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Man-Yeon Choi
United States Department of Agriculture

Russell A. Jurenka
Iowa State University, rjurenka@iastate.edu

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Abstract

Pheromone biosynthesis-activating neuropeptide (PBAN) and pyrokinins belong to a family of insect peptide hormones that have a common FXPRLamide C-terminal ending. The G-protein-coupled receptors (GPCRs) for this peptide family were first identified from a moth and *Drosophila* with sequence similarity to neuromedin U receptors from vertebrates. We have characterized the PBAN-receptor (PBAN-R or PR) active binding domains using chimeric GPCRs and proposed that extracellular loop 3 is critical for ligand selection. Here, we characterized the 3rd extracellular domain of PBAN-R through site-directed point mutations. Results are discussed in context of the structural features required for receptor activation using receptor activation experiments and in silico computational modeling. This research will help in characterizing these receptors towards a goal of finding agonists and/or antagonists for PBAN/pyrokinin receptors.

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Site-directed mutagenesis and PBAN activation of the *Helicoverpa zea* PBAN-receptor

Man-Yeon Choi [a,b,*], Russell A. Jurenka [a]

[a] *Department of Entomology, Iowa State University, Ames, IA 5001-3222, USA*
[b] *USDA-ARS, 1600 SW 23rd Drive, Gainesville, FL 32608, USA*

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ABSTRACT

Pheromone biosynthesis-activating neuropeptide (PBAN) and pyrokinins belong to a family of insect peptide hormones that have a common FXPRLamide C-terminal ending. The G-protein-coupled receptors (GPCRs) for this peptide family were first identified from a moth and *Drosophila* with sequence similarity to neuromedin U receptors from vertebrates. We have characterized the PBAN-receptor (PBAN-R or PR) active binding domains using chimeric GPCRs and proposed that extracellular loop 3 is critical for ligand selection. Here, we characterized the 3rd extracellular domain of PBAN-R through site-directed point mutations. Results are discussed in context of the structural features required for receptor activation using receptor activation experiments and in silico computational modeling. This research will help in characterizing these receptors towards a goal of finding agonists and/or antagonists for PBAN/pyrokinin receptors.

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1. Introduction

Neuropeptides are the largest group of neurohormones produced in the central or peripheral nervous tissues, and released for intercellular communication for various physiological and behavioral regulatory events during development and reproduction in animals. A number of peptide families have been identified and classified based on their functional and/or structural characteristics in insects [1]. Pheromone biosynthesis-activating neuropeptide (PBAN)/pyrokinin family of peptides have been identified and characterized with a conserved C-terminal pentapeptide (FXPRLamide) which represents the minimal sequence required for activity [2,3]. Insects from a variety of Orders and even a crustacean have peptides with the FXPRLamide motif, although not all insects regulate pheromone biosynthesis using this peptide. PBAN-like peptides found in other insects have other functions including contraction of hindgut muscles (pyrokinin activity), melanization in Lepidoptera larvae, induction of embryonic diapause in the silkworm (diapause hormone), acceleration of puparium formation in flies, and termination of pupal diapause in heliothine moths [2,4].

PBAN is produced in the subesophageal ganglion and released into the hemolymph where it stimulates pheromone biosynthesis in the pheromone gland of moths. Since the *Drosophila melanogas-ter* genome was completed, ≈44 genes coding G-protein-coupled receptors (GPCRs) were classified [5]. Four of these were identified as belonging to the neuromedin U-like family of GPCRs in vertebrates and the ligands were identified as belonging to the FXPRLamide peptide family [6]. Subsequently, the PBAN-receptor (PBAN-R or PR) was identified from pheromone glands of *Helicoverpa zea* female moths [7], and from other moths [8–10].

