

Aquaporin-1, Nothing but a Water Channel*

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Aquaporin-1 (AQP1) is a membrane channel that allows rapid water movement driven by a transmembrane osmotic gradient. It was claimed to have a secondary function as a cyclic nucleotide-gated ion channel. However, upon reconstitution into planar bilayers, the ion channel exhibited a 10-fold lower single channel conductance than in *Xenopus oocytes* and a 100-fold lower open probability ($<10^{-6}$) of doubtful physiological significance (Saparov, S. M., Kozono, D., Rothe, U., Agre, P., and Pohl, P. (2001) *J. Biol. Chem.* 276, 31515–31520). Investigating AQP1 expressed in human embryonic kidney cells, we now have shown that the discrepancy is not due to alterations of AQP1 properties upon reconstitution into bilayers but rather to regulatory processes of the oocyte expression system that may have been misinterpreted as AQP1 ion channel activity. As confirmed by laser scanning reflection microscopy, from 0.8 to 1.4×10^6 AQP1 copies/cell contributed to osmotic cell swelling. The proper plasma membrane localization was confirmed by observing the fluorescence of the N-terminal yellow fluorescent protein tag. Whole-cell patch clamp experiments of wild type or tagged AQP1-expressing cells revealed that neither cGMP nor cAMP mediated ion channel activity. The lack of significant CNG ion channel activity rules out a secondary role of AQP1 water channels in cellular signal transduction.

Water permeation across biological membrane is essential to life. Aquaporins (AQP)¹ found in all life forms including bacteria, plants, and mammals, facilitate rapid transmembrane water movement, which is driven by a transmembrane osmotic gradient (1). In humans 11 members of the family (AQP0–10) have been found so far (2). AQP1, abundant in hematopoietic tissue, kidneys, and choroid plexus epithelium, and transiently observed in periosteum, heart, vascular endothelium, and cornea during fetal development (3), is the best characterized member of the family (4–6). Although forming homotetramers, each ~28-kDa subunit with six transmembrane helices constitutes an individual water pathway (7, 8).

Although most aquaporins are widely believed to be selectively permeable to water (9–11), human AQP1 (AQP1) was claimed to have a secondary function as a cAMP-stimulated

cation channel (12) or a cGMP-gated cation channel (13). Whereas the cAMP channel activity was not reproducible by multiple research groups (5, 14), cGMP-mediated ion channel activity has been observed in reconstituted planar bilayers (5). The channel characteristics, however, were totally different. Single ion channel conductivities of 145 or 10 pS and open probabilities of 1:56,000 or 1:10⁶ were, respectively, obtained in the *Xenopus* oocyte expression system or with reconstituted planar bilayers. Whereas the former result suggest an impact on kidney proximal tubule function (15) and a role in transmembrane signaling (16), the latter allows us to conclude that AQP1-mediated ion conductance is of doubtful physiological importance. The tiny fraction of ion-conducting channels corresponds to less than one channel/cell even in AQP1-rich tissue such as red cells (17).

To clarify whether the discrepancy is due to (i) a cofactor required for ion channel activation but missing in the reconstituted system or (ii) a regulatory response of the oocyte that may up-regulate channels along with AQP1 expression, we have used mammalian cells (HEK293) to express AQP1 fused to or cotransfected with the yellow fluorescent protein (YFP). Protein expression and proper plasma membrane localization were controlled by confocal fluorescence microscopy. Water permeability was measured by observing the volume increase of the cells with laser scanning reflection microscopy (LSRM). cGMP-gated cation conductivity of AQP1 was tested with the whole-cell patch clamp technique.

MATERIALS AND METHODS

Plasmid Construction, Cell Culture, and Transfection—Human AQP1 DNA (a kind gift from Dr. Peter Agre) was subcloned into pEYFP-C1 vector (BD Clontech) to create the YFP-tagged AQP1 plasmid at the N terminus of human AQP1 (pST001). Residues 2 and 3 of AQP1 were replaced with SATQISSSSF. A second (wild type AQP1) expression vector was constructed by removing YFP (except the first Met) and the following linker segment from the pST001 plasmid (pST002).

