RESEARCH REPORTS
Biological

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J Dent Res 90(9):1098-1102, 2011

Cold Suppresses Agonist-induced Activation of TRPV1

ABSTRACT

Cold therapy is frequently used to reduce pain and edema following acute injury or surgery such as tooth extraction. However, the neurobiological mechanisms of cold therapy are not completely understood. Transient receptor potential vanilloid 1 (TRPV1) is a capsaicin- and heat-gated nociceptive ion channel implicated in thermosensation and pathological pain under conditions of inflammation or injury. Although capsaicin-induced nociception, neuropeptide release, and ionic currents are suppressed by cold, it is not known if cold suppresses agonist-induced activation of recombinant TRPV1. We demonstrate that cold strongly suppressed the activation of recombinant TRPV1 by multiple agonists and capsaicin-evoked currents in trigeminal ganglia neurons under normal and phosphorylated conditions. Cold-induced suppression was partially impaired in a TRPV1 mutant that lacked heat-mediated activation and potentiation. These results suggest that cold-induced suppression of TRPV1 may share a common molecular basis with heat-induced potentiation, and that allosteric inhibition may contribute, in part, to the cold-induced suppression. We also show that combination of cold and a specific antagonist of TRPV1 can produce an additive suppression. Our results provide a mechanistic basis for cold therapy and may enhance anti-nociceptive approaches that target TRPV1 for managing pain under inflammation and tissue injury, including that from tooth extraction.

KEY WORDS: temperature, cold, TRPV1, capsaicin, antagonist, pain.

DOI: 10.1177/0022034511412074

Received February 8, 2011; Last revision March 16, 2011; Accepted April 23, 2011

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

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INTRODUCTION

Cooling the site of inflammation or injury is one of the most widely used supplemental anti-nociceptive therapies (Oosterveld *et al*., 1992; McCarberg and O'Connor, 2004). Cold pack is frequently used following tooth extraction, which significantly attenuates post-surgical edema and pain (Laureano Filho *et al*., 2005). Multiple mechanisms could be involved in these effects of cold, such as vasoconstriction and reduced generation of action potentials (Algafly and George, 2007). However, the neurobiological mechanisms underlying cold therapy are not completely understood.

TRPV1 is a polymodal receptor that is activated by capsaicin (CAP, the pungent substance of chili peppers), low pH (< 6.8), and noxious heat (> 43°C) (Caterina *et al*., 1997). TRPV1 activation has been implicated in various pain contexts, and TRPV1 is a reliable target for the development of novel approaches to manage pathological pain conditions (Szallasi *et al*., 2007). It has been postulated that cold-induced suppression of TRPV1 may contribute to anti-nociception (Babes *et al*., 2002; Kichko and Reeh, 2004). In this study, we investigated the underlying mechanisms of cold-induced inhibition of TRPV1 and demonstrate the potential utility of cold for suppressing TRPV1 in nociceptors.

Indeed, cold decreases CAP-induced nociception in humans (Kilo *et al*., 1995), neuropeptide release from skin (Kichko and Reeh, 2004), and ionic currents in sensory neurons (Babes *et al*., 2002). However, the suppressive effects of cold temperature on agonist-mediated activation of recombinant TRPV1 have not been assessed. Currently, the suppressive effects of cold on CAP-induced responses are explained by two mechanisms. First, cold attenuates channel conductance, due to thermodynamic reductions in ionic mobility (Grandl *et al*., 2010). Second, cold reduces the rate of agonist association with receptors (Szallasi and Blumberg, 1993). As opposed to these 'passive' mechanisms, it is not known if cold temperature 'actively' suppresses TRPV1. Heat not only activates TRPV1 but also allosterically modulates the activation by agonists of different modalities, resulting in potentiation of agonist-evoked activation (Vlachova *et al*., 2003; Neelands *et al*., 2008). However, it is not known whether cold can allosterically inhibit TRPV1.

