Structural Determinants of the High Affinity Extracellular Zinc Binding Site on Ca\textsubscript{\textit{v}}3.2 T-type Calcium Channels*

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Ho-Won Kang\textsuperscript{1,9}, Iuliia Vitko\textsuperscript{7}, Sang-Soo Lee\textsuperscript{1,9}, Edward Perez-Reyes\textsuperscript{3,5}, and Jung-Ha Lee\textsuperscript{1,9,11,12,13}

From the \textsuperscript{4}Department of Life Science and \textsuperscript{5}Basic Science Institute for Cell Damage Control, Sogang University, Seoul 121-742, Korea and the \textsuperscript{6}Department of Pharmacology and \textsuperscript{7}Neuroscience Graduate Program, University of Virginia, Charlottesville, Virginia 22911

Ca\textsubscript{\textit{v}}3.2 T-type channels contain a high affinity metal binding site for trace metals such as copper and zinc. This site is occupied at physiologically relevant concentrations of these metals, leading to decreased channel activity and pain transmission. A histidine at position 191 was recently identified as a critical determinant for both trace metal block of Ca\textsubscript{\textit{v}}3.2 and modulation by redox agents. His\textsuperscript{191} is found on the extracellular face of the Ca\textsubscript{\textit{v}}3.2 channel on the IS3-S4 linker and is not conserved in other Ca\textsubscript{\textit{v}}3 channels. Mutation of the corresponding residue in Ca\textsubscript{\textit{v}}3.1 to histidine, Gln\textsuperscript{172}, significantly enhances trace metal inhibition, but not to the level observed in wild-type Ca\textsubscript{\textit{v}}3.2, implying that other residues also contribute to the metal binding site. The goal of the present study is to identify these other residues using a series of chimeric channels. The key findings of the study are that the metal binding site is composed of a Asp-Gly-His motif in IS3–S4 and a second aspartate residue in IS2. These results suggest that metal binding stabilizes the closed conformation of the voltage-sensor paddle in repeat I, and thereby inhibits channel opening. These studies provide insight into the structure of T-type channels, and identify an extracellular motif that could be targeted for drug development.

Low voltage-activated (LVA)\textsuperscript{2} T-type Ca\textsuperscript{2+} channels have been extensively studied because of their crucial implication in neuronal excitability, hormone secretion, muscle contraction, and pacemaker activity (1, 2). Molecular cloning studies demonstrated the existence of a family of T-type Ca\textsuperscript{2+} channels that consist of the three isoforms, Ca\textsubscript{\textit{v}}3.1, Ca\textsubscript{\textit{v}}3.2, and Ca\textsubscript{\textit{v}}3.3. Expression studies of the T-type channel \(\alpha_1\) subunits revealed that the activation and inactivation kinetics of Ca\textsubscript{\textit{v}}3.1 and Ca\textsubscript{\textit{v}}3.2 are much faster than that of Ca\textsubscript{\textit{v}}3.3, although all three isoforms are activated at low voltages. It is generally accepted that the electrophysiological properties of currents recorded through T-type channel \(\alpha_1\) subunits are similar to those recorded from isolated cells (1, 3, 4).

