Published in final edited form as:
Peptides. 2014 March ; 53: 243–249. doi:10.1016/j.peptides.2013.11.005.

Molecular cloning and functional characterization of the diapause hormone receptor in the corn earworm *Helicoverpa zea*

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Abstract

The diapause hormone (DH) in the heliothine moth has shown its activity in termination of pupal diapause, while the orthology in the silkworm is known to induce embryonic diapause. In the current study, we cloned the diapause hormone receptor from the corn earworm *Helicoverpa zea* (HzDHr) and tested its ligand specificities in a heterologous reporter system. HzDHr was expressed in Chinese Hamster Ovary (CHO) cells, which were co-transfected with the aequorin reporter, and was used to measure the ligand activities. A total of 68 chemicals, including natural DH analogs and structurally similar peptide mimetics, were tested for agonistic and antagonistic activities. Several peptide mimetics with a 2-amino-7-bromofluorene-succinoyl (2Abf-Suc) N-terminal modification showed strong agonistic activities; these mimetics included 2Abf-Suc-F[dA]PRLamide, 2Abf-Suc-F[dR]PRLamide, 2Abf-Suc-FKPRLamide and 2Abf-Suc-FGPRLamide. Antagonistic activity was found in the ecdysis triggering hormone in *Drosophila melanogaster* (FFLKITKNVPRLamide). Interestingly, HzDHr does not discriminate between DH (WFGPRLamide C-terminal motif) and another closely related endogenous peptide, pyrokinin 1 (FXPRXamide; a C-terminal motif that is separate from WFGPRLamide). We provide large-scale *in vitro* data that serve as a reference for the development of agonists and antagonists to disrupt the DH signaling pathway.

1. Introduction

Neuropeptides are major controllers of various physiological and developmental events in insects, such as metabolism, reproduction, diapause, molting, growth, and metamorphosis. Among more than 40 different types of neuropeptides in insects [5, 10, 24], a large group carrying the C-terminal amino acid motif PRXamide is generally further categorized into three groups: cardioacceleratory peptide (CAPA) [23], ecdysis triggering hormone (ETH) [25], and pyrokinin/pheromone biosynthesis activating-neuropeptide (PK/PBAN) [7].

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Likewise, the receptors for these peptides, G protein coupled receptors (GPCR), are clustered in the phylogeny [19, 22], indicating that the C-terminal motifs are an ancestral signature that is conserved for proper ligand-receptor interactions. Furthermore, the PRXamide C-terminal motif is also found in the mammalian neuropeptide neuromedin U, and its receptor is also clustered with insect PRXamide receptors [21].

The PRXamide peptides are further categorized by variations of the C-terminal motifs: CAPA contains FPRXamide; ETH has PRXamide generally with a K at the −6 position [25]; and PK/PBAN is known for FXPRXamide [7]. The PK/PBAN groups of peptides are further categorized into two subgroups; PK1 includes the diapause hormone (PK1/DH) with the WFGPRLamide motif, and PK2 includes PBAN (PK2/PBAN) with the general consensus FXPRLamide excluding the PK1 motif [7]. While the ligands are categorized by their conserved C-terminal motifs, the receptors for the ligands are similarly distinguished in the phylogeny as coevolutionary events between the ligands and the receptors.

DH was originally described for the induction of embryonic diapause by acting on developing oocytes during the pupal-adult development of the mother in *Bombyx mori* [6, 29]. Subsequently, DH-like peptides were found in heliothine moths, including *Helicoverpa zea* [12], *H. assulta* [3] and *Heliothis virescens* [28]. However, the bioactivity of DH in the heliothine moths was found to break pupal diapause, opposite to the action of DH in *B. mori*. The DH activity that breaks pupal diapause was found in *H. armigera* [32], *H. assulta* [33], *H. virescens* [28] and *H. zea* [30]. The DH signaling pathway, for either inducing or breaking diapause, which enables insects continue life in regularly recurring harsh environments, may serve as an excellent target system for controlling insect pests. This concept has been demonstrated by disruption of the diapause of *H. zea* by using DH agonistic or antagonistic peptidomimetics [30].

Understanding the properties of ligand interaction with the target receptor lays a foundation for rational design of peptidomimetics and potentially simple chemical compounds. Several PRXamide mimetics have previously been tested on *in vivo* systems of lepidopteran species for their various bioactivities [13–15, 17], which may have occurred through several different PRXamide receptors including the DH receptor (DHr). Furthermore, the *in vivo* activities of peptidomimetics are the consequences of complex phenomena, such as biostability, and bioavailability, and receptor specificity of the compounds. In this study, we provide *in vitro* activities of a total 68 peptidomimetics and PRXamide analogues on the DHr of a notorious pest species *H. zea*.

2. Materials and methods

2.1 Chemicals and Insect

All pyrokinin analogs and peptide mimetics were synthesized as described previously by Nachman et al. [13–15, 17]. The peptides from *Tribolium castaneum* were synthesized by Genescript (Piscataway, NJ). For culturing Chinese Hamster Ovary cells, DMEM/F12 medium, fetal bovine serum (FBS), Fungizone® and Penicillin/Streptomycin, and coelenterazine for an aequorin functional assay were purchased from Gibco® Cell Culture at Life Technologies™ (Grand Island, NY). TransIT®-LT1 Transfection Reagent (Mirus Bio

LLC, Madison, WI) was used for transient transfections. The last larval instar of *H. zea* was purchased from Benzon Research (Carlisle, PA).

