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Stimulation of AQP2 membrane insertion in renal epithelial cells in vitro and in vivo by the cGMP phosphodiesterase inhibitor sildenafil citrate (Viagra)

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Bouley, Richard, Nuria Pastor-Soler, Ori Cohen, Margaret McLaughlin, Sylvie Breton, and Dennis Brown. 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, 2005. First published January 11, 2005; doi:10.1152/ajprenal.00337.2004.—Vasopressin-stimulated insertion of the aquaporin 2 (AQP2) water channel into the plasma membrane of kidney collecting duct principal cells is a key event in the urinary concentrating mechanism. The paradigm for vasopressin-receptor signaling involves cAMP-mediated protein kinase A activation, which results in the functionally critical phosphorylation of AQP2 on amino acid serine 256. We previously showed that a parallel cGMP-mediated signaling pathway also leads to AQP2 membrane insertion in AQP2-transfected LLC-PK1 (LLC-AQP2) cells and in outer medullary collecting duct principal cells in situ (Bouley R, Breton S, Sun T, McLaughlin M, Nsumu NN, Lin HY, Ausiello DA, and Brown D. J Clin Invest 106: 1115–1126, 2000). In the present report, we show by immunofluorescence microscopy, and Western blotting of plasma membrane fractions, that 45-min exposure of LLC-AQP2 cells to the cGMP phosphodiesterase type 5 (PDE5) inhibitors sildenafil citrate (Viagra) or 4-[(3',4'-methylene-dioxobenzyl)amino]-6-methoxyquinazoline elevates intracellular cGMP levels and results in the plasma membrane accumulation of AQP2; i.e., they mimic the vasopressin effect. Importantly, our data also show that acute exposure to PDE5 inhibitors for 60 min induces apical accumulation of AQP2 in kidney medullary collecting duct principal cells both in tissue slices incubated in vitro as well as in vivo after intravenous injection of Viagra into rats. These data suggest that AQP2 membrane insertion can be induced independently of vasopressin-receptor activation by activating a parallel cGMP-mediated signal transduction pathway with cGMP PDE inhibitors. These results provide proof-of-principle that pharmacological activation of vasopressin-independent, cGMP signaling pathways could aid in the treatment of those forms of nephrogenic diabetes insipidus that are due to vasopressin-2 receptor dysfunction.

phosphodiesterase type 5; cAMP; nephrogenic diabetes insipidus; vasopressin; LLC-PK1 cells; Brattleboro rats; vasopressin receptor type 2

A CONSIDERABLE AMOUNT OF WORK has shown that aquaporin (AQP) water channels are important for urinary concentration and body fluid homeostasis. AQP2 is expressed in collecting duct principal cells, where its plasma membrane expression is stimulated by the antidiuretic hormone vasopressin (VP) (6, 7, 13, 45, 54). The most widely understood pathway leading to AQP2 membrane accumulation is via vasopressin type 2 receptor (V2R) stimulation of adenylyl cyclase, cAMP-mediated activation of protein kinase A, and phosphorylation of AQP2 on amino acid serine 256. This phosphorylation event is necessary for VP-stimulated membrane accumulation of AQP2 (19, 30). Most cases of hereditary nephrogenic diabetes insipidus (NDI) result from functionally inactivating mutations in the V2R, which leads to the X-linked form of NDI. The rarer autosomal form of the disease is due to mutations in the AQP2 protein itself (2, 8, 14, 25, 44, 61, 62). Because patients with V2R mutations probably express AQP2 in their collecting ducts, it would theoretically be possible to alleviate their symptoms if an alternative, V2R-independent pathway leading to AQP2 membrane insertion could be activated.

Our previous data showed that acute elevation of intracellular cGMP levels by nitric oxide (NO), L-arginine, or atrial natriuretic peptide (ANP) also leads to AQP2 membrane insertion both in transfected epithelial cells and in principal cells in some regions of the kidney collecting duct (4). This effect probably occurs via protein kinase G-mediated phosphorylation of serine 256 on the AQP2 COOH terminus (4). We have also previously shown that this cGMP-mediated AQP2 trafficking occurs with no intracellular cAMP elevation and is thus independent of stimulation of the V2R signaling pathway.