Numerous specific TRPV1 antagonists were developed to selectively suppress pain receptor TRPV1. One of these antagonists has been tested in clinical trials to assess post-surgical pain following third molar extraction (Gavva *et al*., 2008; Wong and Gavva, 2009). However, a major problem of TRPV1 antagonists, revealed in animal studies as well as a clinical trial, is hyperthermia (Steiner *et al*., 2007; Gavva *et al*., 2008). A TRPV1 antagonist also increases the heat-pain threshold in humans (Chizh *et al*., 2007), which could increase the risk of burn injury. Therefore, it is important to selectively inhibit TRPV1 at the site of injury, with few adverse side effects. Although the inhibitory effects of cold on CAP-induced responses are known, whether cold temperature enhances the efficacy of TRPV1 antagonists has not been directly examined.

In this study, we hypothesized that cold temperature suppresses agonist-induced activation of TRPV1 (a null hypothesis: cold temperature does not suppress agonist-induced activation of TRPV1). We tested three predictions: (i) Agonist-induced activation of TRPV1 is suppressed by cold temperature, (ii) cold temperature allosterically inhibits TRPV1, and (iii) the combination of cold and a specific TRPV1 antagonist additively inhibits TRPV1. To investigate these, we examined whether and how cold temperature affects the agonist-induced activation of TRPV1 by electrophysiological, pharmacological, and mutagenic approaches.

MATERIALS & METHODS

To evaluate whether agonist-induced activation of TRPV1 is affected by cold temperature, we used two well-established models for testing the function of TRPV1(Caterina *et al*., 1997; Chung *et al*., 2008). Heterologous expression systems allow us to study the behavior of TRPV1 under the condition when its expression is predominant and also to take a mutagenic approach. The use of cultured sensory neurons is an excellent system for investigating the function of endogenous TRPV1. Since single-channel conductance of TRPV1 is reduced by approximately 45% when the temperature decreases from 25 to 10°C (Grandl *et al*., 2010), the extent of cold-induced suppression above this level will be regarded as an inhibition achieved by mechanisms besides thermodynamic reductions in ionic mobility.

To test whether pathways activated by CAP and heat interact, and if such interaction is necessary for cold-induced suppression, we utilized a mutant TRPV1. Recently, thermal activation was demonstrated to be selectively impaired in TRPV1 N628K/N652T/Y653T with little effects on CAP-evoked activation (Grandl *et al*., 2010). We compared the effects of superimposed application of heat and cold in wild-type and TRPV1 N628K/N652T/Y653T to assess the requirement of allosteric modulation in cold-induced suppression.

Human embryonic kidney (HEK) 293 cells stably expressing rat TRPV1 (TRPV1-HEK) and HEK293/T-antigen cells were cultured and transfected as previously described (Guler *et al*., 2002; Chung *et al*., 2008). Transiently or stably transfected HEK293 cells were re-plated onto poly-L-ornithine-coated coverslips and used for the experiments after 16-24 hrs.

Trigeminal ganglia (TG) neurons were cultured from adult male Sprague-Dawley rats (200-300 g, Harlan, Indianapolis, IN, USA). All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and under a University of Maryland-approved Institutional Animal Care and Use Committee protocol. Rats were sacrificed by a lethal dose of sodium pentobarbital. The ganglia were dissected out and minced in cold DMEM/F12 containing 10% horse serum and penicillin/streptomycin/glutamine (PSG). The ganglia were incubated in 1 mg/mL collagenase (type XI, Sigma, St. Louis, MO, USA) for 30 min at 37°C and triturated with flame-polished Pasteur pipettes. The ganglia were incubated in phosphate-buffered saline containing 0.05% Trypsin and 0.1% EDTA for 2 min. After ganglia were washed with culture medium, dissociated cells were added to a 25% Percoll gradient, centrifuged for 12 min at 900 x *g*, and plated onto poly-L-ornithine- and laminin-coated glass coverslips. The neurons were cultured in DMEM containing 10% horse serum, 1% PSG, and 100 ng/mL nerve growth factor, in 5% CO_2 at 37°C. The neurons were assayed 16 to 48 hrs later.

