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A STRUCTURE-ACTIVITY STUDY OF THE NEUROPEPTIDE PF1, SDPNFLRFamide, USING THE DORSAL BODY WALL MUSCLE OF THE CHICKEN NEMATODE, *ASCARIDIA GALLI**

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The action of a range of N terminally modified peptides structurally related to the nematode peptide PF1, SDPNFLRFamide, has been investigated using a dorsal muscle strip preparation from the chicken nematode, *Ascaridia galli.* Acetylcholine contracts this muscle preparation in a concentration-dependent manner when applied in the range 1–100 μM with an EC50 value of 9 μM. These contractions are reduced in the presence of PF1 and its analogues, with a threshold effect of PF1 of around 1 nM and an IC50 value of 470 nM against 10 μM acetylcholine. All the PF1 analogues tested were less potent than PF1 in reducing the acetylcholine contractions, indicating the importance of the N terminal amino acids in the action of PF1 in this preparation.

Keywords: Ascaridia galli – PF1 – SDPNFLRFamide – acetylcholine – dorsal muscle strip

INTRODUCTION

Neuroactive peptides play key transmitter and modulatory roles in synaptic transmission throughout the animal phyla and attempts have been made to identify evolutionary links between different peptide families [13]. The FMRFamide family of peptides was first sequenced from tissue in lamellibranch molluscs [18]. Subsequently members of this family have been identified not only in all the major molluscan classes but in most, if not all, animal phyla [21]. RFamides are even present in cnidaria (coelenterates), suggesting that members of this family were some of the first compounds to act as synaptic transmitters [10]. FMRFamide-related peptides (termed FaRPs) were first identified in nematodes by Stretton and his group [5]. Since then around 60 members of the family have either been identified or proposed from gene sequences in nematodes [4, 16].

Many of the first FMRFamide peptides to be identified in nematodes were isolated from *Ascaris suum* and were termed AF peptides [6]. Subsequently others were

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isolated from *Panagrellus redivivus* [9] and *Caenorhabditis elegans* [16]. The FMRFamide genes isolated from *C. elegans* were termed flp genes [19] and currently there are 22 flp genes in *C. elegans* [16]. The first flp gene, flp 1, encodes seven distinct FaRPs with FLRFamide sequences at their C terminal [17]. These authors found that flp-1 was expressed in neurones in the anterior region of *C. elegans*, including neurones which have been shown to contain FMRFamide-like immunoreactivity [20]. Nelson et al. [17] found evidence that flp-1 plays a role in co-ordination, activity and sensitivity to high osmolarity. Among the peptides encoded by flp-1 are CF1, SDPNFLRFamide, and CF2, SADPNFLRFamide, which have also been identified in *P. redivivus* and named PF1 and PF2, respectively [9]. Both peptides have been tested on *A. suum* body wall muscle and found to relax the muscle [8]. In addition to a postsynaptic action it is likely that both peptides have presynaptic actions to modify acetylcholine release [11]. Acetylcholine is the excitatory transmitter onto nematode body wall muscle [14]. The actions of PF1 and PF2 have also been tested on pharyngeal pumping in *A. suum* but neither peptide appear to have an observable effect [4]. Both PF1 and PF2 have, however, been found to inhibit contractions and relax the vagina vera of *A. suum* [7]. It has been proposed that the relaxing action of PF1 and PF2 on *A. suum* body wall muscle is mediated through nitric oxide (NO). Compounds which interfere with NO synthesis blocked the effect of PF1 on levamisole-induced contractions. In contrast sodium nitroprusside, which releases NO, mimicked the actions of PF1 and PF2 on levamisol-induced contractions. These authors also demonstrated that NO synthase activity was present in *A. suum* tissue, particularly in the hypodermis [1].

In the present report a structure-activity study has been carried out by altering the N terminal amino acids of PF1 to determine their relative importance in reducing the amplitude of acetylcholine-induced contractions of the isolated body wall muscle of *Ascaridia galli*.