The purpose of the present study was to determine whether elevation of cGMP in these cells, leading to AQP2 membrane insertion, could be achieved by inhibition of cGMP phosphodiesterases, specifically phosphodiesterase-5 (PDE5). This PDE isomorph is expressed in renal collecting ducts along with PDE4 (a cAMP-specific isomorph) and PDE1, a calcium/calmodulin-dependent isomorph (16). As demonstrated by the successful clinical application of the PDE5 inhibitor 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl][sulfonyl]-4-methylpiperazine; sildenafil citrate; Viagra) for erectile dysfunction (1, 12, 40), this enzyme is an attractive target for therapy aimed at bypassing the V2R/cAMP axis that is defective in X-linked NDI. Our results show that acute exposure of both LLC-PK1 cells stably transfected with AQP2 (LLC-AQP2 cells) and collecting duct principal cells in tissue slices of rat kidney to sildenafil citrate and 4-[(3',4'-methylene-dioxobenzyl)amino]-6-methoxyquinazoline (MBMQ), both of which are PDE5 inhibitors,
leads to a vasopressin-like stimulation of AQP2 accumulation in the plasma membrane. Our results also show a similar accumulation of AQP2 in the apical plasma membrane domain of collecting duct principal cells in Brattleboro rats (which lack VP) in vivo on acute exposure to sildenafil. This study provides proof-of-principle data that pharmacologically mediated PDE5 inhibition can potentially be used to bypass the V2R signaling cascade, leading to AQP2 appearance on the plasma membrane of epithelial cells.

MATERIALS AND METHODS

Chemicals and reagents. Lysine VP (LVP), arginine VP (AVP), [deamino-Cys8, d-Arg9]-vasopressin (DDAVP), ANP, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). MBMQ was purchased from Calbiochem (La Jolla, CA). The sildenafil citrate was prepared by solubilizing Viagra tablets (Pfizer, New York, NY).

All cell culture reagents including Geneticin (G418), medium, and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY), the porcine antidiuretic hormone, was used to stimulate pig kidney-derived LLC-PK1 cells, whereas AVP (or DDAVP) was used on rat tissues. For simplicity, both forms of the hormone will be referred to as VP in the remainder of the text. Phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4) was obtained from Boston Bioproducts (Boston, MA).

Experimental animals. Animal experiments were approved by the Massachusetts General Hospital Institutional Committee on Research Animal Care, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) and Brattleboro rats (Harlan, Indianapolis, IN) were used for the tissue slice and in vivo experiments, respectively (see below).

Cell culture. The production of LLC-AQP2 cells by stably transfecting LLC-PK1 cells with c-myc epitope-tagged AQP2 cDNA has been described previously (31). Cells were passaged every 4 days at a 1:10 dilution and maintained in DMEM supplemented with 10% heat-inactivated PBS and 1 mg/ml of G418.

Immunofluorescence on cell cultures. Cells were grown on glass coverslips (Bellco, Vineland, NJ) to ~60% confluence and rinsed with DMEM. Cells were then treated for 10 min with VP (10 nM) or 45 min with either sildenafil (0.5 μM), MBMQ (23 μM), IBMX (1 mM), or ANP (1 μM). The concentration of each PDE inhibitor used for immunofluorescence studies was 10 times greater than that reported to inhibit cGMP PDE activity by 50% (12, 58). For Western blotting of isolated plasma membranes, three concentrations of the drugs were used as indicated in Fig. 2. After incubation, cells were fixed with 4% paraformaldehyde in sodium phosphate buffer (0.1 M, pH 7.4) for 20 min. They were washed three times in PBS, permeabilized with 0.1% Triton X-100 for 4 min, and incubated with an anti-c-myc monoclonal antibody (17) in PBS for 1 h. Cells were then incubated with goat anti-mouse IgG conjugated to FITC at a final concentration of 5 μg/ml (Jackson Immunologicals, West Grove, PA) for 1 h, washed, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and examined using a Bio-Rad Radiance 2000 confocal laser-scanning microscope. Gain and/or laser power was set so that the brightest regions of the images were just below saturation level using the “set color” feature of the Bio-Rad Lasersharp software. Finally, images were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) as TIFF files.

After collection, images were imported into IPLab Spectrum (Scanalytics, Vienna, VA) for analysis. Mean pixel intensity of a fixed area of the cell membrane was quantified and corrected by the subtraction of the mean pixel intensity of the same sized area from the nucleus. The corrected mean pixel intensity of the cell membrane was obtained for each group of cells. Between 20 and 50 measurements were taken from each group, and at least 3 different controls and experimental sets of cells were taken. Results are expressed as the ratio of the average of the mean intensity ± SE (experimental sets vs. the controls). An increase in the mean intensity reflects an increase in AQP2 fluorescence in the plasma membrane area.

