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REVIEW

Aquaporin ion conductance properties defined by membrane environment, protein structure, and cell physiology

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Abstract

Aquaporins (AQPs) are multifunctional transmembrane channel proteins permeable to water and an expanding array of solutes. AQP-mediated ion channel activity was first observed when purified AQP0 from bovine lens was incorporated into lipid bilayers. Electrophysiological properties of ion-conducting AQPs since discovered in plants, invertebrates, and mammals have been assessed using native, reconstituted, and heterologously expressed channels. Accumulating evidence is defining amino acid residues that govern differential solute permeability through intrasubunit and central pores of AQP tetramers. Rings of charged and hydrophobic residues around pores influence AQP selectivity, and are candidates for further work to define motifs that distinguish ion conduction capability, versus strict water and glycerol permeability. Similarities between AQP ion channels thus far include large single channel conductances and long open times, but differences in ionic selectivity, permeability to divalent cations, and mechanisms of gating (e.g., by voltage, pH, and cyclic nucleotides) are unique to subtypes. Effects of lipid environments in modulating parameters such as single channel amplitude could explain in part the variations in AQP ion channel properties observed across preparations. Physiological roles of the ion-conducting AQP classes span diverse processes including regulation of cell motility, organellar pH, neural development, signaling, and nutrient acquisition. Advances in computational methods can generate testable predictions of AQP structure–function relationships, which combined with innovative high-throughput assays could revolutionize the field in defining essential properties of ion-conducting AQPs, discovering new AQP ion channels, and understanding the effects of AQP interactions with proteins, signaling cascades, and membrane lipids.

Keywords Aquaporin · Patch clamp · Ion channel · Phospholipid bilayer · Membrane · Protein structure

Introduction

Aquaporins (AQPs) are multifunctional proteins known for their capacity to facilitate water flux across cell membranes. They are governed by a diverse array of physiologically relevant control mechanisms, including phosphorylation, pH, Ca^{2+} and osmotic gradients (Tyerman et al. 2021). In addition to facilitating water flux, AQPs across phyla have an unexpected breadth of roles in transporting gases such as carbon dioxide ($C0_2$) and oxygen (0_2); ions such as sodium (Na^+), potassium (K^+) and chloride (Cl^-); signaling agents such as hydrogen peroxide (H_2O_2), purines and pyrimidines; metabolites and nutrients including glycine, lactic acid, urea, ammonia (NH_3), glycerol, polyols and more, contributing much more than simple water permeation to cells and tissues (Conde et al. 2010; Hachez and Chaumont 2010; Wagner et al. 2021; Ishibashi et al. 1994; Hara-Chikuma et al. 2015; Bienert et al. 2008; Jahn et al. 2004; Uehlein et al. 2003). Preceding the first discovery that AQPs mediated transmembrane water permeability (Preston et al. 1992), the bovine (*Bos taurus*) Major Intrinsic Protein of lens fiber 26 (MIP26, now classified as AQP0), was shown to conduct ions using black lipid membrane (BLM) bilayer electrophysiological techniques (Ehring et al. 1990; Shen et al. 1991; Zampighi et al. 1985). Since then, more subtypes of AQPs have been identified as either anion or cation permeable channels, including two classes from human, one from *Drosophila melanogaster* and four from plants. Emerging evidence supports the physiological relevance of AQP ion

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channel functions in motility, volume regulation, and adaptive responses to environmental stimuli. In silico and microorganism-based approaches are promising tools that could be used for characterizing AQP ion channel activity that, with electrophysiological approaches, could help address remaining knowledge gaps of AQP-mediated ion channel function.

AQP ion channel activity in native, heterologous and recombinant expression systems

Ion conduction properties are evident from published studies of AQP channels across different phyla, with continuing work beginning to identify the structural components that regulate ion conduction through AQP monomeric and tetrameric pores. Three of the classes of ion-conducting AQPs have been tested in bilayers as well as expression systems; these are described below, summarizing ion selectivity, mechanisms of activation, and regulation of function.

1. Major intrinsic protein of lens tissue (MIP)

Bovine MIP (MIP26, referred to here as BtAQP0) was the first cloned mammalian AQP (Gorin et al. 1984), later redesignated AQP0 (Agre et al. 1993). AQP0 is highly expressed in ocular lens fibre cells, and was initially considered to be a gap junction protein, not a water channel. Cloned BtAQP0 cDNA was predicted to encode a 26 kDa protein comprising six transmembrane segments that formed a channel-like pore (Gorin et al. 1984), features later confirmed as ubiquitous across the AQP family. Ion channel activity was evident within 5 to 10 min after purified BtAQP0-enriched lens membrane proteins were added to a preformed planar lipid bilayer (Zampighi et al. 1985). Channels were voltage dependent with a single channel amplitude of 200 pS in 100 mM KCl (Zampighi et al. 1985). HPLC-purified BtAQP0 was relatively anion selective in planar bilayers (P_{Cl-}/P_{K+} approximately 1.8) and showed two main conductance states (160 and 380 pS) plus multiple subconductance states in 100 mM KCl (Ehring et al. 1990). Further experiments using spectrometry in liposomes and electrophysiology in planar bilayers showed that BtAQP0 was permeable to K^+, sucrose, and Na^+ (Shen et al. 1991). The open probability of the channel was inversely proportional to voltage, and Cs^+ reduced the mean channel opening time (0.13 s) but did not decrease the single-channel conductance in 100 mM KCl (Shen et al. 1991), implying that BtAQP0 gating but not pore permeation is modulated by cations. Phosphorylation of BtAQP0 at serine-243 located near the C-terminus is required for voltage-dependent closure of the channels, reconstituted into planar lipid bilayers (Ehring et al. 1992). Sucrose permeability of BtAQP0 reconstituted into liposomes, measured by osmotic swelling, was blocked in the presence of Ca^{2+} and calmodulin (CaM); Mg^{2+} had no effect (Girsch and Peracchia 1985). Membranes from chicken lens enriched in a MIP homolog (originally named MIP28, referred to here as GdAQP0) also formed channels with functional properties similar to those of BtAQP0 (Modesto et al. 1990); two prominent unitary conductances (60 and 290 pS in symmetric 150 mM KCl) were voltage-dependent, closing at ± 80 mV. Channels were relatively anion preferring, with a permeability ratio (P_{Cl-}/P_{K+}) of 1.87; open probability was reduced in the presence of 5 mM Ca^{2+} (Modesto et al. 1996). However unlike BtAQP0, GdAQP0 channels were not phosphorylated at the C-terminus. A C-terminal truncated variant generated by partial hydrolysis, lacking the equivalent serine-243 of BtAQP0, retained conductance and voltage sensitivity properties similar to those of the full-length GdAQP0 channel (Modesto et al. 1996). These studies showed in liposomes and planar bilayers that AQP0 proteins are non-selective ion channels with long open and closed times, and multiple conductance states that are modulated by phosphorylation (in BtAQP0) and Ca^{2+}.

Although the original studies showed robust and reproducible ion channel activity in artificial bilayers, no currents associated with BtAQP0 expression were detected in *Xenopus laevis* oocytes using the two-electrode voltage clamp (TEVC) technique (Mulders et al. 1995). This work did confirm that BtAQP0-expressing oocytes showed a 4- to fivefold increase in water permeability as compared to water-injected control oocytes (Mulders et al. 1995). The water channel activity of BtAQP0 expressed in oocytes showed positive correlations with the concentrations of Ca^{2+} and protons (H^+) (Németh-Cahalan et al. 2013). Each BtAQP0 monomer was suggested to act cooperatively in the tetrameric AQP0 channel (Németh-Cahalan et al. 2013). A comparison of the single channel properties of AQP0 in oocytes with those previously determined in BLMs would be interesting to assess details of Ca^{2+} and pH modulation, and possible effects of membrane lipid environments.

