Regulation of AQP0 water permeability is enhanced by cooperativity

Karin L. Németh-Cahalan, Daniel M. Clemens, and James E. Hall

Department of Physiology and Biophysics, University of California, Irvine, Irvine, CA 92697

Aquaporin 0 (AQP0), essential for lens clarity, is a tetrameric protein composed of four identical monomers, each of which has its own water pore. The water permeability of AQP0 expressed in *Xenopus laevis* oocytes can be approximately doubled by changes in calcium concentration or pH. Although each monomer pore functions as a water channel, under certain conditions the pores act cooperatively. In other words, the tetramer is the functional unit. In this paper, we show that changes in external pH and calcium can induce an increase in water permeability that exhibits either a positive cooperativity switch-like increase in water permeability or an increase in water permeability in which each monomer acts independently and additively. Because the concentrations of calcium and hydrogen ions increase toward the center of the lens, a concentration signal could trigger a regulatory change in AQP0 water permeability. It thus seems plausible that the cooperative modes of water permeability regulation by AQP0 tetramers mediated by decreased pH and elevated calcium are the physiologically important ones in the living lens.

INTRODUCTION

Aquaporins (AQPs) are a family of proteins that promote the diffusion of water through cell membranes (Chrispeels and Agre, 1994). They are found in native membranes as tetramers, and each monomer has its own water pore (Murata et al., 2000). Two AQPs, AQP0 and AQP1, are expressed in the lens where they play a critical role in maintaining lens transparency. AQP1 is expressed in many tissues and in lens epithelial cells, whereas AQP0 is found only in lens fiber cells. Recently, another AQP, AQP5, was found in the lens epithelial cells and fiber cells, but its role in the lens has not yet been elucidated (Kumari et al., 2012).

In previous studies, we showed that water permeability of AQP0 but not AQP1 is modulated by changes in pH and calcium (Németh-Cahalan and Hall, 2000, 2004). Although the molecular mechanisms of regulation by pH and calcium remain unknown, we do know quite a lot about the structure of AQP0 and have identified parts of the molecule involved in both calcium and pH regulation of water permeability. Fig. 1 illustrates AQP0 water channel structure and highlights the two key sites of modulation by pH and calcium ions at H40 and S235, respectively. Even though each AQP monomer has its own pore, water gating can be cooperative. We previously showed that water permeability of AQP0 is strongly modulated by zinc, with a concentration dependence that was best fitted using a Hill equation with a coefficient of four (Németh-Cahalan et al., 2007). An important consequence of this strong apparent cooperativity is that the zinc concentration dependence of the water permeability is so steep that it acts virtually as a concentration-dependent switch, so that the water permeability of the tetramer doubles over a very narrow range of zinc concentration. This observation resonates with earlier observations suggesting that monomers of AQPs interact with one another (Mathai and Agre, 1999) and sets the stage for the present work.

The fundamental questions we deal with in this paper are: Can protons and calcium regulate the water permeability of AQP0 in a cooperative fashion? Can protons and calcium act on single monomers in a noncooperative fashion, and if either of these two modes of ion concentration gating occurs, could it play a role in the physiology and development of the lens? By coinjection of WT AQP0 with an insensitive mutant for pH or calcium, we investigated a possible cooperativity between monomers that may be physiologically relevant.

MATERIALS AND METHODS

cRNA preparation for *Xenopus laevis* oocytes

The expression construct for bovine AQP0 (pXβG-AQP0) was provided by P. Agre (Johns Hopkins University, Baltimore, MD). WT and mutated AQP0 cRNAs were transcribed in vitro using the T3 RNA polymerase (mMACHINE kit; Ambion). The mutations used in this study were prepared using standard primer methods and have been described previously (Németh-Cahalan and Hall, 2000, 2004, 2007).

Oocyte swelling assay and measurement of water permeability

Oocytes were obtained in two ways. Female *Xenopus* were anesthetized, and stage V and VI oocytes were prepared and injected with 10 ng cRNA as described previously (Németh-Cahalan et al., 2007).

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In some experiments, oocytes were obtained from EcoCyte Bioscience. The day after isolation, oocytes were injected with the appropriate AQP cRNA and maintained in ND96 (mM: 96 NaCl, 2 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, and 5 HEPES, pH 7.5) at 18°C.

2 d after injection, oocyte swelling assays were performed at room temperature (20–21°C) by transfer from 200-mOsM (100% ND96) to 70-mOsm (30% ND96) solution. Water permeability, P$_f$, was calculated from optical measurements of the increase in cross-sectional area of the oocyte with time in response to diluted ND96 using the formula:

$$P_f = \left[\frac{d(V / V_0)}{dt} \right](V_0 \ / \ S) \ / \ (\Delta_{osm}V_w),$$

where V is the volume as a function of time, V$_0$ is the initial volume, S is the geometric surface area, Δ_{osm} is the osmotic gradient, and V$_w$ is the molar volume of water.

The swelling assay is performed either under control conditions of pH 7.5 and 1.8 mM Ca^{2+}, or experimental conditions of altered pH or Ca^{2+}. Each data point is the average of measurements from at least nine oocytes from three different batches. Error bars are shown as ±SEM.

Controls

Under standard conditions, uninjected oocytes had an average water permeability of 9.4 ± 0.5 µm/s ($n = 51$) and showed no change in water permeability under any experimental challenge including changes in calcium or pH.

Fraction of increase and fraction of insensitive monomer

In Figs. 2–5, we plot the fraction of increase versus the fraction of insensitive monomer. The fraction of increase is defined as:

$$FI = \frac{\left[P_f^{test} - P_f^{uninjected} \right]}{\left[P_f^{control} - P_f^{uninjected} \right]},$$

where P_f^{test} is the water permeability under the test conditions, $P_f^{uninjected}$ is the water permeability of uninjected oocytes, and $P_f^{control}$ is the water permeability in solutions of ND96 with 1.8 mM Ca^{2+} and pH 7.4. The error bars of the fraction of increase were calculated using standard propagation of error methods. Reduced χ^2, χ^2, and R^2 for testing goodness of fits were calculated using Origin Software (OriginLab).

