Inactivation of TRPM2 Channels by Extracellular Divalent Copper

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Abstract

Cu²⁺ is an essential metal ion that plays a critical role in the regulation of a number of ion channels and receptors in addition to acting as a cofactor in a variety of enzymes. Here, we showed that human melastatin transient receptor potential 2 (hTRPM2) channel is sensitive to inhibition by extracellular Cu²⁺. Cu²⁺ at concentrations as low as 3 μM inhibited the hTRPM2 channel completely and irreversibly upon washing or using Cu²⁺ chelators, suggesting channel inactivation. The Cu²⁺-induced inactivation was similar when the channels conducted inward or outward currents, indicating the permeating ions had little effect on Cu²⁺-induced inactivation. Furthermore, Cu²⁺ had no effect on single channel conductance. Alanine substitution by site-directed mutagenesis of His995 in the pore-forming region strongly attenuated Cu²⁺-induced channel inactivation, and mutation of several other pore residues to alanine altered the kinetics of channel inactivation by Cu²⁺. In addition, while introduction of the P1018L mutation is known to result in channel inactivation, exposure to Cu²⁺ accelerated the inactivation of this mutant channel. In contrast with the hTRPM2, the mouse TRPM2 (mTRPM2) channel, which contains glutamine at the position equivalent to His995, was insensitive to Cu²⁺. Replacement of His995 with glutamine in the hTRPM2 conferred loss of Cu²⁺-induced channel inactivation. Taken together, these results suggest that Cu²⁺ inactivates the hTRPM2 channel by interacting with the outer pore region. Our results also indicate that the amino acid residue difference in this region gives rise to species-dependent effect by Cu²⁺ on the human and mouse TRPM2 channels.

Introduction

The TRPM2 channel belongs to the melastatin subfamily of the mammalian transient receptor potential (TRP) channels, which share several conserved domains with other TRP channels, such as the TRPM homology domains (MHD domains) in the N-terminus and the TRP box and coiled-coil domain in the C-terminus [1–4]. The TRPM2 channel is a homo-tetramer and each subunit contains six transmembrane segments with a pore-forming region between the fifth and sixth segments and intracellular N- and C-termini [5]. The TRPM2 channel is a non-selective cation channel and permeates calcium ion, and is activated by intracellular ADP-ribose (ADPR) [1,6] or intracellular calcium [7–10]. Accumulating evidence indicates that the TRPM2 channel plays an important role in a number of physiological and pathophysiological processes, including neurodegeneration, immunological functions, insulin release [11–15]. Previous studies showed that the TRPM2 channel can undergo rapid inactivation upon exposure to extracellular proton and Zn²⁺ that interact selective residues in the pore region [9,16,17]. Mutation of the residues in the pore region can strongly alter the channel inactivation. Thus, the disease-associated PI018L mutation conferred rapid inactivation of the hTRPM2 channel, whereas manipulation of the pore region by site-directed mutagenesis resulted in a TRPM2-LDE mutant channel that exhibited no inactivation, suggesting alterations in the conformation and structure of the pore region represent an important molecular mechanisms of the TRPM2 channel inactivation [18,19].

Cu²⁺ is the third abundant trace metal in the human body, and plays a critical role in a variety of physiological and pathological conditions. Cu²⁺ is a cofactor for a variety of enzymes, and relates to the formation of reactive oxygen species. Like zinc, excessive Cu²⁺ is toxic for neurons [20,21]. Cu²⁺ is involved in several human diseases [22–24], and the Cu²⁺ chelators have been intensively used as therapeutic treatments Cu²⁺ related diseases, such as Wilson’s disease and cancer [25]. Several studies suggest Cu²⁺ and Zn²⁺ regulate cell functions via altering the activity of a variety of ion channels [26,27]. For example, Cu²⁺ reduces the tonic inhibition of neurons by blocking the GABAA receptors [28]. Therefore, elucidating the mechanisms regulating ion channels by Cu²⁺ is critical for a better understanding of its physiological and pathological roles in humans.

