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Vasopressin receptor-mediated functional signaling pathway in primary cilia of renal epithelial cells

Malay K. Raychowdhury,[1]* Arnolt J. Ramos,[1]* Peng Zhang,[1]* Margaret McLaughin,[1,2] Xiao-Qing Dai,[3] Xing-Zhen Chen,[3] Nicolás Montalbetti,[4] María del Rocío Cantero,[4] Dennis A. Ausiello,[1,2] and Horacio F. Cantiello[1,4]

[1]*Nephrology Division and Electrophysiology Core, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Charlestown;* [2]*Program in Membrane Biology, Massachusetts General Hospital, Boston, Massachusetts;* [3]*Department of Physiology, University of Alberta, Edmonton, Alberta, Canada; and* [4]*Laboratorio de Canales Iónicos, ININCA, UBA-CONICET, Buenos Aires, Argentina*

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Raychowdhury MK, Ramos AJ, Zhang P, McLaughlin M, Dai, XQ, Chen XZ, Montalbetti N, Cantero MR, Ausiello DA, Cantiello HF. Vasopressin receptor-mediated functional signaling pathway in primary cilia of renal epithelial cells. *Am J Physiol Renal Physiol* 296: F87–F97, 2009. First published October 22, 2008; doi:10.1152/ajprenal.90509.2008.—The primary cilium of renal epithelial cells is a nonmotile sensory organelle, implicated in mechanosensory transduction signals. Recent studies from our laboratory indicate that renal epithelial primary cilia display abundant channel activity; however, the presence and functional role of specific membrane receptors in this organelle are heretofore unknown. Here, we determined a functional signaling pathway associated with the type 2 vasopressin receptor (V2R) in primary cilia of renal epithelial cells. Besides their normal localization on basolateral membrane, V2R was expressed in primary cilia of LLC-PK_1 renal epithelial cells. The presence of V2R in primary cilia was determined by spontaneous fluorescence of a V2R-*gfp* chimera and confirmed by immunocytochemical analysis of wild-type LLC-PK_1 cells stained with anti-V2R antibodies and in LLC-PK_1 cells overexpressing the V2R-Flag, with anti-Flag antibody. Ciliary V2R colocalized with adenylyl cyclase (AC) type V/VI in all cell types tested. Functional coupling of the receptors with AC was confirmed by measurement of cAMP production in isolated cilia and by testing AVP-induced cation-selective channel activity either in reconstituted lipid bilayers or subjected to membrane-attached patch clamping. Addition of either 10 μM AVP (*trans*) or forskolin (*cis*) in the presence but not the absence of ATP (1 mM, *cis*) stimulated cation-selective channel activity in ciliary membranes. This channel activity was reduced by addition of the PKA inhibitor PKI. The data provide the first demonstration for the presence of V2R in primary cilia of renal epithelial cells, and a functional cAMP-signaling pathway, which targets ciliary channel function and may help control the sensory function of the primary cilium.

apical vasopressin; cystic kidney disease; cAMP production; polycystin-2; ciliary function

THE PRIMARY CILIUM IS A SOLITARY, nonmotile, sensory organelle projecting from the apical surface of renal epithelial cells (29, 33). Most renal tubular epithelial cells of the mammalian nephron, with the exception of intercalated cells in the collecting duct, contain a single primary cilium, which responds to fluid flow or mechanical bending by eliciting cytosolic Ca^{2+} signals (29). We recently demonstrated that renal epithelial primary cilia display abundant cation-selective channel activity, consistent with the immunocytochemical observation of several channel proteins in the primary cilium, including the polycystin-2 (PC2, TRPP2) channel, TRPC1, and the α-subunit of the renal epithelial channel complex ENaC (35). The signaling pathways for the regulation of ciliary-located channel function, and in particular the role of the signaling mechanisms associated with second messenger pathways in ciliary channel activity, are heretofore unknown. The type 2 vasopressin receptor (V2R) is a G protein-coupled receptor, which regulates Na^+ and water reabsorption in the mammalian nephron, chiefly in principal cells of the collecting duct (6). V2R localizes mostly to the basolateral aspects of vasopressin-sensitive renal tubular epithelial cells, which is in agreement with the normal effect of circulating vasopressin. After binding with the ligand vasopressin (AVP), V2R activation causes the G protein-mediated activation of closely associated adenylyl cyclase (AC) and the elevation of cAMP. An acute effector signaling pathway is the stimulation of cAMP-dependent protein kinase (PKA), which phosphorylates a number of target proteins, including proteins associated with transepithelial fluid transport. The cAMP-PKA pathway phosphorylates transport proteins, including aquaporin-2 (AQP2) and epithelial ion channel proteins, such as CFTR (11), K^+ channels (9), including RomK (46), and Na^+ channels, including ENaC (17, 32). Less is known, however, about the apical epithelial receptor function in the mammalian nephron. Both cAMP-associated V2R (12, 23) and parathyroid hormone (PTH) receptors (40), as well as vasopressin type 1 receptor (V1R) receptors, which is associated with Ca^{2+} signals (44, 45), have been found in the luminal aspect of tubular epithelia. How these signaling pathways correlate, if at all, to ciliary function is as yet unknown.

In this report, we characterized a signaling mechanism based on the functional activation of a ciliary receptor, V2R, which controls ion channel activity in primary cilia of renal epithelial cells. We provide evidence for the presence of V2R in primary cilia of LLC-PK_1 renal epithelial cells, which is functionally coupled to AC, and the activation of PKA as assessed by the local production of cAMP, and the regulation of cation-selective channel activity. The coupling of V2R to ciliary channel function may contribute to the regulation of sensory function in primary cilia of renal epithelial cells.

* M. K. Raychowdhury, A. J. Ramos, and P. Zhang contributed equally to this work.

Address for reprint requests and other correspondence: H. F. Cantiello, Nephrology Div. and Electrophysiology Core, Massachusetts General Hospital East, 149 13th St., Charlestown, MA 02129 (e-mail: cantiello@helix.mgh.harvard.edu).

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MATERIALS AND METHODS

Cell culture. Wild-type and V2R-*gfp*- and V2R-Flag-overexpressing LLC-PK_1 renal epithelial cells were cultured as described (4) in DMEM supplemented with 10% FBS, with or without 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C, in a humidified atmosphere with 5% CO_2. Cells of fewer than 25 passages were cultured to full confluence before collection. For visualization of primary cilia, cells were typically grown for 3–7 days after confluence.

