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Differential effects of neuropeptides on circular and longitudinal muscles of the crayfish hindgut

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Abstract

Proctolin (Arg-Tyr-Leu-Pro-Thr-OH) and crayfish peptide "DF_2" (Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2) enhance spontaneous contractions of isolated crayfish hindguts. Both peptides increase the frequency and amplitude of spontaneous, rapid contractions. Proctolin induces a slow contraction, which gives the appearance of an increase in overall tonus. DF_2 has no such effect. To determine whether the peptides affect both longitudinal and circular muscles, hindguts were cut into longitudinal strips and into rings, and contractions were recorded from each. The longitudinal strips generated only rapid contractions, and both peptides increased the frequency and amplitude of such contractions without significantly altering tonus. Rapid contractions were observed in only 1 of 14 preparations of rings. Proctolin induced slow contractions in the rings, and DF_2 had no such effect. The results indicate that neuropeptides have different effects on circular and longitudinal muscles of hindgut.

Keywords: Proctolin; FMRFamide; Crustacean; Intestine; Visceral muscle; *Procambarus clarkii*

1. Introduction

As in other species, peristaltic movement of the intestines of decapod crustaceans involves coordinated contractions of circular and longitudinal muscles. These movements are under the control of the central nervous system [22]. The most extensive studies of such neural control have been performed in the lobster, *Homarus*, in which motor output is generated by neurons located in the sixth abdominal ganglion and is sent to the hindgut through the paired seventh abdominal roots [24,25]. Severing these roots, which removes the motor output, causes the hindgut to contract only weakly and in an uncoordinated manner [23]. The neural mechanisms for controlling peristaltic movements have not been studied extensively in crayfish. However, coordinated contractions seem to be mediated by output through the unpaired seventh abdominal root of the sixth abdominal ganglion. Most of the axons in this root can be traced to cell bodies in the sixth abdominal ganglion, but a few axons project to more anterior ganglia [11,18]. Axons from the seventh abdominal root(s) give rise to a dense plexus of fine branches and nerve endings around the hindgut [1,5], and these are presumed to be the sites where neurotransmitters are released onto the circular and longitudinal muscles.

Some progress has been made in identifying transmitters that modulate hindgut contractions. The nerve plexus on the decapod hindgut has long been known to contain catecholamines [2,5,6], and the presence of dopamine in the plexus and in the seventh abdominal root has been confirmed in crayfish [4,14]. Extracts of crayfish hindgut have also been found to contain neuropeptides. These include orcokinin (NFDEIDRSGFGFN; 3), proctolin (RYLPT-OH; 17) and a partially sequenced, FMRFamide-like peptide [17]. Crustacean cardioactive peptide (CCAP) appears to be absent from hindgut extracts of *Orconectes* [19]. All of these substances stimulate contractions in isolated crayfish hindguts. Earlier observations [17] indicated that some substances, such as proctolin, increase both contraction frequency and tonus, while the FMRFamide-like peptides F_1 (TNRNFLRFamide) and F_2 (SDRNFLRFamide) increase contraction frequency but not tonus. The aim of the present work was to account for this apparent difference in effectiveness. Rather than using F_1 and F_2, which are lobster neuropeptides [20], effects of proctolin were compared with those of DF_2 (DRNFLRFamide), a homologous peptide from crayfish [16].

By detecting the movement of probes on the hindgut surface, Winlow and Laverack [23] deduced that during spontaneous movement, contractions of the circular muscles are

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typically much slower than those of the longitudinal muscles. Thus, changes in tonus that we have reported previously [10,14,17] might actually be caused by slow contractions of the circular muscles. The purpose of the present work was to examine effects of neuropeptides on circular and longitudinal muscles separately. Here, we provide evidence that the circular muscles contract slowly, the longitudinal muscles contract more rapidly, and different responses of whole, isolated hindguts to two neuropeptides can be explained by their actions on circular and longitudinal muscles.