Human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 10% fetal calf serum at 37 °C. 1×10^6 cells were plated on a polylysine-coated glass plate (30 mm diameter) in a 35-mm dish 1 day before transient transfections. LipofectAMINE 2000 (Invitrogen) was employed to perform transfections with 1–2 μ g of plasmid DNA. Two different sets of experiments were performed: (i) transfection with YFP-AQP1 (pST001) or (ii) cotransfection of AQP1 (pST002) with the pEYFP-C1 vector. In both cases, protein expression was controlled by exiting YFP at 488 nm and observing its fluorescence with a long-pass emission filter of 570 nm. One or 2 days after transfection, individualized cells were selected for swelling and patch clamp experiments.

Osmotic Permeability Measurement—Osmotic water permeability (P_f) was determined from the time course of cell swelling at 22 °C in response to a 2-fold dilution of the extracellular Dulbecco's modified Eagle's medium (300 mosm) with distilled water. Plasma membrane osmotic P_f was calculated for single adherent cells by Equation 1 (18),

$$P_f = (\tau(A/V)_0 V_w \phi_0)^{-1} \quad (\text{Eq. 1})$$

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¹ The abbreviations used are: AQP, aquaporin; YFP, yellow fluorescent protein; LSRM, laser scanning reflection microscopy; CNG, cyclic nucleotide-gated; CNB domain, cyclic nucleotide binding domain.

FIG. 1. **Plasma membrane localization of YFP-AQP1.** *A*, fluorescence image of cells transfected with YFP-AQP1 (pST001). *B*, trypan blue (0.5%) image of the same cells showing plasma membrane staining. *C*, overlay of images *A* and *B*.

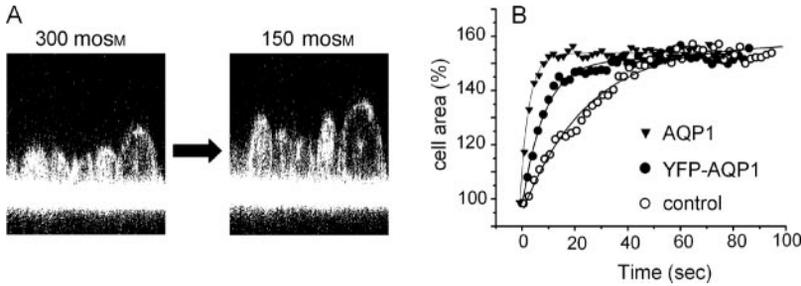
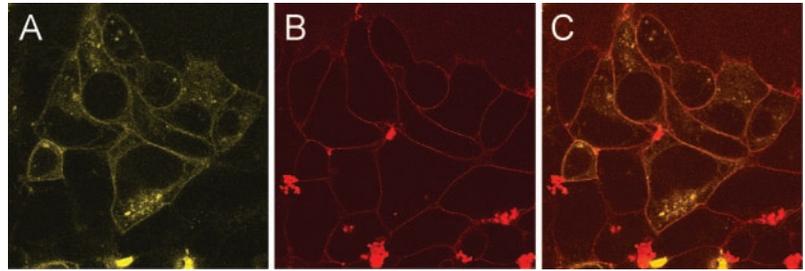


FIG. 2. **Osmotic water permeability measurements of HEK293 cells using LSRM.** *A*, vertical reflection image of cells on a glass plate. The osmolality change of the bath solution (300–150 mosM) induced cell swelling. *B*, time course of cell area increase upon osmotic challenge. *Closed circles*, YFP-AQP1-transfected cells; *triangles*, cells transfected with wild type AQP1; *open circles*, control cells.

where τ , $(A/V)_0$, and V_w are, respectively, the time constant of the exponential function, which describes cell volume change due to challenge by the transmembrane osmotic gradient, the initial ratio of the cell surface area (A) to volume (V), and the molar volume of water ($18 \text{ cm}^3/\text{mol}$). τ was obtained from a time series of cell images captured by LSRM (LSM410 or LSM510, Zeiss, Oberkochen, Germany) using a long-pass emission filter of 575 nm (x 63 water immersion objective). The x - z image of the cell was scanned every 2 s and processed by the KS-400 image analyzing software (Zeiss). The time dependent increase of cell areas a was fitted with the exponential Equation 2,

$$a(t) = a_{\min} + (a_{\max} - a_{\min})(1 - e^{-t/\tau}) \quad (\text{Eq. 2})$$

The x - y images were scanned in an $0.5\text{-}\mu\text{m}$ discrete for z direction to determine A and V . The integrated cell surrounding and cell area of all the images were regarded as A and V , respectively.

