

The Activity of Human Aquaporin 1 as a cGMP-Gated Cation Channel Is Regulated by Tyrosine Phosphorylation in the Carboxyl-Terminal Domain[§]

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ABSTRACT

In addition to a constitutive water channel activity, several studies suggest Aquaporin-1 (AQP1) functions as a nonselective monovalent cation channel activated by intracellular cGMP, although variability in responsiveness between preparations has led to controversy in the field. Data here support the hypothesis that responsiveness of the AQP1 ionic conductance to cGMP is governed by tyrosine phosphorylation. Wild-type and mutant human AQP1 channels expressed in *Xenopus laevis* oocytes were characterized by two-electrode voltage clamp and optical osmotic swelling analyses. Quadruple mutation by site-directed mutagenesis of barrier hydrophobic residues (Val50, Leu54, Leu170, Leu174) to alanines in the central pore induced inward rectification of the ionic current and shifted reversal potential by approximately +10 mV, indicating increased permeability of tetraethylammonium ion. Introduction of cysteine at lysine 51 in the central pore (K51C) in a cysteineless template created new sensitivity to block of the conduc-

tance by mercuric ion. Mutations of candidate consensus sites and pharmacological manipulation of serine and threonine phosphorylation did not alter cGMP-dependent responses; however, mutation of tyrosine Y253C or pharmacological dephosphorylation prevented ion channel activation. Modification of Y253C by covalent addition of a negatively charged group [2-sulfonatoethyl methanethiosulfonate sodium salt (MTSES)] rescued the cGMP-activated conductance response, an effect reversed by dithiothreitol. Results support the proposal that phosphorylation of tyrosine Tyr253 in the carboxyl terminal domain, confirmed by Western blot, acts as a master switch regulating responsiveness of AQP1 ion channels to cGMP, and the tetrameric central pore is the ion permeation pathway. These findings advance resolution of a standing controversy and expand our understanding of AQP1 as a multifunctional regulated channel.

Introduction

Aquaporins (AQPs) are members of the family of major intrinsic proteins (MIPs) found across all forms of life (Agre, 2004; Benga, 2009; Gomes et al., 2009). Vertebrate and invertebrate AQPs facilitate the rapid transport of water across cell membranes, with some subtypes transporting other small molecules and ions (Yool and Weinstein, 2002; Campbell et al., 2008; Gomes et al., 2009). Aquaporins form as tetramers of subunits that each have an individual mono-

meric pore that allows water molecules to move down osmotic and hydrostatic gradients (Preston et al., 1992) containing evolutionarily conserved Asn-Pro-Ala (NPA) signature motifs (Jung et al., 1994; de Groot and Grubmüller, 2001).

In addition to its water channel activity, AQP1 has been shown to function as an ion channel when activated by cGMP (Yool et al., 1996; Anthony et al., 2000; Saparov et al., 2001; Boassa and Yool, 2002; Zhang et al., 2007). Based on ion substitution experiments, the activated AQP1 ion conductance was shown to be nonselective for monovalent cations with a relative permeability sequence of $K^+ \approx Cs^+ > Na^+ > TEA^+$, and showed no appreciable conduction of Cl^- , protons, or divalent cations Ca^{2+} or Mg^{2+} (Yool et al., 1996). In response to cGMP application, AQP1 channels in excised oocyte patches showed large conductance channels with a unitary conductance of approximately 150 pS under physio-

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ABBREVIATIONS: AQP, aquaporin; MIP, major intrinsic protein; TEA, tetraethylammonium; MTSES, 2-sulfonatoethyl methanethiosulfonate sodium salt; DTT, dithiothreitol; bpV, bisperoxo(1,10-phenanthroline)oxovanadate(1-), potassium salt; 8-CPT-cGMP, (Rp)-8-(para-chlorophenylthio)-cGMP.

logical saline conditions with slow on and off gating kinetics and modest voltage sensitivity (Anthony et al., 2000). Molecular dynamic simulation and electrophysiological analyses have supported the proposal that cations permeate AQP1 via the pore formed at the center of the tetrameric subunits and that a conserved intracellular loop between the fourth and fifth transmembrane domains is required for cGMP-dependent gating (Yu et al., 2006). Possible physiological relevance of the AQP1-mediated cationic conductance was supported by results from studies of net fluid transport in primary cultures of choroid plexus (Boassa et al., 2006). However, the ion channel activity of AQP1 has been considered controversial because of variability in the responses between different experimental models and preparations (Saparov et al., 2001; Tsunoda et al., 2004). We hypothesized that the differences between preparations reflected differences in intracellular regulatory pathways that govern AQP1 ion channel availability.

Phosphorylation state is known to govern gating behavior in many classes of channels, resulting, for example, in the switching of a potassium channel (KCNK2) between a voltage-gated and a leak mode of activity (Bockenhauer et al., 2001), transitions between modes of gating in L-type Ca channels (Hashambhoy et al., 2009), amplitude and kinetics of Kv1.3 current in HEK cells (Fadool et al., 1997), and many others (for reviews, see Catterall and Epstein, 1992; Levitan, 1994). Protein kinase C acting at threonine and serine consensus sites was shown to be a positive regulator of the AQP1-mediated ionic conductance but did not modulate the cyclic nucleotide-activated conductance (Zhang et al., 2007).

The purposes of this study were to assess the proposed tetrameric central pore as the pathway for ion permeation in AQP1 and to evaluate the roles of candidate phosphorylation sites for regulating the responsiveness of ionic conductance to cGMP stimulation. Human AQP1 wild-type and mutant channels expressed in *Xenopus laevis* oocytes were analyzed by site-directed mutagenesis, video imaging of swelling, electrophysiology, and pharmacology. Results here show that tyrosine phosphorylation in the carboxyl-terminal domain controls the availability of AQP1 ion channels to be activated by cGMP. We confirmed that threonine/serine phosphorylation does not affect the cGMP-mediated ionic conductance response, in agreement with results of prior work (Zhang et al., 2007). Results here further support the idea that the proposed central pore is the pathway for gated ion flux. Mutation of the hydrophobic barrier residues, identified by molecular dynamic modeling, altered the ionic current properties, causing inward rectification and a positive shift in the reversal potential. We generated a functional cysteine-less AQP1 template into which cysteine residues could be introduced into the carboxyl terminal and the central pore domains, and the effects on functional properties were probed by application of sulfhydryl-reactive agents. Introduction of cysteine in the central pore domain created a gain-of-function block of the ionic conductance by mercury that was not seen in the cysteine-less AQP1 channel.

