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Biochemical and Biophysical Research Communications 334 (2005) 69–78
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Regulatory mechanisms underlying pheromone biosynthesis activating neuropeptide (PBAN)-induced internalization of the *Bombyx mori* PBAN receptor

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Received 8 June 2005
Available online 23 June 2005

Abstract

Internalization of the *Bombyx mori* pheromone biosynthesis activating neuropeptide receptor (PBANR) has been attributed to the presence of a 67 amino acid C-terminal extension absent in PBANRs from *Helicoverpa*. To identify the structural motif(s) responsible for internalization, a series of truncation mutants fused with enhanced green fluorescent protein were constructed and transiently expressed in insect Sf9 cells. Confocal microscopy analyses revealed that truncation at Gly357 severely inhibited internalization while truncation at Gln367 did not, indicating that the PBANR internalization motif resides between Gly357-Gln367. Alanine substitution studies suggest that Tyr360 and Leu363 may constitute a YXXL endosomal targeting motif that facilitates endocytosis, however, this motif does not appear to be the primary determinant; an indication that multiple sites are involved. Furthermore, we determined that internalization of the PBANR proceeds via a clathrin-dependent pathway, is dependent on the influx of extracellular calcium, and likely does not involve a G protein-coupled receptor kinase.

Keywords: PBAN receptor; PBAN; *Bombyx mori*; GPCR; Confocal microscopy; EGFP; Receptor-mediated endocytosis; Sf9; Agonist-induced internalization

For many insects, the proliferation of individual species is often dependent on the production, and subsequent detection, of small molecule signals commonly known as sex pheromones. In most lepidopteran species (i.e., moths and butterflies), sex pheromones are species-specific blends of volatile compounds that are generally released by females to indicate their reproductive receptivity to the corresponding conspecific males. A major class of pheromone components produced by female moths is the C_{10}–C_{18} unsaturated, acyclic, aliphatic compounds that contain an oxygenated functional group such as aldehyde, alcohol, or acetate ester. They are de novo synthesized in the pheromone gland, a functionally differentiated organ located between the 8th and 9th abdominal segments, from acetyl-CoA via fatty acid synthesis and can include desaturation, and limited chain-shortening reactions followed by reductive modification of the acyl group [1,2]. Given the economic and ecological impacts that lepidopteran species have, a more precise understanding of the molecular processes underlying sex pheromone production is of major interest.

In most lepidopteran species, initiation of the sex pheromone biosynthetic pathway is dependent on a molecular interaction between the 33 amino acid pheromone biosynthesis activating neuropeptide (PBAN) and its cognate receptor. This interaction triggers an influx of extracellular calcium which in turn activates a cascade of species-specific enzymatic reactions culminating in sex pheromone production and release (reviewed in [3,4]). The PBAN receptor (PBANR) has recently been cloned,

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doi:10.1016/j.bbrc.2005.06.050

based on predicted structural similarities with the mammalian neuromedin U receptor [5], from the pheromone glands of three lepidopteran species, *Bombyx mori* [6], *Helicoverpa zea* [7], and *Helicoverpa armigera* (GenBank Accession No. AAW47417).

The PBANRs have been characterized as rhodopsin-like G protein-coupled receptors (GPCRs) that mobilize extracellular calcium in a PBAN dose-dependent manner. However, to date only the *B. mori* PBANR has been shown to have an expression profile consistent with a biological role in pheromone biosynthesis. Despite strong sequence similarities, there appear to be species disparities between the PBANRs since the 413 amino acid *B. mori* PBANR contains a 67 amino acid C-terminal extension that is absent in the *H. zea* and *H. armigera* PBANRs, a curiosity reminiscent of the type I gonadotropin-releasing hormone receptors (GnRHRs) [8]. The non-mammalian type I GnRHRs possess a C-terminal tail and exhibit rapid agonist-induced receptor internalization, whereas the mammalian type I GnRHRs, in contrast, lack a C-terminal tail and exhibit significantly different internalization kinetics [8,9].

As a means of controlling the scope and magnitude of the ligand-induced signal transduction cascade, the ligand–receptor interaction also sets in motion a series of molecular events that culminate in removal of the GPCR from the cell surface [10–12]. This desensitization mechanism, which is initiated upon GPCR phosphorylation, often results in G protein uncoupling with the subsequent recruitment of adaptor proteins that facilitate endocytosis of the GPCR, usually via a clathrin-dependent pathway. After entering the endosomal pathway, the receptor is then either sorted to recycling endosomes that transport the receptor back to the cell surface following dephosphorylation or to lysosomes for degradation and eventual down-regulation of the receptor [13]. While components of the desensitization and internalization pathways of many GPCRs have been identified, less is known concerning the specific structural motifs within the GPCRs that mediate the regulatory processes. It is becoming exceedingly clear, however, that the residues that constitute these motifs often reside in the intracellular C terminus [14–20]. A similar mechanism appears to be present in the *B. mori* PBANR as we previously showed that deletion of 67 amino acids from the C terminus negatively impacted PBAN-induced internalization [6].

A number of motifs are present in the *B. mori* PBANR C terminus that could contribute to the internalization process, including 13 potential Ser/Thr phosphorylation sites, a potential endosomal targeting motif (YXXL), and a potential calmodulin-binding site. Consequently, for the present study, we constructed a series of enhanced green fluorescent protein (EGFP) fused C-terminal truncations of the *B. mori* PBANR, which we hypothesized would significantly impair, or compromise, the internalization process. Using confocal microscopy, we determined that the internalization signal resides between Gly357 and Gln367 of the *B. mori* PBANR C terminus and that Tyr360 and Leu363 likely constitute a YXXL endosomal targeting motif that facilitates endocytosis. We have also established that the endocytotic event proceeds via a clathrin-dependent pathway that is dependent on an influx of extracellular calcium and is mediated by a staurosporine sensitive kinase, an indication that G protein-coupled receptor kinases are likely not involved.