Kidney tissue slice preparation. Thin slices of kidney were prepared for in vitro studies as previously described (4). Male adult Sprague-Dawley rats were anesthetized with pentobarbital sodium (65 mg/kg), and the kidneys were briefly perfused via the left ventricle with Hanks’ balanced salt solution (HBSS), pH 7.4, at 37°C equilibrated with 5% CO2-95% O2. When the kidneys were cleared of blood (~1 min), they were removed and thin slices (~0.5 mm) were quickly cut using a Stadie-Riggs slicer (Thomas Scientific, Swedesboro, NJ). Some slices were fixed immediately after the pretreatment incubation period, before the addition of drugs or agonists (control time 0).

Other slices were preincubated at 37°C for 15 or 45 min in equilibrated HBSS. Subsequently, the slices were divided among different treatment groups and incubated as follows: 1) HBSS alone for 15 min (control time 15), 2) VP (10 nM) plus forskolin (10 μM) for 15 min, 3) sildenafil (0.5 μM) for 45 min, 4) MBMQ (23 μM) for 45 min, 5) HBSS for 45 min (control time 45), and 6) HBSS for 45 min followed by VP-forskolin for 15 min. After the assigned incubation, all slices were fixed by immersion in PLP fixative for 5 h at room temperature. Slices were then rinsed several times in PBS and immunostained (see below) to determine the location of AQP2 under the various incubation conditions.

Acute in vivo treatment of rat kidney. Brattleboro rats are Long-Evans rats that are homozygous for a single base pair genetic mutation that impairs their ability to synthesize VP in the hypothalamus (55). This animal model of central DI was chosen to ensure that any observed effect on AQP2 trafficking could not be attributed to endogenous VP. Before anesthesia, the Brattleboro rats had restricted water availability for 6–8 h to reduce any potential effect of surgery-induced oxytocin release on AQP2 location (35). Adult Brattleboro rats (weighing 250 g) were divided into three groups of three animals: group 1 served as controls, group 2 was treated with DDAVP, and group 3 received sildenafil citrate. Anesthesia was induced using pentobarbital sodium (65 mg/kg body wt) ip. The right internal jugular vein was exposed, and a 25-gauge needle was used to infuse the compound of choice into the vein. Control animals were each treated with a 0.5-ml infusion of saline, whereas the DDAVP-treated group each received 10 μg of DDAVP in 0.5 ml of saline. For both groups, infusions were started 50 min after induction of anesthesia. Sildenafil citrate-treated animals received 4 mg/kg (in 0.5 ml) on induction of anesthesia. One hour after the induction of anesthesia, the kidneys of all animals were perfused with PBS via the left ventricle for 1 min, followed by PLP perfusion fixation for 5 min (5). The kidneys were removed, cut into thinner slices, and placed in PLP at room temperature for 4–6 h or overnight at 4°C. Tissues were washed three times in PBS and kept in PBS containing 0.02% sodium azide at 4°C before use. Injected sildenafil solutions were simultaneously tested on LLC-AQP2 cells to confirm the efficiency of the diluted drugs to trigger AQP2 membrane insertion.

Immunocytochemistry. After fixation and washing, kidney samples were cryoprotected in PBS containing 30% sucrose for 2 h. Sections (4 μm) were cut from frozen tissue using a Reichert Frigocut microtome (Leica, Deerfield, IL). After dehydration in PBS for 5 min, sections were incubated in PBS containing 1% bovine serum albumin for 15 min to block nonspecific staining. Sections were incubated with a previously characterized antiserum raised against the second extracellular loop of AQP2 (21) (diluted 1:100 in PBS) for 1.5 h at room temperature, followed by two washes of 5 min each in high-salt PBS (containing 2.7% NaCl) to reduce background staining, and one final wash in PBS. Sections were then incubated with goat anti-rabbit IgG conjugated to FITC at a final concentration of 5 μg/ml (Kirkegaard and Perry Labs, Gaithersburg, MD) for 1 h at room temperature and then rinsed as for the primary antibody. Sections were mounted in Mounting medium (containing 2.7% NaCl) to reduce background staining, and one final wash in PBS. Sections were then incubated with goat anti-rabbit IgG conjugated to FITC at a final concentration of 5 μg/ml (Kirkegaard and Perry Labs, Gaithersburg, MD) for 1 h at room temperature and then rinsed as for the primary antibody. Sections were mounted in