Reagents and immunochemistry. A mouse monoclonal anti-acetylated α-tubulin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies included anti-V2R (A1714, ABR, 1:100 dilution) and anti-AC types V/VI antibodies (1:200, Santa Cruz Biotechnology). Goat anti-rabbit IgG coupled to CY3 (indocarbocyanine) was from Jackson ImmunoResearch Laboratories (West Grove, PA). To immunolocalize ciliary proteins, cells were grown to confluence and fixed for 20 min in freshly prepared paraformaldehyde (4%) and sucrose (2%). Cells were rinsed (×3) with PBS. Cells were blocked with BSA (1%) for 30 min before exposure to the primary antibody. To localize primary cilia, cells were immunolabeled with anti-acetylated α-tubulin antibody (2.8 μg/ml). For immunofluorescence microscopy, isolated cilia were seeded on poly-D-lysine-coated glass coverslips, fixed for 20 min at room temperature with freshly prepared paraformaldehyde (4%), and washed (×3) with PBS. After a washing, isolated cilia were blocked in PBS containing 1% BSA (30 min) and incubated for 1 h with primary antibodies. Cilia were further incubated with either FITC- or CY3-conjugated secondary antibodies and mounted with VectaShield (Vector Laboratories, Burlingame, CA). Fluorescent images were captured with an inverted Olympus (IX71) microscope connected to a digital CCD camera (C4742–80-12AG, Hamamatsu Photonics). Images were collected with IPLab Spectrum (Scanalytics, Vienna, VA) acquisition and analysis software, running on a Dell-NEC personal computer. Final composite images were created using Adobe Photoshop 4.0.1 for size reduction and editing.

Tissue preparation for immunofluorescence. Rats were anesthetized with pentobarbital (50 mg/kg body wt ip, Nembutal, Abbott Laboratories, Abbott Park, IL). The animals were perfused through the left cardiac ventricle with PBS (0.9% NaCl in 10 mM phosphate buffer, pH 7.4), followed by the paraformaldehyde-lysine-periodate fixative (PLP), which contained 4% paraformaldehyde, 75 mM lysine HCl, 10 mM sodium periodate and 0.15 M sucrose in 27.6 mM sodium phosphate. Kidneys were dissected, sliced, rinsed and stored in PBS. For sectioning, kidney slices were cryoprotected in 30% sucrose at 4°C overnight. Sections were embedded in Tissue Tek OCT, frozen at −20°C, and 5-μm cryosections were cut and stored at 4°C until use. Sections were rehydrated in PBS, blocked with 1% BSA for 15 min, and incubated with the primary antibody for 60 min at room temperature, rinsed 3 × 5 min in PBS, followed by the secondary antibody, and rinsed again for 3 × 5 min. Slides were mounted in Vectashield medium for fluorescence microscopy. Sections that were double labeled were incubated with the corresponding anti-rabbit polyclonal primary antibody followed by the corresponding secondary monoclonal antibody conjugated to the fluorophore mentioned above. The Massachusetts General Hospital Institutional Committee on Research Animal Care approved the animal protocols in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Other reagents. Arginine (AVP) and lysine (LVP) vasopressin, forskolin, IBMX, and cAMP-dependent kinase catalytic subunit (PKA) and, unless otherwise stated, all other chemicals were obtained from Sigma. The PKA inhibitor (PKI), 5-24 amide (H5950) was obtained from Bachem Americas (Torrance, CA). A Parameter cAMP assay kit was obtained from R&D Systems (Minneapolis, MN). All cell culture reagents were from GIBCO-BRL (Grand Island, NY).

Isolation of primary cilia from LLC-PK_1 cells. Primary cilia were isolated from either wild-type or V2R-*gfp*-expressing LLC-PK_1 cells, as recently reported (35). Briefly, cells grown to confluence for 2–3 wk (34) were scraped with Ca^{2+}-free PBS and centrifuged for 5 min at 52 *g*. The cell pellet was suspended in a high-Ca^{2+} "deciliation"

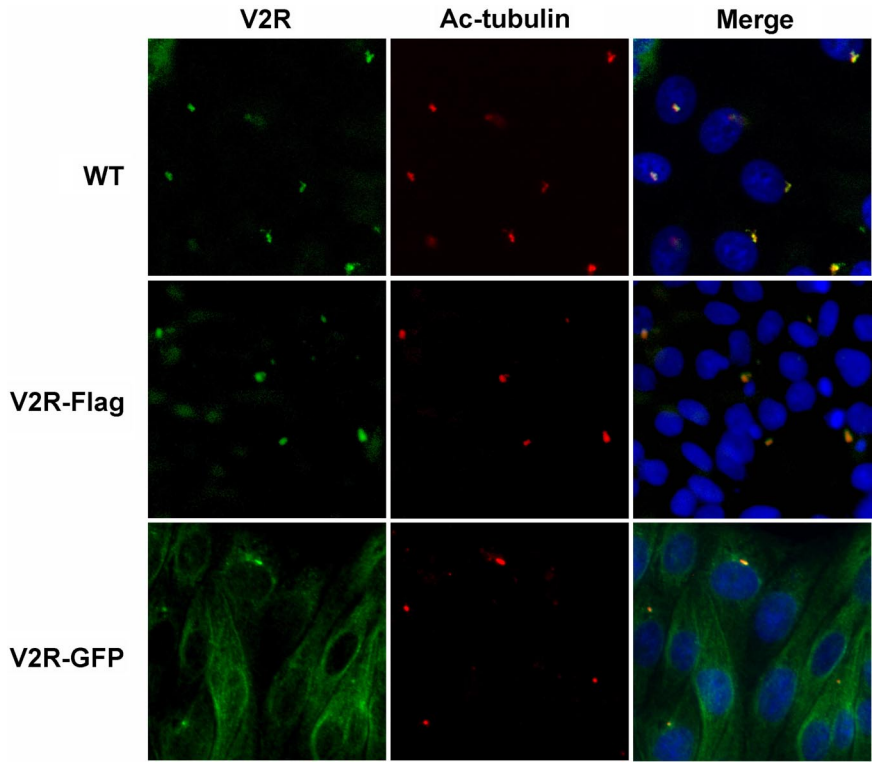


Fig. 1. Localization of type 2 vasopressin receptor (V2R) in primary cilia of renal epithelial cells. V2R was localized to the primary cilia of confluent wild-type LLC-PK_1 renal epithelial cells (WT; ×60) and further evidenced in cells expressing either the V2R-Flag (V2R-Flag; ×32), or the V2R-*gfp* chimeras [V2R-green fluorescence protein (GFP); ×40]. V2R labeling was conducted either with anti-V2R antibody (WT), anti-Flag (V2R-Flag), or spontaneous GFP fluorescence, respectively. Epithelial cells were grown for 7 days after confluence and also stained with mouse anti-acetylated tubulin antibody (red). 4,6-Diamidino-2-phenylindole (DAPI) fluorescence (blue) is shown for contrast, and green labeling is GFP.

solution containing (in mM) 112 NaCl, 3.4 KCl, 10 $CaCl_2$, 2.4 $NaHCO_3$, and 2 HEPES, adjusted to pH 7.0. Resuspended cells were shaken for 10 min at 4°C. Ciliary membranes were separated by centrifugation for 5 min at 7,700 *g*. The supernatant was loaded onto a 45% sucrose solution in high-Ca^{2+} saline solution and centrifuged for 1 h at 100,000 *g*. The sucrose-supernatant interface band was collected and diluted nearly 10-fold and again centrifuged for 1 h at 100,000 *g*. The pellet was resuspended in normal saline solution adjusted to pH 7.0 and supplemented with 2.0 mM EGTA and 0.5 mM sucrose. The resuspended pellet was aliquoted and stored frozen at −80°C until further use. Protein content was assessed by the method of Lowry (19).