Materials and methods

Construction of expression plasmids. Overlap extension PCR was used to construct a series of C-terminal deleted PBANR expression plasmids with the EGFP coding sequence fused in-frame to the terminal PBANR residue. The initial PCRs were performed using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) and entailed amplification of the *B. mori* PBANR coding region lacking the stop codon from the plasmid, pIB-PBANR, using the specific PBANR primer (F: 5′-GAGCTCATATTCGAAATGATG-3′, includes nine nucleotides upstream of the initiation codon and a *Sac*I site) and one of the antisense chimeric primers listed below. The EGFP coding sequence was amplified from pEGFP-1 (Clontech) using one of the chimeric sense primers listed below and the EGFP-specific primer, R: 5′-CTTGTACAGCTCGTCC-3′. The final reactions for each construct were performed with LA Taq (Takara Bio., Otsu, Japan), involved the products from the two previous reactions as templates, and utilized the PBANR gene-specific sense primer and the EGFP-specific antisense primer. The resulting products were sub-cloned into the pIB/V5-His expression vector (Invitrogen) and sequenced to confirm presence and orientation of the insert. Overlap extension PCR was also used to generate the double mutant Y360A/L363A from pIB/PBANR-EGFP using the specific sense PBANR primer and the specific antisense EGFP primer with the alanine substituted primers (F: 5′-GCAAGTGCAGCACTGGCTTCG-3′, R: 5′-AGTGCTGCACT TGCTG ACCGACC-3′; nucleotides changed to generate Ala residues are underlined). As before, the construct was cloned into the pIB/V5-His vector and sequenced to confirm alanine substitution of Tyr360 and Leu363.

Chimeric primers used in preparing the PBANR truncations were: PBANR-357-EGFP—F: 5′-GGGCCTCGCATGGGTATGGTGAG CAAGGGC-3′, R: 5′-GCCCTTGCTCACCATACCCATGCGAGG CCC-3′; PBANR-367-EGFP—F: 5′-CTGCTGGCTTCGCAAATG GTGAGCAAGGGC-3′, R: 5′-GCCCTTGCTCACCATTTGCGAA GCCAGCAG-3′; PBANR-377-EGFP—F: 5′-AATGGCTTGACAG ATATGGTGAGCAAGGGC-3′, R: 5′-GCCCTTGCTCACCATAT CTGTCAAGCCATT-3′; PBANR-387-EGFP—F: 5′-CGAAGACTT CGGCGAATGGTGAGCAAGGGC-3′, R: 5′-GCCCTTGCTCACC ATTCGCCGAAGTCTTCG-3′; and PBANR-397-EGFP—F: 5′-AC GCACCTTTGCGATATGGTGAGCAAGGGC-3′, R: 5′-GCCCTT GCTCACCATATCGCAAAGGTGCGT-3′. Nucleotides corresponding to the *B. mori* PBANR sequence are underlined. A schematic of the C-terminal mutations is shown in Fig. 1.

Transient transfection of Sf9 cells. Transfections were performed according to the Cellfectin (Invitrogen) transfection reagent protocol using adherent monolayer Sf9 cells attached to 27-mm glass-bottomed dishes (Matsunami, Tokyo, Japan) and ~0.75–1 μg plasmid DNA/transfection. Twelve hours after transfection, the medium was replaced with IPL-41 insect medium (Gibco-BRL) supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), and kanamycin (50 μg/mL).

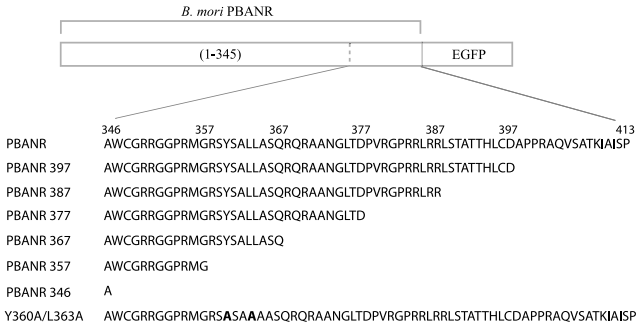

Fig. 1. Schematic representation of the *B. mori* PBANR-EGFP construct and mutations to the PBANR C terminus. The terminal 67 amino acids of the *B. mori* PBANR C terminus, as well as the nature and location of the mutations utilized in this study, are shown using one letter amino acid representations. Bold font indicates the location of residues targeted for alanine substitution. The reporter protein, EGFP, is shown fused in-frame to the terminal PBANR residue.

Cells were incubated for an additional 24–36 h at 27 °C before use in internalization assays.

Preparation of a fluorescent PBAN analog. A fluorescent analog of *B. mori* PBAN was prepared as described previously [6]. In brief, Rhodamine Red-X succinimidyl ester (Molecular Probes) was conjugated under slightly basic conditions to the amino terminus of synthetic PBAN. The conjugated peptide, designated as Rhodamine Red-labeled PBAN (RR-PBAN), was purified by reversed-phase HPLC, lyophilized overnight in a centrifugal vaporizer, and stored covered in foil at 4 °C until needed.