Block of voltage-dependent Ca\textsuperscript{2+} channels by metal ions has been extensively studied as it provides insight into structural changes in channel conformations during gating, and serves as a pharmacological tool to distinguish the various channel types. Among trace metals, Cd\textsuperscript{2+} has been shown to selectively block high voltage-activated (HVA)\textsuperscript{2} Ca\textsuperscript{2+} channels. Combined experiments using molecular biology and electrophysiology revealed that the Cd\textsuperscript{2+} binding site in HVA Ca\textsuperscript{2+} channels is composed of a Glu-Glu-Glu-Glu (EEEE) motif in the pore loops, thereby providing insights into Ca\textsuperscript{2+} channel permeation and selectivity (5, 6). Pharmacological studies have shown that LVA T-type currents are inhibited by much higher concentrations of Cd\textsuperscript{2+} than HVA channel currents (7, 8). Sensitivity to nickel inhibition of T-type currents varies greatly between cell types, implying the existence of multiple types of T-type channels. Molecular cloning of three T-type channel \(\alpha_1\) subunits (Ca\textsubscript{\textit{v}}3.1, Ca\textsubscript{\textit{v}}3.2, and Ca\textsubscript{\textit{v}}3.3) allowed a comparison of nickel sensitivities of the three T-type channel isoforms, revealing that only Ca\textsubscript{\textit{v}}3.2 was sensitively inhibited by low micromolar concentrations of nickel (9). All three of the T-type channel isoforms share the Glu-Glu-Asp-Asp (EEDD) motifs in their pore regions corresponding to those of HVA Ca\textsuperscript{2+} channels. Replacement of the third and fourth aspartate with glutamate residues enhances cadmium block of Ca\textsubscript{\textit{v}}3.1 T-type channels, suggesting that LVA and HVA channels share a similar pore structure (10). Detailed biophysical studies of metal block have also found evidence that LVA and K\textsuperscript{+} channels share an internal activation gate (11), which in the case of Zn\textsuperscript{2+} binding to Ca\textsubscript{\textit{v}}3.3 (12), can lead to a “foot-in-the-door” block reminiscent of Rb\textsuperscript{+} block of K\textsuperscript{+} channels (13).

Pharmacological studies of cloned T-type channels reconstituted in expression systems have shown that Ca\textsubscript{\textit{v}}3.2 is more sensitively inhibited by not only nickel, but also zinc and copper, relative to either Ca\textsubscript{\textit{v}}3.1 or Ca\textsubscript{\textit{v}}3.3 (9, 12, 14). Notably, Ca\textsubscript{\textit{v}}3.2 channels are among the most sensitive targets of ion channel targets to zinc block (12). Our recent experiments using chimeric channels between Ca\textsubscript{\textit{v}}3.1 and Ca\textsubscript{\textit{v}}3.2 channels identified His\textsuperscript{191} in the extracellular loop connecting S3 and S4 of domain I as a major structural determinant for inhibition of the Ca\textsubscript{\textit{v}}3.2 by nickel, zinc, copper, and redox agents (15–17).

Metal binding sites in the voltage-sensor paddle have also been reported in Ca\textsubscript{\textit{v}}2.3, Na\textsubscript{1.2}, K\textsubscript{2.1}, and H\textsubscript{1} channels (18–23). Two histidine residues in the IS3–IS4 loop of the Ca\textsubscript{\textit{v}}2.3 channel were identified to be critical for the nickel-sensitive inhibition (18). The interaction sites of \(\alpha\)-scorpion and sea anemone toxins, which cause slowing of fast inactivation, were

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1 To whom correspondence should be addressed: Shinsu-Dong 1, Mapo-Gu, Seoul 121-742, Korea. Tel: 82-2-705-8791; Fax: 82-2-704-3601; E-mail: jhleem@sogang.ac.kr.

2 The abbreviations used are: LVA, low voltage activated; HVA, high voltage activated; HEK, human embryonic kidney; TEA, tetraethylammonium.
localized to the S3–S4 loop of domain IV of the Na,1.2 channel (19, 20). The ability of hanatoxin to reduce channel activity and shift channel gating was also localized to the S3–S4 loop of K,2.1 channels (21). Hanatoxin interaction with the S3–S4 loop of the K,2.1 channel was thought to stabilize a closed state of the channel thereby reducing channel opening (21, 22). We previously proposed that the mechanism of nickel inhibition of Cav3.2 channel activity may be similar to the mechanism of hanatoxin inhibition (15). These findings establish that not only pore regions including S5 and S6, but also voltage-sensor regions including S1–S4 and their connecting loops could be potential binding sites for inhibitors.

