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Identification of Soluble Binding Proteins for an Insect Neuropeptide

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A photoaffinity analog of *Helicoverpa zea* pheromone biosynthesis activating neuropeptide (Hez-PBAN) was used to identify PBAN binding proteins in various tissues of the corn earworm moth, *H. zea*. Synthetic Hez-PBAN was derivatized on Lys-27 with *p*-benzoyldihydrocinnamoyl-N-hydroxysuccinimide ester (BZDC-NHS ester). The resulting BZDC-PBAN stimulated pheromone production in *H. zea* isolated abdomens at levels comparable to those of the unmodified peptide. Photoaffinity labeling experiments using [^{3}H]BZDC-PBAN with female moth tissues revealed soluble 100 and 115 kDa proteins in the brain-subesophageal ganglia complex, ventral nerve cord, and thoracic muscle that were specifically labeled with the PBAN analog.

Production of pheromone in many moth species is required for sexual attraction and activation of mating behaviors. In most female moths, pheromone biosynthesis is regulated by a pheromone biosynthesis activating neuropeptide (PBAN). This neuropeptide was first identified in the corn earworm moth, *Helicoverpa zea*, as a 33 amino acid peptide with a C-terminal amide necessary for activity [1]. Subsequently, PBANs have been purified and sequenced from the silkworm, *Bombyx mori* [2, 3], and the gypsy moth, *Lymantria dispar* [4]. The minimal sequence required for activity is a C-terminal pentapeptide, FXPRLamide (X = G, S, T, or V) [5], which adopts a well-defined type I β-turn as demonstrated by NMR studies [6]. This C-terminal sequence is similar to the pentapeptide C-terminal of a variety of insect myotropic peptides with functions that include regulation of coloration in phase polymorphism in moth larvae [7, 8], myotropic activity on insect hindguts and oviducts [9], and induction of embryonic diapause in *B. mori* [10].

The gene coding for PBAN has been identified in *B. mori* [11] and *H. zea* [12, 13]. The deduced amino acid sequence from both insects indicated that in addition to PBAN, four other peptides are produced that belong to the PBAN family of peptides. One of these peptides is the diapause hormone required for induction of embryonic diapause in *B. mori* [14]. These peptides are found in posttranslational processed form in *B. mori* [14] and *H. zea* [15]. The subesophageal ganglion (SEG) is the site of PBAN mRNA synthesis based on northern hybridization and *in situ* hybridization histochemistry experiments [12, 14] (Ma, unpublished observation). Immunocytochemistry using polyclonal antibodies indicated that in addition to the SEG, these peptides are found in the corpora cardiaca (CC), in axons projecting through the ventral nerve cord (VNC), and in nerve cells of thoracic and abdominal ganglia [15, 16]. Neurohemal structures from some nerve cells in abdominal ganglia indicated that immunoreactive substances may be secreted into the hemolymph at these sites in addition to the CC [15]. Pheromonotropic activity has also been found in tissue extracts of VNCs including individual ventral ganglia [15, 17].

The presence of PBAN-like immunoreactive material and pheromonotropic activity in ventral nerve tissues indicates a role for the ventral nervous system in regulation of pheromone production. An intact VNC was found to be necessary for pheromone production in the gypsy moth, *L. dispar* [18], and in *Heliothis virescens*

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Abbreviations: **PBAN,** pheromone biosynthesis activating neuropeptide; **Hez-PBAN,** pheromone biosynthesis activating neuropeptide from *Helicoverpa zea;* **BZDC-NHS ester,** N-hydroxysuccinimide ester of *p*-benzoyldihydrocinnamic acid; **BZDC-PBAN,** benzoyldihydrocinnamido-pheromone biosynthesis activating neuropeptide; **PMSF,** phenylmethylsulfonylfluoride; **SEG,** subesophageal ganglion; **CC,** corpora cardiaca; **VNC,** ventral nerve cord; **TFA,** trifluoroacetic acid; **TEA,** triethylamine; **DMF,** dimethylformamide; **MeOH,** methanol; **MALDI-MS,** matrix assisted laser desorption ionization mass spectroscopy; **Tris,** tris(hydroxymethyl)aminomethane; **[^{125}I]ASA-PBAN,** Lys^{27}-[^{125}I]azido-salicylamido-PBAN.

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and *H. zea* [19]. Others have reported that an intact VNC was not required for pheromone production in *H. zea* [20, 21]. These findings present a confusing picture as to the role of these peptides in the VNC.