It is well known that Cu²⁺ can activate, modulate or inhibit ion channels. For example, Cu²⁺ activates the TRPV1 and TRPA1
channels [29,30] and, by contrast, Cu$^{2+}$ inhibits endothelial Na$^+$ channels [31], BK and Shaker K$^+$ channels [32]. A recent study has reported that extracellular Cu$^{2+}$ induces the hTRPM2 channel inactivation [33], but the underlying molecular or structural basis still remains unclear. Here, using site-directed mutagenesis and patch-clamp recording, we identified His995 in the pore region to be crucial in Cu$^{2+}$-induced hTRPM2 channel inactivation. In addition, the mTRPM2 channel is insensitive to Cu$^{2+}$ and such a species-dependent effect by extracellular Cu$^{2+}$ arises from replacement of His995 in the hTRPM2 channel with glutamine at the equivalent position in the mTRPM2 channel.

Materials and Methods

Clones, cells and molecular biology

The cDNAs encoding the hTRPM2 and mTRPM2 were kindly provided by Dr AM Scharenberg (Washington University, USA) and Dr Y Mori (Kyoto University, Japan), respectively. Tetracycline-inducible HEK293 cells stably expressing hTRPM2 was kindly provided by Dr AM Scharenberg. The point mutations were introduced by site-directed mutagenesis [34] and confirmed by sequencing. Human embryonic kidney (HEK) 293 cells were used to transiently express wild-type (WT) and mutant channels. HEK293 cells were maintained in DMEM/F-12 medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA). Cell transfection was described previously [4]. Tetracycline-inducible hTRPM2-expressing HEK293 cells were used in some experiments and the expression of the hTRPM2 channel was induced by adding 1 μg/ml tetracycline in the culture medium for 12–24 h before use.

Electrophysiology

Whole-cell current recordings were performed using an Axonpatch 200B amplifier at room temperature as described previously [4]. The extracellular solution contained (in mM): 147 NaCl, 2 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, and 13 glucose,
pH 7.4. The intracellular solution contained (in mM): 147 NaCl, 0.05 EGTA, 1 MgCl₂, 10 HEPES, and 0.5 ADPR, pH 7.3. Thus, the currents were mainly carried by Na⁺. The cell membrane potential was held at 0 mV. To record ADPR-induced currents, voltage ramps with 500 ms duration from −2100 mV to 100 mV were applied every 5 s. The glass microelectrodes with resistance of 3–5 MΩ were used. Single channel activity recordings were performed using a HEKA EPC10 amplifier controlled with PatchMaster software (HEKA), and carried out in the outside-out configuration as previously described [17]. Data were acquired at 10 kHz and filtered offline at 50 Hz. CuSO₄ stock solution was prepared by dissolving in the extracellular solutions. Changes of extracellular solutions and applications of Cu²⁺, clotrimazole (CLT), 2-ME and EDTA were achieved using an RSC-160 system (Biologic Science Instruments) with a solution change time of 300 ms [4].

Data analysis

The data are presented in the text and figures, where appropriate, as mean ± S.E. values. The inhibition was expressed by the currents in the indicated solutions as the percentage of the currents before the solution changes, and the kinetics were estimated by determining the time required for 90% inhibition (t₉₀%). Single-channel conductance was estimated from all-point histograms constructed from the current events recorded at −80 mV. A double-Gaussian function was fitted to the histograms as previously described [35]. Statistical analysis was performed using Student’s t-test with p<0.05 designated as significant difference.

Results

Extracellular Cu²⁺ induces hTRPM2 channel inactivation

We first investigated the effect of extracellular Cu²⁺ on the open hTRPM2 channels. The activity of the hTRPM2 channels was induced by application of ADPR (500 µM) in the pipette solution, as illustrated by the inward currents at −80 mV (Fig. 1A). Perfusion with 3 µM Cu²⁺ abolished the hTRPM2 channel currents in several minutes, as reported by a recent study [33]. While the hTRPM2 channels were completely inhibited by Cu²⁺ independently of concentrations from 3 µM to 1 mM, the time required for 90% inhibition (t₉₀%) was concentration-dependent from 98.4±16.7 s (n = 4) at 3 µM to 71.2±1.5 s (n = 5) at 1 mM (Fig. 1B). The inhibitory effect of Cu²⁺ was irreversible on washout, which suggests that Cu²⁺ induced channel inactivation like proton and zinc [16,17,36]. Such inactivation could result from either channel inactivation or tight binding of Cu²⁺ to the hTRPM2 channels. To clarify this issue, we applied 2-ME or EDTA to chelate Cu²⁺, after Cu²⁺ induced complete inhibition of ADP-induced currents. The inhibition was still not rescued by treating with the 2-ME or EDTA for up to two min (Fig. 1C, D).
which suggests that Cu$^{2+}$ may not bind with the TRPM2 after TRPM2 inactivation, or alternatively Cu$^{2+}$ tightly binds with the hTRPM2 channels in the small pocket and both EDTA and 2-ME cannot access.

