Eur Biophys J (2011) 40:737–746
DOI 10.1007/s00249-011-0687-2

ORIGINAL PAPER

Water flux through human aquaporin 1: inhibition by intracellular furosemide and maximal response with high osmotic gradients

Marcelo Ozu · Ricardo A. Dorr · M. Teresa Politi · Mario Parisi · Roxana Toriano

Received: 26 August 2010 / Revised: 13 January 2011 / Accepted: 16 February 2011 / Published online: 4 March 2011

Abstract This work studies water permeability properties of human aquaporin 1 (hAQP1) expressed in *Xenopus laevis* oocyte membranes, applying a technique where cellular content is replaced with a known medium, with the possibility of measuring intracellular pressure. Consequences on water transport—produced by well-known anisotonic gradients and by the intracellular effect of probable aquaporin inhibitors—were tested. In this way, the specific intracellular inhibition of hAQP1 by the diuretic drug furosemide was demonstrated. In addition, experiments imposing anisotonic mannitol gradients with a constant ionic strength showed that the relationship between water flux and the applied mannitol gradient deflects from a perfect osmometer response when the gradient is higher than 150 mosmol kg_W^{-1}. These results would indicate that the passage of water molecules through hAQP1 may have a maximum rate. As a whole, this work demonstrates the technical advantage of controlling both intracellular pressure and medium composition in order to study biophysical properties of hAQP1, and contributes information on water channel behavior under osmotic challenges and the discovery of new inhibitors.

Keywords hAQP1 · Intracellular inhibition · Furosemide · Anisotonic gradients · *Xenopus* oocytes

Electronic supplementary material The online version of this article (doi:10.1007/s00249-011-0687-2) contains supplementary material, which is available to authorized users.

M. Ozu (✉) · R. A. Dorr · M. Teresa Politi · R. Toriano
Laboratorio de Biomembranas, Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 7° Piso, C1121ABG Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina
e-mail: mozu@fmed.uba.ar

M. Ozu · R. A. Dorr · M. Parisi · R. Toriano
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

Introduction

Aquaporin-mediated water transport is generally measured in *Xenopus laevis* oocytes using an osmotic swelling assay. This implies a two-compartment system (the inner side of the oocyte and the external medium) where oocyte swelling is driven by decreasing the osmolality of the medium bathing the extracellular side of the membrane. Therefore, the osmotic permeability coefficient (P_f) is calculated by measuring changes in whole-oocyte volume, monitored by video-microscopy, during initial swelling (Zhang et al. 1990; Dorr et al. 2007). Although this method has proved convenient for identifying aquaporins and for studying some of their biophysical properties, it has limitations, such as not allowing direct access to or full control over one of the two system compartments: the cytoplasm of the oocyte. In this way, classic water permeability measurements are based on several assumptions that consider the cytoplasm an ideal mixing model and a simple, homogeneous and well-defined solution. On the contrary, cytoplasm actually constitutes a heterogeneous and viscous medium (Luby-Phelps 2000) that contains diffusion barriers and areas that could be osmotically insensitive (Hill et al. 2005). In an effort to control the cytoplasmic composition of *Xenopus* oocytes, Taglialatela et al. (1992) developed a cut-open technique, used in voltage-clamp studies. A comparable approach, applied to water movement measurement in cells, was virtually lacking, mainly due to unsolved technical difficulties. Pursuits in this field were the use of barnacle muscle cells in the study of intrinsic osmotic properties (Bitner et al. 2001) and the emptied-out oocyte

(EOO) technique in the study of water permeability of native oocyte membranes, without the expression of heterologous aquaporins (Ozu et al. 2005).

Up until now, intracellular aquaporin inhibitors were tested indirectly or by the intracellular injection of the drug. For example, Migliati et al. (2009) tested furosemide, bumetanide and derivatives as possible intracellular inhibitors of rat aquaporin 4 (rAQP4) by means of injection or by developing permeable drugs. This double-injection of both cRNA and drugs alters cellular membrane integrity, which may be inconvenient for water movement measurements, especially in the case of very fast volume changes (Parisi et al. 2008). The EOO technique opened up several new possibilities: among others, the chance to test the inhibitory effect of new drugs acting directly on the intracellular side of membranes expressing aquaporins.

Additionally, the standard technique involves, after a short period, the formation of blebs on the injured area of the membrane and loss of cytoplasmic content before cellular burst, both consequences of increased intracellular pressure and weakening of the membrane. An example of blebbing can be observed in Peter Agre's pioneer work on aquaporin discovery (Fig. 2b from Preston et al. 1992). This artifact can be potentially avoided with the EOO technique, consequently allowing the study of water transport events that take place at longer times.

Evidence in plants shows that the aquaporin function can be modified by the magnitude of the osmotic gradient established between intracellular and extracellular sides (Ohshima et al. 2001; Vandeleur et al. 2005). Furthermore, although aquaporin 1 (AQP1) has been shown to be a bidirectional (Meinild et al. 1998) and strictly selective water channel (Preston et al. 1992), there is no proof indicating whether its function can be modified by the magnitude of the osmotic gradient. Since the EOO technique allows not only strict control over the imposed osmotic gradient but also higher experimental gradients, it could offer useful information on clarifying this unresolved issue.

Despite its advantages, the EOO technique is not intended to replace the easier-to-implement whole oocyte method. Instead, it should be viewed as a complementary technique for specifically studying events that occur on the cytoplasmic side of an intact cellular membrane without interferences caused by the heterogeneous nature of the cytoplasm.

Making use of the mentioned experimental advantages of the EOO technique, the objective of this work was to test, under strictly controlled conditions, the permeability properties of human AQP1 (hAQP1) expressed in *Xenopus laevis* oocyte membranes and to examine the effect of possible new inhibitors added on the intracellular side of the protein.

Materials and methods

Oocyte isolation

Adult female *X. laevis* (Nasco, Fort Atkinson, WI) were maintained in a room with controlled temperature (18°C) and a 12-h light-dark cycle. Each frog was kept in an individual tank with filtered water and was fed twice a week. Frogs were anesthetized for surgery, and oocytes were removed and prepared as previously described (Ozu et al. 2005). Selected cells had 1–1.3 mm diameter, thus belonging to stage VI (Dumont 1972).

