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G protein-coupled receptor for diapause hormone, an inducer of *Bombyx* embryonic diapause

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Abstract

Bombyx diapause hormone was the first chemical substance identified as a maternal control factor that arrests offspring development. However, the molecular mechanisms by which the hormone transduces the signal to the oocyte that induces embryonic diapause immediately after mesoderm segmentation are not fully understood. Here, we describe a cDNA for a G protein-coupled diapause hormone receptor with seven transmembrane domains. Its amino-acid sequence shows a high level of similarity to the receptors of mammalian neuromedin U and insect regulatory peptide, an FXPRL-amide C-terminus. When expressed in a *Xenopus* oocyte system, the receptor exhibited the highest affinity (EC_{50}, ~70 nM) for diapause hormone, when compared with other *Bombyx* FXPR/KL-amide peptides. Diapause hormone without amidation at the C-terminus, which never induces embryonic diapause in vivo, had no effect in this heterologous expression system. The mRNA is expressed in the ovaries during *Bombyx* pupal–adult development. These results strongly indicate that the cDNA encodes the diapause hormone receptor.

Keywords: G protein-coupled receptor; GPCR; Diapause hormone; FXPRL-amide; Neuropeptide; Peptide hormone; *Bombyx mori*; Embryonic diapause; Maternal control factor; Pheromone biosynthesis-activating neuropeptide

During development, maternal effects are known to provide an important degree of phenotypic plasticity to the progeny. Although mothers are able to transmit signals about environmental variability to the next generation and influence the adaptive phenotypic response of their offspring, the molecular bases of this process are yet to be explored [1,2]. Embryonic diapause (the programmed arrest of development) is a good system for studying the mechanisms of these biological events as maternal control of diapause is well documented in insects, mites, crustaceans, and mammals such as rodents, kangaroos, and mustelids [3,4]. One of the best-known examples is the embryonic diapause of the silkworm *Bombyx mori*.

In *Bombyx* bivoltine strain (two generations per year), the environmental signals that mothers experience during their own embryonic development determine whether they produce diapause or nondiapause eggs. If eggs are incubated at 25 °C under illumination, the resultant female moths lay diapause eggs, while eggs incubated at 15 °C in darkness develop into females that lay nondiapause eggs [5–7]. These female brains are believed to determine whether a maternal control factor, an inducer of embryonic diapause, is secreted or not [5–7].

Bombyx ovarian development actively proceeds during the pupal-adult transformation. Shortly after the adult emergence, female moths are capable of copulating with

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males and laying eggs. Those eggs from ovaries attacked by the diapause inducer develop until the gastrula stage (immediately after mesoderm segmentation, at which time the embryo is composed of about 12,000 cells) when the cell divisions arrest in G_2 phase [8–10]. This state persists for about one year if the eggs are kept at 25 °C. In contrast, if they are incubated at 5 °C for about 2 months, diapause is terminated and embryonic development resumes as soon as the eggs are transferred to 25 °C [11,12].

In 1957, Hasegawa [13] proposed that a chemical substance extracted from the *B. mori* maternal subesophageal ganglion (SG) is a diapause inducer, i.e., a diapause hormone (DH) that targets developing ovaries via the blood, thereby inducing embryonic diapause in the progeny. Eggs from ovaries not exposed to DH develop continuously until larval hatching after about 10 days incubation at 25 °C.

DH was purified to homogeneity in 1991 and was found to consist of 24 amino-acids with amidation at the C-terminus; TDMKDESDRGAHSERGALWFGPRL-amide (FXPRLa motif sequence is underlined) [14]. The hormone is synthesized in seven pairs of neurosecretory cells in the SG and is released via the corpus cardiacum–corpus allatum complex into the blood [15,16]. Active DH is processed from a precursor protein that also contains pheromone biosynthesis-activating neuropeptide (PBAN, RLSEDMPATPADQEMYQPDPEEMESRTRYFSPRLa) and three SG neuropeptides (α-, β-, and γ-SGNPs; IIFTPKLa, SVAKPQTHESLEFIPRLa, and TMSFSPRLa, respectively) [17].

