Aquaporins in human platelets: intracellular localization and possible role in granule and lysosome secretion

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This study was undertaken to establish the presence and the role of aquaporins (AQPs) in human platelets. Immunodetection with polyclonal antibodies and fluorescent microscopy suggest the presence of AQP isoforms 0–7 and 9–12—localized (in resting platelets) in the plasma membrane and in the dense and alpha granules. In thrombin- or monensin-treated platelets, the granules’ AQP activities become visible in the whole cell body, indicating the granules’ swelling. In our studies on the role of AQP s in platelet responses we used tetrachloroauric acid (HAuCl₄), a classical water channel blocker. We found that 10–100 µM of Au(III) inhibited the hypotonicity- and monensin (simulating the action of Na⁺/H⁺ exchanger)-, and collagen-evoked platelet swelling and reduced triated water uptake by platelets treated by collagen or monensin, indicating its ability to block water channels in these cells. HAuCl₄, at the concentrations reducing water influx, did not induce cell lysis, alter the plasma membrane shape or the –SH group content. The inhibitor also failed to affect Na⁺ and Cl⁻-related osmotic gradient formation and protein kinase D₂ phosphorylation. In platelets activated by threshold concentrations of collagen (simulating the action of Na⁺/H⁺ exchanger), ATP from dense granules but failed to reduce platelet aggregation. In collagen-stimulated platelets, HAuCl₄ (10–100 µM) completely inhibited secretion of ATP from dense granules but failed to reduce platelet aggregation. In collagen-stimulated platelets, HAuCl₄ (10–100 µM) reduced secretion from dense and alpha granules, as well as lysosomes, in a dose-dependent manner. We conclude that human platelets possess numerous AQPs subtypes localized in the plasma and granule membranes. AQP-mediated water fluxes may be crucial for platelet volume regulation as well as secretion from dense and alpha granules and lysosomes.

Key words: blood, platelets, aquaporins, aggregation, secretion, osmotic swelling

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INTRODUCTION

Aquaporin water channels (AQPs), represent a large family of evolutionarily conserved, integral membrane proteins that mediate rapid and selective transport of water and small uncharged solutes (for example, glycerol) across cell and organelle membranes in response to osmotic gradients generated by ion exchangers and transporters (for instance, the Na⁺/H⁺ exchanger, NHE) (Agre et al., 2002; Kato et al., 2006; Hub & de Groot, 2008; Carbrey & Agre, 2009; Verkman, 2012).

All AQP units can be described as homotrimeric forming four channels within the membrane, resembling an hourglass-like structure, and acting as independent water pores (Sui et al., 2001; Walz et al., 2009; Khalili-Araghi et al., 2009). Monomers contain six transmembrane domains and five interhelical loops (designated A-E). Two of these loops (B and E), containing a highly conserved short hydrophilic region (designated A-E). Two of these loops (B and E), containing a highly conserved short hydrophilic region (designated A-E). Two of these loops (B and E), containing a highly conserved short hydrophilic region (designated A-E). Two of these loops (B and E), containing a highly conserved short hydrophilic region (designated A-E). Two of these loops (B and E), containing a highly conserved short hydrophilic region (designated A-E).

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Aquaporins are divided into two subfamilies depending on their genomic structure and selectivity: (1) water-specific “classical” AQPs (2) aquaglyceroporins, and (3) superaquaporins (S-aquaporins). The aquaglyceroporins have a less constricted pore that allows them to be able to transport larger molecules, besides water, such as glycerol (Hub & de Groot, 2008; Benga, 2012). S-aquaporins are highly deviated from previously known AQPs and have been found exclusively inside the cell, e.g. in the secretory granules (Ishibashi, 2006; Ishibashi et al., 2014).

Mammalian AQPs are expressed in various epithelia and endothelia, involved in fluid transport, as well as in a variety of cell types including erythrocytes, leukocytes, adipocytes, keratinocytes, astrocytes, and skeletal muscle cells (Schwab et al., 2012; Verkman et al., 2014).

Initially, it was thought that the major physiological role of AQPs in humans is restricted to transepithelial and transendothelial water movement, and the adaptation of cells to changes in osmotic pressure (Agre et al., 2002; Verkman, 2014). More recently, it has been discovered that AQPs constitute important components of the cellular migration machinery, where formation of filopodia...
and lamellipodia play a pivotal role (Saoudoun et al., 2005; Papadopoulos et al., 2008; Verkman, 2014).

AQPs have been also proposed to be involved in water uptake by secretory vesicles of the exocrine pancreas cells (Cho et al., 2002; Sugiya & Matsuki, 2006) and in the swelling of synaptic vesicles isolated from rat brains (Kelly et al., 2004; Jeremic et al., 2005). Secretory granule swelling is believed to be a necessary step in the release of their content (exocytosis) (Sugiyama et al., 2008).

The role of AQPs in the physiology of human platelets is not completely understood. A study performed on platelets from three children with a mutation of the AQP7 gene indicated that human platelets possess AQP7 and that this AQP isoform may be important for platelet morphology, aggregation (adrenaline-induced), and secretion (Goubau et al., 2013). A more recent study also revealed presence of AQP1 in human platelets (Agbani et al., 2018). Using AQP1-null mice it was shown that this AQP isoform is crucial for the platelet procoagulant response. However, deletion of AQP1 had minimal effects on collagen-induced platelet secretion and aggregation. Yet, the presence of other AQPs and their relevance in platelet physiology is still not completely known.

Consequently, in this study we systematically examined, for the first time, the expression and localization of AQPs in human platelets and investigated their role in the platelet physiology.

