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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^3 P) 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, TcPKr-A was highly promiscuous with a high number of agonists, while TcPKr-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

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of CAPA ligands. The PRXamide receptor assays with peptidomimetics provide useful insights into the biochemical properties of receptors.

Keywords

GPCR; Pyrokinin; CAPA; PBAN; ETH; pharmacology

1. 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 loss of critical functions and increased 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 (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.

2. Materials and methods

2.1 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]. Peptides from *T. castaneum* were purchased from 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).

2.2 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. 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).

2.3 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-sec 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 ($\text{Lum}_1(\text{X})$) obtained in the first treatment with test-ligands relative to the full strength of the agonistic activity ($\text{Lum}_1(\text{Ligand})$) were regarded as agonistic activity (Fig. 1b). The responses of cells in the second treatment with the model ligand ($\text{Lum}_2(\text{Ligand})$) 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 $1\mu\text{M}$ ligands, which is approximately the lowest dose of the authentic ligands fully activating the GPCRs.

3. 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 receptors 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 $1\mu\text{M}$ 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.

Pyrokinin/DH ligands

The ligands with the intact PK1/DH C-terminal motif WFGPRLamide were generally active on TcPKr-A, B, and C (Fig. 2). Interestingly, the exceptions included the *Tribolium* endogenous ligands TcPK1/DH-1 and -2 and ligand 1417 (*Sarcophaga bullata* PK1/DH) which discriminated against TcPKr-C. Similar lower activities of PK1/DH on PK2/PBAN receptors were reported in *Aedes aegypti* [3] and *Drosophila melanogaster* [36]. Low activities of TcPK1/DHs on the TcPKr-C was also previously shown in a comparison of entire dose-responses, with the EC_{50} resulting in very high values of 10 and $0.5\mu\text{M}$ for TcPK1/DH-1 and -2, respectively. It is possible that the lack of cross-talk between TcPK1/DH-1 and -2 to the endogenous receptors for PK2/PBAN may be a consequence of evolutionary pressures that prevented it from occurring. This selectivity observed for the receptors is likely determined by N-terminal amino acids outside of the otherwise identical C-terminal sequences of the ligands.

The activities of a number of PK1/DHs were expanded to TcCAPAr, although the activities were in general lower than those on TcPKr (Fig. 2). Deletions in the C-terminal PRXamide region significantly reduced or completely abolished the activities on the receptors. Activities of the ligands with alanine scans of the C-terminal 7 amino acids supported the importance of the PRXamide motif at the positions -1 to -3, as has been reported in previous *in vitro* studies [17, 21] and an *in vivo* bioassay [42].

Peptidomimetics with the PK1/DH backbone showed strong agonistic activities on TcPKr-A, including those with N-terminal modifications such as acetylation (marked as 1 in Fig. 2), 2Abf-Suc (marked as 2), and Hex-Suc (marked as 3). It is noteworthy that peptidomimetic agonist analogs 1605 (TcPKr-A: 106% vs TcPKr-B: 4%; Hex-Suc-A[dF]PRLa), 1791 (TcPKr-A: 116% vs TcPKr-B: 1%; Hex-Suc-LWA[dF]PRLa) and 1525 (TcPKr-A: 104% vs TcPKr-B: 3%; 7Abf-Suc-AAAA[dF]PRLa) were particularly effective at distinguishing between the TcPKr-A and TcPKr-B receptors, and represent potentially useful tools for neuroendocrinologists studying these otherwise very similar receptors and the systems they mediate. The presence of D-Ala in addition to the replacement of F with A in the same position of each of these analogs likely provides the reason for this agonist selectivity. A number of the peptidomimetics expanded their activities to TcPKr-B and -C (i.e., compound 1784 and 1895). Interestingly, the *cyclo*-peptide (compound 1740), which did not show activity on the *Helicoverpa* DH receptor [17], showed full activity on TcPKr-A DH-like receptor and moderate activity on TcPKr-C.

A number of peptides and peptidomimetics showed significant levels of antagonistic activities. The antagonistic activity was mainly found against TcPKr-C and was expanded to TcCAPAr. Peptidomimetic analog 1782, carrying an internal beta-proline ([β^3 P] marked as 6), showed significant non-selective antagonistic activities on a number of receptors – TcPKr-A, -B, -C, and TcCAPAr. Conversely, peptidomimetic analog 1894 (7Abf-Suc-FSTRLa) demonstrates antagonist activity on TcPKr-A (64%) and TcPKr-C (84%), but not on TcPKr-B (1%). This analog therefore is able to distinguish between the two related DH-like receptors TcPKr-A and TcPKr-B. This compound was previously also shown to have antagonistic activity on the *Helicoverpa* DH receptor [17]. Interestingly PK1/DH of *S. bullata* showed the strongest antagonistic activity on the TcPKr-C, which must be determined by the variable N-terminal amino acids.

