RESEARCH ARTICLE

Lithobates catesbeianus (American Bullfrog) oocytes: a novel heterologous expression system for aquaporins

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ABSTRACT

Xenopus laevis oocytes are a valuable tool for investigating the function of membrane proteins. However, regulations around the world, specifically in membrane Brazil, render the import of Xenopus laevis frogs impractical, and, in some cases, impossible. Here, as an alternative, we evaluate the usefulness of the North American aquatic bullfrog Lithobates catesbeianus, which is commercially available in Brazil, for the heterologous expression of aquaporin (AQP) proteins. We have developed a method that combines a brief collagenase treatment and mechanical defolliculation for isolating individual oocytes from Lithobates ovari. We find that they have a similar size, shape, and appearance to Xenopus oocytes and can tolerate and survive following injections with cRNA or water. Furthermore, surface biotinylation, western blot analysis, and measurements of osmotic water permeability (Pₒ) show that Lithobates oocytes can express AQPs to the plasma membrane and significantly increase the Pₒ of the oocytes. In fact, the Pₒ values are similar to historical values gathered from Xenopus oocytes. Due to the presence of a mercury sensitive cysteine (Cys or C) in the throat of the water channel, the Pₒ of oocytes expressing human (h) AQP1, hAQP1FLAG [FLAG, short protein tag (DYKDDDDK) added to the N-terminus of AQP1], hAQP8, and rat (r) AQP9 was inhibited with the mercurial compound p-chloromercuribenzenesulfonate (pCMBS), whereas AQPs lacking this Cys – hAQP1C189S mutant [residue Cys 189 was replaced by a serine (Ser or S)] and hAQP7 – were mercury insensitive. Contrary to previous studies with Xenopus oocytes, rAQP3 was also found to be insensitive to mercury, which is consistent with the mercury-sensitive Cys (Cys 11) being located intracellularly. Thus, we consider Lithobates oocytes to be a readily accessible system for the functional expression and study of membrane proteins for international researchers who do not currently have access to Xenopus oocytes.

KEY WORDS: Lithobates catesbeianus, American Bullfrog, Oocytes, Aquaporins, Water permeability, Xenopus laevis, Heterologous expression system

INTRODUCTION

The use of Xenopus laevis oocytes as a viable heterologous expression system was first demonstrated 46 years ago (Gurdon et al., 1971). Eleven years later, it was shown that membrane proteins could also be translated and inserted into the oocyte plasma membrane (Miledi et al., 1982a,b), thus conferring functional activities that are otherwise absent in water-injected control oocytes. Since then, numerous publications have utilized this system to study the transport of a wide variety of substances (Musa-Aziz et al., 2010). One protein family, of particular relevance to this study, which has been studied extensively using Xenopus oocytes, is the aquaporin family of water channel proteins (Geyer et al., 2013a; Preston et al., 1992).

The landmark study by Preston et al. using Xenopus oocytes (Preston et al., 1992) definitively expressed and characterized the water transport properties of CHIP28 (channel-forming integral protein of 28 kDa), which is now known as aquaporin 1 (AQP1), and also showed that this protein is mercury sensitive. By systematically mutating each cysteine (Cys or C) residue in AQP1 to a non-sulfhydryl containing amino acid serine (Ser or S), this group also discovered that Cys 189, located in the throat of the water channel aquapore, near the second asparagine-proline-alanine (NPA) motif, and accessible from the extracellular surface, is the mercury-sensitive Cys residue of AQP1 (Preston et al., 1993). Since this discovery, 12 more aquaporin (AQP) proteins have been cloned and functionally characterized, most of which are also mercury sensitive, with the exceptions of AQP4 (Jung et al., 1994) and AQP7 (Ishibashi et al., 1997b). Despite the similar primary amino acid sequences and highly conserved secondary, tertiary, and quaternary structures, each AQP possesses diverse transport properties. For example, in addition to transporting water, AQPs have been shown to transport glycerol, ions, hydrogen peroxide, dissolved gases (carbon dioxide and ammonia), and arsenic (Geyer et al., 2013a). It has been shown that, in many cases, the localization of AQPs to specific organs or cell types is largely based on the transport specificities of the AQPs. While the Xenopus oocyte system has been instrumental in the growth and progress of the AQP field, it is not readily available to some researchers. For example, the state of California requires researchers to obtain a license to import the Xenopus laevis frog into the state (California Natural Resources Agency Department of Fish and Wildlife, 1984). Additionally, the import of Xenopus frogs and oocytes to the South American country of Brazil is tightly regulated and extremely difficult, due to the fear of the frogs escaping and becoming an invasive species, which could wreak havoc on the tropical ecosystem. Therefore, an alternative to the Xenopus system, using a frog that is not tightly regulated or prohibited needs to be developed and characterized.

