Human AQP1 Is a Constitutively Open Channel that Closes by a Membrane-Tension-Mediated Mechanism

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SUPPORTING MATERIAL

I. GLOSSARY

$hAQP1$: human aquaporin-1
$E(t)$: elastic modulus or elastance of the membrane (dP_i/dV_i)
$T(t)$: membrane tension
$J_W(t)$: water flux
$A(t)$: membrane area
V_w: partial molar volume of water
m_i: total particles of internal solutes
$V_i(t)$: internal volume
V_0: initial internal volume
$V_i(t)/V_0$: relative internal volume
$m_i/V_i(t)$: internal osmolarity
C_e: osmolarity of the external compartment
Δosm: osmotic difference between internal and external compartments
$P_f(t)$: osmotic permeability coefficient
P_f^{INI}: initial osmotic permeability coefficient
$P_i(t)$: internal pressure
P_0: initial internal pressure
$P_i(t)/P_0$: relative internal pressure
CP_fM: Constant P_f Model
VP_fM: Variable P_f Model
VE: vitelline envelope
EOO: emptied-out oocytes

II. MATERIALS AND METHODS

Oocyte isolation

Adult female *X. laevis* (Nasco, Wisconsin, USA) were maintained in a room with controlled temperature (18ºC) and a 12-hour 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 by immersion in 0.3% tricaine (MS222) and oocytes were removed and prepared as previously described (**1**). Selected cells had 1.2 to 1.4 mm of diameter, thus belonging to stage VI (**2**).

Plasmid construction, in vitro synthesis and translation

The complete coding regions of full-length hAQP1 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* (**3**). Capped complementary RNAs (**4**) were synthesized in vitro using T3 RNA polymerase kit (Ambion, Austin, USA) and purified as described by Preston and co-workers (**5**). Synthesized products were suspended in RNAse-free water at a final concentration of 1 μg/μl and stored at -20°C until microinjection.

hAQP1-cRNA microinjection

Oocytes were microinjected with hAQP1-cRNA (50 ng, 1 μg/μl) 24 hours after isolation, using an automatic injector (Drummond Scientific Co, Broomall, PA, USA). For control experiments, oocytes were injected with water. Afterwards, all injected oocytes were kept during 24 hours at 18°C in ND96 solution supplemented with 1 μg/ml gentamicin (GIBCO, Maryland, USA) (**1**).

Gramicidin A incorporation to the membrane of *Xenopus* oocytes

Some oocytes were incubated with ND96 + 10 μM Gramicidin A (Sygma-Aldrich, St. Louis, MO, USA) during 2 hours, as previously published (**1**). These oocytes were tested with the emptied-out technique using hypoosmotic gradients of 200 and 400 mM mannitol.

Determination of the relationship between internal pressure and volume

Determination of the relationship between internal pressure (P_i) and volume (V_i) was performed in both emptied-out and whole oocytes.