In summary, data here provide converging lines of evidence that the central pore is the cation channel in AQP1, and that tyrosine phosphorylation serves as one of the master switches that governs AQP1 ion channel responsiveness to cGMP. Phosphorylation of Tyr253 enhances cGMP-dependent AQP1 ion channel activity, and dephosphorylation pre-

vents the ionic conductance response. Conditions that promoted tyrosine dephosphorylation effectively suppressed the ion channel activity to non-AQP1-expressing control levels, removing the ability to respond to cGMP, whereas conditions promoting tyrosine phosphorylation increased cGMP-dependent activity up to but not exceeding the levels seen in untreated AQP1-expressing oocytes.

The presence of hierarchical levels of regulation of AQP1 would explain the differences that have been reported in ion channel activity across various experimental preparations, resolving a standing question in the field about why an increase in cGMP level alone is not sufficient to guarantee AQP1 ion channel activation. Findings presented here move toward resolving an important controversy in the aquaporin field and further expanding our understanding of aquaporins as complex multifunctional channels.

Materials and Methods

Site-Directed Mutagenesis of AQP1 and cRNA Synthesis. cDNA for human AQP1 was provided by P. Agre [Johns Hopkins Bloomberg School of Public Health, Baltimore, MD (Preston et al., 1992); GenBank accession number NM_198098]. Point mutations were introduced into hAQP1 in the *X. laevis* expression vector construct (pXBGev-AQP1) by polymerase chain reaction using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Forest Hills, VIC, Australia) with custom-synthesized primers (Supplemental Table 1). Construct fidelity was established by replicate DNA sequencing of the full-length products. Wild-type and mutant AQP1 cDNAs were linearized with BamHI and cRNA transcribed with T3 RNA polymerase using the mMessage mMachine kit (Ambion, Austin, TX).

Oocyte Preparation. Unfertilized *X. laevis* oocytes were defolliculated with collagenase (type 1A, 1.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and trypsin inhibitor (15 mg/ml) in OR-2 saline (82 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, and 5 mM HEPES) at 18°C for 1.5 h, washed in OR-2 saline solution, and maintained in ND96 saline (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.55) supplemented with 100 µg/ml penicillin and 100 U/ml streptomycin, and 10% (v/v) heat-inactivated horse serum. Oocytes were injected with 50 nl of water containing 1 ng of AQP1 wild-type or mutant cRNA and were incubated for 2 or more days at 18°C before swelling and electrophysiology. Non-AQP1-expressing control oocytes without cRNA injection were prepared in parallel from the same batches of oocytes and served as baseline water and ionic conductance properties for comparison with AQP1-expressing oocytes. All animal procedures were approved by the University of Adelaide Animal Ethics committee and performed in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Oocyte Swelling Assays. Immediately before quantitative swelling assays, oocytes were rinsed in isotonic ND96 saline (without serum or antibiotics) for 1 to 2 h at room temperature. AQP1-mediated water permeability was measured as the rate of swelling in 50% hypotonic saline (isotonic saline diluted with an equal volume of water). Inhibition by mercury was tested by preincubation of injected oocytes in isotonic saline containing 1 mM HgCl₂ for 15 min before swelling assays. Swelling rates were quantified by relative increases in volume, measured from oocyte cross-sectional area imaged by videomicroscopy (charge-coupled device camera; Cohu, San Diego, CA) at 0.5 frames/s over 30 s using National Institutes of Health ImageJ (<http://rsbweb.nih.gov/ij/>), as described previously (Anthony et al., 2000; Boassa and Yool, 2003) and Prism (GraphPad Software Inc., San Diego, CA) software. Data for oocyte volume (*V*) were standardized to the initial volume (*V*₀) and plotted as a function of

time and fit by linear regression to determine the slope value ($V/V_0 \cdot 10^5 \cdot \text{seconds}^{-1}$).

Electrophysiology. For two-electrode voltage clamp, capillary glass electrodes (1–3 M Ω) were filled with 3 M KCl. Unless otherwise specified, all recordings were done in standard Na⁺ bath saline containing 100 mM NaCl, 2 mM KCl, 4.5 mM MgCl₂, and 5 mM HEPES, pH 7.3. cGMP was applied extracellularly using the membrane-permeable cGMP analog (Rp)-8-(para-chlorophenylthio)-cGMP (8-CPT-cGMP; 10 μ M; Sigma-Aldrich) (Wei et al., 1998) or the nitric oxide donor sodium nitroprusside (4–10 mM; Sigma-Aldrich), which increases cGMP by activation of endogenous soluble guanylate cyclase (Boassa and Yool, 2003). For studies of reversal potential and rectification, extracellular NaCl in the bath saline was substituted with equimolar tetraethylammonium (TEA) chloride; all other components were as per standard Na⁺ bath saline. Agents were added to bath saline during the recording, and ion conductances were monitored throughout by voltage step protocols. Recordings were done with a GeneClamp amplifier and pClamp 8.0 and 9.0 software (Molecular Devices, Sunnyvale, CA). HgCl₂ was applied in the extracellular bath during the recording to a final concentration of 25 μ M to assess effects on introduced cysteine residues. Injection of oocytes with the sulfhydryl-reactive agent 2-sulfonatoethyl methanethiosulfonate sodium salt [(MTSES; Jomar Bioscience, Kensington, SA, Australia); 50 nl of 20 mM solution per oocyte yielded an estimated final intracellular concentration of approximately 500 μ M, injected 1 day before electrophysiological recording] was used to assess effects of covalent attachment of a negatively charged substituent group onto introduced Cys residues. The reducing agent dithiothreitol (DTT; Sigma-Aldrich) was applied on-line during electrophysiological recordings at a final extracellular concentration of 1 mM to reverse covalent cysteine modification.