Confocal microscopy. For imaging of agonist-induced internalization of the chimeric PBANR-EGFP fusion proteins and receptor-mediated endocytosis of RR-PBAN, transfected cells were washed with fresh IPL-41 insect media (Gibco-BRL) and incubated in the presence of 50 nM RR-PBAN for 1 h at 4 °C. The low temperature was utilized to minimize premature internalization. Cells were washed twice with cold phosphate-buffered saline (PBS) to remove unbound label and incubated for various times at 27 °C in 2 mL IPL-41. At the end of the incubation, cells were fixed for 30 min with 10% formalin at 4 °C, washed twice with cold PBS, and then imaged. Fluorescence images were obtained with a Leica TCS NT confocal system using the 488-nm laser line of an argon laser (EGFP) and the 568-nm laser line of an argon/krypton laser (Rhodamine Red). For dual excitation experiments, only one laser line was used with single channel recordings to minimize cross-talk. Images were processed using Photoshop 6.0 (Adobe Systems, San Jose, CA).

Inhibition of PBANR internalization. To further characterize the PBANR internalization pathway, endocytosis was blocked via hypertonic shock, removal of extracellular calcium, and inhibition of second messenger kinases. To assess the effects of hypertonic shock, binding and initiation of internalization were done as before with the exception that the cells were subjected to IPL-41 medium supplemented with 0.45 M (final concentration) sucrose throughout the internalization period. The viability of the sucrose-treated cells was confirmed by washing cells that had been incubated for 30 min at 27 °C in the presence of 0.45 M sucrose with IPL-41 and re-incubating for another 30 min at 27 °C in medium lacking sucrose. The role of extracellular calcium was examined in experiments performed as previously described with the exception that the incubation media were replaced with a modified Ringer's buffer (21 mM KCl, 12 mM NaCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 170 mM glucose, and 10 mM Pipes; pH 7.2) [7] prepared with or without $CaCl_2$ and supplemented with 3 mM EGTA. In experiments designed to examine the effects of second messenger kinases, transfected cells were pre-treated for 30 min with 5 μM staurosporine (Sigma) and assayed for RR-PBAN internalization in the presence of the general kinase inhibitor.

Quantification of RR-PBAN and PBANR internalization. To evaluate receptor internalization, the fluorescence attributable to internalized RR-PBAN (568-nm fluorescence) was calculated from the confocal microscopy data using the Leica LCS Lite 2.0 software package (Leica Microsystems, Heidelberg, Germany). Quantification was performed as described previously [6] by calculating the ratio of intracellular fluorescence intensity to total cellular fluorescence (including plasma membrane associated fluorescence) and adjusting for pixel number.

Data analyses. Data were analyzed in GraphPad Prism 3.0 (Graphpad Software) and statistical significances were determined using a two-tailed paired *t* test, defined as $P < 0.05$.

Results

RR-PBAN internalization

A central feature of GPCR regulation is ligand-induced internalization of the receptor [10–12]. This process often results in co-internalization of the ligand, an event that we previously demonstrated visually via the co-localization of fluorescent signals attributable to the green fluorescent PBANR-EGFP and the red fluorescent RR-PBAN [6]. To determine the time dependence of this PBAN-induced internalization, cDNAs encoding the *B. mori* PBANR and a mutant truncated at residue 346 (PBANR-346) were sub-cloned into the pIB/V5-His insect expression vector and transfected into Sf9 cells. Two days after transfection, cells transiently expressing the PBANRs were exposed to 50 nM RR-PBAN for 1 h at 4 °C (non-permissive for internalization) and examined at various time points (0, 2, 5, 15, 30, 45, and 90 min) at 27 °C for intracellular localization of RR-PBAN. As shown in Fig. 2A, the receptor-mediated endocytosis of RR-PBAN by PBANR is an expeditious event with the formation of clearly visible vesicles at the cytosolic-plasma membrane interface within minutes of being transferred to the permissive temperature. To determine the kinetics and extent of RR-PBAN internalization, the ratio of intracellular fluorescence intensity to total cellular fluorescence (including the plasma membrane) was calculated for the PBANR and PBANR-346 expressing cells. A plot of the internalization kinetics demonstrates that RR-PBAN internalization in PBANR expressing cells is rapid ($t_{1/2}$ 2.1 min) and virtually complete (~90%) but is severely impaired in cells expressing PBANR-346 (Fig. 2B). These data confirm our previous observation that the terminal 67 amino acids of the PBANR C terminus are critical for removal of ligand-occupied receptor from the cell surface [6]. This defect in internalization is likely not the result of structural changes in the global structure of the receptor following deletion of the terminal 67 residues because PBANR-346 localizes to the cell surface and shows no apparent defect in RR-PBAN binding [6]. Rather, given the numerous reports demonstrating that truncations or mutations of the C terminus impair

