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The cDNA for leucomyosuppressin in *Blattella germanica* and molecular evolution of insect myosuppressins

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Abstract

Myosuppressins are a group of 10-residues FMRFamide-related peptides reported in Dictyoptera, Orthoptera, Lepidoptera and Diptera. Myosuppressins inhibit visceral muscle contractions and, in the cockroach *Blattella germanica*, inhibit food intake. In *B. germanica*, the cDNA of leucomyosuppressin (LMS) has been cloned and sequenced. The deduced precursor is 96 amino acids long and contains a single copy of LMS. Brain mRNA levels remain constant during the first reproductive cycle of adult females, whereas those in the gut show a slight decline during the time of maximal food intake. Comparison of myosuppressin precursors of different species reveals that all have the same organization. Phylogenetic analysis suggests that the precursor experienced an accelerated evolution in Lepidoptera and Diptera with respect to Dictyoptera, whereas only Lepidoptera has radical changes in the bioactive peptide.

Keywords: Myosuppressin; Leucomyosuppressin; *Blattella germanica*; German cockroach; Gene expression; Antimyotropic activity

1. Introduction

Insect myosuppressins are a group of FMRFamide-related peptides with a characteristic C-terminal HV/SFLRFamide hexapeptide [25]. The first myosuppressin was isolated from nervous tissues of the cockroach *Leucophaea maderae* by monitoring its ability to inhibit spontaneous hindgut contractions; the sequence turned out to be pQDVDHVFLRFamide and was named leucomyosuppressin (LMS) [12]. Later, LMS was isolated from nervous system tissues of the cockroaches *Periplaneta americana* [28] and *Blattella germanica* [1]. Two peptides similar to LMS have been identified in locusts: PDVDHVFLRFamide in *Schistocerca gregaria* [30] and *Locusta migratoria* [32], and ADVGHVFLRFamide in *L. migratoria* [27]. A similar peptide, TDVDHVFLRFamide, has been isolated and sequenced in the flies *Neobellieria bullata* [7] and *Drosophila melanogaster* [24]. Finally, in the moth *Manduca sexta*, the homologous peptide pQDVVHSFLRFamide has been reported [14,15]. The gene or cDNA encoding for insect myosuppressins has been described in the cockroach *Diploptera punctata* [4] and in the lepidopterans *M. sexta* [18] and *Pseudaletia unipuncta* [17]. The peptides deduced from the conceptual translation of the corresponding cDNAs are identical to those isolated in cockroaches and moths.

All myosuppressins show a powerful inhibitory activity in visceral muscle contractions, e.g. in several gut regions of cockroaches [28,1,10] and in the oviduct of the migratory locust [27,16]. Structure–activity studies on truncated analogues of the myosuppressins PDVDHVFLRFamide and ADVGHVFLRFamide have revealed that the integrity of the C-terminal hexapeptide HVFLRFamide is essential for antimyotropic activity in the locust oviduct, whereas in the cockroach gut, the active core of the molecule is the pentapeptide VFLRFamide [27]. The six C-terminal amino acids of the active core are practically identical in all the insect orders studied so far. However, in Lepidoptera, there is Ser instead of Val in position 5 from C-terminus, which is a curious case of nonconservative change within the active core of myosuppressins.

In the German cockroach, LMS not only inhibits foregut contractions but also food intake in a dose-dependent manner when applied in vivo at doses between 5 and 50 μg [1]. Examination of the digestive tract of treated females

Abbreviations: LMS, leucomyosuppressin; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

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shows that food accumulates in the foregut, suggesting a causal link between antimyotropic and antifeeding activity [1]. In the adult female of *B. germanica*, food consumption is cyclic and parallel to vitellogenesis [26], which indicates that feeding is finely regulated. This and the above evidence of antifeeding activity suggest that LMS is involved in food intake regulation in the German cockroach.