MATERIALS AND METHODS

Materials. Rabbit polyclonal anti-human AQP0 (sc-99059), AQP1 (sc-20810), AQP2 (sc-28629), AQP3 (sc-20811), AQP4 (sc-20812), AQP5 (sc-28628), AQP6 (sc-28627), AQP7 (sc-28625), AQP8 (sc-28624) and AQP9 (sc-28623) antibodies, normal rabbit polyclonal IgG (isotype control), horseradish peroxidase (HRP)-conjugated goat anti-rabbit, and HRP-conjugated goat anti-mouse antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-human AQP11 antibody (PA5-25704), Alexa Fluor 488-conjugated and Alexa Fluor 568-conjugated goat anti-rabbit antibodies, N-(Ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) and enhanced luminescence Super Signal West Pico Substrate were from Thermofisher Scientific (Life Technologies). Rabbit polyclonal anti-human AQP10 (SAB4300715) and AQP12 (SAB4300758) antibodies, mouse anti-phosphoserine antibody (p-Ser), mepacrine, monensin, tetrachloroauric acid tetradecahydrate (HAuCl₄ × 4H₂O, here referred to as Au (III)), [¹⁹F]-5-hydroxytryptamine-5-HT, serotonin) creatinine sulphate, tritiated water (H₂O), thrombin receptor activating peptide (TRAP), epinephrine (adrenaline) bitartate salt, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), protease and phosphatase inhibitor cocktails were purchased from Sigma. Arachidonic acid, Chrono-Par collagen suspension (for incubations with sterile saline and kept at –20ºC for no longer than three weeks. Attention was taken to not exceed 0.25% (v/v) of HAuCl₄ solution in a sample (final concentration). Such additions did not evoke any noticeable changes in the samples’ pH.

Blood collection and platelet preparation. Blood was collected from healthy volunteers into 10 ml polypropylene tubes containing 1 ml of 130 mM trisodium citrate. All procedures were conducted in accordance with the Declaration of Helsinki and the study was approved by the local Ethics Committee on human research. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200×g for 20 min. Washed platelets were prepared as described previously (Miształ et al., 2015). Gel-filtered platelets were prepared using a Sepharose 2B gel filtration method. Platelet preparations were standardized to 2×10⁸ cells/ml with the Tyrode-Hepes buffer.

Fluorescent and confocal cell imaging. Confocal images were acquired using a Nikon Digital Eclipse C1-Plus Modular Confocal System attached to an inverted fluorescence microscope – Nikon Eclipse Ti. The system is equipped with DS-Fi1 digital microscope camera (5-megapixel CCD that captures at a high resolution of 2560×1920 pixels) and the following laser lines: a violet diode laser (405 nm), an argon ion laser (488 nm), and a HeNe laser (543 nm). Images were captured with EZ-C1 software using an oil immersion Plan Apochromat objective lens (60×/1.4 or 100×/1.4).

Immunodetection and localization of AQP isoforms. Gel-filtered platelets were lysed by the addition of an equal volume of ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors cocktail. Lysates were analyzed towards AQP presence by Western Blotting with a chemiluminescent detection system. Lysates (10 µg of protein) of gel-filtered platelets were separated by SDS-PAGE under reducing conditions, followed by transfer onto a nitrocellulose membrane, blocking (with 5% fat-free milk) and overnight incubation (at 4°C) with primary antibody (1:1000 dilution). After washing, blots were incubated for 1 hour at room temperature (RT) with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution). Immunoreactive signals were measured by using an enhanced chemiluminescence reagent. Some adjustments in brightness were made (the same for each lane). Monomer subunits of AQPs are expected to run between 28–37 kDa; higher molecular-weight bands are likely to be stable multimers or highly glycosylated AQPs forms. Data are from four independent experiments, each performed on a separate platelet preparation. To check intraplatelet sublocalization of AQPs, aliquots of non-stimulated platelets were fixed by using an equal volume of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min at RT. Next, the platelets were pelleted (2200×g, 2 min) and resuspended in PBS containing 3% bovine serum albumin (BSA), supplemented (or not) with saponin (0.04% final conc.) followed by incubation for 30 min at RT. After that, the platelets were pelleted again and resuspended in the same buffers as mentioned above, followed by the addition of an appropriate anti-AQP (0-12) antibody (one type of antibody per one sample, 1:500 dilution) and overnight incubation at 4°C with gentle agitation. After 3 washes, the samples were supplemented with a secondary antibody conjugated with Alexa Fluor 488 (1:250 dilution) and incubated...
for 1 h at RT. After 3 washing steps and final resuspension, aliquots (25 μl) of platelets were transferred into microchamber slides (ibidi µ-Slide VI®) and observed under a fluorescent microscope (Nikon ECLIPSE Ti/C1 Plus, ×100/1.4 oil immersion objective) at the same gain value. In some experiments, the platelets were activated by thrombin or exposed to monensin before fixation in PFA. To assess whether AQP5s can be localized within the platelet secretory granules, we used two-color labeling—concomitant detection of AQP5s using Alexa Fluor 568-conjugated secondary antibody (1:250 dilution), and dense or alpha granules detection using mepacrine (10 μM final conc.) or FITC-anti-P-selectin antibody (1:25 dilution), respectively. Orange to pastel yellow color indicates signal co-localization. Isotype control primary antibody (normal rabbit IgG) was used to evaluate potential non-specific bindings. No relevant fluorescence was found when samples were incubated with only secondary antibodies.

**Aggregation.** Platelet aggregation was followed turbidimetrically (in PRP) (Born & Cross, 1963) or electronically (in whole blood) (Mackie et al., 1984) by using the Chrono-Log 700 lumiaggregometer (Chrono-Log Corp.).

**Simultaneous measurement of platelet aggregation and ATP secretion.** ATP release was monitored simultaneously with optical aggregation by using the Chrono-Log 700 lumiaggregometer. All procedures were conducted in accordance with the Chrono-Log Diagnostics Protocol.

**Secretion.** Serotonin (5-HT) secretion was studied by the isotopic method as described in (Tomasiaq et al., 2004). Platelet factor 4 (PF4) release was measured by ELISA. P-selectin exposure on the surface of activated platelets was evaluated by using flow cytometry and by fluorescent microscopy. Hexosaminidase release from lysosomes was assayed colorimetrically as described in (Nakagawa et al., 1978).

