

## pH and Calcium Regulate the Water Permeability of Aquaporin 0\*

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**Aquaporins increase the water permeability in many cell types across many species. We investigated the effects of external pH and  $\text{Ca}^{2+}$  on water permeability of *Xenopus* oocytes injected with aquaporin cRNA by measuring the rate of swelling in hypotonic solutions. Lowering pH to 6.5 increased the water permeability of aquaporin (AQP0)  $3.4 \pm 0.4$ -fold. Diethylpyrocarbonate pretreatment increased water permeability  $4.2 \pm 0.5$ -fold and abolished pH sensitivity, suggesting that the pH regulation is mediated by an external histidine. Lowering  $\text{Ca}^{2+}$  increased water permeability  $4.1 \pm 0.4$ -fold. The effects of  $\text{Ca}^{2+}$  and pH each required the presence of histidine 40, indicating a critical role of this amino acid in facilitating the modulation of water permeability. Clamping intracellular  $\text{Ca}^{2+}$  at high or low values abolished sensitivity to external  $\text{Ca}^{2+}$ , suggesting that  $\text{Ca}^{2+}$  acts at an internal site. Three different calmodulin inhibitors each increased AQP0 water permeability, suggesting that  $\text{Ca}^{2+}$  may act through calmodulin. None of the above altered the water permeability induced by AQP1 or AQP4. Because the greatest change in AQP0 water permeability is in the normal pH range found in the lens (7.2–6.5), this paper provides evidence for regulation of an aquaporin by pH under physiological conditions.**

The major intrinsic protein (MIP, now designated aquaporin 0 and abbreviated AQP0)<sup>1</sup> of the optical lens was the first sequenced member of the aquaporins, an ancient family of proteins found in bacteria, plants, and animals (1–4). Preston *et al.* (5) discovered that CHIP 28 (now called aquaporin 1 (AQP1)), a protein abundant in red blood cells, facilitates the diffusion of water across the plasma membrane when expressed in *Xenopus* oocytes. AQP4 (previously called MIWC for mercurial-insensitive water channel) is expressed strongly in the brain and kidney collecting duct (6, 7). Work in several laboratories subsequently demonstrated that many members of the aquaporin family facilitate the diffusion of water and other nonelectrolytes (3, 8–12). Among the aquaporins, AQP0 forms a water channel with a relatively low water permeability (13, 14); the water permeability per molecule is 40 times higher for AQP1 (13, 15). The structural basis of this large difference in water permeability is unknown. AQP0 and AQP1 form tetrameric arrays in their native membranes and when reconsti-

tuted in lipid vesicles (6–18). AQP0, AQP1, and AQP4 share ~40% sequence identity with each other. Attempts to increase AQP0 water permeability by exchanging parts of AQP0 for corresponding parts of AQP1 have been ineffective (19).

Although low in water permeability per molecule, AQP0 comprises more than 60% of the membrane protein in the normal vertebrate lens and therefore provides the major permeability pathway for water movement across the membranes of lens fiber cells. If it is defective or missing from an otherwise normal lens, a cataract results (20, 21). In a chimeric mouse model, cataract can be prevented by the presence of 20% normal cells, which presumably supply the requisite AQP0 (22). The role of AQP0 in maintaining normal lens conditions is uncertain, but it likely facilitates the intrinsic circulation of fluid in the lens that maintains lens transparency and homeostasis in the absence of blood vessels (23). pH and  $\text{Ca}^{2+}$  are likely candidates for effecting regulatory control of this circulation, because both of these ions seem to play important roles in the lens. The lens interior is more acidic (pH 6.5) than the surface (pH 7.02) (24, 25), and disturbances in  $\text{Ca}^{2+}$  concentration are associated with cataract (26–28). In this paper we show that both pH and  $\text{Ca}^{2+}$  can regulate the water permeability of AQP0 expressed in *Xenopus* oocytes but not the water permeability of AQP1 or AQP4. We localize the molecular site of pH modulation to a single extracellular histidine, His<sup>40</sup>, unique to AQP0. Modulation of water permeability by local ionic changes within the lens interior may play an important role in lens physiology. Some of the results reported here were previously presented in abstract form (29, 30).

### EXPERIMENTAL PROCEDURES

**Preparation of Oocytes**—Female *Xenopus laevis* were anesthetized, and stage V and VI oocytes removed and prepared as described previously (13). The day after isolation, oocytes were injected with either 10 ng of AQP0 or 5 ng of AQP1 cRNA (except as noted) and maintained in ND96 (96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ , 5 mM HEPES, pH 7.5) supplemented with 10 mg/ml penicillin, 10 mg/ml streptomycin, and 2.5 mM sodium pyruvate at 18 °C.

**Expression Constructs and RNA Preparation**—The expression constructs for bovine AQP0 and human AQP1 were gifts from Peter Agre and Greg Preston (Johns Hopkins). The rat AQP4 gene was purchased from ATCC (number 87184) and placed in the same expression vector. RNA was transcribed *in vitro* using T3 RNA polymerase (mMESSAGE mMACHINE kit, Ambion).

