Impaired Pressure Sensation in Mice Lacking TRPV4*

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The sensation of pressure, mechanosensation, in vertebrates remains poorly understood on the molecular level. The ion channel TRPV4 is in the TRP family and is a candidate for a mechanosensitive calcium-permeable channel. It is located in dorsal root ganglia. In the present study, we show that disrupting the Trpv4 gene in mice markedly reduced the sensitivity of the tail to pressure and acidic nociception. The threshold tonoxious stimuli and the conduction velocity of myelinated nerve responding to stimuli were also impaired. Activation of unmyelinated nerve was undetected. However, the mouse still retained olfaction, taste sensation, and heat avoidance. The TRPV4 channel expressed in vitro in Chinese hamster ovary cells was opened by low pH, citrate, and inflation but not by heat or capsaicin. These data identify the TRPV4 channel as essential for the normal detection of pressure and as a receptor of the high-threshold mechanosensory complex.

Stimulation of sensory receptors by pressure due to a weight affects a variety of sensations, including touch, pressure, and pain. The molecular mechanism underlying both touch and pain has been investigated by studies on mice lacking a mechanosensitive channel (1) or a nociceptor (2). A mouse lacking Trpv1 does not respond to stimulation with capsaicin; heat and acid induce nociception, but the knock-out shows response to pressure sensation (2). The ion channel that converts pressure or anisosmolarity into electrical stimuli remains unknown. A clue to its identity came from the discovery of Osm-9 in a genetic screen of high-osmolality-insensitive Caenorhabditis elegans mutants (3). The structure of Osm-9 is quite similar to the vanilloid-receptive channel, VR1 (TRPV1) (4), as well as to TRPV4 (SAC1 (5), OTRPC4 (6), VR-OAC (7), VR12 (8,9)), and TRPV2 (10). TRPV1 is a member of the TRP family; it has six transmembrane segments and ankyrin-like repeats and is responsive to the physical factor of heat. Assuming that the family operates through a mechanically gated channel, we have cloned the incomplete form of Trpv4 (a PCR-based chimera of TRPV1 and TRPV4) as a stretch-inhibitable channel (11). However, we did not confirm that TRPV4 was a mechanosensitive channel at that point. TRPV4 has been reported as a swell-activated but not inflation-activated channel (6). Further it has also been reported to be activated by heat (12).

To investigate the role of TRPV4 in mechanosensation, we disrupted the mouse Trpv4 gene and then examined its role in the sensation of pressure. We reproduced affluent stimuli in vitro.

MATERIALS AND METHODS

Cloning of cDNA and Detection—Homologous cDNA of Trpv4 was screened by the BLAST program on the EST (expressed sequence tag) data base, resulting in two independent clones, Trpv2 and Trpv4 (GenBank™ accession number AB021875). The cloned cDNA was ligated to mammalian expression vector pCMV-SPORT (Invitrogen). A plasmid expressing enhanced green fluorescence protein (pEGFP-N1, BD Biosciences) or red fluorescence protein (pDsRed1-N1, BD Biosciences) was used as a marker for transfection into cells, which was performed using FuGENE™ (Boehringer Ingelheim GmbH). The functional experiments were performed 48 h after the transfection.

Reverse polymerase followed by polymerase chain reaction (RT-PCR)† was performed according to the manufacturer’s protocol (RNA PCR, Takara, Osaka, Japan) with 0.3 μg of RNA as a template. The amplification conditions consisted of incubation at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for a total of 29 cycles. Antibody was raised against C-terminal peptide (CDGHQQGYAPK) in a solution using keyhole limpet hemocyanin as a conjugate. The antigen of 1 mg/ml DW with 1 ml of Freund’s adjuvant was injected intramuscularly into a New Zealand White rabbit followed by a bi-weekly booster injection of the same dose of the antigen in Freund’s incomplete adjuvant. The titer of the serum used was more than 10,000 times higher than the control. To obtain polyclonal anti-TRPV4 antibodies, the IgG fraction was purified with a protein G column (HiTrap, Amersham Biosciences) and affinity-purified with a kit (Prot On™, MPS, San Diego). Western blot analysis with a blocking test by excess of antigen was performed to evaluate the specificity. Histologic staining was performed and detected by fluorescein isothiocyanate-labeled anti-rabbit IgG (Dako, Kyoto, Japan).