2. Nodulin-26

Nodulin-26 (NOD26) is the major protein present in peribacteroid membranes of the nitrogen-fixing root nodules in soybean *Glycine max* (Fortin et al. 1987). Cloned GmNOD26 was noted to show amino acid sequence similarities to AQP0 and AQP1, and proposed to facilitate nutrient fluxes between the plant and the symbiotic bacteria living within the root nodules (Miao and Verma 1993). The first functional characterisation involved the incorporation of purified native GmNOD26 from soybean root membranes into BLMs, revealing GmNOD26 was an ion channel with a large unitary conductance (3.1 nS) in 1 M KCl (Weaver

et al. 1994). Weakly selective for anions, as indicated by a permeability ratio (P_{Cl-}/P_{K+}) of 1.21, GmNOD26 displayed channel open times ranging from 1 to 50 ms, and subconductance states ranging from 0.5 to 2.5 nS. Recombinant poly-histidine tagged GmNOD26 protein produced in *E. coli* showed almost identical biophysical properties to native GmNOD26 in BLMs (Lee et al. 1995). A Ser-262-Asp phosphomimetic mutant of GmNOD26 showed more frequent closure and preferential occupancy of lower subconductance states than wild type, which indicated phosphorylation modulates NOD26 ion conductivity (Lee et al. 1995). The physical properties of GmNOD26 in planar lipid bilayers are strikingly similar to those of MIP channels measured in the same system.

Using *Xenopus* oocytes and proteoliposomes, GmNOD26 was later shown to be a water channel with a relatively low transport rate, as well as a glycerol facilitator (Dean et al. 1999; Rivers et al. 1997). Treatment of GmNOD26-expressing oocytes with the phosphatase type 1 and 2A inhibitor okadaic acid, led to a fourfold increase in water permeability (Guenther et al. 2003). This suggested that Ca^{2+}-dependent protein kinase phosphorylation at Ser-262, which occurs *in planta*, stimulates GmNOD26 water channel activity (Guenther et al. 2003). It is interesting that phosphorylation at Ser-262 stimulated NOD26 water channel activity in oocytes, but inhibited its ion channel activity in BLMs. Supporting the originally proposed role of GmNOD26 in nutrient flux, the recombinant channel reconstituted into proteoliposomes was shown to be NH_3 permeable. NH_3 uptake, protonation, and subsequent alkalinisation of the liposome interior were measured by monitoring decreased fluorescence of preloaded carboxyfluorescein using stopped-flow spectrophotometry (Hwang et al. 2010). Hence GmNOD26 is now considered to be multifunctional.

3. Aquaporin 1 (AQP1)

The Channel-forming Integral Protein of 28 kDa (CHIP28) isolated from erythrocytes showed an amino acid similarity with BtAQP0 that prompted the idea it might have channel-like activity (Smith and Agre 1991). Expressed in *Xenopus* oocytes, CHIP28 showed constitutive water channel activity (Preston et al. 1992) and was renamed AQP1 (referred to here as HsAQP1). A non-selective monovalent cationic conductance with a permeability sequence $K^+ \simeq Cs^+ > Na^+ >$ tetraethylammonium (TEA^+) was shown using TEVC in HsAQP1-expressing oocytes, after indirect activation by forskolin or intracellular injection of protein kinase A catalytic subunit (Yool et al. 1996) via an H7-sensitive kinase signaling pathway proposed to enhance cGMP (Yool and Stamer 2004). Single HsAQP1 channels from inside-out patches of oocyte membranes were directly activated by cGMP, and showed a unitary conductance of 150 pS in symmetrical 0.1 M K^+ with flickery subconductance states (Anthony et al. 2000). To assess native AQP1 ion channels, the rat (*Rattus norvegicus*) homolog (RnAQP1) was examined in primary cultured choroid plexus cells, patch-clamped in whole cell and excised patch configurations. A cGMP-activated, Cd^{2+}-sensitive, monovalent cation conductance of 166 pS was characterized, with properties comparable to those of HsAQP1 channels expressed in oocytes (Boassa et al. 2006; Anthony et al. 2000). The cGMP-dependent AQP1-like channel activity was abrogated in cells transfected with siRNA constructs to knock down RnAQP1 expression (Boassa et al. 2006). However when purified native or recombinant HsAQP1 was reconstituted into planar lipid bilayers, cGMP activation gave rise to channel events with small unitary conductances of 2, 6, and 10 pS in 0.1 M Na^+ or K^+ (Saparov et al. 2001). The small unitary conductances might reflect differences in the physical properties of the bilayers in reconstituted versus eukaryotic cell membranes. The recombinant HsAQP1 channels studied by Saparov et al. (2001) were assessed in bilayers formed with *E. coli* total lipids, consisting predominantly of phosphatidylethanolamine (PE). Preliminary data in our lab (Henderson et al., unpublished) suggest that recombinant HsAQP1 produces high conductance single channels (~75 pS) with long open times when reconstituted into proteoliposomes made with soybean azolectin (Fig. 1) which consists predominantly of phosphatidylcholine (PC), as used successfully for studies of mechanosensitive and other ion channels (Martinac et al. 2010). The difference in headgroups (ethanolamine or choline) between PE and PC can influence transmembrane protein structure, as shown for μ opioid receptors with conformational states controlled by lipid interactions (Angladon et al. 2019). Lipids affect properties of many channel types (see below), and might be expected to influence the structure and function of AQP channels as well. Patch-clamping proteoliposomes with various defined lipid compositions should enable comparisons of environmental effects on ion channel activity of AQP1 and other AQPs to test the hypothesis that membrane lipids modulate AQP channel activity.

Differences in aquaporin channel properties could depend on membrane composition

Membrane protein activity (e.g., of mechanosensitive channels, KcsA K^+ channels, and nicotinic acetylcholine receptors) is affected by the physical properties of the surrounding lipid environment, including thickness, elasticity, viscosity, and tension (Lee 2004). As summarized above, properties of ion conducting AQPs differ between membrane preparations. The sensitivity of AQPs to the lipid environment is supported by results of studies of water channel activity, which also show variability when

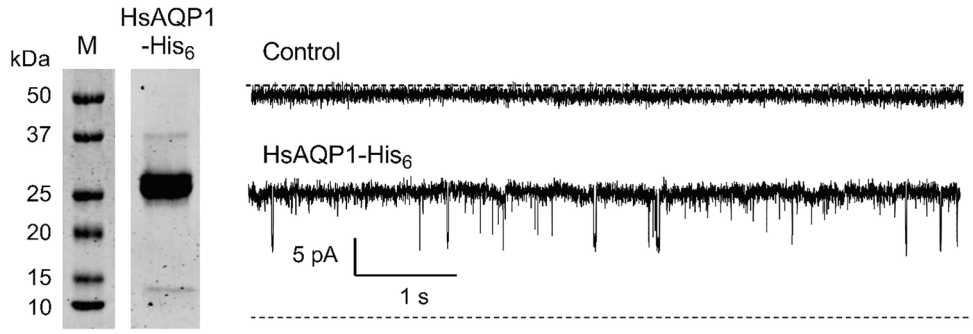

Fig. 1 Single channel activity of recombinant histidine-tagged human AQP1 reconstituted into proteoliposomes measured using the patch-clamp technique. (Left) Coomassie stained SDS-PAGE of purified recombinant HsAQP-His_6. (Right) Upper trace is a patch from a control (empty) liposome showing no channel activity. Lower trace is a patch from an HsAQP1-His_6 reconstituted liposome showing closing events (downward deflections, estimated unitary conductance 75 pS). Both patches were recorded in the presence of 10 µM CPT-cGMP at a holding potential of + 100 mV in symmetrical K^+. Dashed lines indicate the zero current levels

channels are reconstituted into proteoliposomes comprising different lipid types and proportions. Single channel water fluxes in two reconstituted human AQP4 isoforms decreased with increasing membrane bilayer compressibility and thickness, induced by addition of cholesterol and sphingomyelin (Tong et al. 2012). Similarly, the unitary water permeability of purified BtAQP0 was lower in proteoliposomes containing high cholesterol or sphingomyelin (Tong et al. 2013). Liposomes made from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) showed high water permeability when reconstituted with AQPZ from *E. coli*; however, AQPZ did not enhance water permeability in liposomes made with 1,2-Dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) (Zhao et al. 2013). The absence of cardiolipin, which is naturally prevalent in bacterial and mitochondrial membranes, was shown to reduce AQPZ water permeability in proteoliposomes (Laganowsky et al. 2014). Another factor setting the optimal water permeability of AQPZ was the lipid-to-protein ratio, which was maximal at 200:1 in proteoliposomes (Zhao et al. 2013).

