Chapter 1

Aquaporins:
A Family of Highly Regulated Multifunctional Channels

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Abstract

Aquaporins (AQPs) were discovered as channels facilitating water movement across cellular membranes. Whereas much of the research has focused on characterizing AQPs with respect to cell water homeostasis, recent discoveries in terms of the transport selectivity of AQP homologs has shed new light on their physiological roles. In fact, whereas some AQPs behave as "strict" water channels, others can conduct a wide range of nonpolar solutes, such as urea or glycerol and even more unconventional permeants, such as the nonpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the reactive oxygen species hydrogen peroxide and the metalloids antimonite, arsenite, boron and silicon. This suggests that AQPs are also key players in various physiological processes not related to water homeostasis. The function, regulation and biological importance of AQPs in the different kingdoms is reviewed in this chapter, with special emphasis on animal and plant AQPs.

Introduction—The Discovery of Aquaporins

Water is the most abundant substance in cells and organisms and is indispensable for life. Transport of water across phospholipid membranes is a universal requirement for any living organism, but, for decades, transmembrane water flow was explained only by the simple diffusion of water molecules through the phospholipid bilayer. However, this process is very slow and requires a high activation energy (E_a > 10 kcal/mol). The model of simple diffusion failed to explain why the membrane permeability of some cell types is so high that the bulk movement of water across the membrane occurs as fast as if no membrane was present and why the activation energy is comparable to that of water molecules diffusing freely in solution (E_a < 5 kcal/mol). Moreover, plasma membrane water permeability varies greatly for different cell types and, for a given cell type, transient variations in membrane water permeability are observed in response to the application of diuretic and anti-diuretic compounds or blocking agents, such as mercury chloride. These observations could only be explained if water channels existed in the membrane. The existence of such "hydrophilic pores" within biological membranes was first hypothesized in 1956 by Stein and Danielli[1] to account for the high membrane water permeability of red blood cells, which cannot be explained by a simple diffusion model.[2] In plants, the existence of such aqueous pores was first postulated by Ray.[3] However, the exact nature and role of these water channels remained obscure for years. The discovery, in the beginning of the nineties, of

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proteinaceous channels that enhanced the water permeability of the membranes solved this mystery. These water channels, named aquaporins (AQPs), shed a new light on water movement regulation, but, as the number and range of known AQP-permeating compounds constantly increases, it appears that AQPs also play a central role in many other biological processes not related to cell water balance regulation. AQP research has recently demonstrated that AQPs act as multifunctional channels and have unexpected roles. In this chapter, we will describe the nature of AQPs, as well as their regulation and involvement in cell physiology.

The first report of protein-mediated water transport through membranes was by Gheorghe Benga in 1986.[4] In 1987, the archetypal animal water channel was accidentally copurified with the blood group antigen Rh from cell membranes of Rh (D)-positive erythrocytes.[5,6] This 28 kDa protein turned out to be extremely hydrophobic and, after isolation and characterization, was found to present some similarities with other channel-forming proteins[7] and was therefore designated CHIP28 for "channel-like integral protein of 28 kDa".[8] CHIP28 was found to be expressed in tissues with a high water permeability[5] and showed homology to other uncharacterized proteins: a 26 kD major intrinsic protein (MIP) from bovine lens cells, *Drosophila* big brain, soybean nodulin 26 and *Escherichia coli* glycerol facilitator (GLPF) protein. In 1992, evidence that CHIP28 displayed water channel activity when heterologously expressed in *Xenopus* oocytes or inserted in proteoliposomes constituted a major breakthrough in the field and paved the way to AQP research.[9] CHIP28 was renamed AQP1 and MIP, as the original prototype member, was renamed AQP0. In 1999, Agre[10] reported the first high-resolution images of the three-dimensional structure of AQP1 at 4.5 Å resolution. Further studies using computer simulations identified the path of water molecules as they move through the channel and demonstrated how a pore can allow water to pass without allowing the passage of small solutes.[11,12] Peter Agre was awarded the 2003 Nobel Prize for chemistry for his role in the discovery and characterization of AQP1.

AQPs belong to the ancient MIP family, the members of which are found in all kingdoms, with more than 450 AQPs having been identified in organisms ranging from vertebrates and insects to plants, fungi, bacteria[13,14] and even viruses.[15] MIPs form channels through membranes, facilitating the passive transport of water and other small neutral solutes. Although the term "aquaporin" was initially restricted to water-transporting MIPs, it is now used to describe all MIPs. While five to seven AQP homologues have been identified in different insect species[14] and 13 in mammals,[16] higher plants show a higher multiplicity of isoforms, with 35, 33, or 36 homologs in *Arabidopsis thaliana*, *Oryza sativa,* or *Zea mays,* respectively.[17-19] Plant AQPs are classically grouped into four subfamilies on the basis of sequence similarity, these being the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs). Recently, three other subfamilies, the GlpF-like intrinsic proteins (GIP), the hybrid intrinsic proteins (HIPs) and the uncategorized X intrinsic proteins (XIPs), have been discovered in the moss *Physcomitrella patens*.[20,21]

