ANNUAL REVIEWS

Annual Review of Pharmacology and Toxicology

Targeting the Trafficking of Kidney Water Channels for Therapeutic Benefit

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Annu. Rev. Pharmacol. Toxicol. 2020. 60:175–94

First published as a Review in Advance on September 27, 2019

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

https://doi.org/10.1146/annurev-pharmtox-010919-023654

Keywords

aquaporin-2, urine concentration, vasopressin receptor type 2, collecting duct principal cells, endocytosis, exocytosis

Abstract

The ability to regulate water movement is vital for the survival of cells and organisms. In addition to passively crossing lipid bilayers by diffusion, water transport is also driven across cell membranes by osmotic gradients through aquaporin water channels. There are 13 aquaporins in human tissues, and of these, aquaporin-2 (AQP2) is the most highly regulated water channel in the kidney: The expression and trafficking of AQP2 respond to body volume status and plasma osmolality via the antidiuretic hormone, vasopressin (VP). Dysfunctional VP signaling in renal epithelial cells contributes to disorders of water balance, and research initially focused on regulating the major cAMP/PKA pathway to normalize urine concentrating ability. With the discovery of novel and more complex signaling networks that regulate AQP2 trafficking, promising therapeutic targets have since been identified. Several strategies based on data from preclinical studies may ultimately translate to the care of patients with defective water homeostasis.

INTRODUCTION

Aquaporins (AQPs) are highly conserved, integral membrane proteins that facilitate osmotically driven transport of water across cell membranes (1, 2). In humans there are 13 isoforms, and 7 of these are found in the kidney: AQP1, 2, 3, 4, 6, 7, and 11 (3). Among these, AQP2 is the most highly regulated and is the focus of this review. Its expression and cellular trafficking are controlled by the antidiuretic hormone, vasopressin (VP), and it plays a crucial role in water conservation. A decrease in blood volume or an increase in blood osmolality causes VP release from the posterior pituitary gland (4). When VP binds to the vasopressin type-2 receptor (V2R), a G protein–coupled receptor (GPCR), it stimulates adenylyl cyclase and increases intracellular cyclic AMP (cAMP). Elevated cAMP activates protein kinase A (PKA), which is involved in AQP2 phosphorylation. This change in phosphorylation status promotes AQP2 accumulation on the apical plasma membrane of collecting duct principal cells and results in osmotic water reabsorption from urine into the hypertonic interstitium through AQP2 (**Figures 1** and **2**). This restores blood volume and normalizes blood osmolality. Upon restoration of water balance, VP release from the pituitary gland ceases and AQP2 is rapidly sequestered into endosomes within cells, thus reducing collecting duct water permeability.

Dysfunction of the V2R/AQP2 signaling pathway results in diseases of water balance. For example, nephrogenic diabetes insipidus (NDI) (5) is caused by a renal insensitivity to VP. This

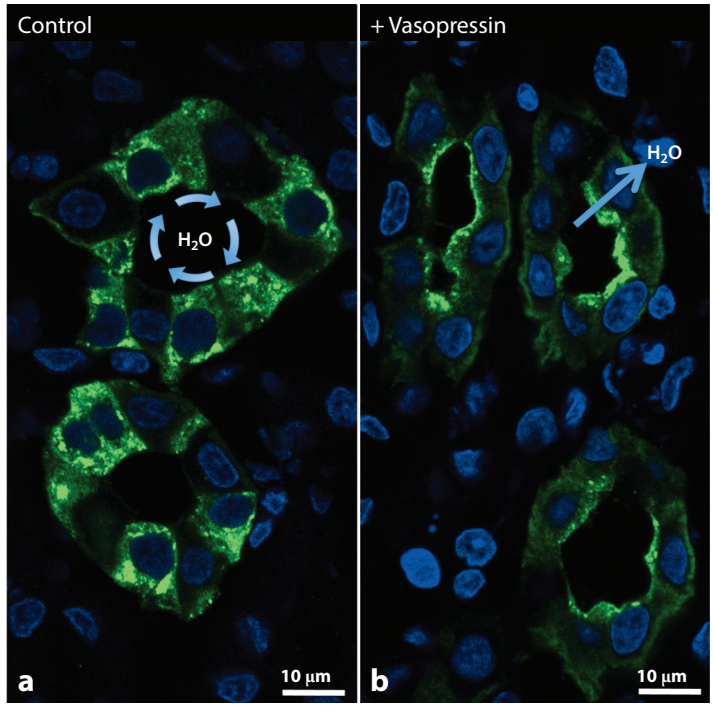

Figure 1

Aquaporin-2 (AQP2) accumulates at the apical membrane of kidney collecting duct principal cells when stimulated by vasopressin (VP), increasing water permeability and reabsorption. (*a*) Under baseline conditions, AQP2 (immunostained in *green*) is distributed mainly in the cytoplasm of the principal cells, and water (H_2O) reabsorption from the lumen is low. (*b*) When cells are exposed to VP, AQP2 accumulates in the apical membrane, increasing its water permeability and allowing reabsorption across the epithelium.

