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Regulation of the water channel aquaporin-1: isolation and reconstitution of the regulatory complex

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Abstract

Aquaporins (AQP) are involved in rapid and active gating of water across biological membranes. The molecular regulation of AQP is unknown. Here we report the isolation, identification and reconstitution of the regulatory complex of AQP-1. AQP-1 and $G_{\alpha i3}$ have been implicated in GTP-induced gating of water in zymogen granules (ZG), the secretory vesicles in exocrine pancreas. In the present study, detergent-solubilized ZGs immunoprecipitated with monoclonal AQP-1 antibody, co-isolates AQP-1, PLA2, $G_{\alpha i3}$, potassium channel IRK-8, and the chloride channel ClC-2. Exposure of ZGs to either the potassium channel blocker glyburide, or the PLA2 inhibitor ONO-RS-082, blocked GTP-induced ZG swelling. RBC known to possess AQP-1 at the plasma membrane, swell on exposure to the G*ai*–agonist mastoparan, and respond similarly to ONO-RS-082 and glyburide, as ZGs. Liposomes reconstituted with the AQP-1 immunoisolated complex from solubilized ZG, also swell in response to GTP. Glyburide or ONO-RS-082 abolished the GTP effect. Immunoisolate-reconstituted planar lipid bilayers demonstrate conductance, which is sensitive to glyburide and an AQP-1 specific antibody. Our results demonstrate a $G_{\alpha i3}$-PLA2 mediated pathway and potassium channel involvement in AQP-1 regulation.

Keywords: Aquaporin; Atomic force microscopy; Electrophysiology; Light scattering

1. Introduction

Water being the most abundant molecule in living cells, its transport within and out of the cell is vital. Water channels or aquaporins have been identified to perform this important cellular function. As opposed to the slow osmotic swelling, aquaporins are a family of membrane proteins that actively and rapidly transport water across cellular membranes. The presence of water conducting channels was first suggested in the mid 1950s by Sidel and Solomon (1957), and later in 1970 by Macey and Farmer (1970). Subsequent experiments using radiolabeled inhibitors of water diffusion in red blood cells (RBC), followed by SDS–PAGE and autoradiography, reveal radiolabeled proteins migrating at the band 4.5 region, suggesting the radiolabeled bands to be possible water channels (Benga et al., 1986a,b). In the past decade, the water channel Aquaporin-1 (AQP-1), has been purified, cloned, and its crystal structure determined (Cheng et al., 1997; Denker et al., 1988; Preston and Agre, 1991; Preston et al. 1992; Walz et al., 1997). A 2.2 angstrom resolution structure of the bovine AQP-1 has recently been obtained by X-ray crystallography (Sui et al., 2001), revealing the site of water molecules in a frozen state. These and other structural studies provide a wealth of information on the molecular arrangement of the water channel in lipid membranes. The movement of ions and solutes through cellular membranes is highly regulated. Under various physiological conditions, the cell is required to pump water into or out of the cell, and hence there must exist some form of regulation of water channel activity.

To understand the transport properties of the water channel, it has previously been reconstituted into lipid bilayers (Saparov et al., 2001), or expressed in oocytes (Anthony et al., 2000). However, these earlier

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reconstitution studies used the purified proteins expressed in yeast (Saparov et al., 2001). There are two major problems with this approach: one, the pure protein would behave differently in the absence of its regulatory components; and two, the recombinant protein may co-isolate with the traces of cation channel from the host. Furthermore, over expressed water channels in oocytes may promote association with ion channels and other proteins, which in its native state, would normally not associate. In a study (Zeidel et al., 1992), where native RBC AQP-1 were isolated and reconstituted into proteoliposomes, it was reported that AQP-1 alone could function and it needed no other proteins. The authors in the study also suggest that AQP-1 has bi-directional water transport capability. If this were the case, the cell would certainly require some form of regulation of the channel. How the cell would otherwise determine directionality of water flow or went to switch-on or switch-off water transport? Additionally, in the study (Zeidel et al., 1992), the native AQP-1 purified from RBC were not assayed for other associated proteins. In figure 1A of that manuscript, a broad band that contains glyCHIP28 (glycosylated form of AQP-1) may also contain potassium channel and the $G_{\alpha i3}$ protein. In the same figure, there appears a high molecular weight band that could correspond to PLA2. The same group has recently published a manuscript (Saparov et al., 2001), where native AQP-1 purified from human RBC could conduct both water and cations. Due to these conflicting reports, AQP-1 and its associate proteins were immunoisolated with high stringency from their native membranes both for biochemical analysis and functional assays, following reconstitution in artificial lipid membranes. Additionally, the aquaporin-1 activity and regulation determined in intact cells and organelles, confirm to those observed in the reconstituted systems. Hence, these studies provide for the first time insights into the composition of the molecular machinery and the mechanism of water channel regulation.

Zymogen granules (ZGs), the membrane-bound secretory vesicles in exocrine pancreas, swell in response to GTP mediated by a $G_{\alpha i3}$ protein (Jena et al., 1997). Recently, AQP-1 was found in ZG membrane and its participation in rapid GTP-induced vesicular water entry and swelling was revealed (Cho et al., 2002a). Isolated ZGs exhibit low basal water permeability, however, exposure of granules to GTP results in a marked potentiation of water entry. Treatment of ZGs with $HgCl_2$ (a nonspecific inhibitor of water channels) is accompanied by a reversible loss in GTP-stimulable water entry and vesicle swelling. Introduction of an AQP-1 specific antibody raised against the carboxyl terminal domain of AQP-1 was found to block GTP-stimulable swelling of ZG (Cho et al., 2002a). These results demonstrate that AQP-1 associated at the ZG membrane is involved in GTP-induced rapid gating of water in ZGs, mediated by a $G_{\alpha i3}$ protein.

