

ORIGINAL RESEARCH

Roflumilast and aquaporin-2 regulation in rat renal inner medullary collecting duct

Ezigiobiara N. Umejiego¹, Yanhua Wang², Mark A. Knepper¹ & Chung-Lin Chou¹¹ Epithelial Systems Biology Laboratory, Systems Biology Center, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1603² Renal Division, Department of Medicine, Emory University, Atlanta, Georgia 30322**Keywords**

cAMP phosphodiesterase, kidney, nephrogenic diabetes insipidus, X chromosome.

Correspondence

A. Knepper, National Institutes of Health, Bldg. 10, Room 6N307, 10 CENTER DR, MSC-1603, Bethesda, MD 20892-1603.
Tel: (301) 496 3064
Fax: (301) 402 1443
E-mail: knepper@nhlbi.nih.gov

Funding Information

The work was funded by the Division of Intramural Research, National Heart, Lung, and Blood Institute (projectZO1-HL001285, MAK)

Received: 7 September 2016; Revised: 29 November 2016; Accepted: 12 December 2016

doi: 10.14814/phy2.13121

Physiol Rep, 5 (2), 2017, e13121,
doi: 10.14814/phy2.13121

Abstract

Roflumilast is a cyclic nucleotide phosphodiesterase inhibitor that is FDA-approved for treatment of chronic obstructive pulmonary disease. With a view toward possible use for treatment of patients with X-linked nephrogenic diabetes insipidus (NDI) due to hemizygous mutations in the V2 vasopressin receptor, this study sought to determine the effect of roflumilast on aquaporin-2 (AQP2) phosphorylation, AQP2 trafficking, and water permeability in the rat inner medullary collecting duct (IMCD). In the presence of the vasopressin analog dDAVP (0.1 nmol/L), both roflumilast and its active metabolite roflumilast *N*-oxide (RNO) significantly increased phosphorylation at S256, S264, and S269, and decreased phosphorylation at S261 (immunoblotting) in IMCD suspensions in a dose-dependent manner (3–3000 nmol/L). Another commonly used phosphodiesterase inhibitor, IBMX, affected phosphorylation only at the highest concentration in this range. However, neither roflumilast nor RNO had an effect on AQP2 phosphorylation in the absence of vasopressin. Furthermore, roflumilast alone did not increase AQP2 trafficking to the plasma membrane (immunofluorescence) or increase water permeability in freshly microdissected perfused IMCD segments. We conclude that roflumilast can be used to enhance vasopressin's action on AQP2 activity in the renal collecting duct, but has no detectable effect in the absence of vasopressin. These findings suggest that roflumilast may not have a beneficial effect in X-linked NDI, but could find useful application in acquired NDI.

Introduction

X-linked nephrogenic diabetes insipidus (NDI) is a rare genetic disorder that is associated with marked polyuria due to mutations in the gene that codes for the V2 vasopressin receptor (gene symbol: *Avpr2*) (Bockenhauer and Bichet 2015)(Boone and Deen 2008). This receptor normally couples to the heterotrimeric G-protein *G_s* and activates adenylyl cyclases in the renal collecting duct. Upon stimulation by binding of vasopressin, the receptor-mediated signaling elicits increases in intracellular cyclic

AMP (cAMP) and secondary increases in intracellular calcium (Star et al. 1988; Yip 2002). These responses regulate the molecular water channel aquaporin-2 (AQP2), which is rate-limiting for transepithelial water transport in the collecting duct epithelium. AQP2 is regulated in the short term by phosphorylation changes. Vasopressin regulates phosphorylation of AQP2 at four serine residues (S256, S261, S264, and S269) located at the carboxyl terminus (Hoffert et al. 2006; Yu et al. 2009). While phosphorylation changes at S256 are necessary for exocytosis of AQP2 (Fushimi et al. 1997; Kamsteeg et al. 2000),

phosphorylation at S261 and S269 controls AQP2 endocytosis (Hoffert et al. 2007, 2008; Moeller et al. 2008). These regulatory processes allow AQP2 to play a central role in the urine concentration mechanism of the kidney.

With a defective V2 receptor in X-linked NDI (Bichet et al. 1993; Holtzman et al. 1993a,b; Moses and Scheinman 1993), other means of regulating AQP2 that bypasses this receptor offer the hope of alleviating and/or treating the symptoms that accompanies this malady. It may be possible to ameliorate the disease through the use of agents that can either activate other G α s-linked receptors in collecting duct cells (Bouley et al. 2000, 2011; Li et al. 2009; Olesen et al. 2011; Procino et al. 2014) or that can increase cAMP signaling by other means (Bouley et al. 2005; Li et al. 2011; Procino et al. 2014; Russo et al. 2006). Although economic driving forces are not conducive to de novo development of drugs to treat X-linked NDI, it is possible that drugs developed and FDA-approved for treatment of other diseases could be employed cross-label for its treatment. One such drug with potential for treatment of X-linked NDI is roflumilast, a FDA-approved agent for treatment of severe chronic obstructive pulmonary disease (COPD) (Beghe et al. 2013; Rabe 2010, 2011). Roflumilast is a cyclic nucleotide phosphodiesterase (PDE) inhibitor that is selective for type 4 PDEs. PDE4 is coded by four different genes, viz. *Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d*, and is one of three cAMP-specific PDEs (with PDE7 and PDE8) expressed in the kidney inner medullary collecting duct (Beavo 1995; Bolger 1994; Conti et al. 1995, 2003; Dousa 1999; Engels et al. 1994; Francis et al. 2011; Maurice et al. 2014; McSorley et al. 2006; Stefan et al. 2007; Uawithya et al. 2008). Accordingly, the PDE inhibitor roflumilast could theoretically ameliorate X-linked NDI by increasing intracellular cAMP in collecting duct cells, despite the absence of a functional V2 vasopressin receptor.

In this study, we do the groundwork to address the feasibility of such an approach, working with isolated inner medullary collecting ducts (IMCDs) from rats. We carry out experiments to test the effects of roflumilast on: (1) phosphorylation of AQP2 at the four vasopressin-regulated sites (S256, S261, S264, and S269), (2) trafficking of AQP2 to the apical plasma membrane in collecting duct cells, and (3) osmotic water permeability in isolated perfused IMCD segments.

Methods

Rats

Untreated Sprague–Dawley rats were used for harvest of renal tissue as described previously (Miranda et al. 2014). Animal procedures were approved by the National Heart,

Lung, and Blood Institute Animal Care and Use Committee (protocol no. H-0110R3). Briefly, pathogen-free male Sprague–Dawley rats (Taconic Farms, Germantown, NY), weighing 190–250 g, were used for these studies. Rats were maintained on autoclaved standard rat diet and ad libitum drinking water in filter-top microisolator cages. The animals received an intraperitoneal injection of furosemide (0.50 mg; Hospira, Lake Forest, IL) on the experimental day, to trigger a rapid diuresis and clear out most of the inner medullary axial solute concentration gradient present in untreated animals (Sands and Knepper 1987). After 20–25 min, the animals were euthanized by decapitation without anesthesia. Kidneys were rapidly removed and chilled on ice-cold normal saline. The inner medulla tissues were subsequently processed for one of three purposes summarized below: (1) preparing inner medullary collecting duct (IMCD) suspensions for immunoblotting; (2) measuring water transport in isolated, perfused tubules; or (3) microdissecting of single IMCD tubules for immunofluorescence.

Preparation of rat kidney IMCD suspensions

IMCD suspensions were prepared as previously described (Miranda et al. 2014). Briefly, kidney inner medullas were dissected, minced, and digested by incubation at 37°C for 70–90 min in digestion solution (250 mmol/L sucrose, 10 mmol/L triethanolamine, pH 7.6) containing collagenase B (3 mg/mL; Roche, Indianapolis, IN) and hyaluronidase (1400 U/mL; Worthington, Lakewood, NJ). The suspensions were centrifuged (70 g, 20 sec), and the supernatant was discarded. The pellet was washed and resuspended with IMCD (bicarbonate-buffered) solution that had been preequilibrated with 95% air/5% CO₂ (repeated twice). The composition of the IMCD solution was (in mmol/L) 118 NaCl, 25 NaHCO₃, 5 KCl, 4 Na₂HPO₄, 2 CaCl₂, 1.2 MgSO₄, 5.5 glucose, and 5 Na acetate (290 mosmol/kg H₂O). IMCD suspensions were allowed to equilibrate with gentle stirring with the aid of a microstirring bar at 37°C with 95% air/5% CO₂ supply for 10 min before use. Samples were incubated with roflumilast (product no. S2131, Selleck Chemicals), roflumilast *N*-oxide (RNO; product no. R639710, Toronto Research Chemicals), or arginine vasopressin (product no. H1780, Bachem) as described in the figure legends. Upon completion of the incubation, the suspensions were pelleted by centrifugation at 16,000 g for 2 min to harvest the IMCD segments. Pellets were resuspended in 1X Laemmli buffer (1.5% SDS, 10 mmol/L Tris, pH 6.8) containing Halt protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL) and passed through a DNA shredder (Qiagen, Valencia, CA) for 1 min. Protein concentration was determined by the BCA method

(Thermo Scientific). Samples were diluted to a protein concentration of 2 mg/mL, glycerol 6%, and DTT 40 mmol/L. Samples were heated at 60°C for 10 min prior to storing at 4°C.

Before immunoblotting, samples were separated on an SDS-PAGE gel (12% Tris-Glycine, precast polyacrylamide, Criterion TGX, Bio-Rad) and stained with Coomassie blue to check for equal loading (Kim *et al.* 1999). This “total-protein-profile” approach has been the standard for equal-loading controls for vasopressin studies in our laboratory, because of studies showing that vasopressin alters the abundances of single proteins frequently used as loading controls, *viz.* GAPDH (Pisitkun *et al.* 2006), beta-actin (Pisitkun *et al.* 2006), and gamma-actin (van Balkom *et al.* 2004).