2. Method

Adult crayfish (*Procambarus clarkii*) of various sizes were obtained from the Atchafalaya Biological Supply Co. (Raceland, LA) and were maintained in tanks of aerated freshwater at 15 °C. They were fed a diet of carrots and "Tender Vittles™" cat food. Prior to dissection, crayfish were placed in ice for 10 min (to reduce sensation) and were euthanized by decapitation and rapid destruction of the cerebral, suboesphageal and thoracic ganglia. Subsequently, the abdomen was removed, the dorsal abdominal shell was dissected away, and the entire abdominal portion of the hindgut was isolated and placed in a petri dish lined with "Sylgard™" and containing crayfish physiological saline [21] with the following composition: NaCl (205 mM); KCl (5.3 mM); $CaCl_2$ (13.5 mM); $MgCl_2$ (2.45 mM); HEPES (5 mM) (pH 7.4).

Contractions were recorded in a chamber with a volume of 0.5 ml. The chamber was perfused continuously with crayfish saline at a rate of 3.0 ml/min using a peristaltic pump to supply saline at one end and a vacuum pump to remove saline at the other end. Effects of peptides were examined by changing the solutions delivered to the chamber by the peristaltic pump. All experiments were carried out at room temperature (21 °C).

Contractions were recorded using a Grass FT03 tension transducer connected to a Grass Model 7B polygraph. A stylus was constructed to help amplify the contractions and to attach hindgut tissue to the transducer. The stylus consisted of a 51 mm long stainless steel dissecting probe with a 0.35 mm diameter dissecting pin glued to the end of it. The sharp end of the insect pin was bent in the shape of a hook, which was attached to the tissue to record contractions. The stylus was glued to the transducer, parallel to the spring (Fig. 1A). This arrangement amplified contractions by effectively extending the length of the spring.

In some experiments contractions were recorded from "whole" hindguts containing both circular and longitudinal muscles. Small sections of hindgut, approximately 5–8 mm long, were placed in the recording dish and pinned at one end with a stainless steel minutien pin. The tension transducer was then attached to the free end of the hindgut by pushing the hook of the stylus through the tissue. Care was taken not to stretch the tissue too much, to avoid tearing. The

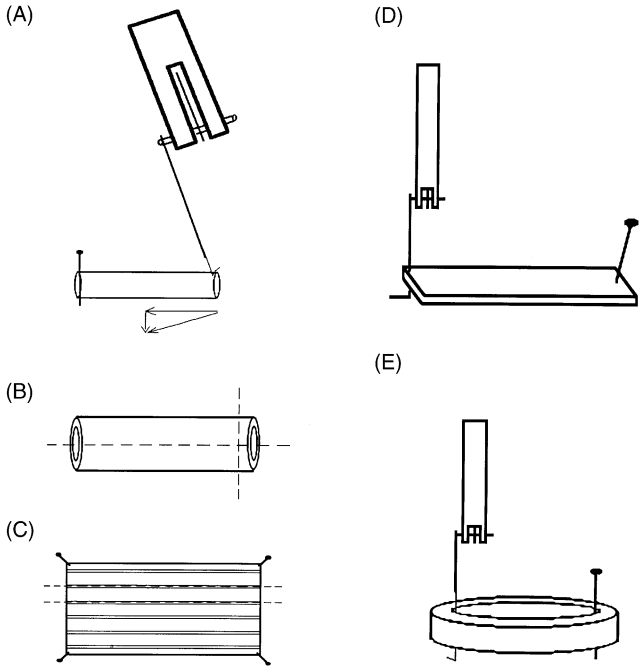

Fig. 1. Schematic diagram showing the methods for recording contractions from sections of "whole" hindgut, longitudinal strips and rings. For "whole" hindgut section (A) a hooked stylus was used to attach the transducer to the hindgut. Arrows indicate force vectors associated with contraction of the longitudinal muscles (horizontal arrow) and the circular muscles (vertical arrow) and the net force generated on the transducer stylus. Sections of hindgut were cut either longitudinally or transversely as indicated by the dashed lines (B). Following longitudinal section, a strip of tissue containing a single band of longitudinal muscles was isolated (C) and was used to record contractions of the longitudinal muscles (D). Following transverse section, a ring of tissue was used to record contractions of the circular muscles (E).

hindgut was kept approximately horizontal and was oriented so that longitudinal contraction produced upward movement of the recorder pen. However, in these experiments the transducer was set at an angle of 75° from the horizontal. Although most of the force detected was generated by contraction of the longitudinal muscles, contraction of the circular muscles also pulled on the transducer and was detected (Fig. 1).

