermectin 0.96 ± 0.14 μS, after ivermectin 1.07 ± 0.19 μS; $n=0.34$; Student's paired t test).

8 These data indicate that PF4 elicits a potent, inhibition of *Ascaris* muscle cells which is partially mediated by chloride and which is independent of the inhibitory GABA receptor.

Keywords: Nematode; neuropeptide; chloride; *Ascaris*

Introduction

Parasitic nematodes are a major cause of morbidity and mortality in man and also cause widespread loss of food production by infection of livestock. The emergence of drug resistant strains of nematode (Prichard, 1994) necessitates the discovery of novel targets for anthelmintic action. We have undertaken a rational approach involving the physiological and pharmacological characterization of processes specific to the parasite. These functional studies have centred on the large parasitic nematode *Ascaris suum* which, unlike many of the smaller nematode species, is well suited to electrophysiolgoical studies (Martin, 1994 for review).

The motonervous system of *Ascaris* has been well characterized anatomically (see Stretton *et al*., 1985 for review) and consists of forty excitatory, cholinergic (Johnson & Stretton, 1985) motoneurones and fifteen inhibitory, γ-aminobutyric acid (GABA)ergic (Johnson & Stretton, 1987) motoneurones. This simplistic outline of the *Ascaris* motonervous system has recently been complicated by the observation that many of the motoneurones also contain peptide-like immunoreactivity (Sithigorngul *et al*., 1990) and in particular FMRFamide-like immunoreactivity (Davenport *et al*., 1988; Cowden *et al*., 1993). This has focused interest on the involvement of peptidergic mechanisms in the nematode motonervous system (Halton *et al*., 1994) and the possibility of exploiting these systems for anthelmintic action.

Over the last few years a number of neuropeptides belonging to the FMRFamide family have been isolated from nematodes and shown to have effects on *Ascaris* muscle (Cowden *et al*., 1989; Geary *et al*., 1992; Cowden & Stretton, 1993; Maule *et al*., 1994; 1995; Keating *et al*., 1995). The first peptide, AF1, was isolated from *Ascaris* (Cowden *et al*., 1989) and has the sequence KNEFIRFamide. Latterly, AF2, with the sequence KHEYLRFamide was isolated by the same group (Cowden & Stretton, 1993). Both these peptides have a potent effect on motor activity when injected into the whole worm (Stretton *et al*., 1991) with an initial stimulant effect followed by paralysis. These observations highlighted the possibility that peptidergic mechanisms may represent novel target sites for anthelmintics.

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Subsequently, similar peptides have been isolated from other nematodes, including free-living species such as *Caenorhabditis elegans* (Rosoff *et al*., 1992) and *Panagrellus redivivus* (Geary *et al*., 1992; Maule *et al*., 1994; 1995). The peptides from *Panagrellus* have been designated PF1, PF2, PF3 and PF4 and have the sequences SDPNFLRFamide, SADPNFLRFamide, KSAYMRFamide and KPNFIRFamide, respectively. In contrast to the predominantly excitatory action of AF1 and AF2 on *Ascaris* muscle, PF1 and PF2 cause a relaxation (Franks *et al*., 1994). This effect is mediated both presynaptically by inhibition of the release of the excitatory neuromuscular transmitter, acetylcholine (Holden-Dye *et al*., 1995), and postsynaptically by inhibition of muscle contraction (Franks *et al*., 1994). PF1 and PF2 elicit a small, chloride-independent, hyperpolarization of *Ascaris* muscle. PF3 has been shown to have an excitatory action on *Ascaris* muscle (Maule *et al*., 1994) but the mechanism has not yet been characterized.

This paper describes the actions of KPNFIRFamide (PF4). PF4 has been found to cause a marked relaxation of *Ascaris* muscle (Maule *et al*., 1995), qualitatively similar to the action of the inhibitory neuromuscular junction transmitter, GABA (Del Castillo *et al*., 1964; Johnson & Stretton, 1987). GABA is known to mediate muscle relaxation by a ligand-gated chloride channel which is not blocked by bicuculline and is only weakly blocked by picrotoxin (Martin, 1980; Holden-Dye *et al*., 1989; Parri *et al*., 1991). This GABA receptor is the target for the anthelmintic piperazine which acts as a weak partial agonist and causes a flaccid paralysis of *Ascaris* (Martin, 1982). The anthelmintic ivermectin also acts at this GABA receptor as a non-competitive antagonist (Martin, 1987; Holden-Dye *et al*., 1988; Martin & Pennington, 1989; Holden-Dye & Walker, 1990).

Here we have used an electrophysiological approach to study the inhibitory action of PF4 on *Ascaris* somatic muscle. The aims of the study were (i) to compare the potency of PF4 with that of GABA, (ii) to compare the ionic mechanism of action for PF4 with that of GABA, (iii) to determine whether the response to PF4 is likely to involve stimulation of GABA release from the inhibitory motoneurones. The mechanism of action of PF4 indicates the presence of a previously uncharacterized and potent inhibitory site on *Ascaris* muscle and thus may be of considerable interest with respect to anthelmintic development.

Methods

Ascaris suum were obtained from a local abattoir and maintained for up to five days in the laboratory in artificial perienteric fluid (APF; composition in mM: NaCl 67, Trizma base 5, Na Acetate 67, KCl 3, $CaCl_2$ 3, $MgCl_2$ 15.7, glucose 3, pH 7.6 with glacial acetic acid at 37°C). An anterior section of the worm, approximately 2 cm, was excised, slit in a longitudinal direction along one lateral line and opened out. The intestine was removed revealing the muscle bag cells. The section was securely pinned out, cuticle side down, in a perspex perfusion chamber, volume 5 ml, and continuously perfused with APF at 10 ml min^{-1}. Bath temperature was maintained at 34 ± 1°C and continuously monitored via a temperature probe placed adjacent to the recording site. Muscle cells were impaled with two glass microelectrodes (10–30 MΩ; 4M KAcetate, 10 mM KCl) and intracellular recordings were made by an Axoclamp 2A (Axon Instruments, U.S.A.) system. For voltage recordings and muscle cell input conductance measurements, current pulses (5–85 nA, 0.1 or 0.2 Hz, 500 ms pulse width) were injected intracellularly through one of the electrodes. It has previously been shown that the current-voltage relationship for *Ascaris* muscle cells in this range is essentially linear (Holden-Dye *et al*., 1989). For low chloride APF, NaCl was replaced by Na isethionate.

