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Structure-Activity Relationships of FMRFamide-Related Peptides Contracting *Schistosoma mansoni* Muscle

TIM A. DAY,*[1] AARON G. MAULE,† CHRIS SHAW† AND RALPH A. PAX*

**Department of Zoology, Michigan State University, East Lansing, MI 48824*
†Comparative Neuroendocrinology Research Group, The Queen's University of Belfast, Belfast BT7 1NN, Northern Ireland

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DAY, T. A., A. G. MAULE, C. SHAW AND R. A. PAX. *Structure-activity relationships of FMRFamide-related peptides contracting* Schistosoma mansoni *muscle*. PEPTIDES **18**(7) 917–921, 1997.—This study reports the potent myoactivity of flatworm FMRFamide-related peptides (FaRPs) on isolated muscle fibers of the human blood fluke, *Schistosoma mansoni*. The turbellarian peptides YIRFamide (EC_{50} 4 *ηM*), GYIRFamide (EC_{50} 1 *ηM*), and RYIRFamide (EC_{50} 7 *ηM*), all induced muscle contraction more potently than the cestode FaRP GNFFRFamide (EC_{50} 500 *ηM*). Using a series of synthetic analogs of the flatworm peptides YIRFamide, GYIRFamide and RYIRFamide, the structure-activity relationships of the muscle FaRP receptor were examined. With a few exceptions, each residue in YIRFamide is important in the maintenance of its myoactivity. Alanine scans resulted in peptides that were inactive (Ala^1, Ala^2, Ala^3 and Ala^4 YIRFamide; Ala^4 and Ala^5 RYIRFamide) or had much reduced potencies (Ala^1, Ala^2 and Ala^3 RYIRFamide). Substitution of the *N*-terminal (Tyr^1) residue of YIRFamide with the non-aromatic residues Thr or Arg produced analogs with greatly reduced potency. Replacement of the *N*-terminal Tyr with aromatic amino acids resulted in myoactive peptides (FIRFamide, EC_{50} 100 *ηM*; WIRFamide, EC_{50} 0.5 *ηM*). The activity of YIRFamide analogs which possessed a Leu^2, Phe^2 or Met^2 residue (EC_{50}'s 10, 1 and 3 *ηM*, respectively) instead of Ile^2 was not significantly altered, whereas, YVRFamide had a greatly reduced (EC_{50} 200 *ηM*) activity. Replacement of the Phe^4 with a Tyr^4 (YIRYamide) also greatly lowered potency. Truncated analogs were either inactive (FRFamide, YRFamide, HRFamide, RFamide, Famide) or had very low potency (IRFamide and MRFamide), with the exception of nLRFamide (EC_{50} 20 *ηM*). YIRF free acid was inactive. In summary, these data show the general structural requirements of this schistosome muscle FaRP receptor to be similar, but not identical, to those of previously characterized molluscan FaRP receptors. © 1997 Elsevier Science Inc.

FMRFamide-related peptide Flatworm Helminth Platyhelminth Trematode *Schistosoma mansoni* Peptide Receptors Muscle

CURRENT evidence suggests a fundamental role for FMRFamide-related peptides (FaRPs) in the regulation of neuromuscular function in flatworms. FaRP immunoreactivity has been found to be abundant and widespread in both the central and peripheral nervous systems of representative species from all major classes of flatworms (19,8), including the human blood fluke *Schistosoma mansoni* (7). Subsequent to such studies, specific FaRP transmitters have been isolated and structurally characterized from a number of flatworms: GNFFRFamide from the sheep tapeworm, *Moniezia expansa* (14); RYIRFamide from the predatory terrestrial turbellarian, *Artioposthia triangulata* (13); GYIRFamide from the aquatic turbellarian, *Dugesia tigrinia* (9); and both GYIRFamide and YIRFamide from the marine ectocommensal turbellarian, *Bdelloura candida* (10). In addition, functional studies have shown that these FaRPs elicit muscle contraction in a number of flatworm preparations, including *S. mansoni* (5), the liver fluke *Fasciola hepatica* (12), and the turbellarain *B. candida* (10). In two of these preparations (*S. mansoni* and *B. candida*), FaRPs induce contraction of isolated muscle fibers, indicative of a direct effect on a muscle receptor. Although a number of transmitters have been implicated as possible neuromuscular transmitters in the flatworms, so far only the FaRPs have been shown to contract non-innervated flatworm muscle.

