Novel Gating and Sensitizing Mechanism of Capsaicin Receptor (TRPV1)

**Tonic Inhibitory Regulation of Extracellular Sodium Through the External Protonation Sites on TRPV1**

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Transient receptor potential V1 (TRPV1) is a nonselective cation channel expressed in nociceptors and activated by capsaicin. TRPV1 detects diverse stimuli, including acid, heat, and endogenous vanilloids, and functions as a molecular integrator of pain perception. Herein we demonstrate a novel regulatory role of extracellular Na⁺ ([Na⁺]o) on TRPV1 function. In human embryonic kidney 293 cells expressing porcine TRPV1, low [Na⁺]o evoked increases of [Ca²⁺]i, that were suppressed by TRPV1 antagonists and facilitated responses to capsaicin, protons, heat, and an endovanilloid. [Na⁺]o, removal simultaneously elicited a [Ca²⁺]i increase and outward-rectified current with a reversal potential similar to those of capsaicin. Neutralization of the two acidic residues which confer the proton sensitivity to TRPV1 resulted in a reduction of low [Na⁺]o-induced responses. In primary culture of porcine sensory neurons, the removal of [Na⁺]o produced a [Ca²⁺]i increase and current responses only in the cells responding to capsaicin. Low [Na⁺]o evoked a [Ca²⁺]i increase in sensory neurons of wild type mice, but not TRPV1-null mice, and in human embryonic kidney 293 cells expressing human TRPV1. The present results suggest that [Na⁺]o negatively regulates the gating and polymodal sensitization of the TRPV1 channel. [Na⁺]o surrounding several proton-sensitive sites on the extracellular side of the pore-forming loop of the TRPV1 channel may play an important role as a brake to suppress the excessive activity of this channel under physiological conditions.

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²The abbreviations used are: TRPV1, transient receptor potential V1; hTRPV1, human TRPV1; pTRPV1, porcine TRPV1; PIP₂, phosphatidylinositol 4,5-bisphosphate; 15(S)-HPETE, 15-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15(S)-HPETE), a lipxygenase product, also activate TRPV1 (4). Because mice genetically lacking the TRPV1 channel exhibit impaired nociception (5, 6) and several TRPV1 antagonists have antinociceptive activity in vivo (7–9), TRPV1 is considered to be a key component of signal transduction pathways in the nociceptive system. Several regulatory mechanisms of TRPV1 function have been elucidated. Sensitization can arise from phosphorylation of TRPV1 at multiple sites via protein kinase C and protein kinase A downstream of activation of GTP-binding protein-coupling receptor by bradykinin, ATP, nerve growth factor, calcitonin gene-related peptide, and prostaglandins (10). Our recent report also showed that the metabotropic 5-HT receptor facilitates TRPV1 functions through protein kinase C/protein kinase A pathways (11). TRPV1 is subject to tonic inhibition by phosphatidylinositol 4,5-bisphosphate (PIP₂) (12), providing an additional biochemical pathway through which the activity of the channel can be regulated after GTP-binding protein-coupling receptor activation. Recently, increases of cationic strength have been shown to contribute to inflammatory pain signaling through modulation of TRPV1 channels, since extracellular cations such as Na⁺, Mg²⁺, and Ca²⁺ gate and sensitize them (13). Hyponatremia, a disease due to reduction of the plasma Na⁺ concentration, is one of the most common electrolyte disorders (14). It occurs via excess loss of plasma Na⁺ as a result of various causes such as chronic renal failure and congestive heart failure. When the serum Na⁺ level falls gradually over a period of several days or weeks, sodium levels as low as 110 meq/liter may be reached with minimal symptomatology. Its symptoms are often concomitant with pain (15). However, the molecular detector of pain perception in hyponatremia has not yet been identified.

We recently cloned a porcine orthologue of TRPV1 (pTRPV1) and analyzed its functional properties using a heterologous expression system (16). We showed that pTRPV1 was a nonselective cation channel sensitive to a number of vanilloid agonists, including capsaicin and endovanilloids, heat, and protons. In pharmacological characteristics of TRPV1, there are remarkable species differences; chicken TRPV1 is not sensitive...
to capsaicin (17), and rabbit TRPV1 is less sensitive to capsaicin (18). Phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), a TRPV1 agonist, can stimulate rodent (19–22) but not human TRPV1 (20). Capsazepine, a TRPV1 antagonist, does not antagonize proton- and heat-induced responses in rodent (20, 21) but does antagonize in human (20) and porcine TRPV1 (16). Therefore, using a porcine orthologue may be more advantageous to predict physiological and pharmacological characteristics in human TRPV1.

