Vasopressin-independent targeting of aquaporin-2 by selective E-prostanoid receptor agonists alleviates nephrogenic diabetes insipidus

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In the kidney, the actions of vasopressin on its type-2 receptor (V2R) induce increased water reabsorption alongside polyphosphorylation and membrane targeting of the water channel aquaporin-2 (AQP2). Loss-of-function mutations in the V2R cause X-linked nephrogenic diabetes insipidus. Treatment of this condition would require bypassing the V2R to increase AQP2 membrane targeting, but currently no specific pharmacological therapy is available. The present study examined specific E-prostanoid receptors for this purpose. In vitro, prostaglandin E2 (PGE2) and selective agonists for the E-prostanoid receptors EP2 (butaprost) or EP4 (CAY10580) all increased trafficking and ser-264 phosphorylation of AQP2 in Madin-Darby canine kidney cells. Only PGE2 and butaprost increased cAMP and ser-269 phosphorylation of AQP2. Ex vivo, PGE2, butaprost, or CAY10580 increased AQP2 phosphorylation in isolated cortical tubules, whereas PGE2 and butaprost selectively increased AQP2 membrane accumulation in kidney slices. In vivo, a V2R antagonist caused a severe urinary concentrating defect in rats, which was greatly alleviated by treatment with butaprost. In conclusion, EP2 and EP4 agonists increase AQP2 phosphorylation and trafficking, likely through different signaling pathways. Furthermore, EP2 selective agonists can partially compensate for a nonfunctional V2R, providing a rationale for new treatment strategies for hereditary nephrogenic diabetes insipidus.

water balance | cell signaling | autocoid | lipid mediators | drug development/discovery

The anti-diuretic hormone vasopressin (VP) controls whole-body water balance mainly by regulating the water permeability of the kidney collecting duct. VP binds to the Gs-protein–coupled VP type 2 receptor (V2R) in collecting duct principal cells, stimulating the accumulation of aquaporin-2 (AQP2) in the apical plasma membrane (1). This process increases the water permeability of the epithelium, allowing water to be reabsorbed from the collecting-duct lumen and increasing the concentration of the urine. The intracellular signaling cascades of VP in the collecting duct involve increased cAMP and protein kinase-dependent phosphorylation of AQP2 at ser-256, ser-264, and ser-269. The ser-256 and ser-269 sites appear to be essential for apical membrane accumulation of AQP2 (2).

Although VP's role is well characterized, there is evidence that alternative mechanisms may also regulate water permeability. For example, functional studies on collecting ducts have shown an EC_{50} of 10^{-11} M for VP-induced increases in water permeability and a requirement of 10^{-8} M for maximal effect (3). Water restriction does not increase plasma VP to these levels (4–6). This leaves room for additional mechanisms to be involved in modulating collecting-duct water permeability, supported by evidence that the urinary concentrating ability of the kidney can increase in the presence of a V2R antagonist during water restriction (7).

Prostanoids are a family of arachidonic acid derivatives produced in most cell types. The biological actions of one class of prostanoids, prostaglandin E2 (PGE2), are diverse. This may result from the ability of PGE2 to stimulate four different E-prostanoid receptors (EP1–4) and therefore potentially initiate different intracellular signaling cascades. Both EP2 and EP4 can signal via increased cAMP (8). PGE2 is synthesized and released in the collecting duct, which expresses all four EP receptors (9–11). Interestingly, at sites where EP1–4 are present, including the collecting duct, PGE2 is known to have opposing effects; e.g., PGE2 has been shown both to increase water permeability and to decrease the effect of high levels of VP in the cortical collecting duct (12).

We hypothesized that selective EP receptor stimulation could mimic the effects of VP. Our main findings were that, at physiological levels, PGE2 markedly increased apical membrane abundance and phosphorylation of AQP2 in vitro and ex vivo, leading to increased cell water permeability. Both EP2 and EP4 selective agonists were able to mimic these effects. Furthermore, an EP2 agonist was able to positively regulate urinary-concentrating mechanisms in an animal model of nephrogenic diabetes insipidus (NDI). These results reveal an alternative mechanism for regulating water transport in the collecting duct that have major importance for understanding whole-body water homeostasis and provides a rationale for investigations into EP receptor agonist use in NDI treatment.