Plasmid construction, in vitro synthesis and translation

The complete coding regions of full-length human AQP1 clone were inserted between the EcoRI and XhoI sites on both ends of a pSP64T-derived Bluescript vector, carrying 5′ and 3′ untranslated sequences of a β-globin gene from *X. laevis* (Abrami et al. 1994). Capped complementary RNAs (Daniels et al. 1994) were synthesized in vitro using a T3 RNA polymerase kit (Ambion, Austin, USA) and purified as described by Preston and coworkers (Preston et al. 1992). Synthesized products were suspended in RNAse-free water at a final concentration of 1 μg/μl and stored at −20°C until microinjection.

AQP1-cRNA microinjection

Oocytes were microinjected with hAQP1-cRNA (50 ng, 1 μg/μl) 24 h after isolation using an automatic injector (Drummond Scientific Co., Broomall, PA). For control experiments, oocytes were injected with water. Afterwards, all the injected oocytes were kept for 24 h at 18°C in ND96 solution supplemented with 1 μg/ml gentamicin (Gibco, Gaithersburg, MD) (Ozu et al. 2005).

Swelling experiments in whole oocytes

Whole *X. laevis* oocytes were used as positive controls to calculate water permeability and to compare with emptied-out oocyte experiments. Oocytes injected with water or hAQP1 were maintained in ND96 solution (between 200 and 215 mosmol kg_W^{-1}) for equilibration before changing the extracellular medium. Next, a hypotonic challenge ($\approx$100 mosmol kg_W^{-1}) was applied by replacing the extracellular solution with a diluted medium. Intracellular osmolality of whole oocytes was assumed to be 200 mosmol kg_W^{-1}. The osmolality of the solutions was measured with a vapor pressure osmometer (VAPRO 5520, Wesco, Logan, UT).

Volume change experiments in emptied-out oocytes

Oocytes were emptied out according to a previously published protocol (Ozu et al. 2005). The membrane of the emptied-out oocytes was used as a diaphragm separating two compartments: a sealed chamber (33.6 µl), representing the "intracellular" medium, and an open compartment (3.5 ml), representing the extracellular medium. This allowed control over media composition on both sides of the membrane. Extracellular medium replacement was accomplished with a system of simultaneous injection and suction of solutions, using two 20-ml glass syringes. Perfusion of 20 ml of the new solution in 10 s ensured the complete replacement of the extracellular medium. Afterward, "inward" and "outward" fluid fluxes across the cellular membrane were estimated from video records of swelling or shrinkage induced by net water movements.

As previously mentioned, the oocytes were injected with the cRNA from hAQP1 or with water. In order to maintain membrane integrity during osmotic experiments, the region where the membrane was injured by the injection had to be clearly identified. Thus, to optimize the technique, injections were systematically done in the center of the vegetal pole for easy identification of the resulting wound. When assembling the oocyte onto the support piece, the membrane lesion was aligned with the hole in the acrylic support (Online Resource 1). The oocyte was then emptied out as previously described (Ozu et al. 2005) and placed in the experimental chamber. The advantage of this procedure is that it avoids the exposure of the damaged membrane area, guarantying membrane integrity throughout the entire experiment. This methodological approach improves the accuracy of volume measurements and allows longer experiments with greater osmotic gradients (see below).

Video-microscopy and image analysis

Video records and image analysis were done as was previously described (Ozu et al. 2005; Dorr et al. 2007).

Intracellular pressure measurements

A disposable clip-on dome prepared to receive an ADInstruments MLT844 physiological pressure transducer (pressure range: −20 to +300 mmHg; sensitivity: 50 µV/V/mmHg; operating temperature range: +10 to +50°C) was connected to a 100-µl Hamilton gas-tight syringe and a catheter coupled to the intracellular side of the measurement chamber. The piezo-resistive transducer was connected via a ML224 Bridge Amp to a PowerLab 4/30 device (both from ADInstruments). LabChart Pro data acquisition software was used for data recording and analysis.

The pressure transducer was connected to the "intracellular" side in order to test system integrity and the absence of leaks. Pressure variations were triggered by changing hydrostatic pressure—that is, by adding a solution to the "intracellular" compartment, with a Hamilton syringe controlled by a horizontal translation stage—or by applying an anisotonic gradient between both sides of the cellular membrane. Pressure variations could be simultaneously followed with volume changes recorded by videomicroscopy (Fig. 1). A drastic fall in "intracellular" pressure occurred in concordance with membrane rupture, also detected by video records (Fig. 1a).

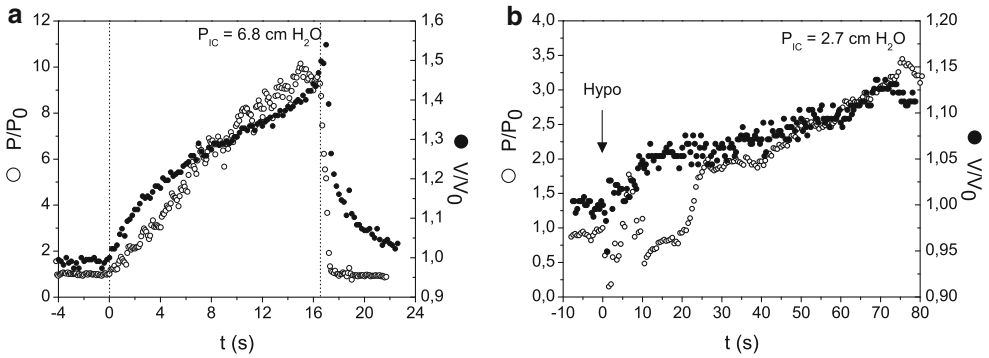

Fig. 1 Simultaneous register of pressure and volume changes in emptied-out oocytes. **a** Changes in pressure (*open circles*) and volume (*filled circles*) simultaneously recorded during the injection of solution into the "intracellular" compartment of an emptied-out oocyte. Volume addition (started at $t = 0$ s) was done by means of a Hamilton syringe controlled by a horizontal translation stage. The fall observed at $t \approx 16$ s indicates membrane rupture. Intracellular pressure (P_{IC}) rose to 6.8 cm H_2O. **b** Changes in pressure (*open circles*) and volume (*filled circles*) simultaneously recorded during an osmotic challenge in an emptied-out oocyte expressing hAQP1. The hypotonic gradient (100 mosmol kg_w^{-1}) was imposed at $t = 0$ s, replacing the extracellular bath with a diluted medium in 10 s. The calculated P_f value was 26×10^{-4} cm s^{-1}. Intracellular pressure (P_{IC}) rose to 2.7 cm H_2O. Initial noise reflects the effect of external solution changes on the intracellular pressure