**Measurement of rapid changes in the platelet volume.** Changes in the platelet volume were followed spectrophotometrically by recording the absorbance at 680 nm in diluted cell suspensions, essentially as described in (Rosskopf et al., 1991). A drop in the absorbance reflects a rise in the cell volume. The changes in absorbance were measured at 73°C in plastic cuvettes (light path 10 mm) without stirring. Changes in the platelet volume were evoked either by hypotonicity or by monensin. Aliquots of standardized PRP (140 μl) supplemented with HAuCl₄ (0–100 μM) were incubated for 2 min at 37°C in the cuvette of the instrument, then 860 μl of an isotonic Tyrode-Hepes buffer (pH 7.4), supplemented or not supplemented with monensin (30 μM final conc.), or a hypotonic Tyrode-Hepes buffer (final osmolality of 200 mOsm), all pre-warmed to 37°C, were added. To ensure rapid mixing, the buffer was added via injector equipped with a silicone tube inserted into the cuvette. The changes in absorbance were recorded for 10 s at 0.1 s intervals using a spectrophotometer (Hitachi F-2900, Hitachi Corp., Japan) connected to a personal computer.

**Measurement of mean platelet volume.** Aliquots of standardized PRP (2×10⁶ cells/ml) supplemented with tirofiban (250 ng/ml), to prevent platelet aggregation, were incubated for 2 min at 37°C with or without HAuCl₄ (10–100 μM). Next, collagen (15 μg/ml) or monensin (30 μM) was added. After 5 min of incubation, platelets were fixed with paraformaldehyde (2% final conc.) and their mean volume (MPV) was measured by using a hematoxic analyzer (Coulter Electronic GmbH).

**Measurement of water uptake.** Aliquots (1 ml) of washed platelets (2×10⁶ cells/ml), supplemented with tirofiban (250 ng/ml), were incubated with stirring for 2 min at 37°C with (10–100 μM) or without (control) HAuCl₄. Afterwards, 7 μl of tritiated water (5 mCi – 185 MBq/ml) were added and after 30 s of stirring, the platelet suspensions were supplemented with collagen (15 μg/ml) or monensin (30 μM). Prior to the addition of stimuli (time zero) and 2 minutes after the addition (uptake), aliquots (0.4 ml) of the incubated mixture were aspirated for filtration. Filtration was performed under the pressure of 10 kPa on Whatman GF/C filters (diameter 25 mm). The filtered sample was immediately washed with 4×5 ml of ice-cold solution containing 0.15 M NaCl, 1 mM EDTA and 5 mM Hepes, pH 7.4. After that, platelets remained on the filters were lysed with Soluene-350. The tritiated water, which remained inside platelets held on the filters, was measured in the scintillation counter (Ballad-1209, LKB) and expressed as disintegrations per minute (dpm). Data are mean values (± S.D.) of three independent experiments.

**Platelet viability and thiok group determination.** Viability of platelets exposed to HAuCl₄ was evaluated by using the Trypan Blue uptake method (Strober, 2015) and by spectrophotometric measurement of the lactate dehydrogenase activity (eventually leaked from damaged platelets) in the fluid left after platelet removal, (Gutmann & Wahlfeld, 1985). Thiol content in the washed platelets exposed to HAuCl₄ was measured by using the Ellman reaction. Sulphydryl group levels were calculated by using the molar absorption coefficient for DTNB (5,5’-dithiobis-(2-nitrobenzoic acid)) at 37°C (DTNB ε₄₁₂=13800 M⁻¹cm⁻¹) (Eyaer et al., 2003).

**Determination of platelet PKD 2, serine residue phosphorylation.** Phosphorylation of serine residues in 115 kDa protein (reference mass of PKD2; Konopatskaya et al., 2011) in platelets was estimated by Western Blotting with a chemiluminescent detection system.

**Measurements of cytosolic Cl⁻ and NHE activity in platelets.** CI influx into collagen-activated platelets was monitored inMQAE-loaded platelets with monochromators set at 355 nm and 460 nm for excitation and emission wavelength, respectively (Koncz & Daugirdas, 1994). The platelets were preloaded with 1 mM MQAE (final conc.) for 45 min at ambient temperature under gentle rotation. Washed platelets were stimulated in a thermostated (37°C) fluorimeter cuvette with stirring (800 rpm). Stimulators were added directly to the stirred platelet suspension through a port in a fluorimeter cover by using a microsyringe. For evaluation of NHE activity, we monitored changes in the cytosolic pH of BCECF-loaded platelets exposed to thrombin (Siffert et al., 1989). The platelets were loaded with BCECF aceotxymethyl ester (10 μM final conc.) mixed with Pluronic 137 (0.005% final conc.) for 60 min at 37°C. Monochromators were set at 495 nm for the excitation wavelength and at 520 nm for the emission wavelength. In-apparatus conditions were as above.