Results

PGE2 Increases Phosphorylation and Apical Targeting of AQP2 in Madin-Darby Canine Kidney Cells. Madin-Darby canine kidney (MDCK) cells stably transfected with AQP2 were stimulated for 40 min with PGE2 (10^{-10} to 10^{-6} M) and apical membrane abundance of AQP2 was assessed by quantitative biotinylation (Fig. 1 *A* and *B*). At each concentration of PGE2, apical membrane abundance of AQP2 was significantly increased compared with control conditions. To assess the physiological levels of PGE2 at the site of the collecting duct in normal and stimulated conditions, PGE2 was measured in urine of control rats or rats stimulated with the bacterial toxin, lipopolysaccharide (LPS) 6 h previously (Fig. 1*C*) to stimulate PGE2 synthesis. PGE2 concentrations of ~1–10 nM were observed in urine, which is within the range that induced membrane targeting of AQP2 in MDCK cells. PGE2 (10^{-7} M) induced a steady increase in AQP2 apical membrane abundance over 2 h (Fig. S1 *A*, *B*, and *I*). In contrast, [deamino-Cys1, D-Arg8]-Vasopressin (dDAVP)-induced apical membrane accumulation of AQP2 reached a maximum after 5 min of stimulation (Fig. S1 *G* and *H*). Ser-264 phosphorylation during PGE2 stimulation exhibited a time-dependant increase reaching a maximum at ~40 min (Fig. S1 *C* and *D*), whereas

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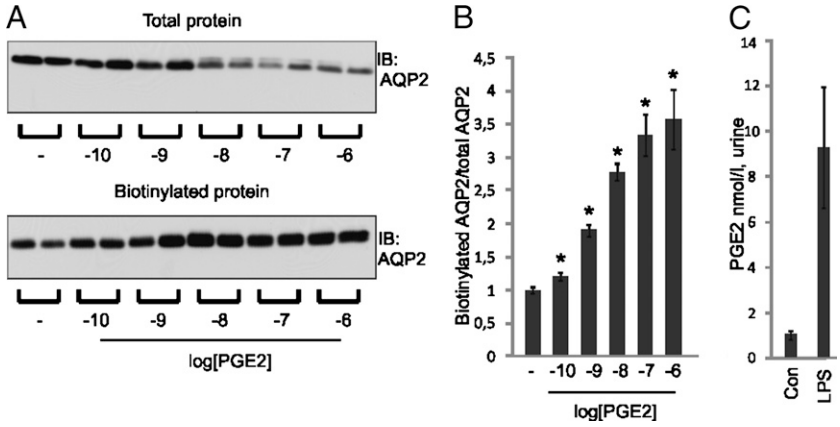

Fig. 1. PGE2 increases targeting of AQP2 in MDCK cells. The effect of PGE2 on AQP2 targeting was assessed by cell-surface biotinylation. (*A*) Representative immunoblot of AQP2 in total and biotinylated protein samples after 40 min PGE2 stimulation at the indicated concentrations. (*B*) Relative AQP2 membrane abundance in control conditions (–) or after PGE2 stimulation at concentrations of 10^{-10} to 10^{-6} M. Results are pooled from experiments done on 3 separate days. An asterisk indicates a significant difference compared with controls. (*C*) PGE2 levels in urine samples from control rats and rats given an i.p. bolus injection of LPS (2.5 mg/kg) 6 h previously.

pS269-AQP2 rapidly increased and reached a maximum after 5 min (Fig. S1 *E* and *F*). PGE2 (10^{-9} to 10^{-7} M) induced a gradual increase in water permeability in MDCK cells (Fig. S2). This increase was comparable in extent to stimulation of MDCK cells with 10^{-5} M forskolin. Ser-256 levels did not significantly increase at any time point (Fig. S3 *A* and *B*), but studies in MDCK cells stably transfected with a mutant form of AQP2 that cannot be phosphorylated at ser-256 (S256A) reveal that, as with VP/forskolin stimulation, this phosphorylation site is required for PGE2-induced targeting (Fig. S3*C*).

EP4 Receptor Agonist CAY10580 Increases Targeting of AQP2 in MDCK Cells. EP2 and EP4 receptors were detected in our MDCK cells using RT-PCR (Table S1 and Fig. S4). To examine the involvement of EP4 in our observations, cells were stimulated for 40 min with CAY10580 (10^{-8} to 10^{-5} M), a specific agonist for the EP4 receptor (13) (Fig. 2). Compared with controls, apical membrane abundance of AQP2 was significantly increased at 10^{-7} to 10^{-5} M (Fig. 2 *A* and *B*). Membrane targeting and ser-264 phosphorylation of AQP2 mimicked the effect of 40 min of PGE2 stimulation (10^{-7} M) (Fig. 2 *A*–*D*). However, our studies with CAY10580 clearly showed that the EP4 receptor cannot fully account for the effect of PGE2 on S269-AQP2 phosphorylation (Fig. 2 *C* and *E*).