Estimation of the osmotic permeability coefficient

The osmotic permeability coefficient (P_f) was determined 24 h after injecting hAQP1-cRNA. All experiments were done at 20°C. Osmotic water permeability coefficient was obtained from the slope of the linear relation between volume and time calculated from the beginning of the osmotic challenge (Ozu et al. 2005). In emptied-out experiments only half of the oocyte is exposed to the solution. Thus, this area was measured, and considerations were made to calculate the volume of a complete sphere. We assumed that the oocyte membrane behaves as osmotically equal along its entire surface. Since the surface area of the effective membrane is actually nine times larger than observed (Chandy et al. 1997), correction for folds and microvilli was done. In all cases, P_f values were expressed in cm s^{-1}.

Experiments with $HgCl_2$ and furosemide

To evaluate the effect of mercurial compounds on water permeability, 0.3 mM $HgCl_2$ (Sigma, St. Louis, MO) was tested separately on both sides of the membrane of oocytes injected with hAQP1-cRNA. $HgCl_2$ was added to the extracellular side of both whole oocytes and emptied-out oocytes, was incubated for 5 min and then was washed out. When $HgCl_2$ was added to the "intracellular" side of an emptied-out oocyte, incubation time was prolonged to the entire time of the full experiment. The applied osmotic difference was 100 mosmol kg_W^{-1}, in all cases.

We also studied the effect of furosemide (10 μM) separately on both sides of the membrane of oocytes injected with hAQP1-cRNA by means of the EOO technique.

To test the intracellular effect of furosemide, emptied-out oocytes were mounted in the experimental chamber with 200 mM mannitol plus 10 μM furosemide in ND96 on the intracellular side. The initial extracellular solution was 200 mM mannitol in ND96. Furosemide concentration did not modify the osmolality of the mannitol solution. Therefore, the initial condition was isosmotic. Since the intracellular chamber cannot be opened during the experiment, furosemide was present in the closed chamber during the entire experiment. The effect of furosemide was tested at two different incubation times by starting the experiment either 5 or 60 min after assembling the experimental chamber. Once the experiment has begun, images were taken at the initial isosmotic condition during 90 s. Then, the extracellular solution was replaced with 50 mM mannitol in ND96.

To test the extracellular effect of furosemide, the internal solution was 200 mM mannitol in ND96, and the initial external solution was 200 mM mannitol plus 10 μM furosemide in ND96. Then this solution was replaced with 50 mM mannitol plus 10 μM furosemide in ND96. Therefore, furosemide was present on the extracellular side for the entire experiment.

Osmotic response to mannitol gradients in hAQP1-expressing EOO

In order to vary osmolality but not ionic strength, solutions were prepared by adding mannitol to ND96. The osmolality of the solutions was measured with a vapor pressure osmometer.

In the first experimental series, consecutive hypo- and hyperosmotic gradients were tested in the same EOO expressing hAQP1. The initial condition was established by adding 100 mM mannitol in ND96 on both sides of the membrane. After 90 s of equilibration, the extracellular solution was replaced with 50 mM mannitol in ND96, establishing a hyposmotic gradient of 50 mosmol kg_W^{-1}. After 60 s, the extracellular solution was replaced with 150 mM mannitol in ND96, establishing a hyperosmotic gradient of 50 mosmol kg_W^{-1} (Fig. 4a).

In the second experimental series, after establishing an initial isosmotic condition (100 mM mannitol in ND96 on both sides of the membrane), the extracellular medium was replaced in order to impose a hyposmotic gradient (extracellular solution: 50 mM mannitol in ND96) or a hyperosmotic gradient (extracellular solution: 150 mM mannitol in ND96). After 60 s, the extracellular medium was changed back to the initial condition (100 mM mannitol in ND96) (Fig. 4b).

In the third experimental series, each EOO expressing hAQP1 was studied under a single osmotic gradient. In this way, we explored the osmotic response to hyposmotic and hyperosmotic gradients of different magnitude (Fig. 5). When studying hyposmotic gradients, the initial condition was established with 250 mM mannitol in ND96 on both sides of the membrane. After 90 s of equilibration, the extracellular solution was replaced with 200, 150, 100 or 50 mM mannitol in ND96. Therefore, the magnitudes of the different hyposmotic gradients were 50, 100, 150 and 200 mosmol kg_W^{-1}. When studying hyperosmotic gradients, the initial condition was established with 50 mM mannitol in ND96 on both sides of the membrane. After 90 s of equilibration, the extracellular solution was replaced with 100, 150, 200 or 250 mM mannitol in ND96. Therefore, the magnitudes of the different hyperosmotic gradients were 50, 100, 150 and 200 mosmol kg_W^{-1}.

In all cases, water flux (J_W, in nl s^{-1}) was calculated from maximal response (10 s) from the beginning of the osmotic challenge.

Experiments with gramicidin A

Oocytes were pre-incubated with 100 μM gramicidin A (Sigma) in ND96. Gradients up to 200 mosmol kg_W^{-1} were

tested in whole oocytes. During osmotic experiments oocytes were placed in a solution of mannitol in ND20 with an osmolality of ~200 mosmol kg_W^{-1}. After 90 s, this solution was replaced with water, ND20 alone or solutions of different concentrations of mannitol in ND20 to create gradients of 200, 146, 107 and 68 mosmol kg_W^{-1}, respectively. Since the intracellular osmolality of the oocyte is ~200 mosmol kg_W^{-1}, it is not possible to create osmotic gradients higher than 200 mosmol kg_W^{-1} by replacing the extracellular solution. Therefore, we used the EOO technique to create hyposmotic gradients of 406 mosmol kg_W^{-1} using solutions of different mannitol concentrations in ND96.

Solutions

ND96 solution (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES; pH = 7.4
ND20 solution (mM): 20 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES; pH = 7.4
OR2 solution (mM): 82.5 NaCl, 2 KCl, 5 HEPES, 1 $MgCl_2$; pH = 7.5
High K^+ solution (mM): 100 K_2HPO_4; pH = 6.5. Then 1 g/l BSA was added.