Contractions were recorded preferentially from either circular or longitudinal muscles in the following manner. The hindgut was cut longitudinally, opened and pinned lumen-side-up to reveal the six discrete bands of longitudinal muscle [23], which were always plainly visible (Fig. 1B and C). A longitudinal strip of tissue was cut, isolating one of these muscle bands. One end of the strip was pinned to the bottom of the recording dish, and the stylus from the force transducer was attached at 90° to the free end (Fig. 1D). Alternatively, the hindgut was cut transversely (Fig. 1B) to produce a ring of tissue approximately 1–2 mm in diameter. A pin was then placed through the ring and secured in the bottom of the dish, and the stylus from the transducer was placed through the ring at the other side

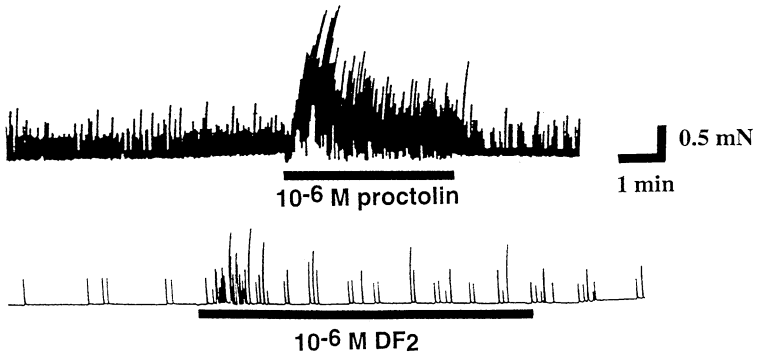

Fig. 2. Recordings of contractions from sections of "whole" hindgut (using the method indicated in Fig. 1). Peptides were present in the bathing solution throughout the period indicated by the solid horizontal bar. The two examples shown were obtained from different preparations.

