Identification of a Novel Prothoracicostatic Hormone and Its Receptor in the Silkworm Bombyx mori*

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The insect brain regulates the activity of the prothoracic glands to secrete ecdysteroids, which affect growth, molting, and metamorphosis. Here we report the identification of a novel prothoracicostatic factor and its receptor in the silkworm Bombyx mori. The prothoracicostatic factor purified from pupal brains of B. mori is a decapeptide with the conserved structure of an insect myosuppressin and thus named Bommo-myosuppressin. Bommo-myosuppressin dose-dependently suppressed the cAMP level and inhibited ecdysteroidogenesis in the larval prothoracic glands at much lower concentrations than the prothoracicostatic peptide, the other prothoracicostatic factor reported previously. In vitro analyses using a prothoracic gland incubation method revealed that Bommo-myosuppressin and prothoracicostatic peptide regulate the prothoracic gland activity via different receptors. In situ hybridization and immunohistochemistry revealed the existence of Bommo-myosuppressin in the brain neurosecretory cells projecting to neurohemal organs in which it is stored. We also identified and functionally characterized a specific receptor for Bommo-myosuppressin and showed high expression of the prothoracic glands. All these results suggest that Bommo-myosuppressin functions as a prothoracicostatic hormone and plays an important role in controlling insect development.

In 1922, it was shown that the insect brain secreted a diffusible factor, now known as the prothoracotropic hormone (PTTH),† necessary for the induction of larval-pupal metamorphosis (1). PTTH stimulates the prothoracic glands (PGs) to synthesize and release ecdysteroids, which directly elicit molting and metamorphosis (2, 3). It is now widely accepted that the insect brain thus activates the PG, thereby controlling the timing of postembryonic development (4).

On the other hand, Carlisle and Ellis (5) demonstrated hormonal inhibition of the PGs by the brain of locusts in 1968. Inhibitory actions of the central nervous system on the PGs have been reported thereafter (6–8). These observations led to our working hypothesis: the brain and other ganglia of the central nervous system exert a prothoracicostatic effect in addition to the tropic effect, thus controlling the complex pattern of the ecdysteroid titer in hemolymph during insect development. In contrast to the extensive studies on PTTH, factors with prothoracicostatic activity have been poorly examined. The prothoracicostatic peptide (PTSP) isolated from larval brains of Bombyx mori is the only cerebral factor elucidated so far to inhibit ecdysteroid synthesis in the PG (9, 10). This peptide inhibits both the basal and the PTTH-stimulated ecdysteroidogenesis in vitro in a dose-dependent manner, but its physiological role has yet to be demonstrated.

In the present study, we purified a novel prothoracicostatic factor from brains of B. mori. The purified factor is a decapeptide with the conserved structure of an insect myosuppressin, which is a member of the FMRFamide-related peptides (FaRPs), and thus named Bommo-myosuppressin (BMS). Moreover its low EC50 value, storage in neurohemal organs, and high expression of its functional receptor in the PG all indicate that BMS plays a role as a prothoracicostatic hormone. To our knowledge, this is the first report on the prothoracicostatic activity of FaRPs, proposing these extensively studied peptides as important candidates for the regulator of insect growth, molting, and metamorphosis.

MATERIALS AND METHODS

Experimental Animals

B. mori larvae were fed on the artificial diet “Silkmate” (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a 16-h light/8-h dark photoperiod and staged after the final (fourth) larval ecdysis. Most larvae started wandering behavior on day 6 of the fifth instar and pupated on day 10.

cAMP Assay

Larvae were anesthetized in water for 10–20 min, and the PGs were dissected rapidly in sterile saline (0.85% NaCl, w/v). The paired glands were first preincubated in 100 μl of Insect Ringer’s solution (154.8 mM NaCl, 13.3 mM KCl, 4.0 mM MgCl2, 4.5 mM CaCl2, 2.1 mM NaHCO3, 0.1 mM Na2HPO4, 67.1 mM glucose, 10.0 mM Hepes, pH 6.8) for 30 min after which each gland of the pair was transferred into 100 μl of the same solution containing 1 mM 3-isobutyl-1-methylxanthine with or without samples. After 30 min of incubation, cAMP was extracted from each gland in 100 μl of acidified ethanol (0.1% 10 N HCl, v/v) with vigorous shaking at room temperature.

