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Research Paper

Intracellular acidification evoked by moderate extracellular acidosis attenuates transient receptor potential V1 (TRPV1) channel activity in rat dorsal root ganglion neurons

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Transient receptor potential V1 (TRPV1) has been suggested to play an important role in detecting decreases in extracellular pH (pH_o). Results from recent *in vivo* studies, however, have suggested that TRPV1 channels play less of a role in sensing a moderately acidic pH_o ($6.0 < pH < 7.0$) than predicted from the *in vitro* experiments. A clear explanation for this discrepancy between the *in vitro* and *in vivo* data has not yet been provided. We report here that intracellular acidification induced by a moderately low pH_o (6.4) almost completely inhibited the effect of extracellular acidosis on TRPV1 activity. In our experiments, sodium acetate (20 mM), which was used to induce intracellular acidosis, attenuated the capsaicin-evoked TRPV1 current (I_{CAP}) in a reversible manner in whole-cell patch-clamp mode and shifted the concentration–response curve to the right. Likewise, the concentration–response curve was significantly shifted to the right by lowering the pH of the pipette solution from 7.2 to 6.5. In addition, application of an acidic bath solution (pH 6.4) to the intracellular side also significantly suppressed I_{CAP} in inside-out patch mode. In cell-attached patch mode, the single-channel activity of i_{CAP} was significantly attenuated by intracellular acidosis that was induced by a decrease in pH_o (6.4). These results suggested that intracellular acidification induced by a low pH_o inhibited TRPV1 activity. When studied in perforated patch mode or by acidifying the intracellular pipette solution, potentiation or activation of TRPV1 by extracellular acidosis (pH 6.4) at 37°C was almost completely inhibited. Likewise, enhancement of neuronal excitability by a moderately acidic pH_o (6.4) at a physiological temperature (37°C) was attenuated by lowering the pH of the pipette solution to 6.5 or using perforated patch mode. Taken together, these results suggest that extracellular acidosis of moderate intensity may not significantly modulate TRPV1 activity in physiological conditions at which intracellular pH can be readily affected by pH_o, and this phenomenon is due to attenuation of TRPV1 channel activity by low-pH_o-induced intracellular acidification.

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Local tissue acidosis often occurs in pathological conditions such as hypoxia/ischaemia or inflammation. The extracellular pH (pH_o) can fall to as low as 6 in these conditions (Rehncrona, 1985; Nedergaard *et al.* 1991), and this is thought to contribute to the pain associated with these conditions (Issberner *et al.* 1996; Pan *et al.* 1999; Stubbs *et al.* 2000; Sutherland *et al.* 2001; Garber, 2003). In isolated primary afferent neurons, extracellular acidosis activates various types of cationic currents (Krishtal & Pidoplichko, 1981; Bevan & Yeats, 1991; Baumann *et al.* 1996; Waldmann *et al.* 1997; Tominaga *et al.* 1998). Among them, the transient receptor potential vanilloid receptor-1 (TRPV1) was cloned as a candidate acid-activated ion channel (Caterina *et al.* 1997; Tominaga *et al.* 1998). This channel is located in small dorsal root ganglion (DRG) neurons that are important in pain processing and whose involvement in mediating inflammatory hyperalgesia has been established (Caterina *et al.* 2000; Davis *et al.* 2000).

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Protons affect TRPV1 function in three ways. First, moderate extracellular acidosis sensitizes this receptor's responses to other stimuli, such as capsaicin (CAP) and heat, leading to pain in conditions that are otherwise non-noxious (Petersen & LaMotte, 1993; Martenson *et al.* 1994; Baumann *et al.* 1996; Kress *et al.* 1996; Caterina *et al.* 1997; Tominaga *et al.* 1998). Second, severe acidosis ($pH \leq 6$) directly activates the channel (Bevan & Yeats, 1991; Baumann *et al.* 1996; Ryu *et al.* 2007; Tominaga *et al.* 1998). Third, moderate acidosis ($6.0 < pH < 7.0$) is not sufficient to activate the TRPV1 channel at room temperature, but at a physiological temperature (37°C), moderate acidosis readily activates TRPV1 (Tominaga *et al.* 1998). These results suggest that TRPV1 may function as an acid sensor by detecting moderate decreases in pH_o in normal physiological conditions. Results from recent *in vivo* studies, however, have been not consistent with the *in vitro* data. For example, Jones *et al.* (2004) showed that skin desensitization by repeated application of CAP had no significant effects on acid-induced pain in humans, suggesting only a minor role for TRPV1 as a mediator of cutaneous acid-induced pain. In addition, other studies reported that pain induced by moderate-intensity acid ($6.0 < pH < 7.0$) was mediated mainly by acid-sensing ion channels (ASICs) rather than TRPV1 channels (Ugawa *et al.* 2002; Deval *et al.* 2008). Moreover, AMG-9810, a potent TRPV1 blocker, failed to suppress mechanical allodynia in a thrombus-induced ischaemic pain model that is closely correlated with tissue acidosis in a pathophysiologically relevant pH range (Seo *et al.* 2010). These data strongly suggest that TRPV1 plays a relatively minor role in signalling acid-induced pain of moderate intensity in peripheral sensory neurons. However, a clear explanation of the discrepancy between the *in vitro* and *in vivo* data is lacking.

Changes in intracellular pH (pH_i) affect various cellular functions (Smith *et al.* 1998; Austin & Wray, 2000). In primary sensory neurons, increased anaerobic glycolysis induced by anoxia/ischaemia leads to lactic acid accumulation, causing dramatic decreases in tissue pH_o (Rehncrona, 1985; Nedergaard *et al.* 1991). This tissue acidosis can readily induce intracellular acidosis, which in turn disturbs cellular functions involving enzymes, transporters and ion channels (Henrich & Buckler, 2008). In fact, intracellular acidosis significantly inhibits current from the G-protein-coupled inwardly rectifying K^+ channel and that from ASICs (Mao *et al.* 2003; Wang *et al.* 2006). Moreover, TRPV1 acts as a proton channel to induce acidification in nociceptive neurons (Hellwig *et al.* 2004). Therefore, intracellular acidosis induced by a decrease in pH_o may affect TRPV1 channel activity and in turn modulate the effect of extracellular protons on TRPV1 activity in primary sensory neurons.

To test this hypothesis, we determined the effects of intracellular acidification transmitted by moderate extracellular acidosis on TRPV1 activity in rat DRG neurons by analysing single-channel activity in whole-cell and perforated patch-clamp modes as well as in current-clamp mode. Our results indicate that intracellular acidosis attenuates CAP-activated TRPV1 activity and that moderate extracellular acidosis (pH 6.4), which causes intracellular acidosis, barely potentiates or activates TRPV1 inphysiological conditions in which pH_i can be readily affected by pH_o.