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Vectashield (diluted 2:1 in 0.1 M Tris·HCl, pH 8.0) and examined using a Nikon Eclipse E800 epifluorescence microscope. Images were captured digitally using a Hamamatsu Orca CCD camera and IPLab Spectrum software. Final images were imported into Adobe Photoshop as TIFF files. For quantitative analysis, apical and cytoplasmic cellular MPI (mean pixel intensity) of AQP2-associated fluorescence in a defined region of interest were measured in between 13 and 32 principal cells from each acute in vivo treated rat. The apical-to-cytoplasmic ratio was obtained per cell, and then an average was obtained for each animal. The average MPI of AQP2-associated fluorescence was obtained for each treatment group, and it was expressed as the mean ± SE.

Plasma membrane preparation and Western blotting. After incubation or without VP and the PDE inhibitors, cells grown on 10-cm dishes were scraped into cold homogenization buffer (20 mM Tris·HCl, pH 7.4, 300 mM sucrose, 1 mM EDTA, protease inhibitor cocktail, Roche) and disrupted using a ball-bearing cell cracker. The resulting membrane suspension was centrifuged at 2,500 g to remove intact cells, nuclei, and other large fragments. A 17,000-g spin resulted in a pellet that was harvested as the “plasma membrane” fraction. Smaller organelles including endosomes remain in the supernatant. Pellets were resuspended and washed three times by centrifugation. This fraction was solubilized (in 50 mM Tris·HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, pH 7.4 containing protease inhibitor cocktail) and separated by SDS-PAGE after protein quantification. Reduced samples (10 μg of protein) were loaded onto each lane of a standard NUPage 4%-12% Bis-Tris Gel and then electroblotted on polyvinylidene difluoride (PVDF) filters (Bio-Rad, Hercules, CA) as previously described (37). Western blotting for AQP2 was performed using a polyclonal antibody against the c-myc tag (0.4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the PVDF sheets were incubated with a donkey anti-rabbit secondary antibody coupled to peroxidase (0.16 μg/ml) in PBS-T buffer for 1 h. Peroxidase activity was detected with the ECL enhanced chemiluminescence system (PerkinElmer, Boston, MA). After detection, antibodies were stripped by washing the membrane for 1 h with 0.2 M glycine-HCl, 0.05% Tween buffer. Membranes were reblotted and exposed for 1 h to a mouse anti-β-actin antibody (0.5 μg/ml; Chemicon, Temecula, CA) as a loading control. After development, films were scanned, and the images were saved in TIFF file format. Images were imported into IPLab Spectrum. The mean pixel intensities (±SE) of AQP2-c-myc and β-actin bands from three independent experiments were quantified. All AQP2-c-myc band intensities were normalized to their respective β-actin bands. The mean pixel intensity from the untreated cell band was subtracted from all intensity values. Results were expressed as the percentage of the mean pixel intensity, where the mean pixel intensity of the AQP2 bands from VP-treated cells was set at 100%.

cAMP and cGMP assays in tissue culture cells. LLC-AQP2 cells were plated onto 96-well plates and grown to confluence. cAMP and cGMP assays were performed with the BioTrak kit (Amersham Pharmacia Biotech) as previously described (4). Briefly, cells were pretreated with 1 mM IBMX for 10 min, washed three times with PBS, and then exposed to the various inhibitors and to VP. To see cGMP elevation, a brief pretreatment with IBMX was used to block other nonselective cAMP and cGMP PDEs that are expressed in LLC-PK1 cells (51). This brief pretreatment increased our ability to detect the effects of subsequent treatment of cells with more specific PDE5 inhibitors and agonists that act via the cGMP pathway. The cells were lysed, centrifuged, and the supernatant was mixed with rabbit anti-cAMP antibody or rabbit anti-cGMP antibody. Aliquots were added to 96-well plates precoated with donkey anti-rabbit antibody. The cAMP or cGMP-peroxidase conjugate was then added for 1 h, the plates were washed, and the peroxidase substrate was added. Sulfuric acid was added to terminate the reaction. The optical density at 450 nm was read on a plate reader. The optical densities were compared with standard curves using 0–3,200 fmol/well of cAMP or 0–512 fmol/well of cGMP. This is a competition assay in which higher optical density values reflect the presence of less cyclic nucleotide in the original cell supernatant. Each intracellular cAMP and cGMP accumulation assay was performed in triplicate.

Statistical analysis of data. Mean values were analyzed by t-test. Where necessary, data were first validated using ANOVA, followed by a t-test. Data were considered significantly different if P < 0.05.