Drugs and the perfusate were applied by a fine bore plastic tube (0.8 mm internal diameter) directed at the cell from which the recording was being made. Drug solutions were separated from the perfusate by air bubbles which escaped through a small hole immediately before entry to the bath. This method of application allows rapid and complete change of the composition of the perfusion stream over the cell being studied. Studies with dye indicated that drugs would be evenly distributed in the bath within 1 min. The concentrations quoted are the concentrations added to the bath. The responses used to construct concentration-response curves are the maximal responses observed within 2 min of drug perfusion.

KPNFIRFamide was synthesized by Dr John Fox at Alta Bioscience, Birmingham (purity 92%). Ivermectin was a gift from Merck Sharp & Dohme. The vehicle for ivermectin was 1% DMSO and control experiments indicated that the vehicle had no direct effect on the muscle cells.

Results are expressed as the mean $\pm$ 1 s.e.mean and significance was tested by use of paired two-tailed Student's *t* test. Concentration-response curves were fitted to the logistic equation by non-linear regression analysis to estimate EC_{50} values (given with 95% confidence limits). This and all other data analyses were performed by use of GraphPad Prism (version 2, GraphPad Software Inc., San Diego, CA 92121, U.S.A.).

Results

The mean resting membrane potential of the muscle cells included in this study was 31.4 ± 0.4 mV ($n = 98$) and the mean input conductance was 1.79 ± 0.06 μS ($n = 98$). PF4 elicited a readily reversible muscle cell hyperpolarization accompanied by an increase in input conductance ($n = 92$; Figure 1a). The threshold for this response was around 10 nM PF4. By comparison, GABA also elicited a hyperpolarization accompanied by an increase in input conductance as previously described (Holden-Dye *et al*., 1989). However, the threshold for the response to GABA was around 10 μM (Figure 1a; $n = 7$).

The EC_{50} values for the hyperpolarizations to PF4 and GABA were 98 nM (2.1 to 461; $n = 6$) and 59 μM (50 to 69; $n = 6$), respectively. The maximal hyperpolarizations elicited by GABA or PF4 were not significantly different ($P = 0.06$; Figure 1b). However, the time-course of the membrane potential responses for PF4 and GABA were different. The membrane potential returned to within $\pm$1 mV of its resting value at a significantly slower rate following 100 μM PF4 compared to 100 μM GABA (581 ± 59 s compared to 325 ± 49 s; $n = 8$; $P < 0.01$). Furthermore, after the initial fast hyperpolarization to concentrations of PF4 greater than 1 μM, there was a slow reduction in the membrane potential to a plateau level before the return to the resting membrane potential (Figure 1a). During this phase of the response the input conductance continued to increase. This suggested that the response to PF4 may consist of two events, an early event (PF4-early) which occurs within 1 min of PF4 application and is similar to the response elicited by GABA, and a late event (PF4-late) which develops after 1 min of PF4 application.

The maximal increase in input conductance elicited by 100 μM PF4 (8.89 ± 1.52 μS; $n = 6$) was significantly greater ($P < 0.001$) than that elicited by either 100 μM GABA (3.87 ± 1.00 μS; $n = 6$) or 10 mM GABA (4.68 ± 1.39 μS; $n = 6$; Figure 1c). An EC_{50} was not determined for the change in input conductance as a maximum response to PF4 was not achieved even at the highest concentration tested. The effect of PF4 and a supramaximal concentration of GABA on muscle cell input conductance were additive. The response to combined application of 10 μM PF4 and 10 mM GABA was 12.05 ± 2.6 μS ($n = 6$) and this was significantly different from the response to either 10 μM PF4 alone (7.58 ± 1.57 μS; $n = 6$; $P < 0.02$) or to 10 mM GABA alone (4.68 ± 1.39 μS; $n = 6$; $P < 0.01$). During prolonged drug applications the change in input conductance was maintained for as long as the drug was present indicating that neither the response to PF4 nor the response to GABA exhibited signs of desensitization (data not shown).

The ionic basis of the early membrane potential response to PF4 (PF4-early) was evaluated by determining the reversal potential for the response in APF containing 40, 60 and 108 nM extracellular chloride within 1 min of application of PF4. For these experiments, fourteen cells were first perfused with APF containing 108 mM chloride, nine of these cells were then perfused with APF containing 60 mM chloride and subsequently all fourteen cells were perfused with APF containing 40 mM chloride. The reversal potential for PF4-early was determined for each of the extracellular chloride concentrations (Figure 2a(i, ii and iii, respectively). In 108 mM chloride the reversal potential was -51 ± 2 mV ($n=14$), in 60 mM chloride it was -33 ± 1 mV ($n=9$) and in 40 mM chloride it was -23 ± 2 mV ($n=14$). Each of these values is significantly different from each other ($P<0.001$; Student's paired t test). The dotted line in Figure 2b shows the theoretical Nernst relationship for a chloride-dependent event (at 34°C):

$$\text{Reversal potential} = 26.5 \ln ([\text{Cl}]_{\text{in}}/[\text{Cl}]_{\text{out}})$$

where $[\text{C}]_{\text{in}}$ and $[\text{C}]_{\text{out}}$ are the intracellular and extracellular chloride concentrations, respectively, and an intracellular chloride concentration of 17 mM was assumed (estimated with chloride-sensitive microelectrodes; Parri *et al*., 1990). Linear regression analysis of the natural logarithm of the extracellular chloride concentration against the reversal potential for PF4-early yielded a straight line with a slope of −29.6 (−34.4 to −24.9, 95% confidence limits; $r^2=0.82$; Figure 2b, solid line).