Like $HgCl_2$, $AgNO_3$ has also been identified to be an aquaporin inhibitor (Niemietz and Tyerman, 2002). However, both $HgCl_2$ and $AgNO_3$ are nonspecific inhibitors, since they interact with the SH-groups of proteins (Niemietz and Tyerman, 2002). No specific inhibitor had been known until the identification of an AQP-1 specific antibody raised against the carboxy-terminal domain of AQP-1, which inhibits AQP-1 function (Cho et al., 2002a). This AQP-1 specific antibody was used in the current study. To identify the minimal regulatory complex of native AQP-1, high stringency AQP-1 associated immunoisolates, were functionally reconstituted into artificial liposomes, to measure vesicle size under different experimental conditions. The AQP-1 specific immunoisolates were also reconstituted into planar lipid bilayers, to monitor changes in membrane conductance. Results from this study demonstrate a $G_{\alpha i3}$–PLA2 mediated pathway and potassium channel involvement in AQP-1 regulation, both in RBC and ZGs. These studies also demonstrate that there is cationic conductance through the native AQP-1 channel, and therefore the transport of hydronium ion (H_3O^+) along with neutral water molecules (H_2O) through the AQP-1 channel is hypothesized.

2. Materials and methods

2.1. Isolation of zymogen granules (ZGs)

ZGs were isolated by using a modification of the method of Jena et al. (1991). Male Sprague–Dawley rats weighing 80–100 g were euthanized using CO_2 inhalation. For every ZG preparation, the pancreas was dissected and diced into 0.5-mm pieces. The diced pancreas was suspended in 15% (wt/vol) ice-cold homogenization buffer (0.3 M sucrose, 25 mM Hepes, pH 6.5, 1 mM benzamidine, 0.01% soybean trypsin inhibitor) and homogenized using three strokes of a Teflon glass homogenizer. The resultant homogenate was centrifuged for 5 min at $300 \times g$ and a temperature of 4 °C to obtain a supernatant fraction. Each volume of the supernatant fraction was mixed with 2 vol. of a Percoll–Sucrose–Hepes buffer (0.3 M sucrose, 25 mM Hepes, pH 6.5, 86% Percoll, 0.01% soybean trypsin inhibitor) and centrifuged for 30 min at $16{,}400 \times g$ at 4 °C. Pure ZGs were obtained as a loose white pellet at the bottom of the centrifuge tube, and used in this study.

2.2. Immunoisolation of the AQP-1 regulatory complex and immunoblot analysis

Native AQP-1 and associated proteins (AQP-1 complex) were immunoisolated from detergent solubilized ZG preparations, using an AQP-1 specific monoclonal

antibody (ABCAM Limited, Cambridgshire, UK). Protein in all fractions was estimated using the Bradford assay (Bradford, 1976). To isolate the AQP-1 complex for immunoblot analysis and reconstitution experiments, the AQP-1 specific antibody conjugated to protein A-sepharose was used. Isolated ZGs were solubilized in Triton/Lubrol solubilization buffer (0.5% Lubrol; 1 mM benzamidine; 5 mM ATP; 5 mM EDTA; 0.5% Triton X-100, in PBS) supplemented with protease inhibitor mix (Sigma, St Louis, MO, USA). AQP-1 specific antibody conjugated to the protein A-sepharose were incubated with the solubilized ZG for 1 h at room temperature followed by washing with wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH=7.5). The immunoprecipitated sample attached to the immuno sepharose beads was incubated in Laemmli sample preparation buffer (Laemmli, 1970), prior to the one dimension 12.5% SDS–PAGE, electrotransfer to nitrocellulose and immunoblot analysis using specific antibodies to PLA2, $G_{\alpha i3}$, the K^+ channel IRK-8, and the Cl channel ClC-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Similarly, for the 2D gel electrophoresis, the immunoprecipitated AQP-1 complex was resolved by the published 2D 16-benzyldimethyl-*n*-hexadecylammonium chloride method (Macfarlane, 1989), followed by 1D 12.5% SDS–PAGE, and stained with coomassie blue. For immunoblot analysis, the 1D SDS–PAGE resolved proteins were electrotransferred to nitrocellulose, which was incubated for 1 h at room temperature in blocking buffer (5% non-fat milk in PBS containing 0.1% Triton X-100 and 0.02% NaN_3), and immunoblotted for 2 h at room temperature with the specific antibody. The primary antibodies were used at a dilution of 1:500 in blocking buffer. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02% NaN_3 and were incubated for 1 h at room temperature in horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000 in blocking buffer. The immunoblots were then washed in the PBS buffer, processed for enhanced chemiluminescence and photographed using a Kodak Image Station 414.

2.3. AQP-1 complex reconstituted liposomes

Large unilamellar lipid vesicles (LUV) were prepared using brain dioleoylphosphatidyl-choline (DOPC), and dioleylphosphatidylserine (DOPS), obtained from Avanti Polar Lipids(Alabaster, AL, USA). A 5 mM suspension of DOPC:DOPS in a ratio of 7:3, was prepared. The lipid suspension was dried in nitrogen atmosphere, and resuspended in solution containing 10 mM HEPES pH 7.5, 140 mM NaCl and 1 mM EDTA. LUVs were generated by mild sonication for 2–3 min. Immunoisolated AQP-1 complex was reconstituted into the lipid vesicles using gentle agitation for 30 min at RT. The reconstituted liposomes were used for AFM studies.