Ion Conductivity Measurements—Conventional whole-cell patch clamp experiments were performed to measure membrane currents of HEK293 cells. The pipettes were fabricated from 0.18-mm -thick wall borosilicate glass tubing with an outer diameter of 1.8 mm (Hilgenberg, Germany). After two pulling steps, the tip of the pipettes was coated with silicone (Sylgard) and then fire-polished. The bath solution contained 10 mM HEPES, 140 mM NaCl, 1 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM glucose adjusted to pH 7.1 with NaOH. The pipette solution consisted of 10 mM HEPES, 10 mM NaCl, 1.5 mM MgCl_2 , 10 mM EGTA, 2 mM CaCl_2 , and 125 mM K-l-glutamate adjusted to pH 7.1 with KOH. Whole-cell current was recorded using a patch clamp amplifier (EPC9, HEKA Elektronik, Lambrecht, Germany) operated by PulseFit software (HEKA). The data were sampled at 10 kHz and filtered at 2.9 kHz .

RESULTS

Expression of AQP1 and YFP-AQP1 in HEK293 Cells—Because the cGMP binding domain is believed to be located at the C terminus (13), YFP was introduced at the N terminus of AQP1. Aquaporins tagged with a fluorescent protein at the N terminus are known to retain their functional activity (19, 20). The fluorescence of the fusion protein indicated its location in the plasma membrane (Fig. 1). The untagged YFP was homogeneously distributed throughout the cell plasma (not shown). Its fluorescence was monitored to identify the HEK cells, which expressed the untagged (wild type) AQP1.

Number of Water Channels Expressed in the Plasma Membrane—Fig. 2*A* shows typical LSRM x - z scanning reflection images of HEK293 cells. Upon osmotic challenge, the cell height immediately began to increase. From a series of reflection images, the time course of cell swelling was calculated (Fig. 2*B*). The P_f of nontransfected cells ($51.9 \mu\text{m/s}$) was trebled by wild type AQP1 expression ($174.8 \mu\text{m/s}$) and doubled by

Cells	P_f	
	$\mu\text{m/s}$	P_f $200 \mu\text{M HgCl}_2$ $\mu\text{m/s}$
Control	51.9 ± 14.9 ($n = 8$)	42.2 ± 7.3 ($n = 3$)
AQP1	174.8 ± 31.6 ($n = 12$)	63.5 ± 10.2 ($n = 4$)
YFP-AQP1	117.5 ± 19.5 ($n = 5$)	44.6 ± 7.1 ($n = 3$)

YFP-AQP1 expression ($117.5 \mu\text{m/s}$). It was reduced to the P_f of control cells by $200 \mu\text{M HgCl}_2$, a known AQP1 inhibitor. HgCl_2 itself did not alter the swelling rate of control cells significantly (see Table I for a P_f summary). From the incremental P_f introduced by AQP1 ($174.8 - 51.9 = 122.9 \mu\text{m/s}$), the number of water channels (n)/cell was calculated using Equation 3,

$$n = P_f A / p_f = 122.9 \times 10^{-4} (\text{cm/s})$$

$$\times A (\text{cm}^2) / 6 \times 10^{-14} (\text{cm}^3/\text{s}) = 1.43 \times 10^6 \quad (\text{Eq. 3})$$

where A is the calculated cell surface area, and p_f is the known single channel water permeability (21). In the case of YFP-AQP1, n was equal to 7.71×10^5 .