Statistical analysis

Data are presented as $\overline{X} \pm$ SEM. Normality and homocedasticity were proven for all data sets. ANOVA and paired or unpaired Student's t test were applied. A P value of $P < 0.05$ was considered statistically significant.

Results and discussion

Effect of cytoplasm disruption

As an initial control, osmotic experiments in oocytes expressing hAQP1 were performed by diluting the extracellular solution. The osmotic response obtained with the EOO technique did not differ from that obtained with the whole oocyte technique (Online Resource 2). As a first conclusion, this control would indicate that the disruption of the cytoplasmic structure with the EOO method does not affect P_f measurements through hAQP1. This is in accordance with previous results indicating that AQP1 is not associated with the cytoskeletal system (Crane and Verkman 2008).

Afterward, we evaluated oocyte volume changes produced by increasing intracellular hydrostatic pressure. In most cases, the maximal volume change tolerated by an EOO expressing hAQP1 was similar to that observed in water-injected oocytes (≈25%). This value concurs with previous measurements performed in native EOO (Ozu et al. 2005). Similar values were obtained with other methods that increased intracellular pressure in whole oocytes that did not express aquaporins (Kelly and Macklem 1991; Zhang and Hamill 2000). Therefore, the maximal tolerated volume appears to be influenced neither by overexpression of hAQP1 nor by the presence or absence of cytoplasm, but to be directly related to membrane characteristics. In addition, intracellular pressure in EOO showed similar values to those reported by Kelly and Macklem (1991) (Fig. 1). In conclusion, the EOO technique could be a valuable procedure for pursuing the study of plasma membrane mechanics by manipulating intracellular pressure.

Intracellular effects of $HgCl_2$ and furosemide

As expected, the addition of $HgCl_2$ to the extracellular compartment produced a significant inhibition of the osmotic response in EOO expressing hAQP1 (Fig. 2). This result, similarly to those presented in Online Resource 2, indicates that hAQP1 was not functionality affected by the emptied-out process.

Nowadays, the mechanism of the inhibitory effect of $HgCl_2$ on the extracellular side of AQP1 is well defined and has been resolved up to molecular levels (Hirano et al.

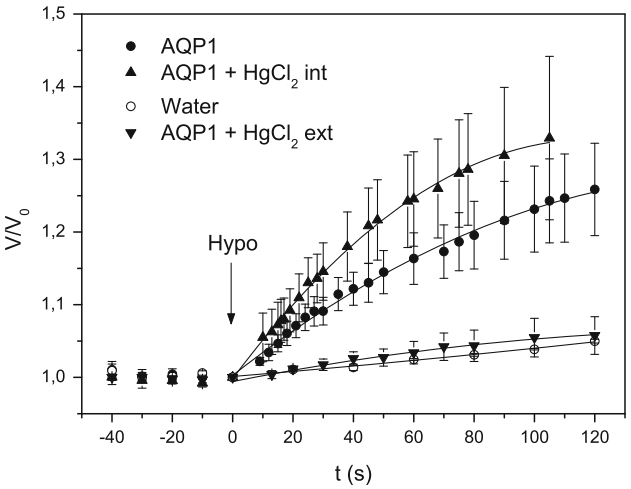

Fig. 2 Intracellular effect of $HgCl_2$. Time course of the osmotic response in EOO expressing hAQP1 (*filled circles*), EOO expressing hAQP1 and treated with 0.3 mM $HgCl_2$ from the intracellular side (*upper triangles*) or from the extracellular side (*lower triangles*), and control EOO injected with water (*open circles*). The P_f values ($\times 10^{-4}$ cm s^{-1}) were 35 ± 8 ($n = 4$), 39 ± 6 ($n = 4$), 7 ± 3 ($n = 4$) and 4 ± 2 ($n = 3$), respectively. In all cases, the hypotonic gradient was 100 mosmol kg_w^{-1}. No significant effect was observed when EOOs expressing hAQP1 were treated with $HgCl_2$ on the intracellular side. On the other hand, significant inhibition was observed when $HgCl_2$ was applied on the extracellular side (one-way ANOVA, $P < 0.05$)

2010). Furthermore, there is evidence of intracellular effects of $HgCl_2$ on AQP4 in reconstituted proteoliposomes (Yukutake et al. 2008).

Since the effect of $HgCl_2$ has never been tested on the cytoplasmic side of hAQP1, we decided to discard any unknown effect of this compound on this channel. Therefore, $HgCl_2$ was added directly into the "intracellular" compartment (Fig. 2). Results show no inhibitory effect on water permeability.

In a different experimental series, the effect of furosemide was tested from the intracellular side of hAQP1. Recently, this inhibitor of the $Na^+/K^+/2Cl^-$ transporter was demonstrated also to be a rat AQP4 inhibitor by acting on the intracellular side after being injected into *X. laevis* oocytes (Migliati et al. 2009). Based on these previous data and since many efforts are being made to discover specific inhibitors for known aquaporins, we decided to test the effect of furosemide on hAQP1 separately on the intracellular or the extracellular side. We first tested the extracellular effect of the drug in whole oocytes expressing hAQP1. Oocytes were pre-incubated in 10 μM furosemide for 2 h (according to Migliatti et al. 2009), then washed and studied with osmotic experiments. No effect was observed (see Online Resource 3). Then we tested the extracellular effect of the drug with the EOO technique. Furosemide (10 μM) was present during the entire experiment (see "Materials and methods"). Also in this case no effect was observed (Fig. 3). On the other hand, when the drug was tested from the intracellular side, a ~66% inhibition of water permeability was observed. P_f values for hAQP1 and hAQP1 + 10 μM furosemide in the intracellular compartment were $(19 \pm 3) \times 10^{-4}$ cm s^{-1} and $(6 \pm 1) \times 10^{-4}$ cm s^{-1}, respectively ($P < 0.05$). Results shown in Fig. 3 demonstrate that furosemide is an intracellular hAQP1 inhibitor. Since experiments with 5 min (Fig. 3) or 60 min (Online Resource 4) of incubation showed the same percentage of inhibition, we can conclude that the effect of furosemide does not depend on the incubation time. Additionally, no effects were registered for DMSO, the vehicle for furosemide (Online Resource 3). According to Migliati and coworkers (2009), the site of action of bumetanide (and analogues) on rAQP4 involves the residues Gly93, His95, Ser180 and Val189. The same authors mentioned that the homologous amino acids involved in the site of action of furosemide in hAQP1 could be Gly72, His74, Tre156 and Ala168. However, further experiments of point mutations are needed to confirm this issue.