2.2 Polymerase chain reaction (PCR) and gene cloning

The total RNA from the heads was isolated using TRI reagent (Ambion), treated by DNase I (Ambion) to eliminate the genomic DNA, and followed by phenol-chloroform extraction. The first strand cDNA was synthesized by a SuperScript™II First-Strand Synthesis System for RT-PCR with oligo(dT) primer in a total volume of 20 μL, according to the manufacturer's instructions (Invitrogen Life Technologies).

For degenerate PCR, the forward primers (Table 1) were designed based on the conserved regions identified in a sequence alignment of the diapause hormone receptors of several moth species, including *B. mori*, *Orgyia thyellina* and *Danaus plexippus* (GenBank accession numbers are BAE93495, BAF36977 and EHJ66507, respectively). The reverse primer covering the 3′ stop codon was designed based on the receptor sequence of a closely related species, *H. virescens* (GenBank accession number EZ407266). PCR reactions consisted of Taq DNA polymerase (NEB) with ThermoPol Reaction Buffer (NEB). The total reaction volume was 25 μL, including 2.5 μL of 10x ThermoPol buffer with Mg^{2+}, 0.6 U Taq DNA polymerase, approximately 50 ng of cDNA, 0.32 mM of each dNTP, and 0.2 μM of each primer. The thermocycler was set for an initial denaturation step of 3 min at 94 °C, and then 30 cycles were run as follows: 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 45 sec with a final extension of 6 min at 68 °C. A nested PCR used the primers DHrDF1 and DHr3′R1 for the 1st round and DHrDF2 and DHr3′R2 for the 2nd round (Table 1). The PCR product was cloned into a pGEMT Easy vector (Promega) and sequenced. The 5′ RACE was performed with the primers GeneRacer™ 5′ Primer and DHr5′R1 and followed by a nested PCR with the GeneRacer™ 5′Nested Primer and DHr5′R2 using a GeneRacer™ RACE Kit (Invitrogen). The target amplicon was purified, cloned, and sequenced followed the Sanger sequencing method [26].

The full open reading frame of the DHr of *H. zea* (HzDHr) was obtained by a PCR using the pair of primers DHr5′F2 and DHr3′R2 (Table 1) and a high fidelity DNA polymerase PrimeSTAR™ HS (Takara). The reaction was in 50 μL and included ~50 ng of cDNA, 10 μL of 5x PrimeSTAR buffer with Mg^{2+}, 0.32 mM for each dNTP, and 0.2 μM of each primer. The PCR program included 35 cycles: 98°C for 10 sec, 58 °C for 10 sec and 72 °C for 90 sec with a final extension of 6 min at 72°C. The amplicon for the ORF was cloned in a pGEMT Easy vector and sequenced.

2.3 Sequence analysis and construction of a phylogenetic tree

The nucleotide sequence and deduced amino acid sequence of HzDHr were analyzed using DNAMAN7 (LynnonBioSoft). Similar sequences were found using BlastP in the non-redundant protein sequences (nr) database of the NCBI website (http://www.ncbi.nlm.nih.gov/). Transmembrane helices were predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM). Multiple sequence alignments were made by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and formatted in BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). A phylogenetic tree was constructed

by MEGA5 [27] applying the neighbor-joining method with a bootstrap test of 1000 replications and complete deletion of the gaps produced by the alignment.

2.4 Heterologous expression and functional assay

The full-length open reading frame (ORF) for HzDHr was inserted into the expression vector pcDNA3.1(+) (Invitrogen). The methods for expressing HzDHr supplemented with aequorin and G-alpha16 in Chinese Hamster Ovary (CHO-K1) cells and for the assays were previously described [1, 9, 21, 22]. For pharmacological assays of the peptides and peptidomimetics, we treated the transfected cells with a test ligand, followed by a model ligand after a 30 min incubation period (Fig. 1). For the model ligand for HzDHr, we chose the peptide with the eight conserved C-terminus amino acids GLWFGPRLamide. The percent luminescence responses of the cells treated with test ligand were obtained from the first treatment (X_1). A 30-minute incubation of the cells with the test ligand was followed by the second treatment with the model ligand ($model_2$). This design allowed discrimination of antagonistic activity from receptor desensitization by the agonists. Internalization of the activated receptors by forming a complex with beta-arrestin is thought to desensitize the cellular response to subsequent treatment with the model ligand. The percent luminescence in the first treatment normalized to that in the model ligand ($Lum(X_1)$) was considered the agonistic activity. One hundred minus the percentage of the sum of agonistic activity and the remaining luminescence activity of the model ligand (% $Lum(model_2)$) was considered the antagonistic activity (ANT). All the ligands in the assays were tested at 1 μM, which is the lowest concentration that induced the maximum response in the model ligand for three biological replications.

3. Results

3.1 Molecular characterization of HzDHr

The *HzDHr* cDNA contained a 1581 bp open reading frame (ORF), encoding a 516 amino acid protein (Fig. S1), with predicted molecular weights of 58.91 kDa and a *pI* of 9.34. The HzDHr sequence was deposited into GenBank (accession number KC182787). The ORF contained seven transmembrane domains, which is a typical signature of GPCRs. Multiple sequence alignment (Fig. 2) with the DHrs of *D. plexippus*, *B. mori*, *O. thyellina* and *Ostrinia nubilalis* [18] showed that DHr is highly conserved in the lepidopteran species, although the C-terminal intracellular tail diverge among different species. Phylogeny of three DHrs together with ten PBANrs (Fig. 3) showed clear clusters of each PK1/DH and PK2/PBAN receptor group with strong bootstrapping supports.

