Zinc Inactivates Melastatin Transient Receptor Potential 2 Channels via the Outer Pore

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Zinc ion (Zn2+) is an endogenous allosteric modulator that regulates the activity of a wide variety of ion channels in a reversible and concentration-dependent fashion. Here we used patch clamp recording to study the effects of Zn2+ on the melastatin transient receptor potential 2 (TRPM2) channel. Zn2+ inhibited the human (h) TRPM2 channel currents, and the steady-state inhibition was largely not reversed upon washout and concentration-independent in the range of 30–1000 μM, suggesting that Zn2+ induces channel inactivation. Zn2+ inactivated the channels fully when they conducted inward currents, but only by half when they passed outward currents, indicating profound influence of the permeant ion on Zn2+ inactivation. Alanine substitution scanning mutagenesis of 20 Zn2+-interacting candidate residues in the outer pore region of the hTRPM2 channel showed that mutation of Lys952 in the extracellular end of the fifth transmembrane segment and Asp1002 in the large turret strongly attenuated or abolished Zn2+ inactivation, and mutation of several other residues dramatically changed the inactivation kinetics. The mouse (m) TRPM2 channels were also inactivated by Zn2+, but the kinetics were remarkably slower. Reciprocal mutation of His995 in the hTRPM2 channel and the equivalent Gln992 in the mTRPM2 channel completely swapped the kinetics, but no such opposing effects resulted from exchanging another pair of species-specific residues, Arg961/Ser958. We conclude from these results that Zn2+ inactivates the TRPM2 channels and that residues in the outer pore are critical determinants of the inactivation.

TRPM2 is member of the melastatin subfamily of the mammalian transient receptor potential (TRP)2 proteins that share the membrane topology of voltage-gated ion channel proteins (1–3). Thus, TRPM2 channels are homo-tetramers assembled predominantly via a C-terminal coiled-coil domain (4–7), with each TRPM2 protein subunit containing intracellularly residing N and C termini and six transmembrane segments (S1–S6) with a re-entrant pore-forming loop between the S5 and S6 (see Fig. 5). TRPM2 channels are known to be present as cell surface Ca2+-permeable non-selective channels in neurons, cardiomyocytes, pancreatic β-cells, epithelial cells, neutrophils, microglia, monocytes, and macrophages and also as lysosomal Ca2+ release channels in pancreatic β-cells (8, 9). They open upon binding of ADP-ribose (ADPR) and structurally related molecules to the TRPM2-specific NUD9-H domain in the distal C-terminal tail (10, 11). TRPM2 channels are also activated by reactive oxygen species (e.g. H2O2), tumor necrosis factor-α, amyloid β-peptide, and concanavalin-A (12–17), most likely as a consequence of ADPR production or similar mechanisms (10, 12). Although the exact biological functions of the lysosomal Ca2+ release channels remain unknown, several recent studies, mainly using gene knock-out or knockdown approaches, have revealed an important role for the TRPM2 channels at the cell surface in mediating oxidative stress-elicited Ca2+ influx that activates intracellular signals leading to generation of cytokines and other proinflammatory mediators from monocytes, microglia, and macrophages, increased interendothelial permeability, and neuronal and cardiomyocyte death (8, 9). Furthermore, TRPM2 channels mediate glucose-evoked Ca2+ influx for insulin secretion from pancreatic β-cells and facilitation of insulin secretion by incretins (18). These findings raise interests in TRPM2 channels as a therapeutic target to treat inflammatory, neurodegenerative, and cardiovascular disorders including diabetes (8, 9).