Here we demonstrate a quite unique property of porcine and human TRPV1; that is, the reduction of [Na\(^+\)]\(_o\), directly activates the TRPV1 channel and facilitates its activation induced by polymodal stimuli in a heterologous expression system and primary culture of sensory neurons. On the extracellular side of the TRPV1, several negatively charged amino acid residues (Glu-600, Glu-648, and Asp-646) have been identified to be critical for regulatory sites by positively charged molecules, such as protons, divalent and trivalent cations, and polyamines (13, 23–25). Thus, in the present experiment we examined the effect of low [Na\(^+\)]\(_o\) on these mutant channels. We hypothesize that [Na\(^+\)]\(_o\) has a novel regulatory role for the TRPV1 channel through its tonic inhibition under physiological conditions. We demonstrate that [Na\(^+\)]\(_o\) appears to act at the same sites as protons and that the specific glutamate residue Glu-600 is particularly important for activation of the TRPV1 channel by [Na\(^+\)]\(_o\). If extracellular Na\(^+\) ionic homeostasis is disturbed by some metabolic causes like hyponatraemia, TRPV1 may contribute to pain signaling in these patients.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation, DNA Cloning, and Transfection**—All protocols for experiments on animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G (Meiji-Seika), and 100 \(\mu\)g/ml streptomycin (Banyu). The expression vectors pcDNA-pTRPV1-DEST53 for N-terminal GFP fusion protein, pcDNA-pTRPV1-DEST47 for C-terminal GFP fusion protein, and pGATE-pTRPV1 were constructed using Gateway Technology (Invitrogen) as described previously (16). Cells were transfected with the expression vector using a transfection reagent (Lipofectamine 2000, Invitrogen) and used 24–48 h after transfection. Human TRPV1 (hTRPV1) was cloned from a human spinal cord cDNA library (Clontech) based on a published sequence (GenBank NM_080704). Complimentary DNA was subjected to PCR amplification with the use of high fidelity polymerase (KOD plus; Toyobo) using two sets of primers corresponding to the open reading frame of hTRPV1 (forward, 5’-CACCAGTAGGAAATGGACACGGACAC-C-3’; reverse, 5’-TCACTTCTCCCGCGAGCGGACCGAGTGAAGAC-C-3’). Single amino acid mutations of TRPV1 were made using the PrimeSTAR® mutagenesis basic kit (TaKaRa). All clones and mutations used were verified by sequencing using an automated sequencer (ABI PRISM 3100, Applied Biosystems). Porcine dorsal root ganglion (DRG) neurons were obtained from male pigs (2–4 weeks after birth, 5–10 kg) as described previously (16). In brief, pigs were deeply anesthetized by sodium pentobarbital (50 mg/kg, intraperitoneal) after tranquilization with ketamine (15 mg/kg, intramuscular) and then killed by bloodletting from the cervical artery. To isolate murine DRG neurons adult mice (C57BL/6) and TRPV1-null (B6.129S4-Trpv1tm1Jui/J, Jackson Laboratories) were euthanized with CO\(_2\) inhalation. Lumbar DRG were removed and enzymatically dissociated with collagenase (1 mg/ml, type II, Worthington) and DNase (1 mg/ml, Roche Applied Science) for 30 min at 37 °C. Thereafter, ganglia were further digested with trypsin (10 mg/ml, Sigma) and DNase (1 mg/ml) for 30 min at 37 °C. Isolated cells were plated onto glass coverslips coated with poly-D-lysine (Sigma) and cultured in M199 (Sigma) supplemented with fetal bovine serum (10%, Sigma), penicillin G (100 units/ml), and streptomycin (100 \(\mu\)g/ml) in a humidified atmosphere of 95% air and 5% CO\(_2\) at 37 °C. The cells were used within 1 day after preparation.

**Measurement of Intracellular Ca\(^{2+}\) and Na\(^+\) Concentrations**—The intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in individual cells was measured with a fluorescent Ca\(^{2+}\) indicator, fura-2, by dual excitation using a fluorescent imaging system controlling illumination and acquisition (Aqua Cosmos, Hamamatsu Photonics) as described previously (16). To load fura-2, cells were incubated for 30 min at 37 °C with 10 \(\mu\)M fura-2 acetoxyethyl ester in normal external solution: 134 mM NaCl, 6 mM KCl, 1.2 mM MgCl\(_2\), 2.5 mM CaCl\(_2\), 10 mM HEPES (pH 7.4 with NaOH). For Ca\(^{2+}\)-free external solution, Ca\(^{2+}\) was omitted, and EGTA (0.5 mM) was added. For extracellular Na\(^+\) ([Na\(^+\)]\(_o\))-free solution, Na\(^+\) was omitted and substituted for by equimolar NMDG\(^+\), various monovalent cations, or isotonic sucrose. For various [Na\(^+\)]\(_o\)-containing solutions (0–126 mM), Na\(^+\) was substituted for by equimolar NMDG\(^+\). The osmolality of all external solutions used was 270–280 mOsm kg (Fiske Osmometer, Fiske Associates). When [Ca\(^{2+}\)]\(_i\) and the intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) were simultaneously measured, cells were loaded with a fluorescent Na\(^+\) indicator, CoroNa Green-AM (26) (10 \(\mu\)M, Molecular Probes) together with fura-2 acetoxyethyl ester. A coverslip with dye-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Olympus IX71, Japan) equipped with an image acquisition and analysis system. To measure [Ca\(^{2+}\)]\(_i\), cells were illuminated every 2.5 s with light at 340 and 380 nm, and the respective fluorescent signals of 500 nm were simultaneously detected. For [Na\(^+\)]\(_i\), cells were excited at 492 nm, and fluorescence above 515 nm was collected. The fluorescence emitted was projected to a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan), and the ratio of fluorescent signals (F\(_{340}/F_{380}\)) for [Ca\(^{2+}\)]\(_i\), and (F\(_{515}\)) for [Na\(^+\)]\(_i\), were stored on the hard disc of a PC (Pro-2500, EPSON). In some experiments the change in [Ca\(^{2+}\)]\(_i\), was measured using cameleon (27), which is a fluorescence resonance energy transfer-based Ca\(^{2+}\) sensor (supplemental Fig. 2A). HeLa cells were transfected with cameleon that was delivered by baculovirus-based transduction (PremoTm Cameleon, Molecular Probes) and porcine TRPV1 cDNA (pGATE-pTRPV1). For measurement of ratiometric fluorescence resonance energy transfer signals, cells were illuminated with light at 450 nm (F450), and yellow fluorescent protein emission (F480) and cyan fluorescent protein emission (F535) were detected. Cells were continuously superfused with...
the external solution at a flow rate of $\sim 1.5$ ml/min through a Y-tube pipette placed close to the cells. All experiments were carried out at room temperature (20–24 °C).

