Characterization of Single Channel Currents from Primary Cilia of Renal Epithelial Cells*

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The primary cilium is a ubiquitous, non-motile microtubular organelle lacking the central pair of microtubules found in motile cilia. Primary cilia are surrounded by a membrane, which has a unique complement of membrane proteins, and may thus be functionally different from the plasma membrane. The function of the primary cilium remains largely unknown. However, primary cilia have important sensory transducer properties, including the response of renal epithelial cells to fluid flow or mechanical stimulation. Recently, renal cystic diseases have been associated with dysfunctional ciliary proteins. Although the sensory properties of primary renal epithelial cilia may be associated with functional channel activity in the organelle, information in this regard is still lacking. This may be related to the inherent difficulties in assessing electrical activity in this rather small and narrow organelle. In the present study, we provide the first direct electrophysiological evidence for the presence of single channel currents from isolated primary cilia of LLC-PK1 renal epithelial cells. Several channel phenotypes were observed, and addition of vasopressin increased cation channel activity. The PC1-PC2 channel complex is not only present in primary cilia of renal epithelial cells (7, 15) but may be a requirement for Ca2+ influx, which is amplified by Ca2+ release from intracellular stores (4, 18). Interestingly, the PC1-PC2 channel complex is not only present in primary cilia of renal epithelial cells (7, 15) but may be a requirement for Ca2+ influx, which is amplified by Ca2+ release from intracellular stores (4, 18). Interestingly, the PC1-PC2 channel complex is not only present in primary cilia of renal epithelial cells (7, 15) but may be a requirement for Ca2+ influx, which is amplified by Ca2+ release from intracellular stores (4, 18). Interestingly, the PC1-PC2 channel complex is not only present in primary cilia of renal epithelial cells (7, 15) but may be a requirement for Ca2+ influx, which is amplified by Ca2+ release from intracellular stores (4, 18).}

EXPERIMENTAL PROCEDURES

Isolation of Cilia from LLC-PK1 Cells—Wild type LLC-PK1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, as reported (20). Primary cilia from LLC-PK1 cells were isolated as follows. Confluent monolayers (2–3 weeks) were scraped with Ca2+-free phosphate-buffered saline and centrifuged for 5 min at 520 × g. The cell pellet was suspended in a high Ca2+-deceloration solution containing 112 mM NaCl, 3.4 mM KCl, 10 mM CaCl2, 2.4 mM NaHCO3, 2 mM HEPES, pH 7.0. Resuspended cells were shaken in this

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† The abbreviations used are: PC, polycystin; TRP, transient receptor potential, TRPC1, TRP-canonical 1; TRPP2, TRP-polycystin-2; pS, picosiemens.

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Cells were immunolabeled with anti-acetylated Image-it (MP) for 30 min prior to exposure to the primary antibody. The pellet was resuspended in normal saline solution, pH 7.0, and supplemented with 2.0 mM EGTA and 0.5 mM sucrose. Samples were aliquotted and stored at −80 °C until further use. Plasma membranes from confluent LLC-PK1 cells were obtained as reported (20). A 50-fold higher protein content in the plasma membranes compared with the isolated cilia (1160 ± 190 μg/ml versus 15.8 ± 4.64 μg/ml, p < 0.004, n = 3) was observed by the method of Lowry.

**Immunocytochemistry**—Confluent cells were fixed with paraformaldehyde (4%) and 2% sucrose, for 10 min. Cells were rinsed three times with phosphate buffer solution and permeabilized with either 0.1% Triton X-100 or 1% Nonidet P-40. Some cells were immunolabeled without permeabilization. Cells were blocked with 1% bovine serum albumin or Image-it (MP) for 30 min prior to exposure to the primary antibody. Cells were immunolabeled with anti-acetylated α-tubulin antibody (mAb, Sigma) at concentrations of 2.8 μg/ml of stock solution. The polyclonal anti-PC2 antibody (0.2 mg/ml stock solution) was obtained from PolyFast (Zymed Laboratories Inc.). The TRPC1 antibody (1:200 dilution from 0.3 mg/ml stock, Sigma) is from rabbit against a peptide corresponding to amino acids 557–571 of human TRPC1. The anti-rat α-epithelial sodium channel antibody was a kind gift from Dr. Tom Kleyman. A goat anti-rabbit secondary Alexa Fluor 594 (Molecular Probes) antibody was used at 2 μg/ml.

