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Structure-activity relationships of insect neuropeptides of the pyrokinin/PBAN family and their selective action on pupariation in fleshfly (*Neobelleria bullata*) larvae (Diptera: Sarcophagidae)

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Abstract. Screening for puparium formation accelerating activity of neuropeptides and/or analogues belonging to 14 different peptide families revealed the strong activity of members and analogues of the pyrokinin/PBAN (pheromone biosynthesis activating neurohormone) family that all share the common C-terminal sequence, FXPRLamide (X = V, T, S, or G). Both pupariation behaviour and cuticular tanning can be accelerated by a C-terminal pentapeptide fragment composed of only the FTPRLamide sequence. Truncation of the C-terminal sequence to the tetrapeptide TPRLa did not diminish either aspect of the activity. Markedly reduced, but still significant, activity was observed after further truncation to the pyrokinin C-terminal tripeptide. The RLa terminal fragment showed no activity. Thus the C-terminal tripeptide appears to be the active core for pupariation acceleration. The core sequence for a maximum response is the C-terminal tetrapeptide TPRLa. This represents a major difference from the activity profile observed in other pyrokinin assays, in which the C-terminal pentapeptide is required. The C-terminal amide group is also of great importance to pupariation acceleration activity, as LPK acid induces a large drop in threshold activity. Periviscerokinin-2 contains a C-terminal tripeptide sequence (PRVamide) that is quite similar to the pyrokinin C-terminal tripeptide PRLamide and, accordingly, elicits a lower level pupariation acceleration activity. The locust pyrokinin Lom-MT-IV preferentially promotes acceleration of the behavioural over the tanning aspects of pupariation and can therefore, in large measure, provide a means of separating the two aspects. Ligation experiments demonstrated that the effect of the LPK analogues on pupariation behaviour is likely mediated through the CNS, while the action on cuticular tanning is of a peripheral nature.

INTRODUCTION

Pupariation in cyclorrhaphous Diptera has been recognised as a complex process involving both behavioural activities (immobilisation, anterior segment retraction, longitudinal body contraction), and cuticular changes known as sclerotisation (deplasticisation and phenolic tanning) (Žďárek & Fraenkel, 1972; Žďárek et al., 1979). This complex morphogenetic process has been shown to be orchestrated by compounds of neurosecretory origin. The active principle was found in extracts of various neural or neurohaemal organs, as well as in the haemolymph of pupariating larvae (Žďárek & Fraenkel, 1969). When an active agent was injected into fleshfly larvae 2–3 h before the onset of pupariation, both

behavioural and cuticular events were greatly accelerated (Fraenkel et al., 1972). The two separate factors were isolated and partially purified and named the puparium tanning (accelerating) factor (PTF) and the anterior (segments) retraction factor (ARF), according to the effects the assayed materials produced in the tests then used (Sivasubramanian et al., 1974). The exact chemical nature of these pupariation factors has never been fully identified, but their proteinaceous nature and neurosecretory origin has been firmly established (Žďárek & Sivasubramanian, 1991 for review). In our present study we have employed a different strategy to approach the problem of the chemical characterisation of the pupariation factors, namely screening of various compounds representing different peptide families for their effects on the process of pupariation.

Comparison of chemical structures of neuropeptides that regulate various aspects of insect homeostasis revealed some common features among many of these compounds. Therefore it is not surprising that the same class of neuropeptides may serve different physiological and behavioural functions. For example, neuropeptides of the pyrokinin/PBAN family, originally identified as myotropins from orthopteroid insects (Holman et al., 1986; Nachman et al., 1986; Schoofs et al., 1991), appear to be widespread in several insect orders. Its members were found to serve several diverse functions including induction of sex pheromone production in various species of female moths (Kitamura et al., 1989), melanisation and reddish colorisation in caterpillars (Matsumoto et al., 1990), and diapause in silkworm moth eggs (Imai et al., 1991). The existence of crossactivity (Abernathy et al., 1995; Fonagy et al., 1992; Kuniyoshi et al., 1992; Nachman et al., 1993a) of the pyrokinin neuropeptides outlined above gave us an impetus to screen various synthetic arthropod neuropeptides which represented 14 different peptide families for their ability to accelerate pupariation behaviour and puparial tanning in larvae of the fleshfly, *Neobelleria bullata* (Parker). As a result we found leucopyrokinin (LPK) and analogues, members of the pyrokinin/PBAN family, highly active in our pupariation bioassay. In the following experiments, we endeavored to evaluate members of the pyrokinin/PBAN peptide family isolated from lepidopteran sources, i.e. the pheromonotropin HezPBAN from *Helicoverpa zea* and the diapause hormone Bom-DH-1[19-Trp] from *Bombyx mori*, as well as several families that have related but incomplete sequence similarities in the C-terminal region of the pyrokinins. These latter peptides would include the periviscerokinins (C-terminal PRVamide) from the cockroach *Periplaneta* (Predel et al., in press) and the myomodulins (C-terminal LRLamide) from molluscan sources (Cropper et al., 1987). In adddition, we have focused on the patterns of activity changes associated with various modifications of the amino acid sequence, particularly in the C-terminus.