Whole-cell Current Recording—Membrane currents were recorded using the conventional whole-cell configuration of the patch clamp technique with a patch clamp amplifier (Axopatch 200B, Axon Instruments). The resistance of patch electrodes ranged from 3 to 4 megohms. The currents were filtered at 1 kHz and sampled at 5 kHz using an A/D converter (Digidata 1322A, Axon Instruments) in conjunction with a personal computer. During the experiments and analysis, pClamp 6 software (Axon Instruments) was used. The pipette solution contained 120 mM CsCl, 20 mM tetraethylammonium chloride, 1.2 mM MgCl$_2$, 2 mM ATP$\gamma$S, 0.2 mM GTP$\gamma$S, 10 mM HEPES, and 0.1 mM fura-2 (pH 7.2 with CsOH). For simultaneous measurement of $[\text{Ca}^{2+}]$, with the current responses, the pipette solution contained 120 mM CsCl, 20 mM tetraethylammonium chloride, 1.0 mM MgCl$_2$, 2 mM ATP$\gamma$S, 0.2 mM GTP$\gamma$S, 10 mM HEPES, and 0.1 mM fura-2 (pH 7.2 with CsOH).

Immunocytochemistry—To identify whether neurons responding to $[\text{Na}^+]_o$ removal express TRPV1 channels, primary cultured murine DRG cells were subjected to immunostaining with an anti-TRPV1 antibody. After $[\text{Ca}^{2+}]$, responses were observed, cells were fixed with 4% paraformaldehyde and incubated with a rabbit antisera for TRPV1 (diluted 1:100, Santa Cruz). Subsequently, the antibody was visualized with Alexa$^\text{TM}$ 488-labeled goat anti-rabbit IgG (10 μg/ml, Molecular Probes) for the detection of TRPV1. Preparations were examined with a confocal laser microscope (FV500, Olympus) using software (Fluoview, Olympus).

Chemicals—Capsaicin from Sigma was dissolved in ethanol at a high concentration as a stock solution (1 mM). Capsazepine, iodoresiniferatoxin, and ruthenium red (from Sigma) were dissolved in dimethyl sulfoxide (Me$_2$SO) to make a stock solution (0.1 mM). 2-[(4-[(4-Nitrobenzyloxy)phenyl]ethyl]sulfoxide mesylate (KB-R7943) was from Tocris. 15(S)-HPETE was from Biomol. The drugs were diluted to their final concentrations using the external solution. The final concentrations of ethanol and Me$_2$SO were less than 0.001% (v/v) and 0.01% (v/v), respectively, which did not affect $[\text{Ca}^{2+}]$, and $[\text{Na}^+]$.

Data Analysis—The data are presented as the mean ± S.E. ($n$ = number of observations). Comparisons were made by the paired Student’s $t$ test, and differences with a $p$ value of less than 0.05 were considered significant. Pairwise associations were examined by Pearson’s linear regression. Values of the 50% effective concentration (EC$_{50}$) and 50% inhibitory concentration (IC$_{50}$) were determined using Origin software (OriginLab). The present data were obtained from at least four different transfections or from three different animals per experiment.