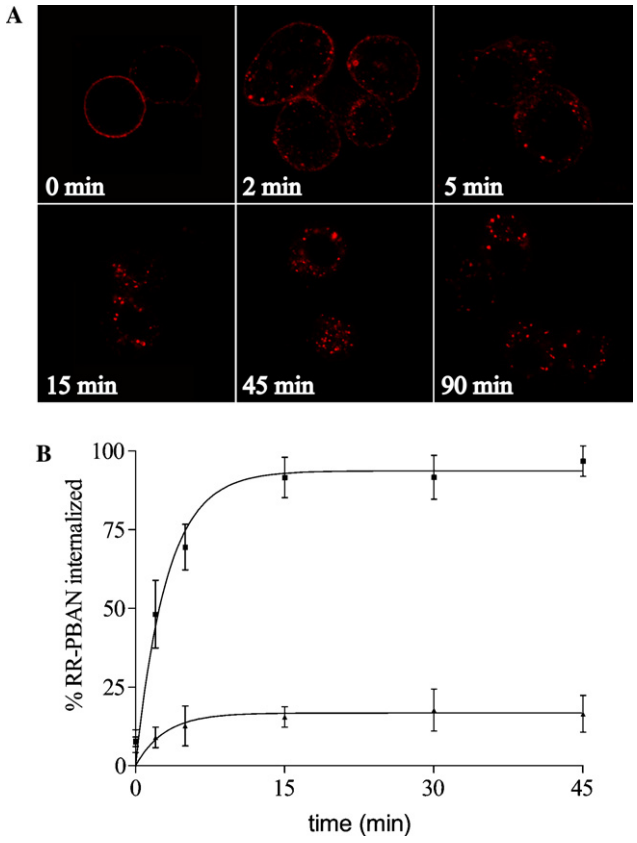

Fig. 2. Time course of receptor-mediated uptake of RR-PBAN. (A) Confocal microscopy images of Sf9 cells transiently expressing the *B. mori* PBANR incubated in the presence of 50 nM RR-PBAN for 1 h at 4 °C and then transferred to 27 °C for 0, 2, 5, 15, 45, or 90 min prior to fixation. Fluorescence was obtained via the 568-nm line of a krypton/argon laser. (B) Plot of the internalization kinetics of Sf9 cells transfected with either PBANR (■) or PBANR-346 (▲). Internalization assays were performed as described above with PBANR expressing cells exhibiting a $t_{1/2}$ of 2.1 min and maximum internalization at ~90%, whereas PBANR-346 expressing cells exhibited a $t_{1/2}$ of 1.8 min and maximum internalization at ~15%. The ratios of the intracellular fluorescence at 568 nm (not including plasma membrane) to the total cellular fluorescence at 568 nm were calculated as a means of quantifying the receptor-mediated endocytosis of RR-PBAN. Values were obtained using confocal microscopy data from multiple images and represent means ± SEM from three separate transfections and have been fit as one-phase exponential associations as described previously [34].

receptor internalization [14–20], it is more likely that one or more sites in the PBANR C terminus mediate regulation.

The internalization motif is bounded by Gly357 and Gln367

To define the site(s) that are important for internalization in the *B. mori* PBANR, a series of C-terminally truncated mutations were constructed with the reporter protein, EGFP, fused in-frame with the C-terminal PBANR residue. These truncations involved deletion of: 67 residues (PBANR-346-EGFP), 56 residues (PBANR-357-EGFP), 46 residues (PBANR-367-EGFP), 36 residues (PBANR-377-EGFP), 26 residues (PBANR-387-EGFP), and 16 residues (PBANR-397-EGFP). A schematic of the various truncations is depicted in Fig. 1. As before, Sf9 cells transiently expressing either PBANR-EGFP or the chimeric mutants were incubated in the presence of 50 nM RR-PBAN at 4 °C, washed to remove unbound and loosely bound ligand, and then incubated for 30 min at 27 °C. Confocal microscopic examinations indicated that the mutants localized primarily to the plasma membrane and showed no apparent defects in RR-PBAN binding (data not shown). Cells transiently expressing the PBANR-EGFP control had numerous vesicles corresponding to the internalized ligand–receptor complex scattered throughout the cytoplasm (Fig. 3G). A similar profile was observed in cells expressing the PBANR-367-EGFP, PBANR-377-EGFP, PBANR-387-EGFP, and PBANR-397-EGFP constructs (Figs. 3C–F). In contrast, the cytosol of cells expressing the PBANR-346-EGFP and PBANR-357-EGFP mutants was practically devoid of these fluorescent vesicles (Figs. 3A and B); an indication that the PBAN-induced internalization mechanism is severely impaired in these two mutants. Quantification of the extent of RR-PBAN internalization in cells expressing PBANR-EGFP indicated that after 30 min at 27 °C, ~90% of the RR-PBAN had been internalized (Fig. 3I). In agreement with the visual confocal microscopy data, removal of residues 368–413 from the PBANR C terminus had no effect on RR-PBAN uptake (Fig. 3I). Receptor-mediated endocytosis in cells expressing the PBANR-346-EGFP and PBANR-357-EGFP constructs, however, was significantly reduced (Fig. 3I). These data suggest that the C-terminal motif(s) essential for internalization of the *B. mori* PBANR resides in the 10 amino acid region spanning Gly357 and Gln367.

Internalization is reduced in the Y360A/L363A double mutant

The 10 residue region between Gly357 and Gln367 in the PBANR C terminus contains a potential tyrosine-based motif, YXXL, which has been shown to be involved in ligand-induced internalization of the thrombin GPCR, protease-activated receptor-1 (PAR-1) [21]. Unlike the PAR-1 motif, YSIL, which is situated approximately 10 residues from the carboxyl end of the 7th transmembrane domain (TM7), the potential *B. mori* PBANR motif, YSAL (residues 360–363), is situated 30–35 amino acids downstream from TM7. This placement is comparable to the location of similar tyrosine-based motifs that function primarily as endocytotic signals, although in these instances the motif is generally not found within the C terminus [22]. To determine what role this potential motif may play in PBAN-induced internalization of the *B. mori* PBANR, we constructed