2.4. AFM volume measurements of ZG and reconstituted liposomes

Isolated ZGs or reconstituted liposome suspensions in 125 mM KCl–Mes buffer, pH 6.5 (25 mM KCl, 100 mM 2-N morpholinoethanesulfonic acid) were plated on Cell-Tak-coated glass coverslips. Ten min after plating, the coverslips were placed in a fluid chamber and washed with the KCl–Mes buffer to remove unattached vesicles, prior to imaging the attached liposomes or ZGs in KCl–Mes buffer or water, in the presence or absence of 40 μM GTP, 20 μM mastoparan, 20 μM glyburide, 20 μM ONO-RS-082, and 10 μM DIDS. AQP-1 antibody raised against the carboxyl-terminal domain of AQP-1, was obtained from Santa Cruz Biotechnology, and had been demonstrated to bind AQP-1 and inhibit its function (Cho et al., 2002a). ZG imaging and dynamics were performed by using the Nanoscope IIIa, an atomic force microscope from Digital Instruments (Santa Barbara, CA, USA). ZGs were imaged both in the "contact" and "tapping" mode in fluid (Jena et al., 1997). However, all images presented in this manuscript 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 <200 pN. Images were obtained at line frequencies of 2 Hz, with 512 lines per image and constant image gains. Time-dependent (resolution in s), morphological changes in ZG and liposomes were obtained by using section analysis. Topographical dimensions of ZG and liposomes were analyzed with the software NANOSCOPE (R) IIIA 4.43r8 supplied by Digital Instruments.

2.5. Light scattering measurements

Kinetics of rat red blood cell (RBC) volume changes were monitored by 90° light scattering with excitation and emission wavelength set at 400 nm in a Hitachi F-2000 spectrophotometer. Seven microliters of erythrocytes suspension was injected into the thermo-regulated cuvette containing 700 μl of PBS buffer (in mM: NaCl 140; KCl 2.5; Na_2HPO_4 0.25; KH_2PO_4 0.25; pH 7.4) at 37 °C. Light scattering was monitored for 5 min after injection of RBC, in the presence and absence of mastoparan, $AgNO_3$ (water channel inhibitor), glyburide (potassium channel blocker) or ONO-RS-082 (PLA2-inhibitor). Values are expressed as percent light scattered over control. Student's *t*-test was used for comparisons between groups with significance established at $P<0.05$ (*).

2.6. Bilayer electrophysiology

Phosphatidylcholine and phosphatidylserine were purchased from Avanti Polar Lipids 10 mg/ml in chloroform and combined 7:3 respectively by weight. Lipids were then dried under N_2 and resuspended in decane. Planar lipid bilayers were formed first and verified to be stable (within limits for capacitance determined by cup hole diameter and free of electrical activity) before incorporation of immunoprecipitated AQP-1 complex using a pipette tip. Current verses time traces were recorded using pulse software, an EPC9 amplifier and probe from HEKA (Lambrecht, Germany). A baseline current was established at −60 mV and baseline conductance was determined before the addition of the AQP-1 complex. The bilayer was either rebrushed if no change was observed or allowed to sit for at least 3 min before an IV curve was taken. After the voltage curve, the bilayer was exposed to a KCl gradient, 20 μM glyburide, and/or the AQP-1 antibody.

3. Results and discussion

3.1. AQP-1 at the ZG membrane associates with $G_{\alpha i3}$, PLA2, IRK8, and ClC-2

Studies using isolated ZGs from the exocrine pancreas and parotid glands demonstrate the presence of Cl^- and ATP-sensitive K^+ selective ion channels at the vesicle membrane, whose activities have been implicated in vesicle swelling (Fuller et al., 1989a,b; Gasser et al., 1988; Gasser and Hopfer, 1990; Segawa et al., 1991; Takuma et al., 1993; Thevenod et al., 1990, 1992, 1996). Furthermore, secretion of ZG contents in permeabilized pancreatic acinar cells requires the presence of both K^+ and Cl^- ions (Curran and Brodwick, 1991). Our earlier studies demonstrated the presence of AQP-1 and $G_{\alpha i3}$ at the ZG membrane (Cho et al., 2002a; Jena et al., 1997). In those studies, AQP-1 and $G_{\alpha i3}$ were implicated in GTP-induced and KCl-sensitive gating of water in ZGs. The possible interaction between AQP-1, $G_{\alpha i3}$, and both K^+ and Cl^- ion channels was therefore hypothesized.

In the present study, detergent-solubilized ZGs were used to immunoisolated AQP-1 and associated proteins, using a monoclonal AQP-1 antibody. In the AQP-1 immunoisolate, AQP-1 and a number of proteins (Fig. 1A), among them PLA2, $G_{\alpha i3}$, the potassium channel IRK-8, and chloride channel ClC-2, were identified as hypothesized (Fig. 1B). The presence of AQP-1 in RBC membrane has previously been reported (Denker et al., 1988). Detergent-solubilized RBC from rat, immunoprecipitated with the monoclonal AQP-1 antibody, also co-isolated with AQP-1, both PLA2 and $G_{\alpha i3}$ (Fig. 1C). This study demonstrates that AQP-1, both in ZG and RBC membrane,