EP2 Agonist Butaprost Mimics Effects of PGE2 on Targeting and Phosphorylation of AQP2. Cells were stimulated for 40 min with the EP2 agonist butaprost at concentrations of 10^{-9} to 10^{-6} M (Fig. 3). Compared with controls, membrane abundance of AQP2 increased at 10^{-8} to 10^{-6} M of butaprost. Membrane targeting and ser-264 phosphorylation of AQP2 with 10^{-7} M butaprost mimicked the effect of 40 min of PGE2 stimulation (10^{-7} M) (Fig. 3 *A*–*D*). In contrast to CAY10580, 10^{-8} M butaprost markedly phosphorylated ser269-AQP2 (Fig. 3 *C* and *E*). To further substantiate this, stimulation of MDCK cells with CAY10580 (10^{-7} M) and butaprost (10^{-8} M), respectively, was performed in the same experiment (Fig. S5). Both agonists increased membrane targeting and pS264-AQP2 levels. CAY10580 had no effect on pS269-AQP2, whereas both butaprost and PGE2 significantly increased pser-269 phosphorylation levels.

Butaprost Increases AQP2 Membrane Accumulation by Selectively Stimulating EP2. To determine whether PGE2 indeed increases AQP2 targeting through both EP2 and EP4 and to assess the selectivity of butaprost for EP2, MDCK cells were stimulated with PGE2 (10^{-8} M) or butaprost (10^{-8} M) with or without 30 min of preincubation with an EP2 antagonist AH6809 (10 μM). This concentration has been shown to induce a 30-fold right shift in the concentration response curve of PGE2-mediated EP2 signaling (14). AH6809 had no effect on PGE2-induced targeting of AQP2 (Fig. S6), which supports our findings that EP2 or EP4 alone can induce the full targeting effect of PGE2. In contrast, blocking the EP2 receptor with AH6809 completely reversed the effect of butaprost on AQP2 membrane targeting, thus suggesting that butaprost acts on AQP2 exclusively through stimulation of the EP2 receptor.

PGE2 and Butaprost, but Not CAY10580, Increase cAMP in MDCK Cells. EP2 and EP4 can signal through cAMP. In our MDCK cells, PGE2 (10^{-8} M) and butaprost (10^{-7} M) significantly increased cAMP levels compared with cells stimulated with control media

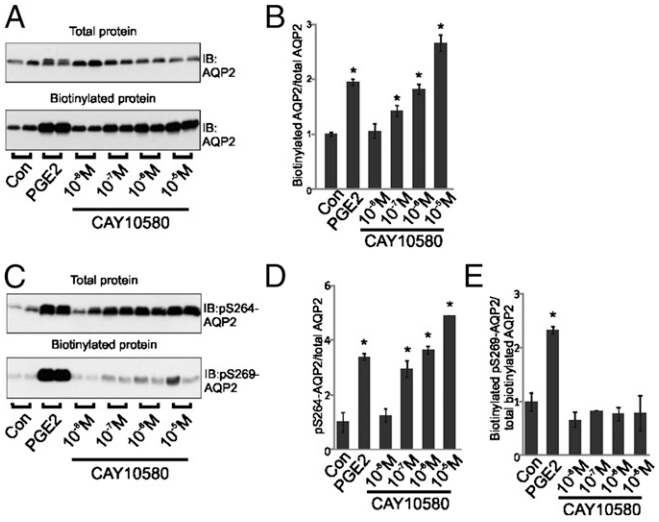

Fig. 2. Effect of EP4 agonist CAY10580 on AQP2 membrane abundance and phosphorylation. Cell-surface biotinylation assay of cells stimulated 40 min with control media, PGE2 (10^{-7} M), or CAY10580 (10^{-8} to 10^{-5} M). (*A* and *C*) Representative immunoblots of biotinylated and total protein samples. (*B*, *D*, and *E*) Relative AQP2 membrane abundance and phosphorylation at ser-264 and ser-269 normalized to control conditions. Experiments were performed on 3 separate days.