MATERIAL AND METHODS

The bioassayed peptides initially screened for their pupariation accelerating activity included members and/or analogues of 14 peptide families, namely (1) insect kinin [Culekinin depolarizing peptide (CDP) – NPFHSWGamide], (2) pyrokinin/PBAN: [Leucopyrokinin (LPK) – pQTSFTPRLamide], (3) FMRFamide-related peptides: [*Leucophaea maderae* myosuppressin (Lem-MS) – pQVDHVFLRFamide], (4) insect tachykinin: (Cus-MT-1 – APSGFMGMR amide), (5) sulfakinin: [4-11, Nle^{9}] *Leucophaea maderae* [$Ser(SO_3)^2$]LSK-II – pQS(SO_3H)DDY(SO_3H)GHMRGamide, (6) diuretic peptide: [CRF-like diuretic peptide (CRF-DP) – NKPSLSIVNPLDVRLRQRLLLEIARRQMKENTRQVELNRAILKNVamide], (7) Adipokinetic hormone / red pigment coloration hormone (AKH/RPCH): *Blaberus discoidalis* (Bld-HrTH – pQVNFSPGWGTamide), (8) eclosion hormone: [(Nle^{11}, Nle^{24}, 4-NaphthylAla^{28}) *Manduca sexta* –

eclosion hormone analog [(Nle^{11}, Nle^{24}, 4-NaphthylAla^{28}) Mas-E – NPAIATGYDRUEICEINCANCK-KULGAZFEGPLCAESCIKFKGKLIPECEDFASIAPPFLNKL-OH; U = Norleucine; Z = 4-Naphthyl-alanine], (9) corazonin: corazonin (pQTFQYSRGWTNamide), (10) allatostatin: allatostatin IV (DRLYSFGLamide), (11) periviscerokinin: periviscerokinin-2 (GSSSGLISMPRVamide), (12) myo-modulin: myomodulin (PMSMLRLamide), (13) proctolin: proctolin (RYLPT-OH), and (14) crustacean cardioaccelerating peptide: CCAP (PFCNAFTGCamide). The chemicals to be bioassayed were dissolved in a buffered Ringer solution.

The bioassay for pupariation factor activity was performed as described earlier (Žďárek, 1980). Briefly, larvae of the fleshfly, *N. bullata* were bred on beef liver and used for all experiments. The tested material was injected by means of a calibrated glass capillary into red-spiracle larvae (2–3 h before pupariation) previously immobilised by chilling on ice. Control larvae were injected with Ringer solution only. After removal from ice the injected larvae were kept at 25°C and the time of anterior retraction (R), longitudinal contraction (C), and onset of tanning (T) was recorded. Normally retraction precedes contraction, and tanning starts after the white puparium has been formed. The effects of a tested compound were expressed as a difference between the mean time after which the developmental change (R, C and T) occurred in the control and experimental groups of larvae. Twelve to 16 larvae were injected in each group. For the evaluation of direct myotropic action the tested peptides were injected into the posterior part of a ligated larva. A ligature placed at the anterior third of the body (i.e. behind the fused central nervous system) causes denervation of all somatic musculature and consequently paralysis of the hind part, but it does not prevent tanning if applied after the critical period for the moulting hormone release. Tanning of the hind part is, however, delayed in relation to tanning of the anterior part containing the CNS and neuroendocrine centres. Peripheral effects of the tested compounds on the neuromuscular system are manifested by spontaneous contractions of the abdominal somatic muscles behind the ligature. The effects on the cuticle can be recognised as an acceleration of posterior tanning in relation to the anterior region.