Experimental pH solutions

For each experimental pH value, 100 and 30% ND96 solutions were made using HEPES for pH 8.0 to 7.0 and MES for pH 6.5 to 6.0. Before the swelling assay in 30% ND96 at the experimental pH, the oocytes were soaked in 100% ND96 at the experimental pH for 5 min. To evaluate only the effect of pH, without the calcium effect, the calcium concentration is adjusted to 1.8 mM in both normal ND96 and in the 30% ND96.

Experimental calcium solutions

For each experimental Ca^{2+} concentration, 100 and 30% ND96 solutions were made as follows: 1 mM EGTA and 1.8 or 5 mM Ca^{2+}. Before the swelling assay was performed in 30% ND96 at the experimental Ca^{2+} concentration, the oocytes were soaked in 100% ND96 at the experimental Ca^{2+} concentration for 5 min.

Hill coefficient curve fitting

We used the statistical package in Origin Software (OriginLab) to fit our data to the modified Hill equation,

$$FI = \Delta FI_{max}\left[\frac{X^n}{\left(X^n + K^n \right)} \right] + 0.9,$$

where FI is the fraction of increase, ΔFI_{Max} is the increment in fraction of increase induced by pH or calcium, x is the concentration of the ion in question, n is the Hill coefficient, K is the concentration of ion at which half of the maximum effect occurs, and 0.9 is the offset of the fraction of increase.

RESULTS

The pH titration curve

pH titration of AQP0 water permeability (Németh-Cahalan and Hall, 2000) indicates a range of water permeability modulation in which water permeability approximately doubles between pH 7.5 and pH 6.5, a 10-fold increase in proton concentration. Fitting the data of Németh-Cahalan and Hall (2000) to the Hill equation indicates a strongly cooperative effect of pH in increasing the water permeability (Fig. 2). The solid black
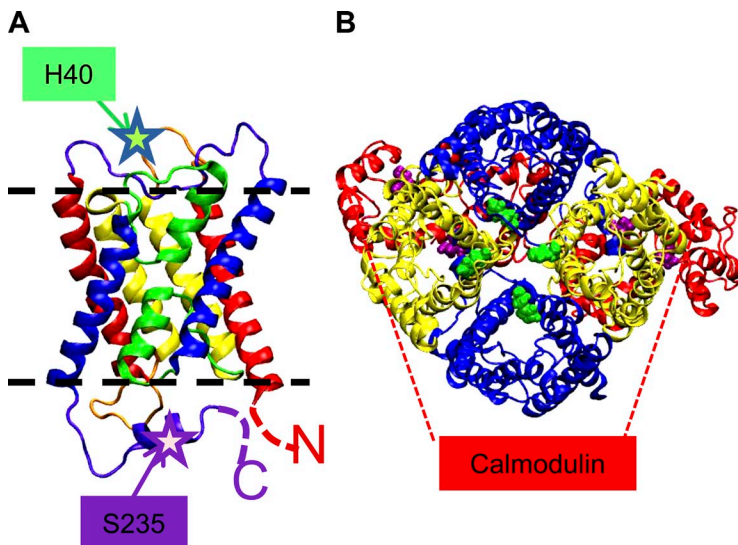

Figure 1. Topology and structure of AQP0. (A) Each AQP0 monomer is composed of six transmembrane domains (1–6) and five loops (A–E) in rainbow color (ROYGIBV). The pore is formed by two half helices and loops B and E (in green) folded back into the membrane. Major players in the cooperativity are H40 in the external loop A and S235 in the C terminus tail. (B) The tetramer is shown from the extracelluar side with the four monomers (blue and mustard) and the two CaMs (red). Both panels were generated with VMD from a molecular dynamics simulation based on crystal structures (deposited in the Protein Data Bank under accession no. 1TM8 for AQP0; Harries et al., 2004; and Protein Data Bank accession no. 1NWD for CaM; Yap et al., 2003) originally modeled together in Reichow and Gonen (2008).

line shows a fit with the Hill coefficient constrained at 4.0, a value consistent with there being four monomers in the tetramer and with the previous observation that the Hill coefficient for titration with zinc is very close to 4 (Németh-Cahalan et al., 2007).

Evaluation of pH cooperativity

We showed earlier that histidine 40 is required for doubling AQP0 water permeability upon acidification to pH 6.5 (Németh-Cahalan and Hall, 2000, 2004). Fig. 3 A shows that WT AQP0 increases its water permeability in response to acid pH, but the mutant H40C loses acid pH sensitivity while increasing its water permeability in response to alkaline pH. To evaluate cooperative interactions, we evaluated the pH sensitivity of mixtures of WT and H40C at varying ratios of expression. A 1:1 mixture of WT and the H40C mutant shows almost no increase in water permeability in response to acid pH but retains a small amount of response to alkaline pH. A 5:1 mixture of WT to mutant shows that the response to acid pH is retained, as is the response to alkaline pH. These data therefore suggest that the dependence of water permeability on pH exhibits both cooperative and noncooperative responses. This is evident from the strong suppression of acid pH sensitivity and intermediate effect of alkaline sensitivity when equal amounts of WT and H40C mutant are mixed. The sensitivity to alkaline pH is directly proportional to the amount of alkaline-sensitive H40C mutant, whereas the sensitivity to acid pH is almost completely eliminated. A reasonable explanation is that a small proportion of acid pH–insensitive monomers in a tetramer could completely shut down the response of the other monomers in the tetramer. In other words, it seems that H40C mutants may respond independently to alkaline pH, but WT AQP0 tetramers respond cooperatively to acid pH.