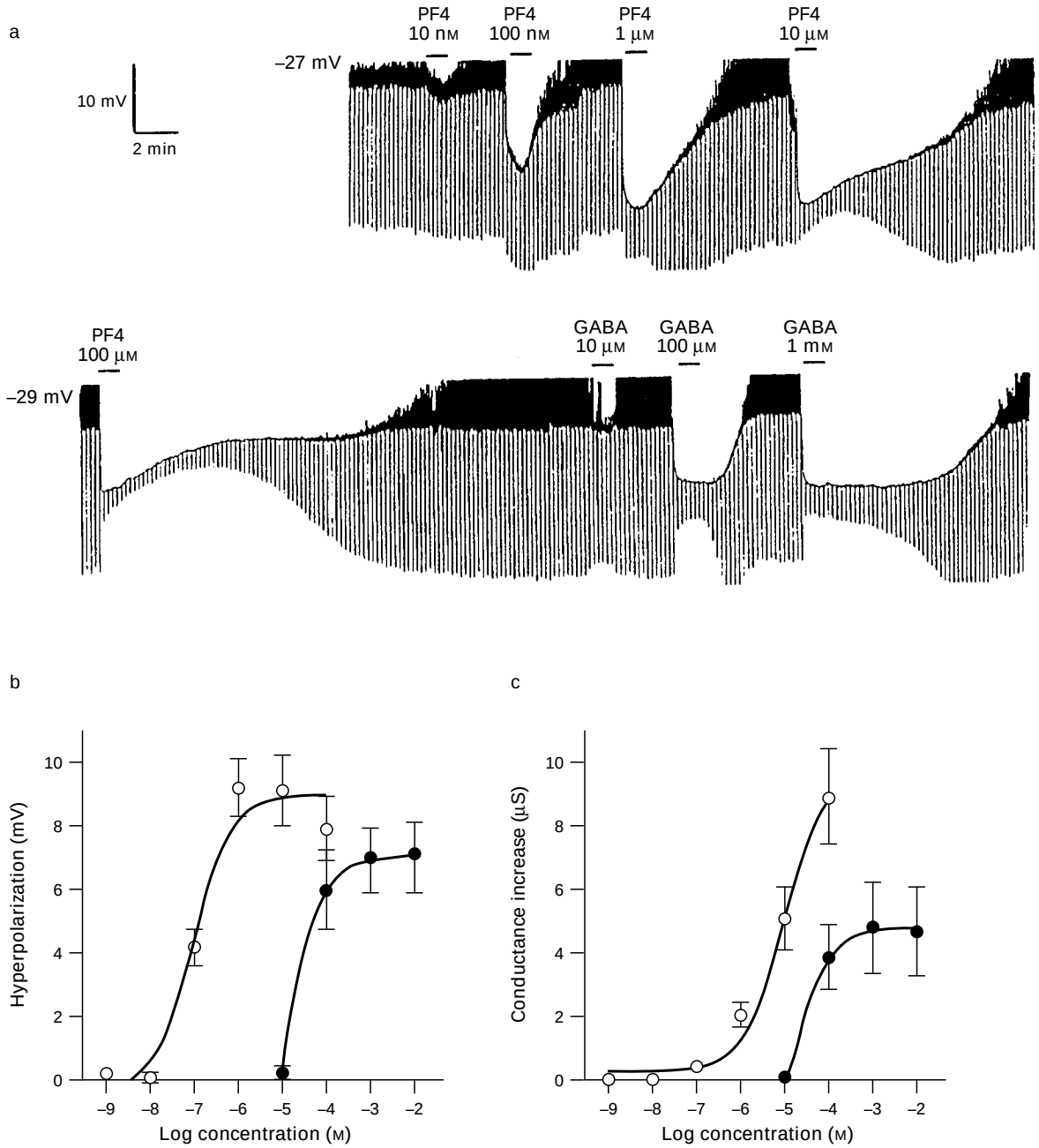

Figure 1 The effects of GABA and PF4 on *Ascaris* muscle cells. (a) Voltage recordings from the same muscle cell. This cell was spontaneously active at resting membrane potential and the amplitude of the muscle cell action potentials were truncated by the chart recorder. The values to the left of each recording are the membrane potentials at the start of each trace. The downward deflections are electronic potentials resulting from injection of current pulses (20 nA, 0.2 Hz, 500 ms). The horizontal bars indicate the duration of bath application of the drug. (b) The concentration-response relationship for the hyperpolarization elicited by GABA (●) and PF4 (○). (c) The concentration-response relationship for the conductance increase elicited by GABA (●) and PF4 (○). Data points are mean and vertical lines show s.e.mean ($n=6$ to 11).

This is close to the slope of −26.5 for a chloride-dependent event, as predicted by the Nernst equation.

It has previously been shown that the GABA hyperpolarization is also a chloride-dependent event (Martin, 1980; Holden-Dye *et al*., 1989; Parri *et al*., 1991). In order to assess whether PF4-early is a chloride-dependent hyperpolarization similar to that elicited by GABA the reversal potentials for GABA and PF4-early were compared. For these experiments the cells were first exposed to 10 μM PF4 and, within 1 min, 100 μM GABA was co-applied (Figure 3a). The reversal potential was determined for PF4-early and GABA (Figure 3b). There was a significant correlation between the reversal potential for PF4 and for GABA (Figure 3c; Spearman correlation coefficient, $r=0.94$; $P<0.0001$; $n=12$).