Photoaffinity labeling studies can provide unique information about neuropeptide-protein interactions and in many cases can be used to selectively identify binding proteins or receptors from a crude protein extract [22, 23]. In this study, we have used a benzophenone-containing analog of Hez-PBAN to identify binding proteins in tissue homogenates. Synthetic Hez-PBAN was derivatized on Lys-27 with the photoaffinity cross-linking reagent, BZDC-NHS ester [24], to give a biologically-active analog, BZDC-PBAN. Radiolabeled BZDC-PBAN was used to identify PBAN binding proteins in several tissues from *H. zea* including thoracic muscle and nervous tissue. Specificity of the [^{3}H]BZDC-PBAN-protein interaction was demonstrated by competitive displacement of the label with excess unlabeled PBAN.

MATERIALS AND METHODS

Equipment and chemicals. PBAN and derivatives (> 1 mg) were HPLC purified (LKB/Bromma 2152 system) on semi-preparative C-4 or C-8 reversed phase columns (Vydac, 10 × 250 mm) using 0 to 80% B in 45 min at 2 ml/min. Mobile phase A: 0% CH_3CN/0.06% TFA; Mobile phase B: 80% CH_3CN/0.052% TFA. Purification and analysis of [^{3}H]BZDC-PBAN was performed on an Aquapore RP-300 C-8 column (Applied Biosystems, 4.6 × 250 mm) using 0 to 80% B in 45 min at 1 ml/min. Eluate absorption was monitored at 220 and 280 nm. MALDI-MS was performed on a Bruker Protein-TOF® system using a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix and manufacturer's instructions. Fluorescent photolabeling lamps with peak emission near 350 nm (Cat. no. RMR-3500) were obtained from Southern New England Ultraviolet Company (Hamden, CT) and mounted in fixtures purchased from a local hardware store.

All organic solvents were HPLC grade. N-(methyl)mercaptoacetamide used for PBAN reduction was purchased from Sigma Chemical Co. (St. Louis, MO). Triethylamine was freshly distilled from CaH_2 before use. BZDC-NHS ester was prepared as described by Olszewski *et al.* [24]. [^{3}H]BZDC-NHS ester was obtained from NEN Life Science Products (Boston, MA). Saline used for dissections, homogenizations, and protein photoaffinity labeling consisted of 21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 170 mM glucose, 3 mM PIPES, pH 6.6.

Synthesis of Lys^{27}-BZDC-PBAN. Hez-PBAN was prepared synthetically using FMOC chemistry with an Applied Biosystems 431a peptide synthesizer. After cleavage and deprotection, the two methionines in PBAN were in various oxidation states [6]. Crude PBAN (10 mg) was chemically reduced [25] and purified on the semi-preparative C-4 HPLC column to give 6 mg of reduced PBAN (60% yield). Vials containing lyophilized peptide were flushed with nitrogen and stored at −80 °C. Product was analyzed by MALDI-MS as described above.

BZDC-PBAN was prepared as described [24]. Thus, crude or reduced Hez-PBAN (5 mg, 1.2 mmol) was mixed with 1 mol eq of BZDC-NHS ester (1.2 mmol) and 15 mol eq of TEA (18.0 mmol) in 500 µl of DMF. After 16 h at room temperature, the reaction was purified on the semi-preparative C-8 column. Mass and purity of products were judged by MALDI-MS and analytical HPLC chromatography. Approximately 2 mg of product, Lys^{27}-BZDC-PBAN, was obtained (40% yield). Peptide was stored at −80 °C under nitrogen as a powder or in solution (1-2 mM in saline).

Lys^{27}-BZDC-PBAN was identified by the inability of endoprotease Lys-C to cleave the lysine modified peptide. Reduced PBAN or BZDC-PBAN products (15 nmol, 2 mM stock in water) were digested with Lys-C (Boehringer Mannheim, Indianapolis, IN) by following the manufacturer's instructions. Cleavage at the single lysine in Hez-PBAN was evaluated by comparing the HPLC chromatograms of the peptides before and after protease treatment.

