Molecular Characterization of a Broad Selectivity Neutral Solute Channel*

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In all living cells, coordination of solute and water movement across cell membranes is of critical importance for osmotic balance. The current concept is that these processes are of distinct biophysical nature. Here we report the expression cloning of a liver cDNA encoding a unique promiscuous solute channel (AQP9) that confers high permeability for both solutes and water. AQP9 mediates passage of a wide variety of non-charged solutes including carboxamides, polyols, purines, and pyrimidines in a phloretin- and mercury-sensitive manner, whereas amino acids, cyclic sugars, Na+, K+, Cl−, and deprotonated monocarboxylates are excluded. The properties of AQP9 define a new evolutionary branch of the major intrinsic protein family of aquaporins and describe a previously unknown mechanism by which a large variety of solutes and water can pass through a single pore, enabling rapid cellular uptake or exit of metabolites with minimal osmotic perturbation.

Transport of solutes such as ions, nutrients, neurotransmitters, and metabolic waste products across cell membranes is of fundamental importance to all mammalian cells. Despite the identification of many selective solute transporters and water channels (1–4), it has remained unclear how transport of large amounts of solutes is coordinated with water movement in metabolically highly active cells such as hepatocytes, spermatocytes, neurons, and glia. The liver is a major site of production and elimination of metabolites such as urea, nucleotides, and ketone bodies, and substantial amounts of these solutes must rapidly cross the hepatocyte plasma membrane with minimal osmotic perturbation (5). In testis, a solute transport mechanism is presumably required to supply nutrients to rapidly growing spermatocytes and to provide an exit pathway for metabolites. In brain, regulation of solute transport is critical because osmolarity changes in extracellular fluids can affect neuronal cell function (6).

Among metabolically active tissues, liver was selected as a target for expression cloning of a new solute-transporting protein because a phloretin-sensitive urea exit mechanism had been described (7–9).

MATERIALS AND METHODS

Expression Cloning—Total RNA was extracted from rats fed a high protein diet (50%, w/w) for 2 weeks. Poly(A)+ RNA purified by oligotAT chromatography was size-fractionated by preparative agarose gel electrophoresis (30). Specific fractions were screened for 1 µl [3H]urea uptake activity in RNA-injected Xenopus oocytes (4, 30). A directional cDNA library was constructed from the positive fraction by using the SuperScript Plasmid System (Life Technologies, Inc.), and cDNA clones were screened for urea uptake (4).

Northern Analysis and in Situ Hybridization—Poly(A)+ RNA (3 µg) from rat tissues was electrophoresed in a formaldehyde-agarose gel and transferred to a nylon membrane. The filter was probed with 32P-labeled full-length AQP9 cDNA, hybridized at 42 °C, and washed with 0.1% SDS, 0.1× SSC, at 65 °C. Autoradiography was performed at −80 °C for 5 days. Digoxigenin-labeled sense and antisense probes were synthesized as described (10) and hybridized on 12-µm crossections of fresh-frozen rat liver and testis. The hybridized probes were visualized using digoxigenin Fab fragment (Boehringer Mannheim) and bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

Oocyte Expression and Radiotracer Uptake Assay—AQP9-cRNA was synthesized from pSPORT1 after linearization with NotI, using T7 RNA polymerase. Human AQP1-cDNA (a gift of Dr. Peter Agre) and rat AQP3-cDNA (a gift of Dr. Gustavo Frindt) were prepared as described previously (11, 12). AQP1-cRNA (10 ng), AQP3-cRNA, and AQP9-cRNA (25 ng) were injected into collagenase-treated, manually defolliculated oocytes maintained at 18 °C for 2–3 days prior to assays. Radiotracer studies were performed as described (10). Briefly, oocytes were incubated for 90 s with Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES, pH 7.4) including 1 µM unlabeled compound and 1–2 µCi/ml radiolabeled compound. Uptake was terminated by adding ice-cold Barth’s solution with 1 µM unlabeled compound, and oocytes were solubilized in 200 µl of 10% SDS. P (where P indicates diffusive solute permeability coefficient (cm/s)) was determined from the following relation: P = N/A × Δc, where N is radiotracer uptake (pmol/s); A is the membrane area (0.045 cm2); and Δc is the concentration difference of the solute (in pmol/cm2). The Arrhenius activation energy was calculated from P values at 4, 22, and 30 °C.

Volumetric Assays—Water permeability was determined by volumetric swelling induced by hypotonic perturbation of oocytes (12). The oocytes were transferred from 200 to 70 mosm of diluted Barth’s solution at 22 °C; oocyte swelling was monitored by video microscopy, and the coefficient of osmotic water permeability (P) was determined. The Arrhenius activation energy was calculated from P values at 4, 22, and 30 °C.

