The effects of FaRPs on the motility of isolated muscle strips from the liver fluke, *Fasciola hepatica*

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SUMMARY

The effects of a range of FMRFamide-related peptides (FaRPs) on isometric contractility were tested using isolated muscle strips from the liver fluke, *F. hepatica*. The neuropeptides tested were the molluscan FaRPs, FMRFamide and FLRFamide, the turbellarian FaRPs, RYIRFamide and GYIRFamide, the cestode peptides, NPF and GNFFRFamide, and the nematode FaRPs, AF-1 (KNEFIRFamide), AF-2 (KHEYLRFamide), AF-8 (KSAYMRFamide), and PF-4 (KPNFIRFamide). Dose–response experiments were undertaken at a concentration range of 5 nM–5 μM for all of the neuropeptides tested. FMRFamide and AF-8 caused statistically significant increases in the amplitude and frequency of contractions at concentrations of 0·5 μM and 5 μM. FLRFamide and AF-2 also caused significant increases in contraction frequency at concentrations of 0·5 μM and 5 μM, although a significant increase in amplitude of contraction was observed only at a concentration of 5 μM. GYIRFamide increased both amplitude and frequency significantly at concentrations of 50 nM, 0·5 μM and 5 μM. RYIRFamide significantly increased frequency of contractions at concentrations of 0·5 μM and 5 μM, but failed to have a significant effect on contraction amplitude. AF-1 at a concentration of 5 μM increased contraction amplitude, but failed to have an effect on frequency at any of the concentrations used. PF-4 caused a statistically significant increase in both the amplitude and frequency of contractions at a concentration of 5 μM. NPF and GNFFRFamide had no effect on the *in vitro* motility of *F. hepatica* over the range of concentrations tested. The results are discussed in the light of possible structure–activity relationships in the FaRPs tested.

Key words: FMRFamide-related peptides (FaRPs), muscle strips, *F. hepatica*, neuropeptides, *in vitro* motility.

INTRODUCTION

In flatworms, a neuroendocrine role is achieved via the peptidergic component of the nervous system. Hence, the peptidergic system will be responsible for controlling and co-ordinating important processes in the liver fluke, such as growth and development, normally controlled by hormones in higher organisms. This is in addition to the putative roles of neuropeptides in neurotransmission. To date, immunoreactivities to more than 30 vertebrate and invertebrate peptides have been demonstrated in both the central and peripheral nervous systems of flatworms (for individual references see reviews by Fairweather & Halton, 1991; Halton *et al*. 1992; Fairweather & Skuce, 1995). In 1977 the peptide FMRFamide was discovered from extracts of ganglia from the clam, *Macrocallista nimbosa*, by virtue of its cardioexcitatory effect (Price & Greenberg, 1977). It is ubiquitous throughout the animal phyla and, since its discovery, numerous related novel peptides have emerged. They have been classified together under the term FaRP (**F**MRF**a**mide-**r**elated **p**eptide) (refer to reviews by Cottrell, 1989; Price & Greenberg, 1989; Greenberg & Price, 1992; Walker, 1992).

The distribution of peptide immunoreactivities in helminths has been widely studied and a number of 'native' or endogenous peptides have been isolated. Neuropeptide F (NPF) and the FaRP, GNFFRFamide have been isolated from the parasitic sheep tapeworm, *Moniezia expansa* (Maule *et al*. 1991, 1993). Several FaRPs have been isolated and structurally characterized from the parasitic nematode *Ascaris suum* (AF peptides) and also from the 2 free-living species, *Caenorhabditis elegans* (CF peptides) and *Panagrellus redivivus* (PF peptides) (Brownlee *et al*. 1996). More recently, the turbellarian FaRPs, RYIRFamide and GYIRFamide have been isolated, respectively, from *Artioposthia triangulata* (Maule *et al*. 1994*b*) and *Dugesia tigrina* (Johnston *et al*. 1995). However, very little physiological experimentation has been undertaken concerning peptides and their functions in parasites.

The aim of the present investigation was to begin to resolve the question of parasite peptide function by undertaking an initial study on one aspect of physiology, namely, neuromuscular activity. As yet no endogenous trematode peptides have been isolated and so the investigation involved the use of a

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number of endogenous helminth peptides, from both flatworms and roundworms. The latter were used to examine the potential cross-phyla activity of the peptides. Previous neurophysiological studies have involved the use of whole flukes to test the effects of classical transmitters. They have established that acetylcholine and noradrenaline may act as inhibitory neurotransmitters in the liver fluke, whilst dopamine and serotonin (5-HT) are potential excitatory transmitters (Holmes & Fairweather, 1984). The use of whole flukes presents a problem as the tegument is present on all sides, making it difficult for pharmacological agents to penetrate. The advantage in using liver fluke muscle strips, as in the present study, is that the tegument is absent from 4 of the 6 surfaces of the strip, enabling neuropeptides to penetrate more readily and gain access to receptors in the fluke. Results with muscle strips from adult flukes have been shown to be consistent with those produced using whole flukes (Tembe *et al*. 1993). In addition to the helminth peptides, the molluscan peptides FMRFamide and FLRFamide were used initially to validate the technique and to serve as comparisons for the actions of the other peptides tested. Comparisons will be made with the results of a separate study involving the effects of flatworm peptides on the activity of body strips of immature liver flukes and using a photo-optic transducer system (Marks *et al*. 1996).

The main findings from the present study have been summarized elsewhere (Fairweather *et al*. 1995).

MATERIALS AND METHODS

Adult (at least 12 weeks old) liver flukes, *Fasciola hepatica*, were recovered from the bile ducts of experimentally infected laboratory rats. The flukes were maintained at 37 °C in sterile NCTC 135 (Flow Laboratories, Irvine, Ayrshire) culture medium containing antibiotics (penicillin, 50 i.u./ml; streptomycin, 50 μg/ml) prior to use. The anterior region of the fluke was removed by a horizontal cut below the ventral sucker, or acetabulum. The posterior end and the lateral edges were trimmed, and the resultant body tissue cut longitudinally into strips, approximately 10 mm long and 3 mm wide. The strips were suspended vertically in 4 ml organ baths and attached to an isometric force transducer (Harvard Instruments, 50 g model) whose output was amplified and recorded on a chart recorder (custom built). The resting tension was set to 2 mN and the tissue strips allowed to equilibrate for 30 min.

