The effects of the peptides AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) on the somatic muscle of the parasitic nematodes *Ascaris suum* and *Ascaridia galli*

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SUMMARY

AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) are endogenous RFamide-like peptides isolated from the parasitic nematode *Ascaris suum*. Here the actions of these peptides on the somatic musculature of *Ascaris* have been investigated and compared to the action of acetylcholine (ACh), the excitatory transmitter at the neuromuscular junction. ACh, AF3 and AF4 contracted muscle with EC_{50}s of 13 ± 1 μM, 24 ± 6 nM and 37 ± 2 nM, respectively ($n = 6$). The muscle cells were depolarized by ACh (3 μM; $5{\cdot}2 \pm 0{\cdot}4$ mV, $n = 42$), AF3 (1 μM; $2{\cdot}6 \pm 0{\cdot}3$ mV, $n = 19$) and AF4 (1 μM; $3{\cdot}3 \pm 0{\cdot}4$ mV, $n = 19$). EC_{50}s were 681 ± 329 nM (AF3) and 901 ± 229 nM (AF4), but an estimate could not be made for ACh due to muscle contraction at concentrations greater than 10 μM. The depolarization to 3 μM ACh was abolished by the nicotinic receptor antagonist mecamylamine (10 μM; $n = 5$) but the responses to the peptides were not (111 ± 7% and 108 ± 17% with respect to control; $n = 5$). The depolarization elicited by ACh was reduced to a greater extent by a 50% reduction in extracellular Na^+ concentration than the response to AF3 and AF4 ($P < 0{\cdot}02$). Cobalt was more effective at blocking the AF3 and AF4 depolarizations than those to ACh. These observations suggest that AF3 and AF4 contract *Ascaris* muscle without an action at the *Ascaris* nicotinic receptor. Furthermore, the ionic mechanism through which AF3 and AF4 depolarize *Ascaris* muscle is different from that for ACh. ACh, AF3 and AF4 were also found to contract *Ascaridia galli* somatic muscle with EC_{50}s of 13 ± 3 μM, 721 ± 236 nM and 371 ± 177 nM, respectively ($n = 7$). The muscle cells were depolarized by ACh ($EC_{50} = 14 \pm 5$ μM, $n = 5$), AF3 ($EC_{50} = 5 \pm 3$ μM, $n = 4$) and AF4 ($EC_{50} = 10 \pm 5$ μM, $n = 4$). Therefore the response to these peptides is not unique to *Ascaris* and they may subserve a functional role in the motor nervous system of parasitic nematodes.

Key words: acetylcholine, *Ascaridia*, *Ascaris*, FaRPs, nematode.

INTRODUCTION

The motor nervous system of the parasitic nematode *Ascaris suum* has been well described (see Stretton *et al*. (1985) for review). The activity of the somatic musculature is controlled by excitatory, cholinergic motoneurones (Johnson & Stretton, 1985), which release acetylcholine, activate a nicotinic non-selective cation channel similar to mammalian nicotinic receptors (Colquhoun, Holden-Dye & Walker, 1991) and thereby cause muscle contraction, and inhibitory GABAergic motoneurones (Johnson & Stretton, 1987) which release GABA (gamma-aminobutyric acid), activate a chloride channel, similar in some respects to the mammalian $GABA_A$ receptor (Holden-Dye *et al*. 1989) and cause muscle relaxation. Furthermore, the presence of neuropeptide-like immunoreactivity in the motoneurones (Cowden *et al*. 1993) indicated that neuropeptides are likely to coexist with ACh and GABA in the motoneurones, and may modulate synaptic transmission in the motor nervous system.

Recent progress has been made in elucidating the role of neuropeptides in the control of the somatic musculature of *Ascaris* following the biochemical isolation of a number of peptides belonging to the FMRFamide family (the FaRPs) from *Ascaris* peptide extracts. The first of these, AF1 and AF2, for '*Ascaris suum* FMRFamide-like' (Cowden, Stretton & Davis, 1989; Cowden & Stretton, 1993), are heptapeptides with sequences KNEFIRFamide and KHELYLRFamide, respectively. Both have potent effects on *Ascaris* motility (Cowden, Stretton & Davis, 1989) and AF2 has been shown to have both pre-synaptic and post-synaptic actions at the neuromuscular junction (Pang, Holden-Dye & Walker, 1992; Pang *et al*. 1995). Cowden & Stretton (1995) isolated 8 further FaRPs from *Ascaris* including AF3 (AVPGVLRFamide) and AF4 (GDV PGVLRFamide), the latter of which in preliminary experiments was shown to contract the dorsal muscle strip preparation of *Ascaris*. In this study these observations have been extended to investigate both

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the pharmacology and ionic mechanisms involved in the responses of somatic muscle to these peptides.

In addition to investigating the effects of AF3 and AF4 upon *Ascaris*, we have also studied their bioactivity in another parasitic nematode, *Ascaridia galli*. This additional approach was adopted to obtain insight into the conservation of peptidergic signalling mechanisms in the motor nervous system between similar parasites of medical and veterinary importance.

MATERIALS AND METHODS

Ascaris suum were obtained from a local abattoir and maintained for up to 7 days in artificial perienteric fluid (APF, composition in mM: NaCl 67, CH_3COONa 67, $CaCl_2$ 3, $MgCl_2$ 15·7, KCl 3, Trizma® base 5, pH 7·6 with glacial acetic acid, 3 mM glucose at 37 °C). *Ascaridia galli* were obtained from experimentally infected fowl (*Gallus gallus*, ♀ brown leghorns), cultured by a method adapted from Isaac *et al*. (1991). They were maintained in APF for up to 48 h.

In vitro *muscle strip tension measurements*

Ascaris dorsal muscle strips (DMS) were prepared by dissecting a 1 cm strip of the body wall muscle immediately anterior to the genital pore of a large (20 cm) female animal. This was obtained by cutting lengthwise along both lateral cords and discarding the ventral portion. Any remaining intestine still attached to somatic muscle cells was carefully removed. The remaining muscle preparation contained the dorsal muscle field, one or two cut commissures and the dorsal cord. It is devoid of motoneurone somata, which are only present in the ventral nerve cord. It does, however, contain the terminals of dorsal inhibitory and dorsal excitatory motoneurones which innervate the muscle and also have reciprocal synapses with each other.