Compounds designed to test structure-activity relationships of LPK-related analogues were synthesised on a Milligen-BioSearch 9600 peptide synthesizer according to previously described procedures (Nachman et al., 1986). The peptide concentrations were determined via amino acid analysis using Phe or Leu as the standard. In order to establish the *minimum threshold dose* (the minimum concentration of the tested compound that accelerated R, C and/or T to 80% or less of the control time), the compounds were serially diluted in the buffered Ringer solution and tested on non-ligated larvae as described above.

RESULTS AND DISCUSSION

Pupariation accelerating activity of representatives of different peptide families

The results of screening for pupariation acceleration activity of synthetic peptides representing 14 different peptide families are given in Table 1. A high pupariation accelerating activity at a level of 1 pmol or less was recorded only for leucopyrokinin (LPK), a peptide of the pyrokinin/PBAN family. A marginal activity was detected in a diuretic peptide isolated from the housefly (CRF-DP), in the adipokinetic hormone from the cockroach *Blaberus discoidalis* (Bld-HrTH), and in periviscerokinin-2 from the cockroach *Periplaneta americana* (R. Predel et al., unpublished data). Peptides of other families did not show any activity at the highest dose tested (250 pmol/larva).

When several synthetic LPK analogues were tested, all of them proved to be active in our pupariation bioassay (Nachman et al., 1997a). However, the activity patterns of the LPK analogues injected at high doses differed from those produced by marginal doses or by haemolymph of pupariating larvae. While in the latter case the highest acceleration activity was recorded for anterior retraction, an excessive dose of synthetic LPK accelerated longitudinal contraction to a greater extent than anterior retraction (Žďárek et al., 1997). This disparity may be explained by a different sensitivities to the analogue of the neuromuscular system causing longitudinal contraction and that responsible for anterior retraction.

TABLE 1. Pupariation accelerating activity of representative members of 14 different peptide families in larvae of *N. bullata*.

Peptide	The threshold doses* (in pmol) for acceleration of	
	pupariation behaviour (R, C)	puparial tanning (T)
CDP	N.A.**	N.A.
LPK	0.25	0.25
Lem-MS	N.A.	N.A.
Cus-MT-1	N.A.	N.A.
[Ser(SO_3H)^2]Lem-SK II	N.A.	N.A.
CRF-DP	N.A.	25
Bld-HrTH	250	250
[Nle^{11},Nle^{24},4-NaphthylAla^{28}]Mas-EH N.A.	N.A.	N.A.
Corazonin	N.A.	N.A.
AST-IV	N.A.	N.A.
PVK-2	25	25
Myomodulin	N.A.	N.A.
Proctolin	N.A.	N.A.
CCAP	N.A.	N.A.

* The lowest dose capable of shortening the latency period to shorter or equal to 80% of that in a control group.
** Not active at 250 pmol level.

The fragment [4-8]LPK produced no appreciable muscular contractions when injected into isolated larval abdomens at 25 pmol level. However, the analogue greatly accelerated tanning of the ligated abdomens (T_p/T_a = 0.46; n = 14,) in comparison with Ringer injected controls (T_p/T_a = 1.36; n = 15 when T_p and T_a are mean times of tanning of the posterior and anterior parts, respectively).

It is significant that only members and/or analogues of one out of 14 peptide families screened, i.e. the pyrokinins, accelerated puparium formation in the fleshfly larvae at pmol levels, i.e. at physiological concentrations. Furthermore, two out of the three peptide families which demonstrate marginal activity contain some sequence similarity to the pyrokinins. The strongly active peptides belong to the pyrokinin/PBAN family, which is characterised by the C-terminal pentapeptide fragment FXPRLamide (X = V, T, S, or G), although they all have different numbers of amino acids (Nachman et al., 1986 and 1993a). The pentamer appears to be the active core required not only for myotropic activity in the cockroach hindgut assay (Nachman et al., 1986), but also for their pheromonotropic (Abernathy et al., 1995; Kuniyoshi et al., 1992; Raina & Kempe, 1990), and melanotropic activity (Matsumoto et al., 1990), as well as for egg diapause-inducing activity in the silkworm (Nachman et al., 1993a). Here we report yet two additional physiological effects of the peptides of the pyrokinin/PBAN family, namely acceleration of pupariation behaviour and subsequent puparial tanning. By accelerating both aspects of puparium formation, the LPK peptides and fragments mimic the effects of the pupariation factors – the ARF that stimulates neuromuscular activity (behaviour), and the PTF that is responsible for the timing of cuticular tanning and, thus, for synchronisation of sclerotisation of the cuticle with morphological changes during transformation of the larval body into an ovoid puparium. Since the ARF is an ethotropic factor (stimulates the performance of stereotypic patterns of pupariation behaviour), the LPK analogues are likely to exert an

