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Aquaporins

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Preface

The mechanisms and physiological functions of water transport across biological membranes are subjects of long-standing interest. Recent advances in the molecular biology and physiology of water transport have yielded new insights into how and why water moves across cell membranes. Aquaporins (AQPs) are a group of water channel proteins that are specifically permeable to water and some other small molecules, such as glycerol, urea, etc. Thirteen water channel proteins (AQP0–AQP12) have been cloned, and gene organization, protein crystal structure, expression localization, and physiological functions of some AQPs have been studied and determined. In recent years, the studies in AQP knockout mouse models suggest that AQPs may be involved in some disease development and be useful targets for drug discovery of selective inhibitors. Our aim in writing this book is to stimulate further research in new directions by providing novel provocative insights into further mechanisms and physiological significance of water and some small molecule transport in mammals.

This book provides a state-of-the-art report on what has been learned recently about AQPs and where the field is going. Although some older work is cited, the main focus of this book is on advances made over the past 30 years on the biophysics, genetics, protein structure, molecular biology, physiology, pathophysiology, and pharmacology of AQPs in mammalian cell membranes. It is likely that advances in understanding molecular biology and physiology of AQPs will yield new insights into biology and medicine.

In listing names, one always lives in fear of having forgotten someone. I thank all authors and colleagues for their contribution to this book.

Beijing, China — Baoxue Yang

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Molecular Biology of Aquaporins

1

Chunling Li and Weidong Wang

Abstract

Aquaporins (AQPs) are a family of membrane water channels that basically function as regulators of intracellular and intercellular water flow. To date, thirteen AQPs, which are distributed widely in specific cell types in various organs and tissues, have been characterized in humans. Four AQP monomers, each of which consists of six membrane-spanning alpha-helices that have a central water-transporting pore, assemble to form tetramers, forming the functional units in the membrane. AQP facilitates osmotic water transport across plasma membranes and thus transcellular fluid movement. The cellular functions of aquaporins are regulated by posttranslational modifications, e.g. phosphorylation, ubiquitination, glycosylation, subcellular distribution, degradation, and protein interactions. Insight into the molecular mechanisms responsible for regulated aquaporin trafficking and synthesis is proving to be fundamental for development of novel therapeutic targets or reliable diagnostic and prognostic biomarkers.

Keywords

Aquaporin • Posttranslational modification • Endocytosis • Exocytosis

1.1 Classification of Aquaporins (AQPs)

1.1.1 Discovery of the First Water Channel

The existence of a water channel protein had been predicted for a long time. In early 1980s last century, people believed that a protein migrating as band 3 on the electrophoretogram of red blood

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cell membrane was a common pore for water and electrolytes [214]. The membrane water channel was not identified until the pioneering discovery of AQP1 by Peter Agre and colleagues around late 1980s and early 1990s. During that period, Agre and coworkers had purified by chance a novel protein from the red blood cell membrane [47], with a non-glycosylated component of 28 kDa and a glycosylated component migrating as a diffuse band of 35~60 kDa, which displayed a number of biochemical characteristics. The 28-kDa polypeptide was found to exist as an oligomeric protein with the physical characteristics of a tetramer. The amino acid sequence was later identified [213] and cDNA was subsequently cloned [190]. The new protein was initially called CHIP28 (CHannel-like Integral Protein of 28 kDa), but was later redubbed aquaporin-1 or AQP1 [2].

The AQP1 was identified by injecting its cRNA into *Xenopus laevis* oocytes, which exhibited remarkably high osmotic water permeability causing the cells to swell rapidly and explode in hypotonic buffer [190]. To test the role of AQP1 as a molecular water channel, highly purified AQP1 protein from human red blood cells was reconstituted with pure phospholipid into proteoliposomes and were compared with liposomes without AQP1 [260, 261]. The unit water permeability (conductance per monomeric AQP1) was extremely high in the liposomes with AQP1 when compared with controls, in addition, AQP1 proteoliposomes were not permeable to various small solutes or protons, thus suggesting that AQP1 was water selective (although later studies found that AQP1 is indeed gas permeable). These results confirmed that AQP1 is a molecular water channel and strongly suggested that AQP1 water channels were of fundamental importance for transmembrane or transcellular water transport in tissues where it is expressed. The discovery of AQP1 also laid the ground for the identification of other water channel family members by homology cloning and other means, which has led to the understanding that aquaporins play essential roles in water transport in tissues.

1.1.2 Classification of AQPs

A large number of evidences have shown an unexpected diversity of AQPs in both prokaryotic and eukaryotic organisms [1, 58] since the discovery of AQP1. More than 300 different aquaporins have been discovered so far in which thirteen isoforms have been identified (AQP0–AQP12) in human. AQPs are integral, hydrophobic, transmembrane proteins that primarily facilitate the passive transport of water depending on the osmotic pressure on both sides of membrane. Subsequent studies showed that AQPs can transport not only water molecules but also other small, uncharged molecules, i.e., glycerol, urea, down their concentration gradients.

Structural analysis of several AQPs has established that these protein channels share common structural features. The functional aquaporin unit is a homotetramer, which comprises six α-helix transmembrane domains with two conserved asparagine–proline–alanine (NPA) motifs embedding into the plasma membrane, a signature sequence of water channels, five loops (A-E) and intracellular N- and C-termini. The amino acid sequences of human AQPs are approximately 30–50% identical. Conformational changes of AQP protein permit other molecules passing through plasma membrane, i.e. urea, glycerol, H_2O_2, NH_3, CO_2, etc.

According to their structural and functional similarities, AQPs are initially subdivided into two subfamilies, classical AQPs (water-selective) and aquaglyceroporins (glycerol channel, Glps) aquaporins. However, this viewpoint was challenged by recent evidence revealing that both subfamilies overlap functionally, for examples, some classical AQPs transport water and other small solutes e.g. glycerol. In addition, a new group of AQPs discovered recently showed that their structure is highly deviated from the previous AQPs especially around the AQP NPA box [95, 104, 107]. This subfamily was later named superaquaporin (also called unorthodox aquaporin) as it has very low homology with the previous two subfamilies [104]. This classification is

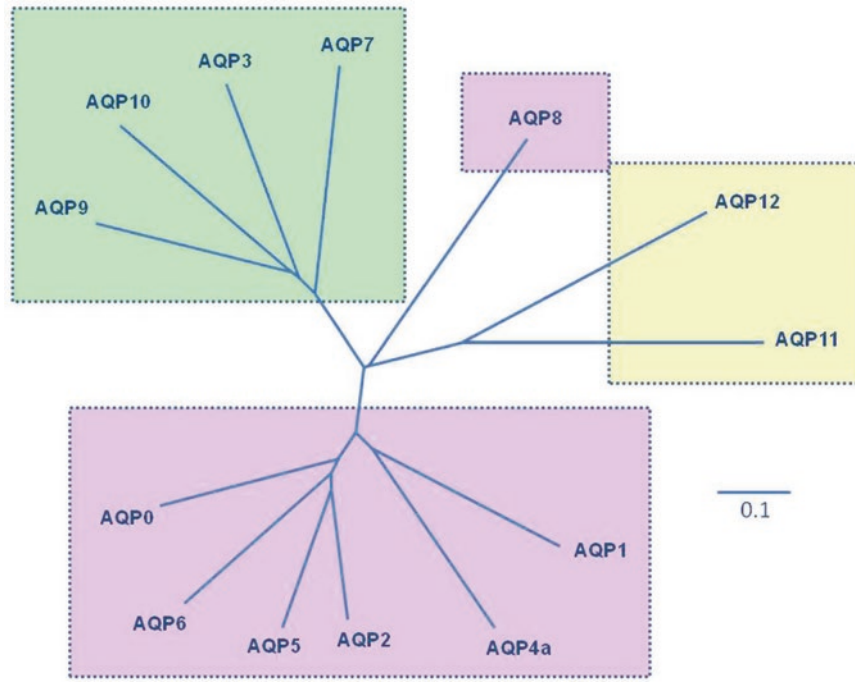

Fig. 1.1 The phylogenetic tree of 13 human AQPs. The tree shows the classical AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8, note that AQP8 is also named as AQP8-related AQPs, as in phylogeny it is different from other classical AQPs, *light pink square*); the aquaglyceroprins (AQP3, AQP7, AQP9, AQP10, *light green square*); and the superaquaporins (AQP11, AQP12, *light yellow square*) (Modified from Ref. [104])

generally accepted and will be discussed in the current review. Aquaporins may also be organized into four categories, classical aquaporins, Aqp8-type aquaammoniaporins, unorthodoxaquaporins, and Glps, according to the phylogenetic tree (Fig. 1.1) or phylogenetic topology inferred from Bayesian inference [58, 104].

The first subfamily is that of aquaporins, the water selective or specific water channels, also named as "orthodox", "classical" aquaporins, including AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. This subfamily of AQPs has been extensively studied, which help us define regulation of AQP expression in the body and their potential roles in physiological and pathophysiological states. Recent literature, however, appears to suggest that AQP6 and AQP8 be classified as unorthodox auquaporins, due to low water permeability of AQP6 [62, 256] and unique, different phylogenetics of AQP8 from others [122, 152].

The second subfamily is represented by aquaglyceroporins that are permeable to water and other small uncharged molecules (ammonia, urea, in particular glycerol). They also facilitate the diffusion of arsenite and antimonite and play a crucial role in metalloid homeostasis [15]. The aquaglyceroporins, including AQP3, AQP7, AQP9 and AQP10, can be distinguished from aquaporins based on amino acid sequence alignments [21]. AQP3 is the first mammalian aquaglyceroporin to be cloned, and it is permeable to glycerol and water [50, 252]. AQP7, AQP9, and AQP10 transport water, glycerol, and urea when expressed in Xenopus oocytes [100, 103, 232]. AQP9 is also permeable to a wide range of other solutes in oocytes [232]. Most aquaglyceroporins which transport glycerol and urea are less understood yet.

The third subfamily of related proteins have low conserved amino acid sequences around the NPA boxes unclassifiable to the first two subfamilies [104]. Mammalian AQP11 and AQP12 are

Table 1.1 Mammalian AQPs and their distributions

	Transport	Distributions
Aquaporins		
AQP0	Water	Eye
AQP1	Water	Brain, eye, kidney, heart, lung, gastrointestinal tract, salivary gland, liver, ovary, testis, muscle, erythrocytes, spleen
AQP2	Water	Kidney, ear, ductus deferens
AQP4	Water	Brain, kidney, salivary gland, heart, gastrointestinal tract, muscle
AQP5	Water	Salivary gland, lung, gastrointestinal tract, ovary, eye, kidney
AQP6	Water, urea (+/−), anion	Brain, kidney
AQP8	Water, urea (+/−), ammonia	Testis, liver, pancreas, ovary, lung, kidney
Aquaglyceroporins		
AQP3	Water, urea, glycerol, ammonia	Kidney, heart, ovary, eye, salivary gland, gastrointestinal tract, Respiratory tract, brain, erythrocyte, fat
AQP7	Water, urea, glycerol, ammonia	Testis, heart, kidney, ovary, fat
AQP9	Water, urea, glycerol	Liver, spleen, testis, ovary, leukocyte
AQP10	Water, urea, glycerol	Gastrointestinal tract
Superaquaporins		
AQP11	Water?	Testis, heart, kidney, ovary, muscle, gastrointestinal tract, leukocytes, liver, brain
AQP12	Unknown	Pancreas

Refs. [43] and [104]

the only two members in this subfamily, which have been called "superaquaporins" or "unorthodox aquaporins". The NPA boxes of these two AQPs are highly deviated from those of other classical AQPs with homology less than 20%, indicating that they belong to a supergene family of AQPs. The structure and function of AQP11 and AQP12 are currently poorly understood.

1.2 Isoforms of AQPs

To date, at least 13 isoforms of AQPs have been discovered in humans (Table 1.1). The biological roles of these proteins have been thoroughly investigated in the past 30 years after the discovery of the first AQP. We have learned substantial base of knowledge on the structure, cellular localization, biological function, and potential pathophysiological significance of these mammalian AQPs, although there are some questions still undetermined.

1.2.1 Classical Aquaporins

1.2.1.1 AQP0

AQP0 is the protein in the fiber cells of the eye lens where it is required for homeostasis and transparency of the lens [40, 65]. AQP0 showed lower water permeability than AQP1, about to 1/40 that of AQP1 [36]. The water transport via AQP0 is regulated by C-terminal cleavage [64], pH and Ca^{2+}/calmodulin (CaM) [168]. Lowering internal Ca^{2+} concentration or inhibiting calmodulin increased AQP0 water permeability. The most latest molecular dynamics and functional mutation studies reveal that binding to calmodulin inhibits AQP0 water permeability by allosterically closing the cytoplasmic gate of AQP0 [198]. A PKC dependent phosphorylation at Ser235 appears to cause the translocation of AQP0 to the plasma membrane [63].

1.2.1.2 AQP1

AQP1 is the first water channel discovered [47, 190, 191] and the first AQP that was found to function as a gas channel [166, 189]. AQP1 is widely distributed water channel in the body [43] where it plays a central role in regulation of water transport through those tissues. AQP1 also plays an important role in angiogenesis, cell migration and cell growth [170], e.g. reduction of AQP1 expression blocked angiogenesis and slowed the progression of tumors [17]. Aside of facilitating water movement, studies have revealed that AQP1 could enhance CO_2 and NH_3 permeability [62, 200] and function as a non-selective

monovalent cation channel when activated by intracellular cGMP [5]. Phosphorylation of tyrosine Y253 in the C-terminus is involved in regulation of AQP1 as a cGMP-gated cation channel [32]. Early evidence showed that threonine and serine kinase activity also regulates AQP1 ion channel activity [264].

1.2.1.3 AQP2

AQP2 is an arginine vasopressin (AVP)-regulated aquaporin which is probably the most thoroughly studied to date. AQP2 displays permeability only to H_2O but not any other small molecules. AQP2 is expressed in principal cells of the collecting ducts and is abundant both in the apical plasma membrane and subapical vesicles [59, 148, 171] in the kidney where it deeply involved in urine concentration. Translocation of AQP2 from intracellular compartment to the apical membrane is dependent on the binding of vasopressin to its V2 receptor [148, 171] located in the basolateral plasma membrane, by which vasopressin increases the water permeability. Regulation of AQP2 expression, posttranscriptional modification, and trafficking is well studied and will be discussed in following parts in the present review (see below). A number of other local or systemic factors or chemicals have been reported to regulate AQP2 expression or trafficking, such as atrial natriuretic peptide (ANP) [22, 23, 243], nitric oxide (NO) [22], protaglanding E2 [262], angiotensin II [17, 131], oxytocin [130], purine [265], statin [17, 194] etc.

1.2.1.4 AQP4

AQP4 is a predominant AQP located in central nervous system and is permeable to water [250, 251] and CO_2 [62]. Both short-term regulation and trafficking have been reported for AQP4. Vasopressin [154] or histamine exposure [33] is suggested inducing translocation of AQP4. Phosphorylation of AQP4 at cytosolic serine residues (Ser111 and Ser180) is indicated mediating water permeability by gating [69], however, recent evidence was not able to support phosphorylation of Ser111 *in vivo* [8]. Studies indeed suggests that phosphorylation at AQP4 C-terminus by protein kinase C (PKC) is required for Golgi transition [8].

1.2.1.5 AQP5

AQP5 is expressed in glandular epithelia, alveolar epithelium and secretory glands where it is involved in the generation of saliva, tears and pulmonary secretions [215, 216]. AQP5 is permeable to water and CO_2 [62, 164]. AQP5 can be directly phosphorylated at Ser156 and Thr259 by protein kinase A (PKA) in the cytoplasmic loop and the C-terminus [78, 246]. However, it is increased intracellular Ca^{2+}, but not PKA-induced phosphorylation, that induces AQP5 trafficking to plasma membrane [106, 223].

1.2.1.6 AQP6

AQP6 colocalizes with the H^+-ATPase in intracellular vesicles in the renal collecting duct type-A intercalated cells [256], indicating that AQP6 may functionally interact with H^+-ATPase in the vesicles to regulate intra-vesicle pH. In response to acid-base changes H^+-ATPase in the intercalated cells is observed translocating from the cytoplasmic vesicles to the apical plasma membrane [239], where no AQP6 is found, indicating that AQP6 lacks intracellular trafficking and functions exclusively at the intracellular sites. The N-terminus of AQP6 seems critical for the trafficking of the protein to the intracellular sites and intracellular vesicles localization [12]. Interestingly calmodulin can bind AQP6 in a calcium-dependent manner at the N-terminus [196], indicating that calcium signals may be involved in internalization of AQP6. AQP6 appears impermeable to H_2O [62, 133], but in the presence of $HgCl_2$ or at acidic pH (<5.5) the water and anion permeability of AQP6 in oocytes was rapidly increased [256]. Moreover, AQP6 also enables transport of urea, glycerol, and nitrate [89, 94].

1.2.1.7 AQP8

AQP8 is a water channel first found in intracellular domains of the proximal tubule and the collecting duct cells [53]. Several studies showed that AQP8 transports water [30, 62] and ammonia [62, 207]. Although AQP8 was shown ultrastructurally localized at inner mitochondrial membrane (IMM) in the liver and functionally permeable to water [30], this was not supported

by a later study that found no difference in water permeability between wild-type and AQP8-deleted mice liver cell inner mitochondrial membrane preparations [253]. In the kidney, mitochondria AQP8 was demonstrated play an important role in the adaptive response of proximal tubule to acidosis recently. AQP8 facilitates transport of NH_3 released from glutamine and glutamate out of the IMM [217] for secretion into the tubule lumen, where the NH_3 buffers acid excreted by epithelia cells, particularly during metabolic acidosis [160]. AQP8 may also facilitate the diffusion of hydrogen peroxide across membranes of mitochondrial in situations when reactive oxygen species is generated, e.g. electron transport chain is highly reduced [14, 16].

1.2.2 Aquaglyceroporins

1.2.2.1 AQP3

AQP3 has a wide tissue distribution. It is permeable to water, glycerol, and urea. Recent studies revealed the pH gating of human AQP3 on both water and glycerol permeabilities using a human red blood cell model and in silico [44]. AQP3 is the most abundant skin aquaglyceroporin, where AQP3 facilitated water and glycerol transport plays an important role in hydration of mammalian skin epidermis and proliferation and differentiation of keratinocytes [18, 25, 165]. In the kidney AQP3 is localized in the basolateral plasma membranes of cortical and outer medullary collecting duct principal cells [101], where they are thought to mediate the basolateral exit of water that enters apically via AQP2. Upon long-term vasopressin stimulation, expression levels of both apical AQP2 and basolateral AQP3 increase, which raises transepithelial water transport capacity of collecting duct principal cells. However, unlike AQP2, there is no evidence for shuttling of AQP3 as no subbasolateral AQP3-containing vesicles have been demonstrated [49]. Emerging evidence showed that AQP3 is regulated on short-term basis likely via cAMP-PKA pathway [90, 109, 147]. In the kidney the increased basolateral diffusion of AQP3 induced by elevated intracellular cAMP likely altered AQP3 interactions with other proteins or lipids in the plasma membrane, which may be a physiological adaptation to the increased water flow mediated by apical AQP2 [147]. AQP3 was recently shown to transport H_2O_2 through the plasma membrane [4, 16], which likely play an important role in initiating intracellular signallings in cell migration [75], inflammation [76], and cancer progression [77, 210].

1.2.2.2 AQP7

AQP7 facilitates transport of water, glycerol, urea, ammonia, arsenite, and NH3 [62, 100, 134]. AQP7 is abundantly expressed in adipose tissue, where it mediates the efflux of newly generated glycerol. Abnormal regulation of glycerol is a remarkable contributing factor to the development of metabolic disease. It is thus plausible that deficiency of AQP7 causes obesity [74, 84] and insulin resistance [201]. It is recently reported that lipolysis activation induces AQP7 translocation from the cortical to inner membranes in white adipocytes [153], in contrast to a previous study showing the opposite translocation of AQP7 [114], probably due to variations in techniques used and different experimental conditions. Six prospective sites of AQP7 for PKA phosphorylation have been identified based on database analysis [143], but the direct regulation by PKA remains to be elucidated.

1.2.2.3 AQP9

AQP9 is expressed at the sinusoidal plasma membrane of hepatocytes [52], where it serves as a conduit for the uptake of NH_3 and mediates the efflux of newly synthesized urea. AQP9 may also function as a glycerol channel to facilitate glycerol uptake in the liver. AQP9 is also permeable to water, glycerol, urea, carbamides, CO_2, and NH_3, moreover, AQP9 is suggested playing a crucial role in metalloid homeostasis by transporting antimonite and arsenite [58, 202]. Interestingly, it also transports much larger substrates such as lactate, purine, pyrimidine [58, 232], probably due to a larger pore size disclosed by a 3D structure analysis [240]. A latest report showed that AQP9 facilitates the membrane transport of H_2O_2 in human and mice cells. Deficiency of AQP9

attenuated H_2O_2-induced cytotoxicity in human and mice cells, indicating that AQP9-mediated H_2O_2 may regulate redox-regulated downstream cell signalling [244]. Human AQP9 has a potential N-glycosylation site at Asn142, a potential PKC phosphorylation sites at Ser11 and Ser222, a potential casein kinase II phosphorylation site at Ser28 [135, 232]. However, little is known about short-term regulation of AQP9.

1.2.2.4 AQP10

AQP10 is an aquaglyceroporin expressed only in the human gastrointestinal tract, but not in the mouse small intestine where it has been demonstrated to be a pseudogene [103, 161]. AQP10 is able to transport water, glycerol and urea when expressed in *Xenopus* oocytes [103]. A recent study provided evidence that AQP10 is another glycerol channel expressed in the plasma membrane of human adipocytes [124]. Silence of AQP10 in human differentiated adipocytes resulted in a 50% decrease of glycerol and osmotic water permeability, suggesting that AQP10, together with AQP7, is particularly important for the maintenance of normal or low glycerol contents inside the adipocyte, thus protecting humans from obesity [124]. Three potential glycosylated sites for AQP10 were predicted, at least one of them Asn133 in the extracellular loop of AQP10 was confirmed. Glycosylation at Asn133 may increase thermostability of AQP10 when challenged with low temperature, indicating a stabilizing effect of the N-linked glycan [179].

1.2.3 Superaquaporins

1.2.3.1 AQP11

AQP11 has a conventional N-terminal Asn-Pro-Ala (NPA) signature motif and an unique amino acid sequence pattern that includes an Asn-Pro-Cys (NPC) motif, which appears essential for full expression of molecular function [95]. Recent evidence strongly suggest that Cys227 of AQP11 plays an important role in formation of its quaternary structure and molecular function [226]. One reconstruction vesicle study has clearly shown that AQP11 is indeed a water channel that transports water as efficient as AQP1 [247, 248]. Although detailed subcellular localization of AQP11 remains unknown, AQP11 has been observed colocalizes with markers of the endoplasmic reticulum in transiently transfected cells [162] and in the kidney from HA-tagged AQP11-transgenic mice [98]. Deficiency of AQP11 is associated with endoplasmic reticulum stress and apoptosis in the kidney proximal tubules [162].

1.2.3.2 AQP12

AQP12 is more closely related to AQP11 than to other aquaporins. With regard to the signature motifs, the first NPA motif of AQP12 is substituted by an Asn-Pro-Thr NPT motif and the C-terminal NPA motif is conserved [31, 107]. AQP12 seems to be expressed specifically in pancreatic acinar cells and retained in intracellular structures, the physiological role of AQP12 remains to be clarified [107]. One study suggests that AQP12 may function as controlling the proper secretion of pancreatic fluid following rapid and intense stimulation [180].

1.3 Gene Structures of AQPs

Table 1.2 shows chromosome localization and numbers of exons of 13 human AQPs.

The gene of AQP0 spans 3.6 kb, contains 4 exons, and is present in single copy in the haploid human genome. Transcription is initiated from a single site 26 nucleotides downstream from the TATA box [186].

Genomic Southern analysis indicated the existence of a single AQP1 gene that was localized to human 7p14 by in situ hybridization [2, 45, 237]. AQP2 cDNA was cloned as the water channel of the apical membrane of the kidney collecting tubule in the rat [59], which shows 42% identity in amino acid sequence to AQP1. Human AQP2 encodes a deduced protein with 89.7–91% amino acid identity to the rat protein [45, 46, 151, 208]. By in situ hybridization, AQP2 gene was mapped to chromosome 12q13 [46, 208], very close to the site of major intrinsic protein (MIP) [65].

Table 1.2 Genes of human AQPs

Aquaporins	Exon numbers	Location	OMIM
AQP0	8	12q13.3	154050
AQP1	7	7p14.3	107776
AQP2	4	12q13.12	107777
AQP3	6	9p13.3	600170
AQP4	6	18q11.2-q12.1	600308
AQP5	5	12q13.12	600442
AQP6	4	12q13.12	601383
AQP7	10	9p13.3	602974
AQP8	6	16q12	603750
AQP9	6	15q21.3	602914
AQP10	6	1q21.3	606578
AQP11	3	11q14.1	609914
AQP12	4	2q37.3	609789

References from www.ncbi.nlm.nih.gov/gene/, and omim.org/entry/

Using a rat AQP3 probe, Ishibashi [99] screened a human kidney cDNA library and isolated a cDNA coding for human AQP3 protein. AQP3 gene is located on 9p13 and appeared to exist as a single copy with 6 exons. The initiation site of transcription was identified to be located 64-bp upstream of the first ATG codon. The 5-prime flanking region contained a TATA box, 2 Sp1 sequences, and some consensus sequences including AP-2 sites [96].

Human AQP4 (initially called mercurial-insensitive water channel, MIWC) cDNA cloned from a fetal brain cDNA library showed that the longest open reading frame encoded 301 amino acids with 94% identity to rat AQP4. Analysis of MIWC genomic indicated 2 distinct but overlapping transcription units from which multiple MIWC mRNAs are transcribed. Later reports revealed that the AQP4 gene is composed of 4 exons encoding 127, 55, 27, and 92 amino acids separated by introns of 0.8, 0.3, and 5.2 kb. Genomic Southern blot analysis indicated the presence of a single MIWC gene, localized on chromosome 18q [140, 250].

Human AQP5 cDNA and gene was isolated and characterized from a human submaxillary gland library, which contained a 795-bp open reading frame encoding a 265-amino acid polypeptide with a transcription initiation site 518 bp upstream of the initiating methionine. AQP5 gene was mapped to chromosome 12q13 [126].

Ma et al. isolated the cDNA by using degenerate PCR from a human kidney cDNA library that was related to AQP2, having 4 exons and was organized similarly to AQP0 and AQP2 and later was referred to this gene as AQP6, assigned to chromosome 12q13 [141, 142].

Human AQP7 gene contains 10 exons. An Alu repetitive sequence and binding sites for several different transcription factors within the AQP7 promoter was determined, including a putative peroxisome proliferator response element (PPRE) and a putative insulin response element, indicating potential involvement of AQP7 in energy metabolism [120].

Like the genes of non-water-selective aquaporins, the AQP8 gene contains 6 exons; however, its exon-intron boundaries are different from the boundaries of those other aquaporin genes. AQP8 gene was mapped to chromosome 16p12 [122, 241].

A partial AQP9 cDNA was isolated by using RT-PCR of leukocyte RNA with primers based on conserved regions of aquaporins [102]. AQP9 shares greater sequence identity with AQP3 and AQP7 than with other members of the family, suggesting that these three proteins belong to a subfamily.

The cDNA encoding AQP10 was isolated from jejunum cDNA library. Sequence analysis predicted that the 264-amino acid protein, which is approximately 53% identical to AQP3 and AQP9, Northern blot analysis revealed expression of a 2.3-kb AQP10 transcript in jejunum but not liver [81].

Human AQP11 gene contains 3 exons and spans 8 kb and was mapped to chromosome 11q14. Human AQP12A gene contains 4 exons and encodes a 1.5-kb transcript only in pancreas [105, 162].

Genetic variants of AQPs may result in disturbance of molecule selection and transport by AQPs; disruption of the formation of tetramers or arrays; and misfolding, faulty sorting of AQPs, or other dysfunction [217]. Cellular and human

studies of naturally-occurring and synthetic mutations have provided great insight into the biology and phenotypic associations of these proteins.

1.4 Crystal Structure of AQPs

Overall AQP structure is largely conserved among the various AQP classes and species isoforms, despite significant differences in sequence similarities (Table 1.3). Structural studies have provided a relevant insight regarding the determining requirements that enable homotetramer formation, the quaternary structure that actually enables water transport activity in animal AQPs [150, 213]. Elucidation of 3-D structures of AQP has confirmed the hourglass fold previously suggested by sequence analysis [110].

The AQPs as a class are tetrameric proteins composed of identical 30-kDa monomers, each of which functions as an independent water channel. The monomer has six transmembrane helices (H1–6, tilted at about 30° with respect to the membrane normally), connected by five loops (A–E), and hydrophilic terminal amino and carboxyl groups always located in the cell cytoplasm [192]. An AQP has three extracellular loops (A, C, and E) and two intracellular loops (B and D) with the asparagine-proline-alanine (Asn-Pro-Ala, NPA) sequences highly conserved [3, 110, 173, 192, 238] (Table 1.3, Figs. 1.2 and 1.3).

The channel is actually a narrow aqueous pathway through the membrane, enclosed by the bundle formed by the six transmembrane domains. The six transmembrane a-helices surround a single, narrow aqueous pore [220], where half transmembrane helices formed by loop B and E (NPA signature motif) fold into the channel from opposite sides of the membrane, associating with substrates selectivity. The positions of loops B and E are stabilized through ion pairs and hydrogen bonds with neighboring transmembrane helices. This pore has electrostatic interactions, where the water molecule, while in Brownian motion (random movement) in the extracellular environment, renders the AQP outer cone walls in a hydrophobic state, causing repulsion [204] (Figs. 1.2 and 1.3a).

Two short helices B and E, together with the tightly stacked NPA motifs, form the central constriction of AQP. The constriction lined with hydrophobic amino acid residues. The diameter of the constriction is about 3 Å , therefore, only one water molecule can pass through the constriction, driven by hydrogen-bond formation with the Asn residues, which lowers the energy barrier for the water molecule entering the constriction [13, 230]. Molecular dynamics simulation of aquaporins revealed how water molecule moves through the channel. The water molecules are forced to pass through the channel in a single fine manner [220] and orientate themselves in the local electrical field formed by the atoms of the channel. Upon entering the channel from the extracellular mouth, the water molecules face with their oxygen atom down the channel. At the level of the central constriction they reverse orientation, facing with the oxygen atom up [13, 163] (Fig. 1.3b).

There is another conserved structural feature of AQP family, which is the aromatic/arginine (ar/R) constriction site located at the extracellular side of the channel. The ar/R constriction site contains highly conserved aromatic and arginine residues [13], acting as a selectivity filter. Having a diameter of 3 Å, which is only slightly larger than the 2.8 Å diameter of the water molecule, the pore constriction prevents permeation of all molecules bigger than water [13, 163]. Therefore the ar/R constriction site is also called the "selectivity filter". In comparison with aquaporins, aquaglyceroporins present much bigger selectivity size (pore size), which can reach ~3.4 Å in diameter [205] (Figs. 1.2 and 1.3a).

Most members of aquaporins possess a cysteine residue in the E loop, which is situated near the pore responsible for functional sensitivity to mercury [192]. In AQP1, the residue Cys189 has been shown to be the site of mercurial binding and water transport inhibition [110, 191]. This inhibition mechanism was elucidated recently by molecular dynamics simulations [85], which shows that the pore is collapsed by conformational changes at

Table 1.3 Sequence alignments of human AQPs at the first and second NPA boxes

	NCBI reference sequence:	Regions of NPA boxes	Fist NPA boxes	Second NPA boxes
AQP0	NP_036196	68–70; 184–186	NISGAHV**NPA**VTFAFLV	YYTGAGM**NPA**SFAPAI
AQP1	NP_001316801.1	76–78; 192–194	HISGAHL**NPA**VTLGLLL	DYTGCGI**NPA**RSFGSAV
AQP2	NP_000477.1	68–70; 184–186	HISGAHI**NPA**VTVACLV	HYTGCSM**NPA**RSLAPAV
AQP3	NP_004916	83–85; 215–217	QVSGAHL**NPA**VTFAMCF	FNSGYAV**NPA**RDFGPRL
AQP4	NP_001304313	97–99; 213–215	HISGGHI**NPA**VTVAMVC	NYTGASM**NPA**RSGPAV
AQP5	NP_001642	69–71; 185–187	PVSGGHI**NPA**ITLALLV	YFTGCSM**NPA**RSFGPAV
AQP6	NP_001643	82–84; 196–198	KASGAHA**NPA**VTLAFLV	HFTGCSM**NPA**SFGPAI
AQP7	NP_001161	94–96; 226–228	RISGAHM**NAA**VTFANCA	MNTGYAI**NPS**RDLPPRI
AQP8	NP_001160.2	92–94; 210–212	NISGGHF**NPA**VSLAAML	PVSGGCM**NPA**RAFGPAV
AQP9	NP_066190	84–86; 216–218	GVSGGHI**NPA**VSLAMCL	LNSGCAM**NPA**RDLSPRL
AQP10	NP_536354	82–84; 214–216	NVSGAHL**NPA**FSLAMCI	ANCGIPL**NPA**RDLGPRL
AQP11	NP_766627	99–101; 216–218	TLVGTSS**NPC**GVMMQMM	SLTGVF**NPA**LALSLHF
AQP12	NP_945349	81–83; 200–202	TLDGASA**NPT**VSLQEFL	PFTSAFF**NPA**LAASVTF

Highly conserved NPAs (asparagine–proline–alanine) are *highlighted* and *underlined*. Sequence was based on NCBI protein database. http://www.ncbi.nlm.nih.gov/protein/

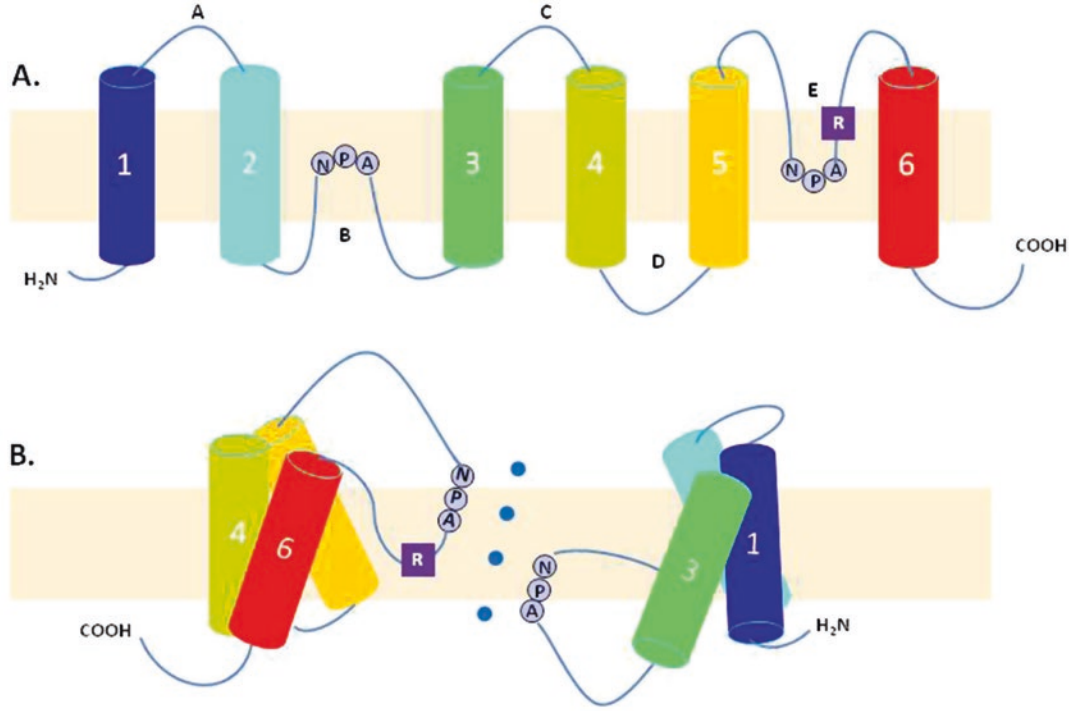

Fig. 1.2 A secondary structure and topology of AQP molecule. (**a**) AQP1 monomer has six membrane-spanning regions (1–6), five loops (*A*–*E*) with intracellular ammino and carboxy termini as well as internal tandem repeats. (**b**) In the monomer, the hydrophilic loops *B* and *E* are bent back into the cavity and meet in the middle to form the putative water-selective gate that contains two consensus NPA motifs (Asn-Pro-Ala). ar/R region is shown close to the entrance of the pore (Modified from Ref. [266])

the ar/R region where the mercury-sensitive cysteine residue is located. However, not all AQPs are inhibited by $HgCl_2$, for examples, AQP4 [251] and AQP6, the water permeability of AQP6, on the contrary, is actually increased in the presence of this mercurial agent [256].

The protein can self-assembles into a tetrameric biological unit by which an additional tetrameric pore is formed, which is thought to be responsible for the translocation of dissolved gasses and ionic species [91]. AQP4, on the other hand, forms larger oligomeric structures in the plasma membrane, also called orthogonal arrays (clusters of intramembrane particles in a special geometric organization) [251], which may suggest a possible role of AQP4 in membrane junction formation *in vivo* [242].

1.5 Protein Modification of AQPs

Proteins can be regulated after translation by the reversible or irreversible addition of functional groups (e.g., phosphorylation, acetylation, and methylation), peptides (e.g., ubiquitination, SUMOylation) or other complex molecules (e.g., glycosylation). Through changes in protein conformation, these post-translational modifications (PTMs) have been shown to modulate the localization, stability, activity, and interacting partners of their substrate proteins, thus playing pivotal roles in intracellular signalling, protein maturation and folding. The precise effect of PTMs depends on the nature of the covalent modification, the identity of the substrate and the residue that is specifically targeted by the chemical

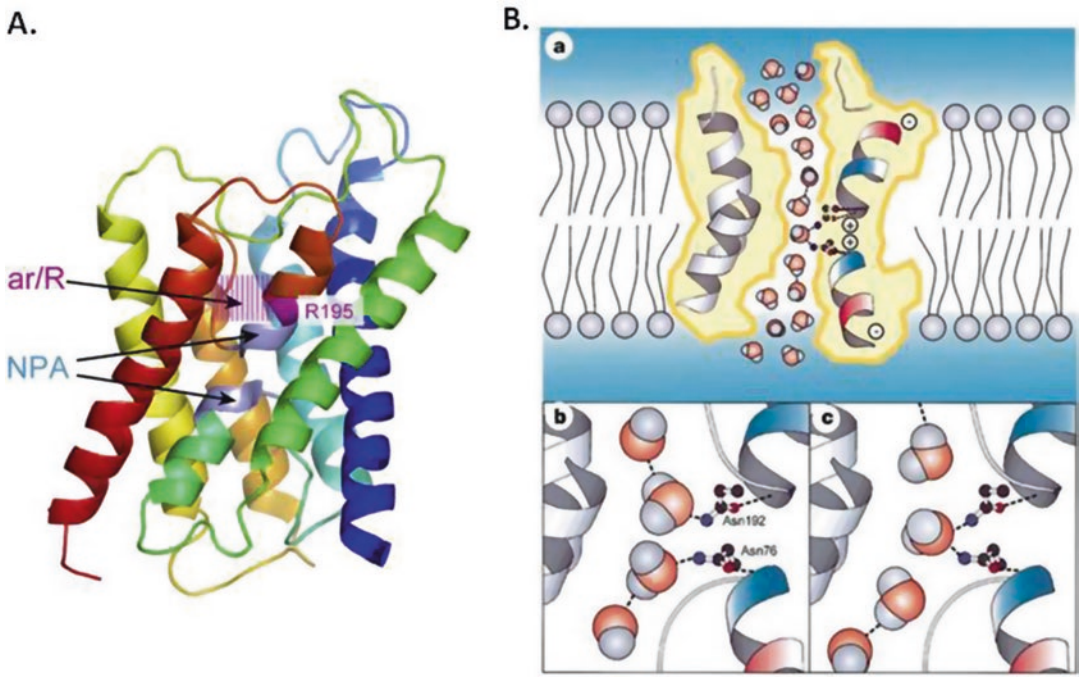

Fig. 1.3 Schematic architecture of AQP1. (**a**) A ribbon model of AQP1 using a rainbow colour scheme from *blue* (N-terminal) to *red* (C-terminal). The narrowest region in the AQP1 pores, previously termed ar/R, is located close to the extracellular entrance of the pore. The Arg195 and NPA motifs are shown in *magenta* and *light blue*, respectively. (Reproduced with permission from Ref. [230]). (**b**) Schematic architecture of the channel within an AQP1 subunit (*sagittal section*). *a*, Diagram illustrating how water molecules pass through the constriction of the pore. *b* and *c*, four water molecules shown represent transient interactions with Asn 76 and/or Asn 192. Note water dipole reorientation. Two partial helices meet at the midpoint of the channel, providing positively charged dipoles that reorient a water molecule as it traverses this point (Reproduced with permission from Ref. [163])

reaction [149]. This section of the review mainly focuses on PTMs of the arginine vasopressin (AVP)-regulated AQP2 that is one the best understood and discusses modification of other AQPs at the end.

1.5.1 Phosphorylation

The modulation of protein abundance in plasma membrane requires a delicately regulated translocation (trafficking) from intracellular compartment to the membrane, which is achieved through multiple sorting signals and PTMs. Phosphorylation is one of the most well-studied PTMs, which often involved in regulation of protein function and cellular distribution, AQP2 phosphorylation is one of the best characterized examples.

AQP2 is expressed in the principal cells of the kidney collecting duct [45, 171, 172], its intracellular distribution is finely regulated by AVP. Stimulation with AVP results in a predominant translocation of AQP2 to apical membrane localization from subapical compartments. This renders the apical membrane highly permeable to water and is a key event in formation of concentrated urine and thus in regulation of body water balance. This intracellular re-distribution of AQP2 induced by AVP is closely associated with phosphorylation/dephosphorylation (and/or ubiquitination) of AQP2.

Phosphorylation of AQP2 at multiple sites in the C-terminus governs its translocation to the apical membrane from intracellular vesicles [86, 236]. AQP2 contains numerous putative phosphorylation sites for various protein kinases, e.g., PKA, PKG, PKC, and casein kinase II [27] based

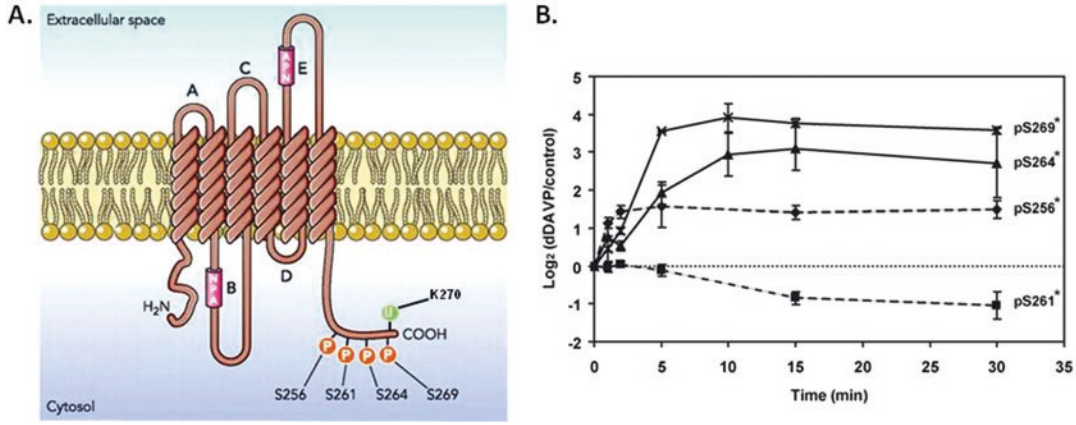

Fig. 1.4 Illustration of the topology of AQP2 and time course studies of AQP2 C-terminal phosphorylation. (**a**) Schematic illustration of the topology of AQP2 and the C-terminal phosphorylation (S256, S261, S264, S269) and ubiquitination sites (K270) of AQP2 (Modified from Ref. [157], originally from Pisitkun T, Physiology (Bethesda), 2007). (**b**) Time course of changes in AQP2 phosphorylation at S256, S261, S264, and S269 in response to 1 nm dDAVP (a V2R agonist) in rat inner medullary collecting duct (IMCD) tubule suspensions. Note that maximal phosphorylation at S256 occurs quickly, whereas it takes longer for maximal phosphorylation to occur at the other S264 and S269. Phosphorylation at the S256, S264, and S269 sites remains high as long as the agonist is present. In contrast, dDAVP stimulation results in decreased phosphorylation at S261 (Reproduced with permission from Ref. [88])

upon bioinformatic analysis. Phosphorylation of the serine at position 256 (S256) of AQP2 in the C-terminal tail of AQP2 was the first to be identified and the best characterized phosphorylation site of AQP2 [59, 112, 236]. Large scale phospho-proteomic analysis later demonstrated that beside S256 phosphorylation site, the polyphosphorylated region of AQP2 contains S261, S264, and S269 (Fig. 1.4a). S256-AQP2 is a target for PKA-induced phosphorylation, which was evidenced by *in vitro* phosphorylation assays of AQP2 C-terminal peptides [88]. Kinases other than PKA may also be involved in AQP2 C-terminal phosphorylation. PKG is proposed to modulate AQP2 trafficking. The agonist of PKG, cGMP, has been shown to mediate translocation of AQP2 to the plasma membrane in AQP2-transfected LLC-PK1 cells and in isolated kidney slices [22]. Activators of the cGMP pathway, such as atrial natriuretic peptide (ANP), L-arginine, cGMP phosphodiesterase type 5 (PDE5) inhibitors sildenafil citrate, elevated intracellular cGMP levels, resulting accumulation of AQP2 in plasma membrane [19, 22, 243]. These data suggests a positive role of PKG on AQP2 trafficking. However, one study showed that ANP and NO (nitric oxide) signalling deceased S256-AQP2 phosphorylation, reduced AQP2 in the plasma membrane, antagonizing vasopressin-mediated water permeability in inner medullary collecting duct cells [116]. In addition, activation of PKC pathway mediates endocytosis of AQP2 that was independent of the phosphorylation state of AQP2 at serine 256 [236]. AKT (also known as PKB) was recently shown to mediate vasopressin-stimulated AQP2 membrane accumulation [113]. The protein kinases responsible for S261, S264, and S269 phosphorylation appears more complex [86–88, 199].

Phosphorylated AQP2 at S256 (pS256) is detected in both intracellular vesicles and the apical plasma membrane in the collecting duct, where its abundance is increased in response to AVP treatment [39]. The expression of phosphorylated AQP2 at S264 was found in plasma membrane-associated compartments and early endocytic pathways. This phosphoform of AQP2 was found to increase in abundance in both the apical and basolateral plasma membrane of principal cells after acute dDAVP treatment [55]. Similar to pS256 of AQP2, pS269 was associated with membrane accumulation of AQP2, indicat-

ing a role in AQP2 plasma membrane targeting [88, 139, 156]. pS261-AQP2 is predominantly localized within the cell in compartments different from the endoplasmic reticulum, Golgi apparatus, and lysosomes [87].

In IMCD tubule suspensions, the specific V2R agonist dDAVP or exogenous cAMP increased phosphorylation of AQP2 at S256, S264, and S269, which remains high as long as the agonist is present [88]. Phosphorylation of S256 increases initially and maximal phosphorylation at S256 occurs rapidly, whereas maximal phosphorylation at the other sites (S264 and S269) occur relatively slow. In contrast, dDAVP stimulation results in decreased phosphorylation at S261 [88] (Fig. 1.4b).

S256 phosphorylation appears required and strongly facilitates phosphorylation of S264 and S269 [88], as the S264 and S269 phosphoforms of AQP2 are not observed in cells expressing the S256 mutated form of AQP2 or in kidney sections from a mouse model with a mutation of S256 to leucine [88, 155]. Recent evidence demonstrated that S256 phosphorylation alone is necessary and sufficient for regulated membrane accumulation of AQP2 induced by AVP (or cAMP), independently of the phosphorylation state of any other sites in the C terminus, e.g. S264 or S269 [7]. These observations strongly suggest that S256 phosphorylation is a priming event for phosphorylation of S264 and S269 and play a critical role in intracellular translocation of AQP2. Both S256 and S269 phosphorylation are involved in the insertion of AQP2 into the apical plasma membrane [157], although the phosphoform of AQP2-pS269 has a distinct cellular localization in the apical plasma membrane [155]. The role of AQP2 phosphorylation at S264 in subcellular distribution of AQP2 in the cell remains unclear [55]. Earlier studies revealed that the increased monophosphorylation of AQP2 at S256 with vasopressin stimulation of rat IMCD coincided with decreased phosphorylation of AQP2 at S261, which was associated with intracellular vesicle distribution, suggesting that phosphorylation of S256 and S261 may inversely regulate AQP2 trafficking [55, 86, 87, 228].

1.5.2 Ubiquitination

It is known that two major protein degradative pathways to function in mammalian cells, the ubiquitin proteasome pathway and lysosomal proteolysis pathway. Ubiquitin (Ub), a 76-amino acid peptide, plays a key role in proteasome-mediated protein degradation. Ubiquitin labels protein through a conjugation system comprising E1 activation, E2 conjugation, and E3 ligation enzymes. Following conjugation to proteins, ubiquitin serves to target them for degradation by cytosolic proteasome complex. Ubiquitination of certain plasma membrane proteins can promote their internalization via endocytotic pathway, followed by their degradation in lysosomes [69]. Protein ubiquitination is reversed by deubiquitinating enzymes (DUBs), which is essential for cellular homeostasis.

The first example of ubiquitination of an AQP was reported more 10 years ago. The studies demonstrated that AQP1 was able to be ubiquitinated and degraded by the proteasome. Exposure to hypertonic medium induced decrease of AQP1 ubiquitination and markedly increased stability of AQP1 protein, thereby contributing to overall protein induction [128].

There are three putative potential attachment sites (cytosolic lysine residues) for AQP2 ubiquitination at positions 228, 238, and 270, but site mutation study revealed that K270 is the only substrate for ubiquitination, with one to three ubiquitins added in a K63-linked chain [111] (Fig. 1.4a). The ubiquitination of AQP2 at the plasma membrane results in the internalization of AQP2, transport to intracellular multivesicular bodies and subsequent proteasomal degradation [111]. Transcriptome analysis and liquid chromatography-tandem mass spectrometry proteomic analysis identified that five common isoforms of E3 ligases (UBR4, UHRF1, NEDD 4, BRE1B, and Cullin-5) are putatively associated with dDAVP (1-deamino-8-D-arginine vasopressin, a vasopressin V2 receptor agonist)-induced AQP2 regulation [127]. For examples, a vasopressin-activated calcium-mobilizing receptor Cullin-5, a member of the cullin gene family of scaffold proteins of the E3 complex [185], was

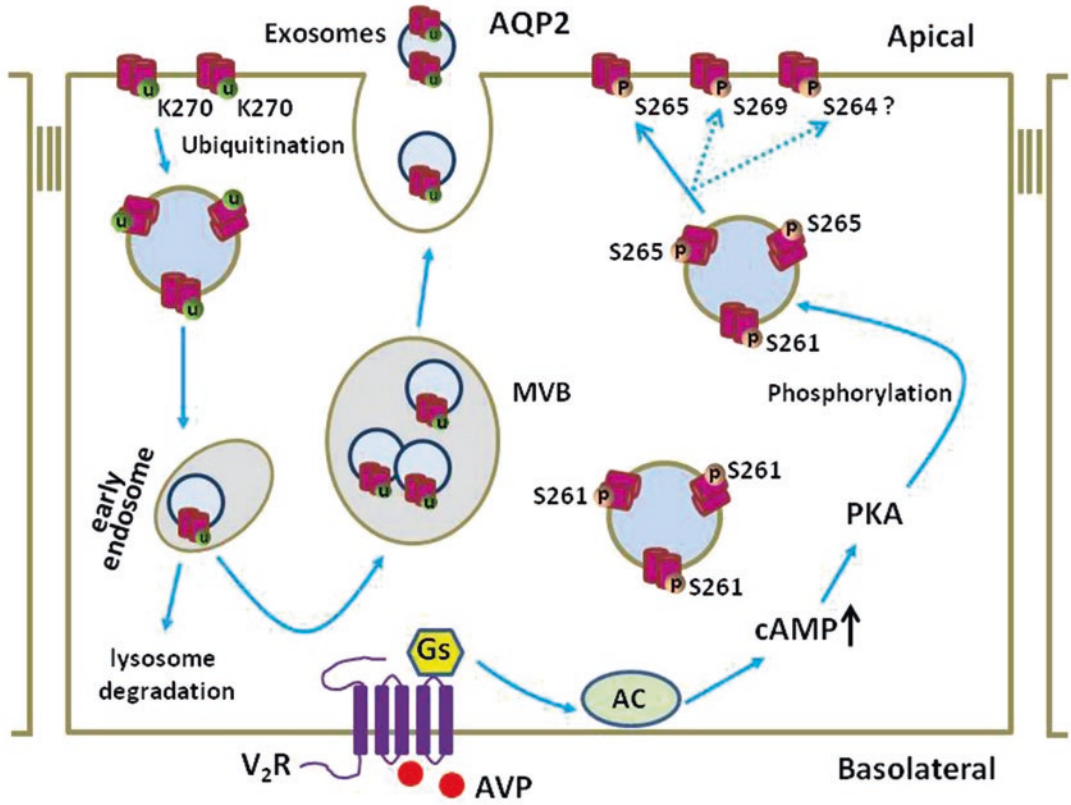

Fig. 1.5 Phosphorylation and ubiquitination of AQP2 determines the intracellular localization. Arginine-vasopressin (AVP) induced phosphorylation at S256 on AQP2 monomers, followed by increased S269 and S264 phosphorylation and reduced S261 phosphorylation, resulting in steady redistribution of AQP2 from intracellular vesicles to the apical plasma membrane. AQP2 is ubiquitinated with one or more ubiquitin proteins at K270. Ubiquitination occurs in the membrane after removal of AVP stimulation and mediates steady redistribution AQP2 to intracellular vesicles. Ubiquitination of AQP2 may be sorted to the multivesicular body (MVB), where AQP2 is either degraded in lysosomes or released in exosomes into the urine via exocytosis

observed to be upregulated during dDAVP withdrawal, which was associated with increased prevalence of AQP2 among the ubiquitinated proteins in intracellular vesicles fractions. This finding suggests that CUL5 may play a role in the attachment of Ub to AQP2, resulting in an ubiquitination of AQP2, internalization of AQP2 and reduction of AQP2 abundance after dDAVP withdrawal, presumably via lysosomal and/or proteosomal degradation [127].

Phosphorylation of AQP2 at S256 and dephosphorylation at S261 cause its translocation from intracellular vesicles to the apical membrane, whereas ubiquitination of AQP2 at K270 induces its internalization and lysosomal degradation, or released in exosomes into the urine via exocytosis. Phosphorylation and ubiquitination are highly dynamic in the cell. A cross-talk between phosphorylation and ubiquitination has been proposed [92]. Phosphorylation and ubiquitination likely act in concert and could greatly increase fine regulation of protein function. Together with the plasma membrane targeting signal of S256, S264, and S269 phosphorylation as well as intracellular S261 phosphorylation, K270 ubiquitination fine tunes the subcellular distribution of AQP2 (Fig. 1.5).

Recently, two studies examined the potential interplay between polyubiquitylation and polyphosphorylation of AQP2 [159, 228]. Stimulation with dDAVP or forskolin induces pS256 on AQP2 monomers, followed by increased S269

and S264 phosphorylation and reduced S261 phosphorylation, resulting in steady-state redistribution of AQP2 from vesicles to the apical membrane, whereas increased AQP2 ubiquitination induced endocytosis and steady-state redistribution of AQP2 to intracellular vesicles. Interestingly, phosphorylation of S261 on AQP2 occurs after ubiquitin-mediated endocytosis, suggesting that phosphorylation of S261 does not induce AQP2 ubiquitination itself, but likely stabilize ubiquitinated AQP2 (Fig. 1.5).

Phosphorylation often occurs as a priming event for ubiquitination and ubiquitination can regulate protein phosphorylation by regulation of kinase activity [92]. AQP2 phosphorylation was demonstrated to be able to override dominant endocytic signal of K63-linked polyubiquitylation. In polarized epithelial cells and kidney tissue, distribution of AQP2 on the plasma membrane is regulated by phosphorylation at S256 and S269. The rate of AQP2 endocytosis was reduced by prolonging phosphorylation specifically at S269. AQP2 phosphorylation at S269 and ubiquitylation at K270 can occur in parallel, with increased S269 phosphorylation and decreased AQP2 endocytosis occurring when K270 polyubiquitylation levels are maximal [159]. The study suggests that site-specific phosphorylation can counteract polyubiquitylation to determine its final localization.

1.5.3 SUMOylation

Beside from ubiquitin, the best-studied ubiquitin-like protein is Small Ubiquitin-like MOdifier (SUMO). SUMOylation is a reversible PTM where SUMOs are covalently attached to lysine residues in the target proteins, similar to ubiquitination. Sumoylation has been found to be involved in multiple nuclear processes, such as chromatin organization, transcription and DNA repair. Sumoylated proteins also play important roles in the regulation of channel activity, receptor function, G-protein signalling, cytoskeletal organization, exocytosis, and autophagy. So far there is no evidence showing involvement of Sumoylation in regulation of AQP expression.

1.5.4 Glutathionylation

As an important PTM, S-Glutathionylation exerts protection of cysteine residues against irreversible oxidation during redox imbalance. The relationship between AQP2 and S-glutathionylation is of potential interest because reactive oxygen species (ROS) may influence the expression and the activity of different transporters and channels, including aquaporins. Recent evidence suggested that in mpkCCD cells, vasopressin stimulated translation of seven glutathione S-transferase (GST) proteins functioning to conjugate the tripeptide glutathione to substrates e.g. cysteine, likely indicating the involvement of redox into vasopressin-activated signal transduction pathway [206]. Glutathione is one of the major cellular antioxidant molecules that are continuously converted into the reduced form of GSH. Topological analysis of AQP2 suggests that Cys75 and Cys79 on cytosolic B-loop might be target of S-glutathionylation [229]. Subsequently, the study later demonstrated that AQP2 is subjected to S-glutathionylation both in kidney tissue and in HEK cells stably expressing AQP2 [229]. The S-glutathionylation of AQP2 is tightly modulated by changes in cellular ROS content both in renal tissue and in HEK cells stably expressing AQP2, specifically, an oxidant inducer caused a significant increase in AQP2 S-glutathionylation secondary to an increases in ROS content, indicating that this redox sensitive PTM is linked to the redox condition of the tissue, however, whether S-glutathionylation affects the localization and the activity of AQP2 is not reported [229].

1.5.5 Glycosylation

In their extracellular loops, AQPs contain N-linked glycosylation consensus sites, some of which are not efficiently recognized during protein synthesis by oligosaccharyltransferase, generating a mixture of glycosylated and nonglycosylated species. N-glycosylation is not believed to be important in the transport function of aquaporins. In AQP1, the site of N-glycosylation

is Asn42, which lies in a potential N-glycosylation consensus sequence. The early study using site-directed mutant of Asn42 showed that the non-glycosylation of AQP1 failed to affect water permeability in oocytes [192]. In AQP2, the glycoslated form has a shorter half-life than the non-glycosylated form [206], indicating that N-linked glycosylation is not necessary for the stability of AQP2. The glycosylation seems important for cell surface expression of AQP2 [82] but is not essential for routing, evidenced by that inhibition of glycosylation does not prevent delivery of AQP2 to the plasma membrane in response to increased cAMP [11]. In addition, glycosylation appears not essential for tetramerization of AQP2 in the endoplasmic reticulum, as part of tetrameric complexes with one or more nonglycosylated AQP2 molecules [82]. However, early data indeed suggested that addition of a single *N*-linked oligosaccharide moiety can partially compensate for ER folding defects induced by disease-related mutations [28].

1.5.6 Several Other PTMs

N-terminal acetylation has been proposed to be a determinant of protein stability [93]. AQP2 was one of the proteins identified with N-terminal acetylation [206]. Recently, N-linked acetylation, carbamylation, and oleoylation was discovered on AQP0 at the N-terminal amino acid residues by using direct tissue profiling method designed for membrane protein analysis [72]. Although biological and physiological significance of these PTM is still undetermined, it might play potential roles in protein-protein interactions and thus regulation of water permeability in the eye. Another study revealed that the N-terminal cysteines of AQP4 are post-translationally modified with palmitic acid, and this palmitoylation likely inhibited the formation of AQP4 square arrays in Chinese hamster ovary cells transfected with AQP4 [222].

A reduction in histone H4 acetylation level of AQP5 induced by TNF-α caused decrease of AQP5 expression and the fluid secretion from salivary acinar cells [249]. Insulin treatment resulted in a marked reduction in the acetylation and a significant increase methylation of histone H3 at the AQP9 promoter in hepatocytes, which was associated with downregulation of AQP9 protein [195]. However, these studies about acetylation and methylation mainly focus on histones and their roles in modulating chromatin structure and gene transcription, which more belongs to epigenetic regulation but not PTM.

1.5.7 PTM of Several Other AQPs

Here are a few examples about phosphorylation regulation of several AQPs, in particular, AQP1, AQP4, AQP5 and AQP8, which have been implicated to trigger membrane specific trafficking.

1.5.7.1 AQP1

AQP1 water permeability has been shown to be dynamically regulated by several hormones. In *Xenopus* oocyte expression system, water permeability of AQP1 was increased by vasopressin and decreased by ANP [184]. Early data from *in vitro* and *in vivo* studies suggest that phosphorylation by PKA result in trafficking of AQP1 from an intracellular compartment to the apical membrane [73, 145, 146]. In addition, PKC positively regulates both water permeability and ionic conductance of AQP1 channels by phosphorylating Thr157 and Thr239 [264]. A quite recent study demonstrated that the signaling molecules cAMP and cGMP promote trafficking of AQP1 into the brush border membrane of proximal tubular cells from intact endosomal compartment [188]. In the same study, cAMP and cGMP have both reduced the ubiquitination of AQP1 and increased AQP1 protein stability, as two potential ubiquitination sites (Lys-243 and Lys-267) were indicated in the AQP1 amino acid sequence [69, 128].

Recent data have shown that a hypotonicity-induced translocation of AQP1 occurs rapidly, which is Ca^{2+}/calmodulin, PKC, and microtubule dependent [41, 42]. On the other hand, interestingly, exposure to hypertonicity also increases AQP1 expression in cultured renal proximal and inner medullary cells [108, 235]. The effect of

hypertonicity may be mediated by promoter-mediated activation of AQP1 synthesis [234] and by inhibition of AQP1 protein degradation [128].

1.5.7.2 AQP4

It is well established that AQP4 water permeability can be regulated by reversible protein phosphorylation. There are several potential phosphorylation sites of AQP4 for PKA, PKC, PKG, casein kinase (CK) and calcium/calmodulin dependent protein kinases (CaMK).

The Ser111 residue of AQP4 is a potential site for both PKA phosphorylation and calcium-dependent CaMKII phosphorylation. The phosphorylation of Ser111 by PKA increases water permeability of AQP4 [69, 71, 263]. Agents that stimulate cAMP production including forskolin, AVP, and V2 receptor agonist were reported to increase the water permeability in a renal cell line transfected with AQP4 [69]. The increased membrane water permeability of an astrocyte cell line transfected with AQP4 cDNA induced by Ser111 phosphorylation was able to be reversed by a Ca^{2+}/CaMKII inhibitor, suggesting that phosphorylation of Ser111 via CaMKII increases the water permeability of AQP4 [70]. It is therefore reasonable to speculate that Ser111 is phosphorylated by PKA in kidney cells and by CaMKII in astrocytes, both phosphorylation leads to increased permeability of AQP4. Early studies have suggested that Ser111 could also be phosphorylated by PKG via CaMKII-NO-cGMP-PKG signalling [71]. In contrast to phosphorylation of Ser111, phosphorylation of Ser180 by PKC downregulates AQP4 water permeability both in *Xenopus* oocyte expression system and in cultured kidney epithelial cells [73, 263], which is previously considered due to a gating effect, since expression of AQP4 in the cytosolic compartment is negligible both under basal conditions and in hormone-stimulated cells [263].

However, recent evidence from crystal structure, functional studies, and molecular dynamics simulations against phosphorylation dependent gating of AQP4 via Ser111 and Ser180 [9, 154, 204]. One latest study by using mass spectrometry demonstrated that AQP4 plasma membrane trafficking or channel gating is not significantly modulated by phosphorylation at COOH-terminal serine residues [9].

1.5.7.3 AQP5

AQP5 membrane trafficking has been shown to be affected by cAMP in a PKA-dependent manner [121, 254]. Elevated intracellular cAMP appears to have distinct acute and chronic effects, which cause a decrease in AQP5 membrane abundance in short-term (minutes) and increased total AQP5 protein in long-term (hours) [211]. Two consensus PKA sites in AQP5 that are able to be phosphorylated have been identified, Ser156 in cytoplasmic loop D [34] and Thr 259 [78, 121] in the carboxy-terminus. However, mutation of these phosphorylation sites resulted in constructs with the same membrane abundance as wild-type AQP5, indicating that phosphorylation may not occur under basal conditions. In contrast, AQP5 phosphorylation at Thr259 by cAMP-PKA was recently shown to be associated with lateral diffusion of AQP5, potentially regulating water flow in glandular secretions [119]. Recent data demonstrated that membrane expression of AQP5 is affected by Ser156 phosphorylation, either by increased targeting or decreased internalization or both [115].

1.5.7.4 AQP8

AQP8 is primarily located within the liver cell in a vesicular compartment [29, 61] and in mitochondria [57]. The expression of AQP8 on cell-surface is very low under basal conditions [61, 67], however, hormone glucagon or its second messenger cAMP strongly induced redistribution of AQP8 to the plasma membrane from intracellular compartment [67]. Thereby, the water permeability of plasma membrane is increased, facilitating osmotic water transport and canalicular bile formation. These studies suggest that both PKA and PI3K pathways are involved in glucagon-induced trafficking of AQP8 [67, 68].

1.6 Expression Regulation of AQPs

AQPs are expressed in a wide range of tissues and usually spatially located within a certain region of the cell. AQPs mediate the bidirectional water flow driven by an osmotic gradient. The transport of water mediating by AQPs is regulated either by gating, a conformational change, or by altering the AQP density in particular membrane. The trafficking of AQPs is regulated at the transcriptional and/or translational level, and also involves shuttles of AQPs between intracellular storage vesicles and the target membrane. PTMs, especially phosphorylation, are one of important mechanisms regulating redistribution of AQPs in the cell. The regulation of AQPs, either through gating or trafficking, allow for rapid and specific regulation in a tissue-dependent manner. There is another relatively long-term regulation by which increased/decreased protein abundance of AQPs is affected by systemic hormones (e.g. vasopressin, insulin, ANP, angiotensin II), local molecules (e.g. purine [265], prostaglandins [182, 262], bradykinin [227], dopamine [20], and other common microenvironment signals including pH [37, 167], divalent cation concentrations [70] and osmolality [125, 129, 235]. These regulations of AQPs are often associated with certain physiological or pathophysiological conditions. In this section, regulatory mechanisms of AQP2 are discussed mainly, based on the fact that regulation of AQP2 is well characterized.

1.6.1 Gating of AQP2

In plants and yeast, the plasma membrane-localized AQPs are gated in response to environmental stress [123]. In mammals, gating regulates the water permeability of AQP0, in a pH-dependent and Ca^{2+}-calmodulin dependent manner [169, 198]. Gating of AQP4 via phosphorylation has also been suggested [71]. Data on gating of AQP2 via phosphorylation is still debatable. Studies from different research groups by using similar systems or different systems have failed to unanimously agree [54, 155, 156]. In fact, the predominant regulatory mechanism of mammalian aquaporins is trafficking. AQP2 trafficking is a canonical example.

1.6.2 Trafficking of AQP2 to the Membrane

Facilitated transport processes across epithelia require an apically to basally polarized distribution of transmembranous transport proteins like AQP. AQPs must be transported in vesicles specifically to the apical or basolateral plasma membrane domain, which requires trafficking machineries, including exocytosis, endocytosis, sorting, clustering, and the maintenance of integral membrane proteins at the plasma membranes [51].

Following translation, AQP2 is folded into its monomeric conformation, and subsequently a tetrameric complex, in the endoplasmic reticulum. These tetramers are later transported to the Golgi apparatus where two monomers are N-glycosylated before they are transported through the trans-Golgi network to different subcellular compartments [158]. A large proportion of AQP2 that exits the trans-Golgi network stored in some form of endosomal vesicles and upon relevant stimulus (e.g. AVP) is transported to the apical plasma membrane [158, 172]. Trafficking of intracellular vesicular containing AQP2 to the membrane, docking and fusion of AQP2 vesicles with the apical plasma membrane (exocytosis), and removal of AQP2 from the membrane (endocytosis) is likely attributed to total plasma membrane abundance of AQP2 [158].

1.6.2.1 The cAMP-Mediated Effect of Vasopressin on AQP2 Trafficking

AQP2 is present in the principal cells of the renal collecting ducts, and its abundance and intracellular localization in response to the AVP determine water reabsorption in this segments [59, 158, 172]. In the absence of vasopressin, AQP2 is localized in subapical vesicles. Upon to stimulation of AVP, a predominantly apical membrane localization of AQP2 is induced. Classically,

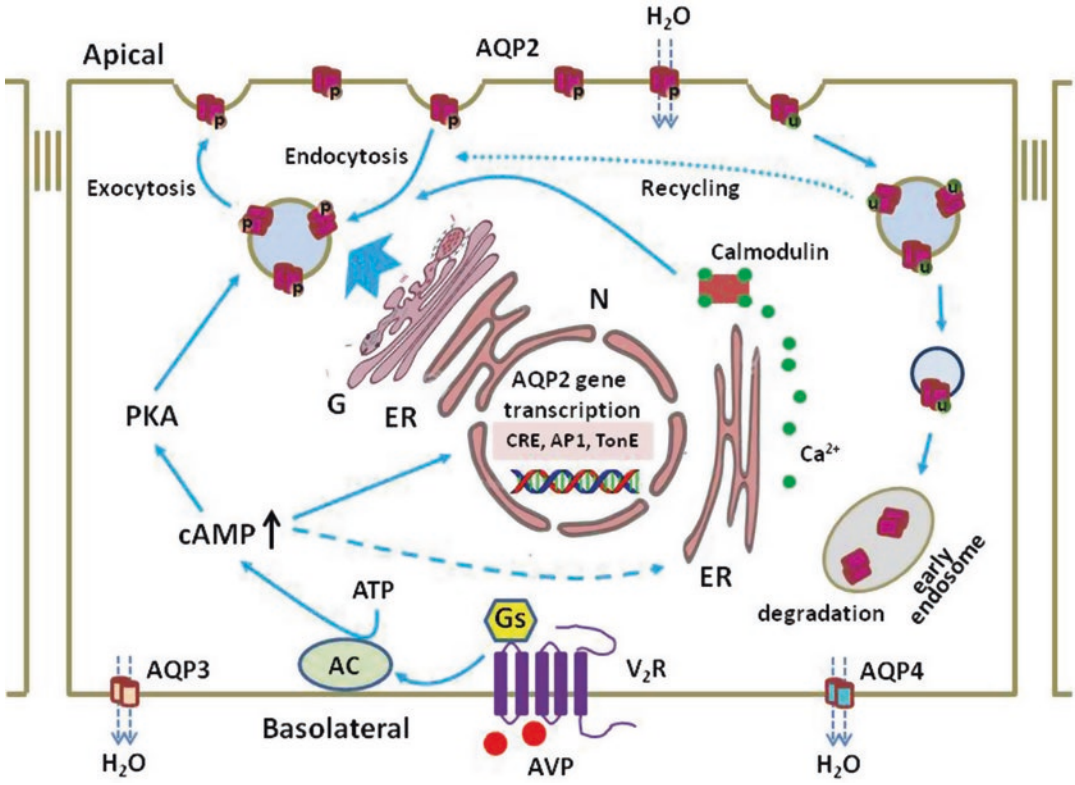

Fig. 1.6 Protein regulation of AQP2 by AVP. AVP binds to the vasopressin type-2 receptor (V2R), present on the basolateral membrane of renal collecting duct principal cells. This induces a signaling cascade, involving Gs protein mediated activation of adenylate cyclase (AC), a rise in intracellular cAMP, activation of protein kinase A (PKA) and subsequent phosphorylation of AQP2. This results in the redistribution of AQP2 from intracellular vesicles to the apical membrane. AVP stimulation also results in increased intracellular Ca^{2+} levels *via* Ca^{2+} release from calmodulin-dependent ryanodine-sensitive intracellular stores, which induces apical membrane expression of AQP2. On the long term, vasopressin increases AQP2 expression *via* activating transcriptional factors, which stimulates transcription of AQP2 at the AQP2 promoter. Once the water balance is restored, AVP levels drop and AQP2 is internalized via ubiquitination. Internalized AQP2 can either be targeted to recycling pathways or to degradation *via* lysosomes. Driven by the transcellular osmotic gradient, water enters principal cells through AQP2 and pass through basolateral plasma membrane *via* AQP3 and AQP4 to the blood. *N* nuclear, *ER* endoplasmic reticulum, *G* Golgi apparatus

AVP binds to the basolaterally located vasopressin V2 receptor (V2R), which is coupled to adenylate cyclase by the heterotrimeric G-protein, Gs. The binding of vasopressin to its receptor causes α subunit of G-protein to release GDP, bind to GTP, and dissociate from the β and γ subunits. This G αGTP complex, in turn, activates adenylate cyclase (AC) to synthesize cAMP which activates PKA. PKA in turn directly or indirectly phosphorylates AQP2 at Ser256, which is important for AQP2 trafficking as described above. The phosphorylation of AQP2 then increases transport or trafficking via the cytoskeleton from the storing cytoplasmic vesicles to the apical membrane [59, 60, 112, 172, 174, 236] (Fig. 1.6).

As PKA has many cellular targets, localization of PKA to specific sites of targets is necessary for a timely and spatially effective phosphorylation of target protein. This is mediated by PKA-anchoring proteins (AKAP). For the phosphorylation of AQP2, anchoring of PKA by AKAP in close proximity to AQP2 is a prerequisite [51, 177]. Several splice variant of

AKAP18, AKAP18delta, and AKAP220 has been reported to be involved in the shuttling of AQP2 [83, 117, 118].

1.6.2.2 The Role of Calcium in Vasopressin-Induced AQP2 Trafficking

Several studies have demonstrated a role of intracellular Ca^{2+} mobilization in vasopressin-mediated AQP2 trafficking. By binding to V2 receptors, vasopressin causes a transient increase in intracellular Ca^{2+} concentration and calcium oscillations in IMCD cells [35, 38, 257]. Removal of extracellular Ca^{2+} in perfused IMCD did not prevent the initial rise of intracellular Ca^{2+} levels induced by vasopressin but inhibited the sustained oscillations [257]. Ryanodine inhibitors, calmodulin inhibitors, or intracellular Ca^{2+} chelators were shown to block vasopressin-stimulated translocation of AQP2 to the plasma membrane and increase of osmotic water permeability in primary cultured IMCD cells [35, 38, 257]. These observations suggest that vasopressin-induced intracellular increase of Ca^{2+} is important for AQP2 translocation to the apical membrane. This involves intracellular Ca^{2+} released from ryanodine-sensitive stores and the influx of extracellular Ca^{2+}. In contrast, data from other studies in primary cultured epithelial cells from renal inner medulla showed that cAMP is sufficient for triggering the exocytic recruitment of AQP2, which is not evoked by vasopressin-induced intracellular calcium increases [136].

1.6.2.3 Vesicles Bearing AQP2 Transport to the Membrane

For the co-ordinated delivery of vesicles to specific sites their transport along the cytoskeleton is needed [51]. In the case of AQP2, the reorganization of microtubules and actin cytoskeletons are essential in its trafficking. The actin cytoskeleton provides a cage anchoring AQP2 in unstimulated cells, preventing their exocytosis. The binding of AVP to V2R causes the depolymerization of F-actin in collecting duct cells, which is critical in promoting the trafficking and fusion of AQP2-bearing vesicles with the apical membrane [212, 259]. Indeed, AQP2 itself can directly modulate the local actin cytoskeleton depolymerization and subsequent exocytosis. PKA-induced phosphorylation of AQP2 at S256 reduced the direct binding of AQP2 to G-actin, but increased the affinity of AQP2 to myosin-Vb, one of the central regulators in apical trafficking. This interaction results in a reduced quantity of myosin-Vb that bounds to F-actin, resulting in F-actin destabilization that allows translocation of AQP2 vesicles to the plasma membrane (Fig. 1.5) [175, 176, 178]. The A-kinase anchoring protein 220 (AKAP220) is a ubiquitously expressed vesicular and membrane-associated anchoring protein that positively regulate actin polymerization and microtubule stability during membrane protrusion [245]. Early studies showed that AKAP220 is physically associated with AQP2 in the principal cells of the kidney collecting ducts [181]. A very lately study demonstrated that loss of AKAP220 leads to accumulation of AQP2 at the plasma membrane and reduces urine-diluting capacity during overhydration [245]. This study supports the role of actin-barrier dynamics in the subcellular localization of AQP2 in the kidney (Fig. 1.7).

It should be noted that there are some binding proteins at C-terminus of AQP2 (or in AQP2-bearing vesicles) that mediate AQP2 sorting and the destination of AQP2. A large-scale proteomic analysis showed that more than 180 proteins were identified, including SNARE proteins, trans-Golgi network markers, motor proteins etc. These proteins interact with AQP2 via direct binding, indirect linkage, forming a protein complex, or colocalization in the same vesicles [10, 209], which is actively involved in regulation of AQP2 dynamics.

1.6.2.4 Docking and Fusion of Vesicles Bearing AQP2 with the Apical Membrane (Exocytosis)

Fusion of AQP2-bearing vesicles with the plasma membrane is a key terminal step in vasopressin-regulated water transport. The docking and fusing of AQP2-bearing vesicles is mediated by SNARE (Soluble N-ethylmaleimidesensitive factor attachment protein receptors) mechanisms [158] which involves vesicle (v) SNAREs (solu-

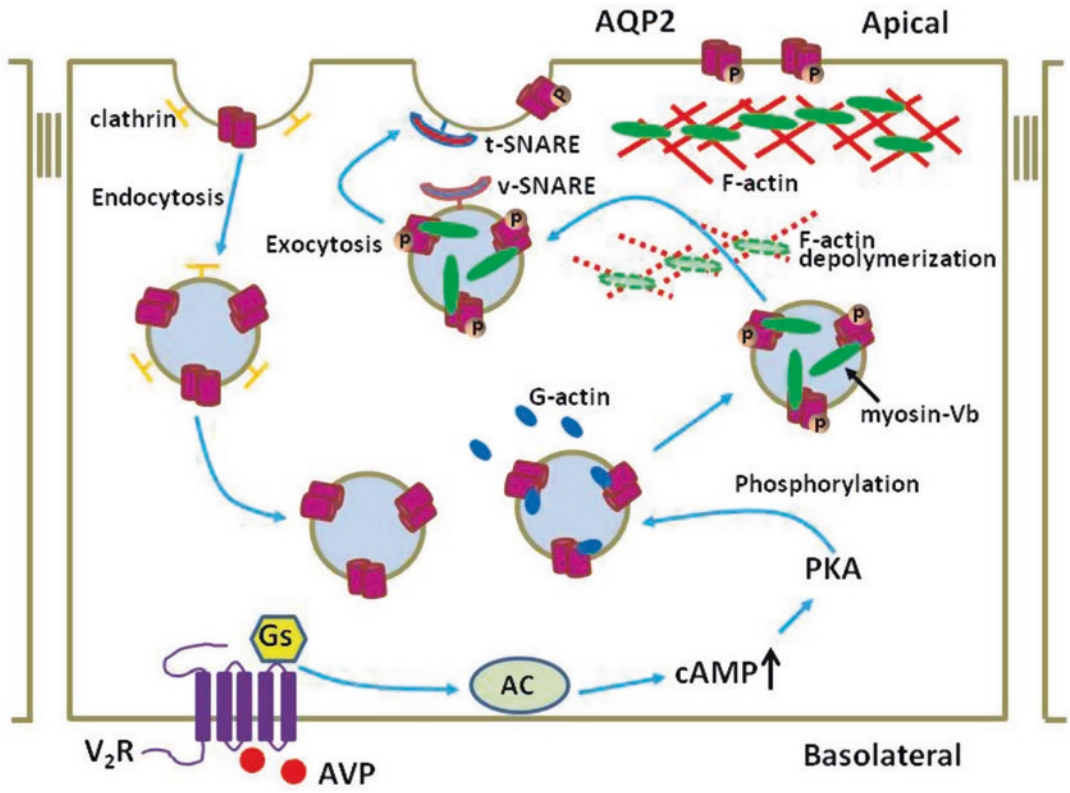

Fig. 1.7 Exocytosis and endocytosis of AQP2. AVP triggers cAMP signaling and induces phosphorylation of AQP2 at S256, which dissociates G-actin from AQP2 and promotes AQP2 interaction with myocin-Vb. This releases myocin-Vb from F-actin and induces destabilization and depolymeriztion of the F-actin network, allowing vesicles bearing-AQP2 transport to the membrane. AQP2-bearing vesicles contain specific v-SNAREs that bind to specific t-SNAREs on the apical plasma membrane, AQP2 is thus fused with the apical plasma membrane. After AVP washout, AQP2 localizes to clathrin-coated pits and undergoes clathrin-mediated endocytosis

ble NSF attachment protein receptors) and target membrane (t) SNAREs. Multiple components of the SNARE system are found in the collecting duct principal cell. The v-SNARE proteins vesicle-associated membrane protein (VAMP)-2 and VAMP-3 are found in AQP2-containing vesicles [10, 172], and t-SNARES (syntaxin-4, syntaxin 3, SNAP23, and SNAP25) are observed in the apical membrane of principal cells [97, 144]. Snapin, an intermediate scaffolding molecule, was found to serve as a linker between AQP2 and the t-SNARE complex and can aid AQP2 trafficking from storage vesicles to the apical plasma membrane [97, 144]. The cleavage of VAMP-2 by tetanus toxin blocked the AVP-mediated AQP2 translocation to the plasma membrane, suggesting a role of v-SNARS in AQP2 docking [66]. Knockdown of Munc18, a protein-inhibiting SNARE mediated membrane fusion, increased AQP2 membrane accumulation, whereas, knockdown of VAMP-2, VAMP-3, syntaxin 3, and SNAP23 inhibited AQP2 fusion at the apical membrane [193]. These studies strongly suggest involvement of SNARE in AQP2 docking and fusion to the membrane. It is noted that many other proteins (e.g. annexin-2, GTPase, AKT substrate) are also involved in AQP2 trafficking and exocytosis, although their precise roles and how they interact with AQP2 (or AQP2-bearing vesicles) remains to be fully established [158] (Fig. 1.7).