The present paper describes the cDNA coding for the *B. germanica* LMS precursor. The LMS precursor sequence of this cockroach adds new data to study the molecular evolution of myosuppressin peptides in insects, which may shed light on the divergent evolution experienced by Lepidoptera. Moreover, these molecular data have allowed studies on LMS gene expression in brain and gut tissues of *B. germanica*, which help to elucidate the physiological significance of this peptide.

2. Materials and methods

2.1. Insect rearing

Adult females of *B. germanica* (L.) (Dictyoptera, Blattellidae) were obtained from a colony fed on dog chow and water and reared in the dark at 30 ± 1 °C and 60–70% relative humidity. Freshly moulted virgin females were isolated daily and used at the appropriate physiological ages for molecular studies or myotropic assays. To study the period of ootheca transport, mated specimens were used because they retain the ootheca attached to the genital chamber throughout embryogenesis. To study species-specificity of the different insect myosuppressins, comparative myotropic assays were carried out on *Spodoptera littoralis*. In this case, we used newly ecdysed fifth-instar larvae which were collected from a laboratory culture, reared on a semiartificial diet [29], at 25 ± 2 °C, 60–70% relative humidity and 18 h of photophase.

2.2. Amplification and cloning of myosuppressin cDNA

An initial PCR was performed using a degenerate primer set (5′-TGAAGMGACARGAYGTBGAYCAC-3′ and 5′-CKBCKRCCGAAICKSAGRAA-3′), derived from the amino acid sequence of *B. germanica* LMS. As a template for this first amplification we used 3′ cDNA (Gibco, BRL RACE kit) synthesized from total RNA from brains of 6- to 7-day-old adult females of *B. germanica*. This yielded a fragment of approximately 45 bp, which was cloned and sequenced. Based on this sequence, specific primers were designed for 5′- and 3′-rapid amplification of cDNA ends (RACE) (Gibco, BRL kits). To complete the cDNA of the LMS precursor we proceed as described elsewhere [9]. To make sure that all amplifications obtained with RACE correspond to the same molecule, the entire cDNA was amplified with a forward primer designed at the 5′ end and a reverse primer designed at the 3′ end just before the Poly $(A)^+$ tail. All PCR products obtained were subjected to electrophoresis on 1.2% agarose gel and subcloned in pSTBlue-1 Acceptor vector (Novagen). Sequence analysis was performed by the dideoxynucleotide chain termination method. Clones were sequenced on both strands using the SP6 and T7 sequencing primers and internal, specific primers, in an automated fluorescence sequencing system ABI (Perkin Elmer).

2.3. Gene expression studies

Total RNA from brain and midgut samples was isolated using the GenElute Mammalian Total RNA kit (Sigma, St. Louis, MO, USA). All RNA samples were treated with RQ1 DNase (Promega, Gaithersburg, MD, USA). cDNAs were prepared as described elsewhere [36]. Aliquots of 0.3 μg of total RNA were retrotranscribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Promega) in a reaction volume of 20 μl. The reverse transcription products were then diluted to 40 μl with TE buffer, pH 8.0. Negative controls without the reverse transcriptase step were used to check for genomic contamination. The resulting cDNAs were used as PCR templates. The primer pair: forward, 5′-TGGCAGTGCTGTTAGCCTGT-3′; and reverse, 5′-GCCGAACCTCAGGAAAAC-3′, were used to amplify a 250 nt fragment of the LMS precursor cDNA that includes the peptide sequence. As a reference, cDNA corresponding to actin-5C RNA of *B. germanica* (unpublished) using forward, 5′-TCGTTCGTGACATCAAGGAGAAGCT-3′; and reverse, 5′-TGTCGGCAATTCCAGGGTACATGGT-3′, was amplified in parallel for each cDNA sample. To obtain semi-quantitative mRNA levels, a non-saturating number of cycles in the PCR system were determined for each experiment, being in all cases between 27 and 30 cycles. After PCR amplification, an aliquot of the total 25 μl reaction was fractionated in a 1.2% agarose gel and visualized by ethidium bromide staining and UV illumination. Pictures were taken with an adapted Polaroid camera.