The organ baths were maintained at 37 °C and perfused with Hédon-Fleig saline, modified from Gatenby's original formula (Gatenby, 1937) by omission of phosphate from the medium. The pH of the saline was adjusted to 7·4 using 1 M hydrochloric acid, in order to relate to experimental estimates of subtegumental pH (Tielens, Nicolay & Van der Bergh, 1982). The saline also contained 0·1 % (w/v) BSA (bovine serum albumin) (Sigma type V) in order to limit non-specific binding of the neuropeptides to the tissue.

Initially, prolonged exposure experiments using FMRFamide at a concentration observed to produce consistent effects, i.e. 5 μM, were carried out to determine the optimal exposure time for the drug. The results showed that the optimal response of the tissue occurred during the initial 5 min of exposure with obvious tachyphylaxis after this time (Ellis, unpublished observations). Hence, in all subsequent experiments drug exposure was limited to 5 min.

A series of dose–response experiments was carried out with the individual peptides. Drugs were diluted 20-fold by injection of 0·2 ml of neuropeptide and a little air (to ensure even mixing) from a 1 ml syringe, into a 4 ml organ bath. Final bath concentrations are quoted throughout. The perfusion pump was switched off 5 min prior to drug injection, and all comparisons were made between this initial 5 min control period and the 5 min after drug addition. The organ bath was perfused with fresh Hédon-Fleig saline for at least 20 min between drug additions. Each experiment was repeated a minimum of 5 times, using tissue strips derived from separate flukes.

The neuropeptides tested were FMRFamide, FLRFamide, KNEFIRFamide (AF-1), KHEYLRFamide (AF-2), KSAYMRFamide (AF-8), KPNFIRFamide (PF-4), RYIRFamide, GYIRFamide, Neuropeptide F (NPF), and GNFFRFamide. All were used at a concentration range of 5 nM–5 μM and all peptide solutions contained 0·1 % (w/v) BSA. FMRFamide and FLRFamide were obtained from Sigma, AF-1, AF-2, AF-8, PF-4 and GNFFRFamide were obtained from Alta Bioscience. The NPF (amino acid sequence YFAIIGRPRFamide), RYIRFamide and GYIRFamide were produced by the peptide synthesis facility in the School of Biology and Biochemistry, Q.U.B.

Changes in the spontaneous mechanical activity of the strips were analysed in terms of the frequency and amplitude of contractions, with mean values during the 5 min control and 5 min drug periods being compared. Only contractions whose amplitude was equal to or greater than 0·5 mN were included in the analysis.

The absolute data were analysed statistically using a single factor, repeated measures analysis of variance and Fisher's probability of least significant difference test. Differences in mean values were accepted as statistically significant at the 95 % level. The raw data were normalized as follows: for each tissue strip the concentration which elicited the greatest response was expressed as unity, with the remaining

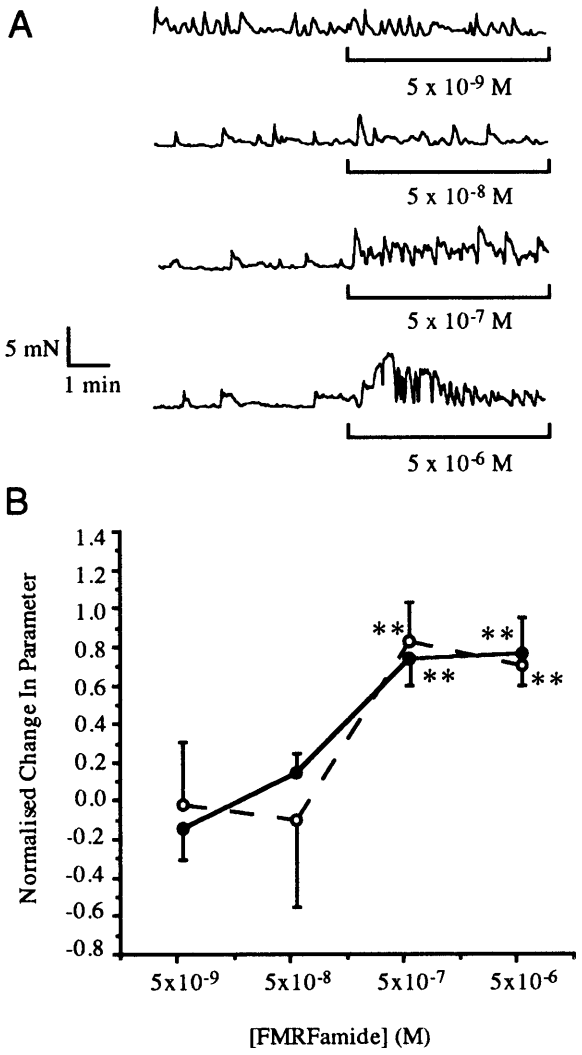

Fig. 1. (A) A trace recording from 1 muscle strip illustrating the effect of increasing FMRFamide concentration (5 nM–5 μM) on the motility of isolated muscle strips from *Fasciola hepatica*. Horizontal line indicates duration of peptide application (5 min). (B) Dose–response curve for FMRFamide at a concentration range of 5 nM–5 μM. The mean normalized changes in frequency and amplitude are plotted, $\pm$ the standard errors of the mean, against neuropeptide concentration. Frequency is shown as solid circles and lines, whilst open circles and broken lines represent amplitude. The double asterisk denotes a statistically significant change ($P < 0{\cdot}01$) for both amplitude and frequency at concentrations 5 μM and 0·5 μM.