Immunoblotting was performed as previously described (Miranda *et al.* 2014). Proteins were resolved by SDS-PAGE on 12% Tris-Glycine polyacrylamide gels (Criterion TGX, Bio-Rad) and transferred electrophoretically onto 0.2 μ m nitrocellulose membranes (Bio-Rad, #162-0112). Membranes were blocked for 1 h with Odyssey blocking buffer (Li-Cor, Lincoln, NE), rinsed, and probed overnight at 4°C with the respective affinity-purified antibodies at an appropriate dilution in Odyssey blocking buffer containing 0.1% Tween 20. After 1 h incubation with secondary antibody (Alexa Fluor 680-nm goat anti-rabbit immunoglobulin G; Invitrogen, Carlsbad, CA) at 1:5,000 dilution, sites of antibody-antigen reaction were detected and quantified using an Odyssey infrared imager (Li-Cor). Band densities were normalized and analyzed statistically as described in the figure legends.

Tubule perfusion

IMCD segments were dissected from the kidneys of pathogen-free male Sprague–Dawley rats weighing 100–200 g using methods previously described (Chou *et al.* 2008). Briefly, coronal slices containing the inner medulla were cut from the kidneys and were transferred to a dissection dish containing chilled dissection solution (12°C) for isolation of collecting ducts. Terminal IMCDs were dissected using a Wild M8 dissection microscope. The dissecting solution contained the following (in mmol/L): 120 NaCl, 25 NaHCO₃, 2 CaCl₂, 2 K₂HPO₄, and 1.2 MgSO₄, 5.5 glucose. This solution was suffused continuously with 95% air/5% CO₂ before and during the dissection.

The microdissected terminal IMCDs were mounted on concentric pipettes and perfused *in vitro* at 37°C as previously described (Chou *et al.* 2008). The perfusion and bath solutions were identical to the dissection medium, except that an additional 111 mmol/L NaCl was added

to the bath solution to create a 200 mOsm transepithelial osmotic gradient (490 vs. 290 mOsm). The osmolalities of the perfusate and bath were measured by vapor pressure osmometry (Wescor, Logan, UT), allowing the calculation of the actual osmolality gradient. One millimolar fluorescein sulfonate (Molecular Probes) was added to the luminal perfusate as a readily measurable, impermeant luminal marker, which is concentrated when water moves from the lumen to the peritubular bath. Fluorescein sulfonate concentrations in perfusate and collected fluid were measured by continuous-flow ultramicrofluorometry (Wall *et al.* 1992), allowing calculation of transepithelial water flux and osmotic water permeability (P_f) according to the equation of Al-Zahid *et al.* (1977).

Immunofluorescence labeling of microdissected renal IMCDs

Tubules were microdissected as described above except that a HEPES-buffered solution was used, composed of (in mmol/L): 118 NaCl, 10 Hepes, 5 KCl, 2.5 Na₂HPO₄, 2 CaCl₂, 1.2 MgSO₄, 5.5 glucose, and 5 Na acetate (pH 7.4). The microdissected IMCD segments were immobilized on glass bottomed culture dishes (MatTek, #P35GC-1.5-14, Ashland, MA) coated with Cell Tak (Corning, #354240, Bedford, MA). The tubules were incubated with or without an agent (dDAVP and/or roflumilast) for 30 min at 37°C and then immediately fixed with 4% paraformaldehyde for 20 min at room temperature, rinsed twice with Dulbecco's PBS with Ca²⁺ and Mg²⁺ (GIBCO, #14040-133), followed with fixative removal using 100 mmol/L glycine for 20 min. The tubules were washed again with PBS three times followed by incubation in 100% methanol at –20°C for 20 min. On completion of the methanol treatment, tubules were washed for 5 min with PBS. Tubules were permeabilized using 0.3% Triton X-100/0.1% BSA/PBS for 10 min, followed with three PBS washes, and blocking (30 min) with 1% BSA/0.2% gelatin. IMCDs were incubated overnight (4°C) in AQP2 antibody (Ab K5007) at an IgG concentration of 17.5 ng/mL or an antibody to AQP1 (L266) at 17.2 ng/mL. Samples were washed three times with 0.05% Tween-20 in PBS for 10 min each, followed with a 2 h incubation with the secondary antibody (1:2,000 dilution, Alexa Fluor 488 goat anti-rabbit immunoglobulin G; Invitrogen, Carlsbad, CA) at room temperature. To label nuclei, tubules were incubated with DAPI (Molecular Probes, D3571, 1:250 dilution in PBS) for 10 min, followed by washing with 0.05% Tween-20/PBS for 10 min (three times). This was followed with a quick wash with PBS (three times), and imaging using confocal microscopy (Zeiss LSM 780).

Antibodies

All primary antibodies used in this study are previously characterized affinity-purified rabbit polyclonal antibodies. The holoprotein-directed antibody, AQP2 (Hoffert *et al.* 2008), was used for both immunoblotting and immunofluorescence. The following phospho-specific antibodies were used for immunoblotting: phospho-S256-AQP2 (Xie *et al.* 2010), phospho-S261-AQP2 (Hoffert *et al.* 2007), phospho-S264-AQP2 (Hoffert *et al.* 2008), and phospho-S269-AQP2 (Hoffert *et al.* 2008). A rabbit polyclonal antibody to AQP1 (Terris *et al.* 1996) was used for immunofluorescence controls.

Statistics

All data were presented as means \pm SE. The *P* value of Student *t*-test $<$ 0.05 was considered statistically significant.

Results

Effect of roflumilast on aquaporin-2 phosphorylation in renal IMCDs

Vasopressin increases phosphorylation of AQP2 at three serines in its COOH-terminal tail (S256, S264, and S269) and decreases phosphorylation at S261 (Hoffert *et al.* 2006; Yu *et al.* 2009). Phosphorylation changes at these sites have been shown to serve an integral role in the recycling and trafficking of AQP2, to and from the apical plasma membrane, and thus its short-term regulation of water permeability (Hoffert *et al.* 2008; Moeller *et al.* 2009). As such, our study began by measuring AQP2 phosphorylation changes at S256, S261, S264, and S269 in response to roflumilast (at the therapeutic concentration, 30 nmol/L (Lambert *et al.* 2014)) in IMCD suspensions from rats (Fig. 1). The left-hand column (Fig. 1A–E) shows examples of the immunoblots. AQP2 is seen as two bands, a lower 29 kDa nonglycosylated band and an upper 33–40 kDa glycosylated band. (AQP2 tetramers contain both forms (Hendriks *et al.* 2004)). To quantify the immunoblots, we measured total density of both bands. The middle column (Fig. 1F–J) shows summary graphs of the effects of roflumilast on band densities in the absence of dDAVP. As can be seen, roflumilast had

no effect in the absence of the vasopressin analog. The last column (Fig. 1K–O) shows summary graphs of the effects of roflumilast in the presence of dDAVP (at a concentration that produces a maximal effect on water permeability (Star *et al.* 1988), 0.1 nmol/L). As can be seen, in the presence of dDAVP, roflumilast significantly decreased phosphorylation of S261, while markedly increasing phosphorylation at S269.

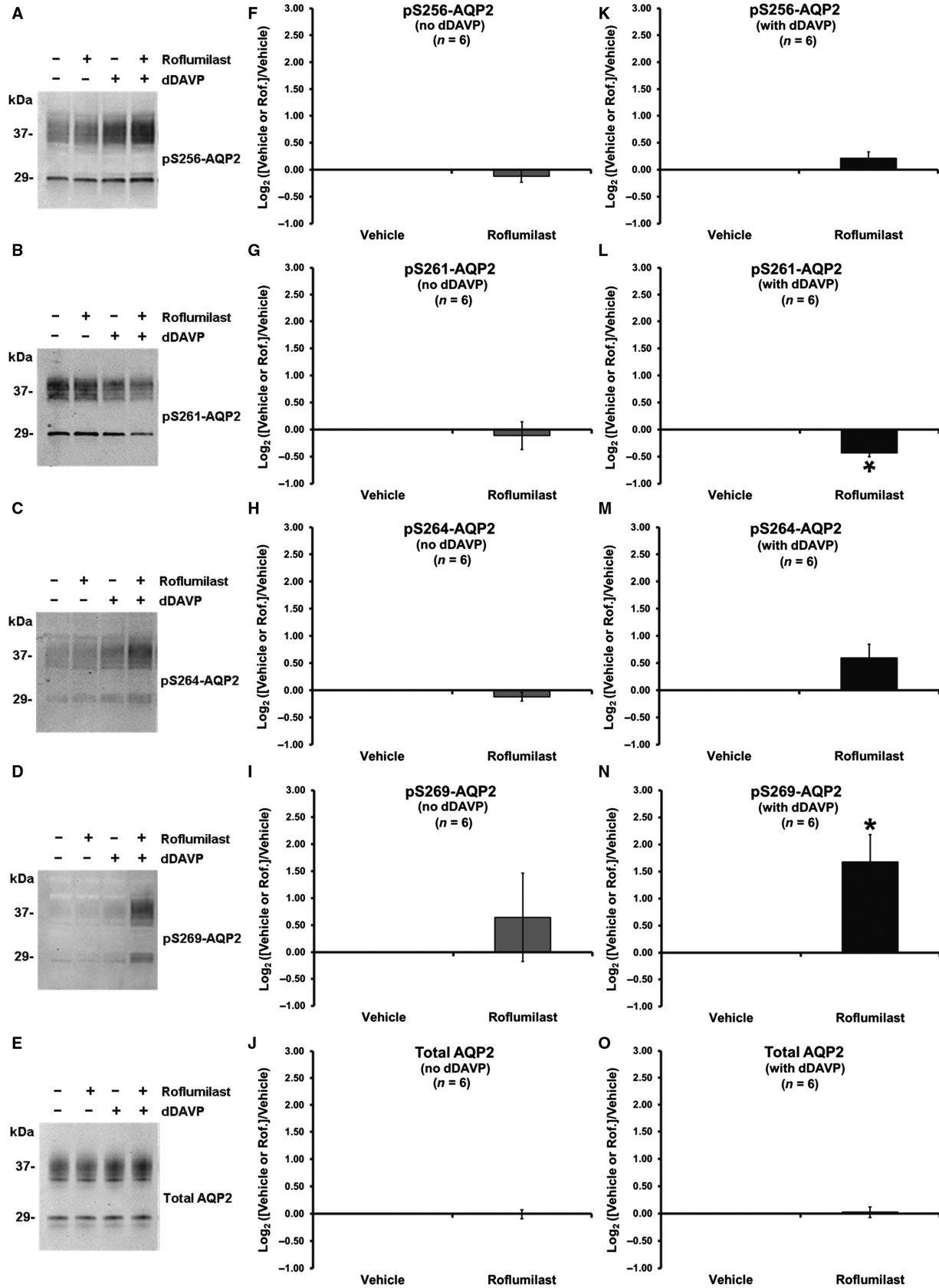
Effect of roflumilast *N*-oxide on aquaporin-2 phosphorylation in IMCD cell suspensions

It could be argued that roflumilast had no effect in the absence of dDAVP because the conversion of roflumilast to its pharmacologically active form, RNO (Hatzelmann and Schudt 2001), may not be optimal in collecting duct cells. Therefore, we carried out studies like those described in the previous paragraph to test the effects of RNO on AQP2 phosphorylation changes at S256, S261, S264, and S269 (Fig. 2). As was seen with roflumilast, 30 nmol/L RNO produced no effects on AQP2 phosphorylation in the absence of dDAVP. However, in the presence of dDAVP, RNO produced a strong effect on AQP2 phosphorylation at all four vasopressin-regulated sites, significantly increasing phosphorylation at S256, S264, and S269, while significantly decreasing phosphorylation at S261. Thus, RNO accentuated the phosphorylation effects of dDAVP at all sites. Taken together, however, the data in Figures 1 and 2 do not support the idea that roflumilast has effects that are independent of vasopressin V2 receptor occupation in the renal collecting duct, although the agent enhanced responses to vasopressin.