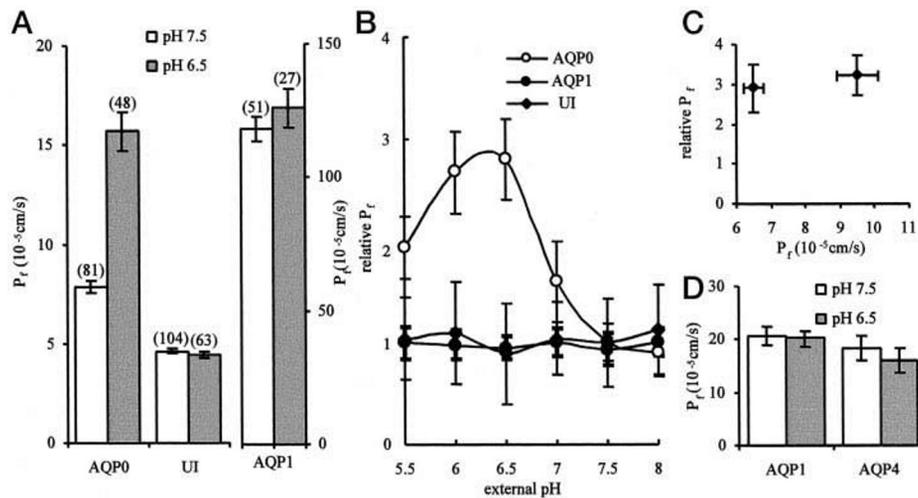
**Mutant Construction**—Histidine was substituted by alanine, aspartate, or lysine at position 40 in AQP0, using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, the mutants were obtained by performing a one step polymerase chain reaction with a set of two appropriate primers overlapping in the region of the mutation using *PfuTurbo* DNA polymerase. The mutations were confirmed by sequencing using fluorescent dye terminators (University of Chicago, DNA Sequencing Facility).

**Swelling Assay and Measurement of Water Permeability**—After 2 days, oocyte swelling assays were performed at 15 °C by transfer from 100% ND96 to 30% ND96. Before the transfer to 30% ND96 at the experimental pH or  $\text{Ca}^{2+}$  concentration, oocytes were always equilibrated for 5 min in 100% ND96 at the same experimental pH or  $\text{Ca}^{2+}$  concentration. Water permeability in cm/s,  $P_p$ , was calculated as described previously from optical measurements of the increase in cross-

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<sup>1</sup> The abbreviations used are: AQP, aquaporin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N, N',N',N'-tetraacetic acid; DEPC, diethylpyrocarbonate; LPA, lysophosphatidic acid; MES, 2-(N-morpholino)ethanesulfonic acid; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.



**FIG. 1. Effect of pH on AQP0 water permeability.** *A*, decreasing pH from 7.5 to 6.5 increases the permeability of AQP0-injected oocytes by a factor of  $3.4 \pm 0.4$ , calculated using Equation 1, but has no effect on the water permeability of AQP1 or uninjected oocytes. *B*, titration curve of the relative permeabilities (calculated using Equation 1) of AQP0, AQP1, and uninjected oocytes from pH 8.0 to pH 5.5. Only AQP0 exhibits a pH-dependent water permeability. *C*, relative permeability of AQP0 at pH 6.5 is not altered by expression level. To obtain a wider range of water permeabilities, we injected 5 or 10 ng of AQP0 cRNA. Expression level, as monitored by permeability under standard conditions with pH 7.5, is shown on the *abscissa*. The *ordinate* is the relative permeability at pH 6.5 calculated using Equation 1. *D*, lowering expression level does not result in pH sensitivity of either AQP1 or AQP4. Lowered expression levels of AQP1 and AQP4 were achieved by injecting different volumes of diluted cRNA, resulting in similar water permeabilities to those induced by injection of 10 ng of AQP0 cRNA. Lowered levels of expression produced no pH dependence of either AQP1 or AQP4 water permeability. Unless otherwise indicated, each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch).

sectional area of the oocyte with time in response to a challenge with diluted ND96 (13). Because the water permeability of uninjected oocytes has a very high activation energy, about 25 kcal/mol, whereas the activation energy of the water permeability induced by the aquaporins is small, 4–8 kcal/mole (13, 14), a signal to noise advantage is obtained by performing these measurements at 15 °C rather than 20 °C. Unless otherwise noted, each data point is the average of experiments using nine oocytes from three different batches. Because AQP0 has a lower permeability than other aquaporins, we used relative permeability to facilitate comparison and to correct for background. We define relative water permeability as:

$$\text{relative } P_f = \frac{P_{\text{exp}} - P_{\text{ct}}}{P_{\text{st}} - P_{\text{UI}}} \quad (\text{Eq. 1})$$

where  $P_{\text{exp}}$  is the permeability measured under experimental conditions,  $P_{\text{st}}$  is the permeability measured under standard conditions of pH 7.5, 1.8 mM  $\text{Ca}^{2+}$ , and  $P_{\text{UI}}$  is the permeability of uninjected oocytes. The standard error of relative  $P_f$  is calculated from the standard errors of the permeabilities using the usual formulae for the propagation of error (31).