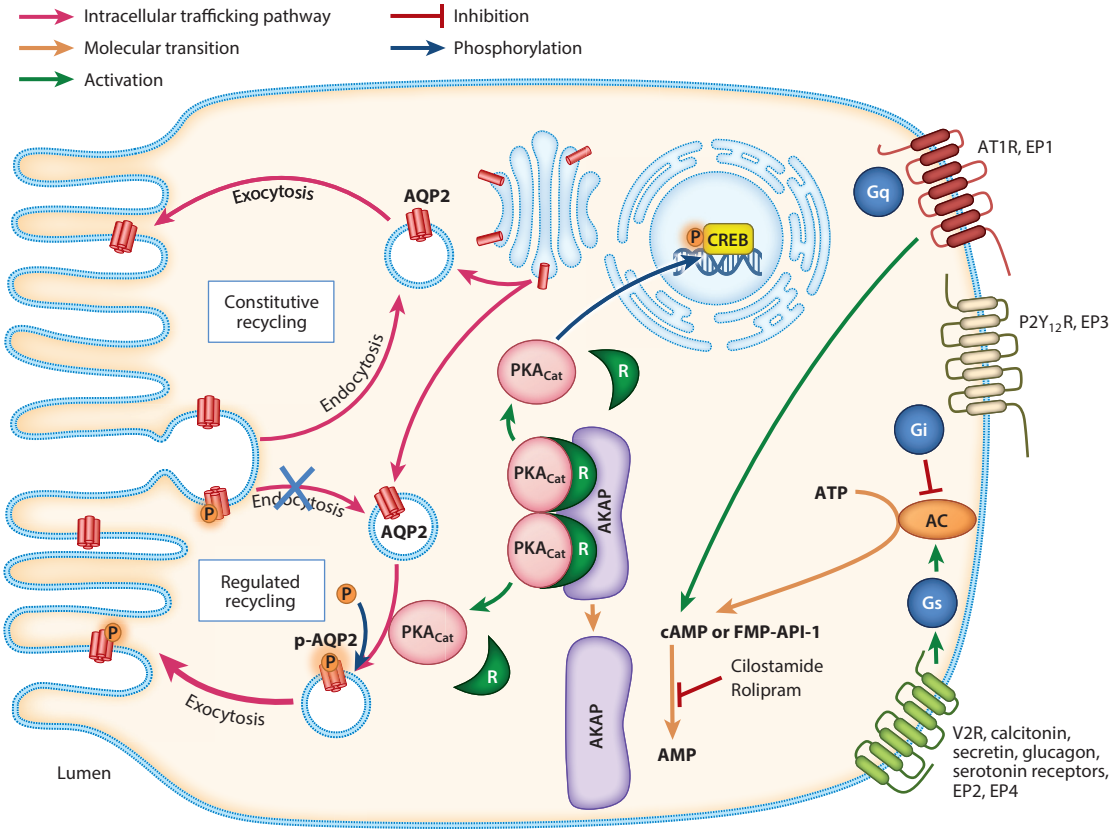

Figure 2

Canonical cyclic AMP (cAMP) signaling pathways involved in aquaporin-2 (AQP2) trafficking. When vasopressin (VP) binds to the vasopressin receptor type 2 (V2R), a G protein–coupled receptor, the Gs (stimulatory) subunit of the G protein activates adenylyl cyclase (AC), which in turn hydrolyzes ATP to increase intracellular cAMP. cAMP activates protein kinase A (PKA) by dissociating its catalytic and regulatory subunits, and PKA then phosphorylates AQP2. A major effect of AQP2 phosphorylation is to decrease its rate of endocytosis, but phosphorylation is not necessary for the constitutive recycling and insertion of AQP2 into the plasma membrane. VP treatment of cells also increases the rate of exocytosis, but this is not AQP2 phosphorylation dependent, and the mechanism remains unclear. This combination of decreased endocytosis and increased exocytosis upon VP exposure results in a net accumulation of AQP2 in the plasma membrane, thus increasing transepithelial water permeability. This schema highlights the potential therapeutic targets along this canonical signaling pathway to increase AQP2 membrane accumulation and increase water permeability and reabsorption. Similar to VP, calcitonin, secretin, glucagon, serotonin, EP2, and EP4 all activate Gs and trigger a similar signaling pathway to stimulate AQP2 membrane trafficking. $P2Y_{12}R$ and EP3 are Gi (inhibitory regulating G protein) receptors, and their antagonists will remove the inhibitory signal that blocks AC activity. Angiotensin II activates AT1R, which belongs to the family of Gq-linked receptors; it activates the phosphatidylinositol signaling pathway and increases cAMP levels. Inhibiting the degradation of cAMP to AMP using phosphodiesterase inhibitors such as cilostamide or rolipram increases cAMP levels and could induce AQP2 membrane accumulation. In the resting state, PKA catalytic subunits are tethered by A kinase–anchoring proteins (AKAPs). An increase in intracellular cAMP causes dissociation of the AKAP-PKA complex, releasing PKA catalytic subunits. Using the AKAP-PKA disruptor FMP-API-1, PKA substrates, including AQP2, are phosphorylated, increasing AQP2 membrane accumulation. PKA also acts in the nucleus by phosphorylating CREB, which in turn activates *AQP2* gene transcription, raising AQP2 levels within the cell over several hours.

condition is characterized by excessive thirst and polyuria—up to 20 L of urine a day. NDI can be congenital or acquired. In the congenital form, 90% of patients have mutations of *AVPR2* (an X-linked disease), and 10% have mutations of *AQP2* (an autosomal disease). Infants and young children with congenital NDI frequently require enteral feeding or intravenous fluid hydration

due to their inability to keep up with urinary loss, and they are often underweight and relatively short. If untreated, NDI can lead to permanent brain damage, impaired mental function, and intellectual disability. In acquired NDI, there is an alteration or dysregulation of the signaling pathway that affects AQP2 expression and/or trafficking. While there are several causes, NDI is most common in bipolar patients taking lithium. Up to 55% of these patients report various degrees of NDI (6). This may require patients to stop lithium, a very effective mood stabilizer, causing suboptimal control of bipolar symptoms. The current treatment for NDI is supportive care, requiring patients to hydrate aggressively to match their urine output, void frequently to avoid hydronephrosis and bladder dysfunction, decrease dietary solutes such as salt and protein to reduce solute excretion and associated water loss, and take thiazide diuretics to excrete salt or nonsteroidal anti-inflammatory drugs (NSAIDs) to increase urine concentrating ability.

On the other end of the spectrum, disproportional water reabsorption caused by excessive VP signaling and overexpression of AQP2 are seen in patients with syndrome of inappropriate ADH secretion (SIADH), heart failure, and liver cirrhosis (5). These diseases are characterized by low blood sodium levels (hyponatremia). Acute hyponatremia can cause seizure, cerebral edema, and death, and when it becomes chronic, patients are at risk of confusion, fall, osteoporosis, and hip fracture (7, 8). Hyponatremia is a significant economic burden in healthcare systems worldwide (9, 10), as it prolongs hospital stay, increases the risk of rehospitalization, and increases morbidity and mortality. The mainstay of treatment is strict free water restriction and a loop diuretic such as furosemide. For heart failure or cirrhotic patients, salt intake is restricted due to concerns of fluid overload, which could result in decompensation of the underlying diseases. On the other hand, patients with SIADH are advised to liberalize salt intake, which often exacerbates hypertension and cardiovascular diseases. Urea powder could also be used to induce nonspecific osmotic water excretion, but its cost is often prohibitory (11). One available treatment for hyponatremia caused by excessive VP production is the selective V2R antagonist tolvaptan. However, its use requires close in-hospital monitoring of blood sodium level and is limited to 30 days due to liver toxicity (12).

