Dual role of the TRPV4 channel as a sensor of flow and osmolality in renal epithelial cells

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The TRPV4 channel [originally named OTRPC4 (54), VR-OAC (29), TRP12 (68), and VRL-2 (13)] has been gaining increasing attention because of the ever-broadening diversity of stimuli, including physical stimuli, that have been found to activate the channel (4, 41, 43) and the wide range of both sensory and non-sensory cells that express the channel. Although the channel is expressed at highest levels in tracheal and kidney epithelial tissues, it also is expressed in various other tissues, thereby implicating the channel in a potential diverse range of functions (13, 29, 54, 68). Expression of TRPV4 in heterologous expression systems demonstrated rapid activation of the channel by hyposmotic stimuli, leading to characterization of the channel as a potential mechanosensitive channel (29, 54, 68). It was subsequently demonstrated that the channel was temperature sensitive, being activated at physiological temperatures (16, 18, 66), which, in turn, sensitized the channel to hyposmotic stimuli (66). Our laboratory (16) further demonstrated that at physiological temperatures, but not at lower temperatures, TRPV4 could be activated by shear stress, at least in overexpression systems, and potentially could act as a molecular sensor of flow. Endogenously expressed TRPV4 may share this sensitivity. Indeed, it was recently shown by Marrelli et al. (35) and Kohler et al. (27) that TRPV4 is expressed in carotid artery endothelial cells and appears to be activated by flow to induce calcium influx leading to vasodilatation (through activation of endothelium-dependent relaxing factor). Low concentrations of 4α-phorbol 12,13-didecanoate (4α-PDD; 1–3 μM), a selective lipid activator of TRPV4 (64), enhanced vasodilatation, whereas ruthenium red, a general blocker of TRPV-type channels, partially inhibited the flow-induced calcium response. Similarly, Suzuki’s laboratory (57) recently demonstrated TRPV4 expression in renal collecting ducts, where supramaximal doses of 4α-PDD (50 μM) enhanced flow-dependent potassium secretion, an apparent calcium-dependent process. In contrast, the effects of flow and 4α-PDD are largely abolished in collecting ducts from TRPV4 knockout animals. Hence, accumulating evidence is consistent with TRPV4 as a potential sensor of flow, or a component of a flow sensor, although a full assessment of this role in defined endogenous settings has not been directly demonstrated.

The purpose of the present study was to assess the potential function of TRPV4 in mouse renal cortical collecting duct M-I cells, which we have shown to endogenously express TRPV4. The renal cortical collecting duct (CCD) is a noted flow-sensitive tissue where the transport of various cations has been shown to be flow dependent (37, 38, 53), including calcium.
influx and the generation of flow-sensitive calcium signaling in native collecting duct cells (31, 37, 70) and cell lines (51). Does TRPV4 act as a flow sensor, or a component of a flow sensor, to control calcium influx in these cells? Furthermore, since the CCD is normally exposed to hypotonic luminal fluid, is the TRPV4 channel in renal cells similarly sensitive to hypotonic fluid? To address these issues, we employed gain- or loss-of-function studies in M-1 cells and TRPV4-transfected human embryonic kidney 293 (TRPV4-HEK) cells that do not express TRPV4 endogenously. TRPV4 was immunolocalized at the plasma membrane, as well as cytoplasmic structures, in M-1 and TRPV4-HEK cells and at the luminal (apical) membrane of mouse kidney CCD cells in situ. In addition to typical stimuli of TRPV4, application of fluid flow/shear stress (parallel plate chambers) or exposure to hypotonic solutions activated calcium influx in both M-1 and TRPV4-HEK cells but not in nontransfected HEK-293 cells. In loss-of-function studies employing small-interfering (si)RNA techniques, TRPV4 was selectively downregulated by TRPV4 siRNA but not by transfection with inappropriate siRNAs, leading to a marked reduction or abolition of induced calcium influx by flow, hypotonicity, or other TRPV4 stimuli. Our study provides strong evidence that TRPV4 may act as a sensor of flow/shear stress and osmolality (hypotonicity), or a component of a sensor complex, in renal collecting duct cells and may be a critical component of mechanosensitive calcium signaling. Part of this study has been presented in abstract form (46).

METHODS

Cell culture and animals. Mouse kidney M-1 (CCD) cells and HEK-293 cells (American Type Culture Collection) were grown in standard DMEM/Ham’s F-12 medium with growth supplements (DB437; Sigma) containing 1 IU/ml penicillin, 1 μg/ml streptomycin, and 5–10% FBS at 37°C, pH 7.4 (16). For most studies cells were seeded onto coverslips and grown to near confluency at 37°C in standard DMEM/Ham’s F-12 medium with growth supplements (D8437; Sigma) containing 1 IU/ml penicillin, 1 μg/ml streptomycin, and 5–10% FBS at 37°C, pH 7.4 (16). For most studies cells were typically grown on coverslips, washed with PBS, fixed in 100% Amine (Invitrogen) with ice-cold wash buffer (0.1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 0.02% sodium azide) containing a protease inhibitor cocktail. After scraping and transfer to a centrifuge tube, the suspension was briefly vortexed and then cleared by centrifugation (15 min, 16,000 g) were resolved by SDS-PAGE and transferred onto actinomycin (Ambion), respectively. Cells were transfected with siRNA constructs as outlined above at a final siRNA concentration of 20–30 nM (from a 20 μM stock). Transfection conditions were maintained for 1–5 days, with periodic addition of culture medium, and the cells were appropriately harvested/employed for each assay (see below).

Immunofluorescence imaging. Mouse kidney M-1 (CCD) cells and HEK-293 cells (American Type Culture Collection) were grown in standard DMEM/Ham’s F-12 medium with growth supplements (DB437; Sigma) containing 1 IU/ml penicillin, 1 μg/ml streptomycin, and 5–10% FBS at 37°C, pH 7.4 (16). For most studies cells were seeded onto coverslips and grown to near confluency at 37°C in a humidified 5% CO2 incubator (Nuaire) at pH 7.4.

C57BL/6 mice were used to obtain kidney tissue samples as described in Immunofluorescence imaging. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical School.

TRPV4 transfection. HEK-293 cells were stably transfected with the mouse TRPV4 cDNA. The full-length TRPV4 cDNA was previously cloned from mouse kidney (16). The cDNA was ligated into the pcDNA3.1/V5-His vector (Invitrogen) and fused with a 14-amino acid V5 epitope (with a downstream polyhistidine tag) on the COOH-terminal end of the TRPV4 cDNA using the TOPO cloning technique (pcDNA3.1/V5-His TOPO TA expression kit; Invitrogen) to provide the pcDNA3.1/TRPV4-V5 plasmid. The pcDNA3.1/TRPV4-V5 plasmid was transfected into HEK-293 cells using the Lipofectamine 2000 kit (Invitrogen) and stable transfections established by selection with Geneticin (Invitrogen) according to the manufacturer’s instructions (80–90% transfection).

siRNA oligoribonucleotide synthesis and transfection. Four separate DNA oligoribonucleotide templates complementary to four separate sites of TRPV4 mRNA were initially selected and constructed. The four template oligonucleotide sequences were 1) 5'-aagctatctctctttccc-3' (nt 31–51), 2) 5'-aagctctgccgtgctgagc-3' (nt 2188–2208), 3) 5'-aagctgtctgttctctctctc-3' (nt 2498–2518), and 4) 5'-aagctctgccgtgctgagc-3' (nt 2500–2520). The siRNAs were constructed for each template oligonucleotide sequence by in vitro transcription using a Silencer siRNA construction kit (Ambion) and labeled with the fluorescent Cy3 probe using the Silencer siRNA labeling kit (Ambion) according to the manufacturer’s instructions. In preliminary studies, the effect of siRNA transfection with each of the four siRNAs was evaluated on TRPV4 protein expression levels in TRPV4 stably transfected HEK-293 cells using an anti-V5 antibody labeled with horseradish peroxidase (anti-V5-HRP; Invitrogen) for detection in Western blots. The siRNA corresponding to the first oligonucleotide sequence, siRNA-1, proved to be the most effective double-stranded RNA to downregulate TRPV4 expression in HEK cells. siRNA-1 was used for all remaining studies and simply called TRPV4 siRNA. In a similar manner, a siRNA was constructed against GADPH mRNA as a positive control and against an inappropriate or scrambled control siRNA as a negative control, and a separate negative control without an siRNA containing transfection medium only also was used (Invitrogen).