The functional properties of several ion channels, for example K^+ ion channels and ligand-gated nicotinicoid receptors, are modified by the surrounding lipids or by direct lipid-protein interactions (Poveda et al. 2014; Tillman and Cascio 2003). It is reasonable to postulate that AQP ion channel activity is also modulated by changes in the lipid environment surrounding the protein, and could explain the variability in single channel conductance values reported for the same AQP in different cell or lipid bilayer preparations (Table 1). Experiments to determine AQP ion channel modulation by lipid composition should be feasible with proteoliposomes and BLMs. Some ion channel AQPs thus far have been tested only in eukaryotic cells such as Xenopus oocytes or human embryonic kidney (HEK) cells, so the potential influence of bilayer composition on channel properties remains to be determined.

Other classes of ion channel AQPs

In addition to ion channel AQP classes presented in the "Introduction," "AQP ion channel activity in native, heterologous and recombinant expression systems," and "Differences in aquaporin channel properties could depend on membrane composition" sections above, several other subtypes have been shown to mediate macroscopic ion currents in expression systems, primarily *Xenopus* oocytes, but have not yet been characterized in purified reconstituted preparations. These classes are summarized below.

4. Aquaporin (AQP6)

Conflicting lines of evidence have been reported for the properties and gating of aquaporin 6 (AQP6). The human AQP6 coding sequence was first isolated from a kidney cDNA library, and denoted hKID (Ma et al. 1996). *hKID* mapped to the 12q13 locus, which also contained *AQP0* and *AQP2*. When expressed in Xenopus oocytes, hKID (referred to here as HsAQP6) elicited a 2.6-fold increase in osmotic water permeability, which was inhibited 72% by mercury (Hg^{2+}), in agreement with the precedent set for Hg^{2+}-inhibition of AQP1-mediated osmotic water permeability (Preston et al. 1992). HsAQP6 expression in oocytes conferred no measurable glycerol or urea flux, suggesting that water was the only permeable substrate (Ma et al. 1996).

The homolog from rat (RnAQP6) initially could not be characterized (Ma et al. 1993) though subsequently was successfully expressed in oocytes. Unexpectedly, it was not blocked but was activated by Hg^{2+}, which enhanced both water and ion fluxes (Yasui et al. 1999a). The ion conductance but not water permeability of RnAQP6 also was activated by acidic pH, which unlike Hg^{2+} has relevance as a physiological stimulus. A positive shift in E_{rev} measured for acid-activated RnAQP6 when external Cl^- was replaced with

Table 1 Comparison of aquaporin ion channel properties measured using different techniques and preparations (*N.D*, not determined; *N/A*, not applicable)

AQP	Species of origin	Single channel cond	Sub-cond. states	Solutions	Gating	Selectivity	Method used	Reference
MIP26	Bovine purified from lens	380 pS and 160 pS	Yes	Symmetrical 0.1 M KCl	Voltage sensitive closure	$P_{Cl-}/P_{K+} < 1.8$	BLM	Ehring et al. (1990)
MIP26	Bovine HPLC-purified from lens	120 pS	No	Symmetrical 0.1 M KCl	Voltage sensitive closure	permeable to K^+, sucrose, and Na^+	BLM and proteo-liposomes	Shen et al. (1991)
MIP26	Bovine Detergent solubilized lens	200 pS	Yes	Symmetrical 0.1 M KCl or NaCl	Voltage sensitive closure	$Na^+ = K^+$	BLM	Zampighi et al. (1985)
MIP28	Chicken (purified lens tissue)	290 pS and 60 pS	Yes	Symmetrical 0.15 M KCl	Voltage sensitive closure	$P_{Cl-}/P_{K+} = 1.87$	BLM	Modesto et al. (1996)
MIP_{tryp}	Chicken purified lens tissue, trypsinated	1.5 nS	N.D	Symmetrical 0.3 M KSO_4	Voltage sensitive closure	N.D	BLM	Modesto et al. (1996)
NOD26	Soybean	3.1 nS	0.5, 1.1, 1.6, 1.9, and 2.5 nS	Symmetrical 1 M KCl	High voltage increased the occupancy of subconductance states	$P_{K+}/P_{Cl-} = 1.21$	BLM	Weaver et al. (1994)
NOD26-His	Soybean Recom-bin	3.1 nS and 2.2 nS	Infrequent occu-pancy of lower subconductance levels	cis 0.2 M/trans 1 M KCl	Kinase-dependent voltage gating	Slightly anionic	BLM	Lee et al. (1995)
NOD26-His S262A	Soybean Recom-bin	3.1 nS and 2.2 nS	N.D	cis 0.2 M/trans 1 M KCl	Infrequent transi-tions to lower subconductance states	Slightly anionic	BLM	Lee et al. (1995)
NOD26-His S262D	Soybean Recom-bin	3.1 nS	1.8 and 0.6 nS	cis 0.2 M/trans 1 M KCl	High voltage increased the occupancy of subconductance states	Slightly anionic	BLM	Lee et al. (1995)
AQP6	Rat	N/A	N/A	NaCl, Na-Glu, NMDG-Cl	Hg, acidic pH	Cl^-	Xenopus TEVC	Yasui et al. (1999a)
AQP6	Rat	49 pS	No	Symmetrical 0.1 M NaCl	Hg, acidic pH	$Na^+ = Cl^-$	Xenopus patch-clamp	Hazama et al. (2002)
AQP6	Rat	N/A	N/A	NaCl, $NaNO_3$	N/A	$NO_3^- > Cl^-$	Xenopus TEVC	Liu et al. (2005)
GFP-AQP6	Rat	N/A	N/A	Various	Acidic pH	$NO_3^- > I^- > Br^- > Cl^- > SO_4^{2-}$ ($P_{NO3-}/P_{Cl-} > 9.8$)	HEK293 whole cell patch-clamp	Ikeda et al. (2002)
AQP1	Human	N/A	N/A	0.1 M Saline	Forskolin, cAMP	$Na^+ = K^+$	Xenopus TEVC	Yool et al. (1996)
AQP1	Human	150 pS	Yes	Symmetrical 0.1 M K^+	cGMP	$K^+ = Cs^+ > Na^+ > TEA^+$	Xenopus, patch-clamp	Anthony et al. (2000)

Table 1 (continued)