Methods

This study was carried out in accordance with NIH regulations for animal care and with the approval of the Institutional Animal Care and Use Committee of Yonsei University, Seoul, Korea.

Acutely dissociated DRG neurons

Dorsal root ganglion neurons were obtained from adult (200–300 g) male Sprague–Dawley rats. Dissociated DRG cells were prepared as described previously with some modifications (Steinhoff *et al.* 2000). Rats were anaesthetized with enflurane (3%, 2–3 min) administered by inhalation in a closed chamber. After induction of general anaesthesia, rats were decapitated, and vertebrae from the thoracic and lumbar regions were extracted and then placed in ice-cold Dulbecco's phosphate-buffered saline. Twenty DRGs were dissected from the thoracic and lumbar regions and incubated at 37°C for 35 min in Earle's balanced salt solution with 0.6 mg ml^{-1} collagenase type 1A (Sigma, St Louis, MO, USA) and 0.3 mg ml^{-1} papain (Sigma). After incubation, the ganglia were washed with Dulbecco's modified Eagle's medium at room temperature. Single neuronal somata were obtained at room temperature by triturating the ganglia in series through three glass pipettes that were fire polished to progressively decreasing diameters. Cells were plated onto a Matrigel (BD Biosciences, Bedford, MA, USA)-coated glass coverslip, placed in a culture dish, and incubated with Dulbecco's modified Eagle's medium, 10% fetal bovine serum and 1% penicillin/streptomycin (50% and 50% each) for at least 6 h and used within 12 h for whole-cell recordings.

Electrophysiology

Recordings were made using standard whole-cell techniques. Electrodes with a resistance between 3 and 5 MΩ when filled with internal solution were pulled from borosilicate glass microcapillary tubes (Sutter Instrument Co., Novato, CA, USA). Recordings were made with an Axopatch 200A patch-clamp amplifier (Molecular Devices, Foster City, CA, USA). Voltage and current commands and digitization of membrane voltages

and currents were controlled using a Digidata 1322A interfaced with Clampex 9.2 of the pCLAMP software package (Molecular Devices), running on an IBM-compatible computer. We analysed data using Clampfit (Molecular Devices) and Prism 4.0 (GraphPad, San Diego, CA, USA). Currents were low-pass filtered at 2 kHz. Capacitance values were taken from automatically calculated recordings using pCLAMP 9.2 software. Action potentials were recorded in the current-clamp mode. Membrane potential measurements were low-pass filtered at 10 kHz.

Perforated patch-clamp mode was used to investigate the effects of extracellular acidosis on TRPV1 activity in physiological conditions in which pH_i can be readily affected by pH_o. For the perforated patch-clamp experiments, amphotericin B (Sigma) was prepared by dissolving it in dimethyl sulfoxide to a concentration of 60 mg ml^{-1} with sonication, and then this stock solution was diluted to 240 mg ml^{-1} with an internal solution, again with sonication. Therefore, in the present study, physiological conditions refer to the conditions in which pH_i can be readily affected by pH_o at a physiological temperature (37°C).

Multiple independently controlled syringes served as reservoirs for a gravity-driven fast drug perfusion system. Manually controlled valves were used to switch between solutions. All experiments were performed at room temperature unless otherwise specified.

Solutions and chemicals

The normal bath solution for the whole-cell, perforated patch-clamp, and current-clamp experiments contained the following (mM): 134 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 14 D-glucose and 10.5 Hepes, adjusted to pH 7.4 with NaOH. The acidic bath solution for the whole-cell and perforated patch-clamp experiments was the same as that used for the normal bath solution, but adjusted to pH 6.4 with NaOH. The Ca^{2+}-free bath solution for the whole-cell and perforated patch-clamp experiments was made by omitting $CaCl_2$ from the normal bath solution. The bath solution and pipette solution for single-channel recording contained the following (mM): 143 KCl, 1.2 $MgCl_2$, 14 D-glucose and 10.5 Hepes, adjusted to pH 7.4 with KOH. The Ca^{2+}-free bath solution was used to prevent Ca^{2+}-induced desensitization of TRPV1. The normal pipette solution used for the whole-cell and perforated patch voltage-clamp recordings contained the following (mM): 140 $CsCl_2$, 1.2 $MgCl_2$, 4 MgATP, 0.4 Na_2GTP, 10 phosphocreatine, 10 Hepes and 10 EGTA, adjusted to pH 7.2 with CsOH. The acidic pipette solution had the same composition as the normal pipette solution, but was adjusted to pH 6.5 with HCl. The pipette solution used for current-clamp recordings contained the following (mM): 113 potassium gluconate, 30 KCl, 1.2 $MgCl_2$, 4 MgATP, 0.4 Na_2GTP, 10 phosphocreatine, 10 Hepes and 0.05 EGTA, adjusted to pH 7.2 with KOH. Dulbecco's phosphate-buffered saline with glucose contained the following (mM): 137.93 NaCl, 2.67 KCl, 1.47 KH_2PO_4, 8.06 $Na_2HPO_4.7H_2O$ and 5.56 D-glucose, adjusted to pH 7.4.

Earle's balanced salt solution, Dulbecco's modified Eagle's medium, fetal bovine serum and penicillin/streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). All other chemicals were purchased from Sigma. Drug stocks were prepared as 1 mM CAP in dimethyl sulfoxide and 10 mM capsazepine (CZP) in dimethyl sulfoxide. Drugs were freshly diluted to the appropriate concentrations at the time of the experiments.

Data analysis

Data are presented as means ± SEM, with the number of experiments indicated in parentheses. The concentration–response curves of sodium acetate-induced TRPV1 inhibition were calculated by fitting the data to a single-site binding isotherm with least-squares non-linear regression, and two curve fits were compared by the F-test using Prism 4.0 (GraphPad). We used Student's unpaired *t* test to compare two groups. We used one-way ANOVA to compare multiple groups using Turkey's *post hoc* test. Differences were considered to be significant at $P < 0.05$.

