Journal of Neuroscience Research 88:95–101 (2010)

Involvement of vH^{+}-ATPase in Synaptic Vesicle Swelling

Leah Shin,[1] Nirukti Basi,[1] Aleksandar Jeremic,[1] Jin-Sook Lee,[1] Won Jin Cho,[1] ZhiHui Chen,[1] Rania Abu-Hamdah,[1] David Oupicky,[2] and Bhanu P. Jena[1*]

[1]Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan
[2]Department of Pharmaceutical Science, Wayne State University, Detroit, Michigan

Secretory vesicle swelling is central to cell secretion, but the underlying mechanism of vesicle swelling, particularly synaptic vesicles, is not completely understood. The $G_{\alpha i3}$-PLA2-mediated involvement of water channel AQP-1 in the regulation of secretory vesicle swelling in exocrine pancreas and the $G_{\alpha o}$-mediated AQP-6 involvement in synaptic vesicle swelling in neurons have previously been reported. Furthermore, the role of vH^{+}-ATPase in neurotransmitter transport into synaptic vesicles has also been shown. Using nanometer-scale precision measurements of isolated synaptic vesicles, the present study reports for the first time the involvement of vH^{+}-ATPase in GTP-$G_{\alpha o}$-mediated synaptic vesicle swelling. Results from this study demonstrate that the GTP-$G_{\alpha o}$-mediated vesicle swelling is vH^{+}-ATPase dependent and pH sensitive. Zeta potential measurements of isolated synaptic vesicles further demonstrate a bafilomycin-sensitive vesicle acidification, following the GTP-$G_{\alpha o}$-induced swelling stimulus. Water channels are bidirectional and the vH^{+}-ATPase inhibitor bafilomycin decreases both the volume of isolated synaptic vesicles and GTP-mastoparan stimulated swelling, suggesting that vH^{+}-ATPase is upstream of AQP-6, in the pathway leading from $G_{\alpha o}$-stimulated swelling of synaptic vesicles. Vesicle acidification is therefore a prerequisite for AQP-6-mediated gating of water into synaptic vesicles. © 2009 Wiley-Liss, Inc.

Key words: synaptic vesicle swelling; vH^{+}-ATPase; AQP6; photon correlation spectroscopy; atomic force microscopy

Earlier studies demonstrated the requirement of secretory vesicle swelling in cell secretion (Kelly et al., 2004). Although recent studies (Jeremic et al., 2005) provide much progress in our understanding of synaptic vesicle swelling, molecular details underlying the process remain to be fully elucidated. The first direct measurement of secretory vesicle swelling at nanometer resolution within live cells was reported in studies on pancreatic acinar cells, using atomic force microscopy (AFM; Cho et al., 2002). In neurons, the water channel AQP-6 and the heterotrimeric GTP-binding protein $G_{\alpha o}$ at the synaptic vesicle membrane participate in rapid water gating and vesicle swelling (Jeremic et al., 2005). It has previously been shown that vH^{+}-ATPases are present at the synaptic vesicle membrane (Stadler and Tsukita, 1984; Hicks and Parsons, 1992) and are responsible for the generation of electrochemical H^{+} gradient (pH 5.2–5.5) within vesicles (Michaelson and Angel, 1980; Füldner and Stadler, 1982), required for transport of neurotransmitters into the vesicle lumen. In addition to the established role of vH^{+}-ATPase in neurotransmitter transport into synaptic vesicles, vH^{+}-ATPase has been suggested to participate in the secretion of the stored neurotransmitters (Morel et al., 2001; Peters et al., 2001). Furthermore, guanine nucleosides have been reported to influence the glutamate-induced cellular response via diverse trophic, proliferative, and modulatory effects of the nucleotide on neurons (Santos et al., 2005). Because synaptic vesicle swelling is $G_{\alpha o}$ mediated and is required for cell secretion (Kelly et al., 2004), the vH^{+}-ATPase in synaptic vesicle membrane (Stadler and Tsukita, 1984; Hicks and Parsons, 1992) may participate in $G_{\alpha o}$-mediated water gating through the AQP-6 channels at the synaptic vesicle membrane, resulting in vesicle swelling. This hypothesis was tested in the present study. In agreement, our results demonstrate that the synaptic vesicle-associated vH^{+}-ATPase is required for GTP-$G_{\alpha o}$-mediated swelling of synaptic vesicles.

Mastoparan, the amphiphilic tetradecapeptide from wasp venom, has been demonstrated to activate the GTPase activity of $G_{\alpha o/i}$ proteins (Higashijima et al., 1988; Vitale et al., 1993; Konrad et al., 1995). Stimulation

The first two authors contributed equally to this work.

Aleksandar Jeremic's current address is Department of Biological Sciences, George Washington University, 340 Lisner Hall, 2023 G Street NW, Washington, DC 20052.