AQP	Species of origin	Single channel cond	Sub-cond. states	Solutions	Gating	Selectivity	Method used	Reference
AQP1	Human (red cells)	2, 6, and 10 pS	N.D	Symmetrical 0.1 M KCl or NaCl	cGMP	$P_{Cl-}/P_{K+} = 12$	BLM	Saparov et al. (2001)
His_{10}-AQP1	Human (recombinant, E. coli)	2, 6, and 10 pS	N.D	Symmetrical 0.1 M KCl or NaCl	cGMP	$P_{Cl-}/P_{K+} = 12$	BLM	Saparov et al. (2001)
AQP1-His_6	Human (recombinant, Yeast)	2, 6, and 10 pS	N.D	Symmetrical 0.1 M KCl or NaCl	cGMP	$P_{Cl-}/P_{K+} = 12$	BLM	Saparov et al. (2001)
AQP1	Rat	166 pS	N.D	Symmetrical monovalent cations	cGMP	Na^+, K^+, TEA^+, and Cs^+	Patch-clamp of choroid plexus cells	Boassa et al. (2006)
PIP2;1	Arabidopsis	N/A	N/A	96 mM NaCl, 2 mM KCl	Not gated. Blocked by Ca^{2+} and Cd^{2+}	Na^+	Xenopus TEVC	Byrt et al. (2017)
PIP2;2	Arabidopsis	N/A	N/A	100 mM NaCl, 2 mM KCl	Not gated. Blocked by Ca^{2+}, Cd^{2+}, and Ba^{2+}	Na^+	Xenopus TEVC	Kourghi et al. (2017a)
PIP2;8	Barley	N/A	N/A	100 mM NaCl or 100 mM KCl	Not gated. Blocked by Ca^{2+}, Cd^{2+}, and Ba^{2+}	Na^+ and K^+ (not Cs^+, Rb^+, or Li^+)	Xenopus TEVC	Tran et al. (2020)
BIB	Drosophila	N/A	N/A	100 NaCl, 2 KCl, 4.5 $MgCl_2$	Not gated. Blocked by Ca^{2+} and Ba^{2+}	$K^+ > Na^+ \gg TEA^+$	Xenopus TEVC	Yanochko and Yool (2002); Yanochko and Yool (2004)

gluconate, but not when Na^+ was replaced with *N*-Methyl-D-glucamine (NMDG) (Yasui et al. 1999a), suggested currents were carried by Cl^- not Na^+. However, Hg^{2+}-activated single channels measured in inside-out patches of RnAQP6-expressing oocytes showed, in contrast, no change in E_{rev} when either Na^+ or Cl^- were reduced, suggesting equal permeability to Na^+ and Cl^- (Hazama et al. 2002). The Na^+ permeability of Hg^{2+}-activated RnAQP6-expressing oocytes was validated with a ^{22}Na radiotracer influx assay (Hazama et al. 2002). Similar tests showed fluxes of ^{14}C-glycerol and ^{14}C-urea in Hg^{2+}-activated RnAQP6-expressing oocytes (Holm et al. 2004), highlighting a potentially remarkable substrate diversity, but also illustrating the diversity of conclusions that have been drawn from different studies. Experimental parameters that influence channel function and baseline membrane permeability apparently remain to be fully defined.

In mammalian cells, the intracellular vesicular localization of RnAQP6 made electrophysiological analyses of ion channel activity difficult until a N-terminal GFP fusion construct was found to localize to plasma membrane in transfected HEK293 cells (Ikeda et al. 2002), enabling whole-cell patch-clamp measurement of acid-activated RnAQP6 currents. The GFP-RnAQP6 fusion protein showed permeability to halide anions with a permeability sequence: $NO_3^- \geq I^- \geq Br^- \geq Cl^-$ (Ikeda et al. 2002). When Asn-60 was mutated to Gly, the anion permeability of RnAQP6 was abolished and the protein became highly water permeable in a Hg^{2+}-independent manner (Liu et al. 2005).

Differences in ionic selectivity of RnAQP6 when gated by Hg^{2+} (selective to water, anions, cations, glycerol and urea) or pH (selective to anions) suggest that Hg^{2+} and protons induce different structural changes that impact the AQP6 monomeric pore (Yasui et al. 1999a). The relevance of acid activation of RnAQP6 anion fluxes aligns with the subcellular localization of this channel in intracellular vesicles of acid-secreting cells of the kidney (Beitz et al. 2006a). Intriguingly, AQP6 anion channel activity has only been demonstrated for RnAQP6, and not for HsAQP6 despite high (77%) sequence identity. Opposing effects of Hg^{2+} on the water permeability of HsAPQ6 and RnAQP6 warrant further clarification, since the cysteine residues that define the Hg^{2+} sensitivity of RnAQP6 have been identified (Cys-158 and Cys-193) (Yasui et al. 1999a), and are conserved in HsAQP6. Residues that determine pH-gating of RnAQP6 remain an area of interest for future investigation.

5. Drosophila big brain

Drosophila BIB amino acid sequence similarities to the AQPs MIP26, NOD26, and the *E. coli* glycerol facilitator GlpF led to speculation that BIB also was a channel-like protein (Rao et al. 1990). Unlike orthologous AQPs, BIB showed no appreciable water channel activity when expressed in oocytes (Yanochko and Yool 2002). Conversely, BIB mediated a voltage-insensitive, nonselective cation conductance in oocytes with a permeability sequence of $K^+ > Na^+ \gg TEA^+$ (Yanochko and Yool 2002). The BIB ion conductance was blocked by divalent cations Ba^{2+} and Ca^{2+} but not Mg^{2+} (Yanochko and Yool 2004). BIB currents were inactivated by tyrosine phosphorylation and activated by a tyrosine kinase inhibitor, consistent with regulated signaling roles for BIB in early nervous system development in the fly (Yanochko and Yool 2004; Rao et al. 1990).

6. Plasma membrane intrinsic protein 2;1 (PIP2;1) and homologs

A renaissance of AQP ion channel research in plants was catalyzed by the characterization of Plasma membrane Intrinsic Protein 2;1 (PIP2;1) from the model plant *Arabidopsis thaliana* as a non-selective cation channel when expressed in *Xenopus* oocytes, measured using TEVC (Byrt et al. 2017). Currents in AtPIP2;1-expressing oocytes resembled a Ca^{2+}-sensitive cation conductance previously measured in isolated *Arabidopsis* root cell protoplasts but not identified at the protein level (Demidchik and Tester 2002). AtPIP2;1 is now thought to be the molecular mechanism for the leak current (Byrt et al. 2017). An ortholog, AtPIP2;2 also elicited an ionic conductance when expressed in oocytes (Byrt et al. 2017). A homolog from barley, HvPIP2;8 in oocytes showed Na^+ and K^+ (but not Cl^-) conductances that were inhibited by divalent cations (Tran et al. 2020). Other plant ion-channel AQPs likely await discovery. Phosphorylation status regulates the ionic conductance of AtPIP2;1 in oocytes (Qiu et al. 2020), fitting the broad theme seen across diverse AQP ion channel types reviewed here. To date, heterologous expression in Xenopus oocytes has been the primary experimental system used for measuring ion channel activity of PIP-type AQPs. Further research on plant AQP ion channel properties using proteoliposome reconstitution is in progress.

In summary, AQP ion channels show a panel of similar features. They are generally permeable to monovalent anions and/or cations, blocked by divalent cations (except Mg^{2+}), can display large unitary conductances in permissive environments, with multiple conductance states and long open and closed times that are gated by subtype-specific signaling mechanisms (including for example pH, voltage, cyclic nucleotides, and phosphorylation). The activation of ion-conducting RnAQP6 intrasubunit pores by Hg^{2+} and the block of intrasubunit water pores in other AQPs relies on covalent modification of cysteine residues likely inducing protein conformational changes, rather than electrostatic interactions that reversibly block the pores (seen for example with Ba^{2+} and Ca^{2+}). The cell type or membrane used

to measure AQP ion conductance can profoundly influence the observed ion channel activity. These properties are summarized in Table 1. Application of classical biophysical approaches, such as reconstitution into artificial membranes, may help further elucidate the functional properties of AQP ion channels, and guide future structure–function studies.