Knowledge about PBAN-R structure is required to understand its functional interaction with PBAN and for the development of antagonists and agonists [11]. The structural analysis of GPCRs for hormones and neurotransmitters is very difficult because they are low in abundance and embedded in the cell membrane. The first solved crystal structure of an intact GPCR from bovine rhodopsin has been used to model the PBAN-R [12]. Recently the crystal structures have been determined for human adrenergic and adenosine GPCRs [13–15]. Based on the identified and crystallized GPCRs and their involvement in many human diseases, computer based-homology modeling methods are now popular [16–18]. Another method that has been utilized is creating chimeric receptors from distant or closely related GPCRs to help understand how these receptors transduce agonist binding into receptor activation [19–21].

The primary sequences of peptide hormone GPCRs in insects have only recently been identified and little information is available about their structures. Testing of chimeric receptors created, from distant but related GPCRs, is a useful approach to understand how specific receptors transduce agonist binding into receptor activation [20]. We were especially interested in the role of

extracellular domains and which ones are critical for agonist activation of the receptor. We have characterized for the first time the active binding domains of an invertebrate GPCR using chimeric receptors where the three extracellular loops or N-terminus of the PBAN-R was swapped with the corresponding sequence in a related *Drosophila* GPCR [19]. We found that extracellular loop 3 (ECL-3) is critical to determine which ligand, PBAN or pyrokinin 1 (PK1) activates the receptor [19]. In the present study, we focused on the specific 3rd extracellular domain of PBAN-R through point mutations and predicted conformational structures using in silico modeling.

2. Materials and methods

2.1. Materials

Synthetic *H. zea* PBAN and leucopyrokinin (LPK: PETSFTPR-NH$_2$) were purchased from Bachem (San Carlos, CA). Fluo-4/acetoxy-methyl ester (AM) was purchased from Molecular Probes.

2.2. Cloning of HezPBAN-receptor

Cloning procedures for HezPBAN-R were followed as described previously [7]. Synthesized cDNA from mRNA extracted from pheromone gland of 1–3-day old *H. zea* female adults was amplified with a primer set shown in Fig. 1, carrying *NotI* and *XbaI* linker in the N- and C-terminal ends, respectively. All cloned receptors and mutants were sequenced and confirmed before transfecting Sf9 cells.

2.3. Site-directed point mutagenesis of the 3rd extracellular loop of the PBAN-R (PR)

The methodology for creating point mutations by PCR amplification is shown in Fig. 1. The mutation site E^{297}, S^{300} or F^{303} in PBAN-R as indicated in Fig. 2 was replaced with A^{297}, A^{300}, or A^{303} by PCR amplification with PBAN-R as template using primer sets in Fig. 1. PBANR-A1 was amplified by PR-A1F and PR-R to produce a partial C-terminal sequence containing A^{297} to stop codon, and using primer set: PR-F and PR-A1R to produce an N-terminal sequence containing ATG to A^{297} of PBAN-R. Then, the full length PBANR-A1 was produced by a second round of PCR using primer set: PR-F and PR-R using the two amplified PCR products from above reactions as templates. The other two mutated receptors, PBANR-A^{300} and PBAN-A^{303} were produced with primer sets: PR-A2F and PR-A2R, and PR-A3F and PR-A3R, by PCR method described above and previously [19].

2.4. Single-cell calcium imaging

Approximately 2–2.5 × 10^4 cells (200 μl per well) from suspension culture were seeded into a 96-well cell-culture plate with black walls and a clear bottom and incubated overnight at 28 °C. Each well was incubated with Fluo-4/AM for 20–25 min and challenged with peptide and then ionomycin to obtain a relative fluorescence intensity as described in the previous study [7,19]. The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) using Statview 5.0 software.