RESULTS

Effect of PDE5 inhibition on AQP2 localization in LLC-AQP2 cells. As shown in Fig. 1, exposure of LLC-AQP2 cells to sildenafil for 45 min resulted in a marked accumulation of AQP2 on the plasma membrane domain (Fig. 1E) compared with the nontreated control cells (Fig. 1A). A similar degree of membrane staining was observed after treatment of the cells with VP (Fig. 1B) or ANP (Fig. 1C), as previously described (4). MBMQ (Fig. 1F), which is a cGMP PDE inhibitor that is structurally distinct from sildenafil, as well as IBMX (Fig. 1D), a cAMP and cGMP PDE inhibitor, also induced significant membrane accumulation of AQP2. Figure 1G shows a quantitative analysis of the AQP2 membrane fluorescence under the different experimental conditions tested. All of the PDE inhibitors tested, as well as VP and ANP, resulted in a significant increase in the level of AQP2 at or close to the plasma membrane, compared with untreated control cells.

PDE5 inhibition stimulates AQP2 membrane accumulation in LLC-AQP2 cells. To determine the effect of the PDE inhibitors on AQP2 membrane accumulation, plasma membranes of drug-treated LLC-AQP2 cells were isolated by density gradient centrifugation. The enriched plasma membranes were subjected to Western blot analysis (Fig. 2A). Using a c-myc antibody, only a small amount of AQP2 was detected in the plasma membrane fraction of untreated cells (Con). After a 10-min exposure to VP, the amount of plasma membrane AQP2 increased significantly (VP). LLC-AQP2 cells were also incubated with increasing amounts of different PDE inhibitors. The maximum effect was seen when cells were incubated with PDE inhibitors at a concentration 10 times greater than that needed to inhibit PDE activity by 50% (IC50). Thus IBMX (1 mM), sildenafil (0.5 μM), and MBMQ (23 μM) all resulted in a marked accumulation of AQP2 c-myc in plasma membrane fractions after 45 min of incubation. This accumulation was dose dependent. β-Actin antibody staining confirmed that the same amount of protein was loaded in each lane. A quantification of the incorporation of AQP2 into isolated plasma membranes from cells treated with sildenafil, MBMQ, and IBMX is shown in Fig. 2B. After 10 min of exposure to VP, the amount of plasma membrane AQP2 increased significantly (VP). LLC-AQP2 cells were also incubated with increasing amounts of different PDE inhibitors. The maximum effect was seen when cells were incubated with PDE inhibitors at a concentration 10 times greater than that needed to inhibit PDE activity by 50% (IC50). Thus IBMX (1 mM), sildenafil (0.5 μM), and MBMQ (23 μM) all resulted in a marked accumulation of AQP2 c-myc in plasma membrane fractions after 45 min of incubation. This accumulation was dose dependent. β-Actin antibody staining confirmed that the same amount of protein was loaded in each lane. A quantification of the incorporation of AQP2 into isolated plasma membranes from cells treated with sildenafil, MBMQ, and IBMX is shown in Fig. 2B. After a 10-min exposure to VP, the amount of plasma membrane AQP2 increased significantly (VP).
compared with controls, as previously reported (4, 53). In contrast, all PDE inhibitors tested (IBMX, sildenafil, and MBMQ) as well as ANP (a stimulator of cGMP production) failed to produce measurably significant levels of cAMP compared with controls (Fig. 3, top). In contrast, all inhibitors tested significantly increased intracellular cGMP levels, whereas no effect of VP alone was detectable (Fig. 3, bottom). The intracellular concentration of cGMP under all conditions

Fig. 1. Confocal immunofluorescence microscopy showing localization of aquaporin 2 (AQP2) in LLC-AQP2 cells before (A) and after treatment with vasopressin (VP; B), atrial natriuretic peptide (ANP; C), IBMX (D), sildenafil (E), or MBMQ (F). In nonstimulated cells, AQP2 is localized in many cytoplasmic vesicles that are concentrated around the nucleus, whereas the plasma membrane is virtually unstained (A). In contrast, AQP2 is located at the plasma membrane domain (arrows) after incubation for 10 min with 10 nM VP (B) or for 45 min with ANP (C), IBMX (D), sildenafil (E), or MBMQ (F). These images are representative of 5 independent experiments (Bar = 5 μm). G: quantification of the effect of phosphodiesterase (PDE) inhibitors on AQP2 membrane accumulation in LLC-AQP2 cells. AQP2 fluorescence closely associated with plasma membranes was analyzed by IPLab Spectrum software on confocal microscope images similar to those shown in A. Results are expressed as membrane mean pixel intensity (MPI), where 100% is arbitrarily set as the value of membrane fluorescence measured in untreated cells. All drugs and hormones tested resulted in a significant increase in AQP2 at the level of the plasma membrane. Values are means ± SE of 3 independent experiments. *P < 0.05 (t-test).
tested was considerably lower than the cAMP levels, as previously reported (4).