HEK-293 cells expressing TRPV4 and M-1 cells were transfected with siRNA using the Silencer siRNA transfection kit and the siPORT Amine and siPORT Lipid agents (Ambion), respectively. Cells were transfected with siRNA constructs as outlined above at a final siRNA concentration of 20–30 nM (from a 20 μM stock). Transfection conditions were maintained for 1–5 days, with periodic addition of culture medium, and the cells were appropriately harvested for each assay (see below).

Immunoblotting. Expression of TRPV4 protein in TRPV4-transfected HEK cells was assessed by immunoblotting by taking advantage of the COOH-terminal V5 epitope built into the TRPV4 cDNA construct. HEK-293-transfected cells were lysed, and protein aliquots were resolved by SDS-PAGE and transferred onto activated polyvinylidene difluoride (PVDF) membranes (Amersham) as described previously (16, 32). After transfer, the PVDF membranes were blocked with TBS-Tween 20 (0.05%) containing 5% nonfat milk for 1 h with gentle rocking. The anti-V5-HRP antibody (Invitrogen) was applied (1:1,000) in blocking buffer for 1 h and then washed with TBS-Tween 20 (0.05%) and visualized on ECL Hyperfilm using the ECL detection reagent chemiluminescence system (Amersham).

Separately, an antibody directed against the rat NH2-terminal 233 amino acids (anti-TRPV4 antibody; Ref. 12) was used to detect endogenously expressed TRPV4 protein in M-1 cells. As described above, following SDS-PAGE separation, transfer to activated PVDF membranes, and blocking, the anti-TRPV4 antiseraum was applied (1:20,000) in blocking buffer for 1 h and rinsed. Goat anti-rabbit IgG secondary antibody labeled with HRP was then applied (1:10,000 or 1:20,000) for 1 h, washed, and visualized with the ECL detection system.

Immunoprecipitation. The specificity of the anti-TRPV4 antibody was further evaluated in an immunoprecipitation protocol. TRPV4-expressing HEK cells in culture dishes were washed in ice-cold PBS and lysed in non-denaturing lysis buffer (1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 0.02% sodium azide) containing a protease inhibitor cocktail. After scraping and transfer to a centrifuge tube, the suspension was briefly vortexed and then cleared by centrifugation (15 min, 16,000 g at 4°C). Protein lysates were precleared by the addition of 30 μl of a 50% protein G slurry and incubated for 30 min at 4°C. Lysates were centrifuged for 5 min (16,000 g, 4°C), and the supernatant was removed.

Protein G-Sepharose beads were labeled with the anti-TRPV4 antibody for at least 1 h at 4°C. After repeated washing, the labeled beads were added to cleared supernatant along with 10 μl of 10% BSA and incubated for 1–2 h at 4°C. Control reactions used beads labeled with phosphate-buffered saline. After incubation, beads were washed with ice-cold wash buffer (0.1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, and 0.02% sodium azide) and then processed for immunoblotting using anti-V5-HRP to detect TRPV4 bands as outlined for the immunoblotting procedure.

Immunofluorescence imaging. The cellular localization of TRPV4 expressed in TRPV4-transfected HEK-293 cells was assessed by immunofluorescent imaging analysis utilizing an anti-V5 antibody conjugated to FITC (anti-V5-FITC; Invitrogen). Briefly, cells were typically grown on coverslips, washed with PBS, fixed in 100%
methanol (room temperature) for 5 min, blocked with 1% BSA in PBS, and then incubated with anti-V5–FITC antibody diluted (1:500) in 1% BSA in PBS for 1 h. After washing with PBS, VectaShield mounting medium (Vector Laboratories) was applied, the coverslip was placed on a glass slide, and the cells were visualized via a Nikon Optiphot epifluorescence microscope (FITC filter set; Nikon ×40 fluoro objective, 0.85 NA). In separate experiments, localization of TRPV4 in TRPV4-transfected HEK cells was also assessed using the anti-V5 antibody. Cells on coverslips were first incubated with wheat germ agglutinin-tetramethylrhodamine (WGA-Rh; 1:200) at 4°C for 30 min to preferentially label the plasma membrane. Cells were washed in PBS at room temperature and then fixed in 3% paraformaldehyde at room temperature for 15 min, washed with PBS, permeabilized with Triton X-100 (0.5% in PBS), and blocked with 3% BSA. Cells were incubated with anti-V5 (1:200) for 1 h in 3% BSA/PBS at room temperature to label the expression TRPV4 construct (with V5 epitope) and incubated with FITC-conjugated anti-mouse IgG secondary antibody (1:400) for 30 min. Cells were washed and mounted with VectaShield mounting medium, whereupon cells were visualized with a Zeiss LSM 510 META confocal microscopy using a ×63 oil-immersion objective (plan achromat, 1.4 NA). Image acquisition was performed in multitracking mode with excitation wavelengths set at 498 nm (argon laser) and 543 nm (helium-neon laser) and emission wavelengths set at 505–550 nm (band-pass filter) and greater than 560 nm (long-pass filter) for detection of FITC (TRPV4, green channel) and WGA-Rh (red channel), respectively. Z-stack optical slices were acquired at 0.35 μm. To evaluate the localization of TRPV4 expression relative to the plasma membrane marker, intensity profiles for the two emission channels were generated along a straight line in the X-Y dimension across a single cell using the Profile function for multichannel images (see Figs. 2 and 3 for details).

Localization of endogenous TRPV4 in M-1 cells was visualized with the anti-TRPV4 (NH2 terminal) antibody (see Immunoblotting and Ref. 12) using fixation/permeabilization and blocking procedures similar to those outlined above for the anti-V5 antibody. The primary anti-TRPV4 antibody was applied to slides (1:500 dilution) for either 1 h at room temperature or overnight at 4°C, and the secondary antibody, FITC-conjugated goat anti-rabbit IgG (1:1,000 dilution), was reapplied for 1 h at room temperature. VectaShield mounting medium was applied, the coverslip was placed on a glass slide, and the cells were visualized with the Zeiss LSM 510 META confocal microscope.