All these neuropeptides possess an FXPR/KL-amide (FXPR/KLa) C-terminus and are thus classified as members of the FXPRLa peptide family [18]. From the biological activities of the FXPRLa peptides, their truncated forms, and analogs, it was concluded that the PRLa tripeptide is indispensable for DH action [19]. In an in vivo assay, DH was shown to be more effective at producing diapause eggs than other hormones, with threshold levels less than 1/100 that of PBAN and other SGNPs [17]. As a result of this sensitivity, it is expected that the DH receptor (DHR) should recognize this tri-peptide amide and distinguish DH from other members of the FXPRLa peptide family.

In order to understand the molecular mechanisms by which DH triggers the phenotypic plasticity of *Bombyx* progeny, we chose to investigate the DHR responsible for receipt of the DH signal. Here, we present a characterization of the cDNA encoding the DHR, which was cloned from developing ovaries.

Materials and methods

Cloning of diapause hormone receptor. The partial cDNA for *Bombyx mori* diapause hormone receptor (BmDHR) was first amplified by polymerase chain reaction (PCR) with first-strand cDNA prepared from the developing ovaries of 5-day-old pupae (Kinshu x Showa strain, diapause-egg producers) using two primers: 5′-ATGCAYACIGCIACIAAYTWYTAYYTITT-3′ and 5′-CKYTGIGCRTGRAAIGGIGCCCA-3′, roughly corresponding to regions MHTATNFYLF and WAPFHAGR (see dotted underlines with arrowheads in Fig. 1), which are conserved in mammalian neuromedin U receptors (human and rat FM-3; GenBank Accession Nos. NM_006056 and AF242873, respectively) and four candidates (CG8784, 8795, 9918, and 14575; GenBank Accession Nos. AF522189, AF522190, AF522191, and AF522193, respectively) from *Drosophila*. The nucleotide sequence of the full-length cDNA was determined using 5′- and 3′-rapid amplification of cDNA ends (RACE) with the SMART RACE cDNA Amplification Kit (Clontech). The nucleotide sequence of the open-reading frame (ORF) was further confirmed by sequencing the PCR product amplified from an ovarian cDNA library using the primers: 5′-CATGACGCCGAATGAAAATTCTGG-3′ and 5′-ATTGCCATCTGAGTAGCTTTCGTG-3′.

BmDHR expression in Xenopus oocytes. The *BmDHR* gene was subcloned into the modified pSPUTK, kindly donated by Dr. K. Touhara (University of Tokyo). After cutting the vector with *Xba*I (Fermentas), *BmDHR* cRNA was synthesized using SP6 RNA polymerase (Invitrogen). *Xenopus laevis* oocytes were treated with collagenase (2 mg/ml, Nitta Gelatin) for 1 h at room temperature. Stage V or VI oocytes were injected with 50 ng *BmDHR* cRNA in 50 nl RNase-free water. Injected oocytes were incubated for 2–4 days at 18 °C in sterile Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.82 mM $MgSO_4 \cdot 7H_2O$, 2.4 mM $NaHCO_2$, and 10 mM Hepes, pH 7.4). Two-electrode voltage clamp recordings of these oocytes were carried out as described previously [20]. Briefly, oocytes were repeatedly subjected to pulses of 200 millisecond from the holding potential of −80 mV to the depolarizing potential of +60 mV every 2 s. Forty microliters of fivefold concentrated stock of each peptide was applied to the recording bath (160 μl) by pipetting. Voltage clamping was performed with the Oocyte Clamp Amplifier Model OC-725C (Warner Co.). Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP 8 (Axon Instruments). EC_{50} values were calculated with Prism software (Graphpad Software). Because the peptides other than DH appeared not to reach saturation at 10 μM, each curve was drawn with the maximum value fixed with that of DH. To avoid receptor desensitization, current recordings at different doses were measured using different oocytes. DH and the three SGNPs were synthesized and purified by high-performance liquid chromatography. *B. mori* PBAN was kindly donated by Dr. S. Matsumoto (RIKEN, Japan).

Expression analysis. For quantification of *BmDHR* mRNA, real-time PCR was carried out using SYBR Green I dsDNA binding-dye chemistry on the GeneAMP 5700 Sequence Detection System (PE Biosystem) with the sense primer 5′-ATGGCATTCGTGCGGCAT-3′ and the antisense primer 5′-GCCGTAATGGAACCCTGTCAAGCTTGACAGGGTTCCATTACGGC-3′. Because at least one intron is present between the two primers based on comparison with the sequence of contig 453681 from the *Bombyx* genome (GenBank Accession No. BAAB01095695; http://sgp.dna.affrc.go.jp/), the real-time PCR product was distinguished based on the size when genomic DNA was contaminated. The mRNA levels were normalized against *Bombyx* ribosomal protein 49 mRNA [12]. Standard procedures were used for the isolation of RNA and the preparation of cDNA [12].