Kinase and Phosphatase Pharmacological Agents. Oocytes expressing wild-type or mutant AQP1 channels were incubated in saline with or without the tyrosine kinase inhibitor 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (10 μ M; Santa Cruz Biotechnology, Santa Cruz, CA) (Bishop et al., 1999), the tyrosine phosphatase inhibitor bisperoxo(1,10-phenanthroline)oxovanadate(1-), potassium salt (bpV; 100 μ M; Santa Cruz Biotechnology) (Posner et al., 1994), the protein kinase G inhibitor (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (1 μ M; KT5823; Sigma-Aldrich) (Gadbois et al., 1992), or the phorbol ester regulator of protein kinase C phorbol 12-myristate 13-acetate (5 μ M; PMA; Sigma-Aldrich) (Matthies et al., 1987) in isotonic saline solution without serum or antibiotics for 4 to 6 h before electrophysiology to evaluate the influence of phosphorylation state on AQP1 ion channel activation. At high doses (≥ 1.6 μ M) as used here, PMA is an inhibitor of protein kinase C (He et al., 1995).

Data Compilation and Statistics. Results compiled from replicate experiments are presented as box plots or scatter plots to show the full range of data points. The box represents 50% of the data, the error bars indicate the full range, and the horizontal bar is the median value. Statistical significance was evaluated by one- or two-way ANOVA, followed by post hoc Bonferroni tests, with significance indicated as $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

Human AQP1 was modified by site-directed mutagenesis to create constructs with alanine, serine, or cysteine substitutions. Figure 1A shows a representation of the tetrameric organization of the AQP1 channel and a diagram illustrating the proposed central pore cation channel, the gating loop (loop D between the fourth and fifth transmembrane domains), and C-terminal residues that were targeted for site-directed mutageneses. A cysteine-less human AQP1 template that retained osmotic water and cGMP-dependent ion

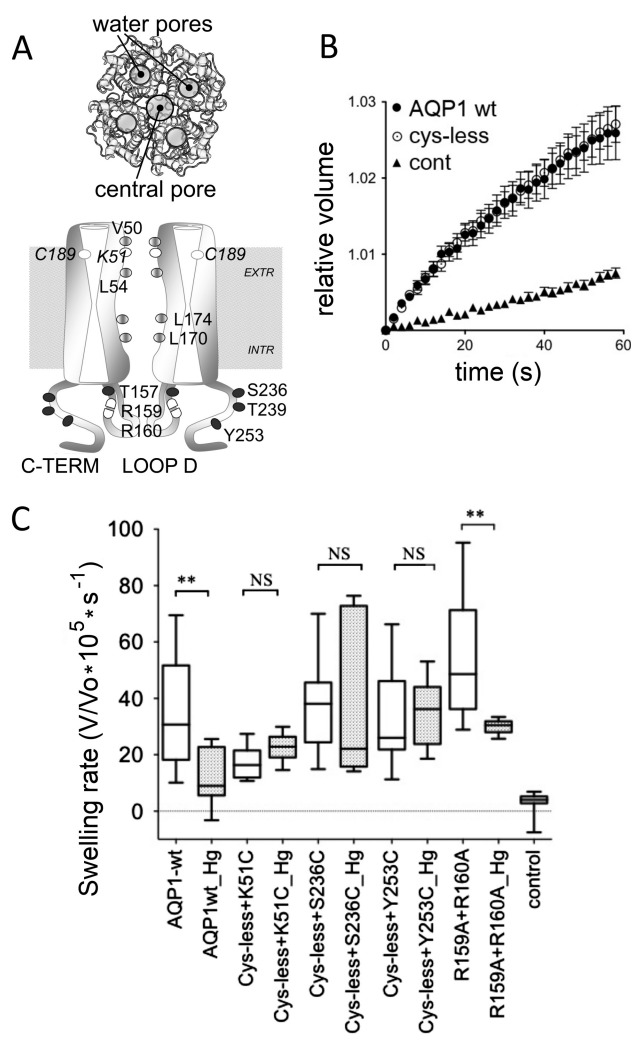


Fig. 1. Location of AQP1 residues analyzed by mutagenesis and confirmation of the functional expression of wild type and mutant constructs as osmotic water channels. A, schematic diagram representing the separate pathways for water and ions in AQP1 wild type channels, organized as tetramers in the membrane lipid bilayer. Critical residues lining the cytoplasmic and periplasmic constriction regions of the central pore are shown along with predicted phosphorylation sites in the C-terminal and loop D regions. B, AQP1 wild type (filled circle) and a mutant construct lacking cysteines (Cys-less; dotted circle) show equivalent high osmotic water permeabilities assessed by quantitative swelling assays compared with nonAQP1-expressing control *X. laevis* oocytes (triangle). Relative volume changes as a function of time were measured from video-imaged cross-sectional areas. Data are mean \pm S.D. for all oocytes in a representative experiment ($n = 6$ –7 per group). C, incubation in 0.1 mM HgCl₂ for 15 min before testing inhibited the osmotic water flux in oocytes expressing AQP1 channels: wild type, mutant constructs in the Cys-less background, and R159A/R160A that contained native Cys189 residues. Mercury had no significant blocking effect on AQP1 Cys-less mutants. Open bars, untreated; shaded bars, with HgCl₂. See Materials and Methods for details. **, $p < 0.01$.

channel functions was generated by the mutation of cysteines at positions 87, 102, and 152 to alanine and at position 189 to serine. The cysteine-less template was then used for site-directed mutagenesis to allow selected cysteine residues to be introduced in the proposed central pore domain and carboxyl terminus. Mutations were located in the external vestibule of the proposed central pore of the tetramer (Lys51), at the four proposed hydrophobic barrier sites lining the central pore (Val50, Leu54, Leu170, Leu174), and at candidate phosphor-

ylation sites in intracellular loop D and the C terminus (Thr157, Ser236, Thr239, Tyr253). The double mutation in loop D (R159A, R160A) was previously shown to knock out the cGMP-dependent ion channel activity without impairing water channel activity (Yu et al., 2006). Coexpression of the mutants K51C and R159A + R160A, neither of which had an ion conductance when expressed alone, rescued the cGMP-induced ion channel activity. All of the AQP1 mutant constructs used in this study were expressed in oocytes and functioned as water channels, indicating that the constructs were assembled and incorporated into the plasma membranes of the oocytes. Introduced cysteines at selected residues in the Cys-less background were used as probe sites for application of the sulfhydryl-reactive agents mercuric ion and MTSES.