The lack of more specific, effective, and safer treatments for these potentially serious water balance disorders highlights the need for further understanding of signaling pathways that regulate AQP2 trafficking. In addition to the canonical V2R signaling pathway described above, a growing number of studies are focusing on modulating AQP2 trafficking and expression by bypassing the V2R, and preclinical studies have shown some promising effects on water regulation. Several of the drugs envisaged are currently used clinically for other indications, and there are scattered case reports of successful amelioration of NDI without significant adverse effects. This review focuses on summarizing these potential therapeutic strategies, based mostly on preclinical data, and on exploring new approaches to treat disorders of water balance. We include a comprehensive table (**Supplemental Table 1**) highlighting the cell, tissue, and animal models available to study AQP2 biology.

Supplemental Material >

TARGETING THE V2R/cAMP/PKA SIGNALING PATHWAY

cAMP is an important second messenger in the regulation of AQP2 trafficking. Forskolin, a direct activator of adenylyl cyclase, which generates cAMP (13), is frequently used as a positive control in AQP2 studies. cAMP elevation has two major roles in the antidiuretic response: First, it stimulates the acute accumulation of AQP2 in the plasma membrane by affecting its trafficking; second, it chronically increases AQP2 expression via a cAMP-responsive element in the *AQP2* gene promoter region (14). Increasing cAMP concentration in target cells in a VP-independent manner is, therefore, an appealing potential therapeutic strategy for the treatment of NDI.

Alternative G Protein–Coupled Receptor Agonists

The use of GPCR agonists similar to VP has been explored as a treatment option for congenital NDI: Endogenous hormones like calcitonin, glucagon, and secretin are such examples (see **Figure 2**). They all have their own specific GPCR on the basolateral surface of kidney principal cells, with differential expression in various segments of the tubules. When bound to their receptors, they trigger cAMP/PKA signaling and increase AQP2 apical expression. They are discussed in more detail below.

Calcitonin. Calcitonin is primarily secreted by thyroid parafollicular cells in response to hypercalcemia, and it was used to correct the concentrating defect in VP-deficient Brattleboro rats by de Rouffignac & Elalouf (15). Our group showed that calcitonin-stimulated AQP2 membrane accumulation was most noticeable in cortical collecting ducts and connecting segments, which express calcitonin receptors. Calcitonin-treated, VP-deficient Brattleboro rats had a reduced urine flow and twofold higher urine osmolality within 12 h of treatment (16), and the effect persisted for over 24 h. However, the urine-concentrating effect diminished thereafter with continuous infusion. The urinary response to calcitonin was restored by spacing the dosing to once daily, avoiding receptor desensitization or downregulation. This daily dosing frequency would probably be easier to adhere to in potentially treating NDI patients. Calcitonin might also be used for short-term amelioration of NDI symptoms, perhaps to allow uninterrupted sleep or for when access to water or toilet facilities is limited. While the effects of calcitonin on serum calcium (Ca^{2+}) levels might be of concern, it is noteworthy that, despite continuous infusion, no significant change in the Ca^{2+} level in rats was noted (16). Calcitonin is approved in patients for the treatment of osteoporosis and bone pain, but its clinical use is limited. Recently, the European Medicines Agency and US Food and Drug Administration (FDA) advised that calcitonin not be used for more than six months due to a small increase in the risk of various cancers (17–20). Clinical trial and post-marketing experiences reported that patients can experience peripheral or generalized edema, which may support the potential role of calcitonin in water retention. However, there are also case reports of polyuria (21), so whether calcitonin would be effective in treating NDI is still unclear.

Glucagon. Glucagon plays a major role in the regulation of blood sugar levels, and its action is mainly on hepatic glycogenolysis and gluconeogenesis (22). Using radiolabeled glucagon, Butlen & Morel (23) showed that the nephron has a specific receptor for glucagon, and glucagon enhances glomerular filtration rate (24) and regulates electrolyte balance (25). Bailly et al. (26) showed that distal nephrons are highly responsive to glucagon, and glucagon produced a dose-dependent stimulation of adenylyl cyclase in cell-free preparations of human renal medulla (27). Maeda et al. (28) showed, however, that glucagon enhances intracellular cAMP levels only in initial parts of the inner medullary collecting duct (IMCD). Glucagon is thought to increase distal water reabsorption via several mechanisms: increasing glomerular filtration rate (24), stimulating urea synthesis in the liver and urea excretion in the kidneys to enhance distal water delivery (24), and increasing AQP2 apical membrane expression by stimulating cAMP generation (25). As a result, glucagon induces a dose-dependent increase in water reabsorption (25).

Secretin. Secretin, a ligand of the secretin receptor, also has a VP-like effect (29). Secretin increased cAMP concentrations in isolated IMCD tubule suspensions from wild-type and tamoxifen-induced V2R knockout (KO) mice (30). Secretin receptor KO mice exhibited mild polyuria, polydipsia, and reduced renal expression of AQP2 (31). Interestingly, combining secretin

and a statin resulted in a significant reduction in urine output and an increase in urine osmolality in NDI mice (30) (see the section titled Combining Therapies).

Serotonin. Fabian et al. (32) reported that paroxetine, a selective serotonin reuptake inhibitor (SSRI), could induce hyponatremia in 12% of elderly patients within 9 days. A meta-analysis of four population-based studies showed that not only SSRIs but also serotonin-norepinephrine reuptake inhibitors caused an increased risk of hyponatremia compared to other classes of antidepressants (33). The findings of urine sodium, urine osmolality, and serum ADH levels are consistent with SIADH, although increased plasma ADH levels have not yet been demonstrated in all cases reported in humans. Fluoxetine, another SSRI, directly increased AQP2 protein abundance by 40% in rat IMCD and increased water permeability and absorption after 10 days of treatment without changing the plasma level of ADH (34). The exact mechanism of SSRI salt and water regulation in the kidney is unclear, but it possibly works by increasing serum serotonin levels and activating serotonin 5-HT1A receptors (GPCRs) (35) that affect multiple transporters and channels in different kidney regions, resulting in hyponatremia.