Animals. Two bivoltine strains of *B. mori* were used: Daizo and Kinshu x Showa. Individuals predisposed to produce either diapause or nondiapause eggs were generated by incubation during embryonic development at 25 °C in light or 15 °C in dark, respectively [5–7]. Larvae derived from these embryos were reared under identical conditions on fresh mulberry leaves or artificial diet (Nosankoh) at about 26 °C. All pupae and adults were kept at 25 °C.

Results

Identification of Bombyx DHR

Based on the knowledge that the structures of *Drosophila* pyrokinins TGPSASSGLWFGPRLa and SVPFKPRLa (PK-1 and -2, respectively) [21,22], which are members of the FXPRLa peptide family, are similar to that of

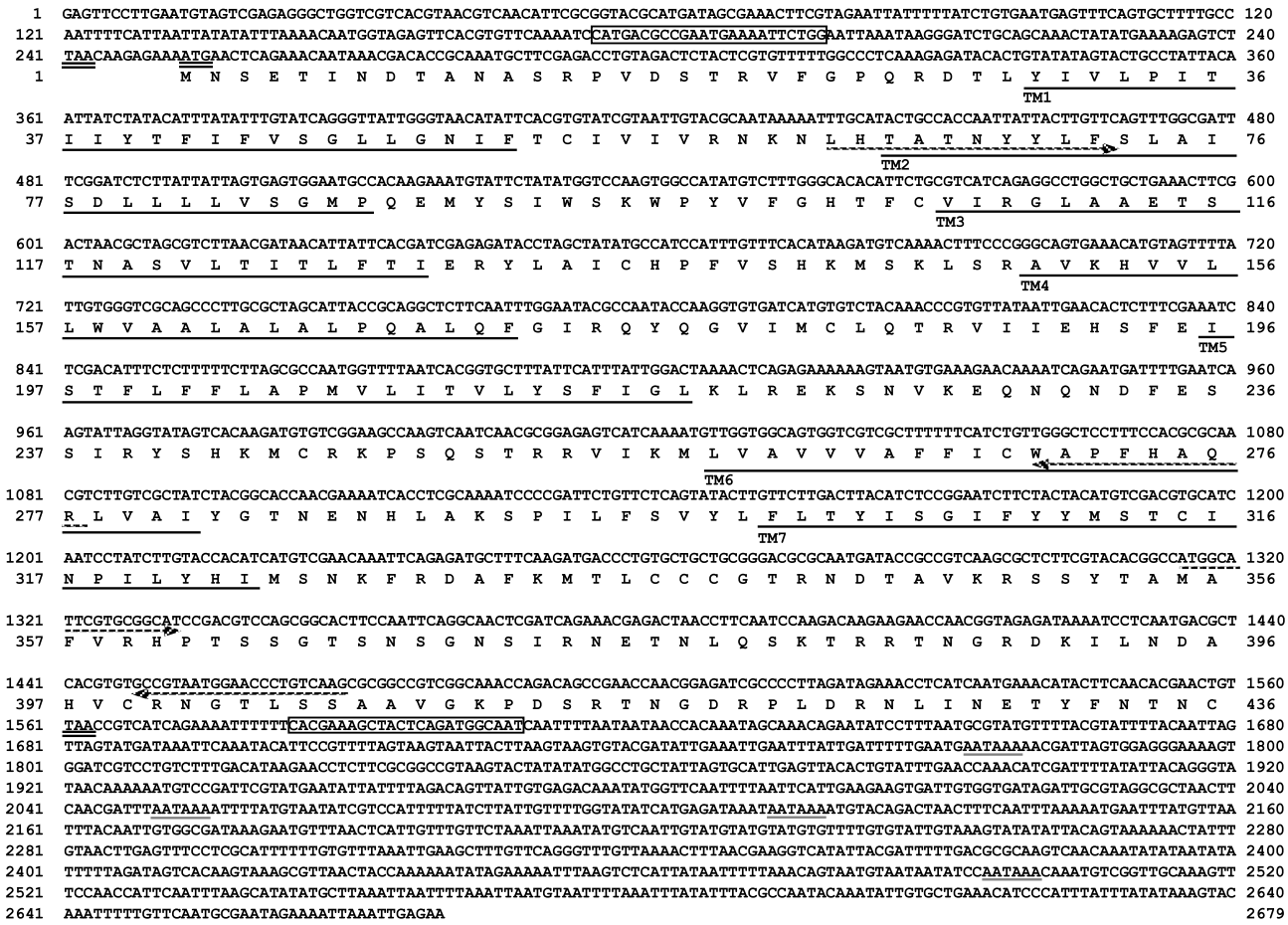

Fig. 1. Nucleotide and amino-acid sequences of *Bombyx* diapause hormone receptor (BmDHR) cDNA (GenBank Accession No. AB164386). Stop and initiation codons are double-underlined. Putative polyadenylation signals (AATAAA) are indicated by gray underlines. The seven transmembrane domains are underlined and labeled TM1-TM7. Dotted-underlines with arrowheads of LHTANYYLF and WAPFHAQR indicate regions corresponding to the primers used for amplification of the predicted receptor cDNA. Boxed nucleotide sequences represent the primers used to PCR-amplify the region containing the ORF. Dotted-underlined nucleotide sequences indicate the primers used for real-time quantitative PCR.

mammalian neuromedin U (NMU), and that PK receptors might be similar in structure to NMU receptors (NMURs) [23], we compared the sequence of DH with mammalian NMUs. We found a strong similarity between DH and human neuromedin U25, FRVDEEFQSPFASQSR GYFLFRPRNa. The alignment of amino-acid sequences of mammalian NMURs (rat and human FM-3, GenBank Accession Nos. AF242873/ AB038649 and NM_006056/ AF272362) and *Drosophila* receptors (CG8784, 8795, 9918, and 14575) generated at least six sets of PCR primers corresponding to several conserved transmembrane helix domains.