Ascaridia dorsal muscle strips were prepared in a similar manner as for *Ascaris*, except that the 1 cm strip consisted of a greater proportion of the entire worm.

Effects on resting tension and phasic activity of the DMS were investigated by securing the muscle strips in a 15 ml organ bath and connecting them by thread to an isometric transducer. The preparation was subjected to a 1 g (*Ascaris*) or 100 mg (*Ascaridia*) load and maintained at 37 °C or 42 °C, respectively. Drugs were added in volumes less than 5% of the bath volume. Rapid mixing of the drugs in the organ bath was ensured by gassing the bath with room air for 30 sec. Drugs were added to the bath for either 30 sec (ACh) or 90 sec (peptides) unless stated otherwise. Drugs were then washed out by at least 3 times the bath volume of APF. A hard copy of the data was obtained on a flat bed chart recorder (BBC, Goerz Metrawatt, Austria).

Electrophysiological studies

Two-microelectrode recording was adopted for electrophysiological studies on *Ascaris* somatic muscle cells. The DMS was pinned cuticle side down on a Sylgard® elastomer 184 (Dow Corning, Wiesbaden, Germany) lined Perspex chamber and continuously perfused with APF at 32–34 °C directed at the cell in question *via* a fine bore tube. The temperature was necessarily lower than that adopted in the organ bath experiments to limit the spontaneous muscle contractions which would cause the cell to dislodge the recording electrodes (Wann, 1987; Franks *et al*. 1994). Individual muscle cell bags close to the nerve cord were impaled with 2 microelectrodes containing 10 mM KCl in 4 M CH_3COOK (10–30 MΩ) connected to an Axoclamp 2A amplifier. One electrode was used to record membrane potential, and the second to pass current pulses (20 nA, 0·2 Hz, 500 msec). The chamber was grounded using a 3 M KCl agar bridge/AgCl electrode. Input resistance could be estimated from the electrotonic potential resulting from current injection. Drugs were added in the perfusate for 60 sec, unless otherwise stated. Bath temperature was continuously monitored with a temperature probe placed adjacent to the muscle strip. Hard copy of data was recorded on a Gould model 35, 2-channel chart recorder (Gould Instruments, Ohio, USA). A similar approach was used to record from *Ascaridia* muscle cells except that only 1 electrode, for recording membrane potential, was used, due to the small size of the cells (50 μm diameter).

Drugs

Drugs were all prepared fresh on the day of the experiment in APF except for peptides which were stored at −20 °C in 10^{-2} or 10^{-3} M 100 μl aliquots, prepared in distilled water. AF3 and AF4 were supplied at greater than 90% purity by Alta Bioscience Ltd, Birmingham, UK. All other drugs were obtained from Sigma Chemical Co., Poole, UK, except mecamylamine hydrochloride (Merck Sharp & Dohme Research, NJ, USA).

Data analysis

The concentration–response relationships for ACh, AF3 and AF4 were obtained and analysed as follows. For each muscle strip or muscle cell, a concentration–response curve was first obtained for ACh. A concentration–response curve was then obtained for either AF3 or AF4 on the same preparation. The data for individual experiments were then normalized with respect to the maximal response obtained to ACh, except for the electrophysiological recordings from *Ascaris* muscle where the responses were normalized with respect to the response obtained by 3 μM ACh. Concentration response curves were

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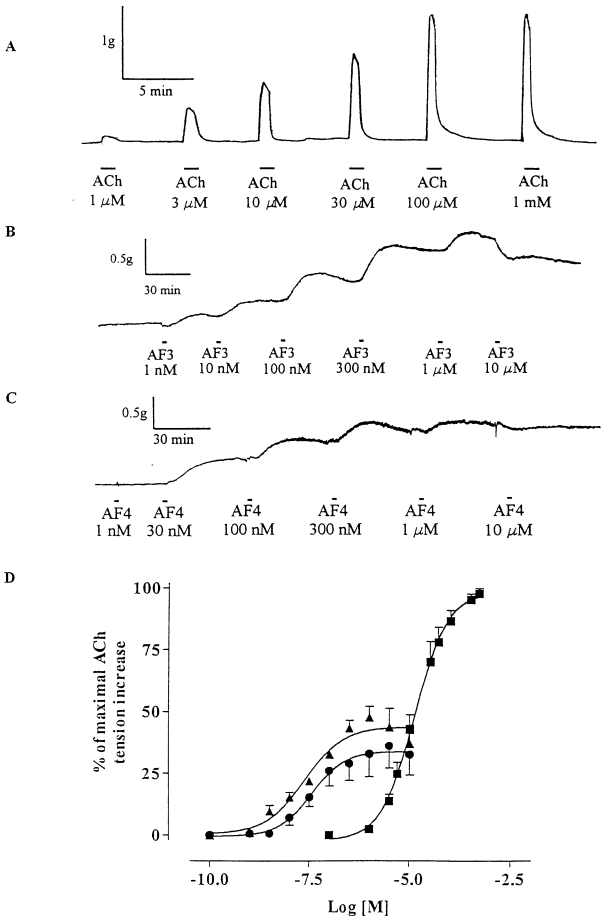

Fig. 1. Representative concentration–response relationship for ACh (A), AF3 (B) and AF4 (C) recorded from the *Ascaris* DMS. The bars indicate duration of drug application. These examples are from different worms. (D) Concentration–response relationship for ACh (■; $n = 12$), AF3 (▲; $n = 6$) and AF4 (●; $n = 6$). The results are the mean ± S.E. mean.