2.5. Protein structure prediction and modeling

Amino acid sequences of HezPBAN-R or mutated GPCRs were submitted to SWISS-MODEL using the Swiss-PdbViewer [22], and requested homology model from the GPCR template library. The web-based Automated Mode of Swiss-Model program provided the homology modeling of HezPBAN-R or mutated GPCRs using the human adenosine A2A receptor (3eml.pdb) as a base template [23,24]. Our previous model [25] used rhodopsin as the template because, at the time, it was the only crystal 3D structure available for GPCRs. Currently the adenosine and adrenergic crystal structures have been solved [13–15]. The Automated Mode of Swiss-Model chose to utilize the human adenosine A2A receptor as a homology model for each mutant HezPBAN-R. This may not necessarily be the best overall model, but provides a prediction about what will happen in the third extracellular loop with specific mutations. The 3-D structures of PBAN-R and mutant GPCRs PDB
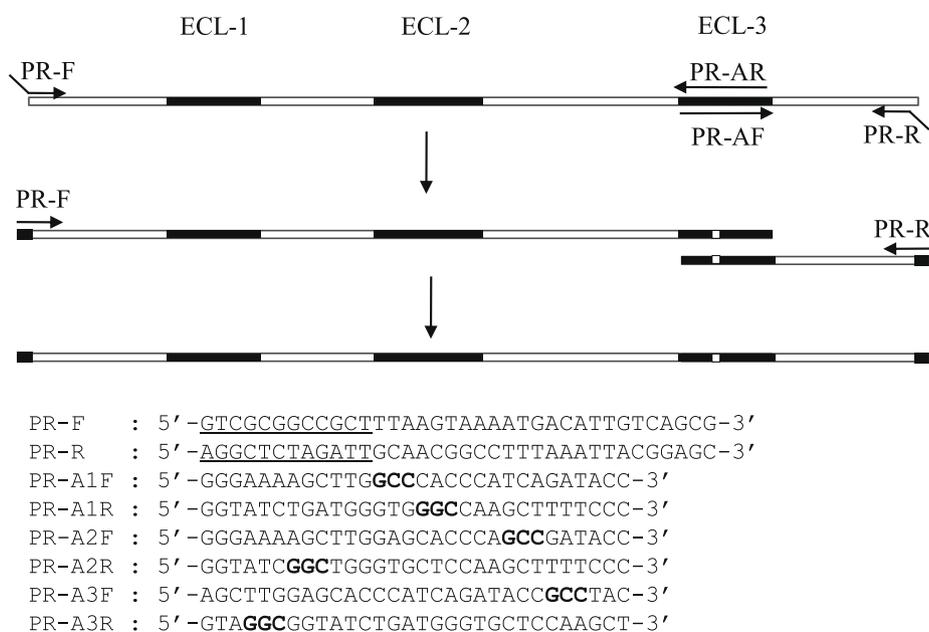