Zinc is one of the most abundant trace metals in the human body (19). In addition to being a structural or catalytic component in hundreds of proteins, mainly enzymes (20), zinc ions (Zn2+) target a wide variety of ion channels and confer them with new functions or more often alter their functions. Zn2+ permeates the ionotropic receptors for glutamate (21) and nicotinic acetylcholine (22, 23), CaV (23–25), and TRPM3 (26) and TRPM7 channels (27, 28). As such, the TRPM7 channels play a role in mediating Zn2+ neurotoxicity (27). Activation of the TRPV1 channels by extracellular Zn2+ is one of the molecular mechanisms underlying metallic taste (29). The large conductance Ca2+-activated K+ channels are activated by intracellular Zn2+ (30), whereas Zn2+ is an extracellular and intracellular opener of the pancreatic KATP channels (31). The most common effect of extracellular Zn2+ is allosteric modulation, potentiating or inhibiting channel activity (32, 33). Examples are numerous, including Kγ (34, 35), NaV (36, 37), CaV (38, 39), ionotropic receptors for γ-aminobutyric acid (40, 41), glycine (42), 5-hydroxytryptamine (43), glutamate (44, 45) and ATP (46–48), two-pore K+ channels (49, 50), acid-sensitive ion channels and epithelial Na+ channels (51, 52), and TRPM1 channel (53).
TRPM2 Channel Inactivation by Zinc Ions

As mentioned above, the TRPM2 channels are functionally expressed in the hippocampal neurons and microglia (8, 9) and pancreatic β-cells (18). It is estimated that the presynaptic vesicles in the hippocampus contain millimolar concentrations of Zn$^{2+}$ and that the local synaptic Zn$^{2+}$ concentrations can reach 300 μM during strong stimulations (54–57). Similarly, millimolar concentrations of Zn$^{2+}$ accumulate in secretory granules of the pancreatic β-cells and form microcrystals of insulin (58, 59), which dissolve during exocytosis to produce large amounts of extracellular Zn$^{2+}$ surrounding the insulin release site (31). It is therefore interesting to know whether and how extracellular Zn$^{2+}$ interacts with the TRPM2 channel. Unlike recent studies reporting substantial Zn$^{2+}$ permeation of the TRPM3 and TRPM7 channels (26–28) and potent reversible Zn$^{2+}$ inhibition of the TRPM1 channel (53), here we provide evidence to show that Zn$^{2+}$ induces an irreversible and concentration-independent inhibition or inactivation of the TRPM2 channels and that residues in the outer pore are critical molecular determinants.

MATERIALS AND METHODS

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells were used to transiently express the wild-type (WT) and mutant TRPM2 channels as detailed previously (60–64). The tetracycline-inducible HEK293 cells stably expressing the WT human TRPM2 (hTRPM2), after being induced with 1 μg/ml tetracycline for 12–36 h, were used in some experiments (see Figs. 2–4). The constructs used were generated in previous studies (61–64). Chemicals and reagents used were purchased from Sigma unless otherwise indicated.

Electrophysiology—Patch clamp recordings were made at room temperature as described previously (61–64), using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). 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 MgCl$_2$, 10 HEPES, 1 ATP, and 1 ADPR, pH 7.3. Thus, the currents were mainly carried by Na$^+$. For the majority of experiments, the whole-cell currents were recorded by applications of a 1-s voltage ramp from −120 to +80 mV every 5 s, with a holding potential of −40 mV. Shown in the figures are the inward currents at −80 mV, denoted by circles. For some experiments, the cells were held constantly at −40 or 40 mV to continuously record the inward or outward currents. Single channel activity recordings were carried out in outside-out configuration. Data were acquired at 10 kHz and filtered offline at 50 Hz. ZnSO$_4$ dissolved in extracellular solution was used. To investigate the effect on the TRPM2 channels in the closed state, the patched cells in the cell-attached configuration were exposed to Zn$^{2+}$-containing solution for 1 or 2 min and washed before being introduced into the whole-cell configuration to activate the TRPM2 channels. N-(p-Amylcninamoyl) anthranilic acid (20 μM) (Calbiochem) was applied to block any substantial residual currents at the end of each recording. The cells in which the ADPR-induced currents showed almost complete inhibition by Zn$^{2+}$ or anthranilic acid were included in analysis. Changes of extracellular solutions, including application of Zn$^{2+}$ and anthranilic acid, were carried out using an RSC-160 system (Biologic Science Instruments) with a solution change time of ~300 ms (65).