**Controls**—Under standard conditions, uninjected oocytes had an average water permeability of  $4.5 \pm 0.2 \times 10^{-5}$  cm/s ( $n = 167$ ) and showed no change in water permeability under any of the experimental conditions (changes in pH,  $\text{Ca}^{2+}$  concentration, DEPC modification, BAPTA, LPA, calmodulin inhibitors). We report a subset of these control data in Fig. 1B, but we do not show the uninjected control results elsewhere. In addition, where appropriate, we show data for oocytes injected with AQP1 and AQP4, two different aquaporins whose water permeability does not change under the experimental conditions.

**Experimental pH Solutions**—For each experimental pH value, 100% and 30% ND96 solutions were made using HEPES for pH 8.0 to pH 7.0 and MES for pH 6.5 to pH 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.

**DEPC Pretreatment**—Oocytes were soaked in a freshly made DEPC solution (0.5 mM at pH 6.0) for 5 min. Then the oocytes were rinsed for 5 min at pH 6.0 to remove the excess DEPC. Finally the oocytes were rinsed for 5 min at pH 7.5, and the swelling assay was performed under appropriate experimental conditions.

**Hydroxylamine Pretreatment**—Oocytes were soaked in a solution of hydroxylamine (20 mM, pH 7.5) for 45 min and then rinsed in ND96, pH 7.5, for 5 min before performing the swelling assay.

**Experimental Calcium Solutions**—For each experimental  $\text{Ca}^{2+}$  concentration, 100% and 30% ND96 solutions were made as follows: 1 mM EGTA; no added  $\text{Ca}^{2+}$ ; 1.8 mM  $\text{Ca}^{2+}$  or 10 mM  $\text{Ca}^{2+}$ . Before the swelling

assay was performed in 30% ND96 at the experimental  $\text{Ca}^{2+}$  concentration, the oocytes were soaked in 100% ND96 at the experimental  $\text{Ca}^{2+}$  concentration for 5 min.

**BAPTA Treatment**—20 nl of 100 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) were injected into the oocyte by pressure injection approximately 30 min before the swelling assays were performed. The final BAPTA concentration in the oocyte was about 2 mM.

**LPA Treatment**—Oocytes were soaked in LPA solution (10  $\mu\text{M}$ ) for 3 min before performing the swelling assay in ND96 pH 7.5 with or without 1.8 mM  $\text{Ca}^{2+}$ .

**Calmodulin Inhibitors**—Oocytes were soaked in 50  $\mu\text{M}$  trifluoperazine, 5  $\mu\text{M}$  calmidazolium, or 100  $\mu\text{M}$  *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), for 30 min in the dark before performing the swelling assay at pH 7.5, 1.8 mM  $\text{Ca}^{2+}$ , with the inhibitor concentration maintained at the value specified above.

## RESULTS

We investigated the effects of varying external pH and  $[\text{Ca}^{2+}]$  on the water permeability induced in oocytes by the lens-specific aquaporin, AQP0, and, for comparison, AQP1 and AQP4.

**pH Modulates Water Permeability of AQP0 Specifically**—Under standard external ionic conditions of pH 7.5 and 1.8 mM  $\text{Ca}^{2+}$ , the water permeability of AQP0 is much lower than that for AQP1. However, when pH is reduced, the water permeability of AQP0 acutely increases, whereas that of AQP1 and uninjected oocytes remains constant (Fig. 1A). The relative permeability, calculated according to equation (1), is  $3.4 \pm 0.4$  at pH 6.5 for AQP0. Fig. 1B shows a titration curve for the water permeabilities of AQP0, AQP1, and uninjected oocytes plotted as relative permeabilities. The curve for AQP0 has an apparent  $\text{pK}_a$  of slightly less than 7.0, a maximum water permeability at pH 6.5, and a minimum at pH 7.5. Permeability is not a monotonic function of pH but decreases as the pH is lowered below 6.0, suggesting the influence of at least one additional titratable site. This decrease toward the value of  $P_f$  found at pH 7.5 may explain why Zeuthen and Klaerke reported pH insensitivity of AQP0 by sampling only at pH 4.5 and 7.4 (32). These effects of pH on AQP0 water permeability were rapidly reversible within the length of time required to exchange solutions.

