TRPP2 and TRPV4 form a polymodal sensory channel complex

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The primary cilium has evolved as a multifunctional cellular compartment that decorates most vertebrate cells. Cilia sense mechanical stimuli in various organs, but the molecular mechanisms that convert the deflection of cilia into intracellular calcium transients have remained elusive. Polycystin-2 (TRPP2), an ion channel mutated in polycystic kidney disease, is required for cilia-mediated calcium transients but lacks mechanosensitive properties. We find here that TRPP2 utilizes TRPV4 to form a mechano- and thermosensitive molecular sensor in the cilium. Depletion of TRPV4 in renal epithelial cells abolishes flow-induced calcium transients, demonstrating that TRPV4, like TRPP2, is an essential component of the ciliary mechanosensor. Because TRPV4-deficient zebrafish and mice lack renal cysts, our findings challenge the concept that defective ciliary flow sensing constitutes the fundamental mechanism of cystogenesis.

Introduction

Primary cilia function as sensory organelles that detect a variety of chemical and physical stimuli. The cilia of renal epithelial cells are exposed to fluid flow that bends this microtubule-based organelle, eliciting an increase in intracellular calcium (Praetorius and Spring, 2001). The calcium transient is contingent on the presence of the polycystin-1–TRPP2 protein complex (Nauli et al., 2003). TRPP2, a member of the transient receptor potential (TRP) family of ion channels, assembles with the PKD1 gene product polycystin-1, a large integral membrane protein with distant homology to TRP channels, to form a receptor–ion channel complex (Hanaoka et al., 2000; Köttgen, 2007). Mutations in polycystin-1 or TRPP2 cause autosomal dominant polycystic kidney disease, and deletion of either of the two proteins causes multiple developmental abnormalities in mice, most notably fluid-filled cysts in the kidney, liver, and pancreas (Lu and Zhou, 1997; Wu et al., 1998; Kim et al., 2000). How polycystin-1 and TRPP2 exert their diverse biological functions has remained largely unknown. Both proteins localize to the primary cilium but assume other distinct subcellular localizations that are regulated by multiple adaptor proteins (Hidaka et al., 2004; Köttgen and Walz, 2005). The flow-mediated Ca²⁺ transient requires the presence of functional TRPP2 (Nauli et al., 2003) and has been implicated in the regulation of tubular polarity and morphology. Because functional and structural defects of the primary cilium cause polycystic kidney disease, it is currently widely accepted that the loss of tubular flow sensing constitutes the fundamental mechanism of cystogenesis (Ong and Wheatley, 2003; Torres and Harris, 2006). Although the localization and calcium permeability of TRPP2 make it an attractive candidate for the ciliary calcium entry pathway, the homomeric channel lacks mechanosensitivity (Giamarchi et al., 2006).
Thus, we hypothesized that TRPP2 assembles with an auxiliary subunit to form a mechanosensitive channel complex.

Results and discussion

TRPV4 localizes to the cilium and interacts with TRPP2

In Caenorhabditis elegans, the TRPV channel OSM-9 is involved in transmitting multiple sensory stimuli, including olfaction, osmosensation, and mechanosensation. Specificity is provided by interaction with specific OSM-9 and capsaicin receptor-related (OCR) ion channels, and signaling depends on the correct targeting of these ion channels to the cilium of sensory neurons (Tobin et al., 2002; Kahn-Kirby and Bargmann, 2006). Because human TRPV4 is related to OSM-9, rescues the avoidance defects of OSM-9–deficient worms in response to hyperosmotic stress and nose touch (Liedtke et al., 2003), and is expressed in the kidney (Tian et al., 2004) as well as the zebrafish pronephros (Mangos et al., 2007), we postulated that TRPV4 may access the ciliary compartment in the kidney of vertebrates. Recently, TRPV4 was found in cilia in cholangiocytes and the oviduct (Teilmann et al., 2005; Gradilone et al., 2007). Consistent with its expression pattern in C. elegans and these mammalian tissues, TRPV4 localized to the cilia of polarized MDCK cells, where it colocalized with TRPP2 (Fig. 1, A–D; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200805124/DC1). Accordingly, we investigated whether TRPV channels are involved in flow-mediated calcium signaling and found that flow-triggered calcium transients in ciliated MDCK cells were completely abrogated by 500 nM of the polycationic ruthenium red (RR), a compound that inhibits TRPV channels (Fig. S2; Voets et al., 2002). These observations led us to speculate that TRPP2 may interact with TRPV4 to mediate tissue-specific functions such as ciliary mechanosensation.