Results

Effects of sodium acetate on the capsaicin-evoked TRPV1 current

We performed experiments using small (23–26 μm) DRG neurons only, because these are known to be involved in nociceptive processing (Coderre *et al.* 1993; Levine *et al.* 1993; Cardenas *et al.* 1995; Snider & McMahon, 1998). A Ca^{2+}-free bath solution was used to determine the effects of intracellular acidification on the capsaicin-evoked TRPV1 current (I_{CAP}) more clearly by preventing Ca^{2+}-induced desensitization of I_{CAP} (Szallasi *et al.* 1994; Liu & Simon, 1996; Petersen *et al.* 1996; Liu *et al.* 1997). When we applied CAP (300 nM) to small DRG neurons with the membrane potential held at −40 mV in whole-cell patch-clamp mode, large inward currents developed. These were completely abolished by 10 μM CZP, a potent antagonist of TRPV1 (Oh *et al.* 1996; Jung *et al.* 1999; Fig. 1*A*). The currents displayed an outwardly rectifying current–voltage relationship and had reversal potentials close to 0 mV, which are typical features of TRPV1 (Oh *et al.* 1996; Fig. 1*B*). To determine whether intracellular acidification affected the TRPV1 current, we applied sodium acetate (20 mM) to the 300 nM CAP-evoked TRPV1 current. This weak acid acetate transports protons and decreases pH_i

in a dose-dependent manner (Li *et al.* 1996; Cho *et al.* 2007). As shown in Fig. 1*A*, sodium acetate (20 mM) attenuated I_{CAP} in a reversible manner by $68 \pm 0.8\%$ ($P < 0.01$, $n = 5$). This attenuation occurred through the entire range of membrane potentials tested ($n = 5$; Fig. 1*B*), and the degree of I_{CAP} inhibition by sodium acetate decreased with membrane potential depolarization (Fig. 1*C*). To determine the effects of sodium acetate on the concentration–response relationship of I_{CAP}, CAP was applied externally to small DRG neurons cumulatively at concentrations of 0.01–10 μM (Fig. 1*D*). As shown in Figs 1*D* and *E*, application of 20 mM sodium acetate resulted in a significant rightwards shift of the concentration–response curve (EC_{50} before sodium acetate treatment, 142 ± 1.3 nM, $n = 5$; and EC_{50} after sodium acetate treatment, 288 ± 22.2 nM, $n = 5$; $P < 0.01$). To test the effects of intracellular acidification on I_{CAP} more directly, we compared the concentration–response curve of I_{CAP} measured using a more acidic pipette solution (pH 6.5) than the normal pipette solution (pH 7.2). Similar to the sodium acetate experiments, CAP was applied externally to small DRG neurons cumulatively at concentrations of 0.01–10 μM (Fig. 1*D*). The concentration–response curve shifted significantly to

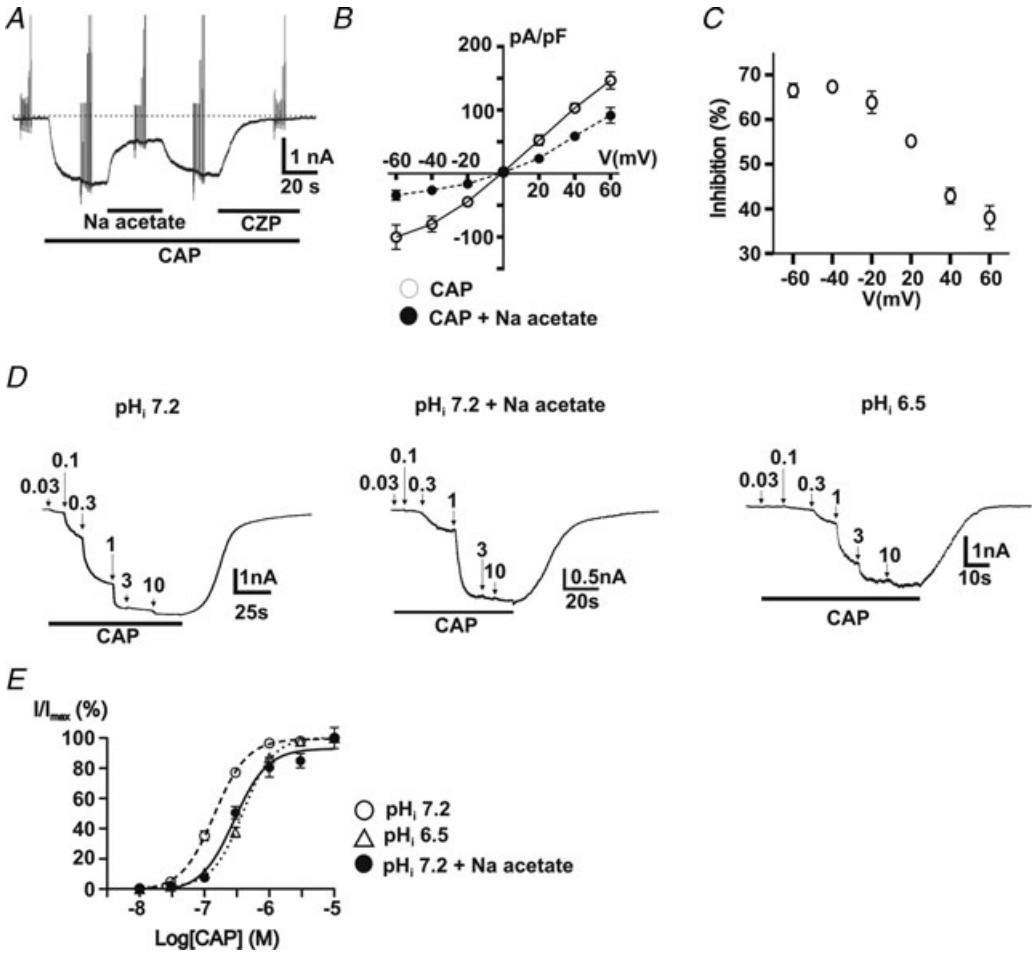

Figure 1. Effects of sodium acetate on the capsaicin-evoked TRPV1 current (I_{CAP}) of rat dorsal root ganglion (DRG) neurons
A, representative traces of I_{CAP} in the presence and absence of 20 mM sodium acetate. The I_{CAP} was evoked by application of 300 nM capsaicin (CAP) at a holding potential of –40 mV after at least 3 min from breakthrough of the patch membrane. The I_{CAP} was completely blocked by 10 micoM capsazepine (CZP). After the I_{CAP} amplitude was stabilized, 20 mM sodium acetate was applied to the cell from a micropipette placed close to the cell. *B*, the current–voltage relationship curve of I_{CAP} measured at depolarizing pulses from –60 to 60 mV in the absence or presence of 20 mM sodium acetate. *C*, voltage dependence of the effect of sodium acetate on I_{CAP}. The ratios of the I_{CAP} amplitudes in the presence sodium acetate (20 mM) to those of control I_{CAP} amplitudes were plotted against membrane potentials using the data from Fig. 1*B*. *D*, representative traces of the effect of intracellular acidification on I_{CAP} by application of 20 mM sodium acetate or lowering pH_i to 6.5. *E*, Effects of intracellular acidification on concentration–response curve of I_{CAP} by application of 20 mM sodium acetate or lowering pH_i to 6.5. The I_{CAP} amplitudes were normalized to those from cells treated with 3 μM CAP without sodium acetate.

the right when acidic pipette solution (pH 6.5) was used compared with normal pipette solution [pH 7.2; EC_{50} in acidic conditions (pH 6.5), 392 ± 4.5 nM, $n = 7$; $P < 0.01$; Fig. 1*D* (right trace) and *E*]. Taken together, these data suggest that intracellular acidosis significantly attenuates the activity of TRPV1.

