

Water channels in platelet volume regulation

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Abstract

The regulation of platelet volume significantly affects its function. Because water is the major molecule in cells and its active transport *via* water channels called aquaporins (AQPs) have been implicated in cellular and organelle volume regulation, the presence of water channels in platelets and their potential role in platelet volume regulation was investigated. G-protein-mediated AQP regulation in secretory vesicle swelling has previously been reported in neurons and in pancreatic acinar cells. Mercuric chloride has been demonstrated to inhibit most AQPs except AQP6, which is stimulated by the compound. Exposure of platelets to HgCl₂-induced swelling in a dose-dependent manner, suggesting the presence of AQP6 in platelets. Immunoblot analysis of platelet protein confirmed the presence of AQP6, and also of G_{α0}, G_{αi-1} and G_{αi-3} proteins. Results from this study demonstrate for the first time that in platelets AQP6 is involved in cell volume regulation *via* a G-protein-mediated pathway.

Keywords: platelets • aquaporin-6 • volume regulation

Introduction

Water, the most abundant molecule in living cells, is required for various vital life functions including the regulation of cell and organelle volume. The volume regulation of cells and subcellular organelles is critical to numerous physiological processes. Changes in cell volume are known to occur during cell secretion [1], in erythrocyte maturation, during cell growth and differentiation [2], and in cell migration and metabolism [3]. Similarly, intracellular swelling of membrane-bound secretory vesicles [4, 5] has been demonstrated in the regulated release of vesicular contents during cell secretion [6]. All these studies demonstrate that rapid swelling of cells or secretory vesicles occurs as a result of rapid water entry through membrane-associated water channels or AQPs [7]. Platelets are 2–3 μm in diameter anuclear cells that contain lysosomes, α granules and dense granules, and secrete a variety of essential molecules including growth factors. Impaired platelet function is causal to bleeding or clotting,

resulting in adverse outcomes such as myocardial infarction, stroke or pulmonary embolism [8]. Because regulation of platelet volume significantly affects its function, this study was undertaken to determine the molecular mechanism of platelet volume regulation.

Volume regulation in cells and secretory vesicles has previously been examined [4, 5]. Live pancreatic acinar cells in near physiological buffer, when imaged using the atomic force microscope (AFM), revealed at nanometre resolution the presence of membrane-bound secretory vesicles called zymogen granules (ZGs) lying immediately below the surface of the apical plasma membrane. Within 2.5 min. of exposure to a secretory stimulus, the majority of ZGs within cells swell, followed by a decrease in size, and a concomitant discharge of intra-vesicular contents [6]. These studies directly demonstrate intracellular swelling of secretory vesicles following stimulation of cell secretion, and vesicle

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deflation following partial discharge of vesicular contents. A similar mechanism of synaptic vesicle (SV) swelling for neurotransmitter release has also been reported [6]. This direct estimation of vesicle size dynamics at nanometre resolution under various experimental conditions has enabled determination of some of the molecular components participating in secretory vesicle swelling. Studies using isolated ZGs demonstrate the presence of Cl^- and ATP-sensitive K^+ selective ion channels at the ZG membrane, whose activities are implicated in ZG swelling [9]. These studies further demonstrate that secretion of ZG contents from pancreatic acinar cells require the presence of both K^+ and Cl^- [10]. Heterotrimeric $\text{G}_{\alpha\beta\gamma}$ protein has been implicated in the regulation of both K^+ and Cl^- ion channels in a number of tissues [11]. Analogous to the regulation of K^+ and Cl^- ion channels at the plasma membrane in cells, the regulation of these channels at the ZG membrane by a $\text{G}_{\alpha\beta\gamma}$ protein has been demonstrated [4]. Isolated ZGs from exocrine pancreas swell rapidly in response to GTP and NaF [4], suggesting the involvement of rapid water entry into ZGs following GTP exposure. As opposed to osmotic swelling, membrane-associated AQP channels are involved in rapid water entry into cells [12]. The presence of AQP1 and $\text{G}_{\alpha\beta\gamma}$ protein at the ZG membrane [4, 5], and AQP6 and $\text{G}_{\alpha 0}$ protein at the SV membrane [4, 5], and their involvement in GTP-mediated vesicle water entry and swelling have previously been reported [4, 5]. Mastoparan, an amphiphilic tetradecapeptide from wasp venom, potentiates the GTPase activity of $\text{G}_{\alpha i}/\text{G}_{\alpha 0}$ proteins [13–15]. Stimulation of G proteins is believed to occur by the peptide inserting into the phospholipid membrane and forming a highly structured α -helix resembling the intracellular loops of G-protein-coupled receptors. Analogous to receptor activation, mastoparan is thought to interact with the COOH-terminal domain of the G protein α subunit [16]. Active mastoparan (Mas7) in the presence of [γ - ^{32}P]GTP demonstrate a significant increase in swelling and GTPase activity in ZG, establishing the presence of $\text{G}_{\alpha i}$ proteins with ZGs [4]. Similarly, mastoparan has also been demonstrated to stimulate SV swelling [17]. Studies further report that vH^+ -ATPase present at the SV membrane [18] is responsible for the generation of electrochemical H^+ gradient (pH 5.2–5.5) within the SV [19] required for transport of neurotransmitters into the SV lumen. In addition to the established role of vH^+ -ATPase in neurotransmitter transport into SV, vH^+ -ATPase has been suggested to participate in the secretion of stored neurotransmitters [20, 21]. Because SV swelling is $\text{G}_{\alpha 0}$ -mediated, and is required for cell secretion [4], the involvement of vH^+ -ATPase at the SV membrane [9] in $\text{G}_{\alpha 0}$ -mediated water gating through the AQP6 channel was hypothesized and tested in a recent study [22]. In agreement, results from the study demonstrated that SV-associated vH^+ -ATPase is required for GTP- $\text{G}_{\alpha 0}$ -mediated swelling of SV [22].