Because TRP channels are known to engage in heteromeric interactions that generate channels with novel functional properties (Schaefer, 2005), we tested whether TRPP2 physically interacts with TRPV4. As shown in Fig. 1 E, the carboxy terminus of TRPP2 immunoprecipitated wild-type TRPV4 as well as the carboxy terminus of TRPV4 (Fig. 1 F). Conversely, precipitation of the carboxy terminus of TRPV4 immobilized wild-type TRPV4 (Fig. 1 G). To confirm these coimmunoprecipitation assays, we used fluorescence resonance energy transfer (FRET) to test whether the carboxy-terminal domains of TRPP2 and TRPV4 associate within living cells. As shown in Fig. 1 (H–J), fusion of CFP and YFP to full-length TRPP2 and TRPV4, respectively, resulted in FRET, which provides further evidence that TRPP2 and TRPV4 form heteromultimers. FRET was not observed when using TRPC6-YFP as a negative control (Fig. S1, G–I).

Functional interaction between TRPP2 and TRPV4

To study whether TRPP2 and TRPV4 also interact functionally, we expressed both channels in Xenopus laevis oocytes for electrophysiological analysis. TRPV4 is activated by a variety of stimuli, including hypotonicity-induced cell swelling, which is akin to a mechanical stimulus (Liedtke et al., 2000; Nilius et al., 2001, 2004). Because a carboxy-terminal acidic cluster precludes the expression of TRPP2 at the cell surface (Köttgen et al., 2005), only TRPV4, but not TRPP2, raised the steady-state inward currents in X. laevis oocytes. Hypotonic cell swelling further increased TRPV4 currents (Fig. 2 A). Coexpression of TRPV4 and TRPP2 significantly augmented the swelling-activated currents in X. laevis oocytes, whereas steady-state currents remained unchanged (Fig. 2 A). The swelling-induced conductance was more than doubled in TRPV4/TRPP2-expressing cells in comparison with TRPV4-expressing cells (Fig. 2 B). Neither water nor TRPP2-injected oocytes showed any swelling-activated currents (Fig. 2, A and B). The current–voltage relationship revealed a slight outward rectification of the TRPV4 currents that was not substantially influenced by coexpression of TRPP2 (Fig. 2, C and D). Increasing the extracellular Ca²⁺ concentration led to a significant increase in whole-cell currents in TRPV4-expressing oocytes. This effect was dramatically augmented in oocytes coexpressing TRPP2 (Fig. 2, E and F). Thus, TRPP2 alters the functional properties of TRPV4. We also observed a functional interaction between TRPP2 and TRPV4 in HEK 293 cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200805124/DC1).

To address the question of whether the TRPP2 effect on swelling-activated currents is simply mediated through increased surface expression of TRPV4, we inserted a V5 tag into the first extra- cellular loop of TRPV4 and TRPP2, respectively, and monitored either ion channel at the cell surface using a modified ELISA system (Wegierski et al., 2006). Interestingly, coexpression of TRPV4 significantly increased TRPV4 surface expression (Fig. 2 G), whereas TRPP2 reduced both TRPV4 surface expression and TRPV4 total protein levels (Fig. 2 I). Thus, the increases in swelling-induced conductance are not the result of increased TRPV4 surface expression but rather caused by altered channel properties through formation of TRPP2/TRPV4 heteromers.