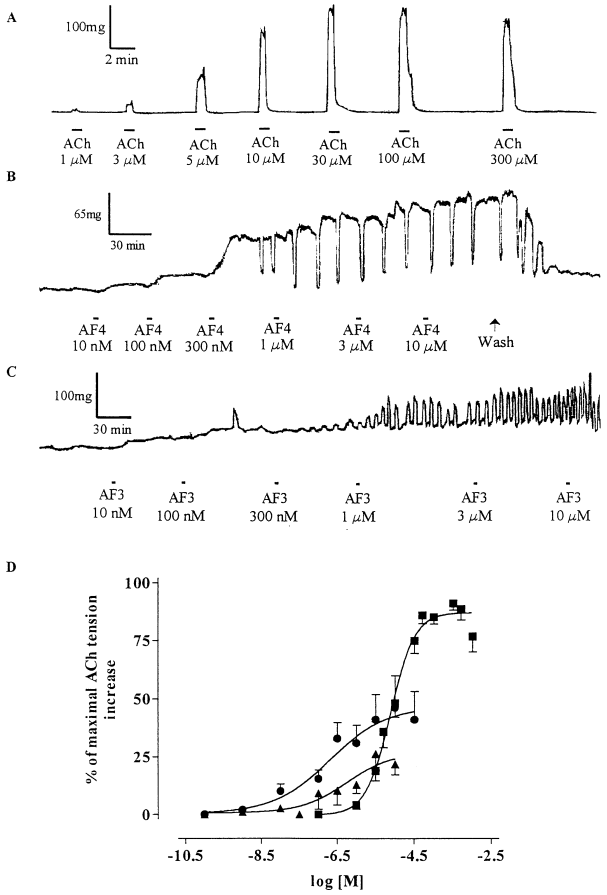

Fig. 2. Representative concentration–response relationship for ACh (A), AF4 (B) and AF3 (C) recorded from the *Ascaridia* DMS. The bars indicate duration of drug application. These examples are from different worms. (D) Concentration–response relationship for ACh (■; $n = 19$), AF3 (▲; $n = 7$) and AF4 (●; $n = 7$). The results are the mean ± S.E. mean.

fitted to the modified logistic equation (response/maximum response = [drug]/[drug] + equilibrium dissociation constant) using GraphPad Prism (version 2; San Diego, California, USA). The results are expressed as mean ± S.E. mean of the normalized data. Significant difference for normalized data was tested using the Mann–Whitney test (two-tailed) and significance was assumed at $P < 0{\cdot}05$. Where stated in the text, the results were not normalized and the Student's *t*-test (two-tailed and either paired or unpaired as appropriate) was used to assess significance.

RESULTS

Concentration response relationships for ACh, AF3 and AF4 on Ascaris *and* Ascaridia *muscle tension using the dorsal muscle strip (DMS) preparation*

The *Ascaris* DMS responded to the excitatory transmitter ACh with a concentration-dependent tension increase (EC_{50} 13 ± 1 μM, $n = 12$), in a similar manner to that previously reported (Baldwin & Moyle, 1949; Fig. 1). AF3 and AF4 also elicited a concentration-dependent contraction, EC_{50} values of 24 ± 6 nM ($n = 6$) and 37 ± 2 nM ($n = 6$), respectively (Fig. 1). Although AF3 and AF4 elicited responses at a lower concentration compared to ACh, the maximum tension increases were significantly less being 48 ± 4% ($P < 0{\cdot}05$) and 36 ± 9% ($P < 0{\cdot}01$) of the maximum ACh response. Concentrations of 10 μM ACh, 1 μM AF3 and 1 μM AF4 elicited the same increase in muscle tension, although the mean duration of the response to the peptides was significantly longer than that for ACh (ACh, 118 ± 12 sec; AF3, 3450 ± 272 sec, $P < 0{\cdot}001$, $n = 14$; AF4, 4329 ± 222 sec, $P < 0{\cdot}001$, $n = 13$; unpaired *t*-test).

In addition to the strong tonic contraction elicited by 1 μM AF3 and AF4, 6 out of 14 (AF3) and 5 out of 16 (AF4) DMS responded with an increase in amplitude and frequency of phasic contractions. This response was not consistent and the basis of this response has not been investigated further here.

The *Ascaridia* DMS responded to ACh with a concentration-dependent contraction, EC_{50} of 13 ± 3

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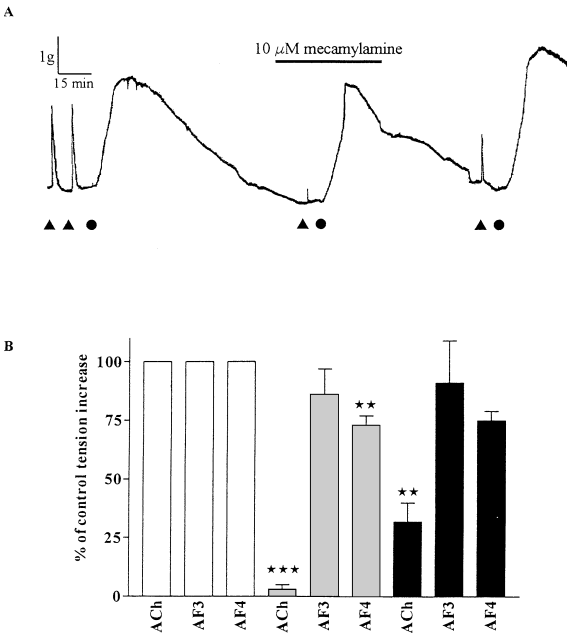

Fig. 3. The effect of 10 μM mecamylamine upon the contractile response of the *Ascaris* DMS to 10 μM ACh ($n = 15$) compared with 1 μM AF3 ($n = 7$) and 1 μM AF4 ($n = 8$). (A) Representative recording of the effect of mecamylamine on 10 μM ACh (▲) and 1 μM AF3 (●) contractions. Each preparation was used to study the effect of mecamylamine on either AF3 or AF4 in comparison with ACh. Similar recordings were obtained when using AF4. (B) Contractions were obtained for ACh, AF3 and AF4 (□). Mecamylamine was added to the muscle for 10 min prior to ACh, AF3 and AF4 (▨) and washed off for 30 min before subsequent application of ACh, AF3 and AF4 (■). The results are the mean ± S.E. mean. Level of significance with respect to control: ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