3.2 Functional expression and dose responses of HzDHr to typical FXPRXaimde ligands

The ligand-mediated GPCR activations were measured by the degree of luminescence in the CHO-K1 cells. DH and the related peptides generated robust responses in the reporter system. The effective concentration for a 50% response (EC_{50}) for the endogenous DH (NDVKDGAASGAMSDRLGLWFGPRLamide) was 40.7 nM (Fig. 4). Similarly, the receptor responded to the peptide carrying only the core C-terminal motif GLWFGPRLamide with an EC_{50} of 48.7 nM. The most active peptide mimetics, 2-amino-7-bromofluorene-succinoyl-FKPRLamide (2Abf-Suc-FKPRLamide), which was modified at

the N terminus with the hydrophobic 2Abf-Suc, showed the lowest EC_{50} of 20.6 nM. Several pyrokinin variants, including the peptide mimetics, showed almost full activity or activity exceeding that of the model ligand (GLWFGPRLamide) (Table 2–4).

3.3 Activity of natural ligands with the PRXamide C-termini at HzDHr

In addition to the three typical ligands that were tested for the full dose response range, 66 variants of peptides or peptidomimetics were tested in the agonist and antagonist assays (Tables 2–4). In the test of HzDHr with the peptides carrying the PRXamide motifs, which were originally identified in other insect species, all the peptides from the pyrokinin group showed almost full activities on the receptor (Table 2). Meanwhile, the natural analogs that encode CAPA and the ecdysis triggering hormone (ETH), which share a common C-terminal PRXamide (X = I/V), did not show significant agonistic activities on HzDHr. Interestingly, the PK2/PBAN having the C-terminal motif of FxGPRLamide (Table 2) at 1 μM, which was the concentration chosen as the lowest concentration for maximal activity of the model ligand, were equally as active as the endogenous ligand PK1/DH. *Drosophila* ETH (FFLKITKNVPRLamide) showed the strongest antagonistic activity with an antagonist index (ANT) of 52.1, whereas none of the other natural ligands showed any antagonist activity.

3.4 Activity of N-terminally truncated DHs and Ala-replacement analogs on HzDHr

Truncations, deletions, or scrambling of the core C-terminal sequence of GLWFPRLamide revealed the important amino acid residues (Table 3). Deletion of any amino acid within PRLamide led to an almost complete loss of activity. ~~GLW~~FGPRLamide (deleted amino acids are indicated by strikethrough) also showed ~50% activity loss, while ~~G~~LWFGPRLamide showed full activity. Interestingly, a DH variant having the W at −7 (WGFGPRLamide, 1643) instead of the W at the −6 position showed almost full activity. In Alanine (Ala or A) replacements of LWFPRLamide, the amino acid residues W, F, P, R, L were all required for the full activity (Table 3). However, the Ala substitutions at −7 replacing L and at −4 replacing G showed even slightly higher activities than that of LWFGPRLamide. Other pyrokinin variants retaining FXPRLamide were all fully active, such as the cases with endogenous PBAN-like peptide carrying 11 C-terminal amino acid residues (Table 2).

3.5 Activity of PK-analogous peptidomimetics

Among the peptide mimetics tested on HzDHr, β-proline substitutions of the proline generally displayed reduced activities (Table 4). Lower agonistic activities are likely caused by the modification of the proline, although another common difference is that this group of mimetics also contained N-terminal acetylation. Other mimetics carrying N-terminal acetylation with modifications other than the modification carrying the beta-proline showed almost full agonistic activities (i.e., 1610-2 and 1784 in Table 4). Another proline variant carrying hydroxyl-proline (1449) also showed a lower agonistic activity. Interestingly, both Ac-LW[β^3F]G[β^3P]RLamide(1782) and Ac-W[β^3F]G[β^3P]RLamide (1780) showed significant antagonistic activities (23.3 and 38.9 ANT, respectively).

N-terminally modified DH analogs with 2Abf-Suc, with the penta C-terminal motifs and amidation, maintained high agonistic activities in general (Table 4). Specifically, 2Abf-Suc-FVPRLamide (1903), 2Abf-Suc-FKPRLamide (1902), and 2Abf-Suc-FGPRLamide (1895) showed activities higher than 110% of the model ligand activities. Of the further modifications with d-amino acid replacements, 2Abf-Suc-F[dA]PRLamide (1534) showed the strongest agonistic activity (144.1%).

Mild degrees of antagonistic activities were observed in several peptidomimetics. The analog 2Abf-Suc-LWA[dF]PRLamide (1790) showed 29.4 ANT, and 2Abf-Suc-F[dF]PRAamide (1604) showed 31.9 ANT. The best antagonist candidate in this category is Ac-W[β3F]G[β3P]RLa (1780), which showed 38.9 ANT. In this case, 30 min of preincubation with the mimetic resulted in a reduction of more than 40% in the activity of the receptor with the model ligand.

4. Discussion

Previous studies have shown that the PK1/DH receptor and the PK2/PBAN receptor generally distinguish their respective ligands DH and PBAN in Lepidoptera. The DHr of *B. mori* expressed in a *Xenopus* oocyte was activated by the DH of *B. mori* and distinguished other *Bombyx* FXP(R/K)Lamide peptides by revealing a 20x~50x higher EC_{50} [4]. Similarly, the PBAN receptor from *H. virescens* showed ~20x higher sensitivity to PBAN than that to the DHs of *B. mori* and *Manduca sexta* [8]. In dipteran insects, *D. melanogaster* and *Anopheles gambiae*, however, significant levels of cross-talk in PK1r and PK2r to the ligands PK1 and PK2 were observed [19, 20].