In view of these earlier studies, the involvement of aquaporin and heterotrimeric G-proteins in the regulation of platelet volume was hypothesized and tested in this study. Mercuric chloride is known to inhibit most AQPs except AQP6 [23], which is stimulated by the compound. Exposure of platelets to HgCl_2 -induced cell swelling in a dose-dependent manner, suggesting the

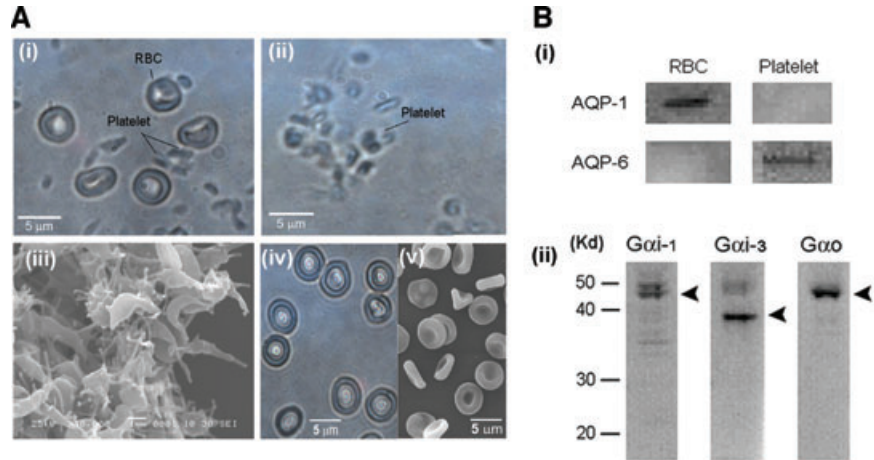
presence of AQP6 in platelets. Immunoblot analysis of platelet protein confirmed the presence of AQP6. To further determine if G proteins were involved in AQP6 function in platelets, G-protein-specific immunoblot analysis and mastoparan-stimulated swelling was examined. Exposure of platelets to mastoparan results in a dose-dependent swelling of platelets, demonstrating for the first time the involvement of G-proteins in platelet volume regulation. Immunoblot analysis further confirm the presence of AQP6 in platelets, and the presence of $\text{G}_{\alpha 0}$, $\text{G}_{\alpha i-1}$ and $\text{G}_{\alpha i-3}$ proteins in platelets as previously demonstrated [24, 25]. Results from this study demonstrate for the first time that AQP6, $\text{G}_{\alpha 0}$, $\text{G}_{\alpha i-1}$ and $\text{G}_{\alpha i-3}$ proteins are involved in platelet volume regulation.

