pH and Calcium Regulate the Water Permeability of Aquaporin 0*

(Received for publication, June 25, 1999, and in revised form, December 3, 1999)

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Aquaporins increase the water permeability in many cell types across many species. We investigated the effects of external pH and 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 ± 0.4-fold. Diethylpyrocarbonate pretreatment increased water permeability 4.2 ± 0.5-fold and abolished pH sensitivity, suggesting that the pH regulation is mediated by an external histidine. Lowering Ca\(^{2+}\) increased water permeability 4.1 ± 0.4-fold. The effects of 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 Ca\(^{2+}\) at high or low values abolished sensitivity to external Ca\(^{2+}\), suggesting that Ca\(^{2+}\) acts at an internal site. Three different calmodulin inhibitors each increased AQP0 water permeability, suggesting that 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) 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 reconstituted 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 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 Ca\(^{2+}\) concentration are associated with cataract (26–28). In this paper we show that both pH and 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\(^{40}\), 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 MgCl\(_2\), 1.8 mM 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, asparagine, or lysine at position 40 in AQP0, using the QuickChange 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 *Pfu* Turbo 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 Ca\(^{2+}\) concentration, oocytes were always equilibrated for 5 min in 100% ND96 at the same experimental pH or Ca\(^{2+}\) concentration. Water permeability in cm/s, *P* \(_{0}\) was calculated as described previously from optical measurements of the increase in cross-

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*This work was supported by Grant EY5661 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 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.
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 nine measurements.

\[ \text{relative } P_t = \frac{P_{\text{exp}} - P_{\text{st}}}{P_{\text{UI}}} \]  
(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 Ca\(^{2+}\), and \( P_{\text{UI}} \) is the permeability of uninjected oocytes. The standard error of relative \( P_t \) is calculated from the standard errors of the permeabilities using the usual formula for the propagation of error (31).

Controls—Under standard conditions, uninjected oocytes had an average water permeability of 4.5 ± 0.2 × 10\(^{-5}\) cm/s (n = 167) and showed no change in water permeability under any of the experimental conditions (changes in pH, Ca\(^{2+}\) concentration, DEPC modification, BAPTA, 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 Conditions—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, the oocytes were soaked in 100% ND96 at the experimental pH of 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.

Hydroxyamine Pretreatment—Oocytes were soaked in a solution of hydroxyamine (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 Ca\(^{2+}\) concentration, 100% and 30% ND96 solutions were made as follows: 1 mM EGTA, no added Ca\(^{2+}\); 1.8 mM Ca\(^{2+}\) or 10 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.

BAPTA Treatment—20 nl of 100 mM BAPTA (1,2-bis(2-aminoethyl)oxy)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 μM) for 3 min before performing the swelling assay in ND96 pH 7.5 with or without 1.8 mM Ca\(^{2+}\).

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

RESULTS

We investigated the effects of varying external pH and [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 Ca\(^{2+}\), the water permeability of AQP0 is much lower than that of 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 ± 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 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 \( P_t \) 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.
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, 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 (His40, His122, and His201). AQP1 has only the third, His201 (as His199), and AQP4 has the second and the third (as His228 and His230). Thus His40 seemed the best candidate histidine.