Previous studies have shown that oocytes from other species of frogs can be utilized for the functional characterization of membrane transporters and channels. For example, Xenopus borealis oocytes have been used to study neuronal ion channels (Cristofori-Armstrong et al., 2015), Bufo marinus – indigenous to south and mainland Central America – oocytes have been employed for evaluating various membrane transporters and ion channels

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(Markovich and Regeer, 1999; Vargas et al., 2004), and *Bufo arenarum* – native to Argentina – oocytes have been characterized for endogenous ion currents and usefulness in expressing urea transporters (Cavara et al., 2003; Silberstein et al., 2003). Here, we have identified a species of frog, *Lithobates catesbeianus* – also known as the American Bullfrog – that is native to North America, but was first introduced to Brazil in the 1930s (Both et al., 2011). Due to the abundance of and accessibility to *Lithobates*, the aim of this work is to evaluate the usefulness and viability of *Lithobates* oocytes as an alternative model system for heterologous expression of AQPs.

As a proof of concept, we developed an oocyte isolation procedure, which employs a brief incubation in purified collagenase to release individual *Lithobates* oocytes, followed by mechanical removal of the innermost follicular cells that create a barrier between the oocyte and the external environment. These oocytes were injected with cRNA encoding for human (h) AQP1, hAQP1FLAG, hAQP1C1N9S mutant (AQP1 with the mercurial target Cys 189 mutated to a Ser), rat (r) AQP3, hAQP7, hAQP8 or rAQP9, monitored for surface expression (Geyer et al., 2013a), assessed for osmotic water permeability ($P_o$), and evaluated for mercury sensitivity. We conclude that *Lithobates* oocytes are a viable heterologous expression system for AQPs, and can be used by researchers that have restricted access to *Xenopus* oocytes, or be employed as an alternative expression system to researchers that study AQPs. Future studies will be geared towards the applicability of *Lithobates* oocytes with other membrane proteins and assays (e.g. voltage clamp, patch clamp, intracellular and extracellular pH measurements).

### RESULTS

#### Blood parameters

Before starting to develop a method for isolating oocytes from *Lithobates catesbeianus* ovaries, it was important to measure some serum chemistry parameters to make sure that (1) the solutions typically used with *Xenopus* oocytes would be compatible with *Lithobates* oocytes, and (2) *Lithobates* oocytes would respond well to the protocols used to isolate the *Xenopus* oocytes. The serum chemistry values of *Lithobates catesbeianus* are also important basic information that needs to be available to other researchers.

The arterial blood gas analysis ($p_{CO_2}$, $HCO_3^-$ and pH), osmolality, and ion concentrations ($Na^+$, $Cl^-$, $K^+$) were measured in arterial blood collected from *Lithobates* frogs and compared to values reported in the literature for *X. laevis*, and a summary of the results are presented in Table 1. Due to the similarities in the values, especially with regards to the osmolarity and pH, it was concluded that the solutions typically used for *Xenopus* oocyte isolation and storage would not need to be modified. Next, we evaluated the effect of using collagenase in the oocyte isolation procedure.

### Oocyte isolation

A typically *Xenopus* oocyte isolation process involves incubating the ovary fragments that include oocytes, connective tissue, blood vessels and follicular cells in 2 mg/ml Type IA collagenase for 40 min. This enzymatic digestion liberates individual oocytes from the ovary and from their follicular layers, leaving the vitelline membrane that surrounds the plasma membrane intact. The vitelline membrane helps to maintain the shape of the oocyte and renders the oocyte more resistant to manipulations.

However, *Lithobates* oocytes lysed and/or died when incubated with 2 mg/ml Type IA collagenase for 40 min. Attempts were made to optimize the Type IA collagenase incubation conditions by reducing the collagenase concentration (from 2 mg/ml to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml or 0.1 mg/ml) and varying the duration of the digestion process (from 40 min to 20 min, 10 min or 5 min (a brief 5 min treatment was applied to both the 0.25 mg/ml and 0.1 mg/ml concentrations of Type IA collagenase). Under all conditions employed, unhealthy nonviable *Lithobates* oocytes were obtained. Therefore, the efficacy and usefulness of other collagenases was investigated. Similar results were obtained with Type II collagenase (Sigma-Aldrich) and Type V collagenase (Sigma-Aldrich) at 1 mg/ml in 0-Ca$^{2+}$ ND-96 solution.