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† The abbreviations used are: PTTH, prothoracotropic hormone; PG, prothoracic gland; PTSP, prothoracicostatic peptide; FaRP, FMRFamide-related peptide; BMS, Bommo-myosuppressin; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; EST, expressed sequence tag; PBS, phosphate-buffered saline; GPCR, G protein-coupled receptor; NCC, nervous corporis cardiace; BMSR, Bommo-myosuppressin receptor.

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For cAMP quantification, the gland extract was dried using a Speed Vac concentrator (GMI, Inc., Albertville, MN) and subsequently dissolved in 10 μl ammonium acetate buffer (pH 5.0). This solution was directly applied onto a DOCOSIL-B 3-SCOD column (2.0 x 250 mm, Sensus Kagaku Co. Ltd., Tokyo, Japan) using a Waters 2695 Separations Module (Waters, Milford, MA). The mobile phase was composed of 90% 0.1 M ammonium acetate (pH 5.0) and 10% acetonitrile/water (70:30, v/v). Each run was performed in the isocratic mode at a flow rate of 0.2 ml/min for 7 min at 40 °C. cAMP was detected by an absorbance at 260 nm using a Waters 996 Photodiode Array Detector and quantified by measuring the peak areas in comparison with external standards with Waters Millennium software. The prothoracostatic activity of a sample was expressed as an inhibition/activation index calculated with the formula: inhibition/activation index = (cAMP content of PG incubated with sample/cAMP content of PG incubated without sample).

Purification of the Prothoracostatic Factor

Approximately 12,000 brains from Bombyx day 0 pupae were extracted in batches of 4,000 brains. Each batch of brains was homogenized in 20 ml of ice-cold 2% NaCl (w/v) with a glass-glass homogenizer, and the homogenate was centrifuged at 9,200 × g for 15 min at 4 °C. The supernatant was pooled, while the pellet was re-extracted three times. The resulting extracts were heated in boiling water for 5 min and cooled rapidly on ice, and then the precipitates were removed by centrifugation at 9,200 × g for 15 min at 4 °C. The aqueous solution was acidified with 2.4 ml of 10% (v/v) trifluoroacetic acid and directly applied to the high performance liquid chromatography (HPLC) purification steps.

Step 1—The acidified extract was loaded onto a PEGASIL-300 ODS-II column (20 × 150 mm, Sensus Kagaku Co. Ltd.) using a JASCO Gulliver series HPLC system (Jasco, Tokyo, Japan). The elution was performed with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid over 40 min at the flow rate of 10 ml/min.

Step 2—The active fraction was aliquoted into three batches and applied onto a PEGASIL-300 C4P column (4.6 × 250 mm, Sensus Kagaku Co. Ltd.) using a Waters 2695 Separations Module. The elution was performed with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid over 60 min at the flow rate of 1 ml/min.

Step 3—The active fractions of step 2 were loaded onto a PEGASIL-300 ODS-II column (4.6 × 250 mm). The elution was performed with a linear gradient of 10–40% acetonitrile in 0.1% heptfluorobutyric acid over 60 min at the flow rate of 1 ml/min.

Step 4—The active fraction was further purified by passing over a PEGASIL-300 ODS-II column (4.6 × 250 mm). The column was eluted with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid over 60 min at the flow rate of 1 ml/min.

Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and MS/MS were performed using an Applied Biosystems 4700 Proteome Analyzer (Applied Biosystems, Foster City, CA). A saturated solution of cyano-4-hydroxycinnamic acid in acetonitrile/water/trifluoroacetic acid (50:49:9.0, v/v/v) was used as the matrix. MS/MS mode was operated without collision gas. DeNovo Explorer software (Applied Biosystems) was used for the sequence analysis.

Synthetic and Recombinant Peptides

BMS, PTSP, and Manduca sexta F7G (FLRFamide II; GNSFLRFamide) and F7D (FLRFamide III; DPPSFLRFamide) were all custom synthesized and further purified by HPLC. Bombyxin-II was synthesized and purified as described earlier (11). Recombinant PTTH was prepared as described previously (12).

Ecdysone Assay

The PGs were dissected rapidly in sterile saline and preincubated in 100 μl of Grace’s Insect Medium (Sigma). After 30 min, the glands were transferred into 100 μl of medium containing various concentrations of samples and further incubated for 3 h. At the end of the incubation, glands were removed, and the remaining medium was diluted with the assay buffer (0.5% bovine serum albumin and 0.05% sodium azide in 50 mM borate buffer, pH 8.4) and analyzed using the ecdysteroid radioimmunoassay as described previously (13). Since 20-hydroxyecdysone was used as the standard, the secreted ecdysteroid amount was expressed in 20-hydroxyecdysone equivalents.