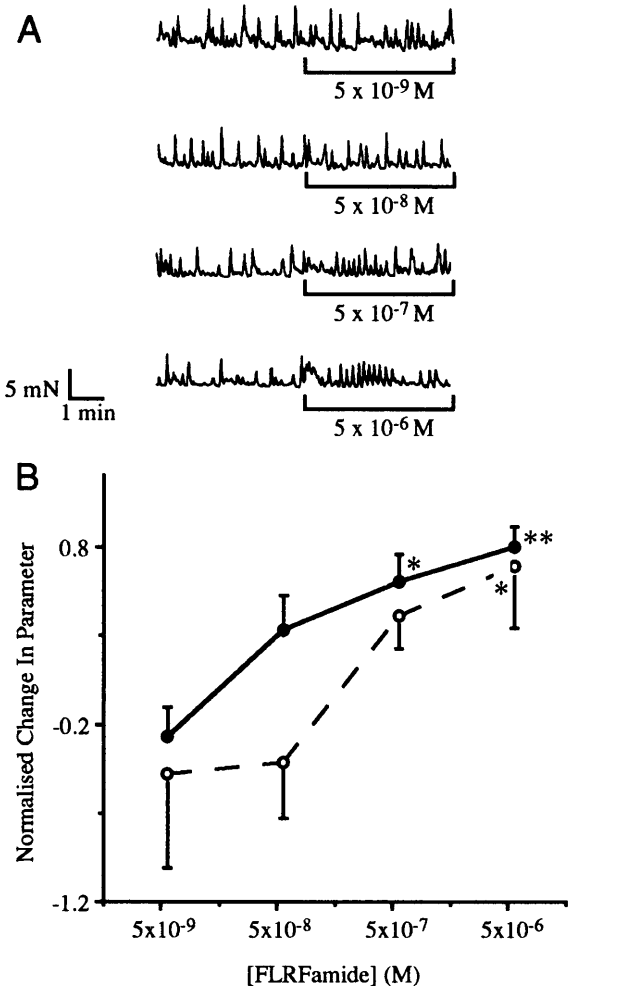

Fig. 2. As for Fig. 1 but illustrating FLRFamide. In (B) the double asterisk denotes a statistically significant change ($P < 0{\cdot}01$) for frequency at 5 μM. The single asterisk represents a significant change ($P < 0{\cdot}05$) for amplitude at 5 μM and for frequency at 0·5 μM.

concentrations expressed as a ratio of this maximum response. The summary graphs presented plot the mean response $\pm$ the standard error of the mean (S.E.M.) for each concentration of neuropeptide.

RESULTS

Normal activity

All tissue strips showed spontaneous, phasic activity. This activity had a mean amplitude of 2·75 $\pm$ 0·15 mN and a mean frequency of 14·15 $\pm$ 0·66 contractions/5 min ($n = 40$).

Controls

Control experiments showed that the activity of fluke tissue strips perfused in saline without BSA and saline containing BSA were no different. Hence BSA has no effect upon *in vitro* motility of the strips. Further experiments involving injection of perfusion medium containing BSA, but omitting the peptide, into the tissue chambers were carried out. The results indicate that the technique used for drug delivery had, in itself, no effect on the activity of the muscle strips.

Molluscan peptides

FMRFamide had an excitatory effect on muscle strip motility (Fig. 1). Prolonged exposure (10 min) experiments showed that the optimal duration of

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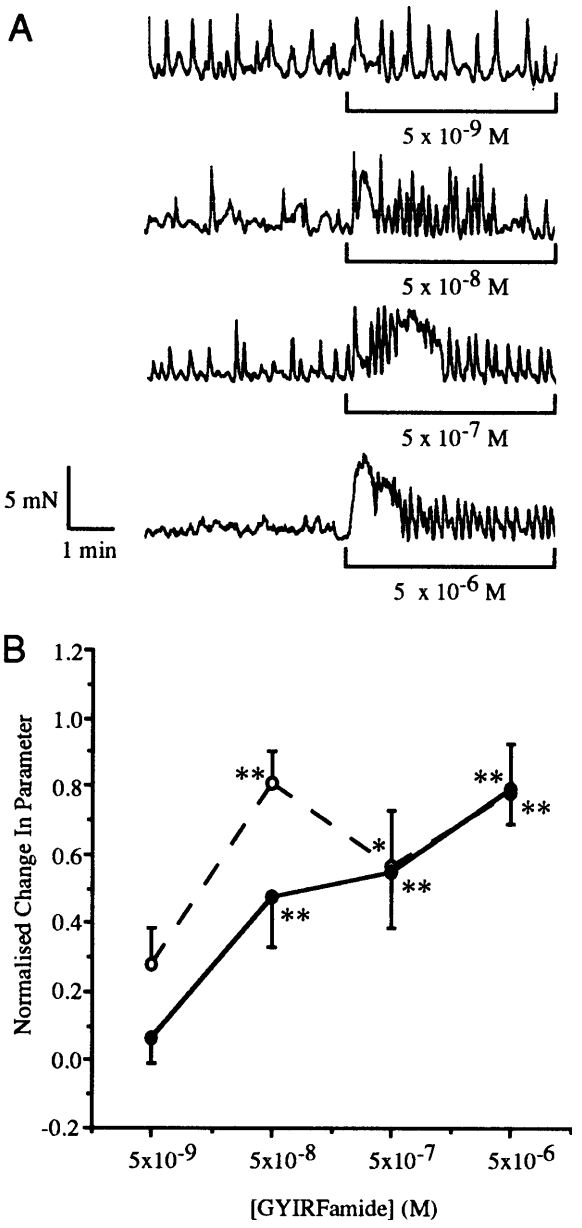

Fig. 3. As for Fig. 1 but illustrating GYIRFamide. In (B) the double asterisk marks a significant change ($P < 0{\cdot}01$) for amplitude at 50 nM and 5 μM, and for frequency at 50 nM, 0·5 μM and 5 μM. The single asterisk illustrates a significant change ($P < 0{\cdot}05$) at 0·5 μM for amplitude. The upper standard error bar corresponds to amplitude and the lower standard error bar to frequency at 0·5 μM and 5 μM concentrations.