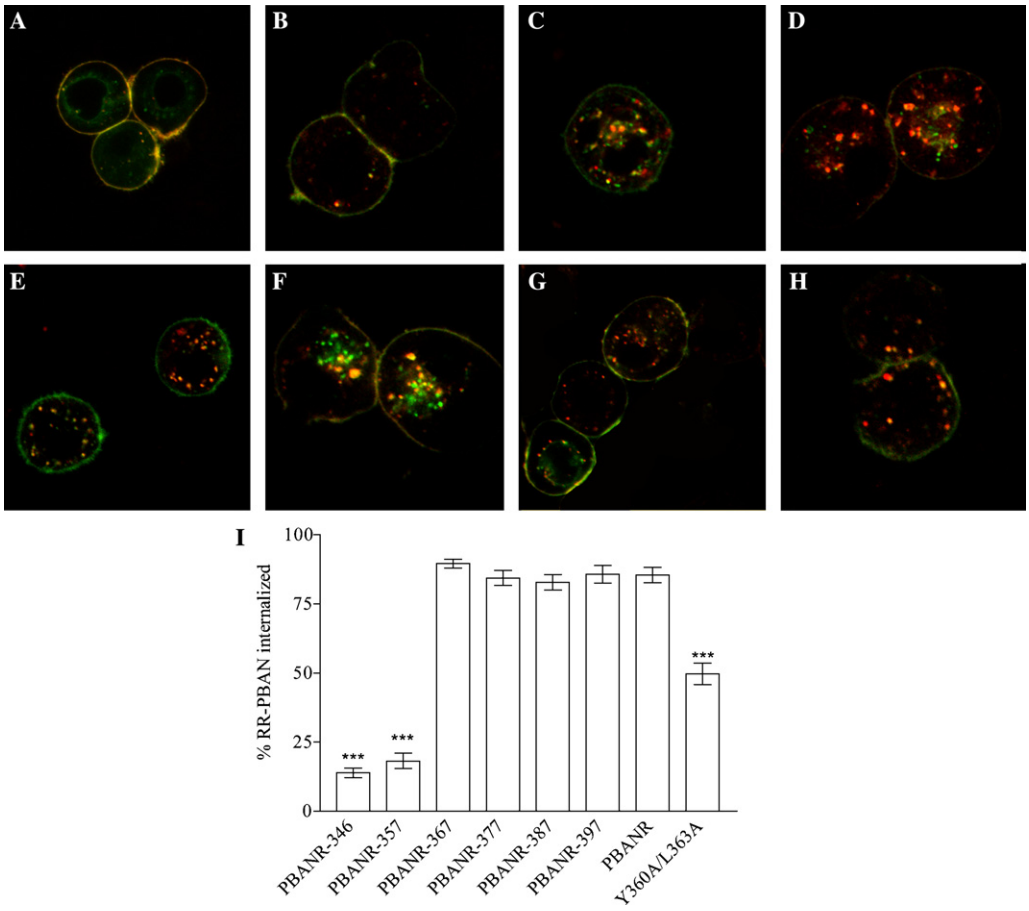

Fig. 3. Effect of mutations in the PBANR C terminus on RR-PBAN-induced internalization. Confocal microscopy images of Sf9 cells transiently expressing PBANR-346-EGFP (A), PBANR-357-EGFP (B), PBANR-367-EGFP (C), PBANR-377-EGFP (D), PBANR-387-EGFP (E), PBANR-397-EGFP (F), PBANR-EGFP (G), or (H) PBANR-EGFP containing the Y360A/L363A double mutation were incubated in the presence of 50 nM RR-PBAN for 1 h at 4 °C and then transferred to 27 °C for 30 min prior to fixation. EGFP fluorescence was obtained via the 488-nm line of an argon laser while Rhodamine Red fluorescence was obtained via the 568-nm line of a krypton/argon laser. Figures were merged using Photoshop 6.0 software. (I) The percentage of RR-PBAN internalized in Sf9 cells transiently expressing the PBANR-EGFP mutations. As before, cells were incubated in the presence of 50 nM RR-PBAN for 1 h at 4 °C and then transferred to 27 °C for 30 min prior to fixation. Values were obtained using confocal microscopy data from multiple images and represent means ± SEM from three separate transfections. Samples exhibiting statistically significant differences were determined using a two-tailed paired *t* test and are indicated by *** with $P < 0.0001$ as compared with PBANR-EGFP.

a double substituted mutant of PBANR-EGFP in which the critical residues of the motif, Tyr360 and Leu363, were converted to alanines (Fig. 1). When transiently expressed in Sf9 cells the double mutant localized to the cell surface and exhibited no apparent defects in RR-PBAN binding (data not shown). While some RR-PBAN associated vesicles were observed in Sf9 cells transiently expressing the Y360A/L363A construct (Fig. 3H) they were not as pronounced as those seen in the PBANR-EGFP construct (Fig. 3G). This difference was more evident upon quantification (Fig. 3I) which revealed that receptor-mediated endocytosis of RR-PBAN was significantly reduced in cells expressing the Y360A/L363A double mutant (~55%) but was not as drastic as that seen in cells expressing either PBANR-346-EGFP or PBANR-357-EGFP (~23%). The ~35% difference in internalization between PBANR-EGFP and the Y360A/L363A double mutant, however, is comparable with that attained by the analogous double mutant (i.e., Y383A/L386A) in PAR-1 [21]. These findings suggest that Tyr360 and Leu363 likely constitute a YXXL endosomal targeting motif that facilitates receptor-mediated endocytosis but that does not function as the primary internalization signal.