Pyrokinin2/PBAN ligands

Endogenous TcPK2/PBAN ligands were equally active on all three TcPKr-A, -B, and -C in the entire range of the dose-response study [16]. This also appears to be the case in expanded PK2/PBAN peptide analogs carrying the motif FXPRXamide (Fig. 3a). Unlike the significant levels of cross activities of PK1/DH ligands to TcCAPAr, PK2/PBAN activities are generally limited only to TcPKr-A, -B, and -C, but not to TcCAPAr, with a few exceptions that feature low activities.

Peptidomimetics in the PK2/PBAN group generally showed selective activities on a subset of TcPKrs, while TcPKr-A was shown to be most promiscuous to a large number of PK2/PBAN ligands. A number of peptidomimetics with N-terminal 2Abf-Suc (marked as 2 in

Fig. 3 a) virtually matched the full efficacy of the endogenous PK2/PBAN peptides on all three TcPKRs (1902, 1868, 1534, and 1535).

A number of peptidomimetics showed strong antagonistic activities on TcPKr-C. Among those, 2Abf-Suc-FSTRLamide (compound 1894) had strong antagonistic activities on both TcPKr-A and -C. 2Abf-Suc-AAAA[dF]PRLa (1525) and 2Abf-Suc-F[dF][β 3P]RLa (1608-2) showed strong agonistic activity on TcPKr-A, but antagonistic activity on TcPKr-C. Ac-YAT[β 3P]RLa (1629) showed agonistic activity on TcPKr-C, but antagonistic activity on TcPKr-A. Many peptidomimetics both in the PK1/DH and PK2/PBAN group showed moderate to high antagonistic activity on TcCAPAr, while none of those showed activity on TcETHr.

CAPA and ETH ligands

CAPA peptides are generally specific only to TcCAPAr and did not show activity on any other receptors (Fig. 3b). This is in good agreement with earlier studies in *D. melanogaster* [2, 13, 36], *An. gambiae* [30], *A. aegypti* [6] and *H. zea* [4, 5, 17]. Interestingly, the TcCAPA-1 lacking F and P at -4 and -3, respectively, is highly active on TcCAPAr. All endogenous CAPA ligands tested showed moderate levels of antagonistic activity on TcPKr-C. Peptides in the ETH group showed significant activities on TcCAPAr. Interestingly, *Drosophila* ETH1 had strong agonistic activities on TcPKr and moderate antagonistic activity on TcCAPAr. Strong cross activities of *Drosophila* ETH1 on PK receptors were also reported previously [2, 36]. These *Drosophila* ETH1 activities, which are different from TcETHs, are likely exerted by amino acids in the variable N-terminal region. A CAPA-like peptidomimetic containing β^3 P (compound 1631) acted like a PK, activating TcPKr, but not TcCAPAr.

TcCAPA receptor

This study is the first to evaluate peptidomimetic analogs on a CAPA receptor system, and it revealed several very interesting analogs with either agonist or antagonist activity. The strongest CAPA agonists were identified as 1784 (Ac-LW[β 3F]GPRLa; 87%), 1895 (7Abf-Suc-FGPRLa; 80%), and 1535 (7Abf-Suc-F[dR]PRLa; 85%). The most effective peptidomimetic antagonists of the TcCAPAr were 1896 (74%; 7Abf-Suc-FTPRIa) and 1902 (67%; 7Abf-Suc-FKPRLa); both showed little or no antagonist activity on the other four PRXamide receptors. While analog 1896 showed selectivity in terms of *antagonism*, it also did exhibit *agonist* cross-activity on TcPKr-A and TcPKr-C receptors. Analog 1902 exhibited agonist cross-activity on TcPKr-A, TcPKr-B and TcPKr-C. Regardless, these are the first peptidomimetic agonists and antagonists described for the CAPA neuropeptide class, and represent important tools for neuroendocrinologists studying CAPA-regulated physiological systems.

Summary

The use of neuropeptidergic systems as targets for pest insect control has been a widely accepted concept and is practiced in limited cases [19, 26, 27, 29, 37, 41]. Peptidomimetics that increase stability and bioavailability have been studied for their use in pest control. For example, a beta-amino acid pyrokinin analog (Ac-Y[β^3 F]TPRLamide) accelerates

pupariation in the flesh fly *S. bullata* at a similar potency (0.2 pmol/larva) to the native pyrokinin, while it induces irregular pupariation behavior patterns at higher concentrations, which indicates potential neurotoxic properties [29]. A linear PK/PBAN antagonist lead (RYF[dF]PRLa) was reported as a potent inhibitor of sex pheromone biosynthesis in *Heliothis peltigera*, and an analog of this lead sequence containing an appended hydrophobic group on the N-terminus demonstrates an ability to penetrate the cuticle of a heliothine insect [27]. Pupal diapause of *H. zea* can be disrupted using DH agonistic and/or antagonistic peptidomimetics [17, 41], and biostable analogs of the insect tachykinin, insect kinin, and pyrokinin families show aphicidal activity that match or exceed the activity of some commercially available aphicides [37].

