Involvement of β-adrenergic receptor in synaptic vesicle swelling and implication in neurotransmitter release

Zhi Hui Chen, Jin-Sook Lee, Leah Shin, Won Jin Cho, Bhanu P. Jena*

Department of Physiology, Wayne State University School of Medicine, Detroit, MI, USA

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Abstract

Secretory vesicle swelling is required for vesicular discharge during cell secretion. The Gαo-mediated water channel aquaporin-6 (AQP-6) involvement in synaptic vesicle (SV) swelling in neurons has previously been reported. Studies demonstrate that in the presence of guanosine triphosphate (GTP), mastoparan, an amphiphilic tetradecapeptide from wasp venom, activates Gαo protein GTPase, and stimulates SV swelling. Stimulation of G proteins is believed to occur via insertion of mastoparan into the phospholipid membrane to form a highly structured α-helix that resembles the intracellular loops of G protein-coupled adrenergic receptors. Consequently, the presence of adrenoceptors and the presence of an endogenous β-adrenergic agonist at the SV membrane is suggested. Immunoblot analysis of SV using β-adrenergic receptor antibody, and vesicle swelling experiments using β-adrenergic agonists and antagonists, demonstrate the presence of functional β-adrenergic receptors at the SV membrane. Since a recent study shows vH+-ATPase to be upstream of AQP-6 in the pathway leading from Gαo-mediated swelling of SV, participation of an endogenous β-adrenergic agonist, in the binding and stimulation of its receptor to initiate the swelling cascade is demonstrated.

Keywords: synaptic vesicle swelling • β-adrenergic receptor • photon correlation spectroscopy • atomic force microscopy

Introduction

In the past decade, the dynamics of intracellular membrane-bound secretory vesicles ranging in size from 0.2 to 1.2 μm in pancreatic acinar cells called zymogen granules (ZGs) [1–5], to the 35–50 nm synaptic vesicle (SV) in neurons [6], have been extensively studied, providing some of the molecular processes involved in secretory vesicle swelling, and its requirement in the discharge of intravesicular contents during cell secretion [5]. Live pancreatic acinar cells in near physiological buffer, when imaged using atomic force microscopy (AFM), reveal at nanometre resolution the size of ZGs lying immediately below the surface of the apical plasma membrane. Within 2.5 min. of exposure to a secretory stimulus, majority of ZGs within cells swell, followed by a decrease in ZG size and a concomitant release of secretory products. These studies directly demonstrated intracellular swelling of secretory vesicles following stimulation of cell secretion in live cells, and vesicle deflation following partial discharge of vesicular contents. A similar mechanism of SV swelling for neurotransmitter release has also been demonstrated [5, 6]. This direct measure of vesicle size dynamics, at nanometre resolution under various experimental conditions, has enabled the determination of some of the molecular mechanisms of secretory vesicle swelling. AFM and photon correlation spectroscopy (PCS) have been greatly instrumental in these earlier studies.

Mastoparan, the amphiphilic tetradecapeptide from wasp venom, has been demonstrated to activate the GTPase activity of Gαo proteins [7–9]. Stimulation of G proteins is believed to occur via insertion of mastoparan into the phospholipid membrane to form a highly structured α-helix that resembles the intracellular loops of G protein-coupled adrenergic receptors. Consequently, the presence of adrenoceptors and the presence of an endogenous β-adrenergic agonist at the SV membrane is suggested. Immunoblot analysis of SV using β-adrenergic receptor antibody, and vesicle swelling experiments using β-adrenergic agonists and antagonists, demonstrate the presence of functional β-adrenergic receptors at the SV membrane. Since a recent study shows vH+-ATPase to be upstream of AQP-6 in the pathway leading from Gαo-mediated swelling of SV, participation of an endogenous β-adrenergic agonist, in the binding and stimulation of its receptor to initiate the swelling cascade is demonstrated.