Measurement of AC activity. AC enzyme activity in isolated cilia was assayed with an in vitro colorimetric method, which assays the amount of cAMP produced in a sample, by competitive binding with a fixed sample of horseradish peroxidase-labeled cAMP, of a monoclonal antibody (Parameter, KGE002, R&D Systems). Briefly, the method was conducted as follows. First, 5 μl of isolated cilia (50 ng/μl) were incubated in a solution containing 25 mM Tris-acetate buffer (pH 7.6), 5 mM Mg-acetate, 1 mM DTT, 0.5 mM ATP. The, the solution containing IBMX (100 μM) in a final volume of 50 μl was incubated at 30°C for 30 min. Whenever indicated, experiments were also conducted in the presence of 10 μM forskolin. The reaction products were used to assay cAMP production in the reaction mixture according to the manufacturer's protocol. Optical density of color developed in the reaction products was measured at 450 nm, with wavelength correction at 540 nm, using a microplate reader Spectramax Plus 384 (Molecular Devices).

Ion channel reconstitution. Ion channel reconstitution of ciliary membranes was conducted as reported (35). Briefly, isolated cilia were combined and sonicated in the lipid mix before reconstitution in the lipid bilayer chamber. The lipid mixture was made of 1-palmitoyl-2-oleoyl phosphatidyl-choline and phosphatidyl-ethanolamine (Avanti Polar Lipids, Birmingham, AL) in a 7:3 ratio in *n*-decane. Both sides

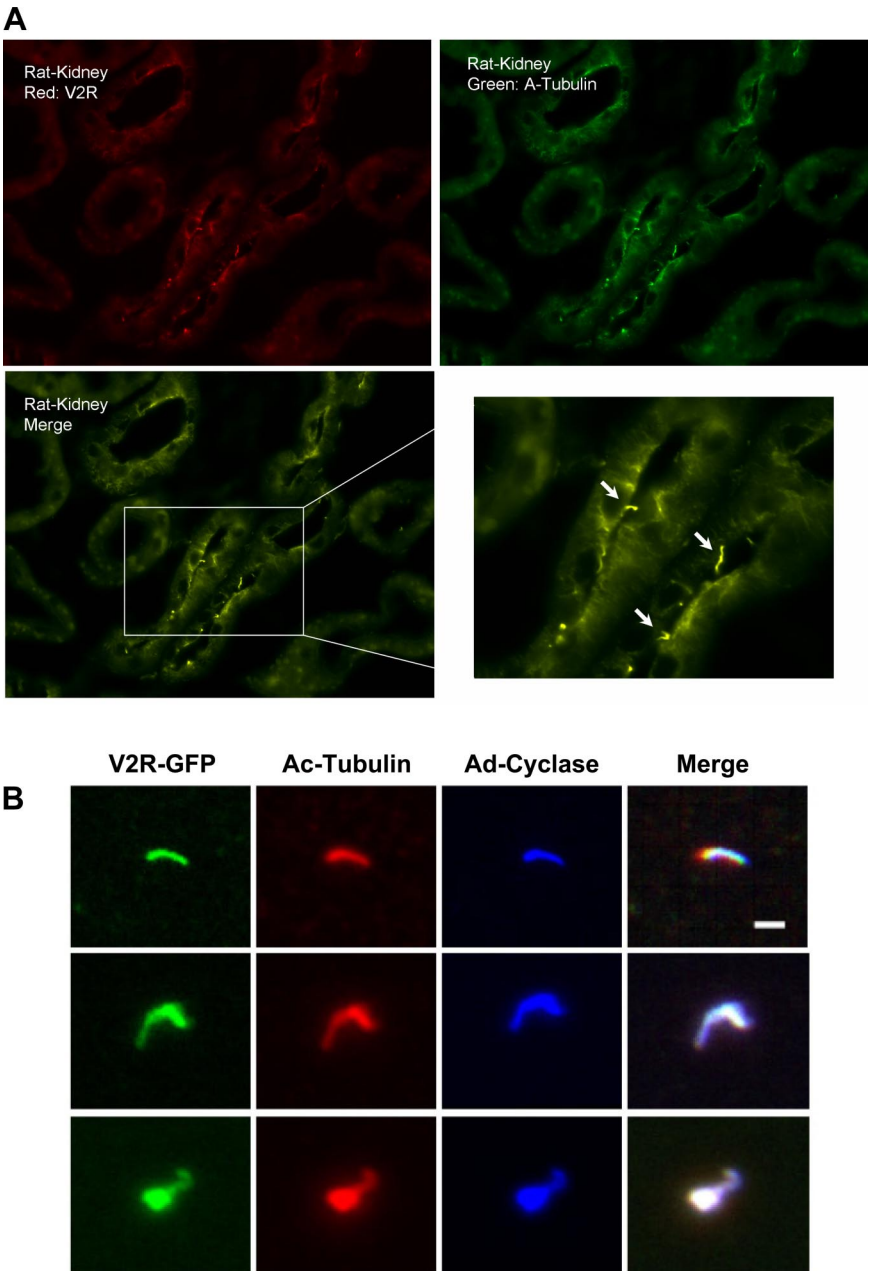


Fig. 2. Localization of V2R and adenylyl cylcase (AC) in primary cilia of renal epithelial cells. *A*: normal rat kidney sections were double-labeled with anti-V2R (red) and anti-acetylated tubulin (green) antibodies. Strong colocalization of both proteins was observed in intercalated cells of medullary collecting duct. *B*: costaining of anti-acetylated tubulin, AC staining, and V2R-*gfp* in isolated primary cilia from V2R-*gfp* expressing cells (×60). V2R-*gfp*-expressing cells were grown as reported (35).

of the lipid bilayer were bathed with a solution containing MOPS-KOH, 10 mM, and MES-KOH, 10 mM, pH 7.40, and 10–15 μM Ca^{2+}. The final K^+ concentration in the solution was ~15 mM. KCl was further added to the *cis* compartment to reach 150 mM.

Data acquisition and analysis. Patch clamping of isolated cilia was conducted as recently reported (35). Whenever indicated, the patch pipette was filled with saline containing 10 μM AVP. Electrical signals were obtained with a PC501A patch-clamp amplifier (Warner Instruments, Hamden, CT) with a 10-GΩ feedback resistor. Single-channel current tracings were further filtered for display purposes only. Unless otherwise stated, pCLAMP Version 5.5.1 (Axon Instruments, Foster City, CA) was used for data analysis, and Sigmaplot Version 2.0 (Jandel Scientific, Corte Madera, CA) for statistical analysis and graphics. Mean currents were calculated as reported elsewhere (21). Unless otherwise stated, statistical significance was obtained by an unpaired Student's *t*-test comparison of sample groups of similar size. Average data values were expressed as means ± SE (*n*) under each condition, where *n* represents the total number of experiments analyzed. Statistical significance was accepted at $P < 0.05$ as calculated by Student's *t*-test (38).

