Cloning and functional expression of an MIP (AQP0) homolog from killifish (Fundulus heteroclitus) lens

LEILA V. VIRKKI, GORDON J. COOPER, AND WALTER F. BORON
Department of Cellular and Molecular Physiology, Yale University
School of Medicine, New Haven, Connecticut 06520-8026

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Virkki, Leila V., Gordon J. Cooper, and Walter F. Boron. Cloning and functional expression of an MIP (AQP0) homolog from killifish (Fundulus heteroclitus) lens. Am J Physiol Regulatory Integrative Comp Physiol 281: R1994–R2003, 2001.—The major intrinsic protein (MIP) of lens fiber cells is a member of the aquaporin (AQP) water channel family. The protein is expressed at very high levels in lens fiber cells, but its physiological function is unclear. By homology to known AQPs, we have cloned a full-length cDNA encoding an MIP from the lens of killifish (Fundulus heteroclitus). The predicted protein (263 amino acids; GenBank accession no. AF191906) shows 77% identity to amphibian MIPs, 70% identity to mammalian MIPs, and 46% identity to mammalian AQP1. Expression of MIPfun in Xenopus laevis oocytes causes an ~40-fold increase in oocyte water permeability. This stimulation is comparable to that seen with AQP1 and substantially larger than that seen with other MIPs. The mercurials HgCl₂ and p-chloromercuribenzenesulfonate inhibit the water permeability of MIPfun by ~25%. MIPfun is not permeable to glycerol, urea, or formic acid but is weakly permeable to CO₂.

major intrinsic protein; aquaporin; glycerol; urea; water permeability

AQUAPORIN (AQP) 1, originally isolated from red blood cells, was the first membrane protein to be described as a functional water channel (21). Since then, numerous AQPs have been identified in animals, plants, and bacteria. The major intrinsic protein (MIP) of the lens fiber cell was actually cloned before AQP1. Subsequent to the identification of AQP1 as a water channel, MIP (now also known as AQP0) was shown to be permeable to water (13, 17, 31), although this permeability is much lower than for most other AQPs.

The vertebrate lens is a transparent avascular organ, the bulk of which consists of highly elongated lens fiber cells surrounded by a lens capsule. An epithelial monolayer lies between the lens fiber cells and the anterior portion of the capsule. The lens fiber cells are highly specialized cells containing high amounts of soluble crystallins. During differentiation from epithelial cells, the fiber cells elongate and progressively lose their nuclei as well as most organellar structures and approach metabolic quiescence. The surface area of the differentiating fiber cells increases markedly, and the cells express a large amount of MIP. In the mature fiber cell membrane, MIP represents >60% of the total membrane protein (4). The functional importance of MIP in lens physiology is underscored by the observation that MIP mutations lead to early cataract formation (2, 24).

The functional role of MIP has long been a matter of controversy. When MIP was first demonstrated in communicating junctions in the vertebrate lens, it was proposed that MIP is involved in cell-to-cell coupling (3). However, after MIP was cloned (11), it was clear that MIP is unrelated to the connexins. Moreover, functional data failed to show that MIP could induce coupling (25, 26). Because MIP is present in specialized “thin” junctions between lens fiber cells, others then proposed that the main function of MIP is to facilitate cell-cell adhesion between the tightly packed lens fiber cells (7, 16, 30). Recent studies using atomic force and cryoelectron microscopy (10) have shown that MIP molecules in lattices of opposing membranes bind together via their extracellular surfaces with a tight “tongue-and-groove” fit. After it was shown that MIP is also permeable to water (17, 31), it was thought that one possible role of MIP is to mediate fluid and nutrient circulation in the avascular lens. According to a model proposed by Mathias et al. (15), the Na⁺-K⁺ pump drives a current that is directed out of the lens equator and back into the anterior and posterior poles of the lens, and fluid circulates along this same path. However, if the main role of MIP is in the microcirculation of fluid in the lens, it is not clear why evolution selected a protein with such a low water permeability.

Of all vertebrate AQPs described to date, the majority are mammalian, with a few representatives cloned from amphibian tissues. MIP homologs have been described in the lenses of rat, mouse, cattle, and humans, as well as in two Anurans, Rana pipiens and Xenopus laevis. No AQPs have been described in fish. We report here the cloning and functional expression of the first fish AQP, an MIP from the killifish Fundulus heteroclitus. This fish MIP is unusual, in that it has a rela-

Address for reprint requests and other correspondence: L. V. Virkki, Dept. of Cellular and Molecular Physiology, Yale University School of Medicine, 333 Cedar St., SHM-B 133, PO Box 208026, New Haven, CT 06520-8026 (E-mail: leila.virkki@yale.edu).