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*Correspondence to: Bhanu P. JENA, Department of Physiology, Wayne State University School of Medicine, 5245 Scott Hall, 540 E. Canfield, Detroit, MI 48201, USA.
Tel.: 313-577-1532
Fax: 313-993-4177
E-mail: bjena@med.wayne.edu

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and is involved in Mas7 and GTP induced swelling. Furthermore, the presence of β-adrenergic receptors in neuronal tissue [11] and the involvement of vH^-ATPase in Gαo-mediating SV swelling have been reported [12]. Results from the Shin et al. study [12] demonstrate that the GTP-Gαo-mediated vesicle swelling is vH^-ATPase dependent, and pH sensitive. A bafilomycin-sensitive acidification of isolated SV following GTP-Gαo-induced swelling is demonstrated. Since water channels are bidirectional, and the vH^-ATPase inhibitor bafilomycin decreases both the volume of isolated SV and GTP-mastoparan stimulated swelling, suggests vH^-ATPase to act upstream of AQP-6, in the pathway leading from Gαo-stimulated swelling of SV. Vesicle acidification is therefore a prerequisite for AQP-6 mediated gating of water into SV [12]. All of the above findings outlined, led us to believe the presence of adrenoceptors, and endogenous β-adrenergic agonist activity, at the SV membrane. This hypothesis was tested in the present study. In agreement, immunoblot analysis of SV followed by vesicle swelling studies using β-adrenergic agonists and antagonists, demonstrate the presence of functional β-adrenergic receptors at the SV membrane. Since vH^-ATPase activity is upstream of AQP-6 in the pathway leading from Gαo-stimulated swelling of SV, binding of the endogenous β-adrenergic agonist to its receptors at the SV membrane, initiates the swelling cascade.

Materials and methods

Brain homogenate, synaptosome and synaptic vesicle isolation, and their solubilization

Brain homogenates (BH), synaptosomes (SS) and SV were prepared from rat brains using published procedure [5, 6]. From Sprague-Dawley rats weighing 100–150 g, whole brain was isolated and placed in ice-cold buffered sucrose solution (5 mM Hepes pH 7.5, 0.32 M sucrose), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The brain tissue was homogenized using 8–10 strokes in a Teflon-glass homogenizer to obtain total BH. The total homogenate was centrifuged for 3 min. at 2500 × g, and the supernatant fraction was further centrifuged for 15 min. at 14,500 × g, to obtain a pellet. The resultant pellet was resuspended in buffered sucrose solution, and loaded onto a 3–10–23% Percoll gradient. After centrifugation at 28,000 × g for 6 min., the enriched SS fraction was collected at the 10–23% Percoll gradient interface. To isolate SV, the SS preparation was diluted using 9 vol. of ice-cold water, resulting in the lysis of SS to release SV, followed by 30 min. incubation on ice. The homogenate was then centrifuged for 20 min. at 25,500 × g, and the resultant supernatant enriched in SV was obtained. Brain fractions were solubilized using Triton/Lubrol solubilization buffer (0.5% Lubral, 0.5% TritonX-100) containing 5% protease inhibitors, and incubated for 2 hrs. Protein concentrations in the solubilized fractions were determined by BCA™ protein assay (PIERCE, Rockford, IL, USA).

Transmission electron microscopy

Isolated SS preparation was fixed in 2.5% buffered paraformaldehyde for 30 min., followed by dehydration and embedding in Unicryl resin. The resin-embedded tissue was sectioned at 40–70 nm. Thin sections were transferred to coated specimen transmission electron microscopy (TEM) grids, dried in the presence of uranyle acetate and methylcellulose, and examined in a JOEL transmission electron microscope (JOEL USA, Inc., Peabody, MA, USA).

Atomic force microscopy

Isolated SS or SV in buffer, was plated on freshly cleaved mica, to be imaged using the AFM. Ten minutes after plating, the mica disk was placed in a fluid chamber and washed with the incubation buffer to remove unattached SS and or SV, prior to imaging. Isolated SS and SV, were imaged using the Nanoscope Illa, Digital Instruments (Santa Barbara, CA, USA). All images presented in this study were obtained in the ‘tapping’ mode in fluid, using silicon nitride tips with a spring constant of 0.06 Nm⁻¹ and an imaging force of less than 200 pN. Images were obtained at line frequencies of 2.523 Hz, with 512 lines per image and constant image gains.