RESULTS

Localization of V2R and AC in primary cilia of renal epithelial cells. To determine the ciliary localization of V2R, LLC-PK_1 cells were grown to confluence and labeled with anti-V2R antibody (Fig. 1). V2R-*gfp*- and V2R-Flag-expressing LLC-PK_1 cells were grown as recently reported (4). V2R localized to primary cilia in all cell types (Fig. 1) and also displayed the expected basolateral plasma membrane staining (data not shown) (see Ref. 12). Primary cilia were immunoidentified by staining with anti-acetylated tubulin antibody (Fig. 1), which colabeled with anti-V2R antibody staining in wild-type LLC-PK_1 cells, and Flag antibody, or spontaneous GFP fluorescence in the V2R-*gfp*-expressing cells, respectively. To confirm that primary cilia indeed express the receptor, immunocytochemistry was also performed in sections of normal rat kidney (Fig. 2*A*). The staining of normal rat kidney with anti-V2R antibody indicated that V2R is present in the apical membrane and some staining in the basolateral membrane of renal epithelial cells (Fig. 2*A*). Immunostaining with V2R antibody also showed its presence in the cytoplasm. Immunostaining with anti-acetylated tubulin indicated the presence of primary cilia mainly in intercalated cells from the medullary collecting duct, which colocalized with V2R (Fig. 2*A*), further confirming the presence of V2R in primary cilia of the normal mammalian nephron. To further determine the presence of V2R in primary cilia, the organelle was isolated from both wild-type and V2R-*gfp*-expressing LLC-PK_1 cells. The receptor segregated to primary cilia, as shown in isolated cilia, which colocalized with AC and acetylated tubulin to identify the cilia (Fig. 2*B*). Thus V2R (when present) and AC colocalized to primary cilia, indicating codistribution in some, but not all cilia. The V2R was also identified by immunostaining with an anti-V2R antibody in wild-type LLC-PK_1 cells, which showed a similar pattern of V2R expression in cilia (data not shown).

Effect of vasopressin on V2R function in primary cilia. To determine whether V2R is functionally coupled to AC, its enzymatic activity was assessed by the production of cAMP in the presence of both 10 μM AVP, MgATP (1 mM), and IBMX (100 μM) in isolated cilia from either V2R-*gfp*-expressing (Fig. 3, *top*) or wild-type (Fig. 3, *bottom*), LLC-PK_1 cells. The amount of cAMP produced, made relative to protein content in the samples, was obtained and compared in the presence or absence of the ligand and substrates. To avoid the possible contaminating basolateral plasma membranes in the cAMP measurements of isolated cilia preparations, we explored by using tubulin/Na^+-K^+-ATPase, and/or V2R/Na^+-K^+-ATPase coimmunolabeling (see Refs. 18 and 35), indicating a minimal contribution to the membrane samples. In the presence of IBMX (100 μM) alone, the cAMP produced by the isolated cilia was 338% ($P < 0.0001$, $n = 5$) higher than their

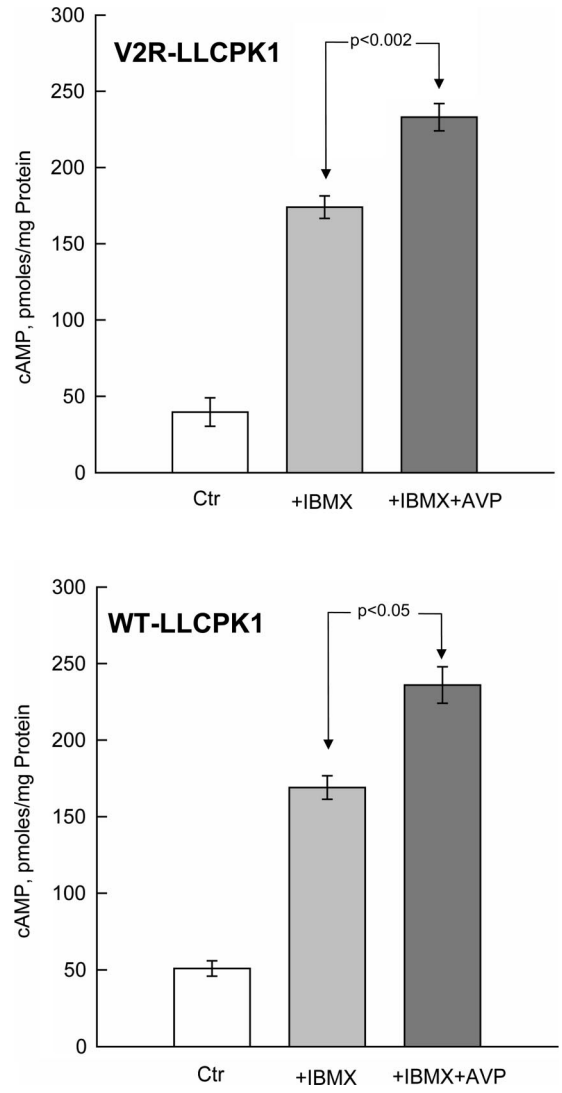


Fig. 3. Effect of vasopressin on cAMP production in isolated primary cilia. The cAMP production of isolated cilia, obtained from V2R-*gfp*-expressing (*top*), and wild-type (*bottom*) LLC-PK_1 cells, was measured by a colorimetric immunoassay (see MATERIALS AND METHODS). Data were corrected by protein content in the samples. Values are means ± SE for 5–6 different samples, each either in duplicate or triplicate. The isolated cilia were incubated for 30 min in the presence or absence of 10 μM AVP and/or 100 μM IBMX. Whenever the primary cilia cAMP production was also measured in the presence of forskolin (10 μM), the contribution of forskolin alone was subtracted from the data in the presence of either AVP or IBMX. The statistical difference between the AVP and AVP+IBMX groups was calculated by Student's *t*-test. Statistical significance was achieved by $P < 0.002$ and $P < 0.05$ for the V2R-*gfp*-expressing (*top*, $n = 5–6$), and wild-type (*bottom*, $n = 7–14$) LLC-PK_1 cells, respectively.

respective controls (Fig. 3). This suggests a large pool of receptor-uncoupled cAMP production, unmasked by diesterase inhibition. Similar results were obtained for either wild-type or V2R-*gfp*-expressing LLC-PK$_1$ cells (Fig. 3). Addition of AVP (10 μM) to V2R-*gfp*-expressing primary cilia increased cAMP production by 33.9% (174 ± 7.36 pmol/mg protein, $n = 4$, vs. 233 ± 8.94 pmol/mg protein, $n = 6$, respectively, $P < 0.002$, Fig. 3, *top*) in the presence of IBMX. We observed quantitatively similar findings by addition of AVP to isolated cilia from wild-type LLC-PK$_1$ cells (Fig. 3, *bottom*). This indicates a functional V2R localization to the primary cilium. Interestingly, we further found that forskolin (10 μM) alone also