Data Analysis—The data are presented in the text and figures, where appropriate, as mean ± S.E. values. The inhibition was determined by expressing the currents in the indicated Zn$^{2+}$ concentrations as the percentage of the currents before solution changes, and the kinetics were determined by the time required for 90% inhibition ($t_{90%}$). Single channel conductance was estimated based on the resolvable unitary currents. Statistical analysis was performed using the Student’s $t$ test with $p < 0.05$ designated as significant.

RESULTS

Effects of Extracellular Zn$^{2+}$ on hTRPM2 Channels—We began with experiments to determine the effect of extracellular Zn$^{2+}$ on the ADPR-induced hTRPM2 channel currents, with the hope that Zn$^{2+}$ in mM concentrations would not cause significant inhibition so that we could test whether the channels are permeable to Zn$^{2+}$ by patch clamp recording as shown previously for the TRPM3 and TRPM7 channels (26, 28). The TRPM2 channel activity, as illustrated by the inward currents at −80 mV in Fig. 1A, rapidly and completely disappeared upon exposure to 1 mM Zn$^{2+}$. Such inhibition was consistently evident at concentrations of down to 30 μM (Fig. 1, B–C). There was no or little effect at 3–10 μM (data not shown). The inhibition kinetics were concentration-dependent; the $t_{90%}$ value was from 251 ± 8 s (n = 3) at 30 μM to 14 ± 2 s (n = 4) at 1 mM (Fig. 1D). More strikingly, the steady-state inhibition was largely not reversed upon washout for minutes (Fig. 1, A and B). Thus, extracellular Zn$^{2+}$ induced an irreversible and concentration-independent inhibition or inactivation, an effect distinct from those previously reported on any other ion channels.

It is known that the state of the ion channels can strongly influence the Zn$^{2+}$ effects (33, 66). To investigate whether Zn$^{2+}$ could interact with the hTRPM2 channels in the closed state to prevent the channel activation, we treated the patched cells in cell-attached configuration with 300 μM Zn$^{2+}$ for 1 min, which was sufficient to inactivate the open channels (Fig. 1B), and washed the cells before breaking them into the whole-cell configuration to activate the channels (Fig. 2A). The ADPR-induced currents in the Zn$^{2+}$-pretreated cells were not significantly different from those in the untreated cells in parallel experiments (Fig. 2B). Furthermore, the inhibition kinetics in the Zn$^{2+}$-pretreated cells, upon subsequent exposure to Zn$^{2+}$, showed no difference to those in untreated cells (Fig. 2B). Virtually the same results were obtained by a 2-min pretreatment (Fig. 2, C and D). These results indicate no irreversible action of Zn$^{2+}$ on the closed channels. Such strong state dependence led us to propose that Zn$^{2+}$ inactivates the channels via interacting with the ion-conducting pathway. We monitored the single channel conductance in membrane patches in outside-out configuration. The single channel conductance remained the same before and after exposure to Zn$^{2+}$ (Fig. 3A), whereas the single channel conductance was strongly reduced by H$^+$ (pH 5.5; Fig. 3B) as we have recently reported (62, 63).

To determine whether the flux of permeant Na$^+$ influences the effects of Zn$^{2+}$, we compared the outward currents by hold-
ing the membrane potential constantly at +40 mV and the inward currents at −40 mV. The results are summarized in Fig. 4. The outward currents were fully inhibited but, surprisingly, 48 ± 10% (n = 4) of the currents were recovered immediately upon washout (Fig. 4, A and C). Subsequent measurement of the inward currents in these same cells showed that the inhibition by Zn²⁺ was virtually not reversed (5 ± 2%, n = 4; Fig. 4, B and C). To distinguish whether such a difference in the reversibility of the inhibition between the outward and inward currents represents bona fide voltage-dependent inactivation or a use-dependent effect (i.e. increased inhibition upon repeated applications), we performed these experiments beginning with the inward currents. As shown in Fig. 4B, the inhibition of the inward currents exhibited negligible reversibility (8 ± 3%, n = 4). The degree of the inward current recovery between the two protocols was not different (p > 0.05; Fig. 4C). Taken together,
these results indicate strong influence by the permeant ions on the Zn$^{2+}$ inactivation, but not the Zn$^{2+}$ inhibition.