**Electron Microscopy**—Isolated cilia were fixed in a solution containing 2% glutaraldehyde and Na+ cacodylate. Samples were with the buffer, spun down at 90,000 × g in a TL 100 ultracentrifuge for 1 h at 4 °C. The pellet was stained for 2 h at room temperature with 2% OsO4. Conversely, aliquots (10 μl) were placed on a gold grid, wick off with Whatman paper (#5), and negatively stained for 10 s with 2% phosphotungstic acid. Samples were observed with a Phillips CM10 electron microscope at 80 kV.

**Electrophysiology of Isolated Cilia**—Cilium-attached patches were obtained from isolated cilia. Currents and command voltages were obtained with a Dagan 3900 patch clamp amplifier (Dagan Corp.) under voltage clamp configuration with leak subtraction adjustments. Ion channel reconstitution of ciliary membranes was conducted as reported for other membranes (21) with some modifications. Briefly, isolated cilia were mixed and sonicated in the lipid mix (21) prior to reconstitution in a lipid bilayer system. All electrical signals were filtered at 5 kHz with the internal four-pole Bessel filter (Dagan Corp.). Data were first analyzed with PClamp 6.03 (Axon Instruments), and the basal line of current records was manually corrected with Clampfit 8.0. The patch pipette was filled with a solution containing 140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl2, 2.5 mM CaCl2, and 10 mM HEPES, adjusted to pH 7.4 with N-methylglucamine. The bathing solution contained an identical solution, and whenever indicated it was replaced with a solution containing 145 mM KCl, 1.0 mM MgCl2, 2.5 mM CaCl2, and 10 mM HEPES, adjusted to pH 7.4 with N-methylglucamine. EGTA was added from a 100 mM stock solution. The patches were intrinsically leaky, which constrains their analyses (22, 23). Thus, the following restrictions apply to the data. Current records were only obtained under a given set of solutions, after offsetting tip potential to “zero current” in the absence of leak subtraction. This procedure was repeated each time that a solution was changed. Changes in baseline currents after experimental procedures prevented quantification. Thus, only identification of channel levels was considered relevant.

**RESULTS**

**Identification and Isolation of Primary Cilia from LLC-PK1 Cells**—The expression of primary cilia in LLC-PK1 cells was determined by acetylated-tubulin immunocytochemical labeling, most particularly in fluid-filled domes and spontaneously formed cysts (Fig. 1a). Most cells contained identifiable cilia as a prominent thick bud and/or more elongated structures in all stages of growth. Primary cilia were detected in cells lining the wall of transporting domes (Fig. 1b). A closer look at cellular localization indicated a clear developement toward the side of the nucleus (Fig. 1c). Some cilia extended several micrometers from the apical membrane, which had the distinctive fragmented pattern of acetylated tubulin (Fig. 1d). Primary cilia from confluent LLC-PK1 cells were isolated with a technique adapted from previous reports (24, 25). Briefly, confluent LLC-PK1 cells were deciliated by a quick exposure to a high Ca2+ solution and concentrated in a sucrose density gradient. Isolated cilia were identified after fixation and immunolabeling with anti-acetylated tubulin antibody (Fig. 2a). Isolated cilia were further visualized and identified at higher resolution (×25,000) by electron microscopy (Fig. 2b) where ciliary stems and tips can be identified as reported (26).

**Patch Clamping of Isolated Cilia**—To obtain direct electrophysiological information, isolated cilia were identified under oil phase contrast (×100) in saline solution, and after location, further visualized at lower resolution (×40) to place the patch pipette in the proximity of the isolated cilium (Fig. 2c and d). Tip resistance was followed by applying 1-mV square pulses before and after suction of the patch pipette against the isolated cilium (Fig. 2e). Most frequently, the resistance increased after touching, indicating that vacuum suction was sufficient to produce a “leaky patch.” “Leak subtraction” compensation followed with the patch clamp amplifier. Despite the intrinsic “leakiness” of the patches, channel activity was clearly observed under spontaneous conditions (n = 22/22, Fig. 3a). The most frequent ion channel observed (Fig. 3, a–d) had a single channel conductance of 83.6 ± 1.0 pS (n = 3) in symmetrical NaCl. Replacement of the bathing solution with either Na+-aspartate (71.5 ± 1.47 pS, Fig. 3d) or KCl (73.2 ± 1.58 pS, Fig. 3d) did not largely modify the single conductance, reversal potential, or rectification properties, suggesting its activity as a non-selective cation channel. Another non-selective cation channel phenotype with a single channel conductance of 173 ± 4.40 pS (n = 3) was observed in...
**Single Channel Currents in Primary Cilia**