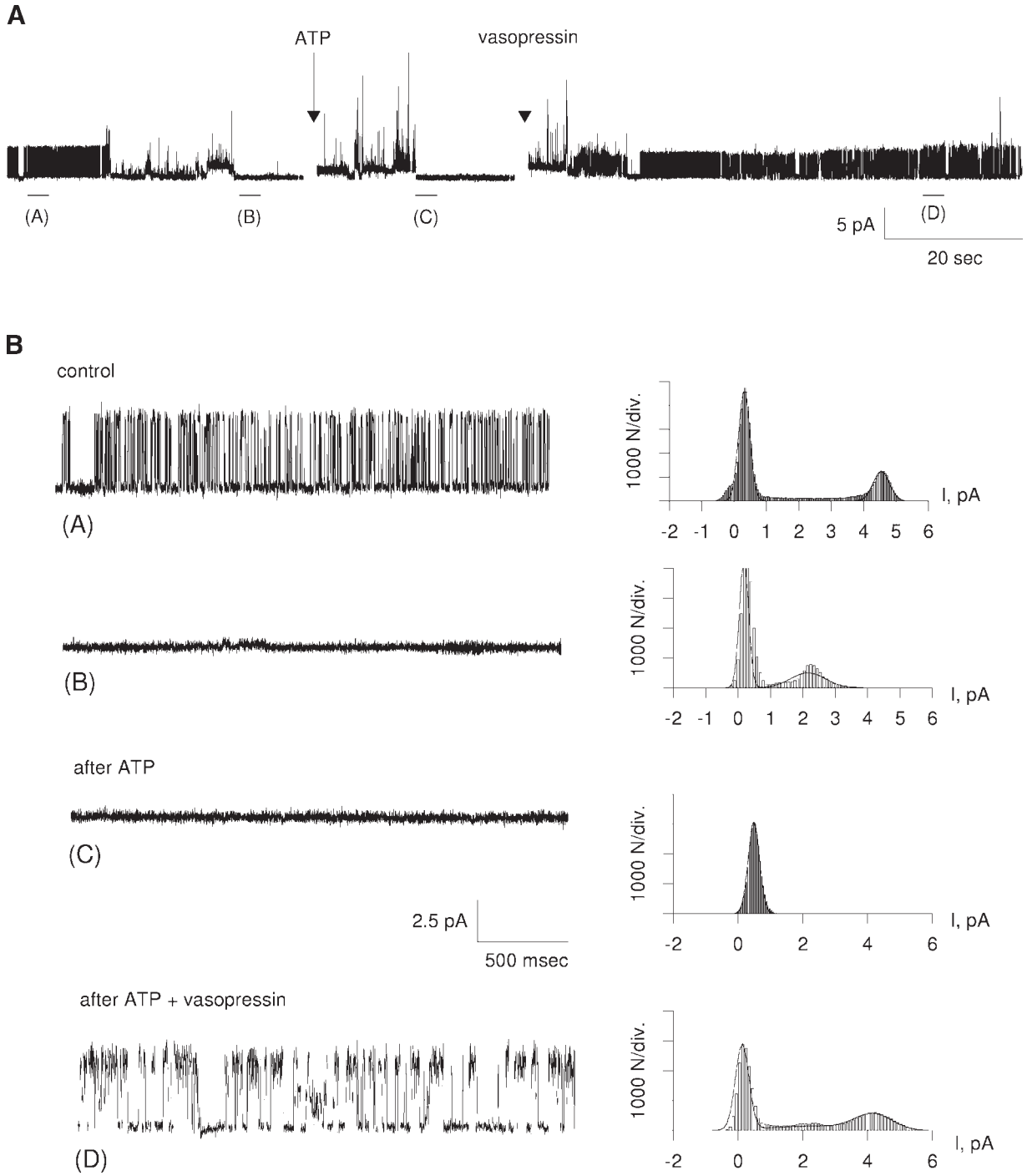
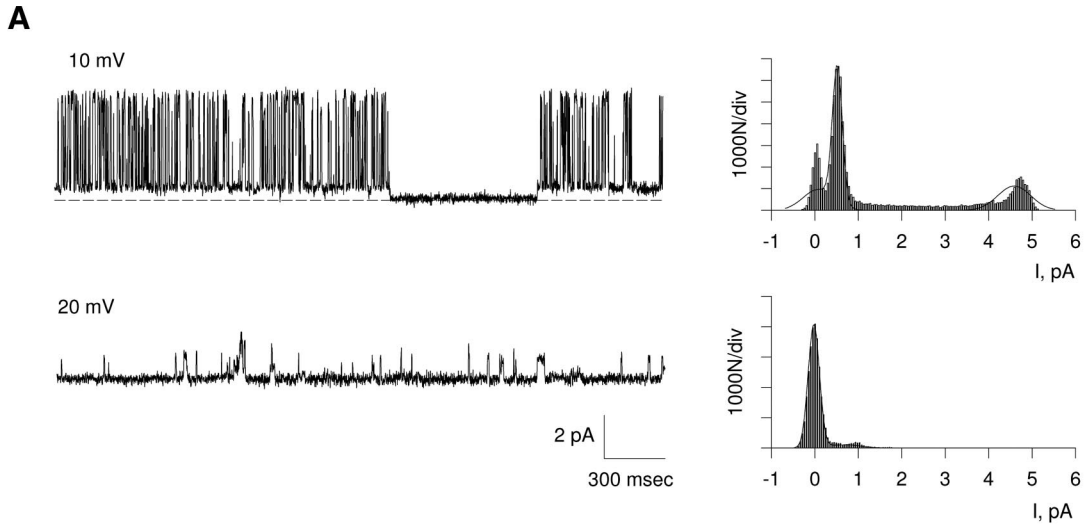
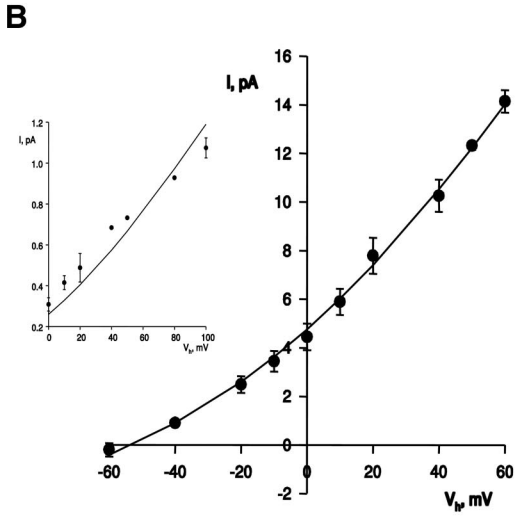
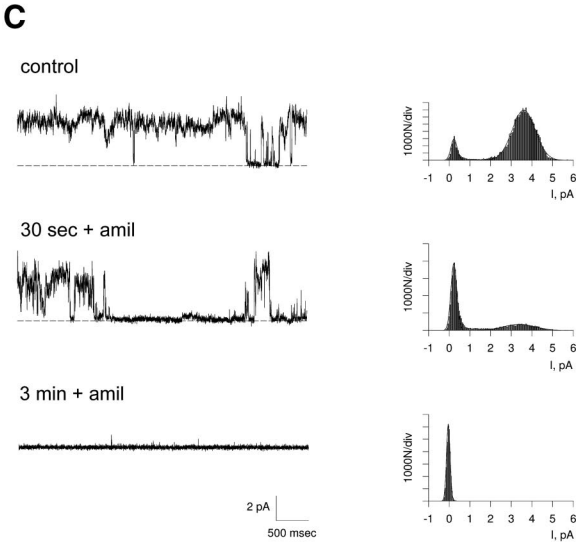
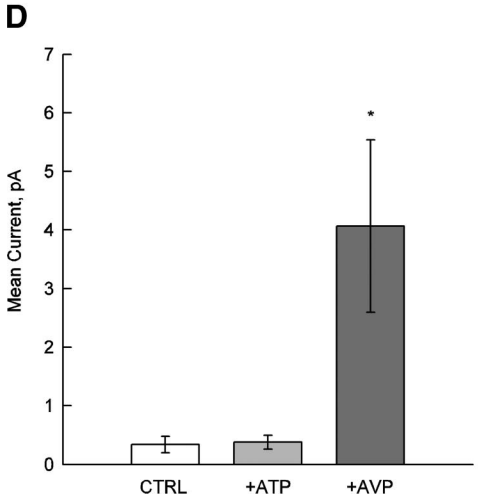