Our previous study showed that Zn$^{2+}$ inactivates the hTRPM2 channel and such inactivation is strongly affected by the permeant ions [17]. It was thus interesting to know whether Cu$^{2+}$-induced hTRPM2 inactivation was similar as that by Zn$^{2+}$. Unlike Zn$^{2+}$, 100 μM Cu$^{2+}$ still inhibited irreversibly the hTRPM2 channel currents at 40 mV (Fig. 2A), which suggests the hTRPM2 inactivation induced by Cu$^{2+}$ may be different from that by Zn$^{2+}$. Moreover, we investigated whether Cu$^{2+}$ affects the single channel conductance using the patch-clamp recording in outside-out configuration. As shown in Fig. 3, the single channel conductance of the hTRPM2 channels was not changed by Cu$^{2+}$.

His995 is a key residue in the outer pore in Cu$^{2+}$-induced inactivation

Metal ions can interact with polar amino acid like histidine, glutamate, aspartate, and lysine [9,16,36,37]. Several studies have identified histidine, cysteine, aspartic acid, arginine and glutamine determine Cu$^{2+}$-induced channel inhibition or inactivation, such as the P2X7 receptor [38], GABA$\alpha$ receptor [28], ENaC channel [31], BK channel [32]. In addition, a previous study of olfactory CNG channel gating suggests that the extracellular pore region is importantly involved in the channel gating [39]. Our previous studies have identified several amino acids which are responsible for channel inactivation induced by proton and Zn$^{2+}$ (Fig. 4A). We hypothesized that Cu$^{2+}$ may interact with similar amino acid residues in the pore region which Zn$^{2+}$ binds to (Fig. 5). We introduced alanine substitution into the candidate amino acid residues as described in previous studies [16,36], and determined the effects of 100 μM Cu$^{2+}$ on the functional mutant channels. All of these mutants did not strongly resulted in strong channel inactivation as illustrated by several examples in Fig. 4. Representative current traces of the hTRPM2 mutant channels are illustrated in Fig. 4A, and the mutational effects on the Cu$^{2+}$-induced inhibition and inactivation are summarized in Fig. 4B. Several mutants, albeit still be inactivated by Cu$^{2+}$, exhibited significantly slower inactivation kinetics, such as K952A (88.8±6.6 s), R961A (92.3±6.7 s), H973A (126.9±10.3 s), D994A (88.7±5.6 s), D1002A (88.8±4.6 s), E1010A (90.6±9.8 s) and R1017A (90.4±7.8 s) as compared to the WT channel (48.5±2.5 s) (Fig. 4A, B). On the contrary, some other mutations accelerated inactivation kinetics, including K1005A (16.7±1.9 s), K1007A (32.9±7.6 s), E1022A (38.2±3.4 s) (Fig. 4B). Strikingly, the H995A mutant channel was insensitive to inactivation by 100 μM Cu$^{2+}$ (Fig. 4A, B). These results suggest that Cu$^{2+}$ inactivates the hTRPM2 channel via engaging the outer pore region.
Figure 4. Alanine substitution of Cu²⁺-binding candidate residues in the outer pore of hTRPM2 channel. (A) The amino acid sequences of the pore region between the S5 and S6 of the hTRPM2 channel; the residues examined in this study are numbered and highlighted in bold. Residues in the extracellular ends of S5 and S6 are indicated in the left and right shading boxes, respectively. Alanine substitutions leading to loss of function are indicated by asterisks. (B) Representative recordings of the ADPR-induced currents in HEK cells expressing WT or the indicated mutant channel before and after exposure to 100 µM Cu²⁺ (denoted by the black bars). The currents for the H995A mutant channels show incomplete inhibition by Cu²⁺ and complete inhibition by subsequent application of 20 µM CLT (denoted by the grey bars). The dotted lines indicate zero currents. (C) Summary of the time for 90% inhibition (left) and the residual currents upon exposure to Cu²⁺ (right). The dotted lines indicate the time or residual currents for the WT channel. The number of cells examined in each case is 3–22. The mutant channels showing significant difference from the WT channels are indicated in parentheses, *, p<0.05, **, p<0.01, ***, p<0.005. doi:10.1371/journal.pone.0112071.g004
Cu²⁺ accelerates the inactivation of hTRPM2 P1018L mutant channel