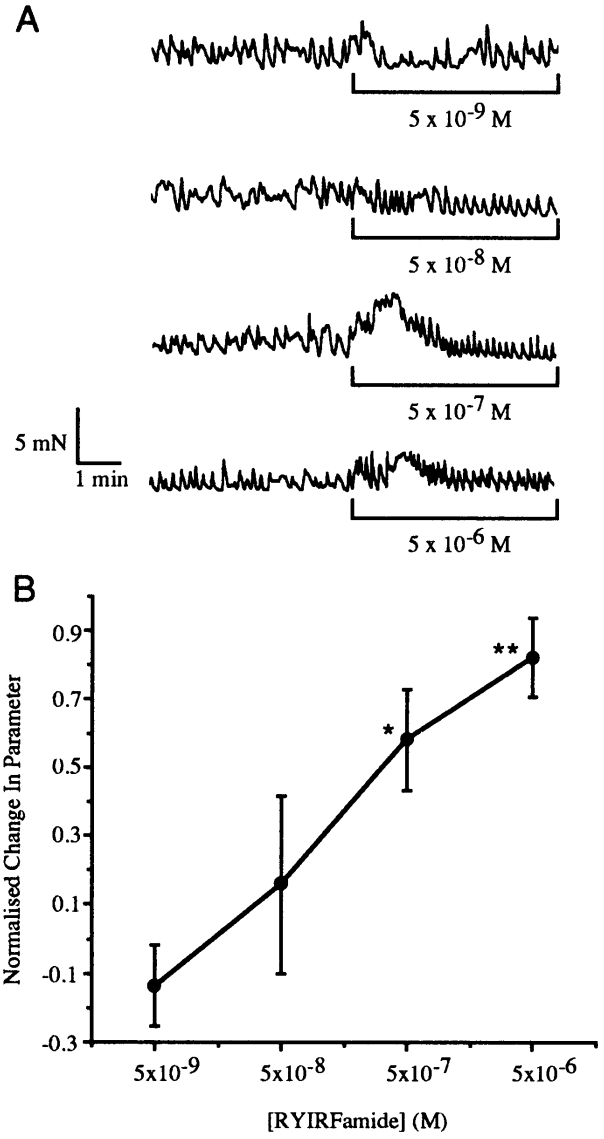

Fig. 4. As for Fig. 1 but illustrating RYIRFamide. In (B) the double asterisk represents a significant change ($P < 0{\cdot}01$) in frequency at 5 μM, and the single asterisk marks a change ($P < 0{\cdot}05$) in frequency at 0·5 μM. Note, non-significant data (for amplitude) are not shown.

tissue exposure to FMRFamide was 5 min. After this time, the amplitude and frequency of contractions decreased toward a pre-drug level. When muscle strips were subjected to repeated exposure (3 or 4 additions for a 5 min time-period) of a high concentration of FMRFamide (5 μM), appreciable tachyphylaxis was observed. Consequently, the order in which the different concentrations of FMRFamide and all other peptides were added during dose–response experiments was randomized. The dose–response experiments using FMRFamide were carried out at a concentration range of 5 nM–5 μM using 6 tissue strips (Fig. 1A). There was a statistically significant increase in the amplitude and frequency of contractions at 5 μM and 0·5 μM ($P < 0{\cdot}01$) (Fig. 1B). Changes at 50 nM and 5 nM were not statistically significant. At the threshold concentration, namely 0·5 μM, amplitude increased from a mean pre-drug level of $1{\cdot}53 \pm 0{\cdot}20$ mN to $2{\cdot}89 \pm 0{\cdot}25$ mN, and frequency of contraction increased from $8{\cdot}00 \pm 1{\cdot}06$/5 min to $14{\cdot}17 \pm 1{\cdot}85$/5 min.

Dose–response experiments using FLRFamide were carried out on 7 tissue strips (Fig. 2A). For contraction amplitude, a significant increase was obtained only at 5 μM ($P < 0{\cdot}05$). Contraction frequency was significantly increased at concentrations of 5 μM ($P < 0{\cdot}01$) and 0·5 μM ($P < 0{\cdot}05$) (Fig. 2B).

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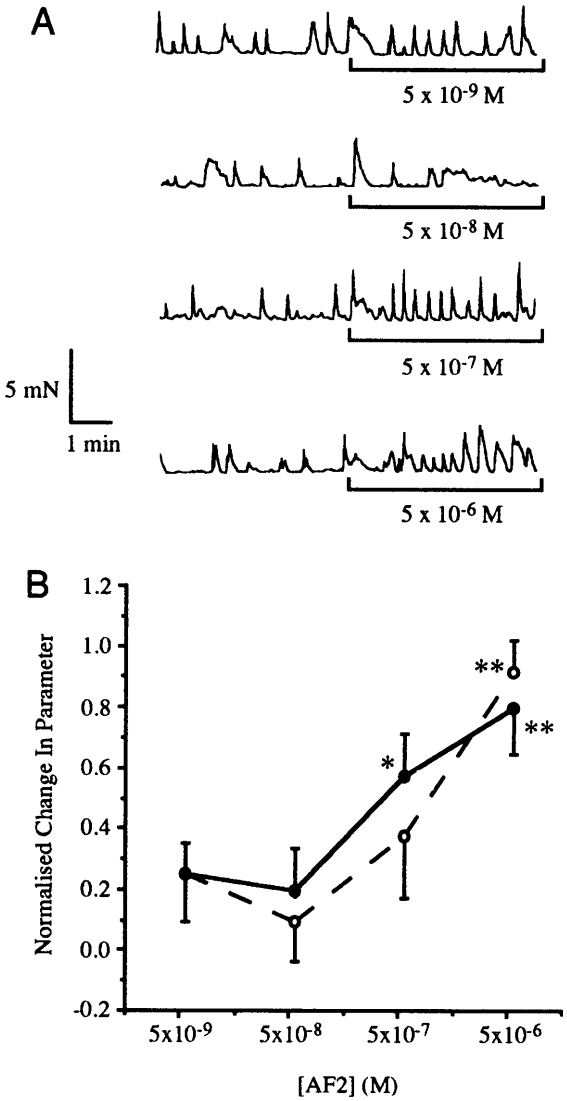

Fig. 5. As for Fig. 1 but illustrating AF-2. In (B) the double asterisk marks a significant change ($P < 0{\cdot}01$) for both amplitude and frequency at 5 μM and the single asterisk denotes a significant change ($P < 0{\cdot}05$) for frequency at 0·5 μM.