Further evidence that PF4-early involved a chloride-dependent hyperpolarization was provided by the observation that exposure of the muscle cells to a supramaximal concentration of PF4 (100 μM) reduced the magnitude of the hyperpolarization that could be elicited by 100 μM GABA (Figure 4a). The resting membrane potential of these muscle cells was -34 ± 1 mV ($n=8$) and the cells hyperpolarized to a potential of -52 ± 2 mV ($n=8$) in the presence of 100 μM GABA. The cells were then perfused with 100 μM PF4 for 1 min, washed, and allowed to return to the resting membrane potential. Subsequent application of 100 μM GABA elicited a hyperpolarization to -44 ± 2 mV ($n=8$), a potential significantly less than that before the exposure to PF4 ($P<0.05$; Figure 4b). In contrast, the increase in input conductance elicited by GABA was the same before and after the application of PF4 (2.1 ± 0.1 μS and 2.1 ± 0.2 μS, respectively; $n=8$). These data are consistent with the idea that the increase in chloride conductance elicited by high concentrations of PF4 caused an accumulation of intracellular chloride and a subsequent shift in E_{Cl}, and thus the reversal potential for GABA.

In contrast to PF4-early, the late response (PF4-late) is not likely to be a chloride-dependent event as the membrane potential during PF4-late is more positive than that in the presence of a maximally effective concentration of GABA (Figure 1a). Therefore, PF4-late is likely to have a different ionic mechanism to GABA. The most convincing evidence was provided by experiments in which GABA and PF4 were sequentially applied to the muscle cells and had opposite effects on the membrane potential (Figure 5a). During the hyperpolarization caused by a supramaximal concentration of GABA the muscle cells depolarized when a supramaximal concentration of PF4 was added to the perfusate (Figure 5a). In a series of six experiments, the membrane potential in the presence of 1 mM GABA was -61.8 ± 4.8 mV and in the presence of 1 mM GABA with 10 μM PF4 was -47.5 ± 1.5 mV (Figure 5b; $P<0.02$; $n=6$). As the membrane potential of *Ascaris* is dependent on the equilibrium potential for chloride (Brading & Caldwell, 1971), and as PF4 can cause intracellular accumu-

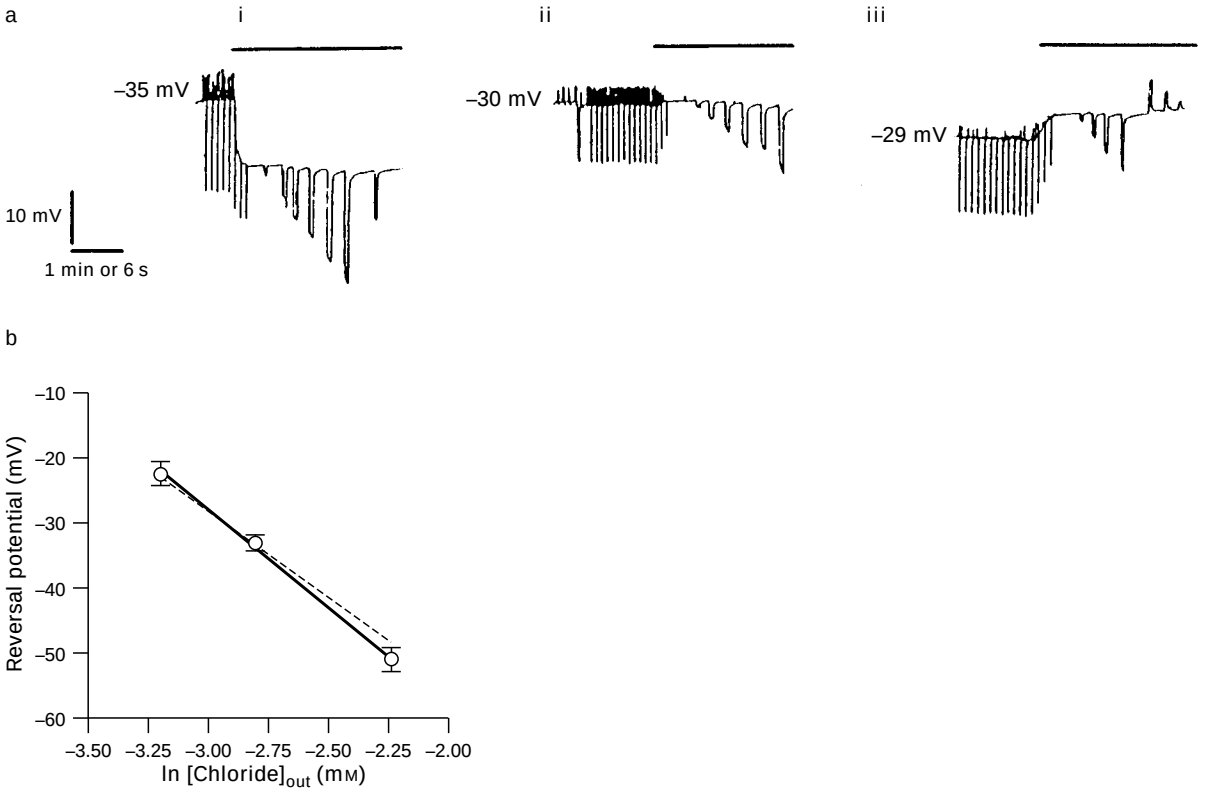

Figure 2 The effect of extracellular chloride concentration on the reversal potential for PF4 (10 μM). Fourteen muscle cells were first perfused with APF containing 108 mM chloride. In nine experiments this was then replaced with APF containing 60 mM chloride, and subsequently all fourteen cells were perfused with APF containing 40 mM chloride. The reversal potential for PF4 was determined in each of the different extracellular chloride concentrations by passing current pulses (5–80 nA, 0.2 Hz, 500 ms), before and during the PF4 application into the cell. The resulting electrotonic potentials were used to construct a current-voltage plot. The intercept of the current-voltage plots constructed before and after the drug application provided an estimate of the reversal potential for the event. (a) An example of the effect of extracellular chloride on the response to PF4. These are voltage recordings from the same muscle cell. The values to the left of each recording are the membrane potentials at the start of each trace. The downward deflections are electrotonic potentials resulting from injection of current pulses. Horizontal bar indicates the duration of application of 10 μM PF4. Note that the chart speed was increased during the measurement of electrotonic potentials, as indicated on the horizontal bar. (i) [Chloride] = 108 mM, (ii) [Chloride] = 60 mM and (iii) [Chloride] = 40 mM. (b) A plot of natural logarithm extracellular chloride concentration against the reversal potential for PF4 is a straight line with a slope of −29.6 (−34.4 to −24.9, 95% confidence limits; $r^2=0.82$; solid line). The data points are the means, and vertical lines show s.e.mean, of 14, 9 and 14 observations for 108 mM, 60 mM and 40 mM chloride respectively. The dotted line indicates the relationship predicted by the Nernst equation for a chloride-dependent event (assuming an intracellular chloride of 17 mM; Parri *et al*., 1990; at 34°C).

