Cellular/Molecular

Ambient Temperature Affects the Temperature Threshold for TRPM8 Activation through Interaction of Phosphatidylinositol 4,5-Bisphosphate

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Cold sensation is an important and fundamental sense for animals and it is known to be affected by ambient temperature. Transient Receptor Potential Melastatin 8 (TRPM8), a nonselective cation channel expressed in a subset of peripheral afferent fibers, acts as a cold sensor, having an activation threshold of ~28°C. Although the cold temperature threshold of TRPM8 is affected by menthol or pH, ambient temperature has not been reported to affect it. Because the cold temperature threshold was thought to be unchanged by alterations in ambient temperature, the relativity of temperature sensing in different ambient temperatures could not be understood at the level of molecular function of thermosensitive TRP channels. Here, we show that ambient temperature changed the temperature threshold for activation of human and rat TRPM8 in a heterologous expression system and cold responses in mouse DRG neurons. Moreover, reducing the level of cellular phosphatidylinositol 4,5-bisphosphate (PIP$_2$) attenuated changes in the cold temperature threshold after alterations in ambient temperature. A single amino acid mutation at position 1008 in the C terminus of TRPM8 (arginine to glutamine) also attenuated changes in the cold temperature threshold induced by ambient temperature. These findings suggest that ambient temperature does affect the temperature threshold for TRPM8 activation through interaction of PIP$_2$.

Introduction

Temperature sensation is affected by initial or ambient temperature, as described concisely in the classical psychophysiological observations of the "three bowls experiment." Water at a neutral temperature causes a warm sensation if one first exposes one's hands to cold water; the same neutral temperature causes a cold sensation if one first exposes one's hands to warm water (Tritsch, 1990). Therefore, our sensation of altered temperature appears to be relative and it changes with the initial or ambient temperature.

Transient receptor potential (TRP) channels contribute to sensory transduction, responding to a wide variety of stimuli, including temperature and nociceptive stimuli. In particular, the involvement of TRP channels in thermosensation has been studied extensively after the cloning of the capsaicin receptor TRPV1 (Caterina et al., 1997; Tominaga and Caterina, 2004). Among

TRP channels, TRPM8 has been proposed for detection of cold temperatures in the innocuous and noxious ranges (McKemy et al., 2002; Knowlton et al., 2010). This channel is also activated by cooling agents such as menthol and icilin (McKemy et al., 2002; Peier et al., 2002; Chuang et al., 2004), voltage (Brauchi et al., 2004; Voets et al., 2004), and phosphatidylinositol 4,5-bisphosphate (PIP$_2$; Liu and Qin, 2005; Rohács et al., 2005; Daniels et al., 2009; Zakharian et al., 2010; Yudin et al., 2011), which regulates the activity of a variety of ion channels (Hilgemann et al., 2001). Direct inhibition of TRPM8 by Gα_q has also been described recently (Zhang et al., 2012). Modulation by PIP$_2$ has been shown for several TRP channel members (Rohács and Nilius, 2007). TRPL (Estacion et al., 2001) and TRPV1 (Chuang et al., 2001) are inhibited through interaction with PIP$_2$, whereas TRPM5 (Liu and Liman, 2003), TRPM7 (Runnels et al., 2002) and TRPM8 (Liu and Qin, 2005; Rohács et al., 2005; Zakharian et al., 2010) are activated.

Temperature thresholds for TRPM8 activation are affected by a variety of factors. Menthol shifts the temperature threshold for TRPM8 activation to higher temperatures (McKemy et al., 2002; Mälkiä et al., 2007). Conversely, in the presence of the TRPM8 antagonists BCTC or SKF96365, the temperature thresholds for TRPM8 activation are shifted to lower temperatures (Mälkiä et al., 2007). The temperature thresholds for TRPM8 activation were shown to be elevated at a higher pH and decreased at a lower pH (Andersson et al., 2004). High concentrations of PIP$_2$ activate TRPM8 channels even in the 32–37°C range, warmer than the