A) Oocytes were emptied-out according to previously published protocol (**6**). The membrane of the EOO 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 (Fig. S1). This allowed control over media composition on both sides of the membrane. Volume changes were triggered by adding an isoosmotic solution to the internal compartment with a 100 μl gastight Hamilton syringe controlled by a horizontal translation stage (**1**). 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 the Hamilton 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, Bella Vista, Australia). LabChart 7 Pro data acquisition software v.7.2.2 was used for data recording and analysis. Internal pressure variations could be simultaneously followed with V_i changes, which were recorded by video-microscopy. Video records and image analysis were done as previously described (**7**).
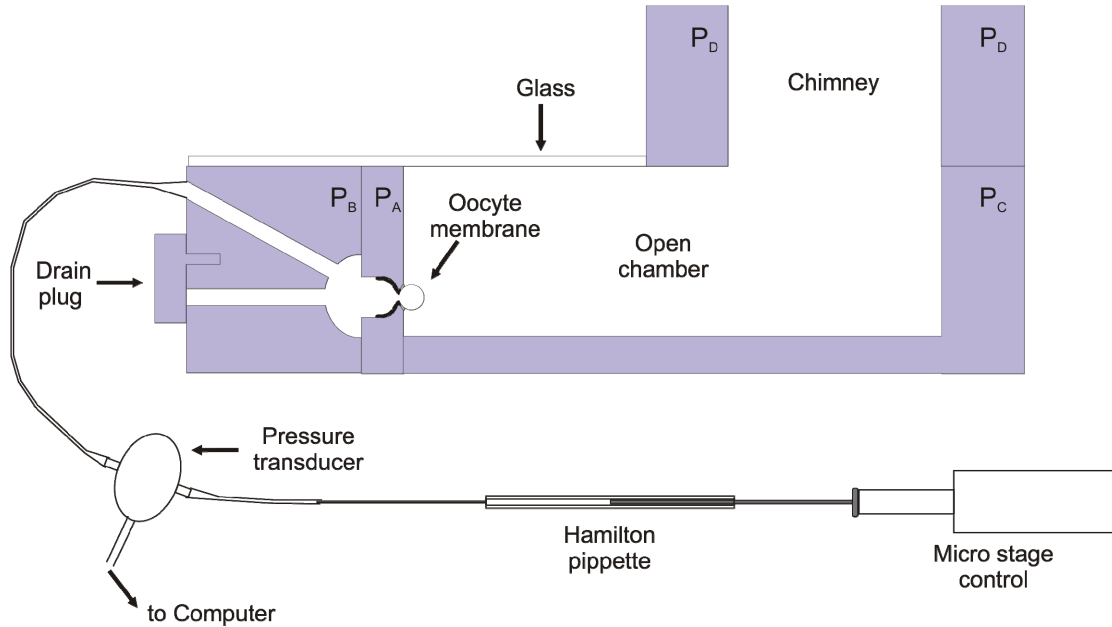
Figure S1. Schematic illustration of the pressure transducer used with the emptied-out oocyte technique. The oocyte is mounted in the experimental chamber as previously published (**6**). A plastic catheter connects the internal chamber –formed by pieces P_B and P_A– to a disposable clip-on dome prepared to receive an ADInstruments MLT844 physiological pressure transducer connected to a computer. Another catheter connects the other side of the clip-on dome to a gas-tight Hamilton syringe. The micro stage control allows micrometer displacement of the syringe piston to modify internal volume. Video records were registered through the glass window of the experimental chamber (*piece P_D*) with a video camera mounted on the microscope, and connected to the computer. Both pressure and images were registered simultaneously using the LabChart 7 Pro software, version 7.2.2 from ADInstruments (details in **1** and **7**).

B) The relationship between internal pressure (P_i) and volume (V_i) was also studied in entire *Xenopus laevis* oocytes (Fig. S2). In this case, the oocyte was puncture with a glass micropipette connected with a catheter to the clip-on dome with the pressure transducer. The Hamilton syringe was connected to this catheter. Like with EOO, volume changes were induced in stepwise injections of solution with the Hamilton syringe. In this case the volume injected in each step was 0.32 µl.

III. SUPPORTING RESULTS

A) Relationship between P_i and V_i in whole oocytes.

Results shown in Fig. S2 are very similar to those presented by Kelly and co-workers (**8**). Table 1 in the body of the paper presents the obtained results with whole oocytes in comparison with those obtained with EOO. Inspection of Table 1 indicates that the initial pressure (P_i) in EOO is almost zero while in whole oocytes is similar to

values previously published (**8**). Although EOO reach higher pressures at rupture, relative volumes (V/V_0) are not significantly different.

Figure S2. Relationship between P_i and V_i in whole oocytes. The procedure was the same used with EOO. Different symbols represent independent experiments (n=5). Each P_i-V_i pair represent mean values obtained in each steady state. Open circle represents mean values of P_i-V_i pairs reached at the end of the experiments. This point indicates the rupture of the system which was evidenced by the beginning of the oocyte content leakage in the injection site of the micropipette. Bars represent SE values. Right axis shows P_i values in Pascal units for easy comparison with data from Kelly and co-workers (**8**).