Angiotensin II

Besides stimulating aldosterone production and increasing salt retention via the angiotensin II type 1a receptor (AT1a), angiotensin II also has a direct role in AQP2 expression (36, 37). Angiotensin II itself increases cAMP levels and increases AQP2-associated water reabsorption (38). It can also enhance VP-induced cAMP accumulation (39, 40), and angiotensin receptor blockade has been shown to decrease urinary concentration and AQP2 expression induced by VP (41). Mice deficient in AT1a demonstrated a VP-resistant NDI, having a threefold increase in 24-h urine excretion that is not reversible by exogenous VP or fluid restriction (42). The potential interaction of these hormones at the molecular and cellular levels has important clinical implications, as they are often dysregulated in human diseases such as heart failure.

Purinergic Receptor $P2Y_{12}R$ Antagonists

The purinergic receptor $P2Y_{12}R$ is an inhibitory GPCR expressed in collecting duct principal cells. Activation of the $P2Y_{12}R$ results in a reduction of cAMP levels, suggesting that the $P2Y_{12}R$ antagonist clopidogrel may increase cAMP levels and improve urine concentration. Indeed, clopidogrel decreased polyuria, increased urine osmolality, and increased AQP2 abundance in mice with lithium-induced NDI (43). However, clopidogrel itself did not increase urine osmolality in VP-deficient Brattleboro rats, indicating that it works by augmenting the effect of VP. Clopidogrel is an antiplatelet agent used to treat and prevent myocardial infarction, stroke, and peripheral artery diseases. The risk of major bleeding events (44–46) associated with this drug probably outweighs its potential benefit as a treatment for NDI.

Prostaglandin E2 Antagonists

Prostaglandin E2 (PGE2) is a ligand of four different G protein–coupled E-prostanoid receptors: EP1–EP4. EP2 and EP4 are classified as stimulatory G protein–coupled receptors, as they are known to elevate intracellular cAMP (47). In MDCK cells, PGE2 and butaprost, a selective agonist of EP2, both increased cAMP levels and AQP2 activity. In addition, butaprost increased urine concentrating ability in a V2R-inhibited NDI rat model (48). In IMCD cells, PGE2 seems to exert its effect on AQP2 via EP4, as selective agonists of EP4 ONO-AE1-329 increased cAMP

levels and AQP2 activity, and ONO-AE1-329 improved urine-concentrating ability in V2R KO mice (49). Collecting duct–specific EP4 KO mice showed impaired urine-concentrating ability (49, 50). On the other hand, EP1 and EP3 seem to have inhibitory effects on water permeability in renal collecting ducts (51). EP1 activation inhibits water reabsorption in isolated perfused collecting ducts via Gq protein regulation (52), and renal EP3 antagonizes VP and AQP2 membrane trafficking by attenuating cAMP production (53). Clinically, indomethacin and other PGE2 inhibitors such as NSAIDs have been used in treating NDI by increasing AQP2 membrane expression and reducing urinary volume (54), but overuse of NSAIDs in even otherwise healthy patients could cause hyponatremia (55). The effect of indomethacin on urine concentration was not observed in EP3-null mice (53). These findings suggest that the baseline activation of EP3 in the collecting ducts plays a more significant role compared to other E-prostanoid receptors in tonically inhibiting AQP2 membrane expression, similar to epidermal growth factor (EGF) (56).

Phosphodiesterase Inhibitors

cAMP-phosphodiesterases (PDEs) degrade cAMP to AMP and decrease intracellular cAMP levels. The PDE4 inhibitor rolipram increased VP-induced cAMP accumulation in IMCDs of NDI mice to near normal levels, and the PDE3 inhibitor cilostamide, although not having a significant effect on cAMP alone, augmented the effect of rolipram when used in combination (57). Interestingly, these NDI mice have an overactive total cAMP-PDE that greatly reduces the intracellular cAMP content of principal cells, even in the presence of VP (57). Further studies in mice showed that rolipram can not only increase cAMP but also promote the translocation of wild-type, but not mutated, AQP2 toward the apical membrane (58). However, when rolipram was used to treat two patients with X-linked NDI, it failed to relieve the polyuria (59). However, this was a very preliminary study, and pharmacokinetics were not followed. In addition, there may be differences in AMP metabolism between mice and humans, and reducing the degradation of cAMP alone might not be sufficient to treat NDI.

Overall, increasing cAMP levels is an attractive strategy to increase AQP2 abundance and apical membrane expression to reduce urine output. However, cAMP is a ubiquitous second messenger that has a plethora of effects, some of which may be potentially undesirable, in many other cell types in the body. Therefore, any therapy that aims to increase cAMP levels should focus on targeting principal cells. In our view, hormones such as calcitonin, glucagon, and secretin are good therapeutic candidates because their actions depend on the cell-specific expression of receptors, and side effects may be more predictable and limited. Because of these considerations, the focus of research then began to move toward a major downstream target of the V2R/cAMP signaling pathway: PKA.

Activation of Protein Kinase A

When PKA is activated, it phosphorylates AQP2 at serine residue 256, which induces AQP2 membrane accumulation (60) (**Figure 2**). In addition, activated PKA also phosphorylates the transcription factor CREB (cAMP response element–binding protein) and increases *AQP2* gene transcription and protein abundance (61). PKA is a tetramer composed of two regulatory (PKA R) and two catalytic (PKA_{Cat}) subunits in its inactive form (62). To phosphorylate downstream targets, PKA_{Cat} subunits need to dissociate from PKA R subunits. In the resting state, PKA subunits are tethered by A kinase–anchoring proteins (AKAPs), scaffolding proteins that keep PKA subunits and other signaling enzymes in close proximity to their target substrates (63). In the renal collecting duct, AKAPs and PKA phosphorylate AQP2 and coordinate AQP2 regulation in the VP

signaling pathway (64, 65). AKAP-PKA disruptors, FMP-API-1 and derivatives, dissociated the binding of AKAPs and PKA R subunits and increased AQP2 membrane activity in mpkCCD cells to the same extent as VP. This drug also attenuated the antidiuretic effect of tolvaptan in mice (66). Directly activating PKA by AKAP dissociation induced the phosphorylation of a very specific set of proteins compared to GCPR agonists or adenylyl cyclase activators such as forskolin, when many more PKA substrates were phosphorylated. Of particular interest is that a renal-specific AKAP-PKA disruptor, FMP-API-1/27, seems to act mainly in collecting ducts without phosphorylating most of the PKA substrates in the whole kidney and heart (66). This specificity would reduce the risk of adverse side effects in other cells and organs.