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The abbreviations used are: AQP, aquaporin; UT, urea transporter; kb, kilobase pairs.
Iso-osmotic swelling assays were performed as described (11). Briefly, the swelling rate of the oocytes was measured by a video microscope every 10 s for 2 min, after transferring the oocytes from standard Barth’s solution to modified Barth’s solution in which 88 mM NaCl was replaced by solutes (adjusted to final osmolality of 200 mosm) or water. Osmolality was measured by freezing point depression.

Electrophysiological Studies—A two-microelectrode voltage clamp (Dagan Clampator-1B) was used to measure currents in water-injected oocytes and oocytes expressing AQP1 or AQP9. Microelectrodes (1–5 MΩ) were filled with 3 M KCl. Oocytes were superfused at 23° C in Barth’s medium and clamped at a holding potential of −50 mV. Step changes in membrane potential (between −150 and −2150 mV, in 20-mV increments) were applied for a duration of 100 ms. Current was filtered at 500 Hz, digitized at 5 kHz, and acquired using the Digidata1200 interface and pCLAMP6 software (Axon Instruments). The steady-state current-voltage relationships were determined with the help of software written by Donald D. F. Loo, UCLA School of Medicine. Functional expression of AQP9 or AQP1 in individual oocytes used for voltage-clamp experiments was subsequently confirmed by radiotracer uptakes or osmotic swelling (lysis) measurements.

RESULTS AND DISCUSSION

Expression Cloning of AQP9 cDNA—We first determined 1 mM [14C]urea uptake in Xenopus oocytes injected with 50 ng of poly(A)+ RNA from rats fed normal and high protein (50% w/w) diets. Amino acid sequence alignment of AQP9, rat AQP1 (GenBank™ P29975), rat AQP3 (GenBank™L35108), rat AQP7 (GenBank™ AB000567), and Escherichia coli GlpF (GenBank™ M55990) was performed using the PILEUP program (Genetics Computer Group). Putative membrane-spanning regions are underlined and numbered 1–6. Conserved residues are indicated by shading. Asn-Pro-Ala (NPA) consensus motifs are highlighted by boxes. The potential sites for N-linked glycosylation (Asn-142) (double line), protein kinase C phosphorylation site (Ser-222), and casein kinase II phosphorylation site (Ser-271) (asterisks) are depicted.

Fig. 1. Expression cloning and deduced amino acid sequence of AQP9. A, uptake of 1 mM [14C]urea in Xenopus oocytes injected with 50 ng of poly(A)+ RNA from rats fed normal and high protein (50% w/w) diets. B, amino acid sequence alignment of AQP9, rat AQP1 (GenBank™ P29975), rat AQP3 (GenBank™L35108), rat AQP7 (GenBank™ AB000567), and Escherichia coli GlpF (GenBank™ M55990) was performed using the PILEUP program (Genetics Computer Group). Putative membrane-spanning regions are underlined and numbered 1–6. Conserved residues are indicated by shading. Asn-Pro-Ala (NPA) consensus motifs are highlighted by boxes. The potential sites for N-linked glycosylation (Asn-142) (double line), protein kinase C phosphorylation site (Ser-222), and casein kinase II phosphorylation site (Ser-271) (asterisks) are depicted. No consensus motifs of protein kinase A phosphorylation were found. C, phylogenetic analysis of AQP9. Deduced amino acid sequences were analyzed using PILEUP. The percent amino acid identity between AQP9 and others is indicated. D, structural model of AQP9. Topology was determined according to the Kyte-Doolittle algorithm. The numbering of putative membrane-spanning regions corresponds to those of B.
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hybridization signals were detected in kidney, colon, heart, and skeletal muscle. Nonradioactive in situ hybridization in rat liver showed that AQP9 is expressed evenly among hepatocytes (Fig. 2B). In testis, AQP9 mRNA was detected in the inner surface of seminiferous tubules and in the interstitial Leydig cells. In seminiferous tubules, signals were obtained in spermatocytes at early developmental stages but not at later stages and not in Sertoli cells. AQP9 mRNA was detected in astrocytes throughout the brain (data not shown).

Functional Expression in Xenopus Oocytes—When expressed in crNA-injected oocytes, AQP9 increased the urea permeability coefficient ($P_u$) from $1.5 \times 10^{-6}$ to $0.2 \times 10^{-6}$ cm/s (water-injected) to $23.5 \times 10^{-6}$ to $2.0 \times 10^{-6}$ cm/s (Fig. 3A). The increase in $P_u$ was similar to that observed in oocytes expressing the urea transporters UT2 and UT3 ($P_u = 25-45 \times 10^{-6}$ cm/s) (cf. Refs. 4, 10, and 18). The activation energy estimated from Arrhenius plots was $7.8 \pm 1.5$ kcal/mol, consistent with movement through a pore. We then determined the selectivity of the AQP9 pore (Fig. 3A) and obtained $P_v$ values for polyols (glycerol, mannitol, and sorbitol), purines (adenine), pyrimidines (uracil and the chemotherapeutic agent 5-fluorouracil), and urea analogues (thiourea) ranging from 15 to $25 \times 10^{-6}$ cm/s, suggesting a promiscuous selectivity profile for AQP9. Radiotracer uptake studies revealed that AQP9 was impermeable to cyclic sugars (D-glucose, D-mannose, and myo-inositol), the nucleoside uridine, and amino acids (glutamine and glycine) (data not shown).