2.4. Sequence comparison and analysis

Amino acid sequences of the myosuppressin precursor used for comparison with that of *B. germanica* obtained in this study were from the dictyopteran *D. punctata* [4] (GenBank number U50341), the lepidopterans *M. sexta* [18] and *P. unipuncta* [17] (GenBank number AF390443), and the dipterans *D. melanogaster* (GenBank number NM_080511) and *Anopheles gambiae* (the sequence was retrieved from the ENSEMBL database, ENSANGESTP00000061721, and it was extended in the N-terminus up to the first Met). Alignments were carried out with ClustalW [34] at the amino acid level. The topology of the known phylogenetic tree

corresponding to the insect orders involved [8], was used to calculate the branch lengths of the tree with Tree-Puzzle [33] using the JTT model of protein evolution [13].

2.5. Synthetic myosuppressins and myotropic assay

Synthetic versions of leucomyosuppressin (LMS) (pQDVDHVFLRFamide) and manducaFLRFa (the lepidopteran myosuppressin, pQDVVHSFLRFamide) were synthesized by Fmoc solid phase methods, purified to near-homogeneity (>95%) by HPLC and characterized by amino acid analysis and MALDI-TOF mass spectrometry.

Synthetic peptides were tested on the whole gut of fifth instar larvae of the lepidopteran *S. littoralis* in a standard organ bath described elsewhere [19]. Selective hindgut or foregut preparations were discarded because these parts of the moth gut are too fragile for myotropic assays. An FSG-01 transducer (Experimetria Ltd., Budapest, Hungary) was used for isometric recording. The activity was calculated as the difference of the mean force produced by the tissue for 1 min after and 1 min before the treatment.

3. Results

3.1. The cDNA of leucomyosuppressin precursor in B. germanica

The complete sequence of the cDNA of the LMS precursor in *B. germanica* is 750 nucleotides long. It contains an open reading frame encoding the LMS peptide within a longer putative precursor. The deduced precursor is 96 amino acids long, with the LMS sequence located at the C-terminal end between potential proteolytic processing sites KR and RRR (Fig. 1). The encoded peptide begins with a glutamine residue, which may be expected to be converted into pyroglutamate, as reported in LMS purified from *B. germanica* brain extracts [1], and ends with a glycine residue, which is necessary for amidation by peptidylglycine α-amidating monooxygenase [6].

The methionine at nucleotides 61–63, which is in-frame with a stop codon [TAG] located just after the C-terminus of the deduced peptide, is considered the first initiation site for translation. There are no in-frame stop codons located upstream in the cDNA characterized, but this start site is

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CCACCACATACACACACACGCACGCACGCACGAGGCAGGCCACCAACACACAGCATCAGA   60

ATGAAGTACGTCAGCGTAGTTCTCATCAGTGTCCTGGCAGTGCTGTTAGCCTGTATGCCA  120
 M  K  Y  V  S  V  V  L  I  S  V  L  A  V  L  L  A  C  M  P    20

CATATGGCCTCTGCAGTCCCTCCGCCTCAGTGCAGTCCCAACATTTTGGACGACGTTCCT  180
 H  M  A  S  A  V  P  P  P  Q  C  S  P  N  I  L  D  D  V  P    40

CCAAGAGTACGCAAGGTCTGCGCAGCACTCTCCACAATCTACGAACTTTCAAATGCGATG  240
 P  R  V  R  K  V  C  A  A  L  S  T  I  Y  E  L  S  N  A  M    60

GAAGCATACTTGGACGATAAAGTTGTTCGTGAGAACACGCCCCTGGTGGACACCGGAGTG  300
 E  A  Y  L  D  D  K  V  V  R  E  N  T  P  L  V  D  T  G  V    80