**Transmission electron microscopy (TEM).** Samples (0.5 ml) of PRP were incubated (or not incubated) with HAuCl₄ (100 μM) for 10 min at 37°C with stirring. Then, collagen was added (15 μg/ml) and the decrease in light transmittance (reflecting shape change) and the ATP release from dense granules (detected luminometrically) were both monitored under the control of a lumigregometer (Chrono-Log 7000). At the moment of substantial shape change and the beginning of dense granules’ release (~45 s after the addition of collagen), 3 volumes of the fixing solution (containing 2% paraform-
Figure 1. Identification and localization of aquaporins in human platelets. (A) Lysates (10 µg of protein) of gel-filtered platelets were separated by SDS-PAGE under reducing conditions, followed by Western Blotting towards AQPs (isoforms from AQP0 to AQP12) presence. Immunoreactive signals were measured by using an enhanced chemiluminescence reagent. Some adjustments in brightness were made (to the same for each lane). Monomer subunits of AQPs are expected to run at 28–37 kDa; higher molecular-weight bands are likely to be stable multimers or highly glycosylated AQPs forms. Data are from four independent experiments, each performed with a separate platelet preparation. (B–D) Aliquots of non-stimulated platelets were fixed in 2% (final conc.) PFA in PBS. After permeabilization (or not) with saponin, samples were mixed with an appropriate anti-AQP (0–12) antibody (one type of antibody per one sample) and incubated overnight at 4°C with gentle agitation. After washing, samples were incubated with a secondary antibody conjugated with AF488 (green color in panel B). After washing and final resuspension, aliquots of platelets were transferred into microchamber slides and observed under fluorescent microscope at the same gain value. To assess whether AQPs can be localized within platelet secretory granules, we used two-color labeling – concomitant detection of AQPs using Alexa Fluor 568-conjugated secondary antibody (red color in panels C–D), and dense or alpha granule detection using mepacrine (green color in panel C) or FITC-anti-P-selectin antibody (green color in panel D), respectively. Orange to pastel yellow color indicates signal co-localization. At least 10 different areas from each sample were imaged. Representative fluorescent microscope images from one (out of four) experiment are presented. Isotype control primary antibody (normal rabbit IgG) was used to evaluate potential nonspecific bindings. No relevant fluorescence was found when samples were incubated only with the secondary antibodies. White bars represent 5 µm (B) and 2 µm (C–D). (E) Dense granules (dense), alpha granules (alpha) and AQPs were labeled and visualized as above. As an illustration of typical AQP localization inside platelets, the result from the AQP6 detection experiment is shown (with the notion that this localization pattern is representative in regard to other AQP isoforms found in platelets). Representative images from one (out of four) experiment are presented. White bar is 5 µm. (F) Gel-filtered platelets were preincubated with or without (+Au) HAuCl₄ (100 µM, 2 min) and exposed to 5 nM thrombin (+Thr) or 40 µM monensin (+Mon) for 5 min at 37°C, followed by fixation, saponization, overnight incubation with specific anti-AQP antibody, and visualized as described above. Control samples were without any (stimulator or inhibitor) additions (unstim). At least 10 different areas from each sample were imaged. Representative images from one (out of four) experiment involving AQP6 detection are presented. White bar is 5 µm.
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aldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer) were added, mixed for 1 min and left for 1 h at 37°C. Next, the platelets were sedimented (2200×g, 2 min) and the obtained pellet was flooded with 2% agarose in PBS. After agarose solidification, a few droplets of the fixing solution were laid on the top of each sample. After 24 h at 4°C, the samples were washed several times with 0.1 M cacodylate buffer followed by post-fixation in 1% OsO4. After 1 h, samples were washed with deionized water, followed by dehydration in a graded series of ethyl alcohol dilutions. The last step was performed in a mixture of 100% ethyl alcohol with propylene oxide (PO) (1:1 v/v) followed by substitution with pure PO. After removing the PO, a series of mixtures containing PO and Epon (3:1, 1:1, and 1:3, respectively) were used for fixation followed by exposure of the samples to pure Epon for 2 h. Next, the samples were transferred into polypropylene capsules and left for solidification at 60°C for at least 48 h. The samples were then cut into 70 nm-thin sections, placed on grids and post-stained using uranyl acetate, followed by post-staining with lead citrate. After a few washings with deionized water, the samples were examined with Zeiss Opton EM 900 transmission electron microscope.

Data analyses. Data reported in this paper are the mean (± S.D.) of the number of determinations indicated.

Figure 2. Inhibitory effect of HAuCl4 on platelet secretion.

(A) PRP samples were incubated for 2 min at 37°C with HAuCl4 (0–100 µM). Secretion was induced by the addition of collagen (15 µg/ml); ADP (10 µM); TRAP (10 µM), or adrenaline (10 µM). ATP released from platelets untreated with HAuCl4 was taken as 100% (control). To determine serotonin secretion, aliquots of [3H]serotonin-loaded platelets were incubated for 2 min at 37°C with HAuCl4 (0–100 µM). The amount of [3H]serotonin released within 4 min after the addition of collagen (15 µg/ml) or monensin (30 µM) was estimated as described under Methods. The maximum amount of [3H]serotonin released from platelets untreated with HAuCl4 was taken as 100% (control). Data are presented as mean values (± S.D.). All experiments were performed at least in quadruplicate using four different platelet preparations. (B) PRP samples were incubated for 2 min at 37°C with HAuCl4 (0–100 µM). Secretion was induced by the addition of collagen (15 µg/ml) or TRAP (10 µM). The amount of PF4 and P-selectin released from platelets within 10 min after the addition of stimulus was estimated by ELISA or flow cytometry, respectively. The maximum amount of PF4 and P-selectin released from activated platelets untreated with HAuCl4 was taken as 100% (control). Data are mean values (± S.D.) of four experiments each performed (at least) in triplicate on separate platelet preparations. (C) aliquots of washed platelets were incubated for 2 min at 37°C with HAuCl4 (0–100 µM). The activity of hexosaminidase (HEX) released from platelets within 4 min after the addition of collagen (15 µg/ml) was estimated by using a colorimetric assay as described under Methods. The maximum activity of HEX released from platelets untreated with HAuCl4 was taken as 100% (control). Data are mean values (± S.D.) of four experiments each performed at least in triplicate on separate platelet preparations. (D) platelets (in PRP) preincubated with (Au+, 100 µM) or without (Au−) HAuCl4 for 10 min at 37°C with stirring, were activated (coll+) or not activated (coll−) for 45 sec with collagen (15 µg/ml). Platelets were then fixed and visualized under a transmission electron microscope. "Filo" – filopodia, "DG" – dense granule, "α" – alpha granule, "OCS" – open canalicular system, and "V" – empty vesicles. Black bar is 500 nm. Original magnification is ×20 000. *P<0.05; **P<0.01; ***P<0.001 vs control.
Human platelets possess several AQP subtypes localized in the secretory granules and in the plasma membrane.