Table 1. Synthetic oligonucleotide primers constructing specific mutations

	Sense primer (5′→3′)	Antisense primer (5′→3′)
K995Q	AGAACAATGACCAGGTCTGGcAGTTCCAGAGGTAC	CCAGACCTGGTCATTGTTCTCCTGGACGGTG
R998Q	ACCAGGTCTGGAAGTTCCAGcaGTACTTCCTGGTG	CTGGAACTTCCAGACCTGGTCATTGTTCTC
R1008Q	TGGTGCAGGAGTACTGCAGCCaaCTCAATATCC	GGCTGCAGTACTCCTGCACCAGGAAGTACCTCTG
Y745H	TCTCCTGGAATGTGGTCTTCACACATCGCCTTCCTCCTG	GAAGACCACATTCCAGGAGAAGACCACGAAG
Y1005F	GGTACTTCCTGGTGCAGGAGTtCTGCAGCCGCCTCAATATCC	CTCCTGCACCAGGAAGTACCTCTGGAACTTCCAGAC
L1009R	TGCAGGAGTACTGCAGCCGCCgCAATATCCCCTTC	GCGGCTGCAGTACTCCTGCACCAGGAAGTACCTC
N799A	ATTATTTTACTGACCTGTGGGcTGTGATGGACACGCTG	CCACAGGTCAGTAAAATAATTCACCCCATTTACG
D802A	CTGACCCTGTGGAATGTGATGGcCACGCTGGGGCTTTTTTAC	CATCACATTCCACAGGTCAGTAAAATAATTCAC
G805A	GGAATGTGATGGACACGCTGGCcCTTTTTTACTTCATAG	CAGCGTGTCCATCACATTCCACAGGTCAG

Lowercase letters indicate substituted bases.

threshold under naive conditions (Rohács et al., 2005). Lipid raft disruption also shifts the temperature thresholds for TRPM8 activation (Morenilla-Palao et al., 2009). Although the molecular mechanisms underlying the three bowls experiment are not known, some of the factors described above could be related to the phenomenon. In contrast, it is generally believed that temperature thresholds for TRPM8 activation are not affected by changes in ambient or initial temperature.

In the present study, we demonstrate that temperature thresholds for mammalian TRPM8 activation are modified *in vitro* by changes in ambient temperature. Moreover, we determined that the interaction of PIP_2 is important for modifying the temperature threshold for activation.

Materials and Methods

Cell culture. Human embryonic kidney-derived 293T (HEK293T) cells were maintained in DMEM (WAKO Pure Chemical Industries) supplemented with 10% FBS (Biowest), 100 units/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and two mM L-glutamine (GlutaMAX; Life Technologies) at 37°C in 5% CO_2. For Ca^{2+} imaging, 1 μg of plasmid DNA containing human TRPM8 (Life Technologies), rat TRPM8 (provided by Dr. David Julius, University of California-San Francisco), mutant human TRPM8 with or without Ci-VSP (provided by Dr. Yasushi Okamura, Osaka University, Osaka, Japan) or rat EP1 (provided by Dr. Shu Narumiya, Kyoto University, Kyoto, Japan) in pcDNA3 in OPTI-MEM medium (Life Technologies) were transfected into HEK293T cells using Lipofectamine Plus Reagent (Life Technologies). After incubating for 3–4 h, the cells were reseeded on coverslips and further incubated at 37°C in 5% CO_2. DRG cells were rapidly isolated from male C57Bl/6NCr mice (4–6 weeks old; SLC; Hamamatsu) and used for the experiments in the following day. All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences and were performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH publication #85–23, revised 1985).

TRPM8 mutants. Vectors containing the full-length mutants were constructed by single amino acid substitutions using a GeneTailor Site-Directed Mutagenesis System (Invitrogen). Synthetic oligonucleotide primers constructing specific mutations are shown in Table 1. The entire sequence, including the desired substitution in the mutants, was confirmed by DNA sequencing.

Ca^{2+} imaging. HEK293T cells on coverslips were mounted in an open chamber and rinsed with standard bath solution containing the following (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4. Cytosolic free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in HEK293 cells was measured by dual-wavelength fura-2 (Invitrogen) microfluorometry with excitation at 340/380 nm and emission at 510 nm. The ratio image was calculated and acquired using the IP-Lab imaging processing system (Scanalytics). Temperature thresholds were defined as temperatures causing ratio increases in menthol-sensitive cells.

