Regular Article

Central Transient Receptor Potential Vanilloid 4 Contributes to Systemic Water Homeostasis through Urinary Excretion

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Intracerebroventricular (icv) injection of transient receptor potential vanilloid 4 (TRPV4) agonists 4 α -phorbol-12, 13-didecanoate (4 α -PDD) and GSK101690A increased urinary excretion under the physiological condition. TRPV4 antagonists ruthenium red and HC-067047 significantly blocked increased urinary volume after intragastric administration of water and 4 α -PDD-induced diuresis. Administration of the TRPV4 agonists did not significantly change the plasma concentration of vasopressin or atrial natriuretic factor. Pretreatment with indomethacin inhibited the diuresis induced by 4 α -PDD. Moreover, icv injection of prostaglandin (PG) F_{2 α} produced diuretic effects. These findings indicate that central TRPV4 regulates urine excretion, which contributes to systemic water homeostasis *in vivo*. The underlying mechanisms are suggested to involve PG synthesis, but not release of vasopressin or atrial natriuretic factor.

Key words transient receptor potential vanilloid 4; systemic water homeostasis; urine excretion; prostaglandin; hypothalamus

INTRODUCTION

In 2000, Liedtke *et al.* demonstrated the possibility that transient receptor potential vanilloid 4 (TRPV4), a member of the TRPV subgroup in the TRP superfamily, is an osmotic sensor in the central nervous system (CNS).¹⁾ This conclusion was derived from the facts that TRPV4 is opened/closed by changes in osmotic pressure and expressed in the vascular organ of the lamina terminalis, the preoptic area, and the subfornical area where the osmosensor is supposed to be present.^{2–4)} Subsequently, some studies have supported the hypothesis that TRPV4 functions as an osmosensor.^{1,3,5,6)} Recently, TRPV4 was shown to possibly function as a volume sensor and not an osmosensor.⁷⁾ In any case, TRPV4 is involved in systemic water homeostasis. TRPV4 knockout (KO) mice were established by some laboratories.^{8–11)} However, because their phenotypes are inconsistent with each other, its role in systemic water homeostasis remains unclear.

Systemic water homeostasis is eventually established by the balance of intake and expulsion of water into and from the body, respectively. Large components of the intake and expulsion of water are the drinking water volume and urinary excretion, respectively, resulted from changes in osmotic pressure of body fluid.¹²⁾ We have already studied TRPV4 ligands in water intake by wild type (WT) animals, which suggested that TRPV4 contributes to systemic water homeostasis through regulation of the drinking water volume under the physiological condition.¹³⁾ The present study was conducted to clarify the role of TRPV4 in regulation of urine outflow. In the CNS, a major mechanism to expel water may be mediated through osmoreceptors detecting changes in systemic osmotic pressure, followed by regulation of Arg-vasopressin (AVP) secretion, resulting in an increase or decrease in urinary volume. Osmoreceptors are present only on the cell bodies of vasopressin-containing neurons in supraoptic and paraven-

tricular nuclei, but also at several sites in circumventricular organs of the CNS.^{2,3)} If TRPV4 is an osmosensor or volume sensor, because TRPV4 is activated by decreased osmotic pressure, its opening should lead to polyuria. In this study, we examined the effects of TRPV4 ligands on urine outflow and AVP release using *in-vivo* system of wild-type animals.

MATERIALS AND METHODS

The study was conducted according to the Guidelines for the Care and Use of Laboratory Animals, and its protocol was approved by the Instructional Animal Care and Use of Committees of Nagoya City University Graduate School of Medical Sciences and Kinjo Gakuin University.

The main methods have been described elsewhere.^{13–17)} Here, they are explained briefly.

Animals Male Wistar rats (9–12-week old; SLC, Hamamatsu, Japan) were used in this study. They were kept in rooms maintained at 23 $\pm$ 2°C and 50 $\pm$ 10% relative humidity with a 12-h light–dark cycle (light on from 8:00 a.m. to 8:00 p.m.).