**PDE5 inhibition with MBMQ and sildenafil stimulates apical membrane accumulation of AQP2 in principal cells of the rat kidney.** Kidney tissue slices prepared from Sprague-Dawley rats were incubated with or without VP, or in the presence of the PDE5 inhibitors sildenafil or MBMQ. Immunofluorescence using an anti-AQP2 antibody revealed that AQP2 is localized throughout the cytoplasm of principal cells in the outer medullary collecting duct after 15 min of incubation in HBSS alone (Fig. 4A). A similar distribution was observed in tissue slices incubated for 45 min in the presence of buffer only (data not shown). After 15 min in the presence of VP, AQP2 is located in a tight band at the apical pole of principal cells (Fig. 4B). MBMQ and sildenafil also resulted in the accumulation of AQP2 in the apical pole of principal cells (Fig. 4, C and D, respectively).

In addition, some of the tissue slices incubated in HBSS for 45 min were then exposed to VP for an additional 15 min. This control tissue was able to respond to VP in a manner that was indistinguishable from the 15-min VP exposure shown in Fig. 4B (data not shown). Therefore, AQP2 was able to accumulate in the apical membrane of principal cells even after a more prolonged period of prior incubation in HBSS.

**PDE5 inhibition with MBMQ and sildenafil stimulates apical AQP2 membrane insertion in principal cells during acute in vivo treatment in Brattleboro rats.** After treatment with intravenous saline (control), VP, or sildenafil, Brattleboro rat kidneys were fixed by perfusion and immersion. Immunofluorescence images revealed that AQP2 staining was mostly intracellular in collecting duct principal cells of controls (Fig. 5A), whereas AQP2 localized to the apical pole of these cells in VP-treated animals and (Fig. 5B). Intravenous sildenafil infusion showed similar effects to VP treatment, although the effects were somewhat less pronounced (Fig. 5C), as with the observations in the tissue slice experiment. The apical redistribution of AQP2 induced by sildenafil was quantified in kidneys from three different animals. Both VP and sildenafil caused a significant apical accumulation of AQP2 in principal cells compared with untreated control rats, although the VP effect was significantly greater than that of sildenafil (Fig. 5B).

**DISCUSSION**

cGMP is central to the signaling cascade of NOS and ANP, and it plays an important hemodynamic role in the glomerulus and other vascular structures (11, 22, 46, 56, 64). cGMP also has indirect and direct effects on the kidney tubular epithelium (4, 26, 49). Previously, we have shown that an increase in intracellular cGMP by activation of the ANP receptor or NOS leads to the phosphorylation of AQP2, and to the membrane insertion of this water channel in LLC-AQP2 cells, as well as in principal cells of the kidney outer medullary collecting duct (4). This observation suggested a cGMP-mediated alternative pathway to increase the amount of AQP2 in the plasma membrane of patients suffering from NDI due to V2R mutations. These patients are unable to maximally concentrate their urine probably because AQP2 water channels remain mainly in intracellular compartments and are not inserted into the plasma membrane (29, 36, 43). Most of the attempts to resolve this problem using alternative methods of increasing intracellular cAMP, including recent work using oxytocin, have not been successful (27). In a strain of mice with NDI that is due to
overexpression of rolipram-sensitive cAMP PDE4 in collecting duct principal cells, administration of rolipram to isolated collecting ducts resulted in morphological changes (seen by freeze-fracture electron microscopy) consistent with an increase in apical membrane water channels (10). These mice also have lower levels of AQP2 protein than normal mice, consistent with the fact that in vivo regulation of AQP2 expression by vasopressin is mediated by cAMP (18). However, administration of a single dose of rolipram to two patients with X-linked NDI failed to increase their urinary concentration ability in one study (3), but no systematic trial of the efficacy of such therapy has been performed using a larger patient base. Increasing knowledge about the PDE family of enzymes, coupled with the recent development of safe, selective, and clinically proven PDE5 inhibitors such as sildenafil, has now opened the door to the alternative solution of stimulating an increase in intracellular cGMP as a potential therapy for NDI (40).