Ultimately, it was found that a brief 5 min treatment with the purified Type VII collagenase (0.25 mg/ml) digested the extracellular connective tissues, while leaving the innermost layer of follicular cells still attached to the exterior of the oocyte. After stopping the enzymatic digestion, the remaining follicular cells were manually removed, without disrupting the vitelline membrane. This combination of methodologies yielded healthy, fully demembranated individual oocytes that appeared the same as they did in the ovary. Fig. 1A and B show the entire isolation process. The isolated *Lithobates* oocytes have about the same size, shape, and appearance as *Xenopus* oocytes: they are large (∼1.0 mm in diameter) with a well-defined dark animal pole (where the nucleus is located), a weakly pigmented vegetal pole (containing the majority of the yolk proteins), and a very faint, sometimes unnoticeable, unpigmented equatorial belt, between the two poles. These *Lithobates* oocytes persisted in OR3 media for 5 days without signs of cell lysis, discoloration or infection. Having established a feasible isolation procedure, it was then necessary to evaluate the usefulness of *Lithobates* oocytes as a heterologous expression system.

Portions of an amino acid sequence alignment of the AQPs relevant to this study are shown in Fig. 2. The conservation of the two NPA motifs (signature of an AQP) and the site of the

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Table 1. Blood parameters of *Lithobates* and *Xenopus*

|            | Lithobates |           |           |           |           |           |
|------------|------------|-----------|-----------|-----------|-----------|-----------|
|            | Average    |           |           |           |           |           |
| Osmolality | 194.71     |           |           |           |           |           |
| pH         | 7.48       |           |           |           |           |           |
| $p_{CO_2}$ | 32.28      |           |           |           |           |           |
| $HCO_3^-$  | 23.60      |           |           |           |           |           |
| $Na^+$     | 94.3       |           |           |           |           |           |
| $K^+$      | 3.72       |           |           |           |           |           |
| $Cl^-$     | 66.0       |           |           |           |           |           |
|            | SE         |           |           |           |           |           |
| N          | 4.22       |           |           |           |           |           |
|           | 0.03       |           |           |           |           |           |
|           | 1.73       |           |           |           |           |           |
|           | 2.16       |           |           |           |           |           |
|           | 1.2        |           |           |           |           |           |
|           | 0.56       |           |           |           |           |           |
|           | 1.3        |           |           |           |           |           |
| $Xenopus$  |           | 66        | 64        | 64        | 67        | 47        |
| Average    | 200–233    | 7.76      | 14.00     | 25.00     | 119.0     | 3.97      |
| $Xenopus$  | NA         | 0.03      | 0.20      | 0.70      | 3.5       | 0.86      |
| SE         |            |           |           |           |           | 5.6       |

*Xenopus* parameters were taken from the literature: osmolality (Green, 2010), pH, $p_{CO_2}$ (Boutilier et al., 1987), $HCO_3^-$ (Boutilier et al., 1987), $Na^+$ (Cathers et al., 1997), $K^+$ (Chang et al., 2015), $Cl^-$ (Cathers et al., 1997). NA, not available; N, the number of individual frogs analyzed for each blood parameter; SE, standard error.
mercury-sensitive Cys (C) residues, which is near the second NPA motif and accessible from the extracellular side of the cell membrane, are highlighted in gray. Cys 189 in AQP1 is accessible from the extracellular surface and responsible for the mercury sensitivity. AQP8 and AQP9 possess an analogous Cys residue located in the throat of the aquapore (Cys 202 in AQP8 and Cys 213 in AQP9). Previous studies have shown that the mercury-sensitive Cys in AQP3 (Cys 11) is located on the intracellular surface of the protein (Kuwahara et al., 1997), and that AQP7 lacks a mercury-sensitive Cys, near the second NPA motif (Ishibashi et al., 1997b).

The western blots in Fig. 3 show that Lithobates oocytes injected with cRNA encoding for hAQP1 (Fig. 3A), hAQP1FLAG (Fig. 3B), hAQP1C189S mutant (Fig. 3C), rAQP3 (Fig. 3D), hAQP7 (Fig. 3E), hAQP8 (Fig. 3F) and rAQP9 (Fig. 3G) express the proteins at the cell surface. The immunoreactive AQP bands are detected at a molecular weight (MW) between 25 and 37 kDa, which is indicative of monomeric AQP (~28 kDa) and glycosylated AQP (~37 kDa), with the exception of AQP7, which has a predicted MW of 37 kDa and does not have any consensus glycosylation sites (Ishibashi et al., 1997b). These results show that the AQP proteins were not only synthesized and posttranslationally modified but also inserted into the plasma membrane.