Although His191 was identified to be critical for rendering the nickel or zinc inhibition sensitivity to Cav3.2 T-type channels (15), reverse introduction of a histidine residue into the corresponding locus (Gln172) of Cav3.1 channels only slightly increased trace metal block, suggesting that other residue(s) are involved in metal block of Cav3.2 channels in addition to His191. Therefore, we investigated additional residue(s) involved in zinc block of Cav3.2, focusing on zinc inhibition rather than copper to avoid complications of redox reactions (16). We found that the residues that precede His191, Asp189, and Gly190 were also critical residues for determining the high zinc sensitivity of Cav3.2. Additionally, we found an important role of negatively charged residues at the outer portion of the IS2 segment in zinc block of Cav3.2. These findings provide the structural basis of the high affinity extracellular metal binding site on Cav3.2, providing a novel therapeutic target for the treatment of neuropsychiatric pain (24).

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Chemicals were purchased from either Sigma or Amresco (Solon, OH). A zinc-chloride stock solution (100 mM; Sigma) was made in deionized water, and then stored at room temperature. A series of zinc solutions (in μM: 0.3, 1, 10, 30, 100, 300, 1000, and 3000) were prepared by diluting the zinc stock solution with 10 mM Ba2+ solution just before experiments and their pH were adjusted to 7.6 if necessary.

**Construction of Chimeras between Cav3.1 and Cav3.2**—The serial chimeric channels constructed between the rat Cav3.1 (α1G; GenBank accession number AF027984) and human Cav3.2 (α1H; GenBank accession number AF051946) channels were previously reported (15). Additional chimeric and point mutant channels were made by the same PCR method described previously (15). All PCRs were performed using Pfu DNA polymerase (Vivagen, Seoul, Korea) and amplified fragments were verified by sequencing analysis. The restriction sites were marked by numbers in parentheses by indicating the 5’-terminal nucleotide generated by cleavage. Silent and non-silent mutations for restriction sites used for construction of chimeric channels are indicated by asterisks and crosses, respectively.

**Cav3.1/3.2,N-IS4**—Construction of plasmid Cav3.1/3.2,N-IS4 was previously reported (15).

**Cav3.1/3.2,N-IS12l**—The fragments used were Clal (5’-polylinker)-Stul (491, Cav3.2) and Stul (491, Cav3.1)-Hindlll (1754, Cav3.1). The plasmid Cav3.1/3.2,N-IS12l was constructed by ligating the above fragments into the ClaI (5’-polylinker) and Hindlll-digested (1755, Cav3.1) plasmid Cav3.1 pGEM-HEA.

**Cav3.1/3.2,S-IS4**—The fragments used were Cav3.1/3.2,N-IS4-Hindlll (1754, Cav3.1/3.2,N-IS4)-Hindlll (1754, Cav3.1/3.2,N-IS4). The plasmid Cav3.1/3.2,S-IS4 was constructed by ligating the above fragments into Clal- (5’-polylinker) and Hindlll-digested (1755, Cav3.1) plasmid Cav3.1 pGEM-HEA.

**Cav3.1/3.2,N-IS12L+L171G+Q172H**—The fragments used were Clal (5’-polylinker)-PmlI (934, Cav3.1) and PmlI (589, Cav3.1)-Hindlll (1754, Cav3.1/3.2,N-IS4). The plasmid Cav3.1/3.2,N-IS12L+L171G+Q172H was constructed by ligating the above fragments into Clal- (5’-polylinker) and Hindlll-digested (1755, Cav3.1) plasmid Cav3.1 pGEM-HEA.

**Cav3.3/3.2,D140A,Cav3.3/3.2,D140E, and Cav3.3/3.2,A141D**—The forward primers used to amplify the fragments for D140A, D140E, and A141D were GAGGCCCTTGGCGCTTTATTTTGCCCTTTTGTG, GAGGCCCTTGGAGGCCTTCATTTTCGCCTTTTGTG, and GAGGCCCTTGGAGGCCTTCATTTTCGCCTTTTGTG, respectively, and the reverse primer was CAGGATCC-GCATGCTAGG. Each point mutant channel was constructed by ligating the Stul- and BamHI-digested PCR fragments and Clal (5’-polylinker)-Stul (491, Cav3.2) fragment into Clal- (5’-polylinker) and BamHI-digested (730, Cav.3.2) plasmid Cav3.2 pGEM-HEA.