Materials and methods

Sprague–Dawley rats weighing 100–120 g were used for the study. Blood was collected by cardiac puncture immediately following CO_2 -induced euthanasia of the animal. All animal studies were approved by the IACUC at Wayne State University. To isolate red blood cells (RBC), the blood sample was diluted in 10 volume of PBS pH 7.4, and spun at $120 \times g$ for 10 min. at 4°C . The resulting pellet was resuspended in 2 volume of PBS, and washed three times, followed by their final resuspension in 10 volume of PBS prior to use. The purity of RBC preparation was examined using light and scanning electron microscopy (Fig. 1A). To isolate platelets, blood was dispersed in acid citrate-dextrose solution (ACD; 0.1M citric acid, 0.2M sodium citrate, 0.4M dextrose, pH 6.8), which functions as an anticoagulant and anti-stimulant for platelets. Blood samples were imaged using light microscopy (Fig. 1A, i). Whole blood with an equal amount of ACD was centrifuged at $100 \times g$ for 15 min. and platelet-rich supernatant was mixed with an equal amount of buffer A (20 mM HEPES-NaOH pH 7.4, 3.3 mM NaH_2PO_4 , 2.9 mM KCl, 1 mM MgCl_2 , 128 mM NaCl, 5.5 mM D-glucose, pH 7.4). The supernatant was centrifuged at $1000 \times g$ for 10 min. to obtain a pure platelet pellet. After washing in PBS, the isolated platelets were imaged using light (Fig. 1A, ii) and electron microscopy (Fig. 1A, iii) to determine their purity. Isolated platelet and RBC preparations for electron microscopy were fixed using 2% paraformaldehyde and 1% glutaraldehyde for 1 hr at room temperature followed by washing in PBS. Isolated pure platelet preparations were used in dynamic light scattering experiments, and both platelet and RBC preparations were solubilized using 1% Triton/Lubrol for immunoblot analysis (Fig. 1B, i and ii).

To perform light microscopy on isolated platelets, 25 μl of platelet preparation was spread on to a glass slide. Cells were allowed to settle and stick to the slide surface at room temperature, and the unattached cells rinsed gently three times using 200 μl of PBS. Cells were imaged using a Zeiss Axiovert microscope (100 \times objective; Fig. 1A, i and ii). Similarly, to perform scanning electron microscopy on isolated platelet preparations, 25 μl of aldehyde fixed isolated platelets was spread on 13 mm Thermoform cover slips coated with 0.2% poly-L-lysine. Cells were allowed to settle and stick to the surface of the cover slip overnight at 4°C , and the excess sample rinsed gently by dipping into four changes of Milli Q dH_2O . The cover slips were loaded into a cylindrical holder and dehydrated for 5 min., each in increasing concentrations of ethanol (35%, 50%, 70%, 85% and 95%) followed by four times 5 min. wash in 100% ethanol. The sample was dried and mounted onto one large aluminium specimen stub in puddles of liquid graphite, sputter coated with gold and palladium and imaged with a JOEL JSM 6060 SEM operating

Fig. 1 Light and scanning electron micrographs of isolated platelets from rat blood, and presence of AQP6, $G_{\alpha o}$, $G_{\alpha i-1}$ and $G_{\alpha i-3}$ proteins in the preparation. (A, i) Light micrograph of platelet-enriched rat blood. Note the presence of both red blood cells (RBC) and platelets. (A, ii) Light and (A, iii) scanning electron micrographs of purified platelet preparation. (A, iv) Light and (A, v) scanning electron micrographs of purified RBC preparation. (B, i) Immunoblot analysis of platelet homogenate, demonstrates the presence of AQP6, and (B, ii) $G_{\alpha o}$, $G_{\alpha i-1}$ and $G_{\alpha i-3}$ proteins. The RBC water channel AQP1 is absent in platelets. Total RBC homogenate demonstrates the presence of AQP1 and the absence of AQP6.



at 25 kV, spot size 30, WD-8-10 mm at various magnifications up to 28,000 \times (Fig. 1A, iii and iv).

Platelet size dynamics were determined using real-time, right angle light scattering in a Hitachi F-2000 spectrofluorimeter. Real-time scattered light intensities at excitation and emission wavelengths of 530 nm were used to measure platelet size dynamics [22]. In these experiments, isolated platelets were suspended in water, and rapid changes in their size was monitored before and after addition of $HgCl_2$ (an AQP1 inhibitor and an AQP6 stimulator), followed by mastoparan exposure. Values are expressed in arbitrary units and presented as percent light scattered over controls. Student's *t*-test was performed for comparison between groups, with significance established at $P < 0.01$.

Immunoblot analysis was performed on total protein content from isolated platelet preparations. Protein concentration was determined by BSA protein assay. Sample aliquots solubilized in Laemmli sample preparation buffer were resolved using 10% SDS-PAGE. Resolved proteins were electrotransferred to nitrocellulose membranes for immunoblot analysis using specific antibodies. The nitrocellulose membranes were incubated in blocking buffer (5% non-fat milk in PBS with 0.1% TWEEN) for 30 min. at room temperature, followed by immunoblotting for 1 hr at room temperature with specific antibodies. Primary polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1:200 (AQP-1, AQP-6, $G_{\alpha o}$, $G_{\alpha i-1}$ and $G_{\alpha i-3}$) in blocking buffer. Immunoblotted nitrocellulose membranes were washed in PBS and incubated in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:1000 for 1 hr at room temperature. After washing in PBS, the nitrocellulose membranes were processed for enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ, USA) and developed using a Kodak 440 image station.

