We have cloned a novel aquaporin (AQP) from *Xenopus laevis* oocytes, which we have provisionally named AQPxlo. The predicted protein showed highest homology (39–50%) to aquaglyceroporins. Northern blot analysis showed strong hybridization to an ~1.4-kb transcript in *X. laevis* fat body and oocytes, whereas a weaker signal was obtained in kidney. We injected *in vitro* transcribed cRNA encoding AQPxlo into *Xenopus* oocytes for functional characterization. AQPxlo expression increased osmotic water permeability ($P_f$), as well as the uptake of glycerol and urea. However, AQPxlo excluded larger polyols and thiourea. An alkaline extracellular pH (pH$_e$) increased $P_f$ and to a lesser extent urea uptake but not glycerol uptake. Remarkably, low HgCl$_2$ concentrations (0.3–10 µM) reduced $P_f$ and urea uptake, whereas high concentrations (300–1000 µM) reversed the inhibition. We propose that AQPxlo is a new AQP paralogue unknown in mammals.

**Experimental Procedures**

**Cloning of AQPxlo**

By searching the GenBank™ data base for sequences with homology to known AQPs, we identified two novel EST sequences (GenBank™ accession numbers AW200125 and AW199821) from *X. laevis* oocytes. To obtain the full-length coding region, we performed PCR on an *X. laevis* oocyte library (X. laevis oocyte 5’-STRETCH cDNA xTriplEx library, Clontech Laboratories, Inc., Palo Alto, CA) using primers specific to the coding sequence and to the vector sequences flanking the 5’ and 3’ ends of the insert. The resulting PCR products were subcloned into a pCR 2.1 TOPO-vector (Invitrogen) and sequenced. We performed end-to-end PCR with primers designed to the 5’ and 3’ regions flanking the open reading frame to obtain a continuous cDNA fragment containing the entire coding region. The sense primer was 5’-AATGACCGATGTTCGCTGCGGACTGG and the antisense primer was 5’-ACTAGTGGCGAATCCAAGAGCGAGATGCCC, where the underlined sequence corresponds to engineered XhoI (sense) and Spel (antisense) restriction sites. The resulting PCR product was cloned into pCR 2.1 TOPO-vector and sequenced. All sequencing was performed by the Keck Biotechnology Resource Laboratory (Boyer Center for Molecular Medicine, Yale University). The cDNA sequence was deposited into the GenBank™ (accession number AY120934). We have provisionally named the clone AQPxlo (for *Xenopus laevis* oocyte).

**Cloning of Rat AQP7**

For the sake of comparing the functional properties of AQPxlo to those of a related mammalian AQP, we cloned rat AQP7. We performed PCR using sense primer 5’-GTGACCCTCGGGGAAATCATG and antisense primer 5’-GGATGCTCATGAGGGG. As a template we used rat kidney cDNA, which was a kind gift from Dr. Inyeong Choi (Yale University). The PCR product was subcloned into a pCR 2.1 TOPO-vector.
directions. Total RNA (10 μg/μl) at a solution flow of 4 ml/min was incubated for 5 min. The area of the oocyte was obtained from the measured volume of each oocyte (calculated from the surface area of a sphere of equivalent size).

Northern Blot

Total RNA from oocytes, lung, skin, fat body, muscle, kidney, urinary bladder, stomach, intestine, and liver was extracted from adult female X. laevis using Trizol reagent (Invitrogen), according to manufacturer’s directions. Total RNA (10 μg) was resolved by formaldehyde-agarose (1%) denaturing gels and blotted to positively charged nylon membrane (Hybond XL, Amersham Biosciences) by capillary elution. Blots were prehybridized by incubation in ExpressHyb hybridization solution (Clontech Laboratories, Inc) at 65 °C for 30 min and then hybridized with an α-32P-labeled probe (Random Primer labeling kit, Invitrogen) corresponding to the full-length AQPd9 sequence at 65 °C for 90 min. The blots were subsequently exposed to film at −70 °C.

Oocyte Expression

The AQPd9 fragment was excised from pCR 2.1 TOPO using XhoI and SpeI and subcloned into a KSM oocyte expression vector, in which the multiple cloning site is surrounded by the 5′ and 3′-untranslated regions for Xenopus β-globin. This expression vector was a kind gift of Dr. William Joiner (Yale University). Rat AQP7 was excised from pCR 2.1 TOPO using XhoI and HindIII and ligated into KSM. Capped cDNA was transcribed in vitro using T3 Message Machine kit (Ambion, Austin, TX).