The structural requirements for the activation of FMRFamide receptors have been investigated in a number of preparations of molluscan tissue (11,15–17). Molluscan FMRFamide receptors appear to share a tolerance for conservative substitutions for Phe^1 and Met^2, a relative intolerance of substitutions for Arg^3, and a strict requirement for *C*-terminal amidation (17). Additionally, for molluscan receptors there is a preference for Phe^1 over Tyr^1

[1] Requests for reprints should be addressed to Tim Day, Department of Zoology, Michigan State University, East Lansing, MI 48824, USA, E-mail: dayt@pilot.msu.edu

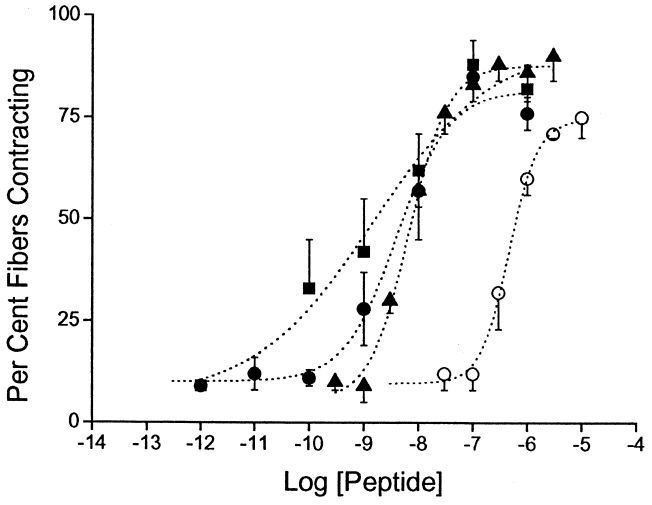

FIG. 1. Dose-response curves for the contraction of *S. mansoni* muscle by flatworm-derived FaRPs: (●) YIRFamide, (■) GYIRFamide, (▲) RYIRFamide, and (○) GNFFRFamide (mean ± SEM). Each of the peptides induces contraction in a dose-dependent manner, though the YIRFamide-containing peptides are all more potent than GNFFRFamide.

(16,17) and some variability regarding preference for Met^2 over Leu^2 (17). In the *S. mansoni* muscle preparation used in the present study, it has already been demonstrated that RYIRFamide is much more active than GNFFRFamide (5).

When FMRFamide was found to be present in a wide range of molluscs, including the earliest forms, and in annelids, it was suggested that this tertapeptide amide was the archetypal member of the FaRP family (20). However, the apparent absence of FMRFamide in flatworms investigated thus far would strongly suggest otherwise, as the turbellarians are generally considered to be the closest extant relatives of the common ancestor of the Bilateria (1,6). As more information becomes available regarding the primary structures of flatworm FaRPs, the putative ancestors of FMRFamide, it is of interest to examine the pharmacological properties of the autologous FaRP receptors that have coevolved with those ligands. To this end, we have investigated the structure-activity relationships for activation of the FaRP receptor on flatworm muscle using isolated muscle fibers derived from *S. mansoni*.

METHOD

Muscle Fiber Dispersion

The muscle fibers were dispersed from a Puerto Rican strain of adult *Schistosoma mansoni* recovered from the portal and mesenteric veins of Swiss Webster mice 45–55 days post-infection. The dispersion was carried out using a combination of light enzymatic digestion and mechanical disruption, as we have described previously (3). The dispersed fibers were plated onto 35 mm petri dishes and incubated at 20°C in a medium which consisted of 82.5 m*M* Na^+, 4.1 m*M* K^+, 3.6 m*M* Ca^{++}, 3.3 m*M* Mg^{++}, 100.4 m*M* Cl^-, 79.9 m*M* glucose, 1 μM serotonin and 15.0 m*M* HEPES (pH 7.4). Each dish of cells was warmed to 35°C 15 min prior to use and throughout experimentation. Following dispersion the fibers were mostly quiescent, with approximately 10% showing spontaneous twitching or intermittent contractions.