Immunoblotting analysis

Solubilized BH, SS and SV fractions were mixed in Laemmli sample preparation buffer and boiled for 1 min. prior to resolving using 10% SDS-PAGE. Resolved proteins were electrotransferred to nitrocellulose membranes, and incubated for 1 hr at 4°C in blocking buffer (containing 5% non-fat milk in phosphate-buffered saline with 0.1% Tween-20 and 0.02%NaNO₃). After blocking, the nitrocellulose membrane was immunoblotted for 1 hr at room temperature with primary antibodies β2AR (1:1000), AQP-6 (1:200), Gαo (1:600) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and VAMP-2 (1:200) (Sigma). Primary antibodies were diluted in PBST washing buffer (phosphate-buffered saline with 0.1% Tween-20 and 0.02%NaNO₃). After washing three times with PBST, the nitrocellulose membrane was incubated for 1 hr at room temperature in goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 in PBST buffer. The immunoblots were then washed ×3 in PBST buffer, processed for enhanced chemiluminescence (GE Healthcare Biosciences Corp., Piscataway, NJ, USA) and imaged using a Kodak 440 image station (Carestream Health, Inc., Rochester, NY, USA).

Immunoprecipitation

The solubilized SV proteins were incubated with primary antibody of Gαo-protein or β2AR and protein A/G agarose beads (Santa Cruz Biotechnology) for 1 hr at 4°C. The pellet containing the isolated Gαo complex was collected after centrifugation at 2000 × g for 2 min. followed by ×3 washing with PBST buffer. The pellet was incubated with Laemmli sample preparation buffer for 1 hr at room temperature, following which, the supernatant containing the immunoprecipitated complex was resolved using a 10% SDS-PAGE. Following electro-transfer procedure, nitrocellulose membranes were probed separately with Gαo-protein primary antibody and the β2AR primary antibody. The immunoblots were then washed ×3 in PBST buffer, processed for enhanced chemiluminescence (GE Healthcare Biosciences Corp.) and imaged using a Kodak 440 image station (Carestream Health, Inc.).

Measurements of synaptic vesicle size

Changes in SV size were determined using PCS. PCS is a well-known technique for the measurement of size of micrometre to nanometre size
particles and macromolecules. PCS measurements were performed in a Zetasizer Nano ZS, (Malvern Instruments Ltd., Worcestershire, UK). In a typical experiment, the size distribution of isolated SV was determined using built-in software provided by Malvern Instruments Ltd. Prior to determination of the vesicle hydrodynamic radius, calibration of the instrument was performed with latex spheres of known size. In PCS, subtle fluctuations in the sample scattering intensity are correlated across microsecond time scales. The correlation function is calculated, from which the diffusion coefficient is determined. Using Stokes–Einstein equation, hydrodynamics radius can be acquired from the diffusion coefficient [8]. The intensity size distribution, which is obtained as a plot of the relative intensity of light scattered by particles in various size classes, is then calculated from a correlation function using built-in software. The particle scattering intensity is proportional to the molecular weight squared. Volume distribution can be derived from the intensity distribution using Mie theory [9, 10]. The transforms of the PCS intensity distribution to volume distributions can be obtained using the provided software by Malvern Instruments Ltd. In experiments, isolated SV were suspended in isotonic buffer containing 0.3 M sucrose, 10 mM Hepes pH 7.5 and 20 mM KCl, and changes in vesicle size monitored prior to and after addition of 40 μM GTP mastoparan, or various concentrations of β2AR agonist isoproterenol, or antagonist alprenolol (Sigma). Student’s t-test was performed for comparison between groups (n = 6) with significance established at P < 0.05(*) and P < 0.01(**).

**Results and discussion**

Electron microscopy (Fig. 1A), atomic force microscopy (Fig. 1B and C), and immunoblot analysis (Fig. 1D), demonstrated a highly enriched SS and SV preparation. Immunoblot analysis demonstrated the SV preparation to be enriched in VAMP-2 and AQP-6 (Fig. 1D), both SV-specific proteins [6]. Additionally, in conformation with earlier findings [6], the GTP-binding Gαo protein was found to associate with the SV preparation. Collectively, these studies demonstrate the isolation of a highly enriched SV preparation from rat brain tissue, for our SV swelling and β2AR immunolocalization assays. To determine the relative concentration of β2AR in SV, immunoblot analysis was performed with 10 μg each of total BH, isolated SS, and SV fractions (Fig. 1D). In conformation with earlier findings [11], β2AR was present both in the BH and the SS fraction. However, for the first time, and in conformation with our hypothesis, β2AR was present and enriched in the SV fraction (Fig. 1D).