Stage V–VI oocytes were defolliculated with collagenase type Ia (Sigma) and stored in OR3 medium (Sigma) as described previously (3). On the day after isolation, oocytes were injected with 50 nl of cRNA encoding either AQPd9 (0.4 μg/μl), rat AQP3 (0.2 μg/μl), rat AQP7 (0.2 μg/μl), the rat urea transporter UT-A2 (0.2 μg/μl), or human AQP1 (0.05 μg/μl). Control oocytes were injected only with water. The cDNA encoding AQP1 (in the Xenopus expression vector pXAG) was a gift from Dr. Peter Agre (The Johns Hopkins University, Baltimore, MD); AQP3 (in pSPORT) was a gift from Dr. Lawrence Palmer (Cornell University, Ithaca, NY); and UT-A2 (in pBluescript) was a gift from Dr. Craig Smith (University of Manchester, UK).

Measurement of Oocyte Water Permeability

We used a volumetric assay to measure the osmotic water permeability (P) of oocytes injected with various cRNAs or water (control). The oocyte was placed in a perfusion chamber and illuminated from below. We acquired images of the oocyte silhouette every 2 s through a video camera attached to a stereomicroscope. The images were saved to a disk and analyzed using Optimas software version 5.2 (Media Cybernetics, Silver Spring, MD). The oocyte volume was calculated from the cross-sectional area of the oocyte, assuming the oocyte to be a perfect sphere. The volume was calibrated using, as an underwater standard, a sphere of equivalent size.

We calculated an apparent rate constant for the uptake of each oocyte, which we used to calculate an initial rate of increase in relative oocyte volume. The initial rate of increase in relative oocyte volume was calculated by subjecting the oocyte to swelling by exposing it to a hypo-osmotic solution (100 mOsm) in ND96 solution. After allowing the oocyte to recover for 90 min, we superfused the oocyte with ND96 solution. Measurement of isotope uptake was started by replacing the ND96 solution with 700 μl of uptake solution, which consisted of ND96 containing 1 μM unlabeled analyte and 1 μM [3H]analyte. Oocytes were incubated on a horizontal shaker for 2 min at room temperature. In preliminary experiments, we monitored the time course of the 3H uptake to ensure that the uptake was linear during the first 2 min. Radioisotope uptake was stopped by washing oocytes three times in ice-cold ND96 containing 10 μM of the unlabeled analyte. Individual oocytes were transferred to scintillation vials and lysed in 400 μl of 10% SDS with continuous shaking. The 3H activity of individual oocytes was assessed by liquid scintillation counting (LKB-Wallac Rackbeta, Turku, Finland).

We calculated the osmotic water permeability (P) of oocytes using the following equation:

\[
P = \frac{d(V_{osm})/dt}{d(V_{osm})/dt} = \frac{d(V_{w})/dt}{d(V_{w})/dt} = \frac{dV_{w}}{dt} = \frac{dV}{dt} = \frac{dV_{osm}}{dt} = \frac{dV_{w}}{dt}
\]

where (d/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.

Oocyte AQP

The osmotic water permeability was calculated using Equation 1 (4, 5), where (d(V_{osm})/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.

\[
P = \frac{d(V_{osm})/dt}{d(V_{osm})/dt} = \frac{d(V_{w})/dt}{d(V_{w})/dt} = \frac{dV_{w}}{dt} = \frac{dV}{dt} = \frac{dV_{osm}}{dt} = \frac{dV_{w}}{dt}
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S = \frac{dV}{dt} = \frac{dV_{osm}}{dt} = \frac{dV_{w}}{dt}
\]

where (d/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.

Radioisotope Uptake—We measured unidirectional influx of urea and glycerol in oocytes using [3H]-labeled urea ([3H]urea) and glycerol ([3H]-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 replacing the ND96 solution with 700 μl of uptake solution, which consisted of ND96 containing 1 μM unlabeled analyte and 1 μM [3H]analyte (37 kBq/μl) of the radioisotopically labeled analyte. Oocytes were incubated on a horizontal shaker for 2 min at room temperature. In preliminary experiments, we monitored the time course of the 3H uptake to ensure that the uptake was linear during this period. Radioisotope uptake was stopped by washing oocytes three times in ice-cold ND96 containing 10 μM of the unlabeled analyte. Individual oocytes were transferred to scintillation vials and lysed in 400 μl of 10% SDS with continuous shaking. The 3H activity of individual oocytes was assessed by liquid scintillation counting (LKB-Wallac Rackbeta, Turku, Finland).