Immunostaining for TRPV4 was also performed in sections of mouse kidney by using standard immunohistochemical procedures similar to those used by others (69). Briefly, a mouse was anesthetized by intraperitoneal injection of 200 mg/kg ketamine with 10 mg/kg xylazine, the left kidney was rapidly removed and placed in ice-cold PBS, and the animal was killed by exsanguination. The kidney was sliced sagittal, and the slices were immediately fixed in 4% paraformaldehyde with 5% sucrose in PBS for 1 h and then neutralized in 50 mM NH4Cl in 5% sucrose/PBS for 10 min, and the cortex was cut into longitudinal blocks (cortical-medullary axis). The cortical blocks were embedded in OCT compound (Sakura Finetek, Torrance, CA), diluted 1:2 with 20% sucrose, frozen in liquid nitrogen, and stored at −80°C until processed further. The blocks were subsequently sectioned, 5 μm thick, from the mid cortical region, incubated in acetone for 10 min at room temperature, hydrated in PBS for 10 min at room temperature, and blocked with 3% BSA in PBS for 1 h. The sections were stained for TRPV4, as described above, incubation first with the primary anti-TRPV4 antibody (1:300 dilution) at 4°C overnight, followed by washing and incubation with FITC-conjugated goat anti-rabbit IgG (1:500 dilution) for 1 h at room temperature in the dark. Sections were analyzed by fluorescence imaging as outlined above.

**Northern blots.** Northern blots were performed by employing standard protocols using Ambion’s NorthernMax kit. The mRNA was isolated from HEK-293 and M-1 cells and subjected to electrophoresis on an agarose gel using standard protocols. The mRNA was transferred to Ambion’s BrightStar-Plus membrane, cross-linked to the membrane, and hybridized to a biotin-labeled full-length TRPV4 cDNA probe. The membrane was washed and blocked, and the cDNA probe was detected using the Strep-alkaline phosphatase assay and chemiluminescence detection system.

**Measurement of intracellular calcium levels using fluorescence imaging.** Intracellular calcium levels ([Ca2+]i) were measured in cells grown on coverslips using the fura-2 fluorescence ratiometric technique described previously (16, 76). Briefly, cells were loaded with fura-2 by incubation of the cells with 2–10 μM fura-2 AM in culture medium for 1 h at 37°C. The cells were washed with control isotonic medium (see Solutions and Chemicals) and incubated for an additional 10–15 min before study. The coverslips, with cells, were attached to the bottom of a perfusion chamber (1.5 ml), and the chamber was attached to the microscope stage of an InCa imaging workstation (Intracellular Imaging) at 37°C. With shear stress studies, the shear stress parallel-plate chamber was attached to the microscope stage (see Shear Stress). Cells were bathed in either isotonic or hypotonic medium (see Solutions and Chemicals). Typically 10–30 cells were simultaneously monitored on a coverslip and the results averaged for each experiment. Intracellular calcium was estimated from the fura-2 fluorescence by excitation at 340 and 380 nm and calculating the ratio of the emission intensities at 511 nm in the usual manner every 1–3 s. Fura-2 fluorescence ratios were converted to intracellular calcium activity, [Ca2+]i, as described by Grynkiewicz et al. (17) using intracellular calibration methods as before (16, 76).

**Shear stress.** The effects of fluid flow/shear stress on TRPV4 channel activation were assessed in cells grown on coverslips. Again, cells were loaded with fura-2 AM as described above. The coverslips were attached to the bottom of a parallel-plate chamber (250-μm height; Flexcell flow chamber, Flexcell International) and the chamber was attached to the microscope stage of the InCa imaging workstation as described above. Cells were typically bathed in isotonic medium. Fluid flow rates through the chamber were regulated by a peristaltic pump (0–75 ml/min) to generate shear stresses from 3 to 20 dyn/cm2. An initial minimal control flow rate was always maintained (control shear stress ~0.3 dyn/cm2) before applying step increases in shear stress to the desired level, thereby avoiding a stop-flow state and the potential buildup of cellular metabolites in the restricted space of the luminal compartment. Intracellular calcium levels in individual cells (typically 10–30 cells) were monitored throughout the experiment and the results averaged for each coverslip. The shear stress was calculated from fluid flow rates for a parallel-plate chamber as

\[
\tau = 6\mu Q bh^2
\]

where \(\tau\) is the shear stress (dyn/cm²), \(\mu\) is the fluid viscosity (0.0069 dyn/cm² at 37°C), \(Q\) is the flow rate (cm³/s), \(b\) is the chamber width (cm), and \(h\) is the chamber height (cm).

**Chemicals and solutions.** The typical isotonic control medium was a modified balanced salt solution (MBBS) that contained (in mM) 140 NaCl, 5.4 KCl, 0.5 MgCl2, 0.4 MgSO4, 3.3 NaHCO3, 2.0 CaCl2, 10 HEPES, and 5.5 glucose, pH 7.4, with an osmolality of 300 mosmol/kgH2O (isotonic medium). In studies using hypotonic medium, the solutions were identical to MBBS (isotonic medium) except that 40 mM NaCl was replaced with 80 mM mannitol (300 mosmol/kgH2O). To make hypotonic medium, mannitol was deleted from isotonic medium to provide a hypotonic solution (225 mosmol/kgH2O). In some studies, the calcium was removed from the standard solutions and 0.5 mM EGTA added, pH 7.4, to chelate calcium (16).

The following drugs, chemicals, and probes were used in the standard studies. PMN (Sigma Chemical) and 4-chloromercuribenzoate (final concentration, 10 μM) were used for Intracellular Imaging. Other chemicals and probes included EGTA (Sigma Chemical), endoglycosidase H (Endo H; Calbiochem), endoglycosidase F2 (Endo F2; Calbiochem), N-glycosidase F (PNGase F; New England Biolabs); WGA-Rh (Molecular Probes), anti-TRPV4...
antibody, obtained from Heller et al. (12), and anti-V5-HRP and anti-V5-FITC (Invitrogen).

RESULTS

TRPV4 expression and detection. Transfection of TRPV4 into HEK-293 cells leads to strong expression of TRPV4 protein over several days. In cells stably transfected with TRPV4, detection of the protein on Western blots typically showed a prominent double band in contrast to a prominent single band typically evident in transient transfection studies (see Ref. 16) when detected with the anti-V5 antibody (Fig. 1A). To verify the specificity of the anti-TRPV4 antibody, protein expression was detected on immunoblots in the presence and absence of a blocking peptide, the antigenic NH2-terminal peptide used to generate anti-TRPV4 antibody (see Ref. 12). Anti-TRPV4 detected a double band of the expected size in both M-1 and TRPV4-transfected HEK cells, although the upper band in M-1 was considerably weaker than that in TRPV4-HEK cells. TRPV4 bands were not apparent in nontransfected HEK cells (Fig. 1A). In the presence of the blocking peptide, no bands were detected, verifying the specificity of the anti-TRPV4 antibody. The two detected bands were identical to that detected by anti-V5 in TRPV4-transfected HEK cells (Fig. 1A), verifying the size of the detected protein. The molecular masses of the two detected bands in the TRPV4-transfected HEK cells were slightly larger than 100 kDa (~102 kDa) and the mid 90-kDa range (~95 kDa), respectively. The two bands from the TRPV4-HEK cell lysates were a few kilodaltons larger than the two bands observed for the endogenous TRPV4 protein from the M-1 cell fractions, reflecting the added COOH-terminal V-5 epitope tag (5 kDa) in the TRPV4-V5 construct. The results are consistent with the predicted size for TRPV4 (98 kDa for the nonglycosylated protein).