**Northern Blot and in Situ Hybridization**

The expressed sequence tag (EST) clone wdV40322 (GenBankTM accession no. AU005976) was obtained from the Bombyx EST project (14) and used as the template for the probe synthesis. Digoxigenin-labeled RNA probes were synthesized using DIG RNA labeling mixture (Roche Applied Science) and T3 or T7 RNA polymerase (Invitrogen) according to the manufacturers’ instructions.

For Northern blot analysis, total RNA was extracted from brains of each stage larvae and pupae using TRIzol reagent (Invitrogen). Ten micrograms of total RNA was loaded on each lane of a 1% formaldehyde gel. After transfer to a nylon membrane (Hybond-N+, Amsham Biosciences), the blots were hybridized to the probe overnight at 68 °C in ULTRAhyb (Ambion, Inc., Austin, TX). The signal of the hybridized probe was detected using anti-digoxigenin-alkaline phosphatase (Roche Applied Science) and CDP-Star (Roche Applied Science) according to the supplier’s protocol. Imaging was performed with a LAS-1000plus imaging system (Fuji Photo Film Co. Ltd., Tokyo, Japan).

For in situ hybridization, whole brains were fixed overnight at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS). Before hybridization, brains were decolorized briefly with a commercially available bleaching solution, stored in methanol for a week at −20 °C, treated with Proteinase K (40 μg/ml) for 20 min, and postfixed with 4% paraformaldehyde in PBS for 20 min. Following a 1-h prehybridization at 50 °C in a solution of 50% formamide, 5 × standard saline citrate, 1 mg/ml yeast tRNA, 50 μg/ml heparin, and 0.1% Tween 20, brains were hybridized overnight at 50 °C with the hydrolyzed probe in the same buffer. The hybridization probe was incubated with digoxigenin-alkaline phosphatase and visualized with 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) and nitroblue tetrazolium chloride (Roche Applied Science) according to the manufacturer’s instructions.

**Antibody Generation and Whole-mount Immunohistochemistry**

[Cys11]BMS (pEDVVHSFLRF where P is pyroglutamate) was synthesized and conjugated with maleimide-activated bovine serum albumin (Fierce). Mice were immunized with the BMS-bovine serum albumin conjugate, splenocytes were fused with NS-I cells, and hybridomas were cultured following the methods described elsewhere (15). Hybridoma culture supernatants were primarily screened by enzyme-linked immunosorbent assay, and the positive wells were next screened by whole-mount immunohistochemistry. Since BMS gene was expressed in two pairs of medial neurosecretory cells in the brain as demonstrated by in situ hybridization, the antibodies that could label these cells were selected, and the eight hybridomas producing these antibodies were cloned and mass cultured. Monoclonal antibodies in the culture supernatant were purified using a Protein A column, and their binding specificity was examined using a competitive enzyme-linked immunosorbent assay. All the antibodies bound to BMS with varying affinity, but none of them recognized Manduca F7G or F7D at all. Since these three peptides share a common C-terminal amino acid sequence (SFLRFamide), the antibodies were presumed to recognize the N-terminal portion of the BMS. One of the monoclonal antibodies with highest affinity to this peptide was used for the analysis of the projection of BMS-producing neurons by whole-mount immunohistochemistry.

Whole-mount immunohistochemistry was performed on the brain and its associated nerves by two methods: fluorescent immunohistochemistry for an overall survey of immunoreactive neurons and peroxidase immunohistochemistry for a detailed analysis of their axonal pathways. For fluorescent immunohistochemistry, dissected blocks of the entire brain, retrocerebral complex, and prothoracic gland were first fixed with 4% paraformaldehyde in PBS for 4 h at room temperature. After washing with PBS, the tissues were treated with 1% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) in PBS for 1 h, washed with 0.5% Triton X-100 in PBS (PBS-T) for 30 min, and blocked with 5% normal goat serum in PBS-T for 30 min. The tissues were then successively incubated with anti-BMS mouse monoclonal antibody (1 μg/ml in PBS-T) and with 1:500 diluted fluorescein isothiocyanate dye-labeled secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, Invitrogen) overnight at 4 °C. The tissues were cleared in 70% glycerol in PBS before observation. Peroxidase immunohistochemistry was conducted as reported previously (16) except for the application of Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) and AEC Substrate Chromogen kit (Zymed Laboratories Inc.) for signal amplification.
cDNA Library Construction, EST Sequencing, and BLAST Analysis