Osmotic response to mannitol gradients in hAQP1-expressing EOO

Bidirectional water movement across membranes expressing hAQP1 was tested using mannitol, a non-permeable, non-ionic solute ($\sigma = 1$).

The first experimental series tested consecutive hypo- and hyperosmotic gradients in the same EOO expressing hAQP1 (Fig. 4a). Volume changes showed the same slope for swelling and shrinkage. The corresponding P_f values were $(30 \pm 10) \times 10^{-4}$ cm s^{-1} and $(28 \pm 8) \times 10^{-4}$ cm s^{-1}, respectively ($n = 6$).

Another experimental series, also in EOO expressing hAQP1, focused on the return to initial conditions (100 mM mannitol in ND96 in both compartments), after swelling or shrinkage driven by a corresponding anisotonic shock (Fig. 4b). Interestingly, the oocyte maintained the volume reached at the end of the anisotonic condition. All together, the results in Fig. 4 indicate that both inward and

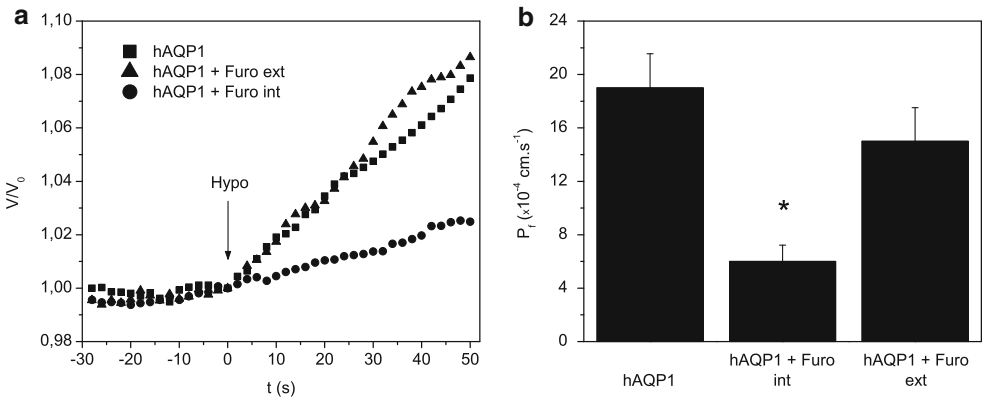

Fig. 3 Intracellular inhibitory effect of furosemide on hAQP1. **a** Volume changes of representative experiments registered 5 min after assembling the experimental chamber. The effect of furosemide on the intracellular or the extracellular side was tested with the drug present during the entire experiment in the corresponding chamber. **b** Average P_f values obtained from the oocytes of 3–5 different frogs (hAQP1: $n = 3$; hAQP1 + furosemide intracellular: $n = 5$; hAQP1 + furosemide extracellular: $n = 3$). * Indicates significant differences ($P < 0.05$) between hAQP1 treated with furosemide on the intracellular side and the two other conditions. The percentage of inhibition was ~66%. The same result was obtained after 60 min of incubation

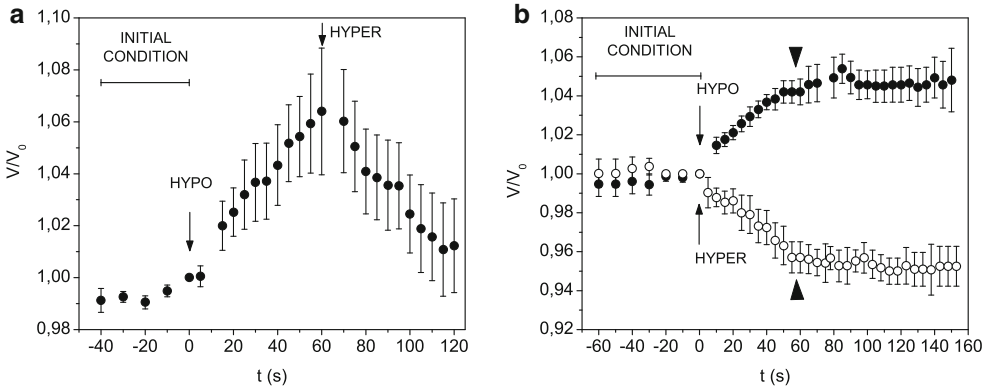

Fig. 4 Osmotic responses to mannitol in emptied-out oocytes expressing hAQP1. **a** The bidirectional osmotic response of EOO expressing hAQP1 was tested by creating successive hypo- and hypertonic 50 mosmol kg_w^{-1} gradients with mannitol in the same oocyte. HYPO and HYPER refer to the solution loaded onto the extracellular side of the membrane. The intracellular solution was maintained throughout the entire experiment. After 90 s of equilibration with the same solution on both sides (initial condition), each EOO was exposed for 60 s to a hypotonic gradient followed by a hypertonic one. The corresponding P_f values ($n = 6$) were $(30 \pm 10) \times 10^{-4}$ cm s^{-1} and $(28 \pm 8) \times 10^{-4}$ cm s^{-1}. **b** Swelling and shrinkage responses were tested separately in EOO expressing hAQP1. After 90 s of equilibration in the initial condition, each EOO was exposed during 60 s to a 50 mosmol kg_w^{-1} hypotonic gradient (*filled circles*) or to a 50 mosmol kg_w^{-1} hypertonic one (*open circles*). Then the extracellular solution was replaced with a solution equal to that used to set the initial condition (*arrowheads*). The P_f values measured under the hypotonic ($n = 6$) and the hypertonic ($n = 5$) gradients were $(24 \pm 5) \times 10^{-4}$ cm s^{-1} and $(17 \pm 6) \times 10^{-4}$ cm s^{-1}, respectively (unpaired *t* test, not significant)

outward water movements across hAQP1 during the first 60 s did not modify the concentration of the "intracellular" compartment of the system. This is in accordance with the fact that the chamber volume is ≈30 times larger than the volume of the oocyte (33.6 µl vs. ∼1.2 µl).