Results and discussion

Analogous to a number of other mammalian cell types, platelet volume significantly affects its function, such as platelet activation and the secretion of a variety of essential molecules. Impaired volume regulation in platelets negatively influences blood clotting,

resulting in adverse outcomes such as myocardial infarction, stroke or pulmonary embolism [8]. Because water channels or aquaporins have previously been implicated in cellular and organelle volume regulation, their presence in platelets and their potential role in platelet volume regulation was hypothesized and investigated.

Platelets were isolated from rat blood, and their purity determined by both light and scanning electron microscopy (Fig. 1). Mercuric chloride inhibits most AQPs except AQP6, which is stimulated by $HgCl_2$. Exposure of platelets to $HgCl_2$ induced swelling in a dose-dependent manner, suggesting the presence of AQP6 in platelets (Fig. 2A, i and ii). $HgCl_2$ induced a dose- and time-dependent increase in platelet size, as demonstrated using dynamic light scattering. Dynamic light scattering further demonstrated that 300 μM $HgCl_2$ was the optimal dose in stimulating platelet size increase. In the first 15 sec. after addition of 300 μM $HgCl_2$, increase of platelet swelling is a linear function of time (Fig. 2A, iii), suggesting that influx of water and ions is unrestricted and rapid at this initial period. After this point, platelet swelling slows down, and changes in platelet volume assume a logarithmic form that can be expressed by a first-order equation, with a rate constant $k = 2.3 \times 10^{-2}/sec$. (Fig. 2A, iv). Similarly, analogous to $HgCl_2$, dynamic light scattering further demonstrated that mastoparan induces a dose- and time-dependent increase in platelet size (Fig. 2B). Dynamic light scattering demonstrated that 40 μM mastoparan was the optimal dose in stimulating platelet size increase (Fig. 2B, i and ii). In the first 10 sec. after addition of 40 μM mastoparan, increase of platelet swelling is a linear function of time (Fig. 2B, iii), again suggesting that influx of water and ions is unrestricted and rapid at this initial period. After this point, platelet swelling slows down, and changes in platelet volume assume a logarithmic form that can be expressed by a first-order equation, with a rate constant $k = 24 \times 10^{-2}/sec$. (Fig. 2B, iv). These studies suggested that both AQP6 and $G_{\alpha i}/G_{\alpha o}$ proteins are present in platelets and participate in platelet volume regulation. This is further confirmed, because