Electrophysiology. Whole-cell patch-clamp recordings were performed 1 d after transfection. The standard bath solution was the same as that

used in the Ca^{2+}-imaging experiments. To make a Ca^{2+}-free bath solution, $CaCl_2$ was omitted and 5 mM EGTA was added. The pipette solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES, pH 7.4 (adjusted with KOH). Data from whole-cell voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software; Molecular Devices). Membrane potential was clamped at −60 mV. All experiments were performed at room temperature. Cold stimulation was induced by lowering the bath temperature with an ice-chilled solution at a constant bath temperature with an inline heater or heating stage. Temperature was monitored with a thermocouple (TA-29; Warner Instruments) placed within 100 μm of the patch-clamped cell. Current densities were plotted on a logarithmic scale against the reciprocal of the absolute temperature in an Arrhenius plot, with the temperature threshold defined as the temperature at the intersection of the two extrapolation lines before and after channel activation. To evaluate the temperature threshold in the presence of m-3M3FBS (Sigma-Aldrich), wortmannin (Sigma-Aldrich), PIP_2 specific antibody (Abcam), or dioctanoyl PIP_2 (diC_8PIP_2, Sigma-Aldrich), the entire experiment was performed in the presence of m-3M3FBS (30 μM), wortmannin (10 μM), PIP_2 specific antibody (1/80), or diC_8PIP_2 (20 μM).

Data analysis. Data are expressed as means ± SEM. Student's *t* test was used to compare the data between two groups. $p < 0.05$ was considered significant.

Results

We first examined whether temperature thresholds for TRPM8 activation could be changed depending on the temperature to which the cells were exposed. We used Ca^{2+} imaging of HEK293T cells expressing human TRPM8 (hTRPM8) or rat TRPM8 (rTRPM8). At 40°C ambient (initial) temperature, temperature thresholds were ~35°C (35.0 ± 0.7°C, $n = 8$ for hTRPM8 and 34.5 ± 0.8°C, $n = 7$ for rTRPM8). In contrast, they were ~28°C (28.3 ± 0.5°C, $n = 7$ for hTRPM8 and 28.6 ± 0.5°C, $n = 7$ for rTRPM8) when the ambient temperature was 30°C (Fig. 1A,B). To confirm the effect of initial temperature on temperature threshold for TRPM8 activation in native sensory neurons, we performed Ca^{2+}-imaging experiments with isolated mouse DRG neurons. In the subset of DRG neurons that showed an increase in $[Ca^{2+}]_i$ when exposed to ice-chilled bath solution, temperature thresholds for a $[Ca^{2+}]_i$ increase were 32.7 ± 0.5°C at an initial temperature of 40°C and 28.8 ± 0.6°C at a 30°C initial temperature ($n = 10$). These results were similar to those observed in HEK293T cells expressing TRPM8 (Fig. 1B). More precise analyses of changes in temperature thresholds for TRPM8 activation were performed by making Arrhenius plots for TRPM8-mediated current responses at various exposed temperatures. The increase in the exposed temperature from 30°C to 40°C increased the temperature thresholds for TRPM8 activation from 28.3 ± 0.4 to 33.8 ± 1.1°C ($n = 6$) in HEK293 cells expressing hTRPM8 (Fig. 1C,D). The fact that lowering the exposed temperature from 40°C to 30°C produced similar changes in tem-

perature thresholds from 32.8 ± 0.9 to 28.2 ± 0.8°C (n = 5; Fig. 1 E,F) indicated that the difference in the change of direction of the exposed temperature was not related to the changes in temperature threshold. The exposed-temperature-dependent changes in temperature thresholds for TRPM8 activation were also observed with rTRPM8 and were further supported by multi-exposed temperature point analysis (Fig. 2A).