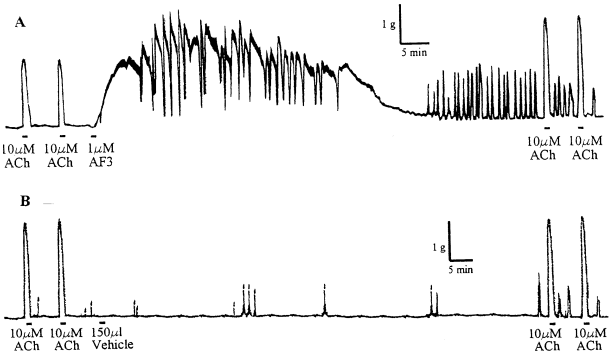

Fig. 4. The effect of 1 μM AF3 treatment upon 10 μM ACh contractions recorded from the *Ascaris* DMS. (A) Consecutive tension recordings from *Ascaris* DMS. The bars indicate duration of drug application. The 10 μM ACh contractions were significantly potentiated by pre-treatment with 1 μM AF3 with respect to parallel controls ($n = 7$, $P < 0{\cdot}05$). Similar responses were obtained when using AF4. (B) Control DMS taken from the same worm as (A); 150 μl APF was added in place of AF3.

μM ($n = 19$). Not all preparations responding to ACh were found to respond to the peptides. Of 13 strips 6 did not respond to AF3, although all 13 responded to ACh. Also, 3 of 10 strips did not respond to AF4 although all 10 responded to ACh (Fig. 2). Due to the long duration of the response to AF3 and AF4, the muscle tension did not return to baseline between the peptide additions. The contractions elicited by AF3 and AF4 were 26 ± 7 % and 46 ± 14 %, respectively, of the maximum ACh response. EC_{50} values were 371 ± 177 nM (AF4) and 721 ± 236 nM (AF3).

Similar to the *Ascaris* DMS, both peptides could elicit an increase in the propagation of phasic spontaneous activity in *Ascaridia*. This response was observed in 3 out of 7 preparations for AF3 and 2 out of 7 preparations for AF4. However, this response was not consistent and has not been investigated further here.

Effects of the nicotinic antagonist mecamylamine on Ascaris *muscle tension*

The nicotinic receptor antagonist, mecamylamine (10 μM), an antagonist of the ACh receptor on *Ascaris* muscle (Colquhoun, Holden-Dye & Walker, 1990), had no consistent effect on resting muscle tension. At this concentration it almost completely and reversibly blocked contractile responses to 10 μM ACh ($P < 0{\cdot}0001$; $n = 15$; Fig. 3) as previously reported (Pang, Holden-Dye & Walker, 1992). In contrast, mecamylamine (10 μM) caused a small (but not significant) reduction in the contraction in response to 1 μM AF3 to 81 ± 11 % compared to the response before the addition of mecamylamine ($P > 0{\cdot}05$; $n = 7$) and a significant, but not complete reduction in the contraction to AF4 to 73 ± 4 % compared to the response before the addition of mecamylamine ($P < 0{\cdot}01$; $n = 8$). However, neither of these reductions of peptide contraction was reversed upon washout of the antagonist.

The potentiation of ACh contractions by AF3 and AF4 in Ascaris *muscle*

Muscle contractions caused by 10 μM ACh were significantly potentiated by pre-treatment of the muscle strips with AF3 or AF4. For these experiments, consistent responses to 10 μM ACh were obtained. The DMS were then exposed to either 1 μM AF3 or AF4 for 1 min. The peptides were washed out and the response to ACh was determined again once the tension had returned to baseline. The response of the muscle to ACh was 127 ± 11 % compared to the response before the addition of AF3

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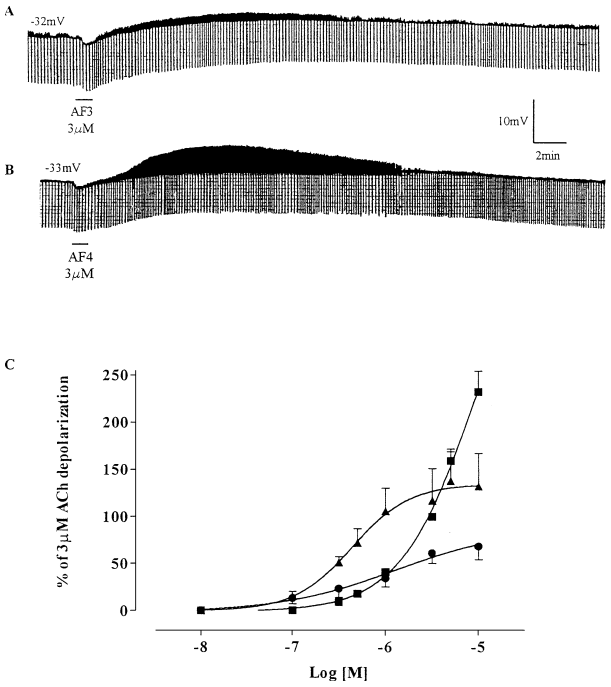

Fig. 5. Effects of ACh, AF3 and AF4 recorded from *Ascaris* muscle cells. (A and B) Representative response to 3 μM AF3 and AF4. Both peptides elicited a transient hyperpolarization followed by a depolarization and an increase in spontaneous electrical activity. The bars indicate duration of peptide application. (C) Concentration response relationship for ACh (■; $n = 11$), AF3 (▲; $n = 7$) and AF4 (●; $n = 5$). The results are the mean ± S.E. mean.

($P < 0{\cdot}05$; $n = 7$) and 157 ± 22 % compared to the response before the addition of AF4 ($P < 0{\cdot}05$; $n = 6$; Fig. 4).