Contract grant sponsor: NIH; Contract grant number: NS39918; Contract grant sponsor: Wayne State University Research Enhancement Program (to B.P.J).

*Correspondence to: Bhanu P. Jena, Department of Physiology, Wayne State University School of Medicine, 5245 Scott Hall, 540 E. Canfield, Detroit, MI 48201. E-mail: bjena@med.wayne.edu

Received 25 February 2009; Revised 14 April 2009; Accepted 24 May 2009

Published online 16 July 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.22180

© 2009 Wiley-Liss, Inc.

of G proteins is believed to occur by the peptide inserting into the phospholipid membrane and mimicking a highly structured helix that resembles the intracellular loops of G-protein-coupled receptors. Analogously to receptor activation, mastoparan is thought to interact with the COOH-terminal domain of the G-protein subunit (Weingarten et al., 1990). In the current study, GTP-mastoparan stimulated synaptic vesicle swelling, which was abrogated in the presence of the vesicular proton pump inhibitor bafilomycin. Isolated synaptic vesicles exposed to various concentrations of ATP had no influence on GTP-mastoparan-stimulated activity (data not shown). The possible reason for this may be that ATP, being a neurotransmitter and present in synaptic vesicles, is there to be utilized by the vH^+-ATPase following stimulation. Our study further demonstrates that the GTP-mastoparan-stimulated swelling of synaptic vesicle is pH sensitive. Because water channels are bidirectional, and bafilomycin exposure decreases the volume of isolated synaptic vesicles, this finding suggests that vH^+-ATPase function is upstream of the water channel AQP-6, in the GTP-$G_{\alpha o}$-mediated pathway. Hence it is inferred from these results that synaptic vesicle acidification is required for AQP-6-mediated gating of water.

MATERIALS AND METHODS

Synaptosome and Synaptic Vesicle Isolation

Synaptosomes and synaptic vesicles were prepared from rat brains using published procedures (Kelly et al., 2004; Jeremic et al., 2005). Whole brain from Sprague-Dawley rats, weighing 100–150 g, 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). The brain tissue was homogenized using eight to ten strokes in a Teflon-glass homogenizer. The total homogenate was centrifuged for 3 min at 2,500*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 synaptosome fraction was collected at the 10–23% Percoll gradient interface. To isolate synaptic vesicles, the synaptosome preparation was diluted using 9 vol of ice-cold water, resulting in the lysis of synaptosomes to release synaptic vesicles, followed by 30 min of incubation on ice. The homogenate was then centrifuged for 20 min at 25,500*g*, and the resultant supernatant enriched in synaptic vesicles was obtained.

Transmission Electron Microscopy

Isolated synaptic vesicle preparations were 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 TEM grids, dried in the presence of uranyl acetate and methylcellulose, and examined in a JOEL transmission electron microscope.

Synaptic Vesicle Size Measurements Using Photon Correlation Spectroscopy

Changes in synaptic vesicle size were determined using photon correlation spectroscopy (PCS). PCS is a well-known technique for the size measurement of micrometer- to nanometer-size particles and macromolecules. PCS measurements were performed in a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, United Kingdom). In a typical experiment, the size distribution of isolated synaptic vesicles was determined with built-in software provided by Malvern Instruments. Prior to determination of the vesicle hydrodynamic radius, calibration of the instrument was performed using latex spheres of known size. In PCS, subtle fluctuations in the sample scattering intensity are correlated across microsecond time scales. The correlation function was calculated, from which the diffusion coefficient was determined. By using the Stokes-Einstein equation, hydrodynamics radius can be acquired from the diffusion coefficient (Higashijima et al., 1988). 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 (Weingarten et al., 1990; Vitale et al., 1993). The transforms of the PCS intensity distribution to volume distributions can be obtained using the provided software by Malvern Instruments. In experiments, isolated synaptic vesicles 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 were monitored prior to and after addition of 40 μM GTP-mastoparan and/or 1 nM bafilomycin. Student's *t*-test was performed for comparison between groups (n = 5), with significance established at $*P < 0.05$.

Measurements of Synaptic Vesicle Size Using Right-Angle Light Scattering

Similarly to PCS, isolated synaptic vesicles were suspended in isotonic buffer (0.3 M sucrose, 10 mM Hepes, pH 7.5, and 20 mM KCl) and changes in vesicle size monitored prior to and following the addition of 40 μM GTP-mastoparan and/or 1 nM bafilomycin. Synaptic vesicle size dynamics were determined using real-time right-angle light scattering, in a Hitachi F-2000 spectrofluorimeter. Scattered light intensities at 600 nm were measured as a function of vesicle radius (Cho et al., 2004). Values are expressed in arbitrary units and as percentage light scattered over controls. Student's *t*-test was performed for comparison between groups (n = 5), with significance established at $*P < 0.05$.