ALTERNATIVE SIGNALING PATHWAYS TO MODULATE AQP2

Targeting the major V2R signaling pathway to treat water balance disorders is a logical approach. However, cAMP and PKA are regulators of many signaling pathways and involved in many biological processes. Therefore, in the absence of cell-specific strategies, modulating cAMP/PKA activity is more likely to cause unwanted off-target effects. As a consequence of this, more efforts are now focused on exploring alternative signaling pathways in principal cells that bypass the canonical cAMP/PKA mechanism to modulate AQP2 trafficking and expression (**Figure 3**).

The Cyclic GMP Pathway

Bouley et al. (67) found that cyclic GMP (cGMP) signaling can phosphorylate AQP2 and induce AQP2 membrane accumulation independent of VP and cAMP. Increasing intracellular cGMP using sildenafil (Viagra), a cGMP phosphodiesterase inhibitor, induced apical membrane accumulation of AQP2 in cultured cells, VP-deficient Brattleboro rats (68), and lithium-induced NDI rats (69). Sildenafil is metabolized much more rapidly in rats than in humans (70), and a recent case report demonstrated the effectiveness and safety of sildenafil in a 4-year-old boy with X-linked NDI who failed standard treatments. Daily sildenafil reduced polyuria significantly (24-h urine output reduced by 46%, 1,764 mL versus 950 mL), doubled urine osmolality, and normalized serum sodium without adverse effects (71). These results suggest that an alternative method to activate AQP2 is effective when the V2R signaling pathway is defective and should inspire the field to pursue more clinical studies. A major benefit of this approach is that sildenafil is an FDA-approved drug, and its target phosphodiesterases have a more limited cellular distribution than cAMP phosphodiesterases (72), thus reducing the risk of side effects.

Activators of Calcium Signaling

Ca^{2+} signaling is important in AQP2 trafficking. VP increases cAMP, which in turn induces intracellular Ca^{2+} oscillations and promotes AQP2 exocytosis (73, 74). Epac (an exchange factor directly activated by cAMP) is a key molecule that mediates cAMP and Ca^{2+} signaling. Epac2, mainly expressed in the apical region of principal cells (75), is thought to enhance Ca^{2+} signaling in response to cAMP in collecting ducts (76). The cAMP analog 8-pCPT-2′-*O*-Me-cAMP, which selectively activates Epac2 but not PKA, mimics the effects of VP on Ca^{2+} oscillations and promotes AQP2 exocytosis (74) and apical membrane accumulation in isolated perfused IMCD (77, 78). In addition, calmodulin, a downstream Ca^{2+}-binding protein, also induces AQP2 activation (79, 80), and calmodulin inhibitors significantly block AQP2 trafficking and transepithelial water transport by reducing VP-induced cAMP production and adenylyl cyclase activity (78, 79). Another key player in Ca^{2+} signaling and AQP2 trafficking is phosphoinositide 3-kinase (PI3K),