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PR-F   : 5'-GTCGCGGCCGCTTTAAGTAAAATGACATTGTCAGCG-3'
PR-R   : 5'-AGGCTCTAGATTGCAACGGCCTTTAAATTACGGAGC-3'
PR-A1F : 5'-GGGAAAAGCTTGGCCCACCCATCAGATACC-3'
PR-A1R : 5'-GGTATCTGATGGGTGGGCCAAGCTTTTCCC-3'
PR-A2F : 5'-GGGAAAAGCTTGGAGCACCCAGCCGATACC-3'
PR-A2R : 5'-GGTATCGGCTGGGTGCTCCAAGCTTTTCCC-3'
PR-A3F : 5'-AGCTTGGAGCACCCATCAGATACCGCCTAC-3'
PR-A3R : 5'-GTAGGCGGTATCTGATGGGTGCTCCAAGCT-3'
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Fig. 1. Outline of method for creating mutant GPCRSs from HezPBAN-R using a PCR based cloning strategy as described in detail in Section 2. White intervals in the ECL-3 indicate point-mutation sites contained nucleotides (in bold) for alanine in each primers, PR-AF and PR-AR. Mutated PBAN-Rs were produced by several additional rounds of PCR with designed primer sets (lower). Primers, PR-F and PR-R were tailed with *NotI* and *XbaI* sites (underlined) that were used for vector insertion. ECL: extracellular loop.
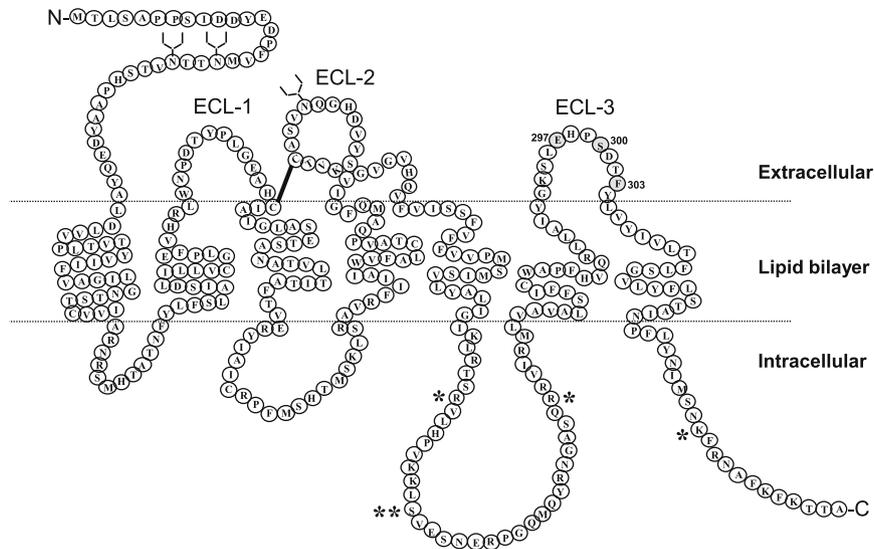
```
ECL-3   : 293 294 295 296 297 298 299 300 301 302 303 304
PR      :  G   K   S   L   E   H   P   S   D   T   F   Y
PR-A297 :  G   K   S   L   A   H   P   S   D   T   F   Y
PR-A300 :  G   K   S   L   E   H   P   A   D   T   F   Y
PR-A300 :  G   K   S   L   E   H   P   S   D   T   A   Y
```

Fig. 2. The schematic diagram of PBAN-receptor, and sites of point mutation. Three site-directed mutants of the PBAN-R (PR) 3rd extracellular loop (ECL) were created by exchanging alanine (A, in bold) with glutamate (E), serine (S), and phenylalanine (F, underlined) of the original PBAN-R (upper). Three protein kinase C phosphorylation sites (*) and one cAMP- or -cGMP-dependent protein kinase phosphorylation site (**) were predicted in the intracellular domains. The schematic diagram is adapted from Choi et al. [19].

files were viewed using RasWin Molecular Graphics software (www.rasmol.org) or Swiss-PdbViewer. We also used a web-base server, PredictProtein (www.predictprotein.org), to predict 3-D modeling and phosphorylation sites. The view of GPCRs was edited by an Adobe Photoshop software ver. 9.

3. Results and discussion

In previous studies [19,25] the extracellular domains of the moth PBAN-R were exchanged with *Drosophila* PK1-R (DrmPK1-R), and tested for binding activity. The results indicated that all extracellular domains to some degree are involved in ligand binding and/or receptor activation. The most interesting finding indicated that the ECL-3 sequence confers selectivity toward peptide ligands as either PBAN-like or diapause hormone-like. These results indicate that several amino acids in the PBAN ECL-3 could be involved in recognizing ligands, and site-directed mutagenesis of these amino acids might interfere with binding activity and receptor activation. Recently, a similar study on the subtype 1 neurotensin receptor revealed that ECL-3 is critical for neurotensin binding and receptor activation [26]. We conducted site-directed mutagenesis of the ECL-3 of the PBAN-R to investigate binding activity and we also predicted their structural models using an in silico computational program.

3.1. Phosphorylation sites for protein kinases

Protein kinase phosphorylation sites can be involved in cellular signal transduction after activation of GPCRs. The Swiss-Model program predicted a cAMP- and cGMP-dependent protein kinase phosphorylation site at serine^{244} (S^{244}) based on the consensus sequence pattern, K^{241}-K^{242}-L^{243}-S^{244} on the 3rd intra-cellular loop. Usually, a consensus sequence pattern of S or T-X-R or -K, predicts a protein kinase C (PKC) site [27], and two PKC sites on the 3rd

intracellular loop and one on the intracellular-C-terminus of HezPBAN-R were located (Fig. 2). These sites could be important for signal transduction from the cell surface to intracellular side after receptor activation.