AAGCGACAGGATGTTGATCACGTTTTCCTCAGGTTCGGACGAAGACGCTAGGCCACTGGA  360
 K  R  Q  D  V  D  H  V  F  L  R  F  G  R  R  R               96

TCGTGATTGTGAAGACTGCATCAAGGACTGAATTCTTGTGTGTTTCGCCTCTCCTCCATT  420
ACGAACTATCTACGTCCATCAATATTATAATTTCTACTTTGGCCAGTACAGTAAACAAAT  480
GTTTGAATAAAGTATTTTTAAGGGAAAATAAGTACGCTTTATGTTTTTTGTTATTCTTTG  540
TTTTATAATTCTGCAGTGTAAGTGGGAAAGCAATGCCAACAGAAACGGGTCTTTATTATT  600
GATTTGTAATTATTGATATGATGTGTAGACCTGCGTGTACATATTTAAAACACGTTTTGA  660
GGTAGGGTATACACAGATAAGTGTCATAATATCTATCCTGTTTCCACAGTAAATCTCATT  720
AGCAAATGGAAAAAAAAAAAAAAAAAAAA                                 750
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Fig. 1. Nucleotide sequence of the cDNA of *B. germanica* leucomyosuppressin (LMS), and deduced amino acid sequence of the protein precursor. The numbering for each sequence in shown on the right. The amino acid sequence of the processed peptide is shown in bold type. Dibasic and tribasic amino acids for potential cleavage sites are underlined and the glycine residue necessary for amidation is shown in italics. Potential polyadenilation signal near the 3′ end of the sequence is double underlined. This sequence has been deposited in the GenBank database (accession no. AJ619986).

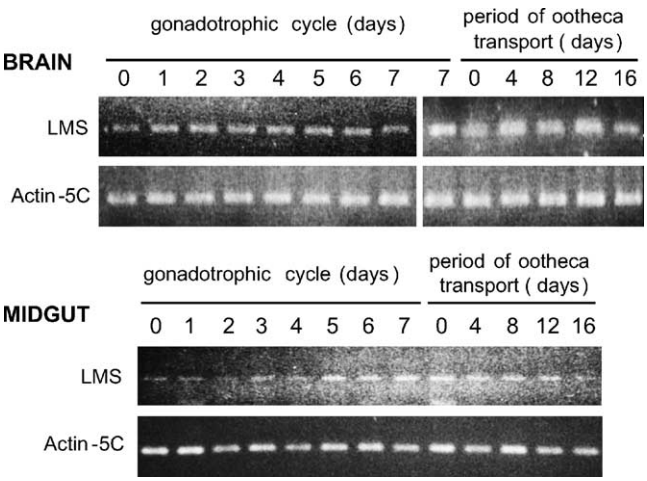

Fig. 2. Leucomyosuppressin (LMS) and actin-5C mRNA levels in brain and midgut of *B. germanica* adult females. RT-PCR amplification of LMS and actin-5C mRNA in brain and midgut of *B. germanica* on each day of the first gonadotrophic cycle and on selected days during the period of ootheca transport. A total of 10 μl of each PCR product was separated on a 1.1% agarose gel (gels showed are representative of two or three replicates).

supported not only by its context but also by the similarity in size of the resulting precursor (96 amino acids) compared with those of *D. punctata* (97 amino acids), *P. unipuncta* (96 amino acids) and *D. melanogaster* (100 amino acids). Finally, a putative polyadenylation site AGTAAA is located at 15 bp from the poly $(A)^+$ tail.

3.2. Gene expression of leucomyosuppressin in brain and gut tissues

Myosuppressin peptides occur either in the central and stomatogastric nervous system as well as in endocrine midgut cells [25,4,18,10,23]. Therefore, we investigated the pattern of LMS mRNA in brain and midgut tissues during the first reproductive cycle of *B. germanica* females. Brain mRNA levels remain approximately constant, whereas those of midgut show a characteristic pattern during the period studied (Fig. 2). In the midgut, mRNA levels are relatively low on the first 4 days of adult life in comparison with the higher levels observed from day 5 to 7 within the first reproductive cycle. During the period of ootheca transport, mRNA levels remain relatively high at least until day 12.