Drug Administration All drugs were administered into the lateral cerebroventricle through a cannula. The cannula was fixed as follows. The rats were set in a stereotaxic instrument (Narishige Co., Ltd., Tokyo, Japan) after anesthesia with pentobarbiturate (50 mg/kg, intraperitoneal). A guide cannula (AG-8; Eicom Co., Ltd., Kyoto, Japan) was inserted into the right lateral ventricle (coordinate: 5.8 mm anterior to lambda, 1.8 mm lateral to the midline and 2.8 mm ventral to the skull surface) according to the Atlas of König and Klippel¹⁸⁾ and fixed using dental cement. A dummy cannula (AD-8; Eicom Co., Ltd.) was placed in the guide cannula when experiments were not carried out. After 1 week of recovery, the rats were used for experiments. The cage for experiments was 30 $\times$ 30 $\times$ 30 cm surrounded by plastic boards. Its bottom was

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wire gauze to collect urine.

On the experimental day, the cannula for drug administration was inserted into the guide cannula instead of the dummy cannula without anesthesia at approximately 10:00 a.m. The drug administration cannula was connected to a 40-cm polyethylene tube containing a drug (10–13 μ L), which was attached to a microsyringe. Then, the rats were placed in the experimental cage. During the experiment, the animals did not receive food or water. The drug solution was injected for 90 s at 10:30–11:00 a.m. A pretreatment drug was injected at 0.5 h before 4 α -phorbol-12,13-didecanoate (4 α -PDD)/vehicle treatments.

Measurement of Urine Volume Urine was collected in a tray under the wire gauze of the experimental cage, and its volume was measured at 0.5, 1.0, 2.0, 4.0, and 6.0 h after drug administration. The measurements were carried out under basal or water-loaded conditions (5 mL/100 g body weight of water was administered using an intra-gastric catheter at 45 min before drug administration).

Measurement of Hormones The measurements were carried out according to the protocols of commercial ELISA kits [AVP: R&D Systems Inc., MN, U.S.A.; atrial natriuretic factor (ANF): Peninsula Laboratories, Inc., CA, U.S.A.]. At 0.1 or 4 h after intracerebroventricular (icv) administration of 4 α -PDD, blood was collected into an ice-cold tube containing ethylenediaminetetraacetic acid (EDTA) and aprotinin from the trunk of ether-anesthetized animals after decapitation. The blood was centrifuged at 1600 $\times g$ and 4°C, and then the plasma was stored at –80°C until measurements. The hormones were extracted through a sep-pak C18 cartridge (Waters Co., MA, U.S.A.), according to the manufacturer's instructions.

Statistics All data are expressed as means $\pm$ standard error. Statistical analyses were carried out using one-way or two-way ANOVA, followed by *post-hoc* tests (Tukey–Kramer test) where appropriate to assess significance across groups (Statcell3 software added in Excel, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

Drugs and Reagents 4 α -PDD (Alexis Biochemicals Co., San Diego, CA, U.S.A.); GSK1016790A and HC-067047 (GSK and HC; Cayman Chemical, Ann Arbor, MI, U.S.A.); ruthenium red (RR; Latoxan, Valence, France); indomethacin (IND) (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.); prostaglandin (PG)_{E2}, PGF_{2 α} , and thromboxane B₂ (Cayman Chemical Co., Ltd., MI, U.S.A.). The chemicals used were the highest grade. 4 α -PDD and PG were dissolved in dimethyl sulfoxide (DMSO) or ethanol and diluted with sterile physiological saline (Otsuka Chemicals, Tokyo, Japan). The final concentrations of DMSO and ethanol were 8 and 5%, respectively. Indomethacin was dissolved in 0.1 N NaOH and diluted by 20-fold with phosphate buffered saline. The other drugs were dissolved in sterile physiological saline.