Muscle Contraction Assay

The assay used to assess the effectiveness of the various peptides on the isolated muscles has been fully described (3–5). The muscle fiber contraction assay involved microperfusion of the peptide to be tested onto individual fibers and observing induced contractions. The observations were recorded on video tape and subsequently analyzed. Each fiber was scored either as a contraction or a non-contraction, since the individual fibers with no imposed load contract in an all-or-none fashion. For each plate, 15–25 fibers were tested and the data shown are the percentages of the tested fibers that contracted. Each datum given is the mean ± SEM for at least 6 different plates, taken from at least 3 different cell preparations. For these trials, only fibers of the class that we have previously described as ''frayed fibers'' were utilized. Spontaneously active fibers were not used.

Microperfusion of the culture medium alone onto the cells elicited contraction of 11 ± 6% of the fibers. We have previously reported that the fibers contract when exposed to the molluscan neuropeptide FMRFamide (5). FMRFamide at 1 μM elicits 80 ± 5% contraction. Each day of experimentation, culture medium and 1 μM FMRFamide were tested as negative and positive controls. Peptides were tested beginning at 1 μM. Any peptide that elicited over 50% contraction at 1 μM was tested at progressively lower concentrations until the response was not significantly different from that of negative controls.

Data Analysis

Potency was determined as the concentration eliciting 50% of the maximal response as determined by a fit of the data for each peptide to the Boltzman equation in the form,

$$\%Contraction = \frac{(y_1 - y_2)}{1 + e^{(x-EC_{50})/k}} + y_2$$

where y_1 and y_2 are the maximum and minimum % contraction fit by the curve, x is the concentration of peptide, k is the slope factor describing the width of the curve, and EC_{50} is the concentration of peptide at which the curve would predict half of the maximal response. If the peptide did not elicit contraction greater than the culture medium controls at 1 μM, it is listed as not effective (NE). If, at 1 μM, the peptide elicited contraction greater than controls but less than 50%, the EC_{50} is listed as >1000 ηM.

RESULTS

Platyhelminth-Derived FaRPs

All of the FaRPs that have been isolated from flatworms elicited contraction of individual *S. mansoni* muscle fibers in a dose-dependent manner (Fig. 1 and Table 1). The three peptides that have been isolated from turbellarians, RYIRFamide, GYIRFamide and YIRFamide, posses the YIRFamide motif which is not present in the only known cestode FaRP, GNFFRFamide. The turbellarian-derived peptides were, on average, 100 times more potent than the cestode-derived peptide. The three turbellarian peptides had similar potencies, each with an EC_{50} between 1 and 7 ηM.

Since YIRFamide is the simplest in structure among the potent flatworm-derived peptides, it was considered the core motif for subsequent structure-activity studies. Therefore, the Arg and Gly residues at the *N*-terminal positions of RYIRFamide and GYIRFamide respectively, are referred to as Arg^0 and Gly^0 such that the tyrosyl residue is Tyr^1 in all three of the potent flatworm peptides used in analog construction.

TABLE 1

MUSCLE CONTRACTING ACTIVITY OF PLATYHELMINTH FaRPS AND ALANINE-SUBSTITUTED ANALOGS

Peptide	EC_{50} (ηM)
Platyhelminth FaRPs	
GYIRFa	1
YIRFa	4
RYIRFa	7
GNFFRFa	500
YIRFamide alanine scan	
YIRFa	4
AIRFa	NE
YARFa	NE
YIAFa	NE
YIRAa	NE
RYIRFamide alanine scan	
RYIRFa	7
AYIRFa	400
RAIRFa	<1000
RYAIFa	<1000
RYIAFa	NE
RYIRAa	NE

NE signifies no effect at 1 μM.

Alanine Scans

An alanine scan was performed in order to probe the importance of each of the four amino acid residues of the YIRFamide tetrapeptide. In each case, alanine substitution resulted in a peptide that was inactive at 1 μM (Table 1), demonstrating that each amino acid residue in the native peptide is independently important for function.