Osmotic water permeability, $P_f$
Having successfully demonstrated that Lithobates oocytes can translate and insert membrane proteins into the membrane, we next evaluated whether or not the expression of AQP increases the $P_f$ of oocytes.

Oocytes were injected with cRNA encoding for the AQP or injected with water, as controls. Four days after injection, the oocytes were transferred from ND96 control solution (~200 mOsm) to a hypotonic ND96 variant solution (~70 mOsm) and the rate of oocyte swelling, from which we computed $P_f$ (cm/s), was monitored using video microscopy. Fig. 4 shows photos of a typical time.
course of cell swelling for H2O-injected control oocytes (upper panel) and AQP1-expressing oocytes (lower panel) exposed to hypotonic ND96 variant solution over the course of 5 min. It is clear that, by the end of the time course, the membranes of oocytes expressing AQP1 started to break due to osmotic pressure. In contrast, the H2O-injected control oocytes did not swell.

The rate of cell swelling, from which we calculate \( P_f \), was performed with each of the AQP-expressing oocytes versus its day-matched H2O-injected controls. Fig. 5 (left side) summarizes these experiments and shows that, oocytes expressing hAQP1, hAQP1FLAG, hAQP1C189S mutant, rAQP3, hAQP7, hAQP8 and rAQP9 (black bars) displayed a mean \( P_f \) value that was significantly higher than that of day-matched H2O-injected controls (gray bars). To confirm that the increased \( P_f \) values were the result of AQP expression, the effect of the mercurial agent pCMBS on \( P_f \) was examined. The same oocytes were incubated with 1 mM pCMBS for 30 min, washed three times in ND96 solution, and then placed in the hypotonic ND96 variant solution (~70 mOsm) and the rate of oocyte swelling, from which we computed \( P_f \), was again monitored using video microscopy. As shown in Fig. 5 (right side), pCMBS treatment reduced the \( P_f \) difference between hAQP1, hAQP1FLAG, hAQP8 and rAQP9 (black bars) and their day-matched H2O controls (gray bars), but had no effect on the \( P_f \) difference between hAQP1C189S mutant, rAQP3, and hAQP7 (dark-gray bars) and the day-matched H2O controls (light-gray bars). Together, these results indicate that all mammalian AQPs are functionally expressed in Lithobates oocytes, and that the enhanced water permeability is AQP-mediated, since the \( P_f \) of hAQP1, hAQP1FLAG, hAQP8 and rAQP9 can be inhibited by the mercurial agent pCMBS.

In Fig. 5, the portion of the \( P_f \) bar that we can ascribe to each AQP is the portion above the H2O-injected control background. Thus, we subtracted the mean \( P_f \) of each AQP-expressing oocyte from the mean \( P_f \) of the day-matched H2O control. This difference represents the channel-dependent \( P_f \) or \( *P_f \) of each AQP. Fig. 6 summarizes these differences and shows that pCMBS significantly reduces the \( *P_f \) of hAQP1, hAQP1FLAG, hAQP8 and rAQP9 by about half, but has no effect on the \( *P_f \) of hAQP1C189S mutant (the mercury-insensitive AQP1), rAQP3 (that has the mercury-sensitive Cys located on the intracellular surface of the protein), and hAQP7 (that lacks a mercury-sensitive Cys).

**DISCUSSION**

In this work, we set out to determine if oocytes isolated from *L. catesbeianus* can be utilized as an alternative to the *Xenopus* oocyte heterologous expression system. We found that the methodologies typically employed with *Xenopus* oocytes are not
entirely compatible with *Lithobates* oocytes. Despite the blood parameters, of the two frogs, being quite similar, there are some unknown properties in the vitelline membrane and/or cellular membrane of the *Lithobates* oocytes. These differences in membrane properties cause the *Lithobates* oocytes to be extremely sensitive to Type IA collagenase, which is commonly used for the isolation of *Xenopus* oocytes (Musa-Aziz et al., 2010). The primary advantage of using the collagenase based method is that a large quantity of truly defolliculated oocytes can be obtained, relatively easily. However, collagenase treatment can affect the metabolic rate, and impact the robustness of the oocytes (Sive et al., 2000). In fact, under all Type IA collagenase concentrations and exposure times the *Lithobates* oocytes lysed and/or died. By evaluating the efficacy of three different types of collagenase, it was found that a brief 5 min treatment with the purified Type VII collagenase digestion was sufficient to effectively releasing individual oocytes, without removing the innermost layer of follicular cells and damaging the vitelline membrane, as
evidenced by longer incubation times with all of the collagenases used, including Type VII. Therefore, following the brief 5 min Type VII collagenase treatment, it was necessary to mechanically or manually remove the follicular layer of cells surrounding the oocytes, so as to ensure that the vitelline membrane, which preserves the integrity and health of the oocyte (Musa-Aziz et al., 2010), remains intact.