B) **Comparison of the elastic properties between emptied-out and whole oocytes.** As mentioned in the main text of the paper, our determinations were made in step controlled conditions, thus pairs of P_i-V_i data points were obtained under steady state conditions. Since P_i and V_i maintain constant values in each step, then the radius is fixed and thus the Laplace Law can be applied to each time interval. Given this equilibrium condition, the mean P_i can be directly associated to a mean value of membrane tension (Fig. S3).
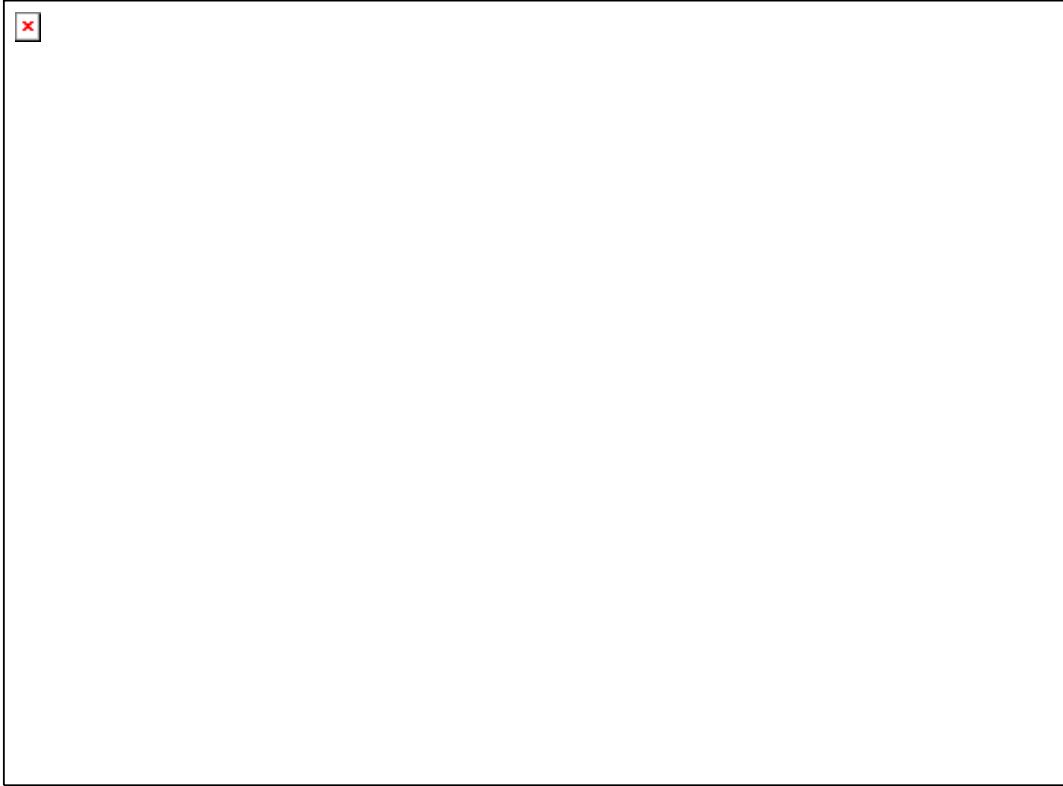
Figure S3. Membrane tension (T) **of emptied-out and whole oocytes represented as function of the experimental relative volume** (V/V_0)**.** Membrane tension (expressed as mean ± SE) was calculated applying the Laplace Law to each experiment like the one shown in Fig. 1 *A* for EOO, and Fig. S2 for whole oocytes. Dark gray area indicates the volume interval (mean ± SE) where rupture of whole oocytes occurred. Light gray area indicates the volume interval (mean ± SE) where rupture of the EOO occurred. As can be observed, initial tensions are very similar in both studied systems.