Using one set of these primers (corresponding to the dotted-underlined amino acid sequences in Fig. 1, as described in Materials and methods) we amplified a 600-bp PCR product, which was then used to obtain full-length cDNA by 5′- and 3′-RACE (Fig. 1). This 2679 nucleotide cDNA with an ORF encoding 436 amino-acids was predicted by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to code for a G protein-coupled receptor with seven transmembrane domains (TM1-7, Fig. 1). The amino-acid sequence showed a high level of similarity to insect receptors for FXPRLa peptide {46.0, 42.8, 40.9, 36.7, and 36.0% identities to *Hz*PBANR [24], CG9918 (*Drosophila* PK-1 receptor) [25,26], *Bm*PBANR [27], and CG8795 and CG8784 (*Drosophila* PK-2 receptors) [22]; see Fig. 2} and mammalian receptors for neuromedin U {27.5 and 26.5% identities to human FM-3 (GenBank Accession No. AF272362) [28] and rat FM-3 (GenBank Accession No. AB038649) [29]}. However, it clearly differed from both insect receptors for PBAN (Fig. 2). The cDNA was tentatively designated *B. mori* (*Bm*) *DHR* (GenBank Accession No. AB164386).

Functional characterization of BmDHR

We next investigated ligand-receptor specificity using a functional expression system of *BmDHR* in *Xenopus* oocytes. Oocytes injected with *BmDHR* cRNA showed Cl^- currents when activated with 100 nM DH (Fig. 3A, left panel), which appeared to result from the Gq protein activation of phospholipase C, liberation of inositol