The structural basis of AQP-mediated transport activity

In addition to the signature asparagine-proline-alanine (NPA) motifs in loops B and E, AQPs also show high conservation of a Glu residue in M1, and a His residue proximal to the first NPA domain in loop B, found with few exceptions across the broad MIP family (Reizer et al. 1993), as illustrated in the subset of ion channel AQPs shown in Fig. 2. An in-depth analysis of the conserved Glu/His pair in the aquaglyceroporin HsAQP10 (Gotfryd et al. 2018) showed glycerol permeability of the intrasubunit pore was regulated by a pH-dependent interaction of the M1 Glu residue with the conserved loop B His residue. Expressed in adipocytes, AQP10 mediates an increased rate of glycerol flux in response to an acidic change in intracellular pH during lipolysis in response to beta-adrenergic stimulation, enabling the physiological release of glycerol. Molecular modeling by Gotfryd and colleagues indicated that protonation of H80 in HsAQP10 at low pH facilitated reorientation and interaction with E27, widening the diameter of the intrasubunit pore to allow glycerol flux, without changing the basal level of water flux. The presence of the Glu/His gate alone is not sufficient to endow glycerol permeability; a comparison with other AQPs which have identical amino acids in equivalent positions showed no glycerol permeability at any pH (for example in the orthodox channel AQP2), or glycerol permeability at neutral not acidic pH in aquaglyceroporins AQPs 3, 7 and 9 (Gotfryd et al. 2018). It is interesting to speculate that this Glu/His pair of residues could be a ubiquitous subcomponent of complex AQP gating mechanisms that would appear to need involvement of other subtype-specific domains to achieve specialized mechanisms of action. A possible role in regulating ion channel AQPs, particularly subtypes such as RnAQP6 and DmBIB thought to allow ion permeation via the intrasubunit rather than the central pore (Yool and Campbell 2012; Yool 2007), remains to be determined.

Differential block of water and ion fluxes by pharmacological agents in mammalian AQP1 supports the presence of independent parallel permeation pathways that use the central pore for ion conduction and intrasubunit pores for water (Pei et al. 2016; Kourghi et al. 2017b). In a subset of AQPs, sites have been identified for reversible block of water flux by extracellular TEA^+ at Tyr (Y), and covalent block by mercuric compounds at Cys (C), as noted in Fig. 2a and covered in prior reviews. Mutation of loop D residues changes ion channel activation and sensitivity to blockers of the ionic conductance (Kourghi et al. 2017b). A comparison of the level of sequence homology for the ion-conducting classes of AQPs is depicted as a phylogenetic tree (Fig. 2b), interestingly showing that the anion-preferring subtypes AQPs 0 and 6 are clustered together, as are the cation-preferring subtypes human AQP1 and plant PIP2;1 along with insect BIB. More distantly related are human AQP8 and plant NOD26, which are both permeable to NH_3 (Hwang et al. 2010; Saparov et al. 2007) and perhaps ammonium (NH_4^+) based on AQP8-induced rescue of NH_4^+-transporter-deficient yeast (Beitz et al. 2006b). The motifs that define the ion-conducting AQP subtypes are yet to be identified, but the process will be complicated for now by the likelihood that not all AQPs capable of conducting ions have been identified, and their potential activating stimuli remain unknown.

The substrate selectivity of the intrasubunit water pore is influenced by an electrostatic barrier formed by Asn side chains from the two NPA motifs, and by residues on the extracellular side of the NPA hourglass known as the aromatic/arginine selectivity filter that forms the narrowest section of the pore (Wang and Tajkhorshid 2007; Beitz et al. 2006b). The intrasubunit pore diameter is narrower in orthodox AQPs as compared with glycerol-permeable classes, accommodating single file water movement. The limiting diameter of the intrasubunit pore creates a selectivity filter, which in the water channel *E. coli* AQPZ, is framed by Phe and His as aromatic residues on one side, faced by a charged Arg on the opposite side. The aromatic residues at equivalent positions in the aquaglyceroporin *E. coli* GlpF are Trp and Phe, comparatively less polar than in the water-selective channels. Combined mutations of residues H-180, R-195, and F-56 in HsAQP1 enhanced permeability to NH_3, urea or glycerol without altering water permeability, whereas mutations neutralizing the Arg charge appeared to allow the passage of protons, reinforcing the role of these residues in AQP substrate selectivity in the intrasubunit pore (Beitz et al. 2006b).

The central pore, located at the four-fold axis of symmetry in the tetramer, mediates cyclic GMP-dependent activation of a nonselective cation conductance in mammalian AQP1, gated by the loop D domain (Yu et al. 2006). In crystal structures presumed to reflect the ion channel closed state, the central pore is lined by inner and outer rings of hydrophobic Leu and Val residues contributed by the M2 and M5 domains of the four subunits. As well, at the intracellular face of the central pore, the distal half (Leu, Gly, Gly) of the four loop D domains covers the cytoplasmic vestibule, flanked by charged Arg-rich proximal half of the loop D domains which serve as the site of interaction with cyclic GMP (Fig. 2c). Binding of cGMP is proposed to facilitate a conformational change in loop D, opening the loops outward