Because TRPM8 is reportedly regulated downstream of the cascade through phospholipase C (PLC) and PIP$_2$ (Liu and Qin, 2005; Rohács et al., 2005; Daniels et al., 2009; Yudin et al., 2011), we hypothesized that they might be involved in the exposed-temperature-dependent changes in temperature thresholds for TRPM8 activation. Therefore, various chemicals affecting this cascade were tested to determine whether the changes in temperature thresholds for hTRPM8 activation were influenced by PIP$_2$-related events. In treatments with a benzenesulfonamide compound, m-3M3FBS (30 μM), which activates all isoforms of PLC (Horowitz et al., 2005; Daniels et al., 2009), the exposed-temperature-dependent changes in temperature thresholds for hTRPM8 activation became significantly lower (26.7 ± 0.2°C at 30°C and 28.5 ± 0.2°C at 40°C, n = 17, p < 0.01; Fig. 2 A,B). Moreover, 10 μM wortmannin, an inhibitor of phosphatidylinositol 4-kinase (Nakanishi et al., 1995), and a PIP$_2$ specific antibody, both of which are supposed to reduce the amount of functional PIP$_2$ (Benedikt et al., 2007), also eliminated the changes in temperature threshold for hTRPM8 activation by exposed temperatures between 30°C and 40°C (24.3 ± 1.3°C at 30°C and 26.6 ± 0.9°C at 40°C with wortmannin, n = 8; 25.5 ± 0.6°C at 30°C and 28.9 ± 1.3°C at 40°C with a PIP$_2$ specific antibody, n = 8; Fig. 2B). However, addition of 20 μM diC$_8$PIP$_2$, a synthetic PIP$_2$ (Rohács et al., 2005), showed significant differences in temperature thresholds for hTRPM8 activation between exposure temperatures of 40°C and 30°C (26.2 ± 0.5°C at 30°C and 33.1 ± 0.9°C at 40°C, n = 7), although temperature thresholds at 30°C exposed temperature were slightly higher than control (Fig. 2B). To determine the involvement of Ca^{2+} influx in the exposed-temperature-dependent changes in temperature thresholds for hTRPM8 activation, we performed patch-clamp experiments in the absence of extracellular Ca^{2+} (Fig. 2B). The exposed-temperature-dependent changes in temperature thresholds for hTRPM8 activation were also observed in the absence of extracellular Ca^{2+} (25.3 ± 0.4°C at 30°C and 30.2 ± 0.4°C at 40°C, n = 10). These results suggest that PIP$_2$, but not Ca^{2+} influx, was involved in the changes in temperature thresholds for hTRPM8 activation.