Synaptic Vesicle Acidification Determined From Zeta Potential Measurements

Zeta potential is the overall surface charge a particle acquires in a certain media. Hence, in the case of liposomes or isolated synaptic vesicles in aqueous media, the zeta potential is a direct reflection of both the internal and the external pH of vesicles. If more alkali buffer is added to the vesicle suspension, then the vesicles acquire more negative charge. In

contrast, if acid is added to the suspension, the vesicles acquire less negative charge. This implies that the pH of the buffer both within and outside the vesicle dictates the zeta potential or the net surface charge of the vesicle. Experiments were performed on isolated synaptic vesicles suspended in isotonic buffer containing 0.3 M sucrose, 10 mM Hepes, pH 7.5, and 20 mM KCl, and changes in vesicle zeta potential were monitored prior to and after addition of 40 μM GTP-mastoparan and/or 1 nM bafilomycin. Zeta potential was determined using the Zetasizer Nano ZS from Malvern Instruments. Student's *t*-test was performed for comparison between groups (n = 8), with significance established at $*P < 0.001$.

Atomic Force Microscopy

Isolated synaptosome membranes or synaptic vesicles in buffer were plated on freshly cleaved mica, to be imaged using the atomic force microscope (AFM). Ten minutes after plating, the mica disk was placed in a fluid chamber and washed with the incubation buffer to remove unattached membrane or synaptic vesicles, prior to imaging in the presence or absence of 40 μM GTP-mastoparan and/or 1 nM bafilomycin. Isolated synaptosome membrane and synaptic vesicles were imaged using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). All images presented here 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. Topographic dimensions of synaptic vesicles were analyzed using the Nanoscope IIIA 4.43r8 software supplied by Digital Instruments.

Immunoblot Analysis

Protein contents in the various brain fractions were determined by the Bradford (1976) method. Sample aliquots solubilized in Laemmli (1970) sample preparation buffer were resolved using 12.5% SDS-PAGE. Five micrograms protein each from total brain homogenate, synaptosome, and synaptic vesicle fractions were resolved using SDS-PAGE. Resolved proteins were electrotransferred to nitrocellulose membrane for immunoblot analysis with specific antibodies to VAMP-2 (Alomone, Jerusalem, Israel), vH$^+$-ATPase, and AQP-6 from the Aquaporins1–9 Kit (Alpha Diagnostic, San Antonio, TX). The nitrocellulose membranes electrotransferred with the resolved proteins were incubated for 1 hr at 4°C in blocking buffer (5% nonfat milk in PBS containing 0.1% Triton X-100 and 0.02% NaN_3) and immunoblotted for 1 hr at room temperature with the specific antibody. Primary antibodies were used at a dilution of 1:3,000 (VAMP-2; or 1:1,000 AQP-6 and vH$^+$-ATPase) in blocking buffer. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02% NaN_3 and incubated for 1 hr at room temperature in horseradish peroxidase-conjugated secondary antibody at a dilution of 1:3,000 in blocking buffer. The immunoblots were then washed in PBS buffer, processed for enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ), and developed with a a Kodak 440 image station.

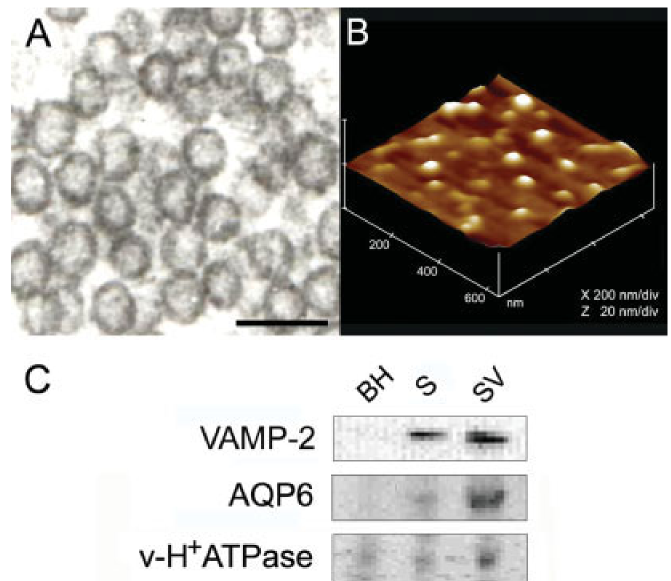

Fig. 1. Association of vH$^+$-ATPase with synaptic vesicles. Purity of synaptic vesicles was determined by using transmission electron microscopy (**A**), atomic force microscopy (**B**), and immunoblot analysis (**C**) on isolated synaptic vesicles. Both electron and atomic force micrographs demonstrate the average size of synaptic vesicles to be 40 nm, which is further confirmed by photon correlation spectroscopy in Figure 2. Immunoblot analysis of 5 μg protein each of total rat brain homogenate (BH), synaptosome (S), and synaptic vesicles (SV) demonstrates the enriched presence of SV proteins VAMP-2 and the water channel AQP6. Note the enriched presence of vH$^+$-ATPase in the SV fraction. Scale bar = 100 nm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS AND DISCUSSION

