Extracellular Zinc Ion Regulates Transient Receptor Potential Melastatin 5 (TRPM5) Channel Activation through Its Interaction with a Pore Loop Domain

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Background: TRPM5 channel is a monovalent cation channel activated by intracellular Ca2++. Result: TRPM5 was inhibited by extracellular Zn2+. The inhibition involves His-896, Glu-926, and Glu-939. Conclusion: TRPM5 is inhibited by physiological concentrations of Zn2+ through interaction with the pore-loop domain. Significance: Zn2+ is a TRPM5 inhibitor and the inhibition might be related to its physiological functions.

The transient receptor potential melastatin 5 (TRPM5) channel is a monovalent cation channel activated by intracellular Ca2+. Expression of this channel is restricted to taste cells, the pancreas and brainstem, and is thought to be involved in controlling membrane potentials. Its endogenous ligands are not well characterized. Here, we show that extracellular application of Zn2+ inhibits TRPM5 activity. In whole-cell patch-clamp recordings, extracellular application of ZnCl2 inhibited step-pulse-induced TRPM5 currents with 500 nM free intracellular Ca2+ in a dose-dependent manner (IC50 = 4.3 μM at −80 mV). ZnSO4 also inhibited TRPM5 activity. Extracellular application of ZnCl2 inhibited TRPM5 activation at several temperatures. Furthermore, inhibition by 30 μM ZnCl2 was impaired in TRPM5 mutants in which His at 896, and Glu at 926 and/or Glu at 939 in the outer pore loop were replaced with Gln. From these results, we conclude that extracellular Zn2+ inhibits TRPM5 channels, and the residues in the outer pore loop of TRPM5 are critically involved in the inhibition.

Transient receptor potential melastatin 5 (TRPM5)3 is a cation-permeable channel. Whereas monovalent cations are permeable, divalent cations are not. TRPM5 is thought to function as a tetramer of each subunit containing intracellular N and C termini, 6 transmembrane (TM) domains, and a pore-forming loop between TM5 and TM6 like other TRP channels (1, 2). This channel is restrictedly expressed in taste cells, the pancreas, and the brainstem, and is thought to be involved in controlling membrane potentials (2). Some reports have shown its physiological functions. TRPM5 activity in pancreatic β-cells controls insulin secretion by modulating the oscillation frequency in the membrane potentials (3). TRPM5 activation in taste cells potentiates sweet taste signals by depolarizing the cells downstream of taste receptor activation (4). These functions are thought to result from activation of TRPM5 mediated by increases in intracellular Ca2+ concentrations ([Ca2+]i). Although most TRP channels have endogenous ligands, there are few reports about endogenous stimuli or inhibitors of TRPM5 except for intracellular Ca2+, PIP2, and increased temperature (stimuli), and extracellular acidification (an inhibitor) (5–8).

Zinc is one of the most abundant metals in organisms. Zinc is an important nutrient and the recommended dietary allowance is 11 mg/day for men and 8 mg/day for women in the United States (9). Zinc deficiency affects about two billion people in the developing world and is associated with many diseases. In children, it causes growth retardation, delayed sexual maturation, susceptibility to infection, and diarrhea, which contributes to the death of about 800,000 children per year worldwide. Zinc is also the only metal used in all enzyme classes including DNA polymerase and alcohol dehydrogenase (10, 11). In addition, many reports have shown that zinc modulates a variety of ion channels such as the NMDA receptor, voltage-gated Ca2+ channels, P2X, voltage-gated K+ channels, two-pore K+ channels, ASIC channel, and epithelial Na+ channels (12–17). These findings suggest that zinc could have more functions than previously thought. Some of the TRP channels were reported to be modulated by Zn2+ or to be involved in zinc-related physiological functions. For example, TRPA1 is activated by about 20 μM ZnCl2, and TRP1 is inhibited by high concentrations (mM) of ZnCl2 (18, 19). TRPM2 is activated by more than 30 μM ZnCl2, and Zn2+ entering cells through TRPM7 channel is involved in Zn2+ -mediated neuronal injury (20, 21). In this study, we found extracellular Zn2+ to be a potential blocker of TRPM5 activity. Furthermore, we determined the amino acid residues required for the inhibition.