3.2. Functional expression and binding activity of mutant PBAN-receptors (PRs)

Three site-directed point mutations of PBAN-R were designed by comparing the HezPBAN ECL-3 sequence with the 3rd extracellular loop of DrmPK1-R. The targeted amino acids were chosen based on functional groups which are charged or uncharged and/or polar and aromatic groups that could potentially interact with the peptide ligands. Mutated receptors were constructed by replacement with an alanine (A) residue on ECL-3 of the HezPBAN-R and PBAN and LPK binding activity was measured in vitro.

3.2.1. PR-A^{297}

The relative florescence intensity of PBANR-A^{297} mutation was not changed compared to PBAN-R binding PBAN or LPK at 300 nM (Fig. 3). The results indicate that this amino acid change did not increase or decrease binding activity of PBAN or LPK. It also indicates that the negative charged amino acid (E^{297}) exchanged with a non-polar residue (A^{297}) did not affect the conformation of PBAN-R ECL-3. The results are supported by in silico PR-A^{297} modeling which was exactly the same as the original PR 3-D structure (Fig. 4).

3.2.2. PR-A^{300}

The polar uncharged residue serine (S^{300}) on the 3rd loop was exchanged with alanine (A^{300}) (Fig. 2). The relative fluorescence intensity decreased by approximately half compared to the native PBAN-R or PBANR-A^{297} after challenge with PBAN (Fig. 3). This reduced activity indicates that the site mutation could interfere with
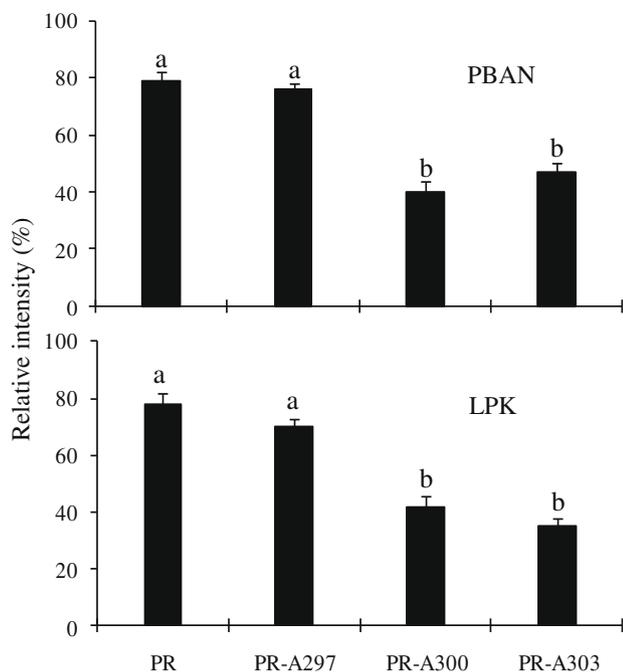
Fig. 3. Relative florescence intensity of activated HezPBAN-R (HezPR) and three mutant receptors expressed in sf9 cells after PBAN or leucopyrokinin (LPK) peptide challenge (300 nM). Values represent the mean + S.E.M. of three replications of at least 10 cells per replication.
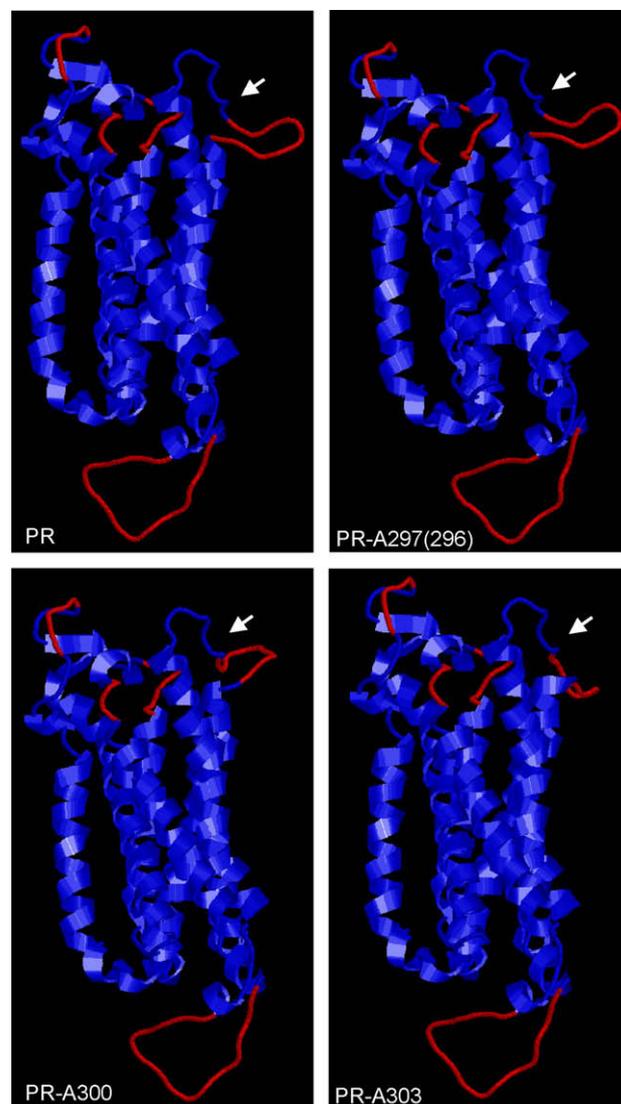