3.3. Comparison of insect myosuppressin precursors

To date, the cDNA coding for myosuppressin precursor has been described in the dipteran *D. melanogaster* (NM_080511, from GenBank), the lepidopterans *M. sexta* [18] and *P. unipuncta* [17], and the dictyopteran *D. punctata* [4]. Additionally, the genomic sequence of the mosquito *A. gambiae* (ENSANGESTP00000061721, from ENSEMBL database) is also available. The alignment of myosuppressin precursor proteins, including that of *B. germanica* (Fig. 3A), reveals that all show the same organization, with a similar length of 96–100 amino acids.

The alignment also shows that the greatest identity is concentrated in the C-terminal region, which corresponds to the peptide. In this region, only the lepidopteran species show amino acid changes in the bioactive core defined in *L. maderae* [22] and *L. migratoria* [27]. Besides this lepidopteran peculiarity, the dipterans have Thr in the first position at the N-terminus, whereas dictyopterans and lepidopterans bear pGlu, although this position is not important for suppression of visceral muscle activity [27,22].

3.4. Phylogenetical analysis of insect myosuppressin precursors

The LMS precursor of *B. germanica* was compared with those of the other species, revealing 76% identity to that of *D. punctata*, 39% and 38% to those of the dipterans *D. melanogaster* and *A. gambiae*, respectively, and 41% and 35% to those of the lepidopterans *P. unipuncta* and *M. sexta*, respectively. These data suggest that the precursor sequence has experienced an accelerated evolution in lepidopterans and dipterans with respect to dictyopterans.

To test this hypothesis, we carried out a phylogenetic analysis with the six precursor sequences available. Since the phylogenetic tree of the three insect orders involved is well established [(Dictyoptera (Lepidoptera, Diptera))] [8], we used this topology and calculated the lengths of the branches in the tree by a maximum-likelihood method (Fig. 3B). The tree shows that branches corresponding to lepidopterans and dipterans are comparatively much longer with respect to the less modified dictyopterans and, in particular, the average length of lepidopterans is slightly bigger. Since the N-terminal part of the protein is very poorly conserved, saturation of substitutions during evolution may obscure a much faster evolutionary rate in lepidopterans. To avoid a possible saturation effect, we used only the best blocks for phylogenetic analysis as selected by the Gblocks method, with the parameter that allows for gaps in half of the sequences [3]. A total of 68 positions selected this way produce a phylogenetic tree with relative branch lengths very similar to those of Fig. 3B (not shown). Finally, we also used among-site rate heterogeneity to account for different rates of evolution in different positions (four different rates), obtaining again similar results with both the original and the Gblocks alignment (not shown). In all cases, confidence limits of the branches indicate that there are no statistical differences in rates between lepidopterans and dipterans. Therefore, we can conclude that both Lepidoptera and Diptera have a similar degree of accelerated evolution in the whole myosuppressin sequence.