MATERIALS AND METHODS

Cell Culture—Human embryonic kidney-derived 293 (HEK293) cells were maintained in DMEM (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS (Biowest...
When analyzing dose-response profiles, the data were fit into a logistic function with IC50 values of 4.3 ± 0.3 μM at −80 mV and 4.3 ± 0.7 μM at +160 mV (Fig. 1B). In addition, we analyzed the peak currents with or without 30 μM ZnCl2 at each membrane potential. As shown in Fig. 1C, significant inhibition of TRPM5 currents by 30 μM ZnCl2 was observed at both positive and negative membrane potentials, indicating that the inhibition by ZnCl2 was independent of membrane potentials. Next we investigated whether Zn2+ acted as a pore blocker. We examined the effect of changing the extracellular and intracellular Na+ concentrations on the inhibition of TRPM5 channel by extracellular ZnCl2 at a concentration of 30 μM. When both extracellular and intracellular Na+ concentrations were 150 mM, we observed TRPM5 currents with an outwardly rectifying current-voltage relationship by 500 nM [Ca2++]i, and the currents were inhibited by 30 μM ZnCl2 without voltage-dependence (Fig. 2A) as in the step-pulse protocol (Fig. 1C). When the extracellular or extracellular Na+ concentration was reduced to 5 mM, similar TRPM5-mediated currents with outward rectification were observed, and the currents were inhibited by 30 μM ZnCl2, similarly without voltage dependence (Fig. 2, B and C). The data indicated that the changes in extracellular and intracellular Na+ concentrations did not affect the inhibition of the TRPM5 channel by 30 μM extracellular ZnCl2. Such voltage-independent inhibition of TRPM5 currents by 30 μM extracellular ZnCl2 was more clearly shown when the extent of inhibition was plotted at each membrane potential (Fig. 2D). Reversal potentials were as follows: (A) −4.0 ± 3.4 mV in 150 mM Na+ intra/150 mM Na+ extra, (B) 26.0 ± 4.0 mV in 5 mM Na+ intra/150 mM Na+ extra, and (C) −33.9 ± 3.4 mV in 150 mM Na+ intra/5 mM Na+ extra, and they were somehow not well shifted as a Na+-selective channel.

Next, to determine whether TRPM5 inhibition mediated by ZnCl2 was due to the effects of Zn2+ itself, we examined the effect of another zinc compound, ZnSO4, on TRPM5 activity.

RESULTS

First, we examined whether zinc ion inhibited TRPM5 using whole-cell patch-clamp recordings. Because TRPM5 is an intracellular Ca2+-activated channel, we used a pipette solution containing 500 nM free Ca2+ to check the inhibition by ZnCl2. In HEK293 cells expressing TRPM5 (but not in mock-transfected cells), step-pulses with 500 nM free intracellular Ca2+ activated currents with slowly activated large outward components and rapidly-desensitized inward components (Fig. 1A). Extracellular application of 20 μM ZnCl2 reduced both outward and inward currents, and this inhibition was partially recovered after washout of ZnCl2 (Fig. 1A). The recovery was observed to a lesser extent after application of high concentrations (more than 30 μM) of ZnCl2 (data not shown). Next, we established a dose-response curve of ZnCl2-induced inhibition of TRPM5, using 0.1 to 100 μM of ZnCl2. TRPM5 activity was inhibited in a dose-dependent manner, similar at both positive and negative potentials, and the dose-dependent curves could be fit to a logistic function with IC50 values of 4.3 ± 3.0 μM at −80 mV and 4.3 ± 0.7 μM at +160 mV (Fig. 1B). In addition, we analyzed the peak currents with or without 30 μM ZnCl2 at each membrane potential. As shown in Fig. 1C, significant inhibition of TRPM5 currents by 30 μM ZnCl2 was observed at both positive and negative membrane potentials, indicating that the inhibition by ZnCl2 was independent of membrane potentials. Next we investigated whether Zn2+ acted as a pore blocker. We examined the effect of changing the extracellular and intracellular Na+ concentrations on the inhibition of TRPM5 channel by extracellular ZnCl2 at a concentration of 30 μM. When both extracellular and intracellular Na+ concentrations were 150 mM, we observed TRPM5 currents with an outwardly rectifying current-voltage relationship by 500 nM [Ca2++]i, and the currents were inhibited by 30 μM ZnCl2 without voltage-dependence (Fig. 2A) as in the step-pulse protocol (Fig. 1C). When the extracellular or extracellular Na+ concentration was reduced to 5 mM, similar TRPM5-mediated currents with outward rectification were observed, and the currents were inhibited by 30 μM ZnCl2, similarly without voltage dependence (Fig. 2, B and C). The data indicated that the changes in extracellular and intracellular Na+ concentrations did not affect the inhibition of the TRPM5 channel by 30 μM extracellular ZnCl2. Such voltage-independent inhibition of TRPM5 currents by 30 μM extracellular ZnCl2 was more clearly shown when the extent of inhibition was plotted at each membrane potential (Fig. 2D). Reversal potentials were as follows: (A) −4.0 ± 3.4 mV in 150 mM Na+ intra/150 mM Na+ extra, (B) 26.0 ± 4.0 mV in 5 mM Na+ intra/150 mM Na+ extra, and (C) −33.9 ± 3.4 mV in 150 mM Na+ intra/5 mM Na+ extra, and they were somehow not well shifted as a Na+-selective channel.