RESULTS

The Removal of External Na$^+$ Increased $[\text{Ca}^{2+}]$, in Porcine TRPV1-expressing HEK293 Cells—The effects of $[\text{Na}^+]_o$, removal on $[\text{Ca}^{2+}]$, in HEK293 cells expressing pTRPV1 were examined using fura-2-based $[\text{Ca}^{2+}]$, imaging. The intracellular Na$^+$ concentration ($[\text{Na}^+]_i$) was simultaneously monitored by loading CoroNa Green as a Na$^+$ indicator. Representative time-lapse $[\text{Ca}^{2+}]$, imaging clearly demonstrated that a $[\text{Ca}^{2+}]$, increase was observed only in the cells responding to capsaicin when the cells were exposed to NMDG$^+$-based $[\text{Na}^+]_o$-free solution (Fig. 1A). The application of $[\text{Na}^+]_o$-free solution elicited a rapid $[\text{Ca}^{2+}]$, increase, which was sustained during the period of $[\text{Na}^+]_o$ removal (Fig. 1B). In this period $[\text{Na}^+]_i$, substantially decreased. Capsaicin (0.3 μM) produced a rapid $[\text{Ca}^{2+}]$, rise concomitant with a sustained rise of $[\text{Na}^+]_i$. The time to peak of the $[\text{Ca}^{2+}]$, increase induced by capsaicin (18.9 ± 1.2 s) was the same as that for the removal of $[\text{Na}^+]_o$, (20.1 ± 1.3 s, $n = 80$). The increments of $[\text{Ca}^{2+}]$, induced by the first application of $[\text{Na}^+]_o$-free solution and that induced by subsequently applied capsaicin were 258 ± 9.4 and 264.9 ± 9.1 nM ($n = 80$), respectively. No additive $[\text{Ca}^{2+}]$, increase occurred with the simultaneous application of $[\text{Na}^+]_o$-free solution.
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In the present experiment we mainly analyzed the TRPV1 function using a recombinant channel protein with an N-terminal GFP fusion protein (GFP-pTRPV1). To avoid the possibility that low [Na\(^+\)]\(_o\) elicited the [Ca\(^{2+}\)]\(_i\) increase in heterologously pTRPV1-expressing cells due to some physicochemical interaction with GFP protein, three expression plasmids were constructed, and their [Ca\(^{2+}\)]\(_i\) responses to low [Na\(^+\)]\(_o\) were analyzed. The [Ca\(^{2+}\)]\(_i\) responses to [Na\(^+\)]\(_o\) removal were observed in cells that expressed pTRPV1 in the C terminus of the GFP fusion protein and even in cells lacking GFP (supplemental Fig. 1). Neither [Na\(^+\)]\(_o\) removal nor capsazepine produced an increase in non-transfected naive HEK293 cells, but ATP produced transient [Ca\(^{2+}\)]\(_i\) responses to low [Na\(^+\)]\(_o\). Moreover, an increase of [Ca\(^{2+}\)]\(_i\), induced by [Na\(^+\)]\(_o\) removal could be detected in pTRPV1-transfected HeLa cells expressing a fluorescence resonance energy transfer-based Ca\(^{2+}\) sensor, cameleon (supplemental Fig. 2). These results completely excluded the possibility that GFP fusion affected the nature of TRPV1, methods for [Ca\(^{2+}\)]\(_i\) measurement, or cellular dependence used for transfection in the [Ca\(^{2+}\)]\(_i\) increase evoked by [Na\(^+\)]\(_o\) removal.

The effects of the replacement of [Na\(^+\)]\(_o\) by various monovalent cations or sucrose on [Ca\(^{2+}\)]\(_i\) were examined (Fig. 2). All monovalent cations and isotonic sucrose, used as a substitute for [Na\(^+\)]\(_o\), caused a sustained increase of [Ca\(^{2+}\)]\(_i\) in cells responding to capsazepine. These [Ca\(^{2+}\)]\(_i\) responses were abolished by the removal of external Ca\(^{2+}\), indicative of Ca\(^{2+}\) influx from the extracellular milieu similar to capsazepin (29). These data suggest that no monovalent cations are able to replace the effect of Na\(^+\) on the TRPV1 channel and that Na\(^+\) removal itself activates the TRPV1 channel. Three TRPV1 antagonists, capsazepine, iodoresiniferatoxin, and ruthenium red, were tested for their ability to inhibit the [Na\(^+\)]\(_o\) together with 0.3 µM capsazepin (data not shown). Under the present experimental conditions, no obvious tachyphylaxis occurred by repetitive application of capsazepin or [Na\(^+\)]\(_o\)-free solution (Fig. 1C). When [Na\(^+\)]\(_o\) was applied at different concentrations, low [Na\(^+\)]\(_o\) evoked a [Ca\(^{2+}\)]\(_i\) increase in pTRPV1-expressing HEK293 cells in a concentration-dependent manner with an EC\(_{50}\) of 30.9 ± 2.0 mM (Fig. 1, D and E). The second application of [Na\(^+\)]\(_o\)-free solution, in which the [Na\(^+\)]\(_o\) level stayed high after capsazepine challenge, produced almost the same amplitude of [Ca\(^{2+}\)]\(_i\) increase (Fig. 1B). The [Ca\(^{2+}\)]\(_i\) increase induced by the removal of [Na\(^+\)]\(_o\) was not affected by KB-R7943 (50 µM), a selective inhibitor of the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (28). These data indicated that the [Ca\(^{2+}\)]\(_i\) increase evoked by [Na\(^+\)]\(_o\) removal was unrelated to the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger.