**Cav3.2/G190A and Cav3.2/G190E**—The fragments used were NotI (341, Cav3.2)-BglII (641, Cav3.2), BglII+ (642, Cav3.2)-BamHI (729, Cav3.2), and BamHI (730, Cav3.2)-Sall (4634, Cav3.2). Plasmid Cav3.2/G190A was constructed by ligating the above fragments into NotI- (342, Cav3.2) and Sall-digested (4635, Cav3.2) plasmid Cav3.2 pGEM-HEA.

**Cav3.2/D190A, Cav3.2/D190E, and Cav3.2/D190D**—The forward primer to amplify the upper cassettes for D190A, D190E, and D190D was TAATACGACTCACTATAGGG (T7 promoter) and the reverse primer was CAGGATCC-GCATGCTAGG. Each point mutant channel was constructed by ligating the Stul- and BamHI-digested PCR fragments and Clal (5’-polylinker)-Stul (491, Cav3.2) fragment into Clal- (5’-polylinker) and BamHI-digested (730, Cav3.2) plasmid Cav3.2 pGEM-HEA.
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GGAAATACTCCAG and TGCCGAGAGGCTGACGTTGTCAGGGTGC, respectively. The forward primers to amplify the lower cassettes were CTGAGCGACACAACGTCGTTCTC and AACGTCAGCTCTCCGAGTCAAGGTC, respectively, and the reverse primer was GAGAAGCTTGCCCAGGTGGCTAGC. The upper and lower cassettes were purified using the PCR purification kit and then combined by second-step PCR. Each point mutant channel was constructed by ligating the ClaI- and HindIII-digested PCR fragments into ClaI- (5’-polylinker) and HindIII-digested (1755, Ca3.1) plasmid Ca3.1 pGEM-HEA.

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Ca3.1/D122A, L171G, Q172H \rightarrow \text{Plasmid } Ca3.1/L171G, Q172H \text{ was used as a PCR template and the forward and reverse primers to amplify the upper cassettes for } D122A + L171G + Q172H \text{ were TATAACGACTCATATAGGG (T7 promoter) and AAAGATGAAGGCATCGAAGGCCTGCAGGAT, respectively. The forward and reverse primers to amplify the lower cassettes were GCCCTTCGATGCTCATCTTTGGCTCCTTTT and GAGAAGCTTGCCCAGGTGGCTAGC, respectively. The upper and lower cassettes were purified using the PCR purification kit and then combined by second-step PCR. Each point mutant channel was constructed by ligating the ClaI- and HindIII-digested PCR fragments into the ClaI- (5’-polylinker) and HindIII-digested (1755, Ca3.1) plasmid Ca3.1 pGEM-HEA.}

Preparation of Oocytes and Expression of T-type Ca2⁺ Channels and Mutant Channels—Preparation of Xenopus oocytes was previously described (15). Briefly, ovary lobes surgically obtained from mature female Xenopus laevis (Xenopus Express, France) were torn into small clusters of 5–7 oocytes in SOS solution (in mM: 100 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 2.5 pyruvic acid, and 50 μg/ml of gentamicin, pH 7.6). Isolated oocytes were then treated with collagenase (10 mg/ml, Invitrogen) and trypsin inhibitor (Type III-O, Sigma) for 50 min in Ca²⁺-free OR2 solution (in mM: 82.5 NaCl, 2.5 KCl, 1 MgCl₂, 5 HEPES, pH 7.6) to remove follicle membranes.

To synthesize capped cRNAs, all cDNAs encoding Ca3.1, Ca3.2, and chimeric channels were linearized by AflIII and in vitro transcribed using T7 RNA polymerase (Ambion, Austin, TX) in accordance with the manufacturer’s instructions. The cRNAs were injected into oocytes at concentrations of 10–50 ng/50 nl using a Drummond Nanoject pipette injector (Parkerway, PA) attached to a Narishige micromanipulator (Tokyo, Japan). The SOS solution was changed daily.

