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Functional characterization of five different PRXamide receptors of the red flour beetle *Tribolium castaneum* with peptidomimetics and identification of agonists and antagonists

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ABSTRACT

The neuropeptidergic system in insects is an excellent target for pest control strategies. One promising biorational approach is the use of peptidomimetics modified from endogenous ligands to enhance biostability and bioavailability. In this study, we functionally characterized five different G protein-coupled receptors in a phylogenetic cluster, containing receptors for PRXamide in the red flour beetle *Tribolium castaneum*, by evaluating a series of 70 different peptides and peptidomimetics. Three pyrokinin receptors (TcPKr-A, -B, and -C), cardioacceleratory peptide receptor (TcCAPAr) and ecdysis triggering hormone receptor (TcETHr) were included in the study. Strong agonistic or antagonistic peptidomimetics were identified, and included beta-proline (β^3P) modification of the core amino acid residue proline and also a *cyclo*-peptide. It is common for a ligand to act on multiple receptors. In a number of cases, a ligand acting as an agonist on one receptor was an efficient antagonist on another receptor, suggesting complex outcomes of a peptidomimetic in a biological system. Interestingly, TcPK-A was highly promiscuous with a high number of agonists, while TcPK-C and TcCAPAr had a lower number of agonists, but a higher number of compounds acting as an antagonist. This observation suggests that a target GPCR with more promiscuity will provide better success for peptidomimetic approaches. This study is the first description of peptidomimetics on a CAPA receptor and resulted in the identification of peptidomimetic analogs that demonstrate antagonism of CAPA ligands. The PRXamide receptor assays with peptidomimetics provide useful insights into the biochemical properties of receptors.

Introduction

Insect neuropeptides and their receptors regulate numerous vital physiological systems. Because of their essential functions in various life stages, the neuropeptidergic signaling systems are considered as potential targets for development of novel insecticides [7,8,11]. Disruption of the signaling systems by using chemical agonists or antagonists may lead to disruption of critical functions and increase mortality among pest insects. One promising approach is the use of peptidomimetics, small modified peptides mimicking the endogenous ligand. Modifications of peptides include changes in the amino acids to synthetic structures with desired molecular properties, such as increased biostability, specificity, and permeability. Knowledge crucial to this biorational approach includes understanding the biochemical properties of neuropeptide receptors, which are generally known as G protein-coupled receptors (GPCRs).

A number of peptidomimetics for endogenous ligands with C-terminal motif PRXamide (hereafter, named as PRXa) were previously developed. PRXa peptides are widely distributed in animals. In insects, PRXa is categorized largely into three groups, pyrokinins (PK), cardio acceleratory peptides (CAPA), and ecdysis-triggering hormones (ETH), while vertebrates have neuromedin U. The pyrokinins are further categorized into diapause hormone (PK1/DH) and pheromone biosynthesis activating neuropeptides

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(PK2/PBAN) by their C-terminal motifs FXPRLamide for the latter and the sequence sub-string WFGPRLamide for the former. CAPA is characterized by the C-terminus FPRXamide and ETH by PRXamide and a K at the -6 position.

The insect PRXa peptides are a group of important neuropeptides involved in crucial physiological processes and could be potential targets for novel insecticide development. PK1/DH is well characterized in a number of lepidopteran insects [18]. DH induces embryonic diapause in *Bombyx mori* [10,12,39], whereas it breaks pupal diapause in heliothine moths [38,41,43,44]. PK2/PBAN stimulates pheromone biosynthesis in adult female moths [34,35]. In addition, pyrokinins induce melanization in Lepidoptera larvae [23], accelerate puparium formation in flesh flies [40], and induce hindgut muscle contraction in cockroaches [9]. CAPA has been isolated from a broad range of insect species and is known for bioactivities such as hormonal diuretic/antidiuretic activity and myotropic effects [33]. ETH is essential for triggering ecdysis behavior by activating multiple peptidergic cells in the central nervous system [20,32]. The GPCRs for this group of ligands form a homologous cluster, indicating coevolution of ancestrally related ligand–receptor partners [31]. We previously characterized five GPCRs for the nine endogenous PRXa peptides belonging to this group in the red flour beetle *Tribolium castaneum* [16]. In this study, we explored the activities of 70 different ligands, including PRXa peptidomimetics, on the five different GPCRs.

Materials and methods

Insects and chemicals

T. castaneum was kept in a 30 °C growth chamber under a 16:8 (L:D) photoperiod and fed a diet of wheat flour and Brewer's yeast (10:1). All pyrokinin analogs and peptide mimetics were synthesized as described previously by Nachman et al. [24,25,27,28]. Endogenous peptides of *T. castaneum* were custom synthesized by Genescript (Piscataway, NJ). Plasmid MIDIprep kit was purchased from Qiagen (CA, USA). The reagents for cell culture including fetal bovine serum (FBS), DMEM/F12 medium, Fungizone® and Penecilin/Streptomycin, and coelenterazine for aequorin functional assay were purchased from Gibco® cell culture at Life Technologies™ (Grand Island, NY). The transfection reagent (TransIt) was purchased from Mirus Bio LLC (Madison, WI).

