

Molecular identification of a myosuppressin receptor from the malaria mosquito *Anopheles gambiae*[☆]

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Abstract

The insect myosuppressins (X₁DVX₂HX₃FLRFamide) are neuropeptides that generally block insect muscle activities. We have used the genomic sequence information from the malaria mosquito *Anopheles gambiae* Genome Project to clone a G protein-coupled receptor that was closely related to the two previously cloned and characterized myosuppressin receptors from *Drosophila* [Proc. Natl. Acad. Sci. USA 100 (2003) 9808]. The mosquito receptor cDNA was expressed in Chinese hamster ovary cells and was found to be activated by low concentrations of *Anopheles* myosuppressin (TDVDHVFLRFamide; EC₅₀, 1.6 × 10^{−8} M). The receptor was not activated by a library of 35 other insect neuropeptides and monoamines, including neuropeptides that resembled myosuppressin in their C-terminal moiety, such as PDRNFLRFamide (*Anopheles* FMRFamide-3), other *Anopheles* FMRFamide peptides, or neuropeptide F-like peptides, showing that the receptor was quite selective for myosuppressin. These results also showed that the myosuppressin receptor needs a much larger portion than the C-terminal FLRFamide sequence for its activation. The insect myosuppressins are often grouped together with the insect FMRFamides under the name FaRPs (FMRFamide-related peptides). However, this is not justified anymore, because the insect myosuppressin receptor/ligand couple is both functionally and evolutionarily fully unrelated to the insect FMRFamide receptor/ligand couple. To our knowledge, this is the first report on the molecular identification of a mosquito neuropeptide receptor.

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The insect myosuppressins are neuropeptides with the C-terminal sequence X₁DVX₂HX₃FLRFamide (where X₁ is pQ, P, T; X₂ is D, G, V; and X₃ is V, S) [1]. The insect myosuppressins obtained their name because they inhibit most insect visceral muscles, including those that are involved in the passage of food along the insect alimentary canal [1–5]. The myosuppressins have been iso-

lated from a variety of insects and it can be assumed that they occur in all insect species [1–4,6–10].

The C-terminal four amino acid moiety of the insect myosuppressins (FLRFamide) resembles that of the FMRFamide neuropeptides. However, the actions of myosuppressins are often different from those of the insect FMRFamides [1,11] and also their preprohormones have a quite different organization (one immature myosuppressin sequence is contained in the myosuppressin preprohormones, while typically a large number of FMRFamide sequences—up to 24—are contained in the insect FMRFamide preprohormones) [12,13], suggesting that the two neuropeptide genes are not evolutionarily related.

[☆] The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with Accession No. AY 345586.

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The FMRFamide preprohormone from the malaria mosquito *Anopheles gambiae* contains a neuropeptide sequence, PDRNFLRFamide (*Anopheles* FMRFamide-3), that resembles a myosuppressin (Cazzamali et al., in preparation). *Anopheles* FMRFamide-3 acts as a genuine FMRFamide on the *Anopheles* FMRFamide receptor (Cazzamali et al., in preparation). It would be important to see whether *Anopheles* FMRFamide-3 also has myosuppressin properties, which would intermingle the insect FMRFamide and myosuppressin systems. This, however, would require the cloning of the *Anopheles* myosuppressin receptor. We have previously cloned and identified (“deorphanized”) the first two insect myosuppressin receptors from the fruitfly *Drosophila melanogaster* [14]. Here, we describe the cloning and functional characterization of a myosuppressin receptor from *Anopheles*. This is, to our knowledge, the first report on the molecular identification of a mosquito neuropeptide receptor.