Electron microscopy (EM; Fig. 1A), atomic force microscopy (AFM; Fig. 1B), immunoblot analysis (Fig. 1C), and photon correlation spectroscopy (PCS; Fig. 2A) demonstrated a highly enriched synaptic vesicle (SV) preparation. Synaptic vesicles were found to have a mean diameter of 35 nm. Immunoblot analysis further demonstrated the SV preparation to be enriched in VAMP-2 and AQP-6 (Fig. 1C), both SV-specific proteins (Jeremic et al., 2005). Collectively, these studies demonstrate the isolation of a highly enriched SV preparation from brain tissue for our SV swelling assays.

To determine the relative concentration of vH$^+$-ATPase in SV, immunoblot analysis was performed with 5 μg each of total brain homogenate (BH), isolated synaptosome (S), and SV fractions (Fig. 1C). In agreement with earlier findings (Stadler and Tsukita, 1984; Hicks and Parsons, 1992), vH$^+$-ATPase was present both in the S and in the SV fraction but was enriched in SV (Fig. 1C). To determine the role of vH$^+$-ATPase in SV swelling, the size of isolated SV was monitored prior to and following addition of the vH$^+$-ATPase inhibitor bafilomycin (Fig. 2A–C,E). Exposure of SV to a bafilomycin concentration as low as 0.5 nM was found to