Electrophysiology and Data Analysis—Ba²⁺ currents were measured using a two-electrode voltage clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) between the 3rd and 5th days after cRNA injection. Microelectrodes were pulled using a pipette puller and filled with 3 M KCl. The electrode resistance was 0.5–1.0 mO. The bath solution contained (in mM) 10 Ba(OH)₂, 50 NaOH, 1 KOH, and 5 HEPES (pH 7.4) with methanesulfonic acid. The currents were sampled at 5 kHz and low pass-filtered at 1 Hz using the pClamp system (Digidata 1322A and pClamp 8; Molecular Devices, Palo Alto, CA). Data analysis and graphs were obtained with Clampfit software and Prism software (GraphPad, San Diego, CA), respectively. Dose-response curves were fitted using the Hill equation in Prism: \[ B = \left(1 + IC_{50}/(Zn^{2+})\right)^{-n}, \] where \( B \) is the normalized block, IC₅₀ is the concentration of Zn²⁺ giving half-maximal inhibition, and \( n \) is the Hill coefficient. Data are presented as mean ± S.E. Statistical significance was measured using Student’s unpaired t-test.

Whole cell patch clamp recordings were obtained at room temperature using an Axopatch 200A amplifier equipped with a CV201A headstage. The amplifier was connected to a computer through a Digidata 1200 A/D converter, and controlled using pCLAMP 9.2 software. Whole cell currents were recorded using the following external solution (in mM): 15 CaCl₂, 155 tetraethylammonium chloride (TEA-Cl), and 10 HEPES, pH adjusted to 7.4 with TEA-OH. The internal pipette solution contained the following (in mM): 125 CsCl, 10 EGTA, 2 CaCl₂, 1 MgCl₂, 4 Mg-ATP, 0.3 Na₃GTP, and 10 HEPES, pH adjusted to 7.2 with CsOH. Pipettes were made from TW-150-3
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**TABLE 1**
Summary for zinc inhibition effects on the Ca₃.1, Ca₃.2, and relevant mutants

IC₅₀ values were estimated by fitting data with the Hill equation. V₅₀ values of activation and inactivation were obtained from fitting data with a modified Boltzmann equation in the absence and presence of zinc that blocked current by about 50%. All data are shown as mean ± S.E. (n = 4–42). Slope factors and Hill coefficients were not statistically different, and thus, are not shown in the table.