Conventional whole-cell voltage clamp recording was performed as described previously (Chung *et al*., 2004, 2008). The bath solution contained 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (in mM, pH 7.4 adjusted with NaOH, 300-310 mOsm). The pipette solution contained 140 KCl, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 2.5 Mg-ATP, 10 EGTA, and 10 HEPES (in mM, pH 7.3 adjusted with KOH, 290-300 mOsm). In experiments testing heat-induced potentiation of CAP-evoked currents, the bath solution contained 140 NaCl, 5 KCl, 2 $BaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES (in mM, pH 7.4 adjusted with NaOH, 300-310 mOsm); and the pipette solution contained 140 NaCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES (in mM, pH 7.4 adjusted with NaOH, 290-300 mOsm). Osmolarity of every solution was measured by means of a vapor pressure osmometer (Wescor, Inc., Logan, UT, USA) and was adjusted with mannitol when necessary. Bath temperature was controlled by means of an in-line heater/cooler (Warner Instruments, Hamden, CT, USA). The actual temperature was recorded throughout the experiment with a thermocouple (Physitemp Instruments, Inc., Clifton, NJ, USA) that had been placed within 4 mm of the patch-clamped cell. Temperature values referred to in the RESULTS section represent the mean of the actual temperatures measured for each group.

Data are expressed as the mean ± standard error. Unless otherwise indicated, statistical comparisons were made by unpaired Student's *t* tests. The level of statistical significance was set at $\alpha = 0.05$.

RESULTS

Effects of Cold Temperature on the Agonist-induced Activation of Recombinant and Native TRPV1

In TRPV1-HEK cells, CAP-induced current (I_{CAP}) was robustly attenuated by decreasing bath temperature to 16°C and 10°C, which is within the range of skin surface temperature achieved by the application of an ice pack (Tomchuk *et al*., 2010). Approximately 70% inhibition was achieved at 10°C (Fig. 1A), which was comparable with the inhibition achieved with 0.3 μM AMG9810, a specific antagonist of TRPV1 (Gavva *et al*., 2005) (Fig. 1B). The extent of cold-induced suppression decreased as CAP concentration increased (Fig. 1C). Cold temperature delayed the activation time course by approximately three-fold at 1 μM (20 to 80% rise time; 9.7 ± 2.2 sec at 23°C, n = 9; 31.2 ± 10.5 sec at 10°C, n = 7; $p < 0.05$), but not at 30 μM (1.5 ± 0.1 sec at 23°C, n = 6; 2.2 ± 0.3 sec at 10°C, n = 6; $p > 0.05$). Such cold-induced suppression of activation also occurred when TRPV1 was activated by piperine or resiniferatoxin (Figs. 1D, 1E).

We also tested the effects of cold temperature on the agonist-induced activation of native TRPV1 in cultured TG neurons. I_{CAP} evoked at 11°C was significantly less than the current evoked at 23°C (Figs. 1A, 1B). Membrane capacitances of the two groups