Materials and methods

Total RNA was isolated from adult *A. gambiae* (strain KWA, kindly supplied by Drs. N. Hill and P. Aiyenuro, London School of Hygiene and Tropical Medicine, UK) using TRIzol Reagent (Life Technologies) and treated with DNase using the DNA-free kit (Ambion). cDNA was synthesized using the SMART RACE cDNA Amplification Kit (Clontech). For 3' RACE, the sense primer 5'-CGTGATGGACGTGCTGGCGTTGGTCAA-3' and the nested sense primer 5'-GGACCGGTGGATGGCGGTGCCG-3' (corresponding to positions 1080–1108 and 1190–1212 of Fig. 1) were used. For 5' RACE, the antisense primer 5'-GCTGCCAAAGATGCACACCAGCAGGCAG-3' followed by the nested antisense primer 5'-GGCCCCATCCTCATCGCCTGCCAG (corresponding to positions 174–201 and 57–81 of Fig. 1) were used. The PCR program was 94 °C for 3 min, then 20 cycles touchdown, 94 °C for 30 s, 68 °C for 45 s, decreasing 0.5 °C per cycle, 72 °C for 3 min, followed by 25 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 3 min.

The coding region was amplified using the sense primer 5'-CACCATGAGCTCGACGGAATTGG-3' and the antisense primer 5'-GGACGTCCCGCGTGGCGCTA-3' (the underlined nucleotides correspond to positions 1–19 and 1281–1301 of Fig. 1), cloned into pCR4-TOPO (Invitrogen), sequenced, and cloned into the pIRES-hrGFP-1a/Neo-module (Stratagene), using *EcoRI*. Cell culture, cell transfection, and the bioluminescence assay were performed as in [14–17].

DNA sequence comparisons were carried out using the Lasergene software package (DNASTAR). ClustalW was used for protein sequence alignments (www.npsa-pbil.ibcp.fr).

Results

We have previously identified two myosuppressin receptors from *Drosophila* [14]. Blasting of their sequences against the genomic database sequences from the malaria mosquito (*A. gambiae*) Genome Project (<http://www.ncbi.nlm.nih.gov/BLAST/Genome/Fly-Blast.html>) revealed the sequence of a putative mosquito

myosuppressin receptor. To obtain the cDNA, encoding this receptor, we carried out PCR, and 5' and 3' RACE PCR, using primers directed against the exons of the putative myosuppressin receptor gene, and cDNA from adult *A. gambiae* as a template.

The receptor cDNA contains a polyadenylation signal and a poly(A)⁺ tail. The start codon is preceded by several in-frame stop codons (Fig. 1). The cDNA codes for a protein of 427 amino acid residues, containing seven transmembrane helices, which is characteristic for G protein-coupled receptors (Fig. 1).

A comparison of the cDNA of Fig. 1 with the genomic sequence of *A. gambiae* revealed a small number of nucleotide differences, which, however, did not result in amino acid residue differences (Table 1). This comparison also showed the presence of two introns in the receptor gene (Table 2).

An alignment of the mosquito receptor with the two *Drosophila* myosuppressin receptors showed 47–48% amino acid residue identity (52–53% in the transmembrane region) and 57–58% similar residues (63–64% in the transmembrane region) (Fig. 2). Furthermore, all three receptors had two introns with the same intron phasings in common, showing that the three receptor genes are evolutionarily closely related (Fig. 2; Table 2).

We stably expressed the coding region of the mosquito receptor gene (Fig. 1) in Chinese hamster ovary (CHO) cells and established clonal cell lines that expressed the receptor efficiently. These cells also stably expressed the promiscuous G protein, G-16 [15]. Two days before the bioassay, we transiently transfected the cells with DNA, coding for apoaquorin and 3 h before the assay, we added coelenterazine to the cell culture medium. An activation of G-16-coupled receptors in these pretreated cells, would lead to an IP₃/Ca²⁺-mediated bioluminescence response, which could easily be measured and quantified [14–17].

We tested a library of 35 insect neuropeptides and biogenic amines on the transfected cells. Low concentrations of *Drosophila* myosuppressin (TDVDHVFLRFamide) activated the receptor (EC₅₀, 1.6 × 10^{−8} M) (Fig. 3), whereas the other neuropeptides and neurohormones, including those that resembled *Drosophila* myosuppressin in their C-terminal moieties, such as the *Anopheles* FMRFamides (we tested SALDKNFMRFamide, PDRNFLRFamide, STGSGYMRamide, and AGNLMRFamide), short neuropeptide F-1 (AQ RSPSLRLRFamide), and perisulfakinin [EQFDDY(SO₃H)GHMRamide] [1,11,18], did not give a response (all tested up to 10^{−5} M). This showed that the receptor is specific for myosuppressin.