Figure 1A demonstrates the presence of 0–7 and 9–12 AQPs in human platelets. AQP3, AQP10 and AQP11 were poorly expressed, whereas AQP8 was untraceable. We found that in the platelets, the AQPs are localized within plasma membrane (Fig. 1B) and in granule-like structures (Fig. 1B–F). Two-color labeling showed that AQP-related immunoreactive signals co-localize with the dense (Fig. 1C) and alpha granules (Fig. 1D).

Given that secretory granules swell following platelet activation and eventually fuse with the plasma membrane/open canalicular system (OCS) (White, 1999), we further studied the subcellular localization of AQPs in platelets activated by thrombin or treated with monensin. Monensin (which simulates the action of the Na+/H+ exchanger), was reported to induce swelling of secretory vesicles isolated from the chromaffin granules (Grinstein, 1982; Geisow & Burgoyne, 1982) and to promote secretion of serotonin from platelets (Tomasiak et al., 2005). As can be seen in Fig. 1F – unlike in resting platelets, where AQPs are visible as distinctly separated spots (unswollen granules) – following stimulation by thrombin or pretreatment with monensin, AQP-related signals were almost randomly distributed in the whole cell body (swollen granules). This signal scattering was not observed following blockage of AQPs by 100 μM concentration of HAuCl₄ (Au(III)) (Fig. 1F), a concentration that almost completely reduces secretion from the dense and alpha granules (Fig. 2A–B and D). A similar picture was observed in the case of P-selectin distribution in platelets activated by thrombin or collagen (Fig. 3). It is well accepted that in quiescent platelets, P-selectin is stored in the α-granules and is exposed on the platelet surface following activation (Ollivier et al., 2014).

Au(III) blocks water channels in human platelets

To assess whether Au(III) blocks AQPs, we studied its impact on the rapid volume changes in platelets. As seen in Figs 4A–B, Au(III) (10–200 μM) reduced the osmotic and monensin-produced swelling of platelets in a dose-dependent manner. In all cases, 100 μM of Au(III) markedly inhibited the platelet swelling, indicating that this concentration of Au(III) is able to block the AQPs in platelets. Similar Au(III) concentrations reduced an increase in the mean platelet volume (Fig. 4C) and inhibited tritiated water uptake (Fig. 4D) in platelets stimulated by collagen or treated with monensin.

Au(III) failed to affect ion fluxes necessary for osmotic gradient formation

Since water influx through AQPs critically depends on osmotic gradient across the plasma membrane, we tested whether Au(III) affects the ion fluxes in platelets stimulated by thrombin or collagen. Au(III) (100 μM) did not modulate the NHE activity (generating Na+ influx) or a rise in cytosolic Cl− concentration following treatment with thrombin or collagen, respectively (Fig. 5A–B).

Lack of cytotoxicity of Au(III) towards platelets at concentrations reducing rapid swelling

Au(III) (up to 1 mM) did not disrupt the plasma membrane of platelets as judged by the trypan blue uptake and lactate dehydrogenase release tests (Fig. 6A–B). Au(III) (up to 100 μM) did not distinctly affect the content of –SH groups in the platelets (Fig. 6C). We also did not observe alterations within the platelet plasma membrane exposed to Au(III) (100 μM), as examined by TEM (Fig. 7).

Blocking of AQPs results in strong inhibition of platelet secretion from the dense granules

Au(III) (10–100 μM) reduced secretion of ATP in a dose-dependent manner from platelets activated by collagen, a thrombin receptor activating peptide (TRAP), adenosine diphosphate (ADP), and epinephrine (Fig. 2A). Blocking of AQPs by 100 μM of Au(III) fully reduced the ATP release from the platelet dense granules stimulated by collagen, ADP, TRAP, adrenaline, arachidonic acid, calcium ionophore A23187, and phorbol 12-myristate 13-acetate (PMA) which was not associated with
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Figure 4. Impact of HAuCl₄ on rapid platelet volume changes. (A–B) PRP samples were preincubated with stirring for 2 min at 37°C with HAuCl₄ added to the final concentrations as indicated. Experiments were started by the injection (arrow) of isotonic Tyrode-Hepes buffer, supplemented or not supplemented with monensin (30 µM final conc.), or low-osmolality Tyrode-Hepes buffer (200 mOsm final osmolality). Changes in volume (swelling) were followed spectrophotometrically (panel A). A drop in the absorbance reflects a rise in the cell volume. Representative traces and bars demonstrating swelling rate (ΔAbs/s) are shown (panel B). Data from four independent experiments (mean values ± S.D.), each in triplicate (at least), are presented. White bar is 5 µm. (C) platelets preincubated with or without HAuCl₄, added to the final concentrations as indicated, were stimulated with collagen or monensin and after 5 min of incubation their mean volume (MPV) was measured. Control samples were without any additions (unstim). The data represent mean values (± S.D.) of four independent experiments (each in triplicate). (D) washed platelets, preincubated with or without HAuCl₄, added to the final concentrations as indicated, were supplemented with tritiated water. Immediately after that and 2 minutes after the addition of a stimulator (collagen or monensin), samples of incubated mixtures were aspirated for filtration. Control samples were without any additions (unstim). The radioactivity (dpm) of tritiated water that remained in platelets held on the filters was measured. Data are the mean values (± S.D.) of three independent experiments (each in triplicate). In all panels: *P<0.05; **P<0.01; ***P<0.001 vs control; #P<0.05; ##P<0.01; ###P<0.001 vs conditions with collagen.