Effects of Site-Directed Mutations in the AQP1 Central Pore on Ionic Conductance Properties. Swelling assays were used to confirm the functional expression of wild-type and mutant AQP1 channels in the oocyte plasma membrane. Mean relative changes in volume of AQP1 wild type, cysteine-less template, and non-AQP1-expressing control oocytes, summarized from all data collected in a single representative swelling experiment with 50% hypotonic saline, showed reproducible responses with no appreciable difference between wild type and Cys-less AQP1 channels for osmotically driven water fluxes (Fig. 1B). Significant increases in relative volumes (standardized to the initial volumes of the same oocytes at time 0) were evident in comparison with non-AQP1-expressing control oocytes lacking aquaporins. The magnitude of the swelling response in the Cys-less AQP1 mutant was comparable with that of wild type for equivalent levels of cRNAs injected into the oocytes. Responses were quantified by optical imaging; swelling rates were determined as the slope of the linear fit of the initial phase (approximately 4–20 s) for standardized volume versus time. Compiled data in Fig. 1C show the swelling rates of AQP1 mutants with and without addition of mercury. The native cysteine residue Cys189 confers mercury sensitivity of osmotic water permeability in wild-type AQP1 (Preston et al., 1993); thus, as expected, swelling rates of oocytes expressing wild-type or the double arginine mutant (R159A, R160A), both of which have native Cys189, were inhibited by HgCl_2 . All AQP1 mutants used in these studies showed osmotic water fluxes at rates that were significantly higher than non-AQP1-expressing control oocytes. None of the mutants designed from the Cys-less AQP1 template showed mercury sensitivity in the swelling assays (Fig. 1C).

Effects of site-directed mutations in the central pore domain were analyzed by electrophysiological two-electrode clamp recordings to test for changes in the properties of the ionic conductance responses to cGMP. The mutation of four residues that were modeled as the hydrophobic barriers to ion permeation in the central pore region (Yu et al., 2006) influenced the current-voltage relationship of the AQP1 cGMP-stimulated ionic conductance (Fig. 2). Quadruple mutation of the four hydrophobic barrier residues (Val50, Leu54, Leu170, and Leu174) to alanines (referred to as "VLLL") did not prevent cGMP-dependent activation of the ionic conductance (Fig. 2A) but did alter the properties of the response (Fig. 2B), inducing inward rectification and causing a small positive shift in the reversal potential measured in bath saline with NaCl substituted by isomolar TEA chloride.

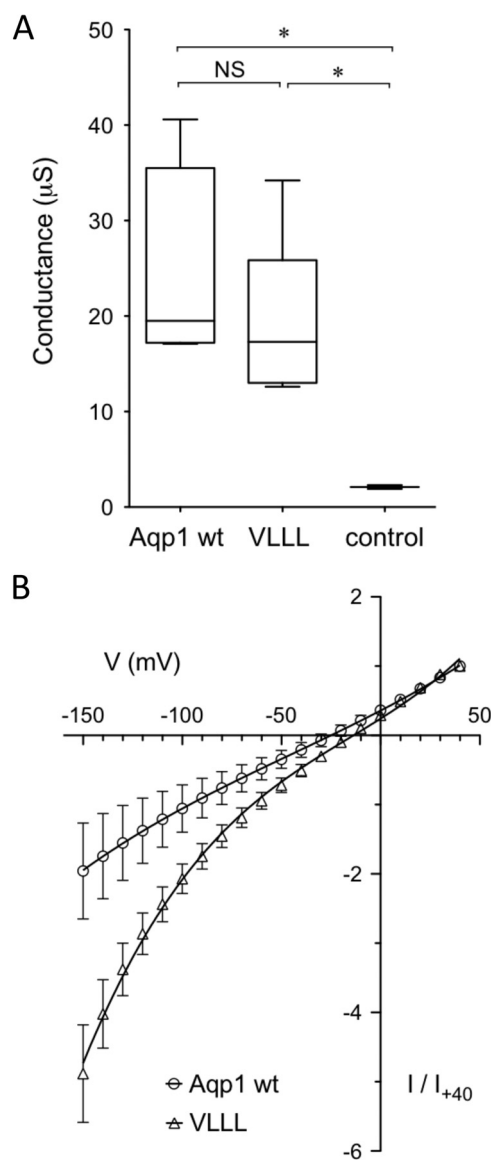


Fig. 2. Removal of hydrophobic residues proposed as barriers in the central pore region influences the AQP1 ionic conductance properties. A, box plot summarizing conductance values measured after activation with 8-CPT-cGMP for oocytes expressing AQP1 wild type, a quadruple mutant lacking the proposed central pore barrier residues (V50A, L54A, L170A, L174A), and non-AQP1-expressing control oocytes. Boxes enclose 50% of data points; vertical bars show the full range; and horizontal bars represent median values. B, mean current amplitudes \pm S.D., standardized to the current at +40 mV, and plotted as a function of voltage illustrate a small positive shift in reversal potential and increased inward rectification in the quadruple central pore mutant construct. The extracellular saline contained isomolar TEA chloride replacing NaCl. **, $p < 0.05$.

These results suggest that modification of the hydrophobic central residues increased the relative permeability of TEA in VLLL compared with wild type, providing evidence that the central pore is the ion channel permeation pathway in AQP1.