In separate experiments, anti-TRPV4 was used in an immunoprecipitation protocol with whole cell homogenates from TRPV4-transfected HEK cells, where the anti-TRPV4 immunoprecipitated protein was immunoblotted and the TRPV4 protein was detected with the anti-V5 antibody (Fig. 1B). Again, a double band was detected of the appropriate size for TRPV4, thereby further verifying the specificity of the anti-TRPV4 antibody.

Since some TRPV channels can mature as glycosylated forms (22), the likely involvement of TRPV4 glycosylation was evaluated. Figure 1C shows the effects of addition of three glycosidases on the anti-V5 detectable protein levels of the transfected HEK cell lysates. Although Endo F2 and Endo H had little affect on the detectable bands, indicating the upper band is not a simple glycosylation product, the upper band was PNGase F sensitive, indicating that the higher molecular weight band reflects a complex, high-mannose, N-glycan glycosylation product of the TRPV4 protein.

permeabilized and stained with the anti-V5 antibody, using a FITC-labeled secondary antibody, to label TRPV4 and visualized with a Zeiss LSM 510 META confocal microscope. As shown in Fig. 2, TRPV4 expression in the stably transfected HEK cells showed high levels of expression at or near the plasma membrane. This is particularly evident in overlay images of anti-V5 and WGA-Rh (Fig. 2C). Intensity profiles of the anti-V5 and WGA-Rh fluorescence channels demonstrated strong coincident V5-epitope labeling at the plasma membrane.

The bands from T-HEK cells run slightly higher because of the added COOH-terminal V5 epitope tag of the TRPV4 cDNA construct. No staining was apparent in nontransfected HEK cell immunoblots or immunoblots blocked with blocking peptide, demonstrating specificity of anti-TRPV4 to the antigen. Furthermore, bands of similar size were detected in T-HEK cells for both the anti-TRPV4 antibody and the anti-V5 antibody. B: T-HEK immunoblots after immunoprecipitation with the anti-TRPV4 antibody. The TRPV4 construct (TRPV4-V5 tag) was detected with the anti-V5 antibody (IB anti-V5) in anti-TRPV4 immunoprecipitated protein (IP anti-TRPV4) and in whole cell homogenates, again demonstrating specificity of the anti-TRPV4 antibody to TRPV4 protein. C: immunoblots of TRPV4 in HEK-293 cells stably transfected with the pcDNA3.1/TRPV4-V5 construct. Cell lysates were digested with 3 separate glycosidases: endoglycosidase F2 (Endo F2), endoglycosidase H (Endo H), and N-glycosidase F (PNGase F). The upper band was PNGase F sensitive, indicating that the higher molecular weight band reflects a complex, high-mannose, N-glycan glycosylation product of the TRPV4 protein.

Fig. 1. TRPV4 immunoblots of M-1 cells, TRPV4-transfected HEK-293 cells (T-HEK), and nontransfected HEK-293 cells (HEK). A: anti-TRPV4 antibody was used to detect TRPV4 protein in the absence (−) and presence (+; 50 μg/100 μl antiserum) of blocking peptide. Note double bands near 100 and 95 kDa in immunoblots of M-1 (weak upper band) and T-HEK cell homogenates. (The bands from T-HEK cells run slightly higher because of the added COOH-terminal V5 epitope tag of the TRPV4 cDNA construct.) No staining was apparent in nontransfected HEK cell immunoblots or immunoblots blocked with blocking peptide, demonstrating specificity of anti-TRPV4 to the antigen. Furthermore, bands of similar size were detected in T-HEK cells for both the anti-TRPV4 antibody and the anti-V5 antibody. B: T-HEK immunoblots after immunoprecipitation with the anti-TRPV4 antibody. The TRPV4 construct (TRPV4-V5 tag) was detected with the anti-V5 antibody (IB anti-V5) in anti-TRPV4 immunoprecipitated protein (IP anti-TRPV4) and in whole cell homogenates, again demonstrating specificity of the anti-TRPV4 antibody to TRPV4 protein. C: immunoblots of TRPV4 in HEK-293 cells stably transfected with the pcDNA3.1/TRPV4-V5 construct. Cell lysates were digested with 3 separate glycosidases: endoglycosidase F2 (Endo F2), endoglycosidase H (Endo H), and N-glycosidase F (PNGase F). The upper band was PNGase F sensitive, indicating that the higher molecular weight band reflects a complex, high-mannose, N-glycan glycosylation product of the TRPV4 protein.
Labeling of the Golgi/endoplasmic reticulum (ER) is also apparent in most cells (see Fig. 4). Anti-V5 labeling at the plasma membrane appeared variable with areas of high and low expression, potentially reflecting areas of clustering of TRPV4 at the plasma membrane. Further structural and biochemical analysis is required to fully define the basis of the localization and apparent clustering.

In a manner similar to that used for TRPV4 localization in TRPV4-HEK cells, endogenous TRPV4 was immunodetected in M-1 cells using the anti-TRPV4 antibody. As described above, cells were first fixed with paraformaldehyde and the cell membranes labeled with WGA-Rh before application of the anti-TRPV4 primary antibody (1:500 dilution) and secondary FITC-conjugated goat anti-rabbit IgG (1:1,000 dilution). Confocal microscopy revealed TRPV4 labeling in both the cytosol and membrane compartments, as is apparent in the immunofluorescence image overlays (Fig. 3, A–C) and intensity profiles of TRPV4 and membrane labels (Fig. 3D). TRPV4 expression at the cell membrane was verified by surface biotinylation labeling of M-1 cells and detection of TRPV4 in immunoblots of the biotinylated membrane fraction (Fig. 3E). It may be that the cytoplasmic TRPV4 labeling represents cytoplasmic pools of TRPV4 that can be readily activated by insertion into the plasma membrane upon the application of appropriate signals. This remains to be verified in future studies.

To determine whether TRPV4 was also present in cell membrane compartments in the mouse kidney collecting ducts, the basis of the M-1 cell line, mouse kidney tissue was fixed and sectioned for localization of endogenous TRPV4 protein expression in CCD using the anti-TRPV4 antibody. Midcortical kidney sections (see Fig. 3F) were stained in a manner similar to that used for M-1 cells above, labeled first with the anti-TRPV4 primary antibody, followed by labeling with the FITC-conjugated goat anti-rabbit IgG. As shown in Fig. 3F, prominent labeling (arrowheads) was apparent at the luminal membrane of most CCD cells, likely reflecting labeling of the dominant cell type, the principal cell. Whether areas of apparently lesser staining of the luminal membrane reflect binding to the luminal membrane of the minority cell type, the intercalated cells, requires further studies. Some labeling was also apparent near the basal aspect of the cells and in some apparent vacuoles.

**siRNA uptake and knockdown of TRPV4 mRNA expression.** We tested four different TRPV4 siRNAs for their efficacy to downregulate TRPV4 protein expression (see METHODS). TRPV4 siRNA-1 was most effective, and we employed this reagent as the siRNA for the studies reported. To evaluate the utility of the siRNA procedure, initial studies assessed the uptake of siRNA into TRPV4-transfected HEK-293 cells by using microscopic fluorescence analysis, taking advantage of the fluorescent Cy3 label incorporated into the siRNA. As

![Image](Fig. 2D). Labeling of the Golgi/endoplasmic reticulum (ER) is also apparent in most cells (see Fig. 4). Anti-V5 labeling at the plasma membrane appeared variable with areas of high and low expression, potentially reflecting areas of clustering of TRPV4 at the plasma membrane. Further structural and biochemical analysis is required to fully define the basis of the localization and apparent clustering.
shown in Fig. 4C, transfection of cells with siRNA for 48 h led to accumulation of siRNA oligonucleotides in the nucleus as evident from the appearance of the C3-siRNA fluorescence (red). The apparent transfection efficiency was typically >75%. In contrast, in control cells that were not transfected with Cy3-siRNA, Cy3 fluorescence was not evident, as anticipated (Fig. 4A).