In addition, since the relationships between P_i and V_i were obtained as a second order polynomial function for each experiment, the elastic modulus (E) of the membrane was determined as the first order derivative (dP_i/dV_i) of the respective P_i-V_i functions. Comparison of mean values obtained with emptied-out and whole oocytes is shown in Fig. S4. These results indicate that E increases faster when the cellular content is absent.
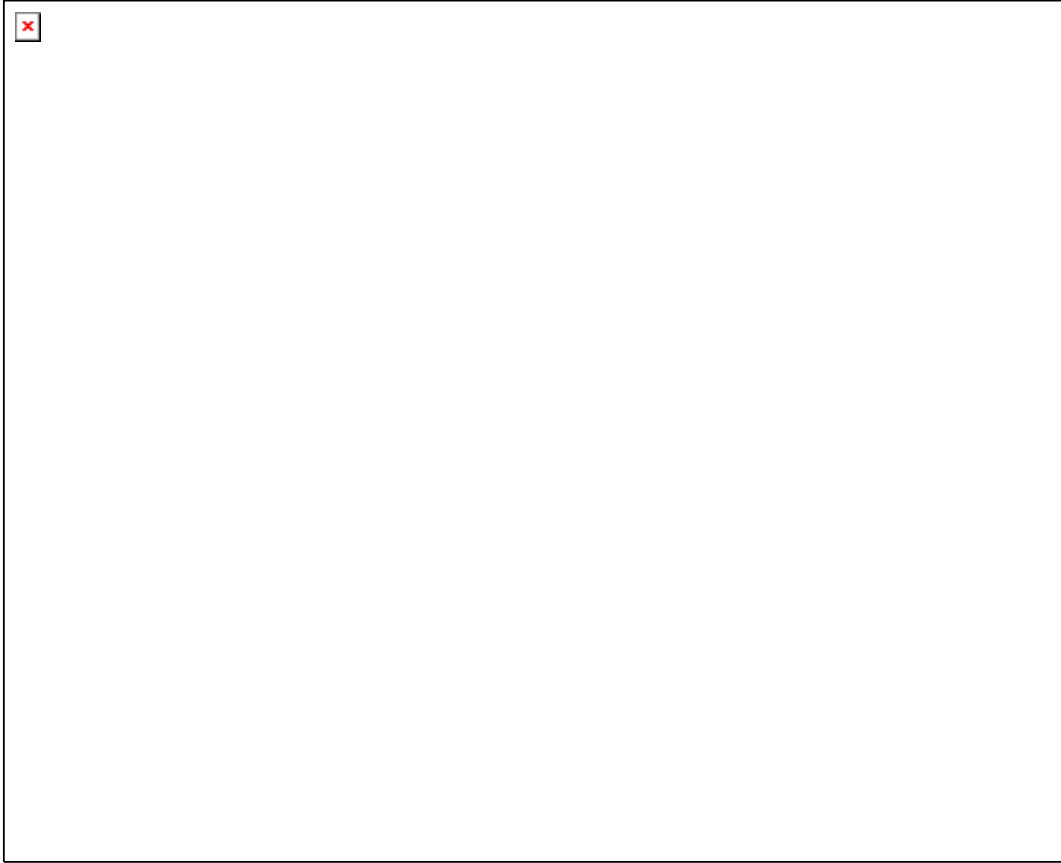
Figure S4. Elastic modulus (E) of the membrane versus the intracellular volume (V_i) of emptied-out and whole oocytes. Comparison of volume ranges indicates the difference between both methodologies. As was previously described, in the EOO technique, the intracellular content is replaced by a defined working solution. The inset shows the slope of the functions that describes the E dependence on volume. ***** indicates significant differences (p<0.05, Mann-Whitney test).

C) **Prediction with the Constant P_f Model**

According to the diffusion model, water moves driven by an osmotic difference (Eq. S1).

