Impaired osmotic sensation in mice lacking TRPV4

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Mizuno, Atsuko, Naoko Matsumoto, Masashi Imai, and Makoto Suzuki. Impaired osmotic sensation in mice lacking TRPV4. Am J Physiol Cell Physiol 285: C96–C101, 2003; 10.1152/ajpcell.00559.2002.—The Ca2+-permeable cation channel TRPV4, which is part of the Trp family located in the circumventricular organs, is activated by cell swelling. To investigate the role of TRPV4 in osmotic sensation, we disrupted the TRPV4 gene in mice and examined the effect on osmotic metabolism. Disruption of the mouse TRPV4 gene did not influence either water intake behavior or serum osmolality. Short-term salt ingestion, however, seemed to impair the transient free water clearance. The level of serum arginine vasopressin (AVP) of TRPV4 gene did not influence either water intake behavior or serum osmolality. Short-term salt ingestion, however, seemed to impair the transient free water clearance. The level of serum arginine vasopressin (AVP) of TRPV4 gene did not significantly change under normal conditions but was significantly increased under stimulated conditions. Incubation of brain slices with graded hyperosmolality suggested an exaggerated response of AVP secretion in TRPV4−/− mice. Thus TRPV4 in the brain may transmit a negative signal to AVP secretion similar to an inhibitory pass through the baroregulatory system. Thus, in the regulation of serum osmolality, TRPV4 is a swell-activated channel that appears to play a role in reversion toward hyposmolality.

Trp; calcium channel; vasopressin; mechanosensitive channel

THE RESPONSE TO OSMOLALITY by vertebrates is poorly understood at the cellular and molecular levels. The ion channel that converts 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 (5). The structure of Osm-9 is similar to that of the vanilloid receptive channel TRPV1 (3) as well as TRPV4 [SAC1 (20), VR1, OTR1C4 (17), VR-OAC (6), TRP12 (26)] and TRPV2 (2). TRPV1 is a member of the Trp family; it has six transmembrane segments and ankyrin-like repeats and is responsive to heat. Assum- ing that the family operates through a mechanically gated channel, we cloned TRPV4’s incomplete form (a chimera of TRPV1 and TRPV4) as a stretch-inhibitable channel (18), but the complete clone of TRPV4 was not directly responsive to the stretch-activated (SA) channel (<30 mmHg) when expressed in Chinese hamster ovary (CHO) cells (20). Recently, TRPV4 was reported to be a swell-activated channel expressed in CHO (6) and human embryonic kidney (HEK) (17) cells. Therefore, TRPV4 may also act as a swell-activated channel in certain cell types.

TRPV4 is abundantly detected in the kidney and lung by Northern blot analysis. When cerebral circumventricular organs are involved, in situ hybridization reveals the localization in detail (6). The localization of TRPV4 in the central nervous system suggests involvement of TRPV4 in regulation of water ingestion or osmolality of the body. To elucidate the osmosensing role of TRPV4, we studied mice lacking this gene and examined its possible functions in the sensation of osmolality.

METHODS

Generation of TRPV4 mutant mice. We disrupted the TRPV4 gene by homologous recombination with the PGK-neo cassette and standard methods (9). Murine genomic clones were obtained from a 129/Sv mouse bacterial artificial chromosome genomic library (Genome Systems, St. Louis, MO) by 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 PGK-neo cassette. The vector was electroporated into RW4 ES cells (Genome Systems), which were selected in G418 (GIBCO-BRL). The resulting chimeras were bred with C57BL/6 females. Mice heterozygous for the TRPV4 mutation (TRPV4+/−) were intercrossed to generate TRPV4-null mice (TRPV4−/−). The genotype was examined by Southern blot or polymerase chain reaction (PCR). Eleven- to nineteen-week-old mice were used in this experiment. Sex-matched wild-type littermates (TRPV4+/+) were used as controls.

Animals. All experiments were performed in accordance with national guidelines for the care and use of research animals. Experiments were performed with mice fed ad libitum and with free access to tap water. Mice were maintained in a balance cage (Sugiyama-Gen, Tokyo, Japan) capable of accurately measuring daily food and water consumption and excretion of urine. Na+ and K+ were measured by flame photometry (IL943; Instrument Lab). Osmolality was measured with an osmometer (One-Ten osmometer; Fiske). All other chemicals were determined with kits designed for colormetric measurements.