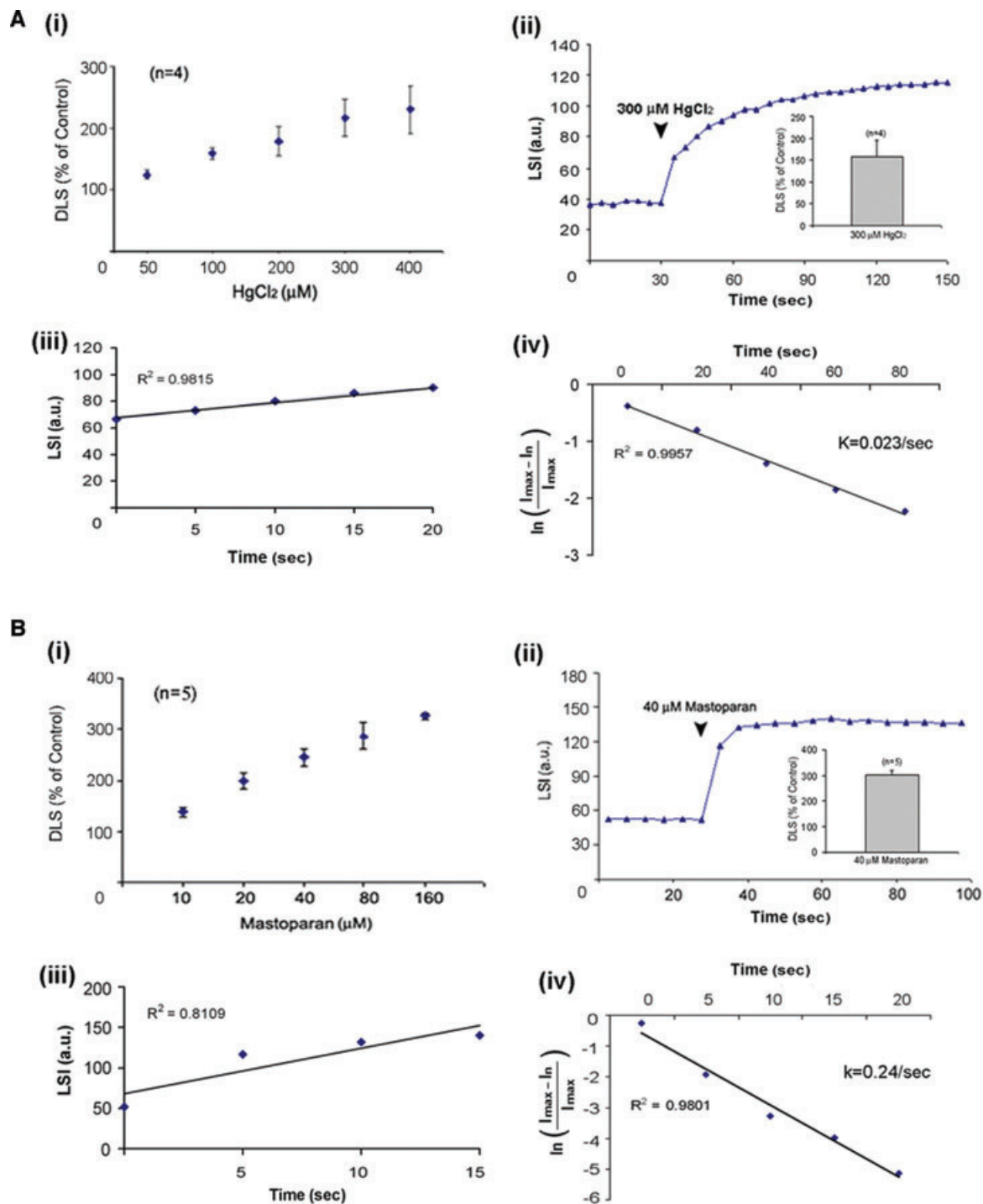


Fig. 2 HgCl₂- and mastoparan-induced dose- and time-dependent increase in platelet size, demonstrated using dynamic light scattering. **(A, i)** Dynamic light scattering, demonstrating HgCl₂ dose-dependent increase in platelet size over control. **(A, ii)** Exposure of 300 μM HgCl₂ to isolated platelets, demonstrates a time-dependent increase in platelet size. **(A, iii)** Initial kinetics of HgCl₂-induced platelet swelling. **(A, iv)** Graph depicts the first-order kinetics of increase in platelet size following exposure to 300 μM HgCl₂. **(B, i)** Dynamic light scattering demonstrating the mastoparan dose-dependent increase in platelet size over control. **(B, ii)** Exposure of 40 μM mastoparan to isolated platelets, demonstrates a time-dependent increase in platelet size. **(B, iii)** Initial kinetics of mastoparan-induced platelet swelling. **(B, iv)** Graph depicts the first order kinetics of increase in platelet size following exposure to 40 μM mastoparan.

immunoblot analysis performed on total platelet proteins using specific antibodies to AQP6, and to $G_{\alpha i}$ and $G_{\alpha o}$ proteins, demonstrate their presence in platelets (Fig. 1B). Immunoblot analysis further demonstrates that AQP6 is present in platelets; however, the RBC water channel AQP1 is absent. Similarly, AQP6 is absent in RBC. To further determine if G proteins are present in platelets, G-protein-specific immunoblot analysis was performed on total platelet homogenate. These immunoblot studies confirm the presence of $G_{\alpha o}$, $G_{\alpha i-1}$ and $G_{\alpha i-3}$ proteins in platelets (Fig. 1B, ii). These results demonstrate for the first time that functional AQP6, $G_{\alpha o}$, $G_{\alpha i-1}$ and $G_{\alpha i-3}$ proteins are present in platelets and are involved in cell volume regulation. It is possible that one or all of the above G-proteins are involved in platelet volume regulation. Analogous to secretory vesicle volume dynamics, we have now demonstrated that the regulation of platelet volume involves both water channels and heterotrimeric G-proteins, driving the active and rapid transport of water through the AQP6 channels at the platelet plasma membrane.

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Authors' contribution

B.P.J. and D.A.W. designed the research and B.P.J. wrote the paper. J.S.L. and S.A., performed majority of the experiments, both contributing equally to the study. M.T. and D.J.T. performed all the E.M. studies.

Conflict of interest

The authors declare no competing financial interests.

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