Dose–response comparison of roflumilast and IBMX effects on aquaporin-2 phosphorylation in the presence of vasopressin

Since 3-isobutyl-1-methylxanthine (IBMX) is a nonselective phosphodiesterase inhibitor commonly used in renal physiology experiments (Bolger 1994), we next compared the efficacy of roflumilast and IBMX in inducing AVP-stimulated AQP2 phosphorylation. Figure 3 shows dose–response data on the effects of roflumilast and IBMX. AQP2 phosphorylation increases were evident from the lowest to highest dose of roflumilast for S256, S264 and

Figure 1. Effect of roflumilast on AQP2 phosphorylation changes in rat IMCD suspensions. IMCD suspensions were incubated with vehicle or roflumilast (30 nmol/L) in the absence or presence of 0.1 nmol/L dDAVP for 30 min. (A–E) immunoblots for the four vasopressin-regulated serine sites (S256, S261, S264, and S269) of AQP2 and total AQP2. (F–O) band density analysis depicted as log₂ value of the effect of vehicle or roflumilast normalized by vehicle in the absence (F–J) or presence (K–O) of dDAVP. *Statistically significant (*P* < 0.05). IMCD, inner medullary collecting duct.



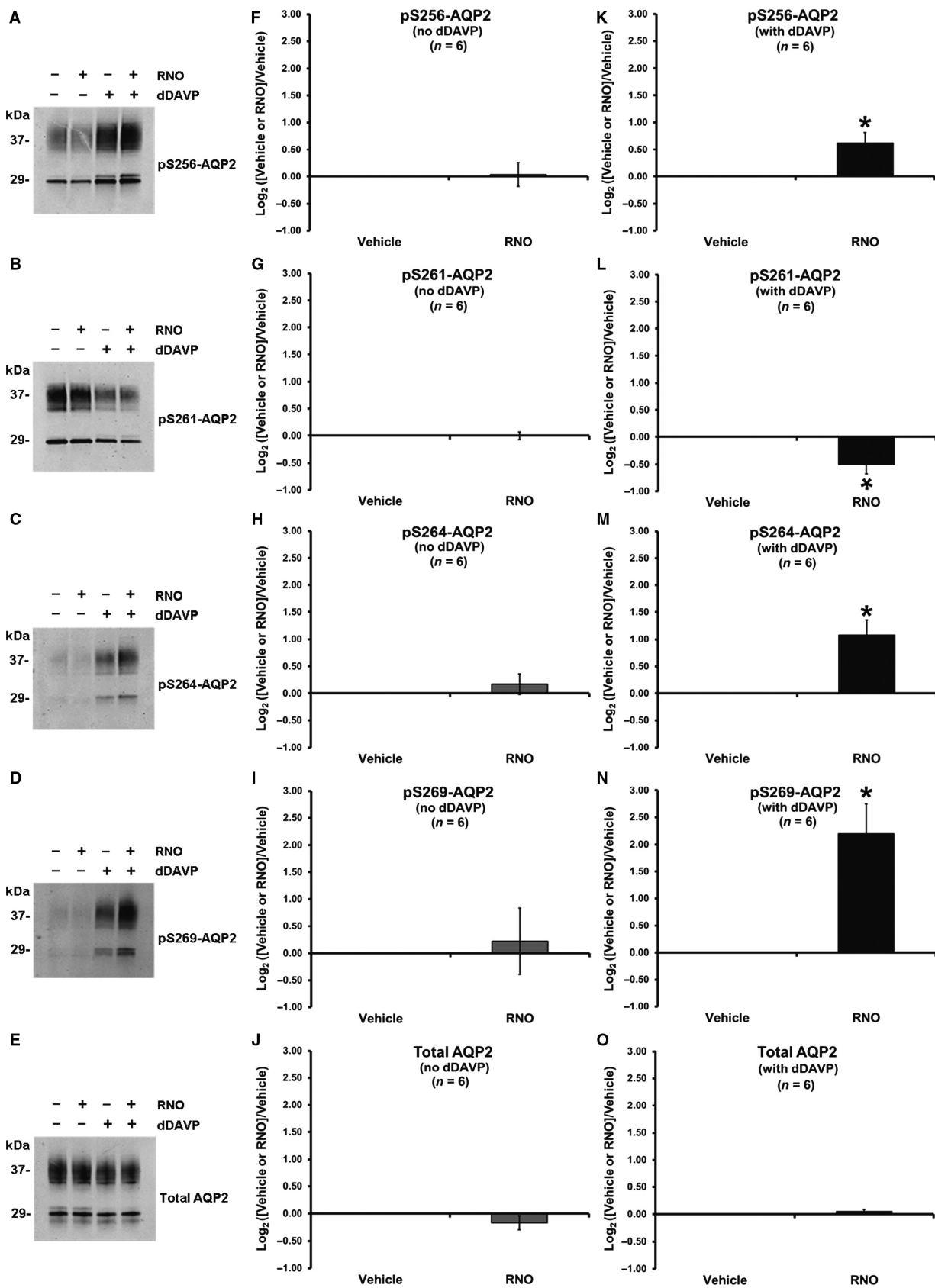


Figure 2. Effect of roflumilast *N*-oxide on AQP2 phosphorylation changes in rat IMCD suspensions. IMCD suspensions were incubated with vehicle or RNO (30 nmol/L) in the absence or presence of 0.1 nmol/L dDAVP for 30 min. (A–E) immunoblots for the four vasopressin-regulated serine sites (S256, S261, S264, and S269) of AQP2 and total AQP2. (F–O) band density analysis depicted as \log_2 value of the effect of vehicle or RNO normalized by vehicle in the absence (F–J) or presence (K–O) of dDAVP. *Statistically significant ($P < 0.05$). IMCD, inner medullary collecting duct; RNO, roflumilast *N*-oxide.

S269, while there were progressive decreases in phosphorylation at S261. However, these phosphorylation changes were minimal in response to IBMX at all but the highest concentration, 3000 nmol/L (Fig. 3D). Thus, roflumilast is much more potent than IBMX in increasing vasopressin-induced AQP2 phosphorylation.

Effect of roflumilast on aquaporin-2 phosphorylation in the presence of EP4 PGE₂ Receptor Agonist, ONO-AE1-329 (ONO)

In a previous study, we showed in mouse cortical collecting ducts that ONO at concentration of 100 nmol/L increased the osmotic water permeability, presumably by activating the EP4 PGE₂ receptor (Li et al. 2009). This increase in osmotic water permeability is thought to be mediated through cAMP actions. We hypothesize that the combination of ONO and roflumilast could have a significant effect on AQP2 phosphorylation. We tested this in IMCD suspensions (Fig. 4). However, there was no significant effect of ONO alone (100 nmol/L) or the combination of ONO and roflumilast (30 nmol/L) on AQP2 phosphorylation at any of the four vasopressin-regulated sites.

Effect of roflumilast on aquaporin-2 trafficking in microdissected inner medullary collecting ducts

Vasopressin regulates the water permeability of collecting duct cells in part by regulating AQP2 trafficking to and from the apical plasma membrane in collecting duct cells (Brown 2003; Nielsen et al. 1995). In theory, this trafficking could be in part independent of AQP2 phosphorylation. To test the ability of roflumilast to redistribute AQP2 to the apical plasma membrane, we exposed microdissected IMCD segments in vitro to roflumilast (30 nmol/L) or dDAVP (0.1 nmol/L). After incubation, the tubules were fixed and labeled for immunocytochemistry. Figure 5 shows representative confocal images. dDAVP caused strong redistribution of AQP2 labeling to the plasma membrane compared to vehicle-treated control tubules. However, there was no evidence of redistribution of AQP2 to the plasma membrane in response to roflumilast ($n = 3$).

Neither roflumilast nor roflumilast *N*-oxide increases osmotic water permeability in isolated perfused IMCD segments

Despite the lack of effect of roflumilast on AQP2 phosphorylation or trafficking, it is conceivable that it could affect water permeability by channel gating or through effects on the basolateral water channels AQP3 or AQP4. Figure 6 shows results of osmotic water permeability measurements in isolated perfused IMCD segments with both roflumilast (upper) and RNO (lower). As can be visualized, neither agent increased osmotic water permeability. However, as expected, dDAVP increased water permeability demonstrating that the tubules are capable of responding.

Discussion

This study tested the effect of roflumilast, a selective PDE4 inhibitor approved by FDA for the treatment of chronic obstructive lung disease, and its active metabolite RNO, on AQP2 regulation in the kidney IMCD. We can draw two main conclusions from this study: (1) although roflumilast alone has no effect on AQP2 phosphorylation, AQP2 trafficking, or water permeability, it enhanced the short-term action of vasopressin to regulate AQP2 in the collecting duct; and (2) roflumilast is significantly more potent than IBMX, and therefore it might be a better choice than IBMX as a phosphodiesterase inhibitor for in vitro physiological studies in renal collecting duct cells. We consider these two conclusions in detail in the following.