We calculated the osmotic water permeability (P) of oocytes using the following equation:

\[
P = \frac{d(V_{osm})/dt}{d(V_{osm})/dt} = \frac{d(V_{w})/dt}{d(V_{w})/dt} = \frac{dV_{w}}{dt} = \frac{dV}{dt} = \frac{dV_{osm}}{dt} = \frac{dV_{w}}{dt}
\]

where (d/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.

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P = \frac{d(V_{osm})/dt}{d(V_{osm})/dt} = \frac{d(V_{w})/dt}{d(V_{w})/dt} = \frac{dV_{w}}{dt} = \frac{dV}{dt} = \frac{dV_{osm}}{dt} = \frac{dV_{w}}{dt}
\]

where (d/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.

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\]

where (d/dt) is the initial rate of increase in relative oocyte volume following a hypo-osmotic challenge; V is the initial oocyte volume; S is the actual surface area; Δw is the osmotic gradient across the oocyte membrane, and Vw is the molar volume of water.
**RESULTS**

**Sequence Data**—The cDNA encoding AQPxlo contains an open reading frame of 894 bp, which encodes 297 amino acids. Fig. 1A shows a sequence alignment with AQPxlo and human AQP3, human AQP10, and the bacterial glycerol facilitator GlpF. Fig. 1B shows a dendrogram containing members of all vertebrate AQPs identified to date (AQP1 to AQP6). The tip of the pH electrode contained a pH-sensitive liquid membrane (Hydrogen Iono-40612.

**Measurement of Intracellular pH**

An oocyte, placed in a perfusion chamber, was superfused at a rate of 4 ml min⁻¹. pH-sensitive electrodes were fabricated and used as described previously (7, 8). Briefly, the oocyte was impaled with two microelectrodes, one for measuring the membrane potential (\(V_m\)) and the other one for measuring intracellular pH (pHi). The pH calibration was performed as described previously (9).

**Effect of pHo Changes on Water Permeability**

The water permeability of oocytes expressing AQPxlo-expressing oocytes increased 6-fold, compared with controls. Thus, AQPxlo appears to function as a water channel when overexpressed in the oocyte. Compared with oocytes expressing AQP1, the water permeability of oocytes expressing AQPxlo is lower. However, because we have no information about the actual number of functional channels at the membrane, our data do not permit a direct comparison of the water permeabilities of the different AQPs.

**Effect of pHi, Changes on Water Permeability**—Zeuthen and Klaerke (10) have shown that the \(P_f\) and the glycerol permeability of AQP3 are virtually 0 at extracellular pH (pHi) values below 6 and rise to maximum values at pHi 6.5–7.0. Fig. 4 shows how changes in pHi affect \(P_f\) in control oocytes and in oocytes expressing AQPxlo. As pHi increases from 5.0, the \(P_f\) of AQPxlo-expressing oocytes increases markedly. Maximal \(P_f\) is observed at pHi 9.5, which is 4-fold higher than the value at pHi 7.5 and 8-fold higher than at 5.0. On the other hand, increasing pHi from 9.5 to 10.5 or 11.5 caused small decreases in \(P_f\). In water-injected oocytes, increasing pHi from 5.0 to 11.5 did not have a statistically significant effect on \(P_f\).

**Northern Analysis**—Fig. 2 shows a Northern blot analysis of total RNA from multiple tissues from *Xenopus*. A strong ~1.4-kb signal is present in oocytes and fat body, and a somewhat weaker signal is seen in kidney. We detected no signal in lung, skin, skeletal muscle, urinary bladder, stomach, intestine, or liver.

**Water Permeability**—Fig. 3 shows the osmotic water permeabilities of control oocytes and of oocytes expressing human AQP1, rat AQP3, rat AQP7, and AQPxlo. For each clone, we injected the minimum amount of cRNA/oocyte required to produce a near-maximum \(P_f\). The water channel pore diameter of these AQPs is 12 Å, and the glycerol permeability to AQPxlo-expressing oocytes was 30-fold higher than that of the archetypal water channel AQP1. \(P_f\) was 30-fold higher in water-injected oocytes, whereas in oocytes expressing the aquaglyceroporins AQP3 and AQP7, \(P_f\) was 15- and 4-fold higher, respectively. The \(P_f\) of AQPxlo-expressing oocytes increased 6-fold, compared with controls. Thus, AQPxlo appears to function as a water channel when overexpressed in the oocyte. Compared with oocytes expressing AQP1, the water permeability of oocytes expressing AQPxlo is lower. However, because we have no information about the actual number of functional channels at the membrane, our data do not permit a direct comparison of the water permeabilities of the different AQPs.
significant changes over the range from 5.0 to 10.5 implies that, over this range, the effects on the $P_f$ of AQPxlo-expressing oocytes are mediated by extracellular protons.