In this study, HzDHr strongly cross reacted to the PK2/PBAN group of peptides in addition to the authentic ligand PK1/DH peptides. The activities at 1 μM of each ligand, which is the lowest concentration that induces the maximum response in the model ligand, have shown that many PK2/PBAN group peptides are equally active on HzDHr, while CAPA and ETH showed significantly lower activities. Although the DH receptor in *B. mori* clearly discriminated those two different ligands in a previous report [4], our results here suggested that HzDHr may be cross active to the endogenous PBAN. Furthermore, our data are consistent with a previous *in vivo* experiment that showed that PBANs were able to terminate pupal diapause in *H. armigera* [32], presumably through cross activity on the DHr.

Previously, in an *in vivo* diapause termination bioassay of *H. zea*, the truncated DHs with an intact C-terminal motif of LWFGPRLamide effectively broke diapause, while ~~L~~WFGPRLamide, ~~LW~~FGPRLamide and LWFGPR ~~L~~amide failed to break diapause by showing activity in less than 40% of the pupae [31]. Our HzDHr assays showed similar results (Table S2). In our *in vitro* assay, ~~LW~~FGPRLamide showed only a moderate level of activity (approximately 50% that of model ligand), while LWFGPR ~~L~~amide failed to show any activity. An exception in the correlation between the *in vivo* [31] and *in vitro* (this study) studies occurs with ~~L~~WGFGPRLamide, which showed no *in vivo* activity but was highly active in the *in vitro* assay (112.8% of model ligand). This difference may have been caused by target accessibility or stability of the compound in the hemolymph.

Alanine substitutions of the C-terminal heptapeptide core sequence LWFGPRLamide revealed the importance of W, F, and P amino acid residues, while the R and the L replacements at −2 and −1, respectively, resulted in almost complete loss of activity. The results are generally consistent with the results of an earlier *in vivo* bioassay [31], except for **A**WFGPRLa (replacement Ala is in bold), which retained *in vitro* activity at HzDHr without *in vivo* activity [7] (Table S2). Interestingly, similar results were reported for *in vitro* assays of the PBAN receptor of *H. virescens* with the alanine substitutions of PBAN in the C-terminal six amino acid residue core; F, P, R, and L were all critical [8]. Therefore, the ligand-binding pockets on the PBAN receptor and the DH receptor may have a similar configuration, except for an additional feature requiring the −6 W recognition in the DH receptor.

Previous *in vivo* studies have shown that structurally modified peptides or peptidomimetics could have equivalent or even more potent activity than that of the endogenous pyrokinins [13, 15, 30]. For instance, PK/PBAN analogs incorporating β-amino acids were evaluated in four different bioassays: pheromone biosynthesis in the moth *H. peltigera*, melanization in the larval *Spodoptera littoralis*, pupariation acceleration in the fly *Neobellieria bullata*, and hindgut contraction in the cockroach *Leucophaea maderae*. In addition, the agonistic activities of peptide mimetics for breaking pupal diapause in *H. zea* have been studied *in vivo* and identified a potent agonist 2Abf-Suc-FKPRLamide with ~50-fold higher potency than the native DH [30]. Similarly, we found that 2Abf-Suc-FKPRLamide has higher activity than native DH at HzDHr (125% AG). We also found that 2Abf-Suc-F-[dA]-PRLamide (chemical 1534) showed the highest potency to HzDHr (144% AG), suggesting that 1534 might be even more effective in breaking the pupal diapause of *H. zea*.

Previous *in vivo* activity assays [2, 13, 15, 16, 30] reported antagonistic peptide mimetics. For example, the PKβA-1 analog that included β amino acids (Ac-YFT[β^3P]RLamide) represents a biostable antagonist in the pheromonotropic assay in *H. peltigera* and the melanotropic assay in *S. littoralis* [13]. A few linear and backbone cyclic (BBC) peptide libraries and the cyclic analogs were tested and demonstrated antagonistic properties for inhibiting PK/PBAN-mediated functions [2]. An amphiphilic PK/PBAN analog, Hex-Suc-A[dF]PRLamide (chemical 1605 in our study), exhibited pure antagonism and was selective for the pheromonotropic physiology in *H. peltigera* [15]. In the current study, however, this analog, PK/PBAN antagonist, did not show significant levels of agonistic nor antagonistic activity on HzDHr. This analog and others featuring the [dF] modification in the variable X position of the PK/DH core region also failed to demonstrate an antagonist response in the previously-reported *in vivo* diapause termination assay [30]. The diverse results observed for the [dF]-modified ‘antagonist’ PK analogs on DH vs the other PK/PBAN bio- and/or receptor assays mentioned above, would seem to point to a further difference between their respective receptors. But, we also speculate that the *in vivo* antagonistic bioactivity of peptides may be a more complex phenomenon than that measured in an *in vitro* assay that aims to detect biochemical antagonism at the ligand-receptor interaction. Antagonistic bioactivities of peptidomimetics may involve more complex phenomena, such as desensitization of the receptor where the functional receptor on the cell membrane is pulled down by GRK (G-protein-coupled receptor kinases) and arrestin [11]. For a better

assessment of the antagonistic activity in a heterologous expression system, the current assay method may require further improvements, such as modifying the pre-incubation method.

In conclusion, N-terminal modification by 2Abf-Suc and further modifications involving replacement of X in the FXGPRLamide motif with d-amino acids was the most efficient method of generating agonists with increased potency. Structure-activity relationship data on HzDHr provide crucial information for the development of potent pseudopeptide and/or non-peptide chemical agonists and/or antagonists of the diapause hormone receptor, which can be developed to disrupt the protective state of diapause in important agricultural insect pests. Overall, our functional assay data were consistent with the data from previous physiological experiments. Therefore, a high-throughput screening with a heterologously expressed cell system for identifying HzDHr agonists and antagonists may be a powerful approach to explore the toxicology of GPCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Ladislav Šimo, Dong Hun Kim, and Joshua Urban for technical assistance. The research was supported in part by a US-Israel Binational Agricultural Research and Development Fund (BARD) grant (IS-4205-09C)(RJN) as well as a USDA-NIFA grant No. 2011-67013-30199(RJN). This paper is contribution no. 14-123-J from the Kansas Agricultural Experiment Station.