No effect of roflumilast alone

For roflumilast to be a good candidate for potential use in treating patients with X-linked NDI, it should have demonstrable effects on AQP2 regulation in vitro. Results from this study provided no evidence that roflumilast alone can alter AQP2 phosphorylation, AQP2 localization, or water permeability in the collecting duct. Similar findings were reported by Stefan et al. (2007) in their studies on AQP2 regulation in primary cultures of renal IMCD cells using the selective PDE4 inhibitor rolipram, wherein they showed that rolipram alone does not alter the trafficking of AQP2 or increase osmotic water permeability.

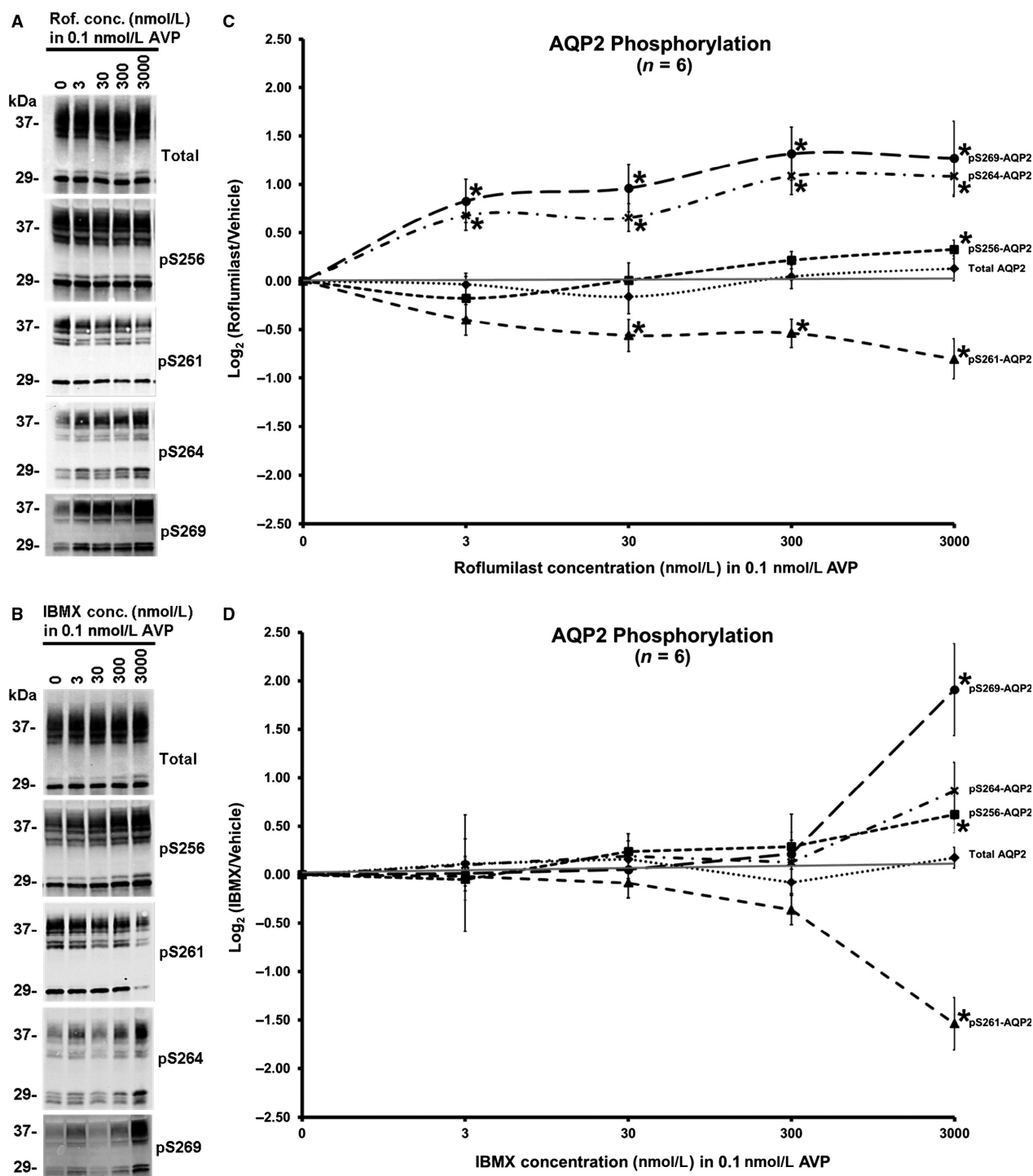


Figure 3. Dose-dependent effect of roflumilast and IBMX on AQP2 phosphorylation changes in IMCD suspensions in the presence of 0.1 nmol/L AVP. The dose range (3 nmol/L, 30 nmol/L, 300 nmol/L, and 3000 nmol/L) and the incubation time (30 min) were the same for both agents. (A&B) immunoblots for total AQP2 and the four vasopressin-regulated serine sites (S256, S261, S264, and S269) of AQP2. (C&D) line graphs summarizing the changes in normalized band densities of the total and four phosphorylation sites of AQP2 elicited by roflumilast (C) or IBMX (D). *n* = 6 for both groups. *Statistically significant (*P* < 0.05). IBMX, isobutyl-1-methylxanthine; IMCD, inner medullary collecting duct.

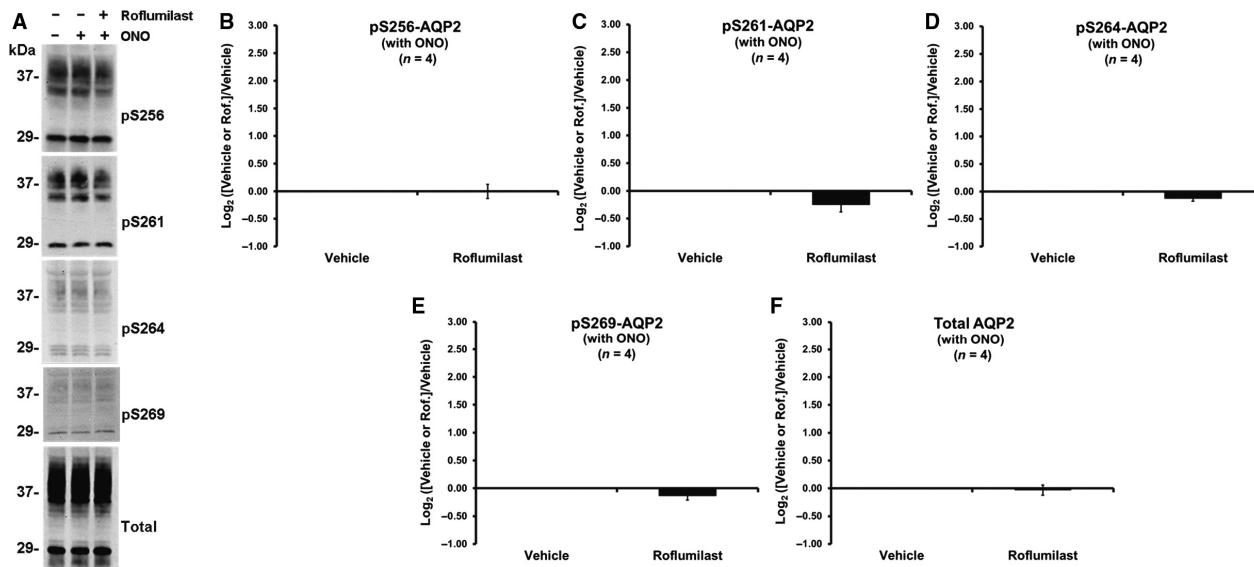


Figure 4. Effect of roflumilast on AQP2 phosphorylation changes in rat IMCD suspensions in the presence of a selective EP4 agonist ONO-AE1-329 (30 min incubation period). (A) immunoblots for the four vasopressin-regulated serine sites (S256, S261, S264, and S269) of AQP2 and total AQP2. (B–F) bar graphs of the normalized band density analysis. IMCD, inner medullary collecting duct.

Likewise, Bichet et al. (1990) found no effect of rolipram when it was used in treating two male patients with congenital NDI. Taking our findings into consideration and the known toxicity of roflumilast in humans (Gross et al. 2010; Izquierdo and Aparicio 2010; Rabe 2010), we therefore concluded that roflumilast alone may not have a beneficial effect in X-linked NDI. This conclusion does not exclude the possibility that roflumilast may be useful for patients with acquired NDI. It is also conceivable that roflumilast alone could have a regulatory effect on AQP2 gene expression over a longer time scale than tested in this study, as is the case with vasopressin (Terris et al. 1996). However, since both the short-term trafficking effects of vasopressin and the long-term effects on AQP2 protein abundance appear to be mediated by cAMP, it is unlikely that roflumilast would trigger an increase in AQP2 abundance with long term exposure.

Why did roflumilast fail to alter AQP2 phosphorylation or trafficking, when theoretically it should have increased cAMP signaling? One possibility is that the degradation of cAMP in the collecting duct is mediated by phosphodiesterases other than the four PDE4 proteins. Indeed, several other phosphodiesterases are expressed in collecting duct cells including *Pde1a*, *Pde1c*, *Pde3b*, *Pde5a*, *Pde6d*, *Pde7b*, and *Pde8a* (Lee et al. 2015; Uawithya et al. 2008). While the functionality of these phosphodiesterases varies from one isoform to another, their nucleotide substrate specificities often overlap. PDE1 is calcium/calmodulin-activated with dual specificity for cAMP/cGMP substrates (Beavo 1995; Beavo et al. 1994; Dousa 1999; Kakiuchi

et al. 1975; Klee and Vanaman 1982; Swinnen et al. 1989), PDE5 and PDE6 hydrolyze cGMP only (Beavo 1995; Beavo et al. 1994; Dousa 1999; Thompson 1991; Yanaka et al. 1998), PDE3 (Beavo 1995; Beavo et al. 1994; Degerman et al. 1997; Dousa 1999; Leroy et al. 1996; Maurice et al. 2014; Weishaar et al. 1985) is cGMP-inhibited but hydrolyzes cAMP, while the isozymes PDE4 (Beavo 1995; Beavo et al. 1994; Conti et al. 2003; Davis 1984; Dousa 1999; Maurice et al. 2014; Schwabe et al. 1976; Swinnen et al. 1989; Wang et al. 1997), PDE7 (Beavo 1995; Bloom and Beavo 1996; Dousa 1999; Gardner et al. 2000; Michaeli et al. 1993), and PDE8 (Dousa 1999; Fisher et al. 1998; Hayashi et al. 1998; Soderling et al. 1998; Soderling and Beavo 2000) selectively hydrolyze cAMP. In addition, studies using isoform specific inhibitors have provided evidence for the expression of PDE1, PDE3, and PDE4 isoforms in renal collecting duct cells (Dousa 1999). Lee et al. has also provided further data on the phosphodiesterases expressed in the different segments of rat renal collecting duct through their deep sequencing studies (see Table 1). Therefore, it is plausible that the other PDE isoforms provide enough cAMP metabolizing activity to maintain low cAMP levels without PDE4 activity in the absence of a stimulus for cAMP production.