Fig. 4. Functional role of vasopressin in cation channel activity of primary cilia. *A*: measuring AVP-sensitive cation-selective channel activity tested the functional role of the AVP-induced cAMP production in primary cilia. Primary cilia from V2R-*gfp*-expressing cells were reconstituted in a lipid bilayer system, where spontaneous channel activity was first observed, in the presence of a KCl chemical gradient, with 150 mM KCl in *cis* and 15 mM in *trans* compartments, respectively. Addition of ATP induced a transient effect, which further increased ciliary channel activity after addition of AVP (10 μM) to the opposite side (*trans*). *B*: representative single-channel tracings are shown for control conditions (*A*), after spontaneous rundown (*B*), and after subsequent additions of ATP (*C*, 1 mM, *cis*) and AVP (*D*, 10 μM, *trans*). All-point histograms are shown on the *right*, respectively. Data are representative of 4 experiments.









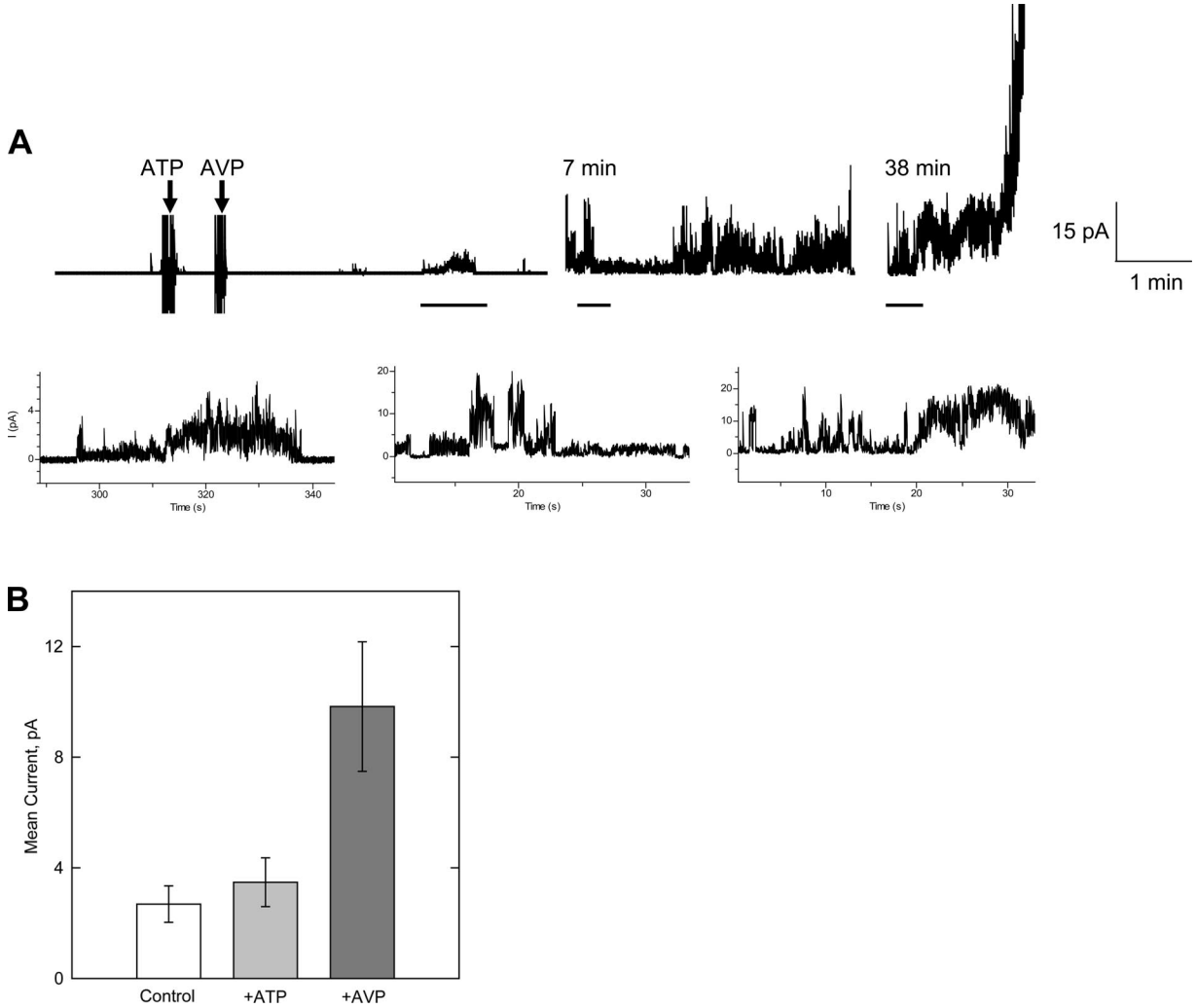


Fig. 6. Functional role of vasopressin in cation channel activity of primary cilia from wild-type LLC-PK$_1$ cells. *A*: primary cilia were reconstituted in a lipid bilayer system, where little spontaneous channel activity was observed, in the presence of a KCl chemical gradient, with 150 mM KCl in *cis* and 15 mM in *trans* compartments, respectively. Addition of ATP (1–5 mM, *cis* chamber) and AVP (10 μM) to the opposite side (*trans*) activated cation-selective channel activity, in agreement with previous results, and the data observed in V2R-*gfp*-expressing cells. Data are representative of 17 experiments. *B*: average data, from mean currents under control conditions, after addition of ATP (1 mM, *cis*), and AVP (10 μM, *trans*). In the presence of both ATP and AVP, there is a significant increase in the average currents (n = 7, 14, and 14, control, ATP, and ATP+AVP, respectively, $P < 0.02$, between ATP alone and ATP+AVP).

increased cAMP production (54.9 ± 7.0 pmol/mg protein, n = 5, vs. 220 ± 29.2 pmol/mg protein, n = 5, $P < 0.05$) in the absence of AVP, confirming the presence of AC activity in cilia as well.

Effect of V2R function on channel activity from primary cilia. To assess a functional role of V2R activation in primary cilia, the organelles were isolated (see MATERIALS AND METHODS, Ref. 35, and Fig. 1*B*), sonicated with lipids, and incorporated into a lipid bilayer reconstitution system. In the presence of ATP (1 mM, *cis* chamber), addition of AVP (10 μM, *trans* chamber) increased ciliary mean currents by 93% (from 0.2 ± 0.1 to 2.9 ± 0.7 pA, n = 7, $P < 0.01$) within 5 min. (Figs. 4 and 5). Addition of AVP (10 μM) alone, however, was without effect (data not shown). Similar experiments were conducted in cilia-attached patches as previously reported (35) in the absence or presence of AVP (10 μM) in the patch pipette with similar results (data not shown). At least two channel phenotypes were identified in the presence of AVP and ATP (Fig. 5), a large-conductance channel (158 ± 3.2 pS, n = 4) and a small-conductance channel (7.06 ± 3.03 pS, n = 2) (both cation selective, Fig. 5).