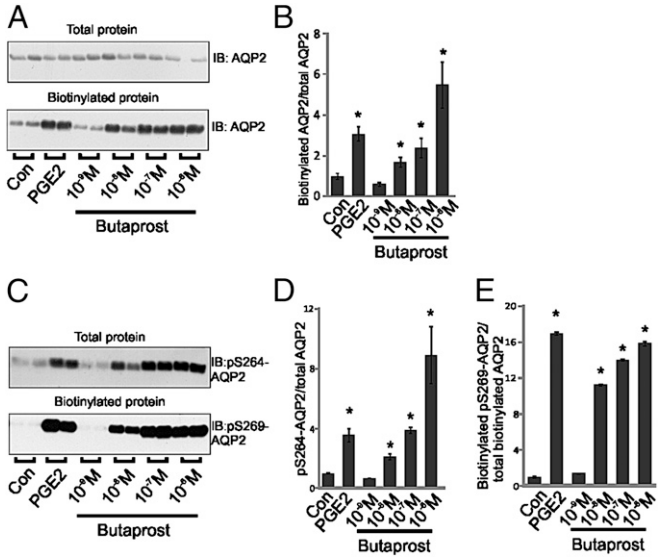

Fig. 3. Effect of EP2 agonist butaprost on AQP2 membrane abundance and phosphorylation. Cell-surface biotinylation assay of cells stimulated 40 min with control media, PGE2 (10^{-7}M), or butaprost (10^{-9} to 10^{-6} M). (*A* and *C*) Representative immunoblots of biotinylated and total protein samples. (*B*, *D*, and *E*) Relative AQP2 membrane abundance and phosphorylation at ser-269 and ser-264 normalized to control conditions. Experiments were performed on 3 separate days.

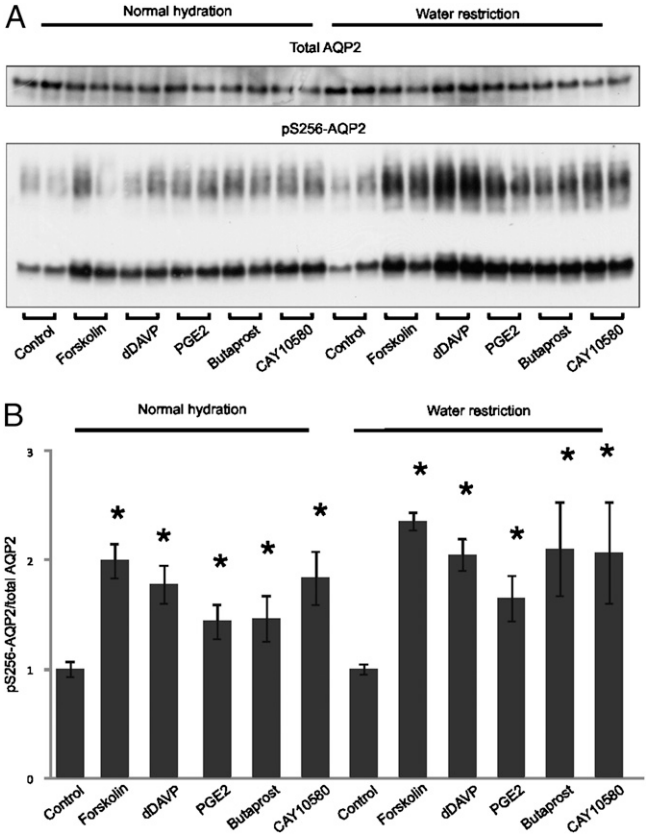

Fig. 4. Stimulation of cortical tubular suspensions from normally hydrated and water-restricted rats. One group of rats was kept on a diet of 7.5 g of food mixed with 15 mL of water/100-g rat/d (normal hydration), and the other group received 7.5 g of food mixed with 7.5 mL of water/100-g rat/d (water restriction) for 2 d. (*A*) Representative immunoblots of total AQP2 and pS256-AQP2 in cortical suspensions after 40 min of stimulation with the indicated agonist. (*B*) Relative ser-256 phosphorylation in cortical suspensions normalized to control conditions in normally hydrated and water-restricted rats, respectively. Experiments were performed on 4 separate days, and results were pooled.

(Fig. S7). Conversely, CAY10580 (up to 10^{-5} M) caused no statistically significant increase in cAMP.

PGE2, CAY10580, and Butaprost Increase AQP2 Phosphorylation in Cortical Tubule Suspensions. To determine whether PGE2, CAY10580, and butaprost affect phosphorylation of AQP2 in native tissue, we performed ex vivo studies on isolated cortical tubules from rat kidney that abundantly express EP2 and EP4 (10). The studies were performed in two groups of rats that had controlled water intake for 2 d before the experiment: one group of normally hydrated rats and one group of water-restricted rats ($n = 4$ in each group, paired experiments performed on 4 separate days). This controlled intake was undertaken to examine whether the response to PGE2 changed according to hydration status. PGE2 (10^{-7} M), butaprost (10^{-6} M), and CAY10580 (10^{-6} M) significantly increased phosphorylation of AQP2 at ser-256 after 40 min of stimulation in normally hydrated rats, an effect retained in water-restricted rats (Fig. 4). We conclude that E-prostanoid receptors EP2 and EP4 increase AQP2 phosphorylation at ser-256 in cortical collecting ducts.