MATERIALS AND METHODS

A. galli were cultured in house, using the domestic chicken as host, *Gallus gallus* White Laying Leghorn male or Brown Leghorn female. Eggs were obtained from adult *A. galli* by chopping the adults into fine fragments which were agitated in 5 ml water to free eggs from the oviduct fragments. The egg suspension was filtered and resuspended in 0.01% potassium dichromate to retard bacterial growth and reduce egg clumping. Eggs were incubated in a Petri dish at 28 °C for 12–14 days in >80% humidity. Approximately 250 eggs in 250 µl were introduced directly into the stomachs of one day old chicks using standard syringe and gavage needle. Chicks were routinely reared for 6–8 weeks. Infected chicks were killed by cervical dislocation and the intestines removed immediately after death. The intestines were opened, the *A. galli* removed, washed in artificial perienteric fluid (APF) and maintained in APF at 41 °C. *A. galli* were used for experiments only on the day of removal from the host.

APF had the following composition: NaC1 67 mM; Na acetate 67 mM; $MgCl_2$ 15.7 mM; $CaC1_2$ 3 mM; KC1 3 mM; Tris Buffer 5 mM; Glucose 3 mM. The pH was adjusted to 7.6 with glacial acetic acid at 37 °C.

Dorsal muscle strips (DMS) were prepared from *A. galli* from the entire dorsal muscle field from 2–3 mm posterior to the mouth up to the genital pore. The tissue was approximately 7–12 mm long and 1–2 mm wide. *A. galli* DMS were suspended in a 10 ml vertical organ bath. At one end a loop of surgical thread (Mersilk twisted silk, Gauge 7.0) was used to secure the DMS at the base of the organ bath. The DMS was then attached in series to an isometric transducer (Harvard) vertically above the preparation. The transducer was connected to a Kipp&Zonnen (BD401) chart recorder via a Harvard student pre-amplifier. The temperature of the organ bath was maintained at around 40 °C. Drugs were applied either by perfusion from a reservoir (application >2–3 minutes) or directly to the organ bath in a 1 ml volume (application 1–3 minutes). To aid mixing of the drugs air was bubbled through the organ bath.

Acetylcholine chloride was obtained from Sigma while the peptides were obtained from Altra Biosciences, University of Birmingham, U.K.

In terms of the statistical analysis, mean values are expressed with standard error of the mean for n determinations. P values were calculated from the Student's *t*-test, paired or unpaired as appropriate.

RESULTS

As in the case of *A. suum* body wall muscle, acetylcholine contracts *A. galli* body wall muscle in a concentration-dependent manner (Fig. 1), with an EC50 value of

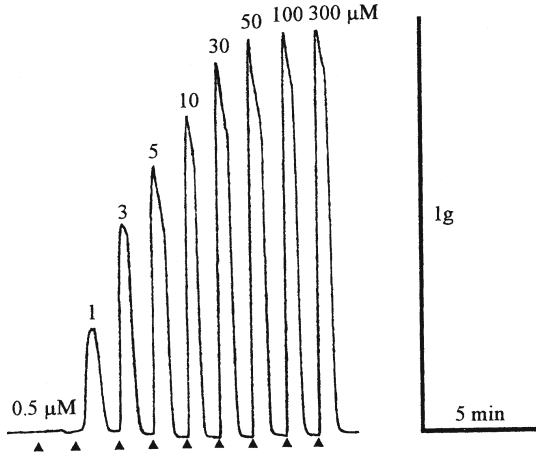

Fig. 1. Chart recorder traces showing the effect of increasing concentrations of acetylcholine on isometric tension in the *A. galli* dorsal muscle strip. Acetylcholine was removed by washing the tissue with fresh APF approximately 30–40 seconds following bath application (▲), ensuring that the response for each concentration had peaked before the wash