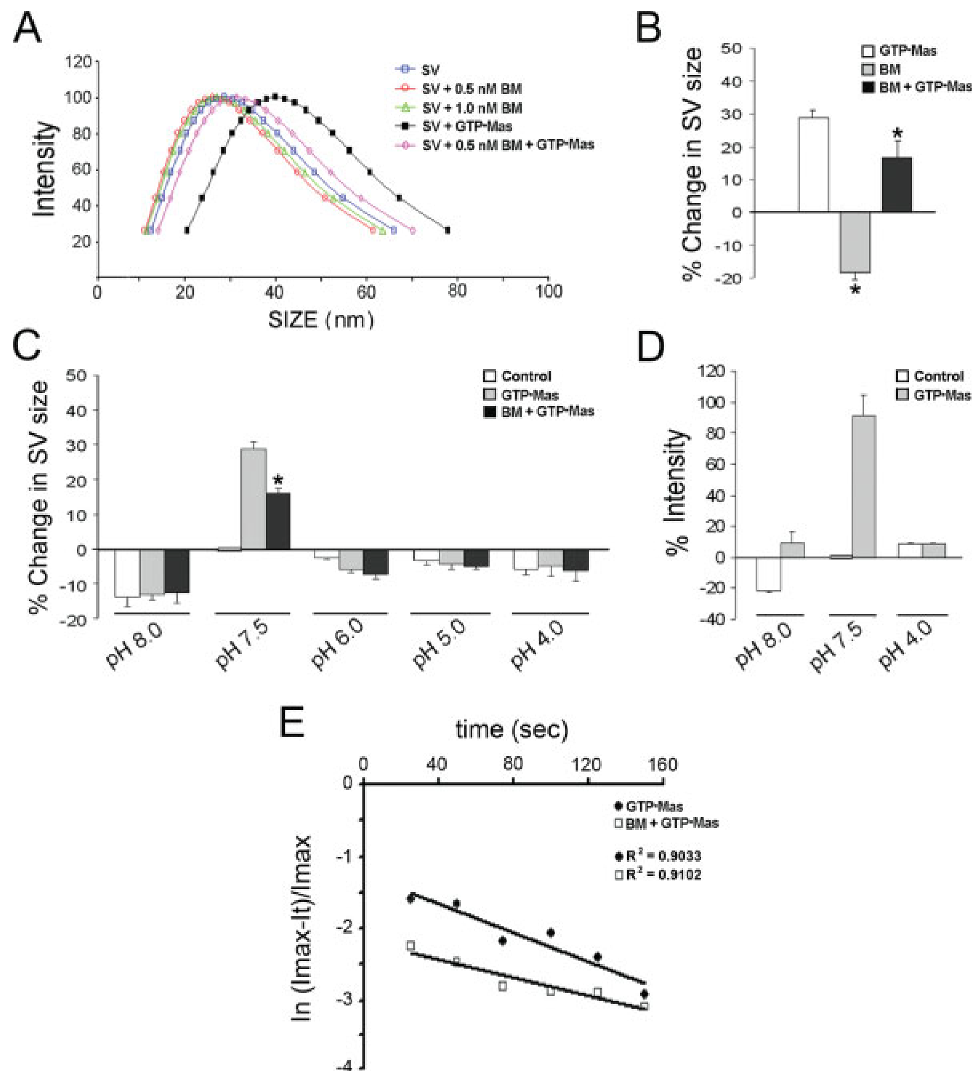

Fig. 2. **A–E:** Guanosine triphosphate-mastoparan (GTP-Mas) induced synaptic vesicle (SV) swelling is both pH and bafilomycin (BM) sensitive. Isolated SVs swell in response to 40 μM GTP-Mas, as demonstrated by using photon correlation spectroscopy (A–C). Similarly, right-angle light scattering also demonstrates an increase in SV size following exposure to the GTP-Mas mixture. Exposure of SV to 0.5 or 1 nM of the vH^+-ATPase inhibitor BM or low pH significantly inhibits GTP-Mas induced vesicle swelling (A–D). Note the loss in SV size following exposure to BM alone (B). The GTP-Mas induced SV swelling is pH sensitive (C), insofar as, except the near physiological pH of 7.5, both alkaline and acidic environments nearly abolish GTP-Mas-induced swelling of SV, as determined both by photon correlation spectroscopy (C) and by dynamic light scattering measurements (D). B–D: n = 5, $*P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibit significantly (~25%, $P < 0.001$) the GTP-mastoparan-stimulated SV swelling. Surprisingly, exposure of unstimulated SV to bafilomycin resulted in an 18–20% ($P < 0.001$) decrease in SV size (Fig. 2B). Because bafilomycin directly inhibits vH^+-ATPase, bypassing the SV membrane-associated $G_{\alpha o}$ protein, the participation of vH^+-ATPase is upstream of AQP-6, in the GTP-$G_{\alpha o}$-stimulated pathway of SV swelling. These results further demonstrate that, in the resting state (unstimulated or steady state), SV volume is in part regulated by the SV membrane-associated water channel AQP-6 and the proton pump vH^+-ATPase. It is highly likely that, at the SV membrane, facilitated water transport into the vesicle via AQP-6 is a consequence of vH^+-ATPase-induced intravesicular acidification.

Earlier studies report that exposure to moderately acidic extracellular pH (pH 6.5) augments plasmalemmal vH^+-ATPase activity in cultured osteoclasts (Nordstrom et al., 1997). In addition, some studies (Bastani et al., 1991, 1994; Chambrey et al., 1994) report that the number of vH^+-ATPase in apical membrane of renal epithelial cells is up-regulated in animals exposed to acidosis (Bastani et al., 1991, 1994; Chambrey et al., 1994). In view of this, the role of pH in vH^+-ATPase activity at the SV membrane and in GTP-$G_{\alpha o}$-mediated SV swelling was explored (Fig. 2C,D). Our studies demonstrate that GTP-