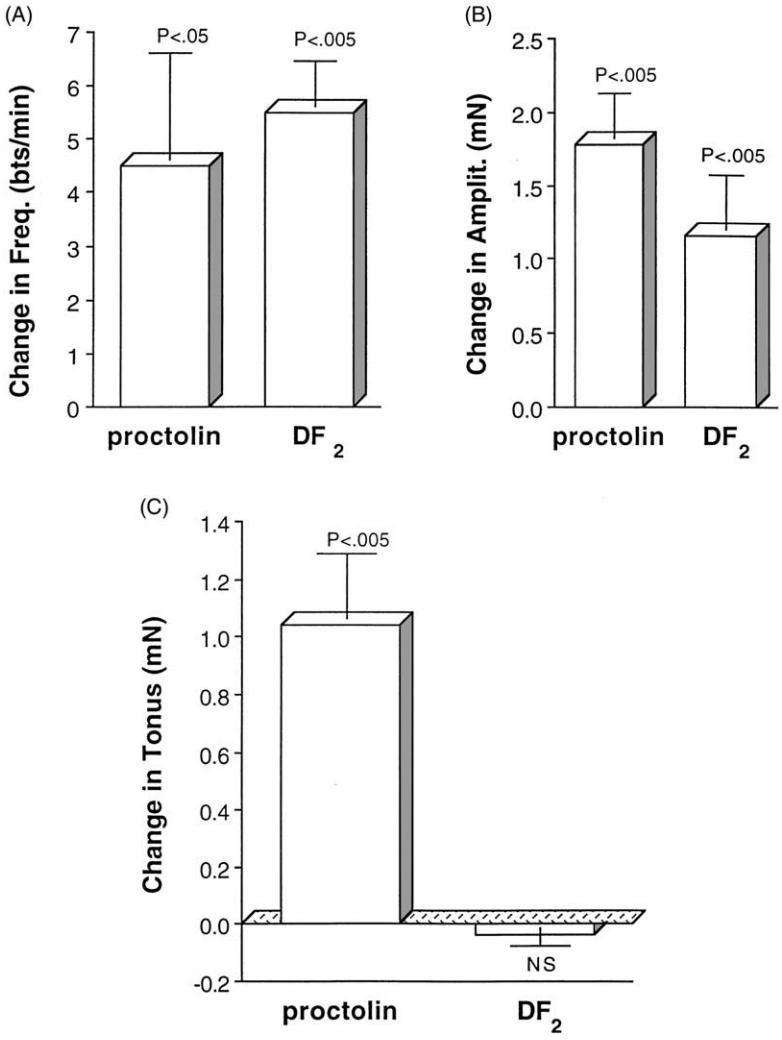

Fig. 3. Effects of peptides on contractions recorded in sections of "whole" hindgut. Changes in contraction frequency (A), contraction amplitude (B) and tonus (C) were determined for experiments with proctolin ($n = 10$ preparations) and DF_2 ($n = 8$ preparations). To quantify effects on rate and amplitude, the maximal response (measured over a 1-min period) was compared with the average rate or amplitude over the 5-min period immediately preceding peptide application, and the numerical difference was taken. Tonus changes were measured as the difference between the highest level of "minimum" contractile force (corresponding to relaxation of the 1–2 s contractions) during the first 1–2 min of peptide application and the average minimum force during the pre-application period. In this figure and in Figs. 6 and 7, the level of statistical significance is indicated, and "NS" indicates that the data were not statistically significant at the 0.05 level.

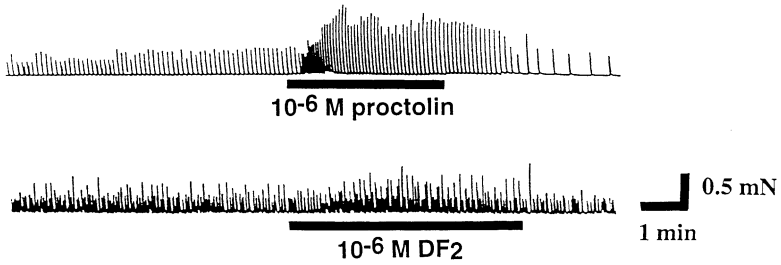

Fig. 4. Contraction recordings obtained from longitudinal strips of hindgut tissue. Peptides were present in the bathing solution throughout the period indicated by the solid horizontal bar. The two examples shown were obtained from different preparations.

and oriented at 90° in order to record contractions of the circular muscles (Fig. 1E). It was necessary to stretch the ring slightly in order to detect contractions.

Levels of statistical significance were determined using a Wilcoxon signed rank test for correlated samples [7]. The value for acceptance was $P < 0.05$.

3. Results

Isolated crayfish hindguts contracted spontaneously, as reported elsewhere [3,8,14,17]. Small sections of hindgut, 5–8 mm in length, generally produced discrete contractions, ranging from 0.8 to 2.0 s in duration. The frequency and