Previous studies with TRPM8 mutants identified the important residues required for interaction with PIP$_2$ (Rohács and Nilius, 2007), menthol (Bandell et al., 2006) or icilin (Chuang et al., 2004). Therefore, we sought to identify the amino acids involved in the exposed-temperature-dependent changes in
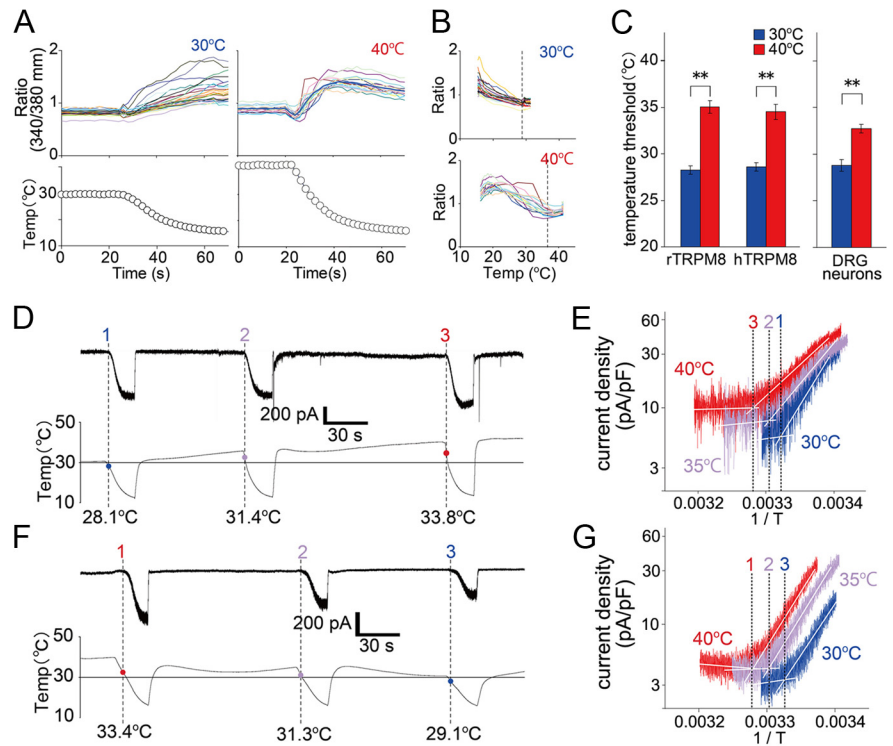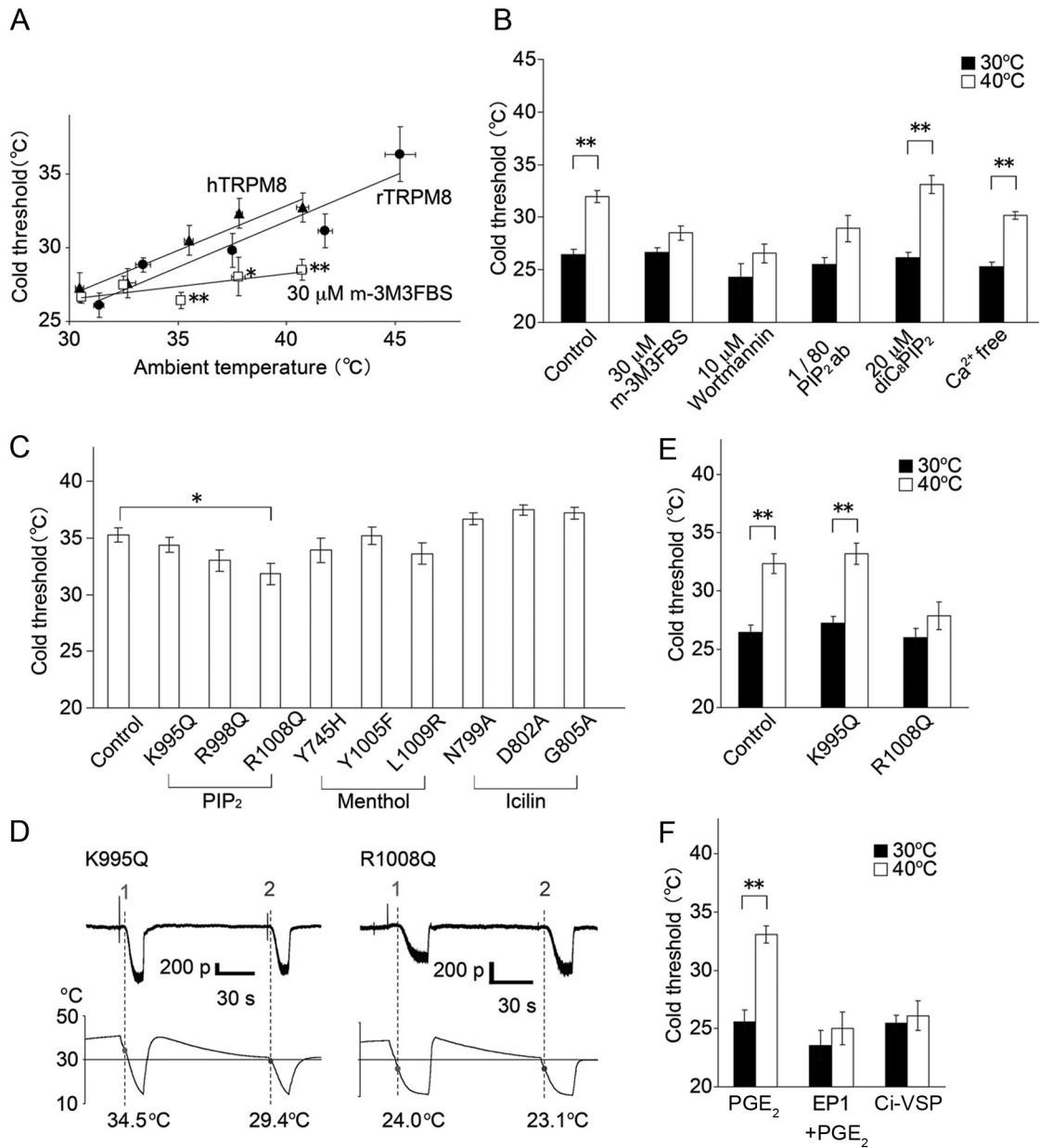