$$\frac{dV}{dt} = A(t) \cdot P_f \cdot V_W \cdot \left(\frac{m_i}{V_i(t)} - C_e \right) \qquad (S1)$$

This is the equation presented in the point 6 of the model section in the main text, where dV/dt represents the water flux (J_W) driven by an osmotic difference (Δosm) across a membrane of surface area $A(t)$. V_W is the molar volume of water (18 ml·mol⁻¹) and P_f is the osmotic permeability coefficient of the membrane. The osmotic difference is expressed as the difference between the internal (C_i) and the external (C_e) solute

concentrations (ΔC). The internal solute concentration is express as the internal mass (m_i) divided by the internal volume $(V_i(t))$. This model is very useful to determine P_f at times near to the beginning of the osmotic challenge. However, as was exposed by Pickard (**9**), application of this model to the entire record of osmotic responses in *Xenopus* oocytes with expression of hAQP1 is not enough to explain the observed behavior (dash line in Fig. S5).
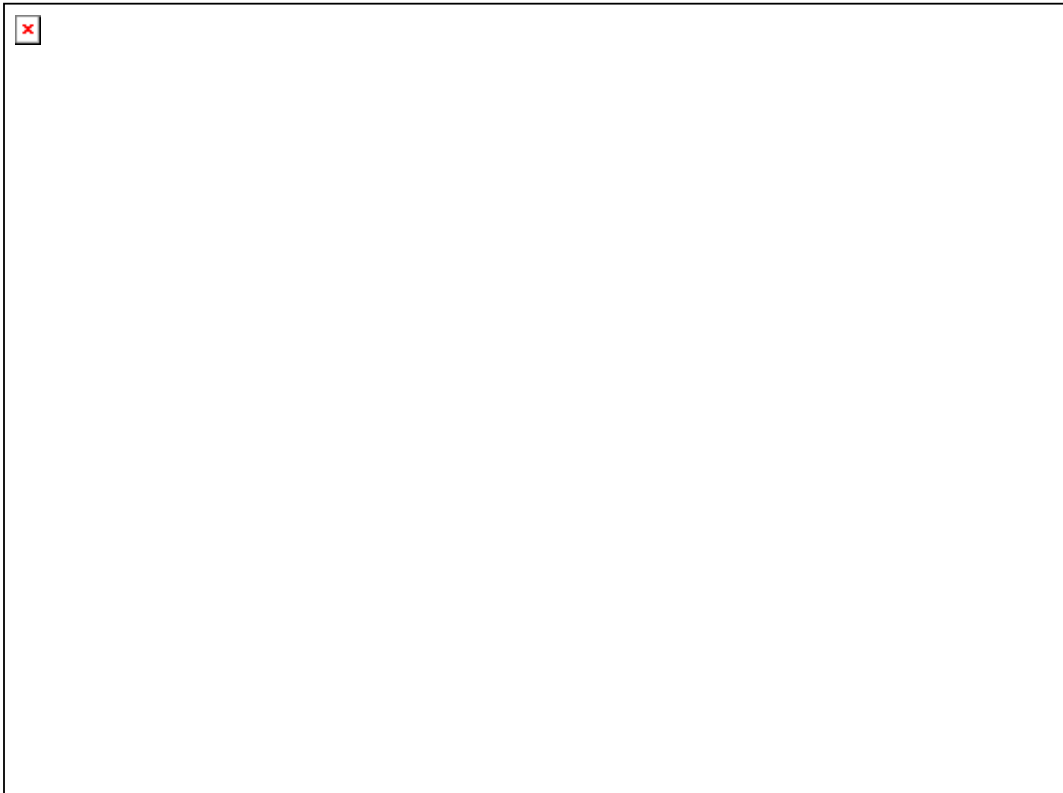