1.6.2.5 Removal of AQP2 from the Membrane (Endocytosis) and Degradation

Regulated endocytosis of AQP2 contributes significantly to final plasma membrane levels of AQP2. Inhibition of endocytosis can increase the amount of AQP2 at the apical membrane [24, 137, 158, 203], indicating another way to increase the water permeability of collecting ducts.

In the endocytotic process, AQP2 accumulates in clathrin-coated pits and is internalized via a clathrin-mediated process in a dynamin-dependent manner [26, 218, 221]. hsc70, a heat shock protein, which is important for uncoating clathrin-coated vesicles, may bind to the C-terminus of non-phosphorylated AQP2 and is reported to be required for AQP2 endocytosis [138]. Mimicking phosphorylation of AQP2 at S256 and S269 decreased their interaction with clathrin, hsp/hsc70 and dynamin alongside a decreased rate of endocytosis [138, 156], thus, phosphorylation of AQP2 may alter the efficiency of pit maturation and clathrin-coated vesicle and modulate quantity of AQP2 in clathrin-coated pits and internalization [56].

Lately, AQP2 was shown to interact with caveolin-1, a principal component of caveolae membranes that are involved in receptor-independent endocytosis [6]. Both AQP2 and caveolin-1 were internalized in response to forskolin removal [6], indicating that AQP2 is internalized through caveolae/caveolin-1 dependent mechanisms. In addition, evidences support the role of membrane rafts in regulation of AQP2 endocytosis. Reagents depleting membrane cholesterol induces plasma membrane accumulation of AQP2 *in vivo* and *in vitro*, likely a result of decreased AQP2 internalization [132, 137, 194, 203].

After endocytosis, AQP2 is retrieved to early endosomes through a PI3K dependent mechanism and then is transferred to Rab11-positive storage vesicles [209, 224, 225]. Ubiquitination works as a signal for endocytosis and subsequent degradation by multivesicular body or proteasome, as discussed above. Some AQP2 transferred to multivesicular body is excreted into the urine as exosomes [183, 187, 219] (Fig. 1.7).

1.6.3 Protein Synthesis of AQP2

Aside from intracellular trafficking and PTM, the protein levels of AQP2 are also regulated transcriptionally. Several different transcription factors, such as CREB, the AP1 [255], NFAT family (TonEBP and NFATc) [79, 80], and NF-γB [80] have been involved in this regulation. Vasopressin treatment or dehydration for a certain time results in increased water permeability of the collecting ducts, a response called "long-term regulation". This response is mainly attributed to an increased abundance of AQP2 protein due to stimulated transcription of the AQP2 gene [231], which is mediated by the vasopressin-V2R signaling cascade [48]. Sequencing of the 5-flanking region of the AQP2 gene revealed several putative cis-binding element motifs including a cAMP-response element (CRE) and an SP-1 site [197, 233]. CRE present in the AQP2 gene regulates transcription of the gene [79, 151]. Hypertonicity affects transcription of many genes through the interaction between the tonicity-responsive enhancer (TonE) and its transcription factor TonEBP. TonEBP knockout mice show down-regulated protein expression of AQP2, confirming the role of TonE/ToneEBP in AQP2 transcription [79] (Fig. 1.6). A Systems-level analysis of cell-specific AQP2 gene expression in renal collecting duct revealed many transcriptional regulators and transcriptional regulators binding elements that were involved in the transcription of the AQP2 gene. The transcriptional regulators that bind to ETS (Ets-like factors), HOX (homeobox-binding factors), RXR(retinoid X receptor family), CREB, and GATA (GATA-binding factors) of the *AQP2* gene are likely to be involved in cell-specific regulation of *AQP2* gene expression [258], providing further insight into the transcription regulation of the AQP2 gene.

1.7 Concluding Remarks

The past three decades have seen substantial progress in our understanding of AQPs. In this review, we have focused on the structure, molecular characteristics, the intracellular trafficking,

posttranslational modification, and regulation mechanisms of aquaporins, in particular, AQP2. Novel technologies and well-designed experimental strategy will continue to improve our understanding on aquaporin structure, function, regulation, and roles in disease at a cell biological, physiological, and pathophysiological level.

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The Evolutionary Aspects of Aquaporin Family

2

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Abstract

Aquaporins (AQPs) are a family of transmembrane proteins present in almost all species including virus. They are grossly divided into three subfamilies based on the sequence around a highly conserved pore-forming NPA motif: (1) classical water-selective AQP (CAQP), (2) glycerol-permeable aquaglyceroporin (AQGP) and (3) AQP super-gene channel, superaquaporin (SAQP). AQP is composed of two tandem repeats of conserved three transmembrane domains and a NPA motif. AQP ancestors probably started in prokaryotes by the duplication of half AQP genes to be diversified into CAQPs or AQGPs by evolving a subfamily-specific carboxyl-terminal NPA motif. Both AQP subfamilies may have been carried over to unicellular eukaryotic ancestors, protists and further to multicellular organisms. Although fungus lineage has kept both AQP subfamilies, the plant lineage has lost AQGP after algal ancestors with extensive diversifications of CAQPs into PIP, TIP, SIP, XIP, HIP and LIP with a possible horizontal transfer of NIP from bacteria. Interestingly, the animal lineage has obtained new SAQP subfamily with highly deviated NPA motifs, especially at the amino-terminal halves in both prostomial and deuterostomial animals. The prostomial lineage has lost AQGP after hymenoptera, while the deuterostomial lineage has kept all three subfamilies up to the vertebrate with diversified CAQPs (AQP0, 1, 2, 4, 5, 6, 8) and AQGPs (AQP3, 7, 9, 10) with limited SAQPs (AQP11, 12) in mammals. Whole-genome duplications, local gene duplications and horizontal gene transfers may have produced the AQP diversity with adaptive selections and functional alternations in response to environment changes. With the above

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evolutionary perspective in mind, the function of each AQP could be speculated by comparison among species to get new insights into physiological roles of AQPs. This evolutionary guidance in AQP research will lead to deeper understandings of water and solute homeostasis.

Keywords
MIP family • Classical AQP • Aquaglyceroporin • Super-gene family • Internal tandem repeat

2.1 Introduction

Aquaporin (AQPs) is an ancient channel that transports water and small molecules such as urea and glycerol for the maintenance of fluid homeostasis in various environments [3, 40, 48, 49, 76, 90, 112]. Moreover, some AQPs transport nutrients, metabolic precursors, waste products, toxins and gases [15, 34, 67, 96]. AQPs are ubiquitous in almost all living systems including a virus [33] with exceptions for some prokaryotes and protozoa. This is consistent with their fundamental roles in homeostasis and long history in evolution. Based on their wide distribution, the evolutionary pathways for AQPs have been proposed by combining a phylogenetic analysis of both AQPs and species [1, 23, 27, 28, 38, 50, 79, 80, 82, 88, 92, 109, 110]. This approach will be insightful to speculate the function and physiological significance of uncharacterized AQPs.

The primary structure of AQP indicates that it has a relatively conserved overall structure with six transmembrane domains and five connecting loops (A–E) (Fig. 2.1). They form a tetramer and the each monomer has a pore that is made of two highly conserved hydrophobic short stretches of ~20 amino acid residues named asparagine-proline-alanine (NPA) motifs in loops B and E to simulate a hour-glass structure (Table 2.1). The NPA motif is highly conserved in AQPs and designated as a signature sequence for AQP family. Before bioinformatic tools were available, this amino acid sequences were exploited to design degenerative primers to clone new AQPs by PCR.

Accumulating databases from genome projects have facilitated the identification of AQPs with powerful bioinformatic tools that automatically find new AQPs using conserved signature sequences: NPA motifs and transmembrane domains [38]. If these AQP-like proteins function as a water channel when properly expressed, they will be included in AQP family. However, most of their function is unknown and they will be grouped as MIP (Major Intrinsic Protein) multigene family. MIP was originally identified as a dominant protein at bovine lens fiber junction membranes with unknown channel function before the identification of AQP1 [30, 35]. This is because AQP was originally proposed as the name for a functionally defined water channel [3].

As many MIP proteins have been identified by genome projects and they transport water, MIP family and AQP family are often used interchangeably. Moreover, the acronym of MIP also comes from a couple of other proteins such as ‘macrophage inflammatory protein’ and ‘mitochondrial intermediate precursor-processing proteinase’. Thus, in this review, AQP family was used instead of MIP family to avoid the above confusion. The usage of AQP family irrespective of its function can also evade the discussion on the functional discrepancies of AQPs in the literature.

In fact, MIP has been renamed to be AQP0 after the discovery of AQP1 although it is a poor water channel [35]. Another member of MIP family cloned and characterized before AQP1 is a bacterial glycerol channel or glycerol diffusion facilitator, GlpF. The homolog of GlpF in mammals was later identified as AQP3. GlpF, however, was not renamed but included in aquaglyceroporin family together with AQP3.

We overview the evolution of AQP family based primarily on the amino acid sequences but

not on the function. However, relevant advantageous functions will be discussed as they are important for the selection bias to survive through evolution. Many detailed reviews on the evolution of AQP family are available in the literature trying to find clues to functionally critical residues and physiological roles through adapting to changing environments through evolution [1, 27, 38, 82, 109]. This review, however, does not intend to provide a detailed analysis of the evolution of AQP family but to present a general and simplified view on the evolution and origins of diversified AQP family through evolution to obtain basic ideas on the history of AQPs. Moreover, the review will update our previous review during five years [50].

2.2 The Classification of AQP Family into Three Subfamilies

AQP has a paired stretch of highly conserved signature sequences, NPA motifs, which form a pore structure and primarily define the selectivity of transport as a charge and size obstacle [10, 43, 66, 103]. Moreover, AQP is composed of an internal tandem repeat of three transmembrane segments (TMS) speculated by the fact that the amino terminal half (hemipore-1) and the carboxyl terminal half (hemipore-2) are related with each other at the level of ~20% amino acid homology to face each other to form a pore (Fig. 2.1) [80, 88]. The amino-terminal half is relatively well conserved among AQPs, while the carboxyl-terminal half is less conserved possibly for the purpose of specifying its unique pore selectivity. Each half has two loops in which the second loop, loop B or loop E, has a highly conserved hydrophobic residues, NPA motif that forms the pore (Fig. 2.1). This structure suggests that AQP originated from a tandem duplication of a half AQP gene (hemipore) [79, 88]. Alternatively, both hemipore may have evolved independently to produce unique structures (hemipore-1 and hemipore-2) and fused to form AQPs (Fig. 2.1).

After the identification of AQP3 as a mammalian homolog of GlpF, a bacterial homolog of AQP1 was found to be AQPZ [17]. With the discovery of these two sets of AQP family in bacteria and mammals, AQPZ vs. AQP1 and GlpF vs. AQP3, the evolutional pathway of AQP family from bacteria to human seemed to be apparent and straight forward [3, 47]. Moreover, the difference in primary sequence also reflects the func-

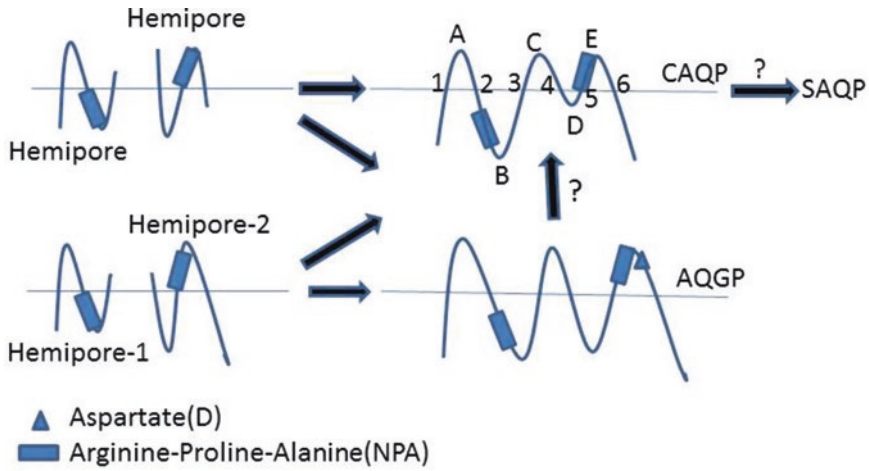

Fig. 2.1 Hypothetical formation of AQPs. AQPs may have been formed by duplication of a hemipore gene [88] or a fusion of two similar genes (hemipore-1 and hemipore-2). AQGP may have been transformed to CAQP by the shortening of loop D and the disrupting mutation of the signature D residue of AQGP in microbes. SAQP may have been produced by the transformation of CAQP in multicellular organisms

Table 2.1 Sequence of AQPs at the first and the second NPA boxes

	First NPA box	Second NPA box
CAQP		
AQPZ	-VGHISGGHF NPA VTIGLWAG-	-SIPVTNTSV NPA RSTAVAIFQG-
Meth	-FGRISGCHI NPA VTIALFAT-	-IGNLTGASL NPA RTFGPYLGDW-
Chl.T	-MGTVSGAHL NPA VTLAFAMR-	-AAPVSGASM NPV RSLAPALVCG-
Chl.P	-MGTVSGAHL NPA VTIAFAMR-	-AAPISGASM NPV RSLAPALVCG-
Cript	-FFRVSGGLF NPA VSLGMVLA-	-GVPYSGGAL NPV RSLGPAVVTH-
Tryp1	-FGYISGGHF NPA VTMAVFLV-	-VGRISGGAF NPA AATGLQLALC-
Tryp2	-FGYISGAHF NPA ITFATFIN-	-VGGFTGGAF NPA VATGTQLVGC-
Leish	-FGYISSSHF NPA VSIAVFLV-	-AGRISGGAF NPA AASGLQVAMC-
D.disA	-VSGVSGCNL NPA VTLANLLS-	-GFNFSGGAL NPV RVLGPSIISG-
D.disB	-ISGISGCQL NPA VTVGCVTT-	-LNLFTGGSL NPA RSFGPAVFSD-
D.disC	-FADVSGAHF NPA VTFATCVT-	-GGSVSGGAF NPA RVFGTALVGN-
D.disD	-CAPVSGGHL NPS ITLATFFA-	-IAPNYIFGF NIA RCLSPAIVLS-
D.disE	-CAPVSGGHL NPS ITIATFFS-	-ISPNYIFGF NMA RCLCPAIVTG-
XIP1.1	-APATSGGHV NPC ITWTEMLT-	-FSGYGGAGI NPG RCIGPAVVLG-
TIP1.1	-GANISGGHV NPA VTFGAFIG-	-GGAFSGASM NPA VAFGPAVVSW-
PIP2.6	-TAGISGGHI NPA VTFGLFLA-	-TIPITGTGI NPA RSFGAAVIYN-
HIP1.1	-TGAISGGHI NPA VTLAFVVA-	-GVPYTGASM NPA RSFGPALVSG-
NIP1.2	-LGHISGAHF NPA VTIAFASC-	-AGPVSGASM NPG RSLGPAMVYS-
SIP1.1	-TVIFGSASF NPT GSAAFYVA-	-GSKYTGPAM NPA IAFGWAYMYS-
LIP	-DIVSGGSQV NPS VSVAMFVH-	-GTPYTGPAM NPM IAFGWAVQSD-
AQP1	-VGHISGAHL NPA VTLGLLLS-	-AIDYTGCGI NPA RSFGSAVLTR-
AQP8	-LGNISGGHF NPA VSLAVTVI-	-GGSISGACM NPA RAFGPAVMAG-
AQGP		
AQPV1	-FGFVS-AHL NPA MCLALFIL-	-MGGVTSIAA NPA R **D** FSPRLAHF-
GlpF	-TAGVSGAHL NPA VTIALWLF-	-MGPLTGFAM NPA R **D** FGPKVFAW-
Entero	-LFVFGGVCI NPA MALAQAIL-	-LGGTTGFAM NQA R **D** LGPRIAYQ-
Ustil	-CATTSGTQF HPA FTIAQVVF-	-CFSSSNVVA NSA R **D** IGARLVCS-
P.viv	-AAKLSGAHL NLA VTVGFATI-	-FGGNTGFAL NPS R **D** LGARLLSL-
GIP	-VGHISG-FF NPA VALAAAVV-	-GGGMTGPAL NPA R **D** LGPALVSG-
AQP3	-AGQVSGAHL NPA VTFAMCFL-	-MGFNSGYAV NPA R **D** FGPRLFTA-
SAQP		
CeAQP9	-IEFQRDAVA HPC PLVTNCYR-	-GINYTGMYA NPI VAWACTFN **C** L-
CeAQP10	-NIFNRGAMT NCA PIFEQFVF-	-LYVVGVPGL NPI VATARLYG **C** R-
CeAQP11	-ALCNRTAFC SPL APIEQYLF-	-VTFVGDQAL DPL VASTLFFG **C** R-
MtAQP11	-TFTFQDGTC DPS ECYEKFCK-	-GLFVSGGYF NPT LSFAMEYG **C** Q
Dros	-GRVWGDASA CPY THMEDVVE-	-AFNFSGGYF NPV LATALKWG **C** R-
Urch1	-LTFDGDSTA NTC MIWQSMLK-	-GLEWTGMMF NPA LAAGITLN **C** G-
Urch2	-NEELSNAGD APL GQAVQVQP-	-GLEYTGAPM NPI LGFASGWG **C** K-
ZF11	-GFSFRGAIC NPT GALELLSR-	-GGRLTGAVF NPA LAFSIQFP **C** P-
ZF12	-TAVMQDVSG NPA VTLLRLLQ-	-ANNYTSGYV NPA LAYAVTLT **C** P-
Xenopus	-GFTFNKASG NSA VSLQDFLL-	-AGSYTGAFF NPT LAAALTFQ **C** S-
Chic11	-GLTLPGSTC NPC GTLQPLWG-	-GGNLTGAIF NPA LAFSLHPH **C** F-
Chic12	-AACANGAAS NPT VSLQEFLL-	-AAPATGAFF NPA LATASTFL **C** A-
AQP11	-GLTLVGTSS NPC GVMMQMML-	-GGSLTGAVF NPA LALSLHFM **C** F-
AQP12	-GVTLDGASA NPT VSLQEFLM-	-AGPFTSAFF NPA LAASVTFA **C** S-

The table was modified from Ref. [50]

tional dichotomy of AQPs: water-selective vs. solute-permeable.

The fact that the dichotomy of AQP family seemed to start early in bacteria suggests that AQP family is ancient and conserved in evolution possibly for its critical roles in water and solute homeostasis for survival since water is vital for every living organism. It is particularly true with GlpF whose gene is located in the functional gene complex, glpFK operon, encoding glycerol kinase as well. However, the role of AQPZ is not clear as its absence produces little defect though controversial [93]. Furthermore, ~90% of bacteria are even devoid of GlpF nor AQPZ as is the case of most archaebacteria [98]. As majority of prokaryotes do not have AQPs, AQPs in bacteria could have been derived from horizontal gene transfer from eukaryotes and accumulated mutations to produce diverse NPA motifs due to the absence of functional constraints on the primary sequences.

The original division of AQP family into two subfamilies was based on their permeability function: water-selective vs. glycerol-permeable. Each was then named a classical AQP (CAQP) or an aquaglyceroporin (AQGP), respectively [40, 90]. Although this classification is based on the function, it is also related to the primary sequence. As the functional data are sometimes controversial and they are not available in most AQPs, the classification is usually conducted based on the primary sequence using signature sequences. The signature sequence for AQGP is the aspartic acid residue (D) in the second NPA motif that is expected to expand the pore to permeate larger molecules such as glycerol [32, 103] (Fig. 2.2). The signature sequence for CAQP, however, is not readily apparent due to several variations in the second NPA motifs. Thus the absence of D suggests CAQPs. It is true that the pore size and characteristics are not determined simply by this D residue alone, but the relevant combination of conserved residues of AQGPs such as a longer loop D always coexist with this D residue, indicating the phylogenetic sequence conservation of AQGP subfamily through evolution. On the other hand, CAQPs may have come from multiple phylogenetic sources although some

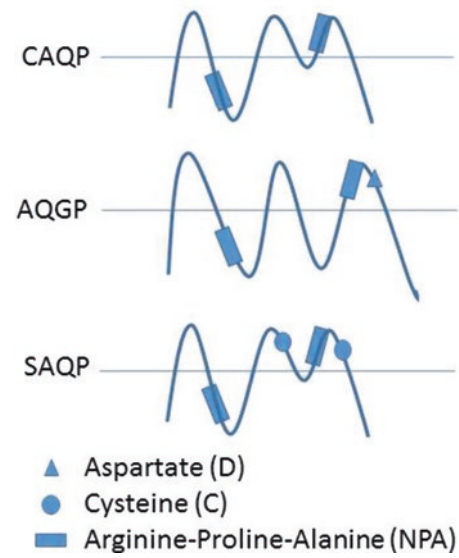

Fig. 2.2 The classification of AQPs into three subfamilies. The signature sequence for AQGP is the aspartic acid residue (D) in the second NPA motif. The signature sequence for SAQP is Cys (C) residue at the downstream of the second NPA motif. The partner Cys residue for disulfide binding is present in the loop C. The absence of these D and C residues indicates CAQP (See text for more details)

critical residues for water-selective transport have been conserved [10, 31].

The amino and carboxyl terminal halves of CAQPs are conserved at the level of ~30% while those of AQGPs are less conserved at the level of ~20% due to the longer residues at the loop D [88] (Fig. 2.1). Nevertheless, as both amino-terminal halves are relatively conserved at the level of 30~40%, CAQP and AQGP may have a common ancestral AQP. It is also possible that the duplication of a half-sized AQP gene may have produced CAQP and AQGP independently with the carboxyl-half diversification [66, 102] (Fig. 2.1). As the role of AQGP with glpFK operon in bacteria is more apparent than CAQP, CAQP could have been produced by the isolation of AQGP from glpFK operon or by a remaining component of deleted glpFK operon. The first AQP, most likely AQGP, may have had a larger pore permitting the uptake of nutrients or the excretion of waste products in bacteria. Then, the mutation of the signature Asp (D) in the second

Fig. 2.3 Schematic presentation of the phylogenetic relationship of AQP subfamilies. SAQP is distantly related to CAQP and further away from AQGP

NPA motif and the deletion in the loop D may have converted AQGP to CAQP specialized for water transport with a newly-formed narrow pore [43] (Fig. 2.1). However, the identification of original AQP in bacteria will be difficult because a major fraction of prokaryotic genes have been derived from horizontal gene transfer among microbes.

The subsequent discovery of AQP11 and AQP12 in mammals has challenged the above dichotomy classification of AQP family because they have highly degenerative NPA motifs with very low overall amino acid homology (<15%), a level of super-gene family [45, 46, 51, 52, 74, 75, 77]. Such AQPs were not identified by PCR cloning because degenerative primers based on NPA motifs did not work. Instead, AQP11 and AQP12 were identified by BLAST search of EST libraries because their first and sixth transmembrane domains are relatively conserved with CAQPs and AQGPs. As previous phylogenetic trees have been drawn from CAQPs and AQGPs with relatively higher amino acid identity (>25%), the inclusion of AQP11 and AQP12 in the tree has broadly expanded AQP phylogenetic trees (Fig. 2.3).

The identification of super-gene family of AQPs stimulated the search for more members in genomic data bases. In fact, such AQPs have been identified in the database of multicellular animals whose amino acid upstream sequence from the first NPA and NPA itself are completely different from other AQPs (Table 2.1). Interestingly, they are absent in bacteria, unicellular eukaryotes, and plants (Table 2.2). Three members in a nematode, *C. elegans* and one in an insect, *D. melanogaster* are particularly deviated and were not previously included in AQP family [42, 55] (Table 2.1). Despite their low homology even among themselves, all have a signature NPA motif in the car-

Table 2.2 The distribution of aquaporins in three subfamilies

Organisms	CAQP	AQGP	SAQP
Microbe			
E. coli	1	1	
P. aeruginosa		1	
S. typhimurium	1		
L. plantarum		6	
M. marburgensis		1	
Chlorella virus	1		
Fungus			
S. cerevisiae	2	2	
S. pombe		2	
A. nidulans	1	4	
U. mydis	2	3	
M. grisea	3	1	
Protist			
L. major	4	1	
T. cruzi	4		
T. brucei		3	
T. gondii	1		
P. falciparum		1	
D. discoideum	5		
Invertebrate			
C. elegans	3	5	3
M. tardigradum	2	8	1
C. intestinalis	4	1	1
Plant			
P. patens	23 (8PIP, 4TIP, 5NIP, 2SIP, 2XIP, 1HIP)		
H. brasiliensis	51 (15PIP, 17TIP, 9NIP, 4SIP, 6XIP)		
A. thaliana	35 (13PIP, 10TIP, 9NIP, 3SIP)		
P. trichocarpa	55 (15PIP, 17TIP, 11NIP, 6SIP, 6XIP)		
Insect			
L. salmonis	2	3	2
D. melenogaster	7		1
G. morsitans	9		1
Vertebrate			
Zebra fish	11	7	2
Salmon	23	13	6
Clawed frog	12	5	2
Green anole	9	5	2
Turtle	10	3	2
Zebra finch	7	4	2
Platypus	8	5	2
Rat	7	4	2
Human[a]	7	4(+4)	2(+1)

The table was modified from Refs. [28, 50]
[a]Human has four pseugogenes of AQP7 and another copy of AQP12

boxyl-terminal half without D residue and in particular they have highly conserved Cys (C) residue at the downstream of the second NPA motif, NPAxxxxxxxxC (x is any amino acid residue) [36, 50] (Fig. 2.2, Table 2.1). This Cys may be important for disulfide binding to construct a 3D structure and will be functionally indispensable as its disruption in AQP11 produced a similar phenotype to that of AQP11-deficient mice [75, 99]. Furthermore, a candidate partner Cys residue for disulfide binding has been identified in the loop C, which remains to be proved [36] (Fig. 2.2). Figure 2.4 shows a simple diagram to classify AQP family into three subfamilies [50].

With this Cys residue as a signature residue, this subfamily will be grouped as a supergene family of AQP family and named superaquaporins (SAQPs) with the deviated NPA motifs [11, 107] (Table 2.1). Although many AQP-like proteins have been identified in bacterial genomes with deviated NPA motifs, they do not have this particular Cys residue and their overall sequences are closer to CAQPs. Therefore, these bacterial AQP-like proteins with different NPA motifs belong to CAQP subfamily. As SAQPs are absent in lower organisms and plants, it could have been obtained in multicellular animals by horizontal gene transfer from cohabitating ancient bacteria with deviated NPA motifs because such mutations may have not adversely affected bacteria as they were less dependent on AQPs. The gain of the Cys residue may have been critical for new or altered functions of SAQPs arising from the deviated NPA motifs to form a unique 3D structure for the benefit of multicellular animals. The presence of cell walls in plants in fact has inhibited to function as multicellular organisms due to cytoplasmic convergence. Consequently, plants might have no need for SAQP even if they had a chance to obtain SAQP by a horizontal gene transfer from symbiotic bacteria. Alternatively, SAQP may have been originated in prokaryotes or even in unicellular eukaryotes but lost through evolution due to too extensive deviations to function as a water channel. In fact, AQP11 is a relatively inefficient water channel [108].

The 3D structural analyses of CAQP and AQGP have revealed that they are highly conserved with each other [103], while such an analysis of SAQP is not currently available. If SAQP indeed has a similar 3D structure as CAQP and AQGP, the validity to include SAQP into AQP family will become robust. Surprisingly, a recent report on 3D structural analysis of a formate channel (FocA) in bacteria has indicated a striking 3D structural similarity to AQPs [21, 106]. Although its primary sequence has no homology with AQP family, it has pore forming hydrophobic amino acid residues similar to NPA motifs with six transmembrane domains in 3D structure. Therefore, this channel could be another water channel although its functional study has failed to show water permeability [106]. It is also composed of tandem internal repeats similar to AQPs. However, the hemipores have a much lower homology with each other at the order of ~7%. Moreover, FocA forms a pentamer rather than a tetramer to permeate formate, nitrite and hydrosulfide. FocA family is only present in prokaryotes and lower eukaryotes. Although AQP family is currently composed of three subfamilies: CAQP, AQGP, and SAQP [11, 50], another novel water channel family could be identified in the future with a similar 3D structure to AQP family with little primary sequence homology.

2.3 Clustering and Evolution of AQPs

Molecular evolution by gene duplication and structural diversification are the basis for the phylogenetic framework for AQP family [5, 16, 19, 24, 44]. However, the presence of horizontal gene transfer from bacteria to eukaryotes or vice versa has been suggested and should be carefully evaluated in the event of missing links between AQPs and species in phylogenetic trees [57]. The most compelling instance of horizontal gene transfer will be the plant acquisition of NOD26-like intrinsic proteins (NIPs) from cohabitant bacteria at root nodule in the absence of glycerol-transporting AQGP in plants [111]. Such a caveat of horizontal gene transfer should be carefully evaluated when comparing AQP sequences from the evolutional point of view [6, 57].

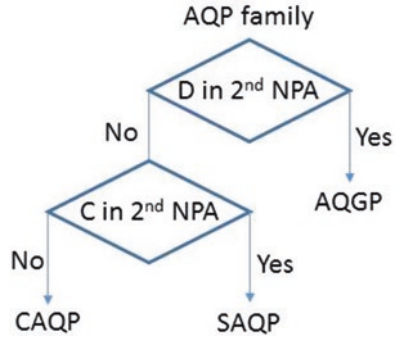

Fig. 2.4 A simple diagram for the classification of AQP family. The characteristics of AQP subfamily shown in Fig. 2.2 is the basis for this diagram (See text for more details)

2.3.1 Microbial AQPs

The first AQP may have been originated in eubacteria as indicated by the presence of two AQPs in *E.coli*, AQPZ and GlpF, which may correspond to the ancestor forms of CAQP and AQGP, respectively. However, most of bacteria do not have a set of AQPs. Generally, gram-negative bacteria have only CAQPs while gram-positive bacteria have only AQGPs. For example, a gram-positive *Lactobacillus plantarum* has six AQPs, all AQGPs. Moreover, only ~10% of bacterial genomes contain AQPs, and the role of AQPs in bacteria is not clear [98]. It is postulated that CAQP may be necessary for freeze tolerance to prevent intracellular icing in the event of freezing by enhancing intracellular osmolality through rapid extrusion of water through CAQP. A similar role has also been reported in the case of insect AQPs [53, 83]. AQGP may serve to transport osmolytes, nutrients or toxins of small molecules rather than water. Therefore, in the event of the transfer to hypotonic environments, AQGP may serve to excrete rapidly glycerol as an osmolyte to prevent cell swelling. The loss of AQGP may have been caused by parasitic life style of microorganisms, in which nutrients were easily obtained and the environment was osmotically stable. The fact that the majority of bacteria do not have any AQP suggests that the presence of AQPs may be harmful in hypotonic environment as the cellular water is rapidly removed through CAQP while its absence will be advantageous to prevent osmotic water loss from the cell body. Moreover, the osmolyte in most bacteria is potassium or amino acids and not glycerol, which may make AQGP unnecessary [15].

Genome projects have revealed the absence of AQPs in many archaebacteria such as thermophilic archaea reflecting limited advantage of AQP as in the case of eubacteria. An exceptional AQP in archaebacteria is AqpM, a member of CAQP, in *Methanothermobacter marburgensis* [63] (Table 2.1). Interestingly, AqpM has a wider pore structure to accept larger molecules such as H_2S instead of water [60]. It could be a primitive non-specialized AQP, an ancestor form of more specialized CAQP and AQGP. Again, much fewer AQPs in archaebacteria suggest minimum roles of AQPs in prokaryotes in general through evolution despite their survival through harsh environments.

It is notable that a chlorella virus has an AQGP, AQPV1, which may have been obtained by a horizontal gene transfer from bacteria to virus, as the virus infect algae at the time of the separation of eukaryotes from prokaryotes [33] (Table 2.1). AQPV1 may play a role in infection and replication of the virus to modulate water transport of the host cell.

2.3.2 Protist AQPs

As eukaryotes have evolved from symbiosis of prokaryotes, they could have inherited several AQPs from prokaryotes to be expressed at the plasma membrane [20, 89]. The number of AQP genes in protists, however, is diverse and may have changed by their environmental constraints through the evolution [9, 26, 104]. A cyst-forming symbiotic *Cryptosporisium parvum* does not have any AQPs while *Plasmodium.falciparum* has only one AQP (PfAQP), AQGP, for the nutrient uptake from erythrocytes [7, 67, 87, 104]. On the other hand, *Toxoplasma gondii* has only one AQP (TgAQP), CAQP, but with a wide range of permeability to uptake nutrients by changing its pore structure through the alterations of functionally critical residues [81]. Some protozoa have

multiple AQPs: *Dictyostelium discoideum* have five AQPs, all CAQPs [50] while *Trypanosoma brucei* has three AQPs, all AQGPs. Three pathogenic Trypanosomatidae (*Leishmania major*,*T. cruzi* and *T. brucei*) have different AQP subfamilies possibly caused by the need for different nutrient uptakes to adapt to their environments beyond evolutional constrains [104]. It seems that eukaryotes need AQPs more than prokaryotes possibly due to their larger size of cell bodies which requires more efficient water transport system at the plasma membrane. The multiple AQPs beyond a genomic AQP subfamily barrier may have been produced by gene duplication or horizontal gene transfer.

2.3.3 Fungal AQPs

The budding yeast *Saccharomyces cerevisiae* has two CAQPs (ScAqy1 and ScAqy2) and two AQGPs (YFL054Cp and ScFps1) suggesting the need for AQPs has been increased in eukaryotes possibly for osmotic adjustment of yeast cells at low temperature [4, 62, 91]. High osmolality induces glycerol accumulation in yeast cells by the high osmolality glycerol (HOG) pathway to accumulate glycerol produced by alcohol fermentation, which is regulated by the closure of an AQGP, Fps1 [41, 84]. On the other hand, the role of CAQPs is not clear as one of them presents inside the cell. A recent comprehensive analysis of AQPs in 38 strains of *S. cerevisiae* has revealed various inactivating mutations in both CAQPs and AQGPs, which may give a competitive advantage in different environmental conditions. Therefore, in selected environments, the presence of AQPs is detrimental for yeasts, suggesting little importance of AQPs even in unicellular eukaryotes.

Alternatively, multiple AQPs in yeast could be caused by horizontal gene transfer and genome fusions of prokaryotes before the separation of eukaryotes [89] and by the subsequent endosymbiosis of prokaryotes into eukaryotes [20]. As the current unicellular yeast used to be a multicellular organism, four AQPs may reflect the combination of multiple cells fused within the cell wall. On the other hand, the baker's yeast *S. pombe* has only two AQGPs [56, 101]. Much wider distribution of AQGP than CAQP in fungi suggests that glycerol is the major osmolyte in yeasts and nutrient imports or toxin exports will be more important than water transport as a function of AQPs.

2.3.4 Plant AQPs

In contrast to animals, plants have developed multiple AQPs due to their polyploids [2, 71]. Interestingly, they have only CAQPs that are diversified in higher plants [65]. For example, *Arabidopsis thaliana* has 35 AQPs, which are further subdivided into four groups: 13 PIPs (Plasma membrane Intrinsic Protein), 10 TIPs (Tonoplast Intrinsic Protein), 9 NIPs (NOD-26 like Intrinsic Protein), and 3 SIPs (Short Intrinsic basic Proteins) (Table 2.2). The absence of AQGPs in plants can be explained by the functional conversion of CAQPs to AQGPs as is the case with protozoa or insects [29, 105]. NIPs can transport small molecules other than water such as glycerol, silicon and boron [96], which are thought to be derived from bacteria by a horizontal gene transfer: symbiotic bacteria in the root may have had ancestors of NIPs for the uptake of nutrients and possibly for the efflux of metabolites and wastes [85, 92, 111]. Some NIPs are expressed at the ER membrane, which may be reminiscent of previous intracellular symbiotic states. The absence of SAQPs in plants is intriguing and may be related to the presence of intracellular TIPs and SIPs, which may have made intracellular SAQPs redundant.

Another subfamily of CAQPs identified in poplar trees, but absent in *A. thaliana*, is XIP (uncategorized X Intrinsic Protein) [37, 39, 69] (Table 2.2). Similar to SIPs, XIPs have less conserved NPA boxes but do have conserved Cys residue after 2nd NPA box (Table 2.1). However, this Cys is located near NPA as NPARC and its functional significance has not been clarified. Thus both SIP and XIPs do not belong to SAQPs. XIPs are expressed at the plasma membrane and have a wider selectivity for permeant substrates

including glycerol [14]. Interestingly, XIPs are also present in protozoa and fungi [22, 37]. More recently, further new subfamilies also belonging to CAQP, HIP (Hybrid Intrinsic Protein, similar to PIPs and TIPs) and LIP (Large Intrinsic Protein, similar to SIP) have been identified in algae but they are absent in higher plants [23, 58].

Interestingly, an AQGP, GIP, has been found in a primitive land plant, a moss *Physcomitrella patens*, functioning as a glycerol channel. However, it may have come from bacteria by a horizontal gene transfer [22, 39]. It is difficult to integrate plant AQPs in evolutionary context as there can be a possible contamination of bacterial horizontal gene transfer such as NIPs and GIP although the absence of AQGPs in plants is intriguing.

It is unique for plants to have seven subfamilies of CAQP based on the primary sequences (PIP, TIP, NIP, SIP, XIP, HIP, LIP), which seems to make plant CAQPs functionally comparable to animal AQPs. For example, the absence of AQGP in plants is in fact compensated by the extended functions of NIPs [85, 96]. The absence of SAQP might be compensated by SIP, XIP or HIP. Detailed phylogenetical analysis of plant AQPs has been reported and such a functional evolution in concert with minute primary sequence changes within CAQP subfamily will give novel insights into evolution paradigm including structure-function relationships of AQPs. Although these results are derived from subfamilies of CAQPs, they will be insightful into the functions of other AQP subfamilies.

2.3.5 Invertebrate AQPs

SAQPs have first appeared in multicellular animals both protostome such as insects and deuterostome such as vertebrates [18, 36]. *Caenorhabditis elegans*, a deuterostome has 3 SAQPs, which are highly deviated from mammalian 2 SAQPs (Table 2.1). They are also not much homologous with each other suggesting the acquisition of SAQPs in *C. elegans* may be through horizontal gene transfers possibly from bacteria. Since SAQPs have first appeared in multicelular nematodes and not in unicellular protozoa, SAQPs may have a role in cellular activities specific to multicellular organisms such as cellular differentiation, apoptosis, organogenesis, mating and intercellular communication. If it is the case, the absence of SAQPs in plants may reflect the unicellular nature of cytoplasmic communication in plants within the cell wall which is absent in animals.

It is notable that *C. elegans* has abundant AQGPs, 5 out of 11 AQPs (45%), suggesting that there is some selection advantage to retain multiple AQGPs [42] (Table 2.2). Although glycerol may be important for its osmoregulation and a nutrient, their functional studies showed that not all AQGPs transport glycerol and one is even water selective, while one CAQPs is water selective but two CAQPs have no transport activities [42]. The function of three SAQPs has not yet been examined and their subcellular localization is not clear. Moreover, the physiological roles of nematode AQPs are still unclear as even multiple AQP knockouts up to quadruple AQP deletion has revealed no abnormalities [42]. Interestingly, aqp-1, an AQGP, has been reported to lengthen the life in a low-sugar diet suggesting that AQPs may play a role in metabolism rather than osmoregulation [64].

The unusual tardigrade survives drying and dehydration by anhydrobiosis, which may be regulated by 11 AQPs: 2 CAQP, 8 AQGPs and a single SAQP [36]. Much more AQGPs in this animal suggest the importance of glycerol in anhydrobiosis. Some of AQGPs in tardigrade may have been derived from horizontal gene transfers to increase their number. As in the case with *C. elegans*, its SAQP is also highly deviated (Table 2.1).

2.3.6 Insect AQPs

Early arthropods, protostomes, have all three AQP subfamilies [18]. A non-insect louse, *Lepeophtherius salmonis*, has 7AQPs: 2CAQPs, 3AQGPs, and 2SAQPs [94] (Table 2.2). A mite, *Rhipicephalus sanguineus*, has an AQGP, RsAQP1, although it has a water-selective

transport [8, 12]. As the osmolyte in insect is not glycerol but trehalose, a remnant AQGP in mites might have been functionally converted to a water-selective AQP, or it may have been gained by a horizontal gene transfer from bacterial AQGPs.

Interestingly, insects with feathers have lost AQGP while insects without larva stages have retained AQGPs. For example, a fruit fly, *Drosophila melanogaster* has lost AQGP from eight AQPs: 7 CAQPs and one SAQP (Table 2.2). SAQP was not initially included as a member of AQP family as the first NPA is unusual CPY with deviated its upstream sequences [18, 55] (Table 2.1). Another fly, Tsetse fly, *Glossina morsitans*, a vector for African trypanosomiasis, has 10 AQPs: 9 CAQPs and one SAQP. The loss of AQGP in higher insects is compensated by the functional conversion of CAQPs to become permeable to glycerol and urea in dipteran order as shown in silkworm larva, *Bombyx mori* [25, 29, 53, 54, 105]. Insect CAQP as a water channel may also be important for freezing tolerance as in the case with bacteria [53, 83].

2.3.7 Vertebrate AQPs

In vertebrates, all three families of AQPs are present. In rats, there are thirteen AQPs in total: CAQPs (AQP0, 1, 2, 4, 5, 6, 8); AQGPs (AQP3, 7, 9, 10); and SAQPs (AQP11, 12) (Table 2.2). Although two rounds of whole-genome duplication (WGD) occurred in the common ancestor of early vertebrates before ray-fined fish [13, 16, 61, 72], the total number of AQPs in mammals is relatively small compared with nematodes with 11 AQPs but may be reasonable from the number of 7 AQPs in *Ciona intestinalis*, an ascidian (sea squirt) from which vertebrates have been derived after a WGD (Table 2.2). Many AQPs might have been lost through the evolution. In fact, the numbers of AQGPs and SAQPs have been decreased in vertebrates compared with nematodes. Alternatively, nematode may have gained these AQP subfamilies by horizontal gene transfers in increase their numbers.

Zebrafish as a teleost have undergone another round of WGD to increase the number of AQP to 20 [30, 100]. Some fish families such as salmon have experienced fourth round of WGD, adding further complexity with 42 AQPs [68]. Interestingly, the orthologue of AQP6 is missing in birds but it has AQP14, CAQP, instead [28]. In lower vertebrates, there are AQP13, AQGP, and AQP14-16, all CAQPs, which, however, have been lost in mammals. Further analyses of the tissue distribution and the hormonal regulation of each AQP orthologue in vertebrates will be useful to obtain an insight into the function and role of each mammalian AQP [95].

Although there is not much divergence of AQGPs in vertebrates, some AQP10 have been lost in turtles [28] or turned to a pseudogene [73]. Not only rodents but also cows have a pseudogene of AQP10 without any authentic AQP10 gene [97]. On the other hand, AQP7 in human has four pseudogenes in addition to the authentic AQP7 gene [28]. Moreover, a nonfunctional splice variant missing sixth transmembrane domain has been identified in AQP10 and possibly in AQP3 [46]. These nonfunctional genes may represent a transitional stage in their way to pseudogenes or deletion. As the role of glycerol in the energy metabolism seems to be small in higher vertebrates, redundant AQGPs may be dispensable and their future lost might be expected in some mammals.

Although the role of SAQPs in the vertebrate is not well clarified, just 2 SAQPs may not be enough as compared with other subfamilies. In fact, *C. elegans* has 3 SAQPs and Atlantic salmon has 6 SAQPs [28] (Table 2.2). Interestingly, there are two copies of AQP12 gene with local gene duplication in human [28]. As AQP12 has limited expression in the acinus of the pancreas [78], the duplication of AQP12 could be in the process of expanding the repertory of SAQPs.

2.4 The Perspectives

The AQP research orientated by an evolutional guidance will be insightful as it may lead to a comparative analysis of osmoregulation and fluid

homeostasis including hormones and their receptors [59, 70, 95]. As water is vital for every living organism, some have expanded the AQP repertories while others have limited its number. Such strategic difference for adapting to environmental changes will be the basis for the expression patterns and functional diversities of AQPs, which may be regulated by osmolarity and hormones. Cooperative evolutions of AQPs and hormones will be expected and may shed a new light on the physiological significance of AQPs [59, 70, 95].

Although AQPs are not usually regulated by open-close states, their functions are sometimes modulated by trafficking to and from the plasma membrane [86]. Such movements of AQPs will be regulated by associated proteins that may also have evolved together. Currently little is known about such associated proteins. Furthermore, the driving force for water movement requires solute transport such as sodium and potassium. Accordingly, co-expression of AQPs with ion channels, pumps or transporters will be important to enhance the water transport efficiency through AQPs. The relevant regulation of ion movements as well as their co-evolution will also be intriguing [33].

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Transport Characteristics of Aquaporins

3

Xiaoqiang Geng and Baoxue Yang

Abstract

Aquaporins (AQPs) are a class of the integral membrane proteins, which are permeable to water, some small neutral solutes and certain gases across biological membranes. AQPs are considered as critical transport mediators that are involved in many physiological functions and pathological processes such as transepithelial fluid transport, cell migration, brain edema, neuro excitation and carcinoma. This chapter will provide information about the transport characteristics of AQPs.

Keywords

Aquaporins • AQPs • Aquaglyceroporins • Water channel

3.1 Water Transport Mediated by Aquaporins

The main physiological function of aquaporins (AQPs) is to facilitate the water transport across plasma membrane of cells. Each AQP channel is composed of six membrane-spanning alpha-helices with a central water-transporting pore (Fig. 3.1) that is permeable to water molecules at various permeability rates [1]. Water transporting property of AQPs was first confirmed via biophysical function studies of AQP1 possessing extremely high water permeability reaching 2~3 $\times 10^9$ water molecules per subunit per second [2].

Almost all of rat AQPs are permeable to water with various single channel water permeability including: AQP0 (0.25×10^{-14} cm^3/s) [3], AQP1 (6.0×10^{-14} cm^3/s) [3], AQP2 (3.3×10^{-14} cm^3/s) [3],

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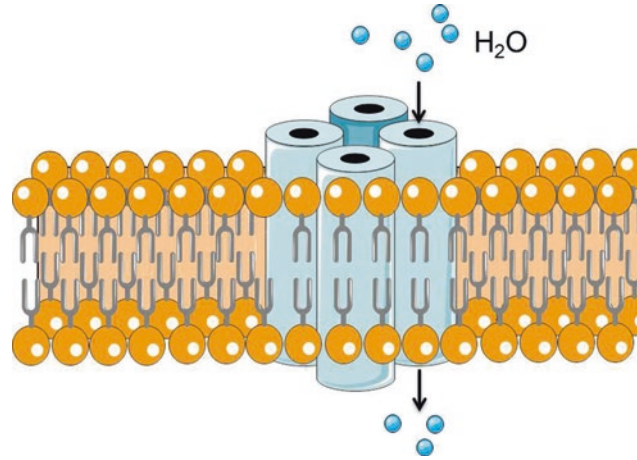

Fig. 3.1 Tetrameric structure of AQP1. AQP1 is organized as a tetrameric assembly of four identical polypeptide subunits. Water molecules pass through a pore of each AQP1 monomer (Reproduction from Ref. [17])

AQP3 (2.1×10^{-14} cm^3/s) [3], AQP4 (24×10^{-14} cm^3/s) [3], AQP5 (5.0×10^{-14} cm^3/s) [3]. The water permeability of other AQPs was measured by various groups: human AQP6 was reported to be inhibited by $HgCl_2$ [4], nevertheless, when the rat AQP6 expressed in oocytes, it was activated by Hg^{2+} to dramatically increase the osmotic water permeability ($P_f = 93.0 \times 10^{-4}$ cm/s) [5]. The mouse AQP6 was identified to have low water permeability activated by Hg^{2+} [6]. Another group confirmed that rat AQP6 lacked water permeability [7]. AQP7 was initially found and cloned from rat testis, and water permeability coefficient of *Xenopus* oocytes injected with rat AQP7-cRNA reached 186 μm/s [8]. The cloning and water transport measurement of mouse AQP8 were performed using *Xenopus* oocytes, and the single channel water permeability of AQP8 was up to 8.2×10^{-14} cm^3/s [9]. Rat AQP9 cRNA-injected *Xenopus* oocytes expressed ~fourfold increase of coefficient of osmotic water permeability (P_f) [10]. The function of AQP10 was also examined in *Xenopus* oocyte expression system, in which the osmotic water permeability increased up to sixfold with AQP10 expression [11]. Using CHO cells transfected with GFP-AQP11 to measure the water permeability of AQP11, the osmotic water permeability P_f value enhanced up to 8.0×10^{-4} cm/s [12]. Another group confirmed that mouse AQP11 was water permeable using stopped-flow analysis of vesicles containing mouse AQP11 [13]. Whether AQP12 could transport water has not been determined yet.

With selective pore for the rapid movement of water across cell membranes, AQPs are crucial for the transport of water and regulation of water homeostasis. In body, there are two trans-tissue water flow routes: transcellular water flow mediated by AQPs and paracellular flow [14, 15]. AQPs are thought to be the specific channels for rapid water transport in response to osmotic gradient, making a critical contribution to the regulation of transcellular water flow [16].

When expressed in *Xenopus laevis* oocytes, AQP1 exhibited significantly high osmotic water permeability that was 20-fold higher than that of the control oocytes [17, 18]. AQP1 protein reconstituted into membrane proteoliposomes caused the 50-fold raise in water permeability [19, 20]. This process occurs with low Arrhenius activation energy and is inhibited by $HgCl_2$ or other mercurial and is reversed by a reducing agent. Water permeability mediated by most AQPs can be inhibited by mercurial reagents such as $HgCl_2$ whose mechanism was elucidated by molecular dynamics simulations [21]. Mercury inhibits water and glycerol transport by mammalian AQPs through binding to cysteine residues [22]. However, AQP4 and AQP6 are not inhibited by $HgCl_2$ [23, 24].

To explain how a simple pore without moving parts could allow rapid transit of water without movement of protons, the groups of Robert

Stroud and Bing Jap solved the atomic structures of AQP1 from bovine red blood cells at 2.2-Å resolution [25]. Later, several groups performed molecular dynamics simulations on the basis of this solved structure [26, 27]. Now, the essence of how AQPs facilitate the movement of water but not protons has been revealed. Peter Agre explained how AQP1 could selectively accommodate water molecules transporting in his Nobel lecture [28]: water maintain the bulk solution condition at the extracellular vestibule and an internal vestibule both have the hourglass structure of the AQP1 molecule. In a single file, water could pass through a 20 Å channel that separates vestibules, the water molecules could interact with pore-lining residues to prevent the formation of hydrogen bonds between the water molecules. Especially near the top of the bridged site, the channel reaches its narrowest constriction of 2.8 Å, thus the pore is so narrow that it just accommodates a single water molecule. The mechanism of repealing proton from its permeation of AQP includes [25, 28–30]: (1) The side chain in loop E forms a fixed positive charge and a conserved histidine residue of another wall forms a partial positive charge, these two positive charges collectively repel protons. (2) Moreover, a single water molecule could forms hydrogen bonds simultaneously when it transiently undergo a transient dipole reorientation, also serves to be the barrier to protons.

3.2 Glycerol Transport Mediated by AQPs

In addition to the primary function of AQPs to facilitate water transport, glycerol transport could be another significant function of AQPs. Aquaglyceroporins, including AQP3, AQP7, AQP9 and AQP10, are a subset of aquaporin family and the exclusive mammalian proteins with the ability to permeate glycerol with their relatively broad solute specificity and sequence homology. One of the physiological functions of aquaglyceroporins is to facilitate the transport of glycerol across the cell membrane. Such glycerol-transporting function of aquaglyceroporins is involved in the movement of glycerol and energy metabolism process.

AQP3 (originally called glycerol intrinsic protein, GLIP, based on its glycerol-transport function) was first cloned by three different groups, respectively [31–33]. AQP3 is a relatively weak transporter of water but functions as an efficient glycerol transporter. Measurements of the 10-min glycerol uptake of *Xenopus* oocytes after microinjection of 5 ng of AQP3 cRNA and a 24~27-h incubation at 18 °C indicate that glycerol uptake is remarkably increased compared with control. Glycerol uptake in oocytes expressing AQP0, AQP1, AQP2, AQP4 or AQP5 is not increased significantly above control [3]. AQP3 is mainly expressed at the basolateral membrane of epithelial cells in kidney collecting duct, airway and intestine, as well as in epidermis, urinary bladder, conjunctiva and cornea [34]. As an aquaglyceroporin, AQP3 mediates glycerol permeability in certain organs, tissues and cells. In skin, the stratum corneum (SC) is the most superficial layer whose hydration determines skin appearance and physical properties [35]. Phenotype analysis of AQP3-deficient mice indicates that AQP3 expressed in epidermal keratinocytes plays essential role in hydration process and maintaining biological functions of skin [36, 37]. Study on AQP3 null mice showed that deficiency of skin AQP3 impaired glycerol transport through basal keratinocyte layer into the epidermis and SC, resulting in the reduced glycerol content of epidermis and SC and therefore impairing hydration and epidermal biosynthetic functions [37]. These data provide us compelling evidence that glycerol-transporting property of AQP3 is important for the skin function [35].

AQP7 is abundantly expressed in human adipose tissue and acts as an adipose glycerol channel [38], it is found to act as a facilitative carrier for water by tenfold (186 ± 15 μm/s), glycerol by fivefold (the calculated $P_{glycerol}$ was 18.9×10^{-6} cm/s) and urea by ninefold (the calculated P_{urea} was 12.0×10^{-6} cm/s), respectively in *Xenopus* oocytes expressing AQP7 [8].

Rodríguez et al. reported that AQP3 and AQP9 were also expressed in omental and subcutaneous fat depots, in addition to the well-known

expression of AQP7 in adipose tissue [39]. AQP3 and AQP9 act as glycerol channels in adipocytes and the hepatocytes respectively, representing novel additional pathways for the glycerol transport in human adipocytes [40, 41]. Studies on AQP7 and AQP9 knockout or knockdown mice demonstrated the pathophysiological relevance of glycerol channels through effects on glycerol metabolism. Impairment or lack of AQP7 function might have a causal role in obesity and diabetes mellitus [41].

AQP9 is mainly expressed in liver, testis, brain, leukocytes, epididymis and spleen [42–44]. By injecting rat AQP9-cRNA into oocytes and determining the permeability profile of AQP9, it is concluded that AQP9 confered high permeability for water as well as other solutes including carbamides, polyols, purines, pyrimidines and monocarboxylates [44]. Subsequent research showed AQP9 mainly facilitated glycerol and urea transport [45]. Further study also showed that AQP9 facilitated glycerol influx and urea efflux in hepatocytes, providing evidence that AQP9 acts as important solute channel associated with energy metabolism [10].

In human adipocytes, AQP3, AQP7 and AQP9 represent the glycerol channels involved in the regulation of lipid and glucose metabolism [38, 40]. AQP3 is present in the plasma membrane and cytoplasm, AQP7 is expressed predominantly in the cytoplasm upon the lipid droplets, AQP9 is constitutively expressed in the plasma membranes [39]. The role of aquaglyceroporins expressed in adipocytes is to control the transport of glycerol into and out of adipocytes, which are critical steps for lipogenesis and lipolysis [41]. In the lipogenic process, AQP9-mediated-glycerol uptake provides glycerol for the glycerol-3-phosphate proceeds, and further involves in the triacylglycerols synthesis of adipocytes (Fig. 3.2) [40]. In the lipolysis, stimulation of adrenergic receptors by catecholamines leads to a translocation of AQP3 and AQP7 to the plasma membrane to facilitate the glycerol release, which parallels with the translocation of HSL to the lipid droplets and its activation, leptin (via the PI3K/Akt/mTOR signalling cascade) and catecholamines downregulate AQP7 expression, which restrict glycerol release from adipocytes (Fig. 3.3) [40].

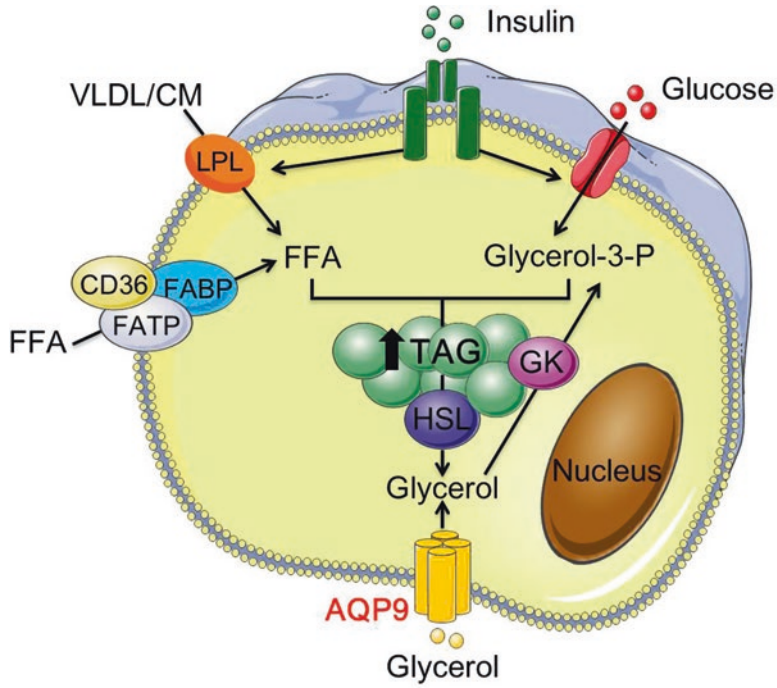

Fig. 3.2 Proposed role of aquaglyceroporins in lipogenesis. Triacylglycerols (TAG) is synthesized from FFA and glycerol-3-phosphate in adipocytes. Glycerol-3-phosphate proceeds from: (1) glucose, (2) glycerol from HSL-dependent lipolysis that is phosphorylated by GK or (3) AQP9-mediated-glycerol uptake (Reproduction from Ref. [39])

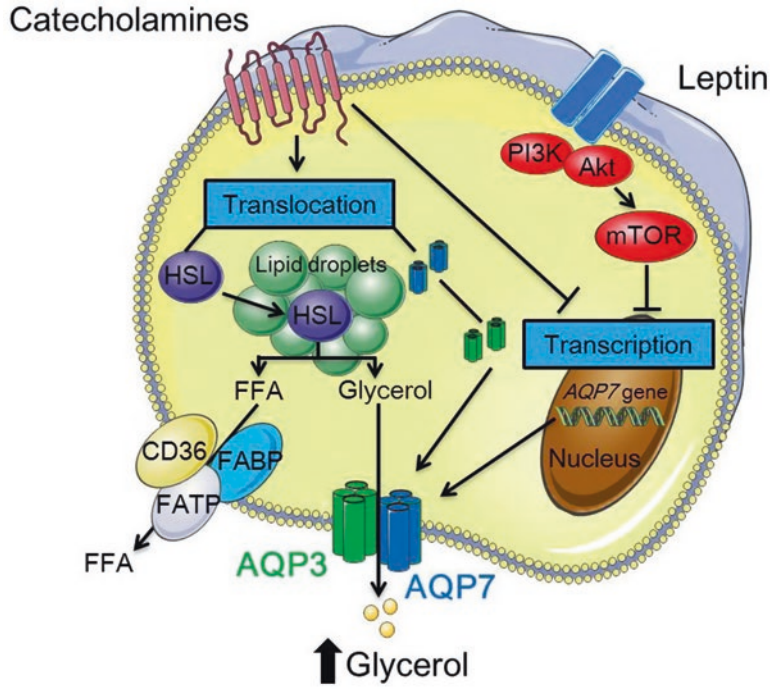

Fig. 3.3 Proposed role of aquaglyceroporins in lipolysis. Translocation of AQP3 and AQP7 to the plasma membrane is led by stimulation of adrenergic receptors by catecholamines to facilitate the glycerol release. AQP7 expression is downregulated by leptin and catecholamines, which represents a negative feedback regulation in lipolytic states to restrict glycerol release from adipocytes (Reproduction from Ref. [39])

AQP10 is abundantly expressed in the duodenum and the jejunum [46], which is also identified as aquaglyceroporin on account of its functional and structural similarity with other aquaglyceroporins AQP3, AQP7 and AQP9 [11]. Using oocytes, mediated isotopic solutes uptakes by AQP10 was detected, resulting that the glycerol permeability was significantly increased threefold with AQP10, which was inhibited by $HgCl_2$ [11].

3.3 Urea Transport Mediated by AQPs

Urea is mainly generated from ammonia in liver as a key role in protein catabolism in mammals. And as a terminal product, approximately 90% of urea is eliminated in urine by the kidney [47]. In kidney, urea transport and cycle are vital in urinary concentrating mechanism [48]. Some of human AQPs are permeable to urea including AQP3 [32], AQP7 [49], AQP9 [42], AQP10 [11], and possibly AQP6 [50], but the physiological significance of these aquaporins in urea transport is not fully revealed.

Whether AQP3 is urea channel remains conflicting. With AQP3 expressing in *Xenopus* oocytes and measurement of the urea permeability, early work suggested that urea uptake was increased to twofold after 30 min incubations with radiolabelled urea, which can be completely blocked by phloretin, the inhibitor of urea transporters [32]. Controversially, subsequent study did not find urea permeability property of rat AQP3 [51]. The difference may be resulted from the use of diverse concentrations of urea, and the AQP3 mediated urea transport was so low that it did not induce significant change in volume under lower urea concentration (20 mM) [52].

AQP6 possesses water permeability under the activation of Hg^{2+} as described by Yasui et al. [5]. AQP6 was determined to be permeable to urea using AQP6-expressing oocytes, the uptake of [^{14}C]urea stimulated by $HgCl_2$ was initially large ($P_{urea} = 21.3 \times 10^{-7}$ cm/s) but decreased with time. However, the uptake of [^{14}C]urea into AQP6-expressing oocytes not stimulated by $HgCl_2$ was about three times less [50].

Ishibashi et al. found that urea uptake was increased up to 9-fold in 5 min and 16-fold in

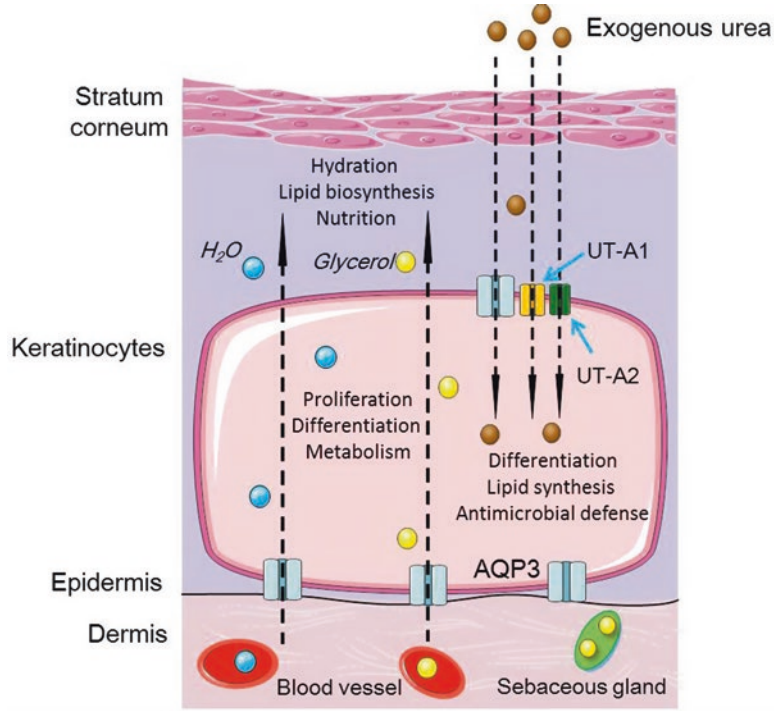

Fig. 3.4 Proposed mechanism of AQP3 function in epidermis. UT-A1, UT-A2 and AQP3 facilitate urea uptake in skin, which may induce keratinocyte differentiation and improve barrier and antimicrobial defense function of skin. Moreover, AQP3 facilitates water and glycerol transport from blood and sebaceous glands to keratinocytes (Reproduction from Ref. [17])

10 min with AQP7 expression in the oocytes, whose stimulation effect of urea uptake was much higher than that of AQP3 [8].

AQP9 expressed in oocytes showed to increase the urea permeability coefficient (P_{urea}) from 1.5 × 10^{-6} cm/s to 23.5 × 10^{-6} cm/s [43].

Urea permeability of AQP10 was measured in *Xenopus* oocyte expression system, and the result showed urea uptake was significantly increased twofold, which was inhibited by phloretin [11].

AQP3, AQP7 and AQP9 appear to play roles in urea transport in skin. AQP3 and AQP9 are expressed in the differentiating layers of human epidermal skin equivalents [53]. AQP7 localizes to superficial epithelial cells of the gastrointestinal tract [54]. Expression of AQP3, AQP7 and AQP9 could be upregulated by urea [55] (Fig. 3.4). Studies revealed that urea transporters and AQPs transport exogenous urea into keratinocyte, playing a critical role in keratinocyte differentiation, lipid synthesis, and maintaining epidermal homeostasis. Moreover, AQP3 is proposed the important channel of epidermis in which AQP3 facilitates water and glycerol transport from blood and sebaceous glands to keratinocytes involved in proliferation and differentiation of keratinocytes [17].

AQP3 null mice have nephrogenic diabetes insipidus under normal conditions. When given a urea load, the concentration of urine reach high level, however, the excretion of other solutes reduces significantly [56]. The capacity of urea to increase the concentration of non-urea solutes relies on AQP3 and its function in transporting both urea and water [57]. AQP10 is only found in duodenum and jejunum [46], and it transports water, urea and glycerol when expressed in *Xenopus* oocytes [11]. Further study of AQP10 in urea transport is less carried out. AQP9 is a urea-permeable protein localized at the basolateral membrane of hepatocytes, since the liver is a major site of urea production [42]. AQP9 also abundantly expresses in the peripheral leukocytes permeable to water and urea [43].

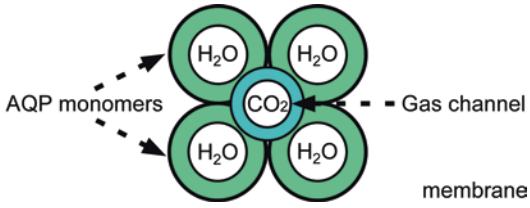

Fig. 3.5 CO_2 passes through the central space of AQP1 tetramer in plasma membrane (Reproduction from Ref. [17])

3.4 Gas Transport Mediated by AQPs

AQPs including AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9 could potentially transport gases such as CO_2, NO, NH_3 and O_2.

3.4.1 Carbon Dioxide (CO_2)

Early study showed that permeability of CO_2 was significantly increased in *Xenopus* oocytes injected with AQP1 cRNA and proteoliposomes containing purified AQP1 [58, 59], supporting the hypothesis that AQP1 is a pathway for CO_2 transport across the membrane. Also, one study adopting ^{18}O-labeled HCO_3^- to examine the CO_2 permeability of AQP1-null human erythrocytes compared with normal ones verified that AQP1 was responsible for 60% of the high P_{CO2} of erythrocytes [60], directly suggesting that AQP1 plays the critical role in mediating CO_2 transport. It is suggested that gases transport plasma membrane through central space of tetramer of AQP1 and do not go through water pore (Fig. 3.5).

Further study on cholesterol-containing membranes reconstituted with human AQP1 mediating CO_2 permeability showed significant increase in membrane CO_2 permeability, suggesting that both cholesterol and AQP1 are necessary in CO_2 permeability across biological membranes [61].

When expressed in oocytes, bovine AQP0, human AQP1, rat AQP4-M23, rat AQP5, rat AQP6, rat $AQP6_{N60G}$, or rat AQP9 exhibited significantly increased permeability to CO_2 measured by microelectrode positioned at the surface of the oocytes [7].

However, some studies suggest that AQP1-dependent CO_2 transport has no physiological relevance [62, 63]. Verkman group reported experiments in which physiological consequences of CO_2 transport by AQP1 were studied by comparing CO_2 permeability in erythrocytes and intact lung of wild-type and AQP1 null mice. Results showed no difference in CO_2 permeability between AQP1 null mice and wild-type mice, providing direct evidence against physiological significance of CO_2 permeability mediated by AQP1 [62, 63].

3.4.2 Nitric Oxide (NO)

As another physiologically important gas, NO plays critical role in cardiovascular system, urinary system, and central nervous system (CNS). Early studies showed that NO produced by the endothelial cells relaxed adjacent vascular smooth muscle cells to regulate blood flow and blood pressure [64–66]. Previous conception that the process of NO transporting from endothelial cells to the vascular muscle cells occurred by free diffusion through the lipid bilayer of the cell membrane was challenged by the discovery of AQP1 in transporting NO. In the vascular system, AQP1 expressed in endothelial cells [67] is involved in vascular function. By transfecting AQP1 into CHO cells and reconstituting purified human AQP1 into the lipid vesicles, transport property of NO by AQP1 was measured. In CHO cells expressing AQP1, NO permeability was identified to be correlated with water permeability, the use of AQP1 inhibitor led to a NO transport reduction by 71% and the NO transport is saturable. In the reconstituted lipid vesicles expressing AQP1, NO influx was increased by 316% [68]. Results all above support the hypothesis that NO is transported by AQP1.

Using AQP1 null mice, Herrera et al. subsequently identified that transport of NO by AQP1 was required in full expression of endothelium-dependent relaxation, though NO free diffusion still occurred in the absence of AQP1 slowly [69, 70].

In addition to AQP1, AQP4 located in brain is also permeable to NO through its central pore, and it even provides a more favorable permeation pathway for gas molecules than AQP1 [71]. Further investigation is required to clarify the role of AQP4 in the control of NO flow in the central nervous system.

3.4.3 Ammonia (NH_3)

Holm et al. first observed a role of AQPs as NH_3 channels [72]. AQP1, AQP3, AQP8 and AQP9 expressing in *Xenopus* oocytes and lipid bilayers have been shown to facilitate NH_3 transport [72–74]. Another group measured NH_3 permeability of the AQPs in *Xenopus* oocytes, and their results indicated that human AQP1, rat AQP3, rat AQP6, rat $AQP6_{N60G}$, human AQP7, human AQP8, and rat AQP9 had a significantly increased permeability to NH_3 [7]. Nevertheless, the physiological significance of AQPs as NH_3 transporters remains unclear [70].

3.4.4 Oxygen (O_2)

Molecular dynamics (MD) simulations on the AQP1-embedded membranes and on the pure lipid bilayers indicated that the central pore of AQP1 was an ideal channel for the permeation of both CO_2 and O_2. The result of MD simulations showed the central pore of APQ1 permeated O_2 with a −0.4~−1.7 kcal/M energy well [75].

3.5 Other Molecule Transport Mediated by Aquaporins

3.5.1 Hydrogen Peroxide(H_2O_2)

Hydrogen peroxide (H_2O_2) belongs to the group of reactive oxygen species (ROS). ROS are generated in a number of key metabolic processes in cells such as the electron transport chain in the inner mitochondrial membrane [76]. Because of the potential damage of ROS on nucleic acids, proteins and lipids, cells have a number of ROS-scavenging systems to remove these molecules and to maintain a relatively low and constant ROS concentration [77]. Although the formation and scavenging of ROS has been studied thoroughly relatively, little is known about their transport mechanism from the site of origin to the place of action or detoxification [77]. The obvious chemical similarity between water and H_2O_2 suggests that AQPs could likely be candidates for H_2O_2 permeation and many studies confirmed that certain AQPs could mediate H_2O_2 transmembrane transport [77–81]. In 2006, human AQP8 was evidenced to facilitate the diffusion of H_2O_2 across membranes adopting fluorescence assay with intact yeast cells and intracellular ROS-sensitive fluorescent dye [77]. And as the H_2O_2 has been revealed to be an important signaling molecule for immune response, growth, differentiation, migration processes, Miller et al. demonstrated that AQP3 and AQP8 promoted uptake of H_2O_2 in HEK-293 cells transfected with AQP3 or AQP8 expression vectors, and that intracellular H_2O_2 accumulation can be modulated by endogenous AQP3 expression that influences downstream cell signaling cascades [81]. Another study showed that the AQP3-meidated H_2O_2 uptake is essentially required for the chemokine-dependent T cell migration during immune response, which revealed a novel physiological role of AQP3-mediated H_2O_2 transport [80].

3.5.2 Some Ions

AQPs were originally regarded as plasma membrane channels that were freely permeable to water or small uncharged solutes but not to ions [82]. There is increasing evidence that certain AQPs have ion channel function [82, 83]. In 1996, Yool group reported that AQP1 acted as a cation channel (K^+, Cs^+, and Na^+ and to a lesser degree tetraethylammonium) [84] that was initially controversial. Subsequent researches by Yool group showed that human AQP1 expressed in *Xenopus* oocytes could mediate cationic conductance gated by the activation of cGMP [85] and defined not only the ion channel function, but also the detailed molecular mechanisms that

govern and mediate the multifunctional capabilities of AQP1 [86]. In AQP1, the central pore at the fourfold axis of symmetry in the tetramer has been proposed as the most likely pathway for cation conduction [83]. AQP1 functions as a non-selective monovalent cation channel when activated by intracellular cGMP, with a large single channel conductance of approximately 150 pS in standard physiological saline conditions [85].

In other AQPs such as AQP0 and AQP6, the possible role of the intrasubunit pores as ionic conductance pathways was proposed by many research groups [5, 86–88]. As the major protein component of isolated lens junctions, AQP0 shows to have ion channel activity when reconstituted in bilayers [89]. Bovine AQP0 has a conductance of 200 pS in unilamellar vesicles with 100 mM saline, which supports ion channel activity [86]. The ion channel of AQP0 was detected to be voltage- and pH-sensitive, opened at acidic pH and closed permanently at neutral pH [87].

Rat AQP6 was found expressed in intracellular vesicles of renal epithelia. As a gated channel, mammalian AQP6 expressed in oocytes shows intermediate conductance (49 picosiemens in 100 mM NaCl) induced by $HgCl_2$ [88]. At pH lower than 5.5, anion conductance is rapidly and reversibly activated in AQP6 oocytes. The cation/anion selectivity changed with the site-directed mutation of lysine to glutamate at position 72 in the cytoplasmic mouth of the pore leaving low pH activation intact [5]. The studies of Ikeda et al. indicated that AQP6 exhibited a form of anion permeation with significant specificity for nitrate [82]. Above all of the researches, the function of AQP as gated ion channel and as water channel is considered to have physiological and potentially translational relevance [90].

3.5.3 Silicon

Silicon is abundantly and differentially distributed in body, researchers showed that unlike silicon transporter existing in plants and algae, human aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) can mediate silicon transport in *Xenopus laevis* oocytes and HEK-293 cells. Further, aquaglyceroporins could act as the relevant silicon permeation pathways in both mice and humans, regulating the Si balance in body. And this study surprisingly found phloretin stimulated the Si transport of AQP9 [91].

3.6 Conclusion

The transport of various kinds of small molecules by AQPs is an interesting topic. However, most data about transport characteristics of AQPs are derived from *in vitro* experiments. The physiological significance of AQPs that are permeable to gases and other small molecules is necessary to be determined by *in vivo* experiments. Studies using knockout mouse models of AQPs have confirmed that AQPs, with water and glycerol permeability, play important roles in urine concentrating mechanism, skin moisture and energy metabolism, etc. Aquaporin-mediated transport of other small gases (CO_2, NH_3 and NO) should be carefully studied, due to that the high intrinsic membrane permeabilities for these gases makes aquaporin-facilitated transport not dominant in physiological mechanism.

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Aquaporins and Gland Secretion

4

Christine Delporte

Abstract

Aquaporins (AQPs) are expressed in most exocrine and endocrine secretory glands. Consequently, summarizing the expression and functions of AQPs in secretory glands represents a daunting task considering the important number of glands present in the body, as well as the number of mammalian AQPs – thirteen. The roles played by AQPs in secretory processes have been investigated in many secretory glands. However, despite considerable research, additional studies are clearly needed to pursue our understanding of the role played by AQPs in secretory processes. This book chapter will focus on summarizing the current knowledge on AQPs expression and function in the gastrointestinal tract, including salivary glands, gastric glands, Duodenal Brunner's gland, liver and gallbladder, intestinal goblets cells, exocrine and endocrine pancreas, as well as few other secretory glands including airway submucosal glands, lacrimal glands, mammary glands and eccrine sweat glands.

Keywords

Aquaporins • Exocrine glands • Endocrine glands • Secretion • Function • Expression

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4.1 The Secretory Glands from Gastrointestinal Tract

AQP expression has been localized to several secretory glands from the gastrointestinal tract (Fig. 4.1).

4.1.1 Salivary Glands

Salivary glands comprise major salivary glands, namely parotid, submandibular and sublingual glands, and minor salivary glands [4, 180]. Salivary glands contains several lobes that further subdivide into lobules. Salivary glands are made of acinar, ductal and myoepithelial cells [136]. The acinar cells are either serous, mucous or seromucous, based on their secretory products and characteristics [136]. The ductal system can be subdivided into intralobular (intercalated and striated), interlobular, interlobar (excretory) ducts.

In all human salivary glands, AQP1 is expressed in myoepithelial [117] and endothelial [10, 50, 158] cells. In addition, AQP3 is located at the basolateral membrane of serous and mucous acini, but not the ducts [10, 50]. AQP4, AQP6 and AQP7 proteins have not been detected in human salivary glands, despite the presence of their mRNA [50]. AQP5 is exclusively expressed at the apical membrane of serous acinar cells [50, 158].

Rat submandibular and parotid glands express AQP1 and AQP5 respectively in endothelial cells [3, 54, 90, 126] and at the apical membrane of acinar cells [45, 120, 125, 135, 158]. The expres-

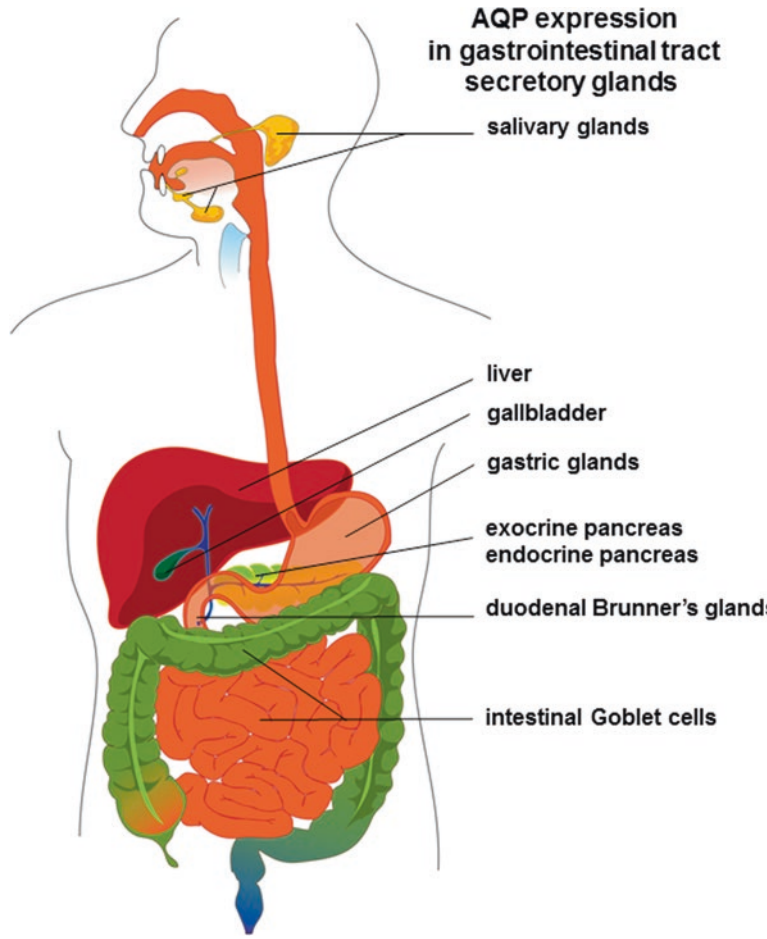

Fig. 4.1 AQP expression in gastrointestinal tract secretory glands. AQP expression has been localized to several secretory glands from the gastrointestinal tract

sion of AQP3 and AQP4 in rat salivary glands remains a subject of controversy [3, 43, 71, 125]. In rat parotid glands, the expression of AQP6 has been localized to secretory granule membrane [101], while that of AQP8 has been located in myoepithelial cells [40, 78, 175].

In all mouse salivary glands, AQP1 is located to endothelial and myoepithelial cells [122]. Acinar and ductal cells express AQP3, AQP4 and AQP8 at their basolateral membrane [122]. AQP5 is expressed at the apical membrane of acinar cells [6, 84, 103]. AQP7 is expressed in endothelial cells [6]. The cell distribution of AQP9 remains to be determined in mouse salivary glands [6, 33, 84]. Ductal cells express AQP11 [6, 84].

Saliva secretion results from the secretion of an isotonic-like fluid rich in NaCl and water by acinar cells and its subsequent modification in composition when entering the ductal lumen [87, 109]. This leads to the secretion of a final hypotonic saliva into the oral cavity.

AQP5, located in acinar cells, plays a major role in saliva secretion [80, 95]. Indeed, AQP5 knockout mice displayed a 60% decrease in pilocarpine-stimulated saliva secretion, as well as a more viscous and hypertonic saliva [80, 95]. In addition, when submitted to hypotonic challenge, water permeability of parotid and sublingual acinar cells from AQP5 knockout mice decreases by 65% and 77%, respectively [80]. Despite the use of AQP1, AQP4 and AQP8 knockout mice, the involvement of these AQPs has not been demonstrated in saliva secretion [95, 169, 178]. The current saliva secretion model suggests that AQP5 is responsible for transcellular water movement occurring in acinar cells [87, 96, 109, 151]. However, an osmosensor feedback model suggest that AQP5 could act as an osmosensor, controlling the tonicity of the transported fluid by mixing transcellular and paracellular water flows [57]. Indeed, in response to stimuli promoting intracellular calcium, AQP5 traffics from intracellular vesicles to plasma membrane [28, 66, 105].

In salivary glands of patients suffering from Sjögren's syndrome, is an autoimmune disease characterized by lymphocytic infiltration of exocrine glands. It has been hypothesized that AQP5 could participate in the pathogenesis of the disease and the reduction of saliva secretion [158]. In salivary glands from Sjögren's syndrome patients and a mouse model of Sjögren's syndrome, AQP5 expression and/or localization has been reported to be either altered [41, 76, 92, 143, 155, 156, 158, 181] or not modified [11, 51, 161]. AQP5 altered distribution could be linked to the presence of inflammatory infiltrates [156]. Therefore, cytokines and autoantibodies directed against muscarinic M3 receptors may play a role in altered distribution and/or expression of AQP5 in salivary glands from patients with Sjögren's syndrome [86, 89, 150, 177, 179]. Even though altered expression and/or localization of AQP5 could not totally account for saliva impairment observed in Sjögren's syndrome patients, nevertheless it could still play a role in the pathogenesis of the disease.

Interestingly, a naturally occurring point mutation of AQP5 has been identified in rats and associated with decreased AQP5 production and saliva secretion [121]. However, no AQP5 mutation has been associated to defects in saliva flow in humans.

