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Oxidation of extracellular cysteines by mercury chloride reduces TRPV1 activity in rat dorsal root ganglion neurons

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Transient receptor potential vanilloid type 1 (TRPV1) receptor plays an important role as a molecular detector of noxious signals in primary sensory neurons. Activity of TRPV1 can be modulated by the change in the environment such as redox state and extracellular cations. In the present study, we investigated the effect of the mercury chloride ($HgCl_2$) on the activity of TRPV1 in rat dorsal root ganglia (DRG) neurons using whole-cell patch clamp technique. Extracellular $HgCl_2$ reversibly reduced the magnitudes of capsaicin-activated currents (I_{cap}) in DRG neurons in a dose-dependent manner. The blocking effect of $HgCl_2$ was prevented by pretreatment with the reducing agent dithiothreitol (DTT). Inhibition of I_{cap} by $HgCl_2$ was abolished by point mutation of individual cysteine residues located on the extracellular surface of TRPV1. These results suggest that three extracellular cysteines of TRPV1, Cys616, Cys634 and Cys621, are responsible for the oxidative modulation of I_{cap} by $HgCl_2$.

Keywords: $HgCl_2$; sulfhydryl oxidation; capsaicin; TRPV1; DRG neuron; rat

Introduction

TRPV1 (transient receptor potential vanilloid receptor-1) is a nonselective cation channel activated by noxious heat and low pH as well as capsaicin (Oh et al. 1996; Caterina et al. 1997; Tominaga et al. 1998). TRPV1 plays an important role as a molecular detector of noxious signals in primary sensory neurons (Ramsey et al. 2006). TRPV1 activity is modulated by various substances involved in inflammation, such as bradykinin (Shin et al. 2002), histamine (Shim et al. 2007), prostaglandins (Lopshire and Nicol 1997), and the pro-inflammatory chemokine CCL3 (Zhang et al. 2005). By activating or inhibiting multiple protein kinases (PKA, PKC, PI3K), these inflammatory mediators modulate TRPV1 responses to stimuli (Levine and Alessandri-Haber 2007). In addition, redox-active substances (Jin et al. 2004; Tousova et al. 2004; Susankova et al. 2006) and extracellular cations regulate the TRPV1 activity (Ahern et al. 2005; Tousova et al. 2005).

Mercury is well known as a highly toxic heavy metal. There are numerous sources of mercury exposure, including seafood, seeds, foodstuffs, disinfectant, electrical equipment and dental amalgam (Aschner and Walker 2002). Acute and chronic exposure to methylmercury (MeHg) and inorganic mercury (Hg^{2+}) may cause consequential damage in various organs, especially in neural tissues. Neurotoxic effects of mercury are partly attributed to their interaction with ion channels of nervous systems (Sirois and Atchison 1996). Hg^{2+} blocked sodium channels (Quandt et al. 1982; Hisatome et al. 2000; Yatsuhashi et al. 2002), potassium currents in outer hair cells (Liang et al. 2003), TWIK-related spinal cord K^+ channel (Czirjak and Enyedi 2006) and calcium channels in cerebellar granule cells (Sirois and Atchison 2000) and in dorsal root ganglia (DRG) neurons (Pekel et al. 1993). GABA-induced current was stimulated by Hg^{2+} in rat DRG neurons (Huang and Narahashi 1996). Hg^{2+} also inhibited CFTR chloride channels in the shark rectal gland (Ratner et al. 2006).

Hg^{2+} and other thiol-avid group IIB cations (Cd^{2+} and Zn^{2+}) have been revealed to have a high affinity for sulfhydryl groups of the critical cysteine residues (Clarkson 1972; Halbach 1989, 1990). It is well known that sulfhydryl groups on cysteines of proteins are the most reactive among all amino acid side chains under physiological conditions (Kenyon and Bruice 1977) and also have been known to play a key role in regulating various ion channel functions. We have reported the critical extracellular cysteine residue of TRPV1 for inhibition by thimerosal, a mercury-containing oxidizing agent (Jin et al. 2004). In this study, we investigated whether $HgCl_2$ can modify the activity of TRPV1 by oxidation of sulfhydryl groups and which cysteine residue(s) is (are) involved in this effect.