To investigate the effect of electric charge on solute permeability, we tested uptake of the monocarboxylates lactate and β-hydroxybutyrate, each of physiological importance in hepatocytes. Their $P_v$ values were significantly increased at physiological pH, but an additional 4-fold increase was observed at pH 5.5 (Fig. 3B), whereas mannitol uptake was independent of pH. In voltage-clamped oocytes expressing AQP9, monocarboxylates at concentrations up to 10 mM evoked no significant currents (less than 5 nA), regardless of pH. Together with pH dependence of radiotracer uptakes, these results indicate that monocarboxylates permeate AQP9 only in their protonated form. The low permeabilities of the purine analogues xanthine and uric acid at pH 7.4 suggest that, likewise, these compounds permeate AQP9 in their protonated form (Fig. 3A).

We measured radiotracer uptake in oocytes expressing the aquaporins AQP1 and AQP3 to compare their solute permeabilities with those of AQP9 (Fig. 3C). AQP1 did not mediate significant uptake of any solute tested, indicating that AQP1 is a selective water channel (1–3). AQP3 facilitated glycerol uptake at levels similar to that for AQP9, but a weak urea permeability was detected in AQP3 (25% of the $P_v$ value for AQP9). In addition, AQP3 was impermeable to the urea analogue thiourea, to polyols larger than glycerol, and to purines and pyrimidines. Our results suggest that the pore selectivity in the aquaporins decreases in the order AQP1 > AQP3 > AQP9. The osmotic permeability coefficient $P_f$ was increased ~30-fold (to 0.014 ± 0.0013 cm/s) in oocytes expressing AQP9 (cf. 0.0005 ± 0.00002 cm/s in water-injected oocytes) (Fig. 3D). AQP9 showed a $P_f$ similar to that for AQP1 (0.016 ± 0.0014 cm/s), but AQP3 exhibited a lower $P_f$ (0.0073 ± 0.0008 cm/s). The Arrhenius activation energy for AQP9-mediated $P_f$ was 3.6 ± 1.3 kcal/mol, again consistent with movement through a pore (1–3).

Notably, unlike other known aquaporins, water permeability was sensitive to 0.1 mM phloretin (86% inhibition). In AQP9, $P_f$ was reduced 61% in the presence of 0.3 mM HgCl2. A cysteine residue thought to be involved in mercurial binding lies 3 amino acid residues N-terminal to the second NPA box (1–3). Thus, AQP9 possesses general features of water channels in

Fig. 2. Tissue localization of AQP9-mRNA. A, Northern blot analysis from various rat tissues. B, AQP9 mRNA in rat liver and testis detected by in situ hybridization. Bright field micrographs of cryosections hybridized to digoxigenin-labeled AQP9 antisense cRNA probe are shown. Upper panel, cross-section of liver (CV, central vein). AQP9 mRNA was found in hepatocytes (arrows). Hybridization of sense AQP9 cRNA probe did not reveal any signal (data not shown). Lower panel, cross-section of testis. Asterisks indicate the lumen of the seminiferous tubules, containing mature spermatocytes with long tails. AQP9 mRNA was detected selectively in immature spermatocytes (large arrows) and also in the interstitial Leydig cells (small arrows). Bar = 100 µm.
FIG. 3. Functional properties of AQP9 expressed in Xenopus oocytes. A, solute permeability in oocytes expressing AQP9. Uptake of 1 mM 14C- or 3H-labeled carbamides, polyols, purines, pyrimidines, nucleosides, and monocarboxylates was measured over 90 s in oocytes injected with water (control) or AQP9 cRNA. Measurements were performed at 22 °C except for lactate uptakes which were performed at 4 °C to minimize the
addition to the distinctive phloretin-inhibitable permeability for a large variety of non-charged solutes.