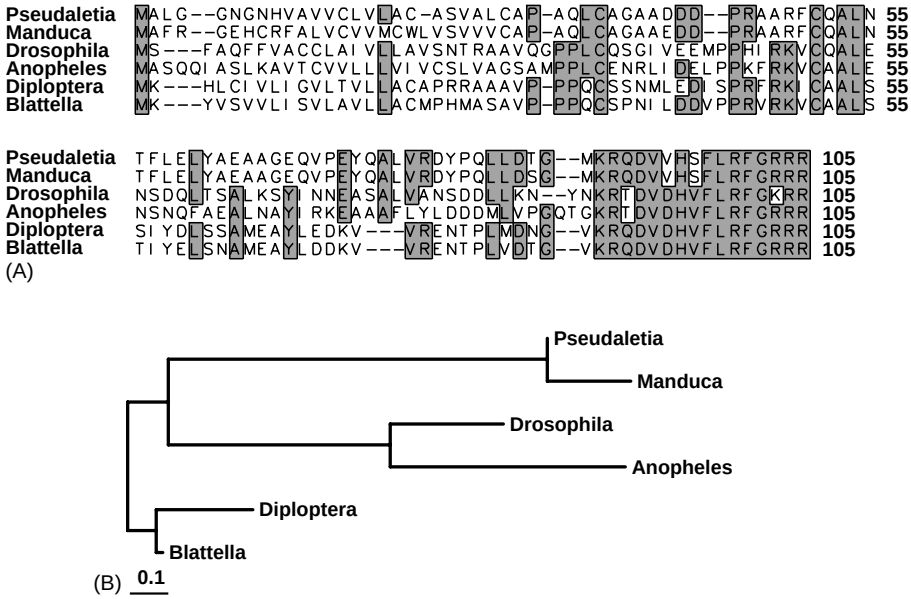

Fig. 3. Alignment (A) and phylogenetic analysis (B) of the amino acid sequence of insect myosuppressins. The sequences highlighted in the alignment have more than 50% of identity. The branch lengths of the tree have been calculated with Tree-Puzzle using the JTT model of protein evolution. The binomial name of all species and the accession number of the corresponding sequences is indicated in Section 2. Scale bar represents 0.1 substitutions per position.

3.5. Antimyotropic activity of insect myosuppressin and species-specificity

The radical changes of lepidopteran myosuppressin may also involve a divergence of the corresponding receptor, which led to predict that the non-lepidopteran myosuppressins should be less active in lepidopteran species, and vice versa. To test this prediction, we compared the antimyotropic activity of LMS and manducaFLRFa in gut tissues of the dictyopteran *B. germanica* and the lepidopteran *S. littoralis*.

Previous antimyotropic assays on the foregut of *B. germanica* had shown that the native peptide, in this case LMS, was significantly more active than all the rest tested, including manducaFLRFa. This difference points to high order-specificity [1]. Here we carried out symmetric assays, testing both peptides in the *S. littoralis* whole gut (Fig. 4). In order to compare the effect of both peptides, we estimated the concentration producing a negative force of 10 mg, and for LMS this concentration was 8×10^{-8} M, that is more than one order of magnitude higher than that obtained for manducaFLRFa (5×10^{-9} M). From a statistical point of view, in the case of manducaFLRFa, the lowest concentration producing inhibitions significantly higher with respect to controls is 10^{-9} M, whereas for LMS the lowest bioactive concentration is 10^{-8} M.

4. Discussion

In the German cockroach *B. germanica*, the cDNA for LMS is 750 nucleotides long and shows an open reading frame encoding a 96 amino acids precursor containing a single copy of LMS. The deduced LMS is identical to the peptide previously isolated from brain extracts [1], and the cDNA shows a length and organization that is very similar to that reported in the LMS cDNA of *L. maderae* [12].

Expression studies during the first reproductive cycle of the adult female revealed that mRNA levels in the brain do not fluctuate, which possibly reflects the constant action and pleiotropic role of this peptide in the central nervous system, where LMS is ubiquitously localized [4,23] and seemingly play roles of neuromodulator [35,20] and neurotransmitter [24]. Conversely, LMS mRNA levels in the midgut are more variable, showing minimal values during the first 4 days of the reproductive cycle, that is when food consumption takes place [26]. This pattern suggests that LMS expression in gut tissues is regulated in connection with alimentary and digestive processes, for example with the stimulation of α-amylase activity described in beetles and cockroaches [11,21], and/or with the modulation of gut contractions which, in turn, may regulate food transit in the digestive tract and food intake [1].