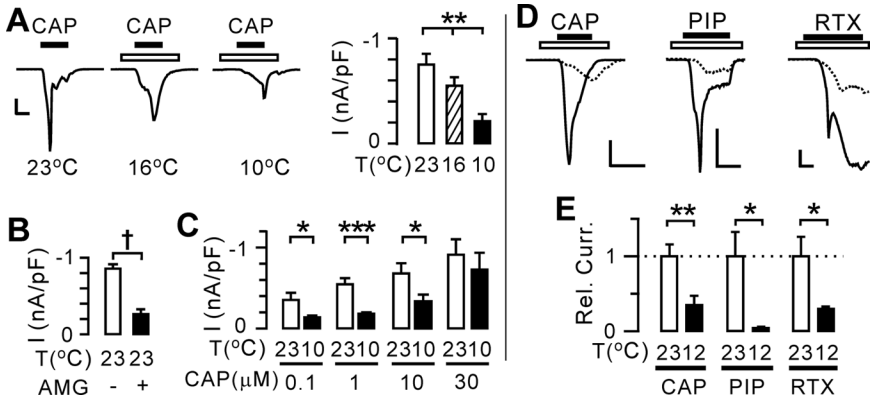

Figure 1. Cold-suppressed agonist-evoked activation of recombinant TRPV1 heterologously expressed in HEK293 cells. (**A**) (left) Representative current traces obtained from HEK293 cells stably expressing rat TRPV1 (TRPV1-HEK) by application of 1 μM capsaicin (CAP) at various temperatures. The duration of each bath temperature is indicated as a white bar above the current traces. Vm = -80 mV; scale bar = 0.1 nA/pF, 30 sec. (right) Current densities evaluated at -80 mV at different temperatures. **p < 0.001 with one-way ANOVA; n = 10; T, temperature. (**B**) Suppression of CAP-evoked currents (I_{CAP}) by 0.3 μM AMG9810 (AMG) under identical recording conditions as in Panel A at 23°C. †p < 10^{-4} with Student's *t* test; n = 6. (**C**) Current densities evoked by different concentrations of CAP at 23°C or 10°C. *p < 0.05; ***p < 10^{-3} with Student's *t* test; n = 5. (**D**) Superimposed whole-cell currents evoked by 1 μM CAP (left), 100 μM piperine (PIP, center), or 10 nM resiniferatoxin (RTX, right) at 23°C or 12°C (dotted). Scale bar = 0.2 nA/pF, 1 min. (**E**) Relative TRPV1 currents evoked by CAP (n = 8), PIP (n = 6), or RTX (n = 4-5) at 23°C or 12°C. Current densities were normalized to the means obtained at 23°C. *p < 0.05; **p < 0.01 with Student's *t* test.

of neurons were not significantly different from one another (16.4 ± 1.4 pF at 23°C *vs*. 16.9 ± 2.5 pF at 11°C, p > 0.8). Since it is well-known that multiple inflammatory mediators sensitize TRPV1 through activation of protein kinase C (PKC) (Szallasi *et al*., 2007), we examined if cold also suppressed the activation of TRPV1 sensitized by phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C. I_{CAP} evoked by a submaximal concentration of CAP (0.3 μM) was significantly enhanced by approximately two-fold following treatment with PMA (p < 0.05). When the ambient temperature was reduced to 10°C during the second application of CAP, I_{CAP} was unchanged from I_{CAP} measured during the first application of CAP (p > 0.9). The extent of sensitization of I_{CAP} by PMA in the 11°C group was significantly different from that in the 23°C group (Figs. 2C, 2D).

Effects of Heat and Cold on TRPV1 Wild-type and TRPV1 N628K/N652T/Y653T

Confirming previous findings (Grandl *et al*., 2010), the ratio of heat-evoked currents at 45°C to I_{CAP} in HEK 293 cells transiently expressing TRPV1 N628K/N652T/Y653T (Mut) was significantly smaller than the ratio obtained for TRPV1 wild-type (WT) (WT, 23 ± 6%, n = 6; Mut, 4 ± 1%, n = 8; p < 0.005 with Mann-Whitney rank-sum test). To test whether heat exerts positive allosteric modulation of I_{CAP} in this TRPV1 mutant, we applied heat (~ 40°C) following receptor activation with a submaximal concentration of CAP. In WT, heat immediately potentiated I_{CAP} by approximately three-fold, which was followed by rapid desensitization below the pre-heat control level. However, in mutant TRPV1, superimposition of CAP and heat did not potentiate I_{CAP} (Figs. 3A, 3B).

Next, we tested whether cold-induced suppression of TRPV1 was affected in mutant TRPV1. Under our recording conditions, I_{CAP} was almost fully recovered from desensitization during the 3-minute washout period, and the ratio of the amplitude of I_{CAP} evoked by the second application of CAP to the first application ($2^{nd}/1^{st}$ I_{CAP}) was 0.88 (Fig. 3Ca). However, when the second application of CAP was performed at 10°C, the $2^{nd}/1^{st}$ I_{CAP} was only 0.22 (Fig. 3Cb), which was significantly less (75%) than the $2^{nd}/1^{st}$ I_{CAP} obtained at 23°C, implying cold-induced suppression of I_{CAP}. Mutant TRPV1 was less likely to recover from desensitization compared with WT following consecutive applications of CAP; however, the effect was not statistically significant (Fig. 3Cc, p > 0.1). When the second application of CAP occurred at 10°C, the $2^{nd}/1^{st}$ I_{CAP} was 0.39, which was only 43% less than the $2^{nd}/1^{st}$ I_{CAP} at 23°C (0.68) (Fig. 3Cd). Consequently, the extent of cold-induced suppression was significantly less in mutant than in WT TRPV1 (Fig. 3D).