Fig. 3 B evaluates the hypothesis that acid pH modulation is cooperative whereas alkaline pH modulation is
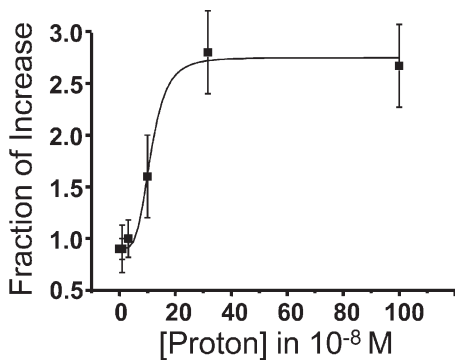

Figure 2. pH dose–response curve of the water permeability of AQP0. The solid black line is a fit to the Hill equation, with $n = 4$ ($\chi^2/\text{DOF} = 0.0068$ and $R^2 = 0.994$).
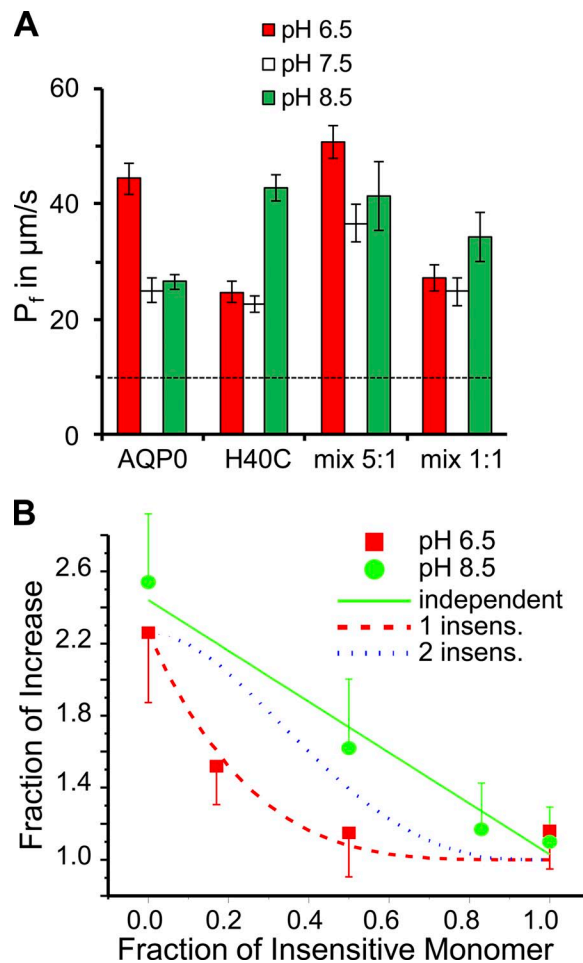

Figure 3. pH cooperativity. (A) Effect of acid and alkaline pH on the water permeability of mixtures of AQP0 (WT) and the H40C mutant. WT responds to acid pH, whereas the H40C mutant responds to alkaline pH. Mix 5:1 represents 10 ng AQP0 and 2 ng of mutant H40C (fraction of insensitive monomer = $2/12 = 0.166$), and mix 1:1 represents 10 ng AQP0 and 10 ng of mutant H40C (fraction of insensitive monomer = $10/20 = 0.5$). The P_f of the mix 1:1 at pH 8.5 is greater than the P_f at pH 7.5, with a p-value of 5×10^{-5} (Student's t test). The horizontal dotted line represents the water permeability of uninjected oocytes. (B) Fraction of increase plotted against the fraction of insensitive monomer. (For acid pH, the H40C mutant is the insensitive monomer. For alkaline pH, WT is the insensitive monomer.) Experimental results for acid pH are plotted as red squares and are well fit by a curve calculated from the binomial distribution of monomers randomly partitioning into each tetramer and the assumption that a single insensitive monomer renders the whole tetramer insensitive to acid pH (dashed red line; $\chi^2 = 0.840$). The curve assuming that two insensitive monomers are required to render the tetramer insensitive to acid pH (dashed black line; $\chi^2 = 8.88$) does not fit the experimental data. Experimental results for alkaline pH are plotted as green circles and are well fit by a straight line (the theoretical prediction assuming each monomer acts independently of the others in the tetramer; $\chi^2/\text{DOF} = 0.071$ and $R^2 = 0.910$; solid green line). Each data point is the average of experiments using nine oocytes from three different batches.

additive by plotting the fractional increase in water permeability induced by either alkaline pH or acid pH as a function of the fraction of insensitive monomer cRNA injected. The response to alkaline pH (when AQP0 WT is the insensitive monomer) shows no cooperativity, and the fraction of increase is simply proportional to the fraction of insensitive monomer (Fig. 3 B, solid green line and green circles). The response to acid pH is, in contrast, very cooperative. The red squares show the fraction of increase induced by acid pH plotted against the fraction of insensitive monomer, which in this case is the H40C mutant. The data are well fit by the dashed red curve, which was calculated using the binomial theorem under the assumptions that insertion of a given type of monomer into a tetramer is random and that a single insensitive monomer in a tetramer is sufficient to render all the monomers in the tetramer insensitive to acid pH. The results of Fig. 3 B show that acid pH response of WT AQP0 is highly cooperative but that the response of the mutant H40C to alkaline pH is not. Interestingly, acid pH in the lens interior is more physiologically relevant than alkaline pH.

The cooperativity of zinc-induced water permeability increase (Németh-Cahalan et al., 2007) may well arise from a cooperative binding of zinc to a site composed of histidines contributed by adjacent monomers while the independent (basic pH) response requires only a single histidine. We tested this hypothesis by investigating the cooperativity of the increase in water permeability induced by alkaline pH in an AQP1 mutant, D48H, which has alkaline pH sensitivity (Németh-Cahalan et al., 2004). We used WT AQP1 as the insensitive monomer and varied its proportion to the alkaline pH–sensitive D48H mutant. Fig. 4 A shows the effects of coinjecting WT AQP1 and mutant D48H cRNAs on acid and alkaline responses. When a mixture of one part WT to one part D48H is used, there is still a small response to alkaline pH but no response to acid pH. Fig. 4 B shows a plot of the factor of increase in AQP1 mixtures as a function of insensitive monomer, in this case WT AQP1. The fraction of increase in water permeability is inversely proportional to the fraction of insensitive monomer, in this case the WT, and is not cooperative at all.