Mandatory ingestion of water (20 ml/kg) or 2% NaCl (20 ml/kg) was performed manually through a tube within a period of 5 min. Arginine vasopressin (AVP) was measured from the serum of three mice (500 µl) or the serum of one mouse (100 µl) after intraperitoneal injection of propylene glycol as an osmotic substrate; mice were decapitated and AVP was measured by radioimmunoassay (Mitsubishi Yuka, Tokyo, Japan). For dialysis, the brain was sliced (2 mm thick) obliquely in the frontal cerebrum and hypothalamus. Bath solution contained (in mM) 127 NaCl, 1.5 KCl, 1.24 KH2PO4, 2.4 CaCl2, 10 glucose equili-
brated with 95% O₂-5% CO₂ (pH 7.4, 300 mosmol/kg H₂O). Osmolality was varied by addition of glycerol to 320, 340, and 360 mosmol/kg H₂O. Each solution was perfused for 10 min. The perfusate was collected and freeze-dried (−50°C) as a sample for AVP measurement (12).

Detection of RNA. Total RNA from the renal cortex was isolated by using a column (RNeasy; Qiagen). Reverse transcriptase (RT)-PCR was performed according to the manufacturer’s protocol (RNA-PCR; Takara, Osaka, Japan) with 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, protein extraction, Western blot analysis, and histochemical analysis. Antibody was raised against the COOH-terminal peptide (CDGHQQGYAPK) in a solution using keyhole limpet hemocyanin (KLH) as a conjugate. The antigen of 1 mg/ml of distilled water with 1 ml of Freund’s adjuvant was intramuscularly injected into a New Zealand White rabbit, followed by a biweekly booster injection of the same dose of the antigen in Freund’s incomplete adjuvant. The titer of the serum used was >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 Pharmacia, Tokyo, Japan) and further affinity-purified with a kit (Prot On; MPS). Western blot analysis was performed with a blocking test by excess of antigen was performed to evaluate specificity (19). Renal extracts were made in (in mM) 300 sucrose, 25 imidazole, 1 EGTA, and 5 EDTA with a cocktail of protease inhibitors by homogenization. Histological staining of the brain was performed and detected by fluorescence isothiocyanate-labeled anti-rabbit IgG (Dako, Kyoto, Japan).

Statistics. The data were analyzed with Student’s t-test or one-way analysis of variance (ANOVA), and the significance was calculated with Scheffé’s analysis. P < 0.05 was considered statistically significant.

RESULTS

We inserted a PGK-neo cassette into the fourth exon encoding the ankyrin-repeat domain (Fig. 1). Southern blot detected an insertion of the neo cassette in the BglII fragment in the TRPV4+/− and TRPV4−/− mice but not in the TRPV4+/+ mouse (Fig. 1). Using RT-PCR with primers downstream from the targeted region and Northern blot, we failed to detect transcripts in the kidney of TRPV4−/− animals (Fig. 1). TRPV4+/−, heterozygous (+/−), and wild-type (+/+ ) responses were produced in the expected Mendelian ratio of 1:2:1. TRPV4−/− mice had normal appearance, growth, size, temperature, and fertility and showed no obvious behavioral (including drinking) abnormalities.

Electrolyte concentrations were determined for the normal diet averaged over 1 wk (Table 1). Blood volumes of 0.2–0.8 ml could be sampled from one mouse. No significant alterations in electrolyte concentration were found with blood chemical analysis. The urinary Na⁺ concentration was low in the TRPV4−/− mouse, but the total amount of Na⁺ excreted into the urine did not change significantly. Blood pressure and water balance were not altered between the two groups (Table 2).

Localization of TRPV4 in osmosensing area. TRPV4 protein was detected in the renal cortical extract in TRPV4+/+ but not in TRPV4−/− mice. An excess of antigen was coincubated with the antibody, resulting in no detectable signal by Western blot analysis of TRPV4 transcripts in renal cortical RNA from TRPV4 wild-type (+/+), heterozygous (+/−), and null (−/−) mice are shown at right. Predicted products are 700 bp for the RT-PCR fragment and 3.2 kb for mRNA.