The effect of siRNA transfection (48 h) on TRPV4 expression in stably transfected HEK-293 cells was first evaluated using immunohistochemical analysis of TRPV4 protein expression detected with the FITC-labeled anti-V5 antibody (Fig. 4). In every cell where siRNA was apparent in the nucleus (verifying transfection with Cy3-siRNA), expression of TRPV4 was not evident, since no anti-V5 antibody fluorescence was apparent (Fig. 4, C and D). In contrast, in the absence of siRNA transfection, TRPV4 expression was routinely observed as evident from the FITC (green) fluorescence in Fig. 4B. Expression of TRPV4 was highest within the Golgi/ER area and at, or near, the plasma membrane, with weak diffuse staining apparent in the cytoplasm (Fig. 4B, inset). Hence, at this level of examination, siRNA transfection appeared to markedly downregulate expression of the TRPV4 protein with no detectable protein apparent within the cytoplasm or at the plasma membrane. A similar assessment of endogenous TRPV4 expression in M-1 cells by immunohistochemical analysis was not undertaken, since our anti-TRPV4 antibody has not been evaluated in this setting.

To evaluate further the actions of siRNA transfection on expression of TRPV4, the effect on mRNA levels was assessed by Northern blot analysis using a TRPV4 mRNA probe (Fig. 5). In HEK-293 cells stably transfected with TRPV4, a transcript of ~3 kb was readily apparent, consistent with the expected size of the mRNA from the TRPV4 construct. The same size transcript was apparent in negative control cells undergoing transfection conditions but without siRNA and in cells transfected with an siRNA against the housekeeping GAPDH. In contrast, in TRPV4-expressing HEK-293 cells transfected with siRNA against TRPV4, there was a time-dependent downregulation of the TRPV4 mRNA. One day after siRNA transfection, TRPV4 mRNA levels had already decreased to <50% of control values and continued to decrease to minimal levels within 2–3 days (>80% reduction). These results were verified by quantitative PCR (SYBR green), where TRPV4 mRNA was shown to be reduced by 70% of control values after 3 days of TRPV4 siRNA transfection (data not shown).

Northern blot analysis of M-1 TRPV4 mRNA levels was assessed after siRNA treatment. As shown in Fig. 5B, TRPV4 siRNA transfection displayed a downregulation of the endogenous TRPV4 mRNA levels similar to that observed for TRPV4-transfected HEK-293 cells. Messenger RNA levels decreased to minimal values within 2–3 days of siRNA transfection (>75% reduction). The results were confirmed by quantitative PCR analysis, where endogenous TRPV4 mRNA levels were reduced by 80% of control values (data not shown). Positive and negative control siRNA transfections had little, or modest, effect on TRPV4 mRNA levels. Hence, TRPV4 siRNA transfection potently, and specifically, downregulated TRPV4 mRNA in both M-1 cells and TRPV4-transfected HEK-293 cells.

siRNA knockdown of TRPV4 protein expression. The studies were extended to assess the effect of TRPV4 siRNA transfe-
tion on TRPV4 protein expression by using immunoblot analysis and the HRP-labeled anti-V5 antibody for detection. In stably TRPV4-transfected HEK-293 cells, the anti-V5-HRP antibody again recognized two protein bands as anticipated for the TRPV4-V5 construct (Fig. 6A). Transfection with siRNA against GAPDH had little influence on TRPV4 protein levels. However, in TRPV4 siRNA-transfected cells, both TRPV4 bands were downregulated in a time-dependent fashion, similar to that observed for TRPV4 mRNA levels, reaching a minimal value within 2–3 days. Normalizing for protein loading using β-actin protein levels (anti-β-actin antibody; Sigma) demonstrated a reduction in TRPV4 protein abundance by 70–80% of control values after 2–3 days (Fig. 6A). GAPDH siRNA transfection had no effect on TRPV4 protein levels.

In a manner similar to that used for HEK cells, the effect of TRPV4 siRNA transfection on endogenous expression of TRPV4 in M-1 cells was assessed using the anti-TRPV4 antibody (Fig. 6B). Again, TRPV4 siRNA transfection showed a similar selective, time-dependent decrease in TRPV4 expression only, reaching a minimum within 2–3 days. Normalizing the abundance to β-actin protein levels demonstrated a reduction similar to that observed for TRPV4-transfected HEK-293 cells within 2–3 days (Fig. 6B). GAPDH siRNA transfection had little influence on TRPV4 protein levels in M-1 cells.

Effect of siRNA knockdown on flow-induced TRPV4 activation. It has been demonstrated previously by our group and others that selected physical and chemical stimuli activate TRPV4 channel currents and calcium influx in TRPV4-transfected HEK-293 cells (16, 18, 54, 64, 68). Furthermore, activation of the TRPV4 channel could readily be monitored from changes in [Ca^{2+}], reflecting calcium influx, by using fluorescence calcium ratiometric imaging techniques (fura-2 fluorescence). In TRPV4-expressing cells, removal of extracellular calcium (Figs. 7 and 9), addition of ruthenium red as a general

**Fig. 4.** Triple-labeled immunofluorescent images of stable T-HEK cells demonstrating the effect of small-interfering (si)RNA transfection against TRPV4. TRPV4 protein expression was identified with the FITC-labeled anti-V5 antibody (green); siRNA accumulation in the nucleus was identified as the Cy3-labeled siRNA (red); and the nucleus was identified by 4,6-diamidino-2-phenylindole (DAPI) labeling of DNA (blue). A: non-siRNA-transfected T-HEK cells (Ctl) showing no labeling of nuclei by siRNA, as expected. B: anti-V5 labeling of non-siRNA-transfected HEK cells demonstrating expression of the TRPV4 V5-tagged protein. Strong TRPV4 labeling was evident (green) in the Golgi/endoplasmic reticulum areas and at the plasma membrane (see inset detail, arrow), with weak diffuse staining in the cytoplasm. C: siRNA accumulation in the nucleus after 48 h of TRPV4 siRNA transfection (red areas). D: anti-V5 labeling of siRNA-transfected cells demonstrating undetectable levels of the TRPV4 V5-tagged protein.
The regulation of TRPV4. The mean influx induced a similar influx of calcium that was abolished in TRPV4 that our laboratory demonstrated previously (16), in application of PMA (100 nM), a PKC-dependent effect on calcium influx (Fig. 9A).

TRPV4 siRNA treatment was 313 ± 462 nM (n = 5) and 22 ± 12 nM (n = 6) in HEK-293 cells and TRPV4 siRNA-transfected cells, respectively (Fig. 9A). Again, transfection with GAPDH siRNA or control siRNA had little, or modest, effect on TRPV4 activation. Removal of extracellular calcium in control TRPV4-HEK cells largely abolished stimuli-induced increases in [Ca$^{2+}$]i (Figs. 7D and 9A), results consistent with the rise in [Ca$^{2+}$]i being due to stimulated calcium influx and not to calcium release from internal stores.