While the technique to manually remove the follicular layer requires some level of training and practice (Sive et al., 2000), it routinely results in several hundred defolliculated and robust oocytes. By combining a brief 5 min treatment with the purified Type VII collagenase, followed by manual removal of the innermost layer of follicular cells, an effective method for isolating Lithobates oocytes was developed, which does not compromise the integrity of the cellular membrane or overall health of the oocyte.

Using methodologies for labeling and isolating proteins at the surface of the oocyte and evaluating AQPs function, not entirely different from those utilized with Xenopus oocytes (Geyer et al., 2013a,b), we have also shown that Lithobates oocytes can express AQPs at the cellular surface and can significantly increase the $P_f$ of the oocytes. Using the membrane-impermeable reagent EZ-link-sulfo-NHS-Biotin, it is possible to selectively modify and surface of the oocyte and evaluating AQP function, not entirely to answer and is beyond the scope of the present study.

The glycosylation of AQP1 and AQP3 was observed by the smeared band at a higher MW (~37 kDa), and is consistent with what others have reported in the literature (Ishibashi et al., 1997a; Preston et al., 1992). AQP7 also displayed a band at ~37 kDa; does not and mercury sensitive, similar to oocytes expressing hAQP1 (Figs 5 and 6). It should also be noted that it appears as though the AQP1C189S mutant had a higher apparent expression level than that of wild-type AQP1. This could be due to a variety of reasons such as higher translational rate of the cRNA, increased stability of the translated protein, or resistance to proteolytic digestion, which will require a more analytical approach to answer and is beyond the scope of the present study.

The glycosylation of AQP1 and AQP3 was observed by the smeared band at a higher MW (~37 kDa), and is consistent with what others have reported in the literature (Ishibashi et al., 1997a; Preston et al., 1992). AQP7 also displayed a band at ~37 kDa; however, this protein has a predicted MW of ~37 kDa, and does not contain a potential consensus glycosylation site (Ishibashi et al., 1997b). Thus, our interpretation of these results, coupled with the functional data, is that the immunoreactive band at ~37 kDa represents monomeric AQP7, and the immunoreactive bands at a lower MW correspond to a proteolytic fragment of AQP7. Unexpectedly, no glycosylation was observed with AQP8 or AQP9, which is inconsistent with previous reports (Calamita et al., 2001; Viadiu et al., 2007). This anomaly could be attributed to the protein concentrations being below the limit of detection for observing the glycosylation product. Despite this discrepancy with AQP8 and AQP9 sites of glycosylation, functional expression (significant increase in $P_f$ and mercury sensitivity) were observed with both proteins (Figs 5 and 6).

When the AQP-expressing oocytes were placed in the hypotonic solution, significant changes in the oocyte volume were observed and reproducible $P_f$ values were calculated (Fig. 5). Therefore, all
AQPs used in this study display significant water permeability when expressed in *Lithobates* oocytes. To ascertain whether or not this significantly increased $P_f$ was due to the presence of AQP channels being expressed at the surface of the oocyte, the mercurial agent pCMBS – which does not permeate cell membranes – was applied to the AQP-expressing oocytes and the day-matched H$_2$O-injected controls. As shown in Fig. 5, the $P_f$ difference between oocytes expressing AQP1, AQP1$_{\text{FLAG}}$, AQP8, and AQP9 and their day-matched H$_2$O controls were all significantly reduced, which is consistent with AQP1, AQP8 and AQP9 having a solvent-accessible mercury-sensitive Cys. The specificity of the pCMBS inhibitory effect was further confirmed with oocytes expressing AQP1C$_{189S}$ mutant (the mercury-insensitive AQP1) and AQP7 (that lacks a mercury-sensitive Cys). Although C189S mutant and AQP7-expressing oocytes had a $P_f$ equivalent to that of untreated oocytes, they were not inhibited by pCMBS (Figs 5 and 6). The results observed with *Lithobates* oocytes expressing AQP1C$_{189S}$ mutant or AQP7 are therefore in agreement with previous studies using *Xenopus* oocytes. Preston et al. (1993) showed that mutating Cys 189 to a Ser had no effect on the AQP1-dependent osmotic water permeability ($P_f$); however, this mutation does make AQP1 insensitive to mercury. Ishibashi et al. showed that AQP7 lacks a mercury-sensitive Cys in the throat of the aquapore, near the second NPA motif, and that it is resistant to mercury inhibition (Ishibashi et al., 1997b). On the other hand, and in contrast to previously published results, it was found that *Lithobates* oocytes expressing AQP3 are not mercury sensitive (Ishibashi et al., 1997a; Kuwahara et al., 1997).