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Materials and methods

Cell culture

Primary cultures of DRG neurons were dissected from all levels of spinal cord of 2-d-old neonatal rats. The dissected ganglia were collected in cold culture medium (4°C). The culture medium contained DMEM/F-12 mixture (Invitrogen, Grand Island, NY, USA), 10% fetal bovine serum (FBS, HyClone, South Logan, Utah, USA), 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The collected ganglia were washed with the DMEM/F-12 mixture and incubated at 37°C for 30 minutes in the culture medium containing 1 mg/ml collagenase (Type II; Worthington, Freehold, NJ, USA). Then ganglia were washed three times with Mg^{2+}- and Ca^{2+} free Hank's balanced salt solution (HBSS, Invitrogen) and incubated in HBSS containing 2.5 mg/ml trypsin (Invitrogen) at 37°C for 30 minutes. After trypsin treatment, ganglia were centrifuged at 1000 rpm for 10 minutes. The pellet was washed two times with the culture medium and resuspended in the culture medium by gentle trituration with a fire-polished Pasteur pipette. The resuspended cells were plated on glass coverslips coated with 0.04 mg/ml polyethylenimine (Sigma). Then, nerve growth factor (25 ng/ml, Alomone labs, Jerusalem, Israel) was added to each Petri dish. Cells were incubated at 37°C in a 95% air-5% CO_2 gas mixture and used 1–3 days after plating.

Site-directed mutagenesis and expression in HEK 293 cells

Mutations of each cysteine residue of rat TRPV1 were introduced into pcDNA3.1–TRPV1 with overlapping mutagenic primers using a site-directed mutagenesis kit (Quick Change™; Stratagene, La Jolla, CA, USA). Mutations were verified by sequencing the mutation sites. Wild-type and mutant TRPV1 were then transfected into HEK293 cells (Lipofectamine™ 2000, Invitrogen/Life Technologies). HEK293 cells were maintained in modified Eagle's medium (MEM, Life Technologies), supplemented with 10% FBS and 100 U/ml penicillin/streptomycin, and were used 1–3 days after plating.

Electrophysiology

Patch clamp recordings in whole-cell configuration were employed to measure membrane currents from DRG neurons. Patch pipettes made of 15 mm standard wall borosilicate glass capillary tubes (Harvard, Kent, UK) were pulled on a two-stage puller (PP-83, Narishige, Japan) to have a resistance of $\sim$3 MΩ. For whole-cell recording, the cell membrane was ruptured by a gentle suction after gigaseals were formed. Then, the capicitative transients were cancelled. Whole-cell membrane currents were recorded using an EPC 7 amplifier (HEKA, Lambrecht, Germany) and filtered at 3 kHz with a low-pass Bessel filter. Data were acquired at a sample frequency of 1 kHz and analyzed using pClamp6 software (Axon Instruments, Union City, CA, USA).

Solutions and chemicals

The Ca^{2+}-free extracellular solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 10 EGTA (pH was adjusted to 7.4 with NaOH). The patch pipettes were filled with an intracellular solution of the following composition (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 BAPTA, 2 ATP-Mg, and 0.1 GTP-Na (pH was adjusted to 7.4 with KOH). Capsaicin was dissolved and stored as 10 mM stock solution in 100% ethanol. Capsaicin concentration used in whole-cell recording was fixed at 0.3 μM throughout the experiments. $HgCl_2$ was dissolved and stored as 1 M and 10 mM stock solution in 100% ethanol. $CdCl_2$, $ZnCl_2$, $CuCl_2$, and $CoCl_2$ were dissolved and stored as 1 M stock solution in distilled water. Dithiothreitol (DTT) was prepared in the Ca^{2+}-free extracellular solution at an appropriate concentration immediately before use. All chemicals were purchased from Sigma-Aldrich.