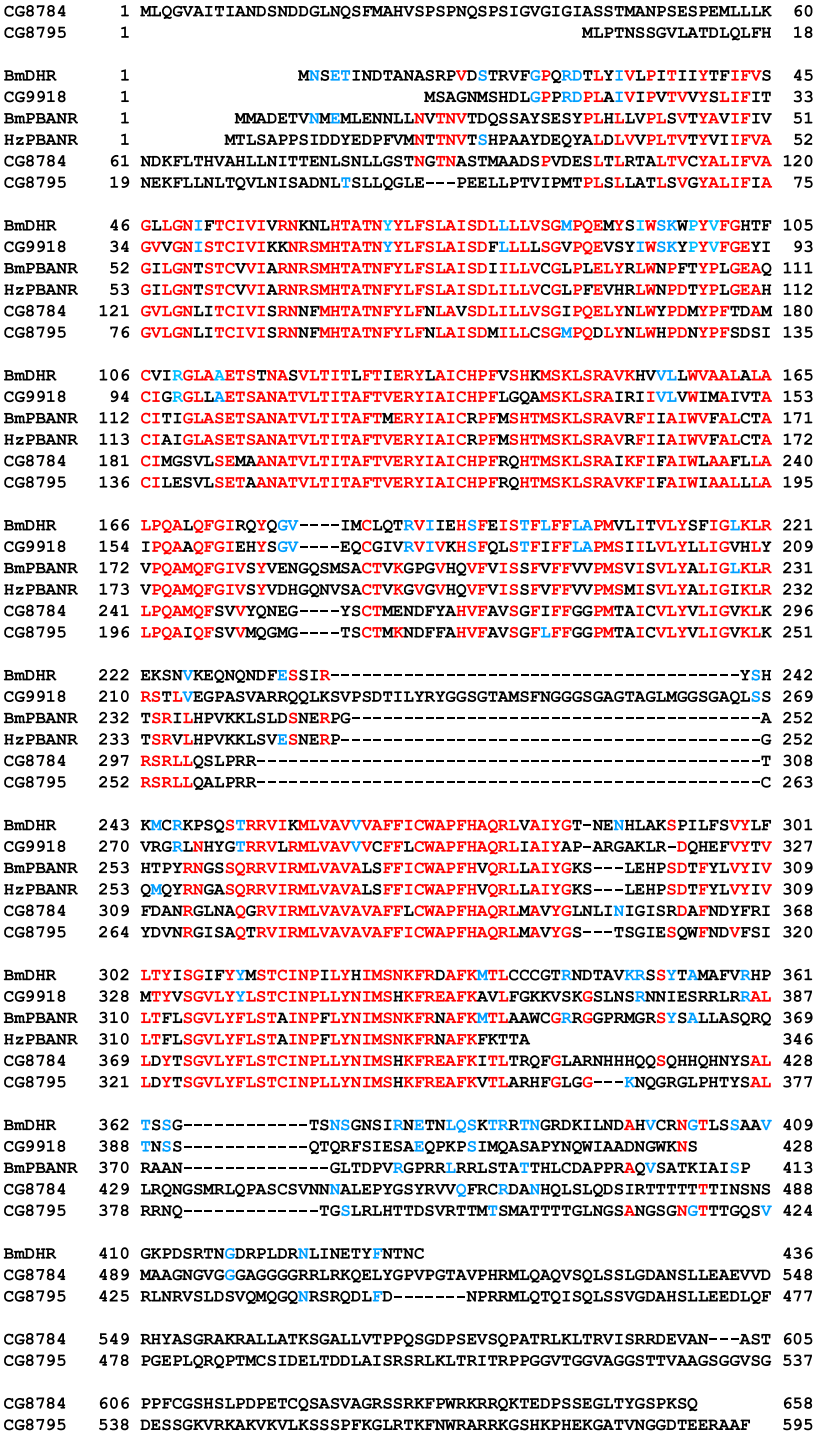

Fig. 2. Comparison of amino-acid sequence of BmDHR with those of insect G protein-coupled receptors for FXPRLa peptide. The accession numbers of the receptor sequences are as follows: BmDHR (GenBank Accession No. AB164386: the present study), CG9918 (GenBank Accession Nos. AF522191, and AF368273: PK-1 receptor/*Drosophila melanogaster*) [25,26], BmPBANR (GenBank Accession No. AB181298: *Bombyx mori*) [27], HzPBANR (GeneBank Accession No. AY319852: *Helicoverpa zea*) [24], CG8784 (GenBank Accession No. AF522189: PK-2 receptor/*Drosophila melanogaster*) [25], and CG8795 (GenBank Accession No. AF522190: PK-2 receptor/*Drosophila melanogaster*) [25]. Amino-acid residues showing 50% conservation or more among the six receptors are shown in red (highlight). When two groups of amino-acid residues shared 50% conservation, the residues identical with BmDHR are shown in red (highlight). Amino acid residues sharing less than 50% identity with BmDHR are shown in blue (box). Multiple sequence alignment was carried out using CLUSTAL W (1.83) of the DNA Data Bank of Japan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