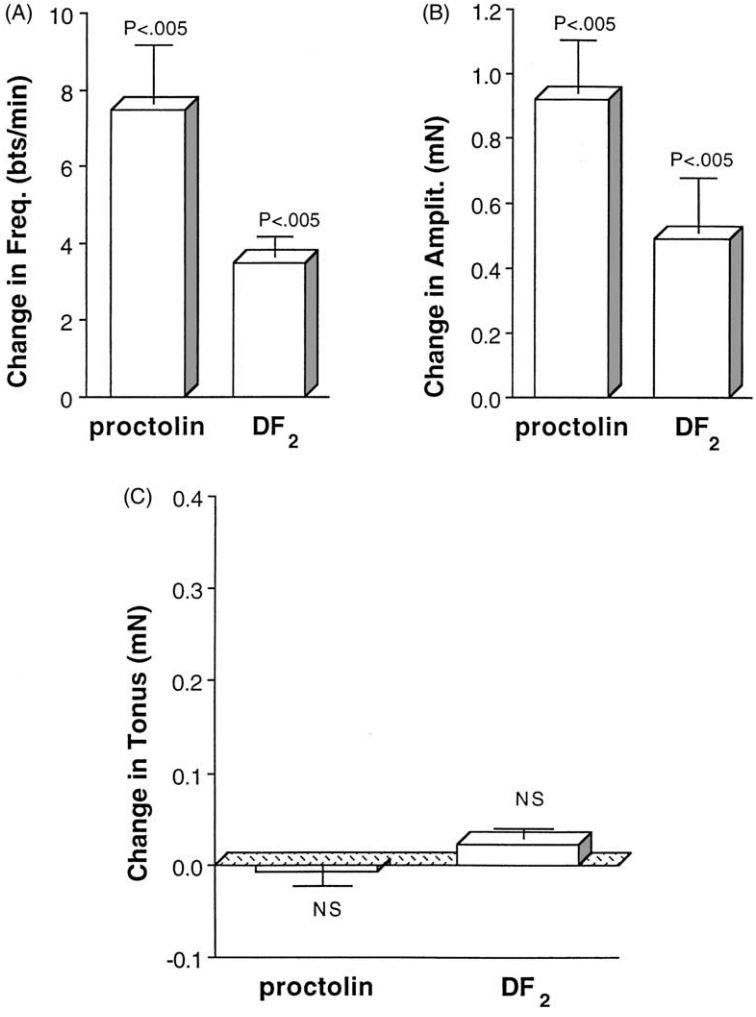

Fig. 5. Effects of peptides on contractions recorded using longitudinal strips. Changes in contraction frequency (A), contraction amplitude (B) and tonus (C) were determined for experiments with proctolin ($n = 12$ preparations) and DF_2 ($n = 10$ preparations). Changes in rate, amplitude and tonus were quantified in the same manner as for "whole" hindguts (see Fig. 4).

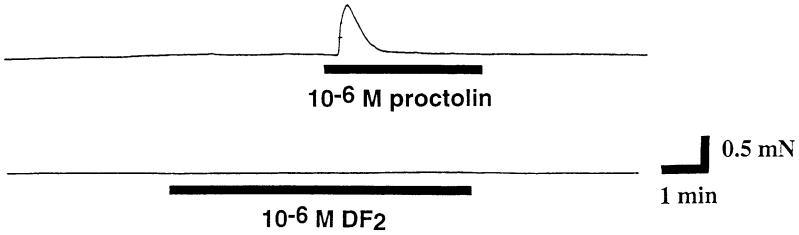

Fig. 6. Recorded contractions from "rings" of hindgut tissue. Peptides were present in the bathing solution throughout the period indicated by the solid horizontal bar. The two examples shown were obtained from different preparations.

amplitude of these contractions were quite variable, both within and between preparations. Longer sections of hindgut invariably produced contractions with more complex waveforms, where contractions frequently summated. This was presumably because muscles in different regions along the length of the hindgut contracted independently, producing summation at random intervals. To simplify the analysis, and to make it feasible to examine the effects of peptides on rate and amplitude of contractions, only hindgut sections less than 8 mm in length were used.

Both proctolin and crayfish peptide DF_2 increased the frequency and amplitude of spontaneous contractions in sections of "whole" hindguts. Thresholds for these were approximately 10^{-9} M for both peptides ([17] and data not shown). For the experiments presented here, the peptides were tested at a concentration of 10^{-6} M (e.g. Fig. 2), which is well above the threshold. Proctolin (but not DF_2) elicited a slow contraction lasting approximately 1–2 min. The threshold for this effect was between 10^{-7} and 10^{-6} M. These observations were in agreement with our earlier work [17], which indicated that proctolin elicits a slow contraction but lobster peptides F_1 and F_2 do not. In our earlier work, we could not determine whether the slow contraction was produced by the circular muscles, whether it was caused by increased tonus in the longitudinal muscles, or whether it represented some combination of both effects. For this reason, it was simply referred to as a change in "tonus," and the same term is used here.

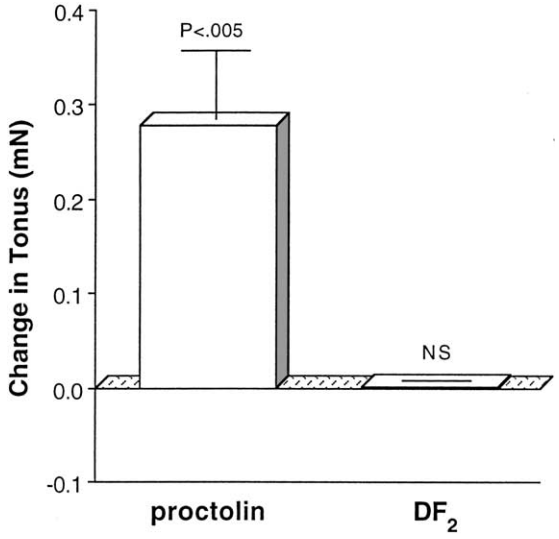

Fig. 7. Effects of peptides on "tonus" in hindgut "rings". The number of preparations used was 14 for experiments with proctolin and 13 for experiments with DF_2. The peak of the contraction was treated as a change in "tonus" and was measured from the peak force in the presence of the peptide to the baseline tension prior to peptide application.