To test this hypothesis, we constructed three different mutants of AQP0, replacing His40 by alanine (H40A), aspartic acid (H40D), or lysine (H40K). Each of these mutants was functional, and although residues replacing His40 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 His40 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 His40 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$^{2+}$ are often functionally linked in modulating properties of membrane transport proteins, and therefore we compared effects of varying extracellular [Ca$^{2+}$] and pH separately and in combination. Nominally Ca$^{2+}$-free solution at pH 7.5 increased relative AQP0 water permeability by a factor of 4.1 ± 0.4 (Fig. 5A) but had no effect on the relative water permeabil-
ity of AQP1-injected oocytes (Fig. 5B), 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\textsuperscript{2+} was quickly restored upon return to standard ionic conditions. Addition of EGTA to chelate Ca\textsuperscript{2+} ions had a smaller effect than simply omitting Ca\textsuperscript{2+}, suggesting a nonmonotonic Ca\textsuperscript{2+} dependence analogous to that seen when pH was reduced below 6.0. Raising Ca\textsuperscript{2+} to 10 mM had no effect, compared with standard ionic conditions. Fig. 5C 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\textsuperscript{2+} compared with standard conditions. These results indicate that reducing external [Ca\textsuperscript{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. 6A shows that the combined effect on AQP0 water permeability of reducing pH and Ca\textsuperscript{2+} together is larger than reducing pH or Ca\textsuperscript{2+} separately, but the effects are not strictly additive. Lowering pH and Ca\textsuperscript{2+} together increased the relative permeability to 5.4 ± 0.6, the largest factor of increase observed in our experiments but smaller than the sum of the relative permeabilities due to pH alone (4.1 ± 0.4) and Ca\textsuperscript{2+} alone (4.1 ± 0.4). These results suggest the involvement of two different interacting sites.

Another way of probing for interdependence of pH and Ca\textsuperscript{2+} is to investigate the effect of reducing Ca\textsuperscript{2+} on the histidine mutants lacking pH sensitivity. Fig. 6B illustrates that all three pH-insensitive histidine mutants also fail to exhibit the increase in water permeability exhibited by wild type under low Ca\textsuperscript{2+} conditions. Results shown earlier suggest that pH or
Ca\(^{2+}\) modulate the water permeability between a low permeability mode, seen under standard conditions, and a higher permeability mode. The failure of low 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 Ca\(^{2+}\) are mediated inside or outside the cell, we clamped the internal Ca\(^{2+}\) at low values using BAPTA and at high values using LPA. BAPTA injection greatly increases the internal Ca\(^{2+}\) buffering in the oocyte and reduces the free internal 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 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 Ca\(^{2+}\) concentration (36, 37). LPA treatment (10 \(\mu\)M in the bath) clamps the water permeability at control values for AQP0 and prevents the rise in permeability normally induced by low external Ca\(^{2+}\) (Fig. 7A). We conclude that BAPTA injection induces and LPA treatment prevents the increased water permeability in AQP0 induced by lowering external 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 Ca\(^{2+}\)-dependent changes in water permeability in AQP0. We tested three calmodulin inhibitors, trifluoperazine (50 \(\mu\)M), calmidazolium (5 \(\mu\)M), and W7 (100 \(\mu\)M) (40) and found that each inhibitor increased the AQP0 water permeability by an average factor of 3.7 ± 1.2 and rendered it insensitive to further alteration of the external 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 Ca\(^{2+}\)-dependent increase in water permeability may be mediated by calmodulin.

**DISCUSSION**

In this paper, we demonstrate that reducing the external pH or Ca\(^{2+}\) increases AQP0 water permeability and that His\(^{40}\) 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\(^{10}\), His\(^{122}\), and His\(^{201}\) in AQP0. Our results raise the possibility that His\(^{40}\) 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 \(Ca^{2+}\) 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\(^{40}\) 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 \(Ca^{2+}\)-sensitive, that interact via a common pathway, requiring His\(^{40}\), to determine the range of modulation.

**An Internal \(Ca^{2+}\) Sensor Coupled to Water Permeability**—The BAPTA and LPA experiments, along with supportive data from the calmodulin inhibitors, require coupling between external and internal \(Ca^{2+}\) concentration in the oocyte system. We cannot identify this coupling mechanism at present. But because clamping the internal calcium concentration at high or low permeability states. This modulation in permeability may be 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\(^{10}\), His\(^{122}\), and His\(^{201}\) in AQP0. Our results raise the possibility that His\(^{40}\) may facilitate switching in AQP3 as well.

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**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 \(Ca^{2+}\) is reduced. Because AQP0 is the major membrane protein of the lens and presents 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.

**Acknowledgments**—We thank Mary Hawley for expert technical assistance including oocyte preparation and Michael Cahalan and K. George Chandy for helpful discussions.