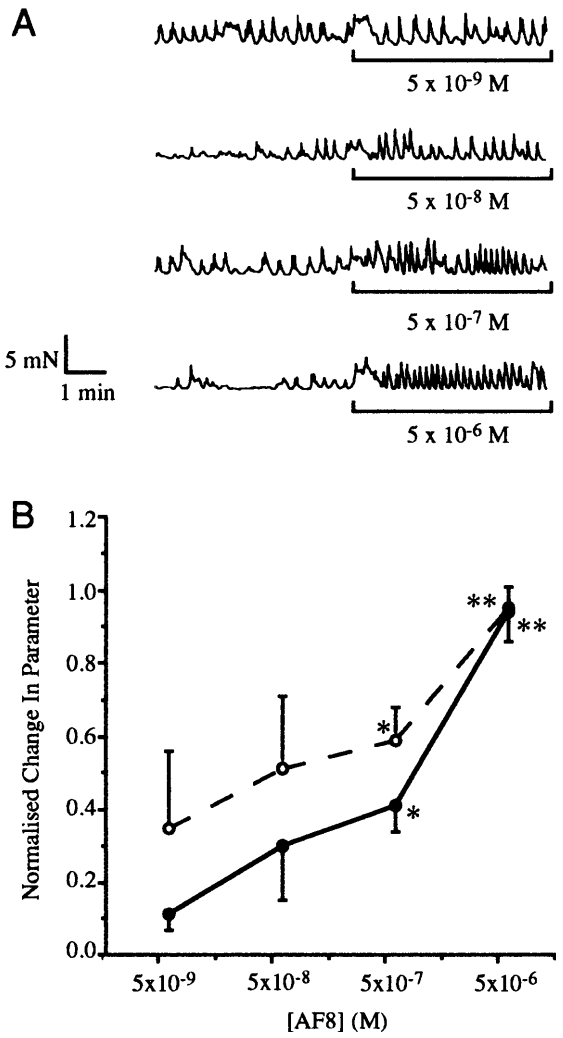

Fig. 6. As for Fig. 1 but illustrating AF-8. In (B) the double asterisk denotes a significant change ($P < 0{\cdot}01$) for both amplitude and frequency at 5 μM and the single asterisk marks a significant change ($P < 0{\cdot}05$) for both amplitude and frequency at 0·5 μM. The upper standard error bar corresponds to amplitude and the lower standard error bar to frequency at an AF-8 concentration of 5 μM.

At 5 μM, contraction amplitude increased from a mean pre-drug level of $2{\cdot}47 \pm 0{\cdot}38$ mN to $3{\cdot}43 \pm 0{\cdot}43$ mN. The frequency of contraction increased from $12{\cdot}71 \pm 2{\cdot}00$/5 min to $16{\cdot}57 \pm 1{\cdot}70$/5 min at a concentration of 0·5 μM.

Turbellarian peptides

GYIRFamide was tested on 6 tissue strips (Fig. 3A). It had a significant excitatory effect on both frequency and amplitude at concentrations of 50 nM, 0·5 μM and 5 μM ($P < 0{\cdot}01$ for all except amplitude at 0·5 μM, where $P < 0{\cdot}05$) (Fig. 3B). At 50 nM, amplitude increased from $2{\cdot}60 \pm 0{\cdot}55$ mN to $4{\cdot}02 \pm 0{\cdot}82$ mN, and frequency increased from $16{\cdot}50 \pm 1{\cdot}80$/5 min to $21{\cdot}67 \pm 1{\cdot}78$/5 min. Seven tissue strips were tested using RYIRFamide (Fig. 4A). It had non-significant effects on amplitude, but significantly increased frequency at 0·5 μM ($P <$ 0·05) and 5 μM ($P < 0{\cdot}01$) concentrations (Fig. 4B). At a concentration of 0·5 μM frequency increased from $17{\cdot}14 \pm 1{\cdot}75$/5 min to $21{\cdot}57 \pm 1{\cdot}69$/5 min.

Nematode peptides

Dose–response experiments using AF-2 were carried out on 7 tissue strips (Fig. 5A). Contraction amplitude was significantly greater at 5 μM ($P <$ 0·01), whilst frequency was increased at 5 μM ($P <$ 0·01) and 0·5 μM ($P < 0{\cdot}05$) (Fig. 5B). At the threshold concentrations, amplitude increased from a mean pre-drug level of $1{\cdot}86 \pm 0{\cdot}27$ mN to $2{\cdot}71 \pm 0{\cdot}31$ mN (at 5 μM), and frequency increased from $9{\cdot}57 \pm 0{\cdot}95$ contractions/5 min to $12{\cdot}43 \pm 1{\cdot}17$ contractions/5 min (0·5 μM). A total of 6 tissue strips was used in the dose–response experiments undertaken with AF-8 (Fig. 6A). Both the amplitude and

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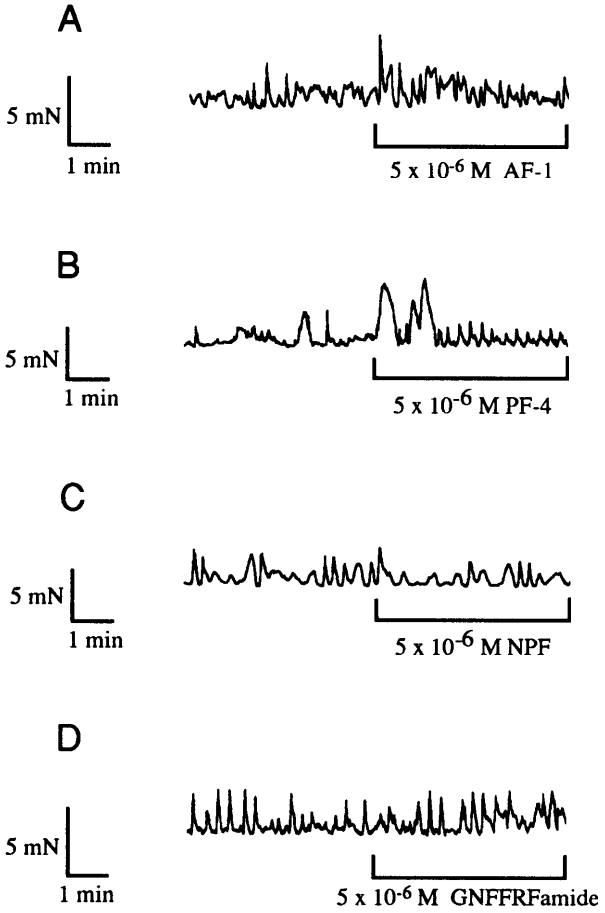