TRPV4 siRNA transfection had little influence on basal intracellular calcium levels, although the basal levels tended to decline in cells where TRPV4 was downregulated. In non-siRNA-transfected (control) stably expressing TRPV4-HEK cells and in M-1 cells, basal calcium levels averaged 85.9 ± 11.7 (n = 9) and 90.9 ± 10.6 nM (n = 8), respectively. Following TRPV4 siRNA transfection, the resting calcium levels appeared to trend downward, averaging 75.4 ± 15.8 (n = 6) and 81.1 ± 18.7 nM (n = 8), respectively, as would be anticipated if TRPV4 were spontaneously active in the control setting as suggested by others (63).

A representative example showing the effect of TRPV4 siRNA on TRPV4 channel activation is shown for TRPV4-expressing HEK-293 cells in Fig. 7 and summarized in Fig. 9A. In control, non-siRNA-transfected HEK-293 cells or in cells transfected for 48 h with GAPDH siRNA or a scrambled control siRNA, the TRPV4 channel was readily activated by application of 4α-PDD (100 nM) as evident from the rapid increase in [Ca$^{2+}$]i, to peak values within a few seconds. The mean 4α-PDD-induced changes in [Ca$^{2+}$]i (Δ[Ca$^{2+}$]i) averaged 462 ± 64 (n = 5) and 67 ± 10 nM (n = 7) in control and TRPV4 siRNA-transfected cells, respectively (Fig. 9A), whereas transfection with control siRNA did not reduce the 4α-PDD-induced calcium influx (Fig. 9A). Similarly, activation of TRPV4 by application of PMA (100 nM), a PKC-dependent effect on TRPV4 that our laboratory demonstrated previously (16), induced a similar influx of calcium that was abolished in TRPV4 siRNA-transfected cells (Fig. 7), demonstrating a PMA-dependent regulation of TRPV4. The mean Δ[Ca$^{2+}$]i following PMA treatment was 313 ± 43 (n = 5) and 22 ± 12 nM (n = 6) in

TRPV4 CHANNEL AS A MOLECULAR SENSOR OF FLOW
with Δ[Ca$^{2+}$], averaging 160 ± 42 (n = 5) and 6 ± 5 nM (n = 4), respectively (Fig. 9A). Transfection with GAPDH siRNA or control siRNA had little influence (Fig. 9A). However, removal of extracellular calcium largely abolished the hypotonicity- or shear stress-induced rise in intracellular calcium (Fig. 9A), again results consistent with stimuli-induced TRPV4 activation.

In a manner similar to that followed for TRPV4-transfected HEK-293 cells, the effect of TRPV4 siRNA transfection on TRPV4 activation in M-1 cells was also tested. As shown in the examples in Fig. 8, 4α-PDD, PMA, hypertonicity (225 mosmol/kgH₂O), and shear stress (15 dyn/cm²) induced a rapid activation of calcium influx in control M-1 cells but displayed a marked reduction in apparent activation (Δ[Ca$^{2+}$]) after TRPV4 siRNA transfection (48 h). The mean Δ[Ca$^{2+}$], following siRNA transfection is summarized in Fig. 9B. In control, non-siRNA-treated cells, application of TRPV4 stimuli led to peak changes in [Ca$^{2+}$] of 621 ± 49 (n = 4), 473 ± 105 (n = 8), 165 ± 18 (n = 9), and 271 ± 99 nM (n = 6) for 4α-PDD, PMA, hypertonicity, and shear stress, respectively. In TRPV4 siRNA-transfected M-1 cells, the change in [Ca$^{2+}$] was markedly reduced in each case to 77 ± 6 (n = 7), 41 ± 6 (n = 5), 34 ± 10 (n = 5), and −5 ± 9 (n = 3) for 4α-PDD, PMA, hypertonicity, and shear stress, respectively. Transfection with GAPDH siRNA or scrambled control siRNA had modest or no effect on TRPV4 activation, although there was a significant decrease for 4α-PDD for unknown reasons (Fig. 9B). As observed with the TRPV4-HEK cells, removal of extracellular calcium largely abolished the rise in [Ca$^{2+}$] upon stimulation, pointing to TRPV4 activation as the source of the rise in intracellular calcium. Hence, TRPV4 siRNA downregulation of endogenous TRPV4 expression in M-1 cells appears to provide an equivalent downregulation of TRPV4 function compared with that observed in TRPV4-transfected HEK-293 cells.

Finally, the time course for activation of calcium influx appeared to differ among some of the stimuli in control cells (see representative examples, Figs. 7 and 8). This was partic-
ularly apparent between the peak response times for 4α-PDD compared with shear stress. Indeed, for TRPV4-transfected HEK cells, the time to peak of the calcium response averaged 66 ± 9 (n = 8) s and 101 ± 15 s (n = 8) for 4α-PDD and shear stress, respectively. Similarly, for M-1 cells, the time to peak response was 36 ± 8 (n = 8) and 87 ± 12 s (n = 8), respectively, again demonstrating a delay to the peak response with shear stress. Often, but not always, the initiation of the response to shear stress was delayed for a few seconds after the onset of the stimulus. Hence, the shear stress response appears to be relatively slow, often taking a few seconds to initiate, especially compared with the rapid onset observed for 4α-PDD. Understanding the basis of this potential difference requires further systematic assessment using well-defined stimuli.

**DISCUSSION**

The properties of the TRPV4 protein have been studied extensively in heterologous expression systems. However, the function of the channel in most cell types where TRPV4 is endogenously expressed remains poorly understood. The current study provides new evidence that TRPV4 may function as a sensor of flow and osmolality, or as a component of a sensory complex, in the flow-sensitive renal CCD cells.

Localization of TRPV4 in renal epithelial cells. The kidney is a dominant site of expression of TRPV4, where it has been shown by in situ hybridization and immunohistochemistry to be associated with the tubular structures, including cortical distal tubular segments (13, 54, 55), although the specific segments have not been fully delineated. Conflicting evidence also has been presented for the cellular site of expression with immunolocalization evidence of a luminal (apical) membrane localization in distal tubules in one study (55) and a basolateral (abluminal) localization in CCD in another study (58). Recently, Heller et al. (12), using an antibody directed against the NH2 terminus of TRPV4 (the anti-TRPV4 used in the present study), provided strong evidence of TRPV4 localization at the apical membrane of distal tubule cells with a clear association.
The renal TRPV4 channel as a sensor of flow and/or shear stress and osmolality. TRPV4 is endogenously expressed in M-1 CCD cells, where it displays mechanosensitive properties consistent with that of a flow- and osmosensor. In both epithelial M-1 cells and TRPV4-transfected HEK cells, elevation of fluid flow rates to produce a shear stress ~15 \text{ dyn/cm}^2 stimulated calcium influx via activation of TRPV4. Initial studies of calcium influx in TRPV4-transfected HEK cells in our laboratory (16) demonstrated that shear stresses from 3 to 20 dyn/cm\(^2\) lead to stimulation of calcium influx. The applied range of shear stresses is within the typical range expected for the mouse CCD in situ, which we estimate in control states to range from ~0.3 dyn/cm\(^2\) at the low end to >20 dyn/cm\(^2\) at the high end (see APPENDIX). These values are similar to those estimated by others (6). TRPV4 expression was selectively downregulated in both M-1 and TRPV4-HEK cells by using siRNA gene silencing. This downregulation was associated with loss of mechano-/osmosensitive calcium influx. In both M-1 cells and TRPV4-transfected HEK cells, hypotonicity- and flow-induced activation of calcium influx could be largely abolished by the TRPV general channel blocker ruthenium red (data not shown) or by removal of extracellular calcium (Figs. 7–9). The current study demonstrates that the endogenous TRPV4 protein expressed in M-1 cells is sensitive to mechanical stimuli including fluid flow/shear stress and hypotonic cell swelling. As such, the TRPV4 channel would appear to function as a molecular sensor of flow and osmolality (hypotonicity) or as a component of a renal epithelial sensing complex.