FIGURE 2. Effects of substitution for [Na\(^+\)]\(_o\) of various monovalent cations or isotonic sucrose on the change in [Ca\(^{2+}\)]\(_i\) in HEK293 cells expressing pTRPV1. A, during the [Ca\(^{2+}\)]\(_i\) increase generated by these treatments, the external Ca\(^{2+}\) was removed (OCa), Vertical scales show 0.2 µM of [Ca\(^{2+}\)]. B, summarized changes in peak [Ca\(^{2+}\)] levels before and during removal of external Ca\(^{2+}\) and after its reintroduction (n = 52–80). cap, capsaicin.
removal-induced \([\text{Ca}^{2+}]_o\) increase. Inhibitory effects of these antagonists were examined by the application of increasing their concentrations with \([\text{Na}^+]_o\)-free solution. All antagonists caused concentration-dependent inhibition of the \([\text{Na}^+]_o\) removal-induced \([\text{Ca}^{2+}]_o\) increase (Fig. 1F). The IC\(_{50}\) values were 6.0 ± 0.1 nM for iodoresiniferatoxin, 30.0 ± 0.2 nM for ruthenium red, and 287.2 ± 12.2 nM for capsazepine. This led to the hypothesis that the removal of \([\text{Na}^+]_o\) relieved the tonic inhibitory action of \([\text{Na}^+]_o\) on TRPV1 channels.

**Sensitization of TRPV1 Function by Low \([\text{Na}^+]_o\)—** We examined the effect of lowering \([\text{Na}^+]_o\) on the capsaicin-induced \([\text{Ca}^{2+}]_o\) increase. HEK293 cells expressing pTRPV1 were repetitively stimulated with a low concentration of capsaicin (0.03 µM) for 30 s at intervals of 5 min. For 112 mM \([\text{Na}^+]_o\)-containing solution, \([\text{Na}^+]_o\) was substituted by equimolar NMDG\(^+\); thus, osmotic pressure was unchanged. By lowering \([\text{Na}^+]_o\) from 134 to 112 mM, the average \([\text{Ca}^{2+}]_o\) response to capsaicin was increased (Fig. 3A). It returned to the original level when \([\text{Na}^+]_o\) was increased to 134 mM. As shown in Figs. 1, D and E, and 3A, lowering \([\text{Na}^+]_o\) to 112 mM did not produce any \([\text{Ca}^{2+}]_o\) response but significantly potentiated the \([\text{Ca}^{2+}]_o\) response evoked by capsaicin. The concentration dependence of capsaicin on the \([\text{Ca}^{2+}]_o\) increase was shifted towarad by lowering \([\text{Na}^+]_o\) from 134 to 112 mM, and the EC\(_{50}\) value of capsaicin was correspondingly significantly reduced from 76.3 ± 2.6 to 33.0 ± 1.8 nM.

TRPV1 functions as a polymodal receptor that is activated not only by the vanilloid agonist capsaicin, but also by protons, noxious heat (2), and endovanilloids (3, 4). We previously reported that these stimuli are effective for activating pTRPV1 (16). Next we examined whether low \([\text{Na}^+]_o\) produced potentiation effects on proton-, heat-, or endovanilloid-induced
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responses. Brief application of protons (pH 6.5), heat (46 °C), and 15(S)-HPETE (1 μM) produces a transient [Ca2+]i increase (Fig. 3C) under which these stimuli cause less than half-maximal responses (16). After the application of 112 mM [Na+]o, the [Ca2+]i response evoked by each stimulus was significantly enhanced (Fig. 3D), suggesting that lowering [Na+]o, not only directly activated but also sensitized pTRPV1 channels.

Membrane Current Responses to the Removal of [Na+]o—It is known that the capsaicin-evoked [Ca2+]i increase results from an influx of divalent cations directly through TRPV1 channels (1). To examine whether low [Na+]o activates functional ion channels and promotes Ca2+ influx through TRPV1 channels, we simultaneously measured [Ca2+]i and current responses to the removal of [Na+]o under voltage clamp conditions in HEK293 cells expressing pTRPV1 (Fig. 4). At a holding potential of −60 mV, capsaicin (0.3 μM) and Cs+-based [Na+]o-free solution evoked an inward current concomitant with a rapid increase of [Ca2+]i. In the same cell NMDG+-based [Na+]o-free solution elicited only a small inward current, if any, together with a prominent [Ca2+]i increase. Presumably, in the former two conditions, a significant proportion of the inward current was carried by monovalent cations (Na+ or Cs+), which was responsible for the much larger inward current. To characterize their current-voltage relationship, a voltage ramp (−80 to +100 mV for 100 ms) was applied every 10 s before and during these stimuli. The current-voltage relation obtained for the [Na+]o removal showed outward rectification similar to capsaicin and exhibited a reversal potential close to 0 mV (Fig. 4B, Erev = +9.1 ± 1.4 mV (NMDG+)) and +8.6 ± 1.2 (Cs+) compared with +8.5 ± 1.4 mV for capsaicin, n = 16). The average currents at −60 and +80 mV and [Ca2+]i, induced by [Na+]o removal and capsaicin were summarized (Fig. 4C). These results suggested that [Ca2+]i responses to the removal of [Na+]o were solely mediated by Ca2+ influx across the plasma membrane and that [Na+]o removal facilitated gating of TRPV1 channels. The effects of capsazepine on current responses to the removal of [Na+]o were examined (Figs. 4, D and E). As expected, capsazepine (1 μM) significantly suppressed changes in membrane currents induced by [Na+]o removal (NMDG+ and Cs+).