Despite decreased AQP1 expression in myoepithelial cells of salivary glands from Sjögren's syndrome patients [10] and reestablished saliva flow in patients with Sjögren's syndrome by drug increasing AQP1 expression [137], further studies will be necessary to better understand its role in xerostomia.

In patients who received ionizing radiation therapy as part of their treatment for head and neck cancer, decrease/loss of AQP5 expression [30, 159] and impaired AQP5 trafficking [5] may participate to xerostomia.

AQPs could be used as either therapeutic targets or therapeutic agents to treat xerostomia. Cevimeline may be a useful drug to treat xerostomia as it restores proper AQP5 trafficking [62, 67, 172]. Drugs finely tuning DNA methylation may also be useful to treat xerostomia as DNA demethylation agent increased AQP5 expression [176]. Gene therapy using delivery of a recombinant adenovirus vector coding for AQP1 (AdhAQP1) to irradiated glands of animals and

human leads to saliva flow restoration [8, 9, 34, 127, 145]. Further studies are required to engineer new adenoviral vectors that would allow more efficient and persistent expression of a transgene, such as for instance hAQP1, in salivary glands. Despite the latter consideration, gene therapy using hAQP1 as a therapeutic agent still represents a promising therapy for patients suffering from xerostomia subsequent to head and neck irradiation therapy, as well as for Sjögren's syndrome.

4.1.2 Gastric Glands

In mammals, the gastric glands are located in distinct regions of the stomach, in gastric pits within the gastric mucosa. The gastric glands comprise fundic glands (located in the cardia), cardiac glands (located in the fundus and body of the stomach) and pyloric glands (located in the antrum of the pylorus). Gastric glands are composed of foveolar cells producing mucous, parietal cells secreting gastric acid and bicarbonate ions, chief cells secreting pepsinogen, G cells secreting gastrin and enterochromaffin-like cells releasing histamine [74].

The human stomach express several AQPs. AQP1, AQP3, AQP4, AQP5, AQP7, AQP8, AQP10 and AQP11 mRNA have been identified in the fundus, while AQP1, AQP3, AQP5, AQP7 and AQP11 mRNAs have been located to the antrum of the pylorus [82]. Both parietal and chief cells express AQP4 protein at the basolateral membrane [43, 44, 58, 79, 114]. In stimulated cells, AQP4 internalization in a vesicle-recycling compartment was followed by phosphorylation [24]. However, AQP4 knockdown mice did not exhibited any change in the rates of both basal or stimulated acid and fluid secretion [174]. Even though other AQPs could compensate for the lack of AQP4, AQP4 is unlikely to play a major role in gastric acid production, but on the other hand may be involved in cell volume maintenance. AQP5 has exclusively been localized to the apical and lateral membranes of pyloric glands [131].

Several AQPs have been found to promote or be involved in stomach pathologies including chronic gastritis and gastric cancer [27, 59, 82, 85, 108, 146, 184].

4.1.3 Duodenal Brunner's Gland

AQP5 was localized to the apical, lateral and secretory granule membranes of the Brunner's gland cells [131]. AQP1 localization was restricted to apical and lateral membranes of the Brunner's glands cells [130]. Vasoactive intestinal peptide (VIP) is known to increase secretion flow rate as well as bicarbonate and protein output from rat Brunner's glands [72]. In both rat and human, VIP induces subcellular redistribution, from intracellular granules to apical plasma membrane, of AQP5 but not of AQP1 in a cAMP and protein kinase-A dependent manner [31, 130]. Co-localization and co-trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and AQP5 provides a parallel pathway for electrolyte secretion and osmotic water movement [31]. In celiac disease-affected Brunner's glands, AQP5 expression was almost absent, while AQP5 expression was reduced in cystic fibrosis [31]. Therefore, AQP5 may participate to the pathophysiology of these diseases characterized by altered duodenal secretion.

4.1.4 Liver and Gallbladder

Liver comprises hepatocytes, as well hepatic ducts made of cholangiocytes, endothelial cells and Kupffer cells [88]. Hepatocytes secrete canalicular bile containing bile salts, organic and inorganic solutes and 95% water. Hepatic ducts facilitates bile flow towards intestine and gallbladder, but also modify the composition of bile [128]. In the gallbladder, bile is concentrated or directly delivered to the intestinal lumen [16]. Humans produce daily about 17 µL/g liver/h, while the production is about 6 times higher in rats [16].

Rat hepatocytes express AQP0 and AQP8 intracellularly and at the canalicular plasma

membrane [19, 40, 60]. Human hepatocytes also express AQP8 [49]. Upon secretin stimulation and intracellular cAMP increase, AQP8, but not AQP0, traffics from intracellular vesicles to the canalicular plasma membrane [22, 47, 48, 60, 162]. AQP8 is also located in rat hepatocyte mitochondria [20]. AQP9 is expressed at the basolateral membrane of both rat, mouse and human hepatocytes [39, 49, 138, 160]. AQP11 mRNA has been detected in mouse and human liver [49, 119]. AQP7 has been located to human hepatocytes [49].

Cholangiocytes express AQP1 in intracellular vesicles and AQP4 at their basolateral membrane [99, 133]. Upon secretin stimulation and intracellular cAMP increase, AQP1 traffics from intracellular vesicles to the plasma membrane of cholangiocytes [157, 163]. Mice cholangiocytes also express AQP3 and AQP8 at their plasma membrane [132, 168].

Endothelial cells express AQP1 [126] and AQP7 [49]. Kupffer cells express AQP3 [49].

Human and mouse gallbladder epithelial cells express AQP1 at their apical and basolateral membranes, while AQP8 is located intracellularly and at the plasma membrane [20, 126].

Hepatocyte secrete bile by a mechanism of filtration in response to osmotic gradients created by the transport of osmotically active solutes in the bile canalicular lumen [16, 20, 22]. Water and small solutes enter passively the canalicular lumen, in response to the osmotic gradient. Water flows through both transcellular and paracellular pathways [16, 22]. Cholangiocytes are responsible for less than 10% bile production in rodents, whereas it humans it may account for about 30% of daily bile production [16]. Cholangiocytes account for secretin-induced ductal bile secretion and extrude Cl^- and HCO_3^- into the canalicular lumen, providing the driving force for osmotic water movement into the ductal lumen [16, 22]. In addition, cholangiocytes reabsorb solutes and fluid from the primary secretion of hepatocytes [16]. Considerable species differences exist with the respect of cholangiocytes in modifying hepatocyte bile [16]. Gallbladder concentrates bile by reabsorbing water, thereby allowing the concentration of bile acids [167]. Gallbladder contracts and expulses bile into the intestinal lumen in response to cholecystokinin [16].

AQP8 subcellular distribution and trafficking suggested its participation in bile formation by the hepatocytes. However, AQP8 knockout mice did not confirm this hypothesis [178]. Compensatory mechanisms and/or species differences could account for this result. While the involvement of AQP8 in mitochondrial volume expansion occurring during active oxidative phosphorylation remains controversial, it is likely to play a role in rat liver mitochondrial ammonia transport [154]. In addition, AQP8 present in smooth endoplasmic reticulum could be involved in glycogen synthesis and degradation in mice [42]. As AQP9 knockout mice display increased plasma glycerol and triglycerides levels as well as decreased hepatocyte glycerol permeability, AQP9 is likely involved in glycerol metabolism and energy balance [21, 97, 110, 139]. AQP11 knockout mice display hepatocyte vacuolization, suggesting that AQP11 is involved in rough endoplasmic reticulum homeostasis and liver regeneration [64, 138]. In cholangiocytes, AQP1 is thought to play a key role in basal and secretin-induced bile secretion [22, 100]. However, AQP1 knockout mice studies do not support a role of AQP1 in bile formation [94, 111]. AQP4 and several other AQPs are expressed in hepatocytes and cholangiocytes, where they could be involved in compensatory mechanism occurring following the deletion of a given AQP. Therefore, further studies are necessary to clarify the roles of AQPs in bile secretion, involving both hepatocytes and cholangiocytes. In gallbladder, AQP1 and AQP8 may participate to water movement. However, their roles in gallbladder function remain unclear due to oppose findings [22, 91]. Therefore, additional studies will be required to address the role played by AQPs in gallbladder function.

4.1.5 Intestinal Goblet Cells

In the intestine, a subset of mucus-secreting goblet cells have been shown to express AQP9 mRNA [129]. However, the presence of AQP9

protein and its function in goblet cells remain to be determined.

4.1.6 Exocrine Pancreas

Exocrine pancreas, accounting for 90% of total pancreatic cells, possesses a morphology very similar to salivary glands, albeit the presence of few differences. Exocrine pancreas is only made of serous acinar cells, centroacinar cells represent an extension of intercalated ducts into each acinus, and the exocrine pancreatic fluid secretion drains into a main collecting duct. Human pancreatic fluid secreted daily (1–2 l) contributes to both the neutralization of the stomach acid and the digestion of food. Several neurotransmitters including acetylcholine, cholecystokinin and secretin are involved in pancreatic juice secretion. They induce both pancreatic enzyme and fluid secretion or mainly fluid secretion, and exert potentiated effects [77].

Human exocrine pancreas has been shown to express AQP1, AQP3, AQP4, AQP8 and AQP12 mRNAs, while only AQP1, AQP5 and AQP8 proteins were detected [17, 68]. AQP1 is expressed in capillaries, centroacinar cells and intercalated ductal cells [17]. In addition, pancreatic zymogen granule membranes express AQP1 [2, 29]. AQP5 and AQP1 are localized to the apical membrane of respectively intercalated ductal cells and acinar cells [17]. The precise localization of AQP12 expression has not yet been determined [68].

Rat exocrine pancreas expresses AQP1, AQP4, AQP5, AQP8, but not AQP12, mRNAs [17, 61, 68]. AQP1 is localized to the apical and basolateral membranes as well as caveolae and vesicle-like structures of intralobular and intralobular ductal cells [46, 73]. In addition, AQP1 is expressed in acinar zymogen granules [29] and in endothelial cells [61]. AQP5 is located at the apical membrane of centroacinar and intercalated ductal cells [18], while AQP8 is expressed at the apical membrane of acinar cells [68].

In mouse exocrine pancreas, interlobular ductal cells express both AQP1 and AQP5 at their apical membrane, while intercalated and intralobular ductal cells only express AQP5 at their apical membrane [18]. Acinar cells express AQP12 intracellularly [69].

The mechanisms leading to pancreatic juice secretion involve a first step during which acinar cells secrete a small volume of isotonic fluid, and a second step in which ductal cells secrete ions (Na^+, Cl^- and HCO_3^-) as well as most of the water [87, 107]. Transcellular water movement to the acinus lumen is ensured by the AQP8 located at the apical membrane, while AQP1 (located at both apical and basolateral membranes) and AQP5 (located at the apical membrane) ensure ductal transcellular movement to the ductal lumen [18]. While AQP8 accounts for 90% of water permeability in rat pancreatic acinar cells [61], AQP8 knockout mice display normal exocrine pancreatic function likely because acinar cells generate small amount of fluid compared to ductal cells [178]. AQP1, expressed in rat pancreatic acinar zymogen granules, contributes to basal and GTP-mediated vesicle water entry and swelling [2, 29]. In rat interlobular ductal cells, AQP1 accounts for 80–90% of secretin-stimulated pancreatic juice secretion [73]. However, AQP1 and AQP5 knockout mice display normal exocrine pancreatic function [17]. Weak level of AQP1 and AQP5 expression or function redundancy may account for this observation. Nevertheless, double AQP1 and AQP5 knockout mice could be valuable to determine the possible contribution of these AQPs to the pancreatic juice secretion. Finally, additional experiments will also be required to elucidate the role of AQP12 in exocrine pancreatic fluid secretion.

4.1.7 Endocrine Pancreas

Endocrine pancreatic cells, accounting for 10% of total pancreatic cells, are organized in islets of Langerhans made of insulin-producing ß-cells surrounded by glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP cells [83]. Human endocrine pancreas is responsible for post-prandial insulin secretion [55, 144].

While no data are currently available concerning the expression of AQPs in human, AQP7 is expressed in both rat and mouse ß-cells [12, 93, 102]. In addition, mouse ß-cells express AQP5 and AQP8 [93].

The classically-described sequential mechanisms involved in insulin secretion in response to increased glucose levels are: massive glucose uptake by the glucose transporter type 2 (GLUT2), glucose metabolization, increase in intracellular ATP concentration, inhibition of ATP-sensitive K^+ channels, membrane depolarization, opening of voltage-dependent Ca^{2+} channels, increase in intracellular calcium concentration, exocytosis of insulin-containing granules [55]. In addition, glucose induces an increase in ß-cell volume that may affect ß-cell activity as well [112]. Indeed, ß-cell swelling in response to hypoosmotic stress induces volume-regulated anion channel (VRAC) activation and cell membrane depolarization leading to activation of voltage-dependent Ca^{2+} channels, calcium entry and insulin secretion [13, 38].

AQP7 emerged as playing a key role in intracellular glycerol content regulation, as well as insulin production and secretion. Indeed, AQP7 knockout mice display reduced ß-cells size, mass, insulin content and cAMP-induced glycerol release [56, 102]. In addition, these mice had increased rates of both basal and glucose-stimulated insulin secretion, glycerol and triglyceride contents and glycerol kinase activity [102]. However, most likely related to the different genetic background of the AQP knockout mice, the mice presented different phenotypes. Some AQP7 knockout mice presented hyperinsulinemia [56, 102] accompanied [56] or not [102] with hyperglycaemia, while other had normal glycaemia with undetermined insulin levels [149]. In both ß-cells and BRIN-BD cells, extracellular isosmotic addition of glycerol induces sequential cell swelling, VRAC activation, membrane depolarization, electrical activity and insulin secretion [12, 35, 171]. Both glycerol entry and glycerol metabolization are likely to contribute to ß-cell activation [12]. In response to increased D-glucose concentration, extracellular hypotonicity or extracellular isosmotic addition of glycerol, AQP7 knockout mice displayed lower insulin released than wild type mice [93]. AQP7 likely plays a dual role in the regulation of insulin released by allowing both glycerol entry and exit, and by acting directly or indirectly at a distal downstream site in the insulin exocytosis pathway [93]. While association between mutations or single-nucleotide polymorphisms of AQP7 with diabetes and/or obesity have been investigated, no clear conclusion has been drawn [25, 26, 75, 113, 134]. Additional studies will be required to further clarify the role of AQP7 in ß-cells physiology and physiopathology.

4.2 Other Secretory Glands

4.2.1 Airway Submucosal Glands

Submucosal glands are present in the trachea and bronchial airways in humans, while only in the trachea in rats and mice [7]. Submucosal glands are made of serous and mucous acinar cells organized is secretory tubules, and ductal cells organized in lateral and collecting ducts. Submucosal gland secrete a mixture of water, ions and mucins ensuring the hydration of the airway surfaces, supporting mucociliary transport, and serving as a fluid matrix for secreted molecules including the gel-forming mucins [7]. Submucosal gland secretion is mainly induced by acetylcholine and VIP stimulations [7].

AQP5 is expressed at the apical membrane of submucosal serous epithelial cells [81, 170].

The mechanisms of submucosal fluid secretion involve the secretion of Cl^- and HCO_3^- creating an electrical gradient for cations such as Na^+ to passively follow through a paracellular pathway. The resulting osmotic gradient drives the passive flux of water across the glandular epithelia [7]. Knockout mice studies have revealed that AQP5 is involved in submucosal fluid secretion, as the latter decreased by more than 50% in AQP5 knockout mice as compared to wild type mice [153]. In submucosal glands from patients suffering from chronic obstructive pulmonary disease, AQP5 expression is decreased and related to the severity of the disease [173]. In

submucosal glands of asthmatic patients, AQP5 is overexpressed [147]. In addition, in an animal model of asthma characterized by hypersecretion of mucins and increase in inflammatory cytokines levels, AQP5 deletion induced a decrease of both parameters [147]. These data suggest that AQP5 is likely involved in the development of mucous hyperproduction and inflammation during chronic asthma [147]. Further studies are required to confirm the possible trafficking of AQP5 and the regulation of such process by physiological stimuli in submucosal glands. In addition, there is a need for a better understanding of the role of submucosal AQP5 in pulmonary diseases.

4.2.2 Lacrimal Glands

Lacrimal glands are composed of several lobules containing acinar cells secreting a fluid into intralobular, interlobular, intralobar, inerlobar and finally excretory ducts. Acinar cells are surrounded by myoepithelial cells. Lacrimal gland secretion is under the control of acetylcholinergic and adrenergic fibers. Lacrimal glands secrete a tear film rich in water and proteins ensuring cornea transparency, as well as the quality of the image projected onto the retina [164].

In rat lacrimal glands, endothelial cells express AQP1 and AQP5, while acinar cells express AQP3 (basolateral membrane), AQP4 (lateral membrane), AQP5 (apical membrane) and AQP11 (intracellularly) [182]. In mouse lacrimal acinar cells, AQP4 and AQP5 are localized to the basolateral and apical membranes, respectively [65, 135]. In addition, AQP5 is also expressed by mouse lacrimal ductal cells [141].

Lacrimal fluid is produced in two steps: formation of a primary isotonic fluid by acinar cells and modification of the primary fluid composition during its transit through the ductal system [37]. Depending on the flow rates, the final lacrimal fluid has much higher K^+ and Cl^- concentrations in rats, and higher K^+ concentration but similar Cl^- concentration in rabbits [37]. In lacrimal glands, electrolytes and water secretion occur in ductal cells [32]. Therefore, AQPs expressed in ductal cells are likely to contribute to tear secretion. However, knockout mice for AQP1, AQP3, AQP4 or AQP5 do not present defects in basal or stimulated tear secretion by lacrimal glands [118, 141]. Direct in situ measurement of major ions in tear fluid in AQP5 knockout mice revealed a significant tear film hypertonicity as compared to wild type mice [140]. It was proposed that AQPs are most likely not required for fluid secretion when fluid transport rates are low, such as in lacrimal glands [165].

In Sjögren's syndrome, characterized by sicca syndrome where tear fluid secretion is decreased, defective cellular trafficking of AQP5 has been shown in acinar cells [166]. In an animal model of Sjögren's syndrome, both AQP5 mRNA and protein levels were increased in ductal cells but decreased in acinar cells and AQP4 expression was decreased in ductal cells [36]. Some of these modifications could be due to autoantibodies directed against muscarinic M3 receptors [52], altered calcium signaling and volume regulation occurring in Sjögren's syndrome [41]. Additional studies will be needed to elucidate the functional role of AQPs in both the physiology and pathology of lacrimal glands.

4.2.3 Mammary Glands

Mammary glands are apocrine glands made of alveoli lined with milk-secreting cuboidal acinar cells surrounded by myoepithelial cells, and lactiferous ducts (intralobular and interlobular ducts) draining milk to the openings in the nipple [98].

Mammalian mammary glands produce and secrete milk. Milk consist of sugars, lipids, proteins, vitamins and minerals dissolved in water [148]. The percentage of water may vary depending on the species and their physiological status [106].

In rat and mouse mammary glands, AQP3 is localized at the basolateral membrane of secretory epithelial cells, as well as intralobular and interlobular ductal cells, while AQP1 is expressed at the apical and basolateral membranes of endothelial cells [104]. In addition, AQP5 is expressed at the apical membrane of acinar cells [123].

In bovine mammary glands, AQP3 is expressed at the basolateral membrane of acinar cells and AQP4 is located at the apical membrane of selected ductal cells [116]. In addition, AQP5 is expressed at the apical membrane of selected acinar and ductal cells and AQP7 is located at the apical membrane of selected acinar cells [116]. Finally, AQP1 is expressed in both endothelial cells, as well as selected myoepithelial cells [116].

AQP3 may be involved in both water and glycerol transport that are essential for milk synthesis and secretion [104]. Glycerol uptake via AQP3 may participate to milk triglycerides synthesis [104]. While AQPs are likely to play a role in milk secretion, additional experiments using knockout mice will be necessary to the understanding of the underlying molecular mechanisms involved in this physiological process.

Altered expression of AQPs has been reported in mammary tumors and breast cancer cells lines [85, 115]. However, it is unclear whether this is a causal or a consequence of neoplasia [110]. In this respect, further studies are required to better understand the role of AQPs in mammary neoplasia and assess if they could be used as either therapeutic targets or diagnostic/prognostic biomarkers.

4.2.4 Eccrine Sweat Glands

Eccrine sweat glands consist of single tubular structure made of secretory acinar cells and ductal cells.

AQP5 is located at the apical membrane of mouse, rat and human eccrine sweat gland acinar cells [63, 124, 183]. In addition, during induced-sweating, AQP5 translocates from intracellular vesicles to the apical plasma membrane of acinar cells [63].

Sweat secretion occurs generally into two steps: a primary fluid is secreted by acinar cells due to the action of active salt transporters followed by movement of water, and the primary fluid draining into ductal lumen undergoes salt reabsorption [142].

Using AQP5 knockout mice, the functional involvement of AQP5 in sweat secretion remains a subject to controversy due to opposing sets of data generated using distinct mouse strains and methodologies [124, 152]. Additional studies are needed to clarify the contribution of AQP5, and possible other AQPs, in sweat secretion.

Modified AQP5 expression is related to various skin pathologies [15, 53, 70]. In addition, mutations of AQP5 gene are responsible for palmoplantar keratoderma [1, 14, 23].

4.3 Conclusions

AQPs are expressed in most exocrine and endocrine secretory glands. By participating to transcellular water flow, AQPs are involved in the mechanisms leading to exocrine glandular fluid secretion. The general mechanism of exocrine secretion usually occurs in two steps (Fig. 4.2). A first step involves the creation of a transepithelial osmotic gradient, formed by the accumulation of ions, mostly NaCl. A second step involves the movement of water from epithelial cells to the gland lumen though AQPs. Water flows from secretory epithelial cells according to a transepithelial osmotic gradient. In addition, AQPs could participate to some endocrine secretion. Despite considerable scientific advances, further studies are still clearly needed to clarify our understanding of the role played by AQPs in glandular secretory processes.

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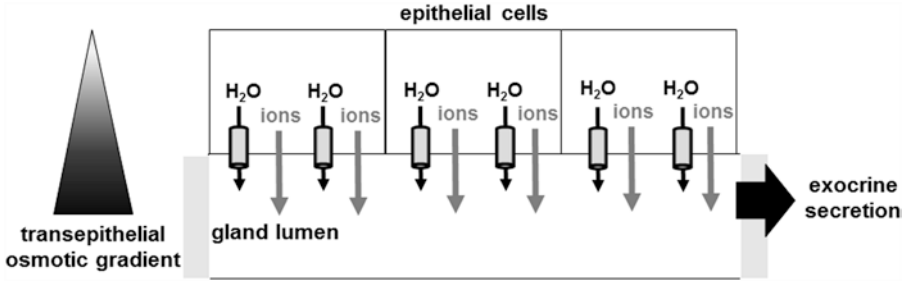

Fig. 4.2 Involvement of AQPs in the general mechanism of exocrine secretion. AQPs are expressed to several exocrine gland epithelial cells. The general mechanism of exocrine secretion usually occurs in two steps. A first step involves the creation of a transepithelial osmotic gradient, formed by the accumulation of ions, mostly NaCl. A second step involves the movement of water from epithelial cells to the gland lumen though AQPs. Thereby, AQPs participate to transcellular water flow

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Aquaporins in Nervous System

5

Mengmeng Xu, Ming Xiao, Shao Li,
and Baoxue Yang

Abstract

Aquaporins (AQPs) mediate water flux between the four distinct water compartments in the central nervous system (CNS). In the present chapter, we mainly focus on the expression and function of the 9 AQPs expressed in the CNS, which include five members of aquaporin subfamily: AQP1, AQP4, AQP5, AQP6, and AQP8; three members of aquaglyceroporin subfamily: AQP3, AQP7, and AQP9; and one member of superaquaporin subfamily: AQP11. In addition, AQP1, AQP2 and AQP4 expressed in the peripheral nervous system (PNS) are also reviewed. AQP4, the predominant water channel in the CNS, is involved both in the astrocyte swelling of cytotoxic edema and the resolution of vasogenic edema, and is of pivotal importance in the pathology of brain disorders such as neuromyelitis optica, brain tumors and Alzheimer's disease. Other AQPs are also involved in a variety of important physiological and pathological process in the brain. It has been suggested that AQPs could represent an important

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target in treatment of brain disorders like cerebral edema. Future investigations are necessary to elucidate the pathological significance of AQPs in the CNS.

Keywords
Aquaporins • Nervous system • Brain disorders

5.1 Introduction

Water homeostasis in the central nervous system (CNS) is of pivotal physiological and clinical importance, since about 80% weight of brain is water [1]. Water transport is linked to a number of brain functions such as the production and drainage of cerebrospinal fluid, cell volume regulation, and the controlling of the dimensions of the extracellular space [2–4]. In a pathophysiological context, water transport plays important role in cerebral edema, which may lead to ultimately cerebral herniation and death due to progressive increase in brain water content [5–7].

In the brain and other organs, water passes through plasma membranes by three distinct mechanisms: mere diffusion through the lipid bilayer, cotransport with organic or inorganic ions, and by way of specialized water channels (aquaporins, AQPs) [2, 8, 9]. It is recognized that AQPs are seen to mediate water flux between the four distinct water compartments existing in the brain: intracellular fluid (ICF), interstitial fluid (ISF), cerebrospinal fluid (CSF), and blood [10, 11], which are driven by osmotic and hydrostatic pressure gradient [12].

At present, 9 AQPs have been identified at distinct brain sites, including AQP1 [13, 14], AQP3 [15, 16], AQP4 [17, 18], AQP5 [15, 19], AQP6 [20, 21], AQP7 [22–24], AQP8 [15, 25], AQP9 [26, 27], and AQP11 [28, 29] (Fig. 5.1). A number of studies have reported the unexpected roles for the three members of this family (AQP1, AQP4, and AQP9) in physiology and pathology of CNS such as cerebral edema [30, 31], tumor angiogenesis [14, 32], autoimmune disease [33], glial scar formation [34], and neuro-excitation [35]. To date, little is known about the function and regulation of AQP3, AQP5, AQP6, AQP7, AQP8 and AQP11 in the CNS [10]. This chapter will provide an update of recent findings in these rarely reviewed AQPs, and further the field of AQPs in the nervous system, and in particular the potential pathophysiological role of AQP4 in the CNS.

5.2 AQPs in the Central Nervous System

5.2.1 Aquaporin Subfamily

5.2.1.1 AQP1

Expression of AQP1 in the CNS

AQP1 is primarily distributed at the apical membrane in epithelial cells of the choroid plexus where the transcellular water movement via AQP1 contributes 25% of CSF production as shown by study on AQP1 null mice [36]. AQP1 has also been found in small diameter sensory neurons in dorsal horn of the spinal cord and trigeminal and nodose ganglia, with a strong implication that AQP1 may be involved in pain signaling [37, 38]. Moreover, intensive AQP1 expression was also detected in neuronal filaments in the septum after juvenile traumatic brain injury [39]. In addition to these locations, AQP1 is also expressed in astrocytes in the white matter and the glia limitans, and neurons innervating the pial blood vessels in the non-human primates [40].

Besides these hereinbefore expression, AQP1 also distributes at specific sites in some brain disorders. For instance, AQP1 is localized in vascular structures of glioblastomas [41] and in microvascular endothelia and astrocytes of astrocytoma and metastatic carcinomas [14].

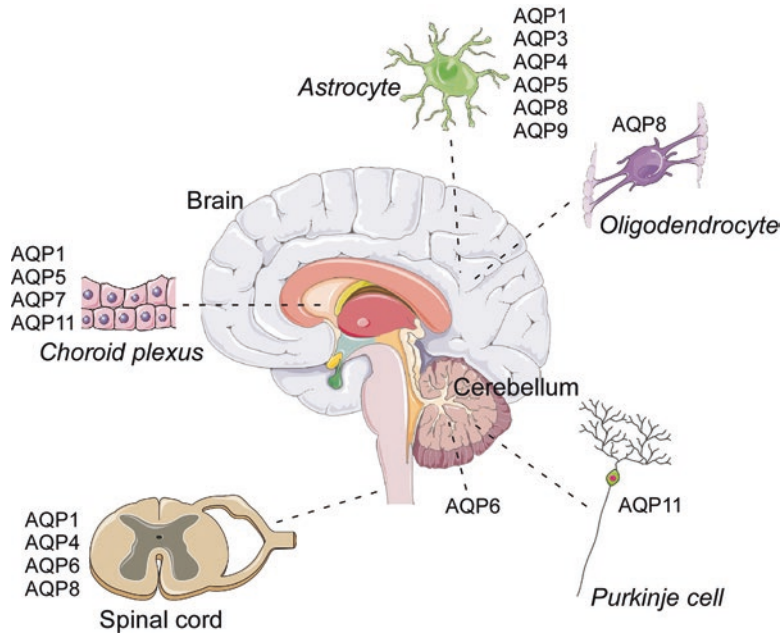

Fig. 5.1 Distribution of AQPs in the central nervous system. Astrocytes express AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9. AQP1, AQP5, AQP7 and AQP11 are expressed in the choroid plexus. AQP1, AQP4, AQP6 and AQP8 are found in the spinal cord. AQP6 is expressed in the cerebellum. AQP11 is found in the cerebellar Purkinje cell. Oligodendrocytes express AQP8

Moreover, in combination with NKCC1 (the Na-K-2Cl cotransporter 1), AQP1 was identified in meningioma cells and capillaries invading the dura [42]. These findings suggest that AQP1 may be involved in the tumor spread [43]. More recently, AQP1 was also detected in astrocytes in the temporal neocortex of patients with Parkinson's Disease (PD), indicating that astrocytes-involving water homeostasis may be disturbed along with the development in PD [44].

Role of AQP1 in Brain Disorders

It has been clearly established that AQP1 expression is up-regulated in brain astrocytomas [14, 45, 46] and positively correlated with the grade of malignancy, which is associated with angiogenesis and tumor invasion [7, 47]. In this case, the AQP1 polymorphisms could be used as a survival prognosticator in patients suffering from glioblastoma multiforme [48]. AQP1 is also upregulated in other brain disorders including choroid plexus tumors [49], spinal cord injury [50], and subependymomas [51]. Based on these findings, many researchers suggest that AQP1 inhibitors could be used as potential drugs in treatment of these brain diseases [7, 36, 52]. Interestingly, AQP1 could be inhibited by melatonin in rodents with spinal cord injury and agmatine in cerebral edema [50, 53], suggesting that melatonin and agmatine agonists could be used as such potent agents. Moreover, it seems that there is a possible link between AQP1 and neuropathic pain sensation, since pain responses were decreased in consistence with reduced AQP1 expression [38, 50].

It has been adequately reported that AQP1 plays important roles in tumor growth [7], cerebral edema [53, 54], angiogenesis [32], neoplastic invasiveness [55], and neurodegenerative disease like Alzheimer's disease (AD) [56] and PD [44]. The possible mechanism could be the induction of cell migration mediated by the water permeation of AQPs in 'Osmotic Engine Model' [57], or the water influx into the cells leading to an expansion of their lamellipodia [58].

5.2.1.2 AQP4

Expression of AQP4 in the CNS

AQP4 is the principal water channel in the CNS, primarily expressed in perivascular astrocyte foot processes, blood vessels, and subarachnoid space

throughout the brain structures [59, 60], such as the spinal cord [61], retina and optic nerve [62], periventricular organs [63], ependymal cells that line the lateral ventricles and cerebellum [17], hypothalamic magnocellular nuclei [64], dentate gyrus [65], and temporal neocortex [44]. The extensive distribution of AQP4 between the brain and various fluid compartments suggests its role in the brain water homeostasis [66]. Interestingly, the expression of AQP4 coincides the location of the potassium channel 4.1 (Kir4.1) [67].

AQP4 expression shows heterogeneous region-specific expression pattern with highest in the cerebellum [60]. AQP4 is also abundant in osmosensory areas, including the supraoptic nucleus and subfornical organ [18]. In the hippocampus, AQP4 expression exhibits laminar-specific pattern, with highest expression in the CA1 stratum lacunosummoleculare and the molecular layer of the dentate gyrus [65]. Activated astrocytes also increase AQP4 expression in the whole astrocyte elements, causing AQP4 depolarized from the vascular end feet to parenchymal process, which occurs in a variety of neurological pathological conditions [56, 68–70]. In addition, reactive astrogliosis occurs in the aging brain or after diffuse injury, such as microinfarction or mild traumatic brain injury, causing the mislocalization of AQP4 from the perivascular end feet to the rest of the astrocyte soma [71, 72].

An interesting finding is that AQP4 can form both homo and hetero tetramers, with the hetero tetramers formed by a longer AQP4-M1 isoform and a shorter AQP4-M23 isoform. The M23-containing tetramers could assemble into orthogonal arrays of particles (OAPs), acting as a critical component of blood-brain-barrier (BBB) [73–75]. Functionally, OAPs may serve to increase water permeability, enable the modulation of AQP4 membrane distribution, and be involved in the development and maturation of the BBB [76–78].

Function of AQP4 in the CNS

Phenotypic analysis of AQP4 knockout mouse model [31, 79–82] has shown that AQP4 facilitates a detrimental cellular water uptake as well as a protective clearance of extracellular fluid in cerebral edema following stroke [30], traumatic brain injury [83, 84], transient focal cerebral ischemia [85], spinal cord injury [86, 87], brain tumors [88], bacterial meningitis [89], and brain metabolic disturbances such as hyponatremia and water intoxication [30, 90]. In accordance with this dual role of AQP4, its overexpression in glial cells accelerates cytotoxic brain swelling in transgenic mice [91] (Fig. 5.2).

In addition to control water movements in the CNS, AQP4 null mice demonstrate other major roles for AQP4 in brain. AQP4 deficiency reduces neuroinflammation, in support of a deleterious role of AQP4 multiple sclerosis pathophysiology [92]. Similarly, AQP4 deletion is neuroprotective after severe global cerebral ischemia [93] and micro traumatic brain injury in mice [94]. However, absence of AQP4 shows more hyperactive microglial inflammatory responses, potentially increasing the severity of PD [44, 95]. Moreover, AQP4 knockout in mice produces several impairments in neuro-excitation phenomena including hearing, vision, olfaction, epilepsy, and cortical spreading depression [96]. Besides, AQP4 deficiency impairs synaptic plasticity and associative fear memory in the lateral amygdala [97], causes impairment of blood-retinal barrier [98], and increases capillary density in the brain [99].

AQP4 is known to be associated with astrocyte migration in glial scar formation [34, 100], and involved in facilitating gas diffusion [101] and cell adhesion between astrocytes [102, 103]. In terms of metal intoxication, AQP4 may act as either a neuro-protector or a mediator during the development of oxidative stress in the brain [104]. Furthermore, interactions of AQP4 and TRPV4 (transient receptor potential isoform 4) could function as an osmoregulatory complex in astrocytes [105].

AQP4 and CNS Water Balance

The high AQP4 polarization at blood-brain and blood-CSF interfaces is crucial for rapid transport of water into and out of the brain parenchyma [66, 106]. The AQP4 deletion causes a slightly increase in the baseline water content in

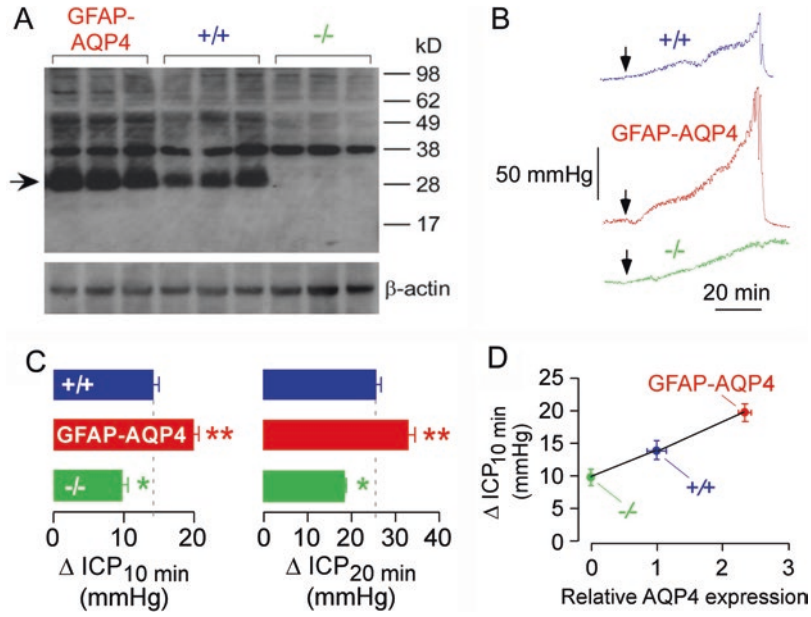

Fig. 5.2 Role of AQP4 in cytotoxic brain swelling. (**a**) Brain AQP4 protein expression detected by immunoblot analysis of whole brain homogenates from AQP4 overexpressing mice (GFAP-AQP4), wild type mice (+/+) or AQP4 knockout mice (−/−). (**b**) Representative intracranial pressure (ICP) curves for mice with indicated genotype in a water intoxication model of cytotoxic brain edema. (**c**) Summary of ICP curve analysis: △ICP at 10 and 20 min, (*, $p < 0.05$, **, $p < 0.01$ *vs.* +/+ mice). (**d**) △ICP at 10 min determined from ICP curve analysis plotted against AQP4 protein expression determined by immunoblot analysis (S.E.) (Adapted from Ref. [91])

the brain and spinal cord of adult mice [86, 87, 107–109], which further supports that AQP4 may facilitate water efflux from the brain parenchyma into the brain vessels, ventricles and subarachnoid space. AQP4 also facilitates the elimination of excess brain water following vasogenic edema [31, 110–112]. However, there is also evidence indicating that AQP4 is responsible for rapid water movement into the brain [113]. AQP4 null mice have reduced brain swelling and improved survival when compared with wild-type littermates following water intoxication, focal cerebral ischemia or controlled cortical impact brain injury [30, 84]. These studies together suggest that AQP4 is a bidirectional water channel that facilitates water transport into and out of the brain.

Apart from maintaining brain water balance under physiological and pathophysiological conditions, AQP4 is also involved in the establishment of brain water homeostasis during the development. Early studies reported that AQP4 expression coincides with the BBB differentiation in the cerebellum of postnatal rat [114, 115] and the optic tectum of embryonic chicken [116]. Subsequent studies revealed that increased AQP4 expression levels partially relate to decreased brain water content in postnatal mice [117]. Systemic or conditional AQP4 knockout mice show a significant delayed decrease in brain water content during the postnatal development, providing the direct evidence for a role of AQP4 in postnatal brain water uptake [117, 118].

AQP4 and Clearance of ISF Substances Including Aβ and Tau Proteins

An imbalance between the production and clearance of Aβ and Tau has been regarded as the central event in AD pathogenesis [119]. Data have accumulated to support that AQP4 is necessary for clearance of interstitial solutes, including Aβ and Tau proteins through the glymphatic system [71, 72, 120].

It is well known that the lymphatic system is responsible for tissue homeostasis clearance via clearance of excess fluid and interstitial solutes.

The lymphatic vessels are present throughout all parts of the peripheral tissues. The CNS has long been regarded as lack of lymphatic network because no conventional lymphatic vessels are found within brain parenchyma. However, this view has been challenged by recent studies that reveal the clearance of ISF with its constituent proteins and other solutes along the perivascular space [121–124]. On the basis of *in vivo* two-photonimaging of small fluorescent tracers, Iliff et al. reported that CSF tracers rapidly enter brain parenchyma along the cortical pial arteries, and then influx into the Virchow-Robin spaces along penetrating arterioles [120]. The tracers rapidly distribute into brain parenchyma and subsequently exit the CNS primarily along the central deep veins and lateral ventral caudal rhinal veins [120]. The ISF within the perivenous space further flows into dural lymphatic vessels, and eventually drains toward the deep cervical lymph nodes [124]. The perivascular pathways within brain parenchyma mainly include eperiarterial space, pericapillary space and perivenous space, all of which are surrounded by astrocyte vascular endfeet [125]. These astrocyte endfeet have 50 nm gaps between each other, creating the outer wall of the perivascular space and forming a donut-shaped tunnel surrounding the vasculature. These unique perivascular pathways, recently entitled the glymphatic system, not only provide efficient routes for rapid interchange of CSF and ISF, but also for clearance of soluble proteins and metabolites from the brain [126].

Particularly, Iliff et al. found that injected fluorescent or radiolabeled $A\beta_{1–40}$ in striaturn is rapidly cleared from the mouse brain along the glymphatic paravenous efflux pathway [120]. Moreover, AQP4 null mice exhibit slowed CSF influx through this system and a ~65% reduction in ISF clearance and a ~45% reduction in clearance of intrastrialtal injected radio-labeled $A\beta_{1–40}$ [120]. These data highly suggest that AQP4-dependent astroglial water fluxes couple the clearance of interstitial solutes, including soluble Aβ from the brain. Further studies have revealed that the paravascular clearance pathways are impaired in the aging brain [71]. Compared to young controls, old mice show dramatic decreases in the efficiency of exchange between subarachnoid CSF and brain parenchyma and clearance of intraparenchymally injected Aβ. Apart from aging brain, impairment of glymphatic pathway function has been observed in traumatic brain injury, ischemic stroke and AD mouse models [72, 127].

AQP4 and Spatial Buffering of Extracellular Potassium

Astrocytes mediated potassium (K^+) homeostasis is of critical importance for the regulation of neuronal excitability. Synaptic activity causes release of K^+ into the extracellular space (ECS). The ECS K^+ is efficiently taken up by astrocytes through the inward rectifier potassium channel Kir4.1, then redistributed through the astroglial syncytium via gap junctions, thereby stabilizing neuronal activity [128]. The early study reported that AQP4 is co-localized with Kir4.1 in the end feet of retinal Müller cells, indicating their functional interaction [67, 129]. By contrast, the subsequent studies on AQP4 null mice provide evidence against functional interaction between AQP4 and Kir4.1 in retinal Müller cells [130]. However, deletion of AQP4 in mice does impair extracellular K^+ clearance, which subsequently affects neuro-excitation with reduced seizure threshold and increased seizure duration [131–133]. These results support that AQP4 contributes to K^+ clearance, although the underling mechanism remains unclear.

Neuronal activity is associated with a shrinkage of the ECS around the active synapses [134, 135], which may be dependent on AQP4-mediated rapid water movement. AQP4 facilitates water entry into astrocyte processes surrounding the synapse, transports water through the astroglial network, and releases distantly into the ECS surrounding micro-vessels, thus subsequently produces a local shrinkage of ECS during the synaptic activity. Certainly, the AQP4-mediated rapid transport of intercellular water would drive reuptake of the ECS solutes including K^+ by astrocytes, because water serves as a transport medium for these substances.

AQP4 and Calcium Signal Transduction

Calcium (Ca^{2+}) signalling serves as a mediator of bidirectional interactions between neurons and astrocytes. Impaired Ca^{2+} signalling plays a critical role in the progression of brain edema [136]. Recent evidence suggests an involvement of AQP4 in astrocyte Ca^{2+} signalling. Deletion of AQP4 reduces hypo-osmotic stress-evoked Ca^{2+} signalling in astrocytes [137]. Subsequent functional studies revealed that AQP4 and TRPV4, a polymodal nonselective cation channel, synergistically regulate cell volume and Ca^{2+} homeostasis [138]. Coimmunoprecipitation and immunohistochemistry further demonstrated that AQP4 and TRPV4 co-localize within astrocytes and retinal Müller glia [138, 139]. Functional analysis of an astrocyte-derived cell expressing TRPV4 but not AQP4 shows that cell-volume control and intracellular Ca^{2+} response can be reconstituted by transfection with AQP4 but not with AQP1 [139]. These data indicate that a TRPV4/AQP4 complex that constitutes a molecular system that finely regulates astroglial volume via integrating Ca^{2+} signalling and water transport, and might exacerbate the pathological outcome when an edema develops.

AQP4 and Regulation of Neurotransmission

Glutamate is the most prominent excitatory neurotransmitter in the CNS. Astrocytes absorb extracellular glutamate via excitatory amino acid receptors [140]. Glutamate uptake is also accompanied by water transport, which causes astrocyte processes to swell around the synapses, subsequently reducing the extracellular synaptic space during synaptic transmission and processing [141]. To restore ECS volume, astrocytes rapidly transport water into the surrounding capillary via AQP4 located in the perivascular end feet. Previous studies demonstrate that the AQP4 deletion downregulates glutamate transporter 1 expression in astrocytes and impairs their ability of glutamate uptake [97, 142–144]. Previous studies also suggest that AQP4 is involved in the metabolism of dopamine, serotonin, and other neurotransmitters [145, 146].

AQP4 and Synaptic Plasticity

There is growing evidence that astrocytes play a role in long-term potentiation (LTP) and long-term depression (LTD) [147–149], which could be regulated by AQP4 [150, 151]. Experiments using mice with a deletion of the astrocyte-specific channel AQP4 on hippocampal synaptic plasticity and spatial memory function has been investigated by Skucas et al. [152]. The mechanism appears to be related to neurotrophins, and especially brain-derived neurotrophic factor (BDNF), because pharmacological blockade of neurotrophin Trk receptors or scavenging BDNF restores synaptic plasticity [152]. However, the underlying mechanism for AQP4 modulating synaptic plasticity still needs more research.

AQP4 and Adult Neurogenesis

A previous study demonstrated that in corticosterone-treated model, AQP4 deficiency aggravated decreased proliferation and survival of new-born cells in the dentate gyrus [153]. Recent studies suggest that the development of depression-like behaviour in corticosterone-treated models is paralleled by hippocampal neurogenesis, and adult hippocampal neurogenesis buffers stress responses and depressive behaviours [154, 155]. Thus, the aggravated neurogenesis inhibition in the hippocampus could also contribute to the exacerbated depressive behaviours in AQP4 null mice. This is consistent with the previous *in vitro* studies demonstrating that deletion of AQP4 impairs proliferation, migration and neuronal differentiation of adult neural stem cells (ANSCs) [156].

The lack of AQP4 could change the intrinsic property of ANSCs and enhance the injurious effects of corticosterone to ANSCs [157]. It has been revealed that AQP4 is essential for the initiation of intracellular Ca^{2+} event, including Ca^{2+} spikes and Ca^{2+} oscillation [156], AQP4 deficiency results in abnormal expressions of Ca^{2+} handling proteins in skeletal muscle cells and cardiac muscle cells [158, 159], and it has been suggested that AQP4 modulates the effects of corticosterone on ANSCs by regulating Ca^{2+} signaling [157]. However, the exact mechanisms still are not fully explored yet.

Role of AQP4 in Brain Disorders

It has been observed that AQP4 is clearly upregulated in several pathological conditions including brain tumors [160, 161], cerebral ischemia [162, 163], traumatic brain injury [39, 164, 165] and neuro-inflammation [166]. In general terms, the upregulation of AQP4 in astrocytes is associated with edema resolution [1, 39, 166, 167]. Most cases, the increase and redistribution of the AQP4 is detected near the lesion site [39, 160, 162]. Indeed, decrease of AQP4 was also detected in some pathological conditions like AD [168–170] and epilepsy [171], the regulation of AQP4 expression might be the changes in rodent strains, injury type, and age at impact [39].

AQP4 and Cerebral Edema

The role of AQP4 in cerebral edema has been extensively established by using AQP4 knockout models [59, 66, 79]. Considering the timeline of the newly observed AQP4 changes in ischemia, some researchers propose that cerebral edema should be divided into three major types: anoxic, ionic, and vasogenic edema, to replace the traditionally two category: cytotoxic and vasogenic edema [1, 172]. The initial anoxic edema, currently used, is characterized as the induces of ions into cells, accompanied by water entry and astrocyte swelling, while ionic edema occurs due to further alternations of the endothelial cell's trans-capillary flux of sodium ion [173]. The development of ionic brain edema is associated with upregulation of AQP4 [1, 162, 163]. The final step termed as vasogenic edema, occurs with the disruption of the tight junction between the cerebrovascular endothelial cells, which comprise the BBB. At this time, a second increase of AQP4 expression is observed [162, 172], the presence of AQP4 is to facilitate clearance of excess fluid in vasogenic brain edema [31] (Fig. 5.3).

The dynamic spatial distribution of AQP4 at the astrocyte membrane is one of the two major modulation following injury. AQP4 becomes more uniformly distributed on the astrocyte plasmalemma, termed as "dysregulation", which seems occur in parallel with cytotoxic edema to counteract early edema formation [39, 174, 175]. Interestingly, the ratio of AQP4-M1 and AQP4-M23 is increased in the ischemic hemisphere [163], the physiological role of this change remains unclear. Dysregulation of AQP4 may be produced via the reduction of the perivascular laminin, agrin and ß-dystroglycan, which facilitate AQP4 to diffuse freely throughout the astrocyte membrane [175, 176]. The true function of AQP4 dysregulation remains largely unknown [176].

The activity of ion transporters or channels that induce AQP4-mediated cytotoxic and ionic edema is the other major modulation following injury [176]. Besides, AQP4 probably integrates with other astrocyte proteins like connexin-43 (Cx43) and the potassium channel Kir4.1 to eliminate the excess fluid [1]. SiRNA to silence the AQP4 expression, used as a potential drug to

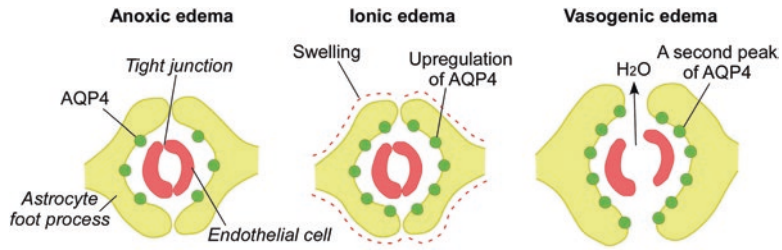

Fig. 5.3 Schematic drawing of AQP4 in 3 different edema phases: anoxic, ionic, and vasogenic edema. Anoxic edema is characterized as a swelling of the astrocytes caused by a disruption of the cellular ionic gradients and the entry of ions followed by water entry and leading to cellular swelling. During the ionic edema, astrocytes become swollen, AQP4 is upregulated. Vasogenic edema is a result of disruption of the tight junctions between the endothelial cells, leading to increased expression of AQP4 and permeability of the cerebral blood-vessels, further contributing to swelling of astrocytes (Adapted from Refs. [8, 43])

block AQP4, contributes to reduction of the edema formation after posttraumatic brain injury [177, 178]. It seems that there is no convective solute flow in the pathology of acute brain edema, as proposed in 'glymphatic' system [179].

AQP4 and Neuromyelitis Optica

AQP4 specific antibodies have been identified as the therapeutic target for neuromyelitis optica (NMO), an autoimmune inflammatory disease of CNS that develops to paralysis and loss of vision [33, 180–182]. The binding of AQP4-IgG to AQP4 on astrocyte end-feet is involved in activation of the complement cascade, a classical inflammatory response that occurs with pronounced granulocyte and macrophage infiltration, followed by oligodendrocyte damage, demyelination and even neuron death [182] (Fig. 5.4). To date, this complement-dependent cytotoxicity may be the most accepted hypothesis for NMO pathogenesis [183]. AQP4 IgG generally has greater binding affinity to OAPs than individual AQP tetramers [184–186], the structural changes in the AQP4 epitope upon array assembly greatly increases complement activation [186]. However, AQP4 water permeability and the size of OAPs are not altered by binding to NMO-IgG [187].

A novel NMO therapeutics that target AQP4, involves using aquaporumab, a monoclonal antibody that blocks the binding of AQP4-IgG to AQP4 and lacks cytotoxic effector functions [188]. Another approach to block the binding of AQP4-IgG and AQP4 involves a small-molecule blocker strategy [189]. AQP-IgG-targeted enzymatic therapeutics involves bacteria-derived endoglycosidase S (EndoS) and the enzyme IdeS, which neutralizes NMO-IgG pathogenicity [190, 191]. Other potential therapeutic strategies for NMO include reducing the entry of AQP4-IgG into the CNS or the expression of AQP4 on astrocytes, as well as preventing the formation of OAPs, or upregulating complement inhibitor proteins such as CD59 [6].

Recently, AQP4 specific antibody was applied for the diagnosis of NMO by using AQP4 extracellular loop-based carbon nanotube biosensor [192]. Since AQP4-targeted therapies are quite

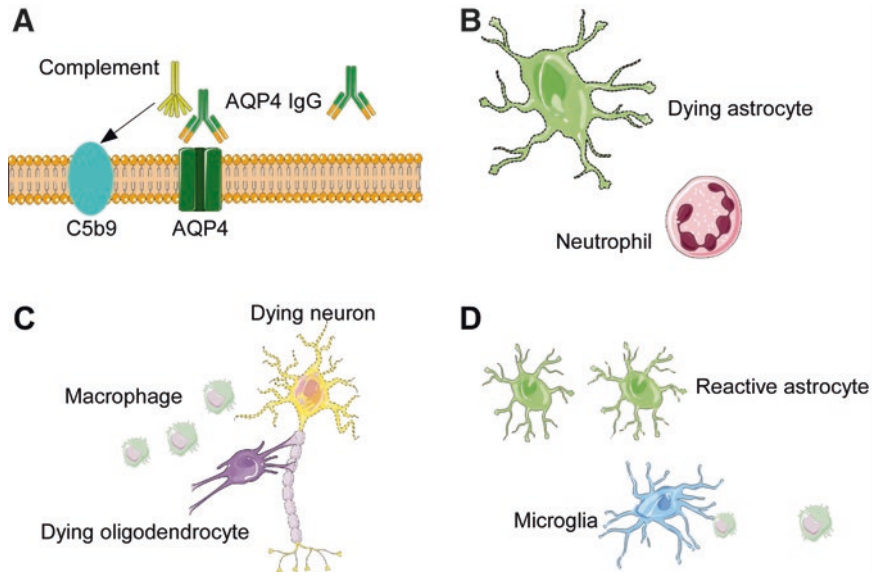

Fig. 5.4 AQP4 and the pathogenesis of neuromyelitis optica. (**a**) AQP4 IgG binds to AQP4 on astrocyte foot processes. Complement is activated via the classical pathway with deposition of C5bC9 complexes in astrocyte cell plasma membranes. (**b**) Activated complement components attract peripheral neutrophils into the lesion, which causing astrocyte death. (**c**) Dying astrocytes attract macrophages, causing death of oligodendrocytes and neurons. (**d**) Microglia enter the lesion as well as reactive astrocytes. The lesion core is necrotic with a macrophage infiltrate

selective, new drugs (like aquaporumab, sivelestat, and eculizumab) entered into clinical trials need to be proved effective for NMO [183]. Moreover, there exist many important unsolved questions about the relationship of AQP4-lgG and NMO. For instance, the role of AQP4-lgG in the classification of NMO remains uncertain [193]. Furthermore, it is largely unknown about the reason why peripheral AQP4-expressing organs cannot be damaged by AQP4-IgG. Further studies in patients worldwide could help to identify more genetic susceptibility factors for NMO [194].

AQP4 and Brain Tumor

AQP4 is expressed in astrocytoma cells and around the tumor [161]. And its expression is upregulated in astrocytoma and glioblastoma [12, 161]. A role for AQP4 in cell migration and cell-cell adhesion suggest its involvement in promoting glioblastoma cell migration, glioma invasion, and glioblastoma cell apoptosis [34, 100, 102, 195–197].

The possible mechanism is that AQP4 induces the cell morphological changes via polarizing to the cell lamellipodia and inducing an increased number or size of lamellipodia in migrating cells [195–197]. Structure of AQP4 (including OAPs) suggests its role in channel-mediated cell adhesion [102]. However, absence of such abnormalities in AQP4 knockout mice raises the argument about whether AQP4 plays a role in cell-cell adhesion [79]. Once, data against involvement of AQP4 in cell adhesion were demonstrated [198]. However, recent experiments display that the larger AQP4-M23 rich OAPs could bind with adhesion complexes, suggesting a role for AQP4-M23 in cell adhesion [73]. So it could be speculated that whether AQP4 plays a role in cell adhesion was determined by the involvement of OAPs.

AQP4 and AD

AD is the most common neurodegenerative disease among the elderly and characterized by Aβ plaque deposition, neurofibrillary tangles, and neuronal and synapse loss in learning and memory related regions [199]. As mentioned earlier, activated astrocytes accompanied with altered polarization of AQP4 occur in the brain tissues of patients with AD and several AD models [56, 68, 69], indicating an involvement in AD pathology. A recent study reported that the AQP4 gene deletion in APP/PS1 transgenic AD model mice impairs exogenous Aβ clearance from brain parenchyma and exacerbates spatial learning and memory defects associated with more severe Aβ plaque deposits and synaptic protein loss [200]. This finding provides the direct evidence for a key role of AQP4 in the pathogenesis of AD. Actually, accumulatively direct and indirect evidences have indicated that AQP4 affect the onset and progress of AD via various mechanisms, such as Aβ clearance, glutamate transduction, synapse plasticity, Ca^{2+} signal transduction, neuroinflammation and neurotrophic factor secretion [71, 72, 120, 127, 152, 200–203]. For example, reactive gliosis with loss of perivascular AQP4 polarization impairs the glymphatic pathway function, causing reduction in CSF-ISF exchange and Aβ deposition in cortical and leptomeningeal vessels [119]. Thus, these clues support that AQP4 may serve as a hopeful target for prevention and treatment against AD.

Modulators of AQP4 in the CNS

It has been proposed that AQP4 modulators have potential utility in the treatment of AQP4 related brain diseases [204, 205]. AQP4 inhibitors such as vasopressin, melatonin, PKC, mercury (Hg^{+}), trombin, dopamine, hypoxia, tetraethylammonium (TEA), bumetanide, acetazolamide (AZA), siAQP4, curcumin, and H_2S may be regarded as potential therapeutic drugs for cytotoxic brain swelling, seizure, glial scar [178, 205–210]; while the AQP4 enhancers including glutamate, syntrophin, dystrophin, connexin 43 (Cx43), K^{+} (Na^{+}, K^{+}-ATPase; NKCC1), Kir4.1, lead (Pb^{2+}), cyclic AMP, and lactic acid have therapeutic potentials in reducing vasogenic brain swelling [131, 205, 211, 212]. AQP4 modulators could offer new therapeutic options for many brain disorders like preventing tumor malignancy in glioblastoma [10]. Notably, many of the AQP4 modulators have been experimentally examined

in isolation. However, these factors are likely to interact after injury [176].

5.2.1.3 AQP5

AQP5 expression in the CNS is similar to that described for AQP3, AQP4 and AQP8, mainly expressed in the astrocytes and neurons of choroid plexus, piriform cortex, hippocampus, and dorsal thalamus [213, 214], and could expand to the nucleus caudatus putamen and globus pallidus in rat ischemic hemisphere [16, 215]. Whether AQP5 facilitates the highest water transport in the body remains uncertain [216, 217].

AQP5 might be an important water channel in astrocytes that is differentially expressed during various brain injuries [215]. AQP5 expression in brain is upregulated both after permanent focal cerebral ischemia [16] and following preterm intraventricular hemorrhage [218]. Upregulation of AQP5 after scratch injury is polarized to the astrocyte processes and cytoplasmic membrane in the leading edge of the scratch-wound, and facilitated astrocyte process elongation [215]. AQP5 expression is also detected near the ischemia-induced infarct border in the rat brain, and AQP5 level could be regulated by hypoxia [219] and protein kinase A (PKA) [19]. Moreover, recent research has also demonstrated that AQP5 expression is associated with the development and intensity of peritumoral edema in meningioma patients [220].

5.2.1.4 AQP6

AQP6 mRNA has been observed in neonatal and adult mouse cerebellum by using reverse transcription PCR [20]. AQP6 gene was found in mouse hind brain (involving cerebellum) and spinal cord [21], while AQP6 protein was detected at synaptic vesicles in rat brains [221]. The role of AQP6 in the CNS remains unknown. Since AQP6 mRNA expression is regulated in a tissue-specific and age-related way, it is likely that AQP6 plays a role in mouse development [20]. In addition, the location of AQP6 in synaptic vesicles might participate in their swelling and secretion [221]. Thus further investigation is needed to understand the function of AQP6 in the CNS.

5.2.1.5 AQP8

AQP8 was early detected in astrocytes, neurons, and oligodendrocytes [219], and in ependymal cells lining the central canal in spinal cords [61]. Recent study showed that AQP8 was primarily expressed in the cytoplasm of astrocytoma cells in piriform cortex, hippocampus, and dorsal thalamus; weakly in ependyma and choroid plexus [25, 213].

AQP8 may play an important role in the development of brain disorders (edema and tumor), and can be used as a potential therapeutic drug for astrocytoma and glioma. For instance, AQP8 expression level is upregulated both along with the severity grade of astrocytoma [25] and gliomas [222]. Furthermore, down-regulation of AQP8 in human glioma cells shows a significant inhibitory effect on cell proliferation and migration [222]. In addition, AQP8 expression is upregulated after brain ischemia, suggesting that AQP8 contributes to the early formation of edema [16]. Even though AQP8 null mice show surprisingly mild phenotype [223], its role in the CNS seems to be pivotal.

5.2.2 Aquaglyceroporin Subfamily

5.2.2.1 AQP3

AQP3, permeable to glycerol and urea, was first found in brain meningeal cells in the CNS [224]. Studies show that similar to AQP5 and AQP8, AQP3 is expressed in astrocytes and neurons of piriform cortex, hippocampus, and dorsal thalamus [213, 219]. However, no expression of AQP3 is found in pig brain [23]. It seems that the distribution of AQP3 in the CNS shows a species-specific model. The role of AQP3 in the CNS remains scarcely investigated, only one research demonstrated that AQP3 expression is upregulated within the first 6 h after ischemia, suggesting a role of AQP3 in the early formation of the cerebral edema and the neuronal swelling [16].

5.2.2.2 AQP7

By using Northern blot analysis, a weak band of AQP7 was first detected in rat brain [225]. Function as a glycerol channel mainly in fat

metabolism, AQP7 is largely localized in the choroid plexus in brain of mice [22, 226]. Similarly, AQP7 mRNA is also detected in pig brain [23]. Recent study shows that AQP7 expression is found to be restricted to the apical membrane of choroid plexus epithelial cells (CPECs) and endothelial progenitor cells (EPCs), in parallel with previous study [22], suggesting that AQP7 could be involved in CSF secretion [24].

5.2.2.3 AQP9

Expression and Function of AQP9 in the CNS

AQP9, a channel permeable to water, glycerol, urea, and monocarboxylates, has been evidenced in rodent and primate brains [40]. AQP9 is present in the ependymal cells lining the ventricles and the tanycytes of hypothalamus [26], astrocytes, endothelial cells of pial vessels, catecholaminergic neurons [27, 227]. The intracellular distribution of AQP9 is in mitochondrial inner membranes of brain cells [228]. It has also been reported that AQP9 is expressed in malignant astrocytic cells and leukocytes, which infiltrate the tumor in glioblastoma [229]. AQP9 knockout mice do not show severe abnormalities [230]. However, silencing of AQP9 in astrocyte cultures contributed to decreased glycerol uptake and increased glucose uptake and oxidative metabolism [231]. In addition, AQP9 expression was decreased under hypoxia and recovered with reoxygenation [219]. It has been suggested that signal transduction via PKA pathway may regulate the expression of AQP9 by some factors induced by dbcAMP [19].

Role of AQP9 in Brain Disorders

AQP9 permeability to various molecules suggests a role in energy metabolism in addition to water homeostasis [78]. AQP9 plays a role in normal cell metabolism, under physiological conditions, and also increases cell stress tolerance, under pathological conditions [232]. For instance, AQP9 expression is upregulated by decreased insulin concentration in diabetic rats [233], after transient focal ceberal edema [27], in astrocytic tumors [234], after permanent middle cerebral artery occlusion [235], and with hirudin treatment after intracerebral hemorrhage [236]. All these findings would suggest that AQP9 is involved in astrocyte energy metabolism and the malignant progression of astrocytic tumors. Changes in AQP9 expression may be the consequence of glial cell attempt to response to hypoxic and ischemic conditions via facilitating clearance of glycerol and lactate [10].

5.2.3 Superaquaporin Subfamily

5.2.3.1 AQP 11

AQP11 is found to be expressed in the CNS in rats [237] and mice [238], appearing in hippocampal and cerebral cortical neurons, purkinje cell dendrites in rat brains [28], and epithelium of the choroid plexus and endothelium of the brain capillary in mice brains [29, 239]. The brain of AQP11 null mice appears normal, without any morphological and functional abnormalities [29, 240]. However, AQP4 expression at the BBB is reduced by half in AQP11 null mice, suggesting AQP11 may functionally interact with AQP4 [29]. It has been proposed that, when osmotically challenged, AQP11 may reduce its expression to protect the brain [239]. Since AQP11 has a unique high affinity mercury ion binding site (tricysteine motif site), AQP11 distributed in Purkinje cells may interact with the cations like mercury in autism, and be the therapeutic target for this cognition-related disorders [241]. Future investigations are necessary to elucidate the physiological role of AQP11 in the CNS.

5.3 AQPs in the Peripheral Nervous System

In the peripheral nervous system (PNS), three AQPs (AQP1, AQP2 and AQP4) are localized to neurons or glial cells in the ganglia and visceral plexuses [242].

5.3.1 AQP1

AQP1 is mainly localized to the cytoplasm and cell membrane of some medium and small-sized trigeminal or dorsal root ganglion (DRG) neurons [37, 243]. The expression pattern of AQP1 in the primary afferent sensory neurons suggests involvement in the specific somatosensory transduction including pain signal transduction [38, 244, 245]. Furthermore, a recent study suggests that AQP1 is mediated in DRG axonal growth and regeneration [246]. Additionally, AQP1 has been found in peripheral trigeminal axons and spinal nerve axons of humans and mice [247]. Interestingly, difference in the cellular localization of AQP1 in the central trigeminal root between humans and mice was detected. AQP1 is specifically expressed in astrocytes of humans, but is restricted to nerve fibres within the central trigeminal root and spinal trigeminal tract and nucleus in mice [247]. In the visceral plexuses, strong AQP1 expression is localized to satellite cells rather than neurons of humans [248]. In contrast, the localization of AQP1 protein in a particular neuronal subtype has been observed in the enteric nervous system of rats [249, 250]. Together, these morphological evidences have revealed a species difference of AQP1 expression in the PNS, but the underlying mechanisms remain to be determined.

5.3.2 AQP2

An early study reported AQP2 expression in rat trigeminal ganglion neurons, with strong labelling in the medium- and large-sized types and weak labelling in the small-size type. After formalin treatment, there was a marked increase of AQP2 expression in small-sized neurons and a decrease in medium- and large-sized neurons [251]. Another study shows that AQP2 expression is not detectable in the DRG of normal rats, but remarkable increase in small-sized DRG neurons in response to chronic constriction injury treatment. These data suggest that AQP2 is involved in pain transmission in the PNS [252]. The cellular localization of AQP2 in the human PNS has not been studied yet.

5.3.3 AQP4

Compared to extensive studies of AQP4 in the normal CNS and neuropsychological diseases, little is known about its expression and function in the PNS. A study by Thi et al. (2008) identified AQP4 protein expression in the myenteric and submucosal nerve plexuses of mice and rats [253]. There are about 12% myenteric neurons positive for AQP4 in the myenteric plexus, while nearly 80% neurons are positive for AQP4 in the submucosal plexus of colon. Glial cells in the rat and mouse enteric plexuses are immunonegative to AQP4. Recently, Kato and colleagues reported that AQP4 is exclusively localized to satellite glial cells surrounding the cell bodies of the primary afferent sensory neurons in the trigeminal ganglia and DRG of mice [254]. Jiang and colleagues reported that there are different patterns of AQP4 expression in the enteric nervous system of human, guinea pig, rat and mouse colon mucosa. In rat and mouse, AQP4 is expressed at a small subpopulation of neurons, while in the guinea pig and human, AQP4 is localized to enteric glial cells [255]. The cellular localization and function of AQP4 in the PNS including in the trigeminal and dorsal root ganglia need further study.

5.4 Future Directions

AQPs are involved in a variety of important physiological processes in the CNS, by coordinating water and solutes trafficking among the different fluid compartments [10]. Specific up-regulation of some AQPs' expression along with their involvement in brain edema formation, has been consistently investigated by many scientists. It has been strongly suggested that AQPs could represent important targets in treatment of brain disorders [6]. However, we are still far from having a full comprehension of the physiological and pathological significance of all AQPs in the CNS

[256] and no specific therapeutic agents have been designed to inhibit and enhance water transport through AQPs [1]. To date, the major challenge is still to facilitate drug delivery across the BBB [257]. Considering the importance of AQPs in brain disorders, it will be of great achievement to find out novel drugs capable to cross the BBB and to suppress AQP up-regulation [10].

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Aquaporins in Cardiovascular System

6

Lu Tie, Di Wang, Yundi Shi, and Xuejun Li

Abstract

Recent studies have shown that some aquaporins (AQPs), including AQP1, AQP4, AQP7 and AQP9, are expressed in endothelial cells, vascular smooth muscle cells and heart of cardiovascular system. These AQPs are involved in the cardiovascular function and in pathological process of related diseases, such as cerebral ischemia, congestion heart failure, hypertension and angiogenesis. Therefore, it is important to understand the accurate association between AQPs and cardiovascular system, which may provide novel approaches to prevent and treat related diseases. Here we will discuss the expression and physiological function of AQPs in cardiovascular system and summarize recent researches on AQPs related cardiovascular diseases.

Keywords

Aquaporin • Cerebral ischemia • Congestion heart failure • Hypertension • Angiogenesis

Aquaporins (AQPs), a family of membrane protein, mediate permeability of water and some small molecules across cell membrane driven by osmotic or concentration gradient. AQPs mainly distribute in epithelial cells and endothelial cells, maintaining water balance in the body. Since the first AQP was identified in red blood cells in 1991, 13 AQPs (AQP0–12) have been identified [1–3]. Functions of AQPs have been gradually revealed, such as the regulation of urine concentration and gland secretions. In recent years, it has been proved that AQPs are also involved in cerebral ischemia, cancer, glaucoma, obesity and

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Table 6.1 AQPs expression in blood vessel of different tissues

	Eye	Brain	Secretory glands	Lung	Liver	Pancreas	Kidney	Skeletal muscle
AQP1	+	–	+	+	+	+	+	+
AQP4	–	–	–	–	–	–	–	–
AQP7	–	–	–	–	–	–	–	+
AQP9	–	+	–	–	–	–	–	–

Table includes integrating data from rats, mice and human experiments [5, 27, 62–65]; +: with AQPs expression; –: without AQPs expression

infection by using gene knockout mice [2]. Studies have shown that AQPs are also involved in the regulation of cardiovascular function and development of related diseases, especially in cerebral ischemia, congestion heart failure, hypertension and angiogenesis. Therefore, further studies are needed to elucidate mechanism accounting for the association between AQPs and cardiovascular diseases, which may lead to novel approaches to the prevention and treatment of those diseases.

6.1 Expression and Physiological Function of AQPs in Cardiovascular System

AQP1, AQP4, AQP7 and AQP9 have been found in cardiovascular system (Table 6.1). They distribute in the heart, endothelial cells and vascular smooth muscle cells [4], participate in water-transportation, glycerol and lactic acid, which play an important role in vascular physiological function. AQPs function may be related to pathological process of vascular diseases.

6.1.1 AQP1

AQP1 is highly expressed in microvascular (capillaries and small veins) endothelial cells (ECs), and also exists in vascular smooth muscle cells (SMCs) and non-vascular endothelia, such as corneal endothelial cells, pulmonary and bronchial endothelial cells, etc., whereas AQP1 has not been found in ECs in the central nervous system [5]. AQP1 is present in cardiomyocytes of rat, but it is not expressed in mouse cardiomyocytes [6, 7]. AQP1 facilitates trans-endothelial water movement in osmotically driven membrane processes. Recently, increasing evidence suggests that AQP1 could also mediate transport of many small molecules, such as urea, NH_3, H_2O_2, NO, CO_2, $Sb(OH)_3$, $As(OH)_3$ [3, 8–10]. In endothelial cells, AQP1 might be involved in the regulation of nitric oxide (NO) entering endothelial cells to regulate vascular tone and blood pressure through controlling NO level, bioavailability and diffusion distance [4, 11]. AQP1 expression is affected by some vascular related diseases. AQP1 is up-regulated in the fibrotic septa of cirrhotic liver and promotes angiogenesis by enhancing endothelial invasion/proliferation [12]. AQP1 knockout attenuated the angiogenesis, fibrosis and portal hypertension that follows bile duct ligation in mice [13]. In retinal vascular endothelial cells, AQP1 has been shown to be involved in hypoxia-inducible angiogenesis through a vascular endothelial growth factor (VEGF) signaling pathway independent manner [14]. However, in oxygen-induced retinopathy microvessel proliferation was not affected in AQP1 knockout neonatal mice [15]. Moreover, AQP1 is highly expressed in microvascular endothelial cells in malignant tumor. Inhibited tumor growth and reduced vascularity with extensive necrosis was found in AQP1 knockout mice after subcutaneous or intracranial tumor implantation [16]. Our previous study demonstrated that a carbonic anhydrase inhibitor acetazolamide could inhibit AQP1 protein expression and angiogenesis in tumor tissues (Fig. 6.1) [17]. AQP1 DNA immunization based on ubiquitin–proteasome system could directly damage melanoma tumor vasculature and suppress the growth of tumor in mice [18]. Therefore, targeting to regulate AQP1 expression in vascular endothelial cells may play a positive role in tumor angiogenesis and treatment.

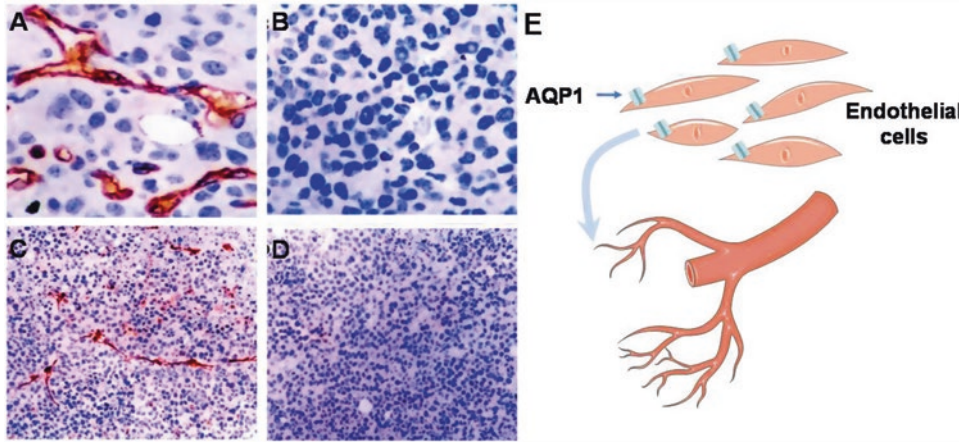

Fig. 6.1 AQP1 and angiogenesis. Expression of AQP1 in capillaries (**a**, **b**) and postcapillary venules endothelial cells (**c**, **d**) of primary tumor; (**a**) and (**c**) untreated group, (**b**) and (**d**) treated with acetazolamide. (**e**) AQP1 in endothelial cells could assist cell migration and promote angiogenesis

6.1.2 AQP4

AQP4 is expressed predominantly in the central nervous system. It is highly expressed in the brain, spinal cord and optic nerve [19–21]. AQP4 mainly exists in astrocytes surrounding cerebral capillaries, and is distributed in the astrocytic foot processes, external glial limiting membrane, ependyma, and subependymal internal glial. Most scientists do not think that AQP4 is expressed in cerebrovascular endothelial cells. However, Amiry-Moghaddam and colleagues (2004) demonstrated that AQP4 is expressed in brain endothelial cells by using immunogold electron microscopy, at lower levels than in astrocytes [22]. A selective knockout of the AQP4 in the astrocytic foot processes delayed cerebral edema, despite the presence of a normal complement of endothelial AQP4. But whether the endothelial AQP4 is involved in maintaining water balance in the brain is still elusive.

AQP4 is an important part of blood brain barrier and blood-cerebrospinal fluid (CSF) barrier. Its major role in the central nervous system is maintaining brain water balance [23]. In fact, the highly polarized AQP4 expression (in glial membranes that are in direct contact with capillaries and pia) indicates that AQP4 mediates the flow of water between glial cells and the cavities filled with CSF and the intravascular space. Another role of AQP4 is to promote astrocyte migration and neural signal transduction. It has been proved that the astrocyte migration is impaired in AQP4 deficient mice, and AQP4 deficiency impairs the migration of cultured astrocytes [24]. In the heart, AQP4 has been found in the cardiomyocytes of mice, not in rats [7].

6.1.3 AQP7

AQP7, an aquaglyceroporin, mainly distributes in renal proximal tubules, testis, cardiac and striated muscle and adipose tissue. A microarray study showed that heart was the second biggest expression tissue of AQP7 mRNA after adipose tissue [25], but studies on the cardiac role of AQP7 are limited. In 2009, Hibuse and colleagues demonstrated that AQP7 knockout mice have lower cardiac glycerol and ATP content than those of wild-type mice [26]. Under basal conditions, AQP7 knockout mice had normal cardiac histology and morphology; when injections of isoproterenol or subjected to transverse aortic constriction (TAC), AQP7 knockout mice developed advanced hypertrophy and lower survival than wild-type mice, indicating the importance of glycerol as a cardiac energy substrate [26]. In addition, AQP7 was expressed in capillary endothelial cells of adipose tissue, but its functions

remain to be fully elucidated [27]. Therefore, it is necessary to clarify the physiological and pathological significance of cardiac and endothelial AQP7 in the future.

6.1.4 AQP9

AQP9 is also an aquaglyceroporin and has permeability to water, monocarboxylate, glycerol, urea and other small neutral solutes. AQP9 has two isoforms: a short isoform located on the inner membrane of mitochondria, and a long isoform located within the cell membrane [28, 29]. AQP9 has been found in brain, liver, spleen, epididymis and testis. AQP9 was observed in the endothelial cells of pial vessels [30]. AQP9, similar to AQP4, has also been suggested to contribute to extracellular water homeostasis and edema formation [31]. Moreover, AQP9 might participate in brain energy metabolism. It is also expressed in neuronal mitochondria and glucose sensitive neurons, and its expression could be negatively regulated by insulin [30]. AQP9 is involved in the transport of lactate and ketone bodies across the blood-brain barrier. It has been suggested that AQP9 may participate in the clearance of excess lactate and other metabolites during cerebral ischemia [32].

6.2 AQPs and Cardiovascular Disease

6.2.1 AQPs and Cerebral Ischemia

In ischemic stroke, a key aggravating factor is the presence of edema. Stroke is a complex and devastating neurological condition with limited treatment options. Brain edema is a serious complication of stroke. Edema is a therapeutic target in cerebral ischemia. Early edema formation can significantly contribute to infarct formation and thus represents a promising target. Seven AQP subtypes, including AQP1, AQP3, AQP4, AQP5, AQP8, AQP9 and AQP12, have currently been identified in the brain. Among them, AQP1, AQP4 and AQP9 are the most abundant AQPs in the brain. The expressions of AQP4 and AQP9 were changed during cerebral edema after ischemic stroke, but AQP1 expression was unchanged [33]. AQP4 expression was found to be increased on astrocyte endfeet in the core and the border of lesion in 1 h after cerebral ischemia, and increased in astrocytes in the border of lesion over the whole cell for 48 h after ischemia; both were coinciding with the peak of cerebral edema [33]. AQP4 was more abundant in the early stage of cerebral ischemia [34]. Studies from various labs have demonstrated that mice lacking AQP4 showed reducing infarct volumes and improved neurological outcomes after cerebral ischemia; absence of AQP4 could partly prevent blood brain barrier disruption and alleviate neuroinflammation induced by cerebral ischemia [35–37]. Hastings and colleagues demonstrated that cerebral hemispheric edema was reduced in AQP4 null mice at 1 h after ischemia [35]. In addition, Hirt and colleagues (2016) reported that AQP4 absence on behavioral outcomes and lesion volume was not associated with the reduction of edema formation on days 3 and 7 after ischemia [38].

The expression of AQP9 showed a significant induction at 24 h after ischemia and gradually increased over time, which was not correlated with the cell swelling [33]. Its functional roles remain to be fully elucidated. A few studies have examined the associations between AQP3, AQP5 or AQP8 and cerebral ischemia. Yang and colleagues (2009) demonstrated that the expression of AQP3, AQP5 and AQP8 enhanced until 24 h after cerebral ischemia in the border region but decreased 6 h after ischemia in the ischemic core, suggesting their involvement in edema formation after cerebral ischemia [39]. Selective regulation of AQPs may serve as an effective strategy for cerebral ischemia.

6.2.2 AQPs and Congestive Heart-Failure (CHF)

Congestive heart failure (CHF) is the typical end-stage of most heart diseases, with impairment of water excretion. Acute exacerbation of CHF

stimulates the pituitary, leads to the activation of renin–angiotensin–aldosterone system (RAAS) and increases release of adrenocorticotropin (ACTH) and arginine vasopressin (AVP). Subsequently the retention of sodium and water is induced. Kidney is essential for the water reabsorption and water and sodium retention. AVP increases the water permeability of the renal collecting duct cells, allowing more water to be reabsorbed from collecting duct urine to blood. In addition, AVP acts on V2 receptors in the renal collecting duct, which regulates the expression and trafficking of AQP2 [40, 41]. AQP2 is a promising marker of the concentrating and diluting ability of the kidney. AVP triggers a reversible translocation of AQP2 from intracellular storage vesicles into the apical plasma membranes (APM) over a period of minutes, and AQP2 protein levels could be elevated by AVP over a period of hours to days [42, 43]. Renal AQP2 expression has been found to be significantly increased in CHF rats, whereas other subtypes of AQP expressions (such as AQP1 and AQP3) were unaltered [44]. Besides its expression, urinary excretion of AQP2 was also markedly increased in CHF patients [45]. There is a close correlation among plasma AVP levels, renal AQP2 expression and the severity of CHF. Administration of V2 receptor antagonist tolvaptan could downregulate renal AQP2 protein levels in the CHF rats [46]. Tolvaptan was approved in 2009 by FDA to treat hyponatremia associated with CHF, however, the ideal responders to tolvaptan have not yet been identified. AQP2 may be served as an ideal predictor of response to tolvaptan and guide its treatment in the future [47].