Fig. 5. Regulation by vasopressin of different cation channels in primary cilia of V2R-*gfp*-expressing cells. *A*: functional role of AVP-induced cAMP production in primary cilia was further tested by measuring AVP-sensitive cation-selective channel activity in reconstituted primary cilia. Addition of AVP (10 μM) to the *trans* side, in the presence of ATP (1 mM, *cis*) increased and/or induced the activity of at least 2 recognizable channel phenotypes. All-point histograms are shown on the *right*, respectively. Data are representative of 7 experiments. These were identified as a large-conductance channel with rapid flickering and a small-conductance channel with much longer open times (see arrows). *B*: cord conductance of the AVP-sensitive large-conductance channel, was ~150 pS, consistent with PC2, while the small-cation channel was ~7 pS, consistent with the epithelial Na^+ channel (ENaC) as previously reported (35). *C*: both channel phenotypes were sensitive to the addition of amiloride (+amil; 100 μM, *trans*). *D*: average data are shown for control conditions before and after addition of ATP (1 mM, *cis*) and AVP (10 μM, *trans*). Values are means ± SE of 6 experiments, which reached statistical significance (*$P < 0.05$) only for the group exposed to both ATP and AVP.

Both channel phenotypes were sensitive to amiloride (100 μM, Fig. 5*C*). The large-conductance cation channel was observed in 19 of 19 reconstituted membranes and displayed several features of polycystin-2 (PC2), while the small-cation channel likely represented a functional ENaC, also observed in primary cilia of these cells (35). The mean currents indicated a 3.5-fold increase in channel activity by addition of AVP (10 μM, *trans*, extracellular side) while in the presence of ATP (1 mM, *cis*, intracellular side, Fig. 5*D*). The effect of AVP on primary cilia channel function was also observed in reconstituted ciliary membranes from wild-type LLC-PK_1 cells (Fig. 6), in agreement with our original findings by patching isolated primary cilia of these cells (35). The localized activation of ciliary AC and the production of cAMP further confirmed the presence of a functional cAMP pathway. Addition of the AC stimulator forskolin (10 μM) and ATP (1 mM) to the *cis* chamber was capable of stimulating cation-selective channel activity (Fig. 7*A*, $n = 3$), which in turn was subsequently inhibited by further addition of PKI (5 μg/ml, *cis*), a selective PKA inhibitor (Fig. 7*B*, $n = 3$).

DISCUSSION

Recent findings revealed that the primary cilium is an important sensory organelle (31), whose dysfunction is implicated in a number of seemingly unrelated diseases (15) which have a common feature, i.e., the development of cysts in a variety of target organs. The nature of the connection between ciliary function and cystic diseases is still largely unknown (15). This is in part due to the lack of understanding as to how the primary cilium works and elicits sensory function (31). Recent studies from our laboratory provided the first direct evidence for the presence of abundant channel activity, and a high cationic permeability, including the presence of functional PC2 and ENaC in the ciliary membrane (18, 35). How this ciliary conductivity is regulated and how it contributes to ciliary function remain open questions. Nonetheless, it is expected that a variety of receptors are present in different cilia, whose function will depend on the cell or tissue with which they are associated. Somatostatin and serotonin receptors, for example, have been identified in primary cilia from neuronal cells in parts of the brain (5, 14). In nonexcitable cells such as MDCK renal epithelial cells, integrin receptors have been found on primary cilia (30), although the precise function of this signal pathway is unknown. In cultured fibroblasts, there is a correlation between the orientation of the primary cilium and the direction of cell migration. Tucker and coworkers (41, 42) found that stimulation with PDGF and addition of Ca^{2+} ionophores induce Ca^{2+} influx and reabsorption of the primary cilium in 3T3 fibroblast cultures, which is an early event in the transition from growth arrest to cell proliferation. Thus cell cycle may also play a role in the transient location of specific receptors to the primary cilium (31). PDGF targets and may actually act directly through receptors in the primary cilium of fibroblasts (37). The findings herein indicate the presence, and a functional coupling, of V2R with AC in the primary cilium of renal epithelial cells. The histological immunostaining of normal rat kidney indicates the presence of V2R in primary cilia of rat kidney, mainly in intercalated cells of the collecting duct. This evidence also demonstrates the colocalization of V2R with type V/VI AC and the entire cAMP pathway, as assessed

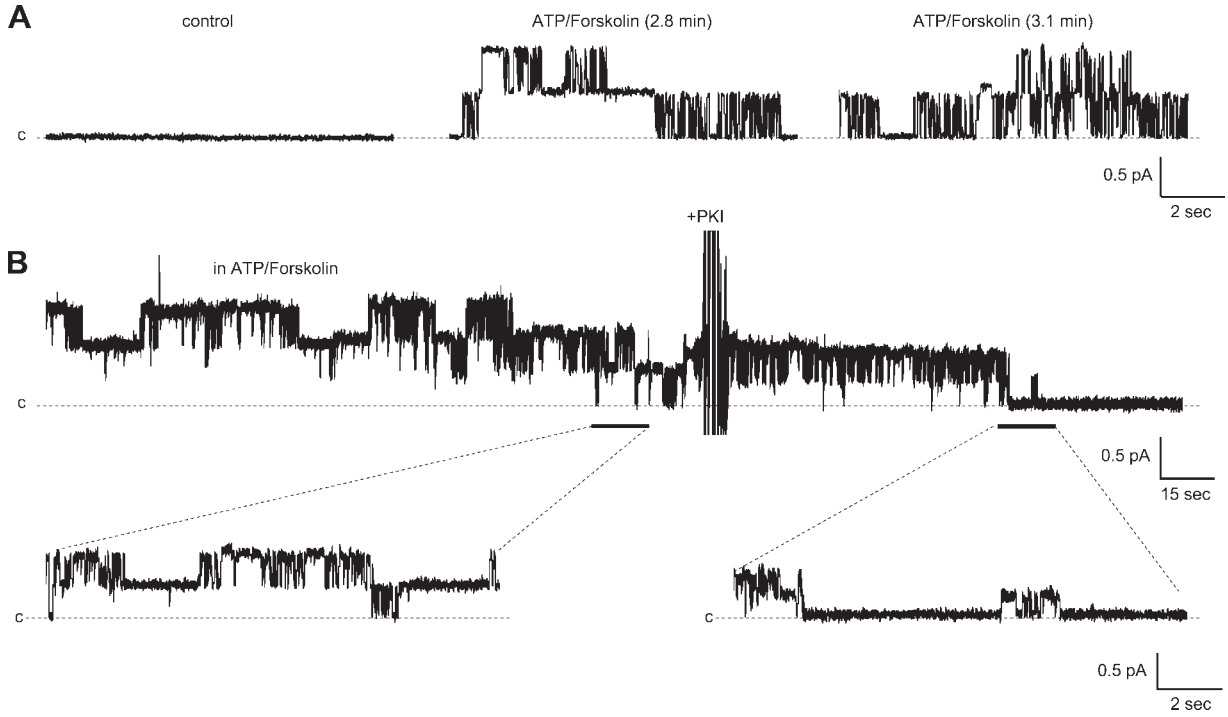


Fig. 7. Regulation by forskolin and ATP of cation channels in primary cilia. *A*: effect of local cAMP production in primary cilia was tested by addition of forskolin and ATP in reconstituted primary cilia of V2R-*gfp*-expressing cells. Addition of forskolin (10 μM) and ATP (1 mM) to the *cis* side increased and/or induced cation-selective channel activity, most predominantly the small-conductance channel phenotype. Data are representative of 3 experiments. *B*: channels activated by forskolin and ATP were readily inhibited by addition of the PKA inhibitor PKI (5 μg/ml) to the *cis* chamber. Data are representative of 3 experiments.