**Ion Conductance of AQP9—** It has been generally considered that none of the aquaporins mediates any type of ion conductance under normal conditions (1–3). A recent report (19) suggested that forskolin induced a nonspecific cation conductance via AQP1, despite the absence of a typical consensus protein kinase A phosphorylation site. This phenomenon has been disputed by others (20) applying the same protocols. By using voltage-clamped oocytes expressing either AQP1 or AQP9, we determined the macroscopic conductance from the slope of the linear current-voltage relationship (between 150 mV and 2150 mV) before and after superfusion with 10 mM forskolin in Barth’s solution for 15 min (Fig. 3E). Only low ionic conductance was observed in the presence and absence of forskolin, in oocytes expressing AQP1 or AQP9, and in water-injected oocytes. The reversal potential was unchanged in all groups following forskolin treatment. Forskolin administered by direct microinjection into the oocytes was also ineffective (not shown). Therefore, neither AQP1 nor AQP9 mediates any ionic conductance under basal or forskolin-treated conditions.

**Inhibition Profiles of AQP9—** A fundamental question is whether or not solutes and water share a single pore in AQP9. To address this issue, we first examined the inhibition profiles of solute permeabilities by phloretin and mercurial compounds. Phloretin (0.1 mM) and HgCl2 (0.3 mM) effectively inhibited the water permeability as well as the permeabilities for mannitol, uracil, adenine, and β-hydroxybutyrate (75–90%) (Fig. 3F). Glycerol and urea permeabilities were inhibited 35–45% by 0.1 mM phloretin. Glycerol permeability was inhibited 50% by 0.3 mM HgCl2. Contribution from endogenous lactate transport (29). 5-FU, 5-fluorouracil; β-HB, β-hydroxybutyrate. Data are mean ± S.E. from 6 to 8 oocytes. B, pH-dependent monocarboxylate permeation. Uptake of 1 mM 14C-labeled lactate and β-hydroxybutyrate was measured at physiological pH (Barth’s solution pH 7.3–7.5) and at pH 5.5. The increase in permeability was obtained as the ratio of the uptake at pH 5.5 to the uptake at 7.3–7.5. C, solute permeability in oocytes expressing AQP1 and AQP3. D, the osmotic water permeability (Pf) was measured in oocytes expressing AQP1, AQP3, or AQP9. Where indicated, oocytes were preincubated in the presence of either 0.1 mM phloretin (10 min) or 0.3 mM HgCl2 (5 min) and during uptake. The reversal of mercurial inhibition was studied by incubation with 5 mM β-mercaptoethanol for 15 min following 10 min of treatment with 0.3 mM HgCl2. Data represent the mean ± S.E. from 6 to 8 oocytes. E, macroscopic ionic conductance in oocytes expressing AQP9 and AQP1 under initial conditions and after 15 min superfusing with 10 μM forskolin. Data are mean ± S.E. from 5 oocytes in each group. F, inhibition of water and solute permeability by phloretin and HgCl2. Percent inhibition of water and solute permeability was examined by incubation with 0.1 mM (or 0.5 mM) phloretin or 0.3 mM HgCl2 for 10 and 5 min, respectively. Asterisks indicate no significant difference from zero (t test).
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The discovery of AQP9 may now highlight the roles that members of the aquaporin family could play in rapid movement of solutes across cell membranes with minimal osmotic perturbation, particularly in tissues that accumulate high levels of metabolites. The liver is a major site of urea production, and a rapid urea exit mechanism is required, and the lack of specialized urea transporters and selective functional water channels in hepatocytes (1-3, 10) emphasizes the need of these cells for AQP9 as an indiscriminate channel. In addition to urea, AQP9 may provide an exit route for purines and pyrimidines derived from nucleotide synthesis de novo, ensuring the plentiful supply of nucleotides to the brain, muscle, and hematopoietic tissues, in which only a salvage pathway is available (25). Purine and pyrimidine analogues such as 5-fluouracil and 6-mercaptopurine are commonly used as chemotherapeutic agents. AQP9 may indirectly affect the chemosensitivity and resistance to these agents in cancer tissues. Permeation of monocarboxylates such as lactate and β-hydroxybutyrate is necessary because liver is a major site of lactate utilization and ketone body formation (5). Hepatocytes secrete lactate under hypoxic conditions and export a large amount of ketone bodies such as β-hydroxybutyrate and acetocetate induced by fasting or diabetic conditions. Notably, AQP9 mRNA was up-regulated in liver from streptozotocin-induced diabetic rats.

In summary, we report the first characterization of a broad selectivity, neutral solute channel, AQP9, that permits solutes to cross cell membranes rapidly and with minimal osmotic perturbation. The observations from inhibition studies (Fig. 3F), and oocyte swelling assays (Fig. 4A), as well as the reflection coefficients (Fig. 4C) strongly indicate that AQP9 provides a common transmembrane pathway for both solutes and water. Our findings have significant physiological implications, particularly in cells that require absorption and excretion of large amounts of metabolites. AQP9's indiscriminate nature, which allows passage of a wide range of structurally unrelated solutes, highlights its pharmacological relevance and the need for structural studies (27, 28) to elucidate the molecular design of this unique promiscuous pore.

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