lation of chloride, as shown in Figure 4, it was necessary to consider the possibility that the depolarization caused by PF4 could occur as a result of chloride redistribution during the drug application (though the rapidity of the effect would suggest otherwise). Therefore, in four of these cells the effect of addition of 1 mM GABA to the muscle during the hyperpolarization to 10 μM PF4 was also subsequently determined (1 mM GABA was added at least 2 min after the addition of PF4). For each cell, GABA elicited a further hyperpolarization (of 1, 2, 6 and 4 mV) in the presence of 10 μM PF4, thus providing evidence that the depolarization caused by PF4 in the presence of GABA may not be explained by intracellular accumulation of chloride.

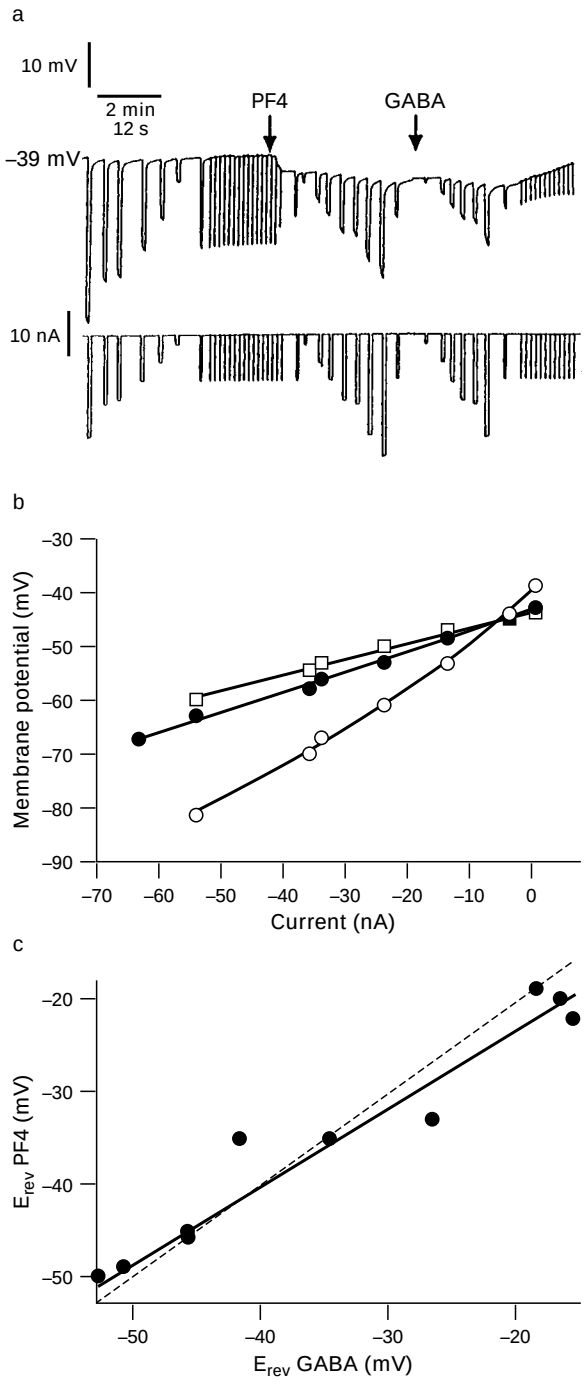

Figure 3 The reversal potential for (E_{rev}) for PF4-early and GABA are similar. (a) The upper trace is the voltage recording from a muscle cell in 108 mM extracellular chloride showing the response to PF4 (10 μM) followed by co-application of 100 μM GABA. The lower trace shows the corresponding current pulses. (b) the current-voltage plots from the data shown in (a). The reversal potentials for PF4 and GABA were determined by passing current pulses (5–63 nA, 0.2 Hz, 500 ms) and measuring the resultant electronic potentials. These were used to construct current-voltage plots. Note that the value for zero nA is plotted, this is the membrane potential. The intercept of the current-voltage plot constructed in the absence of drug and in the presence of PF4 provided the reversal potential for PF4, and the intercept of the current-voltage plot for PF4 and during GABA provided the reversal potential for GABA. Control (○), with 10 μM PF4 (●), with 100 μM GABA (□). (c) Summary of experiments showing the correlation between the reversal potentials (E_{rev}) for PF4 and GABA (solid line; $r = 0.94$; $P < 0.0001$; $n = 12$). The dotted line is that predicted for a direct correlation.