Figure 1. Temperature thresholds of TRPM8 activation are affected by the ambient temperatures in both HEK293 cells expressing TRPM8 and native sensory neurons. **A, B,** Cold stimulus increased the cytosolic Ca^{2+} concentration. Data are presented as the fura-2 ratio (340/380 mm) in HEK293T cells expressing human TRPM8 (hTRPM8) in the presence of extracellular Ca^{2+} after 3 min exposure to either a 30°C or 40°C solution. **C,** Comparison of the temperature thresholds for activation of hTRPM8 (n = 8) and rat TRPM8 (rTRPM8, n = 7) in HEK293T cells, and native mouse sensory neurons (n = 10) between 30°C and 40°C exposed temperatures. **D, E,** Changes in temperature thresholds for hTRPM8 activation determined by Arrhenius plots (right) from the data obtained from an ambient temperature increase from 30°C to 40°C in HEK293 cells expressing hTRPM8 (left). Temperature thresholds for hTRPM8 activation at 30°C, 35°C, and 40°C in the temperature-increasing protocol were 28.3 ± 0.4, 31.4 ± 0.9, and 33.8 ± 1.1°C, respectively (n = 6). **F, G,** Changes in temperature thresholds for hTRPM8 activation determined by Arrhenius plots (right) from the data obtained from an ambient temperature decrease from 40°C to 30°C in HEK293T cells expressing hTRPM8 (left). Temperature thresholds for hTRPM8 activation at 30°C, 35°C, and 40°C in the temperature-decreasing protocol were 28.2 ± 0.8, 31.1 ± 0.7, and 32.8 ± 0.9°C, respectively (n = 5). **p < 0.01.

temperature thresholds for TRPM8 activation. We investigated whether temperature thresholds for TRPM8 activation at an exposed temperature of 40°C were affected in the reported TRPM8 mutants. We used Ca^{2+}-imaging to characterize cells expressing hTRPM8 mutants. Like the wild-type, most of the mutants exhibited high temperature thresholds for activation at an initial temperature of 40°C. However, the R1008Q mutant, in which the 1008th residue, arginine, in the C-terminal region, an interacting site for PIP$_2$ (Rohács et al., 2005), was changed to glutamine, exhibited a significantly lower threshold than wild-type hTRPM8 (35.3 ± 0.6°C in wild-type and 31.8 ± 0.9°C in the R1008Q mutant, n = 5, p < 0.05; Fig. 2C).

We next investigated whether R1008Q preserved the exposed-temperature-dependent changes in temperature thresholds using a patch-clamp method. The exposed-temperature-dependent changes in temperature thresholds observed in the wild-type were lost in R1008Q (26.0 ± 0.8°C at 30°C and 27.9 ± 1.2°C at 40°C, n = 10). However, K995Q, which is also a mutant reported as being an interacting site for PIP$_2$, exhibited changes in the temperature thresholds like that in the wild-type (27.3 ± 0.5°C at 30°C and 33.2 ± 0.9°C at 40°C, n = 9; Fig. 2 D,E). These results suggested that PIP$_2$ interaction with R1008 of hTRPM8 was necessary for the exposed-temperature-dependent changes in temperature thresholds.