effect on the central neurones. Their possible direct myotropic action on the peripheral muscular system has been ruled out by our finding that a ligated hind part (i.e. larval segments deprived of neural connection with the CNS) does not respond to exogenous LPK peptides by muscular contraction. Tanning of the ligated hind parts was, however, significantly accelerated, indicating the peripheral effects of the LPK analogues on cuticular changes.

Despite considerable efforts (see Žďárek & Sivasubramanian, 1991 for review), the chemical nature of pupariation factors still remains elusive. They are believed to be protein molecules with subunits of 26 kD and 90 kD for PTF and ARF, respectively, which are released to the haemolymph presumably from the peripheral nerve endings at the time of puparium formation (Sivasubramanian et al., 1974). Results of the present study suggest that the pupariation factors may contain the C-terminal sequence shared by all members of the pyrokinin/PBAN family and hence belong to this peptide group. If further analysis confirms this contention, it would be possible to explain earlier reports of pupariation accelerating activity of extracts of an amazing variety of neural and neurohaemal organs of different arthropods (see Žďárek, 1985 for review) by the almost omnipresent occurrence of the C-terminal amino acid sequence of the pyrokinin/PBAN peptides in various neural and neurohaemal tissues of insects.

Structure-activity relationships of LPK analogues

A number of structural analogues of LPK were tested for their pupariation accelerating activity (Table 2). Sequential N-terminal truncation of the LPK molecule (pQTSFTPRLamide) did not appreciably decrease the activity of fragments of four or more amino acids. The activity of the pyrokinin C-terminal tripeptide fragment PRLamide demonstrates reduced, but significant, activity at 2.5 pmoles, namely 10% of the response of the parent LPK and the tetrapeptide fragment. The dipeptide RLamide was completely inactive. Hence the tripeptide appears to be the active core for pupariation acceleration, but the core sequence for maximum response is the tetrapeptide TPRLamide. This represents a major difference in the activity profiles observed in other pyrokinin assays, in which the C-terminal pentapeptide fragment is required for activity (Nachman et al., 1997b).

Truncation of the pentapeptide at the C-terminus (i.e. FTPRamide) results in complete loss of activity, as in other pyrokinin assays. The evaluation of a series of alanine replacement analogues (Table 2) supports the conclusion that the two C-terminal residues are most critical for pupariation acceleration. This is because alanine replacements at these two C-terminal positions led to inactive analogues. Replacement analogues containing alanine, which is of intermediate size and polarity among amino acids, are traditionally utilized as a means of identifying residues critical for biological activity. The C-terminal amide group is also of great importance to pupariation acceleration, as LPK acid demonstrates a large drop in potency (Nachman et al., 1997a).

Pupariation acceleration activity was also observed for two naturally-occurring members of the pyrokinin family from lepidopteran sources. These include the diapause induction hormone of the silkworm *B. mori* (Bom-DH-1[19-Trp]) and the pheromonotropic peptide of the corn earworm *H. zea* (HezPBAN), both of which showed activity at a threshold of 25 pmoles/larva. The pupariation acceleration activity was also observed for several naturally-occurring myotropic locust pyrokinins (Nachman et al., 1997a). Lom-

MT II, III and IV all demonstrated about an order of magnitude decrease in potency in terms of accelerating the behavioral aspects of pupariation in *N. bullata*. The three locust peptides showed even more marked decreases in the ability to elicit an acceleration of the tanning aspect of the pupariation response. The observed reductions in potency are probably due to differences between the sequences of the locust peptide in the N-terminal region and those of LPK, LPK fragments and/or the natural factor. These differences may lead to an impairment of the ability of the locust pyrokinins to interact with the receptors regulating the timing of the pupariation response, particularly those concerned with the tanning aspect of the cuticular changes. It is interesting to note that while each of the three locust peptides elicits acceleration of the behavioral aspects of pupariation at a threshold dose of 2.5 pmoles/larva, each demonstrates a successive decrease of one order of magnitude from Lom-MT II to Lom-MT III to Lom-MT IV in potency in the acceleration of the tanning response. The peptide Lom-MT II elicits acceleration of the tanning response at a threshold dose of 2.5 pmoles/larva, whereas Lom-MT IV does so only at the much larger threshold dose of 250 pmoles/larva. The data demonstrate that N-terminal sequence changes in pyrokinin analogs can promote a preference for the acceleration of behavioral aspects over the tanning aspect of the pupariation response. The potential exists for the eventual development of a pyrokinin analogue which can lead to a separation of the two responses by eliciting only the behavioral aspects of pupariation in *N. bullata*.