Construction of Targeting Vector and Generation of Trpv4 Mutant Mice—We disrupted the Trpv4 gene by homologous recombination using the PKG-neo cassette and standard methods (13). Murine genomic clones were obtained from a 129/SV mouse bacterial artificial chromosome (Genome Systems, St. Louis, MO) using Trpv4 cDNA as a probe. The targeting vector contained a 2.2-kb (short) and a 3.7-kb (long) arm of homology flanking a PKG-neo cassette. The vector was electroporated into RW4 embryonic stem cells (Genome Systems), which were selected in G418 (Invitrogen). The resulting chimeras were bred to C57BL/6 females. Mice heterozygous for the Trpv4 mutation were mated to C57BL/6 to the fourth generation. Sex-matched wild-type littermates (Trpv4+/−) were used as controls.

Electrophysiology—Patch clamp recordings were carried out according to methods described previously (8, 11). Currents were recorded at room temperature with an EPC-9 patch clamp amplifier (HEKA, Pflaz, Germany). Current was normalized by capacitance of the individual cell. The bath solution contained 140 mM NaCl, 1.0 mM MgCl2, and 3 mM HEPES. The whole-cell patch pipette contained a filtered solution of

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‡ The abbreviations used are: RT-PCR, reverse transcription followed by PCR; CHO, Chinese hamster ovary; DRG, dorsal root ganglia; RR, rapid response with rapid inactivation.
TRPV4 Is Distributed in Various Sensory Organs—According to Northern blot analysis, TRPV4 is rich in the kidney and lung, and precise detection by antibody or RT-PCR reveals its presence in various other organs than kidney and lung (7, 9). TRPV4 protein was analyzed histologically using Western blot analysis with antibody raised against the C terminus. An excess of antigen supported the specificity of this affinity-purified antibody (Fig. 1, upper left). Using the antibody, neuronal tissues were histologically stained. In the sensory system, neurons in the trigeminal nerve, cauda equina, skin nerve (9), and cochlear hair cells (7) were positively stained. The internal elastic membrane but not the taste bud of the tongue was positively stained (Fig. 1).

Disruption of the Trpv4 Gene—We made a knock-out mouse by a conventional method. We inserted a neo cassette into the fourth exon encoding the ankyrin-repeat domain (Fig. 2A). Southern blot revealed that the neo cassette was successfully inserted into the BglII fragment (Fig. 2B). Using RT-PCR with primers downstream from the targeted region and Northern blot from lung, data not shown), we failed to detect transcripts in the trigeminal root and dorsal root ganglion (DRG) of Trpv4−/− animals (Fig. 2C). Histologically, DRG stains positively but did not do so in the Trpv4−/− mice (Fig. 2D). Therefore, TRPV4 expression was successfully disrupted in the Trpv4−/− mice.

Impaired Pressure and Acid Sensation in Trpv4−/− Mice—Trpv4−/−, heterozygous (+/−), and wild-type (+/+ ) mice were produced in the expected 1:2:1 Mendelian ratio. Trpv4−/− mice had normal appearance, growth, size, temperature, and fertility and showed no obvious behavioral abnormalities. Trpv4−/− and Trpv4+/+ mice avoided the odors induced by acetate, ammonium, benzene, ether, alcohol, pyrazine, and diacetyl. Dietary behavior was determined from the amount of jelly consumed. Over a 2-day period, mice were allowed to eat jelly containing 10% sucrose for 3 h/day. Then we changed the jelly by substituting other tastes. The taste sensation toward sweet (sucrose 10%, 1.2 ± 0.22 g/h), bitter (denatonium benzoate 1 mm, 1.1 ± 0.25 g/h), hot (capsaicin 0.1 mm, 0.01 ± 0.0 g/h), and sour (citrate 10%, 0.6 ± 0.23 g/h) tastes did not differ between Trpv4−/− and +/− mice (n = 8). Serum concentrations of sodium, potassium, and calcium were not different, and other abnormalities suggesting renal or hepatic failure were not found (data not shown).