RESULTS

Effects of the TRPV4 Agonist and Antagonist on Urinary Excretion under the Basal Condition Figure 1A shows the urinary volume after icv injection of the TRPV4 agonist 4 α -PDD under the physiological condition without any treatments. This drug significantly increased urine outflow at 2–6 h after administration compared with vehicle administration. In addition, the 4 α -PDD-induced diuresis was dose

dependent (Fig. 1B). As shown in Fig. 1C, TRPV4 antagonist ruthenium red (RR) completely blocked the 4 α -PDD-induced effect. RR itself did not influence the urinary volume. The selective TRPV4 agonist GSK1016790A (GSK, 50 ng) also produced the same diuresis that was relatively weaker than the diuresis induced by 20 μ g 4 α -PDD (Fig. 1B).

Because TRPV4 is activated by lower osmotic pressure than the physiological pressure, although TRPV4 is possible to detect cell volume, effects of TRPV4 antagonists themselves on the urinary volume were examined under a hypo-osmotic condition after the animals were loaded with water. As shown in Fig. 2A, under the water-loaded condition, the urinary volume was increased after vehicle administration. However, TRPV4 antagonists RR and HC-067047 (HC) significantly inhibited the increased urine outflow at the early period after administration compared with the vehicle-injected group (Figs. 2A, B). From approximately 2 h after administration, antagonist-injected groups gradually increased their urine excretion, and the total volume at 6 h after administration was not different compared with the vehicle-injected group.

Effects of 4 α -PDD on Plasma Concentrations of Hormones AVP and ANF powerfully regulate urine production under the physiological condition. Therefore, these hormones in plasma were measured after 4 α -PDD administration. However, no significant changes were observed between vehicle- and 4 α -PDD-injected groups (Fig. 3).

Effects of a Cyclooxygenase Inhibitor on the 4 α -PDD-Induced Diuresis To investigate mechanisms underlying the 4 α -PDD-induced diuresis, rats were pretreated with a cyclooxygenase (COX) inhibitor, IND. As shown in Fig. 4A, IND significantly blocked the 4 α -PDD-induced diuresis. Next, we examined the influences on urine outflow after administration of some arachidonate metabolites into the lateral ventricle. PGE₂ had a tendency to increase the urine volume, but it was not significant. However, icv injection of PGF_{2 α} significantly induced diuresis, which was a relatively less potent effect with a shorter latency and duration than the 4 α -PDD-induced effect (Fig. 4B). Thromboxane (TX) B₂ did not produce any significant effect on urine volume.

DISCUSSION

TRPV4 is a well-known, non-selective cation channel activated by various stimuli such as temperature, osmotic pressure, shear stress, and cell volume.^{1–3,5,19–21} In terms of osmotic stimuli, this channel is open under a lower pressure than the physiological pressure, and maximum opening is observed at approx. 220 mOsm/kg in TRPV4-transfected cells.^{1,5} Recently, TRPV4 was reported to function as a cell volume sensor and not as an osmosensor.⁷ In any case, TRPV4 open *in vivo* ultimately results in increasing the urine outflow volume and/or decreasing water intake. Our previous study showed that the TRPV4 agonist decreases water intake, and we concluded that TRPV4 participates in systemic water homeostasis through regulation of water intake.¹³ In this study, we examined whether TRPV4 is involved in the homeostasis through regulatory mechanisms of urine outflow using the same methods. Injection of 4 α -PDD and GSK, which is a more selective TRPV4 agonist, into the ventricle resulted in diuresis. Conversely, TRPV4 antagonists induced the opposite effect. In addition, the antagonists inhibited the agonist-induced diuresis.

Fig. 1. Diuretic Effects after Administration of TRPV4 Agonists under the Physiological Condition

A) Intracerebroventricular (icv) injection of TRPV4 agonists, 4α-PDD and GSK1016790A, significantly increased the urinary volume for 2–6 h after injection compared with injection of the vehicle. 4α-PDD: $N=8$; GSK: $N=8$; vehicle: $N=9$. B) The 4α-PDD-induced diuresis was dose dependent at doses of 3–20 μg. C) Pretreatment with ruthenium red (RR), a TRPV4 inhibitor, in the ventricle diminished the 4α-PDD-induced diuretic effect dose-dependently. 4α-PDD, 20 μg: $N=8$; 10 μg: $N=6$; 3 μg: $N=3$; GSK: $N=8$; vehicle: $N=9$; 4α-PDD: $N=8$; RR (1 μg) + 4α-PDD: $N=4$; RR (3 μg) + 4α-PDD: $N=5$; RR (3 μg): $N=5$. * $p<0.05$ by Tukey–Kramer test after two-way ANOVA.