**Effects of Mutating Residues in the Outer Pore on Zn$^{2+}$-Induced Inhibition and Inactivation**—The extracellular regions of the S5 and the S6, the shorter loop connecting the S5 and the putative pore helix (or the small turret), and the longer loop connecting the ion selectivity filter and the S6 (or the large turret) represent the structural components of the outer pore of the TRP channels (67, 68). The corresponding regions in the hTRPM2 channel contain 20 residues that can potentially interact with Zn$^{2+}$, including histidine, cysteine, lysine, aspartate, or glutamate residues (33, 46) (Fig. 5). We introduced alanine substitution into these residues in previous studies examining their roles in the channel activation, permeation, and inhibition by acidic pH (61–64). Although alanine substitution into six positions led to complete loss of function, the other 14 alanine mutants formed functional channels (Fig. 5). To obtain evidence to support that Zn$^{2+}$ inactivates the TRPM2 channels via engaging the outer pore, we determined the effects of 300 μM Zn$^{2+}$ on the functional mutant channels. Representative current recordings are illustrated in Fig. 6A, and the mutational effects on Zn$^{2+}$-induced inhibition and inactivation are summarized in Fig. 6B. Three distinctive mutational effects were observed. The first and most dramatic effect was strong reduction or complete loss of Zn$^{2+}$ inactivation, resulting from the K952A and D1002A mutations (Fig. 6A). The second effect was to slow the inactivation kinetics by the H958A, E994A, H995A, E1010A, and D1012A mutations. The third and last mutational effect was opposite; the inactivation kinetics were accelerated by the D964A, H973A, K1005A, K1007A, R1017A, and E1022A mutations.
mutations (Fig. 6B). In summary, alanine scanning mutagenesis identified multiple residues and diverse mutational effects, suggesting that the outer pore contains critical determinants for Zn$^{2+}$ inactivation.

**Different Zn$^{2+}$ Inactivation Kinetics of hTRPM2 and mTRPM2 Channels**—Previous studies have shown species-dependent interactions of ion channels with ligands including Zn$^{2+}$ (48, 69–74). Thus, we finally examined whether Zn$^{2+}$ inactivated the mouse TRPM2 (mTRPM2) channel in the same way as the hTRPM2 channel. As illustrated in Fig. 7A, the mTRPM2 channels were inactivated by Zn$^{2+}$ to similar degree as the hTRPM2 channels, but the kinetics were threefold slower, clearly indicating species difference.

Three of the 20 Zn$^{2+}$-interacting candidate residues in the outer pore of the hTRPM2 channel identified above (Arg$^{961}$, His$^{995}$, and Arg$^{1017}$) are absent in the mTRPM2 channel.
The alanine substitution experiments with the hTRPM2 channel have thus far shown that the H995A mutation slowed Zn$^{2+}$ inactivation (Fig. 6), hinting a role for His995/Gln992 in determining the different inactivation kinetics. To test this, we characterized the mutant hTRPM2 channels carrying the R961S or H995Q mutation (Fig. 7B) and the mutant mTRPM2 channels carrying the reciprocal S958R or Q992H mutation (Fig. 7C). The results are summarized in Fig. 7D. Clearly, the hTRPM2 R961S and the mTRPM2 S958R mutant channels retained the inactivation kinetics of their respective WT channels (Fig. 7, B–D). In contrast, the hTRPM2 H995Q mutant channel showed slowed kinetics that were similar to the WT mTRPM2 channel (Fig. 7, A–D). Conversely, the mTRPM2 Q992H mutant channel exhibited accelerated kinetics that were virtually the same as the WT hTRPM2 channel (Fig. 7, A–D). Such opposing results provide compelling evidence for a key role of this pair of species-specific residues in the outer pore in determining the difference in the inactivation kinetics between the hTRPM2 and mTRPM2 channels.