Evaluation of calcium cooperativity

We showed previously that varying calcium concentration can increase the water permeability of AQP0 in different ways depending on the state of its phosphorylation (Kalman et al., 2008). When AQP0 is not phosphorylated at serine 235, low calcium (achieved either by adding no calcium to the solution or adding 1 mM EGTA to a solution that contains no calcium) increases the water permeability of AQP0. In contrast, when AQP0 is pseudo-phosphorylated at serine 235 (the mutant S235D), increasing the calcium concentration to 5 mM increases the water permeability, whereas lowering calcium

has no effect. Increased calcium concentration increases water permeability in lens vesicles (Varadaraj et al., 2005), consistent with our observation that increased calcium increases water permeability of the S235D mutant in oocytes. We investigated whether the low calcium-induced increase in water permeability in unphosphorylated AQP0 or the high calcium-induced water permeability increase induced by elevated calcium in phosphorylated AQP0 was cooperative by again coinjecting WT and mutant cRNAs in different proportions.
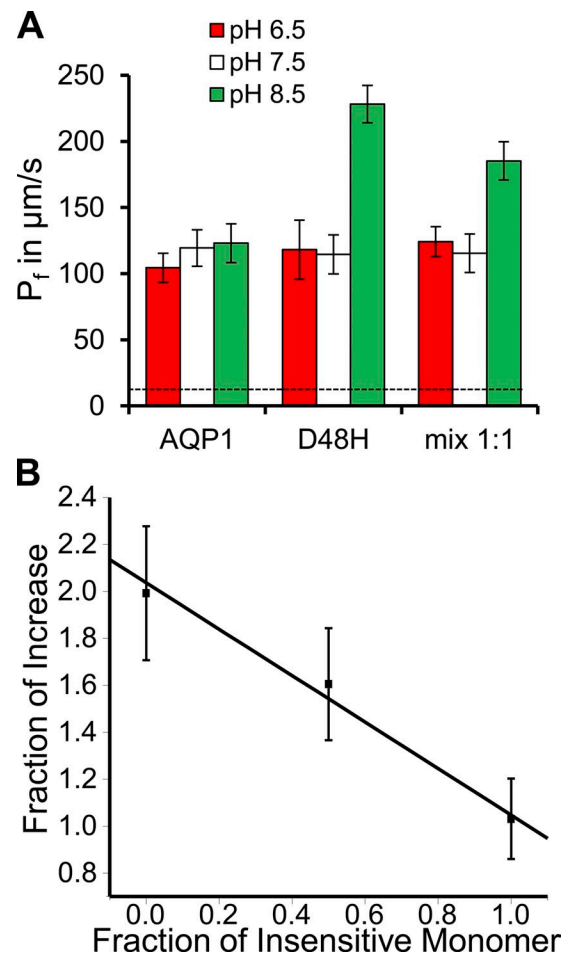

Figure 4. Alkaline pH increase of the P_f of AQP1 D48H is not cooperative. (A) Effect of acid and alkaline pH on the water permeability of AQP1 (WT) and a mutant D48H. WT does not respond to either acid or alkaline pH, whereas the D48H mutant responds to alkaline pH. Mix 1:1 represents 5 ng AQP1 and 5 ng of mutant D48H (fraction of insensitive monomer = $5/10 = 0.5$). The horizontal dotted line represents the water permeability of uninjected oocytes. (B) Fraction of increase plotted against the fraction of insensitive monomer. For alkaline pH, WT is the insensitive monomer. The fraction of increase in P_f induced by alkaline pH is proportional to the fraction of insensitive monomer, indicating a lack of cooperativity between insensitive (WT) and sensitive (D48H) monomers. The data are well fit by a straight black line ($R^2 = 0.99$) indicating that each monomer in a tetramer acts independently of the others.

Fig. 5 A shows the effects of coinjecting WT AQP0 and mutant S235D cRNAs on both the low calcium and the high calcium responses. Note that AQP0 responds to low calcium, but the mutant S235D responds to high calcium (5 mM). When a mixture of one part WT to five parts S235D is used, there is still a small response to low calcium but almost no response to 5 mM calcium. If a mixture of five parts of WT to one part of S235D is used, the low calcium response is still quite large, but the 5-mM calcium response is nearly absent. The data in Fig. 5 A thus suggest that the increase of water permeability induced by 5 mM calcium in the S235D mutant (representing phosphorylated AQP0) is cooperative, whereas the increase induced by low calcium in WT AQP0 is not.

This suggestion is further evaluated in Fig. 5 B, which plots the response to changes in calcium concentration as the fractional increase versus the fraction of the insensitive monomer cRNA injected. Note that for the low calcium response, the insensitive monomer is the S235D mutant and that for the 5-mM calcium response, the insensitive monomer is the WT. Consider the low calcium response (blue squares). The left-hand part of the graph shows that low calcium increases the permeability of pure

WT (fraction of insensitive monomer is zero) by a factor of ~1.9 ± 0.4. When the fraction of insensitive monomer (S235D) is one, low calcium induces no increase of water permeability. Intermediate proportions of insensitive monomer produce fractions of increase that lie along a straight line, indicating that each monomer contributes to the water permeability just as it would if there were no interaction among monomers in a tetramer when calcium is reduced.

The situation is very different for the response to elevated (5 mM) calcium (purple triangles). In this case, at the left side of Fig. 5 B, the fraction of increase is ~2.3 ± 0.5 when the calcium concentration is raised to 5 mM for pure S235D. On the right-hand side, when the fraction of insensitive monomer is one (pure WT), there is essentially no response to 5 mM calcium. But in this case, a very small fraction of WT (even less than 0.2) can almost completely suppress the increase in water permeability. The data lie well below even the one insensitive monomer–required curve. This could be explained if WT monomers associated more strongly with the pseudo-phosphorylated mutants than with other WT monomers (Ding et al., 2005). The dotted purple curve shows the predicted fraction of increase assuming that only one
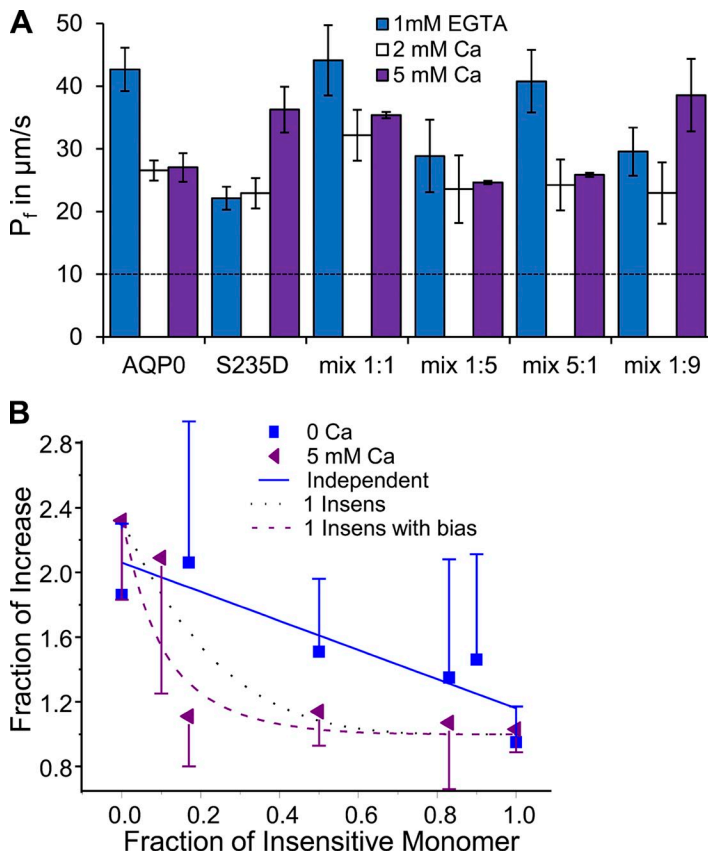