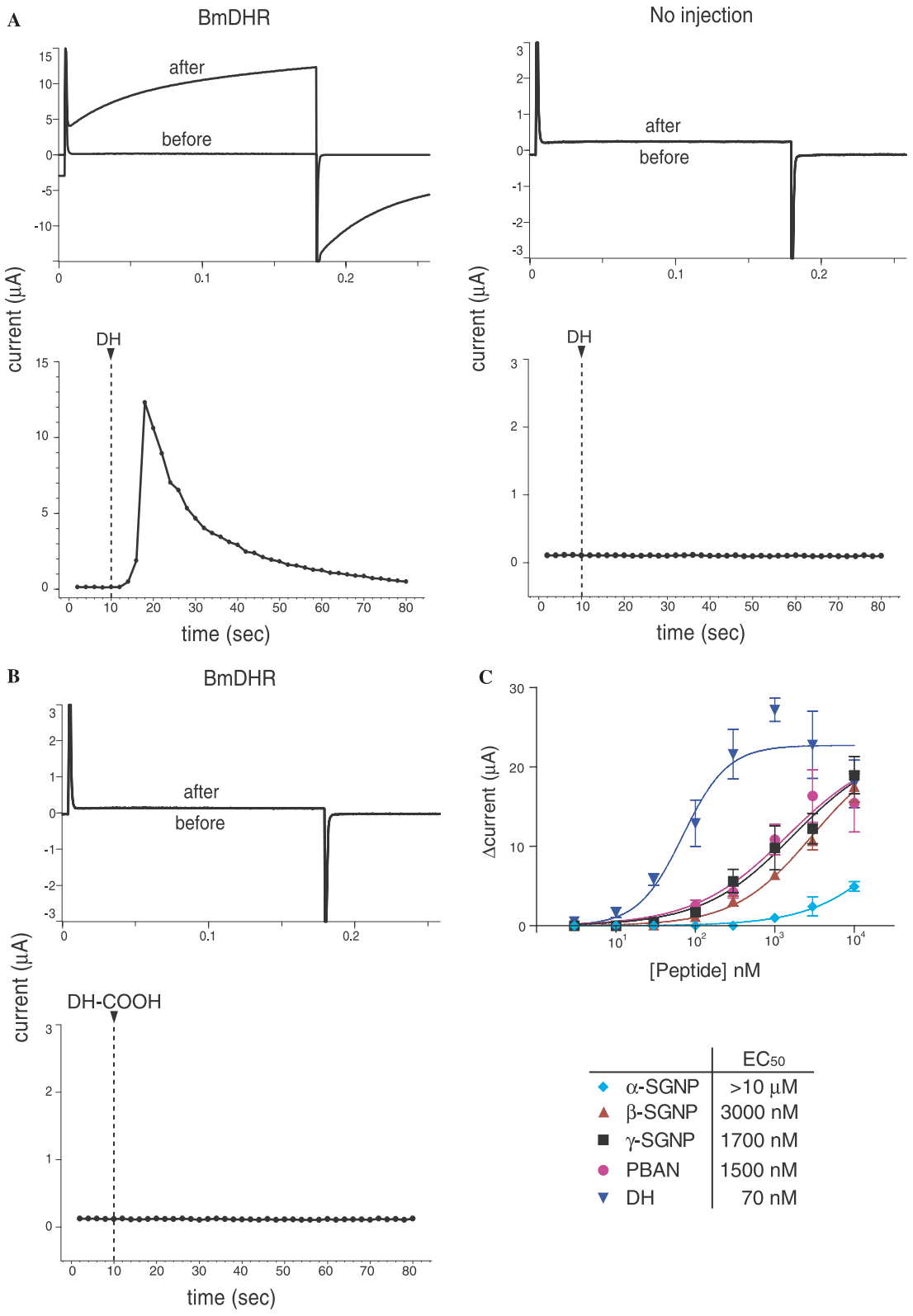

Fig. 3. Heterologous expression of BmDHR in *Xenopus* oocytes. (A) Application of DH generated Ca^{2+}-dependent Cl^- current in oocytes expressing BmDHR. (Upper) Representative current traces of oocytes injected with cRNA of BmDHR (left panel) or noninjected control oocytes (right panel) before and after application of DH (100 nM). (Lower) Time courses of responses plotted as current amplitude at the end of each depolarizing pulse. DH (100 nM) was applied at the time indicated by arrowheads. (B) Nonamidated DH (10 μM) failed to activate oocytes expressing BmDHR. Upper and lower panels show representative data from 4 oocytes. (C) Concentration-response relationships of *Bombyx* FXPR/KLa peptides on BmDHR. Error bars represent standard error ($n = 4$). EC_{50} calculated for each peptide is shown below. None of the peptides (1 μM) activated oocytes not injected with *BmDHR* cRNA (data not shown). α-SGNP, IIFTPKL-amide; β-SGNP, SVAKPQTHESLEFIPRL-amide; γ-SGNP, TMSFSPRL-amide; PBAN, RLSEDMPATPADQE MYQPDPEEMESRTRYFSPRL- amide; DH, TDMKDESDRGAHSERGALWFGPRL-amide; DH-COOH, TDMKDESDRGAHSERGALWFGPRL.

triphosphate, and activation of chloride currents due to mobilization of intracellular calcium [20,25], although oocytes not injected with *BmDHR* cRNA did not respond to DH (Fig. 3A, right panel). Furthermore, oocytes expressing BmDHR were not activated by DH lacking amidation at the C-terminus (Fig. 3B), which never induces embryonic diapause in *Bombyx* pupae [14]. Finally, dose–response relationships were examined using DH and other *Bombyx* FXPR/KLa peptides such as PBAN and three SGNPs (Fig. 3C). The EC_{50} values for BmDHR in *Xenopus* oocytes were estimated to be about 70 nM for DH, 1.5 μM for PBAN, 1.7 μM for γ-SGNP, 3.0 μM for β-SGNP, and >10 μM for α-SGNP (Fig. 3C).