Plasmids and transient expression

The PCR amplicons for full open reading frames (ORFs) of the five receptors were initially cloned into pGEMT easy vector (Promega) and transferred to the expression vector pcDNA3.1(+) (Invitrogen) or pcDNA5/FRT (Invitrogen) by using the common restriction enzymes in the multi cloning site [16]. The sequences of the inserts were confirmed by Sanger sequencing prior to the heterologous expression. High-quality plasmid DNA prepared using the plasmid MIDIprep kit (Qiagen) was used for transient transfection. The methods for transient expression of aequorin and G-alpha16 in Chinese Hamster Ovary (CHO-K1) cells and the procedures for the assays were previously described [15–17]. Thirty hours after the transfection, the cells were collected and preincubated with the coelenterazine (Invitrogen) for the functional assay as previously described [15,17]. GPCR clones used in this study are according to: TcPKr-A (TC011171, KJ435303), TcPKr-B (TC011320, KJ435304), TcPKr-C (TC011318, KJ435305), TcCAPAr (TC007170, KJ435306), and TcETHr-b (TC012494, EF222294).

Agonist and antagonist assay

The luminescence-based calcium mobilization assays were performed as previously described [1,17,22]. For pharmacological characterizations of peptides and peptide mimetics on the receptor, we treated cells sequentially with a test-ligand followed by a model ligand (presumed endogenous ligand) showing the highest activity on the receptor, with a 30 min incubation time between treatments (Fig. 1a). Fifty microliters of test chemicals at a final concentration of 1 μM were prepared in each well of a 96-well plate. The same volume of cells (approximately 2×10^4 cells), transiently transfected for GPCR expression and preincubated with coelenterazine, was applied to the test chemicals. Luminescence of intracellular calcium mobilization was measured for 20 continuous seconds at 100 ms intervals. Thirty minutes after incubation of the cells with the test ligand, the model ligand (final concentration of 1 μM) was applied and a 20-second luminescent response was measured. The model ligands for each of five different GPCRs were TcPK/PBAN-2 for TcPKr-A, -B, and -C, TcCAPA-2 for TcCAPAr, and TcETH-2 for TcETHr-b.

Methods for the assay and data analyses were previously described [17]. Briefly, the percent of the luminescence responses of the cells ($Lum_1(X)$) obtained in the first treatment with test-ligands relative to the full strength of the agonistic activity ($Lum_1(Ligand)$) were regarded as agonistic activity (Fig. 1b). The responses of cells in the second treatment with the model ligand ($Lum_2(Ligand)$) were regarded as the remaining activity. The sum of agonistic activity and remaining luminescence activity was subtracted from 100 to obtain the antagonistic activity (ANT) [17]. Thereby, the value for antagonistic activity discriminates antagonistic activity from the desensitization activity of agonists. All treatments were with 1 μM ligands, which is approximately the lowest dose of the authentic ligands fully activating the GPCRs.

Results and discussion

Tribolium *PRXa receptors*

We recently reported that five *Tribolium* GPCRs for PRXa expressed in the CHO-K1 cell were strongly reactive to endogenous PRXa ligands with EC_{50} in the low nM range, with the exception of TcETHr-b, with an EC_{50} of 20 nM [16]. Analysis of receptor sequences for phylogeny and their ligand specificities to endogenous ligands led to the assignment of TcPKr-A and -B as PK/DH receptors and TcPKr-C as a PK/PBAN receptor, and confirmed that TcCAPAr and TcETHr are relatively specific to their respective endogenous ligands CAPA and ETH [6,13,14,30]. TcPK1/DH-2 (and -1) strongly activated TcPKr-A and -B, but weakly TcPKr-C (Fig. 2). Significant activities (Fig. 2) of TcPK/PBANs on TcPKr-A, -B and -C were apparent (Fig. 3). Therefore, we proposed that TcPKr-A and -B are authentic receptor for TcPK1/DH and TcPKr-C is for TcPK/PBANs, which is consistent with the phylogeny of the receptors [16]. In addition, moderate cross-activities of ETH on TcCAPAr were also described. The current study used a relatively high concentration of 1 μM for each ligand to maximize sensitivities for ligands with low activities. As a consequence of the screening using a high dose, comparisons between high activities of ligands were not meaningful because it may already exceed the dose generating the maximum response of the receptor. The summary of each receptor's responses to the ligands is shown in Fig. 1c.

Pyrokinin1/DH ligands

The ligands with the intact PK1/DH C-terminal motif WFGPRLamide were generally active on TcPKr-A, -B, and -C (Fig. 2).