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Highlights

- The gene encoding Diapause hormone receptor of *Helicoverpa zea* was identified.
- *H. zea* diapause hormone receptor was expressed in a heterologous reporter system.
- The receptor was pharmacologically characterized by 68 ligands including peptidomimetics.
- The ligand activities on the receptor were correlated with previous *in vivo* studies.

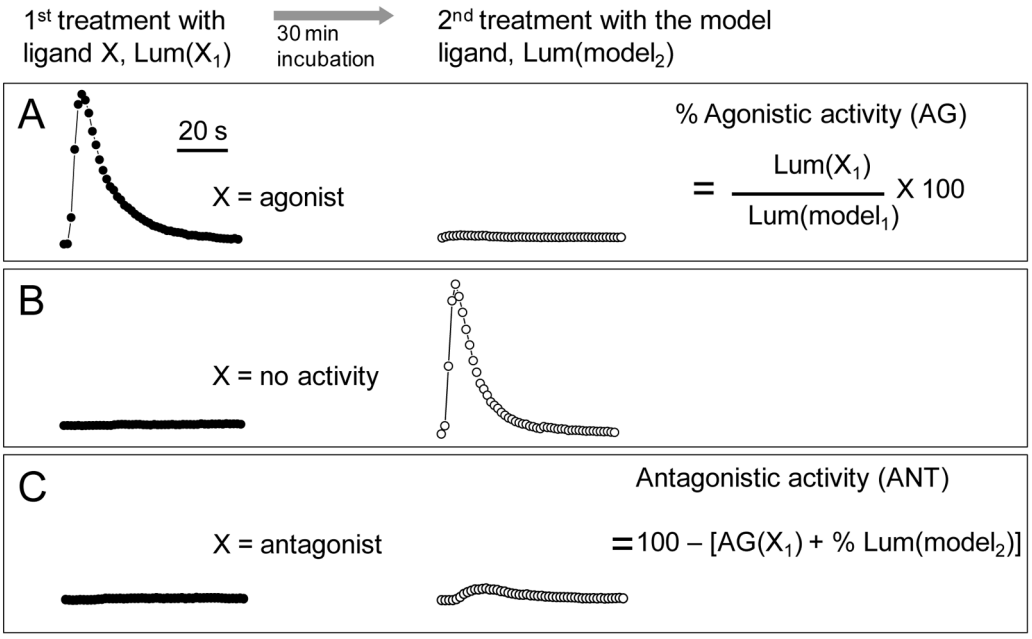

Figure 1.

Schematic diagram showing the procedures of the assays for agonists and antagonists. Three cases of responses for the chemicals were hypothesized and used to calculate each activity. AG, agonist activity in first treatment; RA, remaining activity in second treatment; ANT antagonistic activity of the tested chemicals.

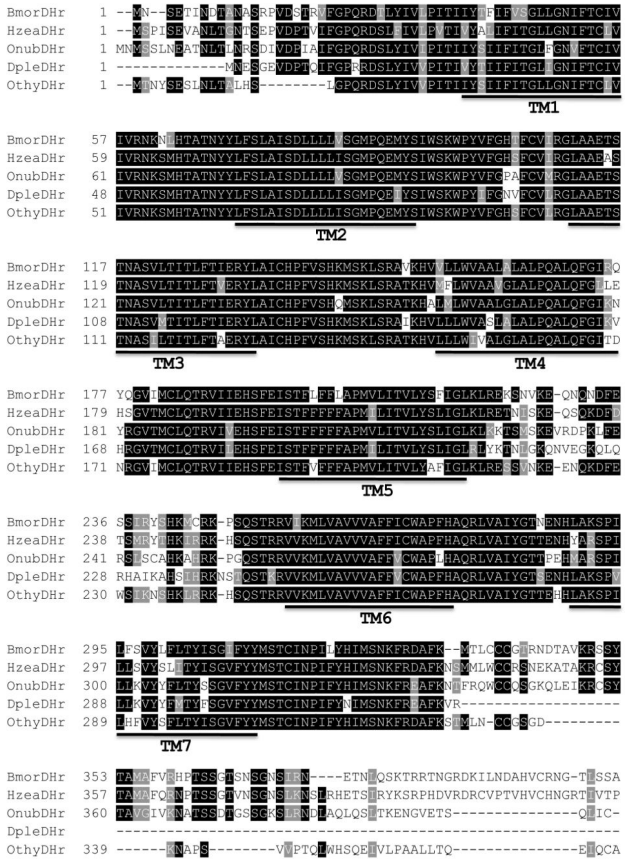

Figure 2.

Amino acid sequence alignment of HzDHr with other insect diapause hormone receptors available in GenBank, including *B. mori*, *O. thyellina*, *Ostrinia nubilalis* and *D. plexippus*. The accession numbers of these receptor sequences are BAE93495, BAF36977 and EHJ66507, respectively. The seven transmembrane domains are numbered TM1-TM7. Identical amino acids are inverted on the black background, and conserved amino acids are on a light gray background, as determined using the 50% majority rule. Dashes are gaps that were introduced for alignment.