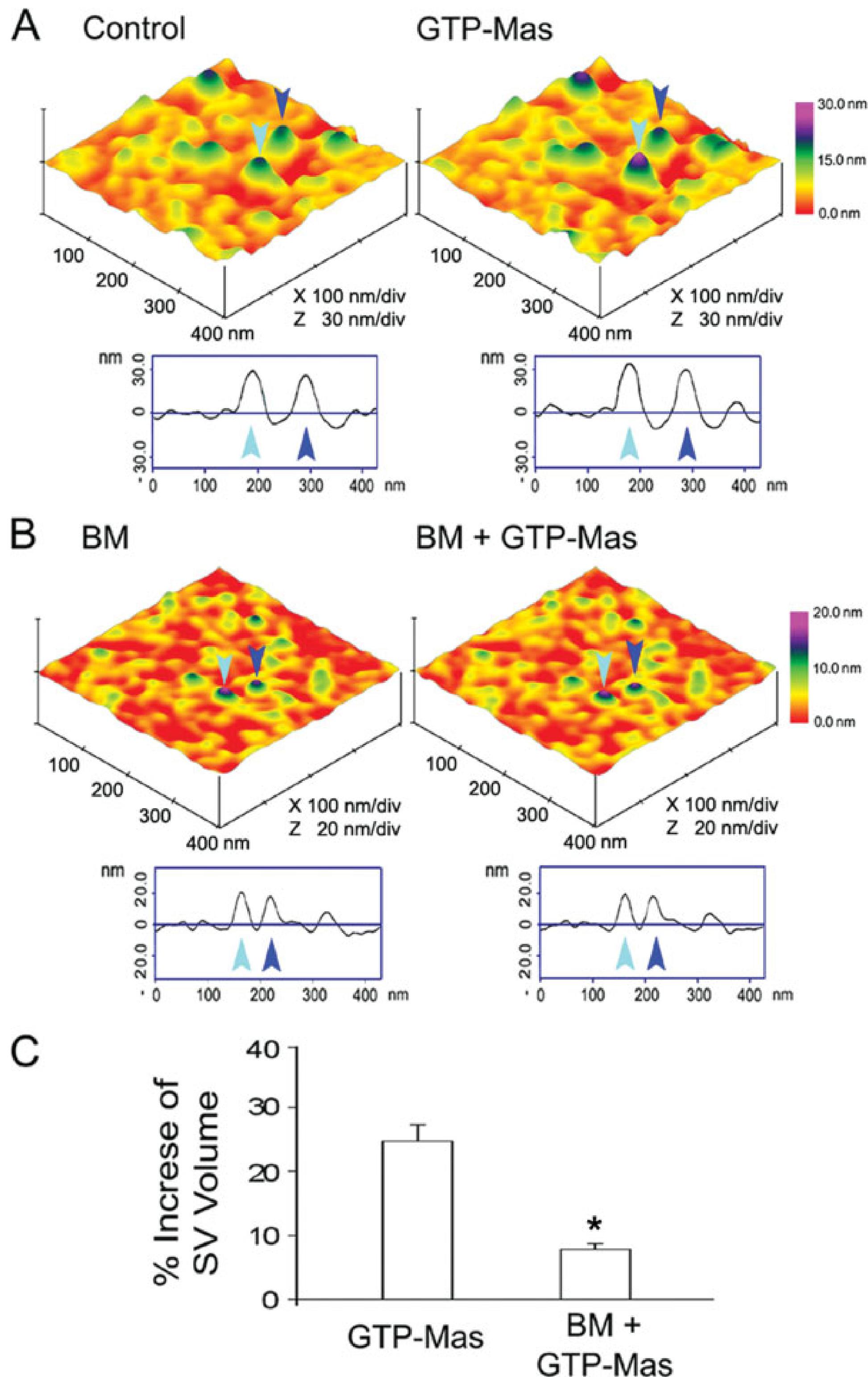

Fig. 3. Atomic force microscopy on synaptic vesicles demonstrating inhibition of GTP-Mas-induced synaptic vesicle (SV) swelling by bafilomycin (BM). In accordance with photon correlation spectroscopy and dynamic light scattering studies (Fig. 2), isolated SVs (**A**, left) swell when exposed to 40 μM GTP-Mas (A, right). Exposure of SV to 1 nM of the vH+-ATPase inhibitor BM (**B**, left) significantly inhibits GTP-Mas-induced vesicle swelling (B, right). Note the loss in GTP-Mas-induced SV volume increase following exposure to BM (**C**); n = 22, $*P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

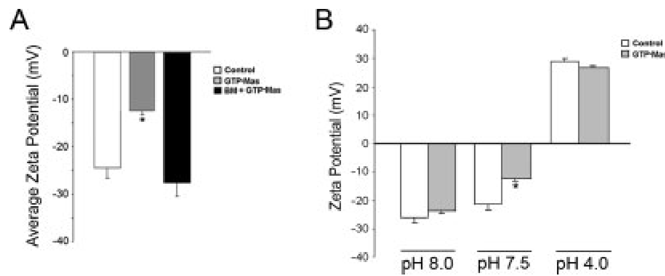

Fig. 4. Exposure of SVs to the vH^{+}-ATPase inhibitor bafilomycin abrogates the GTP-Mas-induced net loss in negative charges at the vesicle membrane by inhibiting vesicle acidification. **A:** With the exception of pH 7.5, vesicles in either acidic or alkaline medium fail to elicit any change in their zeta potential (**B**), demonstrating that both acidic and basic suspension media inhibited vH^{+}-ATPase activity, thereby preventing the entry of protons into the vesicle lumen. $*P < 0.05$.