Characteristics of single-channel currents (i_{CAP}) in inside-out configuration

We characterized the single-channel properties of TRPV1 by applying CAP to the bath (intracellular side) solution in inside-out configuration. Capsaicin and CZP are highly lipid soluble and bind to the cytosolic domain of TRPV1 (Jung *et al.* 1999). Therefore, CAP applied to the bath solution activates TRPV1 in the inside-out configuration. As shown in Fig. 2*A*, channel activity was not observed when the standard bath solution was applied to an inside-out patch held at –40 mV. When CAP (300 nM) was introduced into the bath solution, rapid activation of single-channel currents was observed within seconds, as previously reported (Jung *et al.* 1999; Kwak *et al.* 2000), and this current was almost completely abolished by 10 μM CZP (Fig. 2*A*). These results are summarized in Fig. 2*B*. The NP_o of single channels which was calculated as a product of the number of channel (N) in the patch and channel open probability (P_o) induced by intracellular CAP increased from 0.00 to 0.17 ± 0.06 ($n = 5$). However, this channel activity (i_{CAP}) was completely abolished by CZP (0.01 ± 0.01 for NP_o of CAP plus CZP; $n = 5$). The opening of the single channel at –60 to +60 mV in symmetrical 143 mM K^+ is shown in Fig. 2*C*. The current–voltage relationship curve was obtained by plotting the mean amplitude against the membrane potential (Fig. 2*D*). As shown in Fig. 2*E*, single-channel currents evoked by CAP exhibited outward rectification, and the single-channel slope conductance was 45.6 ± 2.1 pS at –60 mV and 72.1 ± 10.8 pS at 60 mV, consistent with previous reports (Oh *et al.* 1996; Jung *et al.* 1999; $n = 5$).

Effects of intracellular acidification on i_{CAP}

To provide more detailed evidence that intracellular acidification attenuated i_{CAP}, we examined the effects of an acidic bath solution using isolated membrane patches in inside-out configuration. Openings of single channels at –40 mV in symmetrical 143 mM K^+ are shown in Fig. 3*A*. Capsaicin (300 nM) activated i_{CAP} rapidly. When

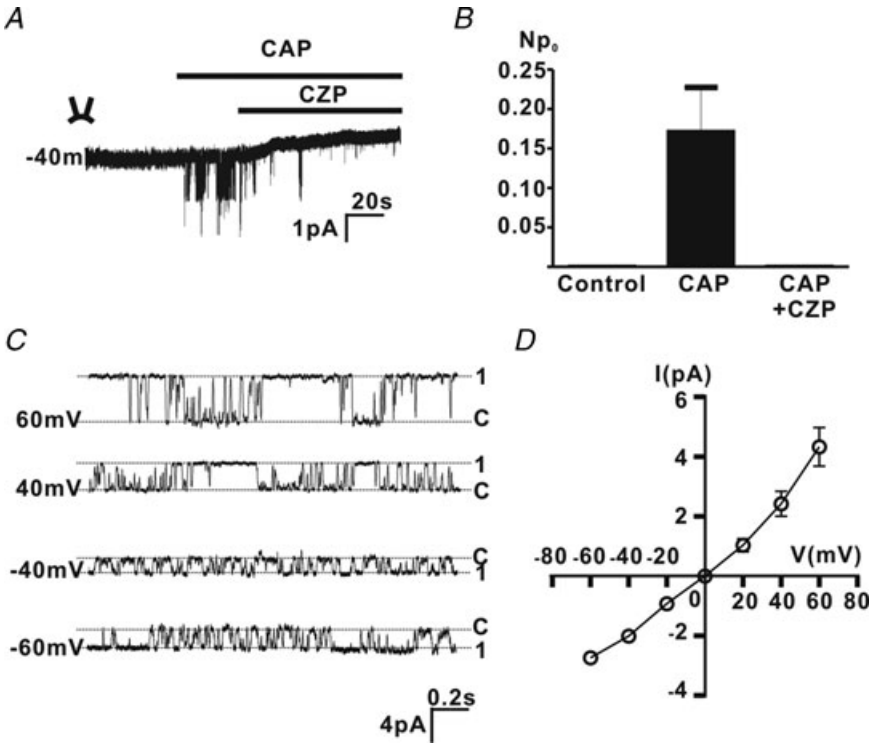

Figure 2. Characteristics of single-channel i_{CAP} in inside-out mode
A, Rrepresentative traces of single-channel i_{CAP} in inside-out mode. An inside-out membrane patch was held at -–40 mV in a symmetrical 143 mM KCl bath solution. After an inside-out patch formed, CAP (300 nM) or CAP plus CZP (10 μM) was applied to the patch from a micropipette placed close to the cell. *B*), NP_o of the channel obtained when CAP or CAP plus CZP was applied to the intracellular surface of patch membranes ($n = 5$). *C*, representative traces of a single-channel i_{CAP} held at different membrane potentials ranging from –60 to +60 mV. *D*, current–voltage relationship of the single-channel i_{CAP}. Each data point is the average of five patches in inside-out mode.

an acidic (pH 6.4) bath solution containing 300 nM CAP was applied to the same patch, channel activity decreased significantly (Fig. 3*A*). These results are summarized in Fig. 3*B*, which shows that the channel activity of i_{CAP}, expressed as NP_o, was significantly attenuated by intracellular acidification (0.69 ± 0.2 at pH 7.4; 0.40 ± 0.1 at pH 6.4; $P < 0.05$; $n = 5$). Amplitude histograms were obtained from channel openings at –40 mV (Fig. 3*C*). Single-channel slope conductance at –40 mV was not affected by intracellular acidification (50.3 ± 4.6 pS at pH 7.4 and 51.0 ± 3.5 pS at pH 6.4; $n = 5$; Fig. 3*D*).

These data suggest that intracellular acidification attenuates TRPV1 channel activity in rat DRG neurons not by decreasing the amplitude of single-channel conductance, but by decreasing the probability of an open i_{CAP}.