Approximately 100 PGs of wandering stage larvae were collected, and total RNA was prepared using TRIzol reagent (Invitrogen). Construction of the full-length cDNA library, named fprP, was commissioned to Hitachi Instruments Service Co., Ltd. (Tokyo, Japan). Sequences from the 5' end of each cDNA were determined, and the ESTs were compiled into a data base. Standard BLAST analysis was performed on the data base to obtain the homologues of Drosophila myosuppressin receptors.

Sequence and Reverse Transcription-Polymerase Chain Reaction Analysis of the BMS Receptor

The amino acid sequence coded by the polymorphic clones fprWP14_F_K14 and fprWP14_F_P21 (GenBank™ accession numbers AB188256 and AB188257) and the related Drosophila G protein-coupled receptor (GPCR) sequences were submitted to the program ClustalW (www.ddbj.nig.ac.jp/search/clustalw-j.html) for phylogenetic analysis.

For reverse transcription-PCR analysis, cDNAs from various tissues were synthesized as described previously (17), and primers (sense primer, 5'-TAATTCGATCTCTGGCTGCTGGCG-3'; antisense primer, 5'-TGCTTTGGTCGGCTCCCTCTCC-3'), which can amplify both the polymorphic transcripts, were used. RpL3 was used as a loading control as described previously (18). Thirty cycles (95 °C for 10 s and 68 °C for 1 min) were carried out for amplification.

Human Embryonic Kidney 293 Cell Expression and Ca²⁺ Imaging Analysis of the Receptor

The open reading frame of the BMS receptor was amplified by PCR performed on the EST clone fprWP14_F_K14. The specific primers had the following sequences: sense primer, 5'-GCAGAATTCGGACCCATGTCGGAGACCTG-3'; antisense primer, 5'-GCATCTGGACAGTACAGGAACATGAGTCATG-3'. The sense primer incorporates an EcoRI restriction site, and the Kozak sequence (GCCACC) (19), while the antisense primer contains an XhoI restriction site. The obtained product was cloned into the pME18S mammalian expression vector and transfected into human embryonic kidney 293 cells with the promiscuous G protein Gaα1, as described earlier (20). Ca²⁺ imaging analysis was performed as reported previously (20).

RESULTS

Purification of the Prothoracicostatic Factor—As a bioassay system to evaluate the prothoracicostatic activity of the brain extracts, we developed a cAMP assay combining in vitro PG incubation and subsequent cAMP quantification because cAMP is a prerequisite second messenger for ecdysteroidogenesis (4). Day 4 fifth instar larvae were used in this assay.

Starting from an extract of 12,000 brains from Bombyx day 0 pupae, four steps of HPLC separation were required to purify the prothoracicostatic factor as a homogeneous peak (Fig. 1). In the first step, the single fraction designated with a bar in Fig. 1 showed strong prothoracicostatic activity (inhibition/activation index = 0.13) when 10 brain equivalents per PG was applied to the assay. In the subsequent three steps, 10 brain equivalents per PG of the active fractions always suppressed the glandular cAMP level almost completely (inhibition/activation index = 0–0.13, data not shown).

Identification of the Prothoracicostatic Factor as Bombyx Myosuppressin—An aliquot of the isolated fraction was subjected to MALDI-TOF-MS analysis, and a single monoisotopic mass of 1229.7 (M + H)⁺ was obtained. Further analysis of this putative peptide with MS/MS gave the sequence pEDVHSHFLRFamide (Fig. 2A). Since the sequence pEDVHSHFLRFamide was identical to Manduca FLRFamide I (21), the sequence of the purified peptide was determined to be pEDVHSHFLRFamide, and the synthetic peptide was prepared. The MS/MS profiles and the HPLC elution times of the purified peptide and the synthetic peptide were completely identical (Fig. 2, A and B, and data not shown). Since this peptide had the conserved structure of an insect myosuppressin (XDVX,HXXLFRFamide) (22), we named this peptide BMS.