All data are expressed as mean $\pm$ S.E.M. The statistical analysis was done using paired Student's *t*-test and ANOVA, and a value of $P < 0.05$ was considered significant.

Results

Mercury chloride ($HgCl_2$) reduced TRPV1 activity in cultured DRG neurons

Figure 1 shows that extracellular $HgCl_2$ reversibly reduced the activity of TRPV1 in cultured DRG neurons. Extracellualr application of capsaicin (0.3 μM) for 20 s evoked capsaicin-activated inward currents (I_{cap}) through TRPV1 in cells at a holding potential of -60 mV. Co-application of capsaicin and $HgCl_2$ (1 mM) decreased I_{cap} to 24.8 $\pm$ 3.4% of the control capsaicin responses ($n = 5$, $P < 0.01$). The I_{cap} decreased by extracellular $HgCl_2$ recovered substantially to 60.0 $\pm$ 7.2% of the control capsaicin response after Hg^{2+} had been washed out. Figure 1B shows the dose-response dependence of the $HgCl_2$ effect on I_{cap}, obtained in four to 12 experiments. The dose-response curve for $HgCl_2$-induced blockade of I_{cap} was fitted

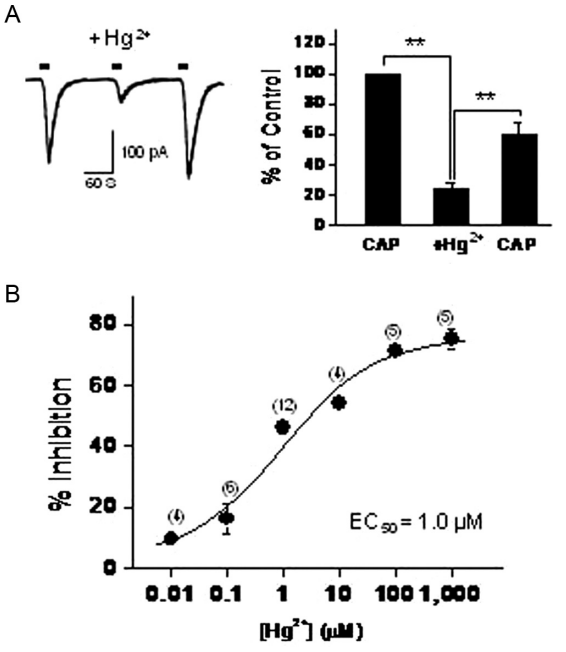

Figure 1. Effects of extracellular mercury ($HgCl_2$) on I_{cap} in cultured DRG neurons. (A) Representative trace shows the reduction of I_{cap} by mercury chloride (1 mM). Bars over the trace indicate the application of capsaicin (0.3 μM). (B) $HgCl_2$ inhibited I_{cap} in a concentration-dependent manner. Data are the mean ± S.E.M. percentile inhibition of I_{cap} at various concentrations (0.01 μM –1 mM) of $HgCl_2$ in 4–12 experiments ($IC_{50} = 1.0$ μM).

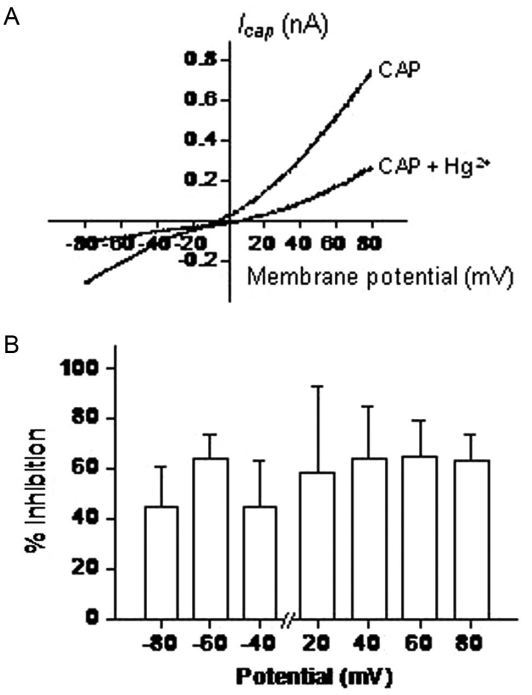

Figure 2. Effect of $HgCl_2$ on I_{cap} was voltage-independent. (A) Current–voltage relationships of I_{cap} in the absence and presence of 1 mM $HgCl_2$. Basal current was subtracted. (B) Voltage-independent inhibition of I_{cap} by $HgCl_2$. The degrees of I_{cap} inhibition by Hg^{2+} were not significantly different at various membrane potentials.

by the Hill equation. The IC_{50} value for $HgCl_2$ was 1.0 μM.