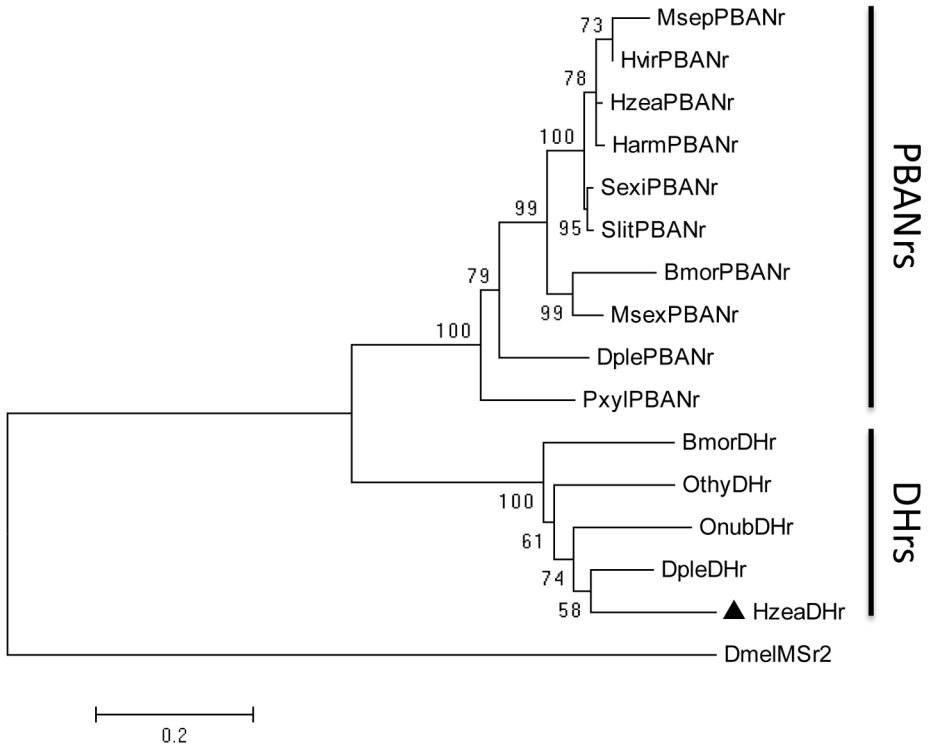

Figure 3.

Phylogenetic tree of HzDHr and its related G protein-coupled receptors. A total fifteen sequences were included in this analysis, in which the *Drosophila* myosuppressin receptor 2 (MSr2) served as the outgroup. The tree was inferred in MEGA 5 applying the Neighbor-Joining method with 1,000 bootstrap tests. The percentage of 1000 bootstrap replicates supporting each node is indicated. Species abbreviations: Msex, *Manduca sexta*, Msep, *Mythimna separate*; Dple, *Danaus plexippus*; Bmor, *Bombyx mori*; Onub, *Ostrinia nubilalis*; Othy, *Orgyia thyellina*; Hzea, *Helicoverpa zea*; Harm, *Helicoverpa armigera*; Hvir, *Heliothis virescens*; Sexi, *Spodoptera exigua*; Slit, *Spodoptera littoralis*; Pxyl, *Plutella xylostella*.