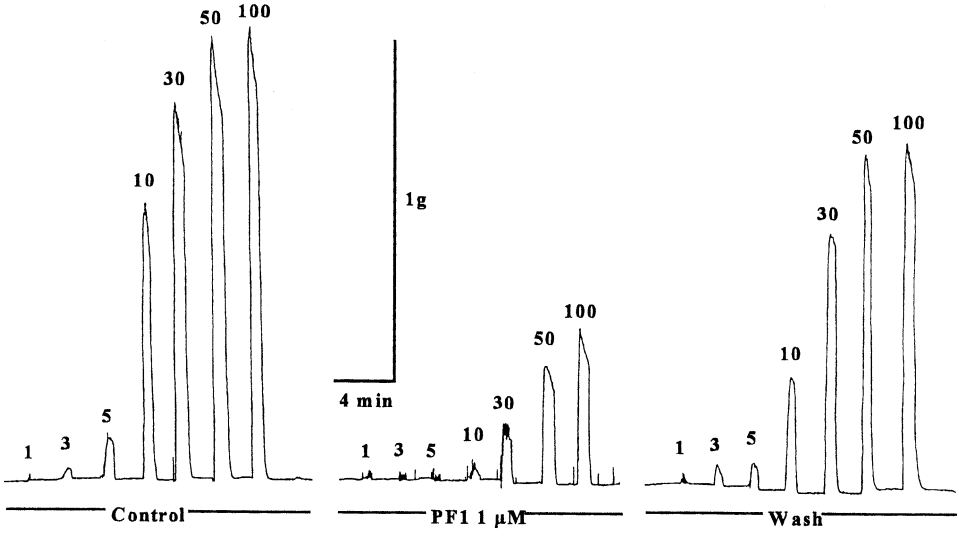

Fig. 2. Chart recording showing the effect of PF1 (SDPNFLRFamide), 1 µM, on the response to increases in isometric tension elicited by acetylcholine in the *A. galli* dorsal muscle strip. Responses to acetylcholine in the presence of PF1, 1 µM, were recorded following pre-incubation of the dorsal muscle strip with PF1, 1 µM, for 5 minutes. Responses were reduced compared to control responses (Control). Following a wash and recovery period of 30 minutes the effect of PF1 was reversed (wash)

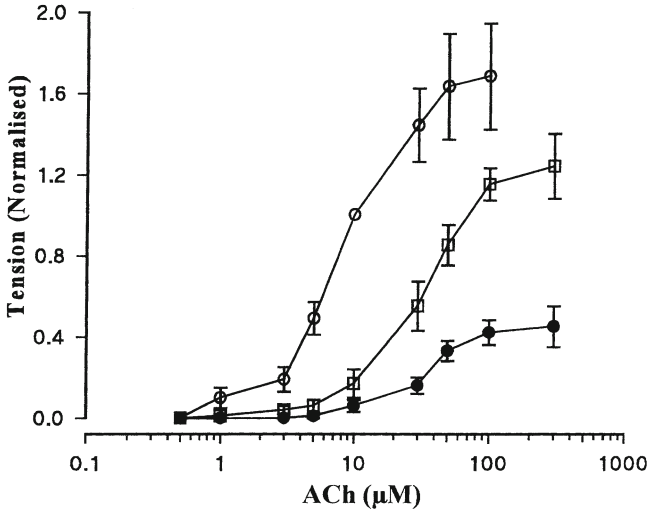

Fig. 3. Increases in isometric tension elicited by acetylcholine (F) in the *A. galli* dorsal muscle strip were reduced in the presence of 1 µM PF1 (SDPNFLRFamide ●). The effect of PF1 was reversed after a wash followed by an approximately 30 minute recovery period (G). n=6; error bars SEM. Data were normalised to the control 10 µM acetylcholine