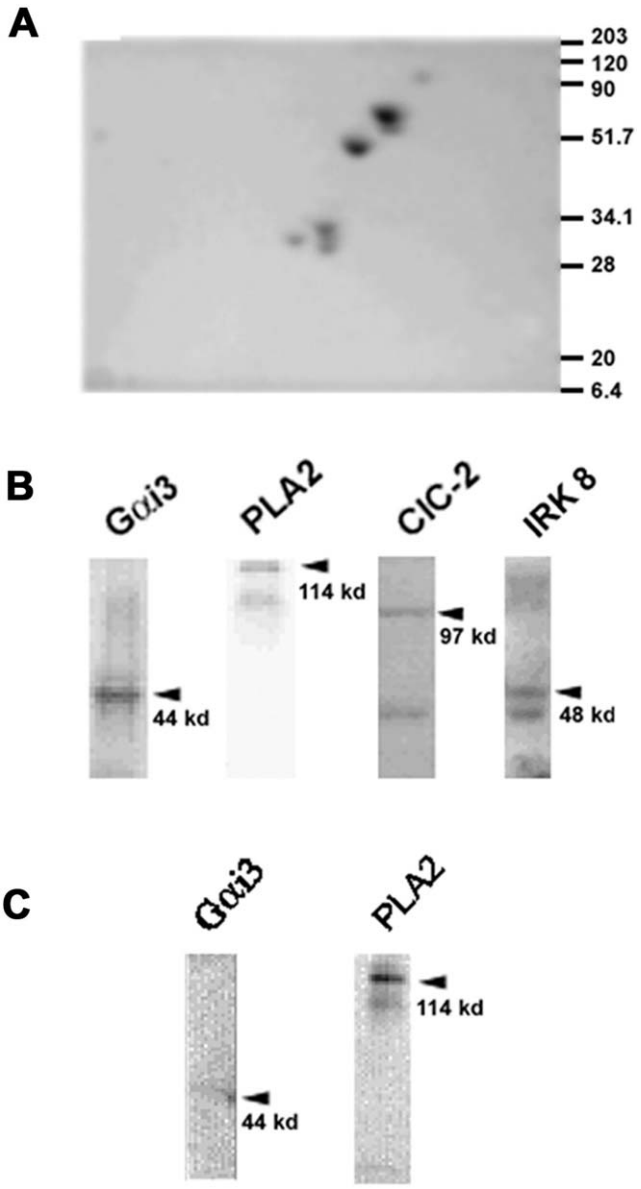

Fig. 1. One (1D) and two dimensionally (2D) resolved, AQP-1 immunoisolated proteins from solubilized pancreatic ZG preparations (A,B) and red blood cells (C), using an AQP-1 specific antibody. The 2D-resolved proteins were coumassie stained and the 1D resolved proteins transferred to nitrocellulose membranes for immunoblot analysis. Note the identification in the immunoisolates of seven spots in the coumassie-stained 2D-resolved gel (A). Immunoblot analysis of the 1D-resolved immunoisolated proteins demonstrate the presence of PLA2, $G_{\alpha i3}$, the potassium channel IRK-8, and chloride channel ClC-2 (B). Similarly, in red blood cells, PLA2 and $G_{\alpha i3}$ immunoreactive bands are detected (C). Lower molecular weight bands may represent proteolytic cleavage products.

interacts with PLA2 and $G_{\alpha i3}$ proteins. Since AQP-1 and $G_{\alpha i3}$ have previously been implicated in GTP-induced gating of water in ZGs (Cho et al., 2002a), it is suggested that $G_{\alpha i3}$ may be regulating AQP-1 activity via PLA2. This hypothesis was tested, and the results described below.