Whereas much of the research effort has focused on characterizing AQPs with respect to cell water homeostasis, recent discoveries about the transport selectivity of MIP homologs suggest that some AQPs are more than just water channels and that AQPs are involved not only in cell water relations, but also in many other major physiological processes.[22,23] For example, in mammals, a role of AQPs in osmotically driven transepithelial fluid transport, as occurs during urine concentration and glandular fluid secretion, has been clearly demonstrated. In addition, involvement of AQPs in other physiological processes has been revealed, e.g., in cell migration, brain and corneal swelling, skin hydration, adipocyte fat accumulation, neural signal transduction and apoptosis.[24-27] In plants, AQPs are involved in many major processes not strictly related to osmoregulation, including nutrient acquisition, nitrogen and carbon fixation, cell signaling and stress responses.[28,29] This sheds new light on their physiological roles in living organisms that will be emphasized throughout this chapter.

Topology of Aquaporins

AQPs are transmembrane proteins composed of 243 to 302 amino acids and typically containing six membrane-spanning α-helices, with the N- and C-termini located on the cytoplasmic side of the membrane. The N- and C-terminal halves form two tandem repeats of three membrane-spanning α-helices (TM1-TM6) showing significant sequence similarity. Each half contains two small hydrophobic loops (loops B and E). This tandem repeat structure probably originates from an ancient intragenic duplication preceding the divergence between pro- and eukaryotes 2.5 billion years ago.[30] The cytosolic loop (loop B) between the second and third transmembrane domains and the extra-cytosolic loop (loop E) between the fifth and sixth transmembrane domains form short helices that are relatively hydrophobic. These two loops contain a asparagine-proline-alanine (NPA) motif, highly conserved in the MIP family, suggesting an essential role for protein function. The two halves of the protein show an obvious structural symmetry, with each of the NPA motif-containing loops dipping into the middle of the lipid bilayer to form two hemipores that, together, create a water-filled channel. The pore formed in AQP1 allows 3×10^9 water molecules per monomer per second to pass through the membrane.[11,31,32] The narrowest part of the pore only measures about 3 Å, which is slightly larger than the 2.8 Å diameter of the water molecule.[11] The highest degree of conservation is found in the transmembrane domains, particularly in the two regions containing the NPA motifs.

The three-dimensional structure of several AQPs has been determined at high spatial resolution (5 to 2.2 Å) by electron microscopy and X-ray crystallography.[11,33-45] The AQP1 channel consists of three topological elements, an extracellular vestibule and a cytoplasmic vestibule connected by an extended narrow pore acting as the selectivity filter. As a result of the pore topology and spatial arrangement of amino acids within the pore region, AQP selectivity results from a combination of mechanisms, including size exclusion at two highly conserved constriction regions (the NPA motif and the aromatic/arginine filter) and stereoscopic recognition of the substrate mediated by spatially defined H-bonding and hydrophobic interactions within the pore.[11,34,46] Analysis of the AQP pore indicates that proton transport through the channel is energetically highly unfavorable. Indeed, whereas water molecules can permeate the pore with a minimal energy barrier, transfer of protons is blocked by a combination of electrostatic repulsion, unfavorable dipole orientation and a H-bond isolation mechanism.[11,12,34,47-49]

AQPs usually form tetramers. This tetrameric assembly seems to be crucial for the proper folding, stability, targeting to the plasma membrane and even functionality of AQPs.[33,42,50,51] According to Murata et al,[11] each monomer interacts with two neighboring monomers through the membrane-spanning α-helices. Nevertheless, the monomer is the functional unit, transporting water independently of the other subunits in the complex.[34,52,53] However, a recent report has shown that, even though each monomer provides its own functional water pore, the properties of this pore are determined by a cooperative interaction of the entire tetramer, allowing stabilization of the water pore structure.[54]

Selectivity of Aquaporins

AQPs were originally characterized as water channels and have been shown to increase the membrane water permeability by up to 20-fold. Their classical role in facilitating transcellular water transport in many fluid-transporting tissues is now well characterized in mammals and plants, as well as in single cell organisms.[27,46] In mammals, involvement of AQP in the urine concentrating mechanism, gland fluid secretion and swelling of tissues under stress is currently being extensively studied using AQP-knockout mice and their unexpected role as water channels in processes such as cell migration has been recently demonstrated.[27,55] In plants, metabolic control of the membrane water permeability by regulation of AQP activity constitutes a way to deal with environmental cues and control tissue water balance.[28,56,57] Plant water balance is a broad concept which encompasses multiple aspects of plant-water relations, such as the regulation of long distance water transport (in the presence or absence of transpiration-driven fluxes), the appropriate water supply for maintenance of cell turgor and elongation and the regulation of cell homeostasis.