Figure S5. Osmotic response of an EOO with expression of hAQP1. The osmotic challenge (~140 mM) was created by dilution of the external solution. Solid dots represent the experimental record. Dash line represents the best fitted simulation obtained by a model-dependent fitting procedure using the *CP$_f$M* described by Eq. S1.

D) **The membrane tension gating mechanism of hAQP1 is reversible**

Reversibility of closure was shown in experiments were consecutive hypo and hyperosmotic mannitol gradients were applied (Fig. 6 of the main text). In these experiments, the hypoosmotic gradient (50 mM) was created with 100 mM mannitol in the internal compartment and 50 mM mannitol in the external compartment. The hyperosmotic gradient was created changing the external solution by another with 150 mM mannitol. Considering the simulated internal osmolarity at the moment of the external solution change (244 mM), then the effective hyperosmotic gradient was 56 mM mannitol. All solutions were created on the base of ND96 (200 mM), so the ionic force was maintained. Osmolalities of solutions were measured with a vapor pressure

osmometer (VAPRO 5520, Wesco, Logan, UT), and expressed as the equivalent osmolarities.

Derivative of simulated P_f values (Fig. S6) shows that changes of P_f are not constant. Instead of, under both hypo and hyperosmotic conditions P_f changes increase their rate from the beginning up to a maximum and then decrease (note that during hypoosmotic condition the derivative is always negative because P_f is diminishing but the absolute values increases and then diminish). This behavior also reflexes the cooperative effect among monomers. The explanation would be that the initial increase of the P_f-change rate could be due to the associated closure (during hypoosmotic conditions) of more and more monomers until a maximal change rate is reached. These first monomers are the most difficult to be closed. After the maximum, close rate lowers while less open monomers left. Then, similar events occur in a symmetrical way, but to open the closed monomers (during hyperosmotic conditions). At the beginning, opening of closed monomers occurs with an increasing rate up to a maximum. During this time, the opening cost decreases, which is reflected by the increasing P_f-change rate. After the maximal rate of P_f-change is easier to open the few monomers that are still in a closed state.
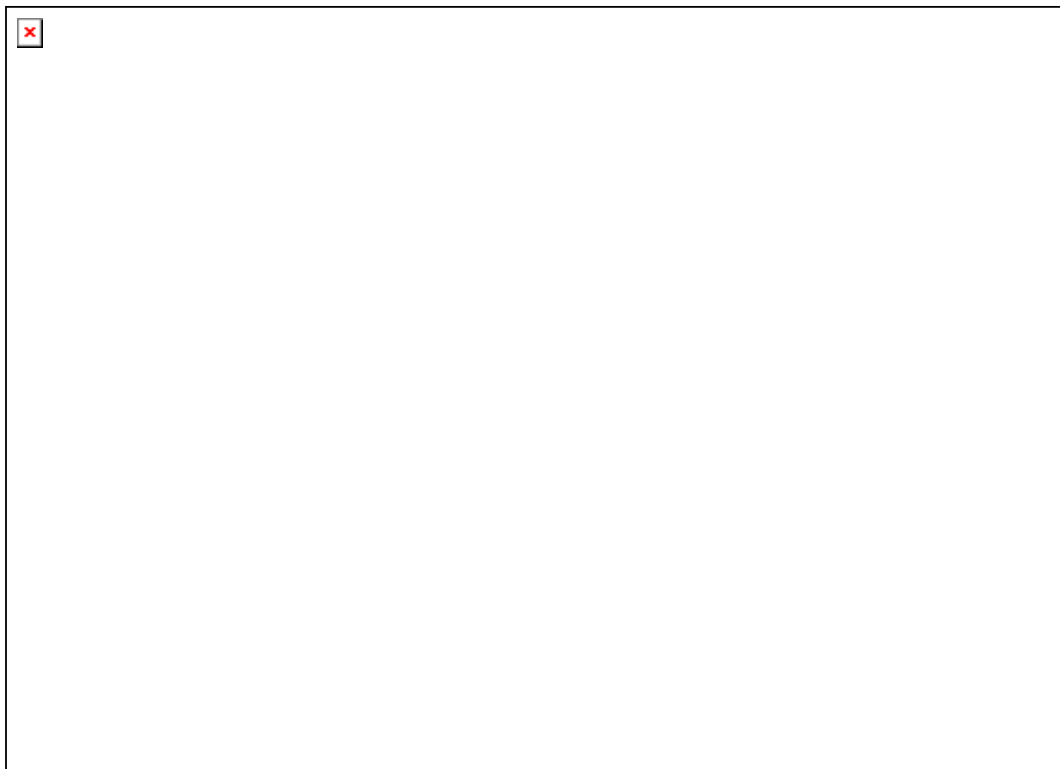

Figure S6. Derivative of simulated P_f values in experiments with consecutive hypo and hyperosmotic gradients. The rate of simulated P_f changes increases up to a maximum and then decreases in both hypo and hyperosmotic conditions. Both responses are symmetrical and reflect the reversibility of the cooperative effect of hAQP1 monomers following conformational changes.

IV. THE VARIABLE P_f MODEL

Simulations were performed to study the osmotic response of *Xenopus* oocytes expressing hAQP1. The used model was based on the general formulation used for fluid fluxes (**10**). The following assumptions are taking into account:

1) The hypotonic shock is modeled employing a step function (Heviside function). Therefore, it is assumed that at time t<0 s the extracellular concentration is equal to the intracellular one. Then, at t=0 the extracellular concentration is instantly lowered to a fix value, which is maintained along the experiment (t>0).
2) A semi-spherical shape is assumed for the intracellular compartment.
3) The oocyte membrane is permeable to water.
4) There are not interactions between the membrane electrical potential and water flux.
5) The membrane area is considered variable and dependent on the intracellular volume according to the following equation:

$$A(t) = \frac{1}{2} \cdot 4 \cdot \pi \cdot r^2 \cdot 9 \qquad (S2)$$

where the factor 9 corrects the observed area for the invaginations of the membrane (**11**).

6) Water can be transported by an osmotic mechanism, according to the following phenomenological expression:

$$J_W(t) = P_f A(t) V_w 10^{-3} \left(\frac{m}{V_i(t)} - C_e \right) \qquad (S3)$$

where P_f, $A(t)$, V_w and C_e, are the osmotic-permeability coefficient of the oocyte membrane, the membrane area mentioned above, the water molar partial volume, and the extracellular osmolarity respectively.

The term $\frac{m}{V_i(t)} - C_e$ represents the osmotic force (ΔC).

7) The rate of internal volume is only affected by the water flux (Continuity Theorem):
$$dV_i(t)/dt = J_W(t)$$
The total solute concentration is computed, as usual, in terms of the total number of osmolytes ($m_i(t)$) and the internal volume ($V_i(t)$):

$$C_i(t) = m_i(t)/V_i(t)$$

The internal volume (V_i) is calculated from the images of the EOO, assuming a semispherical shape

8) Membrane is impermeable to solutes. Thus, the total number of intracellular osmolytes is constant:

$$m_i(t) = m_i$$

Passage of ions (Na$^+$, K$^+$, and Cl$^-$) through the membrane is considered to be depreciated because of their low permeability coefficients (in the order of 10^{-8} cm·s^{-1}, according to Costa and coworkers (**12**).