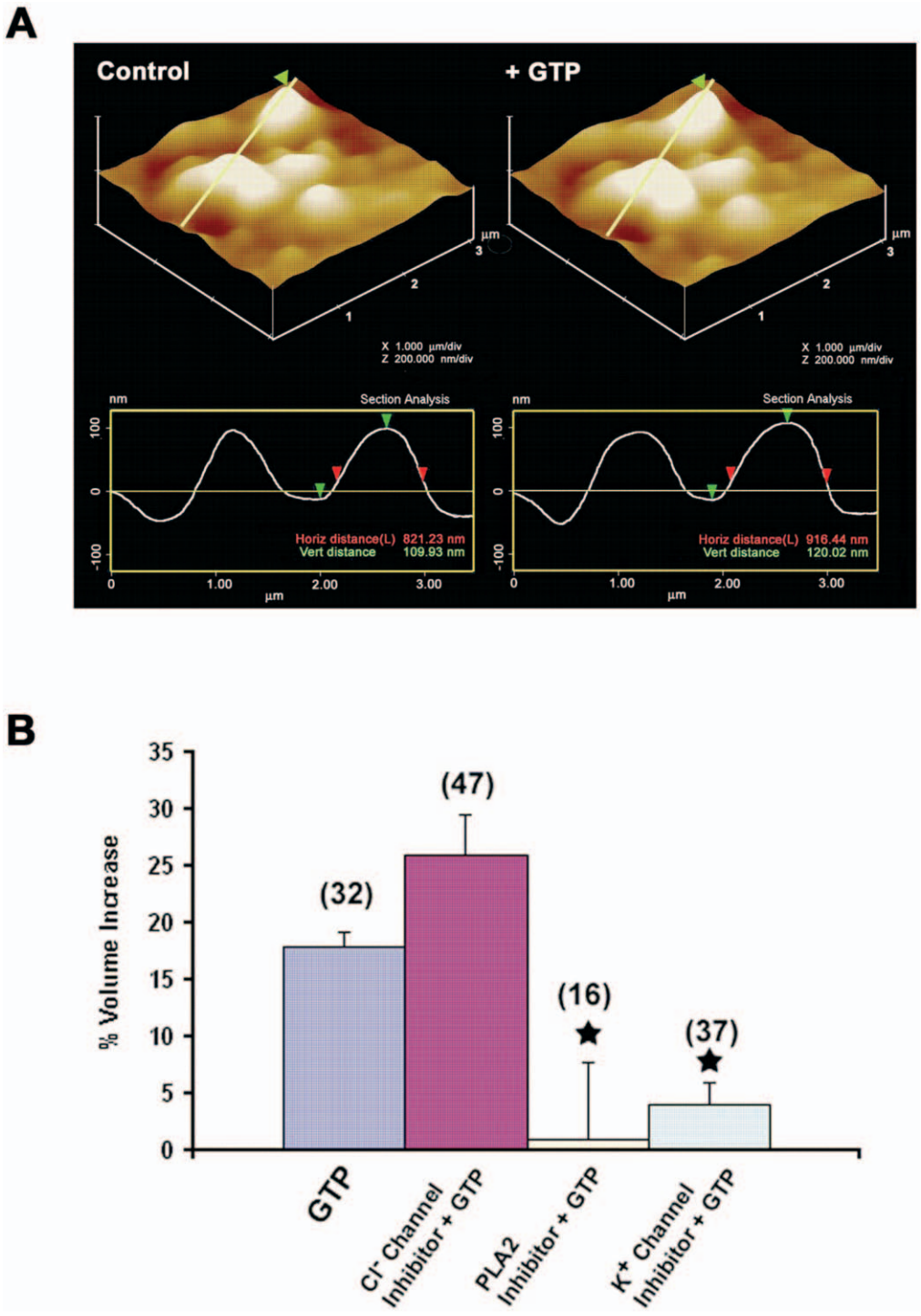

Fig. 2. ZG volume changes measured by AFM. AFM micrographs demonstrating swelling of isolated ZGs in response to 40 µM GTP (A). Histogram showing changes in ZG volume following exposure of isolated ZGs to GTP, and to the inhibitor of the chloride channel DIDS, the inhibitor of the potassium channel, glyburide, or to the inhibitor of PLA2, ONO-RS-082 (B). Note the significant inhibition of GTP-induced ZG swelling, in the presence of glyburide, or ONO-RS-082. DIDS has little effect on GTP-induced ZG swelling. Values represent mean ± SE of mean of the number of ZGs (in parentheses), which were randomly selected. A significant ($P<0.01$) inhibition (□) in the presence of glyburide, or ONO-RS-082 was demonstrated.

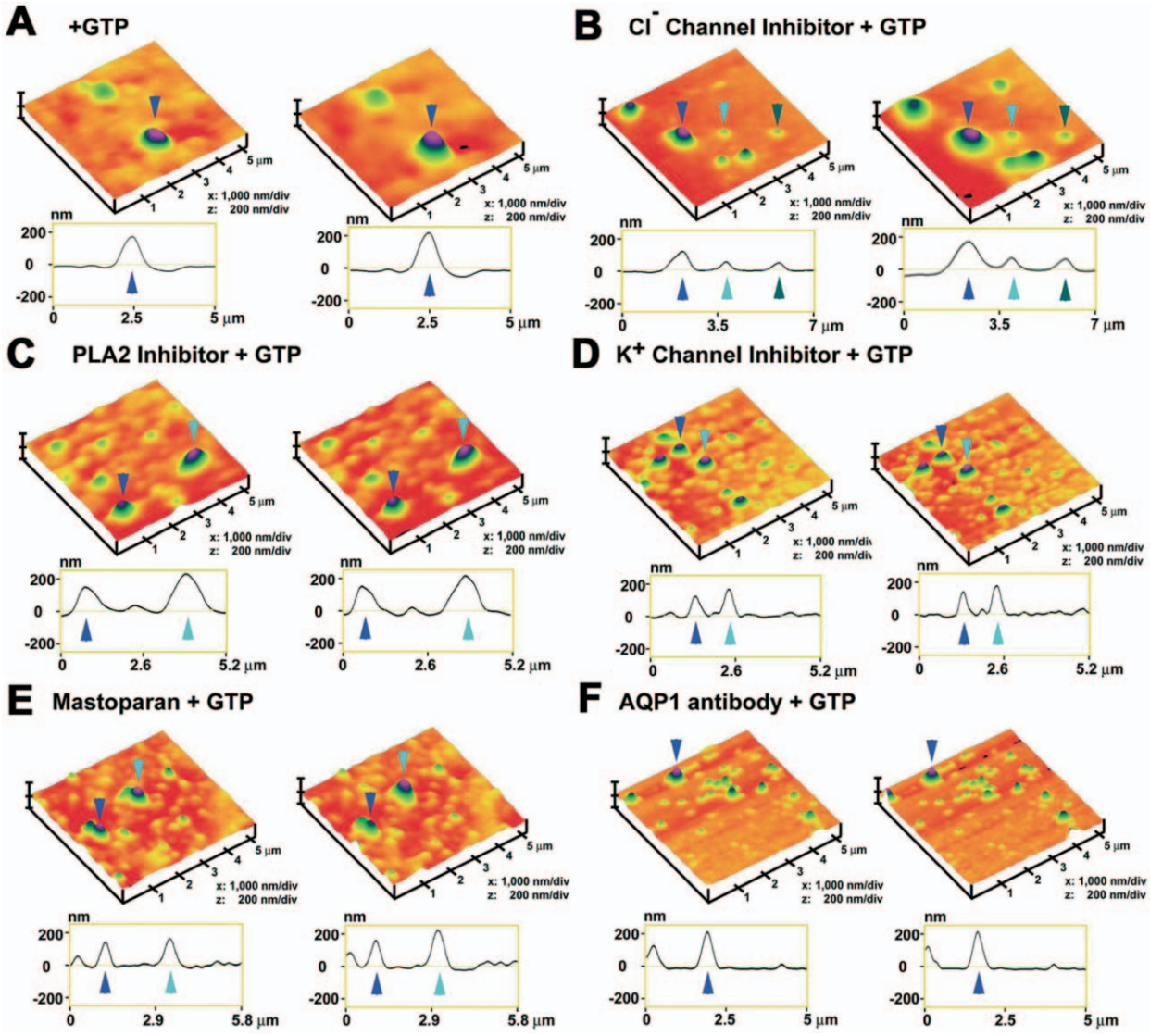

Fig. 3. AFM micrographs of liposomes demonstrating functional reconstitution of the immunoisolated AQP-1 complex. Note swelling of the reconstituted liposomes in response to GTP (A) panel left is control and panel right is 5 min following GTP exposure. Exposure of the chloride channel inhibitor DIDS, did not inhibit GTP-induced swelling (B); however, the PLA2 inhibitor ONO-RS-082 (C), the potassium channel inhibitor glyburide (D), and the inhibitory AQP-1 specific antibody inhibited GTP-induced swelling (F). The $G_{\alpha i}$-stimulable peptide mastoparan, demonstrates no additional effect on swelling over GTP (E), hence the GTP effect is mediated through the $G_{\alpha i3}$ protein.