We also examined $P_f$ at pH$_i$ values of 7.5 and 9.5 in oocytes expressing rAQPxlo. As summarized in Fig. 5A, $P_f$ is significantly increased at alkaline pH$_i$ in AQPxlo-expressing oocytes but not in rAQPx3- or rAQPx7-expressing oocytes. In this batch of oocytes, the $P_f$ of water-injected oocytes is significantly increased at alkaline pH$_i$, suggesting that they may have had a higher level of endogenous AQPxlo expression than those in Fig. 4.

Effect of pH$_i$ Changes on Glycerol and Urea Uptake—Because our sequence analysis placed AQPxlo among the aquaglyceroporins, we measured the uptake of glycerol and urea into water-injected oocytes, as well as oocytes expressing rAQPx3, rAQPx7, or AQPxlo. As shown in Fig. 5B, all of the AQPs tested substantially increased glycerol uptake (6–7-fold compared with water-injected oocytes). By contrast, Fig. 5C shows that only AQPxlo produced a substantial increase in urea uptake (11-fold). This compares to a 2.6-fold increase in urea uptake seen in rAQPx3-expressing oocytes. In contrast to previous reports (11), we did not observe an increase in the rate of urea uptake in rAQPx7-expressing oocytes.

Because raising pH$_i$ from 7.5 to 9.5 substantially increased $P_f$ in AQPxlo-expressing oocytes, we examined the effect of this pH$_i$ on glycerol and urea uptake. As shown in Fig. 5B, increasing pH$_i$ from 7.5 to 9.5 did not affect the rate of glycerol uptake in any of the oocytes expressing an AQP. Curiously, only the water-injected oocytes showed a significant increase in glycerol uptake at alkaline pH$_i$, perhaps reflecting the presence of an endogenous pH-sensitive glycerol transport pathway other than AQPxlo.

In oocytes expressing AQPxlo, the effect on urea uptake of raising pH$_i$ seemed to be intermediate between that on $P_f$ (2.7-fold; see Fig. 5A) and on glycerol uptake (no effect; see Fig. 5B). As shown in Fig. 5C, raising pH$_i$ from 7.5 to 9.5 caused a significant increase in urea uptake (1.5-fold) in oocytes expressing AQPxlo. Raising pH$_i$ failed to increase urea uptake in any of the other groups of oocytes tested.

Effect of HgCl$_2$ on Water Permeability—Fig. 6 shows how HgCl$_2$ affects the $P_f$ of water-injected oocytes and oocytes expressing hAQPxlo or AQPxlo. For AQPxlo-expressing oocytes the interaction with HgCl$_2$ appears biphasic. At low concentrations of HgCl$_2$ (0.3–10 μM) the $P_f$ is low, whereas at high HgCl$_2$ concentrations (300–1000 μM), $P_f$ is similar to the value obtained in the absence of HgCl$_2$. In water-injected oocytes, 1 μM HgCl$_2$ produced a small but statistically significant inhibition of $P_f$. In contrast, the effects of HgCl$_2$ on the water permeability of AQPxlo-expressing oocytes are monophasic. Correcting for the background $P_f$ in water-injected oocytes, 0.5 μM HgCl$_2$ reduced the AQPxlo-dependent $P_f$ by 78%, and 300 μM HgCl$_2$ reduced the AQPxlo-dependent $P_f$ by 82%.

To investigate whether HgCl$_2$ has time-dependent effects on $P_f$, we added 1 or 300 μM HgCl$_2$ and then measured $P_f$ after 2, 5, and 20 min. The time of preincubation did not affect $P_f$ at either concentration of HgCl$_2$ (not shown).