These findings demonstrate that TRPP2 and TRPV4 physically and functionally interact, thereby raising the question of whether TRPP2 nonspecifically alters the activity of TRP channels in vitro. TRPP2 is known to interact with TRPC1 (Tsiokas et al., 1999); therefore, we tested whether TRPP2 influences the channel properties of TRPC1 in X. laevis oocytes. TRPP2 did not alter the whole cell currents mediated by expression of TRPC1 (unpublished data), which indicates that physical interaction with TRPP2 alone does not suffice to alter the functional properties of another TRP channel, at least under the experimental conditions used here.

TRPV4 is an essential component of the ciliary flow sensor in MDCK cells

Calcium transients, triggered by ciliary bending, were first established in MDCK cells (Präetorius and Spring, 2001) and then subsequently validated in other in vitro and in vivo systems (Nauli et al., 2003; Liu et al., 2005). To test whether TRPV4 is a component of the flow sensor in the primary cilium, we studied flow-induced Ca²⁺ transients in MDCK cells. We used a lentiviral siRNA approach to inducibly knock down endogenous TRPV4. After identifying two siRNA oligonucleotides that efficiently reduced expression of canine TRPV4 (Fig. 3 A), we generated polyclonal MDCK cells expressing TRPV4 siRNA under...
flow-induced Ca\(^{2+}\) signals were virtually abolished in the same cells after the induction of TRPV4 siRNA expression by tetracycline (Fig. 3, C, E, G, and H). MDCK cells expressing the second TRPV4 siRNA construct showed similar results, whereas GFP- and siRNA-negative cells displayed the typical, flow-induced Ca\(^{2+}\) signals (unpublished data). Furthermore, robust flow-induced Ca\(^{2+}\) signals were observed in MDCK cells expressing a TRPV4 siRNA with a two-base mismatch, which

the control of the TET repressor; the expression of siRNA in individual cells was monitored by the concomitant expression of GFP (Fig. 3, B and C). Ciliary bending was achieved by superfusing ciliated MDCK cells at physiological flow rates in a laminar flow chamber (Praetorius and Spring, 2001; Kotsis et al., 2007). Noninduced MDCK cells did not express TRPV4 siRNA and consistently displayed Ca\(^{2+}\) signals upon flow stimulation like wild-type MDCK cells (Fig. 3, B, D, and F). However,
Figure 2. Functional interaction of TRPP2 and TRPV4 in *X. laevis* oocytes. (A) Analysis of TRP channel whole-cell currents under voltage clamp (Vc) conditions. Currents were recorded in *X. laevis* oocytes injected with cRNA encoding TRPP2 and/or TRPV4. Mean currents in Ringer or hypotonic solution are shown. (B) Summary of data acquired in A. Asterisks indicate significant differences in the hypotonicity-induced whole-cell conductance (ΔG) compared with water-injected control oocytes; §, significant difference between bars as indicated (n = 21, 15, 38, and 37, respectively). (C and D) Current-voltage (I–V) relations for oocytes expressing TRPV4 or TRPV4 and TRPP2 (D; n = 7). (E) Increasing the extracellular Ca²⁺ concentration from 1.8 to 18 mM on January 29, 2018 jcb.rupress.org Downloaded from
does not alter TRPV4 expression levels (Fig. S2, G–I). These results demonstrate that TRPV4 is an essential component of the ciliary flow sensor in MDCK cells.