Effect of $HgCl_2$ on I_{cap} is voltage-independent

To examine whether $HgCl_2$ affected the current–voltage relationship of I_{cap}, voltage ramps from the membrane potential of -80 to +80 mV were applied to cells for 1 s ($n = 5$). The current–voltage plot (Figure 2A) shows that $HgCl_2$ caused a marked decrease of the membrane current without a significant change of the reversal potential. If mercury chloride binds to a site in the channel pore, its effect should be affected by the driving force across the cell membrane. As shown in Figure 2B, the degree of I_{cap} inhibition by $HgCl_2$ was not significantly different at either negative or positive membrane potentials, indicating that mercury chloride binds to an external site of TRPV1.

Reducing agent reversed I_{cap} decrease caused by $HgCl_2$

Mercury is able to interact with sulfhydryl groups of the cysteine residues. Therefore, we suspected that the inhibitory effect of $HgCl_2$ on I_{cap} might result from the oxidation of the thiol groups that are present in TRPV1. To verify whether the inhibitory effect of $HgCl_2$ on I_{cap} was caused by the oxidation of TRPV1, we tested the effect of a reducing agent, DTT. First, we confirmed the effect of DTT on I_{cap} in DRG neurons. Consistent with the previous report which showed facilitation of heat-activated current (I_{heat}) by DTT (Viklicky et al. 2002), DTT also potentiated I_{cap}. As shown in Figure 3A, co-application of DTT (10 mM) and capsaicin slightly decreased I_{cap} to 80.5 ± 3.9% ($P < 0.01$; $n = 5$), whereas 30 and 50 mM DTT significantly increased I_{cap} to 158.7 ± 9.6% ($P < 0.01$; $n = 5$) and to 211.8 ± 29.2% of the control ($P < 0.05$; $n = 5$), respectively. It was confirmed that the augmentation of I_{cap} by DTT was not the result of sudden increase in osmolarity of the bath solution by using 30 mM mannitol (Jin et al. 2004).

As shown in Figure 3B, the magnitude of I_{cap} blockade by $HgCl_2$ was significantly diminished to 7.0 ± 4.3% ($P < 0.05$, $n = 6$) in the presence of DTT, at a concentration that did not increase I_{cap} (10 mM). These results suggest that DTT protected free sulfhydryl groups in TRPV1 from the oxidation by $HgCl_2$.

We then investigated whether the decrease of I_{cap} by oxidative modification of sulfhydryl groups is a common property of transition metals. Cd^{2+} and Zn^{2+}