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tively high water permeability, consistent with the fluid-circulation hypothesis.

EXPERIMENTAL PROCEDURES

Cloning

Total RNA from the lens of the killifish F. heteroclitus (Marine Biological Laboratory, Woods Hole, MA), extracted using the RNeasy kit (Qiagen, Valencia, CA), was used as the template for reverse transcriptase (Superscript II, GIBCO BRL, Life Technologies, Gaithersburg, MD), primed with oligo(dT). The resulting cDNA was used in subsequent PCR. Degenerate, nested primers were designed to the regions surrounding the highly conserved NPA (Asn-Pro-Ala) motif of known AQPs. The first PCR was carried out using the sense primer 5′-GCRTGATMGCVGATTTRTGCC-3′ and the antisense primer 5′-AARGAYCGCGWQGTTCAT-3′. The second, nested reaction was carried out using the sense primer 5′-AGCGGCGCCACCTBAACCACG-NGTCAC-3′ and the antisense primer used in the first reaction. The resulting PCR products were cloned into a pCR 2.1 TA vector (Invitrogen, Carlsbad, CA) and sequenced. All sequencing was performed by the Keck Biotechnology Resource Laboratory (Boyer Center for Molecular Medicine, Yale University). Homology to known AQPs was confirmed by sequence alignment analysis (BLAST at the National Center for Biotechnology Information). The sequence was extended in the 3′ and 5′ directions using rapid amplification of cDNA ends (RACE, GIBCO BRL). The full coding sequence was obtained from oligo(dT)-primed cDNA using the sense primer 5′-TGTCGACCCCTTTGGTTGATTAGTTGGTAC-3′ (corresponding to the region of the 5′-untranslated region (UTR) just before the start codon) and the antisense primer 5′-CGGT-GACCCGTTGGAAGCTCAAGAAACAT-3′ (corresponding to the portion of the 3′-UTR immediately after the stop codon). The underlined sequences indicate the engineered SalI (sense) and BstEII (antisense) restriction sites. The resulting PCR products were subcloned into a pCR 2.1 TA cloning vector. We sequenced five independent clones in both directions, deriving a consensus sequence that was shared by one vector. We sequenced five independent clones in both directions using rapid amplification of cDNA ends (RACE, GIBCO BRL). The full coding sequence was obtained from oligo(dT)-primed cDNA using the sense primer 5′-GCRTGATMGCVGATTTRTGCC-3′ and the antisense primer 5′-AARGAYCGCGWQGTTCAT-3′. The second, nested reaction was carried out using the sense primer 5′-AGCGGCGCCACCTBAACCACG-NGTCAC-3′ and the antisense primer used in the first reaction. The resulting PCR products were cloned into a pCR 2.1 TA vector (Invitrogen, Carlsbad, CA) and sequenced. All sequencing was performed by the Keck Biotechnology Resource Laboratory (Boyer Center for Molecular Medicine, Yale University). Homology to known AQPs was confirmed by sequence alignment analysis (BLAST at the National Center for Biotechnology Information). The sequence was extended in the 3′ and 5′ directions using rapid amplification of cDNA ends (RACE, GIBCO BRL). The full coding sequence was obtained from oligo(dT)-primed cDNA using the sense primer 5′-TGTCGACCCCTTTGGTTGATTAGTTGGTAC-3′ (corresponding to the region of the 5′-untranslated region (UTR) just before the start codon) and the antisense primer 5′-CGGT-GACCCGTTGGAAGCTCAAGAAACAT-3′ (corresponding to the portion of the 3′-UTR immediately after the stop codon). The underlined sequences indicate the engineered SalI (sense) and BstEII (antisense) restriction sites. The resulting PCR products were subcloned into a pCR 2.1 TA cloning vector. We sequenced five independent clones in both directions, deriving a consensus sequence that was shared by one of the five clones. The consensus sequence was deposited in GenBank (accession no. AF191906). Homology to other known AQPs was determined by aligning multiple AQP sequences using the Megalign module of the Lasergene program suite (DNASTAR, Madison, WI). Hydropathy plots were calculated using the Kyte-Doolittle algorithm. We call the clone MIPfun, i.e., MIP from Fundulus.