This raises the question of a possible synergistic role between PDE4 and other cAMP-selective metabolizing PDE isoforms. Interestingly, number of studies exploring the therapeutic applications of inactivating PDE4, as a means to alleviate different disease states, have revealed

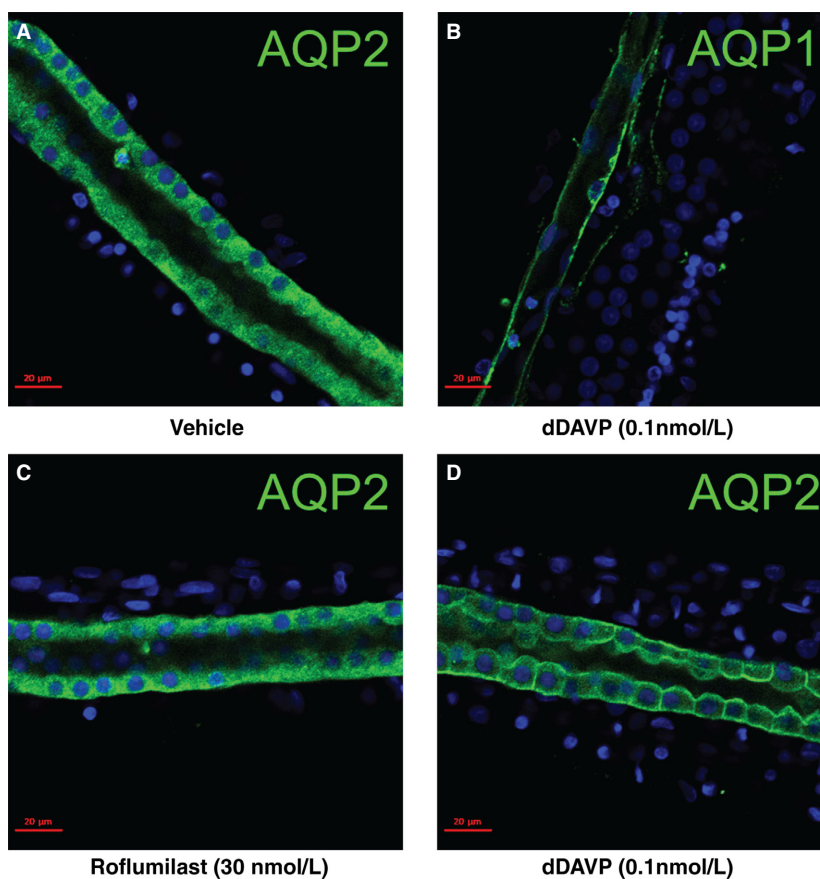


Figure 5. Immunofluorescence labeling of AQP2 in microdissected IMCD segments. Freshly microdissected IMCDs were incubated with vehicle (A) 30 nmol/L roflumilast (C) or 0.1 nmol/L dDAVP (D) for 30 min, followed by fixation and immunofluorescence staining for AQP2. B, using an AQP1 antibody at a concentration similar to that used for AQP2 antibody in A, C and D, AQP1 identified only the thin structures attached to an IMCD segment (pretreated with 0.1 nmol/L dDAVP) but not IMCD. Green: AQP2 or AQP1, blue: DAPI. ($n = 3$). IMCD, inner medullary collecting duct.

the potentiating effects of dual inhibition of PDE4 and one of the other cAMP-hydrolyzing PDE isoforms, chiefly PDE3 and PDE8 (Banner and Press 2009; Giembycz and Newton 2011; Kraynik *et al.* 2013; Matera *et al.* 2014; Maurice *et al.* 2014; Mika *et al.* 2013; Palmer *et al.* 1998; Salari and Abdollahi 2012; Snyder *et al.* 2005; Zhao *et al.* 2008). One such study demonstrated that the simultaneous inhibition of PDE4 and PDE8 significantly increased steroidogenesis in Leydig cells (Demirbas *et al.* 2013; Golkowski *et al.* 2016; Shimizu-Albergine *et al.* 2012). Inactivation of cAMP-selective phosphodiesterase isoforms is not unique to this potential role. Indeed, several studies exploring the inhibition of cGMP-specific PDEs using selective agents such as sildenafil and non-cAMP/cGMP pathways have demonstrated cAMP-independent ways of regulating AQP2 in renal collecting duct (Assadi and Sharbaf 2015; Bonfrate *et al.* 2015; Boone and Deen 2008; Boone *et al.* 2010; Bouley *et al.* 2000, 2005, 2008; Brown 2003; Cheung *et al.* 2016; Sanches and Volpini

2012). As such, this area of research holds potential promise for finding beneficial therapies for patients with X-linked NDI and other types of diabetes insipidus.

Another possibility is that there was insufficient cAMP production in the absence of vasopressin to allow phosphodiesterase inhibition to cause a physiologically significant rise in cAMP to activate protein kinase A and other kinases necessary for AQP2 phosphorylation. Indeed, Stefan *et al.* (2007) had shown that inhibition of PDE4 alone with the selective PDE4 inhibitor rolipram did not produce measurable cAMP levels in whole cell assays of primary cultures of renal IMCD cells. They emphasize that whole cell assays do not necessarily assess cAMP changes in subcellular compartments. Theoretically, other methods of locally increasing the basal levels of cAMP could potentially enhance the effect of PDE4 inactivation on AQP2 regulation. We have previously demonstrated that another G protein-coupled receptor, the prostaglandin E2 receptor EP4, was expressed at significant levels in

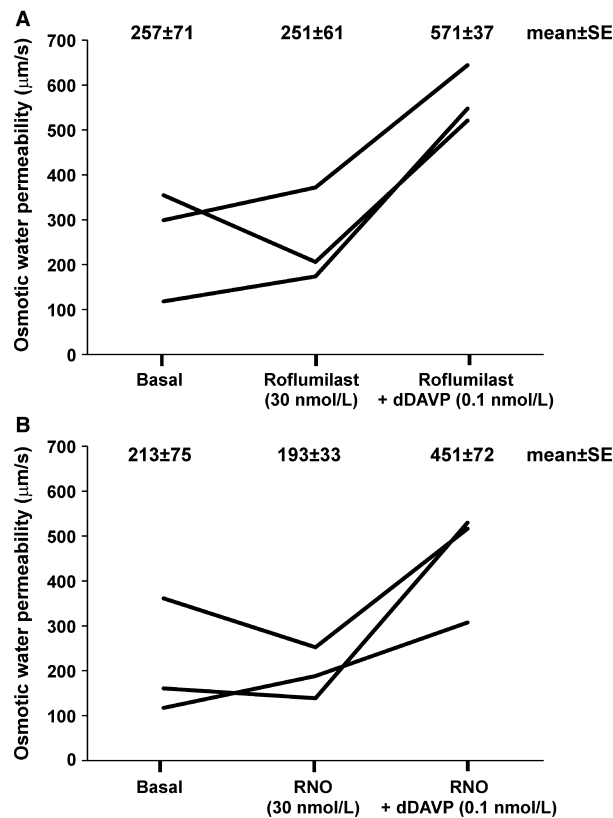


Figure 6. Osmotic water permeability (P_f) measurements in microdissected IMCD segments. IMCD tubules were isolated and perfused for osmotic water permeability measurements in three 30-min experimental periods: basal, roflumilast and roflumilast+dDAVP (upper panel) or basal, RNO and RNO+dDAVP (lower panel). Data from the same experimental period were pooled for statistical analysis. Water permeability measured at 30 nmol/L roflumilast or RNO alone was not statistically different from the basal condition, whereas in the presence of 0.1 nmol/L dDAVP, water permeability was significantly increased ($P < 0.001$, $n = 6$). IMCD, inner medullary collecting duct; RNO, roflumilast *N*-oxide.

mouse IMCD and EP4 agonist (ONO-AE1-329) was capable of increasing cAMP in mouse IMCD (Li et al. 2009). However, in this paper, ONO-AE1-329 produced no change in AQP2 phosphorylation, presumably because it does not affect cAMP in the subcellular compartment relevant to AQP2 phosphorylation which was not assessed in the previous paper (Li et al. 2009).

We also reasoned that the lack of responsiveness of rat IMCD to EP4 agonist (ONO-AE1-329) in the present study, in comparison with previous study (Li et al. 2009), could be related to species (previous in mouse; now in rat) or segment (previous Pf measurements in CCD, now in IMCD). Evidence supporting this speculation came from EP4 localization study in the rat kidney using RT-PCR mRNA in which it was shown that EP4 is

Table 1. Relative transcript abundance of PDE isoforms in rat kidney collecting duct.