These experiments were made possible by the EOO technique, which, unlike whole-oocyte methodology, allows controlling the concentration of all solutes in both compartments and preserving ionic strength even when an anisotonic challenge is being imposed. Although similar tests could be assayed in whole oocytes, the composition of the experimental solutions using non-permeable solutes would depend, in this case, on the intracellular osmolality of the oocyte, thus limiting the osmotic gradient that can be applied. In addition, the presence of osmotically inactive intracellular domains in whole oocytes may sub- or superestimate experimental values of membrane permeability.

As highlighted by Pickard (2008), there are several assumptions needed in order to develop mathematical models to describe swelling: among others, that osmotically active cell contents are ideally mixed so that the osmolality of the entire cytoplasm is homogeneous; that extracellular components are also ideally mixed; and, finally, that oocyte volume changes involve only the simple expansion of a weakly elastic membrane without any blebbing. These are the same suppositions classically used in water permeability measurements.

We assert that the EOO technique allows determining P_f experimentally without considering the previous assumptions. This statement is supported by the results presented in Fig. 4, which highlight the following advantages of the EOO method: (1) the possibility of working with a near-ideally mixed "intracellular" medium, as a result of being free of cytoplasm; (2) an "intracellular" compartment that may be considered a "bulk," whereas its volume is ≈30 times larger than the whole cell volume (33.6 µl vs. ∼1.2 µl); (3) the absence of blebbing, since membrane lesion is avoided; (4) the possibility of establishing a new steady-state after an anisotonic shock and performing a new osmotic challenge in the same experiment since the cellular membrane is not disrupted and, therefore, since there is no cytoplasmic loss.

From results presented up to this point, the EOO technique has proven to be a powerful method for controlling experimental conditions, allowing the study of transport properties of hAQP1.

Effect of high mannitol gradients on water flux

Since the EOO technique allows longer experiments during fast volume changes, we were able to analyze the relation between water fluxes and osmotic gradients using different mannitol concentrations in the "intracellular" or extracellular solutions. Results indicate that the described bidirectional properties of AQP1 (Meinild et al. 1998) show a symmetrical response (Fig. 5). In addition, results also indicate that this animal cellular membrane expressing hAQP1 behaves as a perfect osmometer up to 150 mosmol kg_w^{-1} gradients. Interestingly, there is a deviation from a linear relationship between flux and gradient when

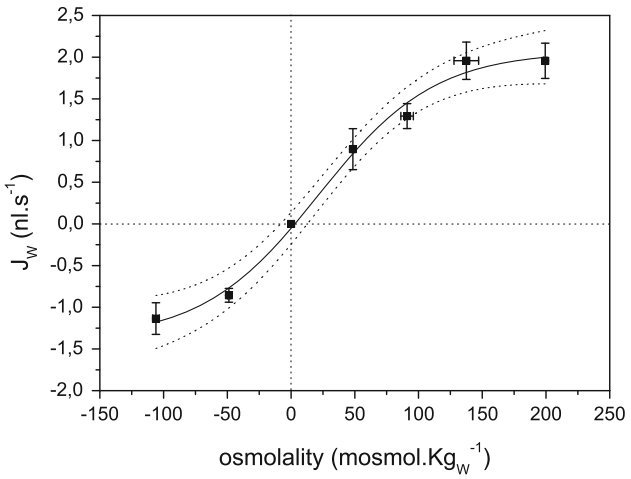

Fig. 5 Water fluxes under different mannitol gradients. Water fluxes (J_W) were measured for 10 s with different osmotic gradients (ΔOsm) imposed across the membrane of EOO expressing hAQP1. Osmotic gradients (50, 100, 150 and 200 mosmol kg_w^{-1}) were created by adding different mannitol solutions on intracellular and extracellular sides, without changing ionic strength. *Negative values* indicate hypertonic gradients and outward water fluxes. Experimental data (*circles*) show a symmetrical response up to 100 mosmol kg_w^{-1} for both hypotonic and hypertonic gradients. Clearly, the J_W values measured with hypotonic gradients higher than 150 mosmol kg_w^{-1} deflect the expected linear relationship between J_W and ΔOsm. *Solid line*: nonlinear fit ± 95% CI, $R^2 > 0.99$

the osmotic gradient is higher than 150 mosmol kg_w^{-1}, a magnitude that lies within the physiological range for animal cells. Thus, the osmotic flux through *Xenopus* oocyte membranes expressing hAQP1 shows a maximum value of approximately 2 nl s^{-1}, under the established experimental conditions. Although in Fig. 5 the scatter is symmetrical between gradients of −100 mosmol kg_w^{-1} and +100 mosmol kg_w^{-1}, water fluxes induced by higher gradients move away from linearity under hypotonic conditions, and probably do so as well under hypertonic conditions. No differences were observed among the water flux values of experiments with 100, 150 or 200 mosmol kg_w^{-1} hypertonic gradients (data not shown). It is possible that this high permeability aquaporin, in the presence of high osmotic gradients, produces such fast shrinkage that it may induce membrane deformations. Due to these deformations, the membrane shape would deviate from the assumed spherical model, which would introduce errors when calculating oocyte volumes from two-dimensional images.

Interestingly, other deviations from linearity between water permeability and the applied mannitol concentration gradient have been described previously in plant membrane vesicles (Ohshima et al. 2001). In addition, a similar deviation of P_f values was reported in plant membranes when the solute concentration was increased (Vandeleur et al. 2005). This deviation could be related to a widespread property underlying the function of water channels. Since the EOO system implies the absence of cytoplasm and the control of solutions that surround both sides of the membrane, a metabolic control over the permeability properties of hAQP1 can be excluded. Therefore, a direct effect of the mannitol osmotic gradient on the channel could be proposed.

There is great consensus that, at the molecular level, hAQP1 functions as a two-step filter: the NPA motifs and the ar/R region (de Groot and Grubmüller 2005). The constriction formed by NPA motifs hinders the collective movement of the single file of water molecules from one side of the channel to the other (Hashido et al. 2007). This is due to the interaction between the water molecule in the center of the channel and the Asp residues of the NPA motifs, resulting in the inversion of the water molecule dipole (de Groot and Grubmüller 2001). However, studies of the ar/R region in different aquaporins and aquaglyceroporins indicate that this region selects by size. The permeability at this site is low (Hashido et al. 2007) and does not depend on the polarity of this region (Beitz et al. 2006). According to Hashido et al. (2007), low permeability values could be due to the strong interaction of the water molecule with the channel. In addition, they propose that the ar/R region determines the value of the flux through the aquaporin.