Northern Blotting

Total RNA from lenses, eye (without lens), blood, brain, gills, heart, intestine, kidney, liver, ovary, spleen, skin, and testis was extracted from adult killifish using TRIzol reagent (GIBCO BRL) according to the manufacturer’s directions. Total RNA (15 μg) was resolved by formaldehyde agarose (1%) denaturing gels and blotted to positively charged nylon membrane (Hybond XL, Amersham) by capillary elution. Blots were prehybridized by incubation in ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA) at 68°C for 30 min and then hybridized with an α-32P-labeled probe (Random Primer labeling kit, GIBCO BRL) corresponding to the full-length MIPfun sequence. As a control, the same membranes were also probed with a similarly labeled 433-bp probe corresponding to F. heteroclitus β-actin (GenBank accession no. AF397164).

Expressing Membrane Proteins in Oocytes

The MIPfun fragment was excised from pCR 2.1 using SalI and BstEII and subcloned into a KSM oocyte expression vector, a derivative of pBluescript in which the insertion site is flanked by the 5′- and 3′-UTRs of Xenopus β-globin. This expression vector was a kind gift from Dr. William Joiner (Yale University). Capped complementary RNA (cRNA) was transcribed in vitro using the T3 Message Machine kit (Ambion, Austin, TX).

Stage V–VI oocytes from Xenopus laevis were defolliculated with collagenase type IA (Sigma, St. Louis, MO) and stored in OR3 medium (Sigma). One day after isolation, oocytes were injected with 50 nl of 0.05 μg/μl cRNA encoding MIPfun or an identical volume of sterile water. For positive controls, oocytes were also injected with 50 nl of 0.05 μg/μl cRNA for human AQP1 (hAQP1) or 20 μg/μl cRNA for rat AQP3 (rAQP3). Bovine MIP (bMIP), or rat urea transporter (UT-A2). The cDNAs of bMIP and hAQP1 (in the Xenopus expression vector pXβG) were a kind gift from Dr. Peter Agre (Johns Hopkins University, Baltimore, MD), rAQP3 (in pSPORT) was a gift from Dr. Lawrence Palmer (Cornell University, New York, NY), and UT-A2 (in pBluescript) was a gift from Dr. Craig Smith (University of Manchester, Manchester, UK). Functional expression of AQPs was verified by placing a test oocyte in deionized water and observing the time taken for the oocyte to rupture under osmotic pressure.

Measuring Oocyte Water Permeability

We used a volumetric assay to measure the osmotic water permeability (P) of oocytes injected with various cRNAs or water. Each oocyte was placed in a perfusion chamber and initially superfused with isotonic ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, pH 7.5, osmolality 200 mosM) at a solution flow of 4 ml/min.

To induce cell swelling, the perfusion solution was switched to a hypertonic solution, prepared by reducing the concentration of NaCl to obtain the desired osmolarity as follows: 70 mosM (16 mM NaCl), 100 mosM (43 mM), and 135 mosM (63 mM). Osmolalities were measured using a pressure vapor osmometer (Wescor, Logan, UT). In experiments where extracellular pH (pHw) or Ca2+ concentration ([Ca2+]w) was varied, the oocytes were initially incubated for 5 min in the appropriate solution before they were switched to the hypertonic solution of otherwise identical composition.

We acquired images of the oocyte silhouette every 2 s using a videocamera attached to a stereomicroscope with illumination from below. The oocyte volume was calculated from the cross-sectional area of the oocyte, with the assumption that the oocyte is a perfect sphere. The volume was calibrated using, as an underwater standard, a brass ball bearing placed near the oocyte.


\[
P_i = \frac{d(V/V_0)/dt}{V_0/5}(\Delta_{sw} V_0)
\]

where \(d(V/V_0)/dt\) is the initial rate of increase in relative oocyte volume after a hyposmotic challenge, \(V_0\) is the initial oocyte volume, \(S\) is the initial surface area, \(\Delta_{sw}\) is the osmotic gradient, and \(V_0\) is the molar volume of water.