A previous study showed that the P1018L mutation in hTRPM2 resulted in channel inactivation [18], it is interesting to know the effect of extracellular Cu²⁺ on this mutant channel. Consistent with the previous study [18], the hTRPM2 P1018L mutant channel exhibited strong channel inactivation (121.6 ± 14.8 s) (Fig. 5A, B). Interestingly, 100 μM Cu²⁺ significantly accelerated the inactivation kinetics of the hTRPM2 P1018L mutant channel (15.3 ± 0.7 s) (Fig. 5C, D).

Mouse TRPM2 channel shows insensitivity to Cu²⁺-induced inactivation

Species difference exists between the human and mouse TRPM2 channels in terms of inactivation of the TRPM2 channels by protons and zinc, as a result of difference in the amino acid sequences of the pore region [17,36]. By comparing with the sequences, three residues in the pore region of the hTRPM2 channel are different with those of the mTRPM2. Arg961, His995 and Arg1017 residues in the hTRPM2 are replaced with Ser958, Gln992, and Ala1014 in the mTRPM2, respectively (Fig. 4A). The results that His995 plays a crucial role in Cu²⁺-induced hTRPM2 channel inactivation suggest that Cu²⁺ may not strongly interact with the pore region of the mTRPM2 channel to induce inactivation of the mTRPM2 channel. To address this hypothesis, we expressed the mTRPM2 channels in HEK293 cells and determined the effect of Cu²⁺ on the mTRPM2 channels. As shown in Fig. 6A, 100 μM Cu²⁺ failed to induce significant inhibition/inactivation of the mTRPM2 channels. We further generated the construct expressing the H995Q hTRPM2 mutant channel. The H995Q mutation almost completely abolished the hTRPM2 channel inactivation induced by Cu²⁺, even better than the H995A mutation (Fig. 6B). These results provide further evidence to indicate that His995 is critical in determining hTRPM2 channel inactivation by Cu²⁺.

Discussion

In this study, we found that extracellular Cu²⁺ inactivates the human but not the mouse TRPM2 channel, and revealed a striking species-dependent effect. Moreover, we identified several residues are involved in this process, particularly His995 which is the key residue in determining the hTRPM2 channel inactivation induced by Cu²⁺.

Cu²⁺-induced TRPM2 inactivation is independent on electrostatic repulsion and channel inhibition

Unlike the actions of extracellular Cu²⁺ on other ion channels, the present study shows Cu²⁺ induced an irreversible and concentration-independent inhibition of the hTRPM2 channel. Moreover, we found that Cu²⁺ induced similar TRPM2 inactiva-
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Figure 6. His^{995} is essential in Cu^{2+} interacting with human TRPM2 channel. (A) Recordings of the inward currents in cells expressing the human, mouse wild-type or mutant TRPM2 channels. (B) Summary of the effects of Cu^{2+} on these three channels. The number of cells examined in each case is 3–5. The significant difference is indicated in parentheses. ***; p<0.005.
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tion at the holding potential of −40 mV driving inward currents, as at 40 mV, driving outward currents. This is different with the membrane potential-dependent action of zinc shown in our previous study [17]. Thus, the action of Cu^{2+} may mechanistically differ from the action of zinc [17]. Remarkably, our data indicate that Cu^{2+}-induced hTRPM2 inactivation is independent on electrostatic repulsion, suggesting that Cu^{2+} binds with the residues outside the electrical field and induces conformational changes that lead to hTRPM2 channel inactivation. Both EDTA and 2-ME failed to reverse the Cu^{2+}-induced channel inactivation. One simple explanation is that Cu^{2+} induces substantial conformational changes that prevent accessibility of such Cu^{2+} chelators.