3.2. Glyburide and ONO-RS-082 blocks GTP-induced ZG swelling

As previously reported (Cho et al., 2002a; Jena et al., 1997), exposure of ZG to GTP results in KCl-sensitive, rapid gating of water and swelling. In agreement with these earlier findings, exposure of ZG to 40 μM GTP, resulted in a significant increase on the percent ZG volume (mean ± SE: 18 ± 2.12), as shown in Fig. 2. Exposure of ZGs to either the potassium channel blocker glyburide, or the PLA2 inhibitor ONO-RS-082, significantly inhibited this GTP-induced ZG swelling. GTP had little effect on the percent of ZG volume increase when ZGs were pre-exposed to either glyburide (mean ± SE: 4 ± 1.97; $P<0.01$), or ONO-RS-082 (mean ± SE: 1 ± 6.75; $P<0.01$). However, exposure of ZGs to the chloride channel inhibitor 4,4′-Diisothiocyanostilbene-2,2′-disulfonic Acid (DIDS), did not inhibit GTP-induced ZG swelling (mean ± SE: 26 ± 3.2). These results demonstrate that PLA2 and the potassium channel IRK-8 are associated with AQP-1 (Fig. 1B) and regulate AQP-1 activity. The $G_{\alpha i3}$-mediated GTP effect (Cho et al., 2002a; Jena et al., 1997) is probably transduced to the water channel via PLA2. The role of the chloride channel ClC-2, in the orchestra of AQP-1 regulation, is unclear at this time. However, it could be speculated that since the chloride channel is associated with the AQP-1 complex, and is not involved in AQP-1 stimulation, it may participate in the

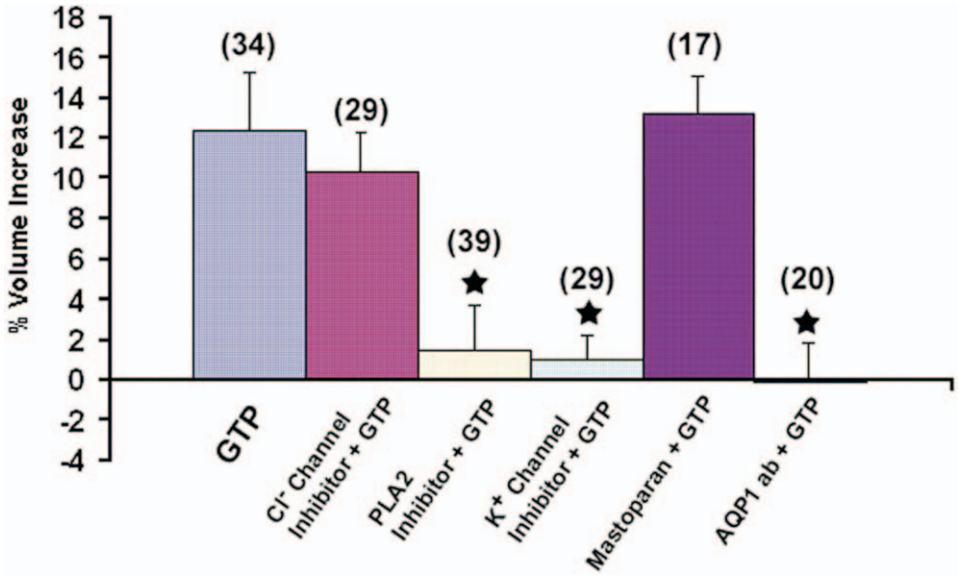

Fig. 4. Percent volume changes in reconstituted liposomes measured by AFM. As observed in the AFM micrographs in Fig. 3, estimation of the changes in liposome volume, demonstrates a 12–15% increase, 5 min following exposure to 40 μM GTP. No significant change in GTP-induced liposome swelling is observed following exposure to the chloride channel inhibitor DIDS or to the $G_{\alpha i}$-stimulable peptide mastoparan. Similar to the responses in ZGs, exposure of the reconstituted liposomes to the PLA2 inhibitor ONO-RS-082, the potassium channel inhibitor glyburide, and the AQP-1 specific antibody, significantly inhibits the GTP effect. Values represent mean ± SE of mean of randomly selected liposomes (in parentheses). A significant (P<0.01) inhibition (□) in the presence of glyburide, ONO-RS-082, and the AQP-1 specific antibody, is demonstrated.

desensitization or switchoff of the channel. Further studies are required to elucidate the role of the chloride channel ClC-2 in AQP-1 regulation.

3.3. AQP-1 specific antibody, glyburide, and ONO-RS-082 block GTP-induced swelling of reconstituted liposomes

To determine the minimal regulatory complex of AQP-1, and to further understand the role of PLA2, $G_{\alpha i3}$, and both K^+ and Cl^- ion channels, the AQP-1 immunoisolates (AQP-1 complex) were reconstituted into liposomes for study. Mastoparan, an amphiphilic tetradecapeptide from wasp venom, has been demonstrated to activate the GTPase activity of $G_{\alpha i}$ proteins (Higashijima et al., 1988; Konrad et al., 1995; Vitale et al., 1993). Stimulation of the $G_{\alpha i}$ protein is believed to occur by the peptide inserting into the phospholipid membrane and forming a highly structured α-helix that resembles the intracellular loops of $G_{\alpha i}$ protein-coupled receptors. Analogous to receptor activation, mastoparan is thought to interact with the COOH-terminal domain of the G protein α subunit (Weingarten et al., 1990). Similar to ZGs, when reconstituted liposomes were exposed to GTP or mastoparan+GTP, a 12–13% increase in vesicle swelling (+GTP, mean ± SE: 12.4 ± 2.09; mastoparan+GTP, mean ± SE: 13.2 ± 1.93) was observed (Figs. 3 and 4). Exposure of reconstituted liposomes to either glyburide (mean ± SE: 1.01 ± 1.25), or ONO-RS-082 (mean ± SE: 1.4 ± 2.28), inhibited significantly the GTP-induced vesicle swelling (P<0.01; Figs. 3 and 4).