Because alkaline pH$_i$ increases the $P_f$ of AQPxlo-expressing oocytes (Fig. 4), we investigated whether HgCl$_2$ at low concentration (1 μM) still blocks $P_f$ when pH$_i$ is increased from 7.5 to 9.5. Because HgCl$_2$ may act as a Cl$^-$-OH$^-$ ionophore (12), we decided not to examine $P_f$ at different pH$_i$ values in the presence of HgCl$_2$. Instead, we first pretreated AQPxlo-expressing oocytes with 1 μM HgCl$_2$ for 5 min in iso-osmotic ND96 at pH$_i$ 7.5. We then exposed the oocytes to either (i) a hypo-osmotic solution at pH$_i$ 7.5 with HgCl$_2$, (ii) a 2-min wash with an iso-osmotic HgCl$_2$-free solution at pH$_i$ 7.5, followed by the comparable hypo-osmotic solution, or (iii) a 2-min wash with an iso-osmotic HgCl$_2$-free solution at pH$_i$ 9.5, followed by the comparable hypo-osmotic solution. Fig. 7A shows that, at pH$_i$ 7.5, HgCl$_2$ reduced $P_f$ by ~70% whether it was present or absent from the hypotonic swelling solution. In contrast, when $P_f$ was measured at pH$_i$ 9.5, HgCl$_2$ pretreatment reduced $P_f$ by only 43%.

Fig. 7A shows that, in AQPxlo-expressing oocytes, inhibition of $P_f$ by HgCl$_2$ is not reversed by washing. We therefore determined whether inhibition by HgCl$_2$ could be reversed using a reducing agent by superfusing an oocyte for 5 min with 5 mM β-mercaptoethanol (after first measuring $P_f$ before and after treatment with 1 μM HgCl$_2$). Fig. 7B shows that β-mercaptoethanol treatment completely reversed the inhibition of AQPxlo by HgCl$_2$.

Effect of Inhibitors on Glycerol and Urea Uptake—We investigated whether HgCl$_2$ has a similar biphasic inhibition profile on glycerol and urea uptake as it has on $P_f$. (Fig. 6). Fig. 8, A and B, shows that a low (1 μM) concentration of HgCl$_2$ reduces both glycerol and urea uptakes of AQPxlo-expressing oocytes. In contrast, a high (300 μM) HgCl$_2$ concentration of HgCl$_2$ inhibits glycerol uptake but has no effect on urea uptake. Thus, HgCl$_2$ acts with a different concentration profile on $P_f$ and urea uptake than on glycerol uptake. In water-injected oocytes or rAQPx3-expressing oocytes, 300 μM HgCl$_2$ had no effect on glycerol uptake. In contrast, in water-injected oocytes, urea uptake was reduced by the low (1 μM) concentration of HgCl$_2$ but was increased by the high (300 μM) HgCl$_2$ concentration.

Of the 11 mammalian AQPs cloned to date, only AQP9 (13) and AQP10 (14) show substantial glycerol and urea permeabilities, raising the possibility that AQPxlo is the amphibian orthologue of one of these. Because phloretin blocks the urea permeabilities of both AQP9 and AQP10, we investigated the effect of phloretin (500 μM) on the urea uptake of AQPxlo. Fig. 8C shows that phloretin strongly inhibits urea uptake in both water-injected oocytes and oocytes expressing the rat urea transporter rUT-A2 but has no effect on AQPxlo-expressing oocytes.

Measurement of Reflection Coefficient $\sigma$—Fig. 9A shows the reflection coefficient of AQPxlo-expressing oocytes for glycerol, xylitol, ribitol, mannotol, urea, and thiourea. Of these, glycerol
is the only solute with a $\sigma$ (0.57 ± 0.09) substantially smaller than 1, indicating that glycerol permeates AQPxlo-expressing oocytes. This result is in good agreement with the isotopic flux data in Fig. 5B. The $\sigma$ obtained for urea in AQPxlo-expressing oocytes (0.95 ± 0.02) is only slightly less than 1, but the difference is statistically significant ($p < 0.0089$). Neither xylose, ribitol, mannitol, nor thiourea had $\sigma$ values significantly lower than 1, indicating that they are not able to permeate AQPxlo-expressing oocytes.

**Fig. 4.** Effect of pH$_o$ changes on $P_f$ and pH$_i$ of AQPxlo-expressing oocytes. A, $P_f$. Oocytes were preincubated for 2 min at the appropriate pH$_o$ before switching to a hypotonic solution with the same pH. Closed circles, AQPxlo-expressing oocytes; closed diamonds, water-injected oocytes. $n$ = 5 (H$_2$O) and 8–14 (AQPxlo). B, pH$_i$. Microelectrodes were used to measure pH$_i$ and $V_m$ of oocytes exposed to solutions of different pH$_o$ values. Similar recordings were obtained in one other AQPxlo-expressing oocyte and two water-injected oocytes. A, asterisk indicates that the difference is statistically significant ($p < 0.05$) compared with the value obtained at pH$_o$ 7.5 in a two-tailed t test. Vertical bars indicate S.E. but are omitted when they are smaller than the symbol.