| Construct          | IC₅₀ µM | n  | V₅₀ for activation | V₅₀ for inactivation |
|--------------------|--------|----|--------------------|---------------------|
|                    | Control | Zn²⁺ | Control | Zn²⁺ |
| Ca₃.1              | 82.2 ± 6.9ᵃ | 13 | -39.9 ± 1.0 | -38.0 ± 1.2 |
| Ca₃.2              | 3.0 ± 0.2 | 42 | -35.3 ± 1.0 | -32.6 ± 0.9 |
| Ca₃.1/C₄₂₁IS₁H     | 5.6 ± 0.4ᵇ | 7 | -38.1 ± 0.5 | -35.7 ± 0.9ᵇ |
| Ca₃.1/C₄₂₁₁₂N-IS₁₅ | 2.6 ± 0.3 | 6 | -40.6 ± 1.1 | -36.8 ± 1.0ᵇ |
| Ca₃.1/C₄₂₁₁₂N-IS₂  | 85.2 ± 9.3ᵇ | 4 | -44.5 ± 2.0 | -41.3 ± 1.5 |
| Ca₃.1/C₄₂₁₁₂SIS₂  | 1.7 ± 0.2ᵇ | 13 | -32.3 ± 0.2 | -30.2 ± 1.0 |
| Ca₃.2/D₁₄₄₄       | 7.9 ± 1.3ᵇ | 8 | -24.6 ± 0.7 | -22.1 ± 0.8ᵇ |
| Ca₃.2/D₁₄₄₆        | 3.4 ± 0.7 | 10 | -30.5 ± 1.3 | -25.1 ± 1.5ᵇ |
| Ca₃.2/A₁₁₁           | 125 ± 2.1ᵇ | 8 | -32.5 ± 1.1 | -30.9 ± 1.1 |
| Ca₃.2/D₁₈₉₁        | 28.2 ± 8.8ᵇ | 13 | -38.0 ± 0.7 | -35.3 ± 0.6ᵇ |
| Ca₃.2/D₁₈₁₄        | 1.0 ± 0.1ᵇ | 6 | -39.3 ± 1.8 | -35.3 ± 1.5 |
| Ca₃.2/G₁₁₀         | 47.8 ± 6.5ᵇ | 12 | -32.3 ± 1.2 | -29.1 ± 1.3 |
| Ca₃.2/H₁₁₀QC       | 103.8 ± 14.3ᵇ | 7 | -36.6 ± 0.7 | -34.9 ± 0.7 |
| Ca₃.1/Q₁₇₂₁H      | 42.5 ± 7.6ᵇ | 4 | -42.8 ± 2.0 | -38.3 ± 2.0 |
| Ca₃.1/L₁₇₂₁-G₁₇₂₁H | 6.4 ± 0.8ᵇ | 4 | -41.5 ± 1.0 | -40.7 ± 0.7 |
| Ca₃.1/Q₁₇₂₁H-F₁₇₂₁ | 56.6 ± 3.3ᵇ | 10 | -42.0 ± 0.8 | -37.3 ± 1.0ᵇ |
| Ca₃.1/₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁ | 1.4 ± 0.2ᵇ | 8 | -35.1 ± 0.3 | -30.0 ± 0.4ᵇ |
| Ca₃.1/L₁₂₂₁₂-₁₄₁₀₁-₁₄₁₂₁-₁₄₁₁₁-₁₄₁₂₁ | 5.5 ± 0.5ᵇ | 10 | -38.9 ± 1.2 | -35.5 ± 1.3ᵇ |

ᵃ Significant differences of the data were analyzed using Student’s unpaired t test, p < 0.01.
ᵇ Significant differences of the data were analyzed using Student’s unpaired t test, p < 0.05.
ᶜ Significant differences of the data were analyzed using Student’s unpaired t test, p < 0.01.

capillary tubing (World Precision Instruments, Inc., Sarasota, FL). Under these solution conditions the pipette resistance was ~2.4 megaohms. Access resistance and cell capacitance were calculated using on-line exponential fits to the capacitance transient induced by a 20-mV depolarization (Membrane Test, pCLAMP software). Cell capacitance averaged 10 picofarads. Access resistance averaged 4 megaohms, and was compensated using a paired Students’ t test. Significant differences of the data were analyzed using Student’s unpaired t test, p < 0.001.

**RESULTS**

Inward Ba²⁺ currents were not detected from control oocytes injected with either H₂O or 0.1 mM KCl, whereas robust inward transient currents were recorded from oocytes injected with cRNA encoding Ca₃.1, Ca₃.2, or chimeric channels. We first confirmed the Xenopus oocyte expression system could be used to measure the higher potency of zinc to inhibit Ca₃.2 over Ca₃.1 channels, which was originally observed in mammalian cells (14, 17). Application of serial zinc solutions inhibited Ca₃.2/H₁₁₀Q currents in a concentration-dependent manner (Fig. 1F, G), and the IC₅₀ value was 103.8 ± 14.3 µM (n = 7). This result indicated that the H₁₁₀Q mutation greatly diminished the zinc sensitivity of Ca₃.2, lowering it to the levels observed with Ca₃.1.

We next tested how reverse mutation of the corresponding glutamine residue to histidine (Q₁₇₂₁H) in Ca₃.1 altered zinc sensitivity of Ca₃.1. The zinc inhibition profile of the Ca₃.1/L₁₇₂₁Q mutant revealed an IC₅₀ value of 42.5 ± 7.6 µM (n = 4), indicating that the mutation increased sensitivity by about 2-fold relative to wild-type Ca₃.1, but still 15-fold less sensitive than Ca₃.2 (Fig. 1E, F). These results suggested that adoption of other residue(s) into Ca₃.1/Q₁₇₂₁H were required to gain the zinc-sensitive inhibition to the levels observed with Ca₃.2 besides His¹⁹¹.