We routinely employed two-electrode voltage clamp to measure the capacitance of oocytes used in these experiments. From the capacitance data, we calculated the actual membrane surface area (the specific capacitance of the membrane was taken to be 0.8 μF/cm²). In cases in which capacitance data were not acquired, we multiplied the geometric area by the appropriate factor (obtained in separate experiments) to approximate the actual area.
Measuring Oocyte Osmolyte Permeability

Isotopic flux measurements. We measured unidirectional influx of urea and glycerol in oocytes using [14C]urea and [14C(U)]glycerol (Moravek Biochemicals, Brea, CA). Four oocytes were placed in a 1.5-ml microcentrifuge tube in ND96 solution. Measurement of isotope uptake was started by aspirating the ND96 solution and replacing it with 700 μl of ND96 containing the unlabeled analyte at 1 mM and 1 μCi/ml (37 kBq/ml) of the radioisotopically labeled analyte. Oocytes were incubated on a horizontal shaker for 5 min at room temperature. In preliminary experiments, we monitored 14C uptake to ensure that the uptake was linear during the first 5 min. Radioisotope uptake was stopped by washing oocytes in ice-cold ND96 solution containing the unlabeled analyte at 10 mM. Individual oocytes were transferred to scintillation vials and lysed in 400 μl of 10% SDS with continuous shaking. The 14C activity of individual oocytes was assessed by liquid scintillation counting (LKB-Wallac Rackbeta, Turku, Finland).

Intracellular pH measurements. We used pH-sensitive microelectrodes to determine the CO2 and formic acid permeability of oocytes expressing MIPfun, AQP1, or AQP3. Control experiments were performed with oocytes injected with water. pH-sensitive electrodes were fabricated and used as described previously (6). Briefly, the vitelline membrane of the oocyte was removed, and the oocyte was placed in an experimental chamber. The oocyte membrane was impaled with two electrodes, one that recorded the membrane voltage and one that contained a proton-selective resin, across which a proton-dependent voltage was generated. Voltages were measured using an electrometer (model PD 223, World Precision Instruments, Sarasota, FL), and data were acquired using software written in-house. Cell pH was obtained by subtracting the signals from the voltage and pH electrodes. The system was calibrated using buffered pH standards at pH 6.0 and 8.0. An additional single-point calibration was performed using the standard ND96 solution of pH 7.5 in the bath before the oocyte was impaled.

The CO2 flux measurement was performed essentially as described by Cooper and Boron (6). Oocytes were superfused in the experimental chamber as described for P1 measurements. The oocyte was initially superfused with nominally CO2-free ND96 solution. CO2-dependent acidification was induced by washing the superfusate to a solution gassed with 1.5% CO2. The composition of this solution was similar to that of ND96 solution, except 10 mM NaHCO3 was added and NaCl was reduced accordingly to maintain osmolality. The initial CO2 flux across the cell membrane was calculated from the initial rapid decrease in intracellular pH (pHi) caused by CO2 entry with subsequent H+ formation according to the following formula

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \]

The CO2 flux (J_{CO2}) was calculated as follows

\[ J_{CO2} = \frac{dH/dt \times \beta \times V}{S} \]  

(2)

where \( dH/dt \) is the initial decrease in pH, \( \beta \) is the buffering power of the oocyte, V is the oocyte volume, and S is the actual surface area (as estimated from capacitance measurements).

The formic acid flux was measured similarly to J_{CO2} flux. The oocyte was initially perfused with ND96 solution, and after the initial equilibration, the solution was rapidly replaced with 50 mM formate-ND96 solution, pH 7.5. This solution was obtained by adding 50 mM formate to ND96 solution (modified by reducing the NaCl concentration to maintain osmolality). We calculated the flux of formic acid through the oocyte membrane from the initial decrease in pH, caused by the entry of the uncharged (protonated) form of the acid. On entering the cell, the acid dissociates, releasing a proton and causing the cell to acidify. In computing the fluxes of CO2 and formic acid, we assume that the oocyte membrane is essentially impermeable to HCO3– and formate, respectively.

Two-Electrode Voltage Clamp

A two-electrode voltage clamp was employed to measure whole cell ionic currents and capacitance in oocytes expressing MIPfun or injected with water (control). Data were collected using a Warner Instruments (Hamden, CT) oocyte clamp controlled by the Clampex module of pCLAMP software (version 8, Axon Instruments, Foster City, CA). Data were analyzed using the Clampfit module of pCLAMP. For measuring whole cell currents, oocytes were held at −50 mV and pulsed from −110 to 0 mV in 10-mV increments. For measuring whole cell capacitance, the area under the elicited capacitive current spike was integrated to give the amount of charge moved by a given voltage step. We plotted charge against voltage and fitted the data with a straight line, the slope of which is a measure of capacitance.