TABLE 2. Structure-activity relationships of LPK-type peptide analogues in the pupariation acceleration assay on *N. bullata* larvae.

Peptide	Threshold activity (pmol/larva) for acceleration of pupariation	
	behaviour	tanning
pQTSFTPRLamide (LPK)	0.25	0.25
FTPRLamide	0.25	2.5
TPRLamide	0.25	0.25
PRLamide	2.5	2.5
RLamide	N.A.*	N.A.
cyclo[NTSFTPRLamide]	2.5	25
FTPdRLamide	N.A.	N.A.
Hydrocinnamyl-TPRLamide	< 0.25	< 0.25
EGDFTPRLamide (Lom-MT II)	2.5	2.5
RNNPFVPRLamide (Lom-MT III)	2.5	25
RLHQNGMPFSPRLamide (Lom-MT IV)	2.5	250
TDMKDESDRGAHSERGALWFGPRLamide (Bom-DH-I[19-Trp])	25	25
LSDDMPATPADQEMYRQDPEQIDSRTKY--FSPRLamide (HezPBAN)	25	25
pQTSFTPRL-OH (LPK-OH)	250	250
YFTPRAamide	N.A.	N.A.
YFTPALamide	N.A.	N.A.
YFTARLamide	2.5	2.5
YFAPRLamide	0.25	0.25
YATPRLamide	2.5	2.5

* Not active at 250 pmol level.

The significant activity elicited by the conformationally constrained LPK analogue *cyclo*[NTSFTPRLamide] provides evidence for the importance of a turn conformation in the C-terminal region for successful interaction with the receptors governing the timing of pupariation (Nachman et al., 1993b, 1997a). The inactivity of the analogue containing a dArg (FTPdRLamide; see Table 2) is consistent with the importance of a turn in the active core for activity, as the presence of this d-amino acid would disrupt the turn. The pseudotetrapeptide hydrocinnamyl-TPRLamide elicits an extremely potent pupariation acceleration response (Nachman et al., 1997a), probably because the absence of an N-terminal amino group eliminates susceptibility to degradation by aminopeptidase M.

The lower level activity observed with PVK-2 and CRF-DP probably arises from the presence of the tripeptide sequences -PRV-NH_2 and -QRL-, respectively, within these peptides. In the first instance, the branched-chain, hydrophobic residues V and L are related. In the second instance, the two most critical residues, R and L, are present in the middle of the CRF-DP sequence. Interestingly, myomodulin contains the RL-NH_2 dipeptide at the C-terminus but is inactive. This provides further evidence that the mere presence of the RL-NH_2 dipeptide is insufficient to elicit pupariation acceleration activity. The inactivity of myomodulin may be due to the fact that the RL-NH_2 dipeptide is preceded by the branched-chain Leu residue.

In summary, members and analogs of the pyrokinin/PBAN family of insect peptides have been shown to be potent accelerators of both the behavioural and tanning aspects of pupariation in the fleshfly *Neobelleria bullata*. The "active core" or minimum sequence for the elicitation of the pupariation response has been identified as the C-terminal tripeptide PRLamide. It is quite possible that the native pupariation acceleration factors found in the larval haemolymph contain this or a related tripeptide within the peptide sequence. A series of pyrokinin peptides from the locust demonstrated a pattern of successive diminution in the ability to accelerate the tanning aspect of pupariation while retaining equipotent activity in terms of the behavioural aspects. This data suggest that some N-terminally modified pyrokinin analogs are capable, in large measure, of successfully distinguishing between the behavioural and the tanning aspects of the pupariation response and that the sequences of the unknown ARF and PTF hormones may likely contain similar sequences to the active cores of the pyrokinin/PBAN molecules.

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