Tris-hydroxymethyl-aminomethane enhances capsaicin-induced intracellular Ca\(^{2+}\) influx through transient receptor potential V1 (TRPV1) channels

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Non-selective transient receptor potential vanilloid (TRPV) cation channels are activated by various insults, including exposure to heat, acidity, and the compound capsaicin, resulting in sensations of pain in the skin, visceral organs, and oral cavity. Recently, TRPV1 activation was also demonstrated in response to basic pH elicited by ammonia and intracellular alkalization. Tris-hydroxymethyl aminomethane (THAM) is widely used as an alkalizing agent; however, the effects of THAM on TRPV1 channels have not been defined. In this study, we characterized the effects of THAM-induced TRPV1 channel activation in baby hamster kidney cells expressing human TRPV1 (hTRPV1) and the Ca\(^{2+}\)-sensitive fluorescent sensor GCaMP2 by real-time confocal microscopy. Notably, both capsaicin (1 \(\mu\)M) and pH 6.5 buffer elicited steep increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), while treatment with THAM (pH 8.5) alone had no effect. However, treatment with THAM (pH 8.5) following capsaicin application elicited a profound, long-lasting increase in [Ca\(^{2+}\)]\(_i\) that was completely inhibited by the TRPV1 antagonist capsaizepine. Taken together, these results suggest that hTRPV1 pre-activation is required to provoke enhanced, THAM-induced [Ca\(^{2+}\)]\(_i\) increases, which could be a mechanism underlying pain induced by basic pH.

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used clinically to reverse acidosis (8–10); however, the effects of THAM on TRPV1 channels have not yet been described.

In this study, we sought to characterize the direct effects of THAM on TRPV1 channel activity in an in vitro experimental system. To this end, baby hamster kidney (BHK) cells expressing human TRPV1 channels (hTRPV1) and the Ca²⁺-sensitive fluorescent sensor GaMP2 were stimulated with receptor agonists, and the resulting intracellular Ca²⁺ ([Ca²⁺]i) signals were then measured by real-time confocal microscopy (11–13).

2. Methods

2.1. Construction and expression of plasmids and chemicals

cDNA encoding full-length human TRPV1 was kindly provided by Dr. M. Tominaga (Division of Cell Signaling, Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki). TRPV1 cDNA was subcloned into the expression vector pCI-neo (Promega, Madison, WI, USA). The pN1-GaMP2 plasmid encoding the intracellular calcium sensor GaMP2 was kindly gifted by Dr. J. Nakai (Saitama University Brain Science Institute, Saitama). Capsaicin and THAM were obtained from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Nacalai Tesque (Kyoto).

2.2. Cell culture and transfection

BHK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL), and sodium bicarbonate (50 mL). The pH was adjusted to 7.4 with NaOH. Culture medium was changed every 2 days.

2.3. Ca²⁺ imaging assay

Ca²⁺ imaging was performed with BHK cells co-expressing TRPV1 using the Ca²⁺ sensor GaMP2 (11–13). The culture medium was discarded and the cells were washed twice with HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM d-glucose, pH 7.4). The HEPES buffer was exchanged with test solution containing capsaicin and/or THAM (pH 8.5), using a perfusion system. When using buffer at pH 6.0, we used Krebs-Ringer Phosphate (KRP)-buffered saline (pH 6.0). The fluorescence of GaMP2 was continuously recorded at 510 nm to measure the fluorescence intensity in each whole cell. Ca²⁺ imaging was performed on a Meta 510 confocal microscope (Zeiss Japan, Tokyo). Data were acquired and analyzed with LSM510 META software (Carl Zeiss, Jena, Germany) and expressed as the fluorescence intensity (510 nm) before and after test solution exposure.

2.4. Capsaicin treatment

BHK cells expressing human TRPV1 were treated with 1 μM capsaicin for 30 s, washed for 10 min, and analyzed to confirm the expression status of both TRPV1 and GaMP2. Test compounds were added in the presence of capsaicin, low pH buffer, high pH buffer, or THAM at various concentrations.

2.5. Statistical analysis

Data were analyzed using Prism 6 (GraphPad Software, San Diego, CA, USA). The Mann–Whitney test was used to compare the data between 2 groups, and non-parametric testing with Kruskal–Wallis one-way analysis of variance was used for multiple comparisons of control and treated groups.

3. Results

3.1. Real-time Ca²⁺ imaging-system optimization

Slight, but significant, green-fluorescent staining was observed in unstimulated hTRPV1/GaMP2-expressing BHK cells. The fluorescent staining rapidly increased to a strong signal once stimulated with 1 μM capsaicin, indicative of an increase in [Ca²⁺]i, and quickly returned to basal levels (Fig. 1A). As expected, in the cells expressing hTRPV1/GaMP2, KRP-buffered saline (pH 6.0) elicited increases in [Ca²⁺]i, as shown in Fig. 1B. Strong signaling elicited with 1 μM capsaicin or low-pH (6.0) KRP was not observed in BHK cells that expressed GaMP2, but not TRPV1 (Fig. 1B).