RESULTS

Sequence Data

The MIPfun sequence contains a 792-bp open reading frame encoding 263 amino acids. Figure 1A shows sequence alignment with bMIP and hAQP1. Sequence alignment with multiple AQP s reveals 77% identity to amphibian and 70% identity to mammalian MIPs. The results of our hydrophy analysis (Fig. 1B) of the deduced amino acid sequence are similar to those for other AQP s (12, 18) and are consistent with six transmembrane segments and five connecting loops (A–E). Loops B and E contain the AQP family signature motif, the amino acid sequence NPA. Two consensus protein kinase C phosphorylation sites are located at residues 231 and 236. In contrast to all other lens MIPs cloned so far, MIPfun lacks a consensus N-glycosylation site. However, other MIPs might not be glycosylated either. For example, although there is a potential glycosylation site on loop E, bMIP in its native environment does not appear to be glycosylated (4).

Northern Analysis

Figure 2A shows a Northern blot analysis of total RNA from multiple tissues from killifish. Figure 2B shows the same blot probed with killifish actin. A strong signal is present in the lens, with transcripts of 2.8 and 1.8 kb. It is likely that the two bands arise from alternatively spliced 3′- or 5′-UTRs, inasmuch as PCR with gene-specific primers designed to areas just outside the open reading frame resulted in only one product size (data not shown). No signal was detected in eye (without lens), brain, gills, heart, intestine, kidney, liver, ovary, spleen, skin, or testis. Thus, as in other species, the fish MIP is exclusively localized to the ocular lens.

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Expression of Water Permeability in Oocytes

Figure 3A shows the time course of $P_f$ of oocytes injected with water (control) or with cRNA encoding MIPfun, bMIP, or hAQP1. It appears that, during the period of observation, the fractional increase in $P_f$ was substantially greater for MIPfun than for bMIP or hAQP1. By day 6, the $P_f$ values of oocytes injected with cRNA (compared with $P_f$ values of water-injected controls) had increased 40-fold for MIPfun, 9-fold for bMIP, and 56-fold for hAQP1 compared with control oocytes.

Capacitance Measurements

A high expression level of exogenous proteins can increase the whole cell capacitance of an oocyte. For example, Chandy et al. (5) demonstrated that expression of bMIP increases whole cell capacitance. If it is assumed that the specific capacitance of the membrane does not change, an increase in whole cell capacitance indicates an increase in total membrane surface area, which could occur as a result of proportional cell growth or an increase in surface area amplification. On the basis of capacitance measurements and electron microscopy, the actual surface area of the oocyte is generally estimated to be eight to nine times larger than the area calculated assuming that the oocyte is a sphere (5, 31). The extra membrane area comes from numerous infoldings and microvilli of the oolemma. Our capacitance measurements yielded slightly smaller values for this amplification of surface area, six to seven times larger than that of a sphere for control oocytes.

Figure 3B summarizes the time course of membrane capacitance, normalized for the calculated surface area, with the assumption that the oocyte is a sphere. In agreement with results obtained by Chandy et al. (5), we found that expression of bMIP, by day 6, leads to an ~80% increase of normalized membrane capacitance ($\mu$F/cm$^2$ membrane area). The normalized capacitance of MIPfun-expressing oocytes increased more slowly, reaching an ~45% increase by day 6, and that of hAQP1-expressing oocytes increased by $<15\%$. 

Fig. 1. Sequence analysis. A: alignment of amino acid sequences of major intrinsic protein (MIP) from Fundulus heteroclitus (MIPfun), bovine MIP (bMIP), and human aquaporin (AQP) 1 (hAQP1). Sequences were aligned using the Megalign module (Clustal method) of the Lasergene program suite (DNASTAR). Identical amino acids are highlighted. Membrane-spanning segments of hAQP1, as determined by high-resolution electron microscopy (18), are indicated by underscoring in the hAQP1 sequence. B: hydropathy analysis of MIPfun. Hydropathy analysis using Kyte-Doolittle algorithm (window size 17) predicts the presence of 6 membrane-spanning segments (1–6) and 5 connecting loops (A–E).
Effect of Mercurial Compounds on $P_f$

Figure 4A shows the effect of 5 min of pretreatment with 0.3 mM HgCl$_2$ on the $P_f$ of oocytes expressing MIPfun or AQP1, and Fig. 4B shows the comparable results for 15 min of pretreatment with 1 mM p-chloromercuribenzenesulfonate (pCMBS). Treatment with 5 mM β-mercaptoethanol for 5 min fully reverses the inhibition by both agents for MIPfun- and AQP1-expressing oocytes. Figure 4C shows that HgCl$_2$ and pCMBS each reduce $P_f$ of MIPfun-expressing oocytes by ~25%. On the other hand, HgCl$_2$ and pCMBS reduce the $P_f$ of AQP1-expressing oocytes by ~90% and ~67%, respectively.