As previously eluded (Cho et al., 2002a), an AQP-1 specific antibody raised against the carboxyl terminal domain of the water channel, was found to block its activity, and inhibit GTP-induced ZG swelling. This AQP-1 specific antibody was used to inactivate the AQP-1 in the reconstituted liposomes in the present study. GTP-induced swelling of the reconstituted liposomes exposed to the AQP-1 antibody inhibited significantly (mean ± SE: 1 ± 1.63) GTP-induced vesicle swelling (P<0.01; Figs. 3 and 4).

Similar to what is observed in ZGs, exposure of reconstituted liposomes to DIDS, had little effect on GTP-induced vesicle swelling (mean ± SE: 10.3 ± 1.93). Hence, in every way, reconstituted liposomes, were able to mimic the responses observed in isolated ZGs. These studies demonstrate that the minimal AQP-1 regulatory complex is comprised of PLA2, $G_{\alpha i3}$, and the K^+ channel IRK-8.

3.4. Mastoparan-induced RBC swelling inhibited by glyburide, ONO-RS-082, and $AgNO_3$

Isolated RBC when exposed to 20 μM mastoparan, swelled as observed in the AFM micrographs (Fig. 5A). Similarly, light scattering studies demonstrated rapid swelling of RBC following exposure to 20 μM mastoparan represented as percent increase in light scattering over control (expressed as mean ± SE: 250 ± 15%, n=7;

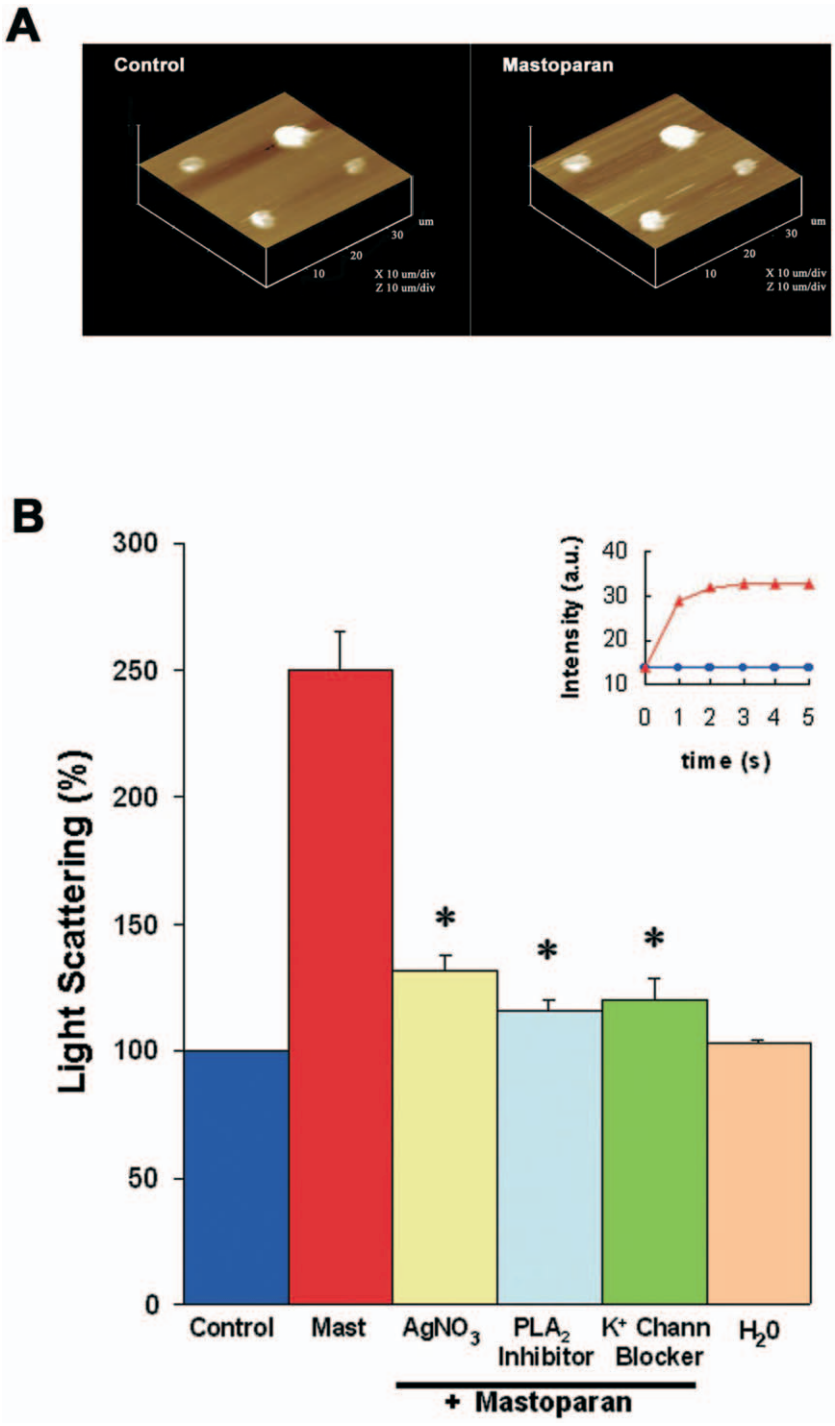

Fig. 5. Measurements of volume changes in RBC by AFM (A) and light scattering (B). AFM micrographs demonstrated swelling of RBCs in response to 20 μM mastoparan (A). Note the rapid swelling of RBC following mastoparan exposure (inset in B), determined by light scattering studies. Light scattering studies (B) show RBC volume changes in response to mastoparan, prior to or following exposure to the AQP-1 inhibitor $AgNO_3$, the potassium channel inhibitor glyburide, and the PLA2 inhibitor ONO-RS-082. Note a significant inhibition (* $P<0.05$) of mastoparan-induced RBC swelling, in the presence of $AgNO_3$, glyburide, or ONO-RS-082. Values represent mean ± SE of 3–7 separate experiments. Exposure of RBC to water for the same period (5 min), had little effect on RBC volume.