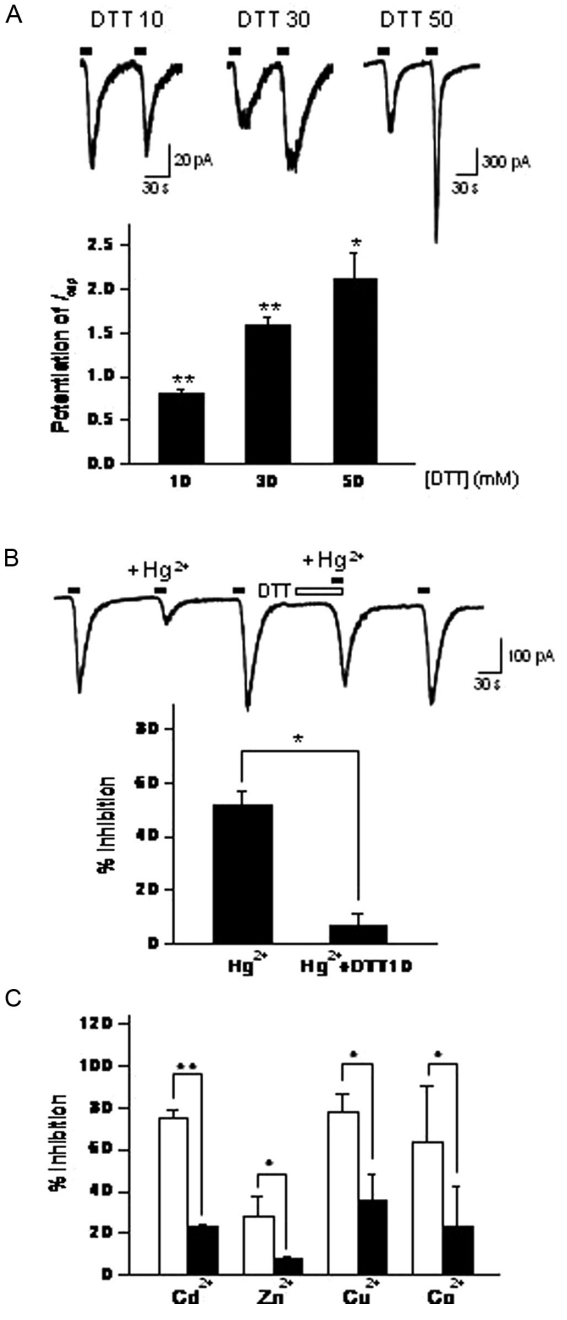

Figure 3. DTT prevented the decrease of I_{cap} by $HgCl_2$. (A) DTT enhanced I_{cap} dose-dependently. The bar graph summarizes the effects of DTT on I_{cap} at a holding potential of -60 mV. Values at the top of each bar represent the number of experiments. (B) Hg^{2+}-induced decrease of I_{cap} was blocked by pretreatment with 10 mM DTT. The bar graph summarizes the relative responses in the presence and absence of 10 mM DTT. (C) The bar graph summarizes the percentile inhibition of I_{cap} by Zn^{2+}, Cd^{2+}, Cu^{2+}, and Co^{2+} in the absence (open bar) and presence (filled bar) of 10 mM DTT. Bars represent the mean $\pm$ S.E.M. Asterisks indicate significant differences at * $p < 0.05$ and ** $P < 0.01$.

from the same group in the periodic table as Hg^{2+} also decreased I_{cap} by $75.1 \pm 4.3\%$ ($P < 0.01$; $n = 9$) and $72.3 \pm 9.5\%$ ($P < 0.05$; $n = 8$) of the control capsaicin response, respectively. Cu^{2+} and Co^{2+} from the same series in the periodic table as Hg^{2+} blocked I_{cap} by $78.0 \pm 8.8\%$ ($P < 0.05$; $n = 5$) and $63.6 \pm 26.5\%$ ($P < 0.05$; $n = 3$) of the control response, respectively. Figure 3C shows that blocking effects of these metals on I_{cap} were also prevented in the presence of DTT (10 mM). Although further studies will be needed, it is possible that various transition metals decrease TRPV1 activity via sulfhydryl oxdation.

$HgCl_2$-induced Inhibition of I_{cap} requires extracellular free sulfhydryl groups