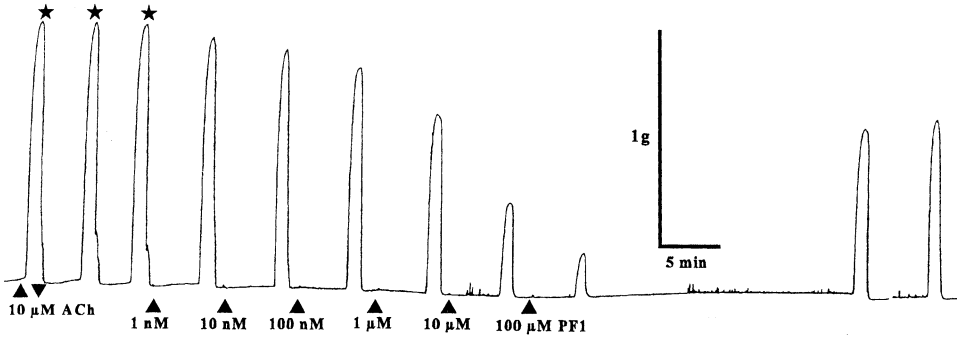

Fig. 4. The *A. galli* dorsal muscle strip was repeatedly given 10 µM acetylcholine as indicated in the first response. Compared to three control responses (★), increasing concentrations of PF1, 1 nM–100 µM, progressively decreased the amplitude of the responses elicited by acetylcholine, 10 µM. Following the final wash and an approximately 30 minute recovery period, the effects of PF1 were partially reversed

9.0 ± 2.0 µM, n = 15. PF1 had a potent effect on contractions elicited by acetylcholine on the dorsal muscle strip of *A. galli* but the effect on resting tension was inconsistent and usually no measureable change in resting tone was observed, even when applied at 100 µM (see Fig. 4). The threshold value for acetylcholine on the muscle was 1–3 µM, (Figs 1, 2). In the presence of 1 µM PF1 these contractions are greatly attenuated (Fig. 2). The acetylcholine contractions return to control following saline wash for 30 minutes (Fig. 2). The results from six separate experiments are shown graphically in Figure 3. It can be seen from this figure that the maximum response to acetylcholine is depressed indicating a non-competitive-type block of the acetylcholine contraction. The mean response to 100 µM acetylcholine in the presence of 1 µM PF1 was 31.0 ± 7.0% of the control. Following wash the response was still reduced compared to control. The effect of PF1 on the acetylcholine contractions was concentration-dependent (Fig. 4). When PF1 was tested against a repeated concentration of acetylcholine, 10 µM, increasing concentrations of PF1, 1 nM–100 µM, progressively reduced the size of contraction (Fig. 4). On two occasions 1 nM PF1 elicited a measureable decrease in the amplitude of the 10 µM acetylcholine response but overall this reduction was not significant. The IC50 value for this effect of PF1 on contractions elicited by 10 µM acetylcholine was 470 ± 22 nM.

The effect of a series of analogues of PF1 where the N terminal amino acids were substituted or removed were tested for PF1-like activity to obtain information regarding the role of the different amino acids in contributing to the potency of the peptide. The results are summarised in Figure 5. PF2, 1 µM, significantly and reversibly reduced the responses to acetylcholine, 1–300 µM, but the effect was less than in the case for PF1 (Fig. 5). In this figure the effect of the peptides against a contraction elicited by 10 µM and 100 µM acetylcholine was tested. One µM PF2 reduced the response to 10 µM to 17.0 ± 6.5% of control and the response to 100 µM to 43.0 ± 6.0% of control, n = 6. The other PF peptide analogues tested all exhibited a