Concentration–response relationships for ACh, AF3 and AF4 on Ascaris *and* Ascaridia *muscle : electrophysiological studies*

AF3 and AF4 elicited a small initial transient hyperpolarization of *Ascaris* muscle cells. The hyperpolarization to 3 μM peptide in 19 different preparations was 0·7 ± 0·1 mV (AF3) and 0·5 ± 0·1 mV (AF4; Fig. 5A and B). This was followed by a concentration-dependent depolarization of duration 1284 ± 112 sec (AF3) and 1358 ± 86 sec (AF4). The depolarization was accompanied by an increase in spontaneous activity (Fig. 5A and B). There were no significant effects upon input conductance in response to either peptide. Resting conductance was 1·34 ± 0·11 μS; and with 1 μM AF3, 1·37 ± 0·12 μS ($n = 14$, $P > 0{\cdot}05$; Student's t-test). Resting conductance was 1·40 ± 0·07 μS, and with 1 μM AF4, 1·43 ± 0·08 μS ($n = 14$, $P > 0{\cdot}05$; Student's t-test). Concentration–response curves were constructed for AF3 (EC_{50} 681 ± 329 nM, $n = 7$), AF4 (EC_{50} 901 ± 229 nM, $n = 5$) and ACh ($n = 11$; Fig. 5C). The EC_{50} value for ACh was not estimated as submaximal

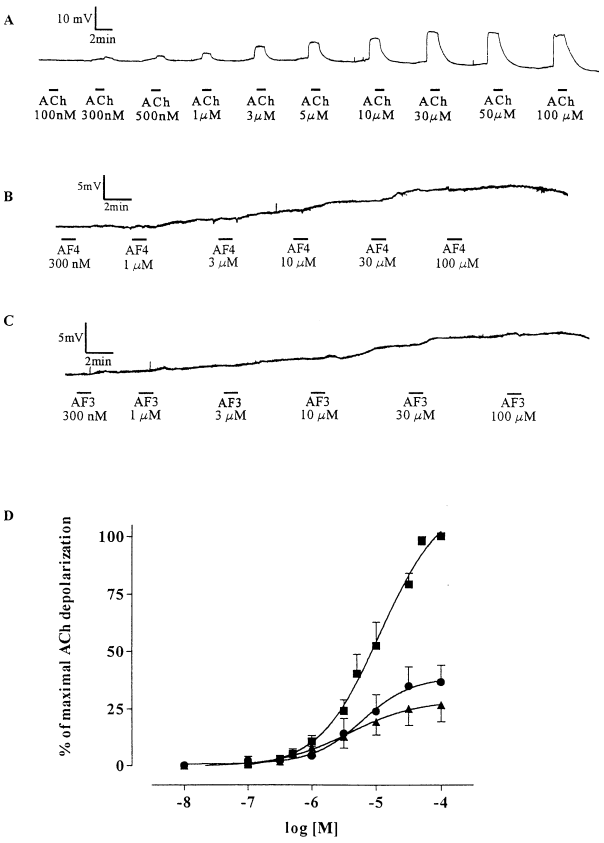

Fig. 6. Representative concentration–response relationship for ACh (A), AF4 (B) and AF3 (C) recorded from *Ascaridia* muscle cells. The bars indicate duration of drug application. These examples are from different worms. (D) Concentration–response relationship for ACh (■; $n = 5$), AF3 (▲; $n = 4$) and AF4 (●; $n = 4$). The results are the mean ± S.E. mean.

concentrations greater than 10 μM were found consistently to dislodge recording electrodes, in a similar manner to that reported by Colquhoun *et al.* (1991).

Ascaridia muscle cells responded to ACh with a concentration-dependent depolarization as previously described (Wann, 1987; Fig. 6). Recording from *Ascaridia* was found to be more stable than *Ascaris* to high concentrations of ACh and therefore a full concentration–response curve was constructed (EC_{50} 14 ± 5 μM; $n = 5$). Both peptides elicited a concentration-dependent depolarization (Fig. 6), with EC_{50} values of 5 ± 3 μM (AF3; $n = 4$) and 10 ± 5 μM (AF4; $n = 4$). In no preparation was an initial hyperpolarization, or propagation of phasic electrical activity observed.

Effects of the nicotinic antagonist mecamylamine on Ascaris *muscle cell responses*

Mecamylamine (10 μM) completely abolished the depolarization in response to 3 μM ACh ($n = 12$) but did not significantly affect the response to either 1 μM AF3 ($n = 6$; $P > 0{\cdot}05$) or 1 μM AF4 ($n = 6$;

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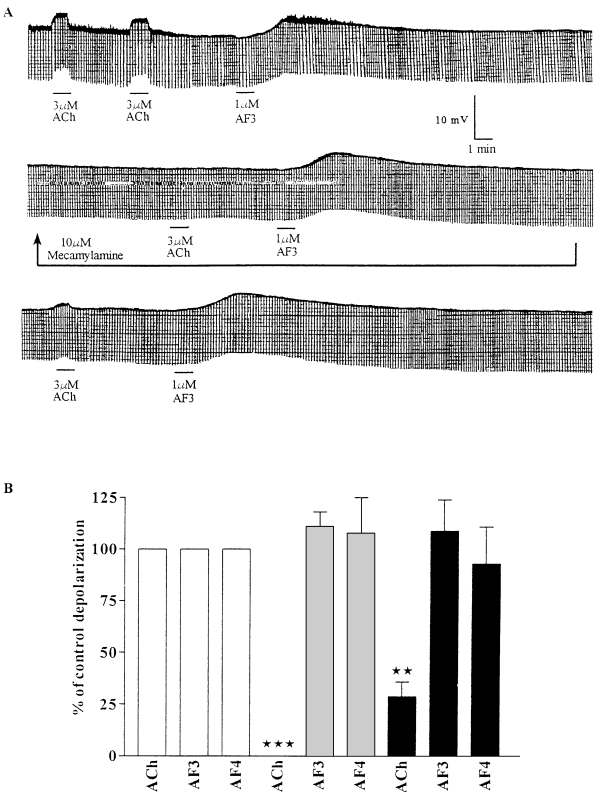

Fig. 7. The effect of 10 μM mecamylamine upon *Ascaris* muscle cell depolarizations in response to 3 μM ACh ($n = 12$) compared with 1 μM AF3 ($n = 6$) and 1 μM AF4 ($n = 6$). (A) Representative recordings (from the same cell) of the effect of mecamylamine on depolarizations by 3 μM ACh and 1 μM AF3. Each preparation was used to study the effect of mecamylamine on either AF3 or AF4 responses in comparison with ACh. The bars indicate duration of drug application. Similar recordings were obtained when using AF4. (B) Depolarizations were obtained for ACh, AF3 and AF4 (□). Mecamylamine was added to the muscle for 10 min prior to ACh, AF3 and AF4 (▨) and washed off for 30 min before subsequent application of ACh, AF3 and AF4 (■). The results are the mean ± S.E. mean. Level of significance with respect to control: ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