Fig. 7. (A and B) Traces from 2 separate muscle strips illustrating the effect of the 2 nematode FaRPs, AF-1 and PF-4 respectively, at the highest concentration used for each i.e. 5 μM. (C and D) Traces from 2 different muscle strips recorded in the presence of the tapeworm peptides, NPF and GNFFRFamide respectively, at a concentration of 5 μM.

frequency of contractions were significantly increased at 5 μM ($P < 0{\cdot}01$) and 0·5 μM ($P < 0{\cdot}05$) (Fig. 6B). At 0·5 μM, amplitude increased from $2{\cdot}38 \pm 0{\cdot}20$ mN to $3{\cdot}22 \pm 0{\cdot}27$ mN, and frequency of contraction increased from $17{\cdot}67 \pm 1{\cdot}43$/5 min to $23{\cdot}00 \pm 1{\cdot}44$/5 min. Analysis of the traces obtained using the neuropeptide AF-1 on 7 tissue strips (Fig. 7A) showed that a significant increase in the amplitude of contractions occurred only at a concentration of 5 μM ($P < 0{\cdot}05$). No significant changes in contraction frequency were recorded. Dose–response experiments were undertaken with PF-4 using 6 tissue strips. At a concentration of 5 μM, both amplitude ($P < 0{\cdot}05$) and frequency ($P < 0{\cdot}01$) were significantly increased (Fig. 7B).

Cestode peptides

The dose–response experiments involving NPF and GNFFRFamide were carried out on 6 and 7 tissue strips, respectively. There were no statistically significant changes, in either contraction amplitude or frequency for either peptide, at any concentration tested (Fig. 7C and D).

A summary of the results is shown in Tables 1 and 2.

DISCUSSION

One of the conclusions drawn from the investigation is that frequency is a more sensitive and more reliable parameter than amplitude for measuring the effects of neuropeptides on *Fasciola* muscle strips. In several preparations, it was noted that the increase in amplitude was substantially greater, after the addition of peptide, in quiescent tissues than in tissues which were relatively active during the 5 min control period. Hence, the true effect of a neuropeptide on contraction may be masked in more active preparations. It was also found that the dose dependence of responses was more consistent using frequency as a parameter. This is particularly evident for GYIRFamide (Fig. 3B). In other experiments using FLRFamide, it was also observed that the effects of repeated application of the same dose of peptide on frequency were more constant than those on amplitude, which demonstrated appreciable tachyphylaxis (Graham, unpublished observations). For these reasons, the biological activity of the peptides will be discussed with an emphasis on their effects on contraction frequency.

The present study provides pharmacological evidence to suggest that FaRPs play a role in the control of motility in *F. hepatica*. In summary, the molluscan FaRPs, FMRFamide and FLRFamide, the turbellarian FaRPs, GYIRFamide and RYIRFamide, and the nematode FaRPs, KHEYLRFamide (AF-2) and KSAYMRFamide (AF-8) exerted an excitatory effect on isolated muscle strips from the fluke. The remaining roundworm peptides, KNEFIRFamide (AF-1) and KPNFIRFamide (PF-4) had a small excitatory effect, but only at a high concentration of peptide (5 μM). The tapeworm peptides NPF and GNFFRFamide had no significant effect on muscle strip motility at the concentrations used.

The excitatory role of FMRFamide is consistent with its role in other animal phyla. In molluscs, FMRFamide generally has been established as being excitatory in various heart, visceral and somatic muscle preparations. For example, when applied exogenously to the tentacle retractor muscle of the land snail, *Helix aspersa*, it evokes rhythmic contractions (Cottrell, Greenberg & Price, 1983). In the clam, *Macrocallista nimbosa*, it has a cardioexcitatory effect (Price & Greenberg, 1977; see also reviews by Cottrell, 1989 and Walker, 1992). In the phylum Annelida, authentic FMRFamide has been demonstrated in the ragworm, *Nereis* (Krajniak & Price, 1990) and FMRFamide induces contraction of the longitudinal muscle of the medicinal leech, *Hirudo*

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Table 1. Summary of the effects of molluscan, flatworm and nematode peptides on the amplitude of *Fasciola hepatica* muscle strip contraction

(Raw data are presented showing the mean amplitude (of 6 or 7 replicates) ± S.E.M. for the 5 min control period and the 5 min period after the addition of 5 μM concentrations of neuropeptide. The level of significance is also shown. *n*, Number of replicates; N.S., non-significant.)

Peptide [*n*]	Threshold concentration	Mean amplitude (mN)		Change (%)
		Pre-treatment level	Post-treatment level (at 5 μM)	
FMRFamide [6]	0·5 μM	1·78 ± 0·24	2·82 ± 0·19 ($P < 0·01$)	58
FLRFamide [7]	5 μM	2·47 ± 0·38	3·43 ± 0·43 ($P < 0·05$)	39
GYIRFamide [6]	50 nM	1·92 ± 0·38	3·24 ± 0·45 ($P < 0·01$)	69
RYIRFamide [7]	—	2·11 ± 0·35	2·73 ± 0·44 (N.S.)	29
KNEFIRFamide (AF-1) [7]	5 μM	2·04 ± 0·21	2·69 ± 0·15 ($P < 0·05$)	32
KHEYLRFamide (AF-2) [7]	5 μM	1·86 ± 0·27	2·71 ± 0·31 ($P < 0·01$)	46
KSAYMRFamide (AF-8) [6]	0·5 μM	1·76 ± 0·23	3·20 ± 0·33 ($P < 0·01$)	82
KPNFIRFamide (PF-4) [6]	5 μM	1·97 ± 0·41	3·01 ± 0·35 ($P < 0·05$)	53
YFAIIGRPRFamide (NPF) [6]	—	2·37 ± 0·40	2·15 ± 0·43 (N.S.)	−9
GNFFRFamide [7]	—	1·91 ± 0·17	2·53 ± 0·35 (N.S.)	32

Table 2. Summary of the effects of molluscan, flatworm and nematode peptides on the frequency of *Fasciola hepatica* muscle strip contraction

(Raw data are shown illustrating the mean frequency (of 6 or 7 replicates) ± S.E.M. for the 5 min control period and 5 min test period after the application of 5 μM concentrations of neuropeptide. The level of significance is also indicated. *n*, Number of replicates; N.S., non-significant.)