To narrow down other regions contributing to the zinc sensitivity of Ca₃.2, we systematically constructed serial chimeric channels by adopting the adjacent regions(s) of His¹⁹¹ into either Ca₃.1 or Ca₃.1/L₁₇₂₁H. We first constructed Ca₃.1/L₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁Q₁₇₂₁H by introducing the region from the amino terminus to the IS₄ segment of Ca₃.2 into the Ca₃.1. The IC₅₀ value of Ca₃.1/L₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁Q₁₇₂₁H was 26.0 ± 3.3 µM (n = 6; Fig. 2), which was similar to that of Ca₃.2. Consistent with our previous report (15), this result strongly suggested that residue(s) determining the zinc sensitivity of Ca₃.2 were present in the voltage-sensor paddle region (IS₁–IS₄) of domain I of Ca₃.2 rather than the pore region. Next, we constructed chimeric channels with smaller substitutions, Ca₃.1/L₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁Q₁₇₂₁H and Ca₃.1/L₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁Q₁₇₂₁H by transferring the NH₂ terminus to the connecting loop between IS₁ and IS₂ of Ca₃.2 and the connecting linker between IS₃ and IS₄ of Ca₃.2 into the Ca₃.1, respectively (Fig. 2). Zinc inhibition profiles of the chimeric channels showed that the IC₅₀ value of Ca₃.1/L₁₂₂₁₂-₁₁₇₁₁-₁₁₇₂₁Q₁₇₂₁H was...
85.2 ± 9.3 μM (n = 4), which was similar to that of Ca,3.1. In contrast, zinc sensitivity of Ca,3.1/3.2:IS34L (IC50 = 5.6 ± 0.4 μM, n = 7) was profoundly increased, but was still 2-fold lower than that of Ca,3.2 (Student’s unpaired t test, p < 0.001; Fig. 2). The 2-fold difference between the wild-type Ca,3.2 and Ca,3.1/3.2:IS34L implied that although residue(s) critically determining the zinc-sensitive inhibition were located in the IS3–IS4 loop of Ca,3.2, additional residues might be present in its neighboring region(s), such as the region preceding the IS3–IS4 loop. Therefore, we tested the zinc sensitivity of Ca,3.1/3.2:IS2-IS4, which was constructed by introducing the region from IS2 to IS4 of Ca,3.2 into Cav3.1 (Fig. 2). The zinc inhibition profiles revealed that Ca,3.1/3.2:IS2-IS4 was 3.3- and 1.8-fold more sensitive to zinc than Ca,3.1/3.2:IS34L and wild-type Ca,3.2, respectively (IC50 = 1.7 ± 0.2 μM, n = 13; p < 0.001; Fig. 2). The enhanced sensitivity suggests that residues in IS2 and IS3 that precede the IS3–IS4 loop may participate in zinc inhibition of Ca,3.2 currents.

The reverse single mutation of Q172H increased the zinc sensitivity of Ca,3.1 by about 2-fold. In contrast, adoption of the IS3–IS4 loop of Ca,3.2 into the corresponding loop of Ca,3.1 increased zinc sensitivity 15-fold, suggesting that not only His191, but also other residues in the IS3–IS4 loop, participate in the zinc sensitivity of Ca,3.2. Comparing the amino acid sequences of the IS3–IS4 loops of Ca,3.1 with those of Ca,3.2 revealed that Leu, Gln, and Phe in Ca,3.1 were different from the corresponding residues, Gly190, His191, and Leu195, in the Ca,3.2 channel (Fig. 3 A). To examine whether these residues influence zinc sensitivity, we additionally introduced individual mutations of L171G and F176L into Ca,3.1/Q172H. Analysis of their zinc inhibition profiles showed that zinc sensitivity of Ca,3.1/Q172H+176L was not significantly different from that of Ca,3.1/IS2-IS4 (Fig. 3, B and