**Role of TRPP2 and TRPV4 in cystogenesis**

TRPV4-deficient mice display defective osmoregulation and thermosensation but fail to develop polycystic kidney disease (Liedtke and Friedman, 2003; Mizuno et al., 2003; Lee et al., 2005). We never observed cystic kidneys in TRPV4-deficient mice up to an age of 12 mo (n = 10). To investigate the role of TRPV4 in another animal model for cystogenesis, we studied the development of the pronephros during early zebrafish embryogenesis. Microinjection of a morpholino oligonucleotide (MO) directed against Zebrafish TRPP2 (pkd2MO) induces nephric cysts that are detectable 48 h postfertilization (hf; Fig. 4, A and B; Sun et al., 2004; Obara et al., 2006). However, neither injection of trpv4MO (Fig. 4, C and E) nor coinjection of trpv4MO in combination with low concentrations of pkd2MO resulted in a significant increase in cyst formation in the zebrafish pronephros (Fig. 4, D and F). The zebrafish results are consistent with the lack of renal cysts in the TRPV4-deficient mice, which suggests that TRPV4, although an essential component of the ciliary mechanosensor in MDCK cells in vitro, does not play an important role in cystogenesis in mouse or zebrafish. The question then arises of whether there is any evidence that TRPV4 is required for flow-induced calcium transients in vivo. Flow-dependent potassium secretion is a calcium-dependent process that is completely abolished in TRPV4 knockout mice (Taniguchi et al., 2007). These findings strongly suggest that flow-induced calcium transients are absent in at least the distal nephrons of adult TRPV4-deficient mice and question the importance of flow-mediated calcium signaling in cystogenesis. We cannot exclude the possibility that flow sensing plays a role in regulating tubular morphology during embryonic development. Yet, to our knowledge, there is no direct experimental evidence supporting a role for flow sensing during development because the role of polycystin-1 and TRPP2 was also studied in cell lines (Nauli et al., 2003).

**The TRPP2-TRPV4 complex functions as a thermosensor in vivo**

Given the lack of epistasis between TRPP2 and TRPV4 in the zebrafish pronephros model, we chose another approach to investigate whether TRPP2 and TRPV4 form a sensory complex in vivo. TRPV4 is activated by warm temperature in addition to osmotic stress (Guler et al., 2002; Watanabe et al., 2002). TRPV4-deficient mice exhibit reduced responses to noxious stimuli and inflammation-induced thermal hyperalgesia (Liedtke and Friedman, 2003; Mizuno et al., 2003; Suzuki et al., 2003; Todaka et al., 2004), as well as a defective avoidance behavior at temperatures between 45 and 46°C, but a normal thermosensation profile at temperatures ≥47°C (Lee et al., 2005). To test whether TRPV4 and TRPP2 form a complex that is activated by warm temperatures, we expressed both proteins in *X. laevis* oocytes. The activation of TRPV4 by warm temperatures (39°C) was doubled in the presence of TRPP2 (Fig. 5, A–D), which is consistent with the hypothesis that the TRPV4–TRPP2 channel complex exerts a thermosensory function. Using a tail immersion assay, we detected that TRPP2+/− mice display a thermosensation profile that closely resembles the abnormalities described for TRPV4+/− mice (Fig. 5, E and F). Latencies in the tail immersion assay were significantly increased at 44°C and 46°C in TRPP2+/− mice (Fig. 5 E) but were comparable to wild-type mice at higher temperatures (Fig. 5 F). No differences were detected between wild-type and TRPV4+/− mice; however, the additional loss of one TRPP2 allele (transheterozygous animals) drastically augmented the thermosensory defect at 44°C and 46°C, exceeding the latencies for either TRPV4+/− or TRPP2+/− mice (Fig. 5, E and F). These findings provide clear genetic evidence that TRPV4 and TRPP2 collectively mediate thermosensation at moderately warm temperatures in the mouse. Our findings demonstrate that TRPP2 and TRPV4 jointly mediate thermosensation at moderate temperatures. Recent observations strongly support the concept that basal body and ciliary proteins play a role in temperature sensation, providing a link between the subcellular localization and function of the ion channels studied here (Tan et al., 2007).

Although many TRP channels form heteromers, the physiological relevance of most interactions is poorly understood. Using progressively complex model systems, we demonstrate that coupling between two TRP channels, TRPP2 and TRPV4, creates a polymodal sensory channel complex, both in vitro and in vivo. Because TRPV4 is expressed in nearly all tissues, our results point toward a mechanism by which TRPP2 promiscuously exploits other TRP channel members to exert tissue-specific functions. This mechanism is also used by *C. elegans*, where OSM-9 provides the multifunctional TRP channel subunit that mediates responses to different sensory stimuli. It is tempting to speculate that TRPP2 utilizes TRPV4 to mediate mechano- and osmosensory signals required for TRPP2 functions such as systemic osmotic and blood pressure regulation that are impaired in polycystic kidney disease before the onset of renal failure. This hypothesis is supported by the recent finding that TRPV4 may also play a role in shear stress detection besides its role as an osmosensor (Wu et al., 2007). Although both channels localize to the cilium of tubular epithelial cells and are required