Intracellular acidification induced by a decrease in pH_o attenuates TRPV1 channel activity

To determine whether intracellular acidification caused by a decrease in pH_o affected the activity of TRPV1, we measured I_{CAP} in cell-attached patches to protect TRPV1 channels located in the membrane patches from potentiating in the acidic bath solution. In this configuration, capsaicin and capsazepine applied in the bath solution can readily activate and block TRPV1 because they are lipid soluble and can cross the membrane to access the channels. In addition, a low pH_o in the bath solution produced intracellular acidosis exclusively without affecting TRPV1 activity. As shown in Fig. 4*A*, when CAP (300 nM) was added to the usual (pH 7.4) bath solution, a rapid inward current developed, similar

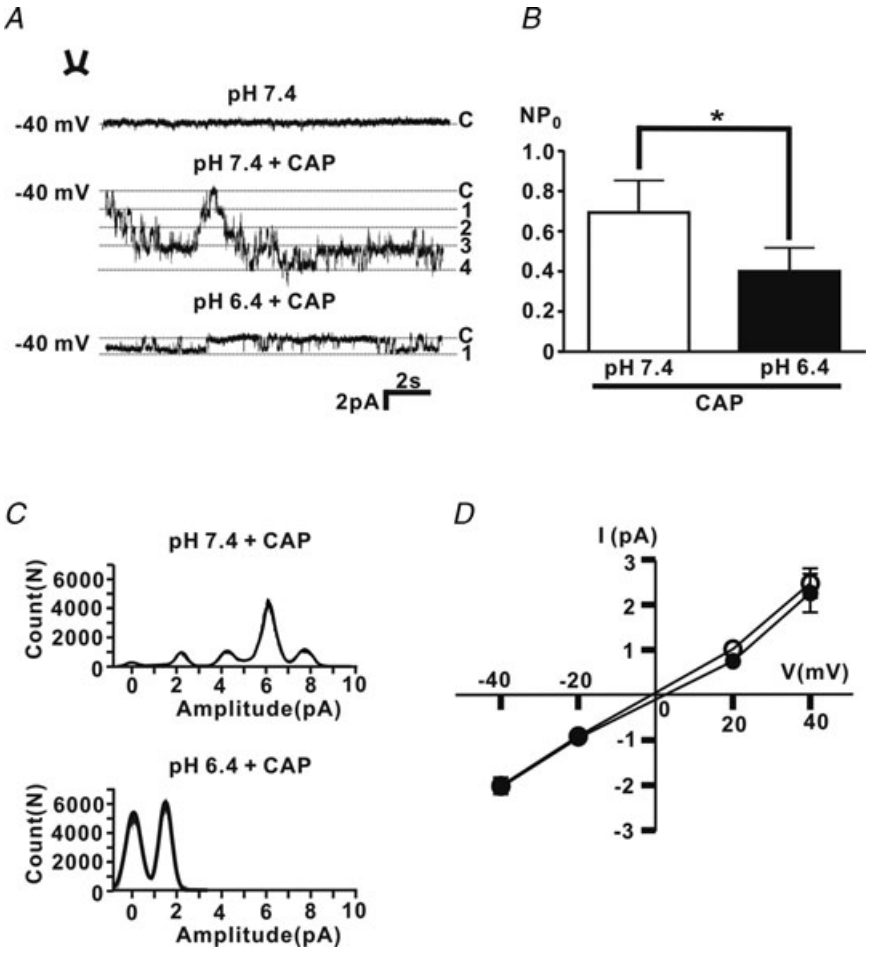

Figure 3. Attenuation of i_{CAP} activity by intracellular acidification
Representative traces of i_{CAP} at pH_i 7.4 and pH_i 6.4 in inside-out mode. After an inside-out patch was formed, control (pH 7.4) or acidic bath solutions (pH 6.4) were applied to the intracellular side of the membrane. *B*, summary of single-channel activity (NP_o) of i_{CAP} at pH_i 7.4 and pH_i 6.4 in inside-out mode. *C*, an amplitude histogram of i_{CAP} channel openings when CAP (300 nM) was applied to the intracellular surface of patch membranes in control (pH 7.4) or acidic bath solutions (pH 6.4). *D*, current–voltage relationship of single-channel i_{CAP} in control (pH 7.4; open circles) or acidic bath solutions (pH 6.4; filled circles). Each data point is the average of five patches in inside-out mode.

to Fig. 2*A*. This current was significantly decreased in a reversible manner by application of the acidic (pH 6.4) bath solution containing CAP (300 nM) and abolished by CZP (10 μM; Fig. 4*A* and *B*). These results are summarized in Fig. 4*C* and suggest that the channel activity of i_{CAP}, expressed as NP_o, was significantly attenuated by intracellular acidification induced by a decrease in pH_o (2.09 ± 1.4 at pH 7.4, 72.0 ± 7.4 at pH 7.4 with or without CAP, 35.71 ± 7.8 at pH 6.4 with or without CAP, 5.53 ± 1.6 at pH 7.4 with or without CAP with or without CZP; pH 7.4 with CAP *versus* pH 6.4 with CAP, $P < 0.05$; $n = 5$).

Moderate extracellular acidosis does not potentiate TRPV1 channel activity in physiological conditions

Extracellular acidosis readily potentiates TRPV1 channel activity (Tominaga *et al.* 1998; Baumann & Martenson, 2000; Neelands *et al.* 2005), while intracellular acidosis attenuates TRPV1 channel activity, as shown in Figs 1 and 3. Therefore, it is possible that intracellular acidification transmitted by a moderately low pH_o may attenuate the potentiating effect of low pH_o on TRPV1 channel activity. To investigate this possibility, we compared the effect of moderate low pH_o on the I_{CAP} at acidic pH_i (6.5) and normal pH_i (7.2). We used the usual bath solution containing Ca^{2+} (2 mM). As shown in Fig. 5*A*, CAP (300 nM) evoked large inward currents with apparent desensitization. The acidic pH (6.4) strongly potentiated I_{CAP} when the usual pipette solution (pH 7.2) was used in whole-cell patch-clamp mode. However, when acidic pipette solution (pH 6.5) was used, the acidic pH (6.4) failed to augment I_{CAP} (Fig. 5*B*), suggesting that intracellular acidosis may offset the potentiating effect of low pH_o on TRPV1 activity [$191 \pm 50.6\%$ increase using the usual pipette solution (pH 7.2), $n = 6$, *versus* $5 \pm 3.7\%$ increase using acidic pipette solution (pH 6.5), $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$].