The results of an alanine scan of RYIRFamide were similar to those of the YIRFamide scan (Table 1). The substitution of alanine for any of the five amino acid residues resulted in a marked decrease in potency, the least dramatic being Ala^0 for Arg^0, where the EC_{50} was decreased from 7 ηM to 400 ηM. The Ala^1 and Ala^2 analogs of RYIRFamide retained some activity, both with EC_{50}'s greater than 1 μM, while Ala^3 and Ala^4 analogs were completely inactive.

Position 1 Substitutions

The substitution of non-aromatic amino acids other than alanine (threonine or arginine) for tyrosine in the first position also resulted in a significant decrease in activity on the schistosome muscle (Table 2). By contrast, tetrapeptides with other aromatic amino acids substituted for Tyr^1 potently elicited contraction. Tryptophan substitution in the first position, i.e. WIRFamide, produced a peptide that was slightly more potent than YIRFamide, but not significantly so (EC_{50} of 0.5 ηM as compared to 4 ηM).

In general, peptides with tyrosine in the first position were more potent than those with phenylalanine. For example, YIRFamide was 25 times more potent than FIRFamide. Similarly, YFRFamide (EC_{50} = 1 ηM) was about 20 times more potent than FFRFamide (EC_{50} = 20 ηM), and YMRFamide was over 10 times more potent than FMRFamide (EC_{50}'s of 3 ηM and 50 ηM, respectively). This difference was less dramatic when leucine occupied the second position, as YLRFa was only slightly more potent than FLRFa (EC_{50}'s of 10 ηM and 30 ηM, respectively).

TABLE 2

MUSCLE CONTRACTING ACTIVITY OF POSITION 1 AND POSITION 2 MODIFIED ANALOGS OF YIRFamide

Peptide	EC_{50} (ηM)
Tyr^1 substitutions	
TIRFa	≈1000
RIRFa	≈1000
FIRFa	100
WIRFa	0.5
Ile^2 substitutions	
YLRFa	10
YFRFa	1
YMRFa	3
YVRFa	200
YARFa	NE

NE signifies no effect at 1 μM.

Position 2 Substitutions

Substitution of the isoleucine in YIRFamide with any of the amino acids more hydrophobic than isoleucine did not have significant effect on bioactivity (Table 2). However, potency was lowered by substitution with valine or alanine, which are less hydrophobic than isoleucine.

RFamide Alterations

Changes in the Arg-Phe-NH_2 structure of the *C*-terminus resulted in a dramatic reduction in bioactivity (Table 3). As noted above, substitution of either of the two *C*-terminal residues with alanine resulted in a complete loss of activity. Likewise, lack of *C*-terminal amidation resulted in an inactive peptide.

TABLE 3

MUSCLE CONTRACTING ACTIVITY OF RFamide ALTERED AND *N*-TERMINALLY TRUNCATED FaRP ANALOGS

Peptide	EC_{50} (ηM)
RFamide alterations	
YIAFa	NE
YIRAa	NE
YIRYa	≈1000
FMRYa	≈1000
YIRF	NE
FMRF	NE
N-terminal truncations	
IRFa	<1000
MRFa	≈1000
nLRFa	20
FRFa	NE
YRFa	NE
HRFa	NE
RFa	NE
Fa	NE

NE signifies no effect at 1 μM. nL = norleucine.

However, when the aromatic amino acid tyrosine was substituted for phenylalanine in the fourth position, some activity was retained, albeit drastically reduced. For example, YIRFamide had an EC_{50} of 4 ηM while YIRYamide had an EC_{50} near 1000 ηM, and FMRFamide was also much more potent than FMRYamide (EC_{50}'s of 50 ηM and 1000 ηM, respectively).

N-Terminal Deletions

The elimination of the *N*-terminal amino acid to produce the tripeptide IRFamide caused a dramatic reduction in bioactivity (Table 3). However, when norleucine was placed in the second position (nLRFamide), much of the activity was retained. Methionine in the second position (MRFamide) formed a more active tripeptide than did isoleucine, but both were relatively impotent. Completely inactive were tripeptide RFamides with *N*-terminal phenylalanine (FRFamide), tyrosine (YRFamide) or histidine (HRFamide). Further truncation to RFamide and Famide also resulted in loss of activity.