Searching of the genomic database from *A. gambiae* (<http://www.ncbi.nlm.nih.gov/BLAST/Genome/Fly-Blast.html>) revealed only one gene coding for a myosuppressin preprohormone, which is in agreement with

| | |
|---|------|
| TGCACCAGCAAACAAATGCTACCGTGAGGCGAAAGTGAAGACACAGCTGTGTGCCTAACTGTGTGCGTTGGTGCCTA | -100 |
| TGTCTGTGGGTGTTTGTAGTGTGTGCGTGCCTGCGTGTACGTTCCGCCAGCAAGCTGTGTGACGAACCAAGCGTAATATTAAACAAACACAGCAC | -1 |
| ATG AGC TCG ACG GAA TTG GGA GAA TTT GTA ATA AAT GTC ACC GAT GGA CCG CAC AGT CTG GCA GGC GAT GAG GAT | 75 |
| Met Ser Ser Thr Glu Leu Gly Glu Phe Val Ile Asn Val Thr Asp Gly Pro His Ser Leu Ala Gly Asp Glu Asp | 25 |
| GGG GCC AAC ATC TCG ACC GCA ACA ACC GCA CAC CTG CTG TAC TGT GGC AAG GCG CTG GAT GAT TTC CAC ACC AGC | 150 |
| Gly Ala Asn Ile Ser Thr Ala Thr Thr Ala His Leu Leu Tyr Cys Gly Lys Ala Leu Asp Asp Phe His Thr Ser | 50 |
| TAC GCC AAA GCG CAC GGG ATC GTC TGC CTG CTG GTG TGC ATC TTT GGC AGC ATT GCC AAC ACA CTC AAC ATT GTG | 225 |
| Tyr Ala Lys Ala His Gly Ile Val Cys Leu Leu Val Cys Ile Phe Gly Ser Ile Ala Asn Thr Leu Asn Ile Val | 75 |
| GTG CTG ACG CGG CGC GAG ATG CGA TCG CCG ACC AAC GCC ATC CTG ACC GGG CTG GCG ATC GCC GAT CTG CTC GTC | 300 |
| Val Leu Thr Arg Arg Glu Met Arg Ser Pro Thr Asn Ala Ile Leu Thr Gly Leu Ala Ile Ala Asp Leu Leu Val | 100 |
| ATG CTC GAC TAC ATG CCC TAC GCA ATC AAC TCC ATC CCG TAC CTG CGG CTG TCG CGC GAG GAG CGG CTC ACG TAC | 375 |
| Met Leu Asp Tyr Met Pro Tyr Ala Ile Asn Ser Ile Pro Tyr Leu Arg Leu Ser Arg Glu Glu Arg Leu Thr Tyr | 125 |
| GGC TGG GCC TGG TAC ATC ATG TTT CAC TCG ATC TTC GCC CAG ATC TGC CAC ACG ATC TCG ATC TGG CTG ACG GTG | 450 |
| Gly Trp Ala Thr Trp Tyr Ile Met Phe His Ser Ile Phe Ala Gln Ile Cys His Thr Ile Ser Ile Trp Leu Thr Val | 150 |
| ACG CTG GCC ATC TGG CGC TAC ATC GCC GTC GCC TAC CCG CAG CGC AAC CGC CAG TGG TGC GAC ATG TCC AAA ACG | 525 |
| Thr Leu Ala Ala Ile Thr Arg Tyr Ile Ala Val Ala Tyr Pro Gln Arg Asn Arg Gln Trp Cys Asp Met Ser Lys Thr | 175 |
| CTG GCC GCG ATC TTC AGC TCC TAC GTC GTG TGC CCG GTC TTT CTG GCC GTA CCG ATC TAC CTC TCC TTC AGC ATC CAG | 600 |
| Leu Ala Ala Ile Phe Ser Ser Tyr Val Val Cys Pro Phe Leu Ala Val Pro Ile Tyr Leu Ser Phe Ser Ile Gln | 200 |
| TCG AAC GTG GAG CTG CTC GGG TGC GAC GGC AAC ACG CTC ACG CCG GAC GCC ATC GGC AAT GTT TCG CAG GGC CGC | 675 |
| Ser Asn Val Glu Leu Leu Gly Cys Asp Gly Asn Thr Leu Thr Pro Asp Ala Ile Gly Asn Val Ser Gln Gly Arg | 225 |