Figure 2. Changes in temperature thresholds of TRPM8 activation depending on the exposed temperature involved PIP_2 interaction with TRPM8. **A**, Temperature thresholds for both hTRPM8 (closed triangles, $n = 16$) and rTRPM8 (closed circles, $n = 7$) activation increased depending on the elevation of the preexposure temperature in HEK293T cells, which was not observed in the treatment with m-3M3FBS, a PLC activator (open squares, $n = 17$) using a patch-clamp method. **B**, Changes in temperature thresholds for human TRPM8 activation between 40°C and 30°C exposed ambient temperatures without (Control, $n = 16$) or with chemicals affecting cytosolic PIP_2 actions (30 μM m-3M3FBS, $n = 17$; 10 μM wortmannin, a specific PIP_2 antibody, $n = 8$; 20 μM diC$_8$PIP$_2$, $n = 8$) in pipette solutions, or a Ca^{2+}-free condition ($n = 10$) using a patch-clamp method. **C**, Temperature thresholds for fura-2 ratio increase at 40°C of exposed temperature in the HEK293T cells expressing different TRPM8 mutants using a Ca^{2+}-imaging method ($n = 5–9$). **D**, Representative traces of the currents activated by cold stimulus in HEK293T cells expressing mutant hTRPM8 (K995Q, R1008Q) at the exposed temperature change from 40°C to 30°C. **E**, Changes in temperature thresholds for wild-type ($n = 11$) and mutant hTRPM8 ($n = 9$ for K995Q, $n = 10$ for R1008Q) in HEK293 cells between 40°C and 30°C exposed ambient temperatures. **F**, Changes in temperature thresholds for hTRPM8 activation in HEK293 cells expressing TRPM8 upon treatment with PGE$_2$ (left, $n = 5$), cells expressing human TRPM8 and rat EP1 upon treatment with PGE$_2$ (middle, $n = 8$), and cells expressing human TRPM8 and rat Ci-VSP upon voltage step ($+100$ mV for 30 s after -60 mV for 3 min; right, $n = 11$). *$p < 0.05$; **$p < 0.01$.

PIP_2 levels can be regulated by different pharmacological approaches from those described above. For example, the amount of PIP_2 is reduced downstream of activation of Gq-protein-coupled receptors. We sought to further confirm the involvement of PIP_2 in the exposed-temperature-dependent changes in temperature thresholds for TRPM8 activation. We treated HEK293T cells expressing mouse EP1 and hTRPM8 with prostaglandin E$_2$ (PGE$_2$, 1 μM). These agents cause TRPV1 sensitization through a Gq-protein-dependent process (Moriyama et al.,

2005). Changes in temperature threshold for TRPM8 activation between 30°C and 40°C were lost after PGE$_2$ treatment (23.6 ± 1.3°C at 30°C and 25.0 ± 1.4°C at 40°C upon PGE$_2$ application in cells expressing EP1, $n = 8$, Fig. 2F). PGE$_2$ application alone caused no changes in cells without EP1 (25.6 ± 1.0°C at 30°C and 33.1 ± 0.7°C at 40°C upon PGE$_2$ application, $n = 5$, $p < 0.01$). However, direct inhibition of TRPM8 by Gα_q (Zhang et al., 2012) could cause similar outcomes. Therefore, we used a different approach with *Ciona intestinalis* voltage-sensor-containing

phosphatase (Ci-VSP), which acts as a phosphatase for the 5-phosphate of $PI(4,5)P_2$ upon membrane voltage changes (Iwasaki et al., 2008). We maintained HEK293T cells expressing Ci-VSP and hTRPM8 at +100 mV for 30 s after being given a voltage of −60 mV for 3 min, which was reported previously to reduce PIP_2 through dephosphorylation (Iwasaki et al., 2008). The exposed-temperature-dependent changes in temperature thresholds for TRPM8 activation were also lost (25.5 ± 0.7°C at 30°C, $n = 11$ and 26.1 ± 1.3°C at 40°C upon voltage step, $n = 9$; Fig. 2F). These results further support the concept that PIP_2 action on TRPM8 is important for causing changes in temperature thresholds for TRPM8 activation.

Discussion

Thermosensitive TRP channels are remarkable for their ability to sense a wide range of temperatures. However, only some of the known thermosensitive TRP channels are thought to sense the ambient temperature in either sensory neurons or skin keratinocytes (TRPV1, TRPM8, TRPA1, TRPV3 and TRPV4; TRPV2 involvement in thermal nociception has been questioned by Park et al., 2011). How do our bodies acquire such a marked ability to sense ambient temperature changes as small as 1°C? It could be achieved by dynamic changes in temperature thresholds for activation. Indeed, capsaicin receptor TRPV1 exhibited a reduction in temperature threshold through serine phosphorylation by protein kinases A and C upon proinflammatory mediator production (Tominaga and Caterina, 2004). It was reported that the lipid environment could change the temperature threshold for TRPM8 activation (Rohács et al., 2005; Morenilla-Palao et al., 2009). In addition, minimal amounts of agonists for TRPV1 or TRPM8 reduce or increase, respectively, their temperature thresholds for activation. Furthermore, we reported recently that temperature thresholds for activation of the temperature-sensitive TRPM2 channel could be reduced through hydrogen-peroxide-mediated oxidation of methionine in the N terminus (Kashio et al., 2012).