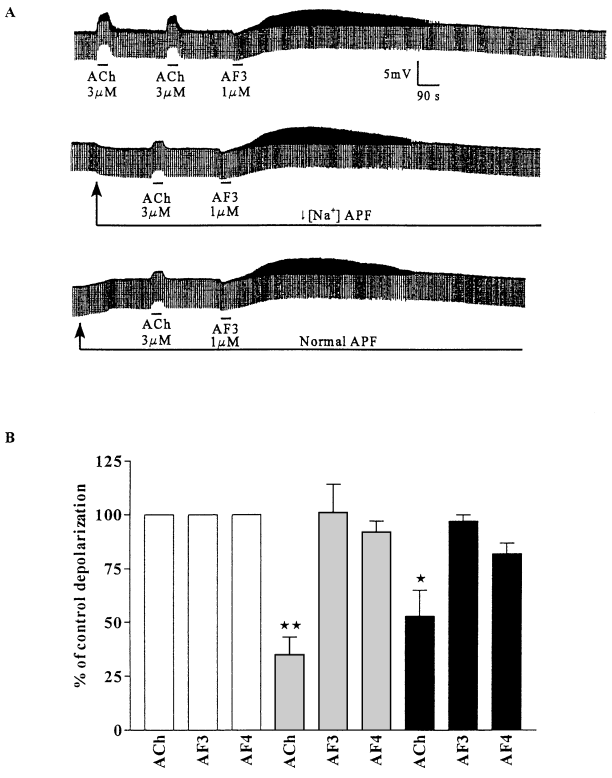

Fig. 8. The effect of reducing extracellular sodium concentration by 50 % (67 mM total) upon *Ascaris* muscle cell depolarizations in response to 3 μM ACh ($n = 11$) compared with 1 μM AF3 ($n = 6$) and 1 μM AF4 ($n = 5$). (A) Representative recordings (from the same cell) of the effect of low sodium on depolarizations by 3 μM ACh and 1 μM AF3. Each preparation was used to study the effect of low sodium on either AF3 or AF4 responses in comparison with ACh.The bars indicate duration of drug application. Similar recordings were obtained when using AF4. (B) Depolarizations were obtained for ACh, AF3 and AF4 (□). Low sodium was added to the muscle for 10 min prior to ACh, AF3 and AF4 (▨) and washed off for 30 min before subsequent application of ACh, AF3 and AF4 (■). The results are the mean ± S.E. mean. Level of significance with respect to control: * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

$P > 0{\cdot}05$). There was no observable effect on the nicotinic antagonist upon resting membrane potential or input conductance (Fig. 7A).

The ionic basis of the AF3 and AF4 response in Ascaris *muscle cells*

In order to investigate the ionic mechanism for the response to AF3 and AF4, and to compare it to that for ACh, the responses to ACh and the peptides were studied in different extracellular solutions. For each of these sets of experiments consistent responses to 3 μM ACh were first obtained in APF. To investigate the role of Na^+, extracellular Na^+ was reduced to 67 mM by replacing NaCl with glucosamine hydrochloride. This modified medium was found to cause a hyperpolarization of $2{\cdot}1 \pm 0{\cdot}3$ mV ($n = 11$) immediately upon addition, with no consistent effect upon input conductance. This low Na^+ APF significantly attenuated the depolarization caused by 3 μM ACh to 35 ± 5 % compared to that in APF containing a normal sodium concentration ($n = 11$; $P < 0{\cdot}0001$) but did not significantly affect the response to 1 μM AF3 ($n = 6$; $P > 0{\cdot}05$) or AF4 ($n = 5$; $P > 0{\cdot}05$; Fig. 8). There was no observable effect of low Na^+ APF upon the initial hyperpolarization in response to either peptide (Fig. 8A).

Cobalt chloride, a selective Ca^{2+} channel blocker (Hille, 1984), was added to normal APF to examine the contribution of extracellular Ca^{2+} to the responses. There was no direct effect upon membrane potential at 1 mM ($n = 9$; $P > 0{\cdot}05$; Student's t-test) or 10 mM ($n = 8$; $P > 0{\cdot}05$, Student's t-test). In these cells there was no significant change in input conductance at 1 mM ($P > 0{\cdot}05$; Student's t-test),

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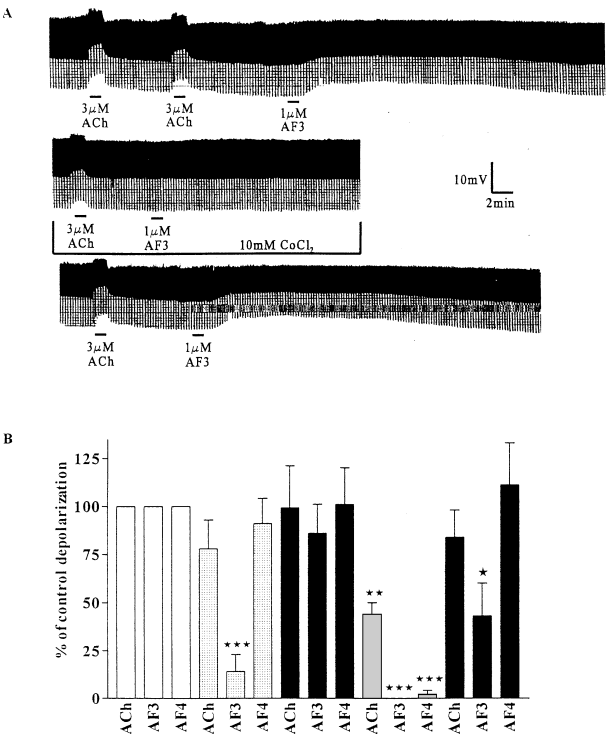