Acute PGE2 and Butaprost Treatment Increases AQP2 Membrane Abundance in Cortical and Outer Medullary Collecting Ducts. Kidney slices (50 μm) from normally hydrated rats were incubated for 30 min in control media, dDAVP (10^{-8} M), PGE2 (10^{-8} to 10^{-7} M), or butaprost (10^{-7} to 10^{-6} M). After stimulation, slices were fixed for subsequent immunohistochemistry. PGE2 and butaprost caused a clear redistribution of AQP2 in cortical and outer medullary collecting-duct principal cells from a predominantly intracellular localization to the apical plasma membrane (Fig. 5).

Butaprost Alleviates the Urinary-Concentrating Defect in a Rat Model of NDI. We conducted in vivo experiments to determine whether the EP2 agonist butaprost was able to increase renal concentrating ability in a rat model of NDI. Rats were given the V2R antagonist OPC-31260 for 12 h before and throughout the study. Butaprost, or saline containing DMSO as a control, was administered as a s.c. bolus injection (4 mg/kg body weight) every 12 h ($n = 6$ in each group). Strikingly, compared with controls given only OPC-31260, rats given butaprost significantly reduced urine volume to 62% of control values on day 1 and 56% on day 2 (Fig. 6*A*). The effect of butaprost on cumulative urine volume was significant at 24 h and remained significant throughout the experiment (Fig. 6*B*). Average urine flow rate was decreased to 59% on day 1 ($P = 0.057$) and 51% on day 2 ($P = 0.059$) (Fig. 6 *C* and *D*). Accordingly, urine osmolality was significantly increased on both days (Fig. 6 *E* and *F*). Butaprost had no significant effect on creatinine clearance (Table S2), suggesting that the observed differences in urine volume are not due to a reduced glomerular filtration rate.

Discussion

VP is well recognized for its importance in maintaining whole-body water homeostasis. Brattleboro rats, which lack endogenous VP production, have a major urinary-concentrating defect, which can be corrected by infusion of VP or the V2R agonist dDAVP. An X-linked hereditary form of NDI, with patients presenting with excessive thirst and excretion of a large amount of dilute urine, is most commonly a result of a defect in the trafficking or function of the V2R (15, 16). These patients do not respond to VP (or dDAVP), and at present there is no specific treatment for this condition. Possible therapeutic strategies may involve activation of the cGMP pathway (17), the use of pharmacological chaperones (18), or, as recently proposed, stimulation of the EP4