**FIGURE 2.** Experimental approach to acquiring electrical information from primary cilia. a, isolated primary cilia from LLC-PK1 renal epithelial cells were obtained with a cell deciliation procedure as described under "Experimental Procedures." Isolated cilia were visualized by immunocytochemistry at ×100 magnification after fixation and labeling with anti-acetylated tubulin antibody. b, electron micrograph of isolated cilia. Arrowheads and arrows indicate cilia and cilia tip structures, respectively, consistent with previous reports (26). Right panels detail cilia and tips. c, the patch pipette was approached under lower magnification (×40) and then switched to higher magnification (×60). e, suction of the cilium to the patch pipette increased the tip resistance, which further increased by leak subtraction with the patch clamp amplifier.

the primary cilium (Fig. 3d, inset). Whether this channel phenotype represents a "dimer" of the 83-pS channel is currently unknown. Further, a small Na\(^+\)-permeable channel (8.07 ± 0.50 pS, n = 3) was also observed after the addition of AVP (10 μM, Fig. 3, e and f). The presence of V2R vasopressin receptors is being currently explored and would suggest that a local second messenger pathway is present in isolated primary cilia (to report elsewhere).

Interestingly, addition of cAMP-dependent protein kinase (50 nM) and MgATP (3–6 mM) also increased cation channel activity (Fig. 3g). This suggests that vasopressin acts on a V2R, instead of a V1R-type of response. However, we do not presently know how the enzyme reaches the intraciliary compartment. It is likely, however, that both the leaky and openended nature of the isolated cilium help diffuse the drugs in place. Channel function was inhibited by addition of amiloride (5 μM, n = 4, Fig. 3h).

**Reconstitution of Isolated Cilia**—Ciliary membrane channels were also observed by reconstitution in a lipid bilayer system (Fig. 3, i and j) in the presence of a Na\(^+\) gradient (150 versus 15 mM, in cis and trans compartment, respectively). Channels were observed in 297 of 303 ciliary membranes. The most frequent cation-selective channel had a single channel conductance of 156 pS, which was inhibited by an anti-PC2 antibody (Fig. 3i). Reconstituted ciliary membranes seldom displayed 75-pS Cl\(^-\)-permeable channels (3/303, Fig. 3j), explaining why it was not observed in ciliumentached patches. The data indicate, however, that abundant cation-permeable channel activity is present in the ciliary membranes. A comparison of reconstituted ciliary versus plasma membranes, indicated as much as 400-fold higher channel activity in ciliary membranes as averaged mean currents were divided by protein content (Fig. 3k).

**Presence of Channel Proteins in the Primary Cilium**—To begin an identification of the channel proteins contributing to the electrical activity of the primary cilium in renal epithelial cells immunolocalization was conducted in isolated cilia identified by co-labeling with acetylated tubulin. Several ion channels were identified in primary cilia. The TRP-type channels TRPC1 and polycystin-2 (TRPP2) were immunodetected in the primary cilium of confluent LLC-PK1 cells (Fig. 4) and also isolated cilia (data not shown). Interestingly, the epithelial Na\(^+\) channel subunit α-epithelial sodium channel was also observed lining the entire surface of the primary cilium (Fig. 4b). The presence of several channel species is in agreement with the high electrodiffusional permeability observed in the primary cilium, with respect to the plasma membrane.

**DISCUSSION**

Recent evidence indicates that renal epithelial cells may sense environmental forces by mechanosensory activity of the primary cilium (4, 15). Little is known, however, about the molecular mechanisms associated with sensory function of renal primary cilia. Electrical activity in this organelle, mediated by functional ion channels, may be an important contributor to this sensory function. Among the most studied ciliary membranes are those from sensory neurons of the olfactory bulb (27–30) and sensory cilia of ciliated invertebrates (31, 32). The reconstitution of olfactory ciliary membranes in planar lipid bilayers originally conducted by Ehrlich et al. (33) demonstrated the presence of voltage-dependent Ca\(^{2+}\) channels from *Paramaecium* cilia. Reconstitution of ciliary membranes from wild type and mutant *Tetrahymena thermophila* also showed the presence of other cation channels (31, 34).

Reconstituted sensory ciliary membranes have also allowed identification of signaling mechanisms involved in ciliary channel regulation. Labarca et al. (35) observed that ion channels from reconstituted ciliary membranes of the *Rana catesbeiana* olfactory epithelium, are sensitive to nanomolar concentrations of odorant ligands. This may have been one of the first demonstrations of TRP vanilloid receptor-type functional channels in cilia. Cyclic AMP activated the 23-pS cation channels in vertebrate olfactory cilia (24) and modulated a high conductance K\(^+\) channel, displaying several open substates with conductances of 34, 80, and 130 pS (24), which is highly reminiscent of PC2 function (21). The presence of cAMP-activated 8 pS chloride channels has also been determined by steady-state noise analysis of ligand-induced currents (36).