An introduced cysteine in the central pore created a gain-of-function block by mercury of the ionic conductance (Fig. 3). Wild-type and Cys-less AQP1 channels showed comparable activation of the cGMP-dependent ionic conductance. The introduction of a cysteine residue at Lys51, located in the outer vestibule of the AQP1 central pore, prevented activation of the ionic conductance response (Fig. 3A), both alone

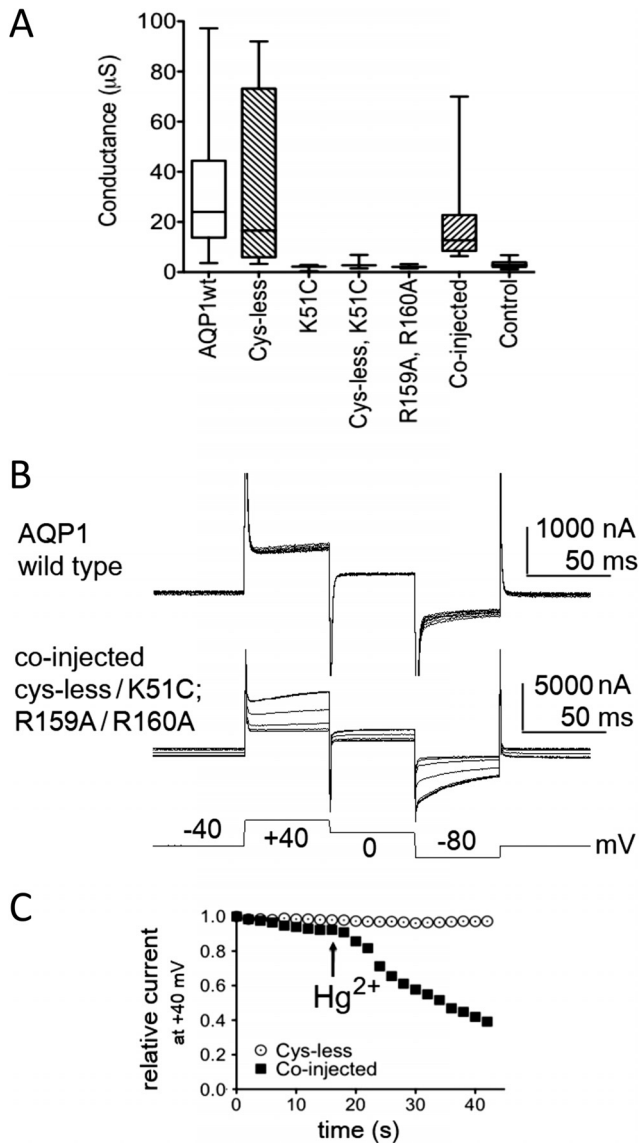


Fig. 3. Introduction of a cysteine residue at position 51 in the central pore induces sensitivity to block by mercury of the AQP1 ion channel conductance. **A**, box plot of compiled data for the final conductance values after application of cGMP measured in oocytes expressing AQP1 wild type or mutant constructs and in non-AQP1-expressing control oocytes. The central pore mutant K51C and the gating loop double mutant R159A,R160A constructs showed no ionic conductance responses when expressed alone but mutually rescued the cGMP-dependent ionic conductance response when coexpressed as heteromeric channels. Open bar, wild-type AQP1 channel; striped bars: mutant AQP1 constructs. **B**, current traces recorded using a brief series of voltage step commands repeated every 6 s, before and during application of mercuric chloride in the bath saline, were used to monitor the sensitivity to block of the cGMP-activated ionic conductance. Rapid block by mercury of AQP1 ion channels containing cysteine at position 51 in the central pore was evident in the decline of outward (at +40 and 0 mV) and inward (at -80 mV) current amplitudes, not seen in wild-type channels. **C**, plot of the current amplitudes at +40 mV for oocytes expressing AQP1 wild type or the co-injected cys-less K51C and R159A,160A constructs, showing the block of the rescued heteromeric channels by mercuric ion.

and in the Cys-less AQP1 background. In agreement with prior work (Yu et al., 2006), the gating-loop double mutation R159A,R160A also disrupted ion channel activation. Coexpression of the two nonfunctional constructs, Cys-less K51C and R159A,R160A rescued cGMP-dependent ion channel activity and offered the opportunity to use the introduced cys-

teine at position 51 to test for an inhibitory effect of mercury. Although the water permeability of wild-type AQP1 (mediated by intrasubunit pores) is mercury-sensitive, the ionic conductance did not show an acute effect of mercury in the wild-type channel (Fig. 3, B and C). However, the presence of cysteine at position 51 induced a novel sensitivity to mercury, seen as a rapid block of the ionic conductance that was not evident for wild type or the cys-less AQP1 alone. Functional ion channels in the coexpression system were necessarily heteromeric subunit assemblies, because neither of the constructs showed an ionic conductance when expressed as a homomeric channel. These data suggest that the presence of cysteine at position 51 is not needed at all four subunits to make the ionic conductance susceptible to block by mercury, consistent with the proposed location of the ionic pore at an interface between subunits.

Effects of Site-Directed Mutations at Kinase Consensus Sites on the Effectiveness of cGMP in Activating the AQP1-Associated Ionic Conductance. Consensus sequences for threonine/serine kinases and tyrosine kinases were modified by site-directed mutagenesis and assessed for effects on cGMP-activated ionic conductance responses. Manipulation of the threonine/serine phosphorylation state caused no appreciable effect on the magnitude of cGMP-dependent activation of the ionic conductance response of AQP1 (Fig. 4). Mutations of candidate threonine and serine phosphorylation sites [Zhang et al. (2007) and predicted by NetPhosK 1.0 Server (<http://www.cbs.dtu.dk/services/NetPhosK/>)] to alanine did not affect the magnitude of the cGMP-activated conductance response (Fig. 4A). Mean conductances were as follows: AQP1wt, 37 μ S; T157A, 20 μ S; T239A, 29 μ S; S236A, 42 μ S. Swelling assays for T157A, T239A, and S236A confirmed that water channel activities were not impaired by the mutations. T157A ($n = 24$) showed a swelling rate of 102 ± 3.5 (mean \pm S.E.M.), and T239A ($n = 20$) showed a swelling rate of 110 ± 3.6 $V_0 \cdot 10^5 \cdot s^{-1}$. S236A swelling rates (data not shown) were comparable with those of S236C (Fig. 1C). Pharmacological inhibition of protein kinase C and protein kinase G signaling pathways did not interfere with cGMP-induced activation of the AQP1 ionic conductance (Fig. 4B). In contrast, the mutation of the candidate tyrosine phosphorylation site Tyr253 disrupted cGMP activation of the ionic conductance (Fig. 4C).