Based on the location of the mercury-sensitive Cys in AQP3 on the intracellular surface of the protein and the properties of pCMBS, which does not permeate cell membranes (Vansteveninck et al., 1965), the mercury insensitivity is not completely unexpected. The first study investigating AQP3 mercury sensitivity was performed by (Kuwahara et al., 1997) and showed that AQP3 contains six Cys residues (at positions 11, 29, 40, 91, 174, and 267). This group showed that the mutation of each of the six Cys to a Ser revealed that only the cysteine at position 11 had resistance to mercury inhibition, which is localized on the intracellular side of AQP3. However, pCMBS is membrane impermeable (Vansteveninck et al., 1965). In fact, it has also been proposed that since mercury has to penetrate the cell membrane to reach its target, the inhibitory effect on AQP3 can be relatively slow (Zelenina et al., 2004). Therefore, inhibition would not be observed with AQP3, unless a significant amount of pCMBS crossed the membrane. It is also plausible that *Lithobates* oocyte membranes are less permeable to pCMBS, as compared to those of *Xenopus*.

CONCLUSION
We found that *Lithobates* oocytes are a useful alternative to *Xenopus* oocytes, when investigating AQP proteins. *Lithobates* oocytes can express native and FLAG-tagged AQPs at the cell surface and increase the $P_f$ of the oocytes. The augmentation of the $P_f$ can be relatively slow (Zelenina et al., 2004). Therefore, inhibition would not be observed with AQP3, unless a significant amount of pCMBS crossed the membrane. Future studies will further evaluate this heterologous expression system with other membrane proteins. Our findings are significant to any researcher that does not have unrestricted access to the *Xenopus* system.

MATERIALS AND METHODS
Expression in *Lithobates* oocytes
Table 2 contains a list of the expression vectors, restriction enzymes and promoters used in this study. The plasmids encoding for hAQP1, hAQP$_{\text{FLAG}}$, hAQP$_{1C_{189S}}$, rAQP3, hAQP7, hAQP8 and rAQP9 before and after treatment with pCMBS (1 mM for 30 min). Subtracting the $P_f$ value for day-matched H$_2$O-injected control oocytes from the $P_f$ of each AQP-expressing oocyte, before and after pCMBS treatment yields the channel-dependent osmotic water permeability ($P_f^*$). Treatment with pCMBS reduces the $P_f^*$ of hAQP1 ($P_f^*=0.01$, $t$-test), hAQP$_{1C_{189S}}$ ($P_f^*=0.03$, $t$-test), hAQP8 ($P_f^*=0.03$, $t$-test) and rAQP9 ($P_f^*=0.02$, $t$-test), but has no effect on $P_f^*$ of hAQP$_{C_{189S}}$ mutant ($P_f^*=0.85$, $t$-test), AQP3 ($P_f^*=0.10$, $t$-test), and AQP7 ($P_f^*=0.54$, $t$-test). A one-way ANOVA, followed by an SNK post hoc analysis to compare $P_f^*$ before and after treatment with pCMBS was also performed. Overall $P$ values: $P_f^*<0.05$ (AQP before pCMBS vs AQP after pCMBS).
hAQP1FLAG, hAQP1C189S mutant, rAQP3, hAQP7, hAQP8 or rAQP9 were transformed into TOP10 competent cells. All of the plasmids were sequenced using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer’s recommendations with some modifications (Geyer et al., 2013a). Prior to surface tagging, the phosphate-buffered saline (PBS, 137 nmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2.0 mmol/L KH2PO4, pH 7.4) was adjusted to pH 7.0 using NaOH or HCl. The OR3 culture medium contained 6.85 g/L of Leibovitz L-15 cell culture medium (L4386-1L, Sigma-Aldrich), supplemented with 10,000 U/ml penicillin G sodium, 10,000 mg/ml streptomycin sulfate (15140-122, Gibco, Gaithersburg, USA), and 5 mM HEPES (pH adjusted to 7.50 with NaOH). The osmolarity of all solutions was adjusted to between 195 and 200 mOsM (milliosmoles/L, the unit of osmotic concentration) with NaCl or water. For osmotic water permeability (Pf) assays, a hypotonic ND96 solution variant (70 mOsM) was prepared by diluting the standard ND96 solution with water. For the inhibitory osmotic water permeability (Pf) studies, the mercurial agent, p-chloromercuribenzene sulfonate (pCMBS) (C367750, Thermo Fisher Scientific) was used.