Figure 5. Calcium cooperativity. (A) Effect of calcium concentration on the water permeability of mixed injection of AQP0 (WT) and a mutant S235D. Mix 1:1 represents 10 ng AQP0 and 10 ng of mutant H40C (fraction of insensitive monomer = 10/20 = 0.5), mix 1:5 represents 2 ng AQP0 and 10 ng of mutant H40C (fraction of insensitive monomer = 2/12 = 0.17), mix 5:1 = 10 ng AQP0 and 2 ng of mutant H40C (fraction of insensitive monomer = 2/12 = 0.17), and mix 1:9 represents 1 ng AQP0 and 9 ng S235D. The left-hand panel shows the response of WT AQP0. Note that P_f increases when the calcium concentration is decreased. In contrast, an increased calcium concentration increases P_f in the S235D mutant. When the two are mixed so as to form hetero tetramers, even 20% of WT is sufficient to suppress completely the P_f increase induced by 5 mM calcium, whereas the proportional increase in P_f induced by lowering calcium remains. When the mixture contains 80% WT, the low calcium response is still present in proportion to the amount of WT, but the elevated calcium response is completely suppressed. The horizontal dotted line represents the water permeability of uninjected oocytes. (B) Fraction of increase plotted against the fraction of insensitive monomer. (For no Ca^{2+} response, S235D is the insensitive monomer. For 5-mM Ca^{2+} response, WT is the insensitive monomer.) Experimental results for 5-mM Ca^{2+} increase are plotted as purple triangles and are well fit by a curve calculated from the binomial distribution assuming one insensitive monomer is sufficient to render the whole tetramer insensitive and with a bias of 0.5 kT for WT to associate with mutants in a tetramer (Ding et al., 2005) (dashed purple curve; $\chi^2 = 1.260$). The dotted black curve is calculated assuming no bias and that one insensitive monomer is sufficient to render the entire tetramer insensitive to Ca^{2+} increase ($\chi^2 = 3.133$). Experimental data for zero calcium are plotted as blue squares and are well fit by a straight line (the theoretical prediction assuming each monomer acts independently of the others in the tetramer; $\chi^2/DOF = 0.18774$ and $R^2 = 0.557$; blue straight line). Positive and negative errors are equal, but error bars are shown in only one direction to avoid clutter.

insensitive monomer in a tetramer is required to eliminate sensitivity to 5 mM calcium, and that S235D monomers bind more strongly to WT monomers than to themselves with an energy difference of ~ 0.5 kT.

In summary, the response to elevated calcium exhibits a strong dependence on the ratio of WT to insensitive mutant, consistent with cooperativity; but the response to low calcium is additive with respect to the ratio of WT to insensitive mutant. As with acid pH sensitivity, the condition of elevated calcium is cooperative and more likely to be encountered by cells in the lens interior.

DISCUSSION

In this paper, we report the surprising finding that both pH and Ca^{2+} modulation of AQP0 water permeability can be either cooperative or noncooperative. But more than that, the cooperative modes of both pH change and Ca^{2+} change are the best candidates for physiologically relevant modes of water permeability control, whereas the noncooperative concentration regimes are much less likely to be of physiological relevance. pH becomes more acidic by nearly one pH unit toward the center of the lens, and calcium concentration also increases toward the center of the lens (Bassnett and Duncan, 1985; Mathias et al., 1991). The calcium concentrations we report are extracellular, but we know that calcium acts intracellularly (Németh-Cahalan et al., 2004), and there is an unknown, but essential, relationship between the external calcium concentration that we can easily control and the internal calcium concentration. Nevertheless, it is clear that variations in calcium concentration with distance into the lens do occur, and the range of reported variation encompasses the range of extracellular calcium concentrations in our experiments (Duncan and Jacob, 1984). We suggest that increases in the concentration of both ions are reasonable candidates for cooperative regulation of water permeability, a more sensitive and physiologically relevant mode of water permeability regulation.

There is precedence for the view that water channel AQP monomers interact with and influence the water permeability of their neighbors in the tetramer. Mathai and Agre (1999) found that although linked dimers of AQP1 formed functional water channels, a linked dimer of AQP1 and AQP1-A73M, an AQP1 mutant with no water permeability, lacked even the water permeability of the functional AQP1. This finding supports the view that individual monomers of AQPs interact and, even more importantly, that cooperation between the monomers in a tetramer might be essential for water permeation. The case for this conjecture was boosted by a different experimental approach showing that mutating a single amino acid could convert an AQP transporting only water to a glyceroporin transporting glycerol

and simultaneously reduce the form of the protein in the membrane from a tetramer to a dimer (Lagree et al., 1998).