Gene	Description	Substrate	CCD	OMCD	IMCD
Pde1a	Calcium/	cAMP,	24.6	50.1	13.6
Pde1c	Calmodulin stimulated	cGMP	1.1	0	0
Pde3b	cGMP-inhibited	cAMP	8.7	10.7	0
Pde4a	cAMP-specific	cAMP	0	0	0.2
Pde4b			0.6	0	0
Pde4c			0	0	0.1
Pde5a	cGMP-specific	cGMP	0.2	0	0.5
Pde6d	cGMP-activated, cGMP-specific	cGMP	32.9	0.2	62.4
Pde7b	cAMP-specific	cAMP	9.9	0.4	0.4
Pde8a	cAMP-specific	cAMP	14.4	15.9	3.3

PDE isoforms, description and substrate is adapted from Maurice et al. (2014) (Maurice). CCD, OMCD, and IMCD are collecting duct segments microdissected from the cortex, outer medulla and inner medulla of the kidney. Numbers are relative transcript abundances of PDE isoforms measured by a single tubule RNA-seq technique (Lee et al. 2015) and expressed as the number of reads per million nucleotides in the whole genome.

expressed very weakly in IMCD but is strongly expressed in the cortical collecting duct (Jensen et al. 2001). Gao et al. have also shown that EP4 protein abundance is very low in the mouse kidney medulla compared to other PGE2 receptor types, although its expression can be enhanced by water restriction to the animals (Gao et al. 2015). Additionally, Olesen et al. (2011) showed that another EP4 agonist (CAY10580) was capable of inducing AQP2 phosphorylation at pS256 in cortical tubule suspensions but it did not cause AQP2 targeting to the plasma membrane in native rat tissue. The absence of significant AQP2 phosphorylation changes in response to EP4 agonist (ONO-AE1-329) in the present study is thus consistent with these previous findings in rats. Investigations of the long-term effect of EP4 agonist have, however, identified soluble (pro)renin receptor (sPRR), by interacting with frizzled-8 (FZD8), as a downstream effector of EP4 or V2R-induced AQP2 expression and antidiuretic response in primary cultures of rat IMCD (Lu et al. 2016; Wang et al. 2016). These findings open potential applications for the use of PDE4 inhibitor in combination with EP4 agonist in a long-term response to enhance the water permeability response in the renal collecting duct.

Roflumilast is significantly more potent than IBMX

Our dose–response studies showed that roflumilast at a range of concentrations from 3 to 3000 nmol/L enhances

the effect of vasopressin to increase AQP2 phosphorylation at S264 and S269. Because S269 plays a critical role in AQP2 trafficking by inhibiting AQP2 endocytosis (Hoffert et al. 2008), this observation reinforces the conclusions of prior studies that PDE4 isoforms play an important physiological role in AQP2 regulation in the collecting duct (Coffey et al. 1991; Feraille et al. 2014; Sohara et al. 2006; Stefan et al. 2007; Wang et al. 2002). In contrast, IBMX only enhanced the vasopressin-dependent AQP2 phosphorylation at 3000 nmol/L. Our results suggest that roflumilast is several orders of magnitude more potent than IBMX.

When compared to rolipram, roflumilast has a significantly higher affinity for PDE4, with an IC_{50} that is over a 1000-fold less than rolipram (Maurice et al. 2014; Rabe 2011). And roflumilast's remarkable efficacy over rolipram has been highlighted by several studies on PDE4 inhibition (Dastidar et al. 2009; Davis et al. 2009; Ikari et al. 2013; Togo et al. 2009; Wollin et al. 2006; Wunder et al. 2013). More importantly, Thompson et al. (2002) have demonstrated through their studies that selective inhibition of PDE4 by high-affinity agents are more effective in increasing cAMP levels than low-affinity ones and nonselective inhibitors. Taking all these into account, we conclude that roflumilast may be a better choice than IBMX or rolipram for the conduct of physiological studies on AQP2 regulation, and could be useful in the treatment of other water transport disorders in the renal collecting duct.

Acknowledgment

Immunofluorescence microscopy was carried out in the NHLBI Light Microscopy Core Facility (Director, Christian Combs). E. N. Umejiego was supported by Biomedical Research Training Program for Individuals from Underrepresented Groups (BRTPUG) of the NHLBI under the leadership of Dr. Helena O. Mishoe.

Conflict of Interest

No conflicts of interest, financial, or otherwise, are declared by the author(s).

References

Al-Zahid, G., J. A. Schafer, S. L. Troutman, and T. E. Andreoli. 1977. Effect of antidiuretic hormone on water and solute permeation, and the activation energies for these processes, in mammalian cortical collecting tubules: evidence for parallel ADH-sensitive pathways for water and solute diffusion in luminal plasma membranes. *J. Membr. Biol.* 31:103–129.

Assadi, F., and F. G. Sharbaf. 2015. Sildenafil for the Treatment of Congenital Nephrogenic Diabetes Insipidus. *Am. J. Nephrol.* 42:65–69.

van Balkom, B. W., J. D. Hoffert, C. L. Chou, and M. A. Knepper. 2004. Proteomic analysis of long-term vasopressin action in the inner medullary collecting duct of the Brattleboro rat. *Am. J. Physiol. Renal. Physiol.* 286:F216–F224.

Banner, K. H., and N. J. Press. 2009. Dual PDE3/4 inhibitors as therapeutic agents for chronic obstructive pulmonary disease. *Br. J. Pharmacol.* 157:892–906.

Beavo, J. A. 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* 75:725–748.

Beavo, J. A., M. Conti, and R. J. Heaslip. 1994. Multiple cyclic nucleotide phosphodiesterases. *Mol. Pharmacol.* 46:399–405.

Beghe, B., K. F. Rabe, and L. M. Fabbri. 2013. Phosphodiesterase-4 inhibitor therapy for lung diseases. *Am. J. Respir. Crit. Care Med.* 188:271–278.

Bichet, D. G., N. Ruel, M. F. Arthus, and M. Lonergan. 1990. Rolipram, a phosphodiesterase inhibitor, in the treatment of two male patients with congenital nephrogenic diabetes insipidus. *Nephron* 56:449–450.

Bichet, D. G., M. F. Arthus, M. Lonergan, G. N. Hendy, A. J. Paradis, T. M. Fujiwara, et al. 1993. X-linked nephrogenic diabetes insipidus mutations in North America and the Hopewell hypothesis. *J. Clin. Invest.* 92:1262–1268.

Bloom, T. J., and J. A. Beavo. 1996. Identification and tissue-specific expression of PDE7 phosphodiesterase splice variants. *Proc. Natl Acad. Sci. USA* 93:14188–14192.

Bockenbauer, D., and D. G. Bichet. 2015. Pathophysiology, diagnosis and management of nephrogenic diabetes insipidus. *Nat. Rev. Nephrol.* 11:576–588.

Bolger, G. B. 1994. Molecular biology of the cyclic AMP-specific cyclic nucleotide phosphodiesterases: a diverse family of regulatory enzymes. *Cell. Signal.* 6:851–859.

Bonfrate, L., G. Procino, D. Q. Wang, M. Svelto, and P. Portincasa. 2015. A novel therapeutic effect of statins on nephrogenic diabetes insipidus. *J. Cell Mol. Med.* 19:265–282.

Boone, M., and P. M. Deen. 2008. Physiology and pathophysiology of the vasopressin-regulated renal water reabsorption. *Pflugers Arch.* 456:1005–1024.

Boone, M., M. Kortenoeven, J. H. Robben, and P. M. Deen. 2010. Effect of the cGMP pathway on AQP2 expression and translocation: potential implications for nephrogenic diabetes insipidus. *Nephrol. Dial. Transplant.* 25:48–54.

Bouley, R., S. Breton, T. Sun, M. McLaughlin, N. N. Nsumu, H. Y. Lin, et al. 2000. Nitric oxide and atrial natriuretic factor stimulate cGMP-dependent membrane insertion of aquaporin 2 in renal epithelial cells. *J. Clin. Invest.* 106:1115–1126.

Bouley, R., N. Pastor-Soler, O. Cohen, M. McLaughlin, S. Breton, and D. Brown. 2005. Stimulation of AQP2