**Fig. 5.** Effect of pH$_o$ changes on $P_f$, glycerol, and urea uptake in oocytes expressing different AQPs. A, $P_f$. B, glycerol uptake. C, urea uptake. Measurements were carried out on oocytes injected with water or expressing AQP3, AQP7, or AQPxlo. Dark gray bars, pH$_o$ 7.5; light gray bars, pH$_o$ 9.5. $n$ = 7–12. Error bars, S.E. Asterisk indicates that the difference between control and AQP-expressing oocytes, at the relevant pH$_o$, is statistically significant ($p < 0.05$). Dagger indicates that the difference between pH$_o$ 7.5 and 9.5, comparing oocytes expressing the same AQP, is statistically significant in a two-tailed t test.

**Fig. 6.** Effect of HgCl$_2$ on $P_f$. Oocytes injected with water (closed diamonds) or expressing either AQP1 (open squares) or AQPxlo (closed circles) were treated with the indicated concentrations of HgCl$_2$. Data are expressed as % of control (i.e. absence of HgCl$_2$, indicated by a dotted line). $n$ = 6–9 (H$_2$O), 4–6 (AQP1), and 5–12 (AQPxlo). Error bars, S.E. Asterisk indicates that the difference between (un-normalized) values in HgCl$_2$ and controls is statistically significant ($p < 0.05$) in a two-tailed t test.

**Fig. 7.** HgCl$_2$ inhibition of $P_f$, effect of pH$_o$ changes, and reversibility. A, effect of pH$_o$. AQPxlo-expressing oocytes were either untreated or pretreated with 1 $\mu$m HgCl$_2$ at pH$_o$ 7.5 for 5 min. Subsequently, $P_f$ was measured (i) in untreated oocytes at pH$_o$ 7.5 or 9.5 (black bars); (ii) in pretreated oocytes in the continued presence of HgCl$_2$ at pH$_o$ 7.5 (light gray bar); or (iii) in pretreated oocytes in the absence of HgCl$_2$ either at pH$_o$ 7.5 or 9.5 (dark gray bars). B, reversibility of HgCl$_2$ inhibition. $P_f$ was measured in the same oocyte before HgCl$_2$ treatment, after treatment with HgCl$_2$ (1 $\mu$m), and after treatment with $\beta$-mercaptoethanol (5 mM). Error bars, S.E. Asterisk indicates that the difference between HgCl$_2$-treated and -untreated oocytes is statistically significant ($p < 0.05$) in a two-tailed t test.
Fig. 8. Effect of HgCl₂ and phloretin on glycerol and urea uptake. A, effect of HgCl₂ on glycerol uptake in water-injected oocytes and oocytes expressing AQPxlo. B, effect of HgCl₂ on urea uptake in water-injected oocytes and in oocytes expressing AQPxlo. Black bars indicate control condition; light gray bar, 1 μM HgCl₂; and dark gray bars, 300 μM HgCl₂. C, effect of phloretin on urea uptake in water-injected oocytes and oocytes expressing UT-A2 or AQPxlo. Black bars indicate control condition; gray bars indicate 500 μM phloretin. A and B, error bars, S.E. N = 6–16; C, n = 4–8. Asterisk indicates that the difference between that value and water-injected control is statistically significant (p < 0.05) in a two-tailed t test. Dagger indicates that the difference between that value and no-drug control (black bar) is statistically significant (p < 0.05) in a two-tailed t test.

Fig. 9. Reflection coefficients σ for osmolytes. A, σ measured for straight chain polyols (glycerol, xylitol, ribitol, and mannitol), urea and thiourea in AQPxlo-expressing oocytes. B, σ measured for glycerol and urea in water-injected oocytes (black bars) and oocytes expressing AQP3 (light gray bars) or AQPxlo (dark gray bars). A, error bars, S.E. N = 6; B, n = 4–6. Asterisk indicates that the value of σ is significantly (p < 0.05) smaller than 1.