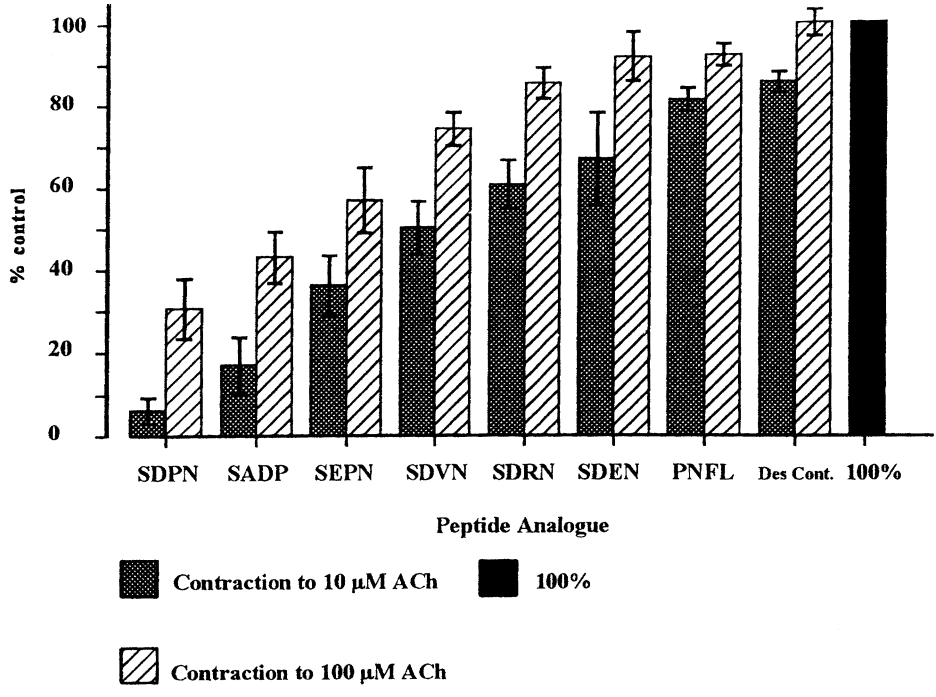

Fig. 5. Summary of PF peptide analogue activity. The histogram shows sample data from each of the *A. galli* organ bath experiments. The analogue labels have been abbreviated to their first four letters. For example SDPN represents the analogue SDPNFLRFamide. For each analogue two bars are shown, representing the mean response elicited by ACh at 10 μM and 100 μM, in the presence of the peptide analogue at a concentration of 1 μM. Each bar represents the mean amplitude of contractions as a percentage of control ACh responses. For PNFLRFamide, n = 4 ± SEM. For all other analogues n = 6 ± SEM. Data from the desensitisation control experiment are shown for comparison. The potency of the peptide analogues was SDPN (SDPNFLRFamide) > SADP (SADPNFLRFamide) > SEPN (SEPNFLRFamide) > SDVN (SDVNFLRFamide) > SDRN (SDRNFLRFamide) > SDEN (SDENFLRFamide) > PNFL (PNFLRFamide)

smaller effect on the acetylcholine contractions compared to both PF1 and PF2 (Fig. 5). Substitution of aspartate (D) by glutamate (E) in PF1, reduced the potency of the peptide in reducing acetylcholine contractions (Fig. 5). SEPNFLRFamide, 1 μM, significantly reduced the tension increase elicited by 10 μM acetylcholine to 36 ± 7.0% control. At 100 μM acetylcholine the peptide reduced the response to 57 ± 8.0%, n = 6, $P < 0.05$. Substitution of the proline (P) by valine (V) to give SDVNFLRFamide also reduced responses to acetylcholine and the effect was significant, $P < 0.05$, at both 10 μM and 100 μM acetylcholine. For example, at 10 μM acetylcholine, the response was reduced to 50 ± 6.0% while at 100 μM acetylcholine the response was reduced to 74 ± 4.0% of control. Following the application of SDVNFLRFamide and subsequent wash there was no recovery in the acetylcholine

responses. However this sustained reduction in the acetylcholine responses, by comparison to the desensitization data, appeared greater that could be accounted for by desensitization alone.

Substitution of proline (P) by arginine (R) to give SDRNFLRFamide significantly reduced the acetylcholine responses. For example, in the presence of 1 μM peptide the response to 10 μM acetylcholine was reduced to 61 ± 6.0% of control and to 100 μM acetylcholine was reduced to 85 ± 4.0% of control, $P < 0.05$, $n = 6$. Again there was no significant recovery following washing away the peptide. Substitution of proline (P) by glutamate (E) to give SDENFLRFamide only significantly reduced the response to 10 μM acetylcholine, $P < 0.05$, $n = 6$. For example, in the presence of 1 μM peptide, the response to 10 μM acetylcholine was reduced to 67 ± 11% of control while the response to 100 μM acetylcholine was only reduced to 92 ± 7.0% of control. The least potent of the analogues was PNFLRFamide where in the presence of 1 μM peptide the response to 10 μM and 100 μM acetylcholine was reduced to 81 ± 3.0% and 92 ± 3.0%, respectively. Responses to acetylcholine following wash were not significantly different from acetylcholine responses in the presence of the peptide. Furthermore the apparent reduction in the amplitude of the acetylcholine responses was similar to those seen in the desensitization control data.