The time when AQPs were considered as simple water conduits is now past, as a constantly increasing number of studies demonstrate that AQPs are multifunctional channels. Although some members of each of the main AQP subfamilies have been shown to act as water channels, AQPs are being found to have an ever-increasing range of substrates.[22,23] Together with the fact that the different subfamilies of AQPs are distributed differently in tissues and cell compartments, the idea that some of these isoforms may not be restricted to water conductance, but are involved in solute transport, has recently found experimental support in the AQP community, with the identification of AQP substrates of physiological importance.[14,28,58,59] Whereas some AQPs, such as SoPIP2;1,[35] behave as "strict" water channels, others can conduct a wide range of nonpolar solutes, such as urea or glycerol and even more unconventional permeants, such as the nonpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the reactive oxygen species hydrogen peroxide and the metalloids antimonite, arsenite [As(III)], boron (B) and silicon (Si).[23,55,58] This indicates that AQPs are key players in various physiological processes not related to water homeostasis. For example, in mammals, there is now strong evidence for the involvement of aquaglyceroporin-facilitated glycerol transport in skin hydration and fat cell metabolism.[58] In plants, GIP1;1, NtAQP1 and Nt-TIPa have been shown to facilitate the passage of urea and/or glycerol.[20,60,61] Absorption of compounds, such as ammonia/ammonium or even formamide, has been shown to be facilitated by TaTIP2.[62] Recently, the diffusion of reduced and noncharged species of the metalloids Si, B, arsenic and antimony was shown to be facilitated by MIPs from different organisms (reviewed in refs. 22,55). For example, Si uptake as silicilic acid is facilitated in rice by NIP Lsi1 (see also chapters by Ma as well as Bienert and Jahn in this book). Facilitated transport of Si is important in higher plants, as Si can account for up to 10% of the shoot dry weight.[63] Boron uptake under conditions of B limitation is achieved through AtNIP5; 1 acting as a plasma membrane boric acid channel (see also the chapter by Miwa et al in this book).[64] AtNIP2;1 transports lactic acid, with a preference for the protonated acidic form and its expression is highly up-regulated in response to water logging and anoxia stress. This suggests that AtNIP2;1 might play a role in adaptation to lactic fermentation under anaerobic stress.[65] The finding that some plasma membrane AQPs facilitate CO_2 diffusion in plants has recently shed new light on their function in carbon bioassimilation in leaves.[29,66] Hydrogen peroxide is used in signal transduction pathways and acts as both an intra- and inter-cellular signaling molecule[55,67] and its diffusion has been shown to be facilitated by mammalian AQP8, but not by AQP1 and by two tonoplast AQPs from Arabidopsis (AtTIP1;1 and AtTIP1;2).[23] Interestingly, another nonwater permeant, ammonia, is conducted by AQP8, but not AQP1,[62] which strongly suggests that close AQP orthologs show different selectivity profiles in a given organism. This fact, together with their tissue localization, is currently believed to be one of the main reasons for the diversity of AQP homologs found in a given organism.

Measurement of Aquaporin Activity and Water Movement

Different methods exist to measure the contribution of AQPs to membrane water permeability, each with advantages and disadvantages. We have chosen to illustrate four of the most commonly used approaches. It is worth noting that these methods are not restricted to measuring the water channel activity of AQPs, but can also be used to measure the passage of other solutes or gases. The different methods do not provide the same kind of information and are often complementary.

Cell Swelling Assays

Cell swelling assays were among the first methods used to investigate the water channel activity of AQPs. Different methods exist, but the most common employs defoliculated oocytes of the African clawed frog *Xenopus*, which exhibit low permeability to water due to the fact that their cell membrane is essentially devoid of water channels. An expression vector containing the *AQP* cDNA of interest (or in vitro transcribed mRNA) is microinjected into defoliculated oocytes, then, after sufficient time to allow the heterologous expression of the AQP protein (usually 48 to 72 hours), the oocytes are examined under a microscope. Following hypoosmotic challenge, their volume change is recorded in real time. This method allows calculation of the water permeability

associated with the expression of a given AQP isoform in the oocyte plasma membrane and has been widely used in the AQP field.[68,69] The *Xenopus* oocyte swelling assay has been used to assess functional consequences of amino acid substitutions or deletion on AQP activity and to measure cooperative effect of AQP proteins in regulating cell water permeability.[68]

The main drawback of this method is that it is laboratory animal-based and so faces increasing legal restrictions aimed at guaranteeing animal welfare. Moreover, heterologous expression of AQPs does not always lead to the insertion of these proteins in the oocyte plasma membrane. Therefore a negative result in terms of swelling needs to be confirmed by localization of GFP fusion proteins or immunodetection. It is worth noting that oocyte swelling assays can be used to assess the selectivity of AQP isoforms by measuring the incorporation of radioactively labeled potential substrates in control and AQP-expressing oocytes.[20] AQP-mediated gas conductance (NH_3, CO_2) as well as AQP-mediated passage of metalloids (Si, As(III) and B) have also been studied using this expression.[63,70-72]