9) Based on this general model, the following modifications were introduced to consider a variable osmotic permeability coefficient $(P_f(t))$:

$$P_f(t) = \frac{P_f^{INI}}{P_i(t)\big/P_0} \qquad \text{(S4)}$$

where $P_i(t)$ is the internal pressure at any time $t \geq 0$, P_0 is the initial internal pressure, P_f^{INI} is the initial osmotic permeability coefficient, and $P_f(t)$ is the osmotic permeability coefficient at any time $t \geq 0$.

The relative internal pressure (P_i/P_0) was determined from the relative internal volume (V_i/V_0) according to the following equation:

$$P_i(t)\big/P_0 = C_2 \cdot \left(V_i(t)\big/V_0\right)^2 - C_1 \cdot \left(V_i(t)\big/V_0\right) + C_0 \qquad \text{(S5)}$$

where $V_i(t)$ is the internal volume at any time $t \geq 0$, V_0 is the initial internal volume, and $C_2 = 976 \pm 8$, $C_1 = 1904 \pm 4$, and $C_0 = 929 \pm 7$ are the mean polinomic coefficients $\pm$ SE (n=4). This equation is the average of 4 independent experiments done in EOO, in which volume increments were induced by injection of solution in steps of 0.16 μl while pressure and volume were simultaneously measured (as was described in the main text of the paper, in the Materials and Methods section).

Equations S4 and S5 establish a simple relationship between P_f and the two variables that define membrane elastance (P_i and V_i).

Equation S4 defines $P_f = P_f^{INI}$ at t=0. After then, P_f would have to diminish due to volume increments produced by the osmotic flux.

V. OTHER TESTED MODELS WERE DISCARDED

From results shown in Fig. 1 of the main text, it can be assumed that there are not significant changes of intracellular pressure (P_i) during the beginning of the volume change. Therefore, for simplification, a linear relationship was assumed for all the experiments. Comparison with whole oocytes (Fig. S2) reflexes that absence of the cytoplasmic content in EOO produces a linear increment of P_i (Fig. S7).
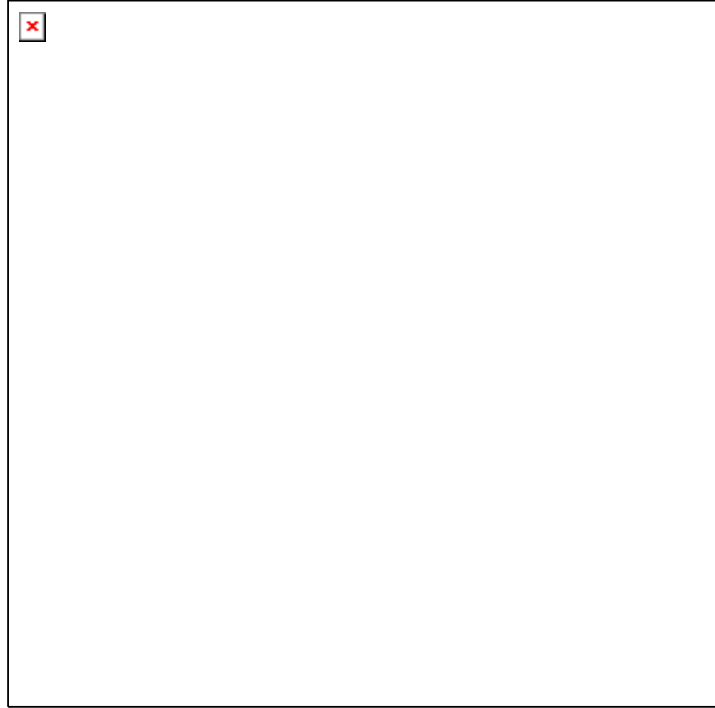
Figure S7. Relationship between P_i and V_i in EOO. All the experiments show depreciable changes of P_i in the first steps of volume change. Thus, if these points are not taking into account, a linear relationship is observed between P_i and V_i. In all cases $R^2 > 0.98$, n=4.