Peptide [*n*]	Threshold concentration	Mean frequency (contractions/5 min)		Change (%)
		Pre-treatment level	Post-treatment level (at 5 μM)	
FMRFamide [6]	0·5 μM	7·83 ± 1·45	14·50 ± 1·06 ($P < 0·01$)	85
FLRFamide [7]	0·5 μM	13·00 ± 0·79	18·71 ± 0·97 ($P < 0·01$)	44
GYIRFamide [6]	50 nM	12·83 ± 0·95	22·33 ± 2·01 ($P < 0·01$)	74
RYIRFamide [7]	0·5 μM	15·71 ± 1·51	23·00 ± 1·35 ($P < 0·01$)	46
KNEFIRFamide (AF-1) [7]	—	15·29 ± 1·29	16·00 ± 1·53 (N.S.)	5
KHEYLRFamide (AF-2) [7]	0·5 μM	8·29 ± 1·08	13·86 ± 0·80 ($P < 0·01$)	67
KSAYMRFamide (AF-8) [6]	0·5 μM	10·67 ± 1·50	23·50 ± 1·95 ($P < 0·01$)	120
KPNFIRFamide (PF-4) [6]	5 μM	10·50 ± 1·52	16·83 ± 1·11 ($P < 0·01$)	60
YFAIIGRPRFamide (NPF) [6]	—	11·86 ± 1·55	9·71 ± 1·11 (N.S.)	−18
GNFFRFamide [7]	—	15·86 ± 1·45	17·71 ± 0·94 (N.S.)	12

medicinalis (Norris & Calabrese, 1990). In the hydrozoan coelenterate, *Polyorchis penicillatus*, it has an excitatory effect on motoneurons (Spencer, 1988). FMRFamide is one invertebrate peptide that has been demonstrated immunocytochemically in *Fasciola*. In the central nervous system, immunoreactivity was located in the paired cerebral ganglia and the 3 pairs of longitudinal nerve cords and their connecting commissures. In the peripheral nervous system, immunoreactivity occurred in the plexuses innervating the subtegumental musculature, the oral and ventral suckers, and the reproductive ducts, especially those associated with the egg-forming apparatus (or ootype/Mehlis' gland-complex) (Magee *et al*. 1989). The distribution of FMRFamide immunoreactivity in the nervous system of *Fasciola* supports the idea that a FaRP has a role to play in the co-ordination of neuromuscular activity.

In a previous investigation on the nematode *Ascaris suum*, AF-8 caused a rapid, concentration-dependent increase in muscle tension when applied to an isolated somatic muscle preparation at a threshold concentration of 0·1 μM (treatment was judged as effective if it induced $\geqslant$ 0·5 g change in baseline tension compared to control treatment, during the period in which the peptide was in contact with the tissue) (Maule *et al*. 1994*a*). In the present study, AF-8 had an excitatory effect identical to that of FMRFamide, in terms of the threshold concentrations required to increase significantly the amplitude and frequency of contractions. Thus, it

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would seem that replacing the phenylalanine (Phe^1) in FMRFamide with a tyrosine at the equivalent position in the longer peptide AF-8, had little functional consequence. Both amino acids are aromatic and hydrophobic, although tyrosine is less hydrophobic due to the presence of a hydroxyl group on the aromatic ring.

FMRFamide and FLRFamide had the same threshold concentrations for increasing contraction frequency (0·5 μM), although FLRFamide had a slightly higher threshold concentration for increasing significantly contraction amplitude in the fluke muscle. The two peptides differ in a single amino acid, the methionine (Met^2) in FMRFamide being replaced with leucine. A characteristic of both amino acids is the possession of hydrophobic side-chains. This may be one of the major factors determining whether they are interchangeable in a peptide sequence. The nematode FaRP, AF-2 had an excitatory effect on the *Fasciola* muscle preparation, with the same threshold concentrations as FMRFamide and FLRFamide (namely, 0·5 μM for frequency). AF-2, used over the concentration range of 10 nM–10 μM, has been shown to have a primarily stimulatory effect on various muscle preparations from *Ascaris* (Cowden & Stretton, 1993; Pang *et al*. 1995) and thus is thought to play an important role in the motonervous system in this parasite. The comparison between FMRFamide and KHEYLRFamide (AF-2) involves the substitution of 2 amino acids, as well as N-terminal extension of 3 amino acids. The effect of AF-2 on frequency supports the idea that the Phe^1 and Met^2 in FMRFamide are interchangeable with tyrosine and leucine residues, respectively. It would also suggest that the additional 3 amino acids have no effect on biological activity in this instance.

The other nematode peptides, PF-4 and AF-1, had little effect on *in vitro* motility. Both are heptapeptides differing from each other in only 2 amino acids at the same location. Thus, AF-1 has the sequence KNEFIRFamide, whilst PF-4 is KPNFIRFamide. Compared to FMRFamide, both AF-1 and PF-4 have an additional 3 N-terminal amino acids (KNE and KPN respectively) and the methionine is replaced with isoleucine. Isoleucine has a hydrophobic side-chain, as does methionine, and is very similar in structure to leucine. Given the observed effects and known structures of the other N-terminally extended peptides tested (AF-2 and AF-8), it might have been expected that AF-1 and PF-4 would have shown greater activity in the *Fasciola* assay. However, even subtle changes in structure can have a dramatic effect on peptide activity. For example, AF-1 is excitatory on the body wall muscle of *Ascaris* at a concentration of 1 μM (Pang, Holden-Dye & Walker, 1992), whilst PF-4 is inhibitory (concentrations equal to, or greater than 0·3 μM induced relaxation in 100% of muscle preparations examined) (Maule *et al*. 1995). Clearly, the change in 2 amino acids in the N-terminal extension parts of these peptides is enough to create sequences which have opposite effects in *Ascaris*.