Cation Channel Activity—Conventional whole-cell patch clamp measurements were performed to test for CNG-channel activity. The current was measured for 200 ms at voltages from -80 to $+40 \text{ mV}$ in 20-mV steps. Current traces and current-voltage (I - V) relationships of transfected cells are indistinguishable from control cells (Fig. 3). The slope at 0 mV corresponded to a conductivity of 0.25 nS ($n = 24$). $100 \mu\text{M}$ cGMP ($n = 7$) or cAMP ($n = 8$) in the internal pipette solution failed to activate ion channels. The membrane-permeable 8-bromo-cGMP (5 mM , Sigma-Aldrich) in the bath solution also did not induce an incremental increase in current ($n = 5$) (Fig. 3).

DISCUSSION

Our studies demonstrate that AQP1 is a water-selective channel lacking CNG channel activity. Expressed in HEK293 cells at a density of about 510 or 275 tetramers/ μm^2 , neither AQP1 nor YFP-AQP1 responded to stimulation by cGMP, cAMP, or 8-bromo-cGMP. Because the expression levels compare well with that in AQP-rich tissues (~ 325 tetramers/ μm^2 in red blood cells) (17), a physiological role of AQP1 in signal transduction (16) seems to be highly unlikely.

The results correspond well to the observation that the num-

FIG. 3. Whole-cell patch clamp recordings. Neither cGMP nor 8-Br-cGMP nor cAMP induced ion channel activity in AQP1- or YFP-AQP1-transfected HEK293 cells. *A*, current traces recorded from YFP-AQP1-transfected cells. Currents were evoked by 200 ms pulses from -80 to $+40$ mV in 20-mV steps. *B*, mean current-voltage (I - V) relationship in control cells (*top*), AQP1-transfected cells (*middle*), and YFP-AQP1-transfected cells (*bottom*).

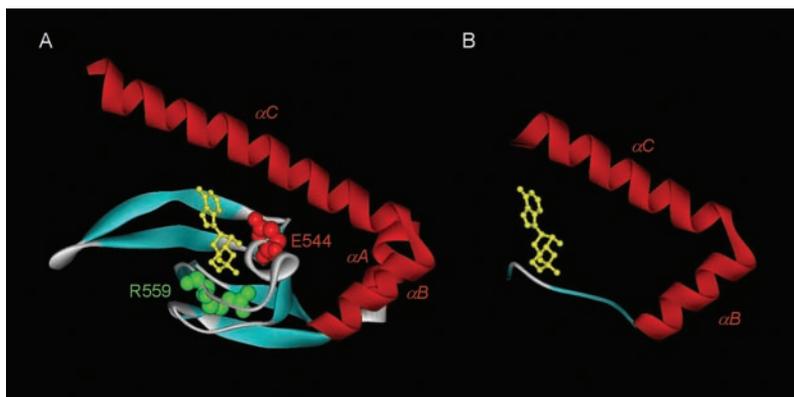
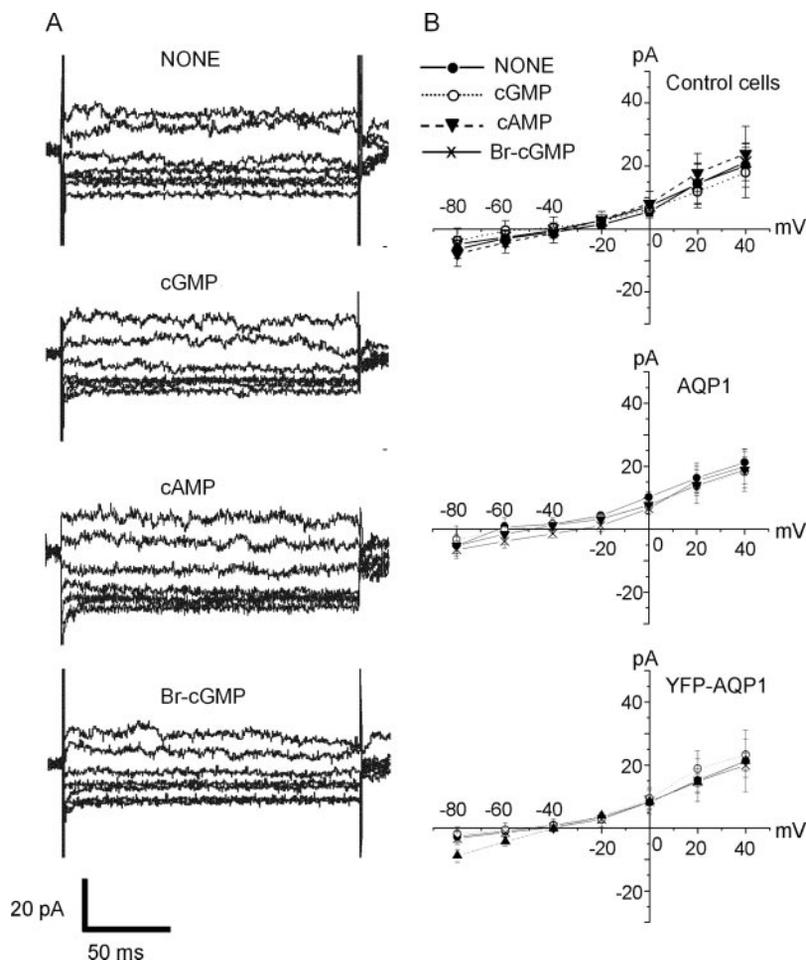