DISCUSSION

Of the FaRPs which have been identified in flatworms, the YIRFamide-containing peptides are all about 100 times more potent than GNFFRFamide in their ability to contract isolated schistosome muscle fibers. This is in accord with other physiological studies of FaRP effects on flatworm muscle. In *F. hepatica* muscle strips, all of the YIRFamide-containing peptides were about two orders of magnitude more potent than GNFFRFamide (12), and in *D. merlangi* GNFFRFamide was completely ineffective (C. Moneypenny, unpublished observations, 1996). The structural differences between the hexapeptide GNFFRFamide and the other flatworm FaRPs, which are all YIRFamide-containing tetra- and pentapeptides, are reflected as a significant functional difference with respect to the excitation of flatworm muscle.

The replacement of any single amino acid of YIRFamide with alanine yielded an ineffective peptide. This reveals that there are at least some structural requirements at every site, despite the fact that there are less restrictions at the first and second positions relative to the *N*-terminus than in the third and fourth positions.

All of the analogs which possessed an aromatic amino acid (tyrosine, phenylalanine or tryptophan) in the first position had potent actions on the muscle fibers. Nevertheless, there was a clear preference for tyrosine over phenylalanine, as YIRFamide, YLRFamide and YMRFamide were each more potent than their respective Phe^1 analog. In the second position, the hydrophobic amino acids isoleucine, leucine, methionine and phenylalanine each produced active analogs. None of the analogs in which the *C*-terminal RFamide was altered retained potency comparable to the parent peptide YIRFamide, demonstrating the importance of the RFamide moiety to myoactivity.

Truncation of the tetrapeptide dramatically reduced potency (minimal loss of potency of 250×), with the notable exception of nLRFamide (which was only 5-fold less potent than YIRFamide). Only a limited number of peptides larger than four amino acids were tested: the two native flatworm peptides RYIRFamide and GYIRFamide, as well as the Ala^0-substituted AYIRFamide. The Arg^0 and Gly^0 peptides were essentially equipotent to YIRFamide, but the Ala^0 peptide had only one thousandth the potency. This demonstrates that some *N*-terminal extensions can be well-tolerated, but others can be quite detrimental.

FMRFamide is not the archetypal FaRP, as has been indicated by the isolation of non-FMRFamide FaRPs in organisms phylogenetically more ancient than molluscs. Nor, then, is the FaRP receptor of molluscs the archetypal FaRP receptor. The results of this study indicate that the structural requirements of the FaRP receptors of the more primitive flatworms are, in broad terms, similar to those of the molluscs. They share a tolerance of conservative changes in the first two positions relative to the *N*-terminus, but they are much less tolerant of changes in the *C*-terminal RFamide, including an apparently absolute requirement for the *C*-terminal amidation. However, whereas the molluscan FaRP receptors consistently prefer Phe^1 over Tyr^1 (17), the schistosome FaRP receptor prefers Tyr^1. Since the molluscan tetrapeptides predominantly contain phenylalanine in this position and flatworm peptides predominantly contain tyrosine in this position, this difference might not be unexpected.

Sufficient comparable data do not yet exist to make useful comparisons between the structural requirements of FaRP receptors in flatworms with those of nematodes and arthropods. Although the native peptides discovered in nematodes and arthropods share some features with those of platyhelminths, they are longer than those isolated from platyhelminths. As such, the SAR studies probing the requirements for FaRP bioactivity in animals of these phyla [(2,21), for example], utilize longer peptides, generating data which are not directly comparable to those from this study.

The potency and the specificity of the schistosome muscle contractile response to FaRPs suggests that the FaRP receptor on this muscle is mediating a transmitter-type of response, as opposed to a more modulatory or trophic response. In contrast, serotonin, which has been long-considered a leading candidate for the role of an excitatory neuromuscular transmitter in flatworms, appears to have a more modulatory role on these muscles. Serotonin itself does not induce contraction of the individual schistosome muscle fibers, but they are much more responsive to elevated potassium in the presence of serotonin than they are in its absence (3). These data make it reasonable to believe that a FaRP is the primary fast transmitter at these muscles and that serotonin has a modulatory role. A FaRP serving a transmitter function in platyhelminths supports the theory that FaRPs originated as transmitters and later filled the role of modulators (18).

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