Expression of BmDHR

The expression of *BmDHR* mRNA was examined in order to clarify its relationship with ovarian development. *BmDHR* mRNA levels in the ovaries were followed from the fifth (last) instar larval through the pupal and adult stages, using real-time quantitative PCR (Fig. 4). In ovaries of pupae producing diapause eggs, mRNA levels rapidly increased after pupation, peaking after 3 days and declining thereafter. In the case of pupae producing nondiapause eggs, mRNA levels peaked in 2-day-old pupae and then rapidly decreased.

Discussion

By carrying out functional expression studies in which *BmDHR* cRNA was injected into *Xenopus* oocytes that were activated by exposure to various concentrations of DH, we used voltage clamping to measure chloride currents (Fig. 3), which probably result from mobilization of intracellular calcium following activation of the G protein-coupled receptor pathway. This strongly suggests that the cloned cDNA is functional in an in vivo system.

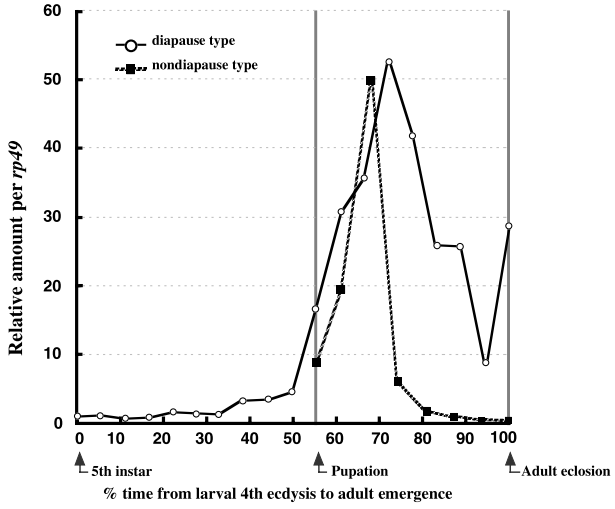

Fig. 4. Temporal profiles of *BmDHR* ovarian mRNA levels from 0-day-old fifth instar larva to 0-day-old adult *Bombyx mori* (Daizo strain). Real-time PCR was used to quantify mRNA, with ribosomal protein 49 (*rp49*) mRNA employed as an internal standard. Each value represents an average of three separate replications (SEMs are within 10% of the average values). As the developmental rates of diapause- and nondiapause-egg producers differ [5,6], the duration of development from the beginning of the fifth instar to adult emergence is here expressed as 100%.