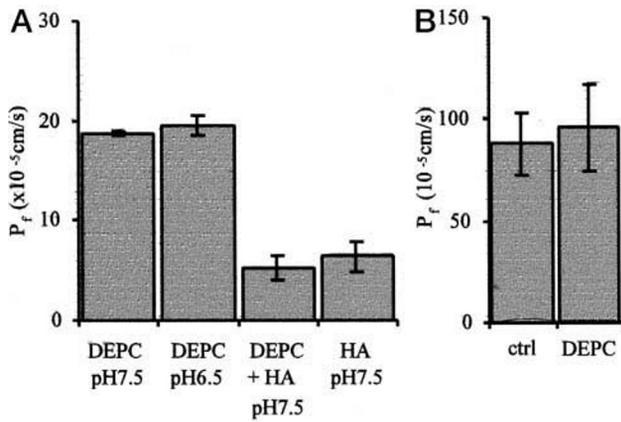


FIG. 2. **Effect of DEPC on AQP0 water permeability.** A, DEPC pretreatment (0.5 mM at pH 6.0 for 5 min) increases water permeability of AQP0 by a factor of  $4.2 \pm 0.5$ , calculated using Equation 1, and eliminates its sensitivity to pH. Hydroxylamine treatment (20 mM in pH 7.5 for 45 min) performed after DEPC treatment reverses the DEPC effect but has no effect alone. B, DEPC has no effect on AQP1. Each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch).

The expression level of AQP0 has no effect on the pH sensitivity. Fig. 1C demonstrates the pH modulation for two different expression levels of AQP0, achieved by varying the amount of mRNA injected. In either case, lowering the pH to 6.5 increased the AQP0 water permeability by a factor of about three. To test whether the absolute level of water permeability might influence the pH sensitivity, we reduced the expression levels of AQP1 and AQP4 to produce levels of water permeability comparable with those seen with 10 ng of injected AQP0 cRNA. Fig. 1D shows that even at reduced expression levels, no effects of pH on AQP1 or AQP4 water permeability were observed. We conclude that the water permeability of AQP0 is specifically increased by low pH and that this increase is not affected by either expression level or absolute level of water permeability.

**Covalent Modification of Histidines Specifically Raises AQP0 Water Permeability**—The pH dependence of AQP0 water permeability suggested the possible involvement of one or more histidine residues. To test this hypothesis, we investigated the effect of DEPC on water permeability. DEPC can covalently modify histidine, lysine, or tyrosine amino acid side chains but preferentially modifies histidine when the reaction is carried out at pH 6.0. Also, the reaction with histidine, but not with lysine or tyrosine, can be reversed by hydroxylamine (33). DEPC pretreatment increased relative  $P_f$  to  $4.2 \pm 0.5$  (compare Fig. 1A with Fig. 2A). In addition, DEPC pretreatment completely abolished the pH sensitivity of the AQP0 water permeability (Fig. 2A), indicating that few, if any, histidines remained unmodified. These results suggest that low pH and DEPC act at the same site or sites to increase AQP0 water permeability. The effects of DEPC on AQP0 water permeability were reversed by incubation with hydroxylamine (20 mM), confirming that the modified side chains are indeed histidines. Hydroxylamine pretreatment itself had no effect on the water permeability of AQP0-expressing oocytes (Fig. 2A). Furthermore, DEPC pretreatment specifically increased the water permeability of AQP0, with no effect on the water permeability of AQP1-injected oocytes (Fig. 2B), AQP4-injected oocytes, or un-injected control oocytes (data not shown). From these chemical modification experiments, we conclude that a histidine residue or residues plays an essential role in pH modulation of AQP0 water permeability.

**Mutations of Histidine 40 Abolish Sensitivity to pH and to DEPC**—Because the increase in water permeability induced by low pH was specific to AQP0 and not seen for AQP1 and AQP4,

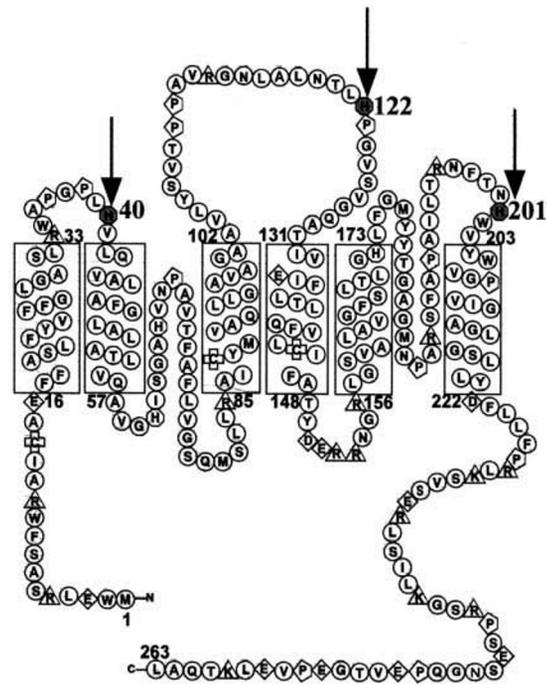


FIG. 3. **The predicted membrane topology of AQP0.** The three histidines found in AQP0, His<sup>40</sup>, His<sup>122</sup>, and His<sup>201</sup> are marked by arrows. His<sup>122</sup> is found only in AQP0 and AQP4, and His<sup>40</sup> is unique to AQP0. Polar amino acids are shown as triangles, prolines as pentagons, nonpolar amino acids as circles, and cysteines as crosses. The conserved NPA boxes are shown as loops into the membrane as suggested by the hour-glass model (41).