Preparation of [^{3}H]BZDC-PBAN. The [^{3}H]BZDC-NHS ester (500 µCi, 34.5 Ci/mmol) in ethyl acetate:hexane (1:1) was transferred to a 1.5 ml polypropylene microfuge tube and concentrated under nitrogen. Reduced PBAN (1.5 mol eq, 22 pmol) and 15 mol eq of TEA (220 pmol) in 20 µL of DMF was added to the reaction tube and mixed with vigorous vortexing. The reaction was incubated overnight at room temperature, diluted with 200 µL of water, and injected directly onto the analytical C-8 HPLC column. Product fractions were identified by comparing elution times with non-labeled Lys^{27}-BZDC-PBAN and by measuring tritium content by liquid scintillation spectroscopy. Total yield of [^{3}H]BZDC-PBAN was 200 µCi (40% radiochemical yield). The desired fractions were pooled into polypropylene microfuge tubes, concentrated using a Speed Vac, and redissolved in saline at a concentration of 1 µCi/µl.

Insects and biological activity. *H. zea* were maintained at 26 ±2 °C with a light:dark cycle of 14:10. Larvae were reared on a wheat germ diet (Bio-serv, Frenchtown, NJ). Sexes were separated as pupae and adult females were segregated daily. Tissues were obtained from adult virgin females in their third or fourth photophase. Peptides were tested by the isolated abdomen bioassay as described [20]. Pheromone amounts were determined as described [26].

Tissue and protein preparations. Tissues were dissected from female moths and placed in ice-cold saline and all subsequent procedures were conducted on ice. Freshly dissected materials were used within a few hours. Brain-SEG and VNC were dissected and stored at −80 °C in saline until used. Tissues were transferred to 2 ml of ice-cold saline containing 1 mM PMSF and then homogenized with a ground-glass homogenizer. Nervous tissues, in groups of twenty, were lysed with a 3 mm diameter stainless steel probe sonicator (Vibra Cell sonic dismembrator from Sonics and Materials Inc., Danbury, CT). Cellular debris was removed by centrifugation at 12000 × g for 10 min and the supernatant was re-centrifuged at 100,000 × g for 1 h. The resulting pellet was resuspended in 0.3 ml of saline by grinding and vortexing. Protein concentrations were determined with a Bradford protein assay kit (BioRad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Protein concentrations were approximately 1 mg/ml for thoracic muscle and ovaries and 0.12 mg/ml for brain-SEG, VNC, gut tissue and pheromone glands.

Photoaffinity labeling. Aliquots (150 µl) of protein samples were transferred to wells in a 24-well tissue culture plate (1.5 cm diameter) kept on ice. To determine specific competitive binding, Hez-PBAN (3 nmol or 30 nmol from 4 mM stock in saline) was added to selected wells for a 30 min preincubation. A 1.0 µCi aliquot of [^{3}H]BZDC-PBAN (30 pmol) was then added to all samples. After 30 min on ice with occasional rocking, samples were placed under UV light. Tissue culture plates were kept on ice with the lamp axis centered over loaded wells and approximately 1 cm from the top of the plate. After 45 min irradiation at 360 nm, 5-µl aliquots were removed for LSC before 17 µl of 10 × SDS-PAGE sample buffer (reducing) was transferred directly into the well.

Electrophoresis and fluorography. SDS-PAGE samples were boiled for 2 min and loaded onto 160 × 160 × 1.5 mm gels (Hoefer 600 SE) composed of either 12% or 7% to 15% gradient acrylamide. After electrophoresis, acrylamide gels were stained with 0.1% Coomassie Blue R250 in 45% MeOH/10% glacial acetic acid and destained in 45% MeOH/10% glacial acetic acid. Gels were transferred to EnHance scintillant (NEN Life Science Products) for 1 h and then transferred to water until a homogeneous white color was apparent. Gel size was reduced in 50% PEG 8000 [27], dried, and fluorgraphed

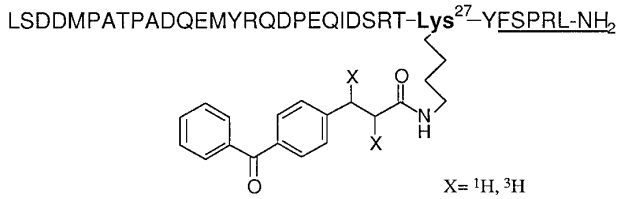

FIG. 1. Structure of Lys[27]-BZDC-PBAN. Chemical details of the benzophenone photophore and tritium-labeling sites are shown on the BZDC group. The C-terminal pentapeptide motif required for pheromone biosynthesis activity is underlined.

on X-ray film (Kodak) at −80 °C for 28 days. Fluorograms were scanned into a computer at 1200 dpi resolution and densitometry was performed with NIH-IMAGE v 1.55.