In the present study, we have provided conclusive evidence that temperature thresholds for activation of mammalian TRPM8 can be changed dynamically by a relatively brief exposure to different ambient temperatures through a mechanism involving PIP_2. We also identified a single arginine residue that could be involved in the exposed-temperature-dependent changes in temperature thresholds for TRPM8 activation. We propose that the PIP_2-mediated mechanism observed in this study could contribute to the three bowls experiment.

Although we are confident that the interaction of PIP_2 with TRPM8 is one of the key events causing the ambient-temperature-dependent changes in temperature thresholds for TRPM8 activation, precisely how PIP_2 behaves in a temperature-dependent manner in response to high exposed temperature is not completely clear. There are several possible mechanisms that could explain the phenomenon. One is a change in the amount of cellular PIP_2 depending on the ambient temperature fluctuation. Indeed, Yudin et al. (2011) found a reduction in cellular PIP_2 upon temperature decrease (from 25°C to 10°C) using fluorescence-based PIP_2 level measurements. Although we tried to investigate the changes in PIP_2 levels using a similar FRET system, both CFP and YFP signals decayed in a temperature-dependent manner at high exposed temperatures (>30°C), making it difficult to measure PIP_2 levels. Alternatively, PIP_2 recruitment near TRPM8 proteins in the plasma membrane or changes in the extent of PIP_2 interaction with TRPM8 could occur depending on the

changes in ambient temperature. Further analysis is required to clarify the precise mechanisms underlying the ambient-temperature-induced events. Nonetheless, it is clear that temperature thresholds for TRPM8 activation can be changed dynamically within a few minutes and that such changes occurred when environmental temperatures increased or decreased (Fig. 1C,E). These results indicate that reversible events are probably involved.

Daniels et al. (2009) reported that rat TRPM8-mediated Ca^{2+} influx activated PLC, thereby decreasing PIP_2 levels and resulting in reduced rTRPM8 activity, although they did not observe the changes in temperature thresholds for activation. There are several differences between our experimental conditions and theirs. The main differences between the two studies are the methods used for measuring TRPM8 activity and the applied temperature ranges. Daniels et al. measured temperature thresholds for rTRPM8 activation using a Ca^{2+}-imaging method with temperatures up to 33°C, whereas we used a patch-clamp method in a temperature range between 30°C and 40°C. We did not observe clear changes in temperature thresholds for hTRPM8 activation at 30°C, consistent with the data by Daniels et al. However, we observed significant differences in the thresholds at temperatures >35°C. Temperature ranges at which experiments were performed seem to be a critical determinant causing different outcomes. In addition, we observed the exposed-temperature-dependent changes in temperature thresholds for hTRPM8 activation regardless of the presence or absence of extracellular Ca^{2+}, making the involvement of Ca^{2+} influx in the phenomenon less likely. The finding that Ca^{2+} influx through hTRPM8 activation upon repetitive cold stimuli increases and decreases the temperature thresholds for TRPM8 activation depending on the direction of solution temperature change supports the idea.

In the present study, we used several methods to change the level of cellular PIP_2. One might imagine that such changes would alter temperature thresholds for TRPM8 activation at both 30°C and 40°C. However, significant temperature threshold changes were observed only when the environmental temperature was 40°C. In addition, exogenous application of diC_8PIP_2 did not cause any change in temperature threshold (Fig. 2). We currently cannot explain these findings. Temperature thresholds in the absence of additional TRPM8 agonists under naive conditions might be tightly regulated to a constant temperature of ~28°C. Alternatively, it might be hard to detect small changes with our system. In any case, the fact that heat-sensing ability is affected by changes in ambient temperature (Tritsch, 1990) suggests that there might be a universal or common mechanism leading to the changes in temperature-sensing ability. More studies are required to better understand the temperature-sensing mechanisms underlying these processes.

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