FIG. 4. Proposed cGMP binding domain in AQP1 lacks an essential part of the CNB domain. *A*, reported x-ray structure of CNB domain (25) from the *Escherichia coli* catabolite gene-activator protein (Protein Data Bank code, 1G6N). The highly conserved residues Glu-544 and Arg-559 (numbered in the sequence of catfish olfactory cyclic nucleotide-gated channel-1) are shown as space-filling models in red and green, respectively. The bound cyclic nucleotide is shown as a ball-and-stick model in yellow. *B*, homology model of the corresponding AQP1 region. The putative nucleotide binding site lacks the residues thought to be responsible for binding in the CNG family. The figure was created by WebLab Viewer software.

ber of ion channels is more than 1,000,000-fold lower than the number of water channels (5). In transfected HEK293 cells, which contain 3.6×10^5 AQP1 tetramers or 1.9×10^5 YFP-AQP1 tetramers/cell, this translates to less than one channel/cell. Given their tiny conductance of 10 pS (5), the opening of a channel would have remained unnoticed because of the background conductivity of the cells. In contrast, the existence of channels with parameters predicted from oocyte experiments (13) can be ruled out easily. Opening of one 145-pS wide ion channel/14,000 tetramers would have translated to 2.1 or 4.1 nS/per HEK293 cell transfected with YFP-AQP1 or AQP1, respectively. In both cases the incremental conductivity would have exceeded the background conductivity of transfected cells by about 1 order of magnitude.

Re-examination of the putative cGMP binding domain at the C terminus (13) revealed similarities with the sequence of

Ca^{2+} -binding proteins belonging to the calmodulin superfamily (22). The cyclic nucleotide binding (CNB) domain of CNG channels (Fig. 4A) includes ~ 120 amino acid residues forming three α helices and 8-stranded β -rolls structure (23). The putative cGMP binding domain of AQP1 lacks a large part of the CNB domain (Fig. 4B), among them such highly conserved and invariant residues as Glu-544 and Arg-559, which interact directly with cyclic nucleotides (23). Thus, the AQP1 C terminus seems to be unable to mimic the full-length CNB. The result is a drastic reduction in binding affinity that, in turn, leads to the activation of only an astonishingly tiny fraction of ion channels (5). Their number is too small to be detected in transfected HEK293 cells or to participate in signal transduction. The regulation of water channel function by cyclic nucleotides is restricted to an adjustment of AQP2 abundance in the plasma membrane by exocytosis (24). There is no indication for a sec-

ondary role of AQP as CNG channels in mammalian cells.

We conclude that the cGMP-triggered AQP1 ion channel characteristics reported in an oocyte expression system (13) were biased by a regulatory response of the oocyte. The function of AQP1 is limited to that of a water channel.

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