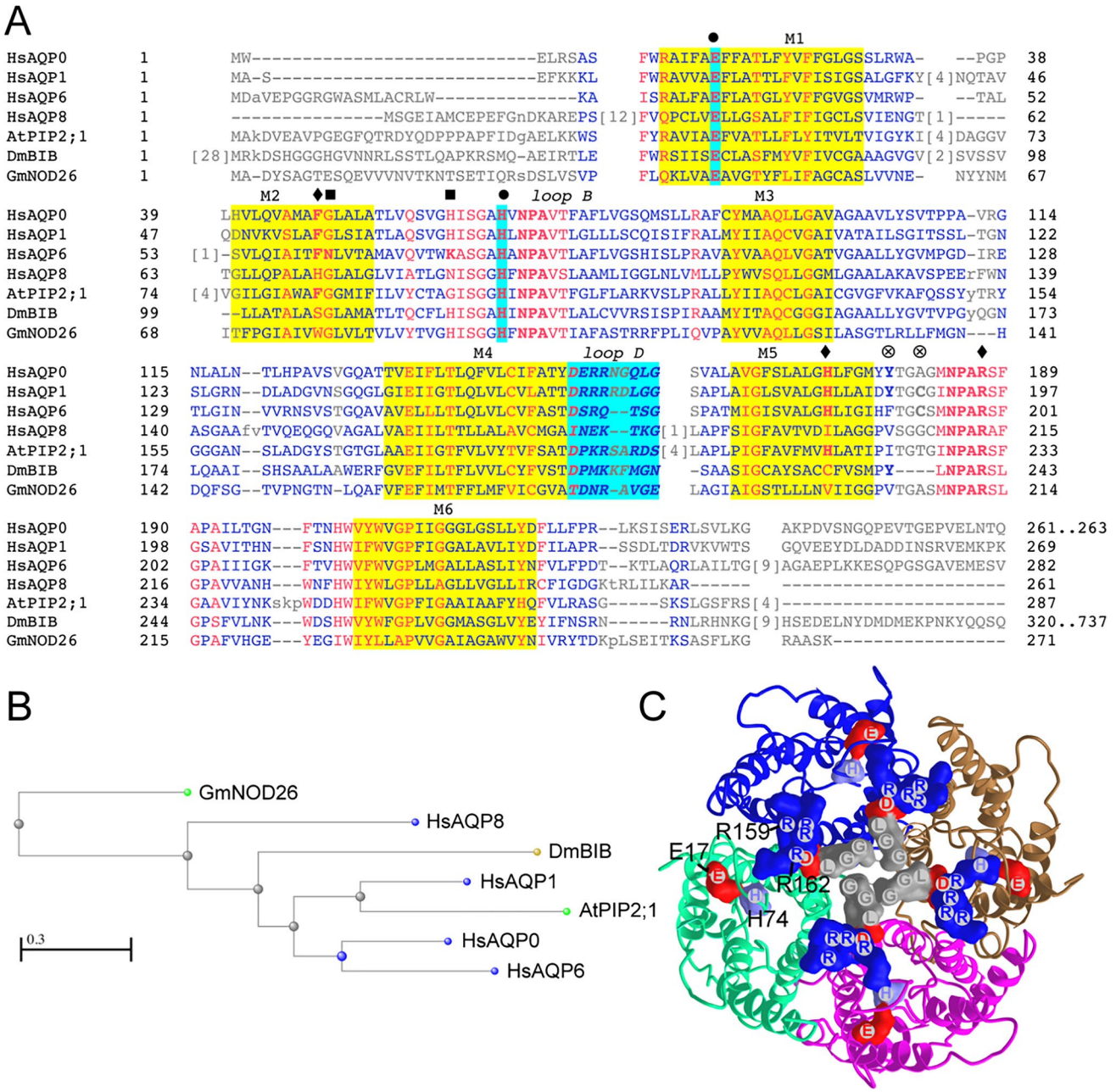

Fig. 2 Amino acid sequence alignment and structure of ion channel aquaporins. **a** Amino acid sequence alignment of ion-conducting mammalian, plant and insect aquaporin channels. Transmembrane domains are M1 to M6 (yellow highlight). AQP channel gating regions are found in M1, loop B, and loop D (teal highlight). Circles (●) mark residues involved pH-dependent gating of glycerol flux through the intrasubunit pore. Diamonds (♦) show residues in the aromatic/arginine filter domain, which influences substrate selectivity of intrasubunit pores. Crossed circles (⊗) show sites for reversible block of water flux by extracellular tetraethylammonium at Tyr (Y), and covalent block by mercuric compounds at Cys (C). Squares (■) mark Asn 60 in AQP6 which when mutated to Gly was reported to eliminate Hg^{2+}-induced anion permeation and enhance otherwise low osmotic water flux (Liu et al 2005), and Lys 72 which when mutated to Glu changed ion selectivity from anionic to cationic. Amino acid sequences are from *Homo sapiens* NP_036196.1 (HsAQP0); NP_932766 (HsAQP1); NP_001643.2 (HsAQP6); NP_001160.2 (HsAQP8); *Arabidopsis thaliana* P43286.1 (AtPIP2;1); *Drosophila melanogaster* NP_001260313.1 (DmBIB, 'Big Brain'), and *Glycine max* NP_001235870.2 (GmNOD26, nodulin-26). COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/) multisequence alignment was run at a 3 bit conservation setting (more stringent than default) to show strongly conserved residues (font red) and moderately conserved residues (font blue). **b** Phylogenetic tree depicting levels of sequence similarities for ion conducting AQP channels by proximity of branchpoints, from COBALT results of the sequence alignment shown in (A). **c** Structural view using the human AQP1 homotetramer (PDB ID 1IH5) as an example to illustrate locations of the different gating domains, generated using the National Center for Biotechnology Information interactive iCN3D viewer (https://www.ncbi.nlm.nih.gov/Structure), with gating regions E17, H74, and loop D depicted in space-fill format with charges indicated by color (red (-), blue (+), gray (neutral))

away from the center of symmetry. In silico modeling suggests the loop D reorientation triggers the opening of the inner hydrophobic barrier, followed by pore hydration, opening of the outer hydrophobic barrier, and Na^+ permeation. Site-directed double mutation of the first two Arg residues in the gating loop to Ala disrupted ion current activation by cGMP without altering osmotic water permeability, confirming the mutant channels were functional and targeted to the plasma membrane but lacked the Arg sites necessary for ion channel activation (Yu et al. 2006). The role of the central hydrophobic rings as barriers to ion permeation was tested by quadruple mutation of Val-50, Leu-54, Leu-170, Leu-174 all to Ala, which induced inward rectification and increased permeability to the bulky cation TEA^+ compared to wild type AQP1. In the same study, mutation of Lys-51 to Cys at the external side of the central pore (in a cysteine-less background) created de novo sensitivity of the ion conductance to block by mercury (Campbell et al. 2012). The C-terminus was found to have a modulatory role for AQP1 ion channel function (Boassa and Yool 2002). Phosphorylation of Tyr-253 in the carboxyl terminal domain, confirmed by Western blot, modulated the availability of the AQP1 channels to be activated by cGMP, suggesting that tyrosine phosphorylation state acts as a master switch (Campbell et al. 2012), perhaps one of many layers of control that sets the proportion of the AQP channel population that are dual water-and-ion channels, versus electrically silent pathways for principally just osmotic water flux. Even if only a tiny proportion (< 0.002%) of AQP1 proteins were functional as ion channels in an epithelium, the outcome was predicted to have a meaningful physiological effect on net fluid and Na^+ transport, based on quantitative modeling of renal proximal tubule function (Yool and Weinstein 2002).

Other AQPs such as AQP6 have been proposed to use the intrasubunit pores as the ion conducting pathways, based on the effects of specific mutations near the intrasubunit NPA domain and correlated effects on water permeation. Asn 60 in the AQP6 M2 domain (Fig. 2a) when mutated to Gly (characteristic of other AQP sequences at this position) was reported to eliminate the Hg^{2+}-induced anion permeation and enhance an otherwise low osmotic water flux as compared with wild type AQP6 (Liu et al. 2005). Lys-72 on the proximal side of the loop B NPA motif (Fig. 2a) when mutated to Glu changed AQP6 ion selectivity from anionic to cationic (Yasui et al. 1999a). A similar possibility has been proposed for the insect DmBIB aquaporin, which does not mediate osmotic water flux, but carries a nonselective cation current with properties that are strongly influenced by mutations of the conserved M1 Glu residue involved in intrasubunit pore gating (Yanochko and Yool 2004; Yool and Campbell 2012; Yool 2007).

In addition to gating the central pore in channels such as mammalian AQP1, loop D domains found in plant AQPs such as spinach SoPIP2;1 are regulated by phosphorylation to control activity of the intrasubunit water pores, in response to changes in pH and salinity (Törnroth-Horsefield et al. 2006). The extended length of the SoPIP2;1 loop D domain allows it to interact in the unphosphorylated state, via H-bonds with the N-terminus, to occlude the intrasubunit pore and impose a closed state. Supported by molecular modeling, phosphorylation was shown to disrupt tethering, unblocking the pore by releasing loop D from the cytoplasmic vestibule site, and also retracting a hydrophobic barrier in the pore-lining domain to promote water flux, as an open state (Törnroth-Horsefield et al. 2006).

In sum, aquaporin channels in the MIP family serve as key mechanisms for transport and homeostatic regulation of diverse processes in prokaryotes and eukaryotes, facilitating transmembrane fluxes of water, glycerol, urea, CO_2, nitric oxide, and other small solutes. Emerging evidence across mammalian, insect and plant classes of AQPs shows that subsets of these channels also can conduct ions, as has been shown thus far for AQPs 0, 1, and 6, *Drosophila* BIB, soybean NOD26, and *Arabidopsis* AtPIP2;1. More classes are likely to be discovered.