In the plant field, a similar approach using plant cell protoplasts has been widely used to measure the activity of endogenous AQPs[57,73-77] or of transiently or constitutively overexpressed isoforms.[51,78] Results using this expression system must be interpreted with caution, as results with protoplasts do not necessarily reflect the cell membrane water permeability measured *in planta*.[77] The advantage for plant biologists is that protoplasts offer an easy-to-handle homologous expression system, but this method has important limitations in terms of the interpretation of the results, as the assignment of the protoplast to a given cell type is difficult and as protoplasting probably modifies the expression of *AQP* genes.[77,79]

Stopped-Flow Spectrophotometry

Stopped-flow spectrophotometry, a technique widely used in AQP research, relies on the analysis of particle light scattering.[80-83] Each suspended particle passing through a laser beam scatters the light in some way. This combination of scattered light is picked up by detectors and, by analyzing fluctuations in brightness at each detector, it is possible to correlate this with variations in the volume of the particle. Cytoplasmic volume variations can be monitored either by fluorescence quenching or by 90° scattered light. Using this technique, the kinetics of plasmolysis or deplasmolysis of AQP-expressing bacterial or yeast cells and of AQP-enriched reconstituted proteoliposomes in response to osmotic challenges are usually recorded in real-time. One advantage of this technique is that, once it has been correctly set up, repeatable results for a large panel of specific AQP isoforms can be rapidly acquired and the process can even be automated. Another is that analyses are performed on easy-to-transform single cell organisms (yeast, bacteria) or even under cell-free conditions (reconstituted proteoliposomes), which can allow the determination of the intrinsic water permeability of a single AQP isoform. Using such expression systems, care should be paid to the correct folding and targeting to the biological membrane, of the heterologously expressed proteins, as mentioned in the case of Xenopus oocytes.

Cell Pressure Probe Measurements

Pressure probe measurements allow determination of the cell hydraulic conductivity, L_p ($m \cdot s^{-1} \cdot MPa^{-1}$), by modulation of the cell turgor. This is currently the technique of choice to probe AQP activity *in planta*,[77,84] but is unfortunately not applicable to cells lacking a rigid cell wall, such as animal cells. After puncturing of the cell using a microcapillary, it is possible to modulate its turgor pressure. The transmembrane water flow results from a hydraulic flow driven by the hydrostatic pressure gradient, ΔP, generated by the pressure probe which causes water to flow out of, or into, the cell. The parameter of interest is the half time ($T^{w}_{1/2}$) value, the time required for half of the turgor change from a given value to the equilibrium value. The elastic modulus (ε) is the other important physiological parameter that must be measured to correlate the volume and pressure of the cell and characterize the mechanical rigidity of the cell wall. High values of ε refer to a low extensibility or a rigid cell wall, i.e., big changes in pressure cause small changes in cell volume. Low values of ε, on the other hand, mean a highly extensible cell wall. The elastic modulus

has the dimensions of pressure and is usually given in MPa. These two parameters, $T^{w}_{1/2}$ and ε are used to calculate the cell hydraulic conductivity L_p according to Steudle et al.[85]

Proton NMR

Proton NMR technology uses the magnetic properties of hydrogen (^1H) nuclei, present mostly in water. This technique detects changes in the properties of water protons as they move from one physical state or chemical configuration to another and thus permits the non-invasive detection of water movement in different compartments within an organism.[86-88] The determination of the transport processes involved in water balance is a subject of considerable interest in many fields of research, e.g., in medicine or in plant and environmental sciences. The advantage of proton NMR over other analytical methods is its non-invasiveness and the possibility of simultaneously acquiring measurements at different organizational levels, from the whole organism to the tissue, or even the cellular, level. Magnetic resonance imaging allows visualization of both static and dynamic water phenomena. Imaging of water movement can be studied either by following the local intensity in time-controlled sequential images (e.g., for monitoring slow de- or rehydration processes) or by directly mapping proton displacement in a well defined time interval based on pulsed field gradient NMR or displacement imaging.[86,88,89]

Aquaporin Inhibition

Blocking of AQPs has been observed with compounds that can oxidize the Cys residues associated with the pore region of the protein or that can bind to protein sulfhydryl groups. A traditional method for determining the involvement of AQPs in physiological processes is the use of AQP inhibitors, such as mercury chloride ($HgCl_2$).[9,46,90] Treatment with a reducing agent, such as β-mercaptoethanol or dithiothreitol, partially reverses the inhibition. It should be noted that the use of this type of blocker can have many side effects, as they also perturb the ionic homeostasis of the cell and the integrity of its membrane. Phloretin, a flavonoid compound, is another transport blocker that has been shown to block AQPs in animals[91,92] and plants.[75,77,93,94] This compound is known to adsorb to lipid surfaces and to alter the dipole potential of lipid bilayers. Its mechanism of inhibition is still unclear, but it might interfere with the conformation of the AQP protein in the membrane.

Most, if not all, plant plasma membrane AQPs are specifically blocked by intracellular acidification due to the presence of charged His residues in the cytosolic loop D that control its conformation and the pore conductance.[35,95] This mechanism can be used to artificially inhibit AQP activity; cell (or tissue) acid loading with propionic acid (20 mM) artificially induces acidosis, which reversibly and specifically inhibits AQPs.[95] This reversible pH inhibition mechanism provides a novel and mild method for probing AQP activity in living organisms.