6.2.3 AQPs and Hypertension

Hypertension is a common cardiovascular disease, that can lead to heart disease, stroke, as well as hypertensive retinopathy and chronic kidney disease. Blood pressure (BP) is affected by various factors, including peripheral resistance, vessel elasticity, blood volume, cardiac output; so the mechanism of hypertension is too complex. Recently, much interest focused on the role of AQPs in the pathophysiology of hypertension. In spontaneously hypertensive rats (SHR), AQP2 expression in renal tubule epithelial cells is upregulated, along with activation of the cAMP pathway induced by AVP [48, 49]. Same results have been found in DOCA-salt hypertensive rat model [50]. In addition, treatment with AVP V2 receptor antagonist would lower BP and urinary osmolarity, and alleviate urinary AQP2 levels both in control and SHR, indicating AQP2 and AVP are involved in the pathogenesis of hypertension in spontaneously hypertensive [51]. Besides AQP2, the expressions of medullary AQP1 and AQP3 were also significantly increased in SHR compared with corresponding control (WKY rats), while that of AQP4 was not [48]. However, Klein Fukuoka and colleagues (2006) demonstrated that medullary AQP2 expression was decreased in response to angiotensin II or norepinephrine-induced acute hypertension [52]. Alterations in the expressions of AQPs in the brain were also found during hypertension. AQP1 expression was increased in choroid plexus epithelium of SHR, and elevated AQP4 expression was found in frontal cortex, striatum, and hippocampus of SHR compared to control WKY rats [53]. The increased AQPs expression may modulate the fluid exchange between blood brain barrier and blood-CSF barrier, and evoked an acute increase in blood pressure and impairment of blood brain barrier.

In 2007, Herrera and colleagues reported that AQP1 mediated transfer of NO at a $K_{1/2}$ (the concentration of NO that produces half of the maximum transport rate) of 0.54 μmol/L, and knockdown of AQP1 by siRNA could prevent NO release by 44% in endothelial cells [11]. They further (2007) demonstrated that AQP1 facilitated transport of NO out of endothelial cells and influx into vascular smooth muscle cells, and got involved in endothelium-dependent vascular relaxation [4]. However, AQP1 null humans and knockout mice are not hypertensive, so additional evidences are needed to confirm the role of AQP1 in hypertension [54].

6.2.4 AQPs and Angiogenesis

Angiogenesis, mechanism of capillaries formation from existing blood vessels, is the main form of vessel formation in the adult. Angiogenesis is regulated by many factors, such as VEGF, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), angiopoietin (ANG), Notch, Wnt, etc. And it relates to a variety of diseases, such as tumor, ophthalmic diseases and wound healing. Recently, AQPs have shown to be involved in angiogenesis, especially in tumor angiogenesis. Tumor angiogenesis includes 3 procedures: (1) matrix breakdown (2) proliferation, migration and differentiation of endothelial cells (3) supplement of periendothelial cells [55].

In the brain cancer and glioblastoma, AQPs expression is positively correlated with tumor histological differentiation [56, 57]. And in other cancers, such as breast cancer, brain cancer and multiple myeloma, high expression of AQPs result in localized edema that aggravated matrix breakdown [58, 59].

AQP-dependent cell migration has been found in a variety of cell types both *in vitro* and *in vivo* (Fig. 6.1). Saadoun and colleagues found upregulated expression of AQP1 in tumor microvascular endothelial cells could assist cell migration and its expression was positively correlated with tumor microvascular density. AQP1 deletion reduces endothelial cell migration, inhibits tumor angiogenesis and growth [16]. AQP1-expressing tumor cells have enhanced metastatic potential and local infiltration. Impaired cell migration has also been seen in AQP1-deficient proximal tubule epithelial cells, and AQP3-deficient corneal epithelial cells, enterocytes, and skin keratinocytes [60]. AQP4 deletion slows the migration of reactive astrocytes, impairing glial scarring after brain stab injury [57]. The mechanisms by which AQPs enhance cell migration are under investigation.

Studies about AQPs and tumor angiogenesis provide a theoretical basis for tumor treatment. Inhibition of AQPs expression and AQP-mediated water influx by acetozolamide, cyclophosphamide, topiramate, thiopenthal, phenobarbital and propofol may affected cancer cells proliferation, migration, metastasis and angiogenic potential [17, 61].

6.3 Conclusion and Prospect

Due to the diversity and complexity of AQP family, blood vessels may require a variety of AQP subtypes to finish its normal physiological function. It is necessary to study the expression and function of AQPs in the blood vessels further from the level of integration.

AQPs are involved in many related disease occurrence, development and blood vessel function regulation. It has important clinical significance to understand the accurate correlation between AQPs expression variation and vascular diseases, which can provide new ideas and methods for vascular disease treatment.

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Aquaporins in Respiratory System

7

Yuanlin Song, Linlin Wang, Jian Wang, and Chunxue Bai

Abstract

Aquaporins (AQPs) are water channel proteins supposed to facilitating fluid transport in alveolar space, airway humidification, pleural fluid absorption, and submucosal gland secretion. In this chapter, we mainly focus on the expression of 4 AQPs in the lungs which include AQP1, AQP2, AQP4 and AQP5 in normal and disease status, and the experience of AQPs function from various model and transgenic mice were summarized in detail to improve our understanding of the role of AQPs in fluid balance of respiratory system. It has been suggested that AQPs play important roles in various physiology and pathophysiology conditions of different lung diseases. There still remains unclear the exact role of AQPs in lung diseases, and thus continuous efforts on elucidating the roles of AQPs in lung physiological and pathophysilogical processes are warranted.

Keywords

Aquaporins • Lung disorders • Fluid transport

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7.1 Introduction

Respiratory system by definition includes respiratory center located in brain stem; respiratory muscle including external and internal intercostal muscle, sternocleidomastoid muscle and diaphragm; airways including upper airway and lower airway; alveolus and surrounding pulmonary and systemic circulation. Each part has specific function and mainly carries the function of ventilation and oxygenation with coordination of ventilation and pulmonary circulation that provides adequate oxygen delivery to distal organs. However, the lungs also have metabolism, defending, immune and fluid transport function. The fetus lung is filled with fluid before the fetus is delivered, and the fluid inside of the lungs is absorbed immediately to keep the lungs relatively dry to maintain adequate ventilation and oxygenation after delivery. When the lungs or airways were insulted, it may bring fluid transport disorders, such as airway and lung edema, pleural effusion, etc. However, if there is extra fluid absorption, the airway may become relative dry and induce thick sputum and subsequent airway inflammation. Thus, it is critical to keep fluid balance in alveolus, interstitial space, airway and pleural space to maintain normal respiratory function.

The fluid transport follows few rules: the osmotic fluid transport due to osmotic gradient; the Starling mechanism due to hydrostatic pressure; and the fluid pinocytosis. It has been a long history for the researchers to discover that the cell membrane express a water channel aquaporin (AQP) to control fluid transport [1]. Since the first report of AQP1 in red blood cells, there were numerous publications addressing expression and function of AQPs in various organs including respiratory system. So far, there are 4 AQPs expressed in the lungs, including AQP1 in the vascular endothelium and pleural membrane, AQP3 in epithelium of large airway, AQP4 in epithelium of small airways, and AQP5 in alveolar type I cells and submucosal glands. In this chapter, the expression of above mentioned AQPs in normal and disease status, and the experience of AQPs function from various model and transgenic mice were summarized in detail to improve our understanding of the role of AQPs in fluid balance of respiratory system.

7.2 Expression of AQPs in the Lungs and Airways

There are 4 AQPs expressed in the lungs including AQP1, AQP3, AQP4 and AQP5. AQP1 is expressed in the endothelium of pulmonary capillary, vein and artery [2, 3], the apical and basolateral membrane of the microvascular endothelium within pleural membrane, including inner and out membrane [4]. AQP3 is located in the basolateral membrane of basal cells of the tracheal epithelium and in submucosal gland cell membranes in rodents and in apical membrane of bronchioles and type-II alveolar epithelial cells (ACEs) of adult humans, while AQP4 is expressed in the basolateral membrane of columnar cells in the bronchi and trachea of rats and in type-I AECs in humans [5–8]. AQP5 is expressed in apical membrane of type I ACEs, as well as apical membrane of serous cells of upper airway submucosal glands, it has also been detected in type-II AECs in mice [8, 9]. Some studies show AQP5 is also expressed at apical membrane of ACEs [10].

Levels of AQPs expression depend on timing of lung development and pathological conditions. There is a dramatic difference of AQPs expression in airway and alveolar epithelium before and after birth delivery. The underlying mechanism might be the accommodation of fluid transport because airway epithelium and alveolar epithelium play an important role in fetal lung fluid secretion before delivery and turn to absorption function after delivery to clear lung fluid for oxygenation. Most of the studies about AQPs in fetal lungs are derived from animal experiments. Fetal sheep have been used as an important animal model for lung developmental studies, particularly of factors regulating the physiological development of the fetal lung [11, 12]. Sheep fetal lungs can express AQP1, AQP3, AQP4 or AQP5 in mRNA and protein levels during midterm gestation [13]. Rat fetal lungs express very

little AQPs before birth, and only AQP1 and AQP4 in rats has been detected at present before birth [14–16]. Although AQP1 expression in mRNA and protein levels in the lungs of fetal and neonatal rats is increased when treated with synthetic glucocorticoids [7, 15], little is known about the physiological factors to control its expression before birth. Besides, Ya sui et al. [15] found that AQP4 could be induced to increase by corticosteroids and β-adrenergic agents. However, AQP5 mRNA expression in very low level was detected before birth in mice [13].

The deletion of one or more AQP genes in the studies of mice suggested that AQPs are not essential for neonatal survival [17]. However, what is true in mice may not be true for all species, including humans [18]. Because the expression and distributions of different AQPs in the lungs are varied from the different species, it is difficult to make a consistent conclusion about the physiological role of AQPs in fetal lung development and the transition to extra-uterine life at birth, especially in the species with long-gestation such as humans.

7.3 AQPs and Lung Fluid Transport

Besides ventilation and oxygenation, the lungs exert other biological functions such as lung fluid transport, metabolism, immune defense, etc. Herein, lung fluid transport refers to the alveolar fluid balance, airway hydration, pleural fluid transport and submucosal glands secretion.

7.3.1 AQPs and Alveolar Fluid Balance

Fluid transport between alveolar and capillary endothelium presents with several forms including the osmotic fluid transport, blood-gas barrier disruption induced fluid leakage and hydrostatic fluid transport. AQP1 and AQP5 are mainly expressed at apical membrane of capillary endothelial cells and type I AECs [8, 9, 19] (Fig. 7.1). The location of these two AQPs suggests possible roles in facilitating water transport. As stated before, AQP expression varies during gestation time, 45 min immediately after delivery do not shown difference of lung wet/dry weight ratio between wild-type and AQP1, 4, 5 knockout mice [21], suggesting slow fluid absorption does not require AQP facilitation, plus these AQPs do not have full expression at the time point of experiment. Several studies have showed that knockout AQP1 and AQP5 could significantly reduce osmotic fluid transport [17, 22]. However, deletion of AQP1 or AQP5 did not alter lung edema formation and resolution difference in acute lung injury model [23, 24], in which increased capillary permeability leads to the fluid accumulation in interstitial and alveolar tissue. This might be explained that AQP-mediated fluid transport is slower than fluid transport through enlarged capillary leakage, and fluid transport through cell membrane is little [23, 24]. Similarly, to study the effects of AQP5 on hydrostatic pressure-induced lung edema, high pressure infusion plus blockage of outflow from left atrium are designed to mimic left heart failure induced lung edema. Deletion of AQP5 did not affect lung edema induced by high pulmonary pressure infusion [22]. These studies further indicate that AQP1 and AQP5 mainly facilitate osmotic fluid transport through the apical membrane of capillary endothelial cells and AECs, but they may not participate in fluid transport driven by capillary permeability and hydrostatic pressure changes.

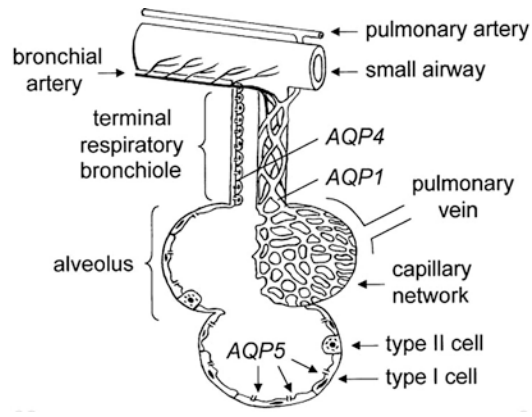

Fig. 7.1 Expression of AQP1, AQP4 and AQP5 in distal airway and alveolar space [20]

Peri-bronchial edema formation was found to decrease in AQP1 mutation patients after bonus saline infusion, for capillary network formation defects after AQP1 mutation, and thus it is unlikely that AQP1 could contribute to hydrostatic pressure induced fluid accumulation [25]. Besides, deletion of AQP4, which is expressed on the epithelium of small airways close to alveolar spaces, does not significantly affect fluid transport compared to wild-type mice. However, AQP4 deletion displays a more decrease in osmotic fluid transport compared with AQP1 knockout in mice, suggesting AQP4 acts as the main role in facilitating fluid transport through small airway epithelium [25]. The potential effect of AQP4 is under covered by AQP1, because function of AQP4 appeared more significant when AQP1 is deleted.

7.3.2 AQPs and Airway Fluid Balance

Airway must keep high humidity to protect airway epithelial cells that work together with submucosal glands to secret fluid to facilitate cilliary movement to expel inhaled exopathogens. Although AQP3 and AQP4 has been found to be expressed on apical membrane of ciliated epithelial cells [26] (Fig. 7.2) and studies showed that AQPs play minor role in airway humidification, ASL hydration, and isosmolar fluid absorption in AQP3 and AQP4 knockout mice [27]. By calculating fluid transport rate, the fluid movement across airway epithelium challenged by dry air is relatively slower compared to salivary gland secretion where AQP5 facilitates fluid transport. Furthermore, the minor effect of AQP3 and AQP4 in airway physiology suggests slow fluid movement does not rely on water channel necessarily unless it is challenged by osmotic fluid movement [27].

A recent study showed AQP3 deletion reduce airway re-epitheliulization [28], the possible role is reduced epithelial cell migration due to water and glycerol transport reduction [29]. The role of AQP3 in airway epithelial growth provide potential role of AQP in tissue repair.

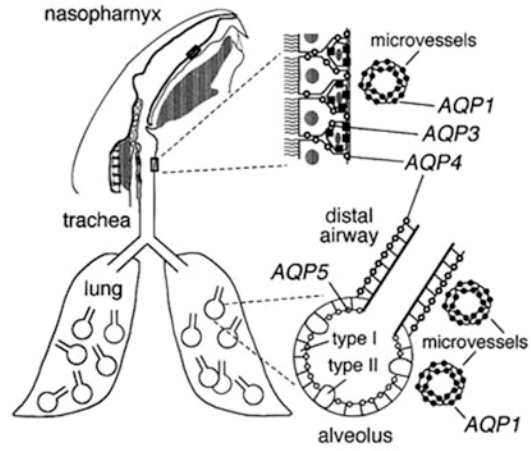

Fig. 7.2 AQP1, AQP3, AQP4 and AQP5 expression in capillary, airway, and alveolar space [26]

7.3.3 AQPs and Pleural Fluid Balance

The pleural space plays an important role in pleural fluid secretion and absorption and lubricating visceral and parietals membrane of pleural space to facilitate lung extension. The fluid is filtered through capillary within visceral membrane and reabsorbed by parietal lymphatic duct located on parietal membrane. In some malignancy, these lymphatic can be blocked to result in fluid accumulation within pleural space. AQP1 is expressed at apical membrane of visceral and parietal pleura, and apical membrane of endothelial cell within visceral membrane [4] (Fig. 7.3). Our group found that AQP1 could facilitate the osmotic fluid transport within pleural space, and deletion of AQP1 could significantly reduce osmotic fluid transport. However, AQP1 did not take part in pleural isosmolar fluid clearance [30, 31]. Similarly, there is no relationship of AQP1 with clinically relevant mechanisms of pleural fluid accumulation or clearance [4].

7.3.4 AQPs and Submucosal Gland Secretion

Submucosal glands are located at upper and lower airway submucosal area, where capillary

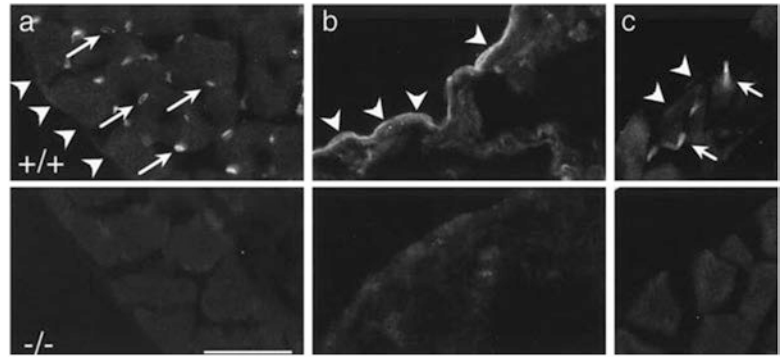

Fig. 7.3 AQP1 expression in parietal and visceral membrane of pleural space [4]

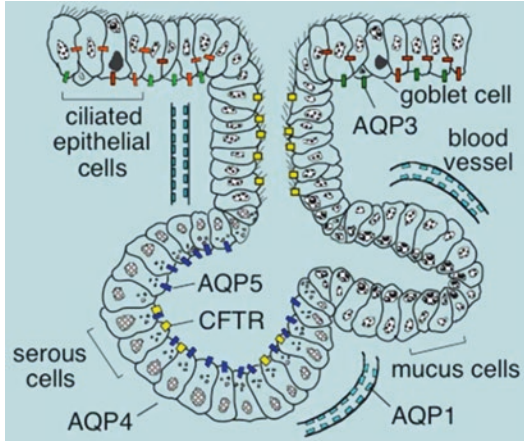

Fig. 7.4 AQP1, AQP3, AQP4 and AQP5 expression in airway submucosla glands [33]

and nerves are surrounded to keep normal function for gland secretion. In general, when glands are stimulated with nerve or chemical through muscarinic receptors, increased cytosolic cAMP level will activate CFTR function, to induce chloride secretion, and sodium will increase in cell to follow the electronic neutralization through intracellular and paracellular pathway, and then water will come out of the cells following the ionic osmotic gradient mainly through AQP5 water channel. This phenomenon was evidenced in airway submucosal glands and salivary glands [32] (Fig. 7.4). Deletion of AQP5 significantly reduced gland fluid secretion and thus made the secreted fluid more viscous [33]. There are few studies showing that dry mouth due to salivary glands radiation or sojoren syndrome are associated with abnormal distribution of AQP5 [34, 35], suggesting AQP5 modulation may potentially improve dry moth syndrome through correction of saliva secretion. It is therefore interesting to test whether AQP5 modulation could be useful to promote airway mucus clearance in COPD or bronchiectasis patients.

7.4 AQPs and Lung Cancer Development

Several studied found that AQP1, AQP3, AQP4 and AQP5 are over-expressed in lung cancer [36–39]. The expression of AQP1 is higher in lung adenocarcinoma (ADCs) and bronchoalveolar carcinoma than that in lung squamous cell carcinoma and normal lung tissue [37]. AQP1 is located in the endothelial cells of capillaries within lung cancer tissue and responsible for tumor angiogenesis [40, 41]. AQP1 is also involved in invasion of lung cancer cells, and reducing AQP1 expression by AQP1-shRNA could inhibit lung cancer cell invasion and migration [41]. Moreover, AQP1 expression is correlated with high postoperative metastasis ratios and low disease-free survival rates in ADCs, especially with micropapillary ADC components [36]. These studies suggest that AQP1 could be a significant prognostic index for stage and histologic differentiation of lung cancer.

AQP3 is over-expressed in non-small cell carcinoma (NSCLC), especially ADCs, well-differentiated bronchioloalveolar carcinomas and papillary subtypes. Some studies found that AQP3 might regulate biological functions of lung cancer cells, in the early stage of lung ADC [36], and even involve in angiogenesis of lung cancer

through HIF-2α-VEGF pathway and lung cancer cell invasion partly by the AKT-MMPs pathway, mitochondrial ATP formation and cellular glycerol uptake [42]. The anticancer effect of shRNA-targeting AQP3 is confirmed in experimental NSCLC models, and further is confirmed in preclinnical studies [42]. Beside, AQP4 wis involved in the invasion of lung cancer cells [41]. Higher transcript and protein levels of AQP4 in well-differentiated lung ADCs suggest an association with a better prognosis [38].

The expression of AQP5 was also detected to dramatically increase in lung ADCs and correlated with poor prognosis of patients with NSCLC [43]. AQP5-expressed cells exhibited a loss of epithelial cell markers and activation of c-Src through SH3 binding domain to promote epithelial to mesenchymal transition (EMT) which might be responsible for the promote metastasis of lung cancer [43]. Over-expressed AQP5 could facilitate lung cancer cell growth and invasion through the activation of the EGFR/ERK/p38 MAPK pathway [43, 44]. The cAMP-protein kinase (PKA) consensus site in AQP5 is also preferentially phosphorylated and promoted cell proliferation ability in tumor. The phosphorylation S156 in PKA consensus site is demonstrated to play an important role in tumor proliferation and invasion [45]. So, S156 in AQP5 may provide a potential therapeutic target by developing small molecules as an inhibitor. Moreover, developing specific monoclonal antibody targeting AQP5 will also be another approach.

7.5 AQPs and Acute Lung Injury and Lung Infection

Several studies have shown that both AQP1 and AQP5 are down-regulated after lung injury [23, 24, 46]. Deletion of AQP1 does not show significant phenotype changes while AQP5 deletion shows worsened lung injury after *P. aeruginosa* challenge [21, 24]. The mechanism may be that AQP1 was expressed in pulmonary capillary endothelium cells, and deletion of AQP1 impairs osmotic fluid transport but not near isosmolar fluid transport during capillary leaking due to increased permeability changes. AQP1 mutation in human does not cause morphology changes, but results in retarding fluid accumulation around airways [23].The underlying mechanism could be a change in capillary networks. It is believed that hydrostatic force could affect isosmolar fluid transport through water channels. Besides, the worsened lung injury in AQP5 null mice after *P. aeruginosa* challenge could be due to airway surface liquid property changes [24], in which AQP5 deficiency leads to reduced mucin production in lung. Moreover, and declined activation of mitogen-activated protein kinase and nuclear factor-kappa B before and after PA infection.

Considering that AQP 1 and AQP5 are expressed at blood-gas barrier, and both of them facilitate osmotic fluid transport, it has been though that AQP1 and AQP5 may play an important role in acute lung injury, especially in the pulmonary edema. Several studies showed that AQP1 and AQP5 are significantly down-regulated after lung injury [23, 24], and deletion of AQP1 does not show any difference of lung edema formation or resolution in LPS induced acute lung injury, suggesting slow fluid transport or fluid leakage from paracellular pathway may not require AQPs for intracellular fluid transport in acute lung injury. Meanwhile, AQPs may facilitate osmotic fluid transport but not near isosmolar fluid movement.

7.6 AQPs and Asthma

Asthma is featured by increased airway constriction, esionphilic infiltration, hypersecretion of airway mucus and small airway epithelium edema formation. Immunostaining study shows AQP1 expressed not only in alveolar type I and type II cells, as well as in airway epithelium. OVA-induced Asthma animal model shows an increase in expression of AQP1 and AQP5 compared to control group, suggesting AQP1 and AQP5 may participate in airway epithelium edema formation [47]. Bronchial provoke test usually shows hyperactivity and hyper responsiveness to methacholine [48]. AQP5 knockout

mice study shows deletion of AQP5 increased airway reactivity challenged by inhalation of methacholine accompanying with increased airway resistance [10]. It is not clear why deletion of AQP5 decreases airway challenge threshold. Besides, same loci of AQP5 and other asthma gene located at chromosome 12q and mouse chromosome 15 further indicated potential role of AQP5 in asthma development [10].

7.7 Summary

AQPs are water channel proteins supposed to facilitating fluid transport in alveolar space, airway humidification, pleural fluid absorption, and submucosal gland secretion. Previous studies suggested the roles of AQPs in various physiology and pathophysiology condition of different lung disease in vivo or vitro. It still remains unclear the exact role of AQPs in lung diseases, and thus continuous efforts on elucidating the roles of AQPs in lung physiological and pathophysiological processes are warranted.

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Aquaporins in Digestive System

8

Shuai Zhu, Jianhua Ran, Baoxue Yang, and Zhechuan Mei

Abstract

In this chapter, we mainly discuss the expression and function of aquaporins (AQPs) expressed in digestive system. AQPs in gastrointestinal tract include four members of aquaporin subfamily: AQP1, AQP4, AQP5 and AQP8, and a member of aquaglyceroporin subfamily: AQP3. In the digestive glands, especially the liver, we discuss three members of aquaporin subfamily: AQP1, AQP5 and AQP8, a member of aquaglyceroporin subfamily: AQP9. AQP3 is involved in the diarrhea and inflammatory bowel disease; AQP5 is relevant to gastric carcinoma cell proliferation and migration; AQP9 plays considerable role in glycerol metabolism, urea transport and hepatocellular carcinoma. Further investigation is necessary for specific locations and functions of AQPs in digestive system.

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Keywords

Aquaporins • Digestive system • Gastrointestinal tract • Water electrolyte balance • Glycerol metabolism • Diabetes

8.1 Introduction

Digestive system includes the digestive tract and digestive gland. The digestive tract is composed of oral cavity, pharynx, esophagus, stomach, small intestine, large intestine and anus. The digestive glands include large digestive glands and plenty of small digestive glands spread over the wall of the digestive tract. The large digestive glands, such as 3-pair salivary glands, pancreas and liver, have secretary portion and ducts formed by gland cells to drain the excreta into the digestive tract. Moreover, pancreas can also perform as an endocrine gland, as A-cells excrete glucagon, B-cells excrete insulin, and D-cells excrete somatostatin, PP-cells excrete pancreatic polypeptide. These endocrine hormones regulate blood glucose and the movement of gastrointestinal tract. Apart from secretion, absorption is an important function for digestive systems, especially for gastrointestinal tract. During a meal, after the primary digestion of saliva, the osmolarities of the food we eat can change rapidly from zero (water) to several hundred millosmoles (solid meal). In response to the rapid change of the osmolality in gastrointestinal tract, cell junctions are tight in the stomach and colon (in order not to lose water when dehydrating feces), and gastric juice or other kind of digestive fluids will be secreted to balance the osmolarity of gastric content [1]. When the content comes to small intestine, most water will be absorbed with solutes and nutrition. When it comes to colon, the content is further dehydrated and forms feces. Totally, about 7.5 L of fluid is secreted into the tract, which includes saliva, gastric secretions, bile, pancreatic juice, and intestinal secretion, and about 9 L fluid is absorbed each day [2, 3]. Moreover, the liver is related to substance metabolism.

Aquaporins (AQPs) are expressed and play physiological roles in the digestive system [2]. The distributions of AQPs are relevant to their functions. Basolateral water channels AQP3 and AQP4 are more expressed in secretive epithelia (e.g, stomach), whereas apical water channels are more localized in absorbing epithelia (e.g, small intestine). In the colon, which can both absorb and secrete water, both apical and basolateral AQPs are expressed [1]. AQP9 is expressed in the liver, and involved in fat metabolism. Here we describe some important isoforms of AQPs in digestive system, and mention others that are not very clearly studied. The general distribution of AQPs in digestive system is summarized in Fig. 8.1 [4–6]. Figure 8.2 presents the possible pathways for transepithelial water transport in digestive system. They mainly consist of paracellular pathway, transcellular pathway, diffusion, and osmolality-dependent AQP pathway. Considering the constant phenotype of specific AQP-knockout mice regarding the fluid secretion [7, 8], the function of AQPs in the digestive system might be limited.

8.2 AQPs in the Gastrointestinal Tract

8.2.1 Aquaporin Subfamily

8.2.1.1 AQP1

In gastrointestinal tract, AQP1 is diversely expressed on the endothelial barriers, while there is no expression in the epithelia and mucosa. It is more expressed in the body of the stomach, duodenum and ascending colon than the pyloric antrum [9]. A moderate amount of AQP1 was also observed in the stromal tissue of the anus, but it is difficult to identify the specific location [10].

In human tissue, AQP1 was demonstrated on the endothelial cells of the lymphatic vessels in the submucosa and lamina, and capillary endo-

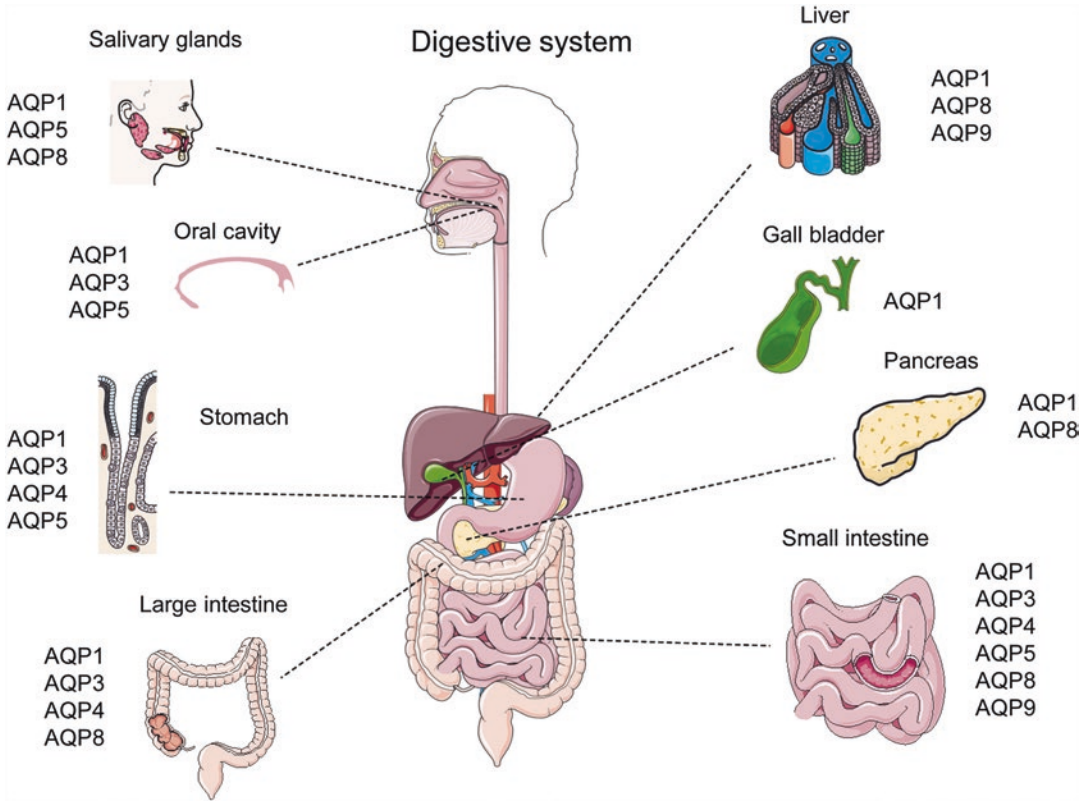

Fig. 8.1 Distribution of aquaporins in the digestive system. AQP1, AQP5 and AQP8 are expressed in salivary glands. AQP1, AQP3 and AQP5 are present in oral cavity. In the stomach, AQP1 is expressed in the endothelial cells of capillaries and small vessels; AQP3 is expressed in the basolateral membrane of surface mucous cells; AQP4 is expressed in the basolateral membrane of parietal cells, and AQP5 is present at the apical membrane of parietal cells. Small intestine expresses AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9. AQP1, AQP3, AQP4 and AQP8 are expressed in large intestine. AQP1, AQP8 and AQP9 are expressed in the liver. AQP1 is diversely expressed in gallbladder, bile duct and pancreas, while AQP8 is present in the pancreas as well [2]

thelial cells in the smooth muscle layer throughout the gastrointestinal tract. For other species, abundant expression of AQP1 was detected in endothelium of capillaries and small vessels in digestive system [11–15].

On the basis of the location of AQP1 in the gastrointestinal tract, speculation is that AQP1 plays an absorptive passage in the water transport between the gastrointestinal mucosa and bloodstream. In addition, AQP1 is present in endothelial cells of central lacteals in the villi of small intestine, which produces chylomicrons when digesting food. Therefore, AQP1 might be involved in the fat digestion process. There is evidence that AQP1 null mice show up a defect in fat absorption [2, 12]. Further studies about functions of AQP1 in gastrointestinal tract are needed.

8.2.1.2 AQP4

AQP4 is selectively expressed in the basolateral membrane of parietal cells of the stomach, especially at the base of gastric pits, which is acknowledged to play the main role in modulating the secretion of the acid. AQP4 null mice were applied to study the role of AQP4 in gastric acid secretion by Verkman's group [7]. There was no apparent difference in morphology in the parietal cells within the gastric pits for AQP4 null mice. And the deficiency of AQP4 showed no difference to the rates of basal or stimulated acid or gastric fluid secretion. Nor did it affect the pH

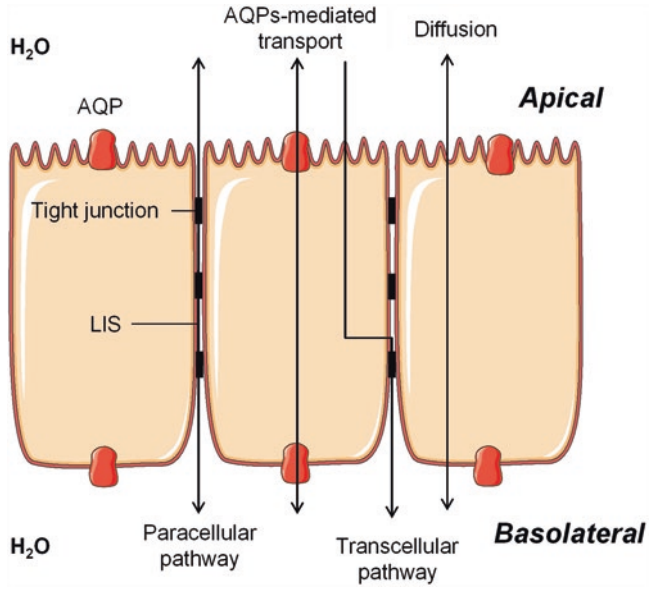

Fig. 8.2 Possible pathways for transepithelial water transport in the digestive system. There are 4 pathways for transepithelial water transport, including paracellular pathway, transcellular pathway, diffusion and osmolarity-dependent AQP pathway

level and fasting serum gastrin concentration in the stomach. These data suggest that AQP4 has little influence on gastric acid production [8].

AQP4 is also expressed in the basolateral membrane of the crypt cells located at the bottom of the crypt in small intestine, and the basolateral membrane of surface epithelial cells in the colon as mentioned above. It is suggested that AQP4 is involved in colonic fluid transport. However, in AQP4 null mice, the water permeability was decreased in the proximal colon but not the distal colon, while the water content of the feces had no difference compared to wild-type mice. All in all, AQP4 in surface epithelial cells has no influence on feces dehydration and colonic fluid secretion [2].

8.2.1.3 AQP5

AQP5 was first isolated from the salivary gland. It is typically expressed in glandular tissues like salivary glands, lacrimal glands, and pancreas. In digestive tract, it is present in the stomach and duodenum in rat. For stomach, it is expressed in apical membrane of secretory cells of the pyloric gland, and there is almost no expression in the fundic gland. In the duodenum, AQP5 is present along the apical membrane of secretory cells in duodenal gland [16]. AQP5 is not detected in other tissues of the digestive system by immunohistochemistry, while the expression of AQP5 was shown by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in the liver [2].

It is reported that AQP5 promotes the progression and invasion of several cancers [17]. It is upregulated in a variety of cancers and associated with the clinicopathological characteristics of patients, which include colon cancer, lung cancer, chronic myelogeous leukaemia, breast cancer, and biliary tract carcinoma. In gastric carcinoma, AQP5 is relevant to the tumorigenesis and progression, such as differentiation, lymph node metastasis and lymphovascular invasion [1, 17].

8.2.1.4 AQP8

AQP8 was first isolated from the pancreas, liver and testis. AQP8 transcript is widely expressed in the digestive system, including the salivary glands, small intestine, colon, pancreas, and liver. In digestive tract, it is mainly present at the

subapical intracellular sites of epithelial cells in the duodenum, jejunum, and colon [2, 18].

AQP8 knockout mice are used to explore AQP8 functions, and they have normal appearance, survival, growth, organ weights and serum chemistries, but larger testis. In small intestine, AQP8 knockout model made no difference in cholera toxin- or agonist-stimulated maximal fluid secretion. In colon, AQP8 knockout model had little effect on the colonic fluid absorption or fecal dehydration. And water content in stool changed little in AQP8 knockout mice. Only mild phenotype differences between the wild-type and AQP8 knockout mice were found. And the function of AQP8 in the water absorption and secretion of small intestine and colon is limited [19].

In 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)- induced colitis model, which mimics human Crohn's disease, AQP8 expression is downregulated with the increase of inflammation and injury [20], indicating that AQP8 is possibly involved in inflammatory bowel disease.

8.2.2 Aquaglyceroporin Subfamily

8.2.2.1 AQP3

In digestive system, AQP3 is highly expressed in the esophagus, proximal and distal colon [21]. According to immunohistochemical results from rat digestive tract, AQP3 is also present in the oral cavity, forestomach, and anus, where AQP3 situates at the basolateral membrane. The functions of AQP3 in tissues mentioned above are still elusive. It is possible that AQP3 is providing water to epithelial cells directly with bad circumstances of feces. And AQP3 in oral and anus is seen as the extension of skin, in which AQP3 has been confirmed to be important in keeping stratum corneum hydration and skin elasticity [2, 22–24].

In human colon, AQP3 is predominantly expressed in the mucosal epithelial cells [24], which indicates its important role in water transport. It is reported that the inhibition of AQP3 in the colon leads to diarrhea. AQP3 inhibitor ($HgCl_2$ and $CuSO_4$) applied for more than 1 h, the fecal water content increased to approximately four times that in the control group, and severe diarrhea was observed [25, 26]. In the meantime, several laxatives present a laxative effect by the upregulation of AQP3 expression. For osmotic laxatives such as magnesium sulfate, previously thought to work by increasing the osmotic pressure in the intestinal tract, AQP3 expression was found upregulated, suggesting that osmotic laxative might play its role in response to the increased AQP3 expression. However, stimulant laxative, such as bisacodyl, works by promoting the peristaltic movements of the bowel. When it was applied to rats, AQP3 expression was found downregulated, and severe diarrhea was observed without osmotic pressure change. Contrary to diarrhea, AQP3 expression is also involved in the constipation. Morphine is clinically used as a narcotic analgesic with usual adverse effect of constipation, which is caused by the decrease of peristaltic movements of the bowel. In this model, AQP3 expression is upregulated, which might take part in the constipation. Generally speaking, deeper investigation for the mechanism of AQP3 involved water transport may provide candidates for new laxatives and antidiarrheal drugs in the future [24].

In TNBS-induced colitis, AQP3 expression was downregulated in accordance with AQP8, accompanied with intestinal inflammation and injury. After small bowel resection and improvement of intestinal functions in IBD rats, AQP3 was upregulated during the adaptation [24]. The evidence indicates that AQP3 might involve in the pathogenesis of inflammatory bowel disease [20, 21].

Looking into the AQP3 null mice, intestinal barrier integrity was impaired based on previous work [27]. The results showed that the AQP3 deletion induced a dramatic increase in *E. coli* C25 translocation [27].

8.2.2.2 AQP10

The specific location of AQP10 in digestive system has not been very clear. Previous studies reported that unspliced AQP10 transcript was detected at the apical membrane of epithelial cells in the small intestine, while some claimed that it was present at endothelial cells of

submucosal capillaries in the duodenum [28]. Moreover, AQP10 performs as a pseudogene in some kind of species [2]

8.3 AQPs in the Digestive Glands

8.3.1 Aquaporin Subfamily

8.3.1.1 AQP1

AQP1 is localized to the basolateral membrane of the gallbladder, intrahepatic cholangiocytes, hepatic ducts, endothelial barriers in the liver, and pancreatic ducts and centroacinar cells in the pancreas [10]. Moreover, AQP1 is specifically expressed in the intralobular and interlobular ducts, modulating the water transport through the cells [2].

8.3.1.2 AQP5

AQP5 is typically expressed in glandular tissues, which include salivary glands, lacrimal glands, and pancreas. It participates in fluid secretion and therefore related to corresponding diseases, such as Sjögren's syndrome (PSS) and diabetes.

In salivary gland, AQP5 is present at the apical membrane, including the intercellular secretory canaliculi of acinar cells. In the AQP5 knockout mice, when compared to wild-type mice, the saliva production was reduced and was hypertonic, which showed that AQP5 played a main role in saliva secretion [2]. In Sjögren's syndrome models, AQP1 expression was increased and AQP5 expression was decreased, suggesting new pathways to explain the disease [29]. Nevertheless there is also oppositions to this hypothesis. The research showed that the distribution and density of AQP5 in salivary glands of PSS patients had no difference. And the role of AQP5 in the pathogenesis of PSS needs to be reassessed [30].

In pancreas, AQP5 is located at the apical membrane of centroacinar and intercalated ductal cells [31]. It might be involved in the diabetes and pancreatitis.

8.3.1.3 AQP8

For digestive glands, AQP8 is mainly expressed in parotid, salivary glands, liver and pancreas. AQP8 is present in myoepithelial cells around the acini and the intercalated duct rather than the acinar or ductal cells of rat parotid, submandibular and sublingual cells [32].

AQP8 knockout mice are applied to study AQP8 functions. In salivary glands, strong AQP8 transcripts were detected while it could not be found at protein level by immunofluorescence or immunoblot analysis. Furthermore, salivary secretion was not affected by AQP8 deficiency, according to the comparison about the phenotypes between AQP8 knockout mice and wild-type mice, nor was it affected in the comparison of AQP8/AQP5 double knockout mice and AQP5 knockout mice. In the liver, AQP8 is predominantly expressed in intracellular vesicles in hepatocytes. Given a high fat diet, AQP8 knockout mice did not show steatorrhea or abnormalities in serum lipid profile, liver function tests, or pancreatic enzymes. Based on the results, AQP8 is not involved in hepatobiliary/pancreatic function, while the plasma triglyceride and cholesterol concentrations rose up a little [24].

Moreover, AQP8 was confined to the apical membrane of acinar cells in human pancreas. It might play a role in the secretion of pancreatic juice.

8.3.2 Aquaglyceroporin Subfamily

8.3.2.1 AQP7

In human gastrointestinal tract, AQP7 was detected on the superficial epithelial cells throughout the small intestine and colon. For rats, it is present on the apical region of the enterocytes in the villi; epithelial cells of the colon and caecum, which suggests its involvement in rapid fluid movement through the villus epithelium [33].

8.3.2.2 AQP9

In liver, AQP9 is located at the basolateral (sinusoidal) membrane of hepatocyte [34, 35]. It has been proved to be the major route of arsenite uptake into the mammalian cells, whose accumulation might result in hepatocellular damage and hepatocellular carcinoma. In addition, bile duct ligation-induced extrahepatic cholestasis has been clarified to induce the downregulation of AQP9 in the hepatocyte basolateral membrane, indicating it might be involved in the bile flow.

AQP9 is also associated with glycerol metabolism in liver. Glycerol, as a product from adipose triglycerides during lipolysis, flows into the liver through the portal vein. And it takes part in gluconeogenesis later. AQP9 is verified as the only glycerol channel in the liver, which selectively localizes at the sinusoidal plasma membrane facing the portal vein. Thus, AQP9 is considered to be the channel for glycerol uptake in the liver [35–39].

AQP9 knockout model was constructed to study its role in glycerol metabolism. The results revealed that AQP9 null mice had evident hyperglycerolemia and hypertriglyceridemia compared to AQP9 heterozygous mice. When AQP9 null mice crossed with $Lepr^{db}/Lepr^{db}$ mice, a model of obese and type II diabetes, it showed that $Lepr^{db}/Lepr^{db}$ AQP9 null mice had lower blood glucose levels than $Lepr^{db}/Lepr^{db}$ AQP9 heterozygous mice. AQP9 null mice had lower plasma glycerol levels than AQP9 heterozytous mice. These results suggest the possible role of AQP9 in the hepatic glycerol absorption as well as glucose metabolism [35].

8.3.3 Superaquaporin Subfamily

8.3.3.1 AQP12

AQP12 is selectively expressed in the pancreas, especially in acinar cells. AQP12 knockout mice were not observed evident abnormality. Further research is needed in its function studies [28].

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Aquaporins in Urinary System

9

Yingjie Li, Weiling Wang, Tao Jiang, and Baoxue Yang

Abstract

Several aquaporin (AQP)-type water channels are expressed in kidney: AQP1 in the proximal tubule, thin descending limb of Henle, and vasa recta; AQP2-6 in the collecting duct; AQP7 in the proximal tubule; AQP8 in the proximal tubule and collecting duct; and AQP11 in the endoplasmic reticulum of proximal tubule cells. AQP2 is the vasopressin-regulated water channel that is important in hereditary and acquired diseases affecting urine-concentrating ability. The roles of AQPs in renal physiology and transepithelial water transport have been determined using AQP knockout mouse models. This chapter describes renal physiologic insights revealed by phenotypic analysis of AQP knockout mice and the prospects for further basic and clinical studies.

Keywords

Water • Urine concentrating mechanism • Polyuria • Knockout mouse • NDI

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The urinary system involves kidney, ureter, bladder and urethra. The upper urinary tract is made up of the kidneys while the other structures are the components of the lower urinary tract [37]. Urine that is formed in the kidney drains into the renal pelvis, ureter and bladder. Finally, urine stored in the bladder is excreted through urethra. Among the whole urinary system, kidney is the core organ to reabsorb water and concentrate urine.

Urine concentration and dilution mainly proceed in the kidney by regulating water excretion and reabsorption [153]. During water excretion and reabsorption, water permeability in the proximal tubules, descending limbs of Henle, late distal tubules and collecting ducts is very important. In contrast, those segments (such as ascending limbs of Henle) of the nephrons that are continuously impermeable to water are also necessary to establish osmotic gradient from renal cortex to inner medulla. Antidiuretic hormone (ADH) regulates the urine concentration by changing water permeability of late distal tubules and collecting ducts. The water permeability in the certain segments of renal tubules, collecting ducts and vasa recta is mediated by water channel aquaporins (AQPs) in the plasma membrane of epithelium and endothelium.

9.1 Expression Localization of AQPs in Urinary System

At least eight AQPs are expressed in kidney: AQP1 in the proximal tubule, thin descending limb of Henle (TDLH), and vasa recta; AQP2-6 in the collecting duct; AQP7 in the proximal tubule; AQP8 in the proximal tubule and collecting duct; and AQP11 in the endoplasmic reticulum (ER) of proximal tubule cells (Fig. 9.1) [71, 95, 111, 157, 167].

Although mammalian urothelium is generally considered impermeable to constituents of urine, some studies indicate urothelial transport of water and solutes under certain conditions [142, 143] and expression of AQP2 and AQP3 in urothelium.

9.1.1 AQP1

AQP1 is localized in the apical and basolateral plasma membranes of the proximal tubule and TDLH and in the micro-vascular endothelium of the medullary descending vasa recta (DVR) [1, 6, 7, 19, 82, 92, 109, 112, 113, 161, 183]. In the proximal tubule, AQP1 is localized from S1 though S3, except for the earliest part of S1 [88, 112].

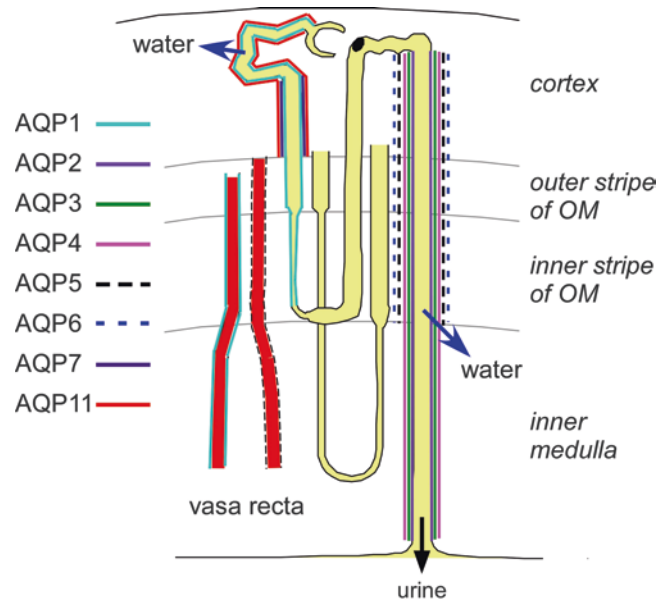

Fig. 9.1 Expression of AQPs in kidney. AQP1 in the proximal tubule, thin descending limb of Henle, and vasa recta; AQP2-6 in the collecting duct; AQP7 in the proximal tubule and AQP11 in the endoplasmic reticulum of proximal tubule cells

AQP1 is also expressed in capillary and arteriole endothelial cells in ureter and bladder [97, 142].

9.1.2 AQP2

AQP2 is expressed in principal cells of whole collecting duct from the connecting tubule and undergoes vasopressin-regulated trafficking between an intracellular vesicular compartment and the cell apical plasma membrane [21, 22, 67, 90, 133].

AQP2 is circumferentially lined the epithelial cell membranes except for the apical membrane of the epithelial cells adjacent to the lumens of both ureter and bladder [98].

9.1.3 AQP3

AQP3, originally called glycerol intrinsic protein (GLIP) based on its glycerol-transport function, is expressed at the basolateral membrane of cortical and outer medullary collecting duct epithelium [5, 30, 31, 39, 44, 56, 74].

AQP3 is shown to be intensely expressed at cell borders in the basal and intermediate layers in urothelium [131].

9.1.4 AQP4

AQP4 is expressed at the basolateral membrane of inner medullary collecting duct epithelium and the proximal tubule S3 region [25, 65, 150, 155].

Orthogonal arrays of particles (OAPs) have been found in basolateral membrane observed by freeze-fracture electron microscopy (Fig. 9.2). It is confirmed that AQP4 forms these OAPs by expressing rat AQP4 in Chinese hamster ovary (CHO) cells [155, 169].

9.1.5 AQP5

Procino *et al.* demonstrated that AQP5 is expressed in type B intercalated cells in the kidney collecting duct, which is the first report about expression of AQP5 in the kidney [123].

9.1.6 AQP6

AQP6 is expressed primarily in the membrane of the intracellular vesicles in type A intercalated cells of the collecting duct [126, 180]. Some AQP6 is colocalized with H^+-ATPase [179].

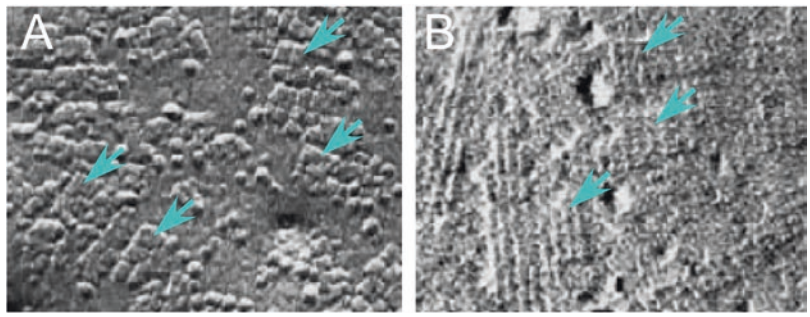

Fig. 9.2 Freeze-fracture electron micrographs of collecting duct principal cell basolateral plasma membranes. (**a**) OAPs in the basolateral membrane P-face of a collecting duct principal cell. (**b**) E-face of the basolateral plasma membrane of a collecting duct principal cell showing the appearance of imprints left in this membrane leaflet by the P-face OAP arrays (Data cited from Ref. [169])

9.1.7 AQP7

AQP7, an aquaglyceroporin, is expressed on the apical membrane of the proximal straight tubules (S3 segment) where AQP1 is also expressed [55, 107].

9.1.8 AQP8

Whether AQP8 is expressed in the kidney is controversial. Elkjær *et al.* first reported that AQP8 was expressed in proximal tubule cells. But ultrastructural localization of AQP8 remains unresolved [32].

9.1.9 AQP9

AQP9, which is another aquaglyceroporin, has been only found in the urinary concentration system of birds [115]. In mammals, AQP9 is present in leukocytes that prevents the transformation of leukocytes when flow through high osmotic environment in the inner medulla [145].

9.1.10 AQP11

AQP11 is an unorthodox AQP. In the kidney, AQP11 is expressed intracellularly in the proximal tubules [3, 104]. AQP11 was identified in ER by fluorescent labeling with ER marker in AQP11-transfected cultured cells and in the kidney from HA-tagged AQP11-transgenic mice [52, 54, 91].

There still may be additional, as yet unidentified, AQPs in kidney and locations of some AQPs are not clear, which will be confirmed in the future.

9.2 Functions of AQPs in Urinary System

9.2.1 AQP1

To evaluate the role of AQP1 in the proximal tubule, Verkman's group generated AQP1 knockout mice by targeted gene disruption [85, 137, 159, 160, 170, 172]. The AQP1 null mice were not obviously different from wild-type mice in survival, gross physical appearance, and organ morphology, except for mild growth retardation.

The measurement of the transepithelial osmotic water permeability (P_f) in S2 segments of proximal tubule showed that P_f in AQP1 null mice was five-fold lower than that in wild-type mice, which indicates that osmolality driven transcellular water permeability is through AQP1. In purified apical plasma membrane vesicles of proximal tubule, P_f (at 10 °C) decreased ninefold in AQP1 null mice compared with wild-type mice. The remaining low water permeability in AQP1 null proximal tubule was not been inhibited by mercurial agents. Furthermore, an intrinsic membrane P_f of approximately 0.006 cm/s (at 37 °C) in AQP1 null proximal tubule, which is similar with water movement exclusively across the lipid portion of the membrane, suggesting that other AQP-type water channel and non-aquaporin transporters play little role in proximal tubule water permeability. Less than 20% of osmotically driven transepithelial water transport in the proximal tubule is paracellular [158].

The higher urine output demonstrates that AQP1 null mice have reduced fluid absorption in the collecting duct. The deletion of AQP1 in the TDLH and DVR likely resulted in a defective countercurrent mechanism, which prevents the formation of a hyperosmolar medullary interstitium. In water-deprived AQP1 null mice, dDAVP stimulation of collecting duct water permeability (that should nearly equalize urinary and medullary interstitial osmolality) did not increase urinary osmolality [15, 85]. Because salt transporters are functional and the collecting duct can be water permeable, AQP1 null mice can mildly concentrate urine.

Ex vivo perfused segments of TDLH were used to study the contribution of AQP1 to TDLH water permeability [19]. P_f was significantly lower in AQP1 null TDLH, indicating that the main water channel in TDLH is AQP1, which also reveals that osmotic equilibration along the TDLH mediated by water transport makes contribution to the renal countercurrent concentrating mechanism.

There is a long-standing controversy regarding the relative contributions of water reabsorption and solute entry to osmotic equilibration along the TDLH [103]. AQP1 null mice showed urine-concentrating defect and a decrease in TDLH water permeability indicating that high water permeability in the TDLH plays an important role in urine concentration. AQP1 deletion does not affect the NaCl and urea permeabilities of the TDLH, so that osmotic water transport out of the lumen of the TDLH is important for the countercurrent multiplication mechanism and that solute entry by itself is not sufficient to permit the formation of maximally concentrated urine [158].

Average body weight decreased by 35% in the AQP1 null mice compared with 20~22% in wild-type mice, and blood osmolality increased to 517 mosm/kg H_2O compared with 311~325 mosm/kg H_2O in wild-type mice after being deprived of water for 36 h. Interestingly, nearly all water-deprived AQP1 null mice that were markedly hyperosmolar could be resuscitated by oral water administration without morbidity [85].

The urine osmolality in AQP1 null mice was similar before and after water deprivation. Measurement of urine osmolalities of the AQP1 null mice every 8 h showed values were consistent less than 650 mosm/kg H_2O because the osmolality gradient could not be established without AQP1 (Fig. 9.3). In contrast, there was a significant increase in the urine osmolality (from 1400 mosm/kg H_2O to 3000 mosm/kg H_2O) in wild-type mice after water deprivation. The urine sodium was less than 10 mM in most of the water-deprived AQP1 null mice. dDAVP, a V2 receptor agonist, treatment did not increase the urine osmolality in AQP1 null mice, indicating urinary concentrating defect in AQP1 null mice was not central osmoreceptor sensing [85]. The reason of urine concentrating deficiency in AQP1 null mice can mainly be the impairment of near-isosmolar water reabsorption in proximal tubule and disruption of the medullary countercurrent multiplication mechanism [58, 69, 85].

9.2.2 AQP2

In the basal state, AQP2 is mainly localized in the intracellular vesicles in collecting duct epithelial cells. Upon stimulation with ADH, AQP2 is translocated from the intracellular compartment to the apical plasma membrane by exocytic fusion of AQP2-bearing vesicles [144]. Water permeability of the apical membrane is regulated by the trafficking of AQP2 to the apical membrane [13, 14, 33, 64, 116, 152, 168, 184]. Since AQP3 and AQP4 are constitutively present at the basolateral membrane, when AQP2 appears at the apical membrane, water is easily reabsorbed by passing through the principal cell layer trans-

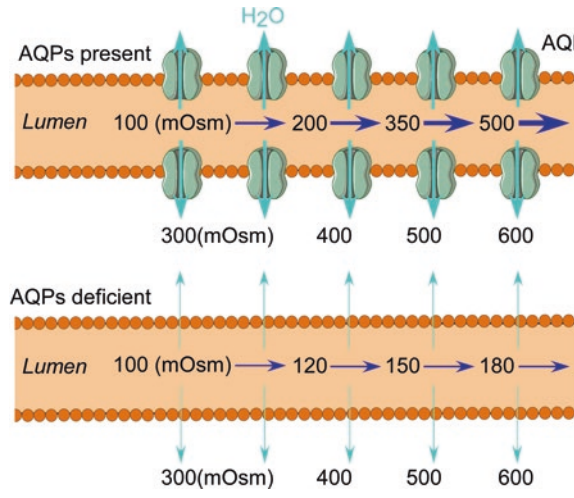

Fig. 9.3 Osmolality gradient in or outside the lumen with or without AQP. *Top*, the osmolality gradient in the lumen was established by water reabsorbing via AQP. *Bottom*, the osmolality gradient could not be established without AQP

cellularly [146]. The physiological importance of AQP2 in urine concentration is obvious considering patients with NDI caused by AQP2 mutation [11, 26]. It was also reported that selective deletion of AQP2 in the mouse collecting duct caused severe urinary concentration defect and mice with global AQP2 gene knockout died within 2 weeks because of serious dehydration [91, 130]. These results suggest that AQP2 is a critical water channel in the kidney.

Dehydration or hypernatremia increases the level of the arginine vasopressin (AVP), which binds to the vasopressin V2 receptor at the basolateral membrane, and activates protein kinase A (PKA) by elevating the cAMP level [8, 10, 38, 72, 129]. This triggers the phosphorylation of Ser256 in the AQP2 C terminus by PKA and flags the protein for trafficking from storage vesicles to the apical membrane [23, 63, 99, 100, 106, 108, 121, 135, 154, 162, 164, 181].

The phosphorylation of AQP2 at Ser 256 is critical in its targeting to the apical cell surface by AVP stimulation [16, 42, 146]. Other possible sites of phosphorylation such as Ser 261, 264, and 269 may also affect the trafficking of AQP2 [18, 34, 46, 120]. Phosphoproteomics analysis of rat inner medullary collecting duct cells revealed AQP2 phosphorylation at Ser 261 [48, 128]. ADH induced an increase in monophosphorylation at Ser 256 and diphosphorylation at Ser 256 and 261, suggesting that phosphorylation of both sites is involved in AQP2 trafficking [49]. Immunofluorescence microscopy showed that AQP2 phosphorylated at Ser 261 was mainly localized intracellularly and distinct from the endoplasmic reticulum, Golgi apparatus, and lysosomes [47]. Point mutation analysis of Ser 261 indicates that the phosphorylation state of AQP2 at Ser 261 does not detectably affect the regulated or constitutive trafficking of AQP2 [80].

Because of the redistribution of AQP2, transcellular water permeability increases and urine is concentrated. When returning to normal condition, AQP2 is internalized through ubiquitin-mediated endocytosis and restored in intracellular vesicles or targeted for degradation [38].

9.2.3 AQP3

AQP3 null mice had normal perinatal survival and postnatal growth but were remarkably polyuric, consuming and excreting ten-fold more fluid than wild-type mice [159]. Average urine osmolality in AQP3 null mice (262 mosm/kg H_2O) was much lower than that in wild-type mice (1270 mosm/kg H_2O). Urine osmolalities in the AQP3 null mice increased significantly in response to dDAVP administration and to a 36 h water deprivation, although to a much less extent than that in wild-type mice. These results define a unique pattern of NDI in AQP3 null mice, which confirms that countercurrent exchange in AQP3 null mice is basically intact, although medullary interstitial osmolalities are probably lower than that in wild-type mice because of diuresis washout. A study on AQP1 and AQP3 double knockout mice demonstrated the different patterns of NDI result from distinct defects in countercurrent exchange (AQP1) and collecting duct function (AQP3) [173].

When P_f was measured in basolateral membrane of cortical collecting ducts using spatial filtering microscopy, the volume of cortical collecting ducts from AQP3 null mice changed slowly with a half-time ($t_{1/2}$) for osmotic equilibration of 2.7 s, which is significantly slower than that from wild-type mice (1.1 s). Because of the underestimation of the rate in tubules from wild-type mice by the finite solution exchange time in the system, AQP3 deletion might decrease the water permeability of the basolateral membrane of cortical collecting duct more than threefold [83]. The experimental results on urinary concentrating defect of the AQP3 null mice indicates that AQP3 plays an important role in the formation of concentrated urine by transporting water across the basolateral membrane of collecting duct epithelium [83].

9.2.4 AQP4

The role of AQP4 in urinary concentration was evaluated using AQP4 null mouse model [84, 156]. No significant difference in urine osmolal-

ity was found in hydrated mice. Serum sodium concentrations and osmolalities were similar in the two genotypes. However, after a 36 h water deprivation, the maximum urinary osmolality in the AQP4 null mice is lower than that in the wild-type mice, indicating a mild urinary concentrating defect in the AQP4 null mice [20, 84, 176].

Transepithelial P_f in *ex vivo* perfused IMCD after 18–48 h of water deprivation and in the presence of vasopressin to make the basolateral membrane P_f rate-limiting are 0.056 cm/s (wild-type) and 0.013 cm/s (AQP4 null), which indicates that AQP4 is responsible for the majority of basolateral membrane water movement in the IMCD.

Although the AQP4 null mice showed greatly reduced IMCD water permeability, there was only a very mild defect in urine-concentrating ability [158]. This is consistent with expectations based on the normal distribution of water transport along the collecting duct. Micropuncture studies of rodents under antidiuretic conditions demonstrate that the main amount of water was reabsorbed in the cortical portion rather than the medulla of the collecting duct [62].

9.2.5 AQP5

AQP5 null mice shows normal renal function as wild-type mice [148]. Further studies are required to uncover an unexpected role for AQP5 in the kidney.

9.2.6 AQP6

Although AQP6 is classified as a classical AQP, it differs from other AQPs. For example, its water and anion permeability is increased by the presence of the well-known AQP inhibitor Hg^{2+} and in acidic condition [2, 45, 50, 125]. These results suggest that AQP6 might be involved in acid secretion in the collecting duct.

However, the role of this transport in the renal tubules is still not clear. It is widely known, that the intercalated cells are characterized by a rich inclusion of mitochondria, which provide energy for the cells necessary for proper function [118]. In these cells there are intracellular vesicles containing H^+ ATPase to transport proton [125]. AQP6 is also localized in the same vesicles. In spite of that several studies have demonstrated that the H^+ ATPase is shuttled from the cytoplasmic vesicles to the apical plasma membrane in response to acid-base changes, AQP6 was not found in plasma membrane of the intercalated cells [86, 126]. Lack of AQP6 in the apical plasma membrane indicates that this protein must function exclusively at the intracellular sites. The accumulating results suggest the possibility that AQP6 may not play a direct role in simple fluid transport, but may do so in maintaining the acid-base balance in cellular regulation [53, 60]. The mechanism of this process, however, has not been explained yet.

9.2.7 AQP7

Compared with the wild-type mice ($20*10^{-3}$ cm/s), P_f of the vesicles obtained from the outer medulla of AQP7 null mice ($18*10^{-3}$ cm/s) significantly decreased [139]. These results indicate that AQP7 makes a small contribution to the water permeability of the proximal straight tubules [139]. Based results from AQP1/AQP7 double knockout mice, the estimated contribution of AQP7 to the water permeability in the proximal straight tubules is one-eighth that of AQP1 [85].

AQP7 null mice do not show a urinary concentrating defect. But under normal conditions urine volume increase significantly in AQP1/AQP7 double-knockout mice compared with AQP1 knockout mice. (AQP1/AQP7 knockout, 7.3 ml *vs.* AQP1 knockout, 5.7 ml). This validates that the amount of water reabsorbed through the effect of AQP7 in the proximal straight tubules is physiologically substantial.

The serum glycerol level in AQP7 knockout mice was mildly lower than that of wild-type mice (AQP7 null, 0.036 mg/ml *vs.* wild-type, 0.042 mg/ml). However, the glycerol concentra-

tion in urine in AQP7 null mice was much higher than that in wild-type mice (AQP7 null, 1.7 mg/ml *vs.* wild-type, 0.005 mg/ml), indicating that AQP7 mediates the glycerol reabsorption in the proximal straight tubules [77, 87, 139, 140]. Although AQP7 plays a minor role in water transport, AQP7 constitutes a major glycerol-reabsorbing pathway in the kidney.

AQP7 has been reported permeable to ammonia [43, 78]. However, the role of this transport remains partly unexplained. It is generally known that there is a process of glutamine metabolism in the proximal tubule resulting in production of HCO_3^- and NH_4^+, which are then excreted into tubular fluid. Some NH_4^+ may exit from the proximal tubule cells and enter to the tubular fluid as NH_3, where it is then protonated [70]. AQP7 may be involved in the secretion of NH_3 or/and NH_4^+, which regulates ammonia concentration on both sides of the membrane in a shorter time.

9.2.8 AQP8

The AQP8 transcript was found in kidney by RT-PCR analysis. Urine osmolality does not differ in AQP8 null mice *vs.* wild-type mice at baseline or after 36 h water deprivation [175]. The urine osmolality is not significantly different even between AQP1 null mice and mice lacking AQP8 and AQP1 together. These results indicate that AQP8 does not play a fundamental role in urinary concentrating function. Some studies reported that AQP8 transport ammonia in the kidney [57, 78, 134, 141]. Molinas *et al.* showed AQP8 knockdown in human renal proximal tubule cell line HK-2 cells decreased ammonia release into culture medium and AQP8 was upregulated in an acidic medium [91, 102].

9.2.9 AQP11

The AQP11 null mice start dying within 2 weeks due to severe renal failure suggesting that this isoform is of fundamental importance [104].

9.3 AQPs in Renal Diseases

9.3.1 AQP1

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common human monogenic diseases, which morbidity is between 1 in 400–1000 worldwide, characterized by massive enlargement of fluid-filled cysts of renal tubular origin. [163]. Renal cyst development in ADPKD is associated with abnormal proliferation and cystic liquid secretion of cystic epithelial cells, mediated by complicated mechanisms. AQP1 is expressed in the epithelia lining 71% renal cysts in human ADPKD, 44% of which are derived from the proximal tubules [4]. Two-thirds of the cysts express either AQP1 or renal collecting duct water channel AQP2 [27, 28].

It is found that AQP1 retard renal cyst development [163]. Kidney size and cyst number are significantly greater in AQP1 null PKD mice than in AQP1-expressing PKD mice (Fig. 9.4a), due to the presence of a greater number of proximal tubule cysts. MDCK cells form cysts with a monolayer of polarized cells enclosing a central lumen under forskolin stimulation. Remarkably, stable AQP1-overexpressing MDCK cells formed cyst-like cell clusters that had no discernible lumens (Fig. 9.4b). AQP1 overexpression decreases β-catenin and cyclin D1 expression, suggesting the down-regulation of Wnt signaling pathway. Phosphorylated-β-catenin, the inactive form of β-catenin, is up-regulated in AQP1 overexpressing MDCK cells, suggesting that AQP1 promotes β-catenin degradation.

Coimmunoprecipitation reveals the interaction of AQP1 with β-catenin, GSK3β, LRP6 and Axin1. Subcellular fractionation experiment shows that β-catenin, GSK3β and Axin1 coexisted in both cytosolic fraction and membrane fraction, while LRP6 and AQP1 are only detected in the membrane fraction (Fig. 9.4c). It is hypothesized that part of the "destruction signaling complex" is located in cell membrane. AQP1 interacts with "destruction signaling complex" and stabilizes the "complex" on the plasma membrane (Fig. 9.4d). Absence of AQP1 could

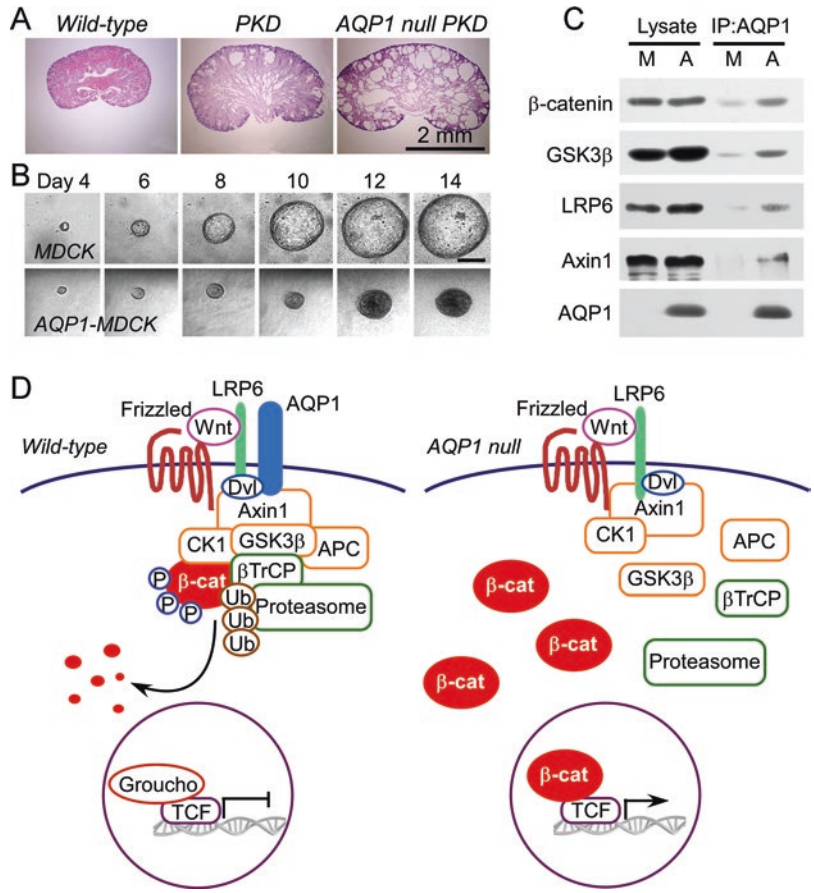

Fig. 9.4 AQP1 retards renal cyst development in polycystic kidney disease. (**a**) Representative images of wild-type, PKD and AQP1 null PKD kidneys. (**b**) Light micrographs of cysts on the days 4–14 formed by non-transfected MDCK and AQP1-MDCK cells. (**c**) Coimmunoprecipitation with anti-AQP1 showing AQP1 interaction with β-catenin, GSK3b, LRP6 and Axin1 in AQP1-MDCK cells. (**d**) Schematic of proposed β-catenin regulation by AQP1 (Data cited from Ref. [163])

decrease the stability of the destruction complex and block the ubiquitination of β-catenin, which leads to β-catenin accumulation and translocation into the nucleus and binding to TCF. The β-catenin/TCF complex upregulates expression of Wnt target genes. Therefore, AQP1 may act as a novel determinant in renal cyst development through inhibiting the Wnt signaling pathway by an AQP1-macromolecular signaling complex.

AQP1 has been shown to be involved in numerous types of tumors, especially those arising from organs where water permeability plays an important role, such as kidney and bladder [66, 79, 105]. AQP1 has been shown to enhance cell growth and migration that plays an important role in tumor angiogenesis [94, 132]. Because of its heterogeneous distribution and expression, AQP1 can not be used as a prognostic factor but as an excellent biomarker [93, 96].

9.3.2 AQP2

A decrease of the AQP2 expression level is often caused by lithium therapy and results in the onset of NDI that is a disorder characterized by the inability to concentrate urine in response to ADH in the kidney collecting ducts [9, 89]. In addition, the mutation of AQP2 is responsible for congenital NDI [12, 17, 29, 40, 51, 68, 73, 75, 101, 110, 122, 128, 138, 147, 174]. Some autosomal recessive and autosomal dominant AQP2 mutations have been found. In the recessive type, AQP2 lose their water channel function, or are misrouted to the endoplasmic reticulum because of mutation [24, 61, 117, 151]. In the dominant type, mutated AQP2 is localized in aberrant intracellular compartments such as the Golgi apparatus, late endosomes, lysosomes, or the basolateral membrane.

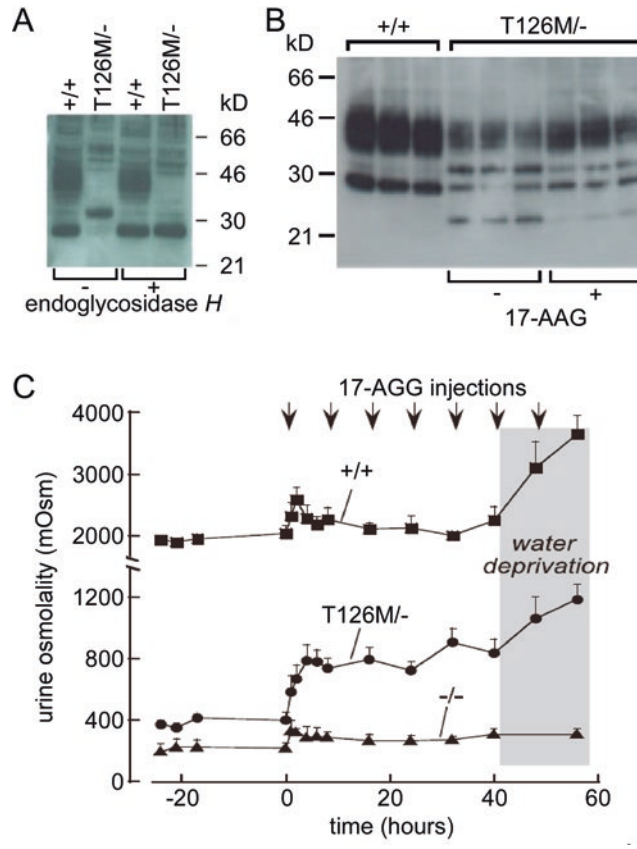

Fig. 9.5 17-AAG partially corrects defective urinary concentrating function in AQP2 mutant mice. (**a**) Immunoblot of AQP2 protein from wild-type (+/+) and AQP2-T126M mutant (T126M/−) mice. (**b**) AQP2 immunoblot of kidney homogenates from wild-type and AQP2-T126M mutant mice treated without or with 17-AAG. (**c**) Urine osmolality in wild-type, AQP2-T126M mutant, and AQP2 null (−/−) mice before and after 17-AAG treatment (Data cited from Ref. [178])

The inducible adult mouse model of human recessive NDI was generated by AQP2-T126M gene mutation and suitable as an *in vivo* model to study candidate AQP2 therapies [136, 171, 177]. A band of 34~40 kDa protein was detected in the whole-kidney of wild-type mice by immunoblot analysis (Fig. 9.5a), which represents fully processed AQP2 with complex glycosylation. A 29 kDa nonglycosylated AQP2 band is also observed. The core-glycosylated form of AQP2-T126M was detected at 31 kDa and largely disappeared following endoglycosidase H treatment [178]. AQP2-T126M mutant mice show marked polyuria, which excretes sevenfold more urine than that in litter-matched wild-type mice. Following an 18 h water deprivation, urine osmolality in wild-type mice increase significantly (from 1840 to 2872 mosmol), not increased in AQP2 null mice, and increased partially (to 1027 mosmol) in AQP2-T126M mutant mice [178]. 17-AAG, an Hsp90 inhibitor, is a novel corrector that partially rescued defective AQP2-T126M cellular processing, indicating that its analogs or other Hsp90 inhibitors may be a potential drug for therapy of some forms of NDI [178] (Fig. 9.5b, c).

AQP2 expression is upregulated in rat kidney with congestive heart failure (CHF) induced by ligation of the left coronary artery [166]. Furthermore, there is a marked increase in the abundance of AQP2 in the apical plasma membrane, consistent with an increase in apical water permeability [114]. Hepatic cirrhosis is another clinically important syndrome associated with water retention. However, different animal models of hepatic cirrhosis showed varied AQP2 expression [35, 36, 41, 59].

9.3.3 AQP4

A clinical study among 45 children with unilateral ureteropelvic junction obstruction and 15 children undergoing nephrectomy for nephroblastoma, shows that the expression of AQP4 is

reduced in proportion with the impairment degree of renal function [76]. Among salt-sensitive hypertension patients, the expression of AQP4 is upregulated while AQP1 and AQP2 is downregulated [124].

9.3.4 AQP5

AQP5 has been found in all of 17 kidney biopsies from patients with diabetic nephropathy but not in 15 normal controls indicating that AQP5 is associated with diabetic nephropathy [165]. Upregulated AQP5 may contribute to polyuria, possibly by impairing AQP2 membrane localization and urine AQP5 is a potential novel biomarker of diabetic nephropathy [81].

9.3.5 AQP6

The expression of AQP6 changed with the development of renal cell carcinoma and oncocytoma and AQP6 could be a marker in renal cancer diagnosis [149, 182].

9.3.6 AQP11

Accurate analysis of the renal phenotype of AQP11 null mice showed that their kidneys are large, anemic and polycystic, which is similar with polycystic kidney diseases (PKD) in human. The cysts are absent in the medulla but abundant in the cortex where AQP11 is highly expressed, which differs from that in PKD.

Vacuolization of proximal tubule cells and enlarged lumen of the ER were also observed in AQP11 null mice [104]. In this process, the expression of ER-stress-responsive genes such as Hspa5 and Hsp90b1 is increased, and TUNEL-positive cells and cleaved caspase-3-positive cells are seen in vacuole-forming proximal tubule cells [119]. Furthermore, Ki-67 is positive and the expression of EGFR is detected in vacuolized cells, suggesting that there is ER stress in the proximal tubule cells, which results in apoptosis and finally in cellular proliferation [91]. These results suggest that deletion of AQP11 that is localized in ER would influence ER function and causes ER stress contributing to renal injury. Inoue *et al.* found that the amount of polycystin-1 increases, the amount of polycystin-2 decreases, primary cilia of the proximal tubules are elongated in AQP11 null mice, as seen in many renal cyst diseases [54]. ER dysfunction may cause the abnormal N-glycosylation of polycystin-1 and impaired trafficking to the primary cilia indicating that the lack of AQP11 resulted in impaired polycystin-1 then indirectly caused PKD [91]. However, there are still so many unresolved questions concerning the relationships between cystogenesis and AQP11. Further analyses are required to clarify these points and finally define the physiological function of AQP11.

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The Physiological Role and Regulation of Aquaporins in Teleost Germ Cells

10

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Abstract

The unicellular germ cells and gametes of oviparous teleosts lack the osmoregulatory organs present in juveniles and adults, yet during development and particularly at spawning, they face tremendous osmotic challenges when released into the external aquatic environment. Increasing evidence suggests that transmembrane water channels (aquaporins) evolved to play vital adaptive roles that mitigate the osmotic and oxidative stress problems of the developing oocytes, embryos and spermatozoa. In this chapter, we provide a short overview of the diversity of the aquaporin superfamily in teleosts, and summarize the findings that uncovered a highly specific molecular regulation of aquaporins during oogenesis and spermatogenesis. We further review the multiple functions that these channels play during the establishment of egg buoyancy and the activation and detoxification of spermatozoa in the marine environment.

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Keywords

Aquaporin • Oocyte • Egg • Sperm • Hydration • Motility • Oxidative stress • Mitochondria

Abbreviations

AQP/Aqp	aquaporin
CNGK	cyclic nucleotide-gated K^+ channel
FAA	free amino acid
IgG	immunoglobulin
LvH	lipovitellin heavy chain
LvL	lipovitellin light chain
MAPK	mitogen activated protein kinase
PKA	protein kinase A
ROS	reactive oxygen species
SAC	stretch-activated channel
Sox	Sry-related high mobility group [HMG]-box
TEA	tetraethylammonium
TRPV	transient receptor potential cation channel subfamily V
Vtg	vitellogenin

10.1 Introduction

Teleost fishes that inhabit marine and freshwater environments are constantly exposed to opposing osmotic gradients that respectively lead to the passive influx or efflux of water and the efflux or influx of ions. These hydromineral perturbances are compensated for by the coordinated control of osmoregulatory organs that mediate the uptake or excretion of water and minerals in order to maintain homeosmotic balance [1]. This situation, however, is extremely challenging for the newly released gametes and embryos of oviparous teleosts, since they are devoid of the juvenile and adult organs that deal with ion and water balance. It therefore seems likely that molecular adaptations have evolved in the reproductive organs and germ cells of these fishes to cope with the external osmotic challenges, and thereby maintain the ability of the gametes to form a zygote that develops into a normal embryo and larva.

In recent years, a growing body of evidence is uncovering the role of transmembrane water channels (aquaporins) that facilitate transepithelial fluid transport in osmoregulatory organs of fishes, such as the gills, intestine or kidney [2, 3]. In marine teleosts, aquaporins have also been shown to play important roles in male and female gametes. This research is revealing unexpected and highly specific aquaporin regulatory mechanisms in germ cells, as well as the function of some of these channels beyond water transport. An overview of the physiological roles and molecular regulation of aquaporins in the oocytes and spermatozoa of marine teleosts is the focus of this chapter.

10.2 Aquaporin Diversity in Teleosts

To understand the physiological roles played by aquaporins in teleost germ cells, it is important to identify the gene copy numbers and establish a framework for their affiliations and functions both within and between the different lineages. This can best be achieved using a phylogenomic approach, which computationally analyses the complete genomic repertoires of the superfamily present in the different species. If sufficient taxonomic diversity is explored, it becomes possible to uncover the evolutionary history of each gene. When these data are combined with experimental evidence for the channel permeation properties, a putative molecular function can be tentatively assigned to each aquaporin subfamily. For teleosts, this approach was first adopted by Tingaud-Sequeira and colleagues [4] who identified 18 aquaporin genes in zebrafish (*Danio rerio*) that are related to 4 aquaporin grades present in eutherian mammals, the classical aquaporins (AQP0, -1, -2, -4, -5 and -6), the aquaporin-8-type channels (AQP8), the aquaglyceroporins (AQP3, -7,

-9 and -10) and the unorthodox aquaporins (AQP11 and -12). These data not only revealed that teleost genomes encode the largest repertoires of functional aquaporins in vertebrates, but that the channels display dual paralogy and substrate specificities similar to the mammalian counterparts [2, 4, 5]. As for the mammalian channels, the teleost classical aquaporins are typically water selective, while the aquaglyceroporins transport water, urea and glycerol, as well as a range of other small molecules [5]. In contrast to the mammalian AQP8 channels, however, some teleost Aqp8 orthologs, such as Atlantic salmon (*Salmo salar*) Aqp8ab and -8bb, are also capable of transporting glycerol in addition to water and urea [6]. The permeability properties of the teleost unorthodox aquaporins have yet to be established, as they are not functional when heterologously expressed in *Xenopus laevis* oocytes [4].