Extracellular acidosis can readily induce intracellular acidosis (Henrich & Buckler, 2008). Therefore, intracellular acidosis transmitted by a low pH_o may offset the potentiating effect of low pH_o on TRPV1 activity. To investigate this hypothesis, we compared the effect of a moderately low pH on TRPV1 in whole-cell patch-clamp mode and perforated patch-clamp mode, in which the physiological intracellular milieu is maintained. As shown in Fig. 5*A* (right panel), the moderately acidic pH (6.4) failed to augment I_{CAP} in perforated

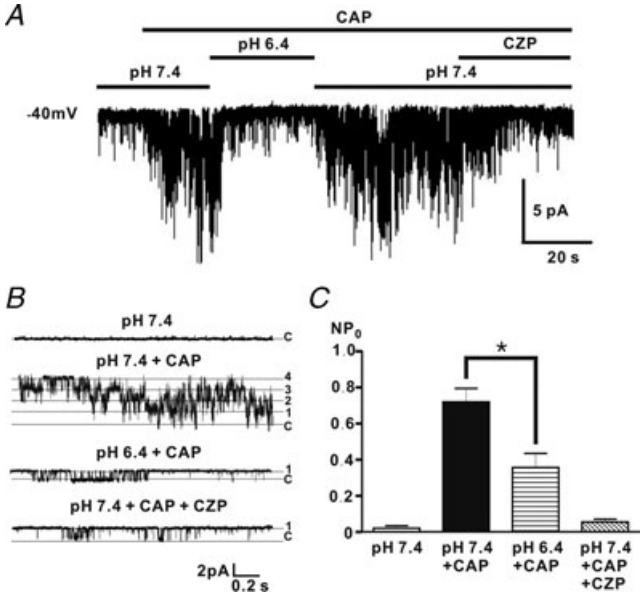

Figure 4. Intracellular acidosis induced by a decrease in pH_o attenuates I_{CAP} activity
A, Representative traces of extracellular acidosis (pH 6.4)-induced I_{CAP} attenuation in cell-attached patch mode. After a cell-attached patch was formed, CAP (300 nM) was applied to the extracellular side of the membrane in control (pH 7.4) or acidic bath solutions (pH 6.4). *B*, single-channel openings of i_{CAP} on an expanded time scale. 'C' represents the closed state of the channel and numbers indicate the count of single-channel openings. *C*, summary of single-channel activity (NP_o) of i_{CAP} at pH_o 7.4 and pH_o 6.4 in cell-attached patch mode.

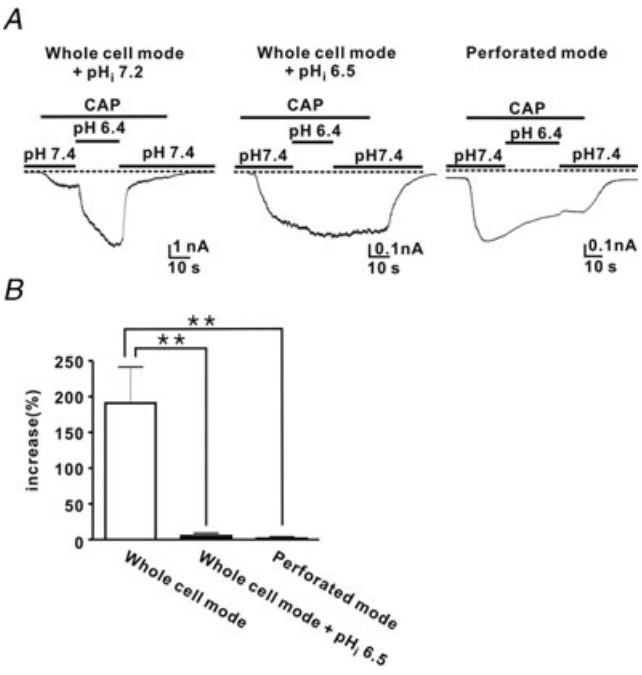

Figure 5. Moderate extracellular acidosis does not potentiate TRPV1 activity in physiological conditions
A, representative traces showing the effect of acidic pH_o (6.4) on I_{CAP} in normal bath solution. The I_{CAP} in whole-cell patch mode or perforated patch mode was evoked by application of 300 nM CAP at a holding potential of –40 mV. *B*, comparison of the effect of acidic pH_o on I_{CAP} between conventional whole-cell and perforated patch-clamp mode. $^{**}P < 0.01$ significant difference between the two groups.

patch-clamp mode (191.02 ± 50.6% increase for whole-cell patch-clamp mode, $n = 6$, *versus* 1.64 ± 2.3% increase for perforated patch-clamp mode, $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$).

These results suggest that intracellular acidosis transmitted by low pH_o offsets the potentiating effects of low pH_o on TRPV1 activity in physiological conditions.

Moderate extracellular acidosis does not activate TRPV1 in physiological conditions

Moderate acidosis ($6.0 < pH < 7.0$) has been suggested only to augment TRPV1 activity, without directly activating TRPV1. However, at a physiological temperature (37°C), moderate acidosis directly activates TRPV1 to provoke an inflammatory pain sensation (Tominaga *et al.* 1998; Jordt *et al.* 2000). Therefore, it is possible that intracellular acidification may inhibit I_{TRPV1} activated directly by protons in physiological conditions. To investigate this possibility, we compared the amplitude of I_{TRPV1} evoked by moderate acidosis (pH 6.4) at a physiological temperature (37°C) as measured by whole-cell patch mode at acidic pH_i (6.5) and normal pH_i (7.2). Subsequently, we confirmed that the DRG neurons tested had TRPV1 channels by recording I_{TRPV1} after CAP (100 nM) application. The amplitude of the proton-activated I_{TRPV1} was normalized by the amplitude of the CAP (100 nM)-evoked I_{CAP}. As shown in Fig. 6*A*, the moderately acidic pH (6.4) activated TRPV1 using the usual pipette solution (pH 7.2) at a physiological temperature (37°C) in whole-cell patch-clamp mode. However, when acidic pipette solution (pH 6.5) was used, the moderately acidic pH (6.4) failed to activate TRPV1 [18 ± 2.7% increase using the usual pipette solution (pH 7.2), $n = 6$, *versus* 2 ± 1.4% increase using acidic pipette solution (pH 6.5), $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$; Fig. 6*A* and *C*]. These results indicate that intracellular acidification may attenuate the TRPV1-activating effect of a moderately acidic pH_o. To investigate whether intracellular acidification transmitted by low pH_o affects the proton-activated I_{TRPV1}, we compared the amplitude of I_{TRPV1} evoked by moderate acidosis (pH 6.4) at a physiological temperature (37°C) as measured in whole-cell patch-clamp mode and perforated patch-clamp mode. As shown in Fig. 6*A* (right trace), the acidic pH (6.5) failed to activate I_{TRPV1} in perforated patch-clamp mode (18 ± 2.7% increase for whole-cell patch-clamp mode, $n = 6$, *versus* 1 ± 0.1% increase for perforated patch-clamp mode, $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$). These results are summarized in Fig. 6*B*.

Taken together, these results demonstrate that moderate acidosis barely evokes the proton-activated TRPV1 current in physiological conditions because of intracellular acidification induced by the moderately low pH_o.

Moderate extracellular acidosis does not enhance neuronal excitability in DRG neurons by affecting TRPV1 activity in physiological conditions

The data in the previous subsection indicate that intracellular acidification induced by a moderately acidic pH_o may readily attenuate augmentation of TRPV1 excitability at a physiological temperature (37°C). To test this hypothesis, we compared the effects of moderate extracellular acidosis (pH 6.4) on neuronal excitability as measured in whole-cell current-clamp mode with acidic pH_i (6.5) and normal pH_i (7.2) at 37°C. As shown in Fig. 7*A* (left trace), a moderately acidic pH_o (6.4) significantly depolarized the membrane potential to trigger burst action potentials when the usual pipette solution (pH 7.2) was used in CAP (100 nM)-responsive neurons. These depolarization characteristics are consistent with TRPV1-mediated depolarization (e.g.