Fig. 9. The effect of 1 μM and 10 mM $CoCl_2$ upon *Ascaris* muscle cell depolarizations in response to 3 μM ACh compared with 1 μM AF3 and 1 μM AF4. (A) Representative recordings (from the same cell) of the effect of 10 mM $CoCl_2$ on depolarizations by 3 μM ACh and 1 μM AF3. Each preparation was used to study the effect of 1 mM or 10 mM $CoCl_2$ on either AF3 or AF4 responses in comparison with ACh. The bars indicate duration of drug application. Similar recordingss were obtained when using AF4. (B) Depolarizations were obtained for ACh, AF3 and AF4 (□). 1 mM $CoCl_2$ (⚄) or 10 mM $CoCl_2$ (▩) was perfused for 5 min prior to application of ACh, AF3 and AF4 and washed off for 30 min before subsequent application of ACh, AF3 and AF4 (■). The results are the mean ± S.E. mean. Level of significance with respect to control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

but at 10 mM there was a small but significant decrease of $0{\cdot}2 \pm 0{\cdot}1$ μS ($P < 0{\cdot}01$; Student's *t*-test). Cobalt chloride at 1 mM was found to reduce significantly the response to both 3 μM ACh ($75 \pm 8\%$ of control; $n = 6$; $P < 0{\cdot}05$) and 1 μM AF3 ($14 \pm 9\%$ of control; $n = 6$; $P < 0{\cdot}005$; Fig. 9) compared to the response in APF containing no cobalt. The difference between these two reductions was also significant ($P < 0{\cdot}05$). Cobalt chloride at 10 mM was found to reduce significantly the depolarization to 3 μM ACh to $27 \pm 9\%$ ($n = 5$; $P < 0{\cdot}0001$) and completely abolished the response to 1 μM AF3 compared to the responses in APF containing no cobalt. There was a significant difference between these two levels of blockade ($P < 0{\cdot}005$). The effect of cobalt was partly reversed on return to normal APF (Fig. 9).

In a further series of experiments, 1 mM cobalt chloride was found to have no significant effect upon the response to either 1 mM AF4 ($n = 5$; $P > 0{\cdot}05$) or 3 mM ACh ($n = 5$; $P > 0{\cdot}05$) compared to the response in APF containing no cobalt. However, at 10 mM there was a significant reduction in both the ACh and AF4 responses to $60 \pm 3\%$ ($n = 5$; $P < 0{\cdot}01$) and $2 \pm 2\%$ ($n = 5$; $P < 0{\cdot}0001$) respectively, compared to the responses in APF containing no cobalt (Fig. 9). The difference between these two reductions was also significant ($P < 0{\cdot}005$). The effect of cobalt chloride was reversed on return to normal APF to values not significantly different from those obtained in APF containing no cobalt at the beginning of the experiment, in all cases.

DISCUSSION

Over the last few years it has become increasingly apparent that the somatic musculature of *Ascaris* is subject to modulation by a number of neuropeptides belonging to the FaRP family. For example, PF1 (SDPNFLRFamide), PF2 (SADPNFLRFamide) and PF4 (KPNFIRFamide) are all potent inhibitors of *Ascaris* muscle and the action of these peptides involves at least 2 distinct mechanisms (Holden-Dye *et al*. 1995; Holden-Dye, Brownlee & Walker, 1996; Brownlee *et al*. 1996). AF1 and AF2 have a predominantly excitatory effect (Cowden *et al*. 1989; Cowden & Stretton, 1993), and for AF2 this involves a marked and prolonged stimulation of phasic contractions (Pang *et al*. 1995). Two further endogenous *Ascaris* peptides, AF3 and AF4, are structurally related N-terminally extended FaRPs sharing a common LRFamide C-terminal with AF2. The effect of AF4 on *Ascaris* muscle tension was observed by Cowden & Stretton (1995) to consist of a potent and strong tonic contraction.

This study has focused on the increase in muscle tension elicited by AF3 and AF4 and the muscle depolarization which accompanies these responses. However, it should be noted that the responses to both AF3 and AF4 are complex compared to the rapidly reversible tonic contraction and depolarization caused by the 'classical' transmitter ACh. For example, in the muscle tension experiments on *Ascaris* the tonic contraction elicited by the peptides was followed by an increase in phasic activity. Furthermore, in the electrophysiological experiments on *Ascaris*, although the dominant feature of the response was a long-lasting depolarization, this was preceded by a small, transient hyperpolarization. This complexity could represent either divergent signalling pathways following activation of a single type of peptide receptor, or interaction of the peptide with multiple subtypes of peptide receptor and remains to be investigated.

Here we have addressed 3 key questions relating to the excitatory action of AF3 and AF4 on the somatic musculature of parasitic nematodes. First, how does

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the potency and efficacy of these peptides at increasing muscle tension compare to that of the excitatory neuromuscular junction transmitter ACh? Second, can the response to the peptides, in whole or part, be due to the release of ACh from the excitatory motoneurones? Lastly, how do the responses of the somatic musculature to AF3 and AF4 compare for two different species of nematode, namely, *Ascaris* and *Ascaridia*? The aim of this latter approach was to assess to what extent there is functional evidence for the conservation of peptidergic signalling mechanisms in the motor nervous system of nematodes.

Qualitatively and quantitatively the contractions and depolarizations to ACh and peptides were dissimilar. For example, the effect of ACh on muscle tension and muscle membrane potential was rapid in onset and offset as may be predicted considering that its effect is mediated by a fast acting ligand-gated ion channel, the nicotinic receptor. Conversely, the peptide responses had a relatively slow time-course. This is consistent with the involvement of a G-protein coupled receptor in mediating the response to the peptides. Furthermore, in electrophysiological recordings in *Ascaris*, there was a qualitative difference in the response to ACh and the peptides. The ACh response consisted of a fast depolarization whereas the peptide responses were biphasic events, consisting of a small, transient hyperpolarization which preceded the sustained depolarization. The same series of experiments demonstrated that both AF3 and AF4 either increased, or in quiescent preparations initiated, the generation of action potentials. This would suggest a post-synaptic site of action of these peptides, the mechanism of which has yet to be elucidated. A similar effect has been observed with AF2 (Pang *et al*. 1995).