pH
The data presented here clearly establish that WT AQP0 shows two types of behavior in response to changes in pH from the normal pH value of ~ 7.5 at the surface of the lens. The response to acidification is cooperative as shown by both direct titration (Fig. 2) and by mixture experiments in which a single acid-insensitive monomer renders the entire tetramer insensitive to acid pH (Fig. 3). The titration curve establishes that the increase in water permeability of AQP0 depends on about the fourth power of the proton concentration. This implies that four protons must bind to allow the increase of water permeability to take place. Our data do not allow us to determine whether or not this binding is itself cooperative, but in any case, the water permeability increases in a switch-like fashion as pH falls from 7.5 to 6.5, exactly the range of pH change from the surface of the lens to its center. The substitution experiments with varying ratios of insensitive monomers to sensitive ones establish that all the monomers in the tetramer must participate to facilitate whatever change occurs in each individual monomer's water pore, implying that the switch-like behavior of the titration curve is related to cooperativity of the gating of the individual monomer pores. On the other hand, the increase in permeability induced by alkaline pH is not cooperative, and each monomer in the tetramer can apparently act independently in increasing its water permeability regardless of what the other monomers in the tetramer do. Recall that the alkaline increase occurs not in a naturally occurring form of AQP0 but in a mutant constructed in the laboratory. In this case, there is no plausible reason for the alkaline response to be physiological. This observation of cooperative and independent changes in water permeability induced by pH suggests that the mechanisms for increasing water permeability in the two cases differ.

We speculate that the increase in water permeability induced by alkaline pH is not physiological and probably arises because of the introduction of a single histidine rather than the pair of histidines present in AQP0. The cooperativity of zinc-induced water permeability increase may well arise from the binding of zinc to a site composed of histidines contributed by adjacent monomers, whereas the independent (basic pH) response requires only a single histidine. We tested this hypothesis by investigating the cooperativity of the increase in water permeability induced by alkaline pH in an AQP1 mutant, D48H, which has alkaline pH sensitivity (Németh-Cahalan et al., 2004). We used WT AQP1 as the insensitive monomer and varied its proportion to the alkaline pH–sensitive D48H mutant. Fig. 4 B shows a plot of the factor of increase in AQP1 mixtures as a function of insensitive

monomer, in this case WT AQP1. The fraction of increase in water permeability is proportional to the fraction of insensitive monomer, in this case the WT. The alkaline pH–induced increase in water permeability conveyed by the D48H mutant is clearly not natural, and the water permeability of WT AQP1 does not depend on pH at all. The specialized cooperativity-based mechanisms of water permeability modulation found in AQP0 are not present and cannot be induced by the introduction of a single histidine. Indeed, the situation here parallels that of the H40C AQP0 mutant, which also exhibits an increase in water permeability with alkaline pH and no cooperativity. We propose that the cooperative mechanisms of both pH- and calcium-induced increase in water permeability of AQP0 depend on allosteric changes of the entire tetramer, which affect the permeability properties of all monomers simultaneously.

Calcium

Depending on the phosphorylation state of AQP0, water permeability can be modulated by either an increase in calcium concentration or a decrease in calcium concentration. Decreased calcium increases water permeability of unmodified AQP0, but increased calcium increases the water permeability of the mutant S235D (to mimic the effects of phosphorylation). Varadaraj et al. (2005) have shown that increasing calcium concentration (or decreasing pH) increases the water permeability of vesicles formed from lens fiber cells. These results were at odds with our observation that eliminating calcium increased water permeability of AQP0 expressed in oocytes. Our subsequent observations showing that increased calcium increases the water permeability in the S235D mutant (which is expected to behave exactly as in vitro phosphorylated AQP0) resolved this discrepancy (Kalman et al., 2008), and the results of Varadaraj et al. (2005) thus support both our pH and calcium results and are consistent with reports that a substantial portion of AQP0 is phosphorylated in vivo (Lampe et al., 1986; Schey et al., 1997; Ball et al., 2004). Varadaraj et al. (2005) suggest that both the acid-induced water permeability increase and the elevated calcium–induced water permeability increase are likely to be physiologically relevant responses, and the current work supports this view.

Fig. 5 shows that the low calcium–induced water permeability increase mediated by unmodified AQP0 is not cooperative: each monomer in the tetramer acts independently. Thus, we do not expect a highly sensitive response to changes in calcium concentration from normal to low. On the other hand, the response to increased calcium is highly sensitive for the S235D mutant, a mimic for phosphorylated AQP0. Thus, like pH-regulated water permeability, calcium-regulated water permeability shows two forms of behavior: one noncooperative and one very cooperative, as demonstrated by the ability of a single insensitive monomer to render the entire tetramer

insensitive to increased calcium. The molecular mixture experiments presented here imply that the increased calcium concentration–induced increase in AQP0 water permeability is highly cooperative, very likely with a Hill coefficient of 4 because here again a single insensitive monomer in the tetramer destroys the ability of any monomer in the tetramer to respond to elevated calcium. This observation combined with the known high level of in vivo phosphorylation of AQP0 and the finding that increased calcium increases the water permeability of lens vesicles strongly argues that this mode of regulation is the physiologically relevant one. This view is supported by observations of Gold et al. (2012) who report that AKAP2 assembles a complex of itself, AQP0, calmodulin (CaM), and PKA promoting the phosphorylation of AQP0 and thus the regulatory mode of increasing water permeability by increased calcium (Hall, 2012). This increase in water permeability by increased Ca^{2+} in a CaM-sensitive fashion might seem paradoxical in light of the observation that phosphorylation greatly reduces the binding of CaM to AQP0 C terminus peptides (Rose et al., 2008), but pseudo-phosphorylation does not eliminate CaM-modulated calcium sensitivity of AQP0 water permeability (Németh-Cahalan et al., 2004). Moreover, the binding of AQP0 C terminus peptides is not the whole story of CaM interaction with the AQP0 tetramer. Our molecular dynamics simulations of AQP0 CaM interactions (unpublished data) indicate that there is a substantial component of electrostatic interaction between CaM and the AQP0 tetramer, which would not be apparent in the binding of CaM to the C terminus fragment alone. We suggest, therefore, that phosphorylation does not eliminate CaM binding, or even drastically reduce CaM binding to the tetramer, but rather alters the binding configuration between CaM and AQP0 so that changes in calcium concentration can either increase or decrease the water permeability. This hypothesis would also account for the effects of AKAP2 on cortical cataract cited above.