Figure 5. HAuCl₄ failed to affect NHE activity, chloride ions influx, and PKD₂ phosphorylation in stimulated platelets. BCECF-loaded (for assessment of NHE activity via pH measurement, (panel A) or MQAE-loaded (for detection of Cl⁻ ions influx, (panel B) washed platelets were preincubated for 2 min with or without HAuCl₄ (0–100 µM f.c.) at 37°C. After that, platelet suspensions were transferred into thermostated (37°C) cuvette containing Tyrode buffer supplemented with CaCl₂ (1 mM f.c.). Thrombin (1–4 nM f.c.) or collagen (15 µg/ml f.c.) was added to the stirred suspension (pointed by the arrow on the left side of each image) after stabilization of basal fluorescent signal. Representative traces of changes in intracellular [Cl⁻] and pH value from one experiment (out of six, each in triplicate) are presented. (C), gel-filtered platelets (1×10⁹/ml) were incubated with HAuCl₄ (100 µM) followed by the addition of thrombin (Thr, 5 nM), without stirring, or collagen (Coll, 15 µg/ml) with initial (30 s) stirring. After 5 min of exposure at 37°C, platelets were lysed by the addition of ice cold RIPA buffer supplemented with phosphatase and protease inhibitor cocktail. Samples (10 µg of proteins) were analyzed to determine the level of phosphorylation of serine residues (p-Ser) in 115 kDa platelet protein (corresponding to protein kinase D₂, PKD₂) by Western Blotting using primary anti-phosphoserine antibody (1:5 000) and secondary HRP-conjugated antibody (1:20 000). Immunoreactive signals were visualized by using an enhanced chemiluminescence detection system. A representative result from one (out of three) experiment is presented.

Figure 6. Effect of HAuCl₄ on platelet viability and free sulfhydryl groups content. (A) samples of PRP were incubated for 60 min at 37°C with or without (control) HAuCl₄ (up to 1 mM) followed by the addition of Trypan Blue in PBS (0.05% final conc.). After 15 min of incubation at 37°C, the samples were transferred into microchamber slides and observed under bright field microscopy. Dark blue color, associated with dye uptake (pointed by arrows), indicates plasma membrane damage. (B) samples of PRP were incubated for 60 min at 37°C with or without (control) HAuCl₄ (up to 1 mM). Next, platelets were centrifuged and the lactate dehydrogenase activity in the supernatant was assayed by a spectrophotometric method. (C) samples of washed platelets were incubated for 20 min at 37°C with or without (control) HAuCl₄ (10–200 µM). The content of thiol (-SH) groups in platelets was measured by using the DTNB assay. The total free-SH groups content in platelets was about 113.9±14 nmoles/2×10⁸ cells. Data are mean values (± S.D.) or representative spectra of three independent experiments (each in triplicate). *P<0.05.
reduction in the platelet aggregatory response (Fig. 8). Au(III) (10–100 μM) inhibited collagen- or monensin-induced serotonin secretion from platelets in a dose-dependent fashion (Fig. 2A). TEM examination revealed that Au(III) (100 μM) reduced the emptying of dense granules in platelets stimulated by collagen (Fig. 2D).

Inhibition of APQs by Au(III) results in reduction of secretion from the platelet α granules

Au(III) (10–100 μM) inhibited secretion of hexosaminidase from collagen-stimulated platelets in a dose-dependent manner (Fig. 2C).

Minor role of AQPs in the platelet aggregation

Blocking of AQPs by Au(III), up to 100 μM, failed to reduce the aggregation of platelets stimulated by the threshold concentrations of collagen, ADP, TRAP, arachidonic acid, or PMA (Fig. 8). Collagen-induced aggregation was not sensitive to Au(III), irrespective of whether it was measured in the whole blood or in PRP. Aggregation induced by the A23187 ionophore was the only one that began with a distinct delay. When aggregation was triggered by adrenaline, 100 μM of Au(III) strongly reduced the second wave of response.

DISCUSSION

The results presented here suggest that besides recently discovered AQP1 (Agbani et al., 2018) and AQP7 (Goubau et al., 2013), human platelets are likely to possess ten additional subtypes of AQP family members belonging to the subfamilies of classical aquaporins (AQP0–2 and AQP4–6), aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) and superaquaporins (AQP11 and 12) (Fig. 1A).
In platelets, AQPs are localized in the plasma membrane and intracellularly, particularly in the dense and alpha granules (Fig. 1B–D). Interestingly, the platelet granules contain superaquaporins (AQPII and 12) i.e. subtypes of AQPs family so far exclusively found inside the cells (10–12). Great diversity of AQPs, as well as their localization in the granules, indicate their importance for platelet function.

To study the role of AQPs in the platelet physiology, we employed 10–100 μM tetrachloroauric acid (here referred to as “Au(III)”) as an inhibitor of water channels. Au(III) in the form of HAuCl₄ is known to inhibit (IC₅₀=14 μM) AQPI in the murine and human erythrocytes (RBC) (Yang et al., 2006). Synthesized gold(III) complexes (like Auphen) have been reported to inhibit AQP3 and AQP7, expressed in human RBC (Martins et al., 2012) and in adipocytes (Madeira et al., 2014), respectively.

Heavy-metal, sulfhydryl-reactive small molecules, such as Hg(II) and Au(III) (classical AQPs inhibitors), inhibit water channels by classical inhibitors (including Au³⁺) is thought to occur via interactions with the -SH group of the cysteine residues protruding into the water channel (Martins et al., 2012; Madeira et al., 2014; Estava-Font et al., 2016). Additionally, Au³⁺, with the ion diameter of ~2.7 Å, matches the predicted AQP pore diameter of 2.8 Å (Niemietz & Tyerman, 2002). This combination of reactivity and size most likely constitutes the basis of the observed inhibitory effect. The disadvantage is that the mentioned ions block water channels at relatively high concentrations and may be toxic (especially Hg) to living cells. Despite numerous efforts to identify novel non toxic small-molecule inhibitors, classical AQP inhibitors, especially Au(III) seem to be currently the best established blockers of different subtypes of water channels (Esteva-Font et al., 2016). Inhibition of water channels by classical inhibitors (including Au³⁺) is thought to occur via interactions with the -SH group of the cysteine residues protruding into the water channel (Martins et al., 2012; Madeira et al., 2014; Estava-Font et al., 2016). Additionally, Au³⁺, with the ion diameter of ~2.7 Å, matches the predicted AQP pore diameter of 2.8 Å (Niemietz & Tyerman, 2002). This combination of reactivity and size most likely constitutes the basis of the observed inhibitory effect. The disadvantage is that the mentioned ions block water channels at relatively high concentrations and may be toxic (especially Hg) to living cells. Despite numerous efforts to identify novel non toxic small-molecule inhibitors, classical AQP inhibitors, especially Au(III) seem to be currently the best established blockers of different subtypes of water channels (de Almeida et al., 2017; Estava-Font et al., 2016).