AQPs appear to play a central role in some human diseases, so it is possible that the manipulation of water channel proteins using compounds developed for this purpose might be exploited for clinical benefit.[27] These could be used to treat clinical disorders in which water depletion or water overload is an important factor, as in brain edema, glaucoma, epilepsy, obesity, or cancer. The search for AQP-targeting drugs using site modeling analysis and systematic screening is currently in progress.

Phenotype Analysis Reveals Involvement of Aquaporins in Key Physiological Processes

In some cases, AQP mutations in man have been used to study the biological significance of a given AQP isoform.[27,96-98] However, reverse genetics, when applicable, provides a more specific and elegant approach to probing AQP activity in vivo and is the strategy of choice for studying the physiological roles of AQPs in living organisms. This approach theoretically makes it possible to alter the expression of a single gene or a set of genes.[99] The role of AQPs in mammalian physiology has been mainly studied by phenotype analysis of transgenic mouse models with *AQP* gene deletions/mutations.

In both mice and humans, phenotype studies support the expected roles of AQPs as water channels mediating kidney fluid transport during urine concentration and fluid secretion from glandular epithelia. As thoroughly reviewed by Verkman et al,[27,100] the analysis of these mutants has revealed that AQPs are involved in many kinds of unexpected physiological phenomena related to their ability to transport water, such as brain and corneal swelling, neural signal transduction, regulation of intracranial and intraocular pressure, tumor angiogenesis and cell migration. The strong expression of some mammalian AQPs in tumor cells, particularly in aggressive tumor cell lines, has received increasing attention regarding their role in cancer development. The involvement of AQPs in cell migration and proliferation processes, as inferred from reverse genetic studies, suggests that they play key roles in cancer biology.[27,100] For example, while AQP1 is expressed ubiquitously in tumor vascular endothelium, AQP1-null mice show defective tumor angiogenesis due to impaired endothelial cell migration.[26] In contrast, enhanced migration of AQP-expressing cancer cells has been recorded in vitro and increased local tumor invasion, tumor cell extravasation and metastases have been found in vivo,[100] showing that AQPs are important in tumor growth and spread. As glycerol facilitators, aquaglyceroporins have been found to play unexpected roles in skin hydration, fat metabolism and tumorigenesis.[58,100] For example, AQP3-null mice have a dry skin, reduced skin elasticity and impaired epidermal biosynthesis.[58] In addition, a basal cell carcinoma was shown to have increased levels of AQP3, AQP3-null mice were found to be more resistant to skin tumorigenesis by a mechanism that might involve reduced tumor cell glycerol metabolism and ATP generation.[100]

In the plant field, given the high multiplicity of isoforms found in a single organism, modulation of AQP expression is considered the strategy of choice for elucidating the role of AQPs in plant physiology.[76,101-105] Silencing of plasma membrane AQPs usually results in a decrease in cell water permeability,[76,101-103] but, although PIP-silenced plants usually show lower cell water permeability than control plants, this does not necessarily result in lower tissue water conductivity, probably as a consequence of the different mechanisms used by plants to deal with lower membrane water permeability (for example, by increasing their root mass).[99] Recently, using antisense lines, tobacco NtAQP1 was shown to facilitate the passage of CO_2 through biological membranes.[66,106] NtAQP1-related CO_2 permeability seems to be of physiological importance under conditions in which the CO_2 gradient across a membrane is small, as is the case between the atmosphere and the plant cell cytosol.[66] A decrease in membrane resistance to CO_2 transport might increase CO_2 bioavailability and improve photosynthesis, ultimately affecting the degree of photorespiration and efficiency of nitrogen and water use. However, the biological significance of AQP-facilitated CO_2 diffusion is still controversial, as this process seems to be mainly governed and limited by the existence of unstirred layers.[107]

Although attractive, reverse or forward genetic approaches are generally complicated by many factors, such as the incomplete characterization of the transport selectivity of the targeted AQP, the fact that AQPs can act as multifunctional channels with multiple physiological roles, possible compensation/interaction mechanisms between close homologs and the number of posttranslational regulation mechanisms. It is also worth mentioning that these strategies can also represent a risk for false interpretation and that results should be supported by complementary approaches. The fact that RNAi-mediated loss of aquaporin AtTIP1;1 was first reported to result in early senescence and plant death[104] and that transposon insertion line, completely devoid of AtTIP1;1 protein did not result in any significant effect on plant metabolism[108] clearly illustrates this latter point.