we examined the sequences of these aquaporins to search for candidate histidine residues. Fig. 3 shows the predicted membrane topology of AQP0, showing the NPA containing loops as folding back into the membrane as proposed by the hourglass model. AQP0 has a histidine residue in each extracellular loop (His<sup>40</sup>, His<sup>122</sup>, and His<sup>201</sup>). AQP1 has only the third, His<sup>201</sup> (as His<sup>209</sup>), and AQP4 has the second and the third (as His<sup>129</sup> and His<sup>208</sup>) (34). Thus His<sup>40</sup> seemed the best candidate histidine. To test this hypothesis, we constructed three different mutants of AQP0, replacing His<sup>40</sup> by alanine (H40A), aspartic acid (H40D), or lysine (H40K). Each of these mutants was functional, and although residues replacing His<sup>40</sup> differed in size and charge, each exhibited water permeability under standard conditions approximately two times larger than that of wild type under comparable conditions of cRNA preparation and injection, and none exhibited pH-sensitive water permeability (Fig. 4A). These experiments show that His<sup>40</sup> plays an essential role in the pH sensitivity of the water permeability of wild-type AQP0.

To test for involvement of additional histidines, we investigated the effects of DEPC pretreatment on the three His<sup>40</sup> mutants. Unlike wild-type AQP0, none of the mutants showed any change in water permeability with DEPC treatment (Fig. 4B). We conclude that histidine at position 40 is necessary for the pH modulation of water permeability in AQP0. The histidines in the second and third extracellular loops are either inaccessible to modification by DEPC or have no functional effect in the modified form.

**Modulation of AQP0 Water Permeability by Calcium**—pH and Ca<sup>2+</sup> are often functionally linked in modulating properties of membrane transport proteins, and therefore we compared effects of varying extracellular [Ca<sup>2+</sup>] and pH separately and in combination. Nominally Ca<sup>2+</sup>-free solution at pH 7.5 increased relative AQP0 water permeability by a factor of  $4.1 \pm 0.4$  (Fig. 5A) but had no effect on the relative water permeabil-

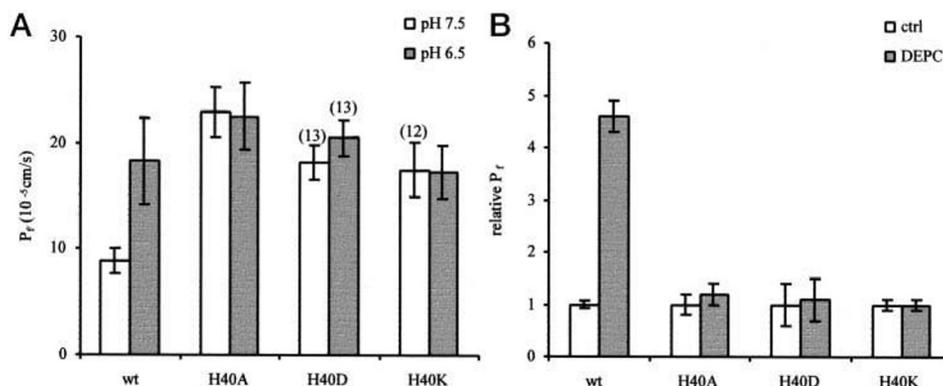


FIG. 4. **Effect of pH and DEPC on AQP0 mutants water permeability.** *A*, all three mutants H40A, H40D, and H40K expressed. Despite having different charges and sizes, all of the mutants had water permeabilities nearly equal to that of AQP0 at pH 6.5. But no mutant showed pH-sensitive water permeability. *B*, DEPC had no effect on the three histidine mutants. Unless otherwise indicated, each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch). *wt*, wild type.

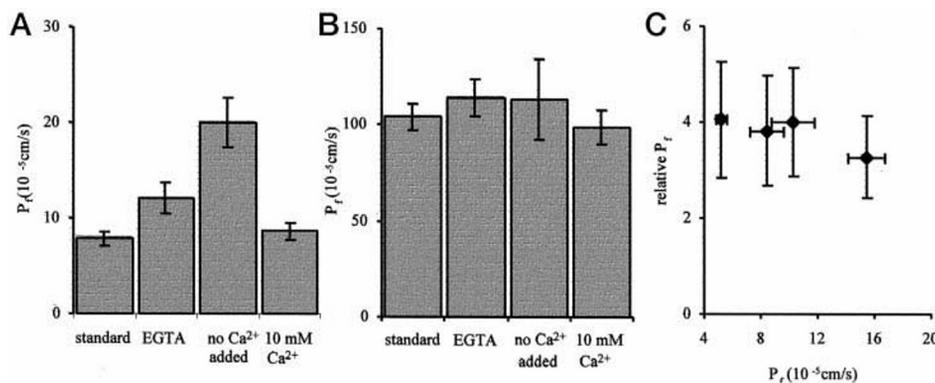


FIG. 5. **Effects of external calcium concentration on water permeability.** *A*, lowering external  $Ca^{2+}$  concentration increases the water permeability induced by AQP0 by a factor of  $4.1 \pm 0.4$ , calculated using Equation 1. *B*, changing external  $Ca^{2+}$  concentration has no effect on the water permeability of AQP1. *C*, the increase in water permeability induced by low  $Ca^{2+}$  does not depend on expression level. To obtain a wide range of water permeabilities, we injected 5, 10, 50, or 125 ng of AQP0 cRNA. Expression level, as monitored by permeability under standard conditions of pH 7.5, 1.8 mM  $Ca^{2+}$ , is shown on the *abscissa*. The *ordinate* is the relative permeability calculated using Equation 1. Each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch).