Mediation by clathrin-coated pits

To further elucidate the molecular mechanisms underlying agonist-induced internalization of the *B. mori* PBANR, we analyzed the effects of hypertonic shock on the receptor-mediated uptake of RR-PBAN by incubating PBANR expressing cells in the presence and absence of 0.45 M sucrose. Hypertonicity has been shown to inhibit the formation of clathrin-coated pits [23] and thus block clathrin-mediated internalization, the predominant route by which most GPCRs undergo

endocytosis [11,12]. In the absence of sucrose, RR-PBAN was rapidly internalized and could be visually discerned via confocal microscopy by the presence of multiple red vesicles distributed throughout the cytosol (Fig. 4A). In contrast, the presence of 0.45 M sucrose reduced receptor-mediated uptake of RR-PBAN (Fig. 4H) to levels seen in the PBANR-346 truncation (Fig. 3I) but with the labeled peptide randomly dispersed throughout the plasma membrane (Fig. 4B), indicating that the PBANR could be sequestered but was incapable of internalization [24,25]. Replacement of the hypertonic solution with media lacking sucrose removed the internalization block as evidenced by the characteristic red vesicles corresponding to internalized RR-PBAN (Fig. 4C) and the return of RR-PBAN internalization to non-treated levels (Fig. 4H). Taken together, these data indicate that ligand-induced internalization of the *B. mori* PBANR occurs via a clathrin-mediated pathway.

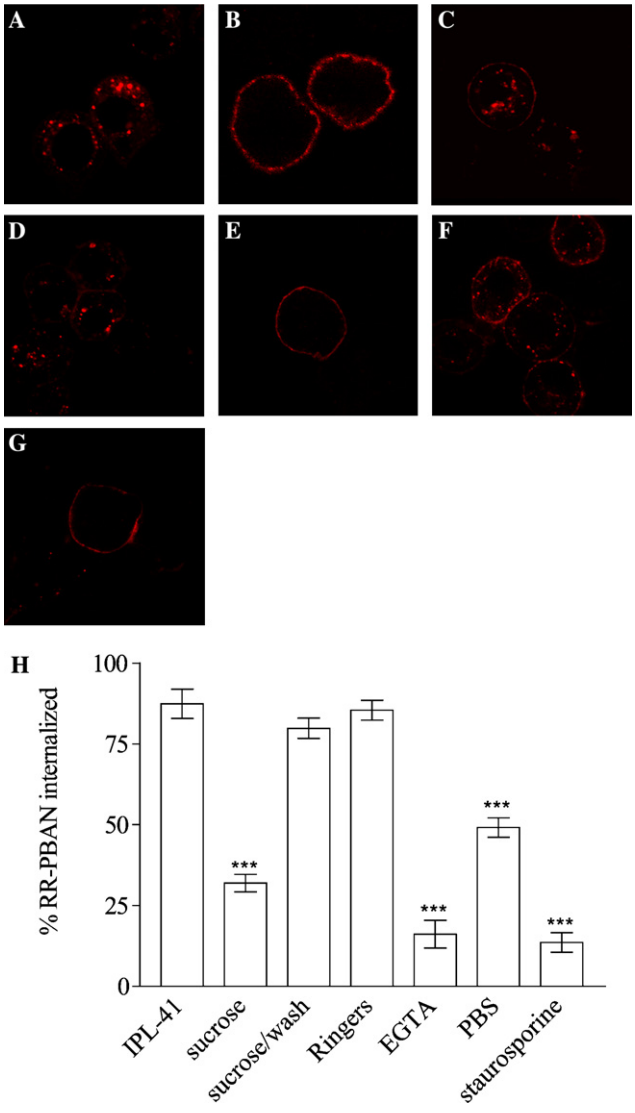

Fig. 4. Inhibition of PBANR internalization and RR-PBAN uptake. Receptor-mediated uptake of 50 nM RR-PBAN in Sf9 cells transiently expressing the *B. mori* PBANR. Confocal microscopy images were taken after 30 min at 27 °C. (A) Incubation with IPL-41 insect medium. (B) Hypertonic shock with IPL-41 medium containing 0.45 M sucrose. (C) Replacement of the hypertonic medium with fresh IPL-41 and re-incubated for 30 min at 27 °C. (D) Incubation with modified Ringer's buffer. (E) Incubation with Ringer's buffer lacking Ca^{2+} and supplemented with 3 mM EGTA. (F) Incubation with PBS. (G) Pre-treatment and incubation with IPL-41 medium containing 5 μM staurosporine (a kinase inhibitor). (H) The percentage of RR-PBAN internalized by receptor-mediated endocytosis in Sf9 cells transiently expressing the *B. mori* PBANR under the varying conditions described above. Values were obtained using confocal microscopy data from multiple images and represent means ± SEM from three separate transfections. Samples exhibiting statistically significant differences were determined using Student's *t* test and are indicated by *** with $P < 0.0001$ as compared with incubations in IPL-41 insect medium.