Effects of proctolin and DF_2 on numerous hindgut preparations were quantified and averaged (Fig. 3). Maximal effects on rate, amplitude and tonus typically occurred within the first 1–2 min of peptide exposure. Accordingly, the maximal response was compared to the average rate, amplitude or tonus level during the 5-min period immediately prior to peptide application. Both peptides caused a significant increase in the frequency and amplitude of hindgut contractions. Proctolin caused a significant increase in tonus, but tonus was not altered significantly by DF_2.

Longitudinal strips of hindgut tissue, containing a single band of longitudinal muscle fibers, were also tested for effects of proctolin and DF_2 (Figs. 4 and 5). These longitudinal strips generated spontaneous, rapid contractions that were approximately 1 s in duration. Both peptides increased the frequency and amplitude of such spontaneous contractions, and these effects were statistically significant. As with "whole" hindgut, thresholds for the two peptides were approximately 10^{-9} M. Neither peptide, however, caused any significant change in tonus in these longitudinal strips. This suggested that tonus changes elicited by proctolin on sections of "whole" hindgut were not caused by prolonged contracture of the longitudinal muscles but were probably caused by contractions of the circular muscles. To examine this possibility, hindguts were cut into "rings," and the effects of proctolin and DF_2 were determined (Figs. 6 and 7). These "ring" preparations did not exhibit spontaneous contractions ($n = 15$). Proctolin, at 10^{-6} M, elicited a slow contraction in 13 of 14 preparations tested. The duration of these "slow" contractions was approximately 1 min. DF_2, on the other hand, had no effect on "ring" preparations ($n = 13$) either at 10^{-6} M or at 10^{-5} M.

4. Discussion

The results of the present study strongly suggest that crustacean neuropeptides are able to exert different effects

on circular and longitudinal muscles associated with the crayfish hindgut. Both proctolin and DF_2 excite the longitudinal muscles; proctolin excites the circular muscles, but DF_2 does not. This interpretation is based on the fact that both proctolin and DF_2 increase the frequency and amplitude of spontaneous, rapid contractions generated by longitudinal strips of hindgut tissue, and that circular rings of hindgut tissue contract when exposed to proctolin but not when exposed to DF_2. The validity of the above interpretation, however, depends on whether or not contractions recorded from the longitudinal strips are generated entirely by the longitudinal muscles and on whether or not the contractions recorded from tissue rings in response to proctolin are generated entirely by the circular muscles.

It was not possible to separate completely the circular and longitudinal fibers in these experiments, due to the small size of the tissue and the proximity of the muscle layers. However, at least three lines of evidence suggest that contractions recorded from longitudinal strips and rings, respectively, reflect contractions of the circular and longitudinal muscles. First, the longitudinal strips generated only rapid contractions, and the rings generated only slow contractions. This agrees with movement recordings from lobster hindgut reported by Winlow and Laverack [23], who showed that the contractions of circular muscles are much slower than those of the longitudinal muscles. Second, the tonus changes in the whole hindgut were very similar in duration to the slow contractions generated by the rings. Similarly, the rapid contractions recorded from longitudinal strips were of similar duration to those recorded in whole hindgut sections. Third, the effects of the peptides on strips and rings correlate with their effects on sections of whole hindgut. Proctolin elicited slow contractions in whole hindgut sections (interpreted as increased tonus) and in rings, but not in longitudinal strips. The simplest explanation for these observations is that the changes in tonus observed in whole hindgut sections are mainly caused by contraction of the circular muscles. Both peptides, on the other hand, increased the frequency and amplitude of spontaneous, rapid contractions in both whole hindguts and longitudinal strips, but no such effect was observed with rings. The simplest explanation for these observations is that the small contractions recorded from whole hindguts were generated by the longitudinal muscles and not by the circular muscles.