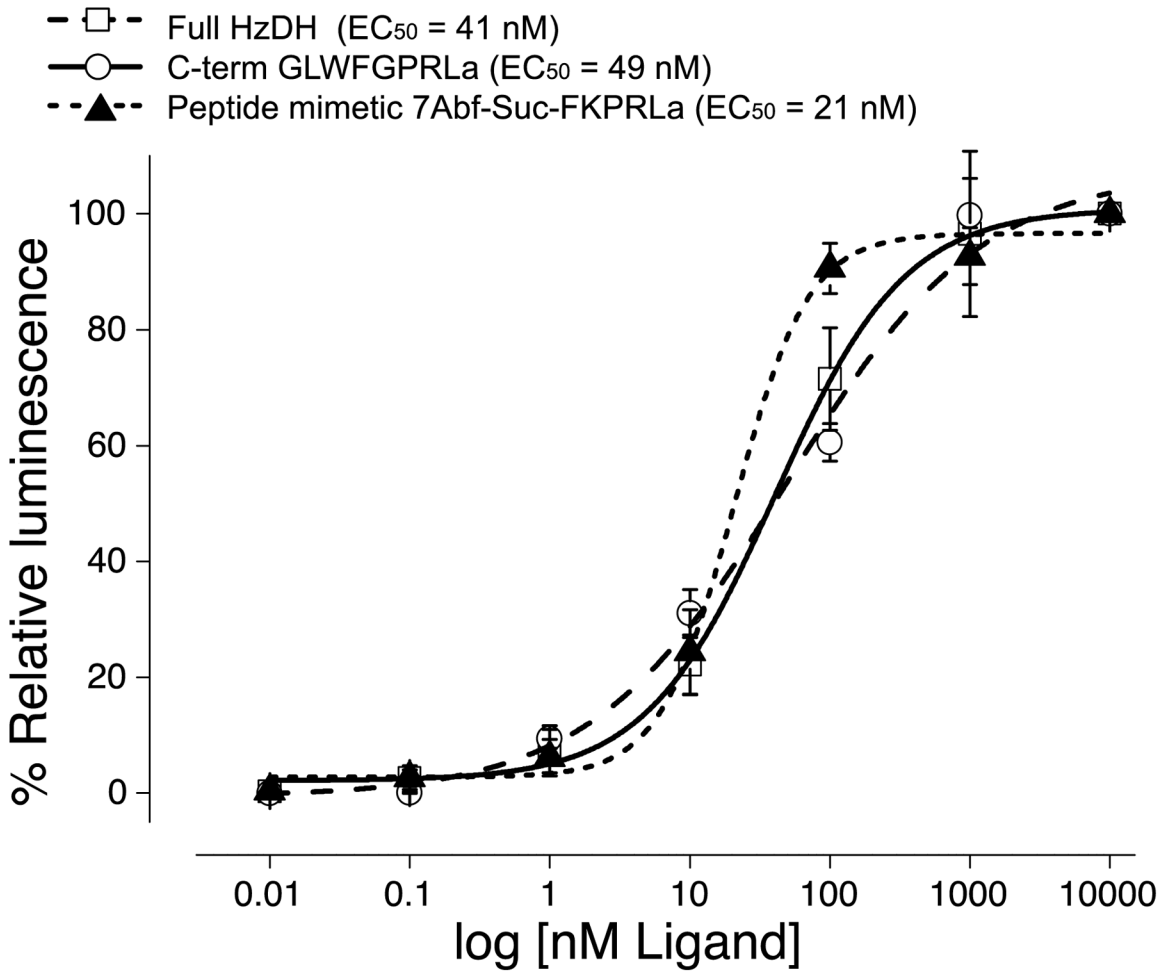

Figure 4.

Dose-response curves of the HzDHr to the three typical ligands. The receptor was expressed in CHO-K1 cells stably co-expressing aequorin and Ga16. Each data point is a mean value ± S.D for RLU in three biological replications. EC_{50}s were shown on the left panel of each curve.

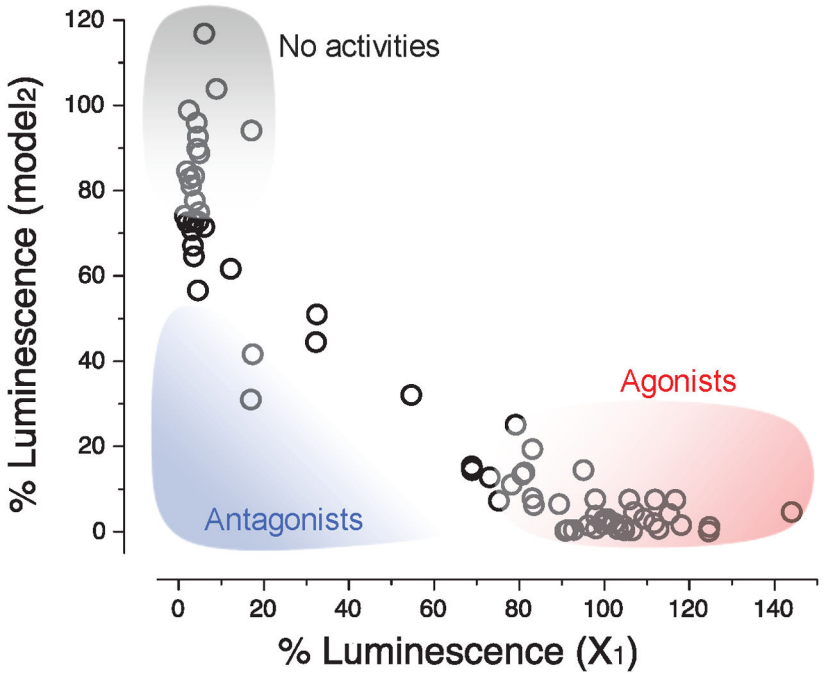

Figure 5.

Agonists and antagonists screening from the 68 chemicals tested. X axis is the percentage of the RLU in antagonists assay, Y axis is the percentage of the RLU in agonists assay. Each point is a mean value generated from 3 biological replicates for RLU. Model ligand 1737 and the endogenous ligand 1720 were marked by the solid arrow heads. The three very efficient agonists (1534, 1535 and 1902) were marked by hollow arrow heads. The only chemical showed antagonist activity was marked by the arrow head with dashed lines.

Table 1

Primers used for the molecular cloning of the HzeaDHr

Experiments	Primer names and sequences	
	Forward	Reverse
Degenerate PCR	DHrDF1: CNAAYTAYTAYYTNTTYWSNYTNGC DHrDF2: CCNCARGARATGTAYWSNATHTGG	DHr3′R1: GGTTGCCTTATTTCAAAGTACAC DHr3′R2: ATCCATACTCTTTCTCACGTTG
5′ RACE	GeneRacer™ 5′ Primer: CGACTGGAGCACGAGGACACTGA GeneRacer™ 5′ Nested Primer: GGACACTGACATGGACTGAAGGAGTA	DHr5′R1: GTTGAGATTTCAAACGAGTG DHr5′R2: GAACTGCAGAGCTTGTGG
Full length confirmation	DHr5′F2: CATCATGAGTCCCATCAGTG	DHr3′R2: ATCCATACTCTTTCTCACGTTG

Table 2

Agonists activity (AG) normalized to model ligand (1737) for those PRXamide peptides from many insects, and their antagonistic activity (ANT)