DISCUSSION

The present study has made three major findings. Firstly, Zn$^{2+}$ induces state- and voltage-dependent inactivation of the hTRPM2 channel. Secondly, multiple residues in the outer pore were identified as critical determinants for Zn$^{2+}$ inactivation. Finally, inactivation of the mTRPM2 channel is noticeably slower than that of the hTRPM2 channel, and such a difference can be attributed to species-specific pore residues His995/
Gln^{992}. Our results suggest that Zn^{2+} inactivates the TRPM2 channels via the outer pore.

**State and Voltage Dependence of Zn^{2+} Inactivation of hTRPM2 Channels**—In addition to being an opener or activator of a few ion channels, Zn^{2+} is far better known to be an endogenous allosteric modulator of a variety of ion channels (see the Introduction). These Zn^{2+} actions are largely reversible and concentration-dependent. The present study shows that although failing to exert any irreversible effect on the closed hTRPM2 channels, Zn^{2+} induced irreversible and concentration-independent inhibition of the open channels (Figs. 1 and 2). Further analysis of the effects of Zn^{2+} on Na^{+}-carrying outward and inward currents indicates that Na^{+} efflux opposes Zn^{2+} to inactivate but not inhibit the open TRPM2 channels (Fig. 4). We interpret such state and voltage dependence of Zn^{2+} effects to suggest that the residues interacting with Zn^{2+} are much more accessible or have higher affinity for Zn^{2+} in the open channels, that interaction with Zn^{2+} induces an initial reversible inhibition, and that conformational changes cause inactivation, as can be briefly summarized as follows:

Closed $\rightarrow$ Open $\rightarrow$ Zn^{2+} $\rightarrow$ Inhibited $\rightarrow$ Inactivated

The Na^{+} efflux hinders Zn^{2+} access to or facilitates Zn^{2+} to disassociate from the Zn^{2+}-interacting residues because of electrostatic repulsion, rendering the open channels to become less prone to transition to the inactivated state. The Zn^{2+} inactivation of the hTRPM2 channel is similar to the recently reported effect of mutating Pro^{1008} located in the outer turret to leucine; the P1008L mutation, identified in Western Pacific amyotrophic lateral sclerosis and parkinsonism-dementia patients, induces strong inactivation by destabilizing the outer pore conformation of the open channel (75).

**Implications to Biological Functions of TRPM2 Channels**—As mentioned in the Introduction, extensive neuronal activities can cause the nerve terminals in the hippocampus to release Zn^{2+} from synaptic vesicles and result in local Zn^{2+} synaptic concentrations of 300 μM (54–57), which have profound effects on the TRPM2 channels as shown here. Recent studies have shown functional expression of the TRPM2 channels in hippocampal neurons (76) and microglia (77). TRPM2 channels mediate oxidative stress-induced neuronal death directly (12) or via activating microglia (77). It is tempting to postulate Zn^{2+} inhibition of the TRPM2 channels as a neuroprotective mechanism. The pancreatic β-cells also contain millimolar concentrations of Zn^{2+} in insulin secretory granules (58, 59). Zn^{2+}, co-released with insulin during exocytosis, has been proposed to constitute a negative feedback on insulin secretion, in part via activation of the K_{ATP} channels (31, 78). A recent knock-out study has shown an important role for the TRPM2 channels in glucose-induced insulin secretion and facilitation of insulin secretion by incretins (18). Thus, inhibition of the TRPM2 channels by Zn^{2+} is another attractive candidate mechanism involved in the negative feedback regulating insulin secretion. Finally, the TRPM2 channels are widely expressed in the immune system (9), whose functions are severely impaired by Zn^{2+} deficiency (79, 80). Accumulating evidence supports a crucial role for the TRPM2 channels in oxidative stress-induced immune and inflammatory responses (9). It would be interesting to examine whether any of the roles of Zn^{2+} in immune cells are associated with the TRPM2 channels. Clearly, the findings reported here will...
facilitate better understanding of TRPM2 channels on the one hand, and Zn$^{2+}$ on the other, in their roles in neurons, pancreatic β-cells, and immune cells.