At the time the zebrafish channels were examined, five genomes from four teleost orders: Cypriniformes (zebrafish), Beloniformes (medaka, *Oryzias latipes*), Perciformes (3-spined stickleback, *Gasterosteus aculeatus*) and Tetraodontiformes (torafugu, *Takifugu rubripes*, and spotted green pufferfish, *Tetraodon nigroviridis*) were analyzed. More recently these analyses were expanded to 28 species of teleost from 13 orders and 18 families that span >300 million years of evolution, confirming that teleost genomes indeed retain the largest copy number of aquaporins in the vertebrate lineage, with the tetraploid Atlantic salmon encoding at least 42 paralogs [7, 8]. The comprehensive data sets further uncovered entirely new aquaporin subfamilies (Aqp14, -15 and -16), revealing that the vertebrate complement consists of 17 subfamilies (Aqp0-16), and that the 4 grades of aquaporin are likely an ancient feature of eukaryotic organisms [9].

New sequencing initiatives continue to provide novel insights to the aquaporin superfamily in teleosts. Recent whole genome sequencing projects include the tetraploid common carp (*Cyprinus carpio*) [10] and diploid European seabass (*Dicentrarchus labrax*) [11]. In the latter case, the aquaporin superfamily was highlighted as an example of an expanded gene family specifically associated with ion and water regulation and adaptation to eurohalinity [11]. As in the initial report on zebrafish [4], 18 aquaporin genes were identified in the seabass genome, which seemingly lacked the *aqp5/1* ortholog described by Zapater et al. [12], but retained 4 copies of *aqp8* [11]. In the same year as the common carp and European seabass genomes were published, the *aqp5/1* gene was identified in selected vertebrates, including sharks, gars, teleosts, turtles and alligators, and was re-annotated as *aqp15* [7].

To illustrate the diversity of aquaporins found in teleosts, we have here assembled the repertoires present in the common carp genome and compared them to recompiled data for zebrafish and European seabass (Fig. 10.1). These data show that zebrafish has 19 paralogs with *aqp15* as a putative pseudogene, while the common carp has 38 paralogs of which 4 are apparent pseudogenes as judged by premature stop codons. The carp data thus precisely reflect duplicated copies of all orthologs in zebrafish, and validate the absence of teleost-specific duplicates such as *aqp4a* and *-11a* in cypriniform fishes. Interestingly, one of the putative carp pseudogenes is a duplicate of *aqp7* (*aqp7-2*), which is only found as a single copy gene in other teleosts, including the tetraploid Atlantic salmon [7, 8]. Considering that the R4 paleotetraploidization in the ancestor of the common carp occurred <10 million years ago [10], the present data indicate that redundant *aqp7* genes are consistently negatively selected through evolutionary time. This contrasts with the positive selection of Aqp0 channels respectively duplicated 320–350 and 80–100 million years earlier in diploid teleosts and tetraploid salmonids, due to their neofunctionalisation and requirement for lens development and transparency [13–17]. The tree presented in Fig. 10.1 further shows that the European seabass genome in fact encodes 22 aquaporin paralogs with 1 putative pseudogene (not shown). The genomic repertoire of European seabass also includes an *aqp15* ortholog, but as in other acanthomorph species, no more than 3 *aqp8* paralogs. The discrepancy between the present data and the earlier analysis of Tine and colleagues [11] can be explained by the

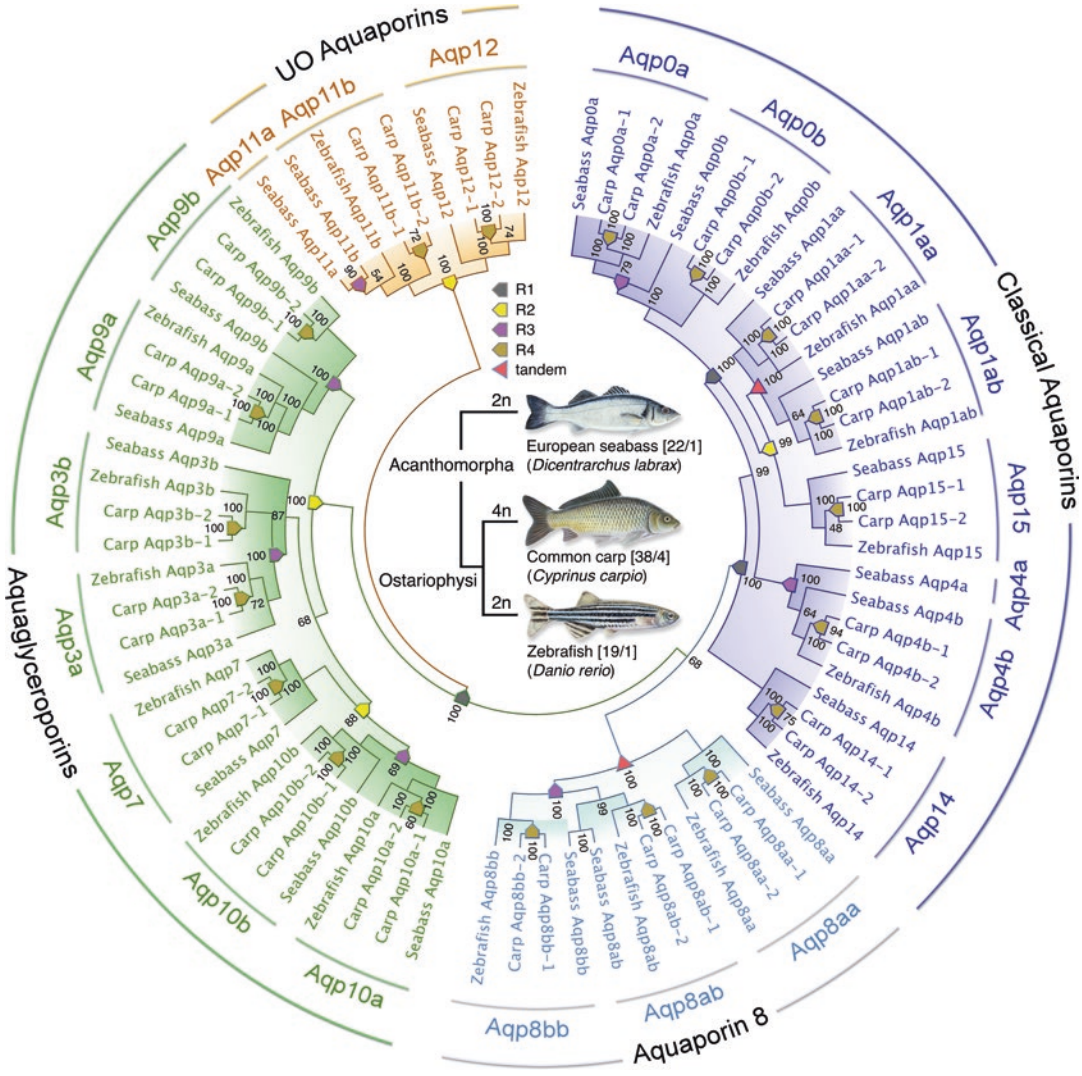

Fig. 10.1 The aquaporin superfamily of teleosts. The channel nomenclature is in accordance with conventions for genomic (-a or -b) and tandem (-aa, -ab, -ba, or -bb) duplicates [2, 4, 5, 7]. The tree is mid-point rooted and generated based upon maximum likelihood analysis of a codon alignment of the assembled transcripts from diploid (2n) and tetraploid (4n) species. Gene origins by serial rounds of whole genome duplication (R1, R2, R3, R4), or tandem duplication are indicated. The genomic number of paralogs/pseudogenes for each species is indicted in square brackets. Bayesian posterior probabilities derived from one million MCMC generations are shown at each node. The aquaporin gene copy for zebrafish is based on Tingaud-Sequeira et al. [4] and the current version of ensembl (v86), which has removed an identical duplicate of the *aqp8bb* gene previously reported by Finn and colleagues [7]

incorrect assignment of the *aqp14* gene as an *aqp8* paralog.

It is well established that the ancestor of extant teleosts experienced an additional round of whole genome duplication (R3) compared to other vertebrates [18–25]. It is also well established that rediploidization typically ensues the polyploidy event, such that many genes are lost through evolutionary time [26–29]. Since current estimates of duplicate gene retention from the teleost-specific genome duplication indicate that 12–24% have survived in modern taxa [30], the high copy

number of teleost aquaporins is intriguing, because it implies strong positive selection of the duplicates, regardless of the mechanism (tandem or genomic) by which they duplicated. Indeed, a direct comparison of the teleost aquaporin complement with the orthologous counterparts in metatherian mammals (AQP0, -1, -3, -4, -7, -8, -9, -10, -11, -12, and -14) shows that 8 out of these 11 subfamilies (73%) are retained as duplicates in teleosts. Only *aqp7*, *-12* and *-14* are found as single copy genes in diploid teleosts, while the *aqp8* subfamily has specifically expanded in the actinopterygian (ray-finned) fishes resulting in up to 4 paralogs in some diploid species, such as zebrafish, 6 in the tetraploid common carp, and 8 in other tetraploid species, such as Atlantic salmon [7, 8]. Despite the increased abundance of water channel genes in teleosts, it has been shown that they lack orthologs of AQP2, -5 or -6, which evolved to play vital roles in the water conservation of extant amphibians, sauropsids and mammals [7, 31–35]. It has therefore been proposed that the differential retention of AQP2, -5 and -6 orthologs in sarcopterygian (lobe-finned) animals represented a permissive condition that facilitated tetrapod terrestrial adaptation [7]. It thus seems reasonable to conclude that the aquatic or terrestrial environment in which vertebrates evolved represented an important selective force in determining the copy number and retention of aquaporins in actinopterygian and sarcopterygian animals. A current challenge is deciphering what roles the increased repertoires of aquaporins play in the vast array of teleost fishes. The next sections provide insight into the molecular function and physiological roles of some of the aquaporin channels in the germ cells and gametes of teleosts.

10.3 Aquaporins in the Female Gamete

10.3.1 The Hydration Process of Pelagic and Benthic Eggs

As an adaptation to the hyperosmotic condition of seawater, ovarian oocytes of oviparous marine teleosts hydrate during meiosis resumption (oocyte maturation) prior to ovulation. Oocyte hydration is more pronounced in marine species producing buoyant eggs in sea water (pelagophils), where water can contribute >95% of the final egg weight, whereas the egg water content is lower (up to 85% in weight) in species that produce benthic, non-buoyant eggs (benthophils) [36, 37] (Fig. 10.2a). The mechanism of oocyte hydration provides a water reservoir for the embryo to compensate for the passive water efflux until osmoregulatory organs develop, and in some benthophil species possibly also facilitates the survival of early embryos exposed to transient periods of environmental desiccation [24, 36, 38]. The high water content of marine pelagic eggs also causes them to float thus giving rise to their pelagic nature, which increases the survival of developing embryos by allowing more efficient oxygen exchange and their dispersal in the ocean [24, 39].

In pelagophil teleosts, it is well established that oocyte hydration correlates with the proteolysis of yolk proteins, with the resulting organic osmolyte pool of free amino acids (FAA) providing much of the osmotic driving force for water influx into the oocyte [37, 40]. In benthophil species, where oocyte hydration is more modest than in pelagophils, limited proteolysis of yolk proteins occurs, resulting in the liberation of a small pool of FAAs [37, 40]. The oocyte yolk proteins are derived from circulating, liver-secreted vitellogenins (Vtgs), VtgAa, VtgAb and VtgC, which are incorporated in the oocyte by receptor-mediated endocytosis and subsequently cleaved in a multi-step acidification process into yolk proteins [24, 41–44]. The linear arrangement of the complete Vtg protein consists of five subdomains corresponding to the different yolk proteins stored in growing oocytes: a lipovitellin heavy chain (LvH), a phosphorylated serine-rich phosvitin, a lipovitellin light chain (LvL), and two Cys-rich C-terminal coding regions (β′ and CT), which are homologous to the mammalian von Willebrand factor type D domain (Fig. 10.2b). During oocyte maturation, the VtgAa LvH, LvL and phosvitins are differentially cleaved by specific proteases such as cathepsins,

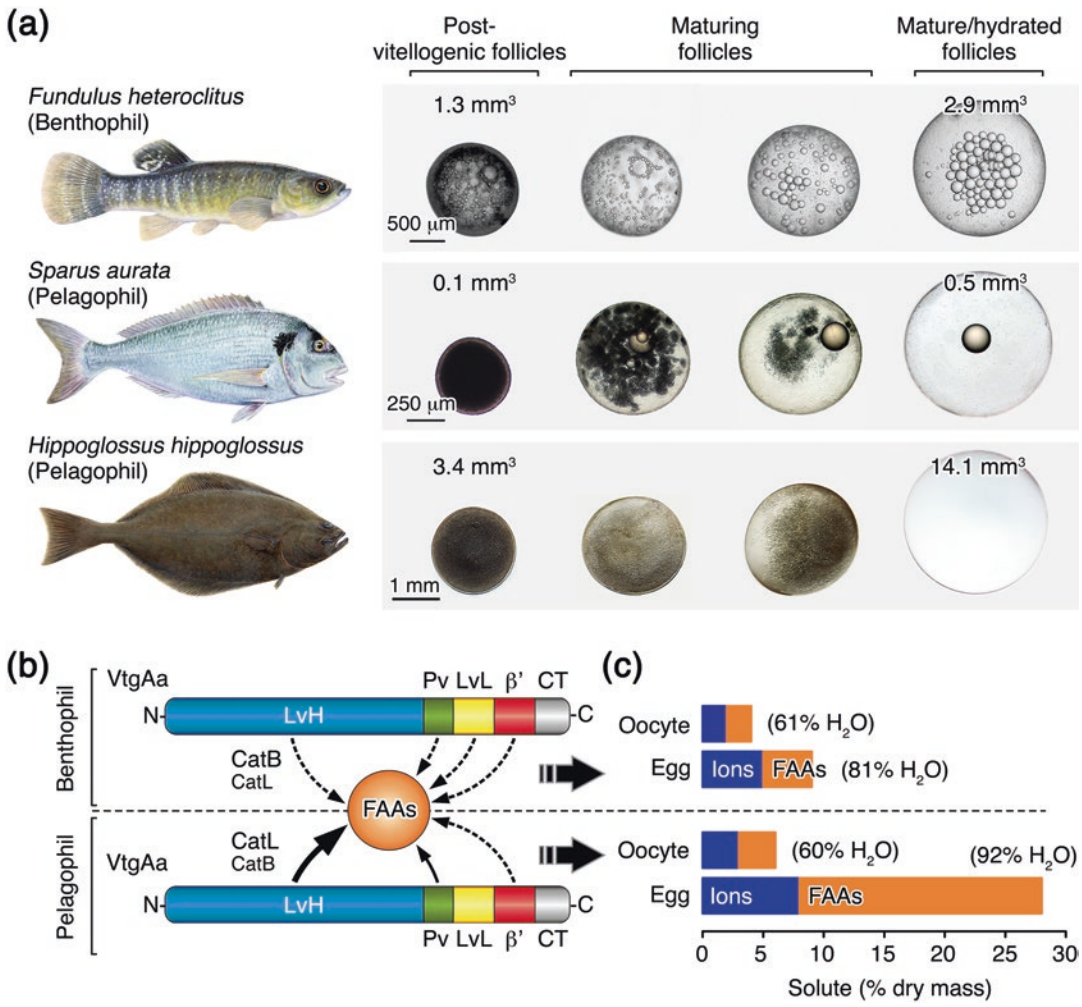

Fig. 10.2 Inorganic ions and free amino acids (FAAs) derived from the hydrolysis of yolk proteins are the major osmotic effectors for oocyte hydration in marine teleosts. (**a**) Follicle-enclosed oocytes from benthophil, common mummichog (*Fundulus heteroclitus*), and pelagophil, gilthead seabream (*Sparus aurata*) and Atlantic halibut (*Hippoglossus hippoglossus*), teleosts undergoing meiotic maturation and hydration. The volume of oocytes before and after hydration is indicated for each species. Parts of this figure were originally published in LaFleur et al. [49]. (**b**) Schematic diagram of a vitellogenin Aa molecule (VtgAa) showing the yolk protein subdomains lipovitellin heavy and light chains (LvH and LvL, respectively), phosvitin (Pv), β′ component (β′), and C-terminal coding region (CT), and their degradation during oocyte maturation by proteases, such as cathepsin B (CatB) and L (CatL), in benthophil and pelagophil teleosts. The relative contribution of each yolk protein to the FAA pool is indicated by arrows. (**c**) Relative ion, FAA and water content in prematurational oocytes and unfertilized eggs from benthophil and pelagophil species

whereas the VtgAb remains predominantly intact as the major yolk protein for embryonic development [45–47]. However, while in most pelagophil teleosts the VtgAa LvH is extensively degraded, this molecule is only partially hydrolized in moronid and benthophil species [24, 46–54] (Fig. 10.2b). Thus, the FAAs resulting from these proteolytic mechanisms, together with the specific accumulation of inorganic ions such as K^+, Cl^-, NH_4^+ during oocyte maturation, as well as of Pi arising from the dephosphorylation of phosvitins, represent the primary osmotic effectors in pelagophils, while inorganic ions such as Na^+ and K^+ seem to be the major osmolytes in

benthophils [37, 55–57] (Fig. 10.2c). Despite these observations, the mechanisms controlling and facilitating the flux of ions in teleost oocytes remain poorly understood.

10.3.2 Role of Aqp1ab During Oocyte Hydration

A number of studies in pelagophil teleosts, such as the gilthead seabream (*Sparus aurata*), Japanese eel (*Anguilla japonica*) and Atlantic halibut (*Hippoglossus hippoglossus*), have now demonstrated that the tandemly duplicated Aqp1 paralog, Aqp1ab (formerly named Aqp1o or Aqp1b), mediates the temporal water permeation and the pre-ovulatory swelling of the oocyte. In these species, *aqp1ab* transcripts are highly expressed in the ovary, and immunocytochemical studies using paralog-specific antibodies indicate that Aqp1ab polypeptides are first detected in the cytoplasm of vitellogenic oocytes, and not in the associated follicle cells, but as vitellogenesis advances, Aqp1ab is shuttled towards the oocyte cortex [12, 58–61] (Fig. 10.3a–h). In gilthead seabream and Atlantic halibut postvitellogenic oocytes, Aqp1ab is completely translocated to a thin layer just below the oocyte plasma membrane, and during meiotic maturation, approximately at the time when maximum proteolysis of yolk proteins and highest ion influx occur [59, 62], Aqp1ab is translocated further into the oocyte microvilli [12, 59] (Fig. 10.3d, e and h). This specific pattern of localization strongly suggested that this channel mediates water influx during oocyte hydration. In the Japanese eel, however, immunostaining analysis has shown that Aqp1ab is mainly observed around the fused yolk masses in oocytes at the migratory nucleus and mature stages (i.e. oocytes undergoing meiotic maturation), and insertion of Aqp1ab in the oocyte plasma membrane is not evident [60]. It has been hypothesized that the differential pattern of Aqp1ab staining in the maturing seabream and eel oocytes might be related to a different mechanism of oocyte hydration in each species [60]. Thus, in the Japanese eel oocyte hydration may be regulated by a two-step mechanism, in which water influx into the oocyte occurs by simple diffusion across the follicle and oocyte plasma membrane, and then into the yolk mass through Aqp1ab inserted in the membranes surrounding the yolk globules, resulting in the swelling of the yolk mass [60]. This hypothesis remains, however, to be demonstrated.

Nevertheless, in all pelagophil teleosts investigated to date it has been found that the swelling of oocytes during meiotic maturation *in vitro* is strongly reduced by mercury, a typical inhibitor of aquaporin water conductance, which is effective at blocking Aqp1ab-mediated water transport in *Xenopus laevis* oocytes [12, 58, 63, 64]. In the gilthead seabream, oocyte hydration *in vitro* is also inhibited to some extent by the quaternary ammonium cation tetraethylammonium (TEA), which also partially blocks the permeability of Aqp1ab and mammalian AQP1 when expressed in *X. laevis* oocytes [59, 65]. Since $HgCl_2$ and TEA can also potentially affect ion channels in ovarian follicles, a more specific inhibition of the Aqp1ab has been obtained in Atlantic halibut oocytes by using specific, affinity-purified antibodies [12]. In this species, follicle-enclosed oocytes undergoing hydration *in vivo* were microinjected with an Aqp1ab antibody, resulting in a dose-dependant inhibition of oocyte hydration, whereas the injection of an IgG control had no effect (Fig. 10.3i, k). Interestingly, the immunological inhibition of Aqp1ab did not affect yolk hydrolysis or meiosis resumption, and could be fully reversed by the artificial expression of the tandemly duplicated halibut paralog Aqp1aa, which is functional when over-expressed in halibut oocytes, but is not recognized by the antibody (Fig. 10.3j, k). These data indicate that the decrease of oocyte hydration of Atlantic halibut oocytes can be directly related to the loss of function of Aqp1ab, and thus provide functional evidence of the essential physiological role of this water channel in the hydration process of pelagic teleosts eggs.

The Aqp1ab paralog may also play a role in some freshwater species, such as the stinging catfish (*Heteropneustes fossilis*), in which oocytes partially hydrate during meiotic maturation although benthic eggs are produced [66]. In this

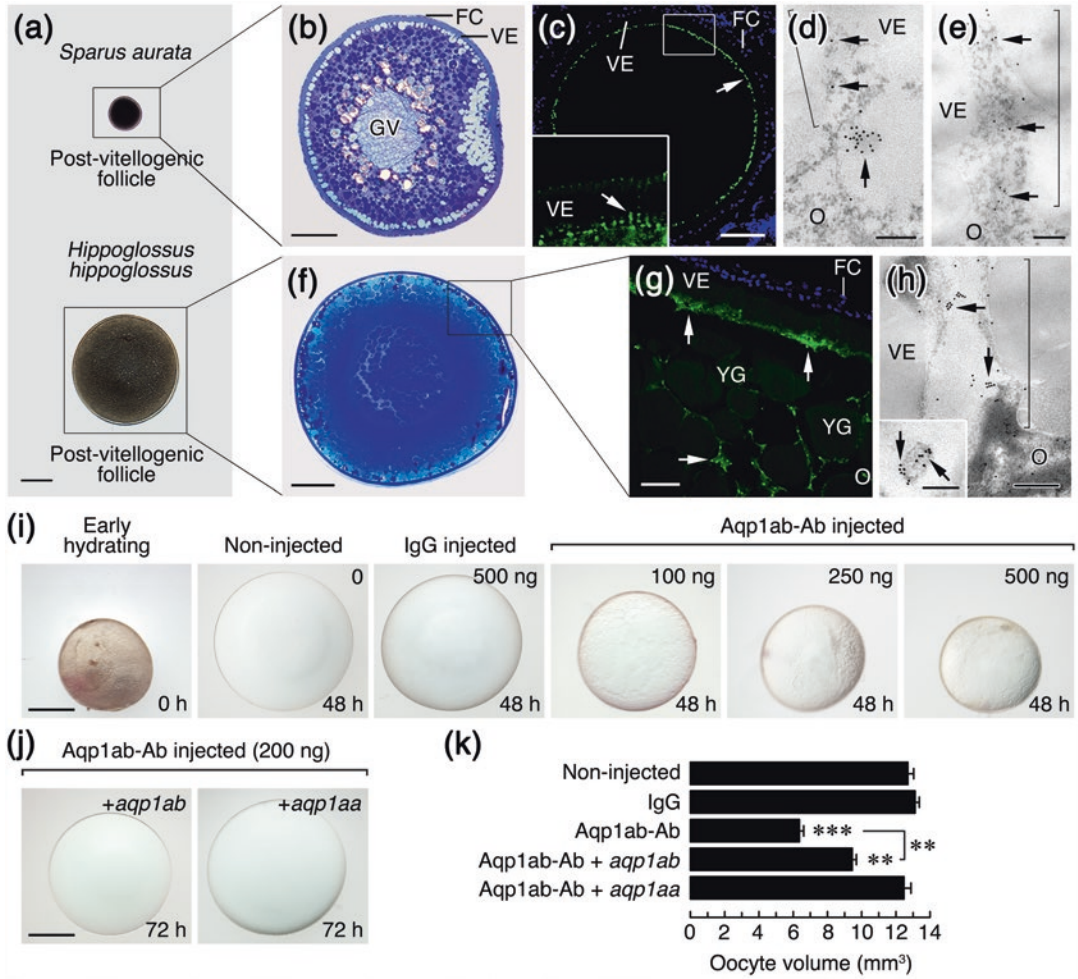

Fig. 10.3 The Aqp1ab paralog mediates oocyte hydration during meiotic maturation in pelagophil teleosts. (**a**, **b** and **f**) Photomicrographs of seabream and Atlantic halibut postvitellogenic follicles, and corresponding histological sections stained with methylene *blue* (which stains yolk). (**c** and **g**) Immunostaining of Aqp1ab (*arrows*) in seabream (**c** and inset) and Atlantic halibut (**g**) oocytes showing the accumulation of the channel close to the plasma membrane. During meiotic maturation (inset in **c**), the channel is translocated into the microvilli crossing the vitelline envelope. (**d**, **e** and **h**) Immunoelectron microscopy micrographs of seabream postvitellogenic oocytes (**d**) and of seabream and Atlantic halibut hydrating oocytes (**e** and **h**, respectively), in which Aqp1ab-positive immunogold particles are indicated by *black arrows*. In d, gold particles are localized within vesicles *below* the oocyte plasma membrane, while in *e* and *h* Aqp1ab appears predominantly in the microvilli extending from the oocyte (*indicated by brackets*). Inset in *h* shows a cross-section of a microvillus. (**i**) Inhibition of Aqp1ab in Atlantic halibut early hydrating oocytes, as a result of the microinjection of an Aqp1ab antibody (Aqp1ab-Ab), which reduces hydration at 48 h of culture *in vitro*. (**j**) Oocyte swelling is partly or fully recovered by the respective overexpression of *aqp1ab* and *-1aa*. (**k**) Inhibition of oocyte hydration (mean ± SEM; $n = 60$ follicles) with 200 ng of Aqp1ab-Ab, and partial or full recovery by coinjection with 25 ng of *aqp1ab* or *-1aa* cRNAs, respectively. $^{***}P < 0.001$; $^{**}P < 0.01$, with respect to non-injected oocytes or as indicated in brackets. *FC* follicle cells, *VE* vitelline envelope, *GV* germinal vesicle, *O* oocyte, *YG* yolk globule. Bars, 500 μm (**a**), 200 μm (**f**), 100 μm (**b** and **c**), 50 μm (**g**), 0.25 μm (**d**, **e** and **h**), 1 mm (**i** and **j**) (Adapted from Fabra et al. [58, 59] and Zapater et al. [12])

species, a functional Aqp1ab ortholog has been isolated from the ovary, in which mRNA is accumulated in response to human chorionic gonadotropin *in vitro* [67], but its function in oocytes is unknown. In atherinomorph species, such as the common mummichog (*Fundulus heteroclitus*)

and Japanese medaka (*Oryzias latipes*), no Aqp1ab paralog has been identified in their genomes [7]. This suggests that the moderate hydration of the oocytes observed in the common mummichog [49, 68] occurs by simple diffusion, or that another aquaporin paralog is involved. It thus remains unknown whether the role of Aqp1ab in oocyte hydration is a specialized feature of modern acanthomorph marine teleosts, or if other channels have evolved in some lineages to play similar roles.

In some salmonid species, a small degree of oocyte hydration of approximately up to 6% occurs around the time of oocyte maturation and ovulation [69], which is very low when compared with the increment observed in pelagophil fishes, which ranges from 14–40% [37]. The physiological significance of such limited hydration is intriguing, since it might increase the oocyte turgency to facilitate the release of the oocyte from the follicle at ovulation. In the Atlantic salmon (*Salmo salar*), Aqp1ab transcripts are expressed in the ovary (Ferré and Cerdà, unpublished data), although at lower levels than in the kidney, which may support a role of Aqp1ab in this process. However, in the rainbow trout (*Onchorynchus mykiss*), microarray analysis has revealed the accumulation of *aqp4*-related transcripts, but not of *aqp1ab*, in the mature ovary, together with mRNAs encoding arginine vasotocin (Avt) and ion transport systems [70]. Since Avt is the non-mammalian counterpart of vasopressin [71], the neurohypophysial peptide that regulates mammalian AQP2 and AQP4 in kidney and brain, respectively [72, 73], it is possible that ovarian Aqp4, instead of Aqp1ab, may mediate the swelling of oocyte during ovulation in salmonids. The existence of this mechanism remains however hypothetical and requires further investigation.

10.3.3 Aqp1ab Transcriptional and Post-translational Regulation in Oocytes

In pelagophil teleosts, the *aqp1ab* transcripts are more highly accumulated in the ovary than in any other adult tissue [58, 60, 61, 63]. In gilthead seabream and Japanese eel, the earliest detection of *aqp1ab* transcripts is noted in the cytoplasm of meiosis-arrested primary growth (previtellogenic) oocytes, whereas oogonia are apparently devoid of these mRNAs [60, 61]. In the seabream, detergent-mediated antigen retrieval and immunofluorescence microscopy also facilitated the detection of the Aqp1ab polypeptides in primary growth oocytes [61]. These findings indicated that the high levels of *aqp1ab* mRNAs and Aqp1ab proteins are stored in the oocytes before entering into the growth (vitellogenic) phase.

Recent studies in the gilthead seabream have uncovered a novel endocrine pathway for the *aqp1ab* transcriptional regulation in the oocytes (Fig. 10.4). Based upon transactivation experiments, incubations of ovarian explants *in vitro*, and chromatin immunoprecipitation (ChIP) assays, the studies suggest that the transcriptional activation of the *aqp1ab* gene in primary growth oocytes is dependent on the classical nuclear progestin receptor (Pgr), which is expressed in the cytoplasm of oogonia and the nucleus of primary oocytes and can bind to two different sites in the *aqp1ab* promoter region [61]. The Pgr is activated by the progestin 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) produced by primordial granulosa cells associated to primary oocytes in response to the follicle-stimulating hormone (Fsh), which up-regulates the expression of steroidogenic enzymes involved in progestin synthesis and down-regulates the major enzymes responsible for estrogen synthesis, resulting in an enhanced production of 17,20β-P in the granulosa cells [74]. However, this mechanism can be potentially modulated by Sry-related high mobility group [HMG]-box (Sox) transcription factors, which can bind to specific sites upstream and downstream of the Pgr binding site in the 5′-flanking region of the seabream *aqp1ab* gene [61]. Interestingly, Sox3 and Sox8b expressed in oogonia elicit a synergic effect to enhance Pgr-mediated transcription in transactivation assays, whereas Sox9b, which is highly expressed in more advanced oocytes, coinciding with a strong depletion of *aqp1ab* transcripts in the oocyte, represses *aqp1ab* transcription [61].

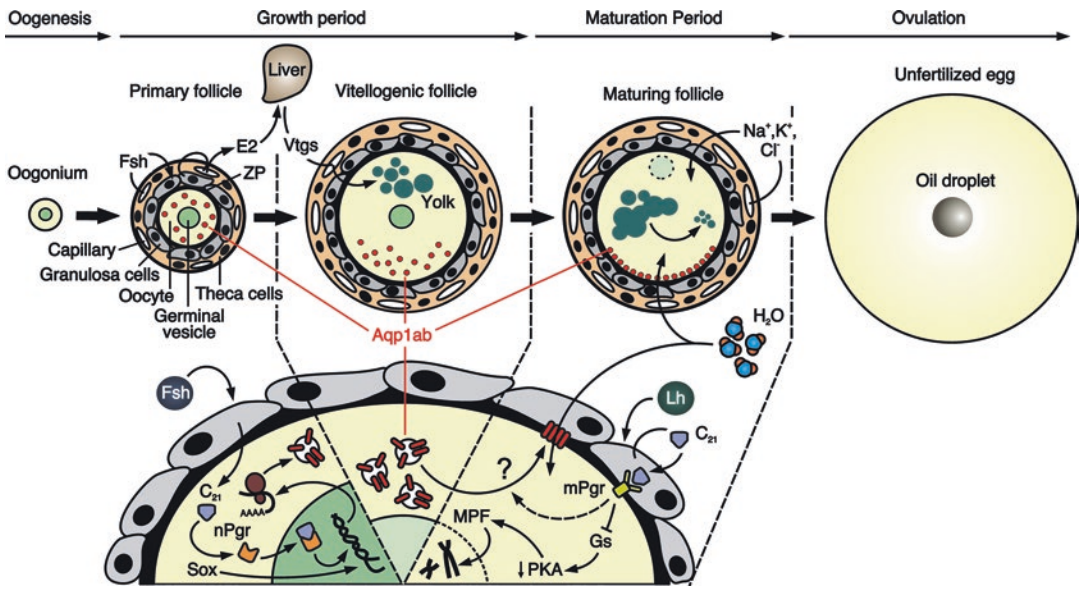

Fig. 10.4 Current model of the regulatory pathways of Aqp1ab in the teleost oocyte. During oocyte differentiation and early growth, follicle cells surrounding the oocytes are presumably stimulated by the follicle-stimulating hormone (Fsh) to produce and release C_{21} steroids (progestins), which activate the nuclear progestin receptor (nPgr) in the oocyte cytoplasm, triggering *aqp1ab* transcription and translation. Sox transcription factors can potentially modulate this mechanism. When oocytes enter into the growth period, vitellogenins (Vtgs) produced by the liver in response to estrogens are incorporated in the oocyte by receptor-mediated endocytosis and processed into yolk proteins that are stored in yolk globules. At the same time, Aqp1ab-containing vesicles are transported toward the oocyte cortex. When vitellogenesis resumes, maturation-inducing progestins synthesized by follicle cells in response to the luteinizing hormone (Lh) activate G protein-coupled membrane progestin receptors (mPgr α and/or β) on the oocyte surface, repressing the stimulatory G protein (Gs) and causing a decrease in cAMP levels followed by the inactivation of protein kinase A (PKA). Decreased PKA activity leads to the activation of the maturation promoter factor (MPF), which triggers meiosis resumption. This process coincides with the proteolytic cleavage of yolk proteins, the accumulation of inorganic ions, and the translocation of Aqp1ab into the microvillar portion of the oocyte plasma membrane through an unknown mechanism

Thus, it is possible that low levels of expression of Sox9b in primary growth oocytes allow Pgr-driven *aqp1ab* mRNA and protein synthesis, whereas increased Sox9b levels in more advanced oocyte stages may inhibit *aqp1ab* expression. However, the seabream *aqp1ab* promoter also shows the presence of putative binding sites for transcription factors other than the Pgr, such as TCF/LEF-1, CREB or PBX-HOX, which might be of relevance during oocyte development [75–77], and also regulate *aqp1ab* transcription. In addition, it remains to be established whether splice variants of the nuclear Pgr, which are expressed in the seabream primary growth stage ovary and can operate as dominant-negative inhibitors of Pgr-mediated transcription [78], can alter *aqp1ab* expression.

During vitellogenesis and oocyte maturation, the major regulatory pathways of Aqp1ab seem to occur at the posttranslational level, resulting in the transport of Aqp1ab-containing vesicles toward the oocyte cortex and eventually into the microvillar portion of the oocyte plasma membrane [12, 59] (Fig. 10.4). The regulatory mechanisms controlling Aqp1ab intracellular trafficking *in vivo* are however largely unknown. Studies in gilthead seabream, Atlantic halibut, and stinging catfish, using frog oocytes as a heterologous expression system for Aqp1ab, suggest a highly divergent nature and function of putative regulatory motifs within the teleost Aqp1ab C-termini that may control intracellular trafficking. Thus, experiments on seabream Aqp1ab employing chimeric constructs and site-directed mutagene-

sis indicate that phosphorylation of Ser^{254} at the cytoplasmic C-terminus, as well as a di-Leu motif located in the same domain, mediate Aqp1ab recycling [53, 79]. However, although the Ser^{254} fulfills a consensus site for a Pro-directed kinase, such as the p38 mitogen-activated protein kinases (MAPK), which is involved in oocyte maturation in vertebrates [80], the physiological significance of these motifs remains unknown. In contrast, Atlantic halibut Aqp1ab only trafficks to the oocyte plasma membrane when it is expressed in native or piscine (zebrafish) oocytes, although membrane trafficking is rescued in *X. laevis* oocytes when halibut Aqp1ab is co-expressed with $polyA^+$ mRNA purified from native postvitellogenic ovarian follicles [12]. These observations thus suggest that Atlantic halibut Aqp1ab may have evolved more specialized mechanisms than the seabream ortholog for its intracellular transport in oocytes. The precise nature of these processes have not yet been clarified, although experimental data suggest that the Aqp1ab C-terminus is likely involved as in the gilthead seabream Aqp1ab [12]. These observations reinforce the hypothesis of the role of the C-terminus in the rapid neofunctionalization of Aqp1ab among teleosts [12, 63].

In the stinging catfish, cAMP can drive the sorting of Aqp1ab to the plasma membrane of *X. laevis* oocytes, most likely through protein kinase A (PKA)-mediated phosphorylation of Ser^{277} [67]. This observation is interesting since oocyte hydration in this species can be triggered by Avt [66], whose mammalian counterpart vasopressin initiates the intracellular transport of tetrapod AQP2 to the plasma membrane of renal epithelial cells through the cAMP/PKA pathway [81]. Accordingly, the catfish oocyte expresses the vasotocin receptor 2 (Avtr2) which activates adenylate cyclase via a Gs protein to increase intracellular levels of cAMP/PKA [82]. Therefore, although the stinging catfish is not a pelagophil species, it offers the first model of an endocrine posttranslational regulation of Aqp1ab in teleost oocytes. In marine pelagophil fish, it is unknown whether a similar vasotonergic system can regulate Aqp1ab trafficking and insertion in the oocyte surface. The investigation of this mechanism in the oocyte of these species may provide novel insights into the transductional pathways activated in the oocyte during meiotic maturation and hydration that coordinate osmolyte generation, Aqp1ab intracellular trafficking, and meiosis resumption.

10.4 Aquaporins in the Male Gamete

10.4.1 Mechanisms of Sperm Motility Activation

Teleost spermatozoa remain quiescent in the testes and efferent ducts with activation of motility only being induced by the aquatic environment into which the sperm are ejaculated. In freshwater teleosts, the release of spermatozoa into the hyposmotic external medium induces membrane hyperpolarization, which leads to a K^+ efflux and a transient increase in intracellular Ca^{2+} and cAMP [83–86]. The subsequent cAMP-dependent phosphorylation of axonemal proteins and dynein light chains triggers the movement of the flagellum [87, 88]. The use of channel blockers has provided indirect evidence for the involvement of Ca^{2+} channels and Na^+/H^+ and Na^+/Ca^{2+} exchangers in intracellular pH regulation or Ca^{2+} influx and therewith sperm activation, although the identity of these putative channels is not known [89]. A recent study in the zebrafish (*Danio rerio*), however, has demonstrated the presence of a novel cyclic nucleotide-gated K^+ channel (CNGK) in the head of the spermatozoa, which surprisingly is not regulated by cyclic nucleotides but by intracellular pH [90]. It has been proposed that alkalization activates the zebrafish CNGK causing membrane hyperpolarization and Ca^{2+} influx, which will induce 'spinning'-like swimming that presumably can guide the sperm into the micropyle [90]. In addition, a volume-sensitive Ca^{2+} channel transient receptor potential vanilloid 1 (Trpv1) channel has been recently found in the head of rohu (*Labeo rohita*) spermatozoa, where its activation increases the duration of motility [91].

For marine teleosts, very few species have been studied to date, but the current models suggest that sperm motility is activated by the osmotic shock caused by exposure to the hyperosmotic seawater [89]. This hyperosmotic shock increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which activates the axonemal machinery directly, or through the Ca^{2+}/calmodulin- or cAMP-dependent protein phosphorylation/dephosphorylation of structural components of the axoneme [92]. In some species, the intracellular Ca^{2+} surge may occur as a consequence of Ca^{2+} influx across the plasma membrane [92, 93], although to date only one reverse-Na^+/Ca^{2+} exchanger has been demonstrated in Pacific herring (*Clupea pallasii*) sperm during ligand-induced motility initiation [94]. In other cases, the increase of $[Ca^{2+}]_i$ may be the result of the stimulation of stretch-activated channels (SACs) in response to osmotic or mechanical changes, or caused by the release of Ca^{2+} from intracellular stores [92]. However, the membrane osmotic and ionic mechanisms and signal transduction pathways triggering and maintaining flagellar motility in marine fish spermatozoa are largely unknown. This scenario is complicated further because the mechanisms for sperm motility initiation in marine teleosts are in many cases species-specific [92].

10.4.2 Biogenesis and Localization of Aquaporins in Teleost Sperm

The osmotic response of marine teleost spermatozoa upon seawater exposure has been estimated to occur within milliseconds [95]. This rapid mechanism suggests the presence of aquaporins in the spermatozoon plasma membrane, which may facilitate a fast water efflux following the osmotic gradient [96]. Accordingly, in different marine species, mercury can inhibit sperm motility, and in some cases the concentrations of $HgCl_2$ that are effective on intact sperm have no effect on the axonemal apparatus of demembranated spermatozoa [89, 96], indicating that the motile apparatus is not directly compromised by low concentrations of $HgCl_2$. In the gilthead seabream, the presence of aquaporins in immotile and motile spermatozoa has recently been confirmed by immunofluorescence microscopy and Western blots with well-characterized, paralog-specific antibodies [97, 98]. In some freshwater teleosts, mercury can also affect sperm motility [99, 100], but in others, such as the rainbow trout (*Oncorhynchus mykiss*), although $HgCl_2$ can reduce the duration of sperm motility in a dose-dependent manner [100], the low plasma membrane permeability of spermatozoa suggest the absence of aquaporins [101]. In addition, no study has yet provided direct evidence for the presence of aquaporins in the spermatozoa of freshwater teleosts. The role of aquaporins in sperm activation in these species is thus uncertain.

Comprehensive studies in the gilthead seabream show that multiple aquaporins, including Aqp0a, -1aa, -1ab, -7, -8bb (formerly named Aqp8b), -9b and -10b, are expressed in both somatic and germ cells of the testis, which are differentially regulated by pituitary gonadotropins throughout the spermatogenic cycle through androgen-dependent and independent mechanisms [102]. These studies have revealed that Aqp0a and -9b are expressed by Sertoli and Leydig cells, respectively, Aqp1ab, -7, and -10b are expressed in all germ cells from spermatogonia to spermatozoa, and Aqp1aa and -8bb are only expressed by haploid spermatids and spermatozoa [98, 102] (Fig. 10.5). During the progression of spermatogenesis, Aqp0a is also transiently expressed in germ cells, but only in spermatogonia and spermatocytes, whereas this aquaporin is no longer present in spermatozoa [102]. Thus, in ejaculated spermatozoa Aqp1aa and -7 are located in the flagellum and the head, respectively, whereas Aqp1ab, -8bb, and -10b are both in the head and the anterior tail [98]. Upon seawater activation, Aqp1ab and -10b are translocated to the plasma membrane of the head and the anterior tail, while Aqp8bb is rapidly phosphorylated and transported to the single spermatozoon mitochondrion, and Aqp1aa and -7 remain unchanged [98, 103] (Fig. 10.5). These findings indicate a complex control of aquaporin expres-

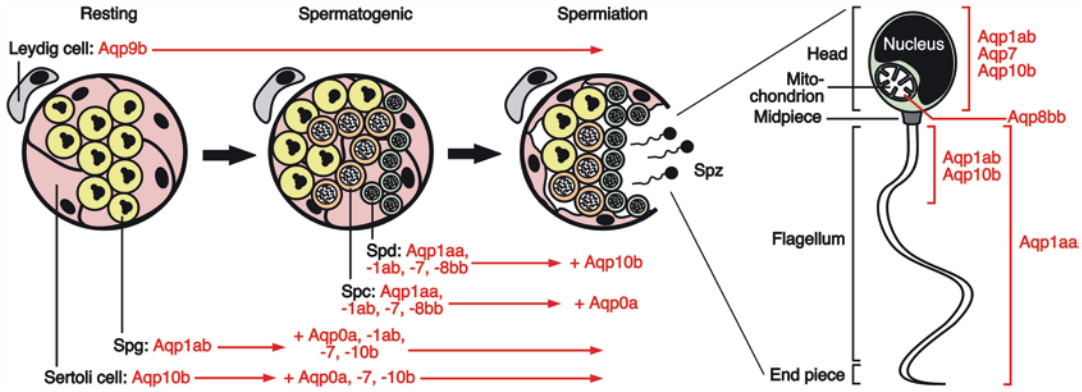

Fig. 10.5 Schematic representation of the cell type-specific expression of aquaporins in the gilthead seabream testis during spermatogenesis and their spatial distribution in activated spermatozoa. *Spg* spermatogonia, *Spc* spermatocyte, *Spd* spermatid, *Spz* spermatozoa

sion during germ cell development, and therefore emphasize the potential importance of aquaporin-mediated water and solute transport for the control of the local fluid balance during teleost spermatogenesis [99]. The observations in the gilthead seabream also suggest an early synthesis of some sperm aquaporins during spermatogenesis, such as Aqp1ab, which may be stored in intracellular vesicles until the spermatozoa differentiate from haploid spermatids.

10.4.3 Role of Aqp1aa During Sperm Activation

In the ejaculated sperm of gilthead seabream, Aqp1aa is distributed along the entire flagellum, and this localization remains unchanged upon seawater activation [98, 104]. The persistence of Aqp1aa in the flagellum may imply an immediate requirement of the channel for the activation of sperm motility. According to this hypothesis, exposure of seabream sperm to mercury completely abolishes seawater-activated motility, which can be reversed by the reducing agent β-mercaptoethanol [97]. Interestingly, *X. laevis* oocyte swelling assays indicate that mercury-inhibited seabream Aqp1aa, but not Aqp10b, can be recovered by β-mercaptoethanol [97, 105], suggesting that Aqp1aa rather than Aqp10b is the channel mediating the water efflux during the hyperosmotic shock, which activates flagellar motility [97]. Since seabream spermatozoa also express Aqp1ab and -7, which can also be rescued by β-mercaptoethanol as in Aqp1aa following mercurial inhibition [58, 98], this view has recently been re-evaluated using Aqp1aa-specific, affinity-purifed antibodies, which can block Aqp1aa water conductance in frog oocytes [104]. These sperm motility assay experiments have shown that the immunological inhibition of Aqp1aa induces a strong decline of the motility, progression and flagellar movement of spermatozoa [104], reinforcing the notion that Aqp1aa is essential for initiating sperm motility.

In the gilthead seabream, as in other marine teleosts [93], Ca^{2+} or K^+ channels may not be required for the initiation of flagellar motility, since sperm can be activated in nonionic solutions and several ion channel blockers have no effect on the initiation of motility [104, 106]. In this species, it is believed that the increase of $[Ca^{2+}]_i$ in spermatozoa during activation is the result of the increased cytosolic concentration due to the massive water efflux following the hyperosmotic shock and/or by Ca^{2+} release from intracellular stores [92, 104]. Under specific altered Aqp1aa function, the elevation of the $[Ca^{2+}]_i$ is partially prevented, and this effect is reversed by exposure of spermatozoa to a Ca^{2+} ionophore, which restores the $[Ca^{2+}]_i$ and the spermatozoon motility [104] (Fig. 10.6). These data therefore provide further support for the current model of sperm activation in the gilthead

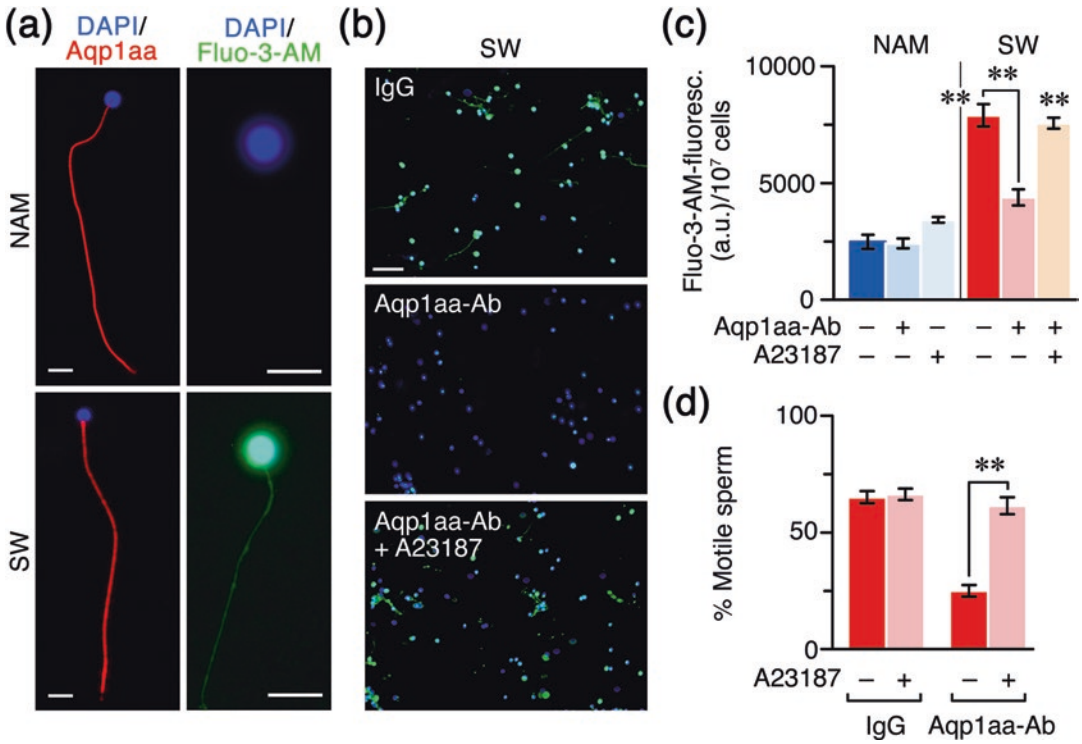

Fig. 10.6 Flagellar Aqp1aa facilitates a rapid increase in $[Ca^{2+}]_i$ and activation of gilthead seabream sperm motility. (**a**, *left*) Immunolocalization of Aqp1aa (*red*) along the flagellum of immotile and SW-activated spermatozoa. (**a**, *right*) Epifluorescence photomicrographs of sperm loaded with 5 μM of the free Ca^{2+} indicator Fluo-3-AM maintained in NAM or activated in SW for 15 min. Originally published in Boj et al. (2015). Bars 1 μm. (**b**) Epifluorescence photomicrographs of sperm loaded with Fluo-3-AM activated in the presence of IgG, an antibody against Aqp1aa (Aqp1aa-Ab), or with Aqp1aa-Ab plus the Ca^{2+} ionophore A23187. Bar, 10 μm. (**c** and **d**) Determination of the $[Ca^{2+}]_i$ (**c**) and the motility (**d**) of spermatozoa treated as in *b* (mean ± SEM; $n = 5–6$ fish). $^{**}P < 0.01$, with respect to sperm in NAM or as indicated in brackets (Data in *a*, *c* and *d* are from Boj et al. [104])

seabream, where the rapid water efflux mediated by Aqp1aa upon the osmotic shock drives an intracellular Ca^{2+} wave, which activates the flagellar movement of spermatozoa [92, 97, 104].

In marine teleost spermatozoa, mechanical activation could be the second signal in response to the first (osmotic) signal via the SACs located in the sperm membrane [95]. As mentioned earlier, SACs are mechanosensitive channels that increase the membrane conductivity to ions such as Ca^{2+} or K^+ when mechanical constraints induce distortion of the membrane. It has been hypothesized that SACs associated with aquaporins may form the signaling pathway of the fish sperm activation [95], although the molecular mechanisms involved are unknown. According to this hypothesis, flagellar Aqp1aa in seabream spermatozoa could not only mediate the first osmotic signal, but also activate SACs and concomitant ion conductivity, which although not required for the induction of sperm motility, may still be necessary to maintain the velocity and flagellar beating of spermatozoa [93]. This mechanism remains however conjectural, although it would be consistent with observations in European seabass sperm, where low concentrations of $HgCl_2$ firstly inhibit the initiation of motility (which would confirm an early role of Aqp1aa), and secondly, induce a 'twist' of the flagellum [95].

10.4.4 Aqp8bb and the Bioenergetics of Flagellar Motility

In the gilthead seabream, Aqp8bb distributed along the anterior flagellum and in vesicles surrounding the nucleus of immotile spermatozoa is rapidly (i.e. in less than one second after activation) phosphorylated and inserted into the inner membrane of the single spermatozoon mitochondrion upon seawater activation [98, 103] (Fig. 10.7a, b and d). Many aquaporin paralogs, including seabream Aqp8bb (Fig. 10.7d), can transport reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) in addition to water [107, 108]. As mitochondrial oxidative phosphorylation seems to be a major source of ATP in fish spermatozoa during the motility phase [89], Chauvigné et al. [103] hypothesized that H_2O_2 produced as a byproduct during oxidative phosphorylation [109], as well as accumulated in the mitochondria due to the hyperosmotic stress [110] (Fig. 10.7c), might be transported out of this compartment through Aqp8bb. To test this hypothesis, these authors employed an affinity-purified antibody specific for seabream Aqp8bb, which blocks the intracellular transport of the channel in the spermatozoa, possibly through steric inhibition of the trafficking mechanism, as well as its permeability once inserted in the mitochondria (Fig. 10.7d, e). When Aqp8bb mitochondrial trafficking and channel activity are immunologically blocked in activated spermatozoa, H_2O_2 levels accumulate in the mitochondria leading to mitochondrial membrane depolarization, the reduction of ATP production, and the rapid arrest of sperm motility (Fig. 10.7f, g and h). However, the decreased sperm vitality underlying Aqp8bb loss of function is fully reversed in the presence of a mitochondria-targeted antioxidant (Fig. 10.7f, g and h).

The discovery of the physiological function of Aqp8bb in the spermatozoon of a marine teleost, has uncovered a role of this channel beyond water transport. Thus, in gilthead seabream activated spermatozoa, Aqp8bb operates as a mitochondrial peroxiporin to allow the efflux of accumulated H_2O_2 thereby maintaining the mitochondrial membrane potential and the production of ATP needed for the maintenance of flagellar motility [103]. These findings thus indicate that mitochondrial Aqp8bb plays an essential role in ameliorating oxidative damage by ROS in activated sperm in order to preserve flagellar motility under hypertonic conditions. Interestingly, it has recently been observed that an Aqp8 ortholog is also accumulated in the mitochondria of activated Atlantic salmon (*Salmo salar*) sperm (Chauvigné et al., unpublished data). These observations suggest that the Aqp8-mediated detoxification mechanism may have evolved in the sperm of teleosts as a selective advantage for increased sperm competition [103].

The signaling pathways and intracellular mechanisms that regulate the trafficking of Aqp8bb to the mitochondria during sperm activation are unknown. It has been found, however, that the inhibition of Aqp1aa function in activated seabream spermatozoa blocks the trafficking of Aqp8bb to the mitochondrion, which can be rescued by a Ca^{2+} ionophore [104]. In addition, mercury treatment of sperm upon seawater activation completely or partially inhibits the phosphorylation of some proteins in the flagellum and head that could control flagellar motility [111]. Altogether these findings suggest a model for the coordinated role of Aqp1aa and -8bb during the activation of seabream spermatozoa, in which an Aqp1aa-triggered intracellular Ca^{2+} surge plays a dual role by initiating flagellar motility through the stimulation of the axoneme and the mitochondrial Aqp8bb-mediated detoxification mechanism for continuous ATP generation (Fig. 10.8). The molecular pathways downstream of Ca^{2+} mobilization involved in these processes remain, however, unknown. Such aquaporin-mediated transduction pathways may involve the Ca^{2+}-triggered activation of adenylyl cyclase, which can elevate the levels of cAMP to cause the phosphorylation of proteins involved in sperm motility activation [92], or of other factors governing the traffick of Aqp8bb. However, direct evidence of this mechanism in seabream is lacking, even though in this species it has been shown that inhibitors of both adenylyl cyclase and PKA can block the initiation of sperm motility [106]. In addition, it has been observed that

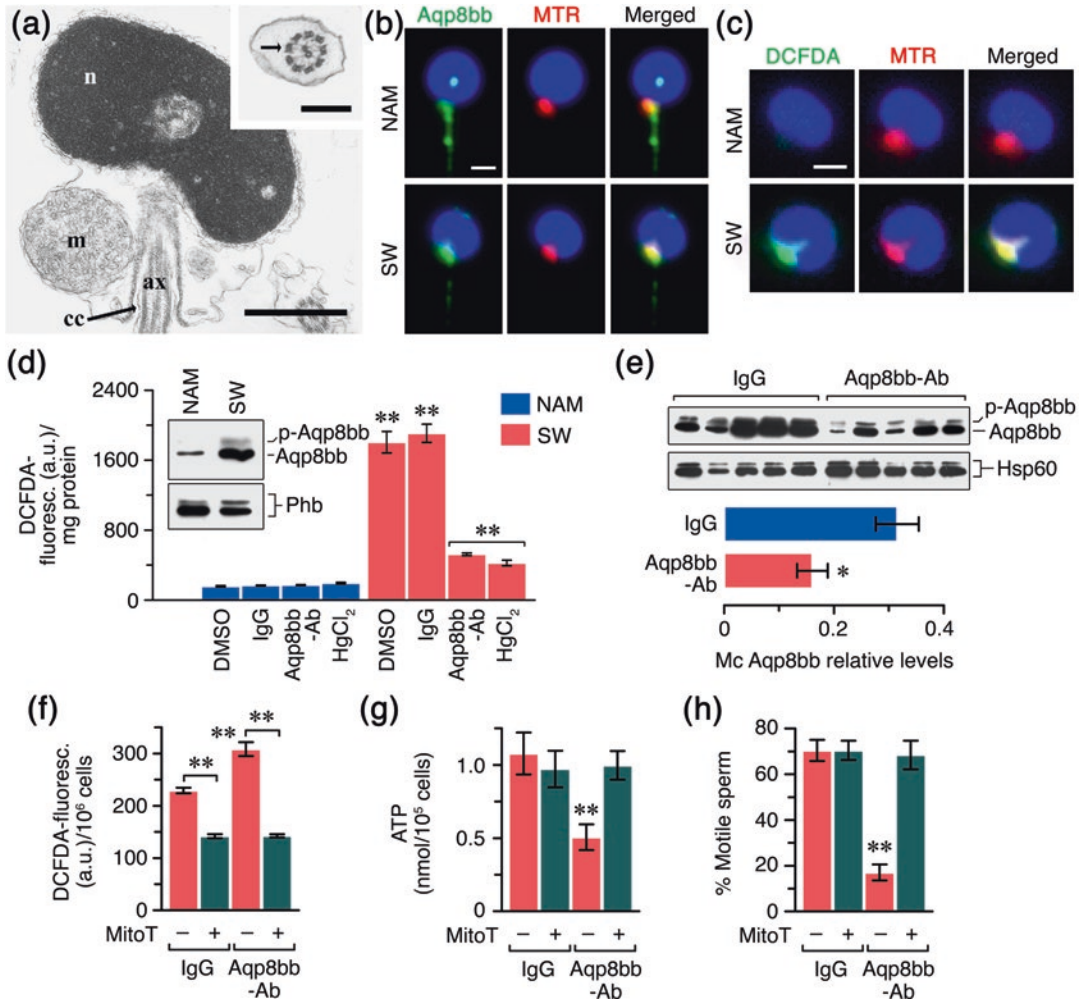

Fig. 10.7 Mitochondrial Aqp8bb mediates H_2O_2 efflux during motility activation in gilthead seabream sperm. (**a**) Sagittal section at head and midpiece level showing the single mitochondrion. Bar, 0.5 μm. a (*Inset*) Cross section through the flagellum showing the 9 + 2 axonemal pattern (*arrow*). Bar, 0.25 μm. ax, axoneme; cc, cytoplasmic canal; m, mitochondrion; n, nucleus. (**b**) Immunolocalization of Aqp8bb (*green*) in non-activated and SW-activated spermatozoa, where the nucleus and mitochondrion were counterstained with DAPI (*blue*) and the mitochondrial dye MitoTracker (MTR, *red*), respectively. Bar, 1 μm. (**c**) Epifluorescence photomicrographs of non-activated and activated live spermatozoa labelled with the reactive oxygen species (ROS)-sensitive, cell permeable fluorescent dye CM-H_2DCFDA (*green*). In this case, the nuclei were counterstained with Hoechst 33,342 (*blue*). Bar, 1 μm. (**d**) H_2O_2 uptake into isolated mitochondria in the presence or absence of 0.5% DMSO, 0.3 μM IgG or Aqp8bb antibody (Aqp8b-Ab), or 10 μM mercury. (**d**, *inset*) Aqp8bb immunoblot of mitochondria from immotile and motile spermatozoa, where phosphorylated (p-Aqp8bb) and dephosphorylated Aqp8bb monomers are indicated. Prohibitin b (Phb) was used as a loading control. $^{**}P < 0.01$, with respect to mitochondria from NAM sperm, or mitochondria treated with DMSO or IgG (*brackets*). (**e**) Mitochondrial Aqp8bb immunoblot of SW-activated sperm treated with IgG or Aqp8bb-Ab, using heat shock protein 60 (Hsp60) as loading control, and corresponding semi-quantitation (mean ± SEM; $n = 5$ fish) of mitochondrial Aqp8bb. $^{*}P < 0.05$. (**f**, **g** and **h**) H_2O_2 levels (**f**), ATP content (**g**) and motility (**h**) of activated spermatozoa (mean ± SEM; n = 4–5 fish) treated with 0.3 μM IgG or Aqp8bb-Ab in the presence or absence of 50 μM of the mitochondria-targeted antioxidant mitoTEMPO (MitoT). $^{*}P < 0.05$; $^{**}P < 0.01$, with respect to IgG-treated sperm, or as indicated in brackets (Panel a is reproduced with permission of the Spanish Society of Histology and Histopathology from Maricchiolo et al. [120]. Rest of data are from Chauvigné et al. [103])

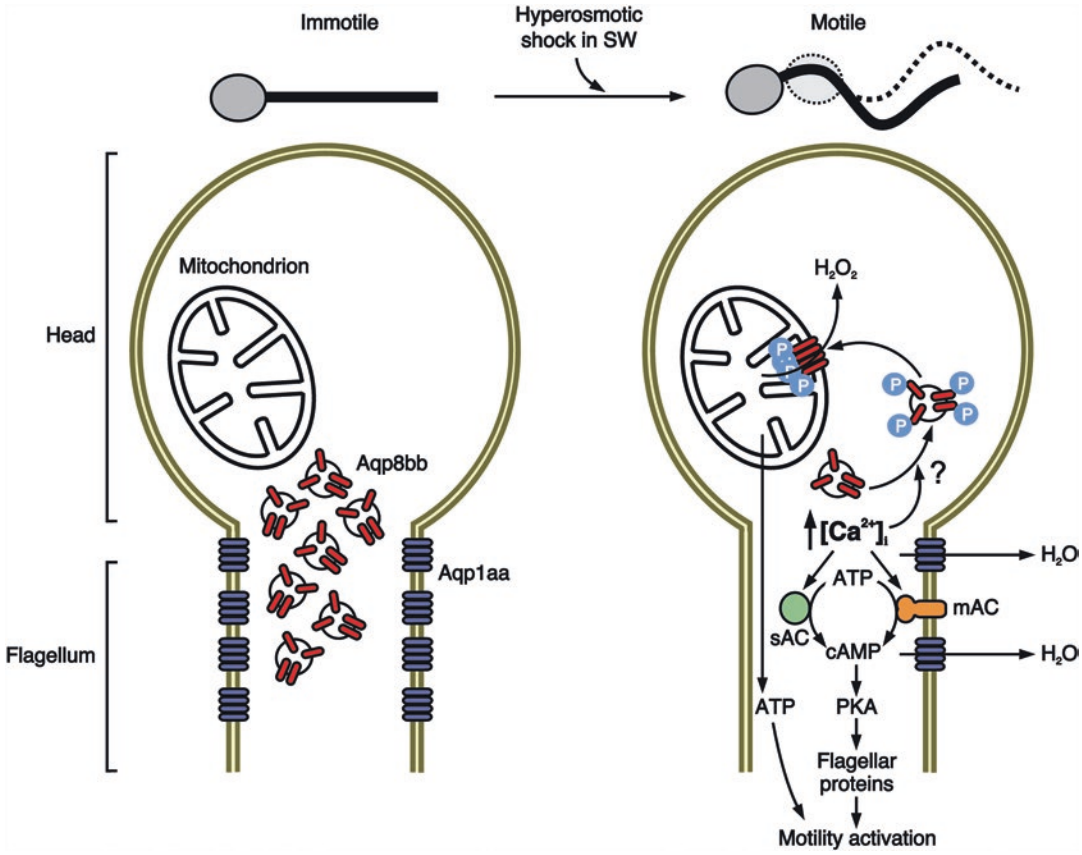

Fig. 10.8 Current model of the coordinated role of Aqp1aa and -8bb during the activation of gilthead seabream sperm motility. The hyperosmotic shock triggers water efflux via flagellar Aqp1aa, which induces a cell volume reduction and the rise in $[Ca^{2+}]_i$. Such a Ca^{2+} increase could lead to the activation of membrane and soluble adenylyl cyclase (mAC and sAC, respectively) and of the cAMP/PKA signaling pathway, causing the phosphorylation of flagellar proteins and the initiation of sperm motility. A Ca^{2+}-dependent pathway through yet unknown factors can also control the rapid phosphorylation of Aqp8bb and trafficking of the channel into the mitochondrion. The Aqp8bb inserted in the inner mitochondrial membrane mediates peroxide efflux from this compartment to avoid the oxidative damage during ATP synthesis, and thus maintaining flagellar motility

while Aqp1aa loss-of-function prevented Aqp8bb mitochondrial accumulation, the phosphorylation of Aqp8bb is unaffected [104], suggesting that both mechanisms may be regulated independently. The elucidation of the Ca^{2+}-dependent signaling pathways involved in Aqp8bb mitochondrial trafficking therefore requires deeper investigation.

10.4.5 Other Sperm Aquaporins

In addition to Aqp1aa and -8bb, the spermatozoa of the gilthead seabream also show spatial segregation of Aqp7 in the head and of Aqp1ab and -10b in both the head and the anterior tail [98]. Upon sperm activation in seawater Aqp1ab and -10b are promptly phosphorylated and translocated mainly to the head plasma membrane, whereas Aqp7 is maintained at the surface of the head [98, 104]. However, in contrast to Aqp1aa, the immunological inhibition of Aqp1ab and -7 in seabream sperm does not affect the motility or progressivity of the spermatozoa, but latently compromises its trajectory and the movement pattern [104]. These data suggest that Aqp1ab and -7 are not involved in the initiation of motility, but they may play a role in controlling the

pattern of sperm motion. Thus, the functional inhibition of these aquaporins may distort the movement of the sperm head and the flagellum, which could underlie an erratic path and the impairment of the flagellar beating. The specific distribution of Aqp1ab and -7 in the sperm head, and of Aqp1ab also toward the anterior and middle portion of the flagellum, where in the latter instance it appears to be localized in different membrane domains as compared to Aqp1aa [104], could allow these effects. However, the function of Aqp10b remains to be investigated.

The molecular mechanisms underlying the possible function of Aqp1ab and -7 controlling the pattern of movement of motile spermatozoa are intriguing. In salmonids, in which sperm motility in the hypotonic environment is mercury sensitive, it has been proposed that aquaporins might play a role mediating the cell volume regulatory mechanism that is likely activated upon ejaculation to preserve the integrity of the sperm plasma membrane and the cell motility [100]. Such a putative function has previously been anticipated for AQP3 and -8 in human and mouse spermatozoa upon release into the relatively hyposmotic microenvironment of the female reproductive tract [112–114]. The molecular processes involved are, however, unclear, although it is possible that some aquaporin channels may function as osmosensors/mechanosensors in sperm to detect early events in cell swelling under hypotonic conditions and convey signals to stimulate the regulatory volume decrease response through interaction with volume-sensitive ion channels or cytoskeletal components [114]. This hypothesis, while not yet directly demonstrated, is supported by the finding that AQP4 or -5 can interact with the TRPV4 channel to regulate cell volume under hypotonic stimulation in mouse astrocytes and salivary glands [115, 116], and by the direct interaction of AQP2 with actin described in rat kidney cells [117]. In addition, TRPV channels have been shown to play functions during sperm capacitation, acrosome reaction, and fertilization in mammals [118, 119] as well as to regulate sperm motility in freshwater teleosts [91]. It is therefore tempting to speculate that aquaporins in marine teleost spermatozoa, such as Aqp1ab and -7, might associate with SACs or other ion channels to drive local osmotic responses and ion transport mechanisms, which can sustain motility and/or control hydrodynamic and haptic interactions governing sperm navigation. The identity of the ion channels present in the spermatozoa of marine fish are largely unknown, and therefore this possibility merits further investigation.

10.5 Conclusions

Current data show that the large repertoires of aquaporins encoded in teleost genomes have evolved to play multiple physiological roles in the germ cells and gametes of both sexes. This includes the specific temporal insertion of Aqp1ab channels in the oocyte plasma membrane to mediate hydration of the egg in preparation for life in the oceanic environment, as well as insertion of Aqp1aa channels in the spermatozoon flagellar membrane to activate motility. Aqp8bb is further rapidly phosphorylated and inserted in the inner mitochondrial membrane where it acts as a peroxiporin to mediate the efflux of reactive oxygen species and thus the maintenance of the mitochondrial membrane potential, the production of ATP and the continuance of sperm motility necessary for fertilization. While these findings represent important advances in our understanding of the roles of aquaporins in teleost germ cells, the observations have only been made in comparatively few species. It thus remains unknown whether the role of Aqp1ab in oocyte hydration is a specialized feature of modern acanthomorph marine teleosts, or if other channels have evolved in different lineages to play similar roles. Similarly the data on aquaporin physiology in teleost spermatozoa are primarily confined to a single marine species, with the roles of many of the paralogs present in sperm, such as Aqp1ab, -7 and -10b, yet to be elucidated. Consequently it will be important to establish what roles the different paralogs play and whether the identified roles are conserved in other species. It seems clear that there is a high divergence in posttranslational regulatory mechanisms

between teleost and tetrapod oocytes, and considering that AQP8 orthologs are not found in the mitochondria of mammalian sperm, a difference in mitochondrial detoxification mechanisms of spermatozoa between mammals and teleosts may exist. However, which pathways and mechanisms govern the intracellular trafficking of aquaporins in teleost germ cells remains completely unknown. Such knowledge will be essential to understand the broader biological relevance of these channels for the development and function of oocytes and sperm.

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Aquaporins in the Skin

11

Ravi Patel, L. Kevin Heard, Xunsheng Chen, and Wendy B. Bollag

Abstract

The skin is the largest organ of the body, serving as an important barrier between the internal milieu and the external environment. The skin is also one of the first lines of defense against microbial infection and other hazards, and thus, the skin has important immune functions. This organ is composed of many cell types, including immune-active dendritic cells (epidermal Langerhans cells and dermal dendritic cells), connective tissue-generating dermal fibroblasts and pigment-producing melanocytes. Comprising the outer skin layer are the epidermal keratinocytes, the predominant cell of this layer, the epidermis, which provides both a mechanical barrier and a water-permeability barrier. Recent data suggest that aquaporins, a family of barrel-shaped proteins surrounding internal pores that allow the passage of water and, in some family members, small solutes such as glycerol, play critical roles in regulating various skin parameters. The involvement of different aquaporin family members in skin function is discussed.

Keywords

Aquaporin • Contact hypersensitivity • Epidermis • Glycerol • Psoriasis • Skin • Skin cancer

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Abbreviations

AQP	aquaporin
BCC	basal cell carcinoma
DNFB	dinitrofluorobenzene
IL	interleukin
PG	phosphatidylglycerol
PI3K	phosphoinositide 3-kinase
PLD-2	phospholipase D-2
PPAR	peroxisome proliferator-activated receptor
PPK	palmoplantar keratoderma
SCC	squamous cell carcinoma

11.1 Introduction to the Skin

The skin is the largest organ in the human body; its main function is to serve as a barrier to the external world. The layers of the skin, from deep to superficial, include the hypodermis, dermis, and epidermis. The hypodermis contains adipocytes and serves as a calorie reservoir, in addition to providing insulation from cold and trauma [1]. The dermis is the vascular portion of the skin and is composed primarily of collagen, elastin, and extracellular matrix. It also contains hair follicles, sweat glands, sebaceous glands, and nerves, with its main role to provide support and nutrition to the epidermis. The layers of the outermost portion of the skin, the epidermis, include, from deep to superficial, the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Fig. 11.1). An additional layer, the stratum lucidum, is located in between the stratum granulosum and stratum corneum in areas of thick skin such as the palms of the hands and soles of the feet. The epidermis is composed primarily of keratin-producing cells, the keratinocytes, although antigen-presenting Langerhans cells, pigment-generating melanocytes, and pressure-sensing Merkel cells are also found.

The stratum basale contains undifferentiated keratinocyte stem cells that proliferate and migrate superficially to regenerate cells in the epidermis. Terminal differentiation of the keratinocytes is known as keratinization and is a process in which the cells produce increasing amounts of keratin to form the physical, impermeable layer of the stratum corneum. In total, it takes about 4 weeks for an undifferentiated keratinocyte to mature and reach the stratum corneum [1]. Early in the cell's life, these undifferentiated basal keratinocytes express markers of their proliferative status, such as the immature keratins, keratin 5 and keratin 14, which dimerize and form the intermediate filaments of the stress-bearing cell cytoskeleton [2].

After leaving the stratum basale, keratinocytes in the stratum spinosum growth arrest and begin to express early markers of differentiation, including the mature keratins, keratin 1 and keratin 10, which form keratin intermediate filaments that provide mechanical stability to the epidermis. These cells also express intermediate markers of differentiation, such as involucrin, before moving into the stratum granulosum or granular layer. In this granular layer keratinocytes synthesize late markers of differentiation such as profilaggrin, loricrin, and various cornified envelope proteins. The many protein constituents of the cornified envelope are cross-linked by the enzyme transglutaminase. In the granular layer the keratinocytes also produce lamellar granules, which contain lipids and lipid-metabolizing enzymes. As the cells mature to form squames in the stratum corneum, the dead surface layer of the skin, they release these lamellar bodies, with the secreted lipids processed by the accompanying enzymes to form a lamellar lipid structure. The transition of granular keratinocytes into cornified keratinocytes of the stratum corneum is marked by the destruction of organelles and maturation of the cornified envelope into an insoluble, highly resistant structure surrounding the cytoskeletal complex and linked to the extracellular lipid milieu [3]. This stratum corneum structure has been compared to a brick wall with the lipid "mortar" surrounding the squame "bricks" to generate the water-permeability barrier of the epidermis [1]. Through this complex differentiation process, the generated stratum corneum plays the main role in forming a barrier against the external environment and preventing internal water loss through evaporation.

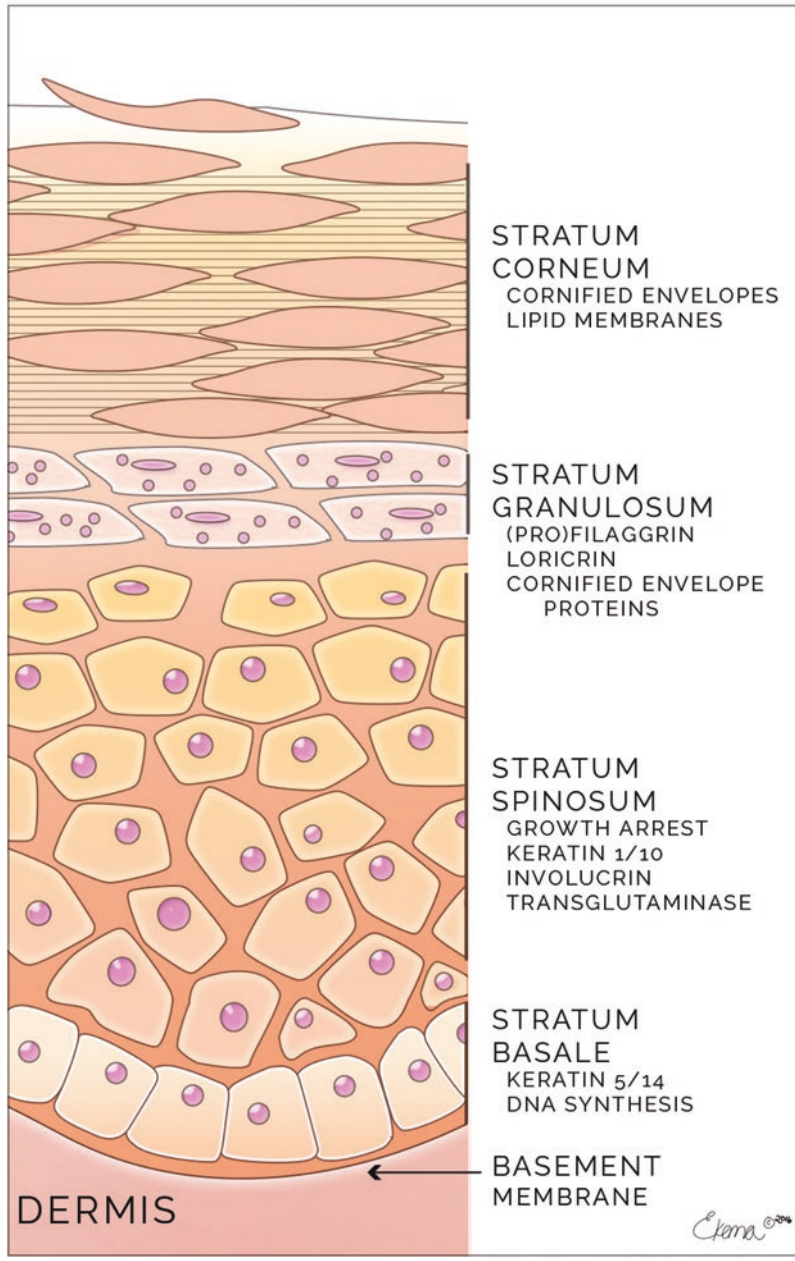

Fig. 11.1 Epidermal structure. The epidermis, the most superficial portion of the skin, is composed predominantly (approximately 90%) of keratinocytes, which stratify to form several layers, with the deepest layer called the stratum basale (or basal layer), in which keratinocytes attached to the basement membrane continuously divide to regenerate the epidermis. As the cells detach from the basement membrane and move up into the next layer, the stratum spinosum or spiny layer, the cells initiate differentiation, becoming growth arrested and expressing the mature keratins, keratin 1 and 10. As the cells continue to migrate through the layers of the stratum spinosum, they express other intermediate keratinocyte differentiation markers, including involucrin. In the last living layer of the epidermis, the stratum granulosum (granular layer), keratinocytes contain keratohyalin granules and lamellar bodies and express late differentiation markers, such as loricrin and filaggrin. As the cells move into the surface layer, the stratum corneum (cornified layer), they terminally differentiate and die, degrading cellular organelles, extruding lamellar bodies and cross-linking proteins of the intermediate filaments and cornified envelope, as well as lipids of the plasma membrane. These crosslinked "cornified envelopes" form a tough "shell" underneath the plasma membrane and serve to provide the mechanical barrier of the epidermis, while the lipids contained in the extruded lamellar bodies are processed to form the water-permeability barrier. The structure has thus been likened to "bricks and mortar", with the corneocyte squames forming the bricks and the lipids the mortar. Other cells present in the epidermis include the pigment-producing melanocytes, the resident dendritic cells called Langerhans cells and pressure-sensitive Merkel cells (not shown)

11.2 Aquaporins

Aquaporins (AQPs) are a family of transmembrane proteins that facilitate the transport of water, and in some cases small solutes, across cell membranes [4].There are currently 13 known AQPs in humans, AQP0 through AQP12. AQP0, 1, 2, 4, 5, and 8 function as water transporters, whereas AQP3, 7, 9, and 10 are known as aquaglyceroporins due to their ability to transport glycerol and other small solutes in addition to water. AQP6, 11, and 12, sometimes referred to as unorthodox AQPs, have as yet largely unelucidated properties [5]. Up to six different AQPs (AQP1, 3, 5, 7, 9, and 10) may be selectively expressed in various cell types in human skin [6]. From deep to superficial, AQP7 is located primarily in the hypodermis (but also in dermal and epidermal dendritic cells [7]), AQP5 is found in the dermis (and in the epidermis of the thick skin of the palms and presumably soles [8]), and AQP9 and 10 are located in the epidermis. AQP1 and AQP3 have been localized to both the dermis and epidermis. This chapter will discuss the complex roles and overall functions of these AQPs in the skin.

11.2.1 AQP1

AQP1 was the first discovered member of the AQP family, and it is expressed in multiple organs throughout the body. In the skin, AQP1 is found in dermal fibroblasts and vascular endothelial cells; it has also been detected in melanocytes, located in the stratum basale of the epidermis [5]. The main function of AQP1 occurs in the vascular endothelial cells, where it exchanges water between the blood and dermis to maintain hydration. Its physiologic role in fibroblasts and melanocytes is less well elucidated, although it has been demonstrated that fibroblasts upregulate AQP1 during periods of hypertonic stress [9]. A similar increase in AQP1 expression has been theorized in melanocytes during periods of osmotic stress, although further research is required [6]. It has also been shown that water transport via AQP1 can mediate keratinocyte migration. In one study, cell migration was restored in keratinocytes derived from AQP3 knockout mice by infecting the deficient cells with either the aquaglyceroporin AQP3 or the water channel AQP1 [10]. Influx of water through either AQP1 or AQP3 was proposed to provide the hydraulic pressure necessary to extend processes for cell movement ([10] and reviewed in [11]). Finally, AQP1 levels are increased in the epidermis, dermis and blood vessels of the skin of infants affected by erythema toxicum neonatorum [12]. Observed only in newborns, this inflammatory skin disease is characterized by papules or pustules on an erythematous base with immune cell infiltration [12]. However, whether there is a causative role for this change in AQP1 levels in the condition is currently unknown.

11.2.2 AQP3

AQP3 is arguably the best-studied, as well as the most abundant [13], aquaporin in the skin. AQP3 is an aquaglyceroporin, able to transport small solutes in addition to water. Indeed, this channel allows efficient flux of glycerol down its concentration gradient and has been recently shown to also transport hydrogen peroxide as well [14–16]. AQP3 immunoreactivity has been reported to localize in the keratinocyte plasma membrane (Fig. 11.2) and to predominate in basal keratinocytes of the epidermis [17]; in fact, however, multiple investigators, including our laboratory, have detected protein expression throughout the stratum spinosum (e.g., [12, 18–20]), in which staining nicely outlines the individual keratinocytes [18], and even into the stratum corneum [21]. AQP3 expression has also been observed in the dermis and in immune cells in the skin, particularly in skin disease (e.g., [7, 12]).