To determine which cysteine residue(s) in TRPV1 was (were) sensitive to extracellular $HgCl_2$, we replaced each of the three extracellular cysteine residues with serine or alanine by site-directed mutagenesis. Wild-type and mutants were transiently transfected into HEK293 cells which do not express endogenous TRPV1 (Caterina et al 1997). Cells that did not respond to capsaicin could be regarded as untransfected and I_{cap} was evoked in approximately 30% of the cells (23 out of 81 cells). Representative responses of the wild-type and each mutant TRPV1 to capsaicin and $HgCl_2$ are shown in Figure 4A. As summarized in Figure 4B, I_{cap} was decreased by 100 μM $HgCl_2$ to $73.3 \pm 2.3\%$ of the control capsaicin response in HEK293 cells expressing wild-type TRPV1 ($n = 3$, $P < 0.05$). It has been postulated that Cys621 is critical for oxidative modification of TRPV1 (Jin et al. 2004; Susankova et al. 2006). As expected, $HgCl_2$ failed to decrease I_{cap} in HEK293 cells expressing the C621A mutant (I_{cap}: $91.9 \pm 6.4\%$ of the control in the presence of Hg, $n = 6$; Figure 4B). However, in HEK293 cells expressing the C616S or C634S mutant channel, the blocking effects of $HgCl_2$ on I_{cap} were also abolished (I_{cap}: $93.7 \pm 5.0\%$, $126.8 \pm 9.2\%$ of the control in C616S and C634S mutants, respectively). These results suggest that inhibition of TRPV1 by $HgCl_2$ requires all the three extracellular sulfhydryl groups to remain free.

Discussion

In the present study, we demonstrated that the oxidation of free sulfhydryl groups in TRPV1 by $HgCl_2$ decreased the channel activity and all three cysteine residues (C616, C621, and C634) on the external surface of TRPV1 were required.

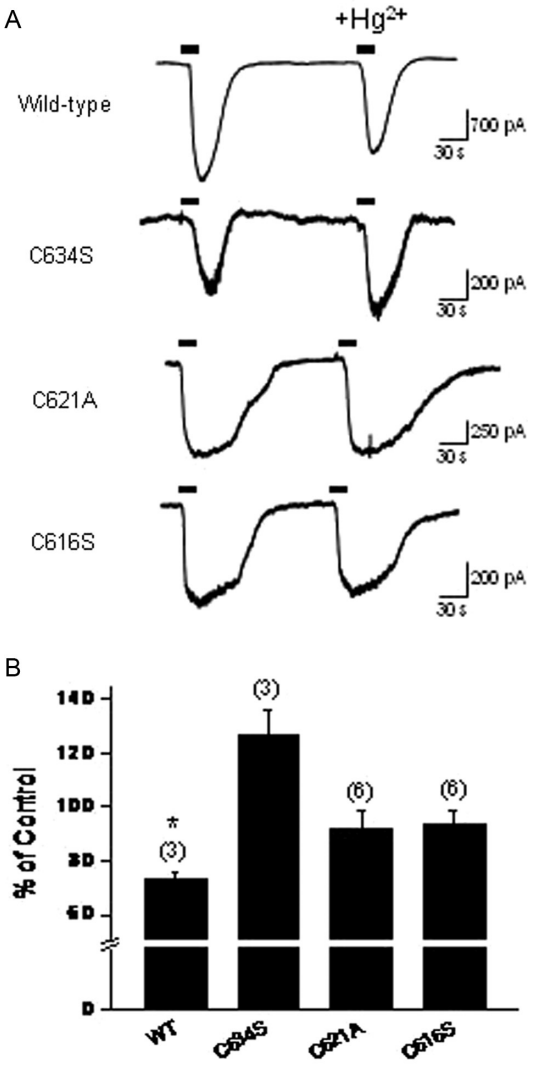

Figure 4. $HgCl_2$-induced down-regulation was abolished by mutations of extracellular cysteine residues of TRPV1. (A) Representative traces show the effect of Hg^{2+} (100 μM) on I_{cap} in HEK293 cells expressing wild-type and mutant TRPV1. Mutations of extracellular cysteines abolished the inhibitory effects of Hg^{2+} on I_{cap}. (B) Summary of the effects of Hg^{2+} on I_{cap} in HEK293 cells expressing wild-type or mutant channels. Values at the top of each bar represent the number of experiments. Bars represent the mean ± S.E.M. Asterisks indicate a significant difference from the control value ($P < 0.05$).