Trpv4−/− mice showed a significantly reduced response to harmful stimuli caused by pressure on the tail and reduced their frequency of writhing for 10 min after application of acetic acid to the abdomen (0.7%, 100 mm Hg). The threshold was measured in triplicate for a single nerve resulting in 120 mm Hg in 16 mice, p < 0.01). The needle was connected to a pulse generator to measure the conduction velocity of the nerve under pressure. Mean conductance of these nerves was 15 and 14 m/s, respectively.
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A slow response (SR) nerve was defined as a nerve that responds slowly to the applied pressure. The nerve began to be activated at a threshold pressure. This pressure was 92 ± 15 mm Hg in Trpv4+/+ and 210 ± 40 mm Hg in Trpv4−/− (n = 8 mice, p < 0.01). The frequency of discharge over a 1-min span was markedly reduced in Trpv4−/− mice (Fig. 4, A and C). Pressure-response relationship, defined as inactivation by reduction of the pressure, occurred similarly in both animals within 1 min. The mean conductance of these nerves was similar to that of RR, 14 and 16 m/s, respectively.

To detect C-fiber, we recorded RR and then measured the conducted pressure to the tail (n = 13) of Trpv4+/+ (open column) and Trpv4−/− (closed column) mice. Lower panels, frequency of writhing behavior after injection of 0.7% acetate into the abdomen was counted over a 10-min span (n = 8). Response latency in the hot plate test and threshold of touch were measured (n = 10) (lower panel). **, p < 0.01, t test.

**FIG. 3.** Response to pressure, acid, heat, and touch in Trpv4+/+ and Trpv4−/− mice. Upper panels, response threshold and latency of avoidance to persistent pressure applied to the tail (n = 13) of Trpv4+/+ (open column) and Trpv4−/− (closed column) mice. Lower panels, frequency of writhing behavior after injection of 0.7% acetate into the abdomen was counted over a 10-min span (n = 8). Response latency in the hot plate test and threshold of touch were measured (n = 10) (lower panel). **, p < 0.01, t test.

**FIG. 4.** Response of neuronal discharges to harmful pressure. A, traces of spikes during a 15-s span in Trpv4−/− (left) and Trpv4+/+ (right) mice. RR (upper panel) is observed on the pressure pattern described below. Slow response with slow inactivation (SR) (lower panel) is observed on the pressure below. The arrow indicates activation of a single fiber responsive to pressure. B, threshold of RR to pressure through a 23-gauge needle is illustrated (n = 16; **, p < 0.01) in Trpv4+/+ and Trpv4−/−. C, frequency of discharge of SR during 1 min is counted in Trpv4+/+ and Trpv4−/− (***, p < 0.01).

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To detect C-fiber, we recorded RR and then measured the conducted pressure, because RR was detected frequently. In 300 pricks, six sites revealed slower (<10 m/s) conductance in Trpv4+/+ but none in Trpv4−/− mice. This difference was significant, using chi square analysis (Fisher).

Finally we touched the foot by von Fray hair (<1 g/mm²) and recorded the nerve activity. Although the conduction was not measured, this procedure evoked a continuous discharge with similar frequency in Trpv4+/+ (n = 12 sites) and Trpv4−/− mice (n = 14 sites).

Electrophysiologic Analysis of TRPV4 Current in Vitro—We evaluated possible stimuli to the ion channel expressed in Chinese hamster ovary (CHO) cells by patch clamping. We then estimated the candidate stimuli of taste and heat by their response to intracellular calcium concentration [Ca²⁺]i, with fluo-3 (data not shown). The following reagents or stimuli inducing a sour, bitter, or hot sensation were tested: 4-aminopyridine, quinidine, denatonium benzoxide, capsaicin, heat at 50 °C, acetate, propionate, lactate, pyruvate, and citrate. Comparable with the structurally similar TRPV2, TRPV4 responded significantly to low pH and citrate (Fig. 5, A–C). Among the organic acids in neutral pH, citrate was a specific activator of the TRPV4 channel, which suggested its involvement in the mechanism of sour detection. The current was activated dose-dependently by H⁺ and citrate (Fig. 5D). The range of pH that activates TRPV4 is similar to that of TRPV1 (15) but lower than the acid-sensing ion channel (16).