Western blot analysis confirmed that immunoprecipitated AQP1 protein was immunopositive for phosphorylated tyrosine. The signal was not detected in a C-terminal truncation mutant, which carried an introduced stop codon (K243Z) located 10 amino acids upstream (5') of the tyrosine phosphorylation site. The presence of higher molecular weight bands representing the glycosylated forms was consistent with plasma membrane localization of both the wild-type and K243Z truncation constructs (Fig. 4D) and furthermore was consistent with other data showing the presence of osmotic water permeability in K243Z-expressing oocytes (data not shown). The presence of faint bands for K243Z in PY11120 condition was likely to be trace residual label remaining after stripping of the anti-AQP1 antibody probe.

In sum, these results showed that AQP1 protein is phosphorylated at Tyr253 and that mutation of this residue to cysteine interfered with ion channel activation by cGMP. The two interpretations of this outcome were that phosphorylation of Tyr253 allowed AQP1 to be available to be gated as an

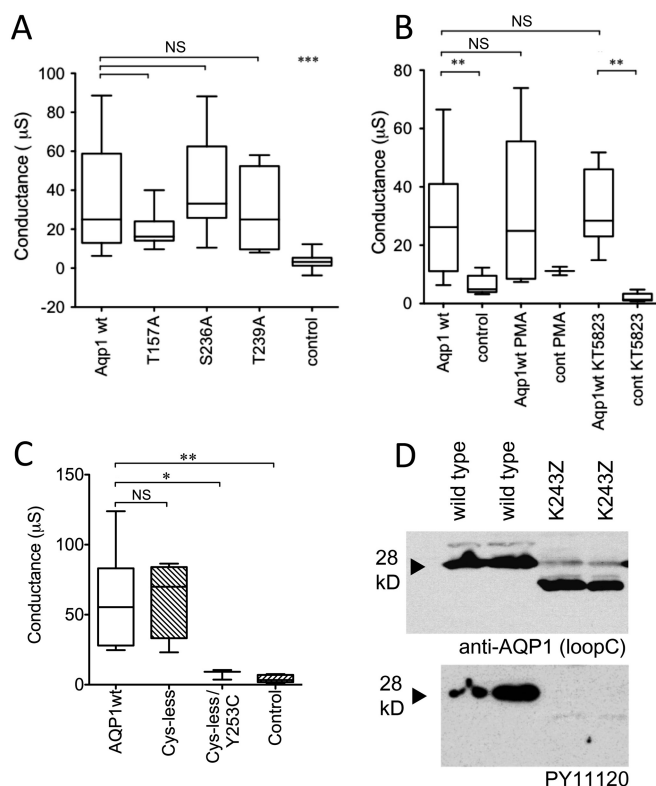


Fig. 4. Tyrosine phosphorylation but not serine/threonine phosphorylation regulates the magnitude of cGMP-dependent ion channel activation in AQP1. **A**, mutations of consensus threonine and serine phosphorylation sites to alanine in AQP1 showed no significant effect on the magnitude of the cGMP-activated ionic conductance responses. Asterisks indicate a significant difference between non-AQP1-expressing control and all other groups ($p < 0.001$). **B**, pharmacological inhibition of protein kinase C (with PMA) or protein kinase G (with KT5823) did not significantly alter the magnitude of the cGMP-activated ionic conductance responses. NS indicates no significant difference from untreated AQP1-expressing oocytes, $p > 0.05$. **C**, mutation of the four native cysteines in AQP1 (Cys-less construct) did not impair ion channel activation compared with wild type, but the additional mutation of a consensus tyrosine phosphorylation site at Tyr253 to cysteine in the otherwise Cys-less background impaired activation of the cGMP-dependent ionic conductance response to a level not different from non-AQP1-expressing control. **D**, Western blot analysis showed that immunoprecipitated AQP1 wild type and C-terminally truncated AQP1 protein subunits, pulled down with antibody to loop C, ran at expected sizes (28 kDa for wild type). Fainter bands above are glycosylated forms. Stripping and reprobing with anti-phosphotyrosine antibody PY11120 showed strong immunopositive labeling of wild type for phosphorylated tyrosine but no phosphotyrosine labeling in a construct with the C-terminal domain truncated by site-directed mutagenesis to create a stop codon at position 243. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ion-channel or, alternatively, that the introduced cysteines at 253 created disulfide bridges between C-terminal domains that prevented the ionic conductance response. Application of the reducing agent DTT did not release any latent conductance response in Y253C-expressing oocytes (data not shown), arguing against a role for C-terminal cysteine cross-bridging as the mechanism of inhibition.

Effects of C-Terminal Tyr253 Phosphorylation and Dephosphorylation on the Magnitude of AQP1 Ionic Conductance Responses. The role of phosphorylation in promoting ion channel activity was tested by pharmacological treatments (Fig. 5) and by using the introduced cysteine at 253 to probe effects of introducing a negatively charged group (Fig. 6). Pharmacological enhancement of the tyrosine

phosphorylation state of the oocyte with a tyrosine phosphatase inhibitor (bpV) increased the responsiveness of AQP1-expressing oocytes to cGMP, whereas treatment promoting tyrosine dephosphorylation (tyrosine kinase inhibitor, naphthyl-1) potently suppressed the ionic conductance activation