In the present study, we show that AQP2 accumulates in the plasma membrane of collecting duct principal cells and LLC-AQP2 cells on exposure to the selective PDE5 inhibitors MBMQ and sildenafil, which inhibit the degradation of cGMP. In cultured cells, this occurs with no detectable increase in cAMP. As previously reported, the actual concentration of cGMP in stimulated LLC-AQP2 cells, while significantly increased over nontreated cells, was considerably less than that seen for cAMP levels in cells exposed to VP (4). However, the increase in cGMP levels in PDE-treated cells was comparable to that observed after exposure to ANP, whose cGMP signaling cascade is well established. Furthermore, the amount of cGMP generated by cells in culture may vary from passage to passage (34), and the distribution of functional pools of intracellular cGMP is unknown. While we have shown in vitro that PKG can phosphorylate the S256 residue of AQP2, cGMP can modulate the activity of kinases other than PKG, and the intracellular mediator of the translocation effect on cGMP elevation remains unclear (23, 47, 66).

Our tissue slice and in vivo assays show that sildenafil alone increases the accumulation of AQP2 in the apical plasma membrane of principal cells, mainly in the outer medulla. The effect of sildenafil on PDE5 is more significant in this area where the basal activity of NOS is higher than in other portions of the tubule (33, 39, 48, 65). From a cell biological point of view, it is interesting that sildenafil results in apical insertion of AQP2 in principal cells, whereas it induces basolateral AQP2 insertion in LLC-AQP2 cells. One condition that influences the polarity of AQP2 insertion is exposure of cells to hypertonicity (60). Long-term DDAVP treatment also caused an increase in basolateral AQP2 expression in the cortical collecting duct (9). Neither of these conditions applies to the cultured LLC-AQP2 cells used here, and the reason that AQP2 is basolateral in these cells remains unclear, as discussed extensively in previous reports (6, 30, 31). Nevertheless, the parallel effects of PDE5 inhibition and VP stimulation on AQP2 membrane accumulation in these distinct cell types illustrate that similar signal transduction pathways are involved in both the apical and basolateral delivery of the water channel.

It is not known whether the sildenafil effect is due to an increase in AQP2 exocytosis, a decrease in endocytosis, or both. Kinetic modeling computations carried out by Knepper and Nielsen (32) before the discovery of AQP2 indicated that the onset and offset of the water permeability response in isolated collecting ducts were best explained by a modulation of both insertion and retrieval pathways of water channels. However, our recent data clearly indicate that blockade of endocytosis is sufficient to induce a rapid plasma membrane accumulation of both wild-type and an S256 phosphorylation-deficient mutant of AQP2 (37). Whether this mechanism underlies all maneuvers that result in increased membrane accumulation of AQP2 remains to be determined.

Several caveats to PDE5 inhibitor treatment of patients with X-linked NDI need to be addressed. Some reports have shown that PDE5 inhibitors increase the glomerular filtration rate (GFR) (38, 52). This effect may initially worsen the polyuria in these patients. Sildenafil treatment in a patient with a kidney dysfunction was performed in triplicate. Each point represents the mean ± SD. *P < 0.05 compared with control values.
allograft caused dilatation of the afferent arteriole, promoting a transient increase in GFR (52). 1,4-Dihydro-5-(2-propoxyphe-nyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one, another PDE5 inhibitor, increases GFR in rats by stimulating regional renal blood flow and increasing the level of intracellular cGMP (20). 1,4-Dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one also increases the level of cGMP in response to a stimulus such as ANP (59). The combination of PDE5 inhibition and ANP stimulation would cause natriuresis and increased urine output by decreasing the activity of the epithelial sodium channel; this natriuretic effect has been shown in rats fed a low-sodium diet (28). In contrast, an upregulation of PDE5 activity in the glomerulus could participate in glomerular resistance to ANP in severe congestive heart failure (57). Recently, other evidence shows new mechanisms by which PDE5 inhibition may have an effect on natriuresis. PDE5 inhibitor treatment may potentiate the effects of interstitial cGMP or the constitutive nitric oxide synthesis of the juxtaglomerular apparatus (26, 49). Therefore, a study of long-term therapy with PDE5 inhibitors is needed to evaluate whether these treated patients would reach a steady state in which PDE5-mediated increases in GFR and in natriuresis could be counterbalanced by AQP2-mediated antidiuresis. In the present in vivo study, no significant change in urine osmolality was detectable over the 60-min treatment period in dehydrated (6- to 8-h water restriction; see MATERIALS AND METHODS), sildenafil-treated Brattleboro rats (548 ± 65 mosmol/kgH2O; n = 3) compared with dehydrated controls (462 ± 84 mosmol/kgH2O; n = 3), although there was a trend toward an increased osmolality after drug treatment. Measurement of acute changes was complicated by the large volume of urine already in the bladder of these animals. This issue will be the subject of further studies using either ureteral catheterization for acute experiments, as well as chronic administration of PDE5 inhibitors. In addition, to see a more positive effect of the AQP2 insertion on water reabsorption, it may be necessary to use combination therapy with drugs that reduce GFR, or to continue to maintain patients on a low-salt diet.