BMS Has a Higher Prothoracicostatic Activity than PTSP—To compare the inhibitory activity of BMS and PTSP on larval PGs, various concentrations of these peptides were subjected to the cAMP assay and the ecysyone assay in the presence or absence of 10⁻² M PTTH (Fig. 3). Only day 4 fifth instar larvae were used in this assay because day 4 larval PGs responded well to PTTH, making it easier than any other stages to assay the prothoracicostatic activities both in the basal and the PTTH-stimulated conditions.

As shown in Fig. 3, A and B, BMS inhibited both the basal and the PTTH-stimulated cAMP accumulation in the PGs in a dose-dependent manner with much lower EC₅₀ values (concentrations that produced 50% of the highest inhibitory activity) than PTSP (0.039 or 0.089 nM for BMS and 54 or 105 nM for PTSP with or without PTTH, respectively). Two relevant Manduca FaRPs (F7G and F7D) also suppressed the glandular cAMP level at higher concentrations than BMS. In the ecysyone assay, BMS inhibited ecdysteroidogenesis in a statistically significant level at around 10⁻⁸ M, again much lower concentrations than the EC₅₀ value of PTSP. Interestingly higher concentrations of BMS rather reduced the inhibitory activity both on the basal and the PTTH-stimulated PGs, inconsistent with the result of the cAMP assay (Fig. 3, C and D).

BMS Inhibits Ecdysteroidogenesis via a Different Pathway from PTTH or PTSP—To elucidate the modes of action of BMS and PTSP, their inhibitory activities on ecdysteroidogenesis in the presence of PTTH were further compared (Fig. 4). First the EC₅₀ value of PTTH in the ecysyone assay using day 4 fifth instar PGs was determined to be 2.8 pm, consistent with prior

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2 N. Yamanaka, T. Namiki, K. Watanabe, R. Niwa, K. Mita, and H. Kataoka, unpublished.
Addition of $10^{-9}$ M or $10^{-7}$ M PTSP suppressed the amount of ecdysteroid secreted at various concentrations of PTTH, but the EC$_{50}$ values of PTTH in the presence of these prothoracicostatic factors remained constant (2.5 and 3.8 pM, respectively; Fig. 4A). This result, that both BMS and PTSP inhibit the PTTH-stimulated ecdysteroidogenesis in non-competitive manners, indicates the existence of receptors for these peptides different from the PTTH receptor.

The additive effect of BMS and PTSP in the presence of $10^{-9}$ M PTTH was examined in Fig. 4B. BMS always had an “optimal” concentration for the inhibition of PTTH-stimulated PGs at around $10^{-9}$ M irrespective of the concentrations of coexisting PTSP. Moreover PTSP dose-dependently inhibited ecdysteroidogenesis even in the presence of $10^{-7}$ M BMS, which is above the optimal concentrations. This result demonstrates that BMS regulates the PG activity via a different receptor from PTSP.

BMS Is Produced in the Neurosecretory Cells of the Brain—The expression pattern of BMS in the brain was determined by Northern blot and in situ hybridization. Northern blot analysis revealed that BMS was expressed throughout the fifth larval instar to the 1st day of the pupal stage but was higher in the early half of the fifth instar (Fig. 5A). Further analysis with whole-mount in situ hybridization on day 3 fifth instar brains showed that BMS was mainly expressed in two pairs of medial neurosecretory cells (Fig. 5, B and C).

To clarify the axonal projection of these neurosecretory cells, whole-mount immunohistochemistry was performed on the brain and its associated nerves from the same stage using an antibody that reacts specifically with BMS. This analysis further confirmed the existence of BMS in the median neurosecretory cells (Fig. 5D). Several pairs of small neurons were also stained in the brain, although their signals were relatively weak, and they did not project to peripheral targets. The median neurosecretory cells projected contralaterally (Fig. 5, D and E) into the corpora cardiaca, one of the well-established neurohemal organs, via nervus corporis cardiaci (NCC) 1 and 2 (Fig. 5F; terminology according to Ref. 24). The strong immunoreactivity of the corpora cardiaca indicates the storage of BMS in these organs. Strong immunoreactive fibers were also found in NCC-recurrens (Fig. 5F). Weak immunoreactivity was also detected in NCC-ventralis (Fig. 5F) and the node (data not shown). In some preparations, fibers running on the corpora allata were also stained weakly, but no immunoreactive materials were observed in the corpora allata. These results suggest that BMS is produced in the two pairs of neurosecretory cells of the brain and released into the hemolymph from the corpora cardiaca and/or NCC-recurrens. Double staining analyses using antibodies against BMS and other cerebral peptides (PTSP, diuretic hormone, and bombyxin) were performed, but no coexistence of any pair of these peptides was detected (data not shown).