Both AF3 and AF4 were effective in *Ascaris* at much lower concentrations than ACh. The threshold for responses to the peptides was in the nanomolar range compared to micromolar for ACh. However, the maximal contraction elicited by AF3 and AF4 was about half of that for ACh. Similarly, the maximal depolarizations caused by AF3 and AF4 were also about half of that for ACh. The EC_{50} values for both peptides were consistently less for increasing muscle tension compared to the values obtained from electrophysiological studies indicating that a maximal contraction can be elicited by a submaximal depolarization, and consistent with the hypothesis that there are 'spare' receptors for AF3 and AF4 in the muscle. A further consideration is that the peptide-induced depolarization may not be directly responsible for the corresponding tension increase. This possibility stems from the observations that 10 nM of either peptide induces a near maximal tension increase in the *Ascaris* muscle strip, yet the same concentration is approximately threshold for depolarization. Furthermore, the concentration response relationships for both peptide contractions in *Ascaris* do not significantly differ. The corresponding relationship for depolarization on the other hand does show marked differences, in terms of both amplitude and shape. A possible explanation for this is that the peptides are likely to act through G-protein-coupled receptors which activate intracellular, divergent signalling pathways. One of these pathways may mediate muscle depolarization (which contributes to the contraction, but is not essential for it) and the other may independently activate muscle contractile proteins.

The nicotinic receptor antagonist mecamylamine was used to test whether the muscle response to AF3 or AF4 involved the stimulation of ACh release from excitatory motoneurones. The concentration of mecamylamine which causes a 50% reduction in the response to ACh has previously been established as $0{\cdot}41 \pm 0{\cdot}04$ μM (Colquhoun *et al*. 1991). In this study 10 μM mecamylamine blocked the increase in muscle tension and the depolarization caused by ACh in a readily reversible manner. In marked contrast, neither the contraction nor depolarization to AF3 and AF4 were blocked by mecamylamine. The only exception to this was a small but significant reduction in the AF4 tension increase. However, this reduction was not reversible and may represent a time-dependent change in the sensitivity of the muscle rather than an action of mecamylamine at nicotinic receptors. From this we conclude that AF3 and AF4 do not exert their excitatory action on the muscle by a pre-synaptic action to increase the release of ACh.

Further evidence that the response to AF3 and AF4 does not involve either stimulation of ACh release or activation of a nicotinic receptor is provided by the experiments which demonstrate that the ionic mechanism mediating the response to ACh is different from that for the peptides. In brief, the depolarization to ACh shows a greater dependence on extracellular Na^+ ions than Ca^{2+}, as would be predicted for an event mediated by a nicotinic receptor and consistent with the earlier findings of Pennington & Martin (1990). However, the magnitude of the depolarizations to AF3 and AF4 were not affected by a 50% reduction in the concentration of extracellular Na^+, although the depolarizations were abolished by the inclusion of the calcium channel blocker, cobalt, in the perfusate. These data indicate that the peptide responses have a greater dependence on extracellular Ca^{2+} than Na^+.

An additional observation with AF3 and AF4 was that, following contraction of *Ascaris* muscle caused by these peptides, the response to ACh was potentiated. A similar effect was observed with the structurally related peptide KHEYLRFamide (AF2; Pang *et al*. 1995) and it was shown that this effect is likely to represent a sensitization of the processes involved in excitation–contraction coupling. As AF3 and AF4 do not potentiate the depolarization elicited by ACh it is likely that the mechanism whereby these

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peptides potentiate the contraction to ACh also involves an increased sensitivity for excitation–contraction coupling.

AF3 and AF4 elicited qualitatively similar effects on *Ascaridia* muscle tension compared to *Ascaris* which consisted predominantly of an increase in muscle tension. However, at peptide concentrations greater than 1 μM, there was a greater propensity for an increase in spontaneous activity in *Ascaridia* compared to *Ascaris*. It is curious that not all *Ascaridia* muscle strips were found to respond to peptide (54% responded to AF3, 70% responded to AF4) even though they all did to ACh. This is an interesting observation, but one which could not be clearly explained in the current study. In the electrophysiological experiments, the actions of AF3 and AF4 on *Ascaridia* muscle consisted of a slow depolarization, similar to that observed in *Ascaris*. However, increased action potential generation was not observed, nor was the initial transient hyperpolarization that was consistently seen in response to AF3 and AF4 in *Ascaris*. The observation that both peptides have a biological action in these two species of nematode indirectly suggests that a peptide similar, or identical, to AF3 and AF4 may also be present in *Ascaridia*. Certainly this would not be without precedent, as AF2 has been isolated from *Caenorhabditis elegans* (Marks *et al*. 1995), *Panagrellus redivivus* (Maule *et al*. 1994) and *Haemonchus contortus* (Keating *et al*. 1995) as well as *Ascaris suum* (Cowden & Stretton, 1993). Although AF3 and AF4 elicit depolarization and contraction in *Ascaridia* similar to *Ascaris*, they are an order of magnitude less potent in *Ascaridia*. This may indicate that the sequence of AF3 and AF4 is not identical to the endogenous peptide in *Ascaridia*. Alternatively, it may reflect a greater degradation of AF3 and AF4 by peptidases in *Ascaridia* muscle compared to *Ascaris* muscle.

In conclusion, the observations made here clearly indicate a biological action for AF3 and AF4 in *Ascaris* and *Ascaridia* motor nervous system. This follows on from the excitatory action previously observed for AF1 and AF2 on *Ascaris* muscle (Cowden *et al*. 1989; Pang *et al*. 1992, 1995; Cowden & Stretton, 1993). The contraction caused by AF3 and AF4 is due to a post-synaptic excitation on the muscle cells, acting independently of the nicotinic ACh receptor. We suggest that the responses to AF3 and AF4 are mediated by different receptors from those that mediate the responses to AF1 and AF2. The reasons for suggesting this are 2-fold. Firstly, the responses to AF3 and AF4 are qualitatively different from those for AF1 and AF2. Secondly, the increased phasic activity caused by AF2 is blocked by mecamylamine (Pang *et al*. 1995) whereas the responses to AF3 and AF4 are not. Considering the similarity in the two peptide sequences for AF3 and AF4, and the close similarity in their biological activity it is likely that their effects are mediated through the same receptor, albeit with different affinities for the two peptides.

As with the other nematode FaRPs, the important question remains as to whether or not these peptides are present in the motoneurones and awaits the results of studies aimed at the cloning and localization of expression of peptide genes.

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