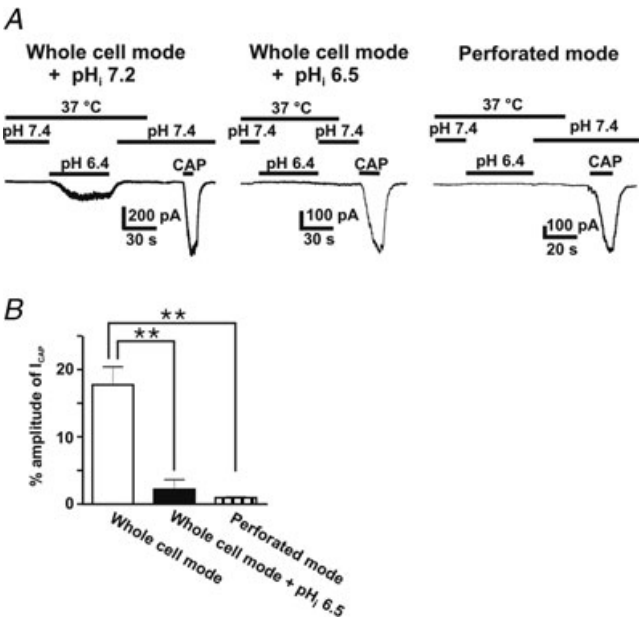

Figure 6. Moderate extracellular acidosis does not activate the TRPV1 current in physiological conditions
A, representative traces showing activation of TRPV1 by acidic pH_o (6.4) at a physiological temperature (37°C) in the usual bath solution. The I_{TRPV1} was evoked by application of moderately acidic bath solution (pH 6.4) at a holding potential of –40 mV in whole-cell or perforated patch-clamp mode. Intracellular acidification in whole-cell patch -clamp mode was performed by using acidic pipette solution (pH 6.5). *B*, comparison of activation of TRPV1 by acidic pH_o in conventional whole-cell and perforated patch modes. The amplitudes of I_{CAP} were normalized to those evoked by 100 nM CAP. $^{**}P < 0.01$ significant difference between the two groups.

slowly developing depolarization without a decay during application of acidic bath solution; Kress *et al.* 1996; Deval *et al.* 2008; Blanchard & Kellenberger, 2011). However, a moderately acidic pH_o (6.4) depolarized membrane potential negligibly without triggering action potentials when acidic pipette solution (pH 6.5) was used (31 ± 5.3 mV depolarization for normal pH_i, $n = 6$, *versus* 10.2 ± 1.8 mV depolarization for acidic pH_i, $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$; Fig. 7*A*, middle trace). To investigate whether intracellular acidification induced by a moderately low pH_o regulated the low pH_o-activated TRPV1 current, we compared the effects of moderate extracellular acidosis (pH 6.4) on neuronal excitability at a physiological temperature (37°C) using whole-cell current-clamp and perforated current-clamp mode. A moderately acidic pH (6.4) depolarized membrane potential minimally without triggering action potentials in perforated current-clamp mode (31 ± 5.3 mV depolarization for whole-cell current-clamp mode, $n = 6$, *versus* 6.6 ± 1.4 mV depolarization for perforated current-clamp mode, $n = 6$; one-way ANOVA, Turkey's *post hoc* test, $P < 0.01$; Fig. 7*A*, right trace). These results are summarized in Fig. 7*B*.

These results indicate that moderate extracellular acidosis does not significantly enhance neuronal excitability through TRPV1 activation in physiological conditions as a result of low pH_o-induced intracellular acidification.

Discussion

As mentioned in the Introduction, the excitatory response of TRPV1 to acidosis results from at least three different mechanisms. First, a moderately low pH_o potentiates TRPV1 activity by lowering the threshold for channel activation by stimuli (Kress *et al.* 1996; Tominaga *et al.* 1998; Neelands *et al.* 2005). Second, severe extracellular acidosis (pH_o less than pH 6.0) directly activates the TRPV1 current. Third, moderate acidosis ($6.0 < pH < 7.0$) is not sufficient to activate the TRPV1 current directly at room temperature, while at a physiological temperature (37°C), the TRPV1 current can be activated by the same pH (Tominaga *et al.* 1998). Consistent with these results, we observed that a pH_o of 6.4 significantly potentiated I_{CAP} (Fig. 5*A*) and directly activated the TRPV1 current at a physiological

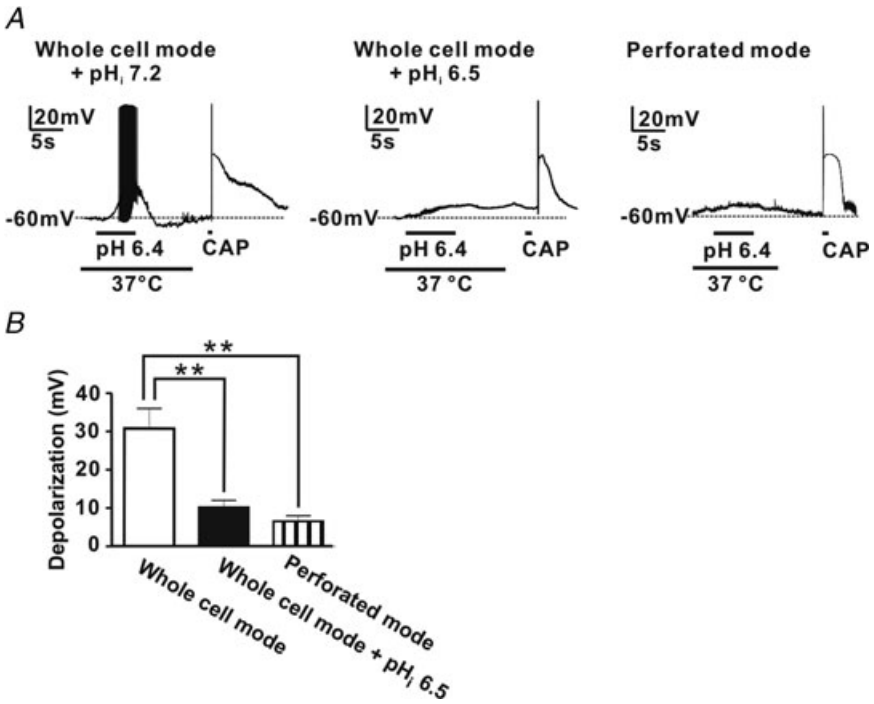

Figure 7. Moderate extracellular acidosis does not enhance neuronal excitability in physiological conditions
A, representative traces of neuronal excitability evoked by a moderately acidic pH_o at a physiological temperature (37°C) in current-clamp mode. Neuronal excitation was induced by application of a moderately acidic bath solution (pH 6.4) in whole-cell or perforated patch-clamp mode. Intracellular acidification in whole-cell patch-clamp mode was performed by using acidic pipette solution (pH 6.5). *B*, comparison of neuronal excitability evoked by a moderately acidic pH_o in whole-cell and perforated patch-clamp modes. $^{**}P < 0.01$ significant difference between the two groups.