Aquaporin Regulation: Gating and Localization

The presence of AQPs in cell membranes makes it possible to regulate water flux between and within cells. This fine regulation is assumed to be under metabolic control. Cells have the ability to alter their water conductivity within a few seconds or minutes in response to stimuli such as hormones (e.g., vasopressin in mammals and abscisic acid in plants) or diuretic or anti-diuretic compounds. Depending on the species and conditions, short-term changes of several orders of magnitude can be seen in membrane water conductivity. In mammals, AQPs are localized in

epithelia that require a high rate of water flux, such as the collecting duct of the kidney, the lung capillaries and the secretory cells of the salivary glands.[109,110] In plants, AQPs have been shown to be expressed in regions where a high cell-to-cell water permeability is required, such as in the vicinity of apoplastic barriers in roots[57] or in leaf vascular bundles[111,112] or reproductive organs.[113] In both mammals and plants, different AQPs in the same organism differ in their transcriptional or posttranslational regulation and subcellular distribution.[97,114] This complex and multilevel regulation allows a finely tuned and rapid control of membrane water permeability mediated by changes in the expression and activation of AQPs.[46,115,116]

The gating of AQPs makes it possible to control the water flow through the pore of the protein and is accomplished by a conformational change in the protein that blocks the channel and thus prevents the flow of water through the pore. In plants and mammals, different gating mechanisms have been observed; these involve the phosphorylation/dephosphorylation of certain serine or threonine residues, the protonation of specific histidine residues and the binding of divalent cations. The same AQP isoform can be simultaneously present in the same membrane in several forms with different isoelectric points.[117] This is proof of the importance of the co or posttranslational regulation of AQP activity. These posttranslational modifications affect the apparent size and isoelectric point of the protein and can be visualized using 2D gel electrophoresis.[117]

Regulation of subcellular localization through AQP trafficking may also represent a means of modulating the membrane water permeability. In fact, the subcellular localization of AQPs should not be seen as a static, but rather as a dynamic, phenomenon. This relocalization of AQP isoforms has been observed in mammalian and plant cells.[118,119] Although the details of the mechanism leading to AQP relocalization differ between these two organizational models, the principle remains the same: the density of functional AQPs in the cell membrane is controlled by the fusion (or endocytosis) of AQP-rich vesicles. As a result, the net density of active AQPs increases (or decreases), leading to enhanced (or decreased) cell membrane water permeability.

Phosphorylation

Phosphorylation of AQPs was first discovered as a posttranslational modification regulating the water channel activity of a TIP in plants[120] but was also shown to play a critical role in the regulation of AQP function in animals and humans.[27,121,122] Phosphorylation was shown to directly affect the water channel activity of the targeted protein, but its effect seems to vary greatly, ranging from stimulation of water transport activity (e.g., SoPIP2;1,[123] ZmPIP2)[69] to its inhibition (e.g., HsAQP4).[122] In addition phosphorylation of AQPs can affect their subcellular localization.[118]

In mammals, phosphorylation of AQPs is mediated by the activity of kinases belonging to the protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG) families.[118,121,124] PKA, also known as cAMP-dependent protein kinase, refers to a family of enzymes, the activity of which is dependent on the level of cyclic AMP (cAMP) in the cell. At low cAMP levels, the kinase is catalytically inactive and activation is triggered by an increase in the cAMP concentration (e.g., activation of adenylate cyclases by G protein-coupled receptors or inhibition of phosphodiesterases that degrade cAMP). The activated kinase then catalyzes the transfer of the terminal phosphate of ATP to serine or threonine residues in protein substrates. PKC is a family of protein kinases that require calmodulin, Ca^{2+}, diacylglycerol and phospholipids for activation. It is currently believed that a given AQP isoform may be the substrate for several kinases belonging to different families. For example, AQP2 is phosphorylated by PKA, but putative phosphorylation sites for PKG, PKC and casein kinase II are also present in the sequence. There is now compelling evidence that PKA-dependent phosphorylation of ser256 in the carboxy-terminal of AQP2 plays a critical role in its trafficking to the apical membrane of kidney duct epithelial cells, whereas activation of the PKC pathway leads to its endocytosis, which is independent of the phosphorylation state of ser256.[125]

In plants, CDPKs seem to be the predominant calcium-dependent kinases phosphorylating AQPs.[69,123,126] A typical CDPK is activated by an increase in free Ca^{2+} concentrations from nM

to μM levels which leads to the phosphorylation of its target. Several plant AQPs have been shown to be phosphorylated in vivo and in vitro at serine residues in the N- or C-terminal regions. For example, in vivo serine phosphorylation has been detected in a variety of plant isoforms, i.e., spinach SoPIP2;1, soybean NOD26, bean PvTIP3;1 and *Arabidopsis* and maize PIP1 and PIP2 isoforms.[69,117,123,127-132]

The effect of the phosphorylation of animal and plant AQPs has been demonstrated by the heterologous expression of mutated proteins in *Xenopus* oocytes, as kinase and phosphatase agonists or antagonists can be added to the medium and their effects measured. [69,120,123,128] For instance, the water channel activity of PvTIP3;1 in oocytes is increased by addition of cAMP agonists, which stimulate oocyte protein kinase A.[120] Similarly, the activity of SoPIP2;1 and GmNodulin26 is increased when oocytes are incubated in the presence of the phosphatase inhibitor, okadaic acid.[123,133] Mutation of Ser to Ala or dephosphorylation of highly conserved Ser residues in the C-terminus or in loops B and D leads to a decrease in water channel activity.[35,69,120,123,133]