The flow-sensitive nature of the TRPV4 channel may underlie certain flow-sensitive properties of the CCD, the most prominent being the flow sensitivity of potassium secretion. Regulation of potassium secretion by the CCD and connecting tubule plays a central role in maintaining potassium balance in mammals. This process has been shown to be highly flow sensitive, with high rates of tubular flow leading to stimulation of potassium secretion (56, 71). The flow-induced activation of potassium secretion is calcium dependent, requiring an increase in intracellular calcium (57, 70). Indeed, luminal calcium influx in CCD is flow sensitive (31, 70) and appears to provide the signal for activation of a luminal membrane potassium channel, likely a calcium-sensitive maxi-K channel (50, 56, 71), leading to flow-induced potassium secretion. The present study provides new evidence that TRPV4 may be the molecular basis of the flow-sensitive luminal calcium influx. This conclusion is further supported by studies in TRPV4 knockout mice, where it was shown that flow-induced potassium secretion was apparent in CCDs isolated from wild-type animals but not in CCDs isolated from mice lacking TRPV4 (57). It was also shown that the flow dependency was enhanced with PACSIN-3 at the luminal membrane. The reason for these differences in expression patterns is not known. Therefore, we have extensively characterized the NH\(_2\) terminus-directed anti-TRPV4 antibody used in the present study to verify its specificity in detecting TRPV4 in both immunoblot and immunohistochemistry protocols. We have provided new evidence that TRPV4 is expressed at the luminal cell membrane and cytoplasm of renal cells in culture. Most importantly, we demonstrated strong luminal membrane staining in mouse kidney CCD in situ. Expression in the kidney CCD segment was apparent in most cells, reflective of expression in both the dominant cell type, the principal cell, and the minority cell type, the intercalated cell. Although low staining was apparent in a few cells, it was not possible without identity markers to know whether this might reflect staining of the luminal membrane of the intercalated cells.
by addition of supramaximal levels of 4α-PDD to the luminal fluid to activate TRPV4, further supporting a role for TRPV4 in the process. However, some question still remains as to the identity of the effector potassium channel in the CCD of mice, since immunolocalization studies by Pluznick et al. (50) demonstrated expression of the maxi-K channel-forming subunit, BK-α, and the dominant regulatory subunit, BK-β1, in upstream connecting tubule segments but apparently not in the CCD of mice. Other β-subunit isoforms (∝–β4) are expressed in rabbit CCD, along with the α-subunit, apparently forming a calcium-sensitive maxi-K channel (71). It may be that a similar expression pattern of isoforms exists in mouse CCD to generate a maxi-K channel, since a functional channel would appear to be present given that potassium secretion in mouse CCD is sensitive to charybdotoxin, a selective maxi-K channel inhibitor (57). Hence, the molecular identity of the potassium secretory channel in mouse CCD remains to be fully elucidated. Nonetheless, the accumulating evidence points to an essential role of TRPV4 as a component of a flow sensor in mediating flow-induced, calcium-dependent potassium secretion in mouse CCD.

The concept of TRPV4 as a flow sensor is also apparent in studies of vascular endothelial cells, where TRPV4 is also endogenously expressed (5, 25, 40). Indeed, it was shown by Marrelli et al. (35), in collaboration with our laboratory, and by Kohler et al. (27) that TRPV4 is expressed in the endothelium of middle carotid artery and carotid artery, respectively. In both vessel types, flow induces calcium influx, leading to a vasodilation, likely as a result of calcium-induced stimulation of endothelium-derived hyperpolarizing factor separately from actions on NO production (35). In both studies, application of low concentrations of 4α-PDD (1–3 μM), a specific activator of TRPV4, enhanced the vasodilatory response, whereas low concentrations of ruthenium red, a general blocker of TRPV4-type channels, partially inhibited both the flow-induced calcium response and arterial vasodilation. Hence, in vascular endothelial cells, TRPV4 again appears to function in a flow-sensing capacity.

The osmosensitivity of the TRPV4 channel, separate from the flow sensitivity, may lead to related or other functions of TRPV4 in the renal CCD. As shown in the current study, the endogenous channel in M-1 cells appears to display a sensitivity to hypotonic swelling similar to that observed in overexpression systems such as TRPV4-transfected HEK cells (Figs. 7–9). The channel would appear to function as a sensor of osmolality, or a component of a sensor, in the renal CCD cells, since this function is abolished following siRNA TRPV4 knockdown. The significance of this osmosensing ability in CCD cells is not known, but could potentially play a critical role in cell volume regulation and the maintenance of the intracellular microenvironment. For example, CCD cells are typically exposed to a hypotonic luminal tubular fluid. In the presence of antidiuretic hormone, such as in volume depleted states, the aquaporin 2 water channel is inserted into the luminal cell membrane of CCD principle cells, rendering the luminal membrane highly water permeable as hypotonic fluid is reabsorbed by the cells (15, 42). This leads to cell swelling which induces most cells, including the CCD cells, to activate efflux of solute, typically potassium and chloride, to return cell volume back to normal, a process referred to as regulatory volume decrease. Cell volume regulation in the CCD cells and other renal epithelial cells can be dependent upon calcium influx to activate solute efflux mechanisms (36, 48, 59). To this end, TRPV4 has been shown to play a role in the process of volume regulation in keratinocyte cells (3). Hence, the osmo-sensitive properties underlying TRPV4 activation may indicate that it plays a role in cell volume regulation in some renal epithelial cells. As with the flow sensory function, the osmo-sensing function also has been noted in mouse aortic endothelial cells (61) and in microvascular endothelial cells of the blood-brain barrier (Brown RC and O’Neil RG, unpublished observations), demonstrating common mechanosensitive functions of TRPV4 in flow-sensitive tissues. Hence, it would appear that the TRPV4 channel plays a multidimensional role relating to both its flow sensitivity and its osmolality sensitivity in controlling cell function. It may be that a common mechanism of activation may underlie the channel sensitivities to microenvironmental stimuli (see Mechanism of TRPV4 activation). Clearly, other functions of TRPV4 in modulating calcium-dependent events may yet remain to be discovered.

Mechanism of TRPV4 activation. The mechanisms of activation of TRPV4 by flow/shear stress and hypotonicity have not been fully elucidated. Although the channel could potentially be directly activated by mechanical stress, indirect regulation by other pathways seems more probable, since the rate of activation by mechanical stresses is rather slow, typically requiring many seconds for full activation (see Figs. 7 and 8). The response to shear stress and hypotonic cell swelling is not likely related to calcium release from calcium storage sites, since release of calcium from intracellular storage sites was either not apparent or rather small (see Figs. 7 and 8). This remains to be more directly tested in future studies.