Identification of the Functional BMS Receptor in PGs—All the above results indicate that the BMS released from the neurosecretory cells inhibits ecdysteroidogenesis via a specific BMS receptor in the PGs. To find this putative receptor, we
constructed a full-length cDNA library from the PGs of wandering stage larvae, sequenced the 5′ end of each cDNA, and compiled a local data base. Standard BLAST analysis of *Drosophila* myosuppressin receptors (*DmsR-1* and -2) (25) using this EST data base showed that two clones (*fprWP14_F_K14* and *fprWP14_F_P21*) share high sequence similarities with *DmsR-1* and -2. Full sequencing of these clones revealed that they coded an identical predicted protein with seven putative transmembrane domains, indicating that these polymorphic genes code a GPCR for BMS (BMS receptor (BMSR)). Phylogenetic analysis of BMSR and related *Drosophila* GPCR sequences (reported in Ref. 26) showed that DMSR-1 and -2 are the most closely related GPCRs of BMSR (Fig. 6A). BMSR expression in various tissues of wandering stage larvae was examined by reverse transcription-PCR. As we expected, BMSR was highly expressed in the PG (Fig. 6B). Functional characterization of BMSR was performed using a heterologous expression system. Human embryonic kidney 293 cells expressing both BMSR and G<sub>a</sub>12 responded to BMS dose dependently in the Ca<sup>2+</sup> imaging analysis, demonstrating this GPCR to be a functional BMS receptor (Fig. 6, C and D). No other peptides tested at concentrations up to 10 μM (PTTH, PTSP, and bombyxin-II) activated BMSR except the two *Manduca* FaRPs (F7D and F7G). These two peptides, which share the common amino acid sequence at the C termini with BMS, activated BMSR weakly but dose dependently, suggesting that the C-terminal structure of BMS is recognized by BMSR. The relative EC<sub>50</sub> values of these two FaRPs in this assay corresponded well to those in the cAMP assay (Fig. 3A).

**DISCUSSION**

In this study, we purified and identified *Bombyx* myosuppressin as a novel prothoracostatic factor. Insect myosuppressins have been isolated from cockroaches, locusts, flies, and moths, and they are now thought to occur in all insects (22, 27–30). As can be anticipated from their name, myosuppressins show inhibitory activities on various kinds of visceral muscles including gut, heart, oviduct, Malpighian tubule, and salivary gland (28, 31–34).

Myosuppressin is a member of a larger peptide family, FaRPs, which are known to regulate a wide range of processes from behavior to physiology in invertebrates (22, 35, 36). However, to our knowledge, these FaRPs including myosuppressins have never been shown to regulate invertebrate development by affecting steroid hormone secretion. Our report thus proposes these extensively studied peptides as important candidates for regulators of invertebrate development, which is a potential target for the development of effective pest management tools.

Using two *in vitro* bioassays (cAMP assay and ecdysone assay), we demonstrated that BMS suppressed the PGs with much lower effective concentrations than PTSP. However, higher concentrations of BMS rather reduced this inhibitory activity in the ecdysone assay, inconsistent with the cAMP assay (Fig. 3). This discrepancy is not the result of the inactivation of the BMS receptor during the longer incubation in the ecdysone assay (3 h) than in the cAMP assay (30 min) because the cAMP level in the PGs remained low even after the 3-h incubation with higher concentrations of BMS (data not shown). If this phenomenon has any physiological importance, it is intriguing to speculate that BMS has different effects on the PG via different second messenger pathways, depending on its concentrations in hemolymph. We are now investigating these putative second messengers other than cAMP that are activated by higher concentrations of BMS (Ca<sup>2+</sup>, for example, has been shown to play another important role in ecdysteroidogenesis) (4).

Its low effective concentration and storage in the corpora cardiaca and NCC-recurrens suggest that BMS functions as a
Northern blot analysis on the developmental changes of the BMS expression in the brain. V0–V8 indicates fifth instar days 0–8, and P0 indicates pupal day 0. 10 μg of total RNA from each stage was loaded. The size of the BMS transcript was estimated to be about 1.0 kb. rRNA serves as a loading control. B and C, whole-mount in situ hybridization analysis on a day 3 fifth instar brain using the antisense probe for the BMS transcript. Arrowheads in B indicate strong signals in two pairs of medial neurosecretory cells, which are shown at higher magnification in C. D and E, whole-mount immunohistochemistry (peroxidase method) on a day 3 fifth instar brain using the antibody against BMS. The boxed area of D is shown at higher magnification in E. Note that the axons of immunoreactive neurosecretory cells in each hemisphere cross at the ventral area of the brain. F, innervations of the BMS-producing neurosecretory cells visualized by fluorescent immunohistochemistry. CC, corpora cardica; CA, corpora allata; RN, recurrents; V, ventralis. Scale bars, 100 μm.