ity of AQP1-injected oocytes (Fig. 5*B*), AQP4-injected oocytes, or uninjected control oocytes (data not shown). Similar to the effects of lowering pH, the increased water permeability induced by lowering  $Ca^{2+}$  was quickly restored upon return to standard ionic conditions. Addition of EGTA to chelate  $Ca^{2+}$  ions had a smaller effect than simply omitting  $Ca^{2+}$ , suggesting a nonmonotonic  $Ca^{2+}$  dependence analogous to that seen when pH was reduced below 6.0. Raising  $Ca^{2+}$  to 10 mM had no effect, compared with standard ionic conditions. Fig. 5*C* demonstrates that varying the AQP0 water permeability over a wide range by varying the amount of cRNA injected did not affect the relative permeability exhibited in low  $Ca^{2+}$  compared with standard conditions. These results indicate that reducing external [ $Ca^{2+}$ ] elevates AQP0 water permeability by about the same factor as reducing pH.

What is the full range of water permeability modulation that can be exhibited by AQP0? Fig. 6*A* shows that the combined effect on AQP0 water permeability of reducing pH and  $Ca^{2+}$  together is larger than reducing pH or  $Ca^{2+}$  separately, but the effects are not strictly additive. Lowering pH and  $Ca^{2+}$  together increased the relative permeability to  $5.4 \pm 0.6$ , the largest factor of increase observed in our experiments but smaller than the sum of the relative permeabilities due to pH alone ( $3.4 \pm 0.4$ ) and  $Ca^{2+}$  alone ( $4.1 \pm 0.4$ ). These results suggest the involvement of two different interacting sites.

Another way of probing for interdependence of pH and  $Ca^{2+}$  is to investigate the effect of reducing  $Ca^{2+}$  on the histidine mutants lacking pH sensitivity. Fig. 6*B* illustrates that all

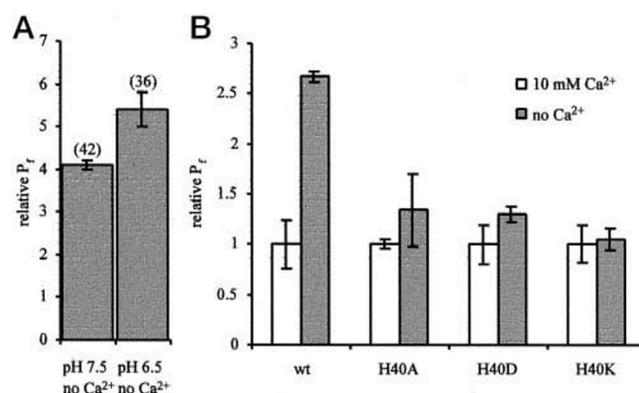


FIG. 6. **Interactions of pH and calcium.** *A*, the relative permeabilities produced by low pH and low  $Ca^{2+}$  are not strictly additive. Lowering  $Ca^{2+}$  at pH 7.5 increased the relative permeability to  $4.1 \pm 0.4$ , calculated using Equation 1. Lowering pH and  $Ca^{2+}$  together increased the relative permeability to  $5.4 \pm 0.6$ , the largest factor of increase observed in our experiments. *B*, low  $Ca^{2+}$  does not change the water permeability of any of the His<sup>40</sup> histidine mutants, suggesting that pH and  $Ca^{2+}$  act through a common mechanism dependent on the presence of His<sup>40</sup>. Unless otherwise indicated, each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch). *wt*, wild type.

three pH-insensitive histidine mutants also fail to exhibit the increase in water permeability exhibited by wild type under low  $Ca^{2+}$  conditions. Results shown earlier suggest that pH or