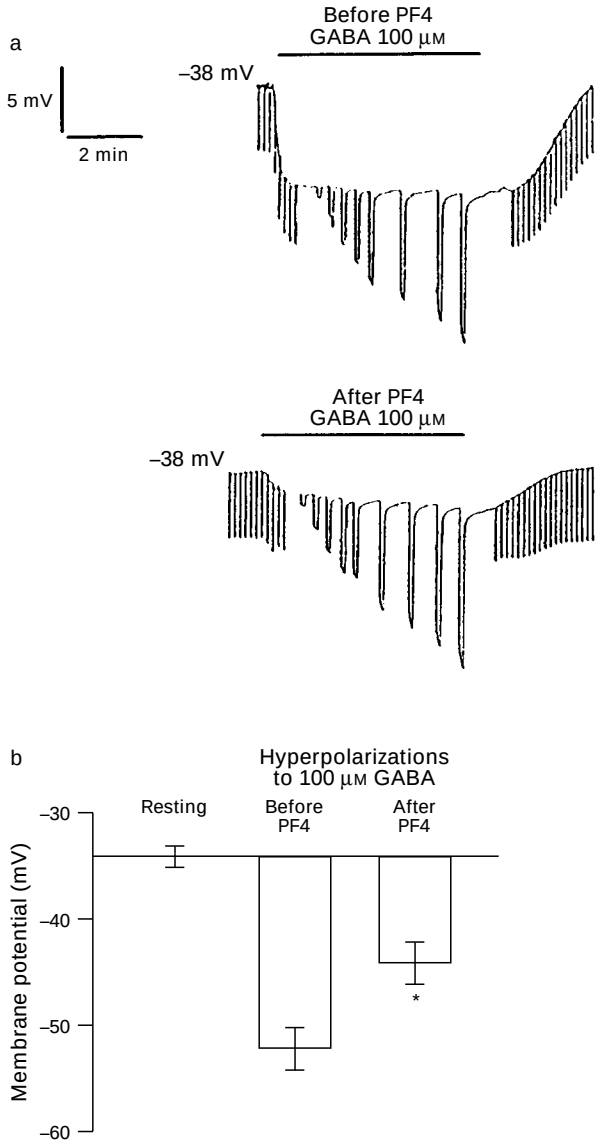

Figure 4 The effect of prior exposure of the muscle preparation to 100 μM PF4 on the hyperpolarization elicited by GABA. For these experiments, nine cells were first exposed to 100 μM GABA and the magnitude of the hyperpolarization determined. The cells were then exposed to 100 μM PF4 for 1 min, washed and then the hyperpolarization to 100 μM GABA was measured again. (a) A voltage recording showing the response to 100 μM GABA before and after the application of PF4. The hyperpolarization elicited by GABA was reduced following application of PF4. The values to the left of each recording are the membrane potentials at the start of each trace. The downward deflections are electronic potentials resulting from injection of current pulses (20 nA, 0.2 Hz, 500 ms). The horizontal bars indicate the duration of bath applications of GABA. (b) a summary of the data (±s.e.mean) showing the resting membrane potential (left-hand column) and the hyperpolarizations elicited by 100 μM GABA before (middle column) and after PF4 (right-hand column). *$P < 0.0001$; $n = 8$.

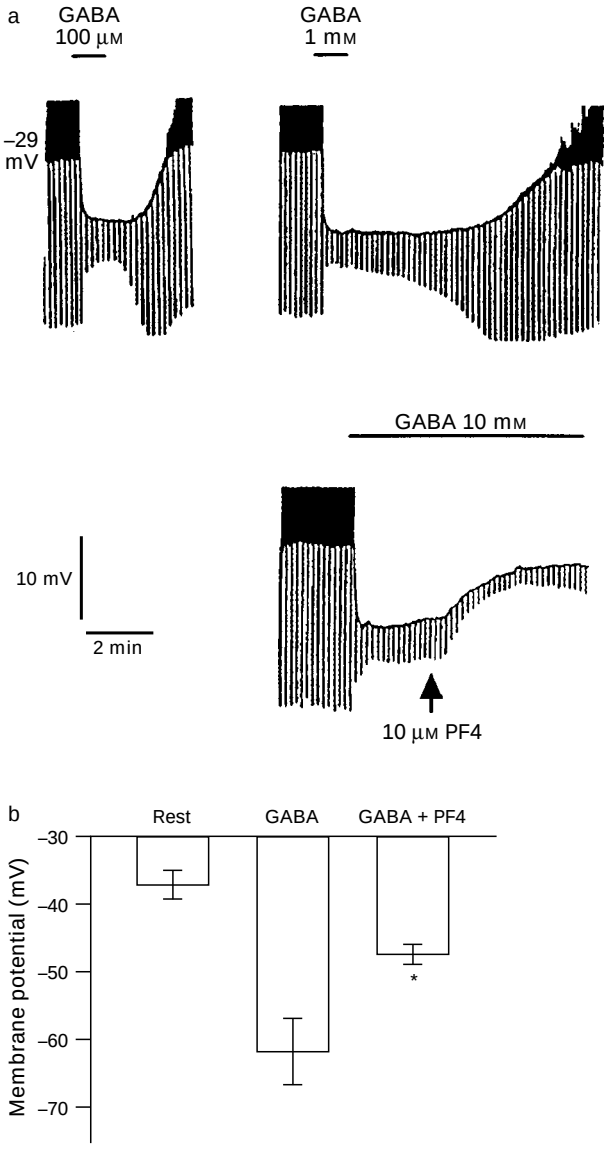

Figure 5 The effect of co-application of GABA and PF4 on membrane potential. This recording is a consecutive recording from the same muscle cell as in Figure 1a. (a) Consecutive intracellular voltage recordings. Resting membrane potential at the onset of the experiment was −29 mV. Downward deflections are electrotonic potentials resulting from current injection (0.1 Hz, 500 mS, 20 nA). The solid bars indicate the duration of application of GABA. The arrow indicates the addition of 10 μM PF4 to the perfusate. (b) A summary of six experiments similar to the example in (a) in which the effect of co-application of 1 mM GABA and 10 μM PF4 on muscle membrane potential was determined. The left-hand column indicates the resting membrane potential ($\pm$s.e.mean), the middle column indicates the membrane potential in the presence of 1 mM GABA and the right-hand column indicates the membrane potential when 10 μM PF4 was added to the perfusate. PF4 caused a significant decrease in the membrane potential ($P<0.02$; $n=6$).