Recently, two kinases acting on the phosphorylation sites ser115 and ser274 of SoPIP2;1 were characterized.[134] The kinase acting on ser274 is bound to the plasma membrane and belongs to the CDPK family. The kinase acting on ser115 is soluble and has still to be characterized. However, its recognition site is typical of that for PKA, CDPK, or PKC.[134]

pH and Divalent Cations

Regulation of AQP permeability by protons and/or Ca^{2+} has been reported for mammalian AQP0, AQP3 and AQP6[135-138] and for plant PIPs.[35,95,139]

The pH effects are different, depending on the isoform. For example, the water permeability of rat AQP3 is decreased by a shift to an acidic external pH (from pH 7.4 to 5.6), but no such effect is observed with rat AQP0, AQP2, AQP4, or AQP5.[140] On the other hand, the water permeability of bovine AQP0 is increased by a shift to an acidic pH.[137] Extracytosolic loops A and C, which contain histidine residues, contribute to this pH sensitivity and the differences were shown to originate from the position of these key His residues in the extracytosolic loops.[138] Mutation of each of these key His residues results in a shift in pH sensitivity. Interestingly, acid or alkaline sensitivity is induced in human AQP1 by adding His residues at positions 48 in loop A and 130 in loop C.[138] In plants, most, if not all, PIPs are blocked by intracellular acidification.[95] Mutagenesis experiments demonstrated the importance of his197 (located in cytosolic loop D) in this pH-dependent regulation mechanism: the H197A mutant shows less pronounced P_f inhibition, while H197D, in which a negatively charged amino acid residue is introduced, is pH-insensitive.[95] This AQP gating in response to intracellular pH acidification plays a role in the response to anoxia (oxygen deprivation) and other environmental stresses.[95] This pH-dependent inhibition of the membrane water permeability is also observed in *Arabidopsis* plasma membrane vesicles, in which acidification to pH 6.0 reduces water permeability to 20% of that seen at pH 8.3.[139] Similar observations were made at low pH on tonoplast vesicles isolated from the storage roots of *Beta vulgaris*.[141] As his197 is conserved in PIPs, this mechanism makes it possible to understand how plant roots decrease their water transport capacity in response to flooding, which leads to anoxia and cytosol acidification.[95] The inhibition mechanism has been modeled in detail by Tornröth-Horsefield et al.[35]

The divalent cation Ca^{2+} modulates the activity of both animal and plant AQPs.[35,83,138,139,142] Whereas some mammalian isoforms, such as bovine AQP0 or MIPfun (killifish AQP0), appear to be inhibited by Ca^{2+}, others, such as rat AQP4 and human AQP1, are not Ca^{2+}-responsive. When observed, this Ca^{2+} inhibition has been shown to be mediated by calmodulin. As the sites of Ca^{2+} inhibition and pH inhibition are distinct, the mechanisms of pH sensing and Ca^{2+} inhibition are completely separable.[138,143]

In plants, Ca^{2+} has also been shown to modulate the activity of AQPs, but the inhibition mechanism is somewhat different from that for mammalian isoforms, as no calmodulin is required for the binding of Ca^{2+} to the AQP. The hydraulic conductivity of *Arabidopsis* cells is reduced by up to 4-fold in the presence of Ca^{2+}.[83,139] Moreover, in the presence of Ca^{2+} chelators, a shift to

higher P_f values compared to control conditions is observed, suggesting downregulation of AQP activity through Ca^{2+} binding. Crystallographic studies and molecular dynamics simulations have demonstrated the importance of loop D in controlling whether the pore is open or closed.[35] Depending on its conformation, loop D acts as a flexible lid to the pore. By altering the conformation of this loop through a network of ionic interactions and hydrogen bonds between specific amino acids in the loop and the N-terminal part of the protein, divalent cations, such as Cd^{2+} or Ca^{2+}, stabilize the closed conformation of the pore.[35] Recently, Cd^{2+} and Mn^{2+} were shown to be potent inhibitors of AtPIP2;1,[83] probably acting via the same mechanism as Ca^{2+}, but the physiological significance of such inhibition needs further analysis.