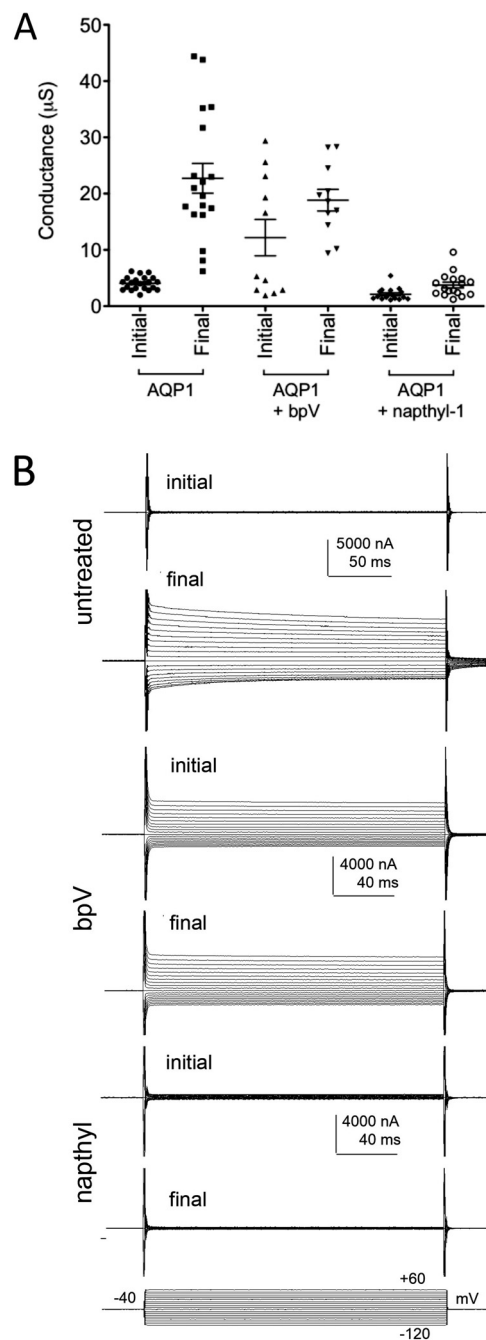


Fig. 5. Pharmacological enhancement of the tyrosine phosphorylated state increases the AQP1 ionic conductance response. **A**, treatment with a tyrosine phosphatase inhibitor, bpV, increased the level of activation of the AQP1 ionic conductance response before application of exogenous cGMP agonist, yielding a range of response amplitudes up to but not exceeding levels seen in untreated AQP1 wild type after cGMP stimulation. Conversely, the tyrosine kinase inhibitor naphthyl-1 disrupted the ability of the AQP1 ionic conductance to be activated in response to cGMP. **B**, representative traces of the currents measured with a set of voltage steps before (initial) and after cGMP stimulation (final) in oocytes expressing AQP1 wild-type channels, with and without pretreatment with naphthyl or bpV.

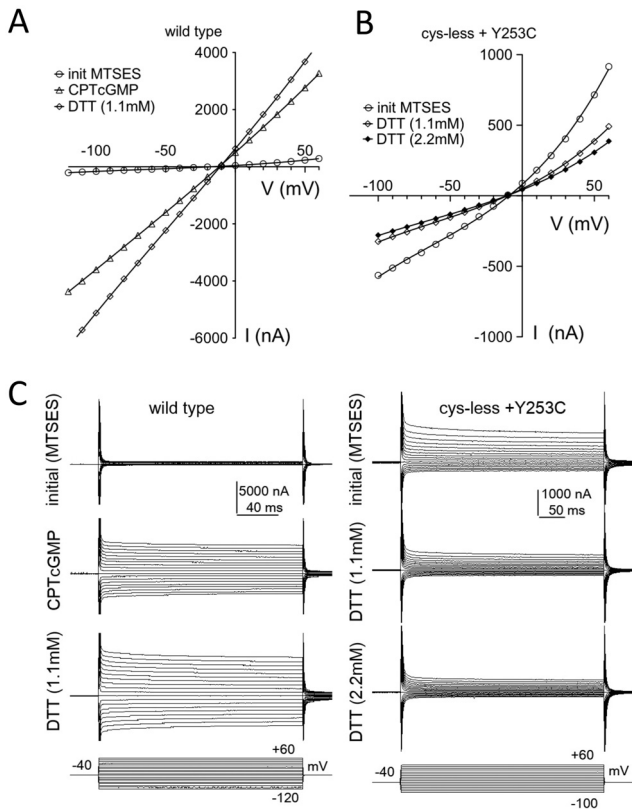


Fig. 6. Covalent modification of Y253C to mimic a phosphorylated state reversibly rescues the ionic conductance response. A, plots of current amplitude as a function of voltage illustrate the low initial conductance in wild type (circle) and the increased final response after activation by cGMP (triangle), which were unaffected by preincubation with intracellularly injected MTSES. The reducing agent DTT did not impair the ionic conductance response. B, pretreatment with MTSES in the Y253C mutant in an otherwise Cys-less background induced a chronically activated ionic conductance, which was reversed by DTT application. C, examples of traces illustrating the current-voltage data shown in A and B.

in response to cGMP (Fig. 5). Conductances were measured by two-electrode voltage clamp in the initial (before cGMP) and final (after cGMP activation) conditions for AQP1 wild-type-expressing oocytes, with and without preincubation in tyrosine phosphorylation pharmacological agents. AQP1-expressing oocytes that were preincubated with bpV had an average 3.5-fold increase in mean initial conductance compared with wild type not treated with bpV. The initial conductance values of bpV-treated AQP1-expressing oocytes ranged from low values equivalent to non-AQP1-expressing control up to levels comparable with but not exceeding those seen after cGMP activation, suggesting that the bpV treatment enhanced the same signaling pathway induced by cGMP, rather than acting via a parallel mechanism. In contrast, AQP1-expressing oocytes incubated in naphthyl-1 were strikingly resistant to activation by cGMP. These results showed that phosphorylation state influences the likelihood of AQP1 ion channel activation, and that tyrosine kinase signaling positively modulates the AQP1 ionic conductance response to cGMP.

Modification of residue 253 in the carboxyl terminal domain to mimic phosphorylation potentiated the cGMP-dependent ionic conductance of AQP1 (Fig. 6). Ionic conductance responses for AQP1 wild type (Fig. 6A) and for Y253C in the Cys-less AQP1 background (Fig. 6B) were quantified from

the slope of current amplitude as a function of voltage, for data as illustrated in corresponding traces in Fig. 6C. Preincubation with intracellularly injected MTSES (see *Materials and Methods*) had no effect on the characteristically low wild-type initial conductance (without cGMP), but induced a chronically activated ionic conductance in Cys-less Y253C channels in the initial condition. The initial conductance of the Cys-less Y253C was reversed by DTT. In contrast, MTSES pretreatment of wild-type AQP1 channels did not affect the response to the cGMP agonist 8-CPT-cGMP, and DTT did not interfere with the response. These results indicate that the incorporation of a negatively charged group at position 253 enhances the ionic conductance induced by cGMP, consistent with a role for phosphorylation at the native tyrosine as a mechanism for controlling availability of AQP1 to function as an ion channel.