**Biolginylation**

The surface protein expression was determined using the EZ-Link Sulfo-NHS-Biotinylation kit (89881, Thermo Fisher Scientific) according to the manufacturer’s recommendations with some modifications (Geyer et al., 2013a). Prior to surface tagging, the phosphate-buffered saline (PBS, 1890535, Thermo Fisher Scientific) and Tris-buffered saline (TBS, 28376, Thermo Fisher Scientific) were diluted to reduce the osmolality of the solutions from 300 mOsm to 200 mOsm, thus matching that of the other oocyte solutions, and ensuring that the oocytes would not be exposed to a hyper-osmotic condition. Briefly, groups of ~20 oocytes – expressing hAQP1, hAQP1FLAG, hAQP1C189S mutant, rAQP3, hAQP7, hAQP8 or rAQP9 or an equivalent volume of sterile water (called ‘H2O-injected control oocytes’). Sterile injection needles were made using a Model P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, USA), as previously described (Musa-Aziz et al., 2010), and aseptically cut to have a diameter of approximately 2 μm. The needles were attached to a Nanoliter 2000 volume microinjector (World Precision Instruments, Sarasota, USA), filled with mineral oil and then filled with cRNA. Oocytes were injected with 25 nl cRNA or sterile water and stored at 18°C in sterile OR3 medium until used for injection.

**Microinjection of cRNAs**

One day after isolation, oocytes were injected with 25 nl cRNA (25 ng (given as 25 nl of a 1 ng/ml cRNA solution)) encoding for hAQP1, hAQP1FLAG, hAQP1C189S mutant, rAQP3, hAQP7, hAQP8 or rAQP9 or an equivalent volume of sterile water (called ‘H2O-injected control oocytes’). Sterile injection needles were made using a Model P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, USA), as previously described (Musa-Aziz et al., 2010), and aseptically cut to have a diameter of approximately 2 μm. The needles were attached to a Nanoliter 2000 volume microinjector (World Precision Instruments, Sarasota, USA), filled with mineral oil and then filled with cRNA. Oocytes were injected with 25 nl cRNA or sterile water and stored at 18°C in OR3 medium. Expression and function were evaluated 3–5 days following injection.

**Solutions**

The blood parameters indicated that no modifications of typical *Xenopus* solutions needed to be made. The ND96 control solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES (pH adjusted to 7.50 using NaOH or HCl). The modified zero calcium ND-96 solution (0 Ca2+ ND-96 solution) was made by replacing CaCl2 with NaCl (pH adjusted to 7.50 with NaOH or HCl). The OR3 culture medium contained 6.85 g/L of Leibovitz L-15 cell culture medium (L4386-1L, Sigma-Aldrich), supplemented with 10,000 U/ml penicillin G sodium, 10,000 mg/ml streptomycin sulfate (15140-122, Gibco, Gaithersburg, USA), and 5 mM HEPES (pH adjusted to 7.50 with NaOH). The osmolarity of all solutions was adjusted to between 195 and 200 mOsM (milliosmoles/L, the unit of osmotic concentration) with NaCl or water. For osmotic water permeability (Pf) assays, a hypotonic ND96 solution variant (70 mOsM) was prepared by diluting the standard ND96 solution with water. For the inhibitory osmotic water permeability (Pf) studies, the mercurial agent, p-chloromercuribenzenesulfonate (pCMBS) (C367750, Toronto 196 Research Chemicals, North York, Canada), was dissolved in ND96 control solution at a concentration of 1 mM (pH 7.50) immediately before the experiments (Geyer et al., 2013b).