by the AVP-induced localized production of cAMP in primary cilia and the AVP- and cAMP-dependent activation of cation-selective channel activity. Interestingly, the effect of AVP, which was disclosed by addition of the phosphodiesterase inhibitor IBMX, was also observed in the presence of the AC activator forskolin. This is consistent with the possibility that transmembrane and V2R-coupled AC are not the only ACs in the primary cilium, and likely a soluble form of the latter may be present in this preparation. Soluble AC has recently been observed in motile cilia of respiratory tract cells (36), where it elicits the localized production of cAMP, which in turn helps control the beating rate of these cilia (36). Soluble AC is a local bicarbonate sensor (10), thus likely serving as a part of a chemical sensory mechanism, while the transmembrane AC may couple more directly to the channel complex. The functional AC complex and the activation of local PKA were further demonstrated by the stimulatory effect of forskolin and ATP on channel function, which was blocked by the PKA inhibitor PKI. Thus the data in this report demonstrate that the entire cAMP second messenger-signaling pathway provides a likely regulatory mechanism for Ca^{2+} signals in this organelle (Fig. 8). This stems from the fact that one of the channel phenotypes observed after AVP activation was consistent with PC2, which we previously determined to be functional in primary cilia of renal epithelial cells (35). PC2 is a nonselective cation channel (8), which acts as a noninactivating Ca^{2+} entry step in primary cilia (35). Its presence and regulation may have important implications in the handling of Ca^{2+} signals, which in turn control ciliary structure, particularly microtubular structures (16, 26).

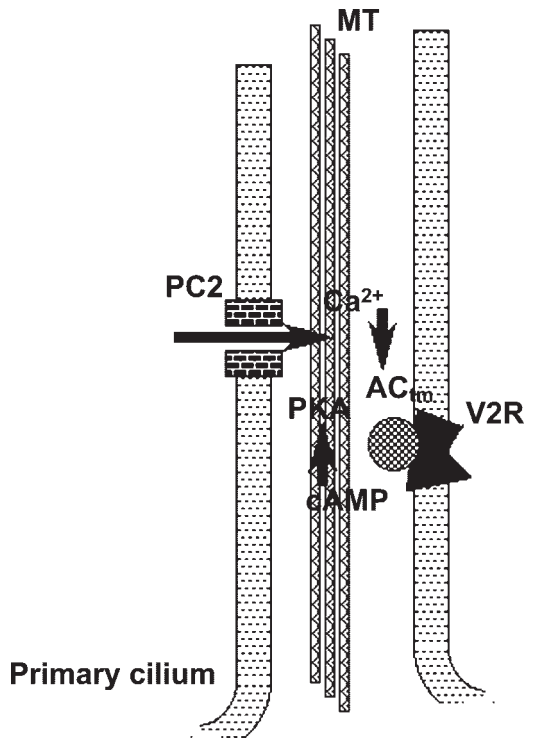


Fig. 8. Schematic model of the role V2R plays in primary cilia. The evidence in this report supports the following model: V2R localizes to the primary cilium of renal epithelial cells, where it couples to AC, which upon activation elicits the localized production of cAMP. The current hypothesis is that primary cilia have a cAMP-dependent second messenger signaling mechanism whose function includes the modulation of ciliary channel activity and thus the control of intraciliary Ca^{2+} signals elicited by PC2 channel function present in these organelles (35). Other potential regulatory signals include the ciliary resting potential by controlling other cation channels, such as ENaC. This ciliary signaling pathway provides a molecular mechanism for microtubular (MT) regulation of channel function, which in turn may help modulate ciliary properties such as length and function.

Vasopressin is an important peptide hormone, central to mammalian hydroelectrolytic homeostasis. AVP exerts its antidiuretic action and regulatory influence on transepithelial transport via V2R-cAMP-mediated responses in the basolateral membranes of target epithelia (6). AVP controls fluid transport by AVP-sensitive Na^+ channel (ENaC)- and AQP2- mediated cation and water transport, respectively. Interestingly, AVP also has a luminal effect in renal epithelia, which is associated with either luminal expression of the V2R receptor (12) or the vasopressin V1R-related rise in intracellular Ca^{2+} (7). Luminal AVP is known to hyperpolarize transepithelial potential and inhibit the hydrosmotic effect of basolateral AVP, which is likely mediated through the luminal V1R AVP receptor (7). cAMP-related signals are also expected from the apical aspect of epithelial cells, as other related pathways, such as the PTH receptor (40), have been found in epithelial apical membranes. Whether any of these receptors localizes to the primary cilium is heretofore largely unknown.

Recent evidence suggests an interesting connection between AVP and the ontogenesis of cystic diseases and primary cilia. Endogenous AC agonists have a stimulatory effect on both cell proliferation and fluid transport, particularly in various forms of cystic renal epithelia (2). This is in agreement with recent evidence indicating that AVP antagonists are effective in ameliorating cystic disease (39, 43). Cystic diseases are linked, however, by dysfunctional proteins, which largely localize to the primary cilium (1). In this context, it is tempting to postulate that the transient expression of ciliary V2R may also have an important role with such signaling pathways, which instead of fluid transport are linked to ciliary-controlled cell cycle and cell proliferation. AVP controls the rate of cell replication, particularly in certain tumors (24, 25, 28), where both normal and abnormal AVP receptors may influence the rate of cell proliferation (24). As the primary cilium is a cell cycle-regulated organelle, signals that control ciliary structure may indeed be associated with the initiation-arrest of cell proliferation (27). This may partly explain the fact that only a fraction of distinguishable cilia displayed V2R localization.

In renal epithelial cells, primary cilia are implicated in the transduction of signals involving Ca^{2+} entry steps and cell activation (22, 29). The presence of a functional PC2 in primary cilia suggests that Ca^{2+} enters the cell, at least in part, through ciliary channels. A functional cAMP pathway in primary cilia suggests a contribution of cAMP-mediated signals to balance and counter Ca^{2+} surges (3, 13, 20) and to regulate function in this organelle by controlling the Ca^{2+} responses, which likely contribute to microtubular stability and thus ciliary length. In summary, herein, we provide the first experimental evidence for the presence of ciliary V2R and a functional cAMP pathway in primary cilia of renal epithelial cells. This, in turn, suggests that in combination with, or in addition to, channel function, receptor-mediated second messenger pathways may be key signaling components in the sensory properties of primary cilia.

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