Dependency on extracellular Ca^{2+}

The principle effector pathway activated by PBAN triggers an influx of extracellular calcium [3,4]. To determine if the PBANR internalization mechanism is dependent on the activation of this effector pathway, we analyzed receptor-mediated uptake of RR-PBAN in the absence of external calcium. Since the insect medium used in the previous experiments was pre-supplemented with calcium, we examined the effects of a medium in which we could more easily eliminate the divalent cation. Consequently, we analyzed RR-PBAN uptake in a modified Ringer's buffer containing 3 mM $CaCl_2$ [7]. In this buffer, RR-PBAN uptake proceeded as before with ~90% RR-PBAN internalization (Fig. 4H) and the concomitant intracellular formation of red vesicles (Fig. 4D). In buffer prepared minus calcium and supplemented with 3 mM EGTA, the uptake of RR-PBAN was effectively blocked (Fig. 4H), resulting in a uniform distribution at the cell surface (Fig. 4E) reminiscent of that seen in PBANR expressing cells maintained at the non-permissive temperature (Fig. 2A, 0 min). In our previous report, we described receptor-mediated endocytosis of RR-PBAN in low-grade PBS [6]. Under these conditions, intracellular vesicles were observed (Fig. 4F) but the internalization process appeared to be less efficient than in media containing physiological amounts of calcium as a portion of the RR-PBAN remained localized at the cell surface (Fig. 4F) with a significant reduction in the extent of RR-PBAN uptake (Fig. 4H). These observations are consistent with our supposition that extracellular calcium plays a role in the ligand-induced internalization of the PBANR. In buffer completely lacking extracellular calcium (i.e., EGTA supplemented), no internalization was observed (Fig. 4E). In buffer containing a low level of calcium (the PBS buffer was prepared from low-grade reagents that were not devoid of the divalent cation), the internalization process was less efficient (Fig. 4F). These results suggest that PBANR internalization proceeds via a

pathway that is dependent on the influx of extracellular calcium.

Involvement of second messenger kinases

A prerequisite for receptor internalization via clathrin-coated pits is the phosphorylation of specific sites within the 3rd intracellular loop and/or C terminus of most GPCRs. While this regulatory event is often mediated by G protein-coupled receptor kinases [10,26,27], there are several reports implicating the actions of the second messenger kinases, protein kinase C (PKC) and protein kinase A (PKA) [28–32]. Examination of the *B. mori* PBANR sequence indicates the presence of four potential PKC sites: Ser233, Ser261, Ser333, and Ser366 as well as two potential PKA sites: Ser243 and Ser389. To determine if either of these kinases plays a role in PBANR internalization, we examined the effects of staurosporine, an inhibitor of second messenger kinases, on the receptor-mediated uptake of RR-PBAN. Pretreatment with 5 μM staurosporine led to a marked decrease in the extent of PBANR internalization (Fig. 4H) with the cytosol of PBANR expressing cells essentially devoid (Fig. 4G) of the distinctive RR-PBAN associated vesicles observed in non-treated PBANR expressing cells (Fig. 4A). These findings suggest that the endocytotic mechanism that mediates the removal of PBANR from the cell surface is dependent on the activation of a second messenger kinase and not the actions of G protein-coupled receptor kinases.

Discussion

In this study, we present a number of new findings regarding the molecular mechanisms underlying PBAN-induced internalization of the *B. mori* PBANR in insect Sf9 cells. The receptor-mediated uptake of RR-PBAN is a rapid internalization event with a $t_{1/2}$ of ~2 min, a rate that is comparable to that observed with other GPCRs that undergo agonist-induced internalization [33,34]. These results also confirmed our previous findings [6] demonstrating a role for the terminal 67 residues of the PBANR C terminus in mediating the internalization event (Fig. 2B). Consequently, we constructed a series of C-terminal truncations to facilitate identification of the site(s) utilized in mediating the internalization event. We were able to determine that the 10 amino acid region bounded by Gly357 and Gln367 is required for internalization (Figs. 3B and C). Identification of Tyr360 and Leu363 as functionally important internalization residues suggests that they may constitute a YXXL endosomal targeting motif that facilitates PBAN-induced internalization (Fig. 3I). The contribution from this conserved motif is likely minor as the double mutant failed to produce a phenotype similar to that of the PBANR-346-EGFP or PBANR-357-EGFP constructs in which internalization was severely impaired (Fig. 3I). While the sensitivity of the PBANR internalization mechanism to hypertonic shock (Fig. 4B) is a hallmark of most pathways mediated by clathrin-coated vesicles, the dependence on extracellular calcium (Fig. 4E) is worthy of note as it suggests that the PBANR endocytotic pathway may be linked via a negative feedback loop to the effector pathway activated by PBAN.

Agonist-induced internalization of many GPCRs via clathrin-coated pits is often dependent on the presence of a motif(s) situated within their cytoplasmic domains (usually the C terminus) [22,35]. The most common endocytotic signals are the tyrosine-based motifs, NPXXY and YXXθ. The NPXXY motif, often located near the TM7-C terminus junction, has been shown to play a role in the internalization of several GPCRs including, β_2-adrenergic receptors [36,37], the platelet-activating factor receptor [38], and the human *N*-formyl peptide receptor [39]. However, this motif does not appear to be universal as mutations of the critical residues have been shown to have little effect on the internalization rate of some receptors [34,40,41]. While this motif is present in the *B. mori* PBANR (residues 325–329; NPFLY), our current study suggests that it is not essential for internalization since the PBANR-346-EGFP and PBANR-357-EGFP constructs, which contain the sequence, are characterized by a severely impaired agonist-induced internalization mechanism (Figs. 3A and B). The YXXθ motif (Y = tyrosine, X = any amino acid, and θ = amino acid with a bulky hydrophobic sidechain) has been shown to be essential for rapid translocation from the cell surface to endosomal compartments of a variety of membrane-bound proteins [22]. A derivative of this motif, YXXL, was recently implicated in the ligand-induced internalization of PAR-1 [21]. Paing et al. [21] further reported that this motif is present in the C terminus of a number of receptors, including several activated by peptides leading to speculation that this motif may be a conserved internalization signal for many peptide GPCRs. This premise is supported by our current findings implicating a role for the motif's critical residues (Tyr360 and Leu363) in the internalization of PBANR (Fig. 3H). However, the motif does not appear to be the primary initiating site since the Y360A/L363A mutation, similar to the analogous double mutant in PAR-1 [21], failed to completely abolish internalization. This suggests that, like numerous other receptors [15,34,42], multiple endocytotic signals may be present in the PBANR C terminus. Indeed, examination of the 10 amino acid region bounded by Gly357 and Gln367 in the PBANR C terminus reveals the presence of several sites that could be involved in PBAN-induced internalization (Fig. 5), including a PKC site (Ser366), two other potential phosphorylation