The present results help to explain earlier observations [17] that proctolin and the FMRFamide-like peptides, F_1 and F_2, increase the frequency and amplitude of spontaneous contractions of the crayfish hindgut, and that proctolin increases tonus of the hindgut while F_1 and F_2 do not. Although the crayfish peptide, DF_2 was used in the present investigation, it is very closely related to F_1 and F_2, and its effects were essentially the same. The ability of all these peptides to increase the frequency and amplitude of spontaneous contractions appears to involve a stimulatory action on the longitudinal muscles. Proctolin appears to increase tonus by stimulating a slow, prolonged contraction of the circular muscles. DF_2, F_1 and F_2, which are all FMRFamide-like peptides with N-terminal extensions containing "Arg-Asn" (i.e. "RNFLRFamides") do not increase tonus in whole hindguts, and this appears to be because they do not elicit contractions of the circular muscles. The results also suggest that in several of our earlier reports [10,14,17], increases in tonus may have been caused by contraction of the circular muscles, which would have generated a force vector detected by the transducer (as in Fig. 1A).

To date, little attempt has been made to study modulation of circular and longitudinal muscles separately, at least in invertebrate preparations. Lange and Orchard [12] examined the modulatory effects of proctolin and several FMRFamide-like peptides on circular muscles of the locust midgut, but not on the longitudinal muscles. Their data for the circular muscles are very similar to those of the present study. The circular muscles of the locust midgut rarely contracted spontaneously, and proctolin elicited a large increase in tonus. Moreover, the circular muscles were not affected by three nanopeptides containing the sequence "RNFLRFamide," and one of these peptides contained the entire sequence of DF_2 [12]. These data support the present findings and suggest that at least some neuropeptides may serve common modulatory functions in arthropod visceral muscles.

The effects of DF_2 (present study) and lobster peptides F_1 and F_2 on crayfish hindgut [17] suggest that "RNFLRFamide" peptides are generally excitatory on this preparation. Such excitatory effects, however, do not extend to the visceral muscles of all arthropods, since *Limulus* midguts are inhibited by lobster peptide F_1 and by the crab neuropeptide, GYNRSFLRFamide [9]. Thus, caution must be exercised when attempting to generalize regarding the physiological actions of invertebrate neuropeptides. Differences between the effects of "RNFLRFamides" on *Limulus* and crayfish intestinal muscles probably reflect differences in the receptors for these peptides or in the intracellular signaling mechanisms to which the receptors are coupled.

Possible effects of combinations of neuropeptides were not examined in the present study. Lange and Orchard [12] reported that the effect of proctolin on circular muscles of the locust midgut is inhibited by three nanopeptides belonging to the myosuppressin sub-group. These peptides contain the common sequence "HXFLRFamide," where "X" refers to either Ser or Val. We have not ruled out the possibility that DF_2 or other FMRFamide-related peptides might suppress the excitatory effect of proctolin on crayfish hindgut muscles. However, Leucomyosuppressin (pQDVD-HVFLRFamide) increases tonus in sections of whole crayfish hindgut [17], which would suggest a stimulatory effect on the circular muscles rather than an inhibitory effect.

Although RNFLRFamide-like peptides can modulate contractions of crayfish hindgut muscle, it is unclear whether they do so under physiological circumstances. Extracts of crayfish hindgut do not contain significant amounts of F_1, F_2, DF_2 or other closely related peptides [17], so it is very

unlikely that such peptides are released directly onto the hindgut. Crayfish pericardial organs contain DF_2 and one other heptapeptide (NRNFLRFamide), both of which can be released into the haemolymph [16]. These peptides might act on hindgut muscles if sufficient amounts of peptide get there through the circulation. Crayfish haemolymph, contains FMRFamide-like immunoreactivity in amounts equivalent to approximately 0.2 nM, and this increases to approximately 0.7 nM 1 h after feeding [13]. This is just below the threshold for effects on contraction of the longitudinal muscles. On the other hand, we have not examined effects of mixtures of proctolin and FMRFamide-like peptides and cannot rule out the possibility that DF_2 may be more effective in the presence of proctolin or some other peptide.

Immunohistochemical evidence indicates the presence of FMRFamide-like material in nerve terminals on the crayfish hindgut [15]. This suggests that FMRFamide-like peptides are released as transmitters directly onto the hindgut muscles. Extracts of crayfish hindgut do contain at least one FMRFamide-like peptide that has only been partially sequenced [17]. It will be necessary to obtain a complete sequence before the effects of this peptide can be elucidated.

Overall, the present results suggest pharmacological differences between the effects of neuropeptides on circular and longitudinal muscles. This suggests the possibility of differential modulation of such muscles by neuropeptides.

Acknowledgments

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