Fig. 5B). In less than a second, the effect of mastoparan on RBC swelling is almost complete (see Fig. 5B, inset). In contrast, exposure of RBC to a hypotonic solution (H_2O) for up to 5 min had little or no effect on RBC size (mean ± SE: 102 ± 2%, n=3; Fig. 5B). However, exposure of RBC to the water channel inhibitor $AgNO_3$

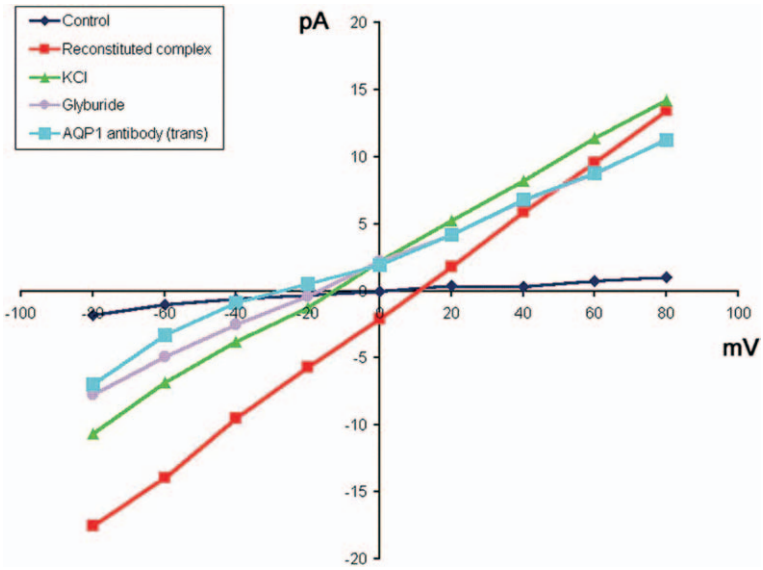

Fig. 6. Electrophysiological properties of AQP-1 complex reconstituted bilayer. AQP-1 complex was reconstituted into a PC:PS bilayer. The bilayer was exposed to a KCl gradient (~410 mM cis, 150 mM trans) following incorporation. Glyburide (20 μM) was then added to the cis side followed by AQP-1 antibody to the trans side of the bilayer chamber. An IV curve (80 mV to +80 mV by 20 mV increments with the mean of 755 points used to determine each voltage point) was taken approximately 3 min after each addition. Initial bath solution was 150 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, pH 7.5 symmetric. Note the decrease in cationic conductance, both following addition of glyburide and the AQP-1 specific antibody.

(mean ± SE: 134 ± 6%, n=4), or to the potassium channel blocker glyburide (mean ± SE: 120 ± 8%, n=3), or the PLA2 inhibitor ONO-RS-082 (mean ± SE: 116 ± 3%, n=3), significantly abrogated mastoparan-induced swelling (P<0.05; Fig. 5B). These studies demonstrate that AQP-1 in the RBC is similarly regulated as in the ZG. Furthermore, since the PLA2 inhibitor ONO-RS-082, inhibited mastoparan-induced swelling, demonstrates that PLA2, like the K^+, and water channels, is down stream of $G_{\alpha i3}$ regulation. Therefore, GTP acts through $G_{\alpha i3}$ and PLA2 to regulate both the K^+ and water channels, whose activities and regulations are closely linked (Fig. 7).

Fig. 7. Schematic outline depicting the regulatory pathway and the main players involved in water channel aquaporin-1 regulation.

3.5. Reconstituted lipid bilayer exhibit decreased conductance on exposure to glyburide or the AQP-1 specific antibody

In order to determine if ionic transport activity was associated with AQP-1, the immunoisolated AQP-1 complex was reconstituted into a planar lipid bilayer (Fig. 6). Incorporation of the complex using the pipette brush technique (Cho et al., 2002b; Jena et al., 2003; Kelly and Woodbury, 2003) led to an increase in conductance in the bilayers. When a KCl gradient was introduced across the bilayer, a shift in reversal potential was observed corresponding to the passage of K^+. Addition of glyburide to the cis side of the chamber resulted in a decrease in overall conductance. Addition of the AQP-1 specific antibody to the trans side of the bilayer (where the carboxyl terminus of AQP-1 should be located) also resulted in a decrease in conductance. These results further confirm the association of a potassium channel with AQP-1 immunocomplex. Since the conductance decreases following addition of the AQP-1 specific antibody, a positively charged ion and not neutral water is being conducted. Sensitivity of the cation conductance by the AQP-1 antibody could be the result of several factors. It has been reported by Anthony et al. (2000) that the AQP-1 channel conducts cations when expressed in *Xenopus* oocytes. AQP-1 expressed in *Xenopus* oocytes is also blocked by the potassium channel blocker, TEA (Brooks et al., 2000). When AQP-1 purified from *Saccharomyces* was reconstituted into planar lipid bilayers, a small percentage of cation channels were observed (Saparov et al., 2001). These channels were much smaller than the ones reported in oocyte membranes. However, these conductances can be explained by channels known to exist in both *Xenopus* oocyte and *Saccharomyces* membranes (*Xenopus* oocytes possesses a c-GMP gated glybencamide (glyburide)-sensitive potassium channel; Sakuta

et al., 1993; Sakuta et al., 1994, and *Saccharomyces* possesses a small potassium channel). The real question is how does an antibody against AQP-1 decrease conductance? There are two possible answers. First, either the potassium channel and AQP-1 channel are directly linked or the channels are capable of intermixing subunits. Indeed, it has been noted by Yool and Weinstein, 2002, the amount of similarity between potassium channels and the AQP-1 pore. On the other hand, we cannot eliminate the possibility that AQP-1 may be directly blocked by the potassium channel blockers as suggested by Brooks et al., 2000, and to some small degree be capable of passing cations. However, crystallography studies (Sui et al., 2001) demonstrate that water clearly passes through the AQP-1 pore. Thus, for a pure AQP-1 channel, the only cation expected to be transported would be the positively charged hydronium ion (H_3O^+). These possibilities will be the focus of further studies (Fig. 7).

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