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A class-A GPCR solubilized under high hydrostatic pressure retains its ligand binding ability

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ABSTRACT

The effect of high hydrostatic pressure (HHP) on the solubilization of a class-A G protein-coupled receptor, the silkmoth pheromone biosynthesis-activating neuropeptide receptor (PBANR), was investigated. PBANR was expressed in expresSF+ insect cells as a C-terminal fusion protein with EGFP. The membrane fraction was subjected to HHP treatment (200 MPa) at room temperature for 1–16 h in the presence of 0–2.0% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM). The solubilization yield of PBANR-EGFP in the presence of 0.6% (w/v) DDM increased to ~1.5-fold after 1 h HHP treatment. Fluorescence-detection size-exclusion chromatography demonstrated that the PBANR-EGFP ligand binding ability was retained after HHP-mediated solubilization. The PBANR-EGFP solubilized with 1.0% DDM under HHP at room temperature for 6 h retained ligand binding ability, whereas solubilization in the absence of HHP treatment resulted in denaturation.

1. Introduction

Pheromone biosynthesis-activating neuropeptide receptor (PBANR), a class-A GPCR with the characteristic seven transmembrane-spanning α-helical domains, controls PBAN-mediated sex pheromone biosynthesis activation in the pheromone glands of many female moths. To better facilitate the development of novel methods for controlling moth mating, we have initiated a number of studies examining the structure, function and interaction of PBANR and PBAN [1–3]. Solubilization of PBANR from the cell membrane is a critical first step for structural and biochemical analyses. A fluorescent PBANR enhanced green fluorescent protein (PBANR-EGFP) chimera localizes to the cell membrane of expresSF+ insect cells. However, a non-negligible portion of the chimera remains associated with the membrane fraction after protein solubilization at ambient pressure (AP) with 1.0% (w/v) DDM, a mild nonionic detergent commonly used for the solubilization of GPCRs [4]. This incomplete solubilization indicates that more efficient methods of extracting membrane proteins such as GPCRs from the cell membrane are required.

Hydrostatic pressure (HP), like temperature, is an important thermodynamic parameter that can profoundly influence molecular systems. While HP is usually regarded as constant and its influence is ignored in many biochemical experiments, the physicochemical effects of high hydrostatic pressure (HHP) on biomolecules, cells and tissues have been studied [5–7]. For example, HHP has been utilized in the solubilization of membrane-associated protein kinase C without detergent [8], and in the preparation of various proteins, lipids and nucleic acids [9]. In addition to static HHP treatment, dynamic HHP treatments,

Abbreviations: AEBSF, 4-(2-aminoethyl)benzensulfonyl fluoride; AP, ambient pressure; CBB, Coomassie Brilliant Blue; CHS, cholesteryl hemisuccinate; CPM, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide; DDM, *n*-dodecyl- β-D-maltopyranoside; EGFP, enhanced green fluorescent protein; FSEC, fluorescence-detection size-exclusion chromatography; GPCR, G protein-coupled receptor; HHP, high hydrostatic pressure; HP, hydrostatic pressure; K_d, dissociation constant; mKO, monomeric Kusabira Orange; NDSB, non-detergent sulfobetain; PBAN, pheromone biosynthesis-activating neuropeptide; PBANR, pheromone biosynthesis-activating neuropeptide receptor; PI, protease inhibitor; RR, Rhodamine Red-X; S.D., standard deviation; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TEV, tobacco etch virus; TM, transmembrane; TtFbpB, *Thermus thermophilus* ferric ion-binding protein B subunit.

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such as pressure cycling, have been developed and utilized for diverse biochemical approaches [9].

To investigate the effect of HHP on the solubilization of cell membrane proteins with detergent, we used various concentrations of DDM to solubilize PBANR-EGFP under HHP (200 MPa) and AP (0.1 MPa), albeit only at room temperature due to the specifications of our instruments. The extent of PBANR-EGFP solubilization from the membrane fraction was greater (~1.5-fold increase) following 1 h at 200 MPa than at 0.1 MPa. Furthermore, fluorescence-detected size-exclusion chromatography (FSEC) revealed that the PBANR-EGFP solubilized at 200 MPa for 1–6 h retained ligand-binding ability, and that a greater percentage of PBANR-EGFP solubilized at 200 MPa co-eluted with a rhodamine red-labeled PBAN derivative (RR-C10PBAN) than that solubilized at 0.1 MPa.

2. Materials and methods

2.1. Protein construction and expression

The *Bombyx mori* PBANR used in this study was modified such that the amino and carboxyl ends were truncated by 27 and 60 residues respectively and the third intracellular loop was replaced with T4 lysozyme. To facilitate detection and purification, a TEV protease cleavage site followed by EGFP and a 10× His-tag were fused to the shortened PBANR carboxyl terminus. This chimeric fusion protein is referred to as PBANR-EGFP in this paper. A recombinant baculovirus harboring the above PBANR-EGFP coding sequence was constructed using the Bac-to-Bac Baculovirus Expression System (Life Technologies). The expression of PBANR-EGFP was performed as follows: (1) expresSF+ cells were grown in SF-900III medium (Life Technologies) to a density of ~$2 * 10^6$ cells/ml; (2) the cells were infected with the recombinant baculovirus; (3) the infected cells were incubated at 27 °C, 110 rpm for 3 d; (4) the cells were collected by centrifugation at 3000 ×*g* at 4 °C for 10 min; and (5) the cell pellets were weighed and stored at −80 °C.