To optimize the capsaicin concentration, we examined a range of capsaicin concentrations from 1 pM to 10 μM and observed robust peaks at 1 μM; however, significant increases in [Ca²⁺]i were detected at a minimal concentration of 1 nM (data not shown). Based on these results, we used 1 μM capsaicin for the remaining studies. Both the capsaicin-induced and low-pH (6.0)-induced increases in [Ca²⁺]i were completely inhibited when the hTRPV1 inhibitor capsazepine (10 μM) was added (Fig. 1C), consistent with previous findings (1–6).

In addition, a previous study showed that alkalization (high pH) was also sufficient to elicit TRPV1 activation (7). Based on these findings, we examined the effect of high pH on hTRPV1 by adding HEPES (pH 8.5) or THAM (pH 8.5) at 0.3 mM, a concentration used clinically to treat acidosis (8, 10). As shown in Fig. 1D, both HEPES and THAM increased [Ca²⁺]i; however, not significantly. Moreover, treatment with 0.3 mM THAM at a higher pH (9.0) still failed to elicit any effects (data not shown).

3.2. Enhanced capsaicin-induced Ca²⁺ influx by THAM

BHK cells were exposed to 1 μM capsaicin for 30 s, followed by 0.3 mM THAM (pH 8.5) for 60 s, as shown in the inset of Fig. 2A. We observed a profound increase in [Ca²⁺]i, which persisted for >20 min (Fig. 2A).

Interestingly, the [Ca²⁺]i increase was dependent on the timing of THAM application following capsaicin exposure. For example, while the simultaneous application of capsaicin with THAM did not induce a secondary increase in [Ca²⁺]i, treatment with THAM 30 s or 60 s after treatment with capsaicin elicited a profound secondary phase of [Ca²⁺]i increases. However, a significant increase in [Ca²⁺]i was no longer observed when THAM was added to BHK cells 90 s after capsaicin exposure (Fig. 2B).

Furthermore, when BHK cells were incubated simultaneously with the hTRPV1 inhibitor capsazepine (10 μM) and capsaicin, both capsaicin- and capsaicin/THAM-induced [Ca²⁺]i increases were completely inhibited, regardless of the timing of the dosage (Fig. 2C, E). When capsazepine was added after capsaicin application, the elevation of [Ca²⁺]i, induced by THAM (Fig. 2A) was completely inhibited (Fig. 2D, E), indicating that opened TRPV1 channels are involved in THAM-induced [Ca²⁺]i increases.
3.3. Enhanced Ca\(^{2+}\) influx is capsaicin/THAM-specific

Acidification itself is reported to activate TRPV1 (5). We showed here that KRP-buffered saline (pH 6.0) induced a significant increase in \([Ca^{2+}]_i\), whereas THAM (pH 8.5) had no significant effect (Fig. 1C). In contrast to the results observed following capsaicin treatment, THAM added simultaneously, or at 30, 60, or 90 s after low-pH buffer application failed to induce significant increases in \([Ca^{2+}]_i\) (Fig. 3).

This effect was THAM-specific, as no additional increase in \([Ca^{2+}]_i\) was observed when HEPES (pH 8.5), instead of THAM (pH 8.5), was added simultaneously or at 30, 60, or 90 s after capsaicin application (Fig. 4).

Moreover, we reversed the order of treatment from that shown in Fig. 2A and B, and examined the effects of THAM (pH 8.5) simultaneously or at 30, 60, or 90 s after application of capsaicin. In contrast to the data shown in Fig. 2B, THAM failed to enhance \([Ca^{2+}]_i\) when applied prior to capsaicin (Fig. 5).

4. Discussion

TRPV1 is activated by a variety of noxious stimuli, including the exposure to capsaicin, heat, low pH, and alkalization (1–6). Here, we found that the alkalizing agent THAM (pH 8.5) potentiated TRPV1 activity if TRPV1 was pre-activated by capsaicin. Moreover, we found that low pH (~6.0) caused a significant and rapid increase in \([Ca^{2+}]_i\), consistent with previous findings (14). We also demonstrated that treatment with THAM or HEPES at pH 8.5 did not independently increase \([Ca^{2+}]_i\): however, THAM (pH 8.5) strengthened and prolonged the capsaicin-induced increase in \([Ca^{2+}]_i\). This effect was time-dependent, as the \([Ca^{2+}]_i\) increase was only enhanced when THAM was added 30 or 60 s after capsaicin application, but not when added simultaneously or 90 s after stimulation. Further, the effect of THAM appeared to depend upon the “open-state” of TRPV1 because the THAM-induced increase in \([Ca^{2+}]_i\) was inhibited by capsazepine just after application of capsaicin (Fig. 2D). The precise mechanisms underlying this enhancement are presently unknown; however, we suggest that capsaicin-induced Ca\(^{2+}\) influx through TRPV1 channels terminates within 60 s because of pore closure. Accordingly, we did not observe a THAM-induced increase in \([Ca^{2+}]_i\) 90 s after capsaicin application since the TRPV1 channel was presumably closed. Furthermore, the simultaneous application of THAM and capsaicin failed to enhance Ca\(^{2+}\) influx, suggesting that the TRPV1 channel pore must be pre-opened by capsaicin for THAM to potentiate its activation.