Physiological relevance of AQP ion conductivity

The biological roles of the subset of aquaporins that can transport both water and ions is a fascinating area of work that has only begun to be explored. AQP0, expressed in lens fibers, functions as an adhesive protein forming thin membrane junctions with low water permeability when expressed in *Xenopus* oocytes (Bloemendal and Hockwin 1982; Zampighi et al. 1985), and has been linked to regulation of gap junction channels, cell–cell adhesion and maintenance of ocular lens transparency (Chandy et al. 1997; Chepelinsky 2009). Reduced membrane expression of AQP0 channels due to missense mutations leads to impaired water flux and congenital cataracts in humans and mice (Berry et al. 2000; Varadaraj et al. 1999). Expression of AQP1 in the lens of $AQP^{-/-}$ knockout mice only partially restored lens transparency, which could reflect the additional role of AQP0 in cell–cell adhesion (Varadaraj et al. 2010). AQP0 but not AQP5 null mice showed a reduced compressive load-bearing capacity in lens, suggesting the cell–cell adhesion function of AQP0 contributes to biomechanical properties (Sindhu Kumari et al. 2015). AQP0 permeability to ions has been demonstrated in bilayers, but the physiological function of the ion conductance remains to be defined. The role of AQP0 ion channels in maintaining optimal lens transparency could involve the generation of osmotic gradients or regulation of transmembrane signals.

In addition to normal physiological roles in fluid transport and homeostasis, some classes of AQPs have been implicated in augmenting cancer cell invasion and metastasis, by mechanisms suggested to involve local volume regulation supporting membrane process extension and cytoskeletal assembly (De Ieso and Yool 2018; McLennan et al. 2020). Expression of HsAQP1 is upregulated in gliomas, mammary, lung and colorectal carcinomas, hemangioblastoma, glioblastoma, ovarian, and gastric cancers and multiple myeloma (Saadoun et al. 2002; El Hindy et al. 2013; Endo et al. 1999; Hoque et al. 2006; Moon et al. 2003; Vacca et al. 2001; Chen et al. 2006; Longatti et al. 2006; Nagashima et al. 2006; Verkman et al. 2008; Wang et al. 2020; Wei and Dong 2015; Zhang et al. 2012). AQP1 plays a significant role in tumor cell metastasis and invasion, which are critical in cancer progression (De Ieso and Yool 2018). Water flux mediated by AQPs has an important role in facilitating formation of lamellipodia involved in cell motility and migration (Oster and Perelson 1987). AQP1-mediated osmotic water flux could occur in response to solute influx and actin depolymerisation at the leading edges of migrating cells (Papadopoulos and Verkman 2013). AQP1 facilitated water fluxes may also enable changes in cell shape and volume of migrating tumor cells, particularly relevant during movement through tight extracellular spaces (Papadopoulos et al. 2008). An 'osmotic engine model' proposed by Stroka and colleagues linked the water flux and ion transport mediated by aquaporins and Na^+/H^+ pumps as keys for rapid cell migration (Stroka et al. 2014). Polarized distributions of ion channels and transporters such as AQP1, K^+ and Cl^- channels in the leading edges of migrating cells are consistent with a direct role in osmotic fluid flow for membrane extension and motility (Schwab and Stock 2014; Stock and Schwab 2015). Inhibiting the AQP1 ion conductance with pharmacological blockers AqB011 and 5-hydroxymethyl furfural impaired invasiveness of colorectal (HT29) and breast cancer (MDA) tumor cells (De Ieso et al. 2019; Chow et al. 2020; Kourghi et al. 2016). Combined block of both ion and water fluxes mediated by AQP1 more potently inhibited cell migration in colon cancer cells than either alone (De Ieso et al. 2019). Both water flux and ion currents mediated by AQP1 contribute to the acceleration of cell migration and invasion in subtypes of cancers that upregulate this class of channel during pathological cancer progression.

AQP6, in intracellular acid-secreting vesicles intercalated in the renal collecting duct, is co-localized with H^+-ATPase (Kwon et al. 2001; Yasui et al. 1999b). The V-type H^+-ATPase causes acidification of intracellular organelles in the kidney renal collecting duct; the Cl^- channel ClC-5 co-localized with H^+-ATPase is thought to provide electroneutral balance (Günther et al. 1998). Interestingly, ClC-5 deactivates at pH values below 6.5 while the anion conductance of AQP6 is turned on at pH values below 6.5, suggesting AQP6 and CLC-5 functions could be complementary (Rambow et al. 2014). AQP6 mediated NO_3^- permeation across the vesicle membrane could be linked to the regulation of H^+-ATPase activity in acid-secreting intercalated cells (Arai et al. 1989; Ikeda et al. 2002). AQP6 is expressed in some ovarian cancers and appears to have a protective role in certain viral pathologies but mechanisms and roles in these processes remain unknown (Ma et al. 2016; Molinas et al. 2016).

Big Brain in *Drosophila*, in parallel with the other neurogenic genes Notch (transmembrane receptor) and Delta (ligand for Notch), regulates early development of the nervous system (Artavanis-Tsakonas et al. 1999), contributing to interactions that govern the neuroblast versus epidermal cell fate by a process of lateral inhibition (Doherty et al. 1997; Rao et al. 1990). Loss of function mutations in this gene cause defects in cell fate determination during neurogenesis, and deleterious overgrowth of the nervous system (Lehmann et al. 1983; Brand and Campos-Ortega 1988; Artavanis-Tsakonas et al. 1999). BIB functions as a voltage insensitive non-selective monovalent cation channel when expressed in *Xenopus* oocytes, but unlike most AQPs shows no water permeability (Yanochko and Yool 2002; Yool and Stamer 2004). Ion conductance is decreased when oocytes expressing BIB are treated with insulin, and enhanced by the tyrosine kinase inhibitor lavendustin A. The level of BIB activity depends on the pattern of phosphorylation at multiple tyrosine kinase consensus sites in the long C-terminal domain. Tyrosine kinases downstream of growth factor receptors mediate multiple aspects of neural development; the cation conductance mediated by BIB has been proposed to lead to membrane depolarisation in turn influencing epidermal cell fate determination (Yanochko and Yool 2002). Mutation of a glutamate residue in the first transmembrane domain to asparagine (E71N) in BIB abolishes ion channel activity, and when E71N is co-expressed with wild type BIB, it appears to impose a dominant-negative effect (Yool 2007).

Nitrogen is an essential plant macronutrient required for growth and reproduction. The ion-conducting AQP GmNOD26 in soybean could contribute to the essential role of the nodule in nitrogen fixation. The symbiosome compartment is acidic due to H^+-ATPase activity, which would compromise neutral NH_3 export out of the symbiosome (Masalkar et al. 2010). As NH_3 is protonated to ammonium NH_4^+, the movement of charged NH_4^+ across the symbiosome membrane is facilitated by the transmembrane potential generated by H^+-ATPase activity (Udvardi and Day 1997). A non-selective cation channel (NSCC) conductance permeable to NH_4^+ was identified in symbiosome membranes in soybean and *Lotus japonicus* but the molecular identity remained unknown (Obermeyer and Tyerman 2005; Roberts and Tyerman 2002; Tyerman et al. 1995). NOD26 has a binding site in the C-terminus for glutamine

synthetase (GS) which carries out the first enzymatic step in the NH_3 assimilation pathway, and uses NH_4^+ as a substrate. It is interesting to consider the idea that NOD26, if shown to be permeable to NH_4^+, could contribute to a protein complex which exports fixed nitrogen (such as NH_4^+) from the nodule into the host plant, supporting the symbiotic relationship (Eisenberg et al. 2000; Masalkar et al. 2010). The proposed role of NOD26 as the NSCC permeable to NH_4^+ remains to be explored.