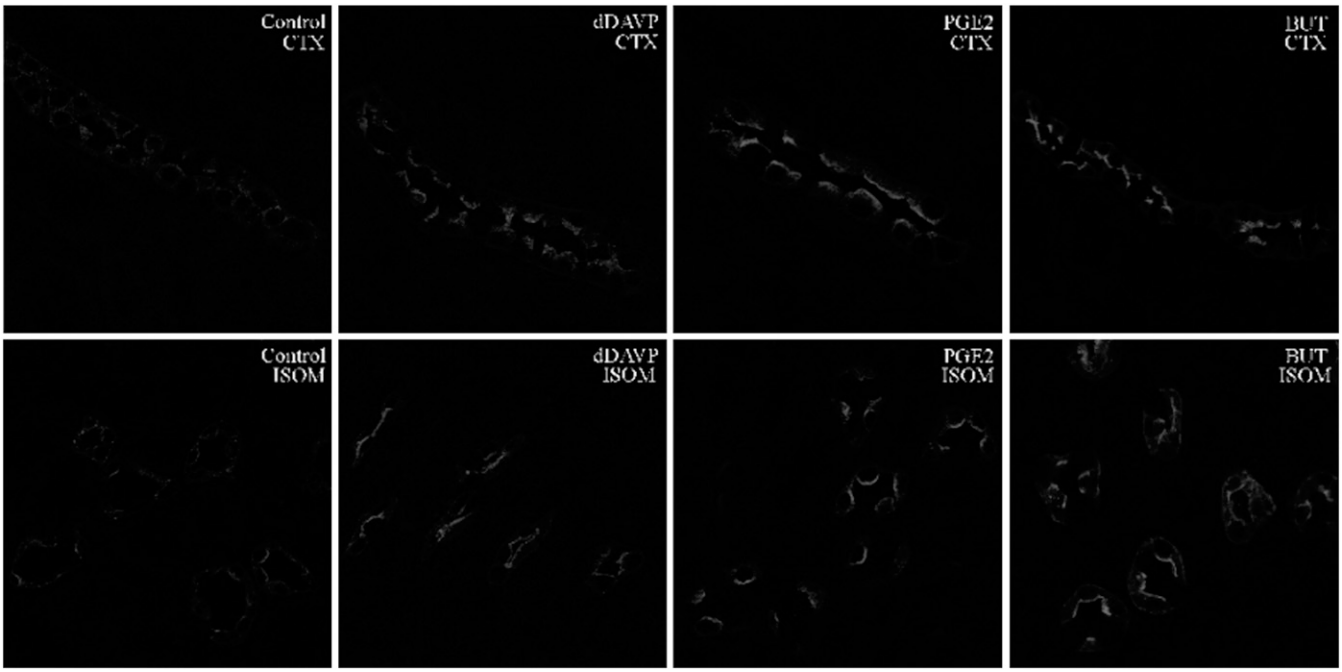

Fig. 5. Confocal laser scanning images of AQP2 trafficking in rat kidney slices. Stimulation of rat kidney slices with dDAVP, PGE2, or butaprost and subsequent labeling for AQP2 for assessment of subcellular distribution.

receptor (19). In the present studies, we demonstrate that direct stimulation of the E-prostanoid receptor, EP2, could be an alternative mechanism to increase kidney concentrating ability.

E-prostanoid Receptors EP2 and EP4 Increase Membrane Accumulation of AQP2. EP2 is present in the cortical portion of the collecting duct (10) and in cell lines derived from the cortical collecting duct (20), although a functional role of this receptor on collecting duct protein trafficking has not previously been investigated. We determined that stimulation of the EP2 receptor with the specific EP2 agonist butaprost increased apical membrane accumulation of AQP2 in vitro and ex vivo. In MDCK cells, this effect is probably a result of the marked increase in intracellular cAMP levels in response to butaprost, which is also the most likely mechanism for AQP2 trafficking in kidney slices. Together, these results demonstrate that EP2 receptor agonists are a potential previously undescribed player in the regulation of AQP2 in the collecting duct.

Recently, a specific agonist for the EP4 receptor (ONO-AE1-329) increased kidney concentrating abilities in a mouse model of NDI (19). We determined that an alternative EP4 receptor specific agonist (CAY10580) induced acute targeting of AQP2 to the membrane in MDCK cells, providing a molecular mechanism for the previously reported effects. Surprisingly, this targeting occurred without significant changes in intracellular cAMP. It has previously been demonstrated that AQP2 can traffic in renal epithelial cells without alterations in cAMP levels, such as following hypertonic challenge (21) or stimulation of the cGMP pathway via nitric oxide (22). The fact that our EP4 selective agonist seemingly acts upon its G-protein–coupled receptor without a significant rise in intracellular cAMP could indicate that the receptor is acting on AQP2 through another pathway, possibly an effect of β-arrestin–dependent signaling (23, 24). This signaling pathway could be an interesting avenue for future studies.

Differential Roles of EP2 and EP4 Stimulation on AQP2 Phosphorylation. In cortical tubule suspensions, PGE2, EP2, and EP4 receptor agonists increased AQP2 ser-256 phosphorylation, effects not easily assessed in our MDCK cells and previous studies (25). Because this phosphorylation site is required for VP-mediated membrane accumulation of AQP2 (2), the increased pS256-AQP2 observed in this study ex vivo solidifies our findings that EP2 and EP4 receptor stimulation increase AQP2 targeting independently of VP. In addition to ser-256 phosphorylation, we demonstrated that both EP2 and EP4 agonists increase AQP2 phosphorylation at ser-264 in MDCK cells. Although the functional role of this phosphorylation site is not known in detail, ser-264 is not essential for AQP2 trafficking (2) but may determine the fate of AQP2 following endocytic retrieval and thereby protein half-life (26). Thus, the previously described effect of an EP4 agonist on AQP2 protein abundance (19) hypothetically could be the result of either stimulation of AQP2 gene transcription or decreased AQP2 degradation.

AQP2 phosphorylation at ser-269 is involved in apical membrane retention of the water channel (2, 27, 28). In our study, *only* EP2 had a significant effect on cAMP levels and subsequent pS269-AQP2 abundance, confirming previous studies that ser-269 phosphorylation is a downstream event to cAMP increases (27). As discussed earlier, an interesting future objective will be to discover which kinases and intracellular signaling pathways are involved in EP4-mediated targeting of AQP2, a mechanism that, in MDCK cells, apparently does not result in increased cAMP or ser-269 phosphorylation.