Fig. 4. Comparison of 3-D models of HezPBAN-R (HezPR) and site-directed point mutants of PR-A297, PR-A300 and PR-A303 using Swiss-Model. The ECL-3 of the PBAN-receptors are indicated with an arrow head. The model of PR-A297 is exactly same as the original PR and PR-A296.

the proper PBAN binding causing a conformationally changed ECL-3. The in silico ECL-3 structural model of PBANR-A^{300} also indicated differences from the original loop of the native HezPBAN-R (Fig. 4). The residue S^{300} of ECL-3 could be an important position to maintain a correct structural conformation of the extracellular domain. This conformational change may not only be residue S^{300} specific but could also change other amino acid conformations.

3.2.3. PR-A^{303}

The 3rd site-directed mutation exchanged A^{303} with the aromatic residue phenylalanine (F^{303}) and a reduced florescence intensity was observed similar to PR-A^{300} after challenge with PBAN or LPK (Figs. 2 and 3). The activation of PR-A^{303} was relatively higher with PBAN and lower with LPK than the activation of PBANR-A^{300}, but they were not significantly different (Fig. 3). An in silico model of PR-A^{303} was proposed and indicates that deleting the aromatic residue F^{303} and replacement with A^{303} resulted in a conformational change (Fig. 4). It is unclear if this interaction is phenylalanine specific or if other functional groups underwent a conformational change.

3.2.4. Proposed models from in silico site-direct mutagenesis on ECL-3 of PBAN-R

The amino acids from 293 to 303 on the ECL-3 of PBAN-R were replaced by A, then submitted into the automated mode of Swiss-Model program to predict models. The conformational model of the point mutation of A^{296} exchanged with L^{296} was well preserved and exactly matched the native PBAN-R and PR-A^{297} (Fig. 4). Therefore, the activation of the mutant PR-A^{296} would be expected to be similar to that of the native PBAN-R or PBANR-A^{297}. Both amino acids, L and A, have similar non-polar and aliphatic functional groups, therefore, A replacement would theoretically not affect a conformational change. Similarly, the residues, G^{293}, K^{294}, S^{295} or H^{298} replaced with A resulted in exactly the same structural model which was well matched to PR-A^{300} ECL-3 (Figs. 4 and 5). Therefore, PBAN binding activity from those mutations is expected to

be similar as PR-A^{300} (Fig. 3). From the modeling and binding results of the mutated receptors, those residues could play an important role to maintain a proper ECL-3 binding site. The in silico mutants-A^{299}(P^{299}), -A^{301}(D^{301}), or -A^{302}(T^{302}) suggested a slightly different model of ECL-3 structure for each mutation (Fig. 5), and PBAN binding activities of these receptors could be reduced.