AQP3 plays a key role in skin hydration, likely by affecting epidermal glycerol content [22, 23]. This idea is supported by findings in AQP3 knockout mice, which exhibit decreased stratum corneum hydration as measured by skin conductance [24]; this defect can be remedied by pharmacologic administration of glycerol (via a topical, intraperitoneal or oral route) [24], consistent with the fact

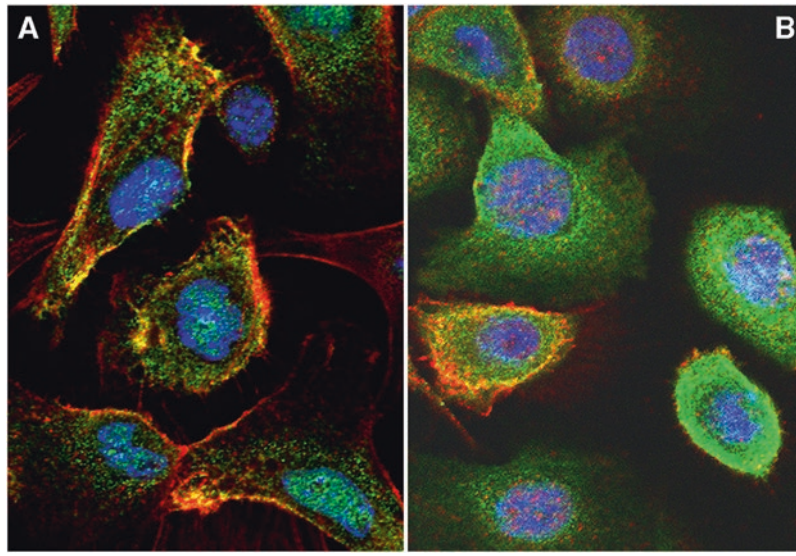

Fig. 11.2 Localization of AQP3 in epidermal keratinocytes. Shown are immuno-fluorescence images of AQP3 localization in primary cultures of mouse epidermal keratinocytes. Keratinocytes were isolated from neonatal mice as described in [106], plated on glass coverslips and incubated at 37 °C in a 5% carbon dioxide-containing incubator until experimentation, with medium replaced every 1–2 days. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 in phosphate-buffered saline (PBS). After blocking with 15% goat serum in PBS, cells were labeled first with primary antibodies and then subsequently with secondary antibodies or labeled phalloidin (to visualize actin). Coverslips were then mounted on slides using Prolong Gold Antifade (Invitrogen) containing DAPI (to visualize nuclei) and analyzed by confocal microscopy on a Zeiss confocal microscope. Results shown are representative of at least 3 experiments. (**a**) Cells were labeled with Alexa Fluor 555-conjugated phalloidin (Invitrogen) and an antibody recognizing AQP3 (LifeSpan Biosciences) followed by Cy3-labeled goat anti-rabbit secondary antibody (Invitrogen) in PBS with 15% goat serum. Panel A represents a merged image with AQP3 staining shown in *green*, actin in *red* and nuclei in *blue*. *Yellow* represent colocalization of AQP3 and actin. (**b**) Cells were labeled with antibodies recognizing AQP3 as in panel A and keratin 5 (K5; Sigma), followed by appropriate secondary antibodies (Cy3-labeled goat anti-rabbit secondary antibody for AQP3 and Alexa Fluor 488-conjugated chicken anti-mouse secondary antibody for K5). Panel B represents a merged image with AQP3 staining shown in *red*, K5 in *green* and nuclei in *blue*; *yellow* represents colocalization of AQP3 and K5

that glycerol can enter cells in the absence of aquaglyceroporins but with greatly reduced efficiency. AQP3 knockout mice show an additional epidermal phenotype that includes decreased skin elasticity, delayed wound healing and impaired barrier recovery after disruption of the epidermal water permeability barrier [22, 23]. The key role of AQP3 in barrier function is also supported by our laboratory's finding of accelerated barrier recovery in transgenic mice overexpressing AQP3 under the control of the human keratin 1 promoter [25], as well as the fact that antenatal corticosteroids increase the water-permeability barrier (i.e., decrease trans-epidermal water loss) in conjunction with an enhancement of AQP3 expression [20]. The alterations in skin barrier function in AQP3 knockout mice can also be corrected by glycerol administration [10, 23]. The delay in skin wound healing observed in AQP3 knockout mice is also restored by glycerol [10]; this result is consistent with the fact that glycerol is often included in wound ointments because this agent is known empirically to improve skin wound healing [26]. For other phenotypes associated with AQP3 ablation, such as decreased tumorigenesis [27], it is not clear whether the changes are sensitive to glycerol, although glycerol did enhance the decreased keratinocyte proliferation observed in AQP3 knockout mice upon wounding [10] or TPA treatment [27].

Recently, Hara-Chikuma and colleagues have reported another effect of AQP3 gene deletion in mice: resistance to the development of psoriasis-like skin lesions in mouse models of psoriasis. Psoriasis is a common skin disease, affecting approximately 3% of the human population (http://www.niams.nih.gov/Health_Info/Psoriasis/default.asp), and while usually not life threatening, the physical and psychological morbidity associated with this disease is significant, with many patients experiencing a decreased quality-of-life and a negative impact from the condition that can be greater even than that created by life-threatening illness [28, 29]. In addition to immune system activation and inflammation [30], psoriasis is characterized by hyperproliferation and abnormal differentiation of keratinocytes [31]. Thus, while immune dysfunction is clearly involved in psoriasis, mounting evidence points to an important role of keratinocytes in various stages of the disease (reviewed in [31]). For example, a transgenic mouse model in which the AP-1 transcription factors c-jun and junB are deleted specifically in epidermal keratinocytes displays psoriasiform lesions as well as a psoriatic arthritis-like phenotype [32]. Interestingly, crossing these mice with Rag2 knockout mice so that they lack functional T and B cells resolves the arthritis but a milder psoriasiform disease persists [32]. These results argue for an important role of keratinocytes in the disease process, as does the fact that several genes associated with the disease in humans are expressed predominantly in keratinocytes (reviewed in [33]). This linkage may be related to the involvement of the permeability barrier, and changes therein, in the development of psoriasis [33]. Indeed, activated keratinocytes are known to produce immune-stimulating cytokines, and T cells secrete keratinocyte-activating factors; increasing evidence suggests that immune cells and keratinocytes establish a "vicious cycle" to initiate and promote psoriasis (reviewed in [34–36]).

With regard to a potential role of AQP3 in the psoriatic disease process, Hara-Chikuma et al. [15] first demonstrated an important role of AQP3 in the transport of hydrogen peroxide, as initially reported by Miller et al. [14], with this transport underlying T cell migration in response to chemokines [15]. In subsequent experiments, these investigators showed that lack of AQP3 in knockout mice inhibited the development of ear swelling and psoriasiform lesions in two mouse models of psoriasis [16]. In one model, interleukin-23, a cytokine that is thought to be involved in psoriasis [37], is injected into the ear and the induced edema monitored; the other uses application of imiquimod (brand name Aldara®). Imiquimod is a toll-like receptor agonist used to treat actinic keratosis, superficial basal cell carcinoma (BCC) and genital warts (http://www.pdr.net/pdr-consumer-monograph/aldara?druglabelid=1348&ConsumerId=2844#1), which can also induce psoriasis in susceptible individuals ([38] and reviewed in [39]). Its application results in ear swelling and psoriasiform skin lesions in mice [40]. However, in these models the psoriasis-like responses of the AQP3 knockout mice are not restored by transplantation of wild-type bone marrow into the mice [16], indicating that the absence of AQP3 expression in immune cells does not contribute to the resistance of AQP3 knockout mice to the development of the psoriasiform skin condition. Instead, AQP3 expression in keratinocytes is the key factor, with this channel transporting hydrogen peroxide into keratinocytes to induce epidermal inflammation and skin lesion development [16]. Thus, AQP3 in keratinocytes seems to play an important role in psoriasis; however, whether AQP3 is up- or down-regulated in psoriasis remains controversial (see below).

Changes in AQP3 protein expression and/or distribution have been demonstrated in many skin diseases, suggesting the importance of this channel in humans as well. For example, down-regulation of this protein has been reported in the non-melanoma skin cancer, BCC [41, 42]; indeed, using immunohistochemistry our laboratory has observed markedly decreased AQP3 levels in BCC compared to the normal-appearing overlying epidermis that is present in these sections (Fig. 11.3) [18]. In another non-melanoma skin cancer, squamous cell carcinoma (SCC), investigators have observed increased AQP3 protein expression, although predominantly in

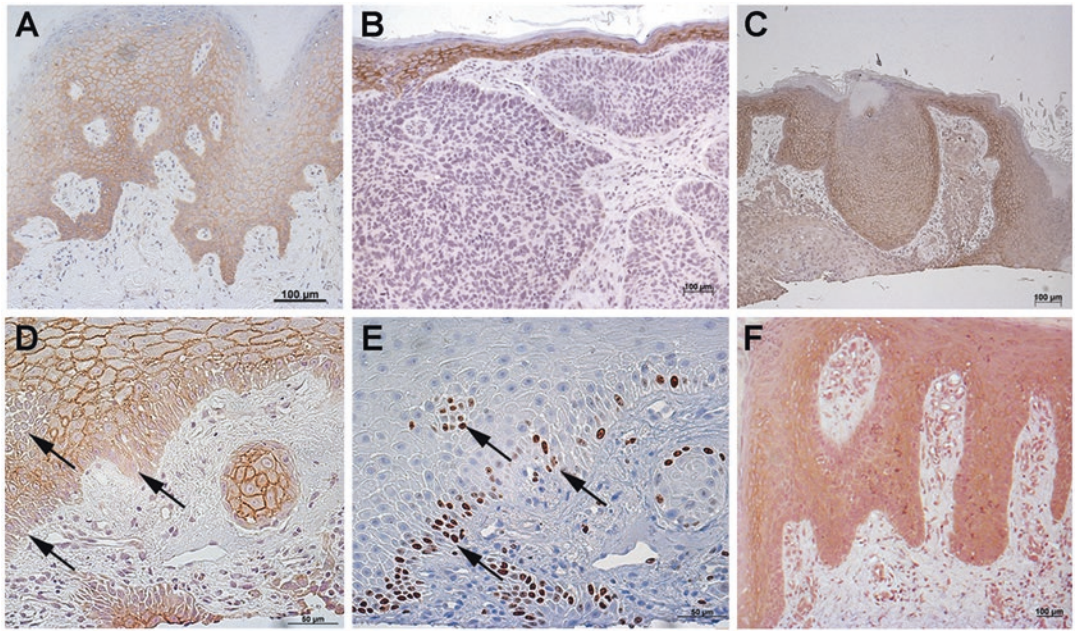

Fig. 11.3 Localization of AQP3 in normal epidermis and in skin diseases. (a) illustrates AQP3 immunoreactivity in normal human epidermis, (b) in basal cell carcinoma, (c) in squamous cell carcinoma and (f) in psoriasis. (d) shows AQP3 staining and (e) corresponding immunoreactivity for Ki67, a marker of proliferation, in sequential serial sections of a squamous cell carcinoma lesion. Immunohistochemical staining was performed on 4 µm sections of formalin-fixed paraffin-embedded samples using a rabbit polyclonal antibody recognizing AQP3 and an ABC staining kit (Santa Cruz Biotechnology), which makes use of biotinylated horseradish peroxidase, avidin and a biotinylated anti-rabbit secondary antibody, with 3,3′-diaminobenzidine (DAB) as the chromogen, as described in [18]. In (e) a mouse monoclonal antibody recognizing KI67 was used in combination with an ABC kit containing an anti-mouse secondary antibody (Figure 11 is reproduced and adapted from Ref. [18])

non-epidermal epithelia (e.g., [43] and discussed in [41]), while others have observed a decrease in AQP3 levels in SCC [41]. Our laboratory observed AQP3 in SCC to be "patchy", with intense immunoreactivity in some portions of the lesion and little or no staining in others [18]. When serial sections of the tumors were stained for AQP3 and Ki67, a marker of proliferating cells, cells that were positive for Ki67 had low levels of AQP3 whereas those with high AQP3 immunoreactivity were Ki67-negative, i.e., there was a negative correlation between AQP3 levels and proliferation observed in SCC [18]. Nevertheless, AQP3 knockout mice exhibit inhibited tumor formation in a mouse model of carcinogenesis [27]. Indeed, in this study Hara-Chikuma et al. [10, 27] determined a role for AQP3 in proliferation, suggesting that the glycerol transported by AQP3 serves as an energy source for ATP production [10, 27]. However, it should be noted that inflammation plays a prominent role in terms of tumor formation in this carcinogenesis model (e.g., [44] and discussed in [45]). As mentioned previously, AQP3 has been shown to be expressed in T cells and to underlie chemokine-induced chemotaxis, and AQP3 knockout mice show diminished contact hypersensitivity reactions [15]. Therefore, the impairment in immune cell infiltration/recruitment could also potentially contribute to the decreased tumorigenesis observed in this mouse model [25, 41].

Although Hara-Chikuma and colleagues have suggested an involvement of AQP3 in keratinocyte proliferation, the effect of AQP3 on proliferation is still somewhat controversial, with some reports (sometimes from the same authors) demonstrating that reductions in AQP3 levels impair

cell growth [10, 27, 46], and others indicating no significant difference in the epidermal proliferation rate (as measured by radiolabeled thymidine incorporation into DNA) [22] or thickness [22, 24] with AQP3 ablation. The difference in these data may relate to the conditions studied, that is, whether proliferation is measured basally or with stimulation. Thus, in the cases for which AQP3 knockout (or knockdown by siRNA) reduces proliferation, the effect is observed predominantly with some type of perturbation: i.e., wounding [10] or treatment with phorbol ester [27], CCL17 or ovalbumin (to induce dermatitis) [46] or retinoic acid [47]. However, proliferation is often monitored by an assay that is sensitive to metabolic conditions, such as the MTT assay. Since the glycerol transported by AQP3 is known to affect metabolism [48], for example, increasing ATP production [10, 27], it seems that alternative methods to monitor cell growth should instead be used.

On the other hand, our laboratory has observed an ability of AQP3 and glycerol to inhibit proliferation and induce keratinocyte differentiation [49, 50]. Thus, increased extracellular glycerol concentrations inhibit radiolabeled thymidine incorporation into DNA, and in experiments using reporter constructs in which promoters of keratinocyte differentiation markers control luciferase expression, co-expression of AQP3 enhances promoter activities [50]. In a recent report we also showed that re-expression of AQP3 in AQP3 knockout keratinocytes increases the mRNA and protein expression of several keratinocyte differentiation markers, either alone or in conjunction with an agent that triggers differentiation [51]. Supporting a pro-differentiative role for AQP3 in keratinocytes, AQP3 expression/levels can be increased by differentiating agents, such as agonists of the nuclear hormone receptor PPARγ [52]. In addition, AQP3 expression is upregulated initially upon high cell density-induced human keratinocyte differentiation, concomitant with increased keratin 1 mRNA levels [53], also suggesting a possible role for AQP3 in differentiation. Similarly, siRNA-mediated knockdown of AQP3 in human keratinocytes results in decreased keratin 10 upregulation in response to a differentiating agent [54], again consistent with an involvement of AQP3 in inducing early keratinocyte differentiation. This group also showed an association of AQP3 with adherens junction complexes, such that siRNA-mediated knockdown of AQP3 levels results in reduced levels of E-cadherin, β- and γ-catenins and phosphorylated (active) phosphoinositide 3-kinase (PI3K) [54]. Since activation of PI3K and its downstream effector Akt is critical for the survival of differentiating keratinocytes [55, 56], and is linked to adherens junctions [56, 57], these results provide an explanation for the reduced keratinocyte survival observed with AQP3 knockdown [54] and argue for an important role for this aquaglyceroporin in maintaining keratinocyte viability during the differentiation process. Further evidence for a role for AQP3 in differentiation is provided by the finding of Lee et al. [58] who observed increased glycerol uptake with early keratinocyte differentiation. Hara-Chikuma et al. [47], however, determined that there was no effect of AQP3 gene ablation on keratinocyte differentiation markers in AQP3 knockout mouse epidermis, suggesting perhaps an ability of the epidermis to compensate for the lack of AQP3 *in vivo*.

A potential explanation for the disparate findings of a role for AQP3 in keratinocyte differentiation versus proliferation may lie in our previous finding that AQP3 is physically and functionally associated with the lipid-metabolizing enzyme, phospholipase D-2 (PLD-2) [59, 60]. PLD-2 can use glycerol, presumably transported by the co-localized AQP3, to produce the phospholipid, phosphatidylglycerol (PG) [25], and PG, in turn, is a second messenger that can inhibit the growth of rapidly dividing keratinocytes and stimulate their differentiation [49, 50]. Indeed, PG production is increased by a differentiating agent (elevated extracellular calcium concentrations) [60], with a dose dependence similar to its described ability to promote mouse keratinocyte differentiation [61]. This idea is also consistent with the fact that the differentiative effect of re-expression of AQP3 in AQP3 knockout keratinocytes depends upon PLD-2 activity [51]. Thus, an inhibitor of PLD-2 and overexpression of a

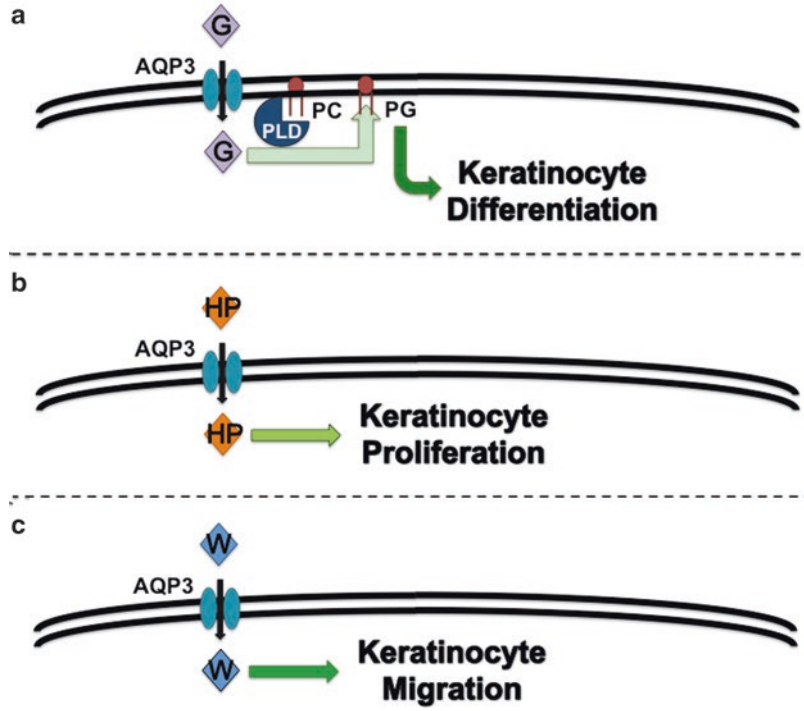

Fig. 11.4 Roles of AQP3 in epidermal keratinocytes. (**a**) AQP3 can transport glycerol (G) into keratinocytes, where this primary alcohol is "funneled" to phospholipase-D2 (PLD-2), which converts it to phosphatidylglycerol (PG). PG, in turn, serves as a lipid signal to inhibit proliferation and promote differentiation of keratinocytes. (**b**) AQP3 can also carry hydrogen peroxide (HP), produced in response to cytokines, to induce proliferation, particularly under conditions mimicking psoriasis. (**c**) AQP3 can also carry water (W); the hydraulic pressure generated is thought to allow extension of lamellopodia resulting in cellular migration

dominant-negative lipase-dead PLD-2 mutant blocks the increase in differentiation marker expression observed upon AQP3 re-expression in AQP3 knockout mouse keratinocytes [51]. In addition, a membrane-permeable caveolin-1 scaffolding domain protein, which disrupts the caveolin-rich membrane microdomains [62] in which AQP3 and PLD-2 co-localize [63], not only inhibits PG production in response to a differentiating agent (an elevated calcium concentration) but also reduces keratinocyte differentiation [62]. Whether AQP3 is pro-differentiative or pro-proliferative may, therefore, be related to its association with PLD-2 (Fig. 11.4) [25, 41].

Alternatively, another factor that may influence AQP3's role in keratinocytes is its localization. Consistent with this finding, our laboratory observed using immunohistochemistry that AQP3 is localized to the plasma membrane in the spinous layers of the epidermis, but can be observed more intracellularly in the basal layer of normal human skin, and in both the basal and suprabasal layers in psoriasis [18], a disease in which growth arrest in the spinous layer is impaired; a similar finding was reported by Seleit et al. [41]. Another group has also noted an intracellular localization of AQP3 in prostate cancer [64]. AQP3 localization to the plasma membrane can apparently be regulated: osmotic stress induces the translocation of AQP3 from a more cytosolic compartment to the plasma membrane in human keratinocytes [19], in addition to inducing its expression [65]. In a colon cancer cell line, AQP3 translocation to the plasma membrane is also observed upon stimulation with epinephrine, through a mechanism involving phosphoinositide turnover and protein kinase C activation [66].

Finally, our laboratory has observed an ability of a differentiating agent (elevated extracellular calcium levels) to decrease AQP3 expression and the levels of the unglycosylated protein [59], but this agent actually increases glycosylated AQP3 protein levels [25]. Since glycosylation is required for surface localization of AQP2 [67], by analogy it seems likely that glycosylated AQP3 represents mature AQP3 at the plasma membrane. Together, these results suggest the possibility that the function of AQP3 in the skin may be modulated by its cellular location, its interaction with PLD-2 or both.

Given all of the many functions of AQP3 in the skin, it is perhaps not surprising that its levels are altered in many human skin diseases in addition to the non-melanoma skin cancers (see above). For example, AQP3 levels seem to be different in psoriasis, although whether AQP3 is up- or down-regulated, or simply abnormally distributed, is unclear. Thus, our laboratory has previously demonstrated that in psoriatic lesions, AQP3 is misdistributed [18], consistent with a recent report describing a cytoplasmic AQP3 staining pattern [41], and likely reduced [18]; another group demonstrated similar results by immunofluorescence, which when quantified detected an approximately 67% decrease in AQP3 protein levels in psoriatic lesions compared to normal skin [58]. In contrast, microarray and RNA-sequencing (RNA-seq) studies have indicated increased mRNA levels in psoriasis [68, 69]. Although these data might suggest a possible divergence between mRNA and protein levels in psoriatic lesions, it seems more likely that the increased mRNA expression observed is due to the fact that proliferating keratinocytes comprise a greater proportion of psoriatic epidermis than of the normal epithelium, and AQP3 expression is decreased with differentiation ([59] and see above). Indeed, this interpretation is supported by results of Hara-Chikuma and colleagues [16] in a mouse model of psoriasis, in which AQP3 protein levels are elevated to a similar extent to the increase observed in the levels of keratins 5 and 14, which mark the proliferating basal keratinocytes. Additional research is needed to clearly define the role of AQP3 in psoriasis.

Changes in AQP3 mRNA and/or protein expression have also been linked to other human skin diseases, such as atopic dermatitis [6, 70], depigmented vitiligo [54] and cutaneous pruritus [71] (reviewed in [25]). In atopic dermatitis, discrepant results suggest both up- and down-regulation of AQP3 [6, 46, 70]. Similar to AQP1, epidermal AQP3 levels are also increased in infants with erythema toxicum neonatorum [12]. In this skin disorder, there was also an increase in the number of AQP3-positive cells in the dermis, with Langerhans cells and other dermal dendritic cells, as well as eosinophils, macrophages and neutrophils near hair follicles, all demonstrating AQP3 immunoreactivity [12]. It has also been proposed that AQP3's transport of water (and possibly glycerol) is involved in the development of pompholyx, a type of eczema characterized by vesicles or blisters on the hands and feet [72]. Interestingly, it appears that AQP3 in epidermal keratinocytes is involved in maintaining the health of the pigment cells, the melanocytes [54], with a reduction in AQP3 levels observed in depigmented vitiligo skin. This result is consistent with the fact that in involved vitiligo skin, as in AQP3 knockout mice [22], delayed barrier recovery is observed [73]. In addition, reduced AQP3 levels are observed in aged skin, both sun-exposed [74] and unexposed [75], suggesting a possible link to the impaired skin function seen with aging [76]. Finally, decreased AQP3 expression has been observed in diabetic rat skin with skin wounding [77]; since diabetes also can result in delayed wound healing (e.g., [78]), as does AQP3 gene ablation in knockout mice [10, 22], this result is again consistent with a role for this channel in a key skin function, wound healing. Thus, data in the literature support an important contribution of AQP3 to skin health, although our understanding of its exact role is as yet incomplete.

The importance of AQP3 in the skin is likely related in part to its ability to transport glycerol. Anecdotal evidence discussed by psoriatic patients on the National Psoriasis Foundation website

suggests a possible therapeutic role for glycerol in psoriasis (https://www.inspire.com/groups/talk-psoriasis/discussion/glycerin-research-important/). Indeed, Pacifico et al. [79] report that a moisturizer containing glycerin (also known as glycerol) improves the symptoms of psoriasis to a greater extent than a Vaseline-based emollient in psoriatic patients treated also with narrow-band ultraviolet B radiation [79]. Additional support for this idea is provided by the asebia mouse model, which exhibits atrophic sebaceous glands and reduced or absent production of sebum [80], which is rich in triacylglycerols and phospholipids. These mice also demonstrate reduced epidermal glycerol content and keratinocyte hyperproliferation that can be corrected by provision of glycerol but not other humectants [81], again showing the key role played by glycerol in epidermal function. Epidermal glycerol content is known to be affected by several factors including the site measured and prior immersion in water [82]. Glycerol is also routinely added to skin lotions and wound salves, based on empirical evidence of improved skin function and enhanced wound healing [26]. Since AQP3 improves the efficiency of entry of glycerol into cells of the skin, increased AQP3 levels may enhance the beneficial effects of this agent in the skin. This idea has led to extensive investigation to identify inducers of AQP3 (e.g., [19, 83]), with the thought that such inducers would improve skin function. Nevertheless, based on the potential role of AQP3 in proliferation, likely in part via effects on ATP production [10, 27], Verkman has urged caution with the use of agents that increase AQP3 levels [84].

In the other cells of the skin in which AQP3 is expressed, this channel seems to play similar roles as in keratinocytes. For example, as discussed above, AQP3 mediates T cell trafficking in the skin such that AQP3 knockout mice show reduced hapten-induced contact hypersensitivity [15]. In addition, in dermal fibroblasts AQP3 mediates epidermal growth factor receptor (EGFR)-induced cell migration [85]. Thus, siRNA-mediated knockdown of AQP3 or inhibition of AQP3 channel activity with divalent cations, such as nickel or copper, delay scratch wound healing of normal human skin fibroblasts. EGFR activation also increases the mRNA and protein expression of AQP3 through a mechanism involving a mitogen-activated protein kinase signaling cascade and PI3K activity [85]. In addition to effects on migration, AQP3 serves other functions in dermal fibroblasts. For example, AQP3 protects these cells from ultraviolet irradiation-induced cell death by up-regulating the levels of the anti-apoptotic protein BCL-2 in normal human dermal fibroblasts [86]. Similarly, AQP3 is expressed in a majority of melanoma cell lines, i.e., transformed melanocytes, and overexpression of AQP3 protects these cells from arsenite-induced apoptosis by increasing the levels of BCL-2 and another anti-apoptotic protein XIAP [87]. (AQP9, which is also detected in approximately half of the tested melanoma cell lines, also protected the melanoma cells from arsenite when overexpressed [87].) Finally, dermal fibroblast AQP3 mRNA and protein expression is induced by bleomycin, which also increases hydrogen peroxide, in a mouse model of scleroderma, an autoimmune skin disorder characterized by fibrosis of the skin and internal organs [88]. Knocking down AQP3 with shRNA reduces the intracellular levels of reactive oxygen species and also inhibits the fibrotic phenotype in the bleomycin model [88]. These results suggest a potential involvement of AQP3 in dermal fibroblasts in scleroderma, although future studies are needed to determine whether the human disease shows a similar increase in dermal fibroblast AQP3 levels.

11.2.3 AQP5

AQP5 plays an important role in the dermis, specifically in the secretion and absorption of sweat in the sweat glands of the skin. Humans have an estimated two to four million eccrine sweat glands located throughout the body [89]. Their secretions help to regulate the internal environment of the body by allowing the passage of water and electrolytes out of the body, maintaining body temperature, and protecting the skin from harmful organisms and bacteria. Humans have an

impressive ability to secrete sweat, with volumes ranging from 100 to 8000 mL per day possible [90]. AQP5 is located in both the apical membrane and the basolateral membranes of the secretory coils of the eccrine sweat glands and translocates to the apical membrane during the active process of sweating to increase plasma membrane water permeability [91]. Acetylcholine is the main regulator of sweating and functions by increasing intracellular calcium thereby stimulating the apical translocation of AQP5 [91]. In addition, AQP5 appears to be necessary for the secretion of sweat, as a loss of AQP5 has been shown to lead to a large decrease in active sweat glands [92]. Research has shown that patients with Sjogren's syndrome retain their AQP5 intracellularly, leading also to decreased saliva and tear production [90]. Drugs modulating AQP5 activity have been suggested as a possible therapy for patients suffering from sweating disorders [91].

Recently, mutations in AQP5 have been identified in patients with a form of diffuse nonepidermolytic palmoplantar keratoderma (PPK) [8, 93], a disease in which the skin of the soles and palms exhibits hyperkeratosis (thickening) as well as a defective water permeability barrier [8, 94]. The phenotype is exacerbated upon exposure of the affected areas to water, with a resulting white spongy appearance of the skin [8, 93, 94]. This experiment of nature suggests a role for AQP5 in regulating keratinocyte function in these areas, and indeed, AQP5 protein expression is observed in the plasma membrane of keratinocytes in the skin of the palms, predominantly in the stratum granulosum [8]. Nevertheless, the mechanism by which mutations in AQP5 result in PPK and how AQP5 contributes physiologically to the formation of the water permeability barrier in thick skin remain unknown, and further studies are needed.

11.2.4 AQP7

AQP7 is an aquaglyceroporin specifically located in the adipocytes of the hypodermis [6]. Adipocytes regulate lipogenesis and lipolysis to provide energy to the human body when needed. When there is an excess of nutrition, adipocytes take in glucose from the blood and metabolize and combine it with fatty acids to make triglycerides in a process called lipogenesis. In times of starvation or exercise when energy is needed, adipocytes activate lipolysis, hydrolyzing triglycerides to glycerol and fatty acids so that the body may use these molecules for energy. AQP7 plays a crucial role during the process of lipolysis by transporting glycerol out of the adipocytes to allow maintained triglyceride breakdown [95].

In adipocytes under basal conditions, AQP7 is found near the periphery of the nucleus, but when lipolysis is required to provide fatty acids (and glycerol) for energy production, epinephrine is secreted and acts on adrenergic receptors to increase the levels of cAMP in the cells [95]. The increased levels of cAMP activate protein kinase A to result in the stimulation of hormone-sensitive lipase activity. Hormone-sensitive lipase hydrolyzes triglycerides to yield glycerol and fatty acids, and AQP7 is translocated to the cell membrane to allow for the transport of glycerol out of the cell and into the bloodstream [95]. This glycerol, in turn, can serve as a substrate for gluconeogenesis [96]. AQP7 expression is partially regulated by insulin, and AQP7 mRNA levels are increased during food deprivation, when lipolysis is induced, due to the decrease in suppressive insulin during these times [95].

AQP7 is also a target of peroxisome proliferator-activated receptor-gamma (PPAR-γ), a regulator of many genes in adipose tissue, and is upregulated when PPAR-γ is activated. PPAR-γ is the main target of the drug class of thiazolidinediones used in diabetes to decrease insulin resistance. These drugs have been shown to sensitize adipocytes to insulin, possibly due partially to the increased expression of AQP7 [95].

On the other hand, an absence of AQP7 has been shown to lead to obesity and insulin resistance due to glycerol accumulation and subsequent adipocyte hypertrophy. Adipocytes in mice lacking AQP7 exhibit increased intracellular glycerol, enhanced uptake of fatty acids, and accelerated triglyceride synthesis [97]. Due to these findings, modulation of AQP7 has been suggested as a possible therapy for obesity [98].

Finally, a recent report suggests that AQP7 is involved in primary cutaneous immune responses [7]. This idea is based on the fact that not only is AQP7 expressed in Langerhans cells (epidermal dendritic cells) and dermal dendritic cells of the skin, but also that AQP7 knockout mice show impairment of their contact hypersensitivity response and decreased sensitization [7]. Further experiments indicate that AQP7 is required for the ability of dendritic cells to take up antigens for presentation; thus, AQP7 knockout dendritic cells demonstrate reduced internalization of antigens of various sizes [7], indicating an impairment of macropinocytosis, and perhaps other cellular uptake mechanisms, in these cells. AQP7 is also necessary for dendritic cell chemotaxis, with the AQP7 knockout dendritic cells exhibiting reduced migration in response to C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal-derived factor-1 or SDF-1) or C-C motif chemokine ligand 21 (CCL21) [7]. Together, these results indicate an involvement of AQP7 in cutaneous dendritic cell function, and thus hypersensitivity reactions in the skin.

11.2.5 AQP9

AQP9 is an aquaglyceroporin that is located in many tissues throughout the human body, including the epidermis. It is specifically located in the upper epidermis, in the layer of the stratum granulosum above the level where AQP3 is expressed [99]. Research has shown that AQP9 expression is regulated in a different manner than AQP3. While retinoic acid increases expression of AQP3 [99, 100] and increases keratinocyte proliferation [99], it actually decreases the mRNA levels of AQP9 [99]. AQP9 facilitates the transport of both glycerol and urea [99], as well as hydrogen peroxide [101]. Urea is an active gene regulator in keratinocytes, affecting keratinocyte differentiation, lipid synthesis, and anti-microbial peptide production [102]. Topical urea has been shown to be beneficial to patients with altered skin barrier function and is used for a variety of conditions such as hyperkeratotic or xerotic skin [102, 103]. Through regulating the transport of urea in keratinocytes, AQP9 plays a role in both antimicrobial defense and barrier permeability function of the skin [102].

Using the hapten dinitrofluorobenzene (DNFB) for sensitization in mice, AQP9 has also been recently shown to play a role in contact hypersensitivity. Despite showing normal numbers of various immune cell types under basal conditions, AQP9 knockout mice exhibited reduced ear edema relative to wild-type animals upon sensitization with DNFB and a subsequent challenge with this agent [104]. Decreased numbers of infiltrating CD4-positive and CD8-positive T cells and neutrophils, but not mast cells, were observed [104]. DFNB-induced ear swelling could be restored in AQP9 knockout mice by reconstituting these mice with wild-type bone marrow, indicating that the relevant cell type in this system seems to be an immune cell rather than keratinocytes. In particular, the response could be restored by reconstituting AQP9 knockout mice with wild-type neutrophils, but not T cells, and was decreased upon antibody-mediated neutrophil depletion (in wild-type mice) [104], suggesting the importance of neutrophils in this contact hypersensitivity response. Skin draining lymph nodes isolated from AQP9 knockout mice under this paradigm also exhibited reduced secretion of interleukin-17A (IL-17A), with no effect on interferon-gamma levels. In addition, AQP9 knockout mouse-derived neutrophils demonstrated reduced chemokine-induced cell migration [104]. Together these results suggest that the AQP9 expressed in neutrophils contributes to the induction of contact hypersensitivity by allowing chemokine-mediated recruitment of IL-17A-producing neutrophils to sites of skin sensitization.

11.2.6 AQP10

AQP10 is another aquaglyceroporin found in the keratinocytes of the epidermis. It has been specifically localized to the stratum corneum in humans through recent *in vivo* studies [21].

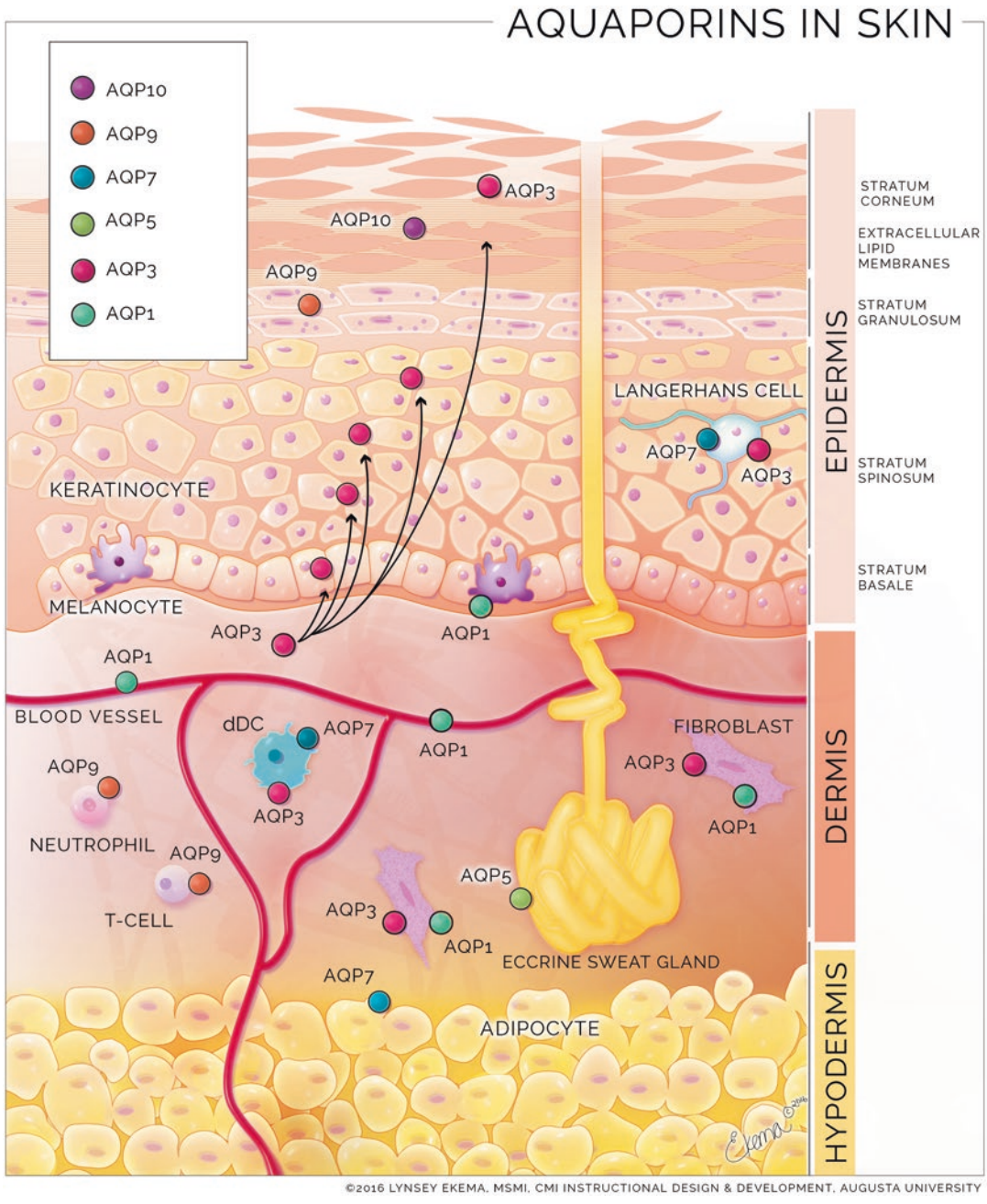

Fig. 11.5 Summary of localization of AQPs in skin. Shown is the structure of skin, consisting of the deepest layer, the hypodermis, the dermis and the most superficial epidermis, along with skin appendages such as eccrine sweat glands. Adipocytes comprise the hypodermis whereas the dermis is made up mostly of connective tissue produced by resident fibroblasts. The dermis is vascularized and also contains infiltrating neutrophils and T-cells (particularly in inflammatory skin conditions) and dermal dendritic cells (dDC). The epidermis is composed of layers as well, with the stratum basale attached to the basement membrane that separates the dermis and epidermis, and the suprabasal differentiated layers, the stratum spinosum, the stratum granulosum and the stratum corneum sitting atop the basal layer. The predominant cell of the epidermis is the keratinocyte, although melanocytes, Langerhans cells and Merkel cells (not shown) are also interspersed among the keratinocytes. Also illustrated is the localization of each AQP in the various cells of the skin

Little information is currently available about the physiologic function of AQP10 outside of its ability to transport water and glycerol, but it is believed to share similar functions as AQP3, and it may play a role in the barrier function of the skin due to its location in the stratum corneum [6, 21]. AQP10, like AQP3, may also be involved in pompholyx [72], although further studies are needed to address this question.

11.3 Conclusion

The skin performs many important functions, serving as a mechanical and water permeability barrier, assisting with thermoregulation, protecting against ultraviolet radiation and chemical hazards and allowing tactile sensation. The skin is also the first organ perceived by fellow humans, such that abnormalities, as with skin diseases, are often immediately apparent and can affect social interactions. For this reason, skin disorders such as psoriasis, which can lead to lesions on various, often exposed, sites of the body, frequently result in a great deal of psychological distress [28, 105]. Aquaporins, channels that transport water and sometimes other small molecules, are expressed in many different cell types in the skin (Fig. 11.5). They play important roles in these various locations, contributing to many key functions of the skin including hydration, formation of the water-permeability barrier, wound healing and immune responses. Nevertheless, many questions remain concerning these important proteins, and studies are in progress in multiple laboratories to better understand the role(s) of each aquaporin in the skin.

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Aquaporins in the Eye

12

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Abstract

The major part of the eye consists of water. Continuous movement of water and ions between the ocular compartments and to the systemic circulation is pivotal for many physiological functions in the eye. The movement of water facilitates removal of the many metabolic products of corneal-, ciliary body-, lens- and retinal metabolism, while maintaining transparency in the optical compartments. Transport across the corneal epithelium and endothelium maintains the corneal transparency. Also, aqueous humour is continuously secreted by the epithelia of the ciliary body and maintains the intraocular pressure. In the retina, water is transported into the vitreous body and across the retinal pigment epithelium to regulate the extracellular environment and the hydration of the retina. Aquaporins (AQPs) take part in the water transport throughout the eye.

Keywords

Water • Water channel • Aquaporin • Eye

Abbreviations

AQP	aquaporin
IOP	intraocular pressure
kDa	kilodalton
Kir4.1	inward rectifying potassium channel
MIP	Major intrinsic protein
mRNA	messenger ribonucleic acid
RGC	retinal ganglion cell
RPE	retinal pigment epithelium
RT-PCR	reverse transcriptase-polymerase chain reaction
TM	trabecular meshwork

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12.1 Aquaporins in the Normal Eye

Precise regulation of ocular fluids is necessary for the optimization of visual function, since the major part of the eye is comprised of water [1].

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Continuous movement of water and ions between ocular compartments and to the systemic circulation is required. The movement of water facilitates removal of the many metabolic products of corneal-, ciliary body-, lens- and retinal metabolism, all while maintaining transparency in the optical compartments [1]. The transport of water across the corneal epithelium and endothelium maintains corneal transparency. Rapid changes in the water content of the iris stroma facilitate changes in shape during pupil constriction and dilatation. In the retina, water is transported transcellularly across the retinal pigment epithelium into the choroid preventing subretinal edema and retinal detachment [1, 2]. The intraocular pressure (IOP) is maintained by the aqueous humour [3]. Aqueous humour is secreted by the pigmented and nonpigmented epithelia of the ciliary body in a concerted action involving active membrane proteins and passive ion and water channels [3]. Aquaporins (AQPs) are expressed in several of these structures and facilitate the transport of water (Fig. 12.1). AQP0 contributes to lens transparency, and AQP1 is involved in the secretion and drainage of aqueous humour. AQP3 and AQP5 have corneal and conjunctival barrier functions, and AQP4 plays an important role in retinal water homeostasis [2, 4, 5]. AQP6–12 have been discovered more recently and the function of them has not been investigated in the human eye.

12.1.1 Aquaporins in the Cornea

The cornea is the first light refractive medium hit by light and the transparency of the cornea relies on the precisely organized stroma consisting of collagen fibrils and glycosaminoglycans. Precise regulation of the water content is required to keep the stroma neatly packed avoiding light scatter. The water content of the cornea is 78% weight and negatively charged glycosaminoglycans and proportionate cations make the cornea slightly hyperosmolar compared to the aqueous humour [6, 7]. Consequently, continuous transport of solutes and steady water expulsion away from the cornea is needed. The corneal endothelium expresses AQP1 and is responsible for transport of the major part of water out of the corneal stroma [5, 6, 8]. The corneal endothelium contains active transporters (Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$, HCO_3^-/Cl^-) that pump solutes from the stroma into the aqueous humour. Water is transported passively, driven by the osmotic gradient, into the anterior chamber partly through AQP1 but also paracellularly [6, 8]. In addition, AQP1 expressed by the keratocytes has been suggested to facilitate volume changes in the keratocytes in response to changing corneal hydration [5, 9].

The outer stratified epithelium of the anterior corneal epithelium expresses AQP3 and AQP5, and facilitates water transport away from the cornea. Deletion of AQP5 in mice increases the

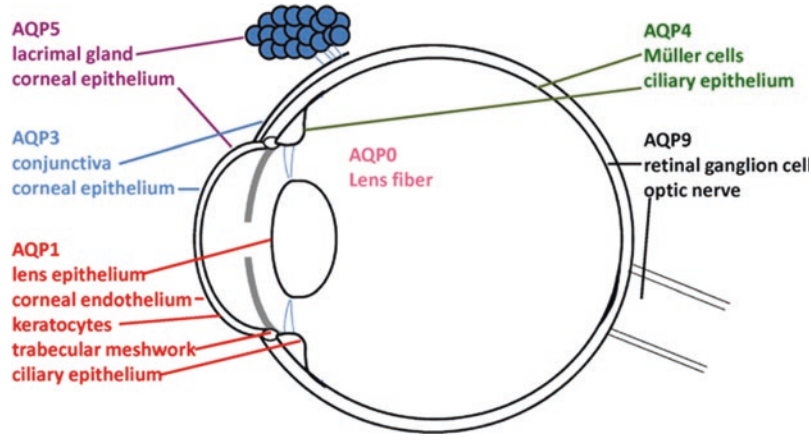

Fig. 12.1 Schematic drawing of the cellular localization of AQPs in the human eye

corneal thickness and the osmotic water permeability across the corneal epithelium is reduced [5, 6]. However, when exposed to hyperosmolar stress no noticeable changes occur during swelling and recovery. Mainly the corneal endothelium maintains the hydration of the cornea, therefore, transport via AQP5 across the epithelium does not affect the overall transparency of the cornea [6].

Maintenance of the corneal epithelium is crucial in providing a smooth and transparent refractive surface [2, 10]. The stratified corneal epithelium expresses the water- and glycerol transporting AQP3 [5, 10]. During reepithelialization AQP3 facilitates the water and glycerol transport in the corneal epithelial cell migration and proliferation (10). AQP3-facilitated cell migration has also been demonstrated in wound healing in skin [11]. In the migration phase of reepithelialization, marginal cells extend lamellipodia and filopodia at the wound's leading edge and AQP3 provides for the local water transport here. Corneal epithelial cell migration also requires mobilization of energy stores, particularly glycogen [10]. Defective glycerol transport in AQP3 deficiency may impair the glycogen synthesis or utilization by direct or indirect effects on glycolysis [11]. AQP3 deletion in mice demonstrated reduced glycerol permeability but the steady state corneal epithelial glycerol content was not significantly affected [10]. However, a significant delay in resurfacing was found and the AQP3-deficient corneas were thinner suggesting impaired proliferation in AQP3 deficiency.

Furthermore, during corneal wound healing AQP1 may facilitate keratocyte migration also through AQP-facilitated water influx into lamellipodia at the leading edge of migrating cells [12].

12.1.2 Trabecular Meshwork

AQP1 is expressed by the endothelial cells of the trabecular meshwork (TM) and Schlemm's canal [5, 13, 14]. In the conventional outflow pathway fluid drainage is predominantly paracellular and therefore AQP1 might not directly regulate the aqueous humour outflow [14]. Rather, AQP1 may regulate TM endothelial intracellular volume and indirectly modulate the paracellular drainage [13]. Also, AQP1 may contribute to endothelial cell survival to resist the mechanical strain during the passage of aqueous humour [14].

12.1.2.1 Ciliary Body

Aqueous humour provides nutrients and removes metabolic waste product from the avascular structures in the anterior eye, namely the cornea and the lens. Furthermore, aqueous humour maintains the intraocular pressure (IOP). Aqueous humour is secreted by the ciliary body through combined actions of active pumps and AQPs in the ciliary epithelia [15]. The ATP-consuming pumps and channels transport ions and major solutes from the ciliary capillaries and into the posterior chamber. AQP1 and AQP4 facilitate the major part of the passive water transport in the apical and basolateral membrane of ciliary nonpigmented epithelial cell [5, 15]. AQPs are not expressed in the ciliary pigmented epithelium, and so water may be actively co-transported into the pigmented layer together with major solutes [16].

12.1.3 Lens

The lens is another important refractive structure and transparency of the lens is equally important. The lens is avascular, and therefore, transport of oxygen, nutrients and ions occur by diffusion and active transport across the epithelial layer from the aqueous humour [17, 18]. The transport of water into the lens is mediated by AQP1 in the epithelial cells and AQP0/MIP (major intrinsic protein) expressed by the lens fibers [17, 18]. AQP0 either functions as a water channel or a structural protein depending on the molecular changes and posttranslational modifications occurring during shifts in the spatial location of the lens fibers [18].

In the cortical fibers, AQP0 interacts with other proteins such as connexins, filensin, phakinin, and crystallins and AQP0 mediates passage of water. However, in the nuclear fibers cleaving of AQP0 results in conformational changes and

closes the water transporting pores of AQP0. The AQP0 function switches from water channel to adhesion molecule, since AQP0 no longer interacts with the surrounding proteins [17, 18]. Instead, AQP0 forms tight junctions and assists in maintaining minimal space between the fibers. Thus, AQP0 facilitates microcirculation and also interfiber adhesion within the lens and consequently contributes to maintaining transparency of the lens.

12.1.4 Retina

In the retina, considerable amounts of water are produced during the large metabolic turnover [4, 19]. Co-transport of water during uptake of metabolic glucose and lactate from the blood also contributes to the water content [19, 20]. Furthermore, hydrostatic forces driven by the intraocular pressure also push water to enter the retina from the vitreous body. Therefore, significant amount of water has to be cleared to maintain local balance of ions for effective signal transduction [4, 21].

In the inner retina the major glial support cells, the Müller cells, redistribute water and ions [4, 19]. Neuronal activity accompanying synaptic transmission results in a transient increase in $[K^+]$ in the plexiform layers of the retina and a decrease in the extracellular $[K^+]$ in the subretinal space. Rapid removal of K^+ is important to maintain neuronal excitability during prolonged light stimulation. The Müller cells, regulates the K^+ balance by up-take of K^+ through Kir4.1, the inwardly rectifying potassium channel, and siphoning K^+ into the vitreous body or retinal capillaries [4, 19]. AQP4, co-localized with Kir4.1, facilitate the accompanying osmotic water transport in response to the K+−flux and together they maintain the spatial buffering of $[K^+]$ [4].

The RPE cells are responsible for clearing metabolic waste product, neurotransmitters, excess ions and water from the outer retina and subretinal space through active transport and solute-linked transport [22, 23]. Cotransport proteins known to facilitate transcellular ion movement elsewhere in the body also function as molecular water pumps in the retina in the absence of an osmotic gradient [16]. AQPs may also contribute to ion- and water elimination at the RPE cells. However, AQP1 has only been identified in the cell membrane of cultured cells, but not in histological sections of human eyes [5, 24]. Therefore, the water transport facilitated by AQPs is unclarified.

AQP9 is expressed by the retinal ganglion cells (RGC) [5, 25, 26], and has formerly been found in the brain, primarily in the astrocytes [27]. AQP9 has been suggested to provide neurons with lactate and glycerol for energy metabolism [19, 27, 28]. The presence of AQP9 in both the brain and retina is not surprising given their close connection. Accordingly, AQP9 may have a similar function in the retina and brain and may facilitate the uptake of lactate or glycerol into the RGCs and photoreceptors [26, 29].

12.1.5 AQP6–12 in the Normal Eye

In the recent study by Tran et al (2013) [30], the localization of AQP6–12 was mapped in the human eye (Fig. 12.2). The transcripts of AQP7, AQP9 and AQP11 mRNA have been detected in various structures of the eye. Immunohistochemical staining demonstrated the specific cellular localization of AQP7, AQP9 and AQP11. AQP7 expression has been observed in the corneal epithelium and endothelium. In the corneal-limbal region AQP7 and AQP11 are expressed in the basal cells of the anterior epithelium. AQP7 is also expressed in the lens epithelium and trabecular meshwork endothelium. In the ciliary nonpigmented epithelium, AQP7, AQP9 and AQP11 are all expressed. In the retina, the Müller cell endfeet showed AQP7 and AQP11 labelling. AQP9 immunolabelling has been found in the RGCs and in the nerve fibers of the optic nerve.

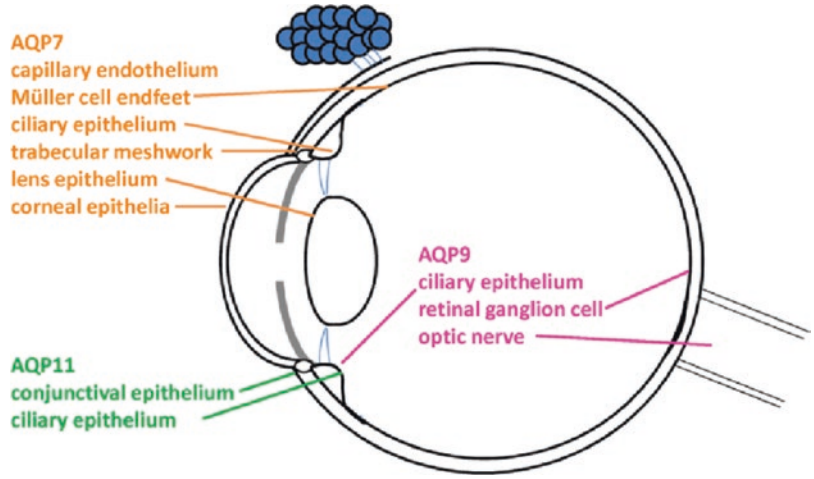

Fig. 12.2 Schematic drawing of the cellular localisation of AQP7, AQP9 and AQP11

12.2 AQPs in Pathological Conditions of the Human Eye

AQPs are present in all structures in the eye that have a water regulating function and this poses the question whether dysfunction or inhibition of AQPs lead to pathology, especially in diseases where oedema is a prominent clinical observation. In Fuch's endothelial dystrophy and psedophakic bullous keratopathy, oedema of the cornea is the central problem. Downregulation of AQP1 in the corneal endothelium has been demonstrated in these keratopathies [31]. Healing of corneal abrasions is linked with AQP3 expression by the corneal epithelium, since deletion of AQP3 delay epithelial cell resurfacing and result in a thinner epithelium [10, 11].

The role of AQP0 in the lens has been clearly demonstrated and several mutations in AQP0 or any of the molecules interacting with AQP0 result in cataract formation [17]. Complex coordination of the lens proteins is required, since each protein contributes to lens transparency, appropriate refractive index and accommodation. All characterized mutations in AQP0 result in autosomal dominant bilateral cataract [17, 18].

In glaucoma, a progressive and vision threatening disease, several structures in the eye are involved. The ciliary body, the trabecular meshwork, the retina and the optic nerve may be coupled with the development of glaucoma. Deletions of AQP1 and AQP4 result in modest reductions in IOP and aqueous humour production, however, the clinical significance is uncertain [32]. In addition, AQP1 and AQP4 null mice show preserved anterior chamber morphology. Downregulation of AQP9 expression by RGC has been shown to be coupled with RGC metabolism and cell survival [26, 33].

Diabetes mellitus and age related macular degeneration (AMD) are two major reasons for impaired vision. Severe stages of both diseases involve neovascularisation resulting in retinal oedema due to leakage from the newly formed capillaries. The excess fluid exceeds the capacity of the glial cells and RPE cell in clearing fluid from the retina. Changes in the expression of ion channels and transporters, and of AQPs may intensify the retinal oedema. Lastly, AQP expression has been demonstrated to be altered in a complex pattern in the diabetic rat retina [34].

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Aquaporins in Fetal Development

13

Nora Martínez and Alicia E. Damiano

Abstract

Water homeostasis during fetal development is of crucial physiologic importance. The successful formation and development of the placenta is critical to maintain normal fetal growth and homeostasis. The expression of several aquaporins (AQPs) was found from blastocyst stages to term placenta and fetal membranes. Therefore, AQPs are proposed to play important roles in normal pregnancy, fetal growth, and homeostasis of amniotic fluid volume, and water handling in other organs. However, the functional importance of AQPs in fetal development remains to be elucidated.

Keywords

Water • Water channel • Aquaporin • Fetal development

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13.1 The Placenta

Placenta and fetus development is a continuous process that begins at the time of fertilization. Placenta is a highly specialized temporary organ exclusive of gestation, which serves as the lung, the kidneys, the liver, the endocrine system, and the immune defence of the baby. Thus, the placenta carries out essential functions and is critical to achieving a successful pregnancy. It provides an interface between the mother and the fetus, prevents the rejection of the fetal allograft, enables the respiratory gas exchange, transports nutrients, eliminates fetal waste products, and secretes peptide and steroid hormones.

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Placenta formation requires the proper growth, differentiation and maturation of its principal cells: the trophoblasts. Trophoblast cells are subdivided in two types of cells: the villous mononucleated cytotrophoblast cells, which proliferate and differentiate into the multinucleated syncytiotrophoblast cells, and the extravillous trophoblast cells. Villous syncytiotrophoblast cells are in direct contact with the maternal blood and facilitate the exchange of nutrients, wastes and gases between the mother and the fetus. In addition, the syncytiotrophoblast also contributes in the biosynthesis of essential hormones for the progression of gestation. Extravillous trophoblast cells invade the uterine tissues and participate in the remodeling of the uterine spiral arterial walls to guarantee the appropriate supply of blood to the fetal-placental unit [1–3].

Anatomically, the placenta is composed of two different surfaces or sides, the maternal surface, facing towards the outside, and the fetal surface, facing towards the inside, or the fetus (Fig. 13.1). The fetal side of the placenta is covered by the amnion or amniotic membrane. Underlying the amnion is the chorion, a thicker membrane. This structure of the placenta is continuous with the lining of the uterine wall. Emerging from the chorion are the chorionic villi, the main functional units of the placenta (Fig. 13.2a, b). The chorionic villi contain a system of fetal capillaries (blood vessels) to allow maximum contact area with the maternal blood (also known as the intervillous space). The umbilical cord also emerges from the fetal side (Fig. 13.1).

The maternal side is composed of the decidua and the lobules known as cotyledons. Each individual cotyledon contains the same chorionic villi emerging from the chorion.

The tissue separating maternal and fetal blood in the placenta is called the placental barrier. According to the Grosser classification [4] the human placenta can be considered hemochorial in which the placental trophoblast is in direct contact with maternal blood (Fig. 13.3).

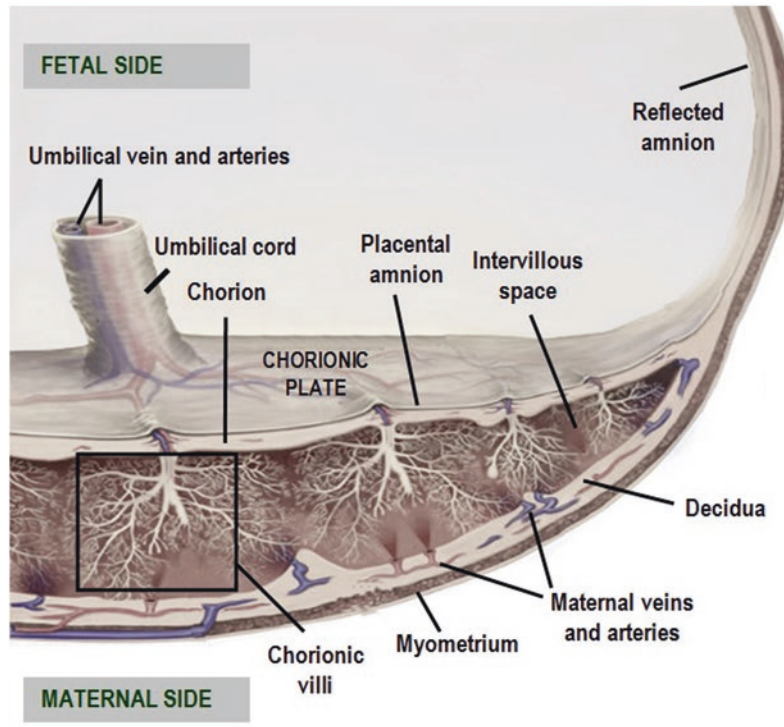

Fig. 13.1 Schematic drawing of a section of the human term placenta

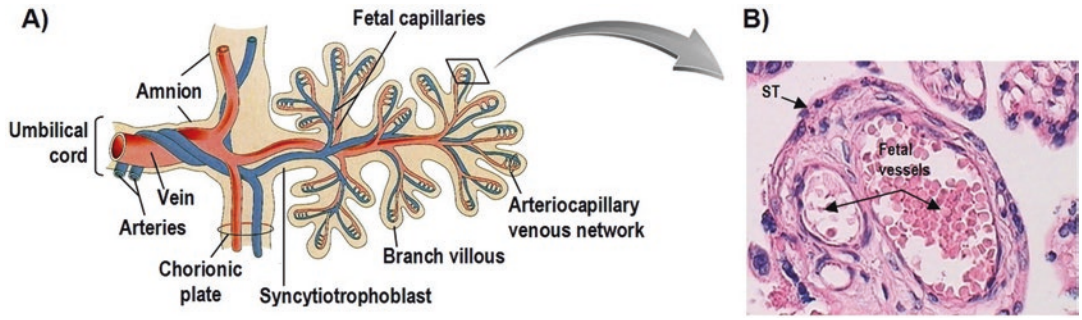

Fig. 13.2 Human chorionic villous. (a) Schematic drawing of a human chorionic villous. **(b)** Hematoxylin and eosin (H&E) staining of a terminal villi tissue section. Fetal *red blood cells* are seen in villous core fetal vessels. ST (syncytiotrophoblast) cells cover the entire surface of the villous. *Magnification* × *400*

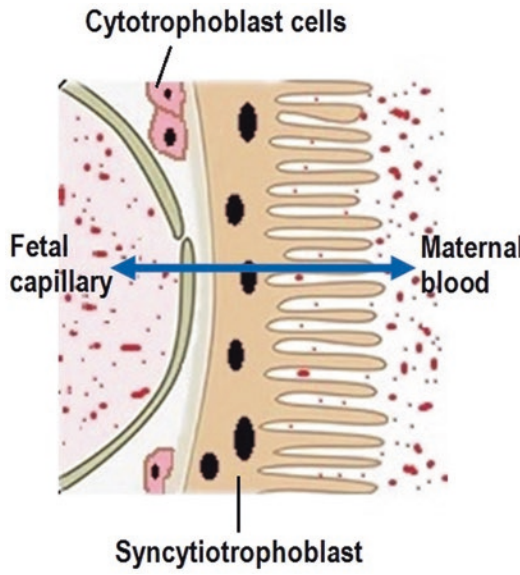

Fig. 13.3 Representation of the human placental barrier. Human placental is classified as hemomonochorial: maternal blood directly bathes the chorionic villi. Note that at term, cytotrobloblast cells do no limit feto-maternal exchange across the placenta and only one layer of syncytyotrophoblast is between the fetal and maternal circulations

13.2 Water Transport Across the Human Placenta

One of the main functions of the placenta is to promote selective transport of nutrients and waste products between the mother and the fetus and to maintain the fetal fluid homeostasis. These processes are controlled by the syncytiotrophoblast, the outermost covering cell layer of fetal placental villi that results from the fusion of the underlying cytotrophoblast cells, and the fetal membranes, which include the chorion and the amnion. Therefore, the successful formation and development of the placenta is critical to maintain normal fetal growth and homeostasis.

Water is the most important component of cells and tissues and water homeostasis during pregnancy is crucial for the normal fetal development. However, the molecular mechanisms of maternal-fetal fluid balance are not fully understood. During pregnancy, fetal water requirements increase markedly due to an exponential growth in fetal weight. Since the placenta behaves like a membrane with a very low permeability, diffusional transfer across the placenta would be not enough to meet the fetal requirements [5, 6]. Physiological data indicate that both a transcellular and a paracellular pathway are available for transfer across the human placenta, but the morphological correlation of the latter is uncertain [7]. In addition, little is known about the molecular mechanisms of these processes. Previous research in isolated membrane vesicles has suggested that water movements across the placenta occur by a lipid diffusion pathway [6, 8 ,9] discarding the transcellular route and the involvement of integral membrane water channel proteins known as aquaporins (AQPs). However, at the beginning of the twenty-first century the expressions of AQP3 and AQP9 were described in the apical membrane of the human syncytiotrophoblast [10]. Even more, using placental

explant cultures significant uptakes of water, urea and mannitol sensitive to mercury (a well-known inhibitor of AQPs) and phloretin (blocker of AQP9), were detected and these results supported the idea that human placental AQPs are functional [11]. Recently, the importance of the actin cytoskeleton in the channel protein regulation has been highlighted and the actin cytoskeleton and its reorganization have been reported to be required for the regulation of both channel activity and its intracellular trafficking [12]. Thus, a possible explanation for the discrepancy in the functional experiments is that in those performed in isolated vesicles, some of the components of the cytoskeleton that may be important for the functionality of AQPs may be excluded.

Nowadays, it is well accepted that water is transferred across the human hemochorial placenta through both the paracellular and transcellular routes, and its transfer may be facilitated by AQPs [13].

13.3 AQPs Throughout Pregnancy

After fecundation, the conceptus develops into the blastocyst that consists of an inner cell mass and a fluid filled cavity surrounded by trophoectoderm epithelium. Previously, it was described in mouse, the expressions of AQPs 3, 8, and 9 in the blastocyst and it was proposed that these AQPs may play a role in the *trans*-trophectoderm water movements occurring during the process of cavitation [14, 15]. In humans, mRNAs of AQPs 1, 2, 3, 4, 5, 7, 9, 11 and 12 were detected in preimplantation embryos, however it was found that only the expression of AQP3 and AQP7 persist from zygote to blastocyst stages, suggesting that both AQPs may be essential for early embryonic development [16] (Table 13.1).

The trophoectoderm gives rise to the placenta and the chorion so the expression of these proteins in both, the placenta and fetal membranes, should be expected. In subsequent experiments, it was reported that AQPs 1, 3, 8, and 9 were present in human, sheep and mice species with anatomically and histologically different placentas [17]. At early stages of human pregnancy, AQPs 1, 3, 4, 5, 8, 9 and 11 are expressed in chorionic villi [17, 18] (Table 13.1). Since AQP11 is an intracellular AQP, it was proposed that its physiological function may be to maintain a suitable environment in the endoplasmic reticulum allowing translation and proper protein folding [17, 18]. Enhanced expression of AQP11 during embryogenesis could be related to the development of certain organs such as salivary glands or kidney and excretory system and slow water transfer to the embryo [19]. However, during fetal development AQP11 expression becomes progressively lower and towards the end of gestation when organ functionality has achieved maturity, other AQPs take over water regulation (AQP1, 3, 8, 9). These findings suggest an important role for AQP11 in intravesicular homeostasis [18–20] and open up new perspectives on the role of AQP11 in fetal membranes during human gestation.

Concerning term placentas, Mann and co-workers found expression of AQP1 and AQP3 in fetal membranes and suggested that AQPs may contribute to amniotic fluid volume regulation [21]. They also localized AQP1 in the capillary endothelium of placental vessels [21]. In addition, Wang and co-workers reported the expression of AQP8 and AQP9 in chorioamniotic

Table 13.1 Expression of AQPs in human gestation

	Location	AQPs expression	References
Blastocyst	Blastocyst	AQP3 and 7	[16]
Early placenta	Chorionic villi	AQP1, 3, 4, 5, 8, 9 and 11	[17, 18]
	Fetal membranes	AQP1, 3, 8, 9 and 11	[17, 21–23]
Term placenta	Placental vessels	AQP1	[21]
	Trophoblast	AQP3, 4, 8 and 9	[10, 18, 23, 24]

Table 13.2 Species variations in aquaporins distributions in fetal organs

Organ	Species				References
	Rat	Mouse	Sheep	Human	
Kidney	AQP1, 2 and 3	–	AQP1 and 2	AQP1 and 2	[26–34]
Lung	AQP1 and AQP4	AQP5	AQP1, 3,4 and 5	–	[35–40]
Skin	AQP3	AQP3	–	–	[41, 42]
Heart	–	–	AQP1, 3, 4 and 8	–	[34]
Brain	AQP4	–	–	AQP1 and 4	[43–45]

membranes [22, 23]. Recently, AQP11 expression was also established in amniotic membranes at term [17]. Regarding the expression of AQPs in the trophoblast, AQP3 and AQP9 were the first AQPs found in the apical membranes of the human syncytiotrophoblast [10]. AQP8 was also localized in the trophoblast but the cell polarity of this protein has not yet been determined [23]. In addition, De Falco and co-workers described the expression of AQP4 in the trophoblast, and its downregulation throughout pregnancy [24] (Table 13.1).

Finally, during fetal growth, fetal organs express AQPs to ensure a proper exchange of fluids. AQPs are essential for the correct development of many organs like heart, skin, lung, central nervous system and so others [25]. Table 13.2 shows the AQPs found in fetuses of different species [26–45]. It is worthwhile to note that there are certain differences between these species and humans, with regard to placentation, length of gestation and maturation of vital organs that can influence the movement of water into and out of the various compartments. Since the major inputs to the amniotic fluid are the fetal urine and the lung liquid the expression of AQPs in these fetal organs may be important in the amniotic fluid formation. The fetal kidney produces big amounts of dilute urine essential for the maintenance of amniotic fluid, and this is possible because this organ express low levels of AQP2 [26, 27]. Others authors have also found weak expression of AQP3 and AQP4 in rat fetal kidneys [28, 29].

In addition, the fetal lung of various species, including humans, rats, mice and rabbits, expresses at least four AQPs (AQP 1, 3, 4 and 5), which maintain this organ in an expanded estate secreting the liquid to the lung lumen and also contribute to the amniotic fluid formation [35]. However, the factors involve in the regulation of the movement of liquid across the fetal pulmonary epithelium have not been entirely explored.

13.4 Role and Clinical Significance of AQPs in Fetal Membranes

Consistent with its development and growth, the fetus produces a varying volume of amniotic fluid throughout pregnancy [46]. Under normal conditions, water flow progressively increases throughout gestation; near term, up to 400 mL per day are transferred from the amniotic cavity across the fetal membranes into the fetal circulation [47]. Because of the osmotic pressure difference between, amniotic fluid (255 mOsm/kg) and fetal blood (280 mOsm/kg), an osmotic gradient drives transport of fluid and solutes from the amniotic compartment into the fetal blood [48]. It has also been reported that the permeability of the human amnion in vitro is 1.5x10−4 cm/s [49]. The regulation of amniotic fluid volume is dependent on a balance between the production and resorption of this fluid and it is crucial for an adequate embryonic and fetal development. The regulation of placental water transfer and intra-

membranous resorption are poorly understood; however, both phenomena support the hypothesis that AQP water channels may be fundamental to the regulation of fetal water flow [50]. In addition, several disorders such as abortion, premature birth, amniotic fluid volume abnormality, malformation and fetal growth restrictions may result when the homeostasis of the maternal-fetal fluid exchange is disrupted.

Remarkably, abnormal placental transfer of fluid may result in excessive (polyhydramnios) or reduced (oligohydramnios) amniotic fluid volume [50–52]. These obstetrical pathologies are known to be associated with fetal abnormalities or significantly increased perinatal mortality and morbidity [52].

Many authors have explored the association between polyhydramnios and oligohydramnios and AQPs. An increase in AQP1 expression particularly in the amnion (33-fold) in pregnancies complicated by idiopathic polyhydramnios was found [53] and it was postulated that this upregulation may be a compensatory response to polyhydramnios. Moreover, Zhu and colleagues found that the expression of AQP8 in the amnion and that of AQP9 in the amnion and in the chorion were significantly increased in idiopathic polyhydramnios, but that their expressions in the placenta were significantly decreased [54]. These authors suggested that when idiopathic polyhydramnios occurs, some modulation factors may be inducing the changes in the expression of AQP8 and AQP9. Consequently, these changes may increase the intramembranous absorption and decrease the maternal-to-fetal water flow to maintain amniotic fluid homeostasis [13].

On the other hand, in pregnancies complicated with oligohydramnios, a decrease in AQP1 expression in the amnion but no significant changes in the chorion and in the placenta have been observed [55]. Decreases in AQP3 in the amnion and in the chorion as well as a significant increase in the placenta have also been found [55].

Although the expression of AQPs has been demonstrated in fetal membranes and has been postulated to have a role in amniotic fluid regulation, to date, no study has examined their functional regulation and significance in human amniotic fluid volume.

It is well-accepted that human amnion has two biologically heterogeneous regions: the placental amnion (amnion on the chorionic plate) and reflected amnion (amnion of the extraplacental fetal membranes) (Fig. 13.1). Recently, it was found regional differences in the expression pattern for the five AQPs in normal human term amnion. This observation implies that each AQP may have a different function with specific roles in transport but further studies are needed to clarify the transport function of individual AQPs in both regions of the amnion [20].

13.5 Role and Clinical Significance of AQPs in Human Trophoblast

Human trophoblast tissue expressed AQP3, AQP4, AQP8 and AQP9 [10, 24] (Table 13.1). AQP3 and AQP9 belong to the aquaglyceroporin family and can permeate urea and glycerol in addition to water. It was postulated that these AQPs could participate not only in the water transport between the mother and the fetus, but also in the rapid movement of solutes across cell membranes, with minimal osmotic perturbation [10].

Although, it was demonstrated that the AQPs expressed in the human trophoblast can mediate transcellular water transport; the hypotheses concerning their roles are still speculative. AQP9 is the most studied AQP found in human placenta. AQP9 is of special interest because, in addition to being permeable to water, it is permeable to neutral solutes such as polyols and purines and pyrimidines [56].

It is well-known that AQPs are not only involved in several physiological processes but also in multiple and diverse clinical dysfunctions [57]. Up to now, the only placenta pathological condition associated to AQPs investigated was preeclampsia. Preeclampsia is one of the most important complications of human pregnancy, and despite its high incidence, its etiology is still unknown. To date, it is considered a syndrome

that occurs in two stages. In the first stage, the reduced placental perfusion, in some, but not in all women, could lead to the maternal multisystemic syndrome of preeclampsia known as second stage [58]. However, what links both stages is not determined yet. Emerging evidence suggest that the dysregulation of the AQPs expressed in the trophoblast may be a crucial step in the development of this syndrome [59].

In preeclamptic placenta it was observed an increase in AQP9 expression by Western blot and a different cellular distribution by immunohistochemical assays [11]. Thus, AQP9 was localized not only in the apical membrane but also in the basal membrane and in the cytoplasm of preeclamptic syncytiotrophoblast [11]. In contrast, the uptake of water and mannitol in preeclamptic placental explants decreased when compared to that observed in normal explants and was not sensitive to $HgCl_2$. These results may suggest a lack of functionality of AQP9 for water and mannitol in preeclampsia. Interestingly, urea uptake sensitive to phloretin and mercury increased 35% in preeclamptic explants. These findings indicate that water and solute permeability of AQP9 is altered under pathological conditions. [11].

Since preeclampsia is not known to be associated with an altered water flux between the mother and the fetus, the role of AQP9 exclusively in placental water homeostasis should be revised.

Although AQPs classical role in facilitation of trans-epithelial fluid transport is widely known, recent studies have revealed unexpected cellular roles of AQPs, including the physiology of organelles, the proliferation, apoptosis and cell migration [60]. All these processes require transient changes in cell volume and activity of certain ion transporters.

In the case of apoptosis, the loss of intracellular K^+ concentration creates an osmotic gradient that draws water out of the cell and forces the cell to shrink. This event is known as apoptotic volume decrease (AVD). In this context, it was proposed that AQPs may take part in the movement of water across the plasma membrane in dying cells [61–63].

It was well documented that placental apoptosis is a physiological event that increase progressively throughout pregnancy. It is an important mechanism of cell death necessary for normal development of human placenta [64, 65]. In addition, fluctuations in O_2 tension are proposed to be a potent inducer of apoptotic changes in human placenta, and an increase of apoptosis as a result of an inadequate trophoblast invasion, has also been found in placentas from pregnancies complicated by preeclampsia [66].

Recently, the role of AQPs in the placental programmed cell death was explored. It was observed that inhibition of these proteins, and in particular the blocking of AQP3 activity, abrogates the apoptotic response [59]. Therefore, changes in the expression and function of placental AQPs in women with preeclampsia may be one of the decisive factors in triggering the clinical manifestations of this gestational hypertensive disorder.

On the other hand, preeclamptic placentas are characterized by insufficient trophoblast invasion and abnormal spiral artery remodeling which lead to placental ischemia, so it is possible that placental AQPs may also participate in energy metabolism.

As it was reported in other tissues, diffusion of glycerol and lactate may be facilitated by AQP9 in human placenta [67, 68]. In addition, lactate may not only act as an energy substrate but also as a source of NADH (the reduced form of nicotinamide adenine dinucleotide), a scavenger of reactive oxygen species (ROS). During ischemic insult, it was supposed that the permeability of AQP9 to monocarboxylates such as lactate increases not only to serve as an energy substrate but also to prevent the accumulation of ROS [69, 70].

In preeclamptic placentas, however, it was observed a loss of AQP9 functionality for water and monocarboxylates [11]. Thus, it is possible that this lack of functionality, adversely affects the survival of the trophoblast cells, at least partly because of the decreased transport of lactate as a substrate for energy and/or ROS scavenger. In addition, the increase of ROS may contribute to the exacerbate apoptosis observed in preeclamp-

sia. So far, whether or not placental AQPs play a direct role either in the pathogenesis or in the adaptive response of preeclampsia is still uncertain and needs further studies.

Finally, it cannot be excluded a possible role for AQP3 and AQP9 channels in the diffusion of urea. It was previously reported the expression of a urea transporter type A (UT-A) in the human placenta [11]. Thus, both AQPs could complement the UT-A function, participating in urea uptake and elimination across the placenta.

13.6 Regulation of Placenta AQPs

Throughout pregnancy, the regulation of amniotic fluid volume depends on a balance between the production and resorption of this fluid [46–51]. It has already been shown that fetal membranes and AQPs take part in this regulation. However, little is known about AQP protein regulation in the human placenta and fetal membranes.

Several lines of evidence suggest that cAMP stimulates AQP1, 3, 8 and 9 mRNA expression in human amnion epithelial cells [71, 72]. However, the response of the individual gene to cAMP stimulation is different. It was observed that cAMP treatment results in rapid but temporary upregulation of AQP3 expression [71]. In the case of AQP8 expression its upregulation was rapid and persistent while the responses of AQP1 and 9 were delayed and long-term after cAMP treatment [72].

Additionally, it was also found a lack of effect of a protein kinase A activator, SP-cAMP, on AQP1, 8, and 9 mRNA expressions suggesting that cAMP upregulates these proteins via a protein kinase A independent pathway [73]. It is noteworthy that besides to their function as a water channel, both AQPs are permeable to neutral solutes including urea and glycerol. Thus, this apparent cooperative upregulation of AQP3 and AQP9 by cAMP may be important to intramembranous absorption of amniotic urea and other solutes to maintain an osmotic gradient between amniotic fluid and fetal circulation while water is being transported intramembranously by various AQP water channels.

AQP8 was also found to be regulated by osmotic stress in amnion epithelial cells. It was observed that a hypotonic media significantly enhanced AQP8 mRNA and protein expression while hypertonic media significantly decreased its expression. These findings proposed that AQP8 may participate in the balance of the amniotic fluid [74].

Furthermore, Belkacemi and co-workers have recently established that in trophoblast-like cells, AQP1 gene expression is also upregulated by vasopressin [75]. These authors demonstrated that a cAMP-dependent pathway is responsible for the vasopressin effect on AQP1 expression. As vasopressin levels may be higher in the amniotic fluid of fetuses with oligohydramnios, these findings may be strong evidence that AQP1 may be important in the regulation of the amniotic fluid volume.

Additionally, recently it was found that AQP3 coding gene was regulated by all-trans-retinoic acid in human amnion and epithelial amniotic cells [76]. Thus, retinoic acid may induce AQP3 transcripts which results in an increase in AQP3 protein and an uptake of glycerol, suggesting an increased in AQP3 function. However, the significance of these results in the amniotic fluid homeostasis is still unknown. It was postulated that since vitamin A is an epithelial wound-healing entity, its active derivative all-trans-retinoic acid, through AQP3, may be important in the fetal membrane environment and play a role in the pathological premature rupture of membranes.

Finally, it was recently reported that insulin represses significantly the transcriptional expression of AQP3 and AQP9 in the amnion, which was blocked by a phosphatidylinositol 3-kinase inhibitor [77].

Regarding the AQPs expressed in the trophoblast, only the regulation of AQP9 expression was widely investigated. The presence of numerous sites of regulation in the gene and on the protein suggests that many mechanisms may be involved in the regulation of AQP9 expression.

13.6.1 Regulation by Human Chorionic Gonadotropin (hCG)

Recent evidence showed that hormones such as human chorionic gonadotropin (hCG) secreted by villous trophoblasts may have a stimulatory effect on the molecular expression and functionality of AQP9 via cAMP pathways [78]. In normal placental explants treated with different concentrations of recombinant hCG or 8-Br-cAMP, a potent analogue of cAMP, AQP9 expression increased significantly compared to the non-treated explants. This effect on AQP9 expression was dependent on hCG and cAMP concentrations. In addition, after both treatments, AQP9 cellular distribution was similar to that observed in preeclamptic placentas. Consequently, it was hypothesized that increased serum levels of hCG found in preeclamptic women, may be involved in the increased AQP9 protein expression in preeclamptic placentas via cAMP pathways. Concerning the effect of placental hormones on transcellular water flux, it was found that hCG may modulate AQP9 activity [78]. In normal explants treated with hCG, consistent with the protein increase of AQP9, it was observed an increase of 1.6-fold in water transcellular flux.

13.6.2 Regulation by Insulin

In placental and trophoblastic cells, insulin is involved in the regulation of several genes at the transcriptional level. [79]. In agreement with earlier studies in liver and brain [80, 81], it has been recently reported that insulin has a concentration-dependent inhibitory effect on the expression of placental AQP9 through negative insulin response elements (IRE) in its promoter gene [79].

Insulin resistance characterizes normal pregnancy, but in preeclamptic pregnant women it is also observed a marked hyperinsulinemia [79]. Therefore, in preeclampsia, the exacerbation of insulin resistance may lead to an impaired insulin signal with low activation of the insulin receptor substrate 1 (IRS-1) through multiple tyrosine phosphorylations. Moreover, it was also found evidence of phosphorylation of serine residues in both IRS-1 and IRS-2, which results in desensitization of insulin action [82].