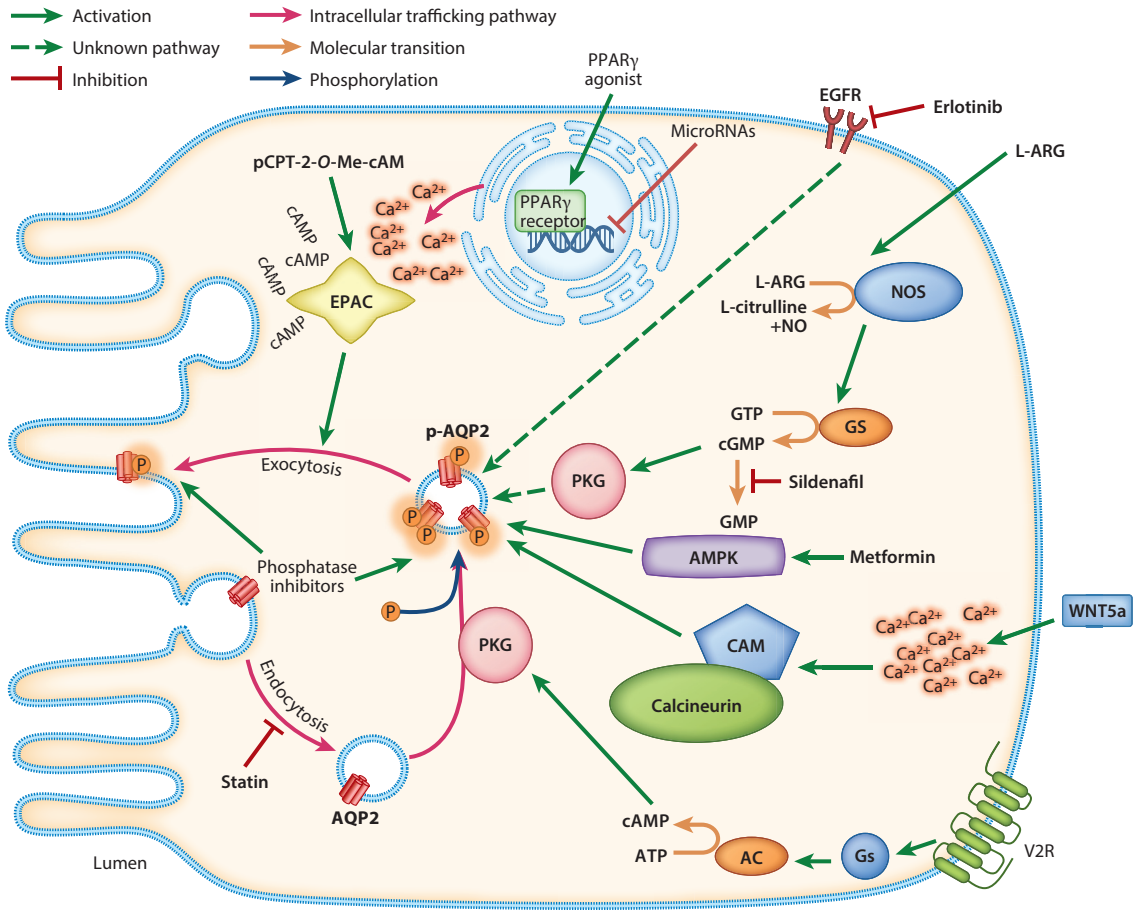

Figure 3

Alternative pathways that contribute to the regulation of aquaporin-2 (AQP2) phosphorylation, trafficking, and membrane accumulation. Calcium (Ca^{2+}) signaling plays a major role in AQP2 activity, and it is activated by cyclic AMP (cAMP) via EPAC. Intracellular Ca^{2+} oscillations increase AQP2 exocytosis. Ca^{2+} (and Wnt5a) activates calmodulin (CAM) and the CAM-dependent protein phosphatase calcineurin, both of which increase AQP2 protein expression and membrane trafficking. PPARγ agonists induce Ca^{2+} influx and stimulate a dose-dependent AQP2 exocytosis and total AQP2 protein expression. cGMP, another second messenger, induces AQP2 membrane accumulation independently of cAMP by inducing AQP2 phosphorylation via PKG activation. L-arginine (L-ARG) stimulates nitric oxide synthase, which increases cGMP levels in cells. AQP2 membrane accumulation can also be achieved by inhibiting the degradation of cGMP using the cGMP PDE inhibitor sildenafil. The epidermal growth factor receptor (EGFR) inhibitor erlotinib causes phosphorylation of AQP2 in a similar pattern to VP, but the kinases involved are not yet known. Erlotinib also increases AQP2 membrane accumulation by increasing exocytosis and decreasing endocytosis. Metformin induces AQP2 phosphorylation by AMPK activation, resulting in membrane accumulation. As AQP2 constitutively traffics to the apical membrane, blocking endocytosis alone with statins also results in AQP2 membrane accumulation. Phosphatase inhibitors, which block dephosphorylation of AQP2, maintain AQP2 in its phosphorylated (active) state, which would theoretically prolong AQP2 membrane retention. Finally, specific microRNAs (miRNAs) decrease AQP2 messenger RNA translation and protein expression.

which can be activated by VP, cAMP, Ca^{2+}, and calmodulin (81); it plays a role in AQP2 endocytosis and membrane retrieval into early endosomes (82).

Moreover, the calmodulin-dependent phosphatase calcineurin also regulates AQP2. Calcineurin and AQP2 are colocalized in the intracellular vesicles of collecting duct cells (83), and although acute treatment with the calcineurin inhibitor cyclosporine did not modify either baseline

or VP-induced AQP2 membrane trafficking in cultured cells or in kidney tissues (84), 4 weeks of cyclosporine treatment in rats decreased AQP2, urea transporter expression, urine osmolality, and free water reabsorption (85). Calcineurin Aα KO mice also had a decreased urine-concentrating response to VP. It was proposed that calcineurin inhibition prevented intracellular AQP2 transport and/or processing in the ER/Golgi pathway by blocking AQP2 dephosphorylation (86). However, in multiple mutant AQP2 S256D cell lines, where AQP2 S256D is constitutively phosphorylated, AQP2 can transit through the ER/Golgi to the plasma membrane, where it accumulates even in the absence of VP stimulation (87–89). Recently, Ando et al. (90) used the Ca^{2+} signaling transducer Wnt5a to activate the calcineurin signaling pathway and found that there is an increase in AQP2 phosphorylation, trafficking, and messenger RNA (mRNA) expression in mpkCCD cells. This was accompanied by increased osmotic water transport in isolated perfused cortical collecting ducts and increased urine-concentrating ability in an NDI mouse. These inconsistencies need to be further explored to establish the role of calcineurin in AQP2 regulation. In humans, calcineurin inhibitors such as cyclosporine or tacrolimus, commonly used to prevent organ transplant rejection, cause peripheral edema and hypertension by upregulating the thiazide-sensitive sodium chloride cotransporter (91). Their direct effect on water balance is not as apparent, as there are no case reports of polyuria suggestive of a urine-concentrating defect. Therefore, the utility of harnessing this pathway for the treatment of NDI in humans remains to be assessed.

Peroxisomal Proliferator–Activated Receptor Subtype γ Agonist

Thiazolidinediones such as rosiglitazone are peroxisomal proliferator–activated receptor subtype γ agonists used to treat diabetes mellitus. Procino et al. (92) showed that rosiglitazone induces an increase in membrane accumulation of AQP2, and they found a dose-dependent Ca^{2+} influx (used as an indirect marker of vesicular exocytosis) via TRPV6 channels triggered by rosiglitazone. In rosiglitazone-treated rats, AQP2 total protein levels and the plasma membrane–associated fraction were upregulated on the third day of treatment, probably contributing to water retention, but then downregulated after five days as a possible physiological response to reestablish water balance (93). Thiazolidinediones unfortunately carry serious side effects, including congestive heart failure, myocardial infarction, bladder cancer, and fracture (94), thus making them unfavorable treatments even for diabetes mellitus and also lowering their practical potential to treat water balance disorders.

Lithium

Lithium is the most common cause of NDI in adults, and dysregulation of AQP2 in the collecting duct is a crucial mechanism for the development of lithium-induced NDI (95). Entering kidney principal cells through epithelial sodium channels (96, 97), the effect of lithium on AQP2 activity is multifaceted. It was initially thought that lithium impairs the stimulatory effect of VP on adenylyl cyclase (98); however, Li et al. (99) later showed that lithium-induced NDI can be dissociated from adenylyl cyclase activity. Glycogen synthase kinase-3 (GSK3) is thought to play a central role in mediating behavioral responses to lithium (100); therefore, it is speculated that GSK3 also plays a role in lithium-mediated renal effects. Rao et al. (101) showed that mice with GSK3β KO in the renal collecting duct have a urinary concentrating defect during water deprivation and under VP treatment. These mice had reduced adenylyl cyclase activity, decreased cAMP generation, and lowered abundance and trafficking of membrane AQP2, suggesting a regulatory role of GSK3β on VP action in collecting ducts. Lithium also inhibits Ca^{2+} signaling by blocking an essential enzyme, inositol monophosphatase, along the PI3K pathway and reducing inositol levels (102),