**Outer Pore as Important Determinant for Zn$^{2+}$ Inactivation—** The extracellular regions of the S5 and S6 and the small and the large turrets form the outer pore in the structural models of the TRPV1 channel (67) and the TRPM8 channel (68), the closest relative of the TRPM2 channel (1–3). Alanine substitution scanning mutagenesis of the Zn$^{2+}$-interacting candidate residues in the hTRPM2 channel outer pore led us to identification of multiple interesting residues (Fig. 5). Substitution of Lys$^{952}$ located in the extracellular end of the S5 or Asp$^{1002}$ in the large turret strongly attenuated or abolished the ability of Zn$^{2+}$ to inactivate the hTRPM2 channel. Interestingly, Zn$^{2+}$ can still exert mild but clear reversible inhibition of these mutant channels (Fig. 6A), suggesting that mutation of Lys$^{952}$ and Asp$^{1002}$ is necessary to remove the reversible inhibition, but mutation of one of them is sufficient to impair the conformational changes for the inactivation. Study of the hTRPM2 channel carrying alanine substitution of both Lys$^{952}$ and Asp$^{1002}$ would be revealing but, unfortunately, such a mutant channel was non-functional (data not shown). Alanine substitution into several other conserved residues in the small and large turrets remarkably altered the inactivation kinetics (Figs. 5 and 6). These findings are consistent with the notion that Zn$^{2+}$ interacts with the outer pore and induces conformational changes inactivating the ion-conducting pathway. Further studies are required to clarify whether these residues are structural and/or mechanistic determinants, i.e. whether they are primarily involved in interactions with Zn$^{2+}$, conformational changes, or both.

During the preparation of this manuscript, a study was published showing that Zn$^{2+}$ induces reversible and concentration-dependent inhibition of the TRPM1 channel but no inhibition of the TRPM3 channel (53). Removal of seven TRPM1-specific residues in the large turret abolished Zn$^{2+}$ inhibition of the TRPM1 channel, whereas insertion of this short stretch into the TRPM3 channel transferred the sensitivity to inhibition by Zn$^{2+}$. Although our study shows different Zn$^{2+}$ effects on and involvement of different residues in the TRPM2 channels, the two studies reveal that the outer pore and especially the large turret are important in mediating the Zn$^{2+}$ actions. Our initial aim was to study using patch clamp recording whether the TRPM2 channel passes Zn$^{2+}$ as shown for the TRPM3 and TRPM7 channels (26, 28). There was little inhibition by Zn$^{2+}$ of the TRPM2 channel at ≤10 µM over several minutes (data not shown). It is still possible to test the Zn$^{2+}$ permeability of the TRPM2 channel using fluorescent Zn$^{2+}$ indicators and low Zn$^{2+}$ concentrations as used in a recent study of the TRPM3 channel (26). Interestingly, as shown by the present and previous studies, the TRPM channel pores show subtype-specific interactions with Zn$^{2+}$ fulfilling distinct properties (permeation versus inhibition), indicating important difference in their structure. Such information should be valuable in developing selective open channel blockers.