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BMS expression and BMS localization in the brain. A. Northern blot analysis on the developmental changes of the BMS expression in the brain. V0–V8 indicates fifth instar days 0–8, and P0 indicates pupal day 0. 10 μg of total RNA from each stage was loaded. The size of the BMS transcript was estimated to be about 1.0 kb. rRNA serves as a loading control. B and C, whole-mount in situ hybridization analysis on a day 3 fifth instar brain using the antisense probe for the BMS transcript. Arrowheads in B indicate strong signals in two pairs of medial neurosecretory cells, which are shown at higher magnification in C. D and E, whole-mount immunohistochemistry (peroxidase method) on a day 3 fifth instar brain using the antibody against BMS. The boxed area of D is shown at higher magnification in E. Note that the axons of immunoreactive neurosecretory cells in each hemisphere cross at the ventral area of the brain. F, innervations of the BMS-producing neurosecretory cells visualized by fluorescent immunohistochemistry. CC, corpora cardica; CA, corpora allata; RN, recurrents; V, ventralis. Scale bars, 100 μm.

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Figure 6. Characterization of the BMSR. A. A phylogenetic tree showing the relationship between BMSR and related Drosophila GPCRs. B, reverse transcription-PCR analysis of the BMSR expression in various tissues from wandering fifth instar larvae. Br, brain; SG, salivary gland; AS, anterior silk gland; MS, middle silk gland; PS, posterior silk gland; FG, foregut; MG, midgut; HG, hindgut; TR, trachea; MT, Malpighian tubule; FB, fat body; MU, muscle; OV, ovary; TE, testis. RpL3 was used as a loading control. C and D, Ca2+ imaging analysis using human embryonic kidney 293S cells expressing BMSR. C, an example of the response patterns of a single cell. Indicated concentrations of BMS were applied for 20 s at the times indicated by arrowheads. D, dose-response curves for the three ligands that activated BMSR. The vertical bars represent S.E. For each ligand, each datum point was calculated from the responses of 150–200 cells in three independent experiments. Relative response was expressed as a percentage of the highest response (induced by 10–6 M BMS).

The expression of BMS in the brain during the fifth instar (Fig. 5A) suggests that BMS is mainly produced and secreted in the feeding period during which the PGs are inactivated and thus the ecdysteroid titer is low (37, 38). Since we have detected the existence of BMS in larval hemolymph by a method combining separation with HPLC and quantification by enzyme-linked immunosorbent assay (data not shown), measurement of the developmental changes of the BMS titer in hemolymph will give important information on the timing when BMS acts on PGs.

In this study, we have also identified and characterized a receptor specific for BMS. BMSR was expressed not only in the PG but in some other tissues including midgut, hindgut, and Malpighian tubule (Fig. 6B). Since myosuppressin actions on these tissues except the PG have been documented in other insects (27, 31, 33), this result indicates that BMS also works as a multifunctional hormone in B. mori.

The EC50 value of BMS determined using the heterologous expression system (32 nM) was higher than that in the cAMP assay (0.089 nM). This discrepancy, however, may be attributed to the relatively low sensitivity of the heterologous expression system because the EC50 value compares well with the cAMP assay (Fig. 3A, 3B). Furthermore, we found two additional ligands that weakly activate BMSR. Since the relative EC50 values of these three ligands in the Ca2+ imaging analysis (Fig. 6D) compares well with the cAMP assay (Fig. 3A), we can see that this receptor actually mediates the inhibitory activity of BMS on the PGs.

Vertebrates including humans have also been shown to possess peptides with an RFamide C terminus (39, 40), and at least one RFamide peptide has been shown to inhibit steroid hormone release in experiments using an in vitro adrenal slice culture system (41). Considering the conservation of RFamide peptides throughout metazoans, our result also indicates that RFamide peptides modulate steroid hormone secretion in various kinds of species, thereby controlling their postembryonic development.

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