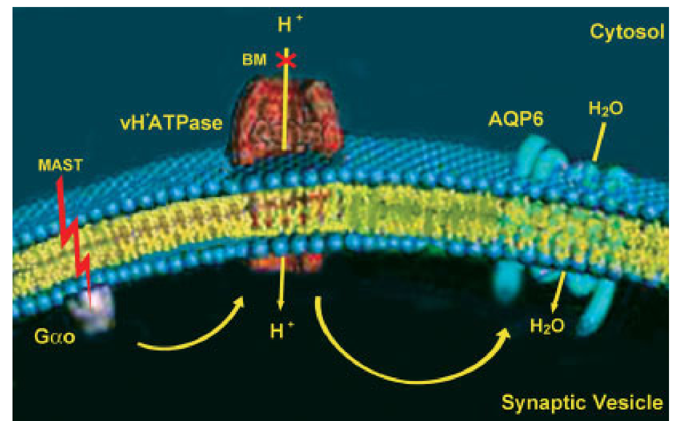

Fig. 5. Diagram of the synaptic vesicle membrane depicting the presence of $G_{\alpha o}$, vH^{+}-ATPase, and the water channel AQP6. Mastoparan (MAST) stimulates GTP-$G_{\alpha o}$ protein. This study demonstrates involvement of vH^{+}-ATPase in GTP-$G_{\alpha o}$-mediated synaptic vesicle swelling. Results demonstrate a bafilomycin (BM)-sensitive (red X) vesicle acidification following the GTP-$G_{\alpha o}$ stimulus, and water channels are bidirectional and the vH^{+}-ATPase inhibitor BM decreases both the volume of isolated synaptic vesicles and GTP-mastoparan-stimulated swelling, suggesting vH^{+}-ATPase to be upstream of AQP-6. Vesicle acidification is therefore a prerequisite for AQP-6-mediated gating of water into synaptic vesicles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$G_{\alpha o}$-mediated SV swelling is pH sensitive, insofar as exposure of isolated SV to both alkaline and acidic buffers resulted in a complete abrogation of both GTP-mastoparan-stimulable SV swelling and bafilomycin-induced SV deflation. The GTP-mastoparan-stimulable SV swelling was found to be rapid (Fig. 2E), as opposed to SV swelling in hypotonic medium. No change in SV size was found up to 105 sec following exposure to a 50% hypotonic buffer (data not shown). Insofar as bafilomycin is specific in its inhibition of vH^{+}-ATPase, this pH sensitivity of SV swelling may be a reflection of the direct inhibition of vH^{+}-ATPase activity under both basic and acidic conditions (Fig. 2B–D).

The role of vH^{+}-ATPase activity in the potency and efficacy of SV swelling was further evaluated by online real-time light scattering studies (Fig. 2E). The kinetics of GTP-Mas-induced swelling of SV in the presence or absence of bafilomycin was therefore determined. GTP-Mas-induced SV swelling takes on a logarithmic form that can be expressed by a first-order equation, with a rate constant $k = 0.0106\ sec^{-1}$ (Fig. 2E). Bafilomycin was found to decrease the rate of GTP-Mas-stimulated SV swelling by ~35% to $k = 0.0068\ sec^{-1}$. Results from the study demonstrate that bafilomycin exposure decreases both the potency and the efficacy of GTP-Mas-induced SV swelling by ~20% and 35%, respectively, suggesting a strong correlation between AQP-6 and vH^{+}-ATPase activity at the SV membrane.

Further conformation of the inhibition of GTP-mastoparan stimulated increase in isolated SV volume following exposure to bafilomycin was demonstrated by using AFM (Fig. 3). Exposure of SV to 40 μM GTP-mastoparan demonstrated a robust increase in vesicle size (Fig. 3A). However, prior exposure of SVs to 1 nM bafilomycin resulted in a significant ($\star P < 0.001$) inhibition of the GTP-mastoparan-induced swelling of synaptic vesicles (Fig. 3B,C).

Next, to determine whether exposure to bafilomycin results in a decrease in vesicle acidification and to test further the hypothesis that exposure of isolated SVs to both acidic and basic pH abrogated vH^{+}-ATPase function, zeta potential measurements were carried out on SV's exposed to bafilomycin and to various pH conditions in the presence and absence of GTP-mastoparan. Isolated SVs are negatively charged, with a zeta potential of approximately –20 to –25 mV. Vesicle acidification was measured as a net loss of negativity of the SV membrane. Exposure of SV to GTP-Mas results in vesicle acidification and a net loss in negative charges at the vesicle membrane (–24.4 ± 2.1 to –12.3 ± 1.0). Exposure of SVs to the vH^{+}-ATPase inhibitor bafilomycin abrogates the GTP-Mas-induced net loss in negative charges at the vesicle membrane (Fig. 4A). With the exception of pH 7.5, vesicles in either acidic or alkaline medium fail to elicit any change in their zeta potential (Fig. 4B), demonstrating that both acidic and basic suspension media inhibited vH^{+}-ATPase activity, thereby preventing the entry of protons into the vesicle lumen. Results from these studies support the idea that luminal acidification of SV is a requirement for AQP-6-induced active water transport and vesicle swelling and that either low (pH 4) or high (pH 8) pH inhibits the activity of SV-associated vH^{+}-ATPase (Fig. 4). However, $G_{\alpha o}$-stimulated SV acidification is completely abrogated (Fig. 4A) and bafilomycin is able to inhibit only partially the $G_{\alpha o}$-stimulated SV swelling (Fig. 2B), suggesting that vH^{+}-ATPase is in part responsible for the $G_{\alpha o}$-mediated SV swelling.

In the current study, an assay system of monitoring nanometer-scale changes in synaptic vesicle size was used