Does cold suppress agonist-induced activation of TRPV1 through a mechanism besides the aforementioned mechanisms? Cold-induced suppression of I_{CAP} was not attributed to desensitization or tachyphylaxis (Appendix Fig., A, B). In support, extracellular Ca^{2+} was not required for cold-induced suppression of I_{CAP} (Appendix Fig., C). It is unlikely that cold depletes any putative environmental factors that are required for agonist-induced activation of TRPV1, since pre-emptive application of cold for 2 min had no effect on TRPV1 activation induced by 1 μM CAP at 23°C (-1117 ± 154 pA/pF following pre-treatment at 10°C *vs*. -862 ± 129 pA/pF following pre-treatment at 23°C, n = 6, p > 0.2).

Effects of Superimposed Application of Cold Temperature and the TRPV1 Antagonist on the Agonist-induced Activation of TRPV1

To demonstrate the utility of cold as a suppressor of TRPV1, we tested whether the combination of cold and the antagonist AMG9810 additively suppressed TRPV1 (Fig. 4). I_{CAP} was slightly suppressed by 0.1 μM AMG9810 at 23°C and only modestly suppressed at 14°C. However, I_{CAP} was strongly suppressed (by 80%) when AMG9180 was applied at 14°C.

DISCUSSION

In this paper, we demonstrated that agonist-mediated activation of recombinant TRPV1 can be strongly suppressed when the ambient temperature is reduced, regardless of the type of agonist. Also, we showed that cold can suppress I_{CAP} in TG neurons under normal or sensitized conditions. Analysis of these data suggests that previous reports of cold-induced inhibition of CAP-evoked responses (Kilo *et al*., 1995; Babes *et al*., 2002;