Effect of pH$_o$ and [Ca$^{2+}]_o$ on $P_f$

We found that the $P_f$ of MIPfun-expressing oocytes fell by ~20% when we lowered pH$_o$ from 7.5 to 5 or 6 (Fig. 5A). Increasing pH$_o$ to 8.5 or 10 had no significant effect on $P_f$. In contrast, for bMIP-expressing oocytes, we observed no effect of lowering pH$_o$ or of raising pH$_o$ to 8.5 (Fig. 5). However, increasing pH$_o$ to 10 caused P$_f$ to increase more than threefold. We also found that the histidine reagent diethyl pyrocarbonate (DEPC) increased the $P_f$ of bMIP by a factor of 4.2 at pH$_o$ 7.5 and made $P_f$ insensitive to pH$_o$ changes. In contrast, we saw no effect of pretreatment for 5 min with 0.1 mM DEPC (pH 6.0) on the $P_f$ of MIPfun- or bMIP-expressing oocytes (data not shown).

Nemeth-Cahalan and Hall (20) also studied the effect of altering [Ca$^{2+}]_o$ on the $P_f$ of bMIP-expressing oocytes. For bMIP expressed in their oocytes, removing extracellular Ca$^{2+}$ increased the $P_f$ of bMIP-expressing oocytes by a factor of 4, whereas increasing [Ca$^{2+}]_o$ to 10 mM had no effect. In contrast, we observed that the $P_f$ of oocytes expressing bMIP and also MIPfun did not change when we lowered [Ca$^{2+}]_o$ (Fig. 5B). However, we did find that raising [Ca$^{2+}]_o$ to 10 mM slightly decreased the $P_f$ of both groups of oocytes.

Isotopic flux measurements. Figure 6A shows the glycerol uptake (measured by $^{14}$C uptake) of oocytes injected with water (control) or expressing MIPfun, bMIP, or rAQP3. The glycerol uptake of MIPfun-expressing oocytes was not different from controls in the

Fig. 3. Osmotic water permeability ($P_f$) and cell membrane capacitance of oocytes as a function of time after cRNA injection. Oocytes were injected with water or cRNA encoding MIPfun, bMIP, or hAQP1. $P_f$ (A) and membrane capacitance (B) were measured 3, 4, 5, and 6 days after injection. Capacitance is expressed as μF/cm$^2$ of oocyte surface area (calculated assuming that the oocyte is a perfect sphere) to compensate for differences in oocyte size. The same oocytes were used in A and B. Error bars, SE. Each data point represents 3–6 oocytes.
absence or presence of pretreatment with 0.3 mM HgCl2 for 5 min. In oocytes expressing bMIP, the glycerol uptake is slightly above background. When we subtracted the glycerol uptakes of water-injected oocytes (with and without HgCl2) from the corresponding uptakes of bMIP-expressing oocytes, the inhibitory effect of HgCl2 was significant (P, 0.01). As found by Echevarria et al. (8), the glycerol uptake of rAQP3-expressing oocytes is high but insensitive to HgCl2 and pCMBS.

Figure 6B shows the urea uptake (measured as 14C uptake) of water-injected controls or oocytes expressing MIPfun or the rat urea transporter UT-A2. MIPfun-expressing oocytes showed no statistically significant change in the urea permeability compared with controls. As expected, expression of UT-A2 caused a fourfold increase in the rate of urea uptake.

pHi measurements. Figure 7A shows the time course of pHi of an oocyte expressing MIPfun. Superfusing the oocyte with a solution equilibrated with 1.5% CO2 (concentration of CO2 in the solution is 384 μM) caused a reversible fall in pHi. As summarized in Fig. 7B, the rate of CO2-induced acidification (expressed as a flux of H+) was ~30% higher in oocytes expressing MIPfun and 46% higher in oocytes expressing AQP1.