The chimeric receptors using two insect GPCRs [19] and their matching computational molecular models [25] have been used to identify the functional binding sites of the PBAN-R. Specific chimera or mutant construction, transfection and expression into cells, then analysis of binding activity is needed. In case of low activity, a green fluorescence protein tag can be used to verify that the GPCR protein is expressed properly or was not on the cell surface. This experimental procedure for each chimeric receptor or mutant is time consuming work. In the present study, a site-directed in silico mutagenesis approach could prove advantageous to save time. Predicted structural models and expected binding activity could be correlated for a specific extracellular functional domain. Specific GPCRs with the most critical conformational changed residue(s) based on structural modeling could be constructed and expressed to determine ligand activation levels. This
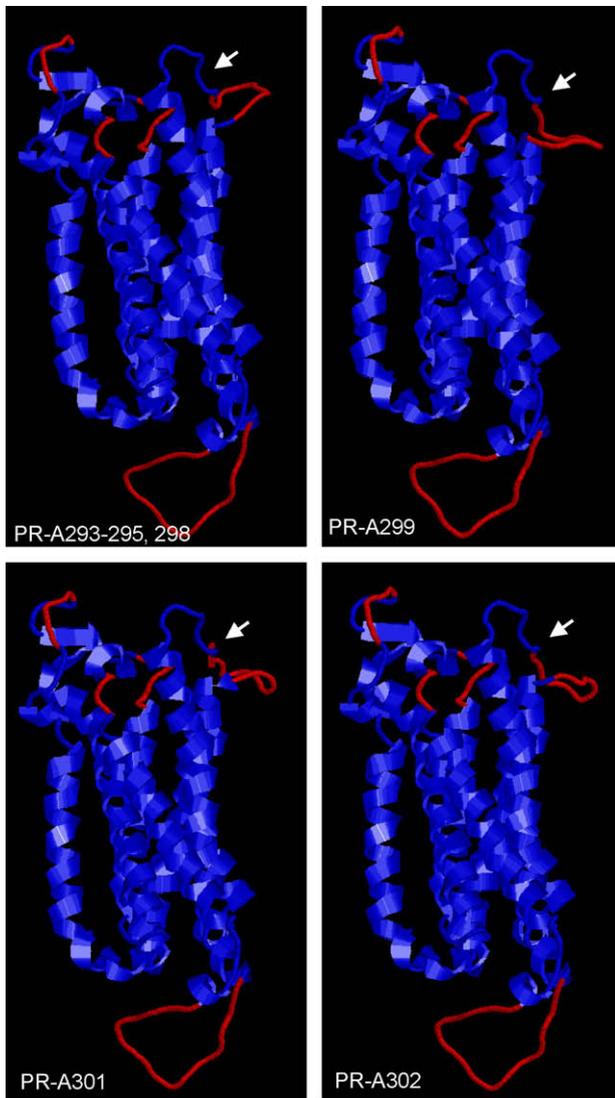
Fig. 5. Putative models of site-directed point mutations of PBAN-receptor (PR) replaced with A (alanine) from amino acid position 293, 294, 295, 298, 299, 301, or 302 in the 3rd extracellular loop (ECL-3) using Swiss-Model program. Each mutant ECL-3 is indicated with an arrow head. PR-A293, -A294, -A295, and -A298 are identical to the structure of PR-A300 as shown in Fig 3.

research will help in the modeling of these receptors towards a goal of finding and screening agonist and/or antagonist for PBAN/pyrokinin receptors, an important class of receptors in insects.

Acknowledgements

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