- membrane insertion in renal epithelial cells in vitro and in vivo by the cGMP phosphodiesterase inhibitor sildenafil citrate (Viagra). *Am. J. Physiol. Renal. Physiol.* 288:F1103–F1112.
- Bouley, R., U. Hasler, H. A. Lu, P. Nunes, and D. Brown. 2008. Bypassing vasopressin receptor signaling pathways in nephrogenic diabetes insipidus. *Semin. Nephrol.* 28:266–278.
- Bouley, R., H. A. Lu, P. Nunes, S. N. Da, M. McLaughlin, Y. Chen, et al. 2011. Calcitonin has a vasopressin-like effect on aquaporin-2 trafficking and urinary concentration. *J. Am. Soc. Nephrol.* 22:59–72.
- Brown, D. 2003. The ins and outs of aquaporin-2 trafficking. *Am. J. Physiol. Renal. Physiol.* 284:F893–F901.
- Cheung, P. W., N. Nomura, A. V. Nair, N. Pathomthongtaweetchai, L. Ueberdiek, H. A. Jenny Lu, et al. 2016. EGF Receptor Inhibition by Erlotinib Increases Aquaporin 2-Mediated Renal Water Reabsorption. *J. Am. Soc. Nephrol.* 27:3105–3116.
- Chou, C. L., M. J. Yu, E. M. Kassai, R. G. Morris, J. D. Hoffert, S. M. Wall, et al. 2008. Roles of basolateral solute uptake via NKCC1 and of myosin II in vasopressin-induced cell swelling in inner medullary collecting duct. *Am. J. Physiol. Renal. Physiol.* 295:F192–F201.
- Coffey, A. K., D. J. O'Sullivan, S. Homma, T. P. Dousa, and H. Valtin. 1991. Induction of intramembranous particle clusters in mice with nephrogenic diabetes insipidus. *Am. J. Physiol.* 261:F640–F646.
- Conti, M., G. Nemoz, C. Sette, and E. Vicini. 1995. Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr. Rev.* 16:370–389.
- Conti, M., W. Richter, C. Mehats, G. Livera, J. Y. Park, and C. Jin. 2003. Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J. Biol. Chem.* 278:5493–5496.
- Dastidar, S. G., A. Ray, R. Shirumalla, D. Rajagopal, S. Chaudhary, K. Nanda, et al. 2009. Pharmacology of a novel, orally active PDE4 inhibitor. *Pharmacology* 83:275–286.
- Davis, C. W. 1984. Assessment of selective inhibition of rat cerebral cortical calcium-independent and calcium-dependent phosphodiesterases in crude extracts using deoxycyclic AMP and potassium ions. *Biochim. Biophys. Acta* 797:354–362.
- Davis, T. G., J. J. Peterson, J. P. Kou, E. A. Capper-Spudich, D. Ball, A. T. Nials, et al. 2009. The identification of a novel phosphodiesterase 4 inhibitor, 1-ethyl-5-{5-[(4-methyl-1-piperazinyl)methyl]-1,3,4-oxadiazol-2-yl}-N-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-b]pyridin-4-amine (EPPA-1), with improved therapeutic index using pica feeding in rats as a measure of emetogenicity. *J. Pharmacol. Exp. Ther.* 330:922–931.
- Degerman, E., P. Belfrage, and V. C. Manganiello. 1997. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J. Biol. Chem.* 272:6823–6826.
- Demirbas, D., A. R. Wyman, M. Shimizu-Albergine, O. Cakici, J. A. Beavo, and C. S. Hoffman. 2013. A yeast-based chemical screen identifies a PDE inhibitor that elevates steroidogenesis in mouse Leydig cells via PDE8 and PDE4 inhibition. *PLoS ONE* 8:e71279.
- Dousa, T. P. 1999. Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int.* 55:29–62.
- Engels, P., K. Fichtel, and H. Lubbert. 1994. Expression and regulation of human and rat phosphodiesterase type IV isogenes. *FEBS Lett.* 350:291–295.
- Feraille, E., E. Dizin, I. Roth, J. P. Derouette, I. Szanto, P. Y. Martin, et al. 2014. NADPH oxidase 4 deficiency reduces aquaporin-2 mRNA expression in cultured renal collecting duct principal cells via increased PDE3 and PDE4 activity. *PLoS ONE* 9:e87239.
- Fisher, D. A., J. F. Smith, J. S. Pillar, S. H. St Denis, and S. H. Cheng. 1998. Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. *Biochem. Biophys. Res. Commun.* 246:570–577.
- Francis, S. H., M. A. Blount, and J. D. Corbin. 2011. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol. Rev.* 91:651–690.
- Fushimi, K., S. Sasaki, and F. Marumo. 1997. Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel. *J. Biol. Chem.* 272:14800–14804.
- Gao, M., R. Cao, S. Du, X. Jia, S. Zheng, S. Huang, et al. 2015. Disruption of prostaglandin E2 receptor EP4 impairs urinary concentration via decreasing aquaporin 2 in renal collecting ducts. *Proc. Natl Acad. Sci. USA* 112:8397–8402.
- Gardner, C., N. Robas, D. Cawkill, and M. Fidock. 2000. Cloning and characterization of the human and mouse PDE7B, a novel cAMP-specific cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 272:186–192.
- Giembycz, M. A., and R. Newton. 2011. Harnessing the clinical efficacy of phosphodiesterase 4 inhibitors in inflammatory lung diseases: dual-selective phosphodiesterase inhibitors and novel combination therapies. *Handb. Exp. Pharmacol.* 415–446.
- Golkowski, M., M. Shimizu-Albergine, H. W. Suh, J. A. Beavo, and S. E. Ong. 2016. Studying mechanisms of cAMP and cyclic nucleotide phosphodiesterase signaling in Leydig cell function with phosphoproteomics. *Cell. Signal.* 28:764–778.
- Gross, N. J., M. A. Giembycz, and S. I. Rennard. 2010. Treatment of chronic obstructive pulmonary disease with roflumilast, a new phosphodiesterase 4 inhibitor. *COPD* 7:141–153.
- Hatzelmann, A., and C. Schudt. 2001. Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J. Pharmacol. Exp. Ther.* 297:267–279.

- Hayashi, M., K. Matsushima, H. Ohashi, H. Tsunoda, S. Murase, Y. Kawarada, et al. 1998. Molecular cloning and characterization of human PDE8B, a novel thyroid-specific isozyme of 3',5'-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 250:751–756.
- Hendriks, G., M. Koudijs, M. Koudijs B. W. Van Balkom, V. Oorschot, J. Klumperman, et al. 2004. Glycosylation is important for cell surface expression of the water channel aquaporin-2 but is not essential for tetramerization in the endoplasmic reticulum. *J. Biol. Chem.* 279:2975–2983.
- Hoffert, J. D., T. Pisitkun, G. Wang, R. F. Shen, and M. A. Knepper. 2006. Quantitative phosphoproteomics of vasopressin-sensitive renal cells: regulation of aquaporin-2 phosphorylation at two sites. *Proc. Natl Acad. Sci. USA* 103:7159–7164.
- Hoffert, J. D., J. Nielsen, M. J. Yu, T. Pisitkun, S. M. Schleicher, S. Nielsen, et al. 2007. Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. *Am. J. Physiol. Renal. Physiol.* 292:F691–F700.
- Hoffert, J. D., R. A. Fenton, H. B. Moeller, B. Simons, D. Tchapyjnikov, B. W. McDill, et al. 2008. Vasopressin-stimulated increase in phosphorylation at Ser269 potentiates plasma membrane retention of aquaporin-2. *J. Biol. Chem.* 283:24617–24627.
- Holtzman, E. J., H. W. Jr Harris, L. F. Jr Kolakowski, L. M. Guay-Woolford, B. Betelho, and D. A. Ausiello. 1993a. A molecular defect in the vasopressin V2-receptor gene causing nephrogenic diabetes insipidus. *N. Engl. J. Med.* 328:1534–1537.
- Holtzman, E. J., L. F. Jr Kolakowski, D. O'Brien, J. D. Crawford, and D. A. Ausiello. 1993b. A Null mutation in the vasopressin V2 receptor gene (AVPR2) associated with nephrogenic diabetes insipidus in the Hopewell kindred. *Hum. Mol. Genet.* 2:1201–1204.
- Ikari, J., J. M. Michalski, S. Iwasawa, Y. Gunji, S. Nogel, J. H. Park, et al. 2013. Phosphodiesterase-4 inhibition augments human lung fibroblast vascular endothelial growth factor production induced by prostaglandin E2. *Am. J. Respir. Cell Mol. Biol.* 49:571–581.
- Izquierdo, J. L., and J. Aparicio. 2010. Roflumilast for COPD. *Drugs Today (Barc)* 46:823–831.
- Jensen, B. L., J. Stubbe, P. B. Hansen, D. Andreasen, and O. Skott. 2001. Localization of prostaglandin E(2) EP2 and EP4 receptors in the rat kidney. *Am. J. Physiol. Renal. Physiol.* 280:F1001–F1009.
- Kakiuchi, S., R. Yamazaki, Y. Teshima, K. Uenishi, and E. Miyamoto. 1975. Multiple cyclic nucleotide phosphodiesterase activities from rat tissues and occurrence of a calcium-plus-magnesium-ion-dependent phosphodiesterase and its protein activator. *Biochem. J.* 146:109–120.
- Kamsteeg, E. J., I. Heijnen, C. H. van Os, and P. M. Deen. 2000. The subcellular localization of an aquaporin-2 tetramer depends on the stoichiometry of phosphorylated and nonphosphorylated monomers. *J. Cell Biol.* 151:919–930.
- Kim, G. H., C. A. Ecelbarger, C. Mitchell, R. K. Packer, J. B. Wade, and M. A. Knepper. 1999. Vasopressin increases Na-K-2Cl cotransporter expression in thick ascending limb of Henle's loop. *Am. J. Physiol.* 276:F96–F103.
- Klee, C. B., and T. C. Vanaman. 1982. Calmodulin. *Adv. Protein Chem.* 35:213–321.
- Kraynik, S. M., R. S. Miyaoka, and J. A. Beavo. 2013. PDE3 and PDE4 isozyme-selective inhibitors are both required for synergistic activation of brown adipose tissue. *Mol. Pharmacol.* 83:1155–1165.
- Lambert, J. A., S. V. Raju, L. P. Tang, C. M. McNicholas, Y. Li, C. A. Courville, et al. 2014. Cystic fibrosis transmembrane conductance regulator activation by roflumilast contributes to therapeutic benefit in chronic bronchitis. *Am. J. Respir. Cell Mol. Biol.* 50:549–558.
- Lee, J. W., C. L. Chou, and M. A. Knepper. 2015. Deep Sequencing in Microdissected Renal Tubules Identifies Nephron Segment-Specific Transcriptomes. *J. Am. Soc. Nephrol.* 26:2669–2677.
- Leroy, M. J., E. Degerman, M. Taira, T. Murata, L. H. Wang, M. A. Movsesian, et al. 1996. Characterization of two recombinant PDE3 (cGMP-inhibited cyclic nucleotide phosphodiesterase) isoforms, RcGIP1 and HcGIP2, expressed in NIH 3006 murine fibroblasts and Sf9 insect cells. *Biochemistry* 35:10194–10202.
- Li, J. H., C. L. Chou, B. Li, O. Gavrilova, C. Eisner, J. Schnermann, et al. 2009. A selective EP4 PGE2 receptor agonist alleviates disease in a new mouse model of X-linked nephrogenic diabetes insipidus. *J. Clin. Invest.* 119:3115–3126.
- Li, W., Y. Zhang, R. Bouley, Y. Chen, T. Matsuzaki, P. Nunes, et al. 2011. Simvastatin enhances aquaporin-2 surface expression and urinary concentration in vasopressin-deficient Brattleboro rats through modulation of Rho GTPase. *Am. J. Physiol. Renal. Physiol.* 301:F309–F318.
- Lu, X., F. Wang, C. Xu, S. Soodvilai, K. Peng, J. Su, et al. 2016. Soluble (pro)renin receptor via beta-catenin enhances urine concentration capability as a target of liver X receptor. *Proc. Natl Acad. Sci. USA* 113:E1898–E1906.
- Matera, M. G., P. Rogliani, L. Calzetta, and M. Cazzola. 2014. Phosphodiesterase inhibitors for chronic obstructive pulmonary disease: what does the future hold? *Drugs* 74:1983–1992.
- Maurice, D. H., H. Ke, F. Ahmad, Y. Wang, J. Chung, and V. C. Manganiello. 2014. Advances in targeting cyclic nucleotide phosphodiesterases. *Nat. Rev. Drug Discov.* 13:290–314.
- McSorley, T., E. Stefan, V. Henn, B. Wiesner, G. S. Baillie, M. D. Houslay, et al. 2006. Spatial organisation of AKAP18 and PDE4 isoforms in renal collecting duct principal cells. *Eur. J. Cell Biol.* 85:673–678.