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\[ y = A_2 + \left( 1 - \frac{A_2}{A_1} \right) \left( \frac{x}{x_0} \right)^p \]

Statistical analysis was performed by Student’s t test or one-way analysis of variance (ANOVA) followed by a two-tailed multiple t test with Bonferroni correction. p values less than 0.05 were considered significant.
**Zn**\(^{2+}\) Modulates TRPM5 Activation

As shown in Fig. 3, 30 \(\mu\)M ZnSO\(_4\) significantly inhibited TRPM5 activity at both positive and negative membrane potentials as did 30 \(\mu\)M ZnCl\(_2\).

It is known that TRPM5 has thermosensitivity and its activity is potentiated by temperature increases (8). Therefore, we checked the effect of Zn\(^{2+}\) on TRPM5 activity at different tem-
Zn$^{2+}$ Modulates TRPM5 Activation

TRPM5 is a monovalent cation permeable channel and its activation can modulate membrane potentials. A proposed endogenous activator is intracellular Ca$^{2+}$, and some molecules such as PIP$_2$ enhance TRPM5 activity. However, inhibitors of TRPM5 are not known. In this study, we found an endogenous inhibitor, Zn$^{2+}$. Zn$^{2+}$-mediated inhibition was observed in the μM range. Under physiological conditions, serum zinc concentrations are about 14 μM (23). However, the precise concentration of free Zn$^{2+}$ is not known because Zn$^{2+}$ binds to many proteins in plasma, such as albumin and transferrin. In addition, many reports have indicated that release of vesicular Zn$^{2+}$ from pre-synapses might cause a transient increase in Zn$^{2+}$ concentrations from one to 100 μM in the brain (24). Given these facts, free Zn$^{2+}$ concentrations in vivo could be high enough to modulate TRPM5 activity under physiological condition.

We identified the Zn$^{2+}$-interacting sites as His at 896 and Glu at 926 and 939 in the outer pore loop of mouse TRPM5. TRPA1, which is activated by extracellular Zn$^{2+}$, reportedly interacts with Zn$^{2+}$ in its intracellular domain (18). In the report, they concluded that Zn$^{2+}$ entered the cell through TRPA1 channels from the extracellular space and activated TRPA1. Intracellular Zn$^{2+}$ levels are thought to be controlled by Zn$^{2+}$ movement through TRPM7 (20). However, TRPM5 is a divalent cation-impermeable channel (25), suggesting that Zn$^{2+}$ acts on the extracellular domains of TRPM5. One report showed that extracellular acidification inhibits murine TRPM5 activity, which is consistent with our findings.

To clarify the amino acids involved in Zn$^{2+}$-mediated inhibition of TRPM5 activity, we examined the effect of Zn$^{2+}$ on TRPM5 mutants. We hypothesized that Zn$^{2+}$ interacted with extracellular domains of TRPM5 because 1) extracellular Zn$^{2+}$ inhibits TRPM5 activity and 2) divalent cations are not permeable to TRPM5. In addition, many reports have shown amino acid residues such as His, Cys, Lys, Asp, and Glu interact with Zn$^{2+}$ (11, 16, 22). Based on these facts, we constructed TRPM5 mutants in which His, Cys, Lys, Asp, and Glu in the outer pore loop, the largest extracellular domain, were mutated. As shown in Fig. 5A, there are twelve such amino acids (His, Cys, Asp, and Glu) in the outer pore loop of TRPM5. First, we constructed single mutants in which Gln was substituted for His and Glu (H894Q, H896Q, E901Q, E923Q, E926Q, and E939Q), and Asn was substituted for Asp (D897N, D922N, and D925N), or Ser for Cys (C931S and C944S). Unfortunately, we had to exclude H894Q, D897N, D922N, C931S and C944S from analysis because these mutants showed no current activation by step-pulses with intracellular 500 nM Ca$^{2+}$ (data not shown). In the single mutants analyzed, inhibition of TRPM5 by 30 μM ZnCl$_2$ was significantly impaired in H896Q, E926Q, and E939Q mutants at +160 mV (Fig. 5B). Some reduction in ZnCl$_2$-induced inhibition was observed in the E923Q mutant although it was not statistically significant (Fig. 5B). Then, we focused on H896, E923, E926, and E939, and constructed double mutants (H896Q/E923Q, H896Q/E926Q, H896Q/E939Q, E923Q/E926Q, E923Q/E939Q, E926Q/E939Q). As shown in Fig. 5C, inhibition of TRPM5 at both +160 mV and −80 mV by 30 μM ZnCl$_2$ was completely lost in H896Q/E926Q and E926Q/E939Q mutants. In the H896Q/E939Q mutant, TRPM5 activity was even increased after application of ZnCl$_2$ especially at −80 mV.