Fig. 5. Effect of changes in extracellular pH (pH0) or Ca2+ concentration ([Ca2+]0) on Pf of oocytes expressing MIPfun. A: pH0 dependence. Pf values of oocytes expressing MIPfun or bMIP were measured at pH0 5.0, 6.0, 7.5, 8.5, and 10.0; values were normalized to Pf measured at pH0 7.5 in the same oocyte. B: [Ca2+]0 dependence. Pf values of oocytes expressing MIPfun or bMIP were measured at different [Ca2+]0, values and normalized to Pf measured in separate oocytes at [Ca2+]0 of 1.8 mM. Error bars, SE; number of observations was 6–10 (A) or 6–16 (B) per data point. *Statistical significance (P < 0.05) of nonnormalized data compared with the pH0 7.5.

Fig. 4. Effect of inhibitors on MIPfun and hAQP1. A: effect of 5 min of pretreatment with 0.3 mM HgCl2 on Pf of oocytes expressing MIPfun or hAQP1. Inhibition by HgCl2 is reversed by incubation for 5 min with 5 mM β-mercaptoethanol. B: effect of 15 min of pretreatment with 1 mM p-chloromercuribenzenesulfonate (pCMBS). Similar to HgCl2, inhibition is reversed by incubation with β-mercaptoethanol. C: percent inhibition of Pf by HgCl2 and pCMBS. For each mercurial, the mean Pf of water-injected oocytes was subtracted from the mean Pf of oocytes expressing MIPfun or AQP1. Error bars, SE; values in parentheses represent number of oocytes. Statistically significant difference (P < 0.05) compared with no-drug condition: *2-tailed t-test; †1-tailed t-test.
Figure 7C summarizes rates of acidification in oocytes exposed to formic acid (50 mM). Because the $pK_a$ of formic acid is 3.75 and $pH_o$ is 7.5, the concentration of the neutral formic acid is $8.9 \times 10^{-3}$ M. The formic acid permeability of oocytes expressing MIPfun was not significantly different from controls. However, in oocytes expressing rAQP3, the rate of acidification was significantly higher than in control oocytes. This is consistent with the observation that rAQP3 has substantial permeability to glycerol (Fig. 6) and other small nonpolar solutes (32).

DISCUSSION

Although fish comprise about half of all vertebrate species, the majority of vertebrate AQPs that have been identified are mammalian or amphibian. This study reports the cloning and functional expression of
the first AQP from a fish, the killifish (*F. heteroclitus*). The novel AQP, termed MIPfun, is an MIP homolog expressed in the ocular lens, as shown by Northern blot. Interestingly, the *P* of MIPfun is substantially higher than that of its mammalian or amphibian homologs.

**Block by Mercurials**

In contrast to its mammalian and amphibian ocular counterparts, MIPfun shows some sensitivity to mercurial compounds. Both pCMBS and HgCl₂ inhibit its *P* by ~25%. MIPfun (as well as other lens MIP homologs) lacks the equivalent of the cysteine in position 189, which is the site of mercurial action in AQP1. MIPfun has three other cysteine residues: Cys⁴⁷ lies near the middle of the predicted transmembrane segment 2, Cys⁹⁴ lies in the middle of the predicted transmembrane segment 3, and Cys¹⁴⁴ (which is conserved in all lens MIPs) lies near the intracellular side of the predicted transmembrane segment 4. The mercurial sensitivity of MIPfun may be due to Cys⁴⁷ or Cys⁹⁴, neither of which has counterparts in other MIPs.

**pH₀ and [Ca²⁺]₀ Sensitivity**

An unexpected outcome of the present study was that, unlike Nemeth-Cahalan and Hall (20), we found bMIP to be relatively unaffected by changes in pH₀ or [Ca²⁺]₀, as well as by the addition of DEPC. We also found that expressing bMIP increases *P* by six- to ninefold (Fig. 3A), whereas Nemeth-Cahalan and Hall only saw a doubling. Starting from this relatively low bMIP-dependent *P*, Nemeth-Cahalan and Hall found that lowering pH₀ and/or [Ca²⁺]₀ raised *P* to levels roughly equivalent to the *P* under control conditions in our studies. Moreover, Nemeth-Cahalan and Hall found that mutating His⁴⁰, which is at the extracellular end of transmembrane segment 2, raised the *P* of bMIP to the same high level observed when they lowered pH₀ or [Ca²⁺]₀ in wild-type bMIP. Finally, the *P* of His⁴⁰ mutants did not increase further with decreases in pH₀ and/or [Ca²⁺]₀.