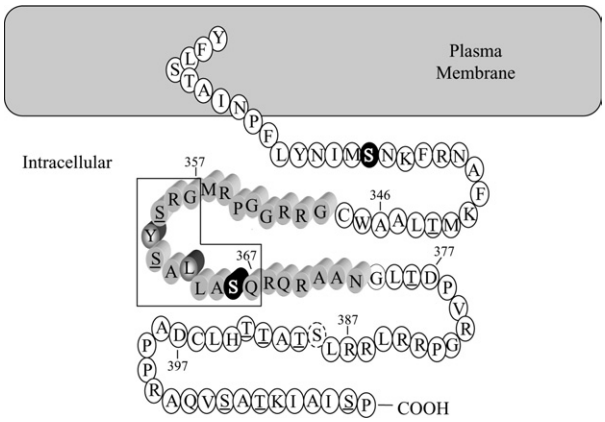

Fig. 5. Schematic diagram of the PBANR C terminus. Shown are residues 318–413 of the *B. mori* PBANR. The functionally important residues bounded by Gly357 and Gln367 are enclosed within the open box. The critical residues (Tyr360 and Leu363) of the tyrosine-based endocytotic targeting motif, YXXL, are shown in dark gray; potential PKC sites are shown in black, a potential PKA site is shown in dashed outline, non-specific Ser/Thr phosphorylation sites are underlined, and a putative calmodulin-binding motif is indicated by the region shown in three dimensions. The calmodulin-binding site was predicted using the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/pub_pages/search/search.htm).

sites (Ser359 and Ser361), and a significant portion of a potential calmodulin-binding site (Gly349-Asn373). This latter point is especially interesting as calmodulin has been reported to play a role in receptor endocytosis [43].

Based on our current findings, we propose two possible mechanisms by which the *B. mori* PBANR is internalized via a direct negative feedback loop requiring the influx of extracellular calcium that is triggered by PBAN binding. This rise in intracellular calcium activates a second messenger kinase which then phosphorylates one or more of the potential kinase sites (PKC—Ser233, Ser261, Ser333, and Ser366; PKA—Ser243 and Ser389) leading to the recruitment of proteins involved in facilitating endocytosis. While the molecular identity of the kinase utilized has yet to be demonstrably shown, current experimental data do not support a role for cAMP in the *B. mori* PBAN signal transduction cascade [3,4], implying that the phosphorylation event is most likely not mediated by PKA. The proposed mechanism is supported by our data which demonstrate that both extracellular calcium and a staurosporine sensitive kinase are necessary to initiate the endocytotic events (Figs. 4E and G). The uniform distribution of RR-PBAN at the cell surface in cells treated with either EGTA or staurosporine indicates that ligand binding has not induced sequestration of PBANR within the lipid bilayer of the plasma membrane and that the recruitment of proteins necessary for facilitating endocytosis has not occurred. Alternatively, the rise in intracellular calcium following PBAN binding could result in the displacement of bound calmodulin from the putative binding site encompassing residues 352–373, an action which would expose both the putative YXXL endosomal targeting motif (Tyr360-Leu363) as well as a potential PKC site (Ser366). This possible mechanism is supported by evidence indicating a reliance on extracellular calcium (Fig. 4E), the role of Tyr360 and Leu363 in internalization (Fig. 3H), and the actions of a staurosporine sensitive kinase, such as PKC, in mediating the endocytotic event (Fig. 4G). Studies are currently underway to determine which, if either, of the two proposed mechanisms is utilized.

In summary, using confocal microscopy-based techniques we have shown that the signal for agonist-induced internalization of the *B. mori* PBANR resides between Gly357 and Gln367. We have also shown that Tyr360 and Leu363 likely constitute a YXXL endosomal targeting motif that facilitates endocytosis and that the internalization mechanism occurs via a pathway mediated by clathrin-coated vesicles, requires an influx of extracellular calcium, and is dependent on the actions of a second messenger kinase rather than the more customary G protein-coupled receptor kinases. While these studies were performed in the artificial system of insect Sf9 cells, they are likely applicable to the pheromone producing cells of the pheromone gland and offer new insights regarding the molecular mechanisms involved in regulating the PBAN:PBANR complex, an interaction that is indispensable for the proficient mating of lepidopteran species.

Acknowledgments

We thank Masaaki Kurihara for his excellent technical assistance. We also thank Drs. C.T. Nomura and K. Moto for comments and useful discussions. This work was funded in part by a post-doctoral fellowship (J.J.H.) from the Japan Society for the Promotion of Science, the Bioarchitect Research Program and the Chemical Biology Research Program from RIKEN, and the Ministry of Education, Culture, Sports, Science and Technology of Japan Grants-in-Aid for Scientific Research (B) 17380041.

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