The results presented here show that Au(III), at relatively low micromolar concentrations (10–100 μM), inhibited hypoosmotically- or monensin-evoked (due to Cl⁻ and Na⁺ influx) rapid platelet swelling (Fig. 4B) – a phenomenon critically dependent on the AQPs action (Verkman, 2012). Since AQPs are likely to be involved in both of the above mentioned types of rapid swelling, this may indicate involvement of Au(III) in the platelet water channel blocking. The observation that Au(III) inhibits tritiated water uptake by platelets treated with collagen or monensin is consistent with this idea (Fig. 4D).

Au(III) compounds are much less toxic than commonly used 0.5 M mercury chloride. Experiments with NaAuCl₄ reveal its relatively low toxicity to mice (Sawalsky et al., 2004). However in the same study it was also suggested that NaAuCl₄ (at 0.1–10 mM concentrations) may affect morphology (shape) of red blood cells suspended in an artificial medium. Specifically, following one hour incubation with 0.1 mM HAuCl₄, some red cells changed their normal discoid shape into stomatocytes (a cup-shaped form with evagination of one surface and a deep invagination of the opposite face). This was suggested to result from interaction of Au(III) with the phospholipids in the inner leaflet of the plasma membrane. However, it should be stressed that Au(III)-related stomatocytosis of RBC was observed in washed cells suspended in buffered saline, i.e. in a highly non-physiological environment. In our experimental model we did not observe stomatocyte or echinocyte (RBC with many small thorny projections) formation following incubation of RBC suspended in plasma (i.e. under conditions much more relevant to the in vivo situation and similar to those used in our study) even following one hour of incubation of whole blood with up to 1 mM HAuCl₄ (observations performed under an optical microscope, results not shown here). Under the same conditions, HAuCl₄ did not induce RBC hemolysis (not shown). These observations make the direct effect of Au(III) on platelet membrane highly unlikely, at least under experimental conditions that we used here, i.e. short (3–5 min) exposure of platelets (suspended in the plasma or in the medium containing albumin) to 10–100 μM HAuCl₄. This notion is further strengthened by the finding that exposure of platelets to 100 μM Au(III) for 10 min did not result in alterations in the platelet ultrastructure (including invagination in platelet plasma membrane) examined under transmission electron microscope (Fig. 7). Accordingly, by using the LDH and trypan blue tests we showed that following one hour incubation of platelets suspended in plasma with 1 mM Au(III), their plasma membrane remains intact (Fig. 6A–B).

To sum up, the substantial influence of Au(III) on platelet membrane is excluded, at least under the experimental conditions that we used for functional studies, i.e. short (3–5 min) exposure of platelets to 10–100 μM Au(III).

Studies performed with several synthetic gold (III) coordinated complexes displaying anti tumor activity reveal that their cytostatic activity can be associated with inhibition of thioredoxin reductase and impairment of mitochondria (Barnard & Berners-Price, 2007; Taotao et al., 2013). Platelets contain a thioredoxin reductase and its inhibition was recently reported to result in attenuation of the platelet-mediated clot retraction (Metcalfe et al., 2016). These effects were proposed to result from interactions of Au(III) with intracellular SH groups (Metcalfe et al., 2016; Taotao et al., 2015). However, treatment of platelets with 100 μM of Au(III) for 20 min did not result in a significant decrease in SH group content in the platelets (Fig. 6C). What is more, treatment of platelets with 100 μM of Au(III) did not result in alteration of kinetics of clot retraction (results not shown). Since kinetics of clot retraction is critically dependent on energy production in the platelet mitochondria (Misztal et al., 2013; Misztal et al., 2014; Misztal et al., 2014), this means that Au(III) does not distinctly affect functionality of the platelet mitochondria. This makes the impact of Au(III) on platelet thioredoxin reductase and mitochondria unlikely, at least under the experimental conditions we used here.

The possibility that Au(III) affects ion fluxes (preceding generation of local osmotic disequilibrium (necessary for AQPs-mediated water influx) (Schwab et al., 2012) is also excluded since the concentrations of Au(III) inhibiting rapid platelet swelling (i.e. up to 100 μM) failed to reduce the influx of osmotically active Cl⁻ and Na⁺ into the cytosol of platelets activated by thrombin or collagen (Fig. 5A–B).

Combined, the findings of this study show that HAuCl₄, at the concentrations of 10–100 μM, may serve as a potent and selective inhibitor of AQPs in platelets. Results presented here show that AQPs are essential for platelet secretion from the dense granules, alpha granules, and from lysosomes. Blocking of AQPs reduced platelet secretion considerably (Fig. 2A–D, Fig. 3, Fig. 8). Notably, blocking of platelet AQPs by 100 μM HAuCl₄ resulted in complete inhibition of secretion from the dense granules (Fig. 2A and 8) induced by all principal physiological and pharmacological activators, including activator of the thrombin receptor. The question arises: how may AQPs contribute to platelet secretion?
The current model of the platelet secretory process considers that a crucial step in exocytotic secretion is fusion of the membranes of secretory granules with the membranes of the open canalicular system (OCS)/plasma membrane (Lemons et al., 1997; Sudhof & Rothman, 2009; Fang & Lindau, 2014; Mellander et al., 2014). It is also recognized that in platelets, membrane fusion is mediated by the assembly of the SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) complex (Sudhof & Rothman, 2009; Peng et al., 2013). However, the central role of SNARE proteins in platelet secretion is still not clear (Golembiewska & Poole, 2015). It is also well documented that membrane fusion, crucial for exocytosis, may be promoted by distending the vesicle membrane (a physical factor raising the surface free energy of the granule membrane, favoring fusion) (Ohrbom et al., 1995; Kelly et al., 2004; Jeremic et al., 2005; Mellander et al., 2014).