In the same way as seen in an earlier study (7), mechanical stresses by hypo-osmolarity induced little TRPV4, and heat failed to activate it. Inflation of cell volume approaching a factor of 1.5 was done by positive pressure through a patch pipette. This inflation significantly increased the evoked current as compared with the basal current (Fig. 5C, *), as well as that of TRPV4 (p < 0.05) compared with the evoked current (Fig. 5C, #). Response to heat (50 °C) was determined by [Ca²⁺]i, which rose in TRPV2 but not in TRPV4 (data not shown). The current encoded by TRPV4 was outwardly rectified, selective for cation (Cs:Na:NMDG:Ca:Cl = 1:1:1.0:0.01:9:0.01) (8), and abolished by 0.1 μM GdCl₃ (Fig. 5B).

**DISCUSSION**

Mice Lacking Trpv4 Showed an Abnormal Sensory Phenotype—Because of the small but significant localization of TRPV4 in the sensory system, we examined various sensations in Trpv4−/− mice. Because TRPV4 is similar to Osm-9 in structure, we examined odor first. An Osm-9 mutant does not avoid pyrazine and diacetyl, but Trpv4−/− did avoid them. Next, we examined taste sensation. Mice lacking Trpv4 do not avoid capsaicin in tap water, whereas Trpv4−/− mice avoided it. Although TRPV4 was detected in the trigeminal ganglion and tongue and it was opened by citrate, citrate did not affect the sour sensation in vitro. Most previous studies have substantiated that the that is channel sensitive to sour is the epithelial sodium channel (17) in taste buds. TRPV1 is expressed in taste buds, sensing capsaicin directly, but TRPV4 was detected in the elastic membrane of the tongue and not in the taste buds, suggesting other mechanical roles in eating behavior.

TRPV4 is known to be sensitive to temperature in *in vitro* HEK293 cells (12). However, chicken but not rodent TRPV4 in CHO cells is sensitive to temperature (7). In both CHO and HEK293 cells, TRPV4 is activated at 34–40 °C but is not activated over 40 °C *in vitro*. This observation is compatible with the present data. TRPV4 detects a physiological range of temperatures rather than noxious heat. TRPV4 is not a nociceptor to heat but may play a role in thermoregulation during energy production, for example.

Writhing is a characteristic response to acidic noiception in a rodent. Like TRPV1, TRPV4 is a kind of nociceptor. Acetic acid administration into the abdominal cavity induces a noxious pain; the pH that opened the channel *in vitro* was lower than the physiologic level. Such low pH damages tissues. TRPV1 is sensitive to this range of pH and plays a role in inflammatory hyperalgesia (18). Thus, TRPV4 may play the
same role in hyperalgesia, whereas the dorsal root acid-sensing channel detects the physiological range of the acidic irritant (19, 20). Touch by von Fray hair is a well known method to examine the sensation of innocuous stress. In BNC1 (brain sodium channel 1) knock-out mice, avoidance of heat is impaired, but harmful pressure can still be detected (1). In Trpv1 knock-out mice, avoidance of heat is impaired, but harmful pressure can still be detected (2). Therefore, we considered that TRPV4 might play its own role in detection of high-threshold mechanosensation or pressure. The hot plate test, tail pressure, and von Fray touch are convenient tests that are easily reproduced. Taken together, impaired acid and pressure but intact hot and touch sensation was observed in Trpv4−/− mice, which was expected from the in vitro electrophysiologic study.