Upon binding to endogenous activators, adrenergic receptors undergo a conformational change that leads to the activation of heterotrimeric GTP-binding proteins [13]. Different groups of
adrenergic receptors couple to and activate only certain G protein types, thus leading to specific intracellular signals [13]. Our immunoblot assays demonstrate the enriched presence of \( \beta_2 \) adrenergic receptors in the SV fraction; therefore, the physical interaction of SV-associated Go\( \alpha \) and \( \beta_2 \) adrenergic receptors was investigated. To determine the co-association of Go\( \alpha \) and \( \beta_2 \)AR at the SV, immunoprecipitation was employed. Detergent-solubilized SV preparation was immunoprecipitated using \( \beta_2 \)AR-specific antibody, and the resultant isolate was then probed with the Go\( \alpha \)-specific antibody, the 68 kD Go\( \alpha \) protein is co-isolated. The dark band represented by (\( * \)) is the heavy chain of the antibody used in immunoprecipitation reaction.

The functional state of the \( \beta_2 \)AR in SV, and its role in regulating SV swelling, was determined using PCS, in the presence and absence of both \( \beta_2 \)-agonist (isoproterenol) and antagonist (alprenolol). Conforming with earlier studies [6], exposure of SV to 40 \( \mu M \) GTP and 40 \( \mu M \) mastoparan results in a significant increase in SV size. However, exposure of SV to both \( \beta_2 \)AR antagonist alprenolol (APN), demonstrates inhibition of the GTP-mastoparan mediated SV swelling. Note the significant (\( n = 6, \cdot \cdot \cdot P < 0.001 \)) inhibition of GTP-mastoparan mediated SV swelling in the presence of 10 nM APN. Exposure of the 10 nM APN-treated SV to the \( \beta_2 \)AR agonist isoproterenol (ISO), demonstrated a dose-dependent increase (\( n = 6, \cdot \cdot \cdot P < 0.05 \)) in SV size.

These studies demonstrate for the first time that functional \( \beta_2 \) adrenergic receptors, and endogenous ligands, are associated with SV, regulating vesicle swelling. Since \( vH^{-} \)-ATPase activity is upstream of AQP-6 in the pathway leading from Go\( \alpha \)–stimulated swelling of SV, binding of the endogenous \( \beta \)-adrenergic agonist to

Fig. 2 Interaction of \( \beta_2 \) adrenergic receptor with Go\( \alpha \) protein in SV. Detergent-solubilized SV preparation immunoprecipitated using \( \beta_2 \)AR-specific antibody, results in co-isolation of the 45 kD Go\( \alpha \) protein, suggesting their physical association in SV. Similarly, when detergent-solubilized SV preparation was immunoprecipitated using Go\( \alpha \)-specific antibody and the resultant isolate was then probed with the \( \beta_2 \)AR-specific antibody, the 68 kD \( \beta_2 \)AR protein is co-isolated.}

Fig. 3 The \( \beta_2 \) adrenergic receptors in SV are functional. PCS used in the determination of SV size. Exposure of SV to 40 \( \mu M \) GTP and 40 \( \mu M \) mastoparan results a significant increase in SV size (control). However, exposure of SV to the \( \beta_2 \)AR antagonist alprenolol (APN), demonstrates inhibition of the GTP-mastoparan mediated SV swelling. Note the significant (\( n = 6, \cdot \cdot \cdot P < 0.001 \)) inhibition of GTP-mastoparan mediated SV swelling in the presence of 10 nM APN. Exposure of the 10 nM APN-treated SV to the \( \beta_2 \)AR agonist isoproterenol (ISO), demonstrated a dose-dependent increase (\( n = 6, \cdot \cdot \cdot P < 0.05 \)) in SV size.
its receptors at the SV membrane, initiates the swelling cascade (Fig. 4). These findings are a significant advancement in our understanding of the molecular regulation of neurotransmitter release and its many possible biomedical applications.

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