Discussion

We have cloned from Xenopus oocytes a novel AQP that is predominantly expressed in fat body, oocytes, and kidney. AQPxlo is permeable to water, glycerol, and urea but excludes the larger polyols xylitol, ribitol, and mannitol, as well as thiourea. Judging from its strong expression in fat body, AQPxlo may play a role in the triglyceride metabolism in this tissue by promoting glycerol transport. The fat body, an organ associated with the anterior testis or ovary, serves as a storage organ for fat and undergoes marked changes in size in many anurans, depending on the season and the nutritional status of the animal.

Sensitivity to Changes in pHᵢ—The Pᵢ of AQPxlo falls sharply as one lowers pHᵢ below 9.5 and more gradually as one raises pHᵢ above 9.5 (Fig. 4A). Regarding other AQPs, both the Pᵢ and glycerol permeability of AQP3 fall sharply at acidic pHᵢ values, with a pK of 6.4 for Pᵢ and 6.1 for glycerol (10). According to one report, the Pᵢ of bovine major intrinsic protein (AQP0) is maximal at pHᵢ 6.5 and falls at both more acidic and more alkaline pHᵢ values (15).

The biphasic pHᵢ dependence of Pᵢ in AQPxlo-expressing oocytes is consistent with the idea that AQPxlo has more than one titratable site that influences the permeability pathway for water, and that the sum of effects is positive in its influence from pH 5.0 to 9.5 and negative above pH 9.5. Because oocyte pHᵢ is remarkably resistant to pHᵢ changes between pH 5.0 and 10.5 (Fig. 4B), it is likely that these sites are located where they are accessible to extracellular protons.

In stark contrast to Pᵢ, which increased 4-fold as we raised pHᵢ from 7.5 to 9.5, glycerol uptake in AQPxlo-expressing oocytes was unaffected (Fig. 5B). The effect of HgCl₂ on urea uptake in AQPxlo-expressing oocytes falls between that of Pᵢ and glycerol uptake in that increasing pHᵢ from 7.5 to 9.5 produced a small but significant increase in the rate of urea uptake (Fig. 5C). Our observation that the movement of the small H₂O is very pHᵢ-sensitive, whereas the uptake of the much larger glycerol is not pHᵢ-sensitive at all, suggests that pHᵢ sensitivity does not depend in any simple way on molecular size. Rather, the complex pattern of pHᵢ sensitivities may reflect interactions between the transported species and specific titratable residues lining the channel.

Sensitivity to HgCl₂—The effect of HgCl₂ on the Pᵢ (Fig. 6) and urea uptake (Fig. 8B) of AQPxlo-expressing oocytes is novel. A low (1 μM) HgCl₂ concentration reduces both Pᵢ and urea uptake, whereas a higher (300 μM) concentration reverses the inhibition. In contrast, HgCl₂ inhibited glycerol uptake equally well at both low (1 μM) and high (300 μM) HgCl₂ concentrations (Fig. 8A).

The simplest way to interpret the data is to assume that the action of HgCl₂ involves at least two reactive groups. One (a high affinity site) reacts at low concentrations of Hg²⁺, resulting in a marked decrease in the permeability of AQPxlo to H₂O, glycerol, and urea. The other (a low affinity site) reacts only when the HgCl₂ concentration is elevated, and its effect is to reverse only the inhibition of Pᵢ and urea uptake. Presumably the reaction of the first Hg²⁺ partially obstructs the pore of AQPxlo, reducing the transport of all substances. The second Hg²⁺ might then cause a conformational change that relieves the obstruction just enough to let H₂O and urea (but not glycerol) pass. We do not know the nature of the Hg²⁺-reaction sites, but they are likely to be thiol groups of cysteine residues, as is the case for several other AQPs. AQPxlo has six cysteines, at positions 24, 40, 74, 105, 174, and 210. Preston et al. (16) showed that of the four cysteines present in AQP1, Cys¹⁸⁹ mediates the blocking effect of mercurials. AQPxlo has a tyro-
sine (Tyr²¹²) at the position equivalent to Cys¹⁸⁹ in AQP1 but has a cysteine nearby (Cys²¹⁰).

Interestingly, Yasui et al. (17) showed that HgCl₂ causes an ~10-fold increase in the Pᵢ of oocytes expressing human AQP6, and also induces an ion conductance. The authors showed that the double mutant Cys¹⁵⁵/Cys¹⁸⁹ is unresponsive to stimulation by HgCl₂. AQP%xlo has a cysteine (Cys¹⁷⁴) at the position equivalent to Cys¹⁵⁵ in AQP6 (Cys¹⁸⁹ in AQP1), but has the aforementioned Tyr²¹² at the position equivalent to Cys²¹⁰ in AQP6 (Cys¹⁸⁹ in AQP1). Thus, of the six cysteine residues in AQP%xlo, Cys¹⁷⁴ and Cys²¹⁰ are good candidates for HgCl₂ action sites.