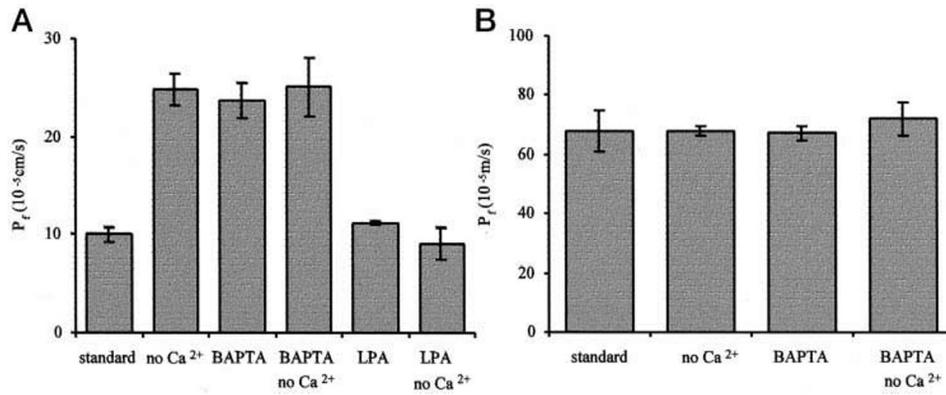


FIG. 7. **Effect of internal calcium on water permeability.** A, injection of BAPTA (2 mM) into the oocyte 30 min before the swelling assay increases AQP0-induced water permeability by a factor of  $3.5 \pm 0.6$ , calculated using Equation 1, and eliminates sensitivity of water permeability to external  $\text{Ca}^{2+}$ . LPA (10  $\mu\text{M}$ ) added to the bath elevates internal  $\text{Ca}^{2+}$  and prevents the increase in AQP0 water permeability normally induced by low external  $\text{Ca}^{2+}$ . B, BAPTA has no effect on AQP1. Each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch).

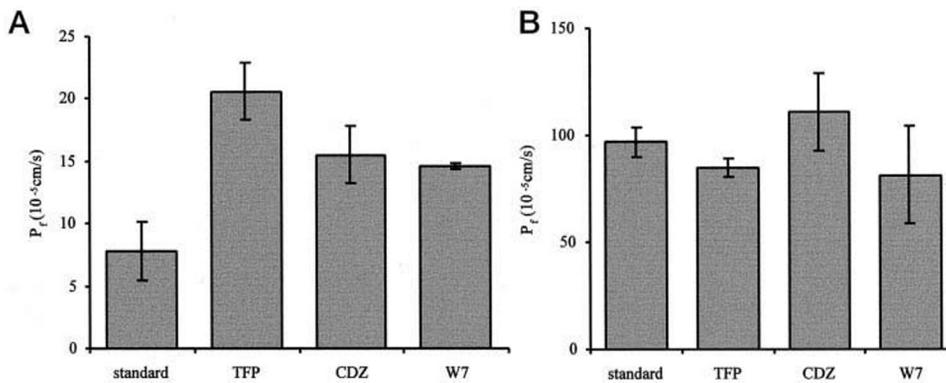


FIG. 8. **Effect of calmodulin inhibitors on water permeability.** A, the calmodulin inhibitors trifluoperazine, calmidazolium, and W7 each increase AQP0 water permeability by a factor of  $3.7 \pm 1.2$ , calculated using Equation 1. B, none of the inhibitors significantly alters the water permeability of AQP1. Each data point is the average of nine measurements (three different batches of oocytes, three oocytes from each batch).

$\text{Ca}^{2+}$  modulate the water permeability between a low permeability mode, seen under standard conditions, and a higher permeability mode. The failure of low  $\text{Ca}^{2+}$  to affect the permeability of any of the mutants demonstrates that this mode switch requires the critical histidine 40 residue.

**Effect of Varying Internal Calcium**—To determine whether the effects of low  $\text{Ca}^{2+}$  are mediated inside or outside the cell, we clamped the internal  $\text{Ca}^{2+}$  at low values using BAPTA and at high values using LPA. BAPTA injection greatly increases the internal  $\text{Ca}^{2+}$  buffering in the oocyte and reduces the free internal  $\text{Ca}^{2+}$  (35). Fig. 7A shows that injected BAPTA (final concentration, approximately 2 mM) increased the water permeability induced by AQP0 under standard ionic conditions. Furthermore, BAPTA rendered the water permeability insensitive to further change induced by lowering external  $\text{Ca}^{2+}$ . Injections of BAPTA did not alter the water permeability of AQP1 (Fig. 7B) or control uninjected oocytes (data not shown). LPA, acting through a G protein, raises internal  $\text{Ca}^{2+}$  concentration (36, 37). LPA treatment (10  $\mu\text{M}$  in the bath) clamps the water permeability at control values for AQP0 and prevents the rise in permeability normally induced by low external  $\text{Ca}^{2+}$  (Fig. 7A). We conclude that BAPTA injection induces and LPA treatment prevents the increased water permeability in AQP0 induced by lowering external  $[\text{Ca}^{2+}]$ .

**Effect of Calmodulin Inhibitors**—In earlier biochemical experiments, two laboratories reported that calmodulin interacts with AQP0 (then called major intrinsic protein) (38, 39), suggesting the possibility that calmodulin might mediate the  $\text{Ca}^{2+}$ -dependent changes in water permeability in AQP0. We tested three calmodulin inhibitors, trifluoperazine (50  $\mu\text{M}$ ), calmidazolium (5  $\mu\text{M}$ ), and W7 (100  $\mu\text{M}$ ) (40) and found that each inhibitor increased the AQP0 water permeability by an average factor of  $3.7 \pm 1.2$  and rendered it insensitive to further alteration of the external  $\text{Ca}^{2+}$  concentration (Fig. 8A). These inhibitors had no effect on the water permeabilities of AQP1 (Fig. 8B) or uninjected control oocytes (data not shown). The fact that three structurally disparate calmodulin inhibitors have the same effect on AQP0-induced water permeability suggests that the  $\text{Ca}^{2+}$ -dependent increase in water permeability may be mediated by calmodulin.