The protocol used to establish whether ivermectin, an antagonist of the *Ascaris* GABA receptor (Holden-Dye *et al.*, 1988; Martin & Pennington, 1989), acts as an antagonist of the PF4 response was as follows. Responses of a similar magnitude to 30 μM GABA and to 1 μM PF4 were obtained in five preparations. The preparations were then perfused with 10 μM ivermectin for 15 min. Following this treatment the responses of the muscle cells to 30 μM GABA and 1 μM PF4 were determined (Figure 6a). The control conductance increase elicited by 30 μM GABA was 0.97 ± 0.20 μS and by 1 μM PF4 was 0.96 ± 0.14 μS. Following ivermectin treatment the response to GABA was reduced to 0.33 ± 0.12 μS representing a significant receptor block ($P<0.05$; $n=5$ throughout). However, in the presence of ivermectin, the response to 1 μM PF4 was 1.07 ± 0.19 μS which is not significantly different from the control response (Figure 6b; $P=0.34$; Student's paired t test; $n=5$). Therefore, ivermectin blocked the GABA, but not the PF4, response.

Discussion

GABA hyperpolarized *Ascaris* muscle as previously described (Martin, 1980; Holden-Dye *et al.*, 1989). This effect is mediated by a GABA-gated chloride channel (Martin, 1980; Parri *et al.*, 1991). The novel peptide PF4 (KPNFIRFamide) also caused a hyperpolarization and the threshold for this effect was three orders of magnitude less than that for GABA. This hyperpolarization consisted of an initial fast hyperpolarization of similar magnitude to that elicited GABA. At concentrations of PF4 greater than 1 μM, the fast hyperpolarization was followed by a slow repolarization to a plateau value before a gradual return to the resting membrane potential. Thus, the effects of PF4 on the membrane potential are biphasic and consist of the early fast hyperpolarization which occurs within 1 min of peptide application, PF4-early, and a slower event, PF4-late. The hyperpolarization elicited by PF4 was accompanied by a marked increase in input conductance which was significantly greater than that caused by a supramaximal concentration of GABA.

Three observations indicate that PF4-early is a chloride-dependent hyperpolarization. First, there was a close dependence of the reversal potential for PF4-early on extracellular chloride concentration. Second, GABA is known to gate a chloride channel on these cells and there was a significant correlation between the reversal potential for PF4-early and GABA. Finally, prior exposure of the muscle cells to PF4 reduced the magnitude of the hyperpolarization elicited by GABA. *Ascaris* muscle cells have a large resting conductance to permeant anions such as chloride and the resting chloride leak is offset by an outwardly directly chloride pump (Del Castillo *et al.*, 1964; Parri *et al.*, 1991). As 100 μM PF4 caused a large increase in chloride conductance, it would be expected to increase chloride influx and lead to an intracellular accumulation of chloride which would shift E_{Cl} and the reversal potential of GABA. This provides an explanation for the significant reduction in the hyperpolarization elicited by GABA after the cells had been exposed to 100 μM PF4.

As both GABA and PF4-early hyperpolarize *Ascaris* muscle by increasing the conductance to chloride, one possible explanation for the inhibitory action of PF4 is that it may act to stimulate the release of GABA from the inhibitory motoneurones. However, the present study has demonstrated that in the presence of a maximally effective concentration of GABA, PF4 was still observed to cause an increase in input conductance indicating that the peptide is unlikely to be causing the release of GABA. Furthermore, Maule *et al.* (1995) showed that PF4 was still able to elicit relaxation in a muscle preparation from which the nerve cord had been removed. As the synapses between muscle and motoneurones occur exclusively at the nerve cord, the inhibitory GABAergic nerve terminals were absent from this preparation and, therefore, PF4 could not be exerting the relaxation by stimulating release of GABA.

Both PF4 and GABA increase muscle cell chloride conductance and it could be proposed that PF4 acts as a GABA receptor agonist, perhaps through an allosteric mechanism. The possibility that PF4 acts on the GABA-gated chloride channel was tested by observing the action of ivermectin on the PF4 response. Ivermectin is a non-competitive, irreversible antagonist of the GABA receptor on *Ascaris* somatic muscle cells (Holden-Dye *et al.*, 1988; Martin & Pennington, 1989; Holden-Dye & Walker, 1990). As the *Ascaris* GABA receptor is bicuculline and picrotoxin insensitive, this is currently the