| AAC GTG ACG CTG TAC CGG CTC GGT ACG TCC CAG CTG GTG CGC GAT AAT CCG GCC CTG CTG AAC GTG AAC TTT TGG | 750 |
| Asn Val Thr Leu Tyr Arg Leu Gly Thr Ser Gln Leu Val Arg Asp Asn Pro Ala Leu Leu Asn Val Asn Phe Trp | 250 |
| ATC TAC AGC GTC GTG TTC AAG CTG ATA CCG TGC ATC GCG CTG ACC ATC CTC AGT CTA CGG TTG ATC GGT GCG CTG | 825 |
| Ile Tyr Ser Val Val Phe Lys Leu Ile Pro Cys Ile Ala Leu Thr Ile Leu Ser Leu Arg Leu Ile Gly Ala Leu | 275 |
| CTA GAG GCA AAG CAG CGC CGC AGC CAA CTG ACC GGC ACG GCC ACC GGC CTG AAG CAG ATC GTC GAC GGC CGG GTG | 900 |
| Leu Glu Ala Lys Gln Arg Arg Ser Gln Leu Thr Gly Thr Ala Thr Gly Leu Lys Gln Ile Val Asp Gly Arg Val | 300 |
| GTG GAC GCA AAG GCC GGC AAG CAG ACG GAC AAG GAG AAG CAG ACG GAC CGG ACG ACG CGC ATG CTG CTC GCG GTG | 975 |
| Val Asp Ala Lys Ala Gly Lys Gln Thr Asp Lys Glu Lys Gln Thr Asp Arg Thr Thr Arg Met Leu Leu Ala Val | 325 |
| CTG CTA CTG TTT CTC ATC ACC GAG TTC CCC CAG GGC ATC CTT GGC TTG CTG AGC GCC GTG CTG AAG AAG GAC TTT | 1050 |
| Leu Leu Leu Phe Leu Ile Thr Glu Phe Pro Gln Gly Ile Leu Gly Leu Leu Ser Ala Val Leu Lys Lys Asp Phe | 350 |
| TTC TTC AAC TGC TAC CTC AAG CTC GGT GAC GTG ATG GAC GTG CTG GCG TTG GTC AAC TCG GCG ATA AAC TTC ATC | 1125 |
| Phe Phe Asn Cys Tyr Leu Lys Leu Gly Asp Val Met Asp Val Leu Ala Leu Val Asn Ser Ala Ile Asn Phe Ile | 375 |
| CTC TAC TGC TCG ATG AGC CGC CAG TTC CGC AGC ACG TTC AAC GAT CTG TTC CGC CCG CGC ATC CTG GAC CGG TGG | 1200 |
| Leu Tyr Cys Ser Met Ser Arg Gln Phe Arg Ser Thr Phe Asn Asp Leu Phe Arg Pro Arg Ile Leu Asp Arg Trp | 400 |
| ATG GCG GTG CCG CAA GGG GAC GAC GAG CAG GCG GGC CGG GGC CAG CAC CAG GAC GCA GCG ACA ACG CAG ATT ACT | 1275 |
| Met Ala Val Pro Gln Gly Asp Asp Glu Gln Ala Gly Arg Gly Gln His Gln Asp Ala Ala Thr Thr Gln Ile Thr | 425 |
| CAG ATA TAG CGCAGCGCGGAGCTCTAACAGTCATTGCCACGGTTGCTTTGGCCAGTTTGCGAACGCAGTGGCGGGAGTTCTAATTAGCAATCGG | 1371 |
| Gln Ile * | 427 |
| CAAACATCGAACCGTGACGACTGTGCAATGCTCTAGCAGCTCCACCCAGCACTCTATAAGCACTGTGTCTGTGTGTGTGTTGTGCATCCGTT | 1470 |
| TTTACCACTGTGCCAGCTCTACTACTAACTAACGTACTTCTCCGCCACCGTATGCAAGCAAGCAAGGCAAACTGCTTCCGTTTTCGCGCCGTCTAA | 1569 |
| TTACTACACTGTGGTGAGCAGGTCCCGTGGCGCTTGCCTACTGTTTGGCGATTAGTCGAAGCACTATTAGCAGATAATGAACACAATATCCACAGTGTT | 1668 |
| CACACACACACACACACATAGACGACACTCACATACTGGGCACAAATGAAATCGAACCATCGAAATGAATGGGTTTCTATTGAAACAAAATAA | 1767 |
| AACTGTTGCTTCCGTGTTACTG (A) _n | 1789 |