Sildenafil is widely used in the treatment of erectile dysfunction. It is contraindicated in patients taking nitrates, yet it is otherwise well tolerated, has few severe side effects, and does not require enzymatic bioactivation. In addition, sildenafil is not known to induce tolerance to its own effects. This characteristic of sildenafil could theoretically offer an
advantage with respect to nitrates when used to induce trafficking of AQP2 in NDI patients (24). PDE5 inhibition is already being used as a therapeutic strategy in pulmonary hypertension and subarachnoid hemorrhage (1, 15). None of these reports, nor pharmacokinetic studies, has described an obvious increase in urine flow rate (41, 42), but the metabolism of sildenafil citrate between human and rat is different (63). It is also possible that in the long term, the beneficial effect of sildenafil may be counteracted by the ability of cGMP to alter gene regulation and gene expression (see Ref. 50 for a review).

Finally, the most obvious effect of sildenafil was seen in outer medullary collecting ducts. Since much of the urinary concentrating mechanism is dependent on water reabsorption in the cortical collecting duct, it is possible that any potential therapeutic effect of increasing cGMP levels in principal cells will result in only a partial improvement in urine concentrating ability. Clearly, our present results need to be considered as a first step in developing therapies for NDI. While the data show proof-of-principle that cGMP PDE inhibition can result in membrane accumulation of AQP2 in some principal cells, they raise many important questions. Further investigation will be needed to determine whether a combined therapeutic approach that includes prolonged cGMP inhibition may have positive clinical implications in patients suffering from NDI.

Fig. 5. Indirect immunofluorescence staining of AQP2 in rat kidney inner stripe collecting duct principal cells after acute in vivo sildenafil treatment of Brattleboro rats. Animals were injected with saline, DDAVP, or sildenafil through the jugular vein. Injection of saline was used as a control (A) and compared with 25 mg/kg DDAVP (B) and 4 mg/kg of sildenafil (C). In the control, AQP2 is diffusely located throughout the subapical cytoplasm of principal cells and shows little apparent membrane staining. DDAVP and sildenafil both induce a marked redistribution of AQP2, which appears as a narrow, brightly stained band at the apical pole of principal cells, consistent with plasma membrane staining (arrows). Interfiled cells in the collecting duct epithelium are unstained. This result is representative of experiments with a total of at least 3 animals/treatment group. Bar = 10 μm. D: quantitative analysis of AQP2-associated apical fluorescence stimulated by sildenafil and VP in principal cells in vivo. Fluorescence intensities of the apical (membrane) region and the cytoplasm were measured in defined regions of interest for between 13 and 32 cells/acute in vivo treated rat kidney. The ratio of the mean pixel intensities (MPI) of the 2 regions was compared to quantify the shift of AQP2 from the cytoplasm to the apical pole of each cell. The apical-to-cyttoplasmic ratio was obtained per cell, and then an average was obtained for each animal. The average MPI ratio of AQP2-associated fluorescence was obtained for each treatment, and it was expressed as the mean ± SE. Both sildenafil and VP treatment caused a significant apical accumulation of AQP2 compared with untreated controls. The VP effect was significantly greater than that of sildenafil. The average MPI of AQP2-associated fluorescence was obtained for each treatment group and analyzed first by ANOVA followed by a t-test (n = 3 untreated, n = 3 AVP-treated, and n = 4 sildenafil-treated). *P < 0.01 vs. control. ** P < 0.05 vs. sildenafil-treated rats (t-test).


63. Walker DK, Ackland MJ, James GC, Muirhead GJ, Rance DJ, Wastall P, and Wright PA. Pharmacokinetics and metabolism of silde-