We suggest that the fundamental difference between the two studies may have been the state of the oocytes in which the bMIP was expressed. Thus bMIP in the oocytes in our study may have already had a maximal *P* under control conditions and, thus, been insensitive to decreases in pH₀ or [Ca²⁺]₀, much as the His⁴⁰ mutants in the oocytes in the study by Nemeth-Cahalan and Hall. What difference between the two populations of oocytes might account for the different behaviors of bMIP? It is well documented that the activity of cyclic nucleotide phosphodiesterases (PDEs) in *Xenopus* oocytes depends on the hormonal state of the mother. Indeed, it is possible that such interpopulation differences in PDE activity might explain why some investigators observed ion channel properties of AQP1 (29) whereas others did not (1). The COOH terminus of bMIP contains a protein kinase A/G consensus site, and protein kinase A alters the channel activity of bMIP reconstituted in bilayers (9).

**Osmolyte Permeability**

**Permeability to glycerol.** The glycerol permeability of the MIPs is controversial. On the one hand, expressing frog MIP (13, 14) or bMIP (this study) in *Xenopus* oocytes increases glycerol permeability. On the other hand, expressing rat MIP (28) or MIPfun (this study) does not. One possibility is that the expression levels of rat MIP and MIPfun were not high enough to engender appreciable glycerol permeability. Alternatively, the expression of certain MIPs may increase the area of oocyte membrane through which glycerol permeates or increase the expression of a glycerol transporter native to oocytes. Regarding the latter possibility, an unusual feature of the glycerol permeabilities of oocytes expressing frog MIP and bMIP is that these permeabilities are reduced by mercurials, whereas the water permeabilities are not. These results are consistent with the hypothesis that another transporter mediates the apparent glycerol permeabilities of frog MIP and bMIP. Schreiber et al. (22) found that activating cystic fibrosis transmembrane conductance regulator (CFTR) with cAMP in CFTR-expressing oocytes also activates a pathway for water and glycerol transport that is distinct from CFTR’s Cl⁻ permeability pathway. The authors hypothesized that activation of CFTR also activates an endogenous AQP homolog that is responsible for the water and glycerol permeability (23).

The glycerol permeability of native oocytes may reflect glycerol transport via a proteinaceous pathway(s), the expression of which may depend on several variables, such as the source and handling of the frogs and the handling of the oocytes. Indeed, some investigators report that oocytes have a substantial pCMBS-sensitive background permeability to glycerol, even in the absence of exogenous proteins (8), whereas we did not detect any HgCl₂-sensitive glycerol transport in our control oocytes.

Finally, experiments by Varadaraj et al. (26) indicate that MIP in its native environment in the lens does not significantly contribute to glycerol permeability. These authors measured similar glycerol permeabilities in vesicles of native membranes made with or without native protein from the lenses of mouse, frog, or rabbit. Their result shows that the native lens fiber cell membrane has very little glycerol permeability, despite high MIP expression.

**Permeability to CO₂.** Of all the analytes tested in our study (water, glycerol, urea, CO₂, and formate), only water had a permeability that increased markedly (i.e., ~40 times) with MIPfun expression. In addition, the JCO₂ flux, computed using the estimated surface area from capacitance measurements, increased slightly (30%) in oocytes expressing MIPfun compared with 46% in oocytes expressing hAQP1. Previously, Nakhoul et al. (19), assuming oocyte surface area to be the same in control and hAQP1-expressing oocytes, found that expressing hAQP1 increases the CO₂-induced...
acidification rate of oocytes by 40%. Cooper and Boron (6) obtained values of 0% (low hAQP1 expression levels) to 100% (high expression levels). We found that hAQP1 expression does not significantly increase oocyte surface area. Thus one cannot attribute the two earlier estimates of the effect of hAQP1 on relative CO₂ permeability to changes in absolute membrane surface area.

**Perspectives**

MIPfun is the first MIP cloned from a phylum more ancient than Amphibia. It is unusual among MIPs in having a high Pᵣ. The structural determinants for the relatively high Pᵣ of MIPfun are not known. Comparing sequences of MIPfun and other cloned MIPs can give a starting point for investigating why they have such different Pᵣ values.

It is intriguing to speculate whether the high Pᵣ of MIPfun is related to the physiology of the killifish. This species is euryhaline and can tolerate large variations in the osmolality of its environment. Perhaps the high Pᵣ of MIPfun allows the ocular lens to withstand osmolality changes and/or allows MIPfun to mediate efficient fluid circulation according to the model of Mathias et al. (15). It would be interesting to measure the rate of fluid circulation in the fish lens and compare it with its mammalian counterpart. This approach could possibly help answer the longstanding question: what is the physiological importance of Pᵣ of MIPs in lens physiology?

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