Chemical ID	Names	Organisms	Sequence information	AG (%)	ANT (%)
DH-like peptides					
1749	DmPK1	*Drosophila melanogaster*	TGPSASSGLWFGPRLa	91.5	8.2
1417	NbPK	*Neobellieria bullata*	AGPSATTGVWFGPRLa	83.5	10.3
1720	HzDH	*H. zea*	NDVKDGAASGAHSDRLGLWFGPRLa	81.3	4.9
PBAN-like peptides					
1736	RpDHL	*Rhodnius prolixus*	NGGGGDGGGLWFGPRLa	92.9	6.7
1750	DmPK2	*D. melanogaster*	SVPFKPRLa	83.2	9.1
	MsPBAN	*Manduca sexta*	DTRTRYFSPRLa	98.0	−5.6
	TcPK2-1	*Tribolium castaneum*	HVVNFTPRLa	100.0	−2.6
1665	TcPK2-2	*T. castaneum*	SPPFAPRLa	99.9	−1.6
	TcPK2-3	*T. castaneum*	HSSPFSPRLa	98.0	−1.7
1217	LmMT-I	*Locusta migratoria*	GAVPAAQFSPRLa	79.2	−4.2
1229	LmPK2	*L. migratoria*	pQSVPTTFTPRLa	95.1	−9.5
CAPA-like peptides					
1735	RpCAPA1	*R. prolixus*	SPISSVGLFPFPRVa	2.0	13.5
	TcCAPA2	*T. castaneum*	RIGKMVSFPRIa	17.2	−11.3
ETH					
1490	DmETH1	*D. melanogaster*	FFLKITKNVPRLa	17.0	52.1
	TcETH1	*T. castaneum*	ENYVLKAAKNVPRIa	6.1	−22.9
	TcETH2	*T. castaneum*	FFMKASKSVPRIa	8.9	−12.7

Peptides. Author manuscript; available in PMC 2015 March 01.

Table 3

Activities of peptide variants of PRX amide on the diapausing hormone receptor of *Helicoverpa zea*. See the Figure 1 for further details of AG (%), Lum_{model2} (%), and Antagonist Index (ANT). The % values are comparative value to the activity of themodelligand (1737 GLWFGPRLa). The gaps of the aligned sequence by deletions are in the font crossed out. Gray box on amino acid sequence indicates the sequence scrambled.

Chemical ID	Sequence information	AG (%)	ANT (%)
Truncated DH and deletion variants			
1739	RIGLWGFGPRLa	98.1	1.2
1643	WGFGPRLa	112.8	−13.5
1738	LGLWGGPRLa	104.6	−5.9
1737	~~L~~GLWFGPRLa	102.4	−4.3
1640	~~LG~~LWFGPR ~~L~~a	17.5	41
1673	~~LG~~LWFGP ~~RL~~a	1.5	24.4
1674	~~LG~~LWFG PR ~~La~~	2.6	14.6
1642	~~LGLW~~FGPRLa	54.7	13.3
1641	~~LGLWF~~GPRLa	3.2	26.2
Pyrokinin 1 variants			
1765	APFSPRLa	104.8	−5.0
1768	FYAPFSPRLa	106.6	−6.8
Capa variant			
1771B	FGNGLIPFPRVa	3.0	15.9
Alanine scan			
1619	LWFGPRLa	109.4	−12.4
1615	AWFGPRLa	116.7	−24.1
1617	LAFGPRLa	75.3	17.5
1618	LWAGPRLa	83.1	−2.4
1614	LWFAPRLa	115.3	−19.5
1620	LWFGARLa	73.1	14.2
1621	LWFGPALa	2.1	25.4
1622	LWFGPRAa	12.3	26.1

Table 4

Agonists activity (AG) normalized to model ligand (1737) of the structurally modified DH or PK analogs, and their antagonistic activity (ANT).

Chemical ID	Sequence information	AG (%)	ANT (%)
1780	Ac-W[β^{3}F]G[β^{3}P]RLa	4.6	38.9
1781	Ac-L[β^{3}W]FG[β^{3}P]RLa	2.4	−1.1
1782	Ac-LW[β^{3}F]G[β^{3}P]RLa	32.3	23.3
1783	Ac-LWFG[β^{3}P]RLa	6.1	22.5
1784	Ac-LW[β^{3}F]GPRLa	96.3	2.3
1629	Ac-YAT[β^{3}P]RLa	4.3	−0.2
1630	Ac-YFT[β^{3}P]RAa	4.9	6.4
1609-2	Ac-YF[dF][β^{3}P]RLa	32.5	16.6
1697	[Aib-Aib-Aib-Aib]FTPRLa	111.9	−19.4
1740	cyclo[GLWFGPRL]	3.9	18.5
1499	Ac-GLWFG[Hyp]RLa	68.9	15.8
1551	Ac-Y[β^{3}F]T[β^{3}P]RLa	80.8	5.9
1610-2	Ac-Y-[β^{3}F][dF]PRLa	105.9	−13.4
1902	2Abf-Suc-FKPRLa	124.7	−25.7
1903	2Abf-Suc-FVPRLa	111.7	−13.6
1894	2Abf-Suc-FSTRLa	3.5	23.8
1895	2Abf-Suc-FGPRLa	124.7	−24.7
1896	2Abf-Suc-FTPRIa	4.5	2.9
1868	2Abf-Suc-[β^{3}F]KPRLa	107.0	−11.6
1790	2Abf-Suc-LWA[dF]PRLa	3.5	29.4
1791	Hex-Suc-LWA[dF]PRLa	100.8	−3.7
1608-2	2Abf-Suc-F[dF][β^{3}P]RLa	3.7	13
1631	2Abf-Suc-TF[β^{3}P]RLa	78.3	10.7
1477	2Abf-Suc-AGPRLa	103.2	−3.8
1478	2Abf-Suc-AGPRAa	4.4	5.9
1604	2Abf-Suc-F[dF]PRAa	3.6	31.9
1605	Hex-Suc-A[dF]PRLa	4.9	20.3
1525	2Abf-Suc-AAAA[dF]PRLa	4.7	22.7
1607	2Abf-Suc-[β^{3}F][dF]PRLa	89.4	4.2
1535	2Abf-Suc-F[dR]PRLa	118.1	−19.6
1534	2Abf-Suc-F[dA]PRLa	144.1	−48.7