Hetero-Oligomerization

Hetero-oligomerization is another way to regulate the activity/trafficking of oligomeric protein complexes and has been observed for many membrane proteins unrelated to AQPs.[144-146] For example, animal voltage-gated K^+ channels of the Shaker superfamily and their plant homologs result from the tetrameric assembly of subunits.[147] The data obtained with these animal and plant channels have demonstrated the existence of heteromerization between different types of subunits, generally, but not always, belonging to the same subfamily.[146,148,149] The case of the AKT2 subunit in plant K^+ channels is representative: hetero-oligomerization with the KAT2 subunit results in relocalization of the protein from the Golgi apparatus to the plasma membrane.[146]

A wide range of experimental data suggests that AQPs usually form homotetramers.[11,43,150] However, several plant isoforms might possibly form heterotetramers.[51,68,151] In *Lens culinaris* seeds, heteromerization of two TIPs was detected in crosslinking experiments, in which dimers, trimers and tetramers were irreversibly crosslinked using dithiobis-succinimidylpropionate.[151] In maize, a cooperative mechanism between ZmPIP1 and ZmPIP2 isoforms was recently demonstrated.[51,68] Whereas all PIP2s exhibit high water-channel activity in *Xenopus* oocytes, plant protoplasts, or yeast vesicles, PIP1s are often inactive or have low activity.[61,82,123,152-155] Interestingly, when ZmPIP2s are co-expressed with increasing amounts of ZmPIP1;2 in oocytes, a synergistic effect on AQP activity is observed, probably resulting from enhanced plasma-membrane targeting.[68] An increased P_f is seen compared to oocytes expressing ZmPIP2 alone. Co-expression of ZmPIP2s with GFP::ZmPIP1;2 improves the targeting of ZmPIP1;2 to the plasma membrane and/or its stability.

A similar effect has been observed in *Z. mays* cells.[51] When expressed alone in maize leaf protoplasts, ZmPIP1 fusion proteins are retained in the endoplasmic reticulum, whereas ZmPIP2s are found in the plasma membrane. Interestingly, when co-expressed with ZmPIP2s, ZmPIP1s are relocalized to the plasma membrane. This ZmPIP1 relocalization results from a physical interaction between ZmPIP1s and ZmPIP2s, as demonstrated using Förster resonance energy transfer/fluorescence lifetime imaging microscopy, as well as immunoprecipitation.[51] The way in which ZmPIP1s interact with ZmPIP2s is not known, but loop E of ZmPIP1s might play an essential role in their ability to interact with ZmPIP2s, as revealed by mutagenesis analysis of ZmPIP1;1 and ZmPIP1;2.[68] In addition to regulation of PIP1 trafficking, heteromerization might activate the channel activity of some monomers.[68] The question whether similar interactions exist between animal AQPs remains to be answered.

Modification of the Subcellular Localization

In mammals, the subcellular localization of AQP2 in the kidney collecting duct is controlled by the anti-diuretic hormone vasopressin, which induces a cascade of signaling events leading to AQP2 phosphorylation.[118,156-158] In the absence of vasopressin, the density of AQP2 in the apical plasma membrane of the collecting duct is quite low and, as a result, the apical membrane displays a low water permeability, preventing water from being reabsorbed from the urine. On the other hand, the basolateral membrane displays a constitutive high water permeability due to the presence of AQP3 and AQP4 in this membrane which facilitates a directional water flux. Binding of vasopressin to its receptor in the basolateral membrane activates adenylate cyclase, causing an increase in cAMP levels, which leads to protein kinase A-mediated phosphorylation of AQP2

and other targets. This phosphorylation event provokes the fusion of AQP2-containing subapical endosomes to the apical membrane, enhancing its water permeability and leading to water reabsorption from the urine. When vasopressin levels drop, AQP2 is endocytotically removed from the apical plasma membrane.[27,158,159] In plants, relocalization of AtPIP and AtTIP from the plasma membrane and tonoplast, respectively, to an endomembrane compartment was recently shown to occur in response to salt stress.[160] PIP2s are subjected to constitutive cycling, their endocytosis being clathrin-dependent.[161] Upon osmotic challenge, maize protoplasts are hypothesized to modulate their water channel activity through the fusion of AQP-rich endosomes with the plasma membrane.[75] Upon osmotic stress in *Mesembryanthemum crystallinum* roots, McTIP1;2 is relocalized from the tonoplast to the endosomes.[162] This process is dependent on AQP glycosylation and a cAMP-dependent pathway and, interestingly, can be inhibited by membrane trafficking inhibitors. As the redistribution of PIPs from the plasma membrane to internal compartments contributes to downregulation of root water uptake,[160] relocalization might act as a stress-response mechanism by rapidly and reversibly modulating the cell membrane water permeability. Alternatively, this relocalization process could lead to the degradation of the protein. Recently, phosphorylation/dephosphorylation of AtPIP2;1 at the C-terminus was shown to regulate its targeting to the plasma membrane or its internalization upon salt treatment.[132]

Conclusion

The roles of AQPs in cell physiology are currently increasing. As pointed out throughout this introduction, the discovery that some AQPs are more than simple water channels has shed new light on their physiological roles in living organisms.[22,23,27,29,55,63-66] Further characterization of the selectivity profile of AQPs and mutant phenotype analysis is being carried out and has already revealed unexpected cellular functions. Given the involvement of AQPs in many key cellular processes and their suggested role in facilitating tumor growth and spread, the quest for specific AQP inhibitors of possible clinical importance is receiving increasing attention. This is of particular interest in the cancer research field, as AQPs could turn out to be interesting drug targets for novel antitumor therapies.[100] Clearly, AQPs offer an exciting field for research and discovery.

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