EP2 Agonist Butaprost Alleviates Symptoms of NDI. Rats treated with the V2R antagonist OPC-31260 had an NDI-like phenotype with major polyuria. Butaprost markedly decreased urine flow rate and increased urine osmolality in these rats, indicating that pharmacological stimulation of the EP2 receptor may be a suitable future target for the treatment of NDI. In particular, an EP2 receptor agonist in combination with EP4 receptor stimulation, which has previously been shown to relieve NDI symptoms in a mouse model of NDI (19), may have major therapeutic potential. Therapeutically, pharmacological stimulation of the EP2 receptor, rather than the EP4 receptor, could have advantages. For example, in our study, butaprost caused a long-term increase

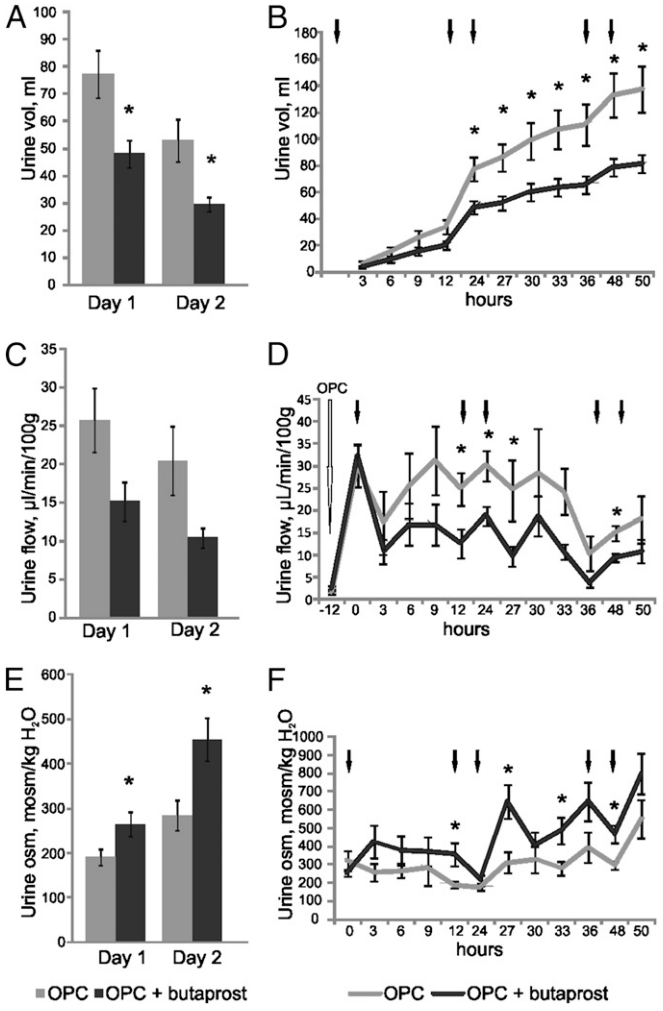

Fig. 6. Effect of EP2 agonist butaprost on kidney concentrating abilities in OPC-treated rats. Rats were treated with V2R antagonist OPC (10 µg/d/rat in 10 mg of food) for 62 h starting at −12 h. Half of the rats were treated with butaprost, 4 mg/kg s.c., starting at time 0 and subsequently every 12 h (indicated with arrows in *B*, *D*, and *F*). (*A*) Urine volume collected from 0 to 24 h (day 1) and from 24 to 48 h (day 2) from rats treated with only OPC and butaprost alongside OPC (OPC + butaprost) (n = 6). (*B*) Cumulated urine volume over the 50-h duration of OPC + butaprost treatment. (*C*) Average urine flow rates on day 1 and day 2 in both groups. (*D*) Urine flow rates over time through the duration of the study. Baseline flow rates were measured before treatment with OPC (from −24 to −12 h) and after 12 h of treatment (from −12 to 0 h). (*E*) Urine osmolality on day 1 and day 2 in the two groups. (*F*) Urine osmolality measured at intervals throughout the study.

in urine osmolality that was sustained for at least 12 h. In contrast, a bolus injection of an EP4 agonist to mice resulted in a peak in urine osmolality after 1 h, an effect that was not apparent 3 h after injection (19). This may be due to elimination properties of the EP4 agonist, but when administered via minipumps, the agonist's effects were also diminished after 24 h, suggesting otherwise. An alternative explanation may lie in the properties of the receptors themselves; that is, the EP4 receptor is internalized and desensitized following ligand binding, whereas the EP2 receptor is not (29, 30). Another advantage of EP2 is that, in comparison with EP4 that is the most widely distributed and abundantly expressed PGE2 receptor, EP2 is the least abundant (31). Thus, EP2 agonists may provide a more restricted effect and thus a more specific treatment of NDI than EP4 agonists. It should also be noted in this context that EP2 agonists have been tested on humans for the treatment of primary dysmenorrhea with good tolerability observed in the subjects (32).