Fig. 2. Decreased Urinary Volume after Administration of TRPV4 Antagonists, RR and HC067047 (HC) under the Water-Loaded Condition

A) RR and HC administered into the ventricle diminished the increased urinary volume after oral administration of water. The same dose of RR inhibited the 4α-PDD-induced diuresis under the physiological condition (Fig. 1C). B) The effects were dose dependent. RR (3 μg): $N=9$; RR (1 μg): $N=6$; HC (5 μg): $N=8$; vehicle: $N=12$. * $p<0.05$ by Tukey–Kramer test after two-way ANOVA.

These results support the above hypothesis that TRPV4 in the CNS contributes to systemic water homeostasis through controlling both urinary and drinking water volumes.

The durations of the TRPV4 agonist- and antagonist-induced effects were 2–6 and 0–0.5 h as shown in Figs. 1 and

2, respectively. There is the time lag between these effects. This phenomenon is supposed to come from the difference in the experimental conditions. The experiments in Figs. 1 and 2 were carried out under the physiological condition and the water-loaded condition, respectively. Because the water-loaded

Fig. 3. Vasopressin (A) and Atrial Natriuretic Peptide (B) Concentrations in Plasma after 4α-PDD Administration under the Physiological Condition

Icv injection of 4α-PDD increased the urinary volume at 2–6 h after injection under the normal physiological condition (Fig. 1). Blood was collected after decapitation at 0.1 and 4.0 h after injection and centrifuged ($1600 \times g$ at 4°C). The plasma was stored at -80°C until measurements. The measurements were carried out using commercial ELISA kits. The plasma concentrations were not significantly influenced by the administration. A) 0.1 h: Vehicle $N=9$; 4α-PDD $N=5$; 4.0 h: Vehicle $N=11$; 4α-PDD $N=11$, B) 0.1 h: Vehicle $N=11$; 4α-PDD $N=6$; 4.0 h: Vehicle $N=9$; 4α-PDD $N=10$.

Fig. 4. Influences of Indomethacin on the 4α-PDD-Induced Diuresis under the Physiological Condition

A) Pretreatments with indomethacin (15 μg; IND) inhibited the 4α-PDD-induced diuresis. 4α-PDD: $N=8$; IND + 4α-PDD: $N=6$; IND: $N=6$; vehicle $N=9$. B) Effects of arachidonate metabolites thromboxane B₂ (TXB₂), PGE₂ and PGF_{2α} on the urinary volume. Icv injection of PGF_{2α} significantly increased the urinary volume. TXB₂: $N=6$; PGE₂: $N=3$; PGF_{2α}: $N=6$; vehicle: $N=9$. * $p < 0.05$ by Tukey–Kramer test after one-way or two-way ANOVA.

condition elicits abnormal hypo-osmotic pressure of body fluid, many sensors, osmosensor, sensor for Na⁺ concentration, cell volume sensor and so on, are already functioning to recover to the normal osmotic pressure.^{6,12,22–24} At this time, the antagonist-induced effects will appear at relatively early time, and even if TRPV4, one of many sensors, is closed by its antagonist, its effect will be limited. On the other hand, be-

cause these mechanisms do not activate under the physiological condition, the agonist-induced function is fully observed, although it took a while for the onset of diuresis from TRPV4 open.