Fig. 1. cDNA and deduced amino acid sequence of the receptor gene. The nucleotides are numbered from the 5' to 3' end and the amino acid residues are numbered, starting with the first ATG (start) codon in the open reading frame. The introns are marked by arrows and numbered 1 and 2. The two nucleotides, bordering each intron, are highlighted in grey. The stop codons in the 5' untranslated region are underlined. The putative polyadenylation signal in the 3' untranslated region is underlined twice. The translation termination codon is marked by an asterisk. The seven transmembrane helices of the receptor protein are boxed and marked TM I–VII. Four potential glycosylation sites, obeying the NXT/S consensus sequence, are marked by filled triangles.

previous findings [18]. The myosuppressin contained in this preprohormone is identical to *Drosophila* myosuppressin. TDVDHVFLRFamide, therefore, is the probable intrinsic ligand for the mosquito myosuppressin receptor.

Blasting of the genomic database from *A. gambiae* with the three receptor sequences from Fig. 2 revealed only one receptor gene with significant amino acid sequence and intron position identities. *A. gambiae*, therefore, appears to have only one myosuppressin receptor.

Table 1

Nucleotide differences between the receptor cDNA and the corresponding genomic sequences from the *A. gambiae* Genome Project

| Position of the nucleotide in the cDNA | Type of nucleotide in the gene | Type of nucleotide in the cDNA | Change in amino acid |
|--|--------------------------------|--------------------------------|----------------------|
| 120 | C | T | — |
| 165 | T | C | — |
| 213 | G | A | — |
| 349 | T | C | — |
| 546 | G | C | — |
| 564 | C | T | — |
| 823 | T | C | — |

Table 2

Intron/exon boundaries of the receptor gene

| Intron | 5'donor | Size (bp) | 3'acceptor | Intron phase |
|--------|-----------------------|-----------|------------------------|--------------|
| 1 | AG gtaagca.... Ser | 3290 | atttcag C Ser | 2 |
| 2 | G gtaagtg.... Gly | 4435 | gtttcag GT Gly | 1 |

Discussion

The insect myosuppressins (X₁DVX₂HX₃FLRFamide) have a C-terminal moiety that resembles those of other insect neuropeptides, such as the insect FMRFamides. Especially the *Anopheles* FMRFamide family has a peptide member, PDRNFLRFamide (*Anopheles* FMRFamide-3), that has five amino acid residues, including the whole C-terminal FLRFamide moiety, in common with the intrinsic *Anopheles* myosuppressin (TDVDHVFLRFamide) [18]. Nevertheless, this FMRFamide was not able to activate the mosquito myosuppressin receptor, clearly illustrating that the mosquito myosuppressin receptor needs a longer peptide sequence for peptide recognition and activation, and perhaps even the whole TDVDHVFLRFamide sequence of *Anopheles* myosuppressin. A similar observation has previously been made for the two *Drosophila* myosuppressin receptors [14]. The *Drosophila* FMRFamide receptor, in contrast, appeared to be less selective and high concentrations of myosuppressin and short neuropeptide F-1 (10⁻⁶ M or ca. 100× higher concentrations than those of the FMRFamides) were able to activate the receptor [17]. Again, a similar observation has