RESULTS

Preparation of Peptide Photoaffinity Labels

The Hez-PBAN amino acid sequence is shown in Fig. 1. The biologically-active C-terminal pentapeptide sequence is underlined and Lys-27 is shown in bold. After synthesis, Hez-PBAN becomes partially oxidized at the methionine (M) residues and is observed as a mixture of peaks in reversed phase HPLC. Reduction and HPLC purification of reduced Hez-PBAN resulted in a single peak [6], thereby simplifying identification of derivatized products during chromatography. The expected molecular mass of the reduced peptide was 3901.3 Da and the observed mass was 3903.4 Da (±0.05%) as determined by MALDI-MS. The peptide has two amine groups (N-terminal and Lys[27] ϵ-amino) available for modification by the BZDC-NHS ester reagent. Lys[27]-BZDC-PBAN (Fig. 1) was the target compound since the photophoric group is only 2 amino-acids (~ 7Å) from the C-terminal pentapeptide sequence known to be the minimal sequence required to stimulate pheromone production [5].

Three BZDC-modified PBAN products were observed by HPLC (Fig. 2A). Optimized conditions with 1 to 1.5 mol eq of BZDC-NHS ester per mole of peptide and 15 mol eq of TEA in DMF resulted in mainly Lys[27]-labeled and double-labeled products. The product at 28 min (Peak 2, Fig. 2A) did not undergo digestion with endoprotease Lys-C indicating the Lys residue was modified. Unlabeled Hez-PBAN was efficiently cleaved under identical conditions (data not shown). A mass spectrum for the purified peptide (Peak 2, Fig. 2A) is shown in Fig. 2B. The expected mass of Lys[27]-BZDC-PBAN was 4138.5 Da and the observed mass was 4139.2 Da (±0.05%). HPLC analysis of the [^{3}H]BZDC-PBAN reaction mixture was essentially identical to that of the non-tritium-labeled coupling reaction. The specific activity of the HPLC-purified photolabel was 34.5 Ci/mmol.

Bioactivity Studies

Lys[27]-BZDC-PBAN was found to stimulate pheromone production in isolated abdomens at levels comparable to native peptide. The pheromone levels per gland, mean ± S.E.M. for n = 4 are as follows: Hez-PBAN, 2 pmol = 56.3 ±14.6; Lys[27]-BZDC-PBAN, 1 pmol = 62.6 ±12.1, 10 pmol = 71.4 ±8.5; 0 pmol control = 2.2 ±0.9. The activity of oxidized and reduced versions of the photolabel was not addressed, though it has been shown the state of oxidation in native PBAN may affect biological activity [28, 29]. Results from the isolated abdomen studies indicated the reduced version of the PBAN photolabel was active and the photophore did not interfere with receptor-activated pheromone production.

Photoaffinity Labeling

Figure 3A shows a complete fluorogram of photolabeled soluble protein obtained from the brain-SEG complex. Competitive labeling of two protein bands at 100 and 115 kDa was clearly observed. Non-specific labeling was observed in lower molecular weight proteins and a 97 kDa protein. Labeling of the 100 and 115 kDa