Although mercury is a nonphysiological heavy metal and a widespread environmental pollutant, people can be exposed to methylmercury and inorganic mercury via various routes such as accidents, occupational and amalgam dental restorations. It has been well known that mercury is able to oxidize sulfhydryl groups on proteins. Although we have briefly mentioned that $HgCl_2$ reduced the activity of TRPV1 (Aschner and Walker 2002; Jin et al. 2004), the underlying mechanism was not determined. Considering the protective effect of DTT on the $HgCl_2$-induced decrease of I_{cap}, the action of $HgCl_2$ appeared to be mediated through promoting oxidation of free sulfhydryl groups of TRPV1. Therefore, we tried to determine the essential cysteine residues on TRPV1 responsible for the modulation. It has been reported that most mercury chloride complexes at a chloride concentration of 142 mM and pH 7.4 are in the charged form, $HgCl_4^{2-}$ (Huang and Narahashi 1996). Because a Ca^{2+}-free bath solution (pH 7.4) containing 147 mM chlorides was used in the experiments, we expected that extracellularly applied $HgCl_2$ could not move through the cell membrane. Therefore, the effects of $HgCl_2$ on I_{cap} might be mediated by the interaction with extracellular cysteine residues in TRPV1. In addition, the blocking effect of $HgCl_2$ on I_{cap} was voltage-independent, also indicating that mercury binds to an external site of TRPV1. Because extracellular cysteine residues are located in the vicinity of the putative p-loop region of TRPV1, interaction of $HgCl_2$ with sulfhydryl groups of these cysteines may impair the ion flow through the channel pore.

We also investigated whether the decrease of TRPV1 activity by oxidation of sulfhydryl groups is a unique property of Hg^{2+}. Cd^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} also decreased I_{cap} reversibly. And the decreasing effects of these metals on I_{cap} were prevented by the pretreatment with DTT. These results suggest that various divalent transition metals can regulate TRPV1 activity by interaction with free sulfhydryl groups. In addition, it has been reported that extracellular cations, Na^+, Mg^{2+}, Ca^{2+}, and Gd^{3+}, sensitized and gated TRPV1 via electrostatic interaction with proton binding sites of TRPV1 (Ahern et al. 2005; Tousova et al. 2005). These results demonstrate that activity of TRPV1 is susceptible to being modulated by various extracellular cations as well as inflammatory mediators.

Next, we tried to identify cysteine residue(s) involved in Hg^{2+}-induced oxidative modification of TRPV1 using mutant channels. Cys621 has been reported (Tousova et al. 2004) to be critical for redox-mediated modulation of TRPV1 (Jin et al. 2004). However, $HgCl_2$ failed to decrease I_{cap} in the HEK293 cells expressing C616S or C634S as well as C621A TRPV1 mutant. Thus, all three extracellular cysteine residues seem to be critical for the oxidative modulation of TRPV1 activity by $HgCl_2$. This differ-

ence may result from the molecular size of $HgCl_2$ and other oxidants such as thimerosal. Although the exact mechanism of inter- or intrasubunit interaction between mercury and free sulfhydryl groups is still unknown, all three extracellular sulfhydryl groups of TRPV1 may be essential for the action of $HgCl_2$.

In contrast to our results, Susankova et al. (2006) have reported that various oxidizing agents potentiated heat-evoked responses (I_{Heat}). They argued that the decrease of capsaicin response by oxidizing agents could result from the reduction in concentration of capsaicin itself by the oxidant. However, their suggestion cannot explain our observations that $HgCl_2$ and thimerosal failed to inhibit the activities of mutant TRPV1. If the effective concentrations of capsaicin were diminished by oxidant, the amplitudes of I_{cap} recorded from mutant TRPV1 must be reduced by co-application of oxidant and capsaicin. We still have no idea why the effects of oxidants on I_{cap} are different from those on I_{Heat}. Of course we cannot exclude the possibility that there might be some differences between capsaicin and heat-evoked responses via TRPV1 activation.

In this study, we demonstrate that Hg^{2+} decreases TRPV1 activity by oxidation and all three extracellular cysteines on TRPV1 may be required for this oxidative modulation. Our observation that trace metals can modulate the capsaicin response of the sensory neurons offers new insights into the neuromodulatory role of heavy metals.

Acknowledgements

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