Despite the fact that it is well documented that secretory granules, in activated platelets, are swollen and connected with the channels of OCS (Escolar & White, 1991; White & Escolar, 1991; White, 1999), the contribution of granule swelling to platelet secretion is currently poorly recognized (Golembiewska & Poole, 2015). Here, we hypothesize that continuous water uptake by platelet secretory granules generates force (hydrostatic pressure) which supports not only their inflation, necessary for fusion, but also determines continuous one-way cargo movement from granules to the OCS channels/plasma membrane and further, to the exterior surface. The role of AQPs in this model is likely to promote a rapid influx of water into secretory granules. In line with this idea is our finding that the platelet dense and alpha granules are rich in AQPs (Fig. 1C–D), and that thrombin- or monensin-evoked changes in the intracellular AQPs distribution (dispersing of AQP-related signals visible under confocal microscope, reflecting granule swelling, (Fig. 1F)) were almost completely reduced following treatment of platelets with 100 μM of Au(III), a concentration of AQP blocker completely reducing platelet secretion from the dense and alpha granules induced by physiological stimulators and monensin (Fig 2A–B). Accordingly, Au(III) also reduces secretion from the dense granules induced by calcium ionophore A23187 (Fig. 8). Experiments performed on isolated platelet granules have revealed that calcium ions trigger secretory vesicle swelling (van der Meulen & Grinstein, 1982; van der Meulen & Grinstein, 1983).

Therefore, it is likely that in platelets AQPs play a role in secretion through the regulation of the vesicle volume. In this context it is of importance to note that the role of AQPs in the swelling of secretory granules was previously demonstrated in chromaffin cells, neurons, and exocrine pancreas cells (Jeremic et al., 2005; Sugiya & Matsuki, 2006; Ohta et al., 2009; Ishibashi et al., 2014). Substantial similarity between platelet and chromaffin cell secretory systems has been recognized (Fitch-Tewfik & Flumenhaft, 2013).

Our results are consistent with the previous report showing that platelets from children with a mutation of AQP7 have impaired ATP secretion from dense granules (Goubau et al., 2013). In the very recently published paper, Agbani et al. demonstrated that platelets from AQP1 knockout mice have only slightly impaired secretion from the dense granules (Agbani et al., 2018). This was interpreted to mean that AQP1 is not directly involved in the platelet secretory response. In the same report, the authors demonstrated that in platelets from wild type mice AQP1 is localized in the membranes of OCS but not in the granule membranes. However, it is highly likely that other types of AQPs may be involved in platelet secretion, especially those which we found to be present in the dense bodies and alpha granules, and those previously reported to play a role in secretion in platelets and other cells i.e. AQP5 (Sugiyama et al., 2008), AQP6 (Jeremic et al., 2005), AQP7 (Goubau et al., 2013), and AQP12 (Ohta et al., 2009).

The results presented here indicate a minor role for AQPs in the platelet aggregatory process (Fig. 8). As reported here, the Au(III)-related inhibition of aggregation induced by epinephrine resulted most likely from impaired secretion rather than from any direct effect on aggregation. This is based on observation that in platelets stimulated by adrenaline, the second wave of aggregation starts simultaneously with secretion. Interestingly, Au(III) did not reduce platelet aggregation evoked by arachidonic acid and PMA (PKC activator), and only slightly inhibited aggregation induced by calcium ionophore A23187, indicating that Au(III) did not affect the platelet responses through the reduction of thromboxane A2 formation, modulation of the PKC pathway or deregulation of calcium signaling.

The important finding of this study is identification of HAuCl4 (10–100 μM) as a new potent and specific inhibitor of platelet secretion. Of importance, 100 μM Au(III) is able to completely block platelet secretion without reducing their aggregation (Fig. 8).

The limitation of this study is the fact that it is not certain whether HAuCl4 equally blocks all of the AQPs channels. It is also difficult to specify which subtypes of AQPs play a role in secretion. Further studies with AQP knockout models are required to completely resolve these questions. The limitations of this study also comprise imaging of AQPs with polyclonal antibodies. The presence of these AQP isoforms should be confirmed in the future by more sensitive detection systems (i.e. monoclonal antibodies, proteomics-related techniques).

In conclusion, this study has revealed that human platelets contain numerous subtypes of AQPs water channels localized both, in the plasma and granule membranes. Rapid water transport through AQPs localized in platelet plasma membrane may be vital for adaptation of these cells to sudden changes in plasma osmolality. AQPs localized in secretory granules play a role in the rapid swelling which seems to be crucial for the release of their cargo. HAuCl4 is emerging as a specific and potent blocker of platelet secretion and a novel tool for examination of the contribution of platelet secretory process to hemostasis. Our finding that blocking of the aquaporin water channels results in complete inhibition of the platelet secretion induced by all physiological stimulators (including activator of thrombin receptor) is of great clinical importance. None of the currently used antiplatelet drug are able to inhibit platelet secretion induced by the strongest platelet stimulus, i.e. thrombin. At pathological conditions, large quantities of thrombin can be produced following sudden rupture of atherosclerotic plaque (Badimon & Vilahur, 2014). Recently published studies strongly indicate that platelet secretion is critical for the thrombus growth (Golembiewska & Poole, 2015; de Witt et al., 2014; Konopatskaya et al., 2009). Our findings thus create basis for projection of new generation of the antiplatelet drugs.

**Conflict of Interest Statements**

All authors declare no competing financial interests.
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