**FIGURE 2.** Potency of zinc to inhibit Ca,3.1, Ca,3.2, and chimeric channels mutated in the domain I. A, schematic diagrams of Ca,3.1, Ca,3.2, and their chimeras were linearly represented for Domain I (left). The transmembrane segments and connecting loops of the T-type channels are displayed with cylinders and lines. White cylinders and thin lines represent the regions from Ca,3.1, whereas gray cylinders and thick lines represent regions from Ca,3.2. Domains II, III, and IV of these chimeras were from Ca,3.1. IC50 values of chimeric channels were exhibited with bar graphs (middle). These values were obtained from dose-response curves (shown in B) for which the percent inhibition data were fitted with the Hill equation. Representative current traces of Ca,3.1, Ca,3.2, and chimeric channels before and after zinc inhibition were exhibited (right panel of A). Scale bars on the x and y axes represent 40 ms and 1 μA, respectively. B, dose-response curves of Ca,3.1, Ca,3.2, and their chimeric channels. The chimeric channels (Ca,3.1/3.2:N4-IS4 (△), Ca,3.1/3.2:IS34L (○), and Ca,3.1/3.2:IS2-IS4 (□)), which contain the linker connecting IS3 and IS4 of Ca,3.2 in common, were inhibited by low micromolar concentrations of zinc. Their zinc inhibition sensitivities were close to that of Ca,3.2 (○). In contrast, Ca,3.1/3.2:N4-IS12L (□) required much higher concentrations to be inhibited and its zinc sensitivity was similar to that of Ca,3.1 (△). All data are presented as mean ± S.E. (n = 4 – 42).
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A

| Residue       | Amino Acid Sequence |
|---------------|---------------------|
| Ca$_{v}$3.1   | TWNRLDFIVIAEMEYSLDQNVSFSAVRTVRVLPLRAINRVSPIR     |
| Ca$_{v}$3.2   | TWNRLDFIVVAGMEYSLDGHNVSLSAIRTVRVLPLRAINRVSPIR     |

B

| Ca$_{v}$3.2   | 3.0 ± 0.2          |
| Ca$_{v}$3.1/Q172H | 42.5 ± 7.6        |
| Ca$_{v}$3.1/L171G+Q172H | 6.4 ± 0.6        |
| Ca$_{v}$3.1/Q172H+F176L | 56.6 ± 3.3        |
| Ca$_{v}$3.2/G190L | 47.8 ± 6.5        |
| Ca$_{v}$3.2/D189A | 28.2 ± 8.8        |
| Ca$_{v}$3.2/D189E | 1.0 ± 0.1         |

C

- [Graph showing percent inhibition vs. Zn$^{2+}$ concentration for various Ca$_{v}$3.2 variants]
In contrast, the zinc sensitivity of Ca3.1/I171G+Q172H was significantly augmented (IC50 = 6.4 ± 0.6 μM, n = 4; p < 0.001; Fig. 3, B and C), suggesting that the double mutation of I171G as well as Q172H into Ca3.1, was critical to the increase in zinc sensitivity of wild-type Ca3.3.1. Consistently, reciprocal mutation of Glu190 of Ca3.2 into leucine strongly reduced the potency of zinc (Ca3.2/I190L, IC50 = 47.8 ± 6.5 μM, n = 12; Fig. 3, B and C), supporting our hypothesis that glycine as well as histidine residues in the IS3–IS4 loop are crucial elements affecting zinc sensitivity of Ca3.3.2.

Sequence comparison of the IS3–IS4 loops of Ca3 isoforms identified an aspartate residue that was commonly present at the preceding position to the identified two critical residues (Gly190 and His191). Because the carboxyl acid side chains of aspartate could be another potential ligand involved in zinc binding, we tested possible participation of Asp189 in the zinc sensitivity of Ca3.2. Point mutation of D189A into Ca3.2 decreased its zinc sensitivity by about 10-fold (IC50 = 