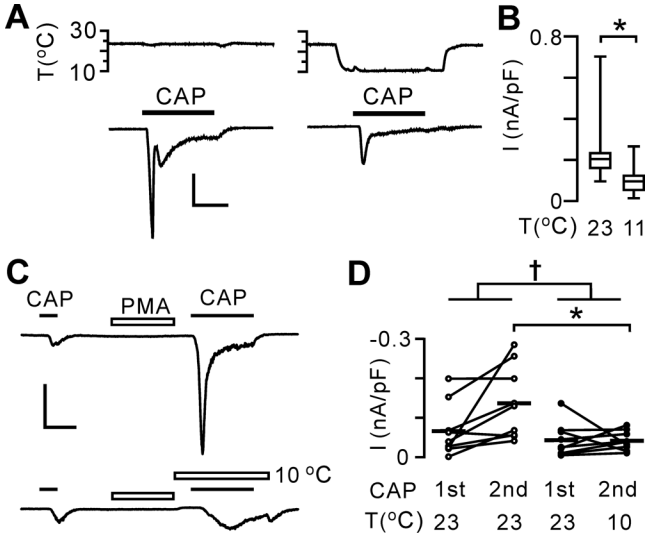

Figure 2. Cold-suppressed I_{CAP} in rat trigeminal ganglia (TG) neurons. (**A**) Representative I_{CAP} traces in small- to medium-sized cultured TG neurons at 23°C (left) or 11°C (right). (upper) Changes in temperature (T) assessed during the current recording. (lower) Whole-cell currents evoked by 3 μM CAP applied during the indicated period. We applied cold stimuli at 11°C for 30 sec prior to the application of CAP at 11°C to identify cold-sensitive neurons, which were excluded from further analysis. Vm = -70 mV; scale bar = 2 nA, 1 min. (**B**) Box and whisker plots of the I_{CAP} densities. Error bars, 5th to 95th percentile; *p < 0.005 with Mann-Whitney U test; n = 12 at 23°C; n = 11 at 11°C. (**C**) (upper) A representative current trace demonstrating sensitization of I_{CAP} by phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, in cultured rat TG neurons. (lower) A representative trace demonstrating the effects of cold temperature on I_{CAP} following sensitization by PMA. CAP, 0.3 μM; PMA, 0.2 μM; scale bar = 1 nA, 1 min. (**D**) Changes in densities of I_{CAP} evoked by the CAP applied before (1st) and after (2nd) the application of PMA. Temperature during the application of CAP is indicated. The mean for each group is marked as a short line. †p < 0.05 with two-way repeated-measure (RM) ANOVA; *p < 0.01 with the Bonferroni *post hoc* test; n = 9.

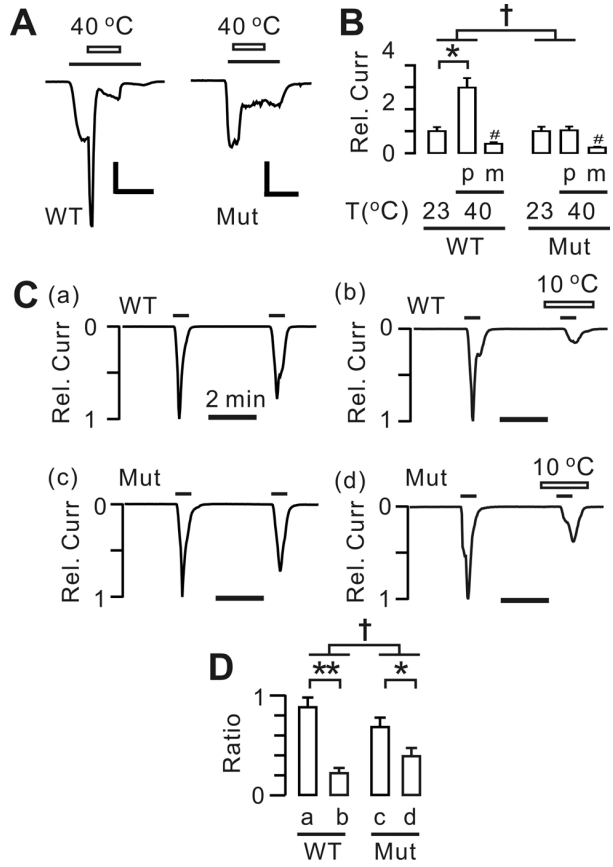

Figure 3. Impaired heat-induced potentiation and cold-induced suppression of I_{CAP} in TRPV1 N628K/N652T/Y653T. (**A**) Representative current traces evoked by CAP (0.1 μM, black bars), superimposed with heat (40°C, white bars), in HEK293 cells transiently transfected with rat TRPV1 wild-type (WT) or TRPV1 N628K/N652T/Y653T triple mutant (Mut). Scale bar = 4 nA, 30 sec in WT; 2 nA, 1 min in Mut. (**B**) Relative current amplitude normalized to the mean I_{CAP} density obtained prior to heat application. p, peak amplitude at 40°C; m, minimum amplitude at 40°C; †p < 10^{-4} with two-way RM ANOVA; *p < 0.001 with the Bonferroni *post hoc* test; #p < 0.0005 with Student's paired *t* test *vs.* peak at 40°C; n = 11 in WT, n = 14 in Mut. (**C**) Normalized current traces evoked by CAP (1 μM, black bars) in WT (**a** and **b**) or Mut (**c** and **d**). For evaluation of the extent of cold-induced suppression, CAP was applied at 23°C the first time and was followed by a second application at either 23°C (a and c) or 10°C (b and d). The duration of cold application is indicated with the white bars above the traces. (**D**) The ratio of currents evoked by the first and second applications of CAP (2nd/1st) in WT and Mut. The letters a to d indicate the groups shown in panel C. †p < 0.05 with two-way RM ANOVA; *p < 0.05 and **p < 0.001 with the Bonferroni *post hoc* test; n = 8-10.