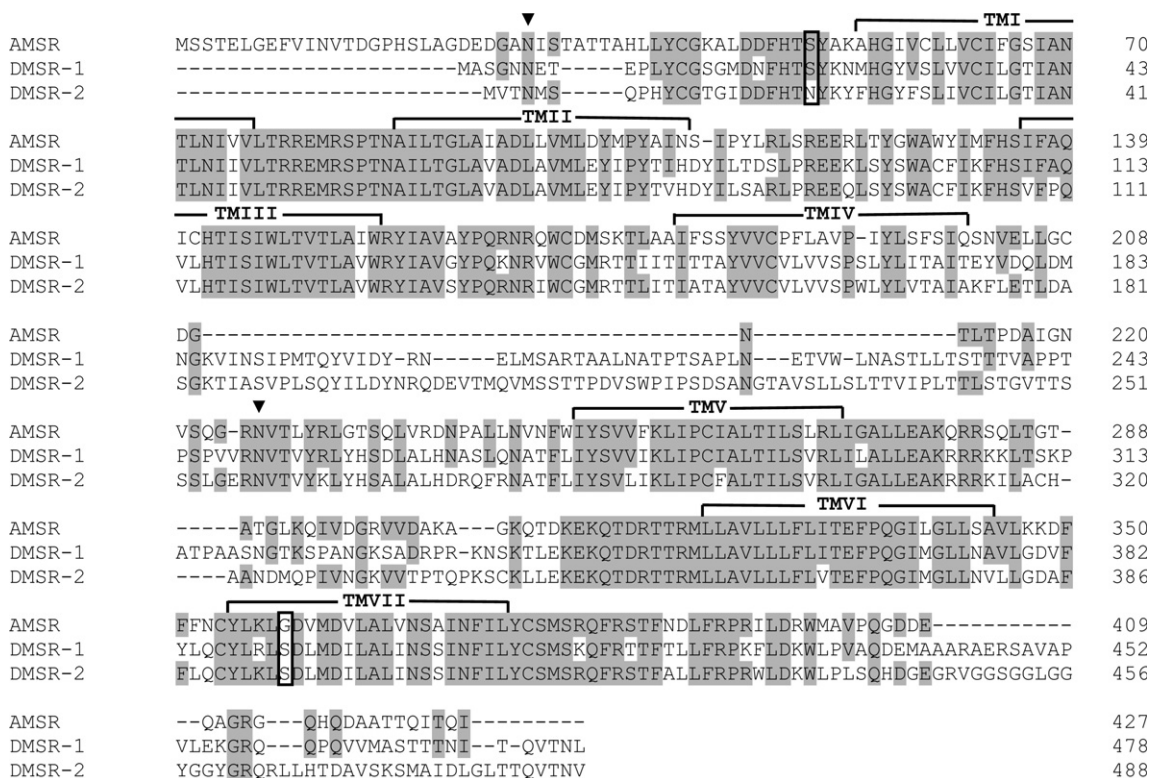


Fig. 2. Amino acid comparisons between the *Anopheles gambiae* myosuppressin-like receptor (AMSR) and the two *Drosophila* myosuppressin receptors (DMSR-1 and -2) published previously [14]. Amino acid residues that are identical between the mosquito and at least one of the *Drosophila* receptors are highlighted in grey. The seven transmembrane helices are indicated by TM I–VII. The positions of the two common introns are indicated by vertical boxes. The filled triangle indicates the glycosylation sites common for all three receptors. Gaps are introduced to optimize the alignments.