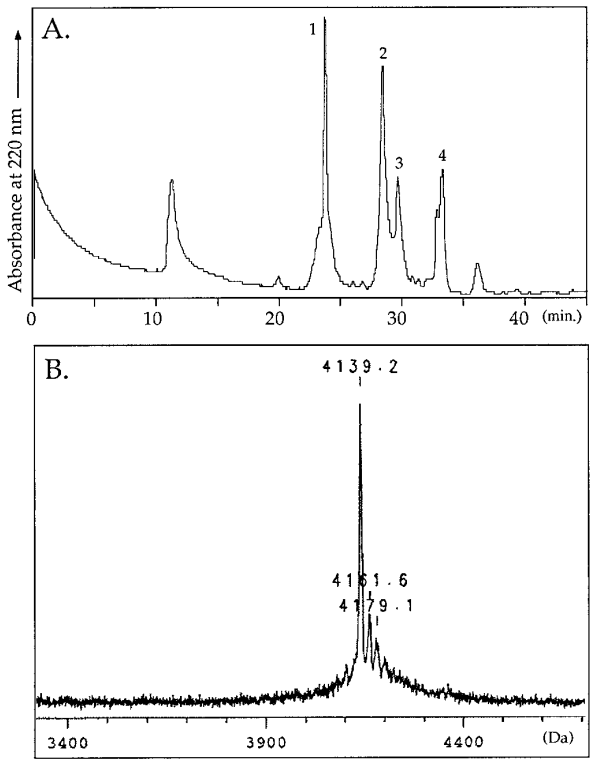

FIG. 2. Purification and analysis of BZDC-PBAN products. **Panel A:** HPLC chromatogram of BZDC-PBAN products: 1, reduced PBAN, R_f = 23 min; 2, Lys[27]-BZDC-PBAN, R_f = 28 min; 3 and 4, N-terminal and double-labeled BZDC-PBAN, respectively. Product under peak 2 was resistant to Lys-C digestion. **Panel B:** MALDI-MS data for peak 2. The expected mass of BZDC-PBAN is 4138.5 Da.

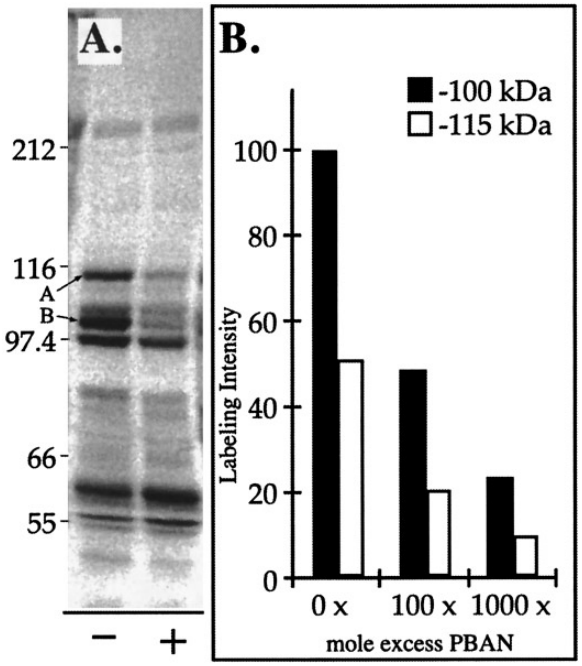

FIG. 3. Results from fluorography after photolabeling experiments. **Panel A:** *H. zea* brain-SEG supernatant after labeling with 30 pmol [^{3}H]BZDC-PBAN, in the presence (+) or absence (−) of a 1000-fold excess of PBAN as competitor. The 100 (labeled B) and 115 kDa (labeled A) PBAN binding proteins are clearly visible. **Panel B:** Displacement of photolabel with increasing concentrations of PBAN. Black, 100 kDa protein; white, 115 kDa protein. Specific labeling is reduced 80% in the 100 and 115 kDa proteins with 1000-fold molar excess of PBAN. Molecular weight markers are in kDa.

proteins by [^{3}H]BZDC-PBAN could be reduced 45% with 100-fold excess PBAN and 80% with 1000-fold excess PBAN (Fig. 3B). The intensity of the 100 kDa labeled band was 50% greater than the 115 kDa band indicating the 100 kDa protein was in greater abundance or had a greater affinity for Hez-PBAN. The nonspecifically labeled 97 kDa protein exhibited a 30% decrease in labeling with 100- or 1000-fold excess PBAN. These data are consistent with a specific interaction between the benzophenone-modified peptide and the 100 and 115 kDa proteins [22, 23].

Similar proteins at 100 and 115 kDa were specifically labeled in thoracic muscle, brain-SEG, and VNC supernatant fractions (Fig. 4). Both proteins were also identified in the membrane fraction of brain-SEG and VNC. Neither of these labeled proteins were observed in gut and ovarian tissues (data not shown). The 100 kDa protein appears to be more abundant in brain-SEG and VNC homogenates.

DISCUSSION

The PBAN photoaffinity analog was developed to characterize the molecular details of the hormonal pathway by which PBAN induces pheromone production. PBAN is thought to be part of a neuroendocrine system, whereby PBAN is secreted into the hemolymph through the CC and transported to the pheromone gland where it binds to receptors on the surface of the glandular cells [30]. Other investigators have proposed PBAN is transported in the VNC to the terminal abdominal ganglion causing release of neurotransmitters that stimulate pheromone production [19]. Although considerable evidence indicates that PBAN is acting directly on pheromone gland cells [20, 31, 32, 33, 34, 35] conclusive evidence for either a neuronal or neuroendocrine passage of PBAN to the pheromone gland cells is lacking.