[Na+]o Interacts at TRPV1 Proton Binding Residues—We next sought to determine the molecular mechanism underlying external [Na+]o regulation of TRPV1. It has been reported that protons and divalent and trivalent cations regulate TRPV1 via extracellular glutamate residues (Glu-600 and Glu-648) (13, 23, 25) and polyamines act on extracellular aspartate (Asp-646) (24), those of which are located in the pore-forming loop of TRPV1 channels. Therefore, we hypothesized that these amino acids are the position where [Na+]o interacts in TRPV1. The effects of [Na+]o removal in single-point mutants of TRPV1 that contained the neutral amino acid glutamine, asparagine, or alanine (E600Q, D646N, and E648A) were examined. These mutant channels showed differing sensitivities to protons. The proton (pH 5.5)-evoked [Ca2+]i, increases of D646N and E648A mutants were reduced significantly, whereas the responses were practically abolished by the mutation of Glu-600 to glutamine (Figs. 5, A and B). In two mutant channels (E600Q and E648A), [Ca2+]i responses to [Na+]o removal were significantly reduced, but those to capsaicin (0.3 μM) remained unchanged. Among these mutants, E600Q showed the strongest inhibitory effects on [Ca2+]i, responses to [Na+]o removal. These results indicated an important role for the acidic residues located near the pore-forming region, Glu-600 and Glu-648, in activation of TRPV1 by lowering [Na+]o.

Because experiments with TRPV1 mutants identified that putative regulatory sites for protons were related to low [Na+]o-induced activation of TRPV1 channels, we hypothesized that [Na+]o interacted with proton binding sites of TRPV1. Thus, we next examined the effects of extracellular pH on [Ca2+]i, responses to various concentrations of [Na+]o. As shown in Fig. 5C, the [Na+]o-dependent response curve was shifted to the right in the external solution with pH 7.8. The EC50 values of [Na+]o were 28.1 ± 5.1 mM for pH 7.4 and 43.4 ± 3.8 mM for pH 7.8. These results suggested that [Na+]o competitively interacted with protons at protonation sites of TRPV1 channels.

[Ca2+]i and Current Responses to [Na+]o Removal in Endogenous TRPV1—To determine whether low [Na+]o activates endogenous TRPV1, the effects of [Na+]o removal on primary culture porcine DRG neurons were examined. Cells were first exposed to NMDG+-based [Na+]o-free solution followed by
capsaicin (0.3 μM) and KCl (140 mM) to identify DRG neurons expressing TRPV1. Time-lapse images showed that the [Ca^{2+}]_{i} response evoked by removal of [Na]_{o} occurred only in the cells responding to capsaicin (cell 1 in Fig. 6A). As depicted in the actual traces (Fig. 6B), application of [Na]_{o}-free solution elicited a rapid [Ca^{2+}]_{i} increase and decreased [Na]_{i}. In the same cell, capsaicin produced a rapid [Ca^{2+}]_{i} rise concomitant with a sustained rise of [Na]_{i}. Subsequently applied KCl elicited [Ca^{2+}]_{i}, in both capsaicin-responding (cell 1) and non-responding cells (cell 2). Among 51 neurons responding to KCl, 32 cells were both [Na]_{o} removal- and capsaicin-sensitive (63%). In capsaicin-sensitive neurons, the peak [Ca^{2+}]_{i} increases evoked by removal of [Na]_{o}, capsaicin, and KCl were 129.7 ± 12.2, 131.4 ± 12.1, and 130.9 ± 9.8 nM (n = 32), respectively. The peak [Ca^{2+}]_{i} response in capsaicin-inensitive, but KCl-sensitive cells was 149 ± 12.6 nM (n = 19). There was a significant positive relation between the amplitude of the [Ca^{2+}]_{i} increase induced by [Na]_{o} removal and capsaicin (r = 0.69, p < 0.01,