The transmembrane subunit of the bacterial iron transporter, *Thermus thermophilus* FbpB (TtFbpB), C-terminally fused with mKO (an orange fluorescent protein) and an 8× His-tag, was expressed in *Escherichia coli* C41(DE3) (Lucigen) using pBAD-His plasmid (Invitrogen) in the presence of 0.02% (w/v) L-arabinose at 25 °C for 24 h. The cells were collected by centrifugation at 6000 ×*g* at 4 °C for 20 min and stored at −80 °C.

2.2. Preparation of membrane suspension

The insect cells were suspended in ice-cold cell disrupting buffer [10 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, 1 mM EDTA, 500 U/ml DNase I and protein inhibitors (PIs) (100 mM AEBSF, 80 μM aprotinin, 1.5 mM E-64, 2 mM leupeptin, 5 mM bestatin and 1 mM pepstain A in DMSO at a final concentration of 1.0% (v/v))], and disrupted with a Multi-Beads Shocker cell disruptor (Yasui Kikai). The resulting disrupted membranes were washed four times with cold wash buffer [1.0 M NaCl, 10 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl and 1 mM EDTA], and once with cold membrane suspension buffer [0.5 M NaCl and 25 mM HEPES-NaOH (pH 7.5)].

The *E. coli* cells were suspended in ice-cold buffer for *E. coli* [20 mM Tris-HCl (pH 8.0) supplemented with 0.6 mg/ml lysozyme, 500 U/ml DNase I, 1 mM EDTA and PIs as before], and disrupted with the same equipment above. The disrupted membranes were washed four times with cold 150 mM NaCl without additives.

The respective membrane suspensions were dispensed in 200-μl aliquots and stored at −80 °C. The amount of PBANR-EGFP or TtFbpB-mKO in each aliquot of the membrane suspension was estimated to be ~20 μg.

2.3. Solubilization of membrane protein with HHP treatment

In each membrane protein solubilization experiment, solubilization buffer (200 μl) was added to the thawed 200-μl membrane suspension. Solubilization buffers for PBANR-EGFP consisted of 0.5 M NaCl, 50 mM HEPES-NaOH (pH 7.5), 40% (v/v) glycerol, 200 μM C10PBAN (Ser-Arg-Thr-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2, chemically synthesized, GenScript), 0.04, 0.4, 4% DDM and 0.004, 0.04, 0.4% CHS, respectively (initial experiments) or 0.4, 0.8, 1.2, 1.6, 2.0% DDM and 0.2% CHS (subsequent experiments), whereas TtFbpB-mKO solubilization buffers consisted of 300 mM NaCl, 40 mM Tris-HCl (pH 8.0) and 0, 0.4, 0.8, 1.2, 1.6, 2.0% DDM. All DDM concentrations are presented as w/v. Each sample was loaded into a 1-ml syringe (Terumo) without air, sealed, and placed in a PreEMT M150 pressure chamber (BaroFold) for HHP treatment without mixing at room temperature (Fig. 1). The hydrostatic pressure in the chamber was raised to 200-MPa over 10 min, maintained at 200-MPa for 1, 3, 6 or 16 h at room temperature, and then returned to AP. The pressure was incrementally lowered in 25-MPa steps with a 5-min pause between each reduction. As a control, an aliquot of the same sample was maintained at AP for an equivalent period at room temperature. Immediately after HHP treatment, each sample was subjected to ultra-centrifugation at 100,000 g at 4 °C for 10 min, and the resulting supernatant collected.

2.4. Quantification of PBANR-EGFP and TtFbpB-mKO

Solubilized membrane proteins were immediately analyzed by SDS-PAGE without heat treatment. Fluorescent gel bands were analyzed on a LAS 4000 imager (GE Healthcare) and quantification was performed with Multi Gauge software (Fujifilm). After fluorescence detection, the SDS-PAGE gels were stained with Bio-Safe Coomassie Stain (Bio—Rad).

2.5. Evaluation of the HHP effect on PBANR-EGFP or TtFbpB-mKO solubilization

To evaluate the effect of HHP on PBANR-EGFP or TrFbpB-mKO solubilization, we used two indices: (1) the percentage of solubilized target protein, and (2) the fold-gain in solubilization efficiency with HHP. The first index is defined as the amount of target proteins divided by the amount of total protein expressed in the cell membrane. The fold-gain in solubilization efficiency is defined as the amount of solubilized target

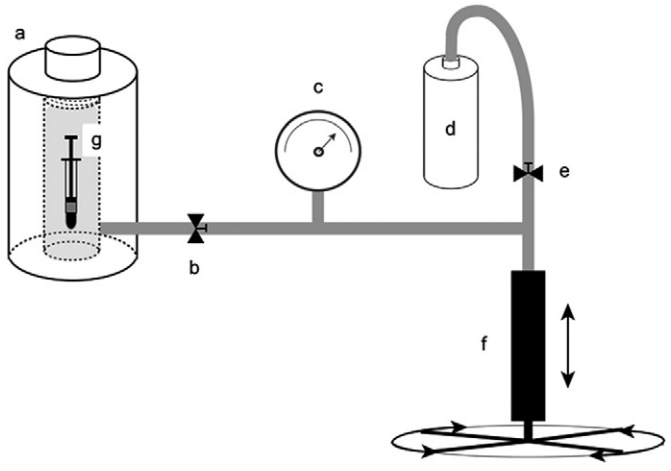

Fig. 1. HHP generation system. PreEMT M150 pressure chamber (BaroFold) is composed of 6 components. a) pressure vessel, b) chamber valve, c) gauge, d) water reservoir, e) intake valve, f) pressure generator. The sample was loaded into a 1-ml syringe without air. The syringe was sealed and placed in the pressure vessel. The lid of the pressure vessel was tightened and the system was filled with water. High hydrostatic pressure was generated by manually rotating the pressure generator (f) and pressure was monitored with the gauge (c).