temperature (37°C) in whole-cell patch-clamp mode (Fig. 6*A*). These results suggest that in rats, moderate extracellular acidosis may regulate the excitability of primary sensory neurons in physiological conditions. However, despite these electrophysiological data, it is still unclear what role TRPV1 plays in moderate-acid-evoked signalling. In fact, although some *in vitro* experiments have suggested that TRPV1 can serve as a sensor of severe acidosis (pH ∼5.0; Santicioli *et al.* 1993; Caterina *et al.* 2000; Davis *et al.* 2000; Leffler *et al.* 2006), others have not (Bevan *et al.* 1992; Bevan & Geppetti, 1994). In addition, clear *in vivo* evidence that TRPV1 is activated in response to a moderately acidic pH_o ($6.0 < pH < 7.0$) in physiological conditions is lacking. Recent results obtained from *in vivo* studies in humans and rats are not consistent with those obtained from the *in vitro* studies reporting that TRPV1 plays a major role in moderate acid signalling. For example, Jones *et al.* (2004) showed that acid-induced pain is significantly attenuated by amiloride and non-steroidal anti-inflammatory drugs, which block ASICs (Waldmann *et al.* 1997; Voilley *et al.* 2001), but skin desensitization by repeated application of CAP had no significant effects on acid-induced pain. In addition, acid-induced pain elicited by direct perfusion of an acidic solution with a pH between 7.0 and 6.0 was significantly associated with ASICs rather than TRPV1 channels (Ugawa *et al.* 2002; Deval *et al.* 2008). Moreover, mechanical allodynia in a thrombus-induced ischaemic pain model, which is closely correlated with tissue acidosis, was not significantly suppressed by AMG-9810, a potent TRPV1 blocker, but by amiloride (Seo *et al.* 2010). These data strongly suggest that TRPV1 in peripheral sensory neurons does not play a major role in signalling a decrease in the external pH. However, a precise mechanism to account for this discrepancy has not been elucidated.

During anoxia/ischaemia-like conditions, a low pH_o induces intracellular acidification in the cells that comprise the peripheral sensory nervous system. This in turn disturbs the cellular functions of enzymes, transporters and ion channels (Henrich & Buckler, 2008). In the present study, sodium acetate (20 mM) induced intracellular acidosis and strongly attenuated I_{CAP} in a voltage-dependant manner (Fig. 1). Furthermore, when the pH_i was maintained at 6.4 by decreasing the pH of the pipette solution, the concentration–response curve shifted to the right compared with when standard pipette solution (pH 7.2) was used (Fig. 1*D* and *E*). In addition, direct application of acidic bath solution (pH 6.4) to the intracellular side significantly reduced I_{CAP} activity (Fig. 3). In cell-attached patch mode, the single-channel activity of i_{CAP} was significantly attenuated by intracellular acidification induced by a decrease in pH_o (6.4). These data indicate that intracellular acidification induced by a moderately acidic pH_o significantly suppressed TRPV1 activity in rat DRG neurons.

At least three models have been suggested to explain the voltage-dependent reduction of current by protons. First, protons can bind to sites within the transmembrane electric field to block ion flow (Woodhull, 1973). Second, voltage-dependent blockage of current may be due to proton-dependent modification of channel gating (Campbell, 1982). Third, protons may titrate surface negative charges to reduce local ionic concentrations near the channel (Drouin & Neumcke, 1974). In the present study, we were not able to determine the precise mechanism underlying the voltage-dependent inhibition of I_{TRPV1} by intracellular protons. However, we argue that proton-dependent modification of channel gating may be involved in the inhibition of I_{TRPV1} by intracellular protons, because intracellular protons significantly reduced I_{CAP} activity by decreasing the probability of an open i_{CAP} without a change in single-channel conductance (Fig. 3).

Taken together, our results suggest that intracellular acidification transmitted by a moderately low pH_o (6.4) may offset the potentiating or activating effect of low pH_o on TRPV1 activity. Most *in vitro* studies have not investigated this possibility, because they were designed to study the effects of acidosis on TRPV1 activity using a whole-cell patch-clamp configuration with a Hepes (5–10 mM)-containing pipette solution (Tominaga *et al.* 1998; Ryu *et al.* 2003, 2007; Neelands *et al.* 2005). Hepes is a potent acid–base buffer, so pH_i changes induced by extracellular acidosis can be artificially prevented by a Hepes-containing pipette solution. Therefore, to determine the effects of extracellular acidosis on TRPV1 activity more precisely, it is essential to examine the effect of extracellular acidosis on TRPV1 in whole-cell patch-clamp mode with the pipette solution clamped to a more acidic pH (6.5). In addition, to determine the effect of extracellular acidosis on TRPV1 in physiological conditions, it is necessary to test the effects of extracellular acidosis on TRPV1 in perforated patch-clamp mode, which allows maintenance of the intracellular milieu. In the present study, a moderately acidic pH (pH 6.4) strongly potentiated I_{CAP} in whole-cell patch-clamp mode (Fig. 5*A*). Surprisingly, however, the acidic pH (pH 6.4) barely affected I_{CAP} in whole-cell patch-clamp mode with acidic pipette solution (pH 6.5) or perforated patch-clamp mode (Fig. 5*B*). In addition, a moderately acidic pH (pH 6.4) potently activated the TRPV1 current at a physiological temperature (37°C) in whole-cell patch-clamp mode, but failed to evoke a TRPV1 current in whole-cell patch-clamp mode with acidic pipette solution (pH 6.5) or in perforated patch-clamp mode (Fig. 6*B*). These results suggest that moderate extracellular acidosis has a minimal effect on TRPV1 activity in rat primary sensory neurons in physiological conditions, because intracellular acidification induced by low pH_o attenuates TRPV1 activity.

In summary, we demonstrated that intracellular acidification significantly attenuates TRPV1 activity, and moderate acidosis does not potentiate or activate the TRPV1 current because of the inhibitory effect of intracellular acidification induced by low pH_o in physiological conditions. These results provide a possible explanation for the discrepancy between the *in vivo* and *in vitro* results. When investigating the effects of external acidosis on the TRPV1 channel, pH_i changes should be taken into consideration for accurate analysis and interpretation.

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