His^{995} is the key residue determining Cu^{2+}-induced inactivation of human TRPM2 channel

As we mentioned before, many studies have identified several residues interact with Cu^{2+}, such as histidine, cysteine, glutamate, aspartic acid and arginine. By alanine scanning, we have shown several residues in of pore region that are involved in the Cu^{2+}-induced inactivation of the hTRPM2 channel (Fig. 4). Particularly, the H995A mutation that is located at the large turret of the pore region strongly attenuated the hTRPM2 channel inactivation by Cu^{2+} (Fig. 4). Although several other residues replaced by alanine did not prevent the hTRPM2 channel inactivation by Cu^{2+}, the kinetics were substantially changed at these mutant channels (Fig. 4). Specifically, K952A, R961A, H973A, D994A, D1002A, E1010A and R1017A mutations slowed down the inactivation kinetics (Fig. 4A, B), whereas K1005A, K1007A and E1022A resulted in faster inactivation kinetics (Fig. 4B). These findings support that Cu^{2+} induces the hTRPM2 channel inactivation by acting on the outer pore region. In future, it is important to uncover the conformational changes that give rise to Cu^{2+}-induced hTRPM2 channel inactivation.

Species specific effects of Cu^{2+} on human and mouse TRPM2 channels

The TRPM2 channels are highly conserved in different species including the human and mouse. Our previous studies found three different residues in the pore region, Arg^{961}/Ser^{956}, His^{995}/Gln^{992}, Arg^{1017}/Ala^{1014}, between the human and mouse TRPM2 channels and showed that His^{995}/Gln^{992} is important in determining the different kinetics of channel inactivation induced by proton or Zn^{2+} between the human and mouse TRPM2 channels [17,36]. Here, we have shown that all of these three sites are critical for the hTRPM2 channel inactivation induced by Cu^{2+}, which is confirmed by the observation that there was no mTRPM2 channel inactivation by Cu^{2+} (Fig. 6). Taken together, the previous and present studies suggest that the hTRPM2 channel is more sensitive to Cu^{2+}, Zn^{2+} and proton than the mTRPM2 channel. Many lines of evidence have indicated that Cu^{2+} is involved in the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease and prion disease [40,41]. On the other hand, accumulating evidence implicates that the TRPM2 channels as an oxidative stress sensor is also related to these mental disorders [5]. In future, it is interesting to investigate whether the role of Cu^{2+} in the neurodegenerative disorders is related to the hTRPM2 channel. However, according to our results, the striking species difference between the human and mouse TRPM2 channels
suggest unsuitable to use mice as animal models to investigate the regulation of TRPM2 channels by Cu\textsuperscript{2+} in relation to the human diseases.

Comparing the effects on TRPM2 channels induced by Cu\textsuperscript{2+} and Zn\textsuperscript{2+}

Both Zn\textsuperscript{2+} and Cu\textsuperscript{2+} are metal ions, but the Cu\textsuperscript{2+}-induced TRPM2 channel inactivation is different with that by Zn\textsuperscript{2+}.

Firstly, the key residues that determine the TRPM2 channel inactivation by Cu\textsuperscript{2+} and Zn\textsuperscript{2+} are different. Secondly, Cu\textsuperscript{2+}-induced TRPM2 channel inactivation is independent on the membrane potential and/or the direction of Na\textsuperscript{+} permeation (Fig. 2). Finally, channel inactivation is independent on the membrane potential.

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These findings open a new way for us to understand the roles of TRPM2 channel in Cu\textsuperscript{2+} or Zn\textsuperscript{2+} treatment.

Our data have shown that the P1018L mutation located at the pore region of hTRPM2 resulted in strong channel inactivation (Fig. 5) as reported previously [18]. We investigated whether hTRPM2 channel inactivation induced by Cu\textsuperscript{2+} and P1018L mutation occurred through similar mechanisms. Our result showed that Cu\textsuperscript{2+} significantly accelerated the inactivation kinetics of the hTRPM2 P1018L mutant channel (Fig. 5), indicating that Cu\textsuperscript{2+} can induce strong channel inactivation of this mutant channel. Taken together, our results suggest that TRPM2 channel inactivation is complex, it will be interesting to know how to induce channel inactivation by these different factors in the future.

In summary, we have shown that extracellular Cu\textsuperscript{2+} inactivates the TRPM2 channel independently of the ion conducting pathway. By introducing point mutation, we identified multiple residues, especially His995, in the outer pore region that are involved in Cu\textsuperscript{2+}-induced TRPM2 channel inactivation. We also discovered that the mTRPM2 channel is insensitive to Cu\textsuperscript{2+}.

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Author Contributions

Conceived and designed the experiments: WY LJH WYY. Performed the experiments: WYY YZ XPH WY. Analyzed the data: WYY WY. Contributed reagents/materials/analysis tools: JHL LHJ WY. Wrote the paper: WYY LJH WY. Revised this manuscript: JHL LJH.

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