FIGURE 6. Effects of external Na$^+$ removal and capsaicin on [Ca^{2+}]$_{i}$ in cultured DRG neurons expressing endogenous TRPV1. A, image under transmitted light of porcine DRG neurons. Pseudocolor time-lapse images for [Ca^{2+}]$_{i}$ (t = 4) and [Na]$_{i}$ (t = 4) corresponding to the time points before and after application of the [Na]$_{o}$-free solution or capsaicin. B, actual changes in [Ca^{2+}]$_{i}$ and [Na]$_{i}$ induced by the removal of [Na]$_{o}$, capsaicin (0.3 μM), and KCl (140 mM) in the same cells marked with the red (cell 1) or green (cell 2) arrowhead in A. C, relationship between the amplitudes of the removal of [Na]$_{o}$- and capsaicin-induced [Ca^{2+}]$_{i}$ increases in individual porcine DRG neurons (n = 32 from 4 animals). D, actual trace and summarized data showing that capsazepine (capsaz, 10 μM) reversibly suppressed the [Ca^{2+}]$_{i}$ increase induced by external Na$^+$ removal in porcine DRG neurons (n = 16 from 3 animals). E, simultaneous measurement of [Ca^{2+}]$_{i}$ and current responses to [Na]$_{o}$ removal and capsaicin in voltage-clamped porcine DRG neurons. At a holding potential of −60 mV, ramp voltages from −80 to +100 mV for 100 ms were applied at the points shown as vertical lines in the current trace and arrowheads in [Ca^{2+}]$_{i}$. Im, membrane current. F, current-voltage relations induced by the removal of [Na]$_{o}$ and capsaicin obtained with a ramp protocol are plotted. These numbers (1–4) correspond to the time points in E. Substantial augmentation of outward current induced by the removal of [Na]$_{o}$ (left) or capsaicin (right) was emphasized by green boxes. G, sensitization of [Ca^{2+}]$_{i}$ responses to capsaicin (0.1 μM) by low [Na]$_{o}$ (112 mM) in porcine DRG neurons. H, summary of changes in [Ca^{2+}]$_{i}$ induced by capsaicin (0.1 μM), before (S1) and after the application of low [Na]$_{o}$ (S2) and capsaicin (cap, 1 μM) (n = 55). *, p < 0.05. I, histogram showing the percentage of cell S2/S1 ratio in the presence of low [Na]$_{o}$.
immunoreactivity to an anti-TRPV1 antibody (Fig. 7A). In contrast, no [Ca\(^{2+}\)]\(_o\) increase occurred after [Na\(^+\)]\(_o\) removal in DRG neurons from TRPV1-null (TRPV1\(^{-/-}\)) mice (Fig. 7A and C). Again, in wild type murine DRG neurons, there was a significant positive relation between the amplitude of the [Ca\(^{2+}\)]\(_i\) increase induced by [Na\(^+\)]\(_o\) removal and capsaicin (\(r = 0.70, p < 0.01\), Fig. 7B), and [Ca\(^{2+}\)]\(_i\), responses to [Na\(^+\)]\(_o\) removal were reversibly inhibited by capsazepine (10 \(\mu\)M, Fig. 7D). These results suggested that the regulatory role of [Na\(^+\)]\(_o\) on TRPV1 activity was endogenous.

**Effects of [Na\(^+\)]\(_o\) Removal on Heterologously Expressed Human TRPV1**—Representative time-lapse [Ca\(^{2+}\)] fluorescence imaging demonstrated that the removal of [Na\(^+\)]\(_o\), elicited a [Ca\(^{2+}\)]\(_i\) increase in HEK293 cells expressing human TRPV1 (Fig. 8, A and B). In the same cells capsazepine induced a [Ca\(^{2+}\)]\(_i\) increase. The average amplitude of the [Ca\(^{2+}\)]\(_i\) increase induced by capsazepine was similar to that elicited by removal of [Na\(^+\)]\(_o\) (Fig. 8C). There was a significant positive relation between these two parameters in each cell (\(r = 0.72, p < 0.01\), Fig. 8D). [Ca\(^{2+}\)]\(_i\) responses to both [Na\(^+\)]\(_o\) removal and capsaicin were reversibly inhibited by capsazepine (Fig. 8E). In addition, the [Na\(^+\)]\(_o\) removal-induced [Ca\(^{2+}\)]\(_i\) increase was completely abolished in the presence of iodoresiniferatoxin (0.1 \(\mu\)M) or ruthenium red (10 \(\mu\)M, data not shown).

**DISCUSSION**

Our data demonstrated that lowering [Na\(^+\)]\(_o\) could directly gate and sensitize porcine and human TRPV1 channels to polymodal stimuli such as capsaicin, protons, heat, and endovanilloids in a heterogeneous expression system. Activation of TRPV1 channels and sensitizing effect on capsaicin-induced responses by low [Na\(^+\)]\(_o\) were also observed in primary culture of porcine DRG neurons. A [Ca\(^{2+}\)]\(_i\) increase induced by [Na\(^+\)]\(_o\) removal occurred in DRG neurons of wild type mice but not in TRPV1-null ones. Experiments with TRPV1 mutants indicated that extracellular negatively charged residues located near the pore-forming region were critical for the activation of TRPV1 by low [Na\(^+\)]\(_o\). These data may imply that [Na\(^+\)]\(_o\) tonically suppresses TRPV1 channels under physiological conditions. To the best of our knowledge, this is the first report that the family of TRP channels is controlled in a [Na\(^+\)]\(_o\)-dependent manner.
This represents a novel mechanism for modulating TRPV1 function and pain signaling.

There is a possibility that the [Na+]o removal activated the reverse mode of the Na+/Ca2+ exchanger and resulted in a [Ca2+]i increase. The amplitude of the [Ca2+]i increase evoked by [Na+]o removal did not change with capsaicin prestimulation, which increased [Na+]i (Figs. 1, A and B). The [Ca2+]i increase induced by [Na+]o removal was suppressed by TRPV1 antagonists in a dose-dependent manner (Fig. 1E) but not by an inhibitor of the reverse mode of the Na+/Ca2+ exchanger. These results clearly indicated that the Na+/Ca2+ exchanger was not involved in the [Ca2+]i increase evoked by [Na+]o removal in HEK293 cells expressing TRPV1 channels. Moreover, since the osmolality of solutions used in the present experiment was almost the same, osmotic effects on TRPV1 (30) were not related to the low [Na+]i-induced TRPV1 activation.