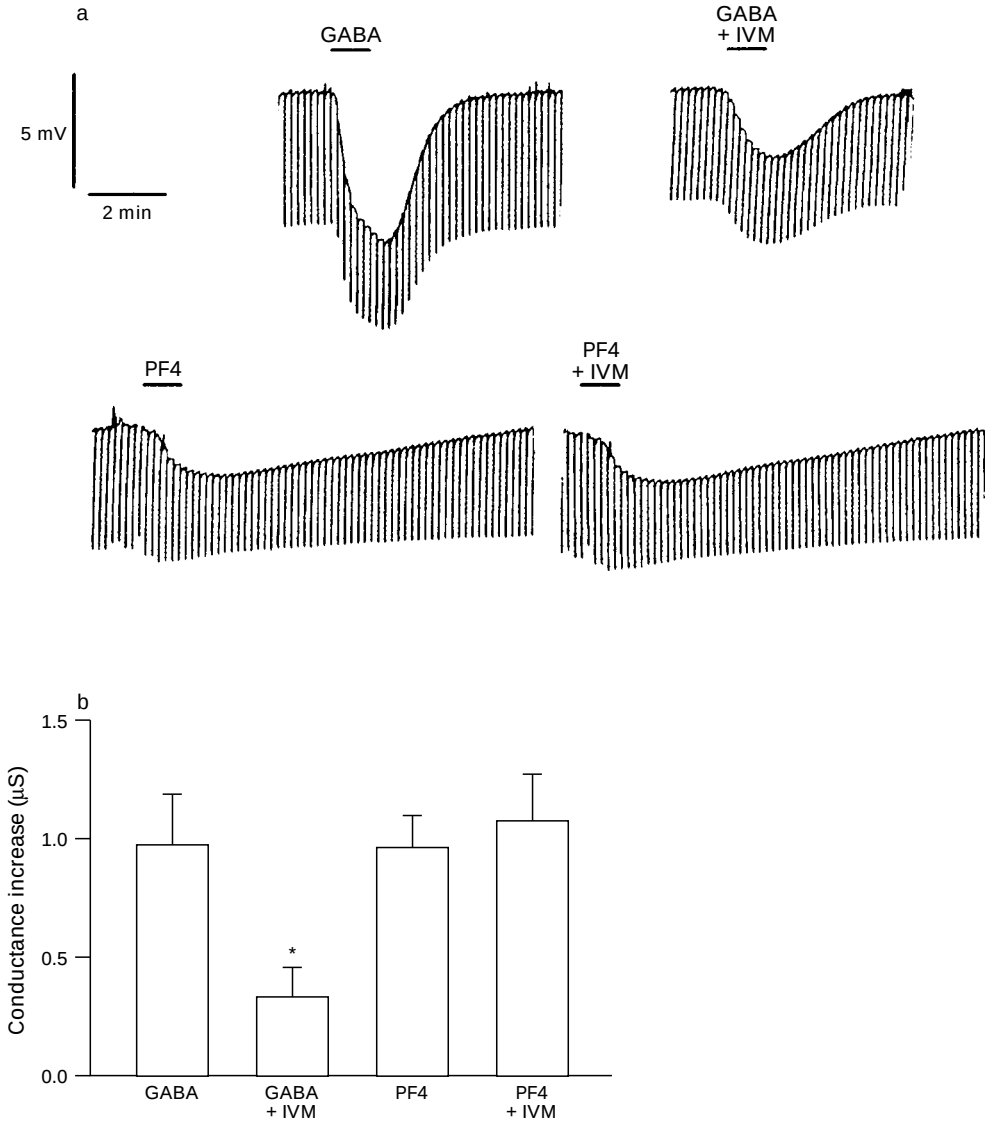

Figure 6 A comparison of the effect of ivermectin (10 μM; IVM) on equieffective concentrations of GABA and PF4 (i.e. concentrations that elicited a similar increase in input conductance). (a) An example of voltage recordings from one *Ascaris* muscle cell. The resting membrane potential was -31 mV at the start of each recording. Control responses to 30 μM GABA and 1 μM PF4 were determined first (left recordings). These concentrations were selected as they elicited a similar increase in input conductance. Ivermectin 10 μM was then included in the perfusate and present throughout the remainder of the experiment. After 15 min the responses to GABA or PF4 were recorded (right recordings). The bar indicates the duration of application of drug. (b) A summary of the results from five experiments as shown in (a). The ivermectin concentration was 10 μM and this decreased the response to GABA ($^*P<0.05$) but not to PF4 ($P=0.34$).

only readily available antagonist for this receptor. Ivermectin has no consistent effect on the resting membrane potential or input conductance of *Ascaris* muscle cells (Holden-Dye & Walker, 1990) although it does inhibit spontaneous depolarizing potentials in spontaneously active muscle cells (Holden-Dye *et al*., 1988). Ivermectin blocked the response to GABA in all five cells studied but it did not block the response to PF4. Therefore, it is unlikely that PF4 exerts its effects on chloride conductance through the GABA receptor.

The membrane potential during PF4-late was less than that during PF4-early. As this study has established that PF4-early is chloride-dependent, the difference in membrane potential before PF4-early and PF4-late suggests that PF4-late is not predominantly dependent on chloride. In order to investigate this further, the effect of PF4 on membrane potential was observed during a maximal hyperpolarization to GABA. As the GABA event is chloride-dependent it would be expected that if PF4-late was also chloride-dependent there would be either no additional change in membrane potential upon addition of PF4, or possibly a small further hyperpolarization. However, addition of PF4 caused a depolarization. Therefore, the ionic basis for PF4-late is not the same as that for GABA and is not predominantly dependent on chloride. The equilibrium potential for cations in *Ascaris* muscle is $+27$ mV for Na^+ and -90 mV for K^+ (Brading & Caldwell, 1971). Therefore, PF4-late could involve either a decrease in K^+ conductance or an increase in Na^+ conductance. Alternatively, the muscle cells of *Ascaris* are known to produce high concentrations of carboxylic acids as a result of the anaerobic metabolism of glucose (Saz & Weil, 1962; Tsang & Saz, 1973). An interesting possibility is that PF4 may activate an anion channel that is permeable to these intracellular carboxylic acids.

We propose that the action of PF4 on the *Ascaris* muscle cells is mediated by a novel and potent inhibitory mechanism. The closely structurally related peptide, PF1 (SDPNFLRFamide), also has potent inhibitory actions on *Ascaris* muscle. However, in contrast to PF4, the hyperpolarization is smaller, not associated with an increase in input conductance and not chloride-dependent (Franks *et al*., 1994).

This brings to six the number of peptides with potent actions on *Ascaris* muscle. AF1 (KNEFIRFamide; Cowden *et al*., 1989) and PF3 (KSAYMRFamide; Maule *et al*., 1994) are excitatory. AF2 (KHEYLRFamide; Cowden & Stretton, 1993; Franks *et al*., 1994; Pang *et al*., 1995) has a biphasic action with the predominant effect being an increase in phasic contractions. PF1 (SDPNLRFamide; Geary *et al*., 1992; Franks *et al*., 1994; Holden-Dye *et al*., 1995), PF2 (SDPNFLRFamide; Geary *et al*., 1992; Holden-Dye *et al*., 1995) and PF4 are inhibitory.

In conclusion, PF4 is the first peptide to be described to have such a marked and potent inhibitory action on *Ascaris* muscle. It remains to be established whether or not PF4 is present in the *Ascaris* motonervous system. In this respect it is of interest to note that a structurally similar peptide, SGKPTFIRFamide, has recently been isolated from *Ascaris* (Cowden & Stretton, 1995) and we are currently assessing whether this is likely to be the endogenous ligand for this peptidergic response in *Ascaris*.

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