The inactivation of IRS in preeclamptic placentas can occur because of the systemic inflammation via the tumor necrosis factor α (TNF-α) [82]. TNF-α was proposed to impair insulin signaling at the level of the IRS proteins. In this regard, the Ser(307) residue in IRS-1 has been identified as a site for the inhibitory effects of TNF-α. Kanety and co-workers have reported that TNF-α induces serine phosphorylation of IRS-1 through inhibition of serine phosphatases or activation of serine kinases other than protein kinase C [83]. This increased serine phosphorylation interferes with insulin-induced tyrosine phosphorylation of IRS-1 and impairs insulin action. Accordingly with these results, in preeclamptic and in normal placental explants previously treated with TNF-α, insulin did not downregulate AQP9 expression [79]. Consequently, it was suggested that a disturbed insulin signaling could explain the poor response of AQP9 to the insulin stimuli observed in preeclamptic placentas.

Unexpectedly, the decrease in the molecular expression of AQP9 showed no link with the functional experiments [79]. It was found in normal placental explants treated with insulin that water uptake was similar to that observed in non-treated explants. These results mean that water is passing through other AQPs expressed in the placenta and may suggest that AQP9 is not exclusively involved in water transport between the mother and the fetus.

13.6.3 Regulation by Oxygen

The human placenta develops initially in a relatively hypoxic environment that is essential for appropriate embryonic development and normal placental angiogenesis [84]. Thus, during normal pregnancies the human placenta is exposed to profound changes in oxygenation and the placenta adapts to these changes by modulation of the hypoxia inducible factor-1α (HIF-1α) and by increasing cellular antioxidant defenses [85].

However, these fluctuations in O_2 tension are critical in pathological conditions in which trophoblast invasion is superficial and placental perfusion is intermittent.

It was found that in normal placental explant culture AQP9 protein decreased abruptly when HIF-1α is expressed by deprivation of O_2 [86]. The same result was observed after $CoCl_2$ treatment. $CoCl_2$ is known to activate hypoxia-dependent pathways under normal O_2 levels by inhibiting prolyl-hydroxylase domain-containing enzymes, a family of enzymes that play a key role in the oxygen-dependent degradation of HIF-1α and consequently stabilizing HIF-1 [86]. In contrast, in explants exposed to hypoxia followed by reoxygenation, HIF-1α was undetectable, while AQP9 increased significantly and changed its cellular distribution, showing the same pattern as that previously described in preeclamptic placentas [11].

It was reported the presence of 14 putative HRE sites (5′-ACGTGC-3′) in the AQP9 gene, but none of them was in the promoter region [86]. Although, in other HIF-1α-inducible genes functional HRE sites were localized outside the promoter region [87–89], it is possible that HREs in the promoter region should be required to induce an upregulation of AQP9 transcription. Therefore, it was proposed that HIF-1α may enhance the expression of some intermediate that impacts directly downregulating AQP9 expression. Thus, the subsequent reoxygenation not only stimulates the synthesis of new AQP9 protein but may also modify the lipid membrane composition of the syncytiotrophoblast as it was observed in preeclamptic placentas [13, 90, 91]. This may probably contribute to create an unfavorable environment for AQP9 insertion in the plasma membrane changing its cell distribution [86].

About water flux, in explants exposed to hypoxia or treated with $CoCl_2$, it was reported a decrease in water uptake which correlates to a decrease in AQP9 expression [86]. Nevertheless, water flux after $CoCl_2$ treatment and not after hypoxia seems to be sensitive to $HgCl_2$ [86]. A probable reason for this discrepancy is that $CoCl_2$ only affects HIF-1α expression while the low- O_2 tension may also modify the intracellular pH (pH_i) of syncytiotrophoblast cells. Even more, in explants exposed to hypoxia followed by a period of reoxygenation, despite the increase in AQP9 expression, water uptake decreased dramatically compared to the control and was not sensitive to $HgCl_2$. This finding confirms that AQP9 may not be exclusively involved in placental water transport [86].

Recently, it was reported that AQP3 protein expression decreased after oxygen deprivation and was mainly found in the cytosol. The latter reoxygenation restored AQP3 expression on the apical membrane of syncytiotrophoblast but failed to restore its expression to basal levels, possibly due to the oxidative damage of the plasma membrane of syncytiotrophoblast, which probably creates an unfavorable environment for AQP3 insertion in the plasma membrane [59].

13.6.4 Regulation by CFTR

Emerging evidence indicates that cystic fibrosis transmembrane conductance regulator (CFTR) is able to interact with various membrane proteins by regulating their transport activity as well as by functioning as a cAMP-regulated chloride channel [92]. In other tissues it was found that water transport mediated by AQPs may be regulated by CFTR [93]. In human placenta, it was reported that CFTR co-localized with AQP9 in the apical membrane of syncytiotrophoblast, nevertheless its expression decreased dramatically (3.8-fold) in preeclamptic placentas where AQP9 is overexpressed but not functional [94]. Since there is no evidence that CFTR could function as a water channel per se, it was investigated whether CFTR protein is required to preserve the normal functionality of AQP9. Water uptake experiments were performed in normal and preeclamptic placental explants treated with diphenylamine-2-carboxylate (DPC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDs) (inhibitors of chloride channels) and glibenclamide (an open-channel blocker of CFTR). Water uptake was significantly reduced by all the inhibitors of CFTR (DIDs and DPC ~35%, glibenclamide ~50% ($P< 0.05$)). In con-

trast, water uptake in explants from preeclamptic placentas showed a decrease of ~51% ($P < 0.05$) compared to normal placentas. Interestingly, water uptake was not modified by the inhibitors tested, possibly due to the reduced expression of CFTR in preeclampsia [94].

These findings are consistent with a synergistic effect of the two proteins suggesting that this decrease may affect water placental transport mediated by AQPs. However, the mechanisms that induce the decrease in the levels of CFTR protein in preeclamptic placentas are not clear yet.

13.6.5 Regulation by NHEs

Three isoforms of the Na^+/H^+ exchanger family (NHEs) –NHE-1, NHE-2 and NHE-3– and one of the Cl^-/HCO_3^- anion exchangers (AEs) –AE1 and AE2– have been identified in human placenta and proposed to be involved in the maintenance of pHi of syncytiotrophoblast [95–97]. Failures in the pHi homeostasis could alter various and critical cellular functions such as water movements and cell volume regulation. AQPs are also a key regulator of cell volume and intracellular ions. As it was observed in other tissues, recently it was described that pHi could modify placental AQPs selectivity [80, 98–100].

In experiments carried out in normal placental explants it was found that after cytosolic acidification**,** water uptake mediated by AQPs did not change in the absence of NHEs inhibitors. Consequently, disturbances of pH do not affect water movement in the presence of functional NHEs that can restore the physiological pH of the cells [101].

On the other hand, when NHEs were blocked water uptake was not only significantly reduced but also not sensitive to $HgCl_2$. Thus, the inhibition of the activity of NHEs may not only modify the syncytiotrophoblast pHi homeostasis but also alter transcellular water transport mediated by AQPs [101].

In conclusion, these results show an interaction between AQPs and NHEs in the regulation of water uptake in human placenta.

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Diabetes Insipidus

14

H.A. Jenny Lu

Abstract

Disruption of water and electrolyte balance is frequently encountered in clinical medicine. Regulating water metabolism is critically important. Diabetes insipidus (DI) presented with excessive water loss from the kidney is a major disorder of water metabolism. To understand the molecular and cellular mechanisms and pathophysiology of DI and rationales of clinical management of DI is important for both research and clinical practice. This chapter will first review various forms of DI focusing on central diabetes insipidus (CDI) and nephrogenic diabetes insipidus (NDI). This is followed by a discussion of regulatory mechanisms underlying CDI and NDI, with a focus on the regulatory axis of vasopressin, vasopressin receptor 2 (V2R) and the water channel molecule, aquaporin 2 (AQP2). The clinical manifestation, diagnosis and management of various forms of DI will also be discussed with highlights of some of the latest therapeutic strategies that are developed from in vitro experiments and animal studies.

Keywords

Diabetes insipidus • Nephrogenic diabetes insipidus • Aquaporin 2 • V2R

14.1 Diabetes Insipidus

Maintaining water homeostasis is essential for mammalian life. Proper water metabolism is responsible for the balance between water intake and secretion. Each side of this balance is important for fluid homeostasis. Diabetes insipidus (DI) is characterized by excessive water loss from the kidney. DI is classified as central diabetes insipidus (CDI) and nephrogenic diabetes

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insipidus (NDI). The former is due to impaired production and/or secretion of the antidiuretic hormone, ADH, also called vasopressin (VP) from the central nervous system. The latter is caused by lack of response of the target tissue, the collecting ducts of the kidney, to circulating ADH/vasopressin. In both cases, the kidney fails to concentrate urine and results in polyuria and polydipsia. DI patients may produce up to 18 liters of urine a day and exhibit a constant need for water intake. Both central DI and nephrogenic DI can be either inherited or acquired. If undiagnosed or improperly managed, diabetes insipidus is associated with a range of clinical symptoms due to severe volume depletion and electrolyte abnormalities. In this chapter we will review the regulatory function of vasopressin for central DI, and the critical role of vasopressin receptor 2 (V2R) and a water channel, aquaporin 2 (AQP2), in modulating water reabsorption in the collecting duct principal cells in nephrogenic DI. Finally, we will discuss the clinical manifestation, diagnosis and treatment for DI, and review some of the most recent progress in developing novel strategies for treating nephrogenic diabetes insipidus.

14.2 Central Diabetes Insipidus and Vasopressin

The antidiuretic hormone, ADH, later known as vasopressin (VP), is the primary determinant of free water excretion or absorption in mammals. Central diabetes insipidus is usually caused by inadequate production and/or secretion of VP from the post pituitary gland in response to osmotic stimulation. Central DI is rarely hereditary in humans. Most frequently it is caused by traumatic or pathological destruction of the neurohypophysis that leads to complete or partial absence of circulating VP.

Vasopressin is a nine-amino acid peptide in most mammals. It is synthesized in the hypothalamus. Substitution of lysine for arginine at position 8 yields lysine vasopressin which is found in pigs. Substitution of isoleucine for phenylalanine at position 3 and leucine for arginine at position 8 yields oxytocin (OT), a hormone with weak antidiuretic activity but a potent smooth muscle constrictor in the uterus. Arginine vasopressin (AVP) and lysine vasopressin (LVP) are the major antidiuretic hormones for mammals. In addition they also cause vasoconstriction, an effect that occurs at concentrations many times higher than those required for antidiuresis. AVP and OT are produced by the posterior pituitary gland [1, 2]. Secretion of AVP can be influenced by many factors, however the most important stimulus under physiological conditions is the plasma osmolality [3–8]. Cells located in the anterior hypothalamus are shown to be able to sense small changes in plasma osmolality and stimulate AVP secretion [2, 8, 9]. Most studies support the existence of a possible osmotic threshold of VP secretion and there is a linear relationship between plasma osmolality and circulating AVP concentration indicating a sensitive regulation of water excretion by vasopressin [5, 6, 8, 10, 11].

The osmolality threshold or set point of vasopressin secretion varies from person to person. Normally, in adults, it ranges from 275 to 295 mOsm/kg H_2O, with an average of 280–285 mOsm/kg H_2O [10]. Many factors are known to affect the set point of the osmoregulation for AVP secretion [4]. For example, aging increases the sensitivity of osmoregulation, and pregnancy dramatically reduces the set point of osmoregulation [4, 12]. Observations have suggested that osmoregulation by AVP secretion is subjected to both stimulatory and inhibitory inputs to the neurohypophysis [6]. Therefore, osmolality sensing is a highly regulated and sensitive process [7, 11]. In addition to the central regulation of production and secretion, VP has a short half-life in circulation from 10 to 20 min, allowing the kidneys to respond to changes in plasma osmolality on a minute-to-minute scale. Therefore, from the VP secretion and its action on the kidneys, this system enables a fine tuned osmoregulation that adjusts the rate of water excretion acutely and accurately to the plasma osmolality [11].

In addition to the primary stimulation of the plasma osmolality, hypovolemia is also a potent stimulus for AVP secretion [13–15]. In rats,

plasma AVP increases as an exponential function of the degree of hypovolemia and hypertension [16, 17]. This hemodynamic influence of AVP secretion is thought to be mediated, at least in part, by neural pathways that originate in the stretch-responsive receptor, the baroreceptor in the central nervous system. This osmolality-independent regulation of VP production was found to be associated with many pathological conditions, such as sodium depletion, hypotension, congestive heart failure, cirrhosis and nephrosis [15, 17, 18].

14.3 Gestational Diabetes Insipidus

This type of diabetes insipidus is caused by a relative deficiency of circulating VP during pregnancy [19]. It is only observed in pregnant women and therefore is termed as gestational diabetes insipidus [20, 21]. It is due to the elevation of a circulating enzyme, cysteine aminopeptidase, also called vasopressinase or oxytosinase that degrades plasma vasopressin and oxytoxin [22, 23]. The vasopressinase or oxytoxinase is normally produced by the placenta to prevent premature uterine contractions induced by oxytocin. Vasopressinase causes accelerated metabolic clearance of circulating VP, and overwhelms the VP-generating capacity of the neurohypophysis, leading to VP deficiency. In addition, the activity of vasopressinase may be abnormally elevated in pathological conditions that associate with pregnancy, such as preeclampsia, acute fatty liver and **HELLP** syndrome (***he***molysis, ***e***levated ***l***iver enzymes and ***l***ow ***p***latelet count). This is due to a decreased metabolism of vasopressinase by the liver [24, 25].

Gestational DI can become overtly symptomatic and poses a serious threat of dehydration and electrolyte imbalance in pregnant women, and therefore should be readily recognized and managed [26]. The pathophysiology of gestational diabetes insipidus is similar to that of central diabetes insipidus, except that gestational diabetes insipidus is resistant to AVP treatment. Like endogenous vasopressin, AVP can be rapidly degraded and cleared from the circulation. However, the synthetic vasopressin receptor V2R agonist, desmopressin is resistant to vasopressinase degradation, and has been used to diagnose and correct gestational diabetes insipidus [21, 27]. Whether there is also a component of nephrogenic diabetes insipidus (due to lack of response to circulating vasopressin) during pregnancy is unclear.

14.4 Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is defined as diabetes insipidus caused by resistance of vasopressin action by the kidney [28, 29]. In contrast to central diabetes insipidus (CDI), in NDI, the plasma VP level is usually normal or elevated. NDI can be either acquired or congenital in origin [29].

14.4.1 Congenital Nephrogenic DI

Congenital NDI was first recognized in the 1950s in several male patients presented with the familial, sex-linked form of the diabetes insipidus (Cannon 1955). Subsequently, it was found that the disorder was due to defects in arginine vasopressin receptor 2 gene (*V2R*) located on the X chromosome (Xq28) [30, 31]. In congenital NDI, patients frequently present with polyuria from birth. The disease manifestation in congenital forms of NDI varies from partial NDI to complete NDI. It affects mostly males, and is usually mild or absent in female carriers. Genetic analysis revealed that more than 90% of cases of congenital NDI are caused by mutations in the arginine vasopressin receptor 2 (*V2R*) [32, 33]. To date, over 225 mutations that result in congenital NDI have been identified in the *V2R* [34–37]. Most of these mutations are missense mutations [34].

The remaining 10% of congenital NDI cases are due to genetic defects in the water channel AQP2 gene that is located on chromosome 12 (12q13) [36]. AQP2 is the major aquaporin that

mediates water transport in principal cells of the collecting ducts (CDs) of the kidney. While congenital NDI due to AQP2 mutations is mostly inherited in an autosomal recessive mode, a few cases have also reported autosomal dominant inheritance [34, 38]. Since the first report of a compound heterozygote of two missense mutations of AQP2 genes (R187C and S217P) in a male NDI patient [39–41], approximately 49 putative disease-causing AQP2 mutations have been described [34, 36, 39, 42–46]. These mutations are roughly grouped into 2 categories, based on the outcome. The first category of mutations affect the formation of the functional channel "pore" structure that allows the translocation of water molecules across the plasma membrane. Most of the autosomal recessive forms of NDI are thought to be due to mutations that fail to form the tetramer with wild type AQP2, therefore causing a defect in "pore" formation. The second category of AQP2 mutations affect the routing or trafficking of AQP2 to the plasma membrane and/or inside the cells. For example, AQP2 is retained in the Golgi apparatus, or sorted to late endosomes, lysosomes or the basolateral plasma membrane instead of the apical membrane [47–54]. This defect is frequently due to mutations that occur in the C-terminal tail of AQP2, which is essential for the correct intracellular routing in response to multiple signaling pathways [47, 48, 50, 52–55].

14.4.2 Acquired Nephrogenic DI

Compared to congenital NDI, the acquired form of NDI is much more common. It can be caused by multiple factors, including lithium toxicity, urinary obstruction, hypokalemia, hypercalcemia, etc. [56–58]. Sometimes, the etiology may not be well defined in the clinical situation. Despite complicating factors that are involved in the pathophysiology of acquired NDI, direct or indirect interruption of VP-V2R and AQP2 signaling and trafficking is evident. Therefore, the VP-V2R/AQP2 regulatory axis is the central component of both congenital and acquired NDI. Indeed, genetic studies of congenital NDI have greatly facilitated the discovery of the vasopressin/ vasopressin V2 receptor and AQP2 signaling pathway that is known to be the major regulatory pathway for water transport in the mammalian kidney.

14.4.3 VP-V2R/AQP2 Axis in Regulating Water Transport

AQP2 is a major water channel expressed in the principal cells (PCs) of the collecting ducts of the kidney [59, 60] (Fig. 14.1). It mediates water transport across the plasma membrane in response to vasopressin. Circulating vasopressin binds to V2R located in the basolateral membrane of the PCs, activates the adenylyl cyclase (AC), and thus causes the elevation of intracellular cyclic AMP. Elevation of cAMP activates the protein kinase A (PKA), leading to the phosphorylation of AQP2. Phosphorylation of AQP2, mainly at the serine 256 residue results in accumulation of AQP2 on the apical membrane. Within the plasma membrane, AQP2 forms tetramers containing a functional "pore" facilitating the passage of water molecules through the channel. Water absorbed from the apical membrane via AQP2 is transported into the interstitium through basolaterally located AQP3 and AQP4 channels. In addition to cAMP, the cGMP and calcium/calmodulin pathways are also involved in regulating AQP2 traffic [43]. Further details of AQP2 trafficking mechanisms are elucidated in Chap. 1.

In addition to regulated trafficking, AQP2 is also constitutively recycling [37, 60]. Blocking AQP2 endocytosis by a cholesterol-chelating reagent, methyl-beta cyclodextrin or by expressing dominant negative dynamin causes a rapid and dramatic membrane accumulation of AQP2 in cultured cells [61, 62]. It suggests that a significant amount of AQP2 is recycling under baseline conditions, and this recycling does not require any phosphorylation, since the phosphorylation "dead" mutation of AQP2, AQP2-S256A recycles as well as the wild type AQP2 [61, 63]. The discovery of the presence of a significant pool of AQP2 that is able to recycle independently

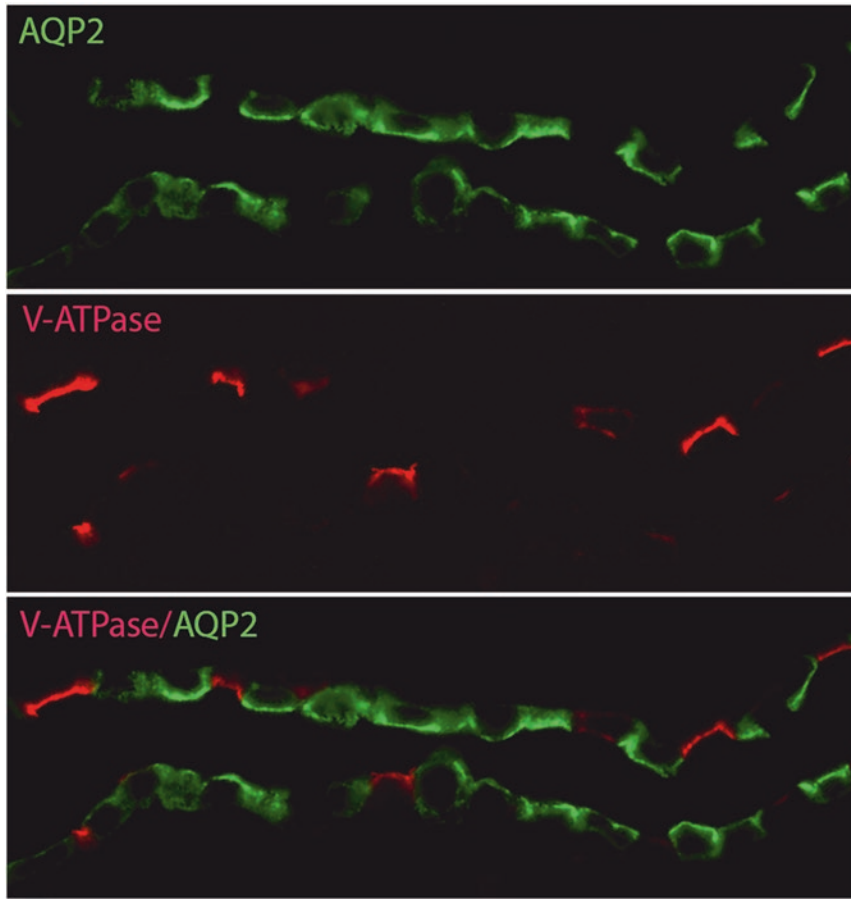

Fig. 14.1 Cellular composition of a kidney collecting duct from the medulla of a mouse kidney. The CD is immunostained for AQP2 (*green*) and V-ATPase (*red*). The merged image is shown in the *lower panel* revealed the presence of principal cells (PCs) that are positive for AQP2 and the intercalated cells (ICs) that are positive for V-ATPase staining. AQP2 staining is present mainly in the subapical region inside cells. AQP2 signal is occasionally seen on the apical membrane without VP stimulation. V-ATPase signal is clearly present on the apical membrane of intercalated cells

of VP stimulation prompted a burst of studies searching for molecular mechanisms and alternative approaches that cause membrane accumulation of AQP2 in the absence of VP-V2R regulation. Many novel targets have been identified to regulate AQP2 trafficking while bypassing the VP/V2R signaling, the commonly defective pathway in most congenital DI patients [43]. This novel strategy has led to several important discoveries that have proven to be effective in treating NDI in animal models [61, 64, 65]. This will be further detailed in the Treatment of NDI section in this chapter.

14.5 Clinical Manifestation, Diagnosis and Treatment of DI

14.5.1 Clinical Manifestation of DI

The primary clinical symptoms that are characteristic for diabetes insipidus are polyuria and polydispsia resulted from the impairment of urinary concentrating mechanisms. Patients with DI frequently describe a strong sensation of thirst (if their thirst sensation is intact) and craving for water, especially cold water [66]. Under normal

circumstances, DI patients have an intact thirst mechanism, and therefore they are able to maintain normal serum osmolality and volume status without clinical symptoms other than polyuria and polydipsia. However, when water deficits occur due to inadequate water intake to compensate for polyuria, symptoms of dehydration and/or hyperosmolality, underlined by hypernatremia develop. Volume depletion leads to hypotension, acute kidney injury, liver injury, muscle injury and shock. Hyperosmolality and dehydration also cause a series of neurological symptoms ranging from irritability, cognitive decline, disorientation, and confusion to decreased levels of consciousness, seizure and coma. These signs are suggestive of hypertonic encephalopathy [67]. Various focal neurological deficits may also develop in this context. Increased incidence of subarachnoid hemorrhage, cerebral infarction and deep venous thrombosis (DVT) are also reported in patients with hyperosmolality. The severity of symptoms is roughly correlated with the degree of hyperosmolality, however the individual variability is marked, and therefore the serum sodium level cannot accurately predict the clinical presentation of a DI patient. The chronicity of the hyperosmolality is important for the development and degree of clinical manifestation. Acute and severe hyperosmolality are frequently associated with marked neurological presentation compared to generally milder symptoms in patients with subacute and chronic hyperosmolality [67].

14.5.2 Differential Diagnosis of DI

Clinical differentiation of central DI and nephrogenic DI, and sometimes primary polydipsia are important for the management [58, 68]. The presence of truly hypotonic polyuria should be established by measuring urine osmolality and volume from a 24-h urine collection. The generally accepted diagnostic criteria of DI is that a 24 h urine volume exceeds 50 ml/kg in adult or 2L/m^2/24 hours in young children, and urine osmolality is less than 300 mOsm/kg H_2O [58, 69]. Meanwhile the presence of hyperglycemia from diabetes mellitus and kidney failure should be ruled out. In patients with hyperosmolality, polyuria and suboptimal urinary concentration (urine osmolality less than 800 mOsm/kg H_2O) define the diagnosis of DI, while primary polydipsia can be ruled out with normal or hypoosmolality of serum and diluted urine. Once the DI is diagnosed, the central DI can be distinguished from NDI by its response to exogenously administrated AVP (1-deamino-8-D-arginine vasopressin), or DDAVP (1–2 μg subcutaneously or intravenously). A significant increase in urine osmolality of more than 50% within 2 h after administration of AVP or DDAVP supports the diagnosis of central DI. In contrast, an increase of less than 10% in urine osmolality indicates NDI. Partial responders (in between these values) are undetermined and need to be further assessed by measuring serum AVP level to aid the diagnosis. Although it has been a subject of debate in the literature, the water deprivation test has been proposed to better distinguish the different types of DI and aid with diagnosis and management [70]. Due to the complexity of this test, it is rarely used clinically. We need to keep in mind that clinical diagnosis of various forms of DI can be complex and confusing. Firstly, measuring serum AVP is difficult and most available assays are not sensitive enough. Secondly, many disorders of DI can overlap and co-exist, and should be kept in mind while formulating a treatment strategy [69].

14.5.3 Treatment of Diabetes Insipidus

The principles for treating all forms of diabetes insipidus are a correction of water deficit and a reduction in the ongoing water loss from the kidney. Theoretically, with an intact thirst mechanism and ability to access water, most DI patients should be able to drink a sufficient amount of water and attain a relatively normal fluid balance [66]. However, polydipsia and polyuria can significantly affect the quality of life of a DI patient. DI treatment becomes necessary in order to manage the symptoms of DI. It becomes especially important in clinical situations when the thirst mechanism is compromised in patients, or patients are unable to access free water due to

their clinical conditions. The specific treatment varies based on the type of DI and the specific clinical situation.

14.5.4 Treatment of Central Diabetes Insipidus

The synthetic form of human AVP, pitressin, has been used for the treatment of acute central DI. It is given intravenously with a short half-life (2~4 h). Desmopressin, a synthetic AVP V2R agonist, has been commonly used for treating both acute and chronic central DI. It has a long half-life (8~20 h), and can be administered intranasally, orally, or by injection based on the clinical situation and the patient's preference [71, 72]. Because it is specific to vasopressin signaling through the V2R, it normally does not affect the blood pressure as AVP does. Although the central DI can be easily managed by DDAVP, one needs to be aware of and closely monitor a critical complication, which is hyponatremia. Hyponatremia is a rare complication of desmopressin therapy, which can cause severe, even fatal sequelae [73]. It is reported in children who are treated with desmopressin for hemophilia and von Willibrand's factor disorders and in children treated for primary enuresis [70]. Therefore, serum electrolytes need to be monitored closely in patients during the initiation of desmopressin therapy. The dose and intervals of administrated desmopressin need to be adjusted to control the symptoms of polyuria and polydipsia while maintaining a safe serum sodium level [35].

Although not classified as central DI, the treatment is the same for gestational diabetes insipidus, which is with desmopressin. The AVP is rapidly degraded by the high level of circulating oxytoxinase or vasopressinase, while desmopressin is resistant to the enzymatic degradation and has been used successfully for the treatment of gestational DI [27]. The dose of desmopressin should be titrated to the individual patient, and fluid administration should be performed with caution. Serum electrolytes should be closely monitored at the time of delivery.

14.5.5 Treatment for Nephrogenic DI

In contrast to the relatively intact vasopressin-V2R and AQP2 pathway in central DI, NDI has a defective VP-V2R and AQP2 axis. Therefore, patients with congenital NDI are resistant to the water concentrating effect medicated by vasopressin. Clinical therapy for treating congenital NDI is limited to restricting sodium intake, administrating a thiazide diuretic alone or in combination with a non-steroid anti-inflammatory drug (NSAID) or amiloride [35, 74]. The thiazide class of diuretics is considered the mainstay for treating NDI. They block the sodium reabsorption in the cortical diluting segment. In combination with restricted sodium intake, it causes modest hypovolemia. Hypovolemia stimulates isotonic solute absorption in the proximal tubule and reduces solute delivery to the distal diluting segment. Thiazide also enhances water reabsorption in the inner medullary collecting ducts independently of vasopressin. However, care must be excised when treating with thiazide diuretics to correct hypokalemia, and to avoid severe volume depletion and resulting kidney injury, especially in combination with NSAIDs. Commonly used NSAIDs to treat NDI are indomethacin and ibuprofen. Administrating high doses of NSAIDs or long term NSAID use increases the risk of developing AKI and chronic kidney disease (CKD) [75, 76]. Therefore, renal function needs to be monitored with chronic use of NSAIDs in DI patients.

Treatment for acquired NDI is focusing on correcting insulting factors if possible. It includes withholding lithium in lithium-induced NDI at the early stage, correcting hypokalemia in hypokalemia-induced NDI, correcting hypercalcemia if it is the cause and relieving urinary obstruction. However, under many circumstances, management of underlying etiology for acquired NDI can be clinically challenging.

One important consideration for treating diabetes insipidus in general is avoiding over correction of hyperosmolality/hypernatremia. The theory is that under a state of hyperosmolality, the brain counteracts osmotic shrinkage by

increasing the intracellular content of solutes, including electrolytes such as potassium and many organic osmolytes. The net effect of this process is to protect the brain against excessive shrinkage during sustained hyperosmolality. However, once the brain has adapted to this new hyperosmolality state, rapid correction of hyperosmolality can cause brain edema since it takes time to re-equilibrate the previously accumulated solutes. Similar to correction of hyponatremia, cautious correction of chronic hyperosmolality or hypernatremia needs to be practiced. Even though severe sequelae from rapid correction of hyperosmolality and hypernatremia are rarely reported, they can occur [77].

Despite that, the clinically proven treatment for congenital NDI is limited. Very excitingly, in recent years, with greater understanding of AQP2 trafficking mechanisms, multiple novel targets have been identified and provided promising new strategies for treating NDI.

14.5.6 Novel Therapeutic Strategy for NDI

Fundamental research on kidney physiology has provided important insights into the development of novel therapeutic targets and strategies to treat human diseases. It is especially true for NDI [78]. As mentioned previously, over 90% of congenital NDI is due to mutations in the *V2R* gene and less than 10% is due to mutations in *AQP2*. The water channel AQP2 remains intact in the majority of congenital NDI patients. Similarly, in many forms of acquired NDI, such as lithium induced NDI, AQP2 is also intact. Therefore, it is possible to develop a strategy to induce membrane accumulation of AQP2 independent of vasopressin stimulation, therefore bypassing the V2R-VP signaling regulation [64]. This has led to great discoveries of multiple new targets for treating NDI [79–83]. Many reagents were proved to be effective in NDI animal models, and a few reagents are subsequently tested in X-linked NDI patients. The results are promising.

14.5.6.1 Phosphodiesterase Inhibitors

It is well known that increasing intracellular cyclic AMP in principal cells leads to membrane accumulation of AQP2. Increasing cyclic guanosine monophosphate (cGMP) by sodium nitroprusside, L-arginine and atrial natriuretic peptide (ANP) also causes an increased AQP2 abundance on the apical membrane. The selective cGMP phosphodieterase (PDE5) inhibitor sildenafil citrate (Viagra) was shown to cause elevation of cGMP and subsequent membrane accumulation of AQP2 in cells and in Brattleboro rat kidney [84]. Sildenafil citrate was also reported to reduce polyuria in rats with lithium induced NDI [85]. More recently, a case study showed that sildenafil improves polyuria and increases urinary osmolality in an X-linked NDI patient [86].

14.5.6.2 Statins

The statin family is a family of 3-hydroxy-3methyglutaryl-coenzyme A reductases. They inhibit the synthesis of cholesterol and are used for treating hyperlipidemia. Simvastatin was shown to increase membrane accumulation of AQP2 in cultured kidney epithelial cells. In addition, simvastatin treatment in Brattleboro rats causes apical membrane redistribution of AQP2 in CDs in parallel with increased urinary concentration in a VP-independent manner (Fig. 14.2) [81]. A later study has also demonstrated that atorvastatin, another member in the statin family, significantly improves urinary concentration in polyuria caused by urinary obstruction in animals [87]. The specific mechanism underlying the effect of statins is not fully understood, but has been attributed to changes in prenylation of RhoA family proteins that are involved in regulating cytoskeleton and AQP2 trafficking [81].

14.5.6.3 Prostaglandins

Prostaglandin E2 is known to increase water permeability in the absence of vasopressin possibly through activating prostanoid receptor EP2 and/or EP4. Both Butaprost, an EP2 agonist, and CAY10580, an EP4 agonist, stimulate AQP2

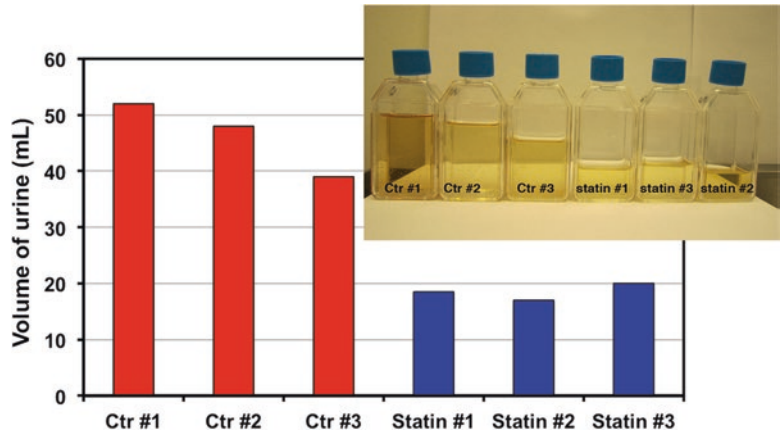

Fig. 14.2 Simvastatin increases urine concentration, reduce urine volume in vasopressin-deficient Brattleboro rats. After 6 h treatment with simvastain, urine volume (*left panel* and inset image) was significantly reduced in simvastain treated Brattleboro rats

trafficking to the apical membrane in cultured MDCK cells [83, 88, 89]. Another EP4 agonist, ONO-AE1–329, increases AQP2 membrane expression, improves polyuria and increases urine osmolality in V2R knock animals. Similarly, Butaprost was shown to reduce urinary volume and increase urine osmolality in rats treated with a V2R antagonist [83]. More interestingly, long-term treatment with ONO-AE1–329 increases AQP2 abundance in V2R knock-out animals. These studies suggest that activating the prostaglandin pathway through EP2 and EP4 holds promise for treating NDI independently of VP-V2R signaling.

14.5.6.4 Metformin

Metformin is an oral antidiuretic drug that stimulates the 5′ AMP-activated protein kinase or AMPK, an enzyme that plays a role in cellular energy homeostasis. A recent study has shown that metformin stimulates AQP2 membrane accumulation in rat inner medullary collecting duct cells and increases urine concentrating ability in two rodent models of NDI, V2R knock-out mice and rats treated with Tolvaptan, the V2R antagonist [90]. Metformin was shown to increase protein abundance of inner medullary urea transporter UT-A1 and AQP2, and membrane accumulation of AQP2 possibly through phosphorylating AQP2 at serine 256. Metformin is able to produce a sustained urinary concentrating effect for up to 10 days in Tolvaptan treated animals. This study suggests that through stimulating AMPK to phosphorylate and activate AQP2 and UT-A1, metformin increases urine concentrating ability, and therefore, is a promising treatment for congenital NDI [90].

Besides the above listed reagents, other studies have uncovered more and more novel targets for therapeutic intervention for NDI. Many of them were proved to be effective in vivo using various NDI animal models. For example, calcitonin, a 32-amino acid peptide produced by the follicular cells of the thyroid, causes an increase of intracellular cAMP and membrane expression of AQP2 in principal cells. More importantly, calcitonin was shown to improve urine concentration in Brattleboro rats [82]. A heat shock protein 90 (HSP90) inhibitor, 17-Allylamino-17-demethoxygeldanamycin, was shown to partially correct NDI in a mouse model of autosomal recessive form of NDI in which the AQP2 mutation AQP2-T126 M is retained in the ER [91]. More recently, Erlotinib, a receptor tyrosine kinase inhibitor that acts on the epidermal growth factor receptor (EGFR), was shown to cause membrane accumulation of AQP2 in a cAMP-independent manner and to alleviate polyuria in lithium-induced NDI animals [92].

In summary, research on water transport disorders including NDI is a fast evolving and exciting field. More and more novel reagents and small molecules will continuously be discovered and will provide more and possibly better therapeutic targets for treating NDI. Clinical trials are

urgently needed to examine and/or confirm the efficacy and validity of many of the novel targets that are identified by in vitro systems and animal models.

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Aquaporins in Obesity

15

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Abstract

Obesity is one of the most important metabolic disorders of this century and is associated with a cluster of the most dangerous cardiovascular disease risk factors, such as insulin resistance and diabetes, dyslipidemia and hypertension, collectively named Metabolic Syndrome. The role of aquaporins in glycerol metabolism facilitating glycerol release from the adipose tissue and distribution to various tissues and organs, unveils these membrane channels as important players in lipid balance and energy homeostasis and points to their involvement in a variety of pathophysiological mechanisms including insulin resistance, obesity and diabetes.

This review summarizes the physiologic role of aquaglyceroporins in glycerol metabolism and lipid homeostasis, describing their specific tissue distribution, their involvement in glycerol balance and their implication in obesity and fat-related metabolic complications. The development of specify pharmacologic modulators able to regulate aquaglyceroporins expression and function, in particular AQP7 in adipose tissue, might constitute a novel approach for controlling obesity and other metabolic disorders.

Keywords

Aquaglyceroporin • Adipose • Glycerol

Abbreviations

AQPs	aquaporins
ATP	adenosine triphosphate
FFA	free fatty acids
G3P	glycerol-3-phosphate
GK	glycerol kinase
IRE	insulin response element
PI3K	phosphatidylinositol-3 kinase

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PPARγ peroxisome proliferator-activated receptor gamma
TAG triacylglycerols

15.1 Obesity

Obesity can be defined as the enlargement and inflammation of adipose tissue and is the most significant metabolic disorder of this century, reaching epidemic proportions [1]. Accumulation of fat in visceral and subcutaneous abdominal adipose tissue and its deposition in internal organs is a major risk for the development of numerous disorders, including insulin resistance and diabetes, dyslipidemia, hypertension, cardiovascular and neurodegenerative diseases among others. Some of these metabolic complications appearing as a cluster were termed as Metabolic Syndrome and have been associated with the most dangerous cardiovascular risk factors. In this way, abdominal obesity, the most prevalent manifestation of this syndrome and a marker of adipose tissue dysfunction, is now recognized as the predominant contributor to type 2 diabetes and cardiovascular risk [2].

15.2 Adipose Biology and Pathophysiology

The adipose tissue is composed by adipocytes, vascular tissue and immune cells, surrounded by an extracellular matrix formed by proteins, mostly collagen. Preadipocytes are the mature adipocyte precursors that undergo differentiation to become mature fully differentiated cells. This process of differentiation has been widely studied in a variety of models [3]. In situations of positive energy balance (increased food uptake or decreased energy expenditure) mature adipocytes increase in number (hyperplasia) and size (hypertrophy) to accommodate excess lipid and their morphology change due to increased free fatty acids (FFA) uptake and triacylglycerols (TAG) synthesis. To allow adipocyte enlargement the extracellular matrix must be adjusted by the action of proteases that hydrolyze the excess of collagen to allow adipose hypertrophy. In addition, the formation of new blood vessels (angiogenesis) is also essential for adipose tissue growth and is a duality between a response to signals emanating from proliferating and enlarging adipocytes and a response to developmental and metabolic signals, preceding the adipocyte proliferation and enlargement [4]. Adipocytes descend from adipose stem cells localized close to the microvasculature of adipose tissue but not in the vasculature of other tissues [5]. These stem cells already committed either prenatally or early in postnatal life, differentiate into adipocytes probably by signals coming from the adipose vasculature that may function as an adipocyte niche [5]. By secreting signaling proteins collectively known as adipokines, adipose tissue is an important endocrine and paracrine organ that communicates with many other organs in the body contributing to the maintenance of energy, lipid and glucose homeostasis, and mediating multiple biological processes such as inflammation, immunity and metabolism.

While it is common to link abdominal obesity with insulin resistance based on population studies, the pathogenicity of obesity and related metabolic complications such as insulin resistance and type 2 diabetes is still not clear. Several hypotheses have been advanced to explain the development of adipose tissue dysfunction and obesity. One of the most accepted that emerged from corroboration of clinical and experimental data, the adipose tissue expandability hypothesis, is based on the limitation of the adipose tissue to expand above a given threshold for a specific individual [6]. When an individual gains weight and increase in fat mass, the adipose tissue enlarges till a point where it may exceed the limit capacity of storage and is no longer able to efficiently accumulate more fat. At this point bloodstream lipids start depositing ectopically in other non-adipose tissues such as liver, muscle and heart, leading to lipid-induced toxicity (lipotoxicity) and resulting in inflammation and insulin resistance (Fig. 15.1) [6]. Importantly, the maximal capacity of adipose tissue expansion is dependent on the type of fat depot, subcutaneous

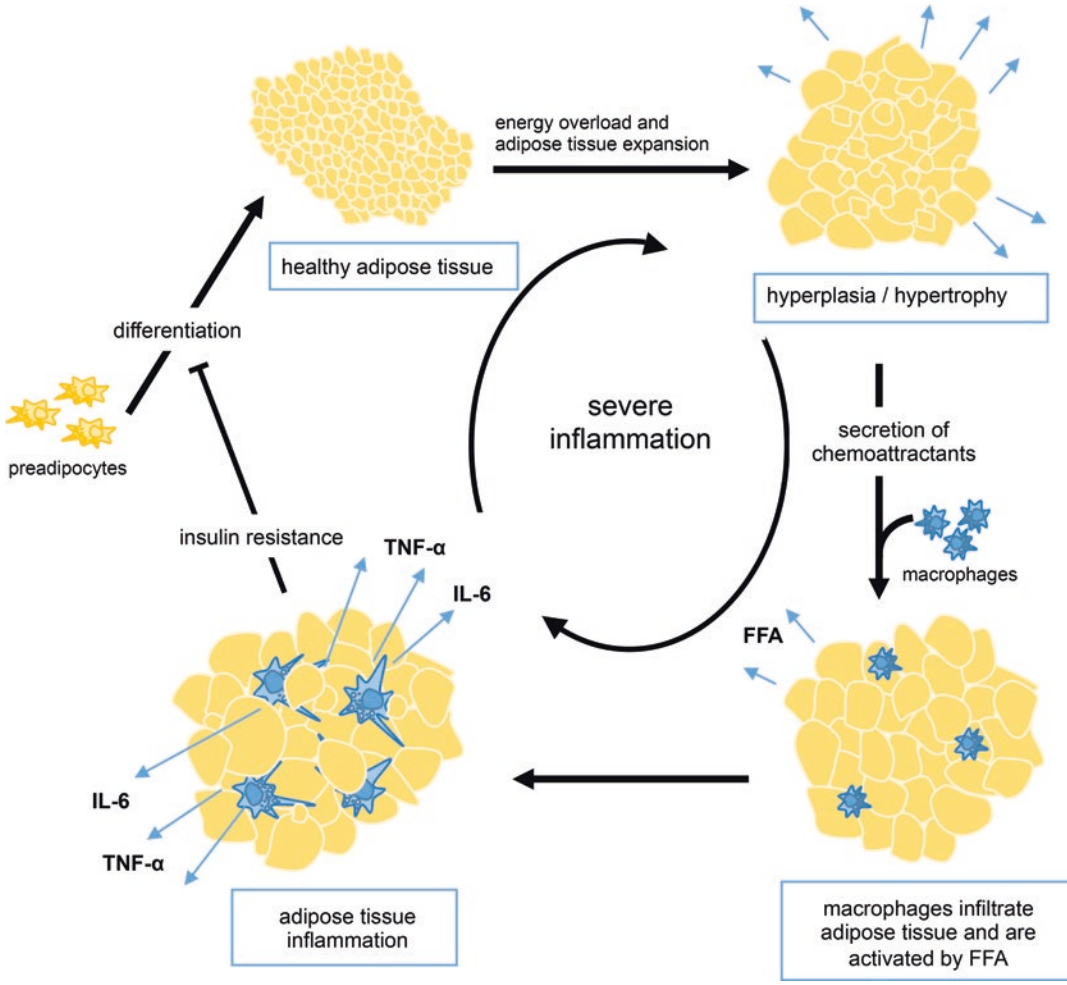

Fig. 15.1 Illustration of cyclic mechanism of adipose tissue inflammation linking to insulin resistance and obesity. When the uptake of nutrients overcomes the energy expenditure, TAG accumulation in adipocytes induces adipose hyperplasia and hypertrophy, secretion of chemoattractants leading to macrophage recruitment. Large adipocytes are induced to secrete more cytokines and FFA, which in turn activate macrophages. Macrophages secrete anti-adipogenic cytokines (TNF-α and IL-6) that inhibit insulin action and lead to adipose tissue inflammation. These cytokines also block the differentiation of preadipocytes into new adipocytes thus inducing the enlargement of insulin resistant-adipocytes, that continue secreting more cytokines and FFA, recruiting macrophages and leading to severe inflammation

or visceral, the first being more adipogenic and with greater expansion capacity and the latter metabolically more active. It is well accepted that in humans, increased visceral fat is associated with increased metabolic complications whereas subcutaneous adiposity is not so harmful and may even be protective [6]. While the reason is not clear, the fact that visceral fat is more closely related with liver through the portal vein than subcutaneous adipose tissue, together with its diminished expansion capability, supports the increased risk of metabolic syndrome strongly associated with visceral obesity. Moreover, evidences that the individual adipose expandability threshold is determined by genetic and environmental factors, may explain why both apparently lean and obese people may develop insulin resistance [7].

In addition to the expandability hypothesis, adipose tissue inflammation mediated by overproduction of pro-inflammatory adipokines and anti-adipogenic cytokines such as TNF-α and

IL-6, is another recognized mechanism linking obesity to insulin resistance. Large adipocytes express and secrete high levels of chemoattractants, thus inducing macrophage infiltration in the adipose tissue and activation by FFA release. These macrophages secrete anti-adipogenic cytokines that inhibit insulin action. Insulin resistant adipocytes continue releasing FFA thus activating macrophages that surround adipocytes to destroy compromised cells and secrete more anti-adipogenic cytokines, increasing insulin resistance in mature adipocytes and blocking maturation of preadipocytes [6]. The cyclic mechanism of adipose tissue inflammation linking to insulin resistance is depicted in Fig. 15.1.

15.3 Aquaporins in Obesity

A number of recent studies evidenced aquaporins (AQPs) as key players in adipose tissue biology and involved in obesity onset. AQPs are transmembrane proteins that facilitate the permeation of water and small solutes across membranes, driven by osmotic or solute gradients [8]. In mammals, the 13 aquaporin isoforms identified so far (AQP0-12) are expressed in a wide range of tissues and are involved in many biological functions, including transepithelial fluid transport, cell migration and proliferation and adipocyte metabolism [9, 10]. AQPs are composed by around 320 amino acid residues with approximately 28 kDa, architected in membranes as tetramers. Each monomer is formed by six transmembrane domains and behaves as an independent pore [11].

Based on their primary sequences and permeation specificities, AQPs are divided into three subfamilies: orthodox aquaporins, considered strict water channels (AQP0, 1, 2, 4, 5, 6, 8); aquaglyceroporins, permeable to water and small uncharged solutes like glycerol (AQP3, 7, 9, 10); and unorthodox aquaporins, found mostly intracellularly, with lower sequence homology and permeability still unclear (AQP11, 12) [12, 13]. Among the three subfamilies, aquaglyceroporins are emerging as important players in adipose tissue homeostasis and insulin response with possible implications in metabolic disorders such as obesity and metabolic syndrome. In fact, their role in glycerol metabolism, mediation of glycerol release from adipose tissue and uptake in liver and heart, reveal that these membrane channels are crucial for glycerol balance and energy homeostasis and may be used for obesity therapy.

15.4 Glycerol Metabolism and Energy Homeostasis

The ability of aquaglyceroporins to facilitate glycerol permeation through adipocyte membranes and its impact in metabolic disorders has raised attention to the involvement of glycerol in metabolism and in a variety of pathophysiological mechanisms.

Glycerol (1,2,3-propanetriol) is a polyalcohol that can be produced intracellularly from various metabolic sources such as glucose, protein and glycerolipid metabolic pathways (endogenous glycerol) as well as taken up from dietary fats released during digestion (exogenous glycerol). Glycerol is in the basis TAG backbone and a precursor for phospholipids synthesis and is also an important intermediate in both carbohydrate and lipid metabolism. In addition, glycerol-3-phosphate (G3P) is a key molecule in the regeneration of NAD^+ from NADH resultant from glycolysis, acting as a shuttle of electrons from the cytosol into the mitochondria [14, 15].

Dietary TAG are digested by lipases in the digestive tract and converted to mono and diacylglycerols by pancreatic lipases in the small intestine that are then absorbed by the duodenum mucosa. In the enterocytes, monoacylglycerols and FFA are reconverted into TAG that are then secreted through the basolateral membrane into the lymphatic system as low density lipoproteins known as chylomicrons. Chylomicrons are released from the lymph to the bloodstream where they circulate till reaching adipose, cardiac and skeletal muscle tissues. Lipoprotein lipases, attached to the surface of endothelial cells of capillaries, hydrolyse TAG components of chylomicrons in FFA, absorbed actively by the tissues,

Table 15.1 Expression of aquaporins by organ/tissue and their implication in glycerol balance

Organ/tissue	Aquaglyceroporin	Glycerol balance
Small intestine	AQP3 [21]	Glycerol secretion and enterocyte proliferation [22]
	AQP7 [23]	Rapid glycerol absorption in villus epithelium [23, 24]
	AQP10 [25–27]	Carrier and channel for glycerol and other solutes transport [24]
Adipose tissue	AQP3 [28]	Glycerol metabolism [28]
	AQP7 [29–31]	Main glycerol transporter; controls glycerol uptake and release [31–33]
	AQP9 [28]	Glycerol influx [28]
	AQP10 [31]	Maintains normal glycerol levels [31]
	AQP11 [34]	Mediates intracellular glycerol movements [34]
Cardiac and skeletal muscle	AQP3 [16, 35, 36]	Glycerol transport for energy production in skeletal muscle [35]
	AQP7 [16, 35, 36]	Glycerol transport for energy production mainly in cardiac muscle [16, 35, 37]
Liver	AQP9 [38]	Uptake of glycerol for glucose production [38]
Endocrine pancreas	AQP7 [39]	Regulation of insulin production/secretion [39, 40]
Kidney	AQP7 [41]	Involved in glycerol reabsorption [42–44]

and glycerol, taken up by the liver and other organs. In the liver, glycerol is used in glycolysis or gluconeogenesis but not before being converted in the intermediate G3P by the enzyme glycerol kinase (GK), which is mainly present in the liver and kidney, but also, in low concentrations, in muscle and brain. G3P is the more important form of glycerol for the cell physiology. In addition, glycerol is an energy substrate via the G3P shuttle, which has a key role in oxidizing glucose rapidly and generating adenosine triphosphate (ATP) in the mitochondria through the oxidation of G3P [16].

In humans, gluconeogenesis occurs mainly in the liver and kidney, sites of greatest measured GK activity. Under normal feeding conditions, glycerol contribution to gluconeogenesis is reduced but it increases considerably during starvation where it becomes the primary source for gluconeogenesis along with lactate, pyruvate, alanine and glutamine [17]. In prolonged fasting, glycerol can be used as the only source for gluconeogenesis, since glycogen reserves are depleted within two fasting days [14].

In situations of negative energy balance, such as fasting or exercise, lipolysis of TAG stored in white adipose tissue yields glycerol and FFA that are released in the bloodstream to be used by other organs as energy source. Lipolysis rates are finely regulated by hormones and by biochemical signals that modulate lipolytic enzymes, allowing the finest response from adipose tissue to changes in energy requirements and availability [18, 19].

Besides the intake of dietary glycerol absorbed in the small intestine, the amount of glycerol circulating in the bloodstream is as well dependent on the amount reabsorbed in kidney microtubules; but its main source results from lipolysis in adipose tissue.

All the above-described metabolic pathways occur exclusively in intracellular compartments forcing glycerol molecules to move across the different tissues. Glycerol permeation through membranes is facilitated by aquaglyceroporins and thus regulation of glycerol transport by AQPs is crucial to control fat accumulation, lipolysis, gluconeogenesis and energy homeostasis [15, 20]. Table 15.1 lists the tissue expression and implication in glycerol balance described for aquaglyceroporins, anticipating its possible implication in fat fate and associated metabolic alterations.

Figure 15.2 depicts a schematic model showing the involvement of aquaglyceroporins in glycerol metabolism and energy balance. The expression and localization of the various aquaglyceroporins in multiple organs is important to assure glycerol fluxes across tissues and is crucial

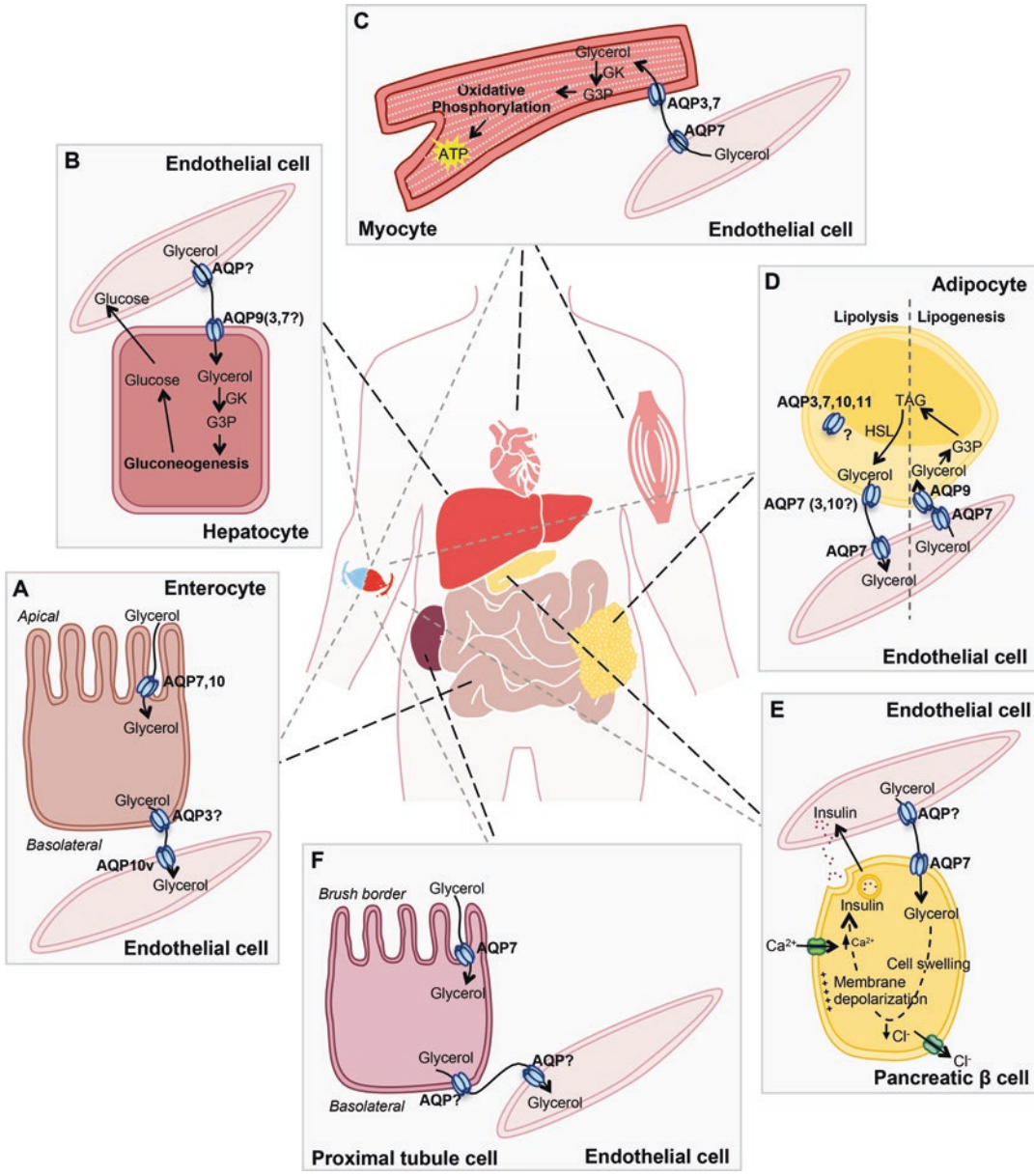

Fig. 15.2 Involvement of aquaglyceroporins in glycerol metabolism and energy homeostasis. (**A**) Glycerol from dietary fat is absorbed via AQP7 and AQP10 in the apical membrane of small intestine epithelial cells, and exits via AQP3 located in the basolateral membrane. Endothelial cells from adipose vasculature express AQP10 that allows glycerol entry to the bloodstream to be used by other organs as energy source. (**B**) Glycerol is taken up to the liver by AQP9 (and possibly also AQP3 and AQP7) expressed at the basolateral sinusoidal membrane of hepatocytes. In the hepatocyte, glycerol is converted to G3P by GK to be used in gluconeogenesis. (**C**) Glycerol is used in cardiac and skeletal muscle as an energy source. It permeates the membrane by AQP3 and 7 and, after conversion in G3P by GK, is used for ATP production. (**D**) In fasting conditions when lipolysis occurs in the adipocytes, TAG hydrolysis yields FFA and glycerol that is released to the blood via AQP7 (and possibly also 3 and 10) expressed in both adipocyte and endothelial cell membranes. In feeding conditions when plasma glycerol reaches high concentrations, glycerol is taken up by adipocytes possibly via AQP9 being converted to TAG and stored in the lipid droplets. (**E**) In response to high plasma nutrient levels, glycerol enters pancreatic β cells via AQP7 and participates in a cascade of events that culminates with insulin exocytosis. (**F**) Glycerol filtered in the kidney is reabsorbed to the blood via AQP7 expressed in the brush border membrane of proximal tubule cells, thus preventing its excretion in the urine

for glycerol metabolism and homeostasis. A detailed description can be found in Fig. 15.2 legend.

15.5 Aquaporins in Adipose Tissue

15.5.1 AQP7 Expression and Role in Fat Accumulation

Among the various mammalian aquaglyceroporins, AQP7 is the most representative glycerol channel and was the first to be detected in human and mice adipose tissue [41, 45, 46] and adipocytes [29, 30]. Despite claimed by other researchers that AQP7 was localized in the vascular tissue surrounding fat rather that in adipose tissue membranes [42, 47], a double localization in adipocytes and endothelial cells was also described [31, 44] and AQP7 expression in both human subcutaneous and visceral adipose tissue was reported [15, 30].

The fundamental role of AQP7 in glycerol release from adipocytes was attained when it was found to function as a glycerol channel [29]. Several experiments were performed to demonstrate AQP7 function. Obese insulin-resistant db+/db+ mice showed higher AQP7 expression compared to control mice [29] and a similar increase in AQP7 mRNA was also observed in adipose tissue of a rodent model of type 2 diabetes with obesity when compared with normal rats [48]. These experiments suggested that the dysregulation of AQP7 can lead to an augmented input of glycerol for hepatic gluconeogenesis and to an increase of glucose in type 2 diabetes [41]. Studies using AQP7 knockout mice showed development of adipocyte hypertrophy and early obesity onset due to an accumulation of glycerol and TAG [32, 33]. In addition, aged AQP7 knockout mice developed insulin resistance, compromising the whole body metabolism. Hibuse et al. [33] proposed a mechanism to explain adipocyte hypertrophy, where an increased accumulation of glycerol in adipocytes stimulates glycerol kinase activity and leads to increased TAG levels in adipose tissue, indirectly favouring the development of obesity and insulin resistance [33]. Yet, susceptibility to develop obesity was not confirmed by other AQP7 null mice lines [42, 49]. Nevertheless, although different phenotypes were reported in distinct AQP7 knockout mice studies, all confirmed the involvement of AQP7 in glycerol metabolism.

Correlation of adipose AQP7 expression with glycerol metabolism and related metabolic complications was not so obvious in human studies [50]. Albeit the link between adipocyte AQP7 expression and insulin resistance, genome-wide analysis found *Aqp7* gene linked to type 2 diabetes [51] and metabolic syndrome [52] as well as associated with obesity but only for the female participants [53]. Gender differences in the role of AQP7 in adipose tissue metabolism were supported by higher fasting circulating levels of glycerol in women than in men, probably due to higher percentage of subcutaneous fat in females, higher lipolytic rates and higher AQP7 expression levels [47, 54].

Three AQP7 missense mutations (R12C, V59L and G264V) and two silent mutations (A103A and G250G) were described in humans. G264V mutation, held by a male homozygous patient, was the only one demonstrating water and glycerol permeability loss; however it did not correlate to obesity nor diabetes, and a lack of increase in plasma glycerol was observed only when stressed by exercise [50, 55]. Three other patients with the same mutation were also diagnosed as neither obese nor diabetic, but they presented increased glycerol excretion in the urine [56].

15.5.2 Regulation of AQP7 Expression

In mice and humans, *Aqp7* gene expression is upregulated by fasting or exercise, leading to glycerol production from endogenous TAG, whereas during the feeding state it is downregulated. AQP7 abundance is inversely related with plasma insulin levels [29]. Transcription of *Aqp7* gene is inhibited by the increase of insulin levels in the bloodstream through a negative insulin

response element (IRE) identified in the promoter region of *Aqp7* gene in mice and humans [55, 57] and by blockage of the phosphatidylinositol-3 kinase (PI3K) pathway [28, 57]. Upregulation of AQP7 by peroxisome proliferator-activated receptor gamma (PPARγ) was also demonstrated in mice and human adipocytes [50, 58, 59]. PPARγ regulates adipocyte differentiation through the regulation of several adipose genes and, in differentiating adipocytes, simultaneous increase in glycerol release to the media and AQP7 mRNA levels were observed [29], suggesting a common regulatory pathway dependent of cell differentiation [60]. In line with this, thiazolidinediones (synthetic PPARɣ) and insulin sensitizers were reported to upregulate AQP7 [48, 55, 57], whereas leptin [28, 61], TNF-α, adrenergic agonists and steroids, being insulin resistance inducers, down-regulate AQP7 expression [62]. Ghrelin, a lipogenic hormone, also interacts in AQP7 regulation, stimulating TAG accumulation in parallel with a decrease in AQP7 expression [63].

In the fasting state, concomitant with lipolysis, low plasma insulin levels and catecholamine stimulation induce *Aqp7* gene transcription and intracellular AQP7 translocation to the plasma membrane, thus potentiating glycerol release from adipocytes [29]. In humans, studies with obese subjects showed a different regulation of AQP7 depending on the type of adipose tissue, subcutaneous or visceral. Obese individuals show low AQP7 expression in subcutaneous fat reflecting fat accumulation and adipocyte hypertrophy but, conversely, show increased AQP7 levels in visceral fat, which can be correlated with increased lipolysis [20, 28]. Since subcutaneous adipose tissue is more insulin sensitive than the visceral, AQP7 downregulation may represent a feedback mechanism attempting to prevent lipid depletion and consequent lipotoxicity and associated disorders [20].

15.5.3 Other Aquaporins in Adipose Tissue

The fact that obese AQP7 null mice still show measurable glycerol secretion and that AQP7 altered expression or dysfunction could not be clearly correlated with obesity and type 2 diabetes, suggested the existence of alternative glycerol pathways in adipose tissue. In fact, the aquaglyceroporins AQP3 and AQP9 were detected in human subcutaneous and visceral adipose tissue, with AQP3 being even more expressed in visceral tissue than AQP7 [28] although other authors did not confirm these same results [29, 30, 64].

AQP3 was found both intracellularly and in the plasma membrane of adipocytes in subcutaneous and visceral adipose tissue, but with a stronger expression in the stromal vascular tissue adjacent to adipose [28, 30, 64]. AQP9 was also detected in the plasma membrane of adipocytes [28]. Described as mostly expressed in the liver, AQP9 is responsible for hepatic glycerol uptake for gluconeogenesis in close coordination with adipose glycerol efflux through AQP7 during fasting [65, 66], and is strongly associated with adipose tissue metabolism and fat accumulation. In addition, non-alcoholic fatty liver disease (NAFLD) has been associated with altered hepatic AQP9 and glycerol permeability [61, 67] that could be reverted by leptin administration [61].

AQP10 was detected in the cytoplasm and in the plasma membrane of adipocytes, in human subcutaneous adipose tissue [31]. Both AQP3 and AQP10 were shown to translocate to the plasma membrane in response to β-adrenergic stimuli [31, 68]. More recently, the unorthodox AQP11 was detected in both subcutaneous and visceral adipocytes being localized in the vicinity of the lipid droplets, possibly associated with the endoplasmic reticulum [34]. AQP11 water and glycerol transport was also demonstrated in an adipose cell model [34] possibly unravelling a facilitated glycerol gateway from the intracellular lipid droplets [20].

15.6 AQPs as Drug Targets of Obesity

The implication of aquaglyceroporins in fat metabolism and obesity indicate that from a pathophysiological point of view these proteins

are promising drug targets. The possibility of regulating the expression of aquaglyceroporins, in particular AQP7 in adipose tissue, offers a potential therapeutic approach for the regulation of fat accumulation and treatment of obesity. Altered AQP7 expression by hormones (insulin, catecholamines) cytokines and adipokines [28, 61–63] is described in the previous section. As for AQP7 channel activity, the only specific modulator described so far is the gold compound Auphen that showed to inhibit glycerol permeability in an adipocyte cell line [69]. However, while Auphen may have a possible application in diagnosis to uncover AQP7 activity and gating [70, 71], strategies to treat obesity point towards activation of AQP7 function rather than inhibition. Thus, the design of small-molecule upregulators of AQP7 expression and function is of utmost interest and would undoubtedly have therapeutic applications.

A recent study reported that supplementation of high-fat diets with apple polyphenols impaired adipocyte hypertrophy and prevented adiposity increase by a mechanism that included increased AQP7 and leptin mRNA levels in rat visceral adipocytes [72]. However promising, the complex mixture of polyphenols included in this functional food may hinder the identification of structure-activity relationships of the phenolic substances. Hence, further investigations to untangle aquaporins interplay with other adipose regulatory molecules, regulation by hormones and possibility of channel gating, are needed to better establish the mechanistic basis of AQPs involvement in the pathogenesis of obesity, which is crucial for the identification of novel modulators or the design of new compounds.

15.7 Final Considerations

Notwithstanding the importance of glycerol as key energy source in multiple vital metabolic processes and in the physiopathology of several disorders, the role of aquaporins on glycerol membrane permeation has only recently been recognised. Given the involvement of aquaglyceroporins in energy and metabolic homeostasis serving as mediators of glycerol delivery and bridging tissues and organs, their targeting might constitute a novel approach for controlling several metabolic disorders. Adipocyte glycerol permeability is a regulator of adipocyte enlargement and weight gain and thus upregulation of AQP7 expression or its functional activation may provide a novel therapeutic approach to prevent or treat obesity. However, potent and selective pharmacologic modulators are still missing. Identification or design of new molecules targeting adipose aquaporins might be seed for drug development and open new perspectives of obesity treatment.

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Aquaporin-Targeted Therapeutics: State-of-the-Field

16

Lukmanee Tradtrantip, Bjung-Ju Jin, Xiaoming Yao, Marc O. Anderson, and Alan S. Verkman

Abstract

Drugs targeting aquaporins have broad potential clinical applications, including cancer, obesity, edema, glaucoma, skin diseases and others. The astrocyte water channel aquaporin-4 is a particularly compelling target because of its role of brain water movement, neuroexcitation and glia scarring, and because it is the target of pathogenic autoantibodies in the neuroinflammatory demyelinating disease neuromyelitis optica. There has been considerable interest in the identification of small molecule inhibitors of aquaporins, with various candidates emerging from testing of known ion transport inhibitors, as well as compound screening and computational chemistry. However, in general, the activity of reported aquaporin inhibitors has not been confirmed on retesting, which may be due to technical problems in water transport assays used in the original identification studies, and the challenges in modulating the activity of small, compact, pore-containing membrane proteins. We review here the state of the field of aquaporin-modulating small molecules and biologics, and the challenges and opportunities in moving forward.

Keywords

Water channel • AQP • Neuromyelitis optica • Brain edema • Drug discovery

16.1 Introduction and Potential Indications of Aquaporin Modulators

As reviewed in elsewhere in this book, more than a dozen mammalian aquaporins (AQPs) have been identified, many of which function as water channels, and some, the aquaglyceroporins,

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also transport various small, polar non-electrolytes including glycerol and hydrogen peroxide. Structurally, AQP monomers are small, membrane-spanning proteins of molecular size ~30 kDa, each containing one narrow aqueous pore. AQP monomers assemble in membranes as tetramers, with some AQPs such as AQP4 assembling further into supramolecular aggregates called orthogonal arrays of particles. There is high-resolution crystal structure data for some AQPs, as well as molecular dynamics simulations of how water and small polar molecules might traverse the aqueous pore. The AQPs have broad tissue expression, which includes absorptive and secretory epithelia, astrocytes, myocytes, adipocytes, epidermal cells, and others. A priori challenges in AQP drug discovery include: (i) the wide distribution and the many structurally similar AQP isoforms; (ii) the structural features of a narrow, drug-excluding pore and a compact tetramer; (iii) the lack of physiological regulation of intrinsic AQP function; and (iv) the unique ability of water, which is present at 55 molar concentration, to circumvent obstacles. Nevertheless, there is no single compelling reason to exclude the possibility of identifying useful inhibitors of AQP function, modulators of AQP expression, or blockers of AQP-targeted pathogenic antibodies in certain diseases. Indeed, mercury-containing and other heavy metal-containing sulfhydryl-reactive compounds inhibit the function of some AQPs by chemical modification of cysteine residues, though their marked toxicity and lack of selectivity preclude their development as drugs.

Notwithstanding the caveats listed above, there are compelling potential opportunities for AQP-targeted therapeutics in human disease. The physiological functions of AQPs, which have been elucidated largely from phenotype studies on knockout mice, are reviewed elsewhere [39]. Some interesting potential applications are mentioned briefly. Inhibitors of AQP1 water transport are predicted to act as unique diuretics, inhibit tumor angiogenesis and growth, reduce intraocular pressure in glaucoma, and potentially reduce nociception [7, 19, 31, 32, 47, 49]. Inhibitors of AQP4 water transport are predicted to reduce brain swelling in ischemic stroke [22]. Inhibitors of AQP3 glycerol and/or hydrogen peroxide transport are predicted to prevent or retard skin tumor growth and inflammatory skin diseases [9, 10]. Though it is unlikely that AQP 'activators' can be identified, as the AQPs are probably already maximally active, selective transcriptional upregulators of AQP7 may reduce adipocyte hypertrophy in obesity, of AQP3 may promote wound healing, of AQP4 may have antiepileptic activity, and of AQP5 might increase glandular fluid secretion. There is evidence that AQP gene transfer may increase epithelial water permeability, promoting saliva secretion in salivary gland disorders [2] and bile secretion in liver diseases associated with cholestasis [23]. Finally, blockers of binding of pathogenic AQP4-targeted autoantibodies to astrocyte AQP4 might prevent or reduce neuropathology and neurological deficit in neuromyelitis optica [28].

Herein, we review approaches to assay for AQP-modulating compounds, the state of the field in the identification and validation of AQP modulators, and potential directions in moving forward.

16.2 Aquaporin Functional Measurements

Reliable assay of AQP function is central to the identification and validation of pharmacological AQP modulators. This section focuses on measurements of AQP water permeability, some of which are suitable for primary high-throughput screening, with discussion of assay limitations and potential artifacts. Alternative assays of transport functions of some AQPs, including glycerol, hydrogen peroxide and gas transport, merit consideration, but are not discussed further as available assays are not sufficiently robust for primary screening applications.

Measurement of water transport across an intact epithelium is accomplished by determination of net volume movement in response to a transepithelial osmotic gradient, which can be accomplished by a variety of methods such as displaced volume and dye dilution as measured

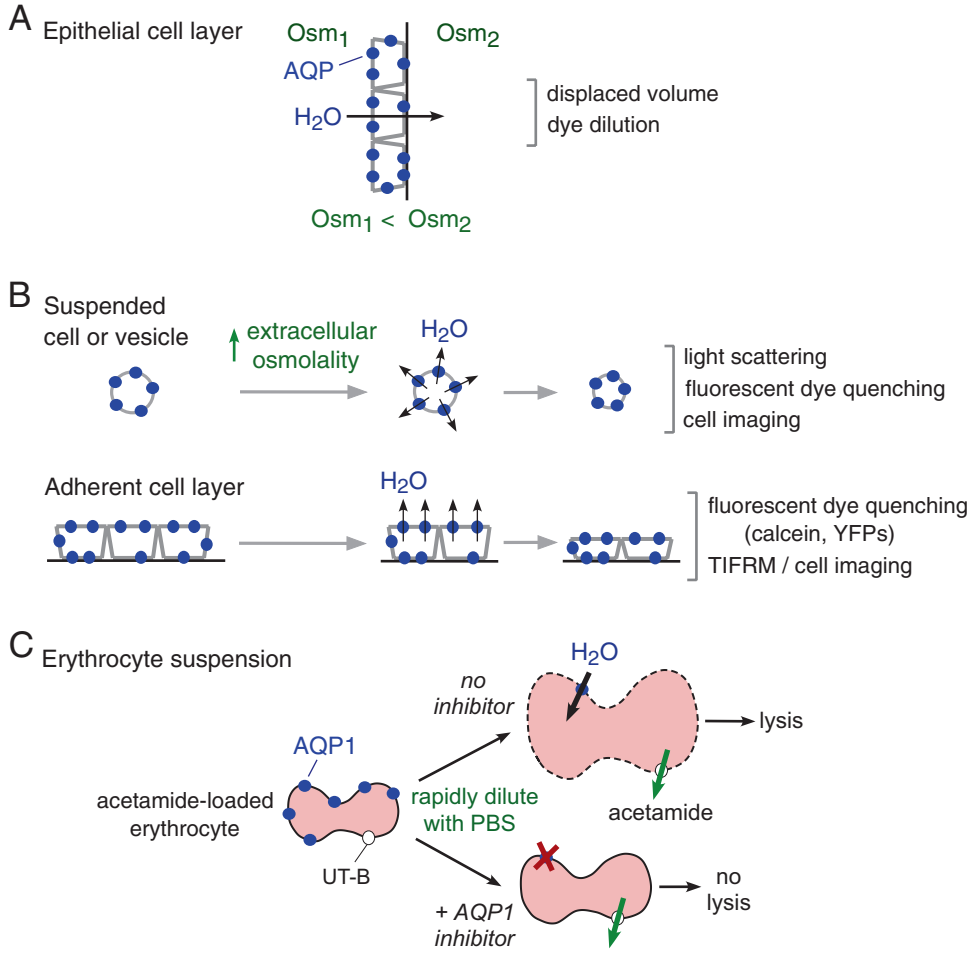

Fig. 16.1 Assays of osmotic water permeability. (**a**) Water transport across an epithelial cell monolayer in response to a transepithelial osmotic gradient produces net volume flux. Osm, osmolality. (**b**) Water permeability across cells or vesicles in suspension (*top*) or across immobilized cells (*bottom*) in response to an osmotic gradient produces time-dependent volume change. Figure depicts water efflux in response to an inward osmotic gradient and cell shrinkage. (**c**) AQP1 water transport measured in erythrocytes, which express AQP1 and the urea / acetamide transporter UT-B. Dilution of acetamide-loaded erythrocytes into an acetamide-free solution drives water influx, cell swelling and lysis, which are reduced by AQP1 inhibition. See text for further explanations

by electrical of fluorescence methods (Fig. 16.1a). The epithelial cell layer can consist of cells cultured on a porous support or a native epithelium such as a kidney tubule or urinary bladder. Measurement of transepithelial water transport is robust and reliable, though not highly precise or suitable for high-throughput measurements. Additionally, because transcellular water transport involves two barriers in series (apical and basolateral membranes) the results probe mainly the rate-limiting barrier.

Osmotic water transport in suspended or adherent cells is measured from the kinetics of cell volume in response to an osmotic gradient that is imposed over a time much less than the osmotic equilibration time (Fig. 16.1b). For suspended cells (or vesicles or liposomes) an osmotic gradient can be imposed using a stopped-flow instrument in which cell-containing and anisomolar solutions are mixed within milliseconds or less. For adherent cells such as AQP-expressing transfected or transduced cells, an osmotic

gradient can be imposed using a perfusion chamber, or, for high-throughput applications, by rapid solution addition in a multi-well plate format. Cell volume readouts include light scattering, fluorescence of a volume-sensing dye, or direct imaging. Light scattering is used mainly to study small cells in suspension, such as erythrocytes, and is subject to various artifacts because the intensity of light scattered from cells depends not only on cell volume but on cell shape, the refractive index of intracellular and extracellular solutions, and membrane optical properties, each of which can be influenced by a putative AQP modulator. In addition, a 'mixing artifact' results from changes in light scattering as flow slows, which is unrelated to cell water permeability. Fluorescence methods are relatively insensitive to cell shape, membrane properties and mixing artifact, but can be affected directly by test compounds and confounded by dye leakage and binding to cell membranes, particularly in the calcein method in which calcein fluorescence is quenched by cytoplasmic proteins as cells shrink [35]. An alternative, genetically encoded chloride-sensing yellow fluorescent protein [3] does not suffer from leakage artifact, but is affected by changes in intracellular pH and anion concentrations, and has limited time resolution.

One particular cell type, the Xenopus oocyte, has been used in many studies to measure water permeability. Xenopus oocytes are injected with cRNA encoding an AQP, and the kinetics of oocyte swelling is measured in response to an osmotic gradient. Generally oocyte volume is inferred from its cross-sectional area (shadow) as measured with transmitted light microscopy using a low magnification lens. Though the oocyte swelling method was valuable in the original identification of AQP1 as a water channel, where large and unambiguous increases in the rate of swelling were seen, leading to oocyte bursting, oocytes have limited value for study of potential AQP modulators because of many potential artifacts. Changes in oocyte cross-sectional area depend not only on oocyte osmotic water permeability, but on oocyte geometry, membrane properties, solute transport, cytoplasmic and extracellular unstirred layers, and other factors. For example, preincubation of oocytes with an ion transport inhibitor can alter oocyte volume and cytoplasmic ionic concentrations, precluding meaningful measurement of osmotic water permeability. A further problem with many published measurements is that oocyte volume responses are measured over minutes, rather than seconds, a time scale where mechanical restrictions can affect swelling and solute transport can affect the osmotic gradient.

For measurement of erythrocyte AQP1 water permeability, we developed a simple method that relies on a single read-out of cell lysis (Fig. 16.1c). Erythrocytes, which natively express AQP1 and urea transporter UT-B, are preloaded with acetamide, a urea analog that is transported by UT-B and equilibrates across the erythrocyte membrane over a time course similar to that of osmotic water transport. Dilution of erythrocytes into an acetamide-free solution produces rapid, AQP1-dependent cell swelling and lysis, as assayed by solution absorbance in a platereader. Inhibition of AQP1 water permeability reduces cell lysis, as water influx is slower than dissipation of the osmotic gradient by acetamide efflux. A variation of this approach has been used to identify inhibitors of UT-B urea transport with low nanomolar potency [18], but a small-molecule screen for AQP1 inhibitors did not produce useful active compounds (unpublished data).