TRPV1 activation induced by [Na+]o removal is not likely due to membrane screening effects, since the [Ca2+]i increase occurred even with replacement by other monovalent cations. These results indicated that no cation used in the present study could replace the inhibitory effect of [Na+]o on TRPV1. Interestingly, application of a sucrose-based [Na+]o-free solution also produced a prompt [Ca2+]i increase in cells expressing pTRPV1. It seems, therefore, that there should be a very close relationship between [Na+]i and TRPV1 channels.

It has been reported that negatively charged residues (Glu-600 and Glu-648) located near the pore-forming region on TRPV1 are critical for opening the channel upon neutralization by protons (23). Another acidic residue, Asp-646, also contributes to the regulatory roles of cations and polyamines (24). From experiments with TRPV1 mutants (E600Q, D646N, and E648A), we confirmed that these residues were important to the sensitivity to protons. Interestingly, the low [Na+]i-induced [Ca2+]i increase significantly decreased in two mutant channels (E600Q and E648A). Particularly, in E600Q the [Ca2+]i response to low [Na+]i was substantially abolished, suggesting that these acidic residues, especially glutamate at position 600, are key negative amino acids responsible for low [Na+]i-induced TRPV1 activation. Similar to our present data, it has been reported that divalent cations and trivalent Gd3+ activate and sensitize TRPV1 via Glu-600 and Glu-648 (13, 25). In addition, positively charged polyamines interact with TRPV1 at Asp-646 and Glu-648 (24). Under extracellular alkali conditions (pH 7.8), the [Na+]o concentration-response curve was shifted to the right, suggesting that the sensitivity of TRPV1 activation by low [Na+]o increased in the presence of a low concentration of extracellular protons. Taken together, these data may indicate that [Na+]o competitively interacts with proton binding sites of TRPV1 channels.
Because the [Ca$^{2+}$] increase and outward current occurred just after the removal of [Na$^{+}$], [Ca$^{2+}$] might directly suppress TRPV1 channels under resting conditions. Moreover, current responses to low [Na$^{+}$]o were significantly suppressed by TRPV1 antagonist. When [Na$^{+}$] becomes low, TRPV1 might be relieved from the suppression of [Na$^{+}$]. This regulatory situation is analogous to the inhibitory mode of PIP$_2$. (12). It has been shown that PIP$_2$ binds to TRPV1 (31) with constitutive suppression of TRPV1, and PIP$_2$-mediated inhibition is released upon activation of phospholipase C by metabotropic receptors, which induce the hydrolysis of PIP$_2$. In addition to direct activation of TRPV1 by the removal of [Na$^{+}$], a low level of [Na$^{+}$], in the pathophysiological range sufficiently sensitized TRPV1 activation induced by not only capsaicin but also by endogenous polypeptide stimuli (protons, heat, and an endovanilloid). Therefore, it may be considered that this sensitizing effect is a cause of pain perception in hyponatremia.

The relative permeability of TRPV1 to Ca$^{2+}$ is high (the pCa/pNa ratio is 9.6) for a nonselective cation channel (1). This Ca$^{2+}$ permeability is very similar to the value (pCa/pNa $= 10.6$) reported for N-methyl-d-aspartate-type glutamate receptors (32). The N-methyl-d-aspartate channel is tonically suppressed by extracellular Mg$^{2+}$ to prevent the [Ca$^{2+}$] increase by excessive activation (33). This concept may be adapted to TRPV1 regulation, which could be constitutively suppressed by [Na$^{+}$]. It is well known that the [Ca$^{2+}$] increase through the activation of TRPV1 channels located on sensory nerve endings leads to secretion of algesic substances such as glutamate and neuropeptides (34, 35). In addition, [Ca$^{2+}$] accumulation after the activation of TRPV1 promotes neural cell death due to the excessive mitochondrial Ca$^{2+}$ overload (36). It is suggested that rapid disassembly of dynamic microtubules due to the increase of [Ca$^{2+}$] upon activation of TRPV1 influences nociception (37). Therefore, it may represent a physiological safety mechanism against a harmful Ca$^{2+}$ accumulation in TRPV1-expressing cells, including sensory neurons, under resting conditions. Similar regulation is proposed in calmodulin (CAM)-dependent desensitization, since some CAM has been suggested to be resident on TRPV1 at all times (38). Therefore, TRPV1 channels seem to be controlled via an elaborate mechanism. A putative model for [Na$^{+}$]o regulation of TRPV1 channels is shown in Fig. 8F. In light of the present data showing competition between [Na$^{+}$]o and protons, the simplest explanation presumably is that [Na$^{+}$]o interaction with Glu-600/Glu-648 prevents TRPV1 protonation. Assuming that protonation of these sites activates TRPV1 channels, the facilitation of channels activity by low [Na$^{+}$]o might arise from protonation. Further study is necessary to certify this hypothesis.

In conclusion, we have demonstrated a novel regulatory role of [Na$^{+}$]o in TRPV1 function. Identification of drugs influencing these interactions may provide the discovery of new clinically relevant drugs affecting TRPV1.

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