Conclusion

Apart from the V2R, two other receptors have the potential to increase AQP2 phosphorylation and membrane targeting in the collecting duct, namely the E-prostanoid receptors EP2 and EP4. EP2 receptor stimulation partially alleviates the concentrating defect in NDI. This sheds light on the involvement of PGE2 in collecting-duct water permeability, with major implications for the understanding of whole-body water homeostasis and for discovering new treatment strategies for NDI.

Materials and Methods

Cells. The generation and characterization of the MDCK cells used throughout the study have been described in detail previously (2, 27). Cell surface biotinylation was performed as described previously (2).

Agonists/Antagonists. PGE2 (Sigma) was dissolved in ethanol (1 mg/mL), butaprost (Sigma) was dissolved in ethanol (5 mg/mL) for in vitro studies and DMSO (10 mg/mL) for in vivo studies, CAY10580 (Cayman Chemicals) was dissolved in ethanol (5 mg/mL), dDAVP (Sigma) was dissolved in H_2O (10^{-4} M), and AH6809 (Cayman Chemicals) was dissolved in DMSO (10^{-2} M).

Animal Studies. The following animal protocols have been approved by the Animal Welfare Division of the Danish Ministry of Justice.

Water restriction studies. Male Wistar rats were placed in metabolic cages for 3 d to acclimatize. Rats then underwent 2 d of water restriction or controlled normal water intake. Control rats were given 7.5 g of food mixed with 15 mL of water, and water-restricted rats were given 7.5 g of food mixed with 7.5 mL of water/100-g rat/d. Rats were then killed by cervical dislocation and kidneys were removed for tissue processing.

OPC-31260 + butaprost studies. Male Wistar rats were placed in metabolic cages for 3 d to acclimatize. OPC-31260 was administered at a dose of 5 µg in 5 g of food twice a day. Twelve hours after the first dose of OPC-31260, butaprost (4 mg/kg body weight) or saline injections were given subcutaneously. Subsequent injections were performed every 12 h. Urine samples were collected every 3 h. Fifty hours after the first injection, a blood sample was taken and rats were euthanized. Urine osmolality was measured by freeze-point analysis. All other plasma and urine analysis was performed at the Clinical Pathology Service Laboratory at MRC Harwell according to their standard procedures.

Cortical Tubular Suspensions. Cortex from both kidneys was dissected, sliced into ~1-mm pieces, and then placed in an enzyme solution containing 0.5 mg collagenase type II (PAN Biotech) and 0.5 mg/mL protease inhibitor pronase (Roche Diagnostics) at 37 °C in buffer B (140 mM NaCl, 0.4 mM KH_2PO_4 1.6 mM K_2HPO_4, 1 mM $MgSO_4$, 10 mM Na-acetate, 1 mM α-ketogluterate, 1.3 mM Ca-gluconate, 5 mM glycine, 48 µg/mL trypsin inhibitor, and 25 µg/mL DNase, pH 7.4). Eight milliliters of enzyme solution was used for each kidney cortex. Samples were mixed continuously at 850 × g at 37 °C. After 20 min, half of the enzyme solution was removed and replaced with buffer B, and samples were incubated for a further 10 min. This procedure was repeated for another 10 min, once for 15 min, and finally for 20 min, where 6 mL of supernatant was transferred to 6 mL of ice-cold buffer B containing albumin (0.5 mg/mL). Samples were then spun at 60 × g for 1 min, and the supernatant was replaced with buffer B. This was repeated and, finally, buffer B was removed and samples were resuspended in Hepes-buffered salt solution (135 mM NaCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 10 mM Na-Hepes, 1.8 mM $CaCl_2$, 1.6 mM K_2HPO_4 × 3 H_2O, 0.4 mM KH_2PO_4, pH 7.4). The tubular suspensions were then transferred into tubes containing agonists diluted in Hepes buffer and mixed continuously for 40 min at 37 °C, 850 × g. Samples were placed on ice and centrifuged for 5 min, 4,000 × g at 4 °C. The supernatant was removed and replaced with sample buffer containing DTT (50 mg/mL).

PGE2 and cAMP Measurements. Commercially available kits were used for measuring urine PGE2 (Amersham) and intracellular cAMP (GE Healthcare).

Statistics. Values are shown as mean ± SEM. Two group comparisons were performed using Student's t test. $P < 0.05$ was considered statistically significant.

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