Discussion

Results here support the proposal that the central pore region of AQP1 is involved in the cGMP-gated conduction of ions through the channel and that tyrosine phosphorylation in the C-terminal region modulates AQP1-mediated ionic conductance responses. These findings are important in moving toward resolution of one of the controversial areas of aquaporin research and in opening new avenues for exploration of the physiological roles of the dual ion and water conductance properties of the AQP1 channel. Understanding the key role of tyrosine phosphorylation as one of the master switches governing AQP1 ion channel availability could reconcile different findings reported in the literature, which have included evidence both for and against AQP1 ion channel function (Anthony et al., 2000; Saparov et al., 2001; Tsunoda et al., 2004; Zhang et al., 2007).

Aquaporin ion channel activity is not unique to AQP1. Tyrosine phosphorylation is involved in regulation of the cationic conductance in the fly aquaporin-related channel Big Brain (Yanochko and Yool, 2002, 2004). Other members of the broad aquaporin family also have been characterized as ion channels (Yool and Stamer, 2004) including lens MIP channel AQP0 (Zampighi et al., 1985; Ehring and Hall, 1988; Ehring et al., 1990), Nodulin-26 (Weaver et al., 1994), and AQP6 (Yasui et al., 1999). An ion channel role for AQP1 was first proposed in 1996 (Yool et al., 1996). Several key properties of the AQP1 ion channel function (including cGMP dependence, a role for the C-terminal domain, and cationic selectivity) were confirmed independently (Saparov et al., 2001) but the extremely low probability of ion channel opening in the reconstituted bilayer was deemed inconsistent with a physiological role. Subsequent work by the same group using AQP1 expressed in human embryonic kidney cells failed to note ionic current responses (Tsunoda et al., 2004). The cyclic-nucleotide activated ionic conductance response was observed independently for AQP1 in the *X. laevis* oocyte expression system, and the finding was extended to demonstrate a separate role for protein kinase C signaling (Zhang et al., 2007). The simplest explanation for the mixture of evidence both for and against AQP1 ion channel function is that levels of permissiveness for ion channel activation vary across preparations as a result of additional regulatory mechanisms not previously recognized.

Results here are the first to show that the responsiveness

of the AQP1 ionic conductance to cGMP is influenced by tyrosine phosphorylation at a carboxyl terminal consensus site. Pharmacological agents promoting the tyrosine-phosphorylated state increased channel activation, whereas dephosphorylation antagonized the ionic conductance response to cGMP. Replacement of Tyr253 with cysteine in a Cys-less background substantially impaired ion channel activation without preventing water channel activity. The impaired conductance response of cys-less Y253C was rescued by intracellularly injected MTSES, an agent that covalently modifies cysteine sulfhydryl to add a negatively charged moiety. Precedent for this approach was the use of MTSES to mimic phosphorylation for the reversible conversion of a potassium channel KCNK2 between leak channel and voltage-gated channel modes (Bockenhauer et al., 2001), reversed by DTT. The potentiating effect of MTSES on the AQP1 ion channel conductance described here similarly was reversed by extracellular application of DTT. Our data are consistent with the idea that phosphorylation of Tyr253 is required for AQP1 ion channels to be available for gating.

Further evidence for the role of tyrosine phosphorylation in creating a permissive state is seen in the effects of bpV treatment, which resulted in a broad, almost bimodal, distribution of initial conductances. One possible mechanism for the observation that approximately half of the AQP1-expressing oocytes having strongly activated initial ionic conductances after bpV (without applied cGMP) is that tyrosine phosphorylation might increase cGMP sensitivity, perhaps enabling endogenous levels of cGMP to become sufficient for ionic conductance activation. This idea is consistent with previous work showing that some batches of *X. laevis* oocytes expressing AQP1 have an ionic current in the absence of applied cGMP, described as "spontaneous activation" and blocked by the broad spectrum kinase inhibitor H7 (Anthony et al., 2000; Bain et al., 2007).

Crystal structural analyses (Sui et al., 2001) and the differential effects of pharmacological agents on water versus ion permeability (Saparov et al., 2001; Yool et al., 2002) argue against conduction of ions through the individual intrasubunit water pores; however, the central pore at the 4-fold axis of symmetry has been proposed as a candidate ion pathway in the subset of aquaporins that have ion channel activity (Fu et al., 2000; Yool and Weinstein, 2002; Yool, 2007). Theoretical modeling illustrated Na⁺ permeation through the central pore, identified four hydrophobic residues in the outer and inner central pore as barriers to hydration and ion permeation, and identified loop D as a candidate gating domain (Yu et al., 2006). New lines of evidence for the central pore as the ion channel in AQP1, as presented here, are the following: 1) modification of the four candidate barrier residues by mutation to alanines resulted in alteration of rectification and ionic selectivity properties of the ionic conductance; 2) coexpression of two nonconducting mutants, one with mutation in the central pore and a second with mutations in the gating loop domain, restored cGMP-induced ion channel activity, consistent with the idea that the ion pore is at a location where multiple subunits interact; and 3) engineering a cysteine in the central pore created a new binding site for mercury at K51C that blocked the ion channel conductance.

In sum, these data support the concept that the central tetrameric pore is the ion channel in AQP1, and that its functionality is regulated by intracellular signaling involving

tyrosine phosphorylation. Based on the high levels of expression of AQP1 channels in many tissues, a multilayered regulatory system for controlling ion channel function is logical. Even when only a small proportion of the total population of AQP1 water channels (1 in 50,000) is available to act as gated ion channels, theoretical considerations in kidney proximal tubule have suggested it is possible to expect physiologically meaningful contributions to net Na⁺ absorption and fluid fluxes (Yool and Weinstein, 2002). Additional studies are needed to address the physiological roles of the proposed dual channel function of AQP1 in transport of fluids and solutes, with potentially important implications for cellular and subcellular control of fluid flux and volume regulation.

Authorship Contributions

Participated in research design: Campbell, Birdsell, and Yool.

Conducted experiments: Campbell, Birdsell, and Yool.

Performed data analysis: Campbell and Yool.

Wrote or contributed to the writing of the manuscript: Campbell and Yool.

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