- Michaeli, T., T. J. Bloom, T. Martins, K. Loughney, K. Ferguson, M. Riggs, et al. 1993. Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient *Saccharomyces cerevisiae*. *J. Biol. Chem.* 268:12925–12932.
- Mika, D., P. Bobin, M. Pomerance, P. Lechene, R. E. Westenbroek, W. A. Catterall, et al. 2013. Differential regulation of cardiac excitation-contraction coupling by cAMP phosphodiesterase subtypes. *Cardiovasc. Res.* 100:336–346.
- Miranda, C. A., J. W. Lee, C. L. Chou, and M. A. Knepper. 2014. Tolvaptan as a tool in renal physiology. *Am. J. Physiol. Renal. Physiol.* 306:F359–F366.
- Moeller, H. B., M. A. Knepper, and R. A. Fenton. 2008. Serine 269 phosphorylated aquaporin-2 is targeted to the apical membrane of collecting duct principal cells. *Kidney Int.* 75:295–303.
- Moeller, H. B., N. MacAulay, M. A. Knepper, and R. A. Fenton. 2009. Role of multiple phosphorylation sites in the COOH-terminal tail of aquaporin-2 for water transport: evidence against channel gating. *Am. J. Physiol. Renal. Physiol.* 296:F649–F657.
- Moses, A. M., and S. J. Scheinman. 1993. Hereditary vasopressin resistance in man and mouse. *Ann. N. Y. Acad. Sci.* 689:233–249.
- Nielsen, S., C. L. Chou, D. Marples, E. I. Christensen, B. K. Kishore, and M. A. Knepper. 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc. Natl Acad. Sci. USA* 92:1013–1017.
- Olesen, E. T., M. R. Rutzler, H. B. Moeller, H. A. Praetorius, and R. A. Fenton. 2011. Vasopressin-independent targeting of aquaporin-2 by selective E-prostanoid receptor agonists alleviates nephrogenic diabetes insipidus. *Proc. Natl Acad. Sci. USA* 108:12949–12954.
- Palmer, D., K. Tsoi, and D. H. Maurice. 1998. Synergistic inhibition of vascular smooth muscle cell migration by phosphodiesterase 3 and phosphodiesterase 4 inhibitors. *Circ. Res.* 82:852–861.
- Pisitkun, T., J. Bieniek, D. Tchapyjnikov, G. Wang, W. W. Wu, R. F. Shen, et al. 2006. High-Throughput Identification of IMCD Proteins Using LC-MS/MS. *Physiol. Genomics* 25:263–276.
- Procino, G., S. Milano, M. Carosino, C. Barbieri, M. C. Nicoletti, J. H. Li, et al. 2014. Combination of secretin and fluvastatin ameliorates the polyuria associated with X-linked nephrogenic diabetes insipidus in mice. *Kidney Int.* 86:127–138.
- Rabe, K. F. 2010. Roflumilast for the treatment of chronic obstructive pulmonary disease. *Expert Rev. Respir. Med.* 4:543–555.
- Rabe, K. F. 2011. Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br. J. Pharmacol.* 163:53–67.
- Russo, L. M., M. McKee, and D. Brown. 2006. Methyl-beta-cyclodextrin induces vasopressin-independent apical accumulation of aquaporin-2 in the isolated, perfused rat kidney. *Am. J. Physiol. Renal. Physiol.* 291:F246–F253.
- Salari, P., and M. Abdollahi. 2012. Phosphodiesterase inhibitors in inflammatory bowel disease. *Expert Opin. Investig. Drugs* 21:261–264.
- Sanches, T. R., R. A. Volpini, M. H. Massola Shimizu, A. C. Braganca, F. Oshiro-Monreal, A. C. Seguro, and L. Andrade. 2012. Sildenafil reduces polyuria in rats with lithium-induced NDI. *Am. J. Physiol. Renal. Physiol.* 302:F216–F225.
- Sands, J. M., and M. A. Knepper. 1987. Urea permeability of mammalian inner medullary collecting duct system and papillary surface epithelium. *J. Clin. Invest.* 79:138–147.
- Schwabe, U., M. Miyake, Y. Ohga, and J. W. Daly. 1976. 4-(3-Cyclopentyl-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. *Mol. Pharmacol.* 12:900–910.
- Shimizu-Albergine, M., L. C. Tsai, E. Patrucco, and J. A. Beavo. 2012. cAMP-specific phosphodiesterases 8A and 8B, essential regulators of Leydig cell steroidogenesis. *Mol. Pharmacol.* 81:556–566.
- Snyder, P. B., J. M. Esselstyn, K. Loughney, S. L. Wolda, and V. A. Florio. 2005. The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis. *J. Lipid Res.* 46:494–503.
- Soderling, S. H., and J. A. Beavo. 2000. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* 12:174–179.
- Soderling, S. H., S. J. Bayuga, and J. A. Beavo. 1998. Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. *Proc. Natl Acad. Sci. USA* 95:8991–8996.
- Sohara, E., T. Rai, S. S. Yang, K. Uchida, K. Nitta, S. Horita, et al. 2006. Pathogenesis and treatment of autosomal-dominant nephrogenic diabetes insipidus caused by an aquaporin 2 mutation. *Proc. Natl Acad. Sci. USA* 103:14217–14222.
- Star, R. A., H. Nonoguchi, R. Balaban, and M. A. Knepper. 1988. Calcium and cyclic adenosine monophosphate as second messengers for vasopressin in the rat inner medullary collecting duct. *J. Clin. Invest.* 81:1879–1888.
- Stefan, E., B. Wiesner, G. S. Baillie, R. Mollajew, V. Henn, D. Lorenz, et al. 2007. Compartmentalization of cAMP-dependent signaling by phosphodiesterase-4D is involved in the regulation of vasopressin-mediated water reabsorption in renal principal cells. *J. Am. Soc. Nephrol.* 18:199–212.
- Swinnen, J. V., D. R. Joseph, and M. Conti. 1989. Molecular cloning of rat homologues of the *Drosophila melanogaster* dunce cAMP phosphodiesterase: evidence for a family of genes. *Proc. Natl Acad. Sci. USA* 86:5325–5329.

- Terris, J., C. A. Ecelbarger, S. Nielsen, and M. A. Knepper. 1996. Long-term regulation of four renal aquaporins in rats. *Am. J. Physiol.* 271:F414–F422.
- Thompson, W. J. 1991. Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol. Ther.* 51:13–33.
- Thompson, W. J., T. Ashikaga, J. J. Kelly, L. Liu, B. Zhu, L. Vemavarapu, et al. 2002. Regulation of cyclic AMP in rat pulmonary microvascular endothelial cells by rolipram-sensitive cyclic AMP phosphodiesterase (PDE4). *Biochem. Pharmacol.* 63:797–807.
- Togo, S., X. Liu, X. Wang, H. Sugiura, K. Kamio, S. Kawasaki, et al. 2009. PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF- β 1-stimulated fibroblasts. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 296:L959–L969.
- Uawithya, P., T. Pisitkun, B. E. Ruttenberg, and M. A. Knepper. 2008. Transcriptional profiling of native inner medullary collecting duct cells from rat kidney. *Physiol. Genomics* 32:229–253.
- Wall, S. M., J. S. Han, C. L. Chou, and M. A. Knepper. 1992. Kinetics of urea and water permeability activation by vasopressin in rat terminal IMCD. *Am. J. Physiol.* 262:F989–F998.
- Wang, P., J. G. Myers, P. Wu, B. Cheewatrakoolpong, R. W. Egan, and M. M. Billah. 1997. Expression, purification, and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. *Biochem. Biophys. Res. Commun.* 234:320–324.
- Wang, W., C. Li, T. H. Kwon, M. A. Knepper, J. Frokiaer, and S. Nielsen. 2002. AQP3, p-AQP2, and AQP2 expression is reduced in polyuric rats with hypercalcemia: prevention by cAMP-PDE inhibitors. *Am. J. Physiol. Renal. Physiol.* 283:F1313–F1325.
- Wang, F., X. Lu, K. Peng, H. Fang, L. Zhou, J. Su, et al. 2016. Antidiuretic Action of Collecting Duct (Pro)Renin Receptor Downstream of Vasopressin and PGE2 Receptor EP4. *J. Am. Soc. Nephrol.* 27:3022–3034.
- Weishaar, R. E., M. H. Cain, and J. A. Bristol. 1985. A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J. Med. Chem.* 28:537–545.
- Wollin, L., D. S. Bundschuh, A. Wohlsen, D. Marx, and R. Beume. 2006. Inhibition of airway hyperresponsiveness and pulmonary inflammation by roflumilast and other PDE4 inhibitors. *Pulm. Pharmacol. Ther.* 19:343–352.
- Wunder, F., R. Quednau, A. Geerts, M. Barg, and A. Tersteegen. 2013. Characterization of the cellular activity of PDE 4 inhibitors using two novel PDE 4 reporter cell lines. *Mol. Pharm.* 10:3697–3705.
- Xie, L., J. D. Hoffert, C. L. Chou, M. J. Yu, T. Pisitkun, M. A. Knepper, et al. 2010. Quantitative analysis of aquaporin-2 phosphorylation. *Am. J. Physiol. Renal. Physiol.* 298:F1018–F1023.
- Yanaka, N., J. Kotera, A. Ohtsuka, H. Akatsuka, Y. Imai, H. Michibata, et al. 1998. Expression, structure and chromosomal localization of the human cGMP-binding cGMP-specific phosphodiesterase PDE5A gene. *Eur. J. Biochem.* 255:391–399.
- Yip, K. P. 2002. Coupling of vasopressin-induced intracellular Ca²⁺ mobilization and apical exocytosis in perfused rat kidney collecting duct. *J. Physiol.* 538:891–899.
- Yu, M. J., R. L. Miller, P. Uawithya, M. M. Rinschen, S. Khositseth, D. W. Braucht, et al. 2009. Systems-level analysis of cell-specific AQP2 gene expression in renal collecting duct. *Proc. Natl Acad. Sci. USA* 106:2441–2446.
- Zhao, H., Q. Guan, C. J. Smith, and J. Quilley. 2008. Increased phosphodiesterase 3A/4B expression after angioplasty and the effect on VASP phosphorylation. *Eur. J. Pharmacol.* 590:29–35.