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**Data Analysis**

- **Statistical Analysis:**
  - Analysis of the relative Ca²⁺ conductance revealed that TRPP2 significantly increased the Ca²⁺ currents (normalized group data, GCa⁺/Gionp from E; n = 10, 10, 11, and 7, respectively). Whole-cell currents of oocytes expressing TRPV4 with or without TRPP2 were inhibited by RR with similar potency (see Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200805124/DC1).
  - Analysis of the surface expression of TRPP2 and TRPV4 (n = 4) using an enzyme-linked assay for detection of both channels at the plasma membrane. Asterisks indicate significant differences between black and grey bars, respectively. (H) Representative Western blot of total protein amount of a representative experiment. (I) Summarized data of total protein amounts (n = 4). Error bars represent mean ± SEM.
for flow-induced Ca\textsuperscript{2+} signals, the lack of renal cysts in TRPV4-deficient animals indicates that activation of the ciliary polycystin-1–TRPP2 complex by a mechanism other than flow-induced deflection of the cilium might be critical for the regulation of tubular morphology.

Materials and methods

Materials

TRPP2 constructs have been described previously (Arnould et al., 1999; Köttgen et al., 2005). The plasmid for mouse TRPV4 was provided by V. Flockerzi (University of Saarland, Saarbrücken, Germany). The carboxy-terminal TRPV4-GFP fusion was a gift of W. Liedtke (Duke University, Durham, NC). The plasmids psGEM-TRPP2 and psGEM-mTRPV4 for cRNA synthesis and the TRPP2 and TRPV4 constructs fused to fluorescent proteins and epitope tags were generated using standard cloning techniques. The TRPV4-V5 loop with a V5 tag engineered into the first extracellular loop for surface detection has been described previously (Wegierski et al., 2006). Similarly, a V5 tag was engineered into the second extracellular loop of human TRPP2 (TRPP2-V5 loop; in the cdm8 vector). For electrophysiology, Ca\textsuperscript{2+} imaging experiments and expression in zebrafish untagged TRPP2 and TRPV4 constructs were used. All other chemicals were obtained from Sigma-Aldrich. Water-insoluble drugs were dissolved in dimethyl sulfoxide or ethanol (final solvent concentration was \textless 0.1\%).

Figure 3. **TRPV4 is required for flow-mediated Ca\textsuperscript{2+} signals in renal epithelial cells.** (A) HEK 293 cells overexpressing V5-tagged canine TRPV4 were transiently transfected with empty vector (none), an ineffective siRNA construct (control), or two different effective siRNA constructs. TRPV4 expression levels were monitored by Western blotting against V5 (with tubulin as a loading control). (B and C) MDCK cells stably expressing tetracycline-inducible TRPV4 siRNA. siRNA expression in the absence [B] and presence of tetracycline [C] can be monitored by concomitant expression of GFP. (D) Flow-mediated Ca\textsuperscript{2+} signaling was studied in ciliated MDCK cells using Fura-2. The time course of cytosolic Ca\textsuperscript{2+} increase in response to laminar flow (ciliary bending) is shown in representative Fura-2 pseudocolor images reflecting the Ca\textsuperscript{2+} concentration in MDCK cells (340/380 nm fluorescence ratio; blue/green: low [Ca\textsuperscript{2+}]; yellow/red: high [Ca\textsuperscript{2+}]; time is indicated in mins). (E) Flow-induced Ca\textsuperscript{2+} signals in cells expressing TRPV4 siRNA. Although the flow-induced response was abolished upon knockdown of TRPV4, 10 μM ATP still elicited a robust Ca\textsuperscript{2+} increase. (F) Single-cell analysis of the Ca\textsuperscript{2+} signals (ratio 340/380 nm) of the same experiment as shown in D (each trace represents an individual cell and the bold line depicts the mean; for better visibility, only 50% of cells in the visual field are represented). (G) Effect of TRPV4 siRNA on flow-induced Ca\textsuperscript{2+} signals. Single-cell analysis of the same experiment as shown in E; again, 50% of cells in the visual field are represented. (H) Grouped data from three independent experiments (n = 3; –tet: 75 cells per n; +tet: 93 cells per n). All cells in the visual field were included to calculate the flow-induced Ca\textsuperscript{2+} peak baseline. *, statistical significance. Error bars represent mean values ± SEM. Bars, 10 μm.
MDCK or HEK 293 cells were grown at 37 °C in DME supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected with FuGene 6 (Roche) and calcium phosphate, and experiments were performed 2–5 d after transfection. Transgene expression was monitored by fluorescence detection and Western blot analysis.