Does AQP%xlo Contribute to Transport in Native Oocytes?—Because the water permeability of native Xenopus oocytes is, at most, low. For example, the other hand, the AQPxlo protein may not be expressed at the cell membrane or at all. Indeed, oocytes contain large amounts of stored mRNA that are not translated into protein until the egg starts developing. Our data indicate that native AQP%xlo expression in the oocyte is, at most, low. For example, the Pᵢ of water-injected oocytes showed little (Fig. 5A) or no (Fig. 4) change in response to changes in pHᵢ. Also, glycerol uptake in water-injected oocytes was not blocked by HgCl₂. On the other hand, HgCl₂ at 1 μM reduces both Pᵢ and urea uptake in water-injected oocytes, consistent with native AQP%xlo expression. It is possible that if oocytes express the AQP%xlo protein at the cell membrane, then the level of expression might vary among batches of oocytes.

However, native AQP%xlo might contribute significantly to transport in oocytes heterologously expressing other membrane proteins. Several groups (18–20) have reported examples in which the heterologous expression of a membrane protein in Xenopus oocytes has led to the up-regulation of native Xenopus membrane proteins. For example, the heterologous overexpression of any of several membrane proteins leads to the appearance of a native nonspecific cation channel in Xenopus oocytes (18). In addition, expression of rBAT or 4F2hc in Xenopus oocytes induces amino acid transport, although the proteins themselves do not possess intrinsic transport activity (19). Instead, they associate with native Xenopus membrane proteins to form a heteromeric amino acid transporter (20).

The up-regulation of endogenous AQP%xlo could account, at least in part, for novel transport activity observed in oocytes heterologously expressing two other membrane proteins. First, expressing major intrinsic protein (AQP9) in oocytes leads to an increase in glycero permeability (21–23), even though major intrinsic protein in its native environment in the lens does not contribute to significant glycero permeability (24). Second, Schreiber et al. (25) have shown that IBMX triggers an increase in water and glycero permeability in oocytes expressing CFTR. In principle, the up-regulation of AQP%xlo could account for this observation. On the other hand, Schreiber et al. (2) eliminated the IBMX-stimulated water and glycero permeabilities by injecting antisense oligonucleotides directed against a Xenopus AQP3 and concluded that CFTR up-regulates endogenous xAQP3. We were unable to obtain from oocytes a PCR product corresponding to xAQP3²⁶; however, it is possible that the Xenopus used by Schreiber et al. (2) contained a significant amount of mRNA encoding xAQP3, whereas those used by us did not.

Is AQP%xlo a New Aquaporin?—Our sequence and functional data indicate that AQP%xlo is an aquaglyceroporin. Four paralogous aquaglyceroporins are known in mammals, AQP3s 3, 7, 9, and 10. The dendrogram in Fig. 1 suggests that AQP%xlo is most closely related to AQP3 and AQP10, sharing ~45% amino acid sequence identity with mammalian and Xenopus AQP3 and ~45% identity with AQP10. This level of sequence similarity is less than seen between mammalian AQP2 and AQP5 (~60%), which are recognized as distinct AQP paralogues. Thus, a comparison of deduced amino acid sequences suggests that AQP%xlo represents a new paralogous AQP. However, searching the human and mouse genome data bases has thus far failed to reveal a mammalian orthologue of AQP%xlo.

The permeability properties of AQP%xlo are unique. Unlike AQP3 and AQP7, AQP%xlo is permeable to urea. Unlike AQP9 (26), AQP%xlo does not serve as a pathway for mannitol or thiothrea and is not sensitive to inhibition by phloretin. Unlike the recently cloned AQP10 (14), which takes up glycerol 7-fold faster than urea, AQP%xlo takes up glycerol and urea equally fast (as measured by ¹⁴C uptake). Also, unlike AQP10, whose urea uptake is blocked by phloretin, AQP%xlo-mediated urea uptake is not inhibited by phloretin. Thus, the conclusion that AQP%xlo is a new AQP parologue is supported by our observation that the permeability properties of AQP%xlo are unlike those of any of the known mammalian aquaglyceroporins.

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