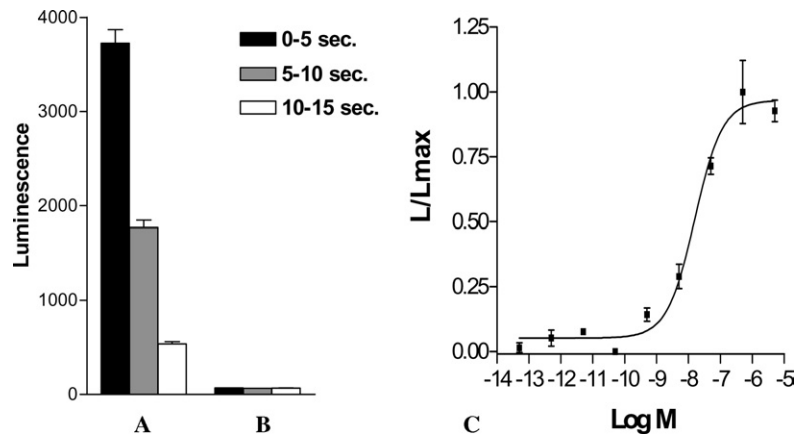


Fig. 3. Functional expression of the mosquito receptor gene. CHO cells, expressing G-16 and apoaeguorin (CHO/G-16); or CHO cells, expressing G-16, apoaeguorin and the receptor cDNA (CHO/G-16/AMSR), were compared. The vertical bars indicate SEMs, which sometimes are smaller than the symbols used. In these cases only the symbols are given. (A) Strong bioluminescence responses 0–5, 5–10, and 10–15 s. after addition of 5×10^{-7} M *Anopheles* myosuppressin to CHO/G-16/AMSR cells. (B) Lack of bioluminescence responses 0–5, 5–10, and 10–15 s. after the addition of 5×10^{-7} M *Anopheles* myosuppressin to CHO/G-16 cells. (C) Dose–response curve of the effects measured in (A). About 35 insect neuropeptides and biogenic amines did not activate the receptor. For a list, see [14] and the peptides mentioned in the text. The biogenic amines were octopamine, tyramine, L-dopa, dopamine, adrenaline, noradrenaline, histamine, and serotonin (all tested at 10^{-5} or 10^{-4} M).

been made for the *A. gambiae* FMRFamide receptor (Cazzamali et al., in preparation). These observations in two insect species, then, suggest that the insect FMRFamide receptor is less specific and that it only needs smaller portions of the peptide sequence for activation, whereas the insect myosuppressin receptor clearly is quite selective for myosuppressins.

The insect myosuppressin receptors are not evolutionarily related to the insect FMRFamide receptors (there are no significant sequence identities). Furthermore, as explained in the Introduction, the insect myosuppressin preprohormones are not evolutionarily related to the insect FMRFamide preprohormones [1,11]. The myosuppressin and FMRFamide receptor/ligand couples, therefore, are two separate systems that have originated independently during evolution. This is important to mention, because the two peptide systems have often been grouped together (as the FaRPs or FMRFamide-related peptides [1,11]). To avoid any further confusion, however, one should abandon the term “FaRPs”. Furthermore, the insect myosuppressins should not be named FLRFamides (as has often been done [1,11]), since *Anopheles* FMRFamide-3 (PDRN FLRFamide) cannot activate the *Anopheles* myosuppressin receptor and, in fact, is a genuine FMRFamide peptide, activating the *Anopheles* FMRFamide receptor (Cazzamali et al., in preparation).

Anopheles has apparently only one myosuppressin receptor and a BLAST search of the recently published genomic database from the honeybee *Apis mellifera* (<http://www.ncbi.nlm.nih.gov/genome/guide/bee/>) gave a similar result for the honey bee. The two myosuppressin receptors found in *Drosophila* [14], therefore, have probably originated by a recent gene duplication after

the split of the mosquito and fruitfly ancestors (approximately 250 million years ago) [19].

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