In our study, THAM or HEPES buffer at pH 8.5 did not independently activate TRPV1, indicating that a high pH alone was insufficient to activate TRPV1 channels. However, combination treatment with THAM and capsaicin caused persistent increases in \([Ca^{2+}]_i\), most likely because THAM entered the cytosol through the capsaicin-activated TRPV1 channels. Thus, we theorize that
cytosolic THAM potentially binds to intracellular residues of TRPV1 that mediate TRPV1 activation by intracellular alkalization (7).

Previous data have shown that TRPV1 is activated by basic pH and have suggested that alkalinization agents, such as ammonia, elicit a distinct, pungent sensation in the nose and airways, causing mucous membrane irritation that can develop into acute pneumonitis and chronic bronchitis with chronic exposure (15). Our results showed that, although THAM (pH 8.5) alone did not elicit marked [Ca^{2+}]_i increases in hTRPV1-expressing BHK cells, it did cause a robust and persistent increase in [Ca^{2+}]_i in cells with pre-activated hTRPV1 channels that were opened by the noxious stimulus capsaicin. While ammonia can permeate cell membranes and activate TRPV1, THAM is likely hard to enter the cells because of its size and positive polarity (6). The physiological significance of this phenomenon is unclear at present; however, we postulate that THAM causes a noxious sensation if TRPV1 is pre-activated by capsaicin, but not at a low pH (Figs. 2 and 3).

![Fig. 2. Enhanced TRPV1 channel activity by THAM following the application of capsaicin.](image-url)

**Fig. 2.** Enhanced TRPV1 channel activity by THAM following the application of capsaicin. (A) Increase in [Ca^{2+}]_i, following stimulation with 1 μM capsaicin for 30 s followed by 0.3 mM THAM (pH 8.5) for 30 s. The time between capsaicin and THAM applications was 30 s. (B) Time-dependent effects of THAM application after capsaicin application. [Ca^{2+}]_i data are expressed as the area under the curve (AUC) and are shown as the mean ± SEM. (C) Capsazepine (10 μM)-dependent inhibition of increased [Ca^{2+}]_i after a 30 s stimulation with capsaicin (1 μM) and a subsequent 30 s stimulation with 0.3 mM THAM (pH 8.5). (D) Calcium influx following sequential 30 s stimulations with 1 μM capsaicin, 10 μM capsaicpine, and 0.3 mM THAM (pH 8.5). (E) Effects of capsazepine on the capsaicin/THAM-induced increase in [Ca^{2+}]_i in cells expressing hTRPV1. Data are expressed as the AUC of [Ca^{2+}]_i and are shown as the mean ± SEM. *p < 0.05; **p < 0.001 vs. control; ***p < 0.001 vs. 1 μM capsaicin; CPZ, capsazepine; n.s., not significant; n, number of experiments.
Some findings have suggested that THAM may effectively compensate for acidosis, ameliorate the deleterious effects of prolonged hyperventilation, and may be beneficial in intracranial pressure control [16]. Previously, it was concluded that THAM administered at 0.55 mmol/(kg·h) to acute lung-injury patients with acidosis was associated with significantly increased arterial pH and base deficits, and triggered a reduction in arterial carbon dioxide tension that could not be fully accounted for by ventilation [8].

Several physiological conditions give rise to alkalinity, some of which are associated with pain sensations. A previous report demonstrated that rabbits infused with 0.3 M THAM showed necrosis around the site of infusion into the marginal ear vein [17]. In addition, respiratory alkalosis due to hyperventilation can cause a tingling sensation in the extremities and lower peripheral nerve thresholds [18]. It has also been suggested that alkaline pH causes pain sensation via TRPA1 activation and may provide a molecular explanation for some human alkaline pH-related sensory disorders.
However, the role of TRPV1 in such disorders has yet to be reported.

Our data showed that THAM prolonged TRPV1 channel activity if the channels were pre-activated. Although the significance of prolonged TRPV1 channel activation is unclear, our results suggest that THAM may cause pain sensations in some circumstances. Thus, it is important to determine how THAM can be used more effectively and promptly in treating pathophysiological conditions involving TRPV1 channel pre-activation.

Conflict of interest

None.

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References


Fig. 5. Effects of varying the timing of capsaicin application following THAM (pH 8.5) treatment. Data are expressed as the area under the curve (AUC) of [Ca2+]i and are shown as the mean ± SEM. *** p < 0.001 vs. control; n.s., not significant; n, number of experiments.