Mechanism of cooperativity

The mechanism of cooperativity remains uncertain, but it is likely that the cooperative modes of water permeability increase by pH and calcium share a common gating mechanism. The first suggestion that this might be the case was the finding that pH- and calcium-induced water permeability increases were not additive (Németh-Cahalan and Hall, 2000). Moreover, zinc and pH exhibit a steep concentration dependence on water permeability (Németh-Cahalan et al., 2007). The interfaces between water-transporting AQP monomers with known structures are unique to each specific AQP and prevent the formation of heterotetramers between different types. In addition, chimeras of different AQP types often fail to express functionally (Mathai and Agre, 1999), supporting the view that interfaces between monomers form restrictive lock-and-key surfaces. This tight and highly

specific coupling between monomers may imply that allosteric cooperative movement induced by calcium or pH is the initial event in cooperative alteration of water permeability. The manner in which CaM, essential for the cooperative modulation of water permeability by increased calcium, binds to AQP0 is suggestive of a cooperative connection between monomers. NMR structures of CaM and C terminus peptides of AQP0 show that a single CaM molecule binds two C terminus peptides in an antiparallel fashion, and that tetrameric AQP0 binds two CaM molecules (Reichow and Gonen, 2008). These results suggest a structural basis for the calcium-related cooperativity because a single CaM molecule would tie two AQP0 monomers in a tetramer together. Also, CaM could induce structural changes in one monomer that would have to be transmitted to all the other monomers in the tetramer through the interfaces between monomers. Moreover, we suggest that a switch-like concentration dependence of water permeability on calcium or pH mediated by cooperative interactions between monomers could serve an important regulatory function in the lens.

Physiological role

The absence of AQP0 or mutations that alter its water permeability lead to cataract or defective lens development (Shiels and Bassnett, 1996). Although AQP0 may exhibit several functions in the lens (Varadaraj et al., 1999; Engel et al., 2008; Kumari and Varadaraj, 2009), its role in providing water permeability is essential. If AQP0 is knocked out, water permeability supplied by AQP1 can partially, but not completely, restore normal lens structure and clarity in the mouse (Varadaraj et al., 2005).

AQP0 assumes multiple forms and may exert several functions in the lens. It exhibits a junctional form resembling gap junctions formed by connexin proteins (Gonen et al., 2004a,b). It underlies transport within the lens circulation (Mathias et al., 2007; Donaldson et al., 2010). It undergoes multiple posttranslational modifications including phosphorylation (Schey et al., 1997, 2010; Ball et al., 2004; Grey et al., 2009), and it loses its C terminus toward the center of the lens so the cortical forms of AQP0 are quite different from its nuclear ones. Thus, the calcium regulation we discuss in this paper, which is dependent on an intact C terminus for binding CaM, is likely relevant only in cortical and near cortical regions, a view supported by the observation that eliminating the ability of AKAP2 to bind to AQP0 and thus promote phosphorylation by PKA results in a cortical cataract (Gold et al., 2012). pH regulation of water permeability, however, may persist into the nucleus as it is mediated by external histidines not cleaved or altered by posttranslational modification.

We find that protons and calcium ions, at concentrations found toward the center of the lens, exhibit cooperative and noncooperative regulation of AQP0 water permeability. In both cases, the cooperative mode of water permeability increase is a plausible candidate for a physiologically relevant mode of water permeability regulation. The total water permeability of all the AQP0 in the lens is estimated to be on the order of the total water permeability of the AQP1 in the lens epithelial cells (Chandy et al., 1997). Changing the water permeability of a significant proportion of the AQP0 in the lens by a factor of two, as the regulatory mechanisms we discuss would do, would be a significant increase relative to the base levels of both AQP0 water permeability and the aggregate water permeability of AQP1.

REFERENCES

Ball, L.E., D.L. Garland, R.K. Crouch, and K.L. Schey. 2004. Post-translational modifications of aquaporin 0 (AQP0) in the normal human lens: spatial and temporal occurrence. *Biochemistry*. 43:9856–9865. http://dx.doi.org/10.1021/bi0496034

Bassnett, S., and G. Duncan. 1985. Direct measurement of pH in the rat lens by ion-sensitive microelectrodes. *Exp. Eye Res*. 40:585–590.

Chandy, G., G.A. Zampighi, M. Kreman, and J.E. Hall. 1997. Comparison of the water transporting properties of MIP and AQP1. *J. Membr. Biol*. 159:29–39. http://dx.doi.org/10.1007/s002329900266

Chrispeels, M.J., and P. Agre. 1994. Aquaporins: water channel proteins of plant and animal cells. *Trends Biochem. Sci*. 19:421–425. http://dx.doi.org/10.1016/0968-0004(94)90091-4

Ding, S., L. Ingleby, C.A. Ahern, and R. Horn. 2005. Investigating the putative glycine hinge in Shaker potassium channel. *J. Gen. Physiol*. 126:213–226.

Donaldson, P.J., L.S. Musil, and R.T. Mathias. 2010. Point: A critical appraisal of the lens circulation model—an experimental paradigm for understanding the maintenance of lens transparency? *Invest. Ophthalmol. Vis. Sci*. 51:2303–2306. http://dx.doi.org/10.1167/iovs.10-5350

Duncan, G., and T.J. Jacob. 1984. Calcium and the physiology of cataract. *Ciba Found. Symp*. 106:132–152.

Engel, A., Y. Fujiyoshi, T. Gonen, and T. Walz. 2008. Junction-forming aquaporins. *Curr. Opin. Struct. Biol*. 18:229–235.