The occurrence of a single copy of LMS in the precursor protein seems characteristic of the myosuppressin family of peptides. This contrasts with the large multipeptide precursors encoding other FMRFamide related peptides [31]. Other precursors of insect peptides such as allatostatins also contain multiple copies of structurally similar bioactive peptides, which have been probably generated by intragenic duplication and homogenization from a single ancestral peptide sequence [2]. Possible duplications of truncated or imperfect copies of the ancestral myosuppressin unit may have occurred during evolution in myosuppressin genes, but

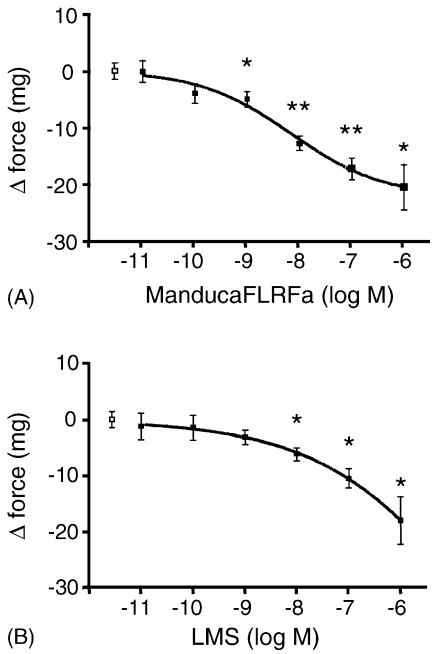

Fig. 4. Inhibitory effect of myosuppressins on gut motility in homologous and heterologous assays. *M. sexta* myosuppressin (manducaFLRFa) (A) and leucomyosuppressin (LMS) (B) tested on gut motility in *S. littoralis* fifth instar larvae. Results (mean ± S.E.M.; $n = 5$–7) are expressed as the difference of the mean of the force produced by the tissue during 1 min after and before the treatment. Empty squares indicate values for water control experiments. The asterisks indicate significant differences with respect to water controls (*t*-test, * $P < 0.05$, ** $P < 0.001$).

it seems that they had not been fixed. Indeed, the fact that myosuppressin precursors known to date contain only one copy of the peptide suggests that sequence requirements for receptor interaction and bioactivity are strict. The locust *L. migratoria*, in which two myosuppressins have been isolated from brain extracts [32,27] may be an exception, although it is not known whether these two peptides are encoded by one or two genes.

Phylogenetic analysis suggests that the myosuppressin precursor experienced an accelerated evolution in Lepidoptera and Diptera with respect to Dictyoptera. Nevertheless, only lepidopterans present radical changes in the active peptide, indicating that a decoupling of the evolution of the active peptide from the precursor protein may have occurred. First, the sequence of lepidopterans has Val at position 4 whereas all other species bear Asp. In *L. maderae*, the substitution of Asp^4 decreases the threshold concentration required to inhibit hindgut contractions by two orders of magnitude compared with the native peptide [22]. The same report describes that the minimum sequence required for myoinhibitory activity is VFLRFamide [22], but lepidopteran sequences have Ser instead of Val in this core, which involves a modification in the secondary structure of the peptide, given that these two amino acids greatly differ from a chemical point of view, Ser being polar, with a hydroxyl group in the side chain, and Val being non-polar.

Finally, heterologous antimyotropic assays revealed that lepidopteran myosuppressin are less active in a cockroach model than in a moth model. The present assays and those previously reported testing lepidopteran and non-lepidopteran peptides on cockroach midguts [1], indicate that the threshold concentration required to inhibit hindgut contractions is one order of magnitude higher in heterologous than in homologous assays. These observations point to the relatively high species-specificity for the receptor, which should be different in dictyopterans and lepidopterans, having coevolved with the ligand. Receptor characterization in these groups would be illuminating but, to date, only two myosuppressin receptors have been cloned, both in *D. melanogaster* [5], and they seem to be specific for *Drosophila* myosuppressin, TDVDHVFLRFamide, since they are not activated by other similar insect peptides, such as FMRFamide, *D. melanogaster* short neuropeptide F-1 and perisulfakinin [5].

Acknowledgments

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