Kichko and Reeh, 2004) may be primarily due to the direct modulation of agonist-mediated activation of TRPV1 by cold temperatures. Cold also efficaciously suppressed I_{CAP} in sensory neurons following sensitization by PMA, suggesting that cold may also inhibit TRPV1 under inflammatory conditions.

Temperature affects ion mobility and therefore reduces the conductance of ion channels, which is a phenomenon reported not only for TRPV1 but also for other thermosensitive TRP channels, including TRPV3 and TRPA1 (Chung *et al*., 2004; Karashima *et al*., 2009). Cold also strongly decreases the association rate of [^{3}H]RTX with native vanilloid receptors (Szallasi and Blumberg, 1993), which can also suppress agonist-induced activation of TRPV1. In this paper, we aimed to demonstrate that cold suppresses the activation of TRPV1 through allosteric inhibition. We demonstrated that TRPV1 N628K/N652T/Y653T is not only impaired in heat activation but also shows complete defect in heat-induced potentiation of I_{CAP}, suggesting that positive allosteric modulation of I_{Cap} by heat is also impaired. Interestingly, this mutant TRPV1 still showed robust heat-induced desensitization of I_{CAP}, indicating that heat-sensing mechanisms *per se* may not be impaired. Importantly, we found that cold-induced suppression of I_{CAP} was partially diminished in the mutant TRPV1. Therefore, we postulate that cold-induced suppression of TRPV1 shares a common molecular basis with heat-induced potentiation of TRPV1, and that such allosteric mechanisms contribute, at least in part, to cold-induced suppression.

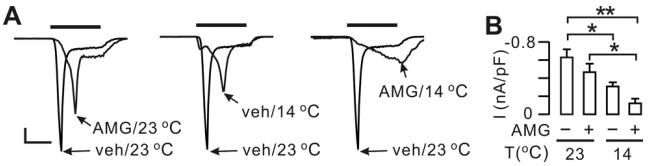

Figure 4. Additive suppression of TRPV1 by the combination of cold and AMG9810, a specific antagonist of TRPV1 in TRPV1-HEK. (**A**) Representative current traces evoked by CAP (1 μM, black bars) at 23°C or 14°C in the presence of AMG (0.1 μM AMG9810) or veh (vehicle, DMSO), as indicated. Scale bar = 0.1 nA/pF, 1 min. **B**) Mean I_{CAP} density. †p < 0.001 with one-way ANOVA; *p < 0.05 and **p < 0.001 with the Bonferroni *post hoc* test; n = 9 in each group.

Selective suppression of TRPV1 localized to an injury site could serve to minimize adverse side effects of TRPV1 antagonists such as hyperthermia (Gavva *et al*., 2008) and an altered heat-pain threshold (Chizh *et al*., 2007). We demonstrated that cold and AMG9810 additively suppressed agonist-induced activation of TRPV1. These results suggest that localized cooling could be a supplemental approach to increase the efficacy of selective TRPV1 antagonists at the site of injury, which may reduce the dose of antagonists and adverse systemic side effects.

In this paper, we focused our analysis on the effects of cold temperature on the activation of TRPV1 at the receptor level. Therefore, the efficacy of the application of cold alone or combined with TRPV1 antagonists for analgesia needs to be verified at the systems level in experimental animals and in clinical trials. The relative contribution of suppression of TRPV1 to cold-induced analgesia also needs to be determined.

In conclusion, we rejected the null hypothesis based upon our results. Overall, our results elucidate detailed mechanisms involved in cold-induced suppression of TRPV1 and provide a mechanistic basis for cold therapy, which is widely used as a supplemental anti-nociceptive approach. Also, we suggest a potential utility of cold therapy as a supplemental method for targeting TRPV1, which may improve our ability to manage pain under inflammation and tissue injury, including that from tooth extraction.

ACKNOWLEDGMENTS

The authors thank Dr. Ardem Patapoutian for kindly providing the mutant TRPV1 cDNA construct, and John Joseph for reading the manuscript. This work was supported by NIDCR (DE019694 to MKC). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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