We have been developing microfluidics methods to measure water permeability, as microfluidics can provide a technically robust platform for rapid assays using very small samples. In one study, a perfusion channel was developed to measure volume changes of epithelial organoids in which the organoids are entrapped by pillars and volume measured by dye exclusion [16]. In another study [15], a microfluidic channel was designed to mimic rapid stopped-flow mixing methodology, in which cells are subjected to an osmotic gradient in milliseconds by solution mixing inside a ~ 0.1 nL droplet surrounded by oil (Fig. 16.2a). Rapid mixing of cells with an anisomolar solution is accomplished in a mixing channel, which then deposits the cell-containing droplets in an observation area in which time

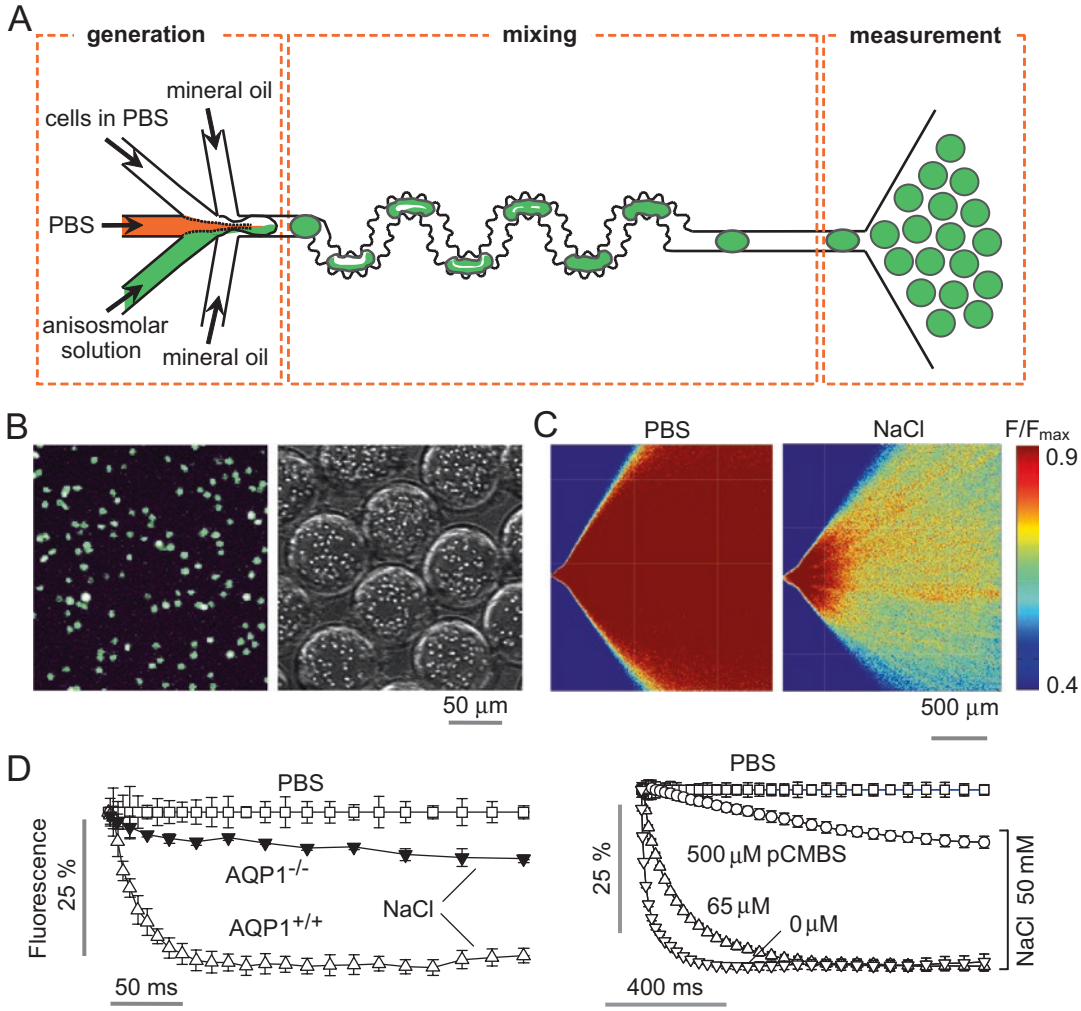

Fig. 16.2 Microfluidic 'stopped-flow' approach to measure water permeability. (**a**) Microfluidic channel design, in which mixture of cells with an anisomolar solution in droplets drives osmotic water transport and cell volume change, as measured by fluorescence in a measurement area. (**b**) Fluorescence micrograph of calcein-loaded erythrocytes on a coverglass (*left*) and in aqueous droplets in the microfluidic channel (*right*). (**c**) The microfluidic channel was perfused with an erythrocyte suspension in PBS, PBS (in central channel), and PBS containing 500 mm NaCl (bottom channel), to give a 200-mM NaCl gradient. Fluorescence micrographs of the measurement region for zero gradient (*left*) or a 200-mM NaCl gradient (*right*). (**d**) Deduced time course of erythrocyte calcein fluorescence in erythrocytes from wild-type and AQP1-knockout mice for a 200-mM NaCl gradient, and in the absence of an osmotic gradient (PBS) (*left*). Measurements on human erythrocytes preincubated with indicated concentration of the mercurial AQP1 inhibitor pCMBS (*right*) (Adapted from Ref. [15])

after mixing is determined by spatial position. Osmotic water permeability is then determined from a single, time-integrated fluorescence image of the observation area. As an example, water permeability was measured in calcein-labeled erythrocytes (Fig. 16.2b). Fluorescence of the observation area showed reduced fluorescence in the presence of an osmotic gradient (Fig. 16.2c), from which the kinetics of water transport can be deduced. Figure 16.2d shows the deduced kinetics data from wildtype and AQP1-null mouse erythrocytes (left) and from control and pCMBS (a mercurial)-treated human erythrocytes (right), which agree with results using the conventional

stopped-flow light scattering method. Compared with costly stopped-flow instrumentation, this microfluidics platform utilizes sub-microliter blood sample volume, does not suffer from mixing artifact, and replaces kinetic measurements by a single image capture using a standard laboratory fluorescence microscope. However, microfluidics methods are in general not yet suited for automated high-throughput screening and do not obviate potential measurement artifacts in conventional suspended cell measurements.

16.3 Aquaporin Inhibitors – State of the Field

16.3.1 Older Literature on Aquaporin Inhibitors

It has long been known that mercurial sulfhydryl-reactive compounds, including mercuric chloride and p-chloromercuribenzene sulfonate (pCMBS), inhibit water transport in erythrocytes and various epithelia [20]. After the discovery of AQPs the cysteine(s) involved in mercurial water transport inhibition were identified, such as Cys-187 in AQP1 [48]. More recently, gold-containing compounds were reported to inhibit AQP3, with auphen being the most potent [24]. Various non-metal-containing small molecules were reported to inhibit water permeability in some AQPs, including the K^+ channel blocker tetraethylammonium (TEA^+), the carbonic anhydrase inhibitor acetazolamide, several anti-epileptic drugs and dimethylsulfoxide (DMSO) [5, 12, 34]. Subsequent testing, however, did not confirm AQP inhibition by these small molecules [34, 43–45], suggesting measurement artifact in the oocyte swelling studies used to identify the compounds, which, as discussed above, are prone to artifact, especially for compounds that inhibit ion transport processes.

16.3.2 Screening to Identify Aquaporin Inhibitors

Additional putative small molecule AQP inhibitors have emerged from experimental and computational screens, with structures of 12 proposed AQP1 inhibitors, and one AQP1 activator, shown in Fig. 16.3. Compounds #1, #2, and #3 were identified by virtual (computational) screening involving docking to the extracellular surface of human AQP1, and testing 14 compounds for inhibition of osmotic swelling in AQP1-expressing Xenopus oocytes [33]. These compounds reduced osmotic swelling of oocytes by ~80% with IC_{50} of 8–20 μM, but were reported not to inhibit AQP1 in erythrocytes. Compound #4 (AqB013), an analog of the NKCC1 inhibitor bumetanide, came from a small synthesis study, and was claimed to inhibit AQP1 and AQP4 with IC_{50} ~20 μM [25], though it did not show the predicted in vivo beneficial effect when tested in a brain injury model [27]. The same group also reported that an analog of the loop diuretic furosemide, compound #5 (AqF026), activated AQP1 by ~20% in the oocyte assay [46], which is probably well below the limited reliability of such assays. Compounds #6, #7, #8 and #9, identified in small screen using the calcein fluorescence assay, were reported to inhibit AQP1 with IC_{50} values of 25–50 μM [26]; however the organolead and organotin structures are not drug-like and likely toxic, and compound #7 (trichopolyn I) is a 10-residue fungal, pore-forming lipopeptide. More recently, compounds #10 and #11 emerged from a small screen using a yeast freeze-thaw assay, of unclear rationale, done in *E. coli* expressing AQP1 [36]. Compounds #12 and #13 emerged from a small screen [29], though their reported activities were quite variable in oocyte, erythrocyte ghost and AQP1 proteoliposome assays. As described below, we have retested each of these compounds using several sensitive assays of AQP1 water permeability [6].

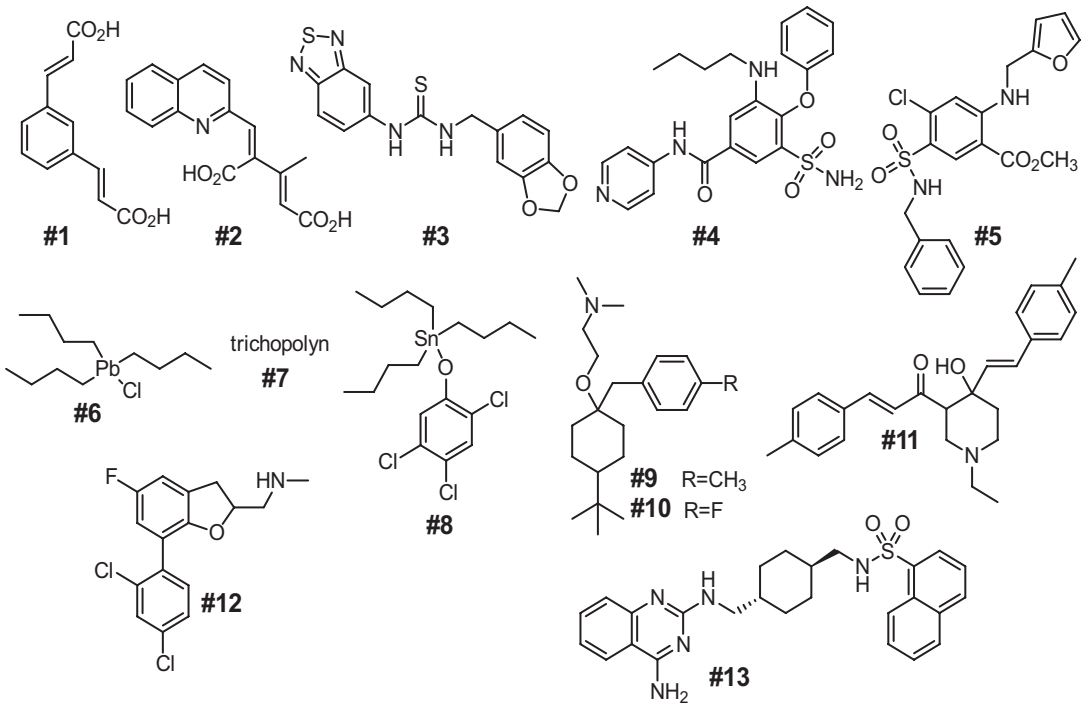

Fig. 16.3 Chemical structures of putative small-molecule AQP1 inhibitors and an AQP1 activator (Compounds shown are reported in Refs. [25, 26, 29, 33, 36, 46]. See text for further explanations)

16.3.3 Screening by Computational Chemistry

Several reports utilize computational methods (virtual screening, some with molecular dynamics (MD) simulations) to identify putative inhibitors of various AQPs. Surprisingly, multiple chemically unrelated antiepileptic drugs, which were selected from docking computation using an electron diffraction structure of rat AQP4, were reported to inhibit oocyte swelling [12]. The same investigators reported non-antiepileptic drugs as AQP4 inhibitors with IC_{50} of 2–11 μM, including 2-(nicotinamido)-1,3,4-thiadiazole, sumatriptan, and rizatriptan [13]. However, retesting of the compounds in Refs. [12, 13] did not confirm activity [45]. As mentioned above, several compounds emerged from a docking screen of ~10^6 compounds from the UCSF-ZINC library against an MD-refined structure of human AQP1 at a site near the ar/R selectivity filter [33]; docked conformations of two of the more promising structures were subjected to several hundred-ns MD simulations to confirm the stability of the docked poses. In a recent study, docking and MD simulations were done using homology models of mouse AQP9 [41], which identified a small set of inhibitors with IC_{50} <50 μM from a shrinking assay in AQP9-expressing CHO cells, though compound activities have not been independently tested to date. In our lab, we carried out large-scale docking studies against high-resolution structures of AQP1 and AQP4, with testing of the best-scoring ~2000 compounds, which, disappointingly, showed <20% inhibition at 50 μM (unpublished data). An example of a well-scored compound of the benzoxazin-3-one class is shown in Fig. 16.4a bound to the cytoplasmic pore region of mouse AQP1. A surface depiction of the complex (Fig. 16.4b) shows a complementary fit, with the nonpolar cyclohexyl substituent projecting deep into the channel, positioned to interact with residues Ile-60, Leu-149, and Val-79.

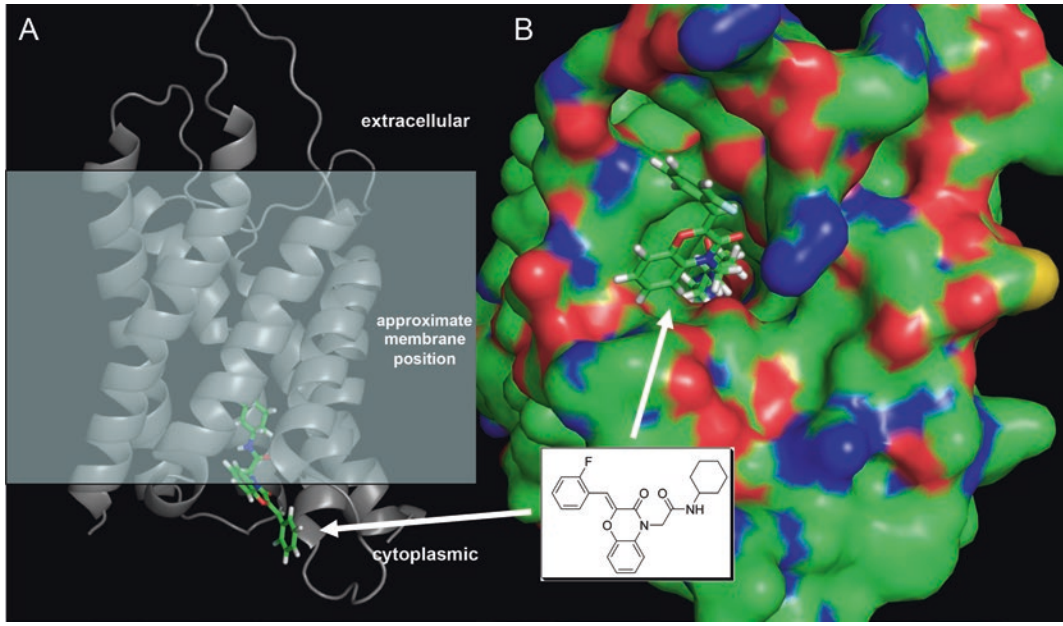

Fig. 16.4 Computational approach to identify aquaporin-interacting small molecules. Docking computation using a homology model of mouse AQP1. (**a**) Side view of an AQP1-ligand complex with the approximate membrane position indicated. (**b**) Surface view of the same complex, showing the cyclohexyl group of the ligand projecting deep into the channel, interacting with a hydrophobic surface

16.3.4 Reevaluation of Proposed AQP1 Inhibitors

In a recent study [6] we reevaluated the 13 compounds shown in Fig. 16.3 for AQP1-modulating activity. The compounds were tested at 50 μM, a concentration predicted from published data to strongly inhibit (or weakly activate) AQP1 water permeability. One approach was stopped-flow light scattering in freshly obtained human erythrocytes. Representative light scattering curves are shown in Fig. 16.5 (left), with averaged data summarized in the right panel. Whereas $HgCl_2$ strongly inhibited osmotic water permeability in erythrocytes, no significant effect was seen for 12 of the 13 test compounds, with the small apparent effect of compound #13 related to cell toxicity. In addition, to rule out the possibility that the lack of inhibition might be due to hemoglobin, which might bind compounds, similar studies done in sealed, hemoglobin-free ghost membranes also showed no inhibition (or activation). Several of the compounds (#6, #9, #10, #12 and #13) showed toxicity as evidenced by erythrocyte crenation and aggregation. Multiple additional assays supported the conclusion that compounds #1 to #13 do not inhibit (or activate) AQP1 water permeability, including erythrocyte swelling assays, erythrocyte water transport assays using calcein fluorescence, and water transport assays in plasma membrane vesicles from AQP1-transfected CHO cells.

It is uncertain why activity could not be confirmed of the many putative AQP modulators reported in the literature. As discussed above the oocyte swelling or calcein fluorescence assays used in most of the studies are subject to considerable artifact in which apparent inhibition of osmotic cell swelling could result from changes in cell size or shape, cell volume regulation, activities of non-AQP ion or solute transporters, etc. Inhibitors of known cell membrane transporter processes, such as bumetanide, acetazolamide and tetraethylammonium, may affect resting cell volume and volume regulation. Because of the very low probability of identifying AQP inhibitors, as suggested from screening

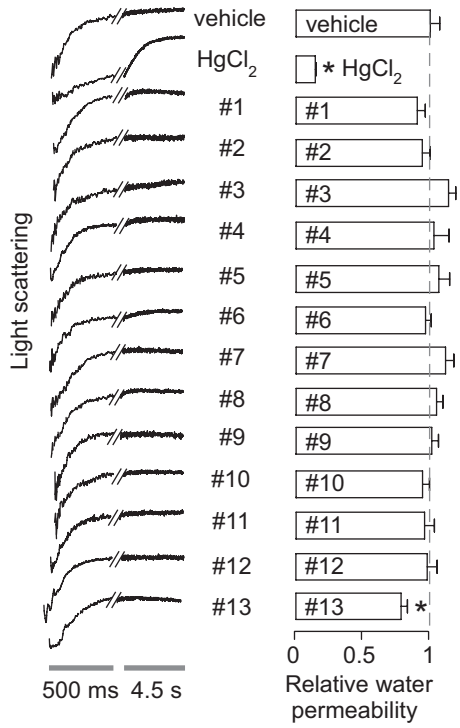

Fig. 16.5 Testing of putative AQP1 modulators in human erythrocytes. Osmotic water permeability was measured in human erythrocytes from the time course of scattered light intensity at 530 nm in response to a 250-mM inwardly directed sucrose gradient. Representative original light scattering data shown on the left for negative control (DMSO vehicle alone) and positive control ($HgCl_2$), and indicated compounds at 50 μM. Summary of relative osmotic water permeability shown on the right (S.E., n = 4, *P < 0.05 compared to control) (Adapted from Ref. [6])

work, it is unlikely that testing of common drugs, such as loop diuretics, carbonic anhydrase inhibitors, and antiepileptics, without large-scale screening, would yield bona fide AQP inhibitors.

16.3.5 Antibodies as AQP Therapeutics

Given the challenges and limited progress in small molecule AQP-targeted therapeutics, consideration of biologic therapeutics is warranted. Of particular interest are IgG1 anti-AQP4 autoantibodies ("AQP4-IgG") in neuromyelitis optica (NMO), an inflammatory demyelinating disease of the central nervous system that can cause paralysis and blindness. It is thought that AQP4-IgG produces neuropathology by binding to AQP4 on astrocytes (Fig. 16.6a) to cause complement- and cell-mediated astrocyte cytotoxicity, which produces inflammation, blood-brain barrier disruption, oligodendrocyte injury, demyelination and neurological deficit [28]. The AQP4-IgG autoantibodies are directed against various 3-dimensional epitopes on the AQP4 extracellular surface. Though it was initially proposed from oocyte swelling studies that inhibition of AQP4 water permeability plays a causal role in NMO [11], subsequent studies showed that AQP4-IgGs, even at saturating concentrations, do not inhibit AQP4 water permeability [30]. Interestingly, autoantibodies against AQP2 [17] and AQP5 [1] have been found recently in interstitial nephritis and Sjogren's syndrome, respectively, though their involvement in disease pathogenesis is not known.

Though the identification of a neutralizing anti-AQP antibody is unlikely because of its large molecular size and binding to extracellular loop regions far from the narrow AQP pore, AQP-binding antibodies have other therapeutic applications. In one application, we generated a high-affinity anti-AQP4 antibody ("aquaporumab") in which the antibody Fc portion was mutated to eliminate effectors functions involved in complement- and cell-mediated cytotoxicity (Fig. 16.6b, left) [37]. The antibody prevented cytotoxicity from NMO patient sera in cell cultures (Fig. 16.6b, right) and prevented pathology and demyelination in animal models of NMO, suggesting its application for primary therapy of NMO. Screening and computational analysis of small molecule blockers of AQP4-IgG binding to AQP4 produced candidate molecules (Fig. 16.6c) [21, 38]; however, their affinities are too low for development as NMO therapeutics, which is not unexpected given the recognized challenges in identifying potent small molecule blockers of protein-protein interactions.

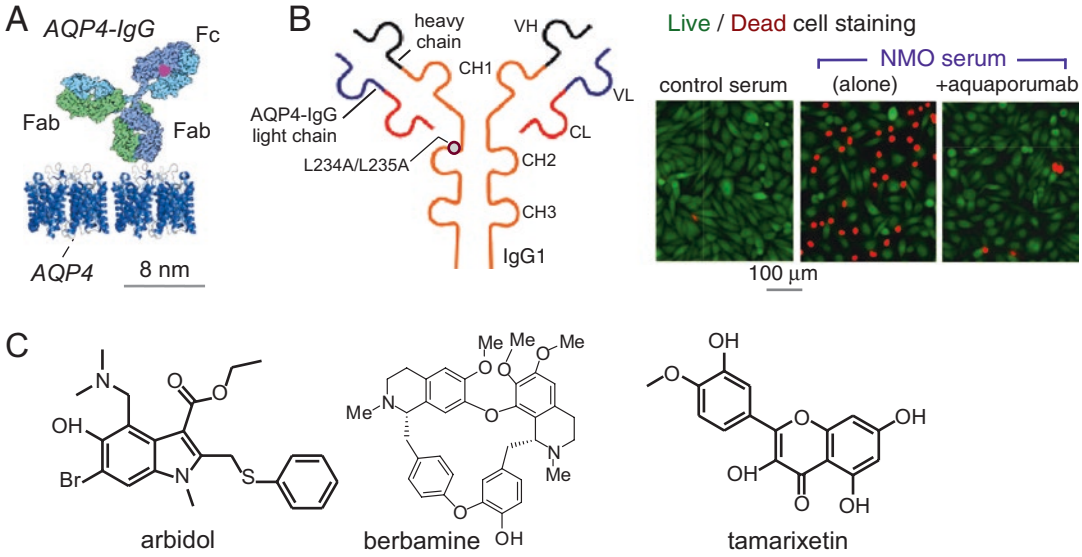

Fig. 16.6 Antibody and small molecule blockers of AQP4 binding of anti-AQP4 autoantibodies causing neuromyelitis optica. (**a**) To-scale diagram of IgG antibody binding to AQP4 on membranes. (**b**) Anti-AQP4 IgG1 antibody with L234A/L235A mutations that eliminate effector functions (left). Live/dead (green/red) cell staining of AQP4-expressing cells exposed to control or human NMO serum, showing protection by aquaporumab. (**c**) Structures of small molecule blockers of autoantibody binding to AQP4 (Adapted from Refs. [37, 38])

16.4 Perspective and Future Directions

Though there is much speculation about the utility of AQP-targeted therapeutics [4, 8, 14, 40, 42], as well as compelling animal data to support AQP drug development, progress in the field has been disappointing. Reports of AQP inhibition by common ion transport inhibitors, such as loop diuretics and antiepileptics, have confused the literature, as have reports of small molecule AQP inhibitors that could not be confirmed on retesting. The potential pitfalls in assays of AQP function merit appreciation, as does the importance of showing robust, cell context-independent compound action. Well-conceived, large-scale functional screens of random, drug-like small molecules may yield useful, bona fide AQP inhibitors, as might smaller screens of compounds collections biased by computation chemistry. A relatively unexplored subject is antibody- and peptide-based AQP therapeutics, and small molecule transcriptional regulators of AQP expression. The interest in commercializing AQP-targeted therapeutics, and the need for useful research tools to overcome the limitations of transgenic animal models, will likely drive further developments in the field.

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Water Transport Mediated by Other Membrane Proteins

17

Boyue Huang, Hongkai Wang, and Baoxue Yang

Abstract

Water transport through membrane is so intricate that there are still some debates. (Aquaporins) AQPs are entirely accepted to allow water transmembrane movement depending on osmotic gradient. Cotransporters and uniporters, however, are also concerned in water homeotatsis. Urea transporter B (UT-B) has a single-channel water permeability that is similar to AQP1. Cystic fibrosis transmembrane conductance regulator (CFTR) was initially thought as a water channel but now not believed to transport water directly. By cotranporters, water is transported by water osmosis coupling with substrates, which explains how water is transported across the isolated small intestine. This chapter provides information about water transport mediated by other membrane proteins except AQPs.

Keywords

Urea transporter B • CFTR • Cotransporter • Water transport

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17.1 Introduction

Although the aquaporin (AQPs) family have been identified to allow water transmembrane movement depending on osmotic gradient, there is water transport mediated by proteins exclusive of AQPs. Water is either transported through AQPs driven by transmembrane difference in water osmosis, or cotransported with some other substrates. Water is also uphill transported by some proteins, called water pump under specific circumstances (Fig. 17.1). The most representative non-AQP water channel is urea transporter B (UT-B). Some cotransporters that not only transport specific solutes or organic molecules across the cell membrane, but also act as water transporters. In this chapter, various modes of water transport and their physiological roles are reviewed.

17.2 Urea Transporter B

UT-B is widely expressed in many tissues, such as kidney, brain, liver, colon, small intestine, pancreas, testis, prostate, bone marrow, spleen, thymus, heart, skeletal muscle, lung, bladder, and cochlea [1]. UT-B transports urea and several chemical analogues of urea, such as methylurea, formamide, acetamide, acrylamide, methylformamide, and ammonium carbamate. Several studies suggested that UT-B functions as an efficient water channel [2–4].

In 1998, Yang et al. found that UT-B was permeable to water when they measured osmotic water permeability in *Xenopus* oocytes expressing UT-B (originally called UT3 and UT11) [4] (Fig. 17.2a). Quantitative measurement of single-channel osmotic water permeability (P_f) of UT-B gave a value of 1.4 $cm^3 \cdot s^{-1}$. UT-B medicating water and urea transport were weakly temperature-dependent, and mostly inhibited by the urea transport inhibitors [2, 4, 5], but not inhibited by the AQP inhibitors $HgCl_2$ [2].

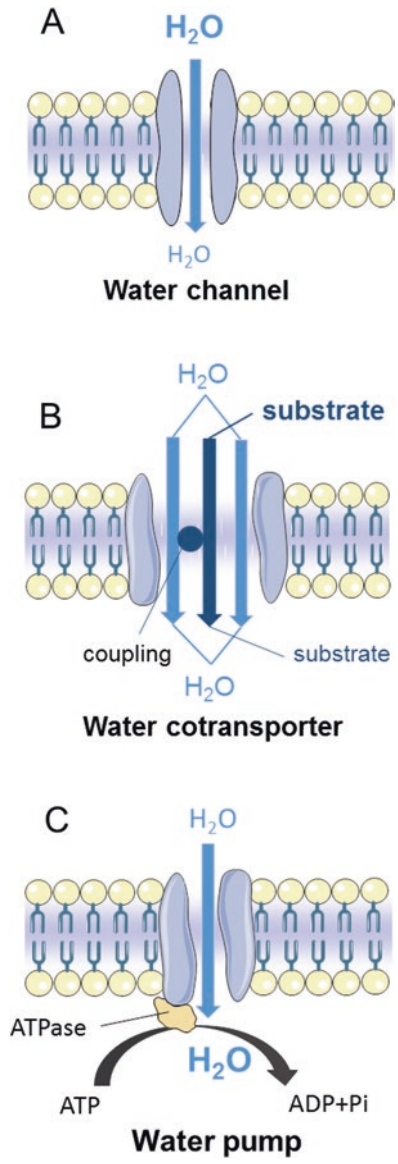

Fig. 17.1 Three ways of water transport. (**a**) Water is transported through a simple channel, driven by osmotic driving force, such as AQPs and UT-B. (**b**) Water is cotransported with substrate through a cotransporter that is bimodal, a passive component transport and a secondary active component transport. (**c**) Water is transported by a pump that actively transports water across membranes relying on ATP hydrolysis (A *darker* and *larger font* indicates more substrates)

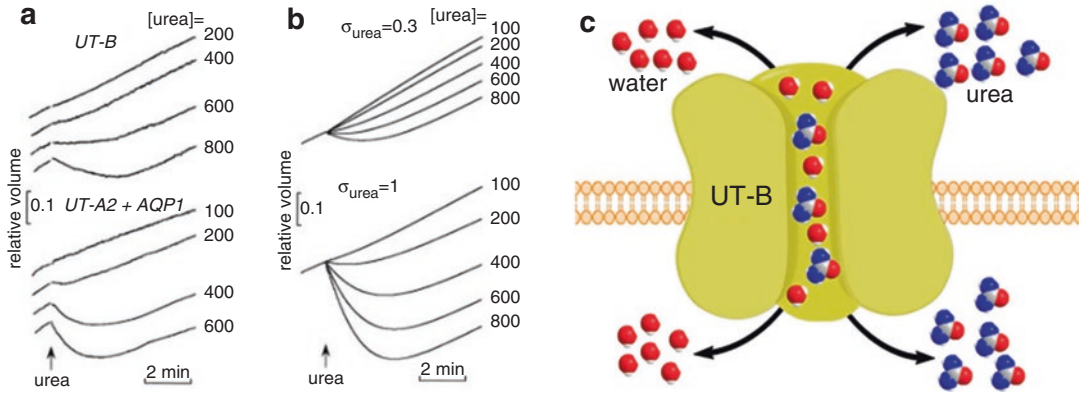

Fig. 17.2 Urea transporter UT-B functions as a water channel. (**a**) Urea reflection coefficient of the UT-B pathway determined in *Xenopus laevis* oocytes. (**b**) Predicted urea reflection coefficient of the UT-B pathway in mathematical model (Derived from Ref. [4]). (**c**) Schematic diagram of UT-B as a urea/water channel utilizing a common aqueous pathway (Derived from Ref. [5])

The most direct evidence for a common water/solute pathway is the low solute reflection coefficient. In the induced osmosis method, oocytes were briefly swelled in 100 mM Barth's buffer and then the external solution was switched to 50 mM Barth's buffer containing different concentrations of urea [4]. As seen in Fig. 17.2a (*top*), oocytes expressing UT-B initially swelled for external 200 and 400 mM urea and shrunk for 600 and 800 mM, suggesting σ_{urea} (the urea reflection coefficient) << 1. The measurements were simulated numerically using the Kedem-Katchalsky equations of coupled water and solute transport for different values of σ_{urea} (Fig. 17.2b). There was good agreement between the simulated and experimental data set for σ_{urea} ~0.3. An important control study was done with oocytes coexpressing water channel AQP1 that is permeable to water but not urea, and urea transporter UT-A2 (originally called UT2) that is permeable to urea but not water. Figure 17.2a (*bottom*) shows little initial oocyte swelling or shrinking for external 200 mM urea, suggesting that σ_{urea} ~1, which was confirmed by the simulated curves in Fig. 17.2b (*bottom*). These results suggest that the UT-B is an aqueous channel that transports water and urea in a coupled manner (Fig. 17.2c).

Sidoux-Walter et al. confirmed increased water permeability in *Xenopus* oocytes expressing UT-B. However, they concluded that UT-B facilitated water transport did not occur under physiological conditions. They proposed that UT-B associated water permeability occurs only when UT-B expressed at non-physiologically high levels [6].

To quantify UT-B-mediated water transport in physiological conditions, double knockout mice lacking both UT-B and the major erythrocyte water channel AQP1 was generated [2]. Osmotic water permeability in erythrocytes from mice lacking both AQP1 and UT-B is 4.2-fold lower than in erythrocytes from mice lacking AQP1 alone. Similar low water permeability was found in erythrocytes from AQP1 null mice after UT-B inhibition by phloretin and in erythrocytes from UT-B null mice after inhibition of AQP1 by $HgCl_2$. The single-channel (per molecule) water permeability of UT-B in erythrocytes is very similar to that of AQP1 (7.5×10^{-14} $cm^3 \cdot s^{-1}$) [2].

In 2013, Slim Azouzi et al. suggested that UT-B should be considered as a new member of water channel family, on the basis of the results that osmotic water unit permeability of UT-B (pf_{unit}) is similar to that of AQP1. Five water molecules were found inside the UT-B pore to form a single-file, which moved rapidly along a channel by hydrogen bond exchange involving two critical threonines [7]. UT-B is a homotrimer and each protomer contains a urea conduction pore with a narrow selectivity filter [8]. The selectivity

filter is divided into three regions: So, Si and Sm sites. When the water molecules cross the region Sm, the water-water (W-W) hydrogen bonds decreases remarkably, and the number of hydrogen bonds with the residues lining the pore increases concomitantly [7]. The fact that urea and water share the same pathway through the pore of UT-B also indicates that UT-B acts as a water channel.

17.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

CFTR is a membrane protein and chloride channel in vertebrates [9]. CFTR is expressed in the apical membrane of epithelial cells in the airway, pancreas, and intestine [10]. It conducts bicarbonate [11], interacting with other Cl^-/HCO_3^- exchangers to provide a recycling pathway [12]. CFTR is different from other Cl^- channels. As a unique member of ABC transporter family, it is a primary active transporter that relies on ATP hydrolysis to actively pump the substrates across membranes [9]. It contains five domains: two membrane-spanning domains forming chloride ion channel, and two nucleotide-binding domains regulating channel to open or to close, and one regulatory domain regulating channel activity [13].

CFTR has been shown to be a regulator of Na^+, K^+, and Cl^- channel, and it also enhances osmotic water permeability when activated by cAMP [14]. Because of the CFTR dependent activation of a water permeable membrane conductance, the osmotic water permeability is activated through stimulation of CFTR in *Xenopus* oocytes. CFTR has a calculated single channel water conductance of 9×10^{-13} $cm^3 \cdot s^{-1}$, suggesting a pore-like aqueous pathway [15].

In 2000, Schreiber et al. demonstrated functional coupling between Cl^- transport performed by the CFTR Cl^- channel and water channel performed by AQP3 [16]. Besides, other members of AQP family interact with CFTR to regulate osmotic water permeability in various cellular systems, including in the epididymis [17]. Considering that AQP7 and AQP8 are expressed in tar testis with a remarkable similar distribution of CFTR [18, 19], several studies attempted to describe their molecular interaction. The interaction between AQP4 and CFTR occurs *in vivo* on condition of a fully intact blood-testis barrier [20, 21]. The same mechanism was found between AQP9 and CFTR as well [22]. Pietrement et al. found water secretion maybe driven by a CFTR-dependent mechanism in the distal regions of the epididymis [23]. These studies showed that CFTR controls the seminiferous tubular fluid in close association with AQPs, providing new sights of counteract male subfertility/infertility. CFTR also acts as a regulator of other membrane transporters. However, the regulation of AQP-mediated water transport is poorly understood, we think that CFTR can not transport water directly but through the establishment of cAMP-stimulated aqueous pore or by interaction with AQPs [14].

17.4 Cotransporters

Some cotransporters not only transport their specific substrates but also water (Table 17.1). Because of the large number of cotransporters per cell and the considerable unit water permeability, the water transport medicated by cotransporters may well be significant. The process and mechanism of water cotransport has been studied in cultured mammalian cells, native tissue, and by heterologous expression in *Xenopus laevis* oocytes. A variety of techniques have been used in this area and added the new vitality to it, for example, fluorescence, immunoprecipitation, electrophysiology, ion-selective micro-electrodes and other sensitive optical methods for volume measurements.

17.4.1 K-Cl Cotransporter (KCC)

KCC has been proposed to play a role in the maintenance and regulation of cell volume [24] and the movement of chloride and water in

Table 17.1 Number of water molecules co-transported per transport cycle

Cotransporters	The coupling ratio	Passive water permeability per transporter (10^{-14} $cm^3 \cdot s^{-1}$)	References
KCC4	500	0.01	[29]
NKCC1	59	4	[38]
SGLT1 (human)	230	–	[50]
GAT1 (human)	330	0.7	[45, 46]
EAAT1	425	0.2	[49]
NaDC-1	176	1.5	[68]
MCT1	500	0.3	[41]

erythrocytes, endothelial cells, trout hepatocytes, ascites tumour cells, and mammalian kidney epithelial cells [25]. KCC is exclusively localized in the same membrane as the Na^+/K^+ ATPase. Four isoforms of KCC (KCC1–4) have been found, and shown different expression pattern depending on tissue type and stage of ontogenesis with no cell-specific expression [26]. Although KCC4 is weakly expressed in the mammalian brain but abundant in the apical membrane of choroid plexus and peripheral neurons, the water transport medicated by KCC4 was mostly studied with circumstance [27].

Thomas et al. studied the interaction between the K^+, Cl^-, and H_2O fluxes in the membrane of the choroid plexus epithelium from *Nectyrus maculosus* [28]. They built a hypothesis of the cotransport of K^+, Cl^-, and H_2O. Hydration of the binding of K^+ and Cl^- induces a conformational change in the KCC, which causes the permeability barrier to shift from one side of the membrane to the other [29]. The external osmolality is higher than the intracellular osmolality by 100 mOsm; the intracellular concentrations of K^+ and Cl^- changed only a few millimolar during the exposure to KCC. When the KCC was blocked by furosemide, the cell shrank osmotically in response to the addition of 50 mM of KCl [29]. Water transport by KCC is abolished with the absence of the Cl^-, and the passive water permeability is lower than other cotransporters (of the order of 10^{-16} $cm^3 \cdot s^{-1}$).

However, KCC1 from kidney inner medulla does not perform secondary active transport, with the evidence no water transport occurred in the absence of osmotic pressure [30], contrary to the water flux 1:500 stoichiometry of K^+: H_2O ratio in the choroid plexus [28].

These data of KCC clarified some unexplained findings for water transport and questioned the simple osmotic models. As a water pump, the KCC well explains the ability of the epithelia, such as small intestine and gallbladder absorb water against osmotic gradients of up to 200 mOsm [31]. What surprises us is that at physiological osmolality, the KCC contributes to half of the capacity for water transport across the exit membrane.

17.4.2 Na-K-Cl Cotransporters (NKCC)

The Na-K-Cl cotransporters are a class of membrane proteins that transport Na^+, K^+, and Cl^- ions into and out of a wide variety of epithelium and other cells [32]. So far, two distinct Na-K-Cl cotransporter isoforms have been identified, i.e. NKCC1 and NKCC2. NKCC1 is present in basolateral and the apical membrane of choroid plexus, the small intestine and the kidney proximal tubule [33]. NKCC2 is expressed only in the kidney epithelial cells of the thick ascending limb [34, 35]. Interestingly, *in situ* plasma membrane distribution of the blood-brain barrier endothelial cell NKCC1 is asymmetrical and most residing into the luminal membrane [36].

NKCC1 transports both ions and water, but NKCC2 only transports ions. The water transport by NKCC1 proceeds uphill against osmotic gradients. In the pigmented epithelium, NKCC1 contributes to half of the passive water permea-

bility of basolateral membrane at normal properties [37]. Different from the conventional channel-mediated osmotic transport, the activation energy is higher than that of aqueous pores (21 kcal·mol^{-1}) [38]. Water permeability per protein is rather high about 4×10^{-14} cm^3·s^{-1}. The NKCC1-dependent influx of water is voltage insensitive, temperature dependent and Na^+, K^+-ATPase independent. Around 115 water molecules are transported per turnover cycle in NKCC1 with one Na^+, one K^+, and two Cl^- ions [39].

As a water pump, the Cl^--dependent influx proceeds inwards against the osmotic gradient of 50 mOsm imposed by the mannitol, which indicates that in NKCC1, ion fluxes are tightly coupled to water influxes [38]. The selective NKCC1 inhibitor (bumetanide) reduces the cytotoxic brain edema during middle cerebral artery occlusion. NKCC1, as an important molecule in the water permeability of the blood-brain barrier, contributes to formation of cerebral edema during ischemia [36]. The function that NKCC1 controls Cl^- and water transport also has clinical relevance about disorders of chloride transport and fluid absorption that mainly causes blindness.

Not all isoforms of NKCC1 transport water. The NKCC1 in renal medullary thick ascending limb cells has no capacity to cotransport water due to its low hydraulic conductance [37].

17.4.3 Moncarboxylate Transporter (MCT)

MCTs catalyse the facilitated of lactate with a proton and transport other metabolically important monocarboxylates such as pyruvates, the branched-chain oxo acids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate and acetate [40]. There are nine MCTs and they distribute in different tissues. MCT1 and MCT4 are expressed ubiquitously in most tissues but MCT2 restrictively distribute in testis; MCT3 is exclusive expressed in the retinal pigment epithelium (RPE) [40]. Little consideration for MCT5-MCT9 made it unclear for the function.

It has been proposed that MCT1 on the apical surface of the RPE plays an additional role in regulating the volume of the subretinal space, since lactate-H^+ transport is accompanied by water transport [41, 42]. The water cotransport properties of MCT1 have also been found in the human fetus, and the water permeability is gated by lactate. The interdependence of the fluxes of MCTs cotransport had a fixed ratio of about 109 mmol of lactic acid per litre of water, that is to say MCT1 cotransports 500 water molecules with each lactate molecule, and exhibited saturation for increasing driving forces [43].

It is worth raising that NKCC1 co-localized with the MCT1 contributes to the uphill transport of water against the osmotic gradient resembles in the apical membrane [44]. The ability of MCT-1 to transport rapidly both lactic acid and water across the RPE and into the blood prevent an accumulation of lactate, which would cause osmotic swelling and the retina detaching from the RPE. It suggests the physiological significance of MCT1.

17.4.4 GABA Transporter (GAT)

GABA is removed from the synaptic cleft by means of Na^+-Cl^- coupled re-uptake. Four different GABA transporter subtypes have been described (GAT-1, GAT-2, GAT-3 and the betaine–GABA transporter-1 (BGT-1)). GAT1 behaved as an SKF89976A-sensitive water channel [45]. Using the *Xenopus laevis* oocyte expression system, the water permeability of the GAT-1 in the oocyte was about 3×10^{-6} cm·(s·osmol·l^{-1}) or 1.6×10^{-4} cm·s^{-1}. Data showed the strict proportionality between the GABA transport and the instant influx of water. The coupling ratio was 330 water molecules per cycle. Cotransport of water was composed of two parts: the cotransport component and the osmotic component. It was constant and independent of external osmotic gradients.

The GAT-1 also works as a Li^+ channel in the absence of GABA and Na^+. However, the water permeability was reduced by 40% when Na^+ was replaced by Li^+ in the bathing solution [46].

Linked with the debates with SGLT, that also supports the water cotransport, is dependent on Na^+-medicated cotransporter and not by the accumulation of ions in an unstirred layer. Otherwise, when Li^+ replaced Na^+ in the bathing solution, cotransport of water was also observed.

17.4.5 Na^+-Coupled Glutamate Transporter EAAT

The different five Na^+-coupled glutamate transporter isoforms (EAAT1-5) have distinct expression. The human EAAT1 was primarily found in glial cells [47] and also localized in peripheral tissue [48]. There are two modes of water transport in human EAAT1, which are separated and proceed in parallel. Every unit charged with about 436 water molecules is co-transported along with glutamate and Na^+ by a mechanism within the protein. The transporter also sustains passive water transport in response to osmotic challenges [49]. Cotransport of water processes uphill against the water chemical potential difference. Glutamate increases the osmotic water permeability of the EAAT1 irrespective of the rate of cotransport. Unlike the high external hyperosmolarity of Na^+–glucose cotransporter SGLT1 (15 mosmol·l^{-1}), in ETAA1, it takes a lower external hyperosmolarity (5 mosmol·l^{-1}) to match osmosis with cotransport [50].

17.4.6 Sodium Glucose Transporters (SGLT)

Twelve members are found in the human SGLT family. Except for SGLT1-5 co-transporting for sugars, they include Na^+ cotransporters for myo-inositol, iodide, short-chain fatty acids, and choline. SGLT6 is also known as Na^+/inositol cotransporter 2 (SMIT, sodium myo-inositol [51]; CHT, choline; SMVT, sodium multivitamin [52]; SMCT, sodium monocarboxylic acid; NIS, sodium iodide cotransporters [53]).

As a multifunctional protein, SGLT1 works as a water channel and transporter, which couples water and glucose. The passive osmotic permeability of the hSGLT1 plays an important role in the final achievement of isotonic transport. And the water cotransport (4 L of water with 1 M of glucose) in the human small intestine plays a vital important role in reuptake (total 9 L per day) [54].

SCLT1 has three modes in isotonic water transport. First, water influx is directly correlated with Na^+ and glucose in the ratio of 260 H_2O/2Na^+/1glucose with no delay in human [55]. Second, it acts as a water channel [50]. Last, it generates an osmotic driving force that is employed by other pathways. Water permeability is increased more than 10-fold in the circumstances of co-expression of AQP1 with SGLT1 [56]. The initial rate of water transport varies with the membrane potential, temperature. Arrhenius plots of Na^+/glucose cotransport is as high as water flow (26 kcal·mol^{-1}) [55]. The cotransport of water is independent of the osmotic gradient and even occurs in the presence of adverse osmotic gradients.

However, Charron et al. agreed with that the water transport mediated by SGLT1 was osmotic and proposed to arise as an unstirred layer effect [57]. The cotransport hypothesis and the osmotic hypothesis explain some numerical analysis at the same time, but the cotransport hypothesis gave a better fit to the volume changes [58].

Study of the Na^+-coupled iodide transporter (NIS), in which thiocyanate (SCN^-), substituting for iodide (I^-), was conducted in the cotransporters expressed in *Xenopus* oocytes. Less water was co-transported along with the larger substrates [59]. For example, the coupling of rabbit SGLT1, human SGLT1, NIS, and a plant H^+/amino acid cotransporter (AAP5) ranged from 50 to 425 water molecules per turnover [60].

17.4.7 Sodium Borate Cotransporter

As a member of the Slc4 family, sodium borate cotransporter is an extremely important protein for both yeast and plant. Because borate plays an significant role to cross-link vicinal diols to stablize the structure of cell walls in bacteria, plants and fungi [61]. However, it is still confused about

what role biochenmocal serves for borate in mammals. Sodium borate cotransporter is abundantly expressed in the renal descending loop of Henle [62] and localizes basolaterally in the corneal enthothelium. Also, it is broadly expressed in salivary glands, thyroid and testis [63].

Sodium borate cotransporter mediates water flux driven exclusively by an osmotic gradient when expressed in *Xenopus laevis* oocytes and HEK293 cells. Water-flux through Slc4a11 is 10^3-fold faster than water movement reported for SGLT1 [64].

Sodium borate cotransporter localizes on the opposite surface from apical AQP1, functioned the basolateral pathway for the water transport from the corneal stroma into the endothelium, and AQP1 mediates water transport out of the corneal stroma into the aqueous humour. The studies suggest that AQP1 and sodium borate cotransporter are coefficient in mediating trans-endothelial water reabsorption [64].

17.4.8 Na^+-Dicarboxylate Cotransporter (NaDC-1)

NaDC1 belongs to the Slc13 family of anion transporters [65]. Na^+-dependent anion transporters contain the Na^+-dependent dicarboxylate transporter and the renal Na^+-sulfate cotransporter [66]. Na^+-dicarboxylate cotransporter was found in the apical membrane of the kidney proximal tubules and contributed to the reabsorption of tricarboxylic acid cycle intermediates [67].

NaDC-1 mediates both passive and solute-coupled water transport, and contributes to fluid reabsorption across the proximal tubule. Many studies suggest that SGLT1 and NaDC-1 share a common mechanism for passive water transport. Water transport medicated by NaDC-1 occurs in the absence or even against an osmotic gradient. The ratio between Na^+, citrate (or succinate), and water is 3:1:176 per transport cycle [68].

NaDC1 plays an important role in regulating succinate and citrate concentrations in the urine. Single nucleotide polymorphisms in the human Na^+-dicarboxylate cotransporter affect transport activity and protein expression, which contributes to human diseases such as kidney stones [69].

17.4.9 Glucose Transporter (GLUT)

Twelve different isoforms of GLUTs have been found in mammalian cells. GLUT1 was the first cloned and most extensively studied. It is abundantly expressed in erythrocytes and endothelial cells of blood-brain barrier [70]. GLUT1, GLUT2 and GLUT4 have been shown to support osmotic water fluxes in addition to its own role as glucose transporters.

In 1989, by investigating the effects of inhibitors of glucose transport on membrane osmotic water permeability, Fischbarg et al. concluded that glucose transporter serves as a water channel in some cells and is sensitive to the specific inhibitor phloretin [71]. *Xenopus laevis* oocytes injected with mRNA encoding the glucose transporters exhibited an average of 4.8-fold of the osmotic water permeability [72]. However, GLUTs serve as water channel in brain, skeletal and liver but not in kidney or intestine epithelia [73].

Water transport in the GLUT1 and GLUT2 has been demonstrated to be bimodal. They act as a water channel and water is co-transported together with the glucose. The water permeability of GLUT2 in oocytes is 0.11×10^{-5} cm·(s·osmol·l)$^{-1}$, equivalent to 6.1×10^{-5} cm·s^{-1}. The GLUT2 co-transports less water in the inward than in the outward. Compare with the Na^+-coupled glucose transporter, the coupling ratio of GLUT2 is six times smaller [74].

Molecular mechanism of water transport for GLUT1 is unclear [75], but GLUT2 is based upon the alternating access model. Conformational changes occur when glucose is applied to the GLUTs. Glucose together with a number of water molecules is occluded, and an aqueous cavity opens to trans side with the glucose exited [59]. The other three-compartment model for transepithelial water transport suggests the coupling space is associated with a static aqueous cavity with substrate binding site [76].

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Methods to Measure Water Permeability

18

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Abstract

Water permeability is a key feature of the cell plasma membranes and it has seminal importance for a number of cell functions such as cell volume regulation, cell proliferation, cell migration, and angiogenesis to name a few. The transport of water occurs mainly through plasma membrane water channels, the aquaporins, who have very important function in physiological and pathophysiological states. Due to the above the experimental assessment of the water permeability of cells and tissues is necessary. The development of new methodologies of measuring water permeability is a vibrant scientific field that constantly develops during the past three decades along with the advances in imaging mainly. In this chapter we describe and critically assess several methods that have been developed for the measurement of water permeability both in living cells as well as in tissues with a focus in the first category.

Keywords

Aquaporin • Cell volume • Osmotic water permeability • Plasma membrane

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18.1 Introduction

Water transport in cell and tissue systems is a necessary function for their homeostasis [1]. Water is the most abundant component of the human body therefore mechanisms that regulate its transport are essential for life. Water permeability is a key feature of the cell membranes that is of critical importance for a number of cell functions that among others include cell fluid secretion and absorption, cell volume regulation, cell proliferation, cell migration, tumor spread,

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angiogenesis, and other processes [2, 3]. There are several different pathways involved in the transport of water across the cell plasma membrane that involve water transport by simple diffusion, transport of water molecules through water channels by facilitated diffusion and water transport with hydrated solutes. Facilitated diffusion of water through plasma membrane channels is mediated through aquaporins (AQPs), a class of membrane water channels whose primary function is to facilitate the passive transport of water across the cell plasma membrane [4]. AQPs have attracted huge research interest during the past 25 years and currently there is a sufficient amount of evidence that they have important roles in nervous, respiratory, renal, cardiovascular, gastrointestinal, reproductive and sensory system physiology [5–15]. Furthermore, various studies have shown that AQPs are involved in a number of pathologies such as cancer, brain edema, brain injury, epilepsy, obesity, glaucoma and others [4]. Finally, it is important to note that in humans there is a class of diseases recently termed aquarinopathies and refer to rare cases of loss-of-function mutations in human AQPs. More specifically, loss-of-function of AQP0 causes congenital cataract and of AQP2 causes nephrogenic diabetes insipidous (NDI) [16, 17]. Serum autoantibodies against AQP4 is a hallmark of neuromyelitis optica (NMO), a rare autoimmune disease [17]. Due to the involvement of the channels that mainly mediate the transport of water through cell plasma membranes in so many physiological and pathophysiological states researchers need to have the appropriate tools in order to assess and study water permeability. The fact that a lot of focus is placed in identifying modulators of AQPs for the treatment of several diseases demonstrates the importance of having precise and reliable experimental techniques for water permeability measurements [18].

Cell volume is the most sensitive parameter to the changes of the total water current across the cell plasma membrane [19]. In general water permeability is measured from the kinetics of the cell volume changes in response to an osmotic gradient. A variety of experimental approaches have been developed to subject cells to an osmotic gradient and subsequently record the resultant kinetics of cell volume from which water permeability is deduced. The problem of water permeability determination is a problem of volume-dependent physical parameters measurement.

The methods used to measure osmotic water permeability are technically challenging, subject to various mixing/flow-related artifacts, and not easily applied to highly water-permeable cells. A very critical characteristic of an experimental setup is the time needed for the establishment of the desired osmotic gradient. All the modern experimental approaches for measuring cell water permeability underestimate this parameter and the degree of the underestimation is dependent on the lag time of the establishment of the osmotic gradient during the experiment. Many types of cells are highly permeable to water because of the abundant expression of AQPs in their plasma membrane [5, 20]. Studies of the water permeability conducted in highly water permeable cells require methods that have very short osmotic gradient formation time. Failure in the incorporation of such a parameter in water permeability measurement studies compromises the validity of the results and subsequently their translation to clinically useful therapies. Indeed, although AQPs are considered to be important drug targets for a variety of diseases and great effort is put in the identification of appropriate AQP modulators there is poor progress targeted therapeutics partially due to challenges and artifacts in measurement of cell membrane water permeability [18].

The purpose of this review is to survey the modern experimental approaches for the measurement of water permeability of living cells and multicellular tissues. We will discuss the principles of each methodology, technical details and limitations. Strategies for the measurement of the plasma membrane osmotic water permeability (P_f) in living cells that are described involve image analysis, light scattering, total internal reflection fluorescence microscopy, confocal

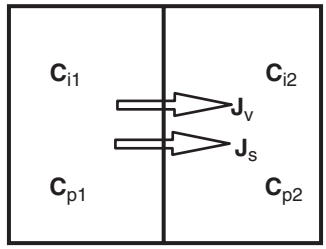

Fig. 18.1 Volume flow across a single barrier separating compartments 1 and 2. *ci*1 and *ci*2 are the osmolalities of impermeant solutes on sides 1 and 2, and *cp*1 and *cp*2 the osmolalities of permeant solutes, V_w the partial molar volume of water, S the membrane surface area, σ_p the solute reflection coefficient, P the hydrostatic pressure. Barrier permeability properties include the osmotic water permeability coefficient P_f, the solute permeability coefficient P_s and the solute reflection coefficient σ_p. Water flux (J_v) and solute flux (J_s) are defined as positive in the *left-to-right* direction as indicated

microscopy, interferometry, spatial filtering microscopy, scanning probe microscopy, bioelectrical impedance methods and volume-sensitive fluorescent indicators. The fundamental physical principles of the cell membrane osmotic water permeability were reviewed in detail earlier [21]. In short, the osmotic volume flow (J_v, cm^3/s) across selectively permeant membrane is described by the following equation and Fig. 18.1:

$$J_v = P_f S V_w[(c_{i2} - c_{i1}) + \sigma_p(c_{p2} - c_{p1}) + (P_1 - P_2)/RT] \quad (18.1)$$

The interpretation of the measured P_f assumes accurate definition of the cell membrane surface area S, the absence of significant unstirred layer effects and types of transporters, which carry water with or without hydrated solutes. As defined by the Eq. (18.1), P_f is measured from the volume flux produced by a defined osmotic gradient or hydrostatic driving force. In animal cells that have just a soft plasma membrane the hydrostatic driving force is considered insignificant.

Molecular transporters that contribute to the total water flow could be temperature dependent according to their Arrhenius activation energy. The Arrhenius activation energy (E_a, kcal/mol) is defined by the relation $\boldsymbol{ln}\ P_f = -E_a/RT + A$, where R is the gas constant, T is absolute temperature, and A is an entropic term. E_a provides a measure of the energy barrier to the water movement by the molecular transporter. For water movement through aqueous channels, E_a is generally found to be low (3~6 kcal/mol). The low E_a associated with water pores is assumed to be related to the weak temperature dependence of the water self-diffusion [21]. In the case that water is transported along with hydrated solvents and contributes significantly to the cell volume changes, the E_a of the specific solute transporters could produce temperature dependence of the P_f of the living cell plasma membrane. In such a case, the P_f values should be obtained at the temperature that is normal for the cells under study in order to be adequate for the purpose of a physiological study.

18.2 Water Permeability Methods Based on Cell Volume Measurements

18.2.1 Water Permeability Measurement by 2D Image Analysis

In cells observed under wide field microscopy the changes in their cell volume are often accompanied by small changes in their size that can be measured in the images captured during the experiment. To estimate the cell volume, the area of a cell is measured by tracing its outline on the video images. This method of cell volume measurement is based on the analysis of two-dimensional (2D) images and on the assumption that the cells change its sizes to the same extent in all three dimensions. Polarizing, phase contrast, and various forms of interference microscopy are the most frequently used microscopic methods for observing living cells and simultaneously recording images continuously using a video camera [22–26]. The relative volume of the cell was estimated as $V/V_0 = (A/A_0)^{3/2}$, where V is the volume, A is the area, and the subscript 0 indicates the control value. This method using differential interference contrast microscopy and

digital imaging was created for measurement of the cell volume and to examine the effects of muscarinic stimulation on single rat salivary gland acinar cells [27]. The advantage of this approach is the use of unlabeled cells and the possibility to measure the reaction of cells in small tissue structures [28]. The disadvantages of the several variations of this method are that they are mainly limited by: (a) the resolution power of the lens system used, (b) the contrast that the specimen generates in the image, and (c) the time required for an image to be recorded. As mentioned above in order to estimate the cell volume, the area of a cell is measured by tracing its outline on the video images, therefore this method is not adequate for cells that have high water permeability and thus fast kinetics of cell volume changes [25].

Based on the principles of this methodology in the recent years image-based cytometry systems have been developed that use bright-field and fluorescent imaging. These image-based systems have been demonstrated to perform numerous assays, such as quantitative cell size and morphology analysis and potentially can be used for measurements of cell water permeability but no such experiments are currently published [29, 30].

18.2.2 Water Permeability Measurement by Scanning Probe Microscopy (SPM)

The most accurate measurements of cell volume is achieved by the direct scanning of living cells and the use of Scanning Probe Microscopy (SPM) and in this context both Atomic Force Microscopy (AFM) or Scanning ElectroChemical Microscopy (SECM) have been used to image live cells [31]. In these studies 3-dimensional reconstruction of the cell shape is achieved by scanning confocal microscopy and different modifications of SPM such as real-time AFM [32, 33]. One of the drawbacks of AFM is the fact that an external mechanical force is applied to the cell because the detection of the cell surface is performed with the deflection of a cantilever. Such an external force is not acceptable in physiologically relevant experiments with mechanosensitive live eukaryotic cells. Scanning ion conductance microscopy (SICM) is form of SPM that overcomes this problem by allowing the imaging of the cell surface under physiological conditions without any physical contact and with a resolution of 3~6 nm [34, 35]. Even SICM though like the other SPM methods are more fit for relatively flat surfaces given that in cases of convoluted surfaces there is a collision of the probe that interferes with cell integrity again. This problem is not an issue in hopping probe ion conductance microscopy (HPICM) where the probe never touches the surface of the cell. In this case the probe that senses current fluctuations and translates it in sample height even at a reduction of 1% is at a Z-position that still does not interfere with the cell membrane at the imaging point. Using HPICM non-contact imaging of the 3-dimensional surfaces of live cells can be achieved with resolution better than 20 nm [36]. These methods are quantitative and provide the best spatial resolution of living cells but scanning is a relatively slow process and like all other SPM techniques their use is restricted to imaging relatively flat surfaces with the exception of HPICM. These limitations make SPM probably not practical for studying the kinetics of cell volume fluctuations in living cells.

18.2.3 Water Permeability Measurement by Light Scattering and Spatial Filtering Microscopy

Cell volume changes lead to changes of the elastically scattered light (Rayleigh scattering) and this effect is the basis of the method of measurement of the cell water permeability by light scattering. The method is simple to apply and requires very small sample quantity however, there is no practical theory that reflects the relationship of scattered light intensity and cell volume because of complexities in the cell optical configuration and interference phenomena. Therefore, assessment of light scattering provides

a semi quantitative index of the cell volume changes in relatively large and adherent cells such as the macrophages [37]. Quantitative data interpretation for this method is efficient for a limited kind of objects that have a relatively simple shape such as vesicles or erythrocytes [38, 39]. Nevertheless the value of P_f of the cells can be calculated from the time course of the light scattering, the cell surface-to-volume ratio, and an empirical calibration of the cell volume and the intensity of the light scattering [40]. An advantage of this method is that it has satisfactory temporal resolution and the main drawbacks are that a calibration step is required in order to evaluate the cell volume and the values that are obtained are relative values.

The angular dependency of the intensity of light scattering by a moving individual particle was the basis for the Flying Light Scattering Indicatrix method (FLSI) using a scanning flow cytometer (SFC) [29]. The FLSI method was used for the measurement of individual particle characteristics from light-scattering data, in order to determine the particle size of polystyrene, latex, milk fat, and spores of *Penicillium levitum, Aspergillus pseudoglaucus*. Measuring cell volume in a flow cytometer could potentially be a prospective approach for high-throughput screening studies of the distribution of P_f values in populations of cells in suspension.

Methods based on the measurement of scattered light intensity are suitable for investigations of homogeneous suspensions of uniform objects like cells or vesicles. They have been used for suspensions of erythrocytes [41], in flow cytometers [29, 42, 43], in suspensions of membrane vesicles and liposomes reconstituted with water channels [40, 44, 45]. Due to the sufficient temporal resolution light scattering was used to determine the P_f of lung alveolar epithelial cells that have very high cell membrane water permeability [46]. The method was also applied to calculate the plasma membrane P_f of cells in micro-dissected fragments of mouse kidney collecting duct. More specifically, the time course of the light scattering intensity measured in dark field microscope on individual fragments positioned in a thermo-stabilized flow chamber was used to study regulation of P_f by vasopressin [47, 48].

A theory relating the signal intensity to the relative cell volume was developed based on the spatial filtering and the changes in the optical path length associated with cell volume changes. It was found that the integrated intensity of monochromatic light in a phase contrast or dark field microscope was dependent on the relative cell volume. The method was applied to characterize transfected cells and tissues that natively express water channels and the results established light microscopy with spatial filtering as a technically simple and quantitative method to measure water permeability in cell layers (Fig. 18.2) [49]. The method is simple, accurate and robust if used in cell layers or uniform cells, however it may work in systems with heterogeneous cells.

Other approaches to follow osmotically induced changes in the cell volume include tracking of immobilized fluorescent beads at the cell surface and laser reflection microscopy [50, 51]. In another method the measurement of cell volume and osmotic water permeability in Madin Darby Canine Kidney (MDCK) cell layers has been performed by interferometry based on the cell volume dependence of the optical path length (OPL) of a light beam passing through cells. The time course of relative cell volume in response to an osmotic gradient was computed from serial interference images. To relate the interference signal to cell volume a mathematical model was developed for this purpose [52]. However, these methods are less practical due to the fact that are experimentally challenging, require complex instrumentation while at the same time may not work in many systems.

18.2.4 Water Permeability Measurement by Bioelectrical Impedance Analysis

Impedance data obtained from microfluidic impedance flow cytometry enable the characterization of cellular sizes, membrane capacitance and cytoplasm resistance in a high-throughput manner [53]. Bioelectrical impedance analysis is

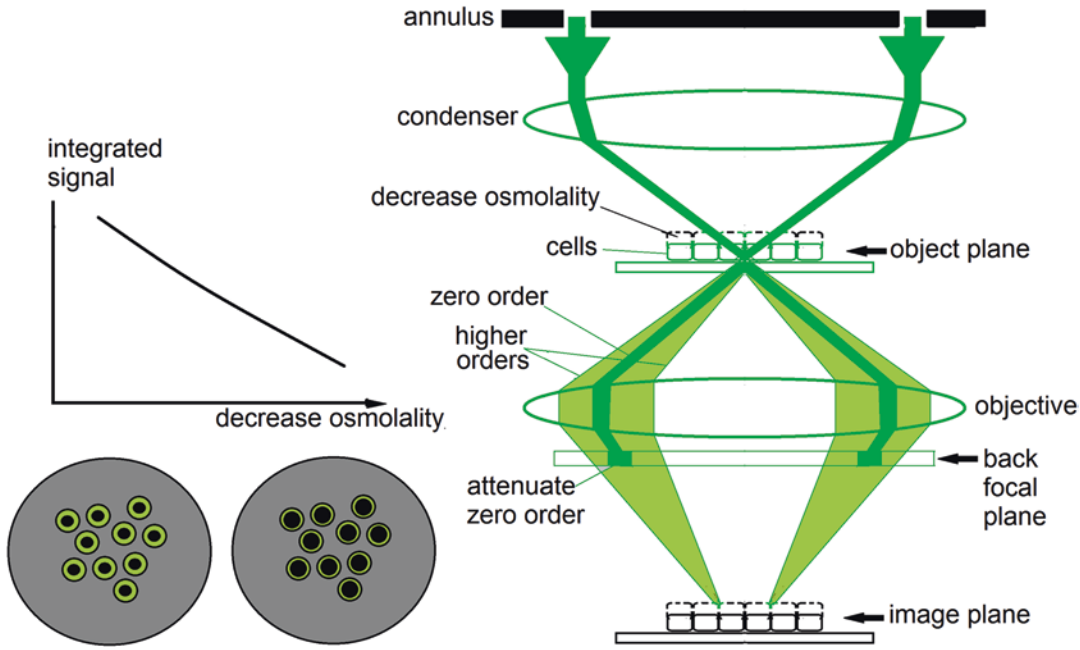

Fig. 18.2 Schematic of the principles of plasma membrane water permeability measurement by means of spatial filtering microscopy. A cell layer under phase contrast or dark field microscopy is shown to change its cell volume under corresponding changes in the medium osmolality that lead to changes in the relative intensity of the higher and zero order beams due to optical path length changes. Attenuation of the zero order beam at the back focal plane of the objective results in light intensity dependency from the cell volume fluctuations (Adapted from Ref. [49])

used in flow cytometry, for example a Coulter counter measures the changing of DC resistance between two electrically isolated fluid-filled chambers when microparticles act as an insulating layer as DC passes through a small connecting orifice [54]. Microfluidics is the technology of the processing and manipulation of small amounts of fluids (10^{-9} to 10^{-18} liters) in channels with dimensions of tens of micrometers. These advantageous features of microfluidic technologies have been used for characterizing the biochemical and/or biophysical properties (mechanical and electrical) of cells at the single-cell level [55]. This technology is prospective for high-throughput single-cell characterization. Electrical impedance based noninvasive cell and tissue-characterizing techniques have become more and more popular in several fields of application [53, 56, 57]. Microfluidic technology includes manipulation with laser tweezers to drag a single cell to a certain position for the measurement of cell characteristics in experiments of electrorotation (ROT) [58]. ROT operates at a single-organism level and does not require extensive cell preparations while the technique is a noninvasive and allows for sequential investigations. A significant disadvantage of the ROT technique for analyzing single cells is that it takes approximately 30 min per test and also requires a skilled operator to position a single cell in the middle of a rotating electric field. Therefore, the temporal resolution that it can offer at the moment is far from being suitable for measurements of cell volume changes kinetics. On the other hand methods with high temporal resolution based on light scattering, bioelectrical impedance changes and cell labelling with fluorescent dyes are the most adequate to study the cells with high water permeability.

Several optical approaches are applied to measure the fluorescence intensity of an aqueous-phase fluorophore in the cytoplasm. Cell-loadable

fluorescent dyes are available with various properties and wavelengths. Semi-quantitative information about the cytoplasmic fluorophore concentration can be obtained using partial confocal optics in which the z-point spread function of a wide-field optical system is increased by the use of a high numerical aperture objective and a limiting aperture in the back focal plane of the emission path [21]. The confocal methods have limited utility in making quantitative P_f measurements in polarized cell sheets. The z-point spread function of a wide-field optical system could not be increased inside most of the flat cells where thickness of the edges could be about 0.5 micrometer. This means that the volume where the fluorescent signal is registered from is both undefined and unstable. The ratio signal/noise in such a system could be low and unstable. This limitation has been overcome by the use of laser confocal systems with z-size of registering the volume inside the cell less than 0.5 micrometer [59]. This method is extremely sensitive to proper focusing and requires high quality optics. This limitation is the reason why it has been scarcely used for the measurement of cell water permeability.

A more robust method is based on the effect of Total Internal Reflection (TIR) that could be applied using a conventional microscope. However, a more sophisticated approach to measure the concentration of an aqueous-phase fluorophore in the cytoplasm is the Total Internal Reflection Fluorescence Microscopy (TIRFM). TIRFM involves the excitation of fluorophores in a cell membrane area in great proximity with the adjacent cytosol near a high-to-low refractive index interface [60]. Fluorescence excitation is usually accomplished using a laser source and a glass prism to illuminate the sample at a subcritical angle at a glass-aqueous interface (Fig. 18.3). It is not difficult and costly to equip a conventional epifluorescence microscope with a laser source and a prism in order to perform TIRFM measurements. The procedure involves loading cells with an aqueous-phase dye and the cell swelling in response to an osmotic gradient results in the cytosolic fluorophore concentration dilution and subsequently to decreased fluorescence signal [61].

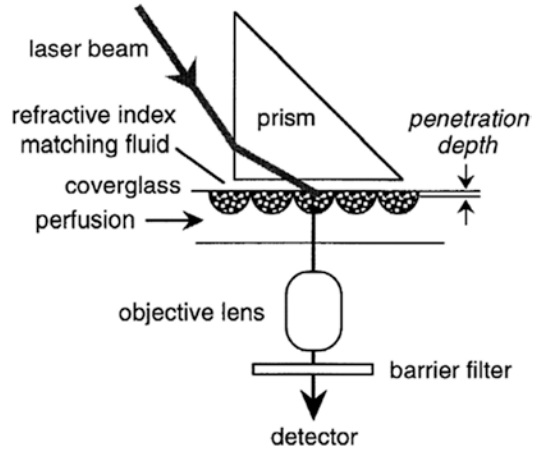

Fig. 18.3 Measurement of osmotic water permeability in adherent cells by total internal reflection fluorescence microscopy. Cells are loaded with a membrane impermeant volume marker. A thin (50~200 nm) layer of cytosol (labeled "penetration depth") is illuminated by a laser beam directed through a glass prism at a subcritical illumination angle. As the cell shrinks in response to an osmotic gradient, fluorophore concentration in the illuminated region increases, producing an increase in detected signal (Reprinted from Ref. [21])

Optical near fields have been successfully used to confine observation volumes of surface-confined and solution fluorescence correlation spectroscopy (FCS). The standard confocal FCS has the immanent problem that the ellipsoidal observation volume has a low axial confinement. TIR-FCS uses objective-type TIRF illumination to restrict the excitation to a thin section less than 200 nm above the interface in combination with standard confocal detection to improve the lateral confinement of the detection volume. The quantitative study of cellular dynamics even in the level of cellular compartments may be useful for the study of processes close to a surface/solution interface. Potentially, it can give access to a local fluorophore concentrations, or kinetic rate constants for reversible association of fluorophores with specific substrates in interface [62].

The methods for measurement of rapid osmosis in cultured cell monolayers using confocal and spatial filtering microscopy may not produce acceptable signal changes in cells that have a low water permeability profile and complex shape. Hamann and coworkers evaluated a calcein

self-quenching method for water transport measurements [63]. In this method, high concentrations of calcein are loaded into the cells in order to produce volume-dependent changes in the total cell fluorescence in response to changes in cytoplasmic calcein concentration due to cell swelling or shrinkage. A more practical modification of the calcein fluorescence quenching method was created to measure osmotic water permeability in highly differentiated cultures of primary brain astrocytes from wild-type and aquaporin-4 (AQP-4)-deficient mice [64]. Cell swelling resulted in a reversible increase in the calcein fluorescence, which was independent of cytosolic calcein concentration at levels well below where calcein self-quenching occurs. The method is based on the quenching of calcein fluorescence by cytosolic proteins. The fluorescent signal in cells is sensitive to osmotic challenge because of changes in the cytosolic protein concentration that alter calcein quenching, explaining the increased fluorescence with cell expansion after exposure hypotonic medium. A significant advantage of this method is that it is simple experimentally without expensive instrumentation requirements. A conventional fluorescent microscope equipped with a sensitive light detector, such as a photo multiplier tube is sufficient. However, the method provides relative results since it is not quantitative and therefore needs calibration as far as the adaptation of calcein loading protocol and the fluorescent signal is concerned for every kind of cells of tissue specimens [65].

Modern microfluidic technologies promote the improvement of the methods for measuring P_f in cells and membrane vesicles. As mentioned previously the water permeability is generally measured from the kinetics of the cell volume changes in response to osmotic gradients. To diminish potential mixing artifacts the temporal aspect of osmotic gradient establishment should be minimized. This is an area where microfluidic technologies can provide new potential for the rapid creation of a gradient. Indeed recently it was demonstrated that a very fast development of an osmotic gradient was reached in a microfluidics platform in which cells labeled with a cytoplasmic, volume-sensing fluorescent dye, were rapidly subjected inside a ~ 0.1 nL droplet surrounded by oil with a solution mixing time of < 10 ms. The osmotic water permeability was deduced from a single, time-integrated fluorescence image of an observation area in which time after mixing is determined by spatial position. Water permeability was accurately measured in aquaporin-expressing erythrocytes with half-times for osmotic equilibration down to <50 ms [66]. A similar approach was used for measuring the quantitative volume changes of immobilized intestinal enteroids in a microfluidics platform. The enteroids were trapped in a "pinball machine-like" array of polydimethylsiloxane posts for measurement of the volume changes in unlabeled enteroids by imaging of an extracellular, high-molecular weight long-wavelength fluorescent dye. The enteroids volume was deduced quantitatively from area-integrated fluorescence of an excluded extracellular dye. Changes in the enteroid volume altered the total amount of dye in the enteroid-containing area [67].

Finally, the method of Light Sheet Fluorescence Microscopy (LSFM) uses a plane of light to optically section and view tissues with subcellular resolution (Fig. 18.4) as compared to confocal and two-photon microscopy. This method is well suited for imaging deep (1 cm) within transparent tissues or within whole organisms. The development of the technology and existing LSFM devices are described in the review of P.A. Santi (2011) [68]. Uncoupling of the illumination and detection axes of the microscope, so that only the part of the specimen that is imaged gets illuminated, provides the ability to image biological systems for extended periods of time with minimal phototoxicity and photobleaching of the specimens and the fluorophores respectively. LSFM produces optical sections that are suitable for three-dimensional image reconstruction. Stability is a critical issue in light-sheet microscopy but due to the low phototoxicity, one can image a sample for hours or days. Photobleaching is not a significant problem for repeated imaging as compared to wide-field fluorescent microscopy. Only a 13% reduction in the fluorescence over 475 s occurs [69].

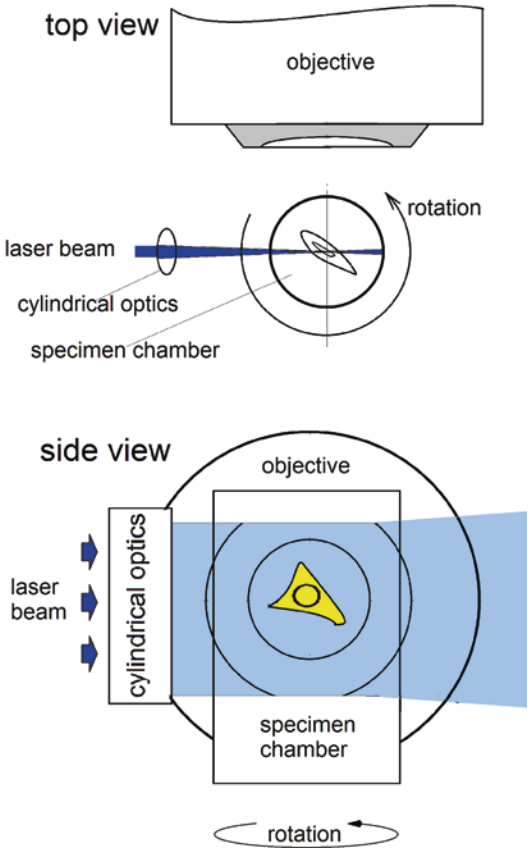

Fig. 18.4 Schematic of the principles of a light sheet fluorescence microscope (LFSM) arrangement demonstrating the top and side views. A light sheet is formed by a laser, collimated and expanded by a beam expander and projected through an illuminative objective through a cylindrical lens. The focal point of the light sheet is centrally positioned on the specimen chamber made out of clear glass walls with an opening on the top-side for specimen insertion purposes. The containing fluid in the specimens chamber depends on the experimental modality (live cell imaging or fixed tissue) therefore contains either physiological solution or clearing fluid respectively. The specimen chamber is attached to rotating and translating stages while the specimen is intersected by the light sheet and a fluorescent plane collected by an horizontally positioned microscope (Adapted from Ref. [68])

This advantage on the other hand necessitates thinking about the optimal 'physiological' conditions that need to be established during the imaging. The detection of fluorescent signals with a wide-field detection device such as a charge-coupled device (CCD) camera allows high-speed imaging [70]. The LSFM technology may be used in a variety of applications spanning all the scales of biological systems from molecules to organisms while the high speed of image recording is an advantageous feature for P_f measuring. Current developments in LSFM by diagonal scanning have improved even further the temporal resolution and the potential of the method [71]. As far as the disadvantages of LSFM are concerned, the light-sheet microscopes are complex and expensive hardware and typically produce enormous amounts of data (in the range of terabytes) streamed from digital cameras to computer hard discs when the experiment lasts for hours or even days. These properties render it a powerful tool from the one hand but not one that can be widely used from the other. Despite their potential utility in making quantitative P_f measurements in heterogeneous cell populations and tissue specimens, LSFM technologies have been used little to measure cell water permeability nowadays.

18.3 Water Permeability Methods for *In Vivo* and *Ex Vivo* Studies

Experimental data on fluid-absorbing epithelia are obtained in *in vivo* and *ex vivo* experiments. An example of water permeability measurements of more complex systems than living cells involve *in vivo* studies of bodily cavities that contain certain physiological amount such as the pleural or peritoneal cavity. The balance of fluid turnover of the pleural compartment occurs in normal chest physiology and is changed in pathophysiological conditions associated with pleural effusions, thus excess accumulation of water, solutes, proteins and cells within the cavity. Comparative physiological studies were performed on wild-type vs. AQP1 null mice. Osmotically driven water transport was measured in anesthetized, mechanically ventilated mice from the kinetics of pleural fluid osmolality after instillation of hypertonic or hypotonic fluid into the pleural space. Water permeability is determined assuming that the pleural barrier is a single barrier separating the pleural

cavity and the blood compartment. The volume flux across of the pleural mesothelium (J_v) is given by $J_v = P_f V_w S[C_o - C_i(t)]$, where V_w is the partial molar volume of water (18 cm^3/mol), S is pleural surface area (cm^2), C_o is plasma osmolality (320 mosmol/kgH_2O), $C_i(t)$ is pleural fluid osmolality, and P_f is the osmotic water permeability. The same experimental model used for measurement of isosmolar pleural fluid clearance and modeling of hydrostatically driven pleural effusions. Hydrostatically driven pleural fluid accumulation was induced by bilateral renal artery ligation and infusion of saline in the peritoneal cavity (40% body wt) to induce acute volume overload. The pleural fluid was collected in all cases and analyzed accordingly reaching the conclusion that AQP1 mediated water permeability is critical only in cases of aniso-osmolality [72]. This type of experimental approach involving or not AQP knockout animals and cavitary effusion formation is useful in a range of medical disciplines for the elucidation of water permeability kinetics of water transporting membranes in the whole body level. The rate of cavitary fluid absorption can be used for identifying therapeutic drugs for effusion resolution [72–76].

Regarding the *ex vivo* assessment of water permeability in isolated tissues the application of the Ussing System has provided a lot of insight to such processes. Initially several isolated tissue from frog have been studied for their water transporting capacities, such as frog skin as well as frog and toad urinary bladder [77–81]. The activation energy (E_a) for the diffusion of water across the epithelial cell layer of the toad bladder was determined in the absence and presence of vasopressin. It was shown that the hormone did not influence the activation energy, thus the conclusion that vasopressin was not changing the molecular structure of water channels was made [78]. More recently the transport properties of pleural, peritoneal, pericardial and leptomeningeal membranes in human and sheep models in *ex vivo* studies are investigated using the Ussing system [82–87]. Ussing Chamber experiments using human and animal transporting epithelia have established many of the biophysical processes involved in the regulation of liquid homeostasis. For understanding the physiology that regulates biophysical processes involved in epithelial liquid homeostasis a biophysical model for water and ion transport to quantify the permeabilities of all pathways apical, basolateral and paracellular was created using Ussing Chamber data reported in the literature [88].

Another imaging method that was developed to map thickness changes in viable spinal cord and brain slices was applied to measure osmotically induced water transport in spinal cord slices from wildtype and aquaporin knockout mice. Changes in the slice thickness were mapped from the amount of light passing through a thin (~100 μm) layer of perfusate bathing the slice, in which hemoglobin (6 mg/ml) was added as an inert absorbing chromophore. In response to osmotic challenges imposed by changing perfusate osmolality by steps of 100 mOsm, the transmitted light intensity changed reversibly with approximately mono-exponential kinetics whose initial rate depended upon the position in the slice [89].

Understanding the mechanisms of fluid absorption and secretion by the kidney epithelia has a long story of intense studies on water transport in isolated perfused tubules [90]. In this type of experiments the tubule segments were perfused *in vitro* in a modified version of the technique described by Burg et al. [91]. The tubules were placed into a temperature controlled flow chamber with a glass bottom and were observed with an inverted compound microscope. The upstream portion of the tubule was drawn into a holding pipette, which contained a concentric pipette. The downstream end of the tubule was drawn into a holding pipette that had inside a tip of collecting pipette. The theory and experimental design of this class of studies was described by Schafer et al. [92]. The osmotic P_f was calculated from the net transepithelial fluid flux (J_v) determined in the presence of an imposed osmolality gradient. Measurements were conducted by collecting the perfusate, which contained an impermeant volume marker. The marker could be a radioisotope [93] or a fluorescent probe using continuous fluorescence measurement and photo bleaching as shown in Fig. 18.5 [94]. The fluorescein sulfonate concentration was measured in the

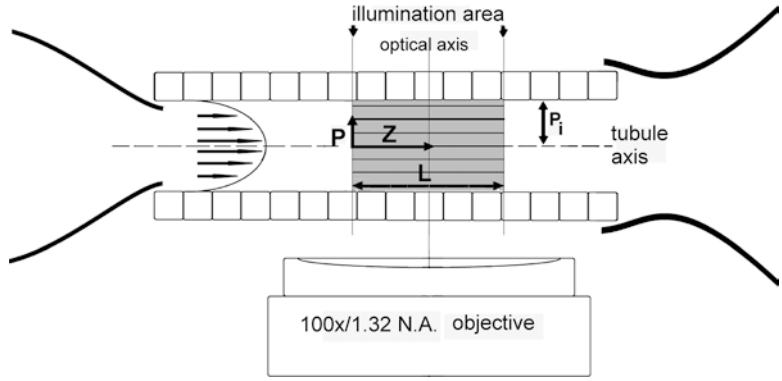

Fig. 18.5 Schematic of a cross-section of an isolated perfused tubule demonstrating the illumination and fluorescence collection geometries. The grey are represents the volume of the illuminating fluorophore while P and Z are the radial and axial coordinates, L is the length of the illuminated area (defined by the illuminating window) while Pi is the inner radius of the tubule. The horizontal lines in the illuminating volume represent the stack of optical sections used to assess the spatial distribution of the light intensity and photobleaching the tubule (Adapted from Ref. [94])

perfusate and the collected fluid using a continuous flow fluorometer (coefficient of variation, 2%) [95]. The theoretical basis for P_f calculation in these experimental approaches is the equation formulated by Al-Zahid et al. [96].

18.4 Conclusion

In conclusion a wide variety of methods for measuring osmotic water permeability have been developed each one of which takes advantage of distinct biophysical principles of the process or the instrumentation. The selection of the most suitable method for water permeability measurement depends on the purpose of physiological study that will be designed. When measurement accuracy and high resolution of water transporting pathways across cell membranes or in contralateral membranes of polarized cells then the experimental approach needs to be carefully planned based on the most suitable method and instrumentation. However, there are fundamental limitations imposed by the unstirred layer effects and the complex composition of tissues where the absolute water permeability coefficients and the activation energies cannot be even defined. Still, comparative measurements may be informative for understanding the physiological and pathophysiological mechanisms involved. An important application of the methods for water permeability measurement is in high-throughput screening assays for the discovery of modulators of water and electrolyte balance in range from a cell to a whole organism.

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