TABLE I

## Modulation of AQP0 water permeability

This table summarizes the conditions that produce high or low water permeability states of AQP0. The numbers in parentheses show the relative permeability calculated according to Equation 1 plus or minus the standard error.

Low permeability	High permeability
1.8 mM $\text{Ca}^{2+}$ and pH 7.5	Low pH ( $3.4 \pm 0.4$ )
DEPC + hydroxylamine	DEPC ( $4.2 \pm 0.5$ )
LPA	His <sup>40</sup> mutants ( $3.5 \pm 0.9$ ) <sup>a</sup>
LPA and low $\text{Ca}^{2+}$	Low $\text{Ca}^{2+}$ ( $4.1 \pm 0.4$ )
	Low $\text{Ca}^{2+}$ and low pH ( $5.4 \pm 0.6$ )
	BAPTA ( $3.5 \pm 0.6$ )
	BAPTA and low $\text{Ca}^{2+}$ ( $3.7 \pm 0.5$ )
	Cam inhibitors ( $3.7 \pm 1.2$ )

<sup>a</sup> For mutants the reference standard permeability in Equation 1,  $P_{\text{std}}$ , was that of wild-type AQP0 at pH 7.5, 1.8 mM  $\text{Ca}^{2+}$ .

zidium (5  $\mu\text{M}$ ), and W7 (100  $\mu\text{M}$ ) (40) and found that each inhibitor increased the AQP0 water permeability by an average factor of  $3.7 \pm 1.2$  and rendered it insensitive to further alteration of the external  $\text{Ca}^{2+}$  concentration (Fig. 8A). These inhibitors had no effect on the water permeabilities of AQP1 (Fig. 8B) or uninjected control oocytes (data not shown). The fact that three structurally disparate calmodulin inhibitors have the same effect on AQP0-induced water permeability suggests that the  $\text{Ca}^{2+}$ -dependent increase in water permeability may be mediated by calmodulin.

## DISCUSSION

In this paper, we demonstrate that reducing the external pH or  $\text{Ca}^{2+}$  increases AQP0 water permeability and that His<sup>40</sup> is

essential for allowing the protein to switch between high and low permeability states. This modulation in permeability may be physiologically important in the lens, allowing increased water circulation during times of increased metabolic activity. Our results contrast with a previous report that AQP0 undergoes no change in water permeability and that AQP3 water permeability decreases essentially to zero at acid pH (30). Interestingly AQP0 and AQP3 are the only aquaporins that share the three histidines His<sup>40</sup>, His<sup>122</sup>, and His<sup>201</sup> in AQP0. Our results raise the possibility that His<sup>40</sup> may facilitate switching in AQP3 as well.

**Molecular Basis for Modulation of AQP0 Water Permeability**—We summarize the conditions favoring the high or low permeability states of AQP0 in Table I with the relative  $P_f$  in parentheses. Each experimental condition increased AQP0 water permeability by a factor of three to five. The combined effects of lowering pH and Ca<sup>2+</sup> together were less than strictly additive, suggesting saturation of a common final pathway, such as single channel permeability or the probability of channel opening. Furthermore, the His<sup>40</sup> mutants not only lost pH sensitivity but also calcium sensitivity, suggesting that the AQP0 water permeability depends upon two sites, one pH-sensitive and one Ca<sup>2+</sup>-sensitive, that interact via a common pathway, requiring His<sup>40</sup>, to determine the range of modulation.

**An Internal Ca<sup>2+</sup> Sensor Coupled to Water Permeability**—The BAPTA and LPA experiments, along with supportive data from the calmodulin inhibitors, require coupling between external and internal Ca<sup>2+</sup> concentration in the oocyte system. We cannot identify this coupling mechanism at present. But because clamping the internal calcium concentration at high or low values over rides the effect of any external Ca<sup>2+</sup>, whereas the converse is not so, we conclude that the Ca<sup>2+</sup> concentration must be sensed internally.

**Possible Role of pH Regulation of Water Permeability in the Lens**—The most significant finding of this paper is that the water permeability of AQP0 is regulated by ions in the physiological range found in the lens. Our data indicate that AQP0, but not AQP1 or AQP4, can switch from low to high permeability states as pH or Ca<sup>2+</sup> is reduced. Because AQP0 is the major membrane protein of the lens and present at a very high density, there is the potential to increase dramatically the water permeability of a fiber cell. Low pH would elevate the water permeability of AQP0 in the interior of the lens. Because lowered pH is a consequence of metabolic activity, it would provide a feedback signal to increase fluid flow to areas of increased metabolic activity or to areas under-supplied by the intrinsic circulation.

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