The linear relationship between P_i and V_i was used to obtained a unique value of membrane elastance (E). If we define E as the pressure change induced by the volume change (Eq. S6), and if we assume E to be constant, then E can be defined according to Eq. S6. Thus, the experimentally determined mean elastance is 4.42 x 10^7 dyn.cm^{-5}.

$$E = \frac{\Delta P}{\Delta V} \qquad \text{(S6)}$$

The general diffusion model for water (Eq. S7) considers two possible forces to generate water movements. These are the osmotic difference (ΔC) and the hydrostatic pressure (ΔP).

$$\frac{dV}{dt} = A \cdot P_f \cdot V_W \cdot \left(\Delta C - \frac{\Delta P}{RT} \right) \qquad \text{(S7)}$$

If we express ΔC as in Eq. S3 and we replace ΔP by the product between E and the volume change, then we obtain Eq. S8, which express the hydrostatic pressure changes induced by water entering the cell in relation to the membrane elastance.

$$\frac{dV}{dt} = A \cdot P_f \cdot V_W \cdot \left(\left(\frac{m_i}{V_i} - C_e \right) - \frac{E \cdot (V_i - V_0)}{RT} \right) \qquad \text{(S8)}$$

We used Eq. S8 to create a model to simulate the osmotic response and find the values of E and P_f that showed the best fit to experimental data. Results show that Eq. S8 predicts a close fit to experimental results when E is a free parameter of the model (continuous line in Fig. S8). However, the predicted value of E is three orders of magnitude higher than the value experimentally determined (Table S1). In addition, when the experimental E value is introduced in the model (instead of leaving E as a free parameter of the model) the dash line shown in Fig. S8 is obtained. This result is very similar to that predicted by Eq. S3 (shown in Fig. S5), indicating that –as is expected– the hydrostatic effect of the water that enters the cell is depreciable against the osmotic pressure. As consequence this model was discarded.
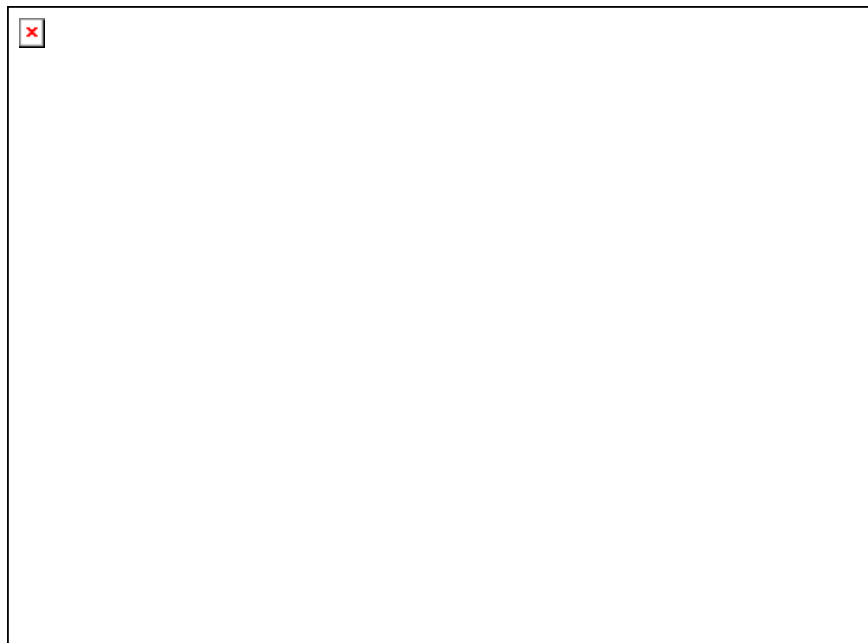

Figure S8. Osmotic response in EOO with hAQP1 predicted by Eq. S8. Circles represent the experimental record of an osmotic experiment in EOO with hAQP1 under a hypoosmotic challenge. The osmotic gradient (~140 mM) was created by dilution of the external solution. Continuous line represents the response predicted by Eq. S8, obtained by a model dependent fitting procedure. The obtained parameters are shown in Table S1. Dash line represents the response predicted by the same equation using the experimentally determined E value.

	P_f	E	
	$(cm.s^{-1})$	$(dyn.cm^{-5})$	$(mmHg.nl^{-1})$

E (fit)	0.00392	1.23×10^{10}	~ 9.23
E (exp)	0.00229	4.42×10^{7}	~ 0.033

Table S1. Parameter values obtained with the model based on Eq. S8. *E (fit)* refers to simulations run to find the best fitting parameters E and P_f . *E (exp)* refers to simulations run with the experimentally determined value of E (4.42×10^{7} dyn.cm^{-5}) as a fixed data of the model. E are expressed in mmHg.nl^{-1} in order to get a quickly interpretation of the results.

Other possible model considered the movement of a unique uncharged solute. This model demonstrated to be inadequate to predict and reproduce the osmotic response (**13**).

Supporting References
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