RNA interference
Canine TRPV4 was cloned from MDCK cDNA into the HindIII and NotI sites of pCDNA6.V5 and sequenced (GenBank/EMBL/DDBJ accession no. EF561643). The efficacy of TRPV4 siRNA sequences was tested by coexpressing different siRNA-expressing pSuper plasmids with cTRPV4.V5 in HEK 293T cells and testing the amount of cTRPV4.V5 protein by Western blotting. Two siRNAs showing consistent high level suppression of TRPV4 were cloned into pLVTH, which in addition to the H1 promoter – driven GFP reporter (Wiznerowicz and Trono, 2003). Lentivirus produced with the pLVTH clones was used to transduce MDCK cells expressing the KRAB Tet repressor, which represses both expression of the whole seventh coding exon (93 bp) and therefore the loss of most of the second transmembrane domain. Higher trpv4MO doses led to substantial increase of lethality in the injected embryos. To examine an interaction between the two proteins with respect to cyst formation, the embryos were injected with both morpholinos. (D) With two morpholinos, pkd2MO and trpv4MO, the cojected embry (55 hpf) shows a pronephric cyst (arrow). For the cojection, the dose of the pkd2MO was titrated to levels at which the occurrence of cysts is very low, and trpv4MO was added in a medium dose with low lethality. The additive effect showed no significant increase in incidence of cysts, as is shown in the bar graph (F).
Images were recorded with 600–800-ms exposure time at acquisition rates of 2–20/min. Excitation was alternated between 340 nm and 380 nm, and emission was collected by using a filter combination DC430 and BP480-520 (AHF Analysentechnik AG). Experiments were recorded and analyzed with the Metafl uor software (MDS Analytical Technologies).

For statistical analysis, ratio values for all the cells (66–104) in one field of view were measured, and the mean was referred to as one independent experiment.

Zeiss, Inc.). Cells were incubated with 5 μM Fura-2 AM in a solution containing 1 mM probenecide for 20–40 min at 37°C. The presence of cilia was demonstrated by differential interference contrast microscopy (Kotsis et al., 2007). The inverted microscope was equipped with a fast-switching monochromator (Cairn Research Limited), a cooled charge-coupled device camera (Coolsnap HQ; Photometrics), and fast shutters in the excitation and transmitted light beam path. A 63×/1.2W C-Apochromat lens (Carl Zeiss, Inc.) was used for image acquisition.

Figure 5. TRPP2 and TRPV4 form a thermosensory complex in vitro and in vivo. (A) Analysis of TRP channel whole-cell currents under voltage clamp (V_{c}) conditions. Currents were recorded in X. laevis oocytes injected with cRNA encoding TRPP2 and/or TRPV4. Representative inward currents at 20°C or 39°C are shown. (B) Summary of data acquired in A. Asterisks indicate significant differences in the temperature-activated whole cell conductance (ΔG) compared with water-injected control oocytes; §, significant differences between data as indicated (n = 4, 4, 4, and 6, respectively). (C) Current–voltage (I–V) relations for oocytes expressing TRPV4 or TRPV4 and TRPP2 (D; gray: 20°C; black: 39°C). (E) Tail withdrawal latencies after immersion into a water bath at moderately hot temperatures were measured in mice of the indicated genotypes (n = 10 per genotype; asterisk indicates significant difference compared with wild-type [WT] and TRPV4+/− mice; §, significant difference from TRPP2+/− mice). (F) Tail withdrawal latencies at noxiously hot temperatures (n = 10 per genotype). Error bars represent mean values ± SEM.
Electrophysiology