Table 1. Electrolytes in TRPV4+/+ and TRPV4−/− mice

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<th>Cr, mg/dl</th>
<th>Na, meq/l</th>
<th>K, meq/l</th>
<th>Ca, mg/dl</th>
<th>Cl, meq/l</th>
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<td><strong>Plasma (n = 10)</strong></td>
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<tr>
<td>TRPV4+/+</td>
<td>0.10 ± 0.03</td>
<td>137.9 ± 1.0</td>
<td>5.4 ± 0.1</td>
<td>9.8 ± 0.4</td>
<td>115 ± 2.8</td>
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<tr>
<td>TRPV4−/−</td>
<td>0.11 ± 0.02</td>
<td>140.7 ± 0.8</td>
<td>5.4 ± 0.4</td>
<td>8.8 ± 0.3</td>
<td>110 ± 1.8</td>
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<tr>
<td><strong>Urine (n = 10)</strong></td>
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<tr>
<td>TRPV4+/+</td>
<td>36.2 ± 3.8</td>
<td>164.9 ± 20.6</td>
<td>277.5 ± 29.2</td>
<td>20.9 ± 1.9</td>
<td>178 ± 27.3</td>
</tr>
<tr>
<td>TRPV4−/−</td>
<td>32.5 ± 3.2</td>
<td>123.1 ± 14.6</td>
<td>217 ± 27.7</td>
<td>17.1 ± 1.5</td>
<td>136.9 ± 18.6</td>
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Values are means ± SE. Urinary chemical data were determined in triplicate in mice on normal diet and averaged over 1 wk. Blood was sampled at the end of the maintenance period. *P < 0.05.
in the absence of the band (Fig. 2). In the central nervous system, TRPV4 is expressed in cells around the ventricle, whereas neurosensory cells are responsive to systemic or cerebrospinal fluid (CSF) osmotic pressure (21). Figure 2 shows a histologically positive signal of TRPV4 protein. The frontal part of the circumventricular area, especially the lateral portion, was positively stained. Choroid plexus of the third ventricle was also positively stained, whereas TRPV4 was not detected in other parts (cerebrum to cerebellum).

Osmotic response in TRPV4−/− mice. Using a water or 2% NaCl ingestion test, we measured serum and urine osmolality. In the water loading test (20 ml/kg at 20°C within 5 min), no significant change in urinary Na⁺ concentration, K⁺ concentration (data not shown), or osmolality was observed between the two groups (Fig. 3). However, urinary osmolality was higher at 2 h after ingestion of the 2% NaCl solution (20 ml/kg at 20°C within 5 min) in TRPV4−/− mice compared with TRPV4+/+ mice. During the test, urinary Na⁺ concentration was increased at 1 h and then decreased, but there was no difference between the two groups. Thus unknown osmotic substrate(s) induced this change. Although abnormality of the osmotic regulation was suggested as a reason for this change, it was not determined until measurement of AVP.

Serum AVP concentration in TRPV4−/− mice. The level of serum AVP was measured by radioimmunoassay. To determine exact values, we diluted control samples and measured them repeatedly. We found that internal error for the measurement was within 0.15 pg/ml with 0.2 ml of serum. The amount of serum from each mouse was around 0.2 ml, whereas 0.5 ml of serum was required for this assay. We therefore used sera of three mice for one sample in the basal condition. To induce AVP secretion, we next measured serum osmolality in various stressful conditions, including salt overload and water deprivation for 2 days. We injected propylene glycol into the peritoneal cavity of a conscious mouse to increase serum osmolality (plus water deprivation) and sampled the whole blood within 10 s. Serum osmolality was stable under these conditions (410 ± 8 mosmol/kgH₂O). Values of AVP in both conditions were compared (Fig. 4). A significant rise in AVP concentration in the TRPV4−/− mouse was observed under the latter stimulated condition (n = 11 vs. 13; P < 0.01, t-test), whereas a significant difference was not obtained under the steady-state control condition.

AVP excretion in vitro by brain slice. Because serum hyperosmolality accompanied water deprivation in vivo, the direct effect of hyperosmolality on AVP secretion through TRPV4 remained obscure. To elucidate the response of AVP to osmolality, we measured the amount of secreted AVP released from sliced brain in vitro during a stepwise increase in perfusate osmolality. Secretion of AVP during 10 min was collected, and it was shown to be increased by hyperosmotic bath solution. The rate of increment rather than the set point was exaggerated in TRPV4−/− mice (Fig. 5; n = 5 in each, ANOVA).

DISCUSSION

TRPV4 is known to act as a Ca²⁺-permeable cation channel in response to mechanical stress including swelling (6, 17, 20). During recovery various forms of TRPV4, including VR12, OTRPC4, VR-OAC, TRP12, and SAC1 are present (6, 17, 20, 26), but Southern blot analysis suggests that all of these forms are located as a single copy in chromosome 5 (Fig. 1).