potentially further reducing AQP2 exocytosis. In addition, lithium exposure remodeled the entire structure of the collecting ducts and increased the ratio of intercalated cells to principal cells (103). This reduction in the absolute number of principal cells may further contribute to NDI that is frequently irreversible in humans, despite lithium withdrawal. Using proteomic analysis, Nielsen et al. (104) showed that there are as many as 77 different proteins within the IMCD that are affected, either directly or indirectly, by lithium treatment. The proteins identified have a variety of functions, including signal transduction, regulation of gene expression, cytoskeletal organization, cellular reorganization, apoptosis, and cell proliferation, highlighting the complexity of the network of pathways affected by lithium that may cause polyuria (104). Using lithium directly to downregulate AQP2 expression does not appear feasible, considering its narrow therapeutic index, potential interactions with other medications, and considerable neuro- and renal toxicity (105).

Epidermal Growth Factor Receptor Inhibitors

EGF plays a role in water balance, as first suggested by Gow et al. (106) who showed that EGF infusion in sheep resulted in brisk water diuresis. Breyer et al. (107) subsequently showed that EGF administration inhibited the stimulatory effect of VP on water permeability in isolated perfused collecting ducts. Our group further demonstrated the crosstalk between EGF and VP, as we showed that EGF attenuated VP-induced AQP2 phosphorylation. Inhibiting the epidermal growth factor receptor (EGFR) using erlotinib stimulated apical membrane accumulation of AQP2 in principal cells and partially rescued lithium-induced NDI by increasing renal water reabsorption (56). The effect of erlotinib bypassed cAMP and PKA and induced a similar AQP2 phosphorylation pattern as VP, suggesting that an as yet unknown kinase(s) can also phosphorylate AQP2 while simultaneously dephosphorylating AQP2 on residue S261. We recently identified the phosphatase that targets S261 as PP2C (84). This potent effect of EGFR inhibitors suggests that tonic activation of the EGF signaling pathway in principal cells in some way inhibits changes in the phosphorylation state of AQP2 and prevents its accumulation on the cell surface. This tonic inhibition can be overcome by either activating the V2R pathway or simply inhibiting the inhibitory process with erlotinib, tipping the balance toward membrane accumulation of AQP2.

The discovery that EGFR inhibitors can mimic VP action opens up many new potential therapeutic targets to treat disorders of water balance. The EGF signaling pathway is complex, and identifying the unknown kinase(s) not only would be informative in expanding our understanding of AQP2 biology but also might reveal a new therapeutic target that is more focused and specific than inhibiting the entire EGF signaling cascade. EGFR inhibitors such as erlotinib and gefitinib and monoclonal antibodies against EGFR such as cetuximab are used in the treatment of non-small-cell lung cancer and pancreatic and colon cancers. There are frequent reports of hyponatremia in patients receiving these treatments (108, 109), suggesting that one consequence of EGFR inhibition is to provoke a SIADH-like syndrome in humans due to its stimulatory effect on AQP2 trafficking. While the beneficial effects of erlotinib on AQP2 and urine osmolality are encouraging in terms of NDI therapy, erlotinib has several side effects, including diarrhea (110); skin rash (111); and, rarely, interstitial pneumonitis and lung diseases (112, 113). However, as further mechanisms are delineated, more specific therapeutic targets are likely to be defined and utilized to avoid unwanted off-target effects.

Adenosine Monophosphate Kinase Activator: Metformin

Another approach to treating NDI is the activation of adenosine monophosphate kinase (AMPK) by metformin. AMPK is an energy-sensing serine/threonine kinase that phosphorylates the

Na-K-2Cl cotransporter NKCC2 in the outer medulla. Klein et al. (114) showed that metformin increases osmotic water permeability by increasing AQP2 phosphorylation and accumulation in the apical plasma membrane. Metformin also increases urea permeability by increasing the phosphorylation of urea transporter A1 (UT-A1). In two rodent models of NDI, V2R antagonist tolvaptan-treated rats and V2R KO mice, metformin increased both AQP2 and UT-A1 protein abundance in the inner medulla and increased urine osmolality. The effect of metformin was sustained for 10 days in tolvaptan-treated rats. Recently, the urine-concentrating effect of metformin was tested on 12 healthy volunteers after 1 week of 500 mg metformin 3 times per day. Metformin did not increase urine osmolality 4 h after water loading compared to subjects not receiving metformin (115). This result, however, should not refute the therapeutic potential of metformin in NDI, in which renal AQP2 expression and abundance are much lower compared to healthy patients. Notably, of the 12 healthy volunteers discussed above, five subjects experienced diarrhea and eight subjects experienced nausea (115). Although metformin has been widely used to treat diabetes mellitus, polycystic ovarian syndrome, and obesity and is considered to have a good safety profile, the gastrointestinal side effects may cause significant dehydration in NDI patients who are already vulnerable to volume loss from urination. A phase 1 clinical trial launched in 2015 to study the effect of metformin on congenital NDI patients was terminated in 2017 due to lack of effect, but only two patients were enrolled for the study (116).