DISCUSSION

The results described in this study using body wall muscle from *A. galli* in terms of responses to acetylcholine are similar to those previously described for *A. suum*. However under the recording conditions used in this investigation *A. galli* body wall muscle exhibited very little spontaneous activity and relaxations which are typically seen with *A. suum* preparations. There have been relatively few previous studies using *A. galli* muscle apart from the electrophysiological studies of Wann [22]. The absence of direct relaxations were observed following the application of PF1 and its analogues in the current study. In contrast PF1 has a marked relaxing effect on *A. suum* dorsal wall muscle [1, 8]. This difference may reflect the physiological state of the muscle under the recording conditions used.

The current studies demonstrate the importance of the N terminal amino acids in conveying the potency of PF1 and PF2. Since removal of SD resulted in almost complete loss of activity. The introduction of an alanine between the N terminal serine and aspartate to give PF2 resulted in a small loss of potency. All the amino acid substitutions resulted in a loss of potency, including the replacement of aspartate by glutamate. A slightly greater loss of potency was seen when proline was replace by valine. The presence of a proline bends a straight chain of amino acids and so slightly distorts the shape of the peptide in relation to its receptor. It is therefore not surprising that SDVNFLRFamide is considerably less potent compared to SDPNFLRFamide. The replacement of proline by arginine results in an even greater loss of potency. In this case there is not only a change in the shape of the peptide but a change in its charge since arginine is basic and would reduce the influence of the

negative charge conveyed by aspartate. In addition proline and valine are non-polar while arginine is polar. The replacement of proline by glutamate not only changes the shape of the peptide but enhances the negative charge and clearly further reduces the interaction between the peptide and its receptor, making SDENFLRFamide the least potent of the octapeptides tested in this study. Finally the removal of the two N terminal amino acids renders the peptide virtually inactive in terms of reducing the response to acetylcholine.

There has been one other recent structure-activity study based on manipulating the PF1 amino acid sequence [2]. These authors used the level of relaxation as a test of relative potency where they increased the muscle tone with levamisole and then added the peptide while the muscle, in this case from *A. suum*, was still contracted to levamisole. Secondly they used a denervated preparation. They also approached the structural modifications slightly differently, viz, the use of 'D' amino acids and also substitution nearer the C terminal. Interestingly they found that substitution of proline by alanine did not result in any loss of potency while replacing either asparagine or phenylalanine 5 with alanine resulted in an increasing loss of potency. Replacing leucine with alanine actually enhanced potency. While as expected replacing either arginine or the C terminal phenylalanine with alanine resulted in loss of potency. As expected removal of the C terminal amide resulted in a complete loss of potency. In contrast to the present study, Bowman et al. [2] found that PNFLRFamide was still active, about half the potency of PF1 and more potent that DPNFLRFamide. Replacement of the L-isomer by the D-isomer for phenylalanine 5 or leucine resulted in peptides that were excitatory. The D-isomer in the cases of proline, asparagine, arginine and the C terminal phenylalanine resulted in almost complete loss of activity, indicating the importance of these amino acids.

Finally another peptide related in structure to PF1, viz, KPNFLRFamide, has also been studied by Bowman et al. [2]. This peptide is very similar in structure to PF4, KPNFIRFamide, the latter being the subject of studies by Kubiak et al. [15] and Holden-Dye et al. [12]. Interestingly KPNFIRFamide and KPNFLRFamide are physiologically indistinguishable in *A. suum* bioassays [15]. PF1 and KPNFLRFamide act through different receptors and use different ions, using potassium and chloride, respectively [2] while PF4 acts through chloride channels [12]. The substitution of proline in PF4 by alanine greatly reduced potency while KANFLRFamide and KANFIRFamide were equipotent [15].

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