We have identified two soluble PBAN binding proteins in brain-SEG and VNC. The proteins have identical molecular weights suggesting they are the same proteins in each tissue. The presence of these two proteins in pheromone gland homogenates could not be unambiguously demonstrated. The 100 and 115 kDa proteins were also found in muscle tissue suggesting PBAN-like peptides may be involved in muscle physiology. Alternatively, these PBAN binding proteins may interact with other neuropeptides, i.e., other PBAN gene products or myotropins, that share a common C-terminal pentapeptide motif. In earlier preliminary PBAN photolabeling studies with [^{125}I]ASA-PBAN, a 115 kDa protein was identified in female *H. zea* VNC, brain-SEG, thoracic muscle, pheromone gland and ovarian tissues (M.-T.B. Davis, unpublished results). This protein was not observed in hemolymph. The 115 kDa protein labeled with Lys^{27}-[^{3}H]BZDC-PBAN is likely the same protein labeled with the [^{125}I]ASA-PBAN probe.

A number of hypotheses can be drawn from the identification of PBAN binding proteins in the soluble fraction of different *H. zea* tissues. If the labeled proteins are

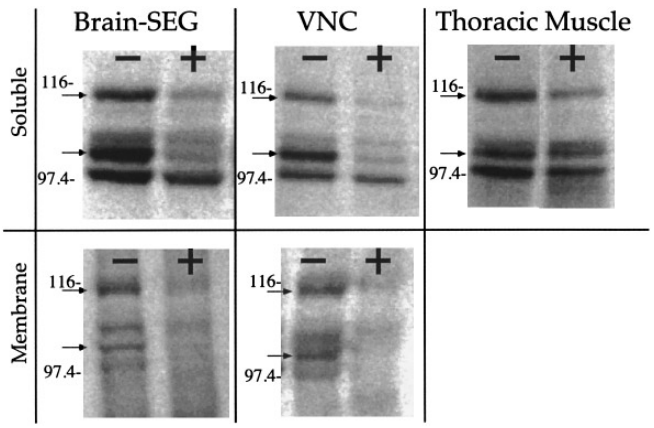

FIG. 4. Photolabeling results from a variety of female *H. zea* tissues. Fluorograms show the 100 and 115 kDa proteins in brain-SEG, VNC and thoracic muscle tissues, labeled in the presence (+) or absence (−) of a 1000-fold excess of PBAN as competitor. Molecular weights are shown in kDa.

cytoplasmic, they may behave as PBAN transporters acting to redistribute, stabilize and store PBAN and similar neuropeptides within neuronal cells. Identification of the same proteins in muscle and pheromone gland supernatants complicates the function of these proteins by suggesting PBAN and similar neuropeptides can be stored in target tissues that do not synthesize hormones. Intracellular storage proteins indicate PBAN must traverse the plasma membrane. M.-T.B. Davis (unpublished data) postulated that the soluble PBAN binding proteins are part of a system similar to that seen for mammalian enkephalin [36] and other hormone transport [37] across the blood brain barrier. It is unknown how these transport proteins would directly participate in PBAN-stimulated pheromone production.

Membrane proteins were probed with the PBAN photolabel in an attempt to identify a membrane receptor capable of transducing the PBAN signal. PBAN may activate a Ca^{2+} channel-linked hormone receptor, thereby explaining the extracellular Ca^{2+}-dependence of PBAN-stimulated pheromone production [20]. The N-terminal sequence of PBAN has homology with human insulin-like growth factor II suggesting it may bind tyrosine kinase-type receptors [38]. The 100 and 115 kDa proteins observed in the supernatant were also competitively labeled in membrane preparations although their abundance was lower than in the soluble protein preparations (Fig. 4).

It should be noted that the PBAN binding proteins on the Coomassie-stained gel could not be identified over the background staining. If the lower detection limit of Coomassie Blue R250 is 0.04 $\mu g/mm^2$ [39], the binding protein concentrations are less than 0.2 μg/20 moths in the respective tissues. The soluble binding proteins were also identified in male moths (data not shown).

The PBAN photoaffinity label, Lys^{27}-[^{3}H]BZDC-PBAN, was prepared and used to identify two soluble PBAN binding proteins in *H. zea* brain-SEG, VNC, and thoracic muscle. Although the function of the 100 and 115 kDa PBAN binding proteins in the PBAN hormonal pathway is unknown, their identification poses new questions about the mechanism of PBAN action. The use of this neuropeptide photoaffinity analog will be beneficial for future studies of these proteins and their function in insect neurohormone pathways.

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