Modulators of AQP2 Trafficking: Statins

AQP2 constitutively traffics to the apical membrane (117), and its membrane abundance is a regulated balance of endocytosis and exocytosis (118). Inhibiting endocytosis alone tips the balance toward membrane accumulation of AQP2 even in the absence of VP. This was originally shown experimentally by blocking clathrin-mediated endocytosis of AQP2 using either dominant negative dynamin, a GTPase that is required for endocytosis, or the chemical methyl-β-cyclodextrin, a drug that inhibits endocytosis by depleting membrane cholesterol (119). In searching for an FDA-approved drug to inhibit endocytosis, we learned that statins blocked endocytosis in proximal tubule cells (120). While statins are best known for their cholesterol-lowering effect, their inhibitory effect on HMG co-A reductase also blocks prenylation and subsequent activation of the RhoA GTPase, whose activity is required for actin polymerization. In many cell systems, actin polymerization is necessary for endocytosis to occur (121), so we hypothesized that statins would result in an accumulation of AQP2 on the plasma membrane, bypassing the need for V2R stimulation. One of the statins, simvastatin, was tested in Brattleboro rats and indeed enhanced the apical membrane expression of AQP2 through the downregulation of Rho GTPase activity and inhibition of endocytosis. It also transiently increased urine osmolality (122). Independent studies using other statins showed a reduction of endocytosis in renal cells in vitro (123), increased AQP2 apical membrane expression in mouse collecting duct cells, and greatly increased kidney water reabsorption in vivo (124). A study that followed 24 patients treated with simvastatin for hyperlipidemia may support its antidiuretic effect. Within 1 week of simvastatin treatment, patients showed an increase in urinary AQP2, an increase of urine osmolality by 36%, and a significant decrease in urine volume. The antidiuretic effect was maintained for at least 12 weeks and was observed in some patients after 1 year of treatment (125). While the study did not control food and water intake (which can affect urine volume and osmolality), another study that enrolled 12 healthy volunteers to take simvastatin again showed a significant increase in urinary osmolality after an acute water-loading test compared to subjects not receiving simvastatin (115). These promising clinical results with FDA-approved statins should prompt a larger study on NDI patients, who may experience even more substantial improvement of their urinary symptoms.

Phosphatase Inhibitors

Net protein phosphorylation is a balance between the action of protein kinases and phosphatases, and these processes are also essential for AQP2 activation and trafficking. Besides activating kinases to phosphorylate AQP2, inhibiting phosphatases could be a potential approach to maintain AQP2 in a phosphorylated and active state. The effect of phosphatase inhibition on AQP2 activity has been primarily tested in vitro, and the results are mixed. Valenti et al. (126) were the first to report that okadaic acid, a combined protein phosphatase 1 (PP1) and PP2A inhibitor, increased AQP2 phosphorylation as might be predicted, and this induced AQP2 membrane accumulation and increased the osmotic water permeability coefficient in AQP2-transfected renal CD8 cells. Subsequently, Ren et al. (127) showed that calyculin, another PP1 and PP2A inhibitor, increased AQP2 apical membrane accumulation in isolated rat inner medulla. Our group used okadaic acid on LLC-PK1 cells but could not find a change in AQP2 membrane expression. Okadeic acid was then tested on rat kidney tissue slices, and again no AQP2 translocation was noted. We expanded the study using the PP2B inhibitor cyclosporine A and the PP2C inhibitor sanguinarine, and neither of the inhibitors induced a noticeable AQP2 apical membrane expression compared to controls (84). However, sanguinarine completely prevented the usual VP-induced dephosphorylation of residue S261 in the AQP2 C terminus (84). Clearly, further studies on the effects of phosphatase inhibitors on AQP2 activity in vivo are needed to establish the importance of phosphatases in water regulation and their therapeutic potential.

Transcriptional Regulators: microRNAs

MicroRNAs (miRNAs) are small, single-stranded noncoding RNAs with an important function in the posttranscriptional control of gene expression. miRNAs target mRNAs (128), leading to mRNA degradation and translational repression. miRNA dysregulation has been increasingly recognized in human diseases, and miRNAs are now being tested in clinical trials to downregulate or block the function of oncogenes or to upregulate the expression of tumor suppressor genes (129, 130). There are two AQP2-targeting miRNAs, miR-32 and miR-137, that could decrease AQP2 expression in kidney collecting duct cells independently of VP regulation (131). miR-137 is correlated with an impaired response to VP and a reduction of urine concentration via the Ca^{2+}-sensing receptor (CaSR) (132). Once activated by high external Ca^{2+}, CaSR promotes the synthesis of miRNA-137 and increases AQP2 ubiquitination and proteasomal degradation, resulting in reduced AQP2 mRNA translation. With current advances in gene therapy and its application in fields like oncology, these exciting new findings may allow us to make a leap forward in the field of water balance to regulate AQP2 expression in conditions such as NDI (for therapeutic upregulation) and heart failure (for therapeutic downregulation).

COMBINING THERAPIES

In addition to targeting one pathway, combination therapies modulating different mechanisms have been attempted to prolong or increase effects on AQP2 activity. For example, drugs that increase membrane accumulation of AQP2 may not work optimally if the cellular content of AQP2 is too low. This may be the case in NDI patients where VP signaling is defective and gene transcription is reduced due to low cAMP levels. To address this, the coadministration of a GPCR agonist secretin and a statin has been attempted in V2R KO mice. The mice were first infused with secretin for 14 days, with the goal of increasing AQP2 abundance in the cytosol of collecting duct principal cells. A single dose of statin was then injected, inducing a nearly 90% reduction of urinary volume and doubling urine osmolality (30). Considering similar underlying mechanisms,

other drug combinations come to mind, such as calcitonin and a statin, especially since calcitonin may be best administered once a day to avoid desensitizing receptors. This might encourage better adherence of medications if translated to patient care. Assuming that AQP2 levels in all kidney regions were adequate to allow a response, the combination of calcitonin and sildenafil may be appealing, because calcitonin induces AQP2 membrane accumulation largely in the renal cortex (16), whereas sildenafil increases AQP2 translocation in the medulla (68). This combination might augment urinary concentrating ability in the kidneys by maximizing the membrane expression of AQP2 in all regions of the kidney parenchyma.

In summary, VP signaling and AQP2 trafficking play a significant role in the pathogenesis of diseases of water balance, and a deeper understanding of the various mechanisms that modulate AQP2 trafficking has helped to design potential therapies. Despite some robust results in preclinical studies, human studies are so far limited. Part of the reason is that congenital NDI is a rare disease, affecting only 1 in 200,000 live births. However, acquired lithium-induced NDI is a much more widespread problem that affects an already compromised patient population. Its management often requires patients and their health-care providers to make difficult decisions that balance the benefit of lithium on mood disorders against its risk to kidney function. In order to move the field forward, we need to think beyond the conventional therapies and consider utilizing novel strategies on patients based on the increasing body of preclinical data that, in many cases, involves the use of drugs that are already FDA approved.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We would like to thank all of our colleagues, past and present, who have contributed significantly to the results from our group described in this review. We would also like to thank all of our scientific colleagues working in the field of aquaporin biology who have made this such an interesting and exciting area of research over the years. The work from our laboratory was supported most recently by National Institutes of Health grants R01DK096586 (to D.B.) and K08DK115901 (to P.C.).

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