**A Key Role of His$^{995}$/Gln$^{992}$ in Species-specific Zn$^{2+}$ Inactivation of TRPM2 Channels—** Experiments with rodent animals including cells from those animals are critical in inferring the functions of human proteins and in testing preclinical drugs. The underlying assumption is that the homologue proteins have the same structure-function relationships. However, an increasing number of ion channels in human and rodent exhibit profound differences in interaction with their ligands (48, 69–74). The opposite effects of Zn$^{2+}$ on the P2X2 receptors represent one such example; Zn$^{2+}$ potentiates the rat receptor but inhibits the human counterpart due to the presence of species-specific Zn$^{2+}$-interacting residues (48). Here we showed that Zn$^{2+}$ inactivated both the hTRPM2 and the mTRPM2 channels but that the mTRPM2 channels exhibited significantly slower kinetics (Fig. 7A). Reciprocal mutation of one pair of species-specific residues in the large turret, His$^{995}$/Gln$^{992}$, completely swapped the kinetics (Fig. 7). No such opposing effects resulted from exchanging another pair of species-specific residues in the small turret, Arg$^{961}$/Ser$^{958}$ (Fig. 7). Thus, His$^{995}$/Gln$^{992}$ essentially governs the species difference. Such a finding provides further evidence to support that the outer pore is an important determinant for Zn$^{2+}$ inactivation.

**Similarities and Differences between TRPM2 Channel Inactivation by Zn$^{2+}$ and H$^{+}$—** The inactivation by Zn$^{2+}$ of the TRPM2 channels described here bears a resemblance in several aspects to the inactivation by H$^{+}$ we previously reported (62, 63). The inactivation in both cases is state- and voltage-dependent and strongly attenuated by the K952A and D1002A mutations, accelerated by the D964A, K1005A, and R1017A mutations, or slowed by the H995A, E1010A, and D1012A mutations. In addition, His$^{995}$/Gln$^{992}$ is critical in determining the species difference in the inactivation kinetics. However, there are some noticeable differences. The Zn$^{2+}$ inactivation is slowed by the H958A and E994A mutations, whereas the H$^{+}$ inactivation is accelerated by the H958A mutation and unaffected by the E994A mutation. The most striking difference is that the single channel conductance is not altered by Zn$^{2+}$ but strongly reduced by H$^{+}$ (Fig. 4). Simultaneous exposure to 100 µM Zn$^{2+}$ and pH 6.0, each on its own inducing slow inactivation of the hTRPM2 channel with $t_{1/2}$ of >250 and >120 s respectively, showed no synergy (data not shown). Taken together, these results suggest overlapping, but not the same, structural and mechanistic determinants for inactivation by Zn$^{2+}$ and H$^{+}$. This is similar to the reversible inhibition of the P2X7 receptors by Zn$^{2+}$ and H$^{+}$ we previously reported (46, 81). Such differences are not unexpected considering the differences in the physical and chemical properties of Zn$^{2+}$ and H$^{+}$. H$^{+}$ protonates charged residues including aspartate, glutamate, histidine, lysine, and arginine, binding being dependent on the pK$_{a}$ of the target residue and the ambient pH. By contrast, Zn$^{2+}$ has the ability to coordinate up to four amino acid side chains presenting nucleophilic ligands (oxygen, nitrogen, and sulfur). Amino acid residues capable of coordinating Zn$^{2+}$ include histidine, cysteine, glutamate, and aspartate. The affinity of Zn$^{2+}$ to a protein depends on the number and nature of the available residues available for coordination. Affinity of Zn$^{2+}$ involving two residues ranges from $10^{-5}$ to $10^{-4}$ M, and affinity involving three residues ranges from $10^{-8}$ to $10^{-6}$ M, whereas sites involving four coordinates range from $10^{-9}$ to $10^{-8}$ M (82). The fact that we saw that Zn$^{2+}$-induced inhibition of TRPM2 occurs at concentrations exceeding $3 \times 10^{-5}$ M suggests the involvement of at least two residues. In the absence of the three-dimensional
structure, it is hard to predict which residues identified participate in coordinating Zn\(^{2+}\).

In summary, we have shown that extracellular Zn\(^{2+}\) inactivates the TRPM2 channels and that multiple residues in the outer pore are critical molecular determinants of the inactivation. Such Zn\(^{2+}\) actions may bear significant consequences on the biological functions of the TRPM2 channels.

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