Beckstein and Sansom (2003) proposed a two-state model for simulated narrow pores. This model assumes that water translocation through the pore occurs in bursts, resulting from the oscillation of water between liquid and vapor states. This behavior is not observed when the radius of the pore is higher than a limit value. In all, simulation studies indicate that the geometrical features of the channel and electrostatic interactions with water molecules could be determinants of the flux.

Because the aquaporin water channel has an hourglass shape (Jung et al. 1994), properties in the single file region of the pore are different from those in the atria. Thus, shape could influence water molecule packaging in different regions of the channel.

Since deviations from linearity were observed in AQP-expressing systems, we tested the relationship between J_W and ΔOsm in oocytes with gramicidin A incorporated into the membrane. Gramicidin A forms channels with a ∼4 Å diameter where water molecules pass in single file but do not invert their dipole (contrary to what happens inside hAQP1). Therefore, interactions between water molecules and gramicidin A are different from interactions with hAQP1 (Chiu et al. 1989). As mentioned in Materials and methods, this experimental series was done in whole oocytes with gradients up to 200 mosmol kg_w^{-1}. Results in Fig. 6 show an ideal osmometer behavior up to 200 mosmol kg_w^{-1}. The linear fit extrapolated up to >400 mosmol kg_w^{-1} shows the expected values of flux up to such gradient magnitudes.

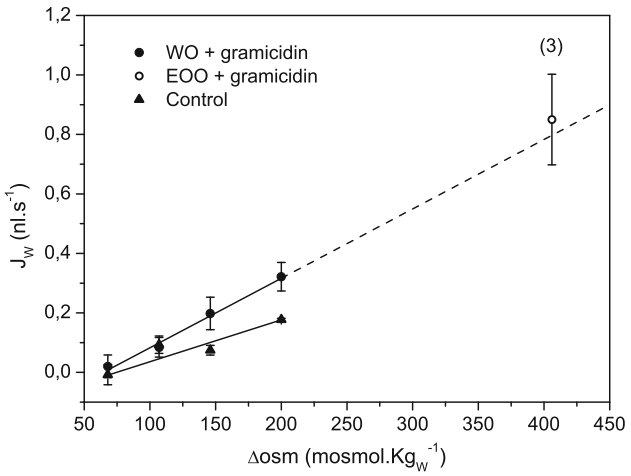

Fig. 6 Relationship between J_W and ΔOsm in oocytes with Gramicidin A. Data up to 200 mosmol kg_w^{-1} gradients are from experiments with whole oocytes ($n = 6$). The linear fit for this scatter ($R^2 \approx 0.98$) was extrapolated up to >400 mosmol kg_w^{-1}. The datum point for 406 mosmol kg_w^{-1} is from experiments using the EOO technique. Comparison of this point to linear fit extrapolation indicates that Gramicidin A incorporated into the membrane of *Xenopus* oocytes behaves as a perfect osmometer. The number of independent experiments is presented between *brackets*. *Upper triangles* represent control experiments with native oocytes ($n = 4$)

Because of the impossibility of creating hyposmotic gradients higher than 200 mosmol kg_w^{-1} in whole oocytes, we used the EOO technique to test gradients up to ~400 mosmol kg_w^{-1}. Results obtained with 406 mosmol kg_w^{-1} show good alignment with the extrapolated fit. As shown in hAQP1-inyected and native oocytes, volume changes in whole and emptied-out oocytes are equivalent (Online Resource 2). Since experiments from Figs. 5 and 6 differ in the nature of the channel, it is possible that the osmotic behavior observed in Fig. 5 depends on an intrinsic property of hAQP1.

If the results of Fig. 5 are not a consequence of the interaction between water molecules and the protein channel, then could they be a consequence of a regulating mechanism gated by membrane mechanics? Kelly et al. (1997) demonstrated that the specific membrane elastance of native oocytes is different in swelling and in shrinkage when plotted against intracellular pressure. Nevertheless, they also showed that it is similar when plotted against cellular volume. These authors thus suggest that volume—and not pressure—is the critical determinant of resistance to swelling. The increased rigidity of the membrane in response to volume changes could offer protection against excessive swelling. Additional evidence proposed that membrane tension would also have an effect on the function of water channels. Studies in yeasts under hyposmotic conditions suggested that the rapid increase in cell volume and consequent development of membrane tension would inhibit the water permeability of the membrane, slowing water fluxes in order to maintain a suitable cell volume, while mechanisms of cell volume regulation would be triggered in order to protect the cell from excessive swelling (Soveral et al. 2008). Furthermore, experiments on algae aquaporins showed a reduction in the activity of water channels after a pulse of positive pressure, indicating that these channels close in response to high turgor pressures (Wan et al. 2004). This implies a gating mechanism triggered by changes in membrane tension. Other proposed gating mechanisms imply conformational changes of the protein after phosphorylation events (Törnroth-Horsefield et al. 2010).

Although several explanations are possible, further experiments and new simulation approaches are needed to elucidate the causes of the experimental evidence presented in this work.

Conclusions

The emptied-out oocyte technique has the advantage of using the membrane of an animal cell where foreign water channels can be overexpressed to measure water movement under isolated and controlled experimental conditions with great stability and for long periods of time.

The possibility to access and control the intracellular compartment opened up the opportunity to uncover the not yet reported inhibitory effect of furosemide on the intracellular side of hAQP1. Future applications of this unique experimental approach may allow even more intracellular inhibitors to be discovered.

The characteristics of this technique guarantee the experimental conditions to confirm that water flux across hAQP1 is symmetrical. Finally, our study demonstrates that, in this system, the magnitude of the mannitol gradient has a direct influence on water flux under constant ionic strength conditions. This response shows a deflection from linearity with gradients higher than 150 mosmol kg_w^{-1}, tending to a maximal value. Although the cause of this response is still unknown, it is clearly evidence of an intrinsic property of hAQP1.

Acknowledgments We thank Drs. Moira Sutka, Karina Alleva and Paula Mut for their participation in the cRNA synthesis, Dr. Adrian Roitberg for kindly reading our preliminary manuscript and Dr. Basilio Kotsias for his valuable advice. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-PICT 32988), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-PIP 5926) and Universidad de Buenos Aires (UBACyT M824).