The EC_{50} value (about 70 nM) of DH estimated from the *Xenopus* oocyte system is physiologically plausible, as injecting 100 pmol DH into 3.5-day-old pupae of a nondiapause strain (N_4, corresponding to 400–1000 nM in the blood because blood volume is estimated as 20–25% of pupal weight) leads to the production of 60–70% diapause eggs in emerging female moths [30]. In addition, PBAN, and three SGNPs are required with threshold levels 100-fold higher than that of DH in order to express similar levels (see Introduction) [17]. Furthermore, nonamidated DH never induces embryonic diapause in *Bombyx* pupae [14]. The sensitivity of *Bombyx* oocytes to DH, nonamidated DH, PBAN, and three SGNPs strongly coincides with the responses of *Xenopus* oocytes expressing BmDHR. These results suggest that BmDHR recognizes the C-terminus amide of DH and distinguishes DH from other FXPR/KLa peptides.

DH, but not nonamidated DH, is also shown to activate the gene expression of ovary trehalase (EC3.2.1.28) [31], promoting the incorporation of blood trehalose via enhanced trehalase activity on oolemma [10,32]. Using the resultant glucose, the oocyte accumulates higher levels of glycogen when compared with ovaries not exposed to DH. With the initiation of diapause, glycogen is converted into polyols such as sorbitol and glycerol [11], which may function as cryoprotectants.

When vitellogenic ovaries were dissected from the pupae, from which the SG had been removed at pupation, and were incubated in vitro with DH for 4–6 h, ovary trehalase activity and mRNA levels were found to increase [30,32,33]. Half-maximal stimulation of trehalase activity or mRNA levels occurred with 20 or 80 nM DH, respectively [30,33], which was similar to the EC_{50} value (70 nM) we observed in the *Xenopus* oocyte system (Fig. 3C). Removal of Ca^{2+} from the in vitro medium, or addition of a specific protein kinase C inhibitor, suppresses the DH-stimulated activity of ovary trehalase [30] (unpublished data), suggesting that BmDHR is coupled to a Gq protein with downstream signaling action involving Ca^{2+} and protein kinase C in *Bombyx* oocytes.

In order to elucidate the relationship between *BmDHR* mRNA levels and ovarian development, we used real-time PCR and showed that levels peaked more rapidly in non-diapause-egg producing pupae (2-day-old pupae) when compared with diapause-egg producers (3-day-old pupae) (Fig. 4). In both cases, mRNA levels rapidly declined after peaking. When DH is injected into nondiapause- or diapause-egg producing pupae at various developmental stages, from which SG has been extirpated at pupation in the case of diapause-egg producers, middle-stage pupal ovaries are the most sensitive to exogenous DH [34]. The pattern of *DHR* mRNA expression strongly coincides with

that during the DH-sensitive stage. This also confirms that DHR is expressed in ovaries from nondiapause strains, as previously demonstrated by induction of diapause-egg production following injection of DH into pupae of a non-diapause strain.

These results suggest that BmDHR transmits DH signals to the oocyte and triggers trehalase gene activation and finally diapause in the gastrula-embryo. Therefore, the present identification of the DHR should help elucidate the signal network by which female moth controls the phenotypic plasticity of her progeny.

Although members of the FXPRLa peptide family are ubiquitous insect neuropeptides, their physiological function has not been clarified for each insect species, with the exception of sex pheromone production, melanization of the larval body, induction of embryonic diapause and pupariation, and midgut motility [18,24,27,35,36]. Recently, gene-silencing experiments using the *Drosophila* PK-2 receptor gene have shown that PK-2 is involved in general embryonic development [22]. Furthermore, a lepidopteran homolog (*Helicoverpa armigera* NDVKDGAASGAHSD RLGLWFGPRLa) of *Bombyx* DH has been suggested to stimulate the prothoracic glands to secrete the molting hormone ecdysone [37,38], although removal of *Bombyx* SG immediately after pupation does not appear to affect pupal-adult development, except for production of diapause eggs and sex pheromone. These results strongly support the view that insect FXPRLa peptide functions as a signal in a more general developmental role than DH and PBAN.

The identification of *Bombyx* DHR should contribute to better understanding of the molecular relationship between receptor structure and novel members of the FXPRLa peptide family involved in the control of general development in insects.

Acknowledgments

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