The peptide GYIRFamide, initially isolated from the free-living flatworm, *Dugesia tigrina* (Johnston *et al*. 1995), had the greatest effect on muscle strip motility using our assay. It was the only peptide tested to have significant effects on the parameters measured at 50 nM concentrations. The other free-living flatworm peptide tested, RYIRFamide, from the terrestrial turbellarian, *Artioposthia triangulata* (Maule *et al*. 1994*b*) also had significant effects on contraction frequency but at a higher threshold than GYIRFamide (namely, 0·5 μM). The biological activity of these peptides on fluke tissue is not surprising as it is believed that trematodes evolved from a free-living flatworm ancestor (Rohde *et al*. 1990). RYIRFamide has been shown to stimulate contraction of muscle fibres of the human blood fluke, *Schistosoma mansoni* (Day *et al*. 1994). In a separate study on *F. hepatica*, GYIRFamide and RYIRFamide have been shown to have stimulatory effects on motility, although RYIRFamide had a greater effect than GYIRFamide, with a threshold of 1 nM as against 3 μM (Marks *et al*. 1996). This result is in direct contrast to the present results. The difference between the two studies is not readily apparent, although direct comparisons are difficult to make. A number of factors have to be taken into account, including differences in methodology (with measurement of muscle contraction based on quasi-isotonic movement in the study by Marks, and recording of isometric tension in the current study), parameter of motility being tested, method of data collection and experimental design, statistical treatment of data, developmental status of fluke, mammalian host used and ionic composition of medium. The difference in activities between GYIRFamide and RYIRFamide, hinging on a glycine/arginine substitution, is not easy to explain. However, it is probable that arginine would be more sensitive than glycine to the actions of enzymes which cleave amino acid residues off the N-terminus of peptides (aminopeptidases). Thus, the actions of RYIRFamide would be terminated more rapidly than GYIRFamide and this would be reflected in their corresponding threshold concentrations.

Neuropeptide F (NPF), first isolated and sequenced from the sheep tapeworm *Moniezia expansa*, was identified as a 39-amino acid peptide with a C-terminal phenylalaninamide. It shows high sequence homology with the vertebrate neuropeptide Y superfamily and was the first regulatory peptide to be fully sequenced from a platyhelminth (Maule *et al*. 1991). Localization within *M. expansa* has been achieved using antisera raised to intact NPF (amino acids 1–39) and to the C-terminal decapeptide of NPF (amino acid sequence YFAIIGRPRFamide) (Maule

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et al. 1992). Immunostaining for NPF has also been demonstrated throughout the central and peripheral nervous systems of *F. hepatica* using the C-terminally-directed NPF antiserum. The pattern of staining is comparable to that previously described for FMRFamide (Magee *et al*. 1989). Due to the above findings, the C-terminal decapeptide of NPF was used in the motility investigation on *F. hepatica* muscle strips, where it was found to have no physiological effect. NPF was found to have a slightly excitatory effect on liver fluke muscle strips in a previous investigation (Marks *et al*. 1996), but only at concentrations of 10 μM or above. In a separate physiological assay, NPF has been shown to inhibit protein and nucleic acid synthesis at concentrations of 0·1 μM–10 μM, indicating a possible morphogenetic role for the peptide in *F. hepatica* (Fairweather *et al*. 1995; Fairweather, unpublished observations). NPF shows little sequence homology with FMRFamide, the only similarity being the C-terminal RFamide, and so it is likely to act on different receptor type(s). In other organisms NPF and NPY have been found to play inhibitory roles (McDonald, 1988; Rajpara *et al*. 1992).

The tapeworm peptide, GNFFRFamide, the first flatworm FaRP to be isolated (Maule *et al*. 1993), has been shown to cause a dose-dependent induction of contractions in individual muscle fibres from the human blood fluke, *S. mansoni in vitro* at concentrations of 0·1 μM–10 μM (Day *et al*. 1994), but would appear to be ineffective in *F. hepatica*. The importance of exhibiting caution when making comparisons between studies where experimental techniques differ has already been discussed. In the experiments on schistosome muscle fibres, the data were presented as the percentage of the tested fibres that contracted. No statistics were applied to the data. In the *Fasciola* muscle strip investigation, the methodology allowed rigorous quantitative analysis of the data through the use of statistics. Both parasites are digenetic trematodes; nonetheless, they have been shown to respond differently to some pharmacological agents: for example, the drug praziquantel causes a very rapid (within 1 min) spastic paralysis in schistosomes at a concentration of 1 μM (Fetterer, Pax & Bennett, 1980), but is ineffective in *F. hepatica* up to 0·3 mM (Fairweather, Holmes & Threadgold, 1984). Previously, GNFFRFamide has been seen to be ineffective in *F. hepatica* at the concentrations used in the present study, having an excitatory effect only at 10 μM concentrations and above (Marks *et al*. 1996). Immunoreactivity to GNFFRFamide has been reported throughout the central and peripheral nervous systems of *F. hepatica*. However, the GNFFRFamide antiserum was not highly specific and showed some cross-reactivity with both NPF and FMRFamide (Marks *et al*. 1995). From the structure–function point of view it is interesting to note that GNFFRFamide contains a sequence with 2 adjacent phenylalanine residues. There may be interactions between the 2 aromatic rings which make it impossible to substitute the methionine in FMRFamide for a phenylalanine, and retain the ability to interact with receptors in *F. hepatica*. In this connection, it is also worth noting that, while FMRFamide itself causes a stimulation of protein and nucleic acid synthesis in *F. hepatica* at concentrations of 1 nM–1 μM, GNFFRFamide has no effect on these parameters at similar concentrations (Fairweather *et al*. 1995; Fairweather, unpublished observations).

This study illustrates cross-phyla activity of neuropeptides, demonstrated by the excitatory actions of the nematode FaRPs, AF-2 and AF-8 on trematode tissue. It is also consistent with a previous study undertaken using *F. hepatica*, in that the FaRPs, GYIRFamide and RYIRFamide were shown to be excitatory (although their activity thresholds differed), whilst NPF and GNFFRFamide had no effect on motility at the concentrations used (Marks *et al*. 1996). Further research is required to gain more knowledge on receptor specificity and the minimum requirements for biological activity. There is a need to test defined series of FMRFamide analogues and deduce the effect of N-terminal extensions in a more rigorous fashion. Pharmacological findings alone do not establish a transmitter role: additional criteria, such as their isolation and characterization from *Fasciola* itself, are required, as well as information on their sites and intracellular signalling pathways. Experiments in these areas are currently in progress.

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