Table 2. Blood pressure and water balance in TRPV4+/+ and TRPV4−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Blood Pressure 11 wk, mmHg</th>
<th>Blood Pressure 20 wk, mmHg</th>
<th>Water Intake, mlday</th>
<th>Urine Output, mlday</th>
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<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
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<tr>
<td>TRPV4+/+</td>
<td>116.1 ± 5.2</td>
<td>123.2 ± 2.7</td>
<td>4.0 ± 0.8</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>TRPV4−/−</td>
<td>104.8 ± 4.2</td>
<td>120.1 ± 8.1</td>
<td>4.5 ± 1.0</td>
<td>1.8 ± 0.5</td>
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Values are means ± SE. Tail blood pressure was measured in duplicate at the ages indicated. Water balance was determined in triplicate in mice on normal diet and averaged over 1 wk.

Fig. 2. Immunohistological localization of TRPV4 in the circumventricular region. Top: Western blot detects 98 kDa of TRPV4 protein from the renal cortical extract with (Ag+) or without (Ag−) excess of the peptide (0.001 mg/ml). Bottom: immunohistological staining of the circumventricular region was performed on TRPV4+/+ mice. Third ventricle (left, ×100) and ventricle of the frontal region are shown (right, ×200). An arrow indicates a positively stained cell.
This observation may predict that knockout of this channel alters cell volume regulation in the expressed cell de novo. An example of the response of cells to hyposmolality is believed to be a two-step process. First, mechanically gated ion channels open in concert with a gain of cell volume from water influx, which generates a rise in intracellular free Ca\(^{2+}\) concentration and/or activation of protein kinase C. Second, subsequent activation of Ca\(^{2+}\)-dependent K\(^+\) and volume-sensitive Cl\(^-\) channels enhances the outflux of K\(^+\) and Cl\(^-\), resulting in hyposmolality in the cell interior and leading to recovery of cell volume. This recovery is termed regulatory volume decrease (RVD) and is observed widely in the renal tubular segments (7). Previously, the mechanosensitive channels working during RVD were believed to be SA channels (15). TRPV4 appears to be an SA channel: from the current-voltage curve of the TRPV4 single-channel currents in cell-free patches, the single-channel slope conductance is 61.4 pS for inward currents and 98.9 pS for outward currents (25), whereas the slope conductance of an SA channel in cell-attached distal lumen is \(100\) pS (23). TRPV4 may not be a mechanosensitive channel: Strotmann et al. (17) explicitly state that TRPV4 is not mechanosensitive because in cell-attached distal lumen does not activate TRPV4; however, cell swelling activates TRPV4. Although the controversy has not been resolved, many studies have stated that cell volume regulation in renal tubules is coupled with Na\(^+\)/H\(^+\) transport or Ca\(^{2+}\) signal transduction (7, 13). Thus we examined the influence of mineral metabolism. Surprisingly, we did not find any difference in Na\(^+\) balance (Table 1) and body weight (data not shown). Therefore, another channel may support the Na\(^+\) balance without TRPV4, or TRPV4 may play a role only in stressful conditions.

Fig. 3. Mandatory ingestion test in TRPV4\(--/\) mice. Mandatory ingestion of water (A and C) or 2% NaCl (B and D) was performed manually through a tube over a period of 5 min (\(n = 10\)). Osmolality (A and B) and Na\(^+\) concentrations (C and D) were then measured hourly until recovery in TRPV4\(+/+\) (\(\bullet\)) and TRPV4\(--/\) (\(\ast\)) mice. \(*P < 0.05\), Student’s t-test.

Fig. 4. Serum and brain arginine vasopressin (AVP) level in TRPV4\(--/\) mice. TRPV4 single-channel currents in cell-free patches, the single-channel slope conductance is 61.4 pS for inward currents and 98.9 pS for outward currents (25), whereas the slope conductance of an SA channel in cell-attached distal lumen is \(100\) pS (23). TRPV4 may not be a mechanosensitive channel: Strotmann et al. (17) explicitly state that TRPV4 is not mechanosensitive because in

Fig. 5. Amount of secretion of AVP from brain slice. Brain slice (2 mm thick) was perfused by 300- to 360 mosmol/kgH\(_2\)O solutions, and perfusate throughout 10 min was collected and freeze-dried. Total amount of AVP of the sample was measured. \(*P < 0.05\), \(**P < 0.01\), analysis of variance (ANOVA).

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**Fig. 4.** Serum and brain arginine vasopressin (AVP) level in TRPV4\(--/\) mice. AVP was measured from serum of 3 mice (each \(n = 9; 310 \pm 2.5\) mosmol/kgH\(_2\)O) or serum of 1 mouse (\(+/+\), \(n = 11\); \(--/\), \(n = 13\); 410 \(\pm 5.2\) mosmol/kgH\(_2\)O) after injection of propylene glycol. \(*P < 0.01\), (Student’s t-test).