Because AVP plays an important role in regulating urine production, the AVP concentration was measured after TRPV4 agonist administration. During the TRPV4 agonist-induced

diuresis, AVP concentration is expected to decrease in the plasma, if the diuresis will be brought from its change. However, there was no significant change in the plasma concentration after administration of the TRPV4 agonist. Therefore, it is suggested that AVP is not involved in the TRPV4-induced regulation of urinary volume in WT animals. Studies of TRPV4 KO mice have measured the AVP concentration and/or urinary volume, and these in TRPV4 KO mice has been reported to be similar to that in WT mice under the physiological condition.^{11,18)} However, under hypertonic stimulation, Mizuno *et al.* found a higher AVP concentration in the plasma of KO mice than that in WT mice,¹¹⁾ and the other reported opposing results.^{8,10)} Although they insist that TRPV4 is needed for systemic water homeostasis through urinary excretion, its role remains unclear. The discrepancy is probably due to differences in compensative functions, DNA damage in KO mice, and/or the hypertonic condition. TRPV4 is mostly closed, may be slightly opened by body temperature under physiological temperature and osmotic pressure, and open under the hypotonic condition. Thus, the effects under the hypertonic condition, which closes TRPV4 and renders the channel non-functional, may result in an increased compensatory function. Therefore, experiments using WT animals injected with agonists and antagonists are possible to exclude the compensatory function. This study showed that neither AVP nor ANP, which influence the urinary volume, were significantly changed after administration of the TRPV4 agonist. On the other hand, pretreatment with IND inhibited the 4α -PDD-induced effect, and icv injection of PGF_{2 α} produced diuresis to the same degree as 4α -PDD. In the cultured aorta endothelial cells, an increase in PGF_{2 α} , not PGE₂ or TXB₂, after TRPV4 activation is reported.²⁵⁾ Therefore, PGF_{2 α} is suggested to play an important role in this TRPV4-induced effect.²⁵⁾ However, because icv injection of PGE₂ showed a tendency to produce diuresis, but not significantly, PGE₂ may contribute in the effect. These suggest that increased PG biosynthesis, but not hormone release, is involved in TRPV4-mediated regulation of systemic water homeostasis through urine outflow. TRPV4 activation increases Ca²⁺ influx into cells, and this is supposed to activate phospholipase A₂ and/or cyclooxygenase, followed by increases in biosynthesis of PGs.^{25–27)} TRPV4 is expressed in glial cells (astrocytes and microglia), neurons, and the endothelium^{20,28,29)} that are able to release PGs.^{30,31)} These cells are probably responsible for PG production after TRPV4 opening. In hypotonic or hypertonic condition, osmotic pressure changes not only in blood, but also in the cerebrospinal fluid and extracellular fluid. Therefore, glial cells are possible to detect its changes. Increases in PG production is possible to increase blood flow in the kidney, followed by diuresis. There are some reports that central administration of PGs increased urinary excretion, blood pressure, heart rate and sympathetic activity.^{32–34)} Also, it has been demonstrated that PGE₂ increases aquaporin 2 expression through EP2 and/or EP4 receptors and then participates in the urinary concentration.^{35,36)} In addition, TRPV4, which was recently demonstrated to function as an anchor protein, binds to aquaporin and Kca channels.^{37–41)} These proteins may underlie the TRPV4-induced effect.

Osmosensation and its regulation involve many kinds of molecules.^{6,12,22–24)} Some recent reports demonstrate that TRPV1 play a role in systemic water homeostasis. TRPV1

may also detects changes in osmotic pressure as osmoreceptor.^{9,29,42–44)} In addition, TRPV1 is present in magnocellular neurons, and influences AVP release.^{20,37,42)} Therefore, TRPV1 is supposed to function in the regulatory mechanism in systemic water homeostasis with TRPV4. Especially, mechanism involving AVP release may need TRPV1.

In summary, TRPV4, at least, partly contributes to systemic water homeostasis of body fluid through regulation of urinary excretion in cooperation with regulation of water intake. The diuresis after TRPV4 open probably requires an increase in PG production, but not release of AVP or ANP.

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Conflict of Interest The authors declare no conflict of interest.

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