It has been shown that TRPV4 regulation by hypotonic cell swelling is associated with the production of arachidonic acid, leading to activation of TRPV4 by downstream epoxycyclo-trienoic acid metabolites (63, 65). Inhibiting the production of arachidonic acid markedly reduces hypotonicity-induced activation of TRPV4 in the HEK overexpression system (63). Furthermore, it was recently demonstrated in ciliated oviductal cells, which endogenously express TRPV4, that increasing the viscosity of the extracellular luminal fluid, thereby increasing the viscous load/mechanical stress to the cell and cilia, activated TRPV4 to enhance ciliary beating (2). Again, inhibiting the production of arachidonic acid and its metabolites reduced viscosity-induced enhancement of ciliary beating in oviductal cells (2). Hence, a role of arachidonic acid metabolism in mechanical control of TRPV4 seems highly probable. The apparent slow response of TRPV4 activation by shear stress in the current studies would be consistent with such an indirect mechanism of activation. However, whether all mechanical stimuli work through metabolites of arachidonic acid to modulate TRPV4 activity or whether other pathways of modulation may be involved in this process, such as the PLC-PKC signaling pathway as shown in other cells (see Refs. 16, 45, 73), is not known. Furthermore, direct mechanical regulation of the channel has not been rigorously tested and cannot be unequivocally ruled out with current evidence.

Role of polycystins and other TRP channels in sensing fluid flow/shear stress. In recent years it has become apparent that other TRP calcium-permeable channels may also participate in mechanosensitive calcium influx in renal cells. Specifically, it has been shown that polycystin-2, the protein that when defec-
In summary, the TRPV4 channel appears to be regulated by mechanical stimuli in renal epithelial cells (and numerous other cell types). The channel can be activated by mechanical stresses, including fluid flow/shear stress and hyposmotic swelling of the cell. The channel’s function as a sensor of fluid flow and hypotonicity would be consistent with the known flow-dependent and swelling-induced calcium signaling characteristics in renal CCD and vascular endothelial cells. However, the channel is expressed in numerous other cells, both sensory and nonsensory. Since other evidence indicates that the channel is sensitive to a diverse range of chemical and physical stimuli (41, 44), the channel would appear to function more as a point of convergence or integration of diverse stimuli (44). Future studies are needed to begin to unravel the apparent complex nature of regulation and signal transduction associated with this channel. The diversity of gating sensitivities and broad range of distinct cell types expressing TRPV4 indicate that the channel function, including its role as a flow sensor, will vary depending on the cell type and microenvironmental setting of the channel. Furthermore, association of TRPV4 with other flow-sensitive channels may provide additional functional modulation of the channel. Hence, the function of the channel is likely to be highly dependent on the cellular and physiological setting or context, leading to context-dependent regulation.

APPENDIX: ESTIMATES OF SHEAR STRESS IN MOUSE CCD

The typical shear stress associated with tubular fluid flow in the mouse CCD in situ can be estimated from flow rates and tubular diameters. Although flow rates in CCD have not been directly measured, we have estimated these values from literature measurements of distal tubule (DT) flow rates, based on micropuncture studies of single-nephron glomerular filtration rate (SNGFR) and fractional delivery (FD) to the DT in the mouse kidney. The measured SNGFR rates in mouse kidney are variable, with control values from individual nephrons ranging from <5 to >20 nl/min (average of all studies = 11 nl/min; Refs. 21, 24, 28, 34, 60, 72). The FD was also variable, with average values ranging from 0.20 for low SNGFRs (34) to >0.35 for high SNGFRs (24) (average = 0.27). Using this “normal” range of SNGFRs and FDs, we calculated the DT flow rate (SNGFR × FD) to range from <1 to >7 nl/min (average = 3 nl/min). If it is assumed that fluid reabsorption is minimal along the distal convoluted tubule and connecting tubule, as a first approximation (a small fraction of fluid can be reabsorbed, especially in hypotonic or volume-depleted states), the DT flow rate provides a reasonable approximation of the flow to the CCDS. Furthermore, since several distal nephrons coalesce into a single CCD, the number of mergers is needed to estimate CCD flow rates. From developmental studies it has been shown that in newborn mouse kidney, 10.6 converging sites can be identified along the entire length of the cortical and medullary collecting duct (8). Assuming the mouse ureteral bud branching is similar to that in the rat, there should be on average of 2.5 branching sites in the cortex, providing for the merger of 5 distal tubules/connecting tubules into each CCD (67). Hence, fluid flow entering each CCD will be on average five times that of a single DT, providing an approximate range of CCD flow rates of 5–35 nl/min (average = 15 nl/min) as summarized in Table 1.

Table 1. Estimated flow rates and shear stresses in mouse CCD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Average or Typical</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT flow rate, nl/min</td>
<td>1–7</td>
<td>3</td>
</tr>
<tr>
<td>CCD flow rate, nl/min</td>
<td>5–35</td>
<td>15</td>
</tr>
<tr>
<td>CCD diameter, μm</td>
<td>12–25</td>
<td>18</td>
</tr>
<tr>
<td>τ at 20°C, dyn/cm²</td>
<td>0.54–34.5</td>
<td>4.4</td>
</tr>
<tr>
<td>τ at 37°C, dyn/cm²</td>
<td>0.37–23.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Cortical collecting duct (CCD) flow rates were estimated from distal tubule (DT) flow rates (21, 24, 28, 34, 60, 72) by assuming 5 DTs merge into 1 CCD (see Appendix for details). CCD diameters were estimated from published micrographs (33, 52). τ. Shear stress.
The other important variable needed for estimating shear stress is the diameter of the lumen of the CCD. Based on literature micrographs, the CCD luminal diameter can vary from ~10 to 25 μm or more (33, 52). If we limit the range to more conservative estimates of ~12–25 μm, then the expected range of shear stresses in the CCD under normal control conditions can be estimated with the Hagen-Poiseuille equation for flow of a Newtonian fluid through a cylinder (10, 14) as
\[
\tau = 4\mu Q/(\pi R^4),
\]
where \(\tau\) is the shear stress at the cylinder wall (dyn/cm²), \( \mu \) is the dynamic viscosity of the tubular fluid (using the viscosity of water: 0.01002 Poise at 20°C, 0.006915 Poise at 37°C), \( Q \) is the volume flow rate (ml/min), and \( R \) is the tubule luminal radius (cm). Using CCD flow rates of 5–35 ml/min and tubular diameters of 12–25 μm, the range of shear stresses is estimated to be 0.54–34 dyn/cm² at 20°C and 0.37–24 dyn/cm² at 37°C (Table 1). This is similar to the normal range of estimates made by others (6). If the CCD luminal diameter is assumed to be constant at 18 μm and flow rate constant at 15 ml/min, the shear stress acting on the CCD wall would average ~4.4 dyn/cm² at 20°C and 3.0 dyn/cm² at 37°C. Since in our studies we typically employ shear stress values of 3–20 dyn/cm² (16), with 15 dyn/cm² used in the current study, the shear stresses employed are within the range anticipated for the mouse CCD under normal conditions. It should be noted, however, that in altered pathophysiological states, where DT volume flow is either enhanced, such as in volume expansion, or depressed, such as in volume-depleted or hypodrpic states, the shear stresses experienced in the CCD will be higher and lower, respectively, than the range estimated for the control conditions of the present study.

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REFERENCES