References

Abrami L, Simon M, Rousselet G, Berthonaud V, Buhler JM, Ripoche P (1994) Sequence and functional expression of an amphibian water channel, FA-CHIP: a new member of the MIP family. Biochim Biophys Acta 1192:147–151

Beckstein O, Sansom SP (2003) Liquid-vapor oscillations of water in hydrophobic nanopores. PNAS 100:7063–7068

Beitz E, Wu B, Holm LM, Schultz JE, Zeuthen T (2006) Point mutations in the aromatic/arginine region in aquaporin 1 allow passage of urea, glycerol, ammonia, and protons. PNAS 103: 269–274

Bitner JB, Peña-Rasgado C, Ruiz J, Cardona J, Rasgado-Flores H (2001) Osmotic properties of internally perfused barnacle muscle cells. I. Isosmotic conditions. Cell Mol Biol 47:855–864

Chandy G, Zampighi GA, Kreman M, Hall JE (1997) Comparison of the water transporting properties of MIP and AQP1. J Membrane Biol 159:29–39

Chiu SW, Subramaniam S, Jakobsson E, McCammon JA (1989) Water and polypeptide conformations in the gramicidin channel. A molecular dynamics study. Biophys J 56:253–261

Crane JM, Verkman AS (2008) Long-range nonanomalous diffusion of quantum dot-labeled aquaporin-1 water channels in the cell plasma membrane. Biophys J 94:702–713

Daniels MJ, Mirkov TE, Chrispeels MJ (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol 106:1325–1333

de Groot BL, Grubmüller H (2001) Water permeation across biological membranes: mechanism and dynamics of aquaporin-1 and GlpF. Science 294:2353–2357

de Groot BL, Grubmüller H (2005) The dynamics and energetics of water permeation and proton exclusion in aquaporins. Curr Opin Struct Biol 15:176–183

Dorr R, Ozu M, Parisi M (2007) Simple and inexpensive hardware and software method to measure volume changes in *Xenopus* oocytes expressing aquaporins. J Neurosci Methods 161:301–305

Dumont JN (1972) Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J Morphol 136:153–180

Hashido M, Kidera A, Ikeguchi M (2007) Water transport in aquaporins: osmotic permeability matrix analysis of molecular dynamics simulations. Biophys J 93:373–385

Hill WG, Southern NM, MacIver B, Potter E, Apodaca G, Smith CP, Zeidel ML (2005) Isolation and characterization of the *Xenopus* oocyte plasma membrane: a new method for studying activity of water and solute transporters. Am J Physiol Renal Physiol 289:F217–F224

Hirano Y, Okimoto N, Kadohira I, Suematsu M, Yasuoka K, Yasui M (2010) Molecular mechanisms of how mercury inhibits water permeation through aquaporin-1: understanding by molecular dynamics simulation. Biophys J 98:1512–1519

Jung JS, Preston GM, Smith BL, Guggino WB, Agre P (1994) Molecular structure of the water channel through aquaporin CHIP. The hourglass model. J Biol Chem 269:14648–14654

Kelly SM, Macklem PT (1991) Direct measurement of intracellular pressure. Am J Physiol 260:C652–C657

Kelly SM, Yan LJ, Macklem PT (1997) Measurement of elastic properties of *Xenopus* oocytes. Comp Biochem Physiol 118A:607–613

Luby-Phelps K (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol 192:189–221

Meinild AK, Klaerke DA, Zeuthen T (1998) Bidirectional water fluxes and specificity for small hydrophilic molecules in aquaporins 0–5. J Biol Chem 273:32446–32451

Migliati E, Meurice N, DuBois P, Fang JS, Somasekharan S, Beckett E, Flynn G, Yool AJ (2009) Inhibition of aquaporin-1 and aquaporin-4 water permeability by a derivative of the loop diuretic bumetanide acting at an internal pore-occluding binding site. Mol Pharmacol 76:105–112

Ohshima Y, Iwasaki I, Suga S, Murakami M, Inoue K, Maeshima M (2001) Low aquaporin content and low osmotic water permeability of the plasma and vacuolar membranes of a CAM plant *Graptopetalum paraguayense*: comparison with radish. Plant Cell Physiol 42:1119–1129

Ozu M, Dorr R, Parisi M (2005) New method to measure water permeability in emptied-out *Xenopus* oocytes controlling conditions on both sides of the membrane. J Biochem Biophys Methods 63:187–200

Parisi M, Dorr RA, Ozu M, Toriano R (2008) From membrane pores to aquaporins: 50 years measuring water fluxes. J Biol Phys 33:331–343

Pickard WF (2008) Modelling the swelling assay for aquaporin expression. J Math Biol 57:883–903

Preston GM, Carroll TP, Guggino WB, Agre P (1992) Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. Science 256:385–387

Soveral G, Madeira A, Loureiro-Dias MC, Moura TF (2008) Membrane tension regulates water transport in yeast. Biochim Biophys Acta 1778:2573–2579

Taglialatela M, Toro L, Stefani E (1992) Novel voltage clamp to record small, fast currents from ion channels expressed in *Xenopus* oocytes. Biophys J 61:78–82

Törnroth-Horsefield S, Hedfalk K, Fischer G, Lindkvist-Petersson K, Neutze R (2010) Structural insights into eukaryotic aquaporin regulation. FEBS Lett 584:2580–2588

Vandeleur R, Niemietz C, Tilbrook J, Tyerman SD (2005) Roles of aquaporins in root responses to irrigation. Plant Soil 274:141–161

Wan X, Steudle E, Hartung W (2004) Gating of water channels (aquaporins) in cortical cells of young corn roots by mechanical stimuli (pressure pulses): effects of ABA and of $HgCl_2$. J Exp Botany 55:411–422

Yukutake Y, Tsuji S, Hirano Y, Adachi T, Takahashi T, Fujihara K, Agre P, Yasui M, Suematsu M (2008) Mercury chloride decreases the water permeability of aquaporin-4-reconstituted proteoliposomes. Biol Cell 100:355–363 Erratum in: Biol Cell (2008) 100:561

Zhang Y, Hamill OP (2000) On the discrepancy between whole-cell and membrane patch mechanosensitivity in *Xenopus* oocytes. J Physiol 523:101–115

Zhang R, Logee K, Verkman AS (1990) Expression of mRNA coding for kidney and red cell water channels in *Xenopus* oocytes. J Biol Chem 265:15375–15378