to determine the role of vH^{+}-ATPase in the regulation of SV volume. Unlike slow water entry by diffusion, GTP-mastoparan-induced rapid swelling of SVs via facilitated transport of water molecules through water channels or aquaporins at the SV membrane has been previously reported (Jeremic et al., 2005). In these earlier studies (Jeremic et al., 2005), AQP-6 at the SV membrane was demonstrated to be involved in GTP-mastoparan-induce SV swelling (Jeremic et al., 2005). Results from the current study further demonstrate that the $G_{\alpha o}$-stimulated SV swelling is vH^{+}-ATPase dependent and pH sensitive. Water channels including AQP-6 are bidirectional, and the vH^{+}-ATPase inhibitor bafilomycin decreases the volume of resting synaptic vesicles, suggesting vH^{+}-ATPase to be upstream of AQP-6, in the pathway leading from $G_{\alpha o}$-stimulated swelling of synaptic vesicles (Fig. 5). In Figure 5, the involvement of vH^{+}-ATPase in GTP-$G_{\alpha o}$-mediated synaptic vesicle swelling is summarized. Our results demonstrate a bafilomycin-sensitive vesicle acidification following GTP-$G_{\alpha o}$ stimulus, and water channels are bidirectional and the vH^{+}-ATPase inhibitor bafilomycin decreases both the volume of isolated synaptic vesicles and the GTP-mastoparan-stimulated swelling, suggesting vH^{+}-ATPase to be upstream of AQP-6. Vesicle acidification is therefore a prerequisite for AQP-6-mediated rapid gating of water into synaptic vesicles. To our knowledge, this is the first direct demonstration of the involvement of synaptic vesicle membrane-associated vH^{+}-ATPase in $G_{\alpha o}$-stimulated SV swelling.

REFERENCES

Bastani B, Purcell H, Hemken P, Trigg D, Gluck S. 1991. Expression and distribution of renal vacuolar proton-translocating adenosine triphosphatase in response to chronic acid and alkali loads in the rat. J Clin Invest 88:126–136.

Bastani B, McEnaney S, Yang L, Gluck S. 1994. Adaptation of inner medullary collecting duct vacuolar H-adenosine triphosphatase to chronic acid or alkali loads in the rat. Exp Nephrol 2:171–175.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254.

Chambrey R, Paillard M, Podevin RA. 1994. Enzymatic and functional evidence for adaptation of the vacuolar H-ATPase in proximal tubule apical membranes from rats with chronic metabolic acidosis. J Biol Chem 269:3243–3250.

Cho SJ, Cho J, Jena B. 2002. The number of secretory vesicles remains unchanged following exocytosis. Cell Biol Int 26:29–33.

Cho WJ, Jeremic A, Rognlien K, Zhvania M, Lazrishvili I, Tamar B, Jena B. 2004. Structure, isolation, composition and reconstitution of the neuronal fusion pore. Cell Biol Int 28:699–708.

Füldner H, Stadler H. 1982. ^{31}P-NMR analysis of synaptic vesicles. Status of ATP and internal pH. Eur J Biochem 121:519–524.

Hicks B, Parsons S. 1992. Characterization of the P-type and V-type ATPases of cholinergic synaptic vesicles and coupling of nucleotide hydrolysis to acetylcholine transport. J Neurochem 58:1211–1220.

Higashijima T, Uzu S, Nakajima T, Ross E. 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J Biol Chem 263:6491–6494.

Jeremic A, Cho WJ, Jena B. 2005. Involvement of water channels in synaptic vesicle swelling. Exp Biol Med 230:674–680.

Kelly M, Cho WJ, Jeremic A, Abu-Hamdah R, Jena B. 2004. Vesicle swelling regulates content expulsion during secretion. Cell Biol Int 28:709–716.

Konrad R, Young R, Record R, Smith R, Butkerait P, Manning D, Jarett L, Wolf B. 1995. The heterotrimeric G-protein Gi is localized to the insulin secretory granules of beta-cells and is involved in insulin exocytosis. J Biol Chem 270:12869–12876.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Michaelson D, Angel I. 1980. Determination of pH in cholinergic synaptic vesicles: its effect on storage and release of acetylcholine. Life Sci 27:39–44.

Morel N, Dunant Y, Israël M. 2001. Neurotransmitter release through the V0 sector of V-ATPase. J Neurochem 79:485–488.

Nordstrom T, Shrode L, Rotstein O, Romanek R, Guto T, Heersche J, Manolson M, Brisseau G, Grinstein S. 1997. Chronic extracellular acidosis induces plasmalemma vacuolar type H^{+} ATPase activity in osteoclasts. J Biol Chem 272:6354–6360.

Peters C, Bayer M, Bühler S, Andersen J, Mann M, Mayer A. 2001. Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature 409:581–588.

Santos T, Souza D, Tasca C. 2005. GTP uptake into rat synaptic vesicles. Brain Res 1070:71–76.

Stadler H, Tsukita S. 1984. Synaptic vesicles contain an ATP-dependent proton pump and show ''knob-like'' protrusions on their surface. EMBO J 3:3333–3337.

Vitale N, Mukai H, Rouot B, Thierse D, Aunis D, Bader MF. 1993. Exocytosis in chromaffin cells. Possible involvement of the heterotrimeric GTP-binding protein G_o. J Biol Chem 268:14715–14723.

Weingarten R, Ransnas L, Mueller H, Sklar L, Bokoch G. 1990. Mastoparan interacts with the carboxyl terminus of the alpha subunit of Gi. J Biol Chem 265:11044–11049.