Arabidopsis PIP2;1 in plant cell plasma membranes is highly permeable to water, and regulated by divalent cations, Ca^{2+} and low pH (Alexandersson et al. 2005; Verdoucq et al. 2008). Expression of AtPIP2;1 in *Xenopus* oocytes confers cation conductance (Na^+) also sensitive to Ca^{2+} and low pH (Byrt et al. 2017). Two related PIPs AtPIP2;1 and AtPIP2;2 from Arabidopsis and one from barley HvPIP2;8 also serve as ion-permeable channels (Qiu et al. 2020; McGaughey et al. 2018; Tran et al. 2020). The NSCC identified in roots (Demidchik and Tester 2002) appears to be mediated by the AtPIP classes of non-selective cation channels (Byrt et al. 2017).

The adaptive benefits of dual ion and water transport by plant aquaporins are likely to include improved tolerance of salinity stress. Increased salinity leads to reduced phosphorylation, internalization of AtPIP2;1 into intracellular vesicles, and recycling in vacuoles (Boursiac et al. 2005; Ueda et al. 2016; Luu et al. 2012). Na^+ taken in during the endocytosis is sequestered in vacuoles as a mechanism to reduce Na^+ toxicity (McGaughey et al. 2018). Regulation of AQP ion and water conducting states via phosphorylation is likely to be a key mechanism for modulating relative ion fluxes (Na^+ and K^+) and water across cell membranes in response to salinity, limiting water loss as a mechanism for survival in saline soils. Among the kingdoms of life, plants have the greatest diversity of classes of aquaporins, leaving open the possibility that there are many aquaporins with yet unidentified functions that might provide a portfolio of highly specialized ion and water conducting states, which can be tuned for optimal growth promoting responses to diverse environmental conditions.

Future directions

Functional insights from heterologous hosts

Heterologous expression systems, such as yeast or bacteria, are useful approaches for studying ion channel function (Tomita et al. 2017; Locascio et al. 2019; Fairbairn et al. 2000). Examples include screening of known ion channels for channel modulators (Zaks-Makhina et al. 2004; Kawada et al. 2016), mutagenesis to identify key residues (Ros et al. 1999), and evaluation of intracellular trafficking mechanisms (Bernstein et al. 2013; Bagriantsev et al. 2014). Heterologous expression systems also can be adapted as through-put screening tools to enable discovery of new classes of ion channels. The main challenges are in establishing a robust phenotype that shows ion selective effects, and in developing tools for correlating the ion channel function with measureable parameters such as cell growth or optical probe signal intensity.

Yeast and bacteria have evolved selective transport systems for acquiring K^+ and excluding Na^+ (Yenush 2016; Stautz et al. 2021). Inactivation of K^+ uptake genes in the *S. cerevisiae* yeast strain Δ *trk1* Δ *trk2* (Sentenac et al. 1992) and in *E. coli* (Epstein et al. 1993) has yielded cell lines that survive only when supplemented with high concentrations of external K^+. Expression of plant (Rubio et al. 1995) and mammalian (Tang et al. 1995) K^+ channel genes rescues cell growth in standard low extracellular K^+, enabling the mass-throughput characterization of channel activity and K^+ conduction. Fluorescence-based monitoring of ion flux kinetics also in possible using heterologous expression systems (Zhou et al. 2007). A few studies have used heterologous expression in yeast to study the potential ion permeability of AQP1 (Wu et al. 2009) and plant PIP aquaporins (Byrt et al. 2017; Qiu et al. 2020). Yeast mass-throughput assays could provide a rapid tool for the future evaluation of novel classes of AQPs as possible cation channels, and identify candidate pharmacological and regulatory compounds as tools for further research.

In silico predictions

Molecular dynamics (MD) simulations have been used to provide insight into channel functions that are difficult to dissect with conventional methods. The number of MD studies focused on investigating AQPs is increasing steadily in parallel with those on other channels (Fig. 3). Solving the structural architectures of AQP1 (Murata et al. 2000; Sui et al. 2001), bacterial aquaglyceroporin (GlpF) (Fu et al. 2000), and others has allowed in depth analyses of substrate permeation through AQP pores at the atomic level (Tajkhorshid et al. 2002). The first AQP shown by MD simulation to be permeable to ions was HsAQP1 (Yu et al. 2006). Sodium and Cl^- transport via monomeric and central pores of HsAQP4 has also been predicted (Bernardi et al. 2019). MD simulations of AQPs have provided views into the molecular basis of features such as substrate selectivity (Hub and De Groot 2008), proton exclusion (Chakrabarti et al. 2004), gating mechanisms (Törnroth-Horsefield et al. 2006; Fischer et al. 2009), and the conduction of gases and signaling molecules (Wang et al. 2007; Yusupov et al. 2018). MD simulations have provided insights into the critical residues (Hadidi and Kamali 2021), post-translational modifications (Sachdeva and Singh 2014), drug interactions (De Almeida

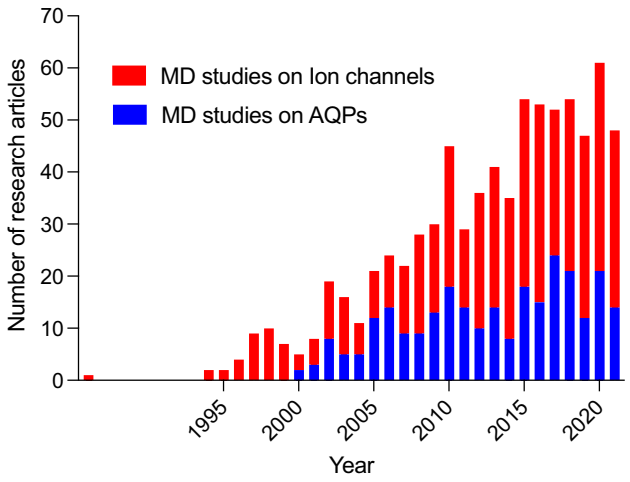

Fig. 3 Comparison of Molecular Dynamics Simulation studies in ion channel and aquaporin fields. The number of publications per year from Web of Science that includes the term "molecular dynamic" and "ion channels" or "aquaporins" in either the tittle, abstract or keywords. (https://www.webofscience.com/) in August 2021

et al. 2017), and voltage-dependence properties (Hub et al. 2010; Mom et al. 2021) that impact the channel function. Given the large number of structures (~50) determined for aquaporins by X-ray diffraction and cryo-electron microscopy, computational techniques to simulate permeation of ions or coupling of water and ion transport via these channels are within reach as exciting new areas of research. Further applications might include simulating protein channel activity in different lipid mixtures relevant to a variety of cell membranes (Aponte-Santamaría et al. 2012; Gu et al. 2017), modeling multiple aquaporins isoforms simultaneously (Pei et al. 2019; Kapilan et al. 2018), and evaluating mechanisms of block and potentiation of ion conductance and other substrate fluxes by pharmacological AQP modulators that have been derived from synthetic (Chow et al. 2020; Huber et al. 2007; De Ieso et al. 2019) and natural (Aung et al. 2019) sources.

Conclusions

The first AQP ion channels were discovered almost 40 years ago. Their biophysical properties are likely to be defined not only by their tetrameric architecture, but also by the lipid environments in which the AQP tetramers reside, as well as interacting proteins and signaling mechanisms. Residues involved in the selectivity, gating, and permeation pathways of the central and intrasubunit pores of ion-conducting AQP tetramers are being uncovered. These candidate motifs can be investigated with the classical array of electrophysiological methods, but continuing work will benefit from incorporating novel approaches using microorganisms (e.g., yeast, bacteria) and computational methods such as MD simulation, to further define the functional roles of ion conductivity in classes of AQPs across all the biological kingdoms of life.

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Declarations

Ethics approval and consent to participate This study did not use animals or human subjects and did not require ethics approval. This study did not involve human participants. Informed consent is not applicable.

Consent for publication This manuscript does not contain any individual person's data in any form. Informed consent is not applicable.

Conflict of interest The authors declare no competing interests.

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