Assays on five different PRXamides receptors in *T. castaneum* with 70 different compounds including the endogenous ligands provided general guidelines for the biorational design of peptidomimetics. In brief, we conclude the following:

1. Unnaturally modified amino acids that comprise the peptidomimetics were all highly successful at acting as agonist, antagonist, or both on different GPCRs for PRXamide. The beta proline at the -3 position was the most successful antagonist on many receptors, while they generally showed agonistic activities on TcPKr-A. Modifications of the N-terminal region, presumably conferring resistance to aminopeptidases, lower the activity in some cases, i.e., as observed for some of the peptidomimetics on TcPKr-B.
2. Evaluations conducted on the TcCAPAr receptor provided the identification of the first antagonists of this critical diuretic neuropeptide family, peptidomimetic analogs 1896 (7Abf-Suc-FTPRIa) and 1902 (7Abf-Suc-FKPRLa); both showed little or no antagonist activity on the other four PRXamide receptors. In addition, the first peptidomimetic agonist analogs were also identified, the most active being 1784 (Ac-LW[β3F]GPRLa) and 1535 (7Abf-Suc-F[dR]PRLa). These antagonists and agonists provide important tools to insect physiologists studying critical CAPA regulated systems in either *in vitro* and/or *in vivo* bioassays.
3. Peptidomimetic agonist analogs 1791 (Hex-Suc-LWA[dF]PRLa) and 1525 (7Abf-Suc-AAAA[dF]PRLa) are particularly effective at distinguishing between the TcPKr-A and TcPKr-B receptors, and represent potentially useful tools for neuroendocrinologists studying these otherwise very similar DH-like receptors and the systems they mediate.
4. Because a ligand acting on multiple receptors is common, our observation appears to be, true among many of the peptidomimetics. Furthermore, we often find that a compound that acts as an agonist on one receptor can act as an antagonist on another receptor. Therefore, one compound can disrupt multiple physiological events in the case of the presence of closely related multiple receptors for diverse physiological downstream events in a single species. This principle may be applicable for the selection of insecticidal target systems in addition to the importance of the systems for insect survival.

5. There appears to be a GPCR that can be categorized as having a more promiscuous ligand binding pocket. Many compounds were highly promiscuous on the DH-like receptor TcPKr-A, while only a few mimetics showed antagonistic activity on the same receptor. However, many other peptidomimetics showed antagonistic activities on TcPKr-C and TcCAPAr. A previous study has shown that the receptor sensitivities to their respective endogenous ligands were similar between 1 and 6 nM, indicating that the difference in the peptidomimetic activities on different receptors occurred without bias caused by downstream coupling efficiencies in the reporter system. This observation suggests that the target GPCRs that feature greater promiscuity will prove more successful for peptidomimetic approaches to the development of novel neuropeptide-based pest insect control strategies.

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Highlights

- 70 different analogues of PRXamide peptide were tested on five different receptors.
- A ligand acting on multiple receptors is common.
- Agonistic or antagonistic peptidomimetics on CAPA receptor are first described.
- TcPKr-A is more promiscuous to agonists, while TcPK-C is more so to antagonists.

Figure 1.

Assay methods used to obtain the % agonistic and antagonistic activities of various ligands and a summary of the results. (a) Experimental procedure showing sequential treatments of the cells transiently transfected for GPCR expression. (b) Formulae used for calculation of agonistic activity and antagonistic activities. (c) Summary of the results showing relative luminescence in the first treatment ($\text{Lum}_1(\text{X})$ in Fig. 1a) and in the second treatment ($\text{Lum}_2(\text{Ligand})$). Data points are for each ligand's activities on four different receptors, which are also presented in Figures 2 and 3. The three colored zones are the ligands approximately categorized for no activity (gray), antagonist (blue), and agonist (red). Note that we proposed that TcPKr-A and -B are authentic receptor for TcPK1/DH and TcPKr-C is for TcPK/PBANs [16].

1=AC, 2=7Abf-Suc, 3=Hex-Suc, 4=[β 3F], 5=[dF], 6=[β 3P], 0=Cyclo[], ^=[β 3W]

Figure 2. Agonistic activities and antagonistic activities of the compounds that are similar to the PK1/DH sequence. The values for agonistic activities and antagonistic activities were calculated by the method described in Fig. 1b. Left column shows conserved sequences highlighted in yellow, while deletions, alanine scans, and amino acid residue variations within the conserved motifs are shown with a gray background. Unnatural amino acid variants in the peptidomimetics are numbered and feature a green background. The number codes are explained at the bottom.

Figure 3.

Agonistic activities and antagonistic activities of the compounds that are similar to the PK2/PBAN sequence (a) and to the CAPA and ETH sequences (b). The values for agonistic activities and antagonistic activities were calculated by the method described in Fig. 1b. Left column shows conserved sequences highlighted in yellow, while deletions, alanine scans, and amino acid residue variations within the conserved motifs are shown with a gray

background. Unnatural amino acid variants in the peptidomimetics are numbered and feature a green background. The number codes are explained at the bottom.

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