Sensory cilia membranes also contain inositol 3-phosphate-gated channels (37). In that study, two types of non-selective cation channels were observed by current fluctuations in rat olfactory cilia membranes fused onto phospholipid bilayers. Direct electrical information from sensory cilia in situ was obtained by Frings and Lindemann (19), who conducted highly demanding studies to determine the biophysical properties and regulation of ionic conductances in cilia from frog olfactory bulb. The authors managed to pull sensory cilia from olfactory receptor cells into a patch pipette. Despite the fact that the pipette did not form a tight electrical seal with the ciliary membrane, transient record currents driven by action potentials arising from the olfactory neuron were collected. With this method, odorant thresholds in the piconmolar range were obtained (19), indicating that ligand affinity is much higher in situ than with reconstituted preparations. This finding also suggests that, however difficult, these experiments may provide more reliable information not available by other methods (28, 38). The encompassed evidence suggests an abundance of ion channels in sensory cilia. Several G protein receptors, including those for somatostatin and serotonin have also been found in primary cilia of brain neurons (13, 14). Thus sensory function in cilia is intrinsically related to the presence of functional channels in these membranes. To date, no information is available on either membrane receptors and/or ion channel activity in primary cilia from renal epithelial cells. Recent studies indicate, however, that the autosomal dominant polycystic kidney disease gene products PC1 and PC2, a novel TRP channel member (TRPP2), are present in the primary cilium of renal epithelial cells (7, 9, 15). A functional interaction between the two may seem central to environmental cell signaling events leading to Ca\(^{2+}\) transport regulation and subsequent cell signaling (15).

In the present study we described a method to acquire single channel data from isolated primary cilia and provided the first direct evidence for the presence of functional channels in the membrane coating the primary cilium of renal epithelial cells. At least three cation-selective channel phenotypes were observed, although the ~80-pS cation-selective channel may be the most prevalent subconductance state of a large channel as observed...
FIGURE 3. Single channel currents from isolated cilia of LLC-PK1 cells. a, spontaneous single channel currents in a cilium-attached patch. b, the all-point histogram shows the main conductance, and a superimposed substate (asterisk). c, single channel currents at different holding potentials. d, current-to-voltage relationships for single channel currents in the presence of symmetrical NaCl (n = 17), asymmetrical Cl⁻/aspartate (inverted triangles, n = 3), and asymmetrical Na⁺/K⁺ (diamonds, n = 2). A large conductance channel, 173 pS was seldom observed (n = 3, circles, Na⁺/Na⁺, and Na⁺/K⁺, diamonds, inset). e, vasopressin-induced small single channel currents (asterisks), with an 8-pS single channel conductance in symmetrical NaCl (f, g). f, channel activity increased after addition of cAMP-dependent protein kinase and MgATP (n = 3). h, cation channel activity was completely inhibited by addition of amiloride (2 μM, n = 4). i, reconstituted cation channels in a lipid bilayer system. The high conductance channel showed several substates and was inhibited by an anti-PC2 antibody. j, anion-selective single channel currents were also observed in reconstituted LLC-PK1 ciliary membranes. The single channel conductance of this Cl⁻-permeable channel was 75 pS (n = 3). k, comparison of average channel activity (as mean current, pA) between LLC-PK1 plasma (Mbs) and ciliary membranes (Cilia). There is a statistically significant >400-fold higher channel activity in ciliary membranes when corrected by protein content (p < 0.001, see “Experimental Procedures”).
in both ciliary patches and reconstituted membranes. The present data are in agreement with the presence of TRP-type channel activity in primary cilia from renal epithelial cells. An ability to patch and identify single channel currents in these narrow organelles may likely lie in the fact that the ciliary channel activity increases the membrane conductance, thus depolarizing the primary cilium and the cytoplasm. Electrical depolarization ($\Delta V$), between the primary cilium and the cytoplasm provides a sensory mechanism for the primary cilium, which acts as an antenna.

FIGURE 4. Presence of ion channel proteins in primary cilia of LLC-PK1 cells. a, the PC2 channel protein is highly expressed along the length of the primary cilium of LLC-PK1 cells. TRPC1 (right), however, was seldom localized to the base of the cilium. b, the presence of PC2 and $\alpha$-ENaC (green) was largely consistent with the presence of the cAMP-dependent protein kinase-regulated $\alpha$-enepithelial sodium channel protein in this organelle. c, hypothetical model of ciliary function. Abundant ciliary channel activity increases the membrane conductance, thus depolarizing the primary cilium. The membrane depolarization (<i>V</i>), between the primary cilium and the cytoplasm provides a sensory mechanism for the primary cilium, which acts as an antenna.

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