Gold, M.G., S.L. Reichow, S.E. O'Neill, C.R. Weisbrod, L.K. Langeberg, J.E. Bruce, T. Gonen, and J.D. Scott. 2012. AKAP2 anchors PKA with aquaporin-0 to support ocular lens transparency. *EMBO Mol Med*. 4:15–26. http://dx.doi.org/10.1002/emmm.201100184

Gonen, T., Y. Cheng, J. Kistler, and T. Walz. 2004a. Aquaporin-0 membrane junctions form upon proteolytic cleavage. *J. Mol. Biol*. 342:1337–1345. http://dx.doi.org/10.1016/j.jmb.2004.07.076

Gonen, T., P. Sliz, J. Kistler, Y. Cheng, and T. Walz. 2004b. Aquaporin-0 membrane junctions reveal the structure of a closed water pore. *Nature*. 429:193–197. http://dx.doi.org/10.1038/nature02503

Grey, A.C., L. Li, M.D. Jacobs, K.L. Schey, and P.J. Donaldson. 2009. Differentiation-dependent modification and subcellular

distribution of aquaporin-0 suggests multiple functional roles in the rat lens. *Differentiation*. 77:70–83. http://dx.doi.org/10.1016/j.diff.2008.09.003

Hall, J.E. 2012. Through a glass darkly. *EMBO Mol Med*. 4:1–2. http://dx.doi.org/10.1002/emmm.201100188

Harries, W.E., D. Akhavan, L.J. Miercke, S. Khademi, and R.M. Stroud. 2004. The channel architecture of aquaporin 0 at a 2.2-Å resolution. *Proc. Natl. Acad. Sci. USA*. 101:14045–14050.

Kalman, K., K.L. Németh-Cahalan, A. Froger, and J.E. Hall. 2008. Phosphorylation determines the calmodulin-mediated Ca2+ response and water permeability of AQP0. *J. Biol. Chem*. 283:21278–21283. http://dx.doi.org/10.1074/jbc.M801740200

Kumari, S.S., and K. Varadaraj. 2009. Intact AQP0 performs cell-to-cell adhesion. *Biochem. Biophys. Res. Commun*. 390:1034–1039.

Kumari, S.S., M. Varadaraj, V.S. Yerramilli, A.G. Menon, and K. Varadaraj. 2012. Spatial expression of aquaporin 5 in mammalian cornea and lens, and regulation of its localization by phosphokinase A. *Mol. Vis*. 18:957–967.

Lagree, V., A. Froger, S. Deschamps, I. Pellerin, C. Delamarche, G. Bonnec, J. Gouranton, D. Thomas, and J.F. Hubert. 1998. Oligomerization state of water channels and glycerol facilitators. Involvement of loop E. *J. Biol. Chem*. 273:33949–33953.

Lampe, P.D., M.D. Bazzi, G.L. Nelsestuen, and R.G. Johnson. 1986. Phosphorylation of lens intrinsic membrane proteins by protein kinase C. *Eur. J. Biochem*. 156:351–357. http://dx.doi.org/10.1111/j.1432-1033.1986.tb09590.x

Mathai, J.C., and P. Agre. 1999. Hourglass pore-forming domains restrict aquaporin-1 tetramer assembly. *Biochemistry*. 38:923–928. http://dx.doi.org/10.1021/bi9823683

Mathias, R.T., G. Riquelme, and J.L. Rae. 1991. Cell to cell communication and pH in the frog lens. *J. Gen. Physiol*. 98:1085–1103.

Mathias, R.T., J. Kistler, and P. Donaldson. 2007. The lens circulation. *J. Membr. Biol*. 216:1–16. http://dx.doi.org/10.1007/s00232-007-9019-y

Murata, K., K. Mitsuoka, T. Hirai, T. Walz, P. Agre, J.B. Heymann, A. Engel, and Y. Fujiyoshi. 2000. Structural determinants of water permeation through aquaporin-1. *Nature*. 407:599–605. http://dx.doi.org/10.1038/35036519

Németh-Cahalan, K.L., and J.E. Hall. 2000. pH and calcium regulate the water permeability of aquaporin 0. *J. Biol. Chem*. 275:6777–6782. http://dx.doi.org/10.1074/jbc.275.10.6777

Németh-Cahalan, K.L., K. Kalman, and J.E. Hall. 2004. Molecular basis of pH and Ca^{2+} regulation of aquaporin water permeability. *J. Gen. Physiol*. 123:573–580. http://dx.doi.org/10.1085/jgp.200308990

Németh-Cahalan, K.L., K. Kalman, A. Froger, and J.E. Hall. 2007. Zinc modulation of water permeability reveals that aquaporin 0 functions as a cooperative tetramer. *J. Gen. Physiol*. 130:457–464. http://dx.doi.org/10.1085/jgp.200709826

Reichow, S.L., and T. Gonen. 2008. Noncanonical binding of calmodulin to aquaporin-0: implications for channel regulation. *Structure*. 16:1389–1398. http://dx.doi.org/10.1016/j.str.2008.06.011

Rose, K.M., Z. Wang, G.N. Magrath, E.S. Hazard, J.D. Hildebrandt, and K.L. Schey. 2008. Aquaporin 0-calmodulin interaction and the effect of aquaporin 0 phosphorylation. *Biochemistry*. 47:339–347. http://dx.doi.org/10.1021/bi701980t

Schey, K.L., J.G. Fowler, J.C. Schwartz, M. Busman, J. Dillon, and R.K. Crouch. 1997. Complete map and identification of the phosphorylation site of bovine lens major intrinsic protein. *Invest. Ophthalmol. Vis. Sci*. 38:2508–2515.

Schey, K.L., D.B. Gutierrez, Z. Wang, J. Wei, and A.C. Grey. 2010. Novel fatty acid acylation of lens integral membrane protein aquaporin-0. *Biochemistry*. 49:9858–9865. http://dx.doi.org/10.1021/bi101415w

Shiels, A., and S. Bassnett. 1996. Mutations in the founder of the MIP gene family underlie cataract development in the mouse. *Nat. Genet*. 12:212–215.

Varadaraj, K., C. Kushmerick, G.J. Baldo, S. Bassnett, A. Shiels, and R.T. Mathias. 1999. The role of MIP in lens fiber cell membrane transport. *J. Membr. Biol*. 170:191–203.

Varadaraj, K., S. Kumari, A. Shiels, and R.T. Mathias. 2005. Regulation of aquaporin water permeability in the lens. *Invest. Ophthalmol. Vis. Sci*. 46:1393–1402. http://dx.doi.org/10.1167/iovs.04-1217

Yap, K.L., T. Yuan, T.K. Mal, H.J. Vogel, and M. Ikura. 2003. Structural basis for simultaneous binding of two carboxy-terminal peptides of plant glutamate decarboxylase to calmodulin. *J. Mol. Biol*. 328:193–204.