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We next examined various conditions that cause Na⁺ metabolism to fluctuate in the body, such as salt overload or restriction. However, no significant differences were observed in TRPV4−/− mice (data not shown). Short-term salt overload or restriction may induce an altered response of osmolality in urine. Traits in TRPV4−/− mice are suggestive of the syndrome of inappropriate secretion of AVP (SIADH). SIADH can develop when the set point of AVP release is lower than normal (280–300 mosmol/kg H₂O). Addition of hypertonic saline (rather than water restriction) is a useful test to detect subtle differences in the regulation of osmolality (10). In our experiment, abnormality of AVP response in TRPV4−/− mice was obviously a response to brief hypertonic saline loading. The results of this test and the higher level of AVP under a condition of hypertonic dehydration (Fig. 4) support the finding that the TRPV4−/− mice exhibited an exaggerated AVP response.

However, hyponatremia was not observed in TRPV4−/− mice. AVP concentrations in the steady-state condition were not high in TRPV4−/− mice compared with TRPV4+/+ mice, suggesting that the set point to release AVP was not altered in TRPV4−/− mice. However, hyponatremia may not be apparent in AVP transgenic mice even when the AVP level is 5 (22) or 100 (4) times higher than normal serum osmolality. Furthermore, constant infusion of AVP does not substantiate hyponatremia in rats (11). Therefore, the absence of hyponatremia or normal AVP level alone did not necessarily indicate an abnormality in the set point. We therefore examined AVP release in vitro. The results strongly suggested that the response rather than the set point was altered in TRPV4−/− mice.

The mechanism of altered response of AVP to hypertonicity remains unclear. The choroid plexus is considered to be the sensor of hydrostatic or osmotic CSF (21). To our knowledge, neurological events that follow injection of hypotonic solution directly into CSF have not been examined. Therefore, the neuron network of the downregulation of AVP is not yet mapped. A rise in AVP concentration in TRPV4−/− mice suggests that TRPV4 in choroid plexus cells and paraventricular cells plays a negative role in sensing hyperosmolality to release AVP. TRPV4 possessing neurons may connect the AVP-secreting network. As to the anatomic connection of TRPV4 with AVP secretion, at least two pathways are possible, directly or indirectly connecting to AVP-secreting cells (Fig. 6). Basically, it is shown in HEK-293 and endothelial cells that TRPV4 is temperature sensitive and thereby constitutively active in the range of 34–40°C (25). If hyperosmolality (320 mosmol/kg H₂O) inhibited the activity of the channel and reduced intracellular calcium in TRPV4-expressing cells, TRPV4 may also play an inhibitory role in AVP secretion. TRPV4 thereby connects to the network of AVP secretion directly (connection A; Fig. 6). TRPV4 may be constitutively active in the isosmotic condition to regulate the tuning of AVP secretion by hyperosmolality. TRPV4 thereby connects to the hyperosmolality-sensitive mechanism (connection B; Fig. 6) rather than directly to the secretion of AVP. Interestingly, the baroregulatory system provides a similar influence on the AVP-osmolality curve, which acute hypovolemia shifts without changing the resetting point in vivo (14). However, the baroregulatory system generally involves neurological afferents that arise in pressure-sensitive receptors in the heart, aorta, or carotid sinus. Thus the brain slice model in the present study did not reproduce the reaction of acute hypovolemia. Nevertheless, it is possible that TRPV4 plays a terminal role in transmitting the inhibitory baroregulatory system through connection B. Regulation of TRPV4 other than by osmolality, such as by protein kinase C, was recently reported (24), which suggests a further possibility for the mechanism.

The mechanism underlying TRPV4 mirrors that of a well-known mechanism whereby the stretch-inhibitable or shrinkage-activated channel responds to hyperosmolality, transmitting a signal that enhances the secretion of AVP (1). It is assumed that AVP secretion reflects a balance of inhibitory and stimulatory inputs (1, 14). Investigation of the connection of TRPV4-positive neurons will clarify the network.

The presence of TRPV4 was also indicated in the lamina terminalis and subfornical organ (6), but we did not find a positive staining of these nuclei. Both are regarded as osmoreceptive sensory organs related to drinking behavior (8, 16). However, we did not find any difference in water intake during the salt restriction or overloading treatments.

In conclusion, TRPV4 is a swell-activated Ca²⁺-permeable cation channel detected in the osmosensing area in the brain. TRPV4−/− mice do not exhibit abnormalities in Na⁺ metabolism but show an abnormal response of AVP. Thus central TRPV4 may play an inhibitory role in AVP secretion.
REFERENCES