**DISCUSSION**

TRPM5 is a monovalent cation permeable channel and its activation can modulate membrane potentials. A proposed endogenous activator is intracellular Ca$^{2+}$, and some molecules such as PIP$_2$ enhance TRPM5 activity. However, inhibitors of TRPM5 are not known. In this study, we found an endogenous inhibitor, Zn$^{2+}$. Zn$^{2+}$-mediated inhibition was observed in the μM range. Under physiological conditions, serum zinc concentrations are about 14 μM (23). However, the precise concentration of free Zn$^{2+}$ is not known because Zn$^{2+}$ binds to many proteins in plasma, such as albumin and transferrin. In addition, many reports have indicated that release of vesicular Zn$^{2+}$ from pre-synapses might cause a transient increase in Zn$^{2+}$ concentrations from one to 100 μM in the brain (24). Given these facts, free Zn$^{2+}$ concentrations in vivo could be high enough to modulate TRPM5 activity under physiological condition.

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through interaction with the extracellular domain (Glu at 830 in TM3–4 linker and His at 934 in the pore loop) (5), different from those involved in Zn\(^{2+}\)/H11001 action in our study. The Zn\(^{2+}\)/H11001-mediated inhibition of TRPM5 was only partially reversed after washout. A similar phenomenon was observed in the inhibition of TRPM5 by protons (5). Protons irreversibly inhibited TRPM5 activity, and His at 896, which is a critical amino acid residue for TRPM5 inhibition by Zn\(^{2+}\), was also critically involved in the reversibility of TRPM5 inhibition by protons. Although the same His residue was involved in the action of both Zn\(^{2+}\) and protons, the mechanisms of inhibition might be quite different. The fact that the involvement of Glu at 926 is close to the putative selective filter (26, 27) raises the possibility that Zn\(^{2+}\) could act as a pore blocker. Pore blockers generally act in a membrane potential-dependent manner and the inhibition could be modulated by permeating ions. In this study, we found that the inhibition of TRPM5 activity by Zn\(^{2+}\) did not depend on membrane potentials and was not modulated by changes in either extracellular or intracellular Na\(^{+}\) concentrations. These results suggest that Zn\(^{2+}\) is not a channel pore blocker.

**FIGURE 5.** His and Glu residues in the pore region are necessary for Zn\(^{2+}\)-mediated inhibition of TRPM5 activation. 

A, candidate residues for Zn\(^{2+}\) interaction in the outer pore region. Top: schematic diagram. Bottom: amino acids in the region. TM: transmembrane domain.

B, summary of the residual currents upon exposure to 30 μM extracellular ZnCl\(_2\) in HEK293 cells expressing single mutants of TRPM5, expressed as the ratio of the currents to those before application of ZnCl\(_2\), at +160 mV (left) and −80 mV (right) potentials. Dotted lines indicate the ratio of residual currents in wild-type TRPM5 channel (wt). Each bar represents the mean ± S.E. from 6 to 8 cells. *, p < 0.05; **, p < 0.01.

C, summary of the residual currents upon exposure to 30 μM extracellular ZnCl\(_2\) in HEK293 cells expressing double mutants of TRPM5, expressed as the ratio of the currents to those before application of ZnCl\(_2\), at +160 mV (left) and −80 mV (right) potentials. Solid and dotted lines indicate the ratios of 1.0 and residual currents in wild-type TRPM5 channel (wt). Each bar represents the mean ± S.E. for 6 to 8 cells. *, p < 0.05; **, p < 0.01.
TRPM5 is restricted in its distribution to taste cells, the pancreas and the brain stem in mammals. Zn$^{2+}$ is reportedly released from pre-synapses like a transmitter in neurons and pancreatic β-cells in response to insulin. Zn$^{2+}$ released by glutamate following pre-synapse activation could modulate synaptic transmission through the modulation of receptors such as the NMDA receptor (12, 24). In the case of the pancreas, Zn$^{2+}$ released from β-cells might modulate glucagon secretion from pancreatic α-cells after increases in blood glucose levels (28, 29). Therefore, it would be intriguing to speculate that modulation of TRPM5 by Zn$^{2+}$ in neurons and the pancreas in an autocrine or paracrine manner could have important roles under physiological conditions. Recently, one report showed that TRPM5 might be involved in cholecystokinin secretion stimulated by linoleic acid in an enteroendocrine cell line (30), suggesting the possibility that TRPM5 could have more physiological functions than those reported to date.

In conclusion, we found that Zn$^{2+}$ is an effective inhibitor of TRPM5. Furthermore, His and Glu residues in the pore-loop domain are critical for the inhibition. Such inhibition by Zn$^{2+}$ could be important for the biological or physiological functions of TRPM5.

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