Two-electrode voltage clamp experiments were performed as described previously (Köttgen et al., 2005). In brief, oocytes were isolated by partial ovariectomy from X. laevis frogs. cRNAs were synthesized in vitro using the mMessage mMachine kit (Ambion). Stage V and VI oocytes were injected with 30 nl of water containing 10 ng of TRPV2 and/or TRPV4 cRNA, or water as a control. Voltage clamp experiments were performed 2–5 d after injection of cRNAs. Whole-cell currents of oocytes were recorded using the Turbo TEC OX3 amplifier (NPI Electronic GmbH). All whole-cell voltage clamp experiments were performed at 20°C in ND96 frog Ringer (control) solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM Hepes; in the hypotonic solution, the NaCl concentration was reduced by 30%). Oocytes were clamped at 0 mV, and voltage ramps from −100 to +50 mV were applied every 1.2 s. Alternatively, voltage steps from 0 mV to −100 mV were applied as indicated. For temperature activation experiments, oocytes were superfused with ND96 at 39°C (control 20°C). Temperature was monitored by a thermometer in the bath chamber. Whole-cell conductance was determined in the voltage range from −100 to 0 mV. Data are presented as original recordings or as mean values ± SEM (n = number of experiments).

ELISA for surface detection

HEK 293T cells transfected by the calcium phosphate method were split on the following day into poly-lysine–coated 48-well dishes. 2 d after transfection, the cells were fixed with 3.7% paraformaldehyde and blocked with 2% horse serum in PBS. The plasma membranes were labeled with mouse anti-V5 (2 μg/ml) and anti–mouse alkaline phosphatase (AP)-coupled antibodies. After three washes with PBS, the enzymatic reaction was performed in 0.1 M glycine, 1 mM MgCl₂, and 1 mM ZnCl₂, pH 10.4, using 1 mg/ml pNitrophenyl phosphate as substrate. The absorbance was read at 405 nm. For each experiment, all conditions were tested in triplicate. The mean absorbance obtained for mock-transfected cells was subtracted as background. In contrast to the TRPV4-V5 loop protein, expression of which stimulated efficient conversion of the AP substrate, the enzymatic reaction on cells expressing the HEK 293T cells was spectrally unmixed into the CFP- and YFP-related components.

Western blotting

Western blot analysis and coimmunoprecipitations were performed as described previously (Arnould et al., 1999; Köttgen et al., 2005). In brief, 24 h after transfection, cells were lysed in 1 ml/10 cm dish of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM NaPO₄, 2 mM NaVO₃, and protease inhibitors) for 15 min on ice. Lysates were centrifuged at 4°C for 15 min at 14,000 rpm. Supernatants were used for Western blotting and coimmunoprecipitation studies. Representative results of at least three independent experiments are shown.

Zebrafish experiments

Morpholino antisense oligonucleotides were designed to target an exon splice donor site causing splicing defects of the mRNA. The MOs were obtained from Gene Tools, LLC. The following morpholinos were used: pkd2MO (5'-TAACTCTCCGAGAAGTCTCATG-3'; Obara et al., 2006) targeting the splice donor of the third exon coding for part of the first transmembrane domain and part of the first extracellular loop, and trpv4MO (5'-GTACAAAAAAGAAGTCTCAGAC-3') targeting the splice donor of the seventh exon coding for the second transmembrane domain. The morpholinos for single and double injections were diluted in 100 mmol/liter KCl, 10 mmol/liter Hepes, and 0.1% Phenol red (Sigma-Aldrich).

The injections were performed using a microprocessor-controlled nanoliter injector (Nanoliter 2000; World Precision Instruments, Inc.) under stereo-microscopic control (MZ16; Leica).