**Surface expression measurements**

The surface protein expression was determined using the EZ-Link Sulfo-NHS-Biotinylation kit (89881, Thermo Fisher Scientific) according to the manufacturer’s recommendations with some modifications (Geyer et al., 2013a). Prior to surface tagging, the phosphate-buffered saline (PBS, 137 nmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2.0 mmol/L KH2PO4, pH 7.4) was adjusted to pH 7.0 using NaOH or HCl. The OR3 culture medium contained 6.85 g/L of Leibovitz L-15 cell culture medium (L4386-1L, Sigma-Aldrich), supplemented with 10,000 U/ml penicillin G sodium, 10,000 mg/ml streptomycin sulfate (15140-122, Gibco, Gaithersburg, USA), and 5 mM HEPES (pH adjusted to 7.50 with NaOH). The osmolarity of all solutions was adjusted to between 195 and 200 mOsM (milliosmoles/L, the unit of osmotic concentration) with NaCl or water. For osmotic water permeability (Pf) assays, a hypotonic ND96 solution variant (70 mOsM) was prepared by diluting the standard ND96 solution with water. For the inhibitory osmotic water permeability (Pf) studies, the mercurial agent, p-chloromercuribenzenesulfonate (pCMBS) (C367750, Toronto 196 Research Chemicals, North York, Canada), was dissolved in ND96 control solution at a concentration of 1 mM (pH 7.50) immediately before the experiments (Geyer et al., 2013b).
Spun X column (8163, Corning, Pittston, USA) containing 180 µl NeutAvidin Gel (Thermo Fisher Scientific). The samples were mixed on a rocker platform for 1 h at room temperature and washed three times with lysis buffer. Finally, 180 µl of the 1 × sample buffer containing 50 mM DTT was applied to the NeutAvidin Gel-biotin labeled protein mixture, and continuously mixed on a rocker platform for 1 h at room temperature. Samples were collected by centrifugation at 1000×g for 1 min. This final eluted sample represents the surface fraction.

**Western blot analysis**

The total and surface biotinylated samples from oocytes AQP injected with cRNA encoding for AQP5s or H2O-injected controls, were separated by SDS-PAGE on 12% Tris-glycine gels. The samples were transferred to PVDF membranes and incubated in TBST with 5% of milk for 1 h at room temperature. The membranes were probed overnight at 4°C with polyclonal anti-AQP1 (AQP11-A, Alpha Diagnostics, San Antonio, USA), anti-AQP3 (AQP31-A, Alpha Diagnostics), hAQP7 (AQP71-A, Alpha Diagnostics), anti-hAQP8 (AQP81-A, Alpha Diagnostics), anti-hAQP9 (AQP91-A, Alpha Diagnostics) or monoclonal anti-FLAG (F3165-2MG, Sigma Aldrich) antibodies. The protein expression was detected using ECL plus Western Blotting Detection Reagents (32132, Thermo Fisher Scientific).

**Physiological measurements**

Measurement of osmotic water permeability of oocytes, $P_f$

We determined $P_f$ by using a volumetric assay (Preston et al., 1993; Virkki et al., 2002). Briefly, we placed a group of up to six oocytes into a Petri dish containing the aforementioned 70 mM N96D variant solution and a metallic sphere, set next to the oocytes served as a size reference. Osmotic swelling was monitored with a Nikon stereoscopic microscope (SMZ745T) equipped with a digital camera (Optix Cam, Roanake, USA) connected to a computer to monitor the projection area of each oocyte over time. Video images were acquired every 10 s for 5 min. The change in the projected area over time was used to calculate the osmotic water permeability ($P_f$, cm/s) of the oocytes (Preston et al., 1993). For the inhibition experiments, the oocytes were preincubated in N96D plus 1.0 mM of the mercurial agent pCMBS (195 mM, pH 7.50), which is known to reduce the $P_f$ of AQP1 (Preston et al., 1993), for 30 min and, then, exposed to the hypotonic N96D variant solution (70 mM, pH 7.50).

In order to compute $P_f$, we assumed the oocyte to be a sphere with a true surface area (S) eightfold greater than that of the idealized sphere (Chandy et al., 1997), according to the equation:

$$P_f = \frac{V_o \cdot d(V_o)/dt}{S \cdot \Delta Osm \cdot V_o},$$

where $V_o$ is the initial oocyte volume, $d(V_o)/dt$ is the maximal fractional rate of volume increase, $\Delta Osm$ is the osmotic gradient across the plasma membrane (195 mM N96D - 70 mM N96D(out) = 125 mM(N96D)), and $V_o$ is the molar volume of water (18 cm³/mol) (Preston et al., 1992).

**Statistics**

All data are presented as mean±s.e., with numbers of oocytes in parentheses. When comparing the difference between two means, Student’s t-tests (two tailed) were performed. When comparing the difference among more than two means, one-way ANOVA was followed by a Student–Newman–Keuls (SNK) post hoc analysis was performed using KaleidaGraph (version 4; Synergy Software, Reading, USA). $P<0.05$ was considered significant.

**Author contributions**

Conceptualization: R.R.G., R.M.-A.; Methodology: J.K., O.B.-S., R.R.G., R.M.-A.; Formal analysis: R.R.G., R.M.-A.; Investigation: J.K., R.R.G., R.M.-A.; Writing - original draft: J.K., R.R.G., R.M.-A.; Writing - review & editing: J.K., R.R.G., R.M.-A.; Supervision: R.R.G., R.M.-A.; Project administration: R.R.G., R.M.-A.; Funding acquisition: R.M.-A.

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