Detection of Functional Pressure-sensing Nerves—We found the presence of TRPV4 in DRG neurons histologically by fluorescence. We also detected it using hematoxylin to visualize the cell body. Less than 10% of the neurons contained TRPV4. Most of the TRPV4-positive cells (38 of 50 cells counted) were less than 20 μm in diameter. The results are matched with the previous findings with in situ hybridization (21). Smaller cells spread nonmyelinated axons corresponding to C-fiber, which is believed to be nociceptive. Therefore, we expected that the impaired axon in Trpv4−/− would be C-fiber. Frequency of detection of the mechanosensitive C-fiber by the present method is rare (about 1 in 50 measurements) (14). However, no C-fiber responding to pressure was detected in 300 measurements in Trpv4−/−, suggesting that pressure-sensitive C-fiber was actually absent in Trpv4−/− mice. This result should be clarified in the future by another method.

The impaired pressure-sensing fibers in Trpv4−/− were myelinated fibers according to their conductance. Burgess and Perl (22) discovered a substantial population of high-threshold mechanosensory sensory units in myelinated afferent fibers. The impaired conduction velocity was around 15 m/s, corresponding to Aβ-fiber or Aγ deflection. The high-threshold mechanosensation is transmitted through Aβ-fiber, innervated mostly to Ruffini endings, or through Aγ with nonspecialized endings (14, 19). TRPV4 is located in skin nerve endings as well as in mechanosensitive small bodies (9). Therefore, pressure-sensing TRPV4 may be located primarily in these endings, transmitting the harmful input through myelinated fibers.

Electrophysiological Characterization in Vitro in CHO Cells—TRPV4 is known as a swell-activated but not an inflation-activated channel (6). We expressed TRPV4 in CHO cells but failed to find significant activation by hypo-osmolarity. We could recognize an infrequent swell-activated current in TRPV4-expressed CHO. The level of the activated current was 400 pA in hypotonic solution, which is consistent with the results of our previous work (7). Thus a lack of significance in our expression may be due to a statistical reason. In contrast, a recent study showed that TRPV4 was expressed as an osmosensing channel when transfected in HEK293 cells (6). We thought that TRPV4 expressed differently in different cells and examined the current again. In the setting of 150 mM NaCl bath solution and 150 mM CsCl in the pipette fill, TRPV4

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FIG. 5. TRPV4 current in CHO cells. A, mean currents are plotted to voltage in CHO cells transiently expressing TRPV4 before (open circles) and after (closed circles) inflation (n = 4). Bath pH was altered from 7.5 to 4.0 (n = 4). B, representative whole-cell currents are shown during 100-ms voltage steps from −100 to +90 mV by 10-mV increment in the given bath pH and in GdCl3. Inflation of the volume was performed through pipette by +50 mm Hg in control CHO cells and TRPV4. C, current density was calculated as the magnitude of the current at +90 mV/cell capacitance. Mean ± S.E. values of four untransfected controls (TRPV2 or TRPV4) are illustrated: *p < 0.05, compared with the control current; #p < 0.05 compared with the experimental control with reagents (10−5 M 4-aminopyridine, 10−5 M quinidine, 10−5 M capsaicin, and 1 mM organic acids in pH 7.4) or with manipulation. D, dose-response results with citrate are plotted with S.E. (n = 6). Current density is plotted against bath pH. 

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endowed a larger current compared with the control in HEK293 cells, which was activated by hypo-osmolarity and decreased by hyperosmolarity (data not shown). Strotmann et al. (6) explicitly indicate that TRPV4 is not activated by inflation of the HEK cell. However, elasticity of the cell membrane is different in individual cells, and response of the cytoskeleton to hypo-osmolarity may be dependent on the enrichment of water channel and elasticity. Thus inflation of the cell can be a stimulus to open TRPV4 when it is expressed in other cell types.

The vanilloid receptor, TRPV1, is a channel that is sensitive to acid, capsaicin, and heat, where heat activation is the least sensitive stimulus in heterogeneously expressed cells using measurement of the currents (15). Likewise, the TRPV4 channel is sensitive to acid, citrate, and swelling, where swelling is the least sensitive stimulus in heterogeneously expressed cells using measurement of the currents (15). Likewise, the TRPV4 channel is sensitive to acid, citrate, and swelling, where swelling is the least sensitive stimulus. In conclusion, TRPV4 detected a nociceptive level of mechanical stimuli, which was substantiated in vivo in the sensory system of Trpv4−/− mice.

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