The effect of the trpv4 splice morpholin was verified by RT-PCR from single embryo total RNA with nested primers in flanking exons.

Embryos were fixed in BT-Fix at 4°C overnight (Westefelder, 2000). After being washed in PBS and taken through an ethanol dehydration series, they were embedded in Technovit 7100 resin (Heraeus Kulzer) and sectioned (2 μm) in methylene blue/azure II (Humphrey and Pittman, 1974), mounted, and examined using an Axiosvert microscope (Carl Zeiss, Inc.).

Total RNA from single embryos was obtained by using an RNAeasy kit (QIAGEN). The zebrafish trpv4 sequence was derived primarily by TBLASTN searches (Sanger Institute, Cambridge, UK). Part of the coding sequence was obtained by reverse transcription and nested PCR of wild-type total RNA (outer primer set: forward, 5'-TTCAGCAGGGTTTTCCTCTC-3'; reverse, 5'-TCTCCCTCCATCAGCAGTAGTTAACA-3'; nested primer set: forward, 5'-ATCGTCTGGCCATGACAGAGTCCT-3'; reverse, 5'-GAATAGAGAAAGACGCGGCAGATACG-3') and subcloned into pCRII using TA cloning (Invitrogen). The morpholino effect was verified by RT-PCR using the following set of primers: outer primer pair forward (5'-TGGGCCTATGGCACGAG-3') and reverse (5'-GGCCCTAATGCGTACGAGACG-3').

Tail immersion assay in mice

TRPV4 knockout mice (Suzuki et al., 2003) were backcrossed five generations onto a C57BL/6 background. TRPV2 conditional knockout mice were generated by homologous recombination using standard techniques. In brief, a loxP site was inserted in intron 10 of Pkd2 and a loxP-FRT-PGKneo-FRT cassette in intron 13 (Pkd2lox10/13lox13). After removal of Pgkneo, Cre-mediated recombination (B6. 129S4-Mexlox1010/th; Jackson Laboratory) between the loxP sites was verified in exons of the carboxy-terminal tail of the protein (Pkd2lox10/13lox13). Animals of different genotypes were obtained by mating of TRPP2+/− animals with TRPV4−/− or TRPV4+/− animals, and genotypes were determined by PCR. Age-matched male mice 11–17 wk of age were individually housed at 11 °C before behavioral assays. Ambient temperature was maintained at 25 ± 1°C, with a light cycle from 8:00 a.m. to 8:00 p.m. All behavioral assays were performed between 12 p.m. and 8 p.m. A single cohort of animals was used in all experiments. All experiments were conducted according to protocols approved by The Johns Hopkins Animal Care and Use Committee.

The tail immersion assays were performed as described previously (Lee et al., 2005). After a 30-min acclimation in a Plexiglas box, the tail of a gently restrained mouse was immersed in a water bath set at a single temperature from 44–52°C, and the time to tail flick was recorded. This assay was performed with the investigator blinded to genotype. Latencies at a given temperature were recorded during three trials separated by at least 1 d and averaged. Mice were allowed to rest at least 1 d between temperatures. The cutoff time was 300 s, after which the tail was removed from the bath regardless of response.

Statistical analysis

Data are presented as original recordings or as mean values ± SEM (n = number of experiments). Unpaired and paired t tests as applicable were used for statistical analysis. A p-value of <0.05 was accepted to indicate statistical significance. For zebrafish experiments, a stratified χ² test (Cochran-Mantel-Haenszel test) was used, and a p-value of <0.05 was accepted to indicate statistical significance.
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Online supplemental material

Fig. S1 shows subcellular localization of native and overexpressed TRPV4 in polarized MDCK cells and FRET negative controls. Fig. S2 shows that flow-mediated Ca2+ signals are inhibited by RR and are not affected in cells expressing a two-base mismatch TRPV4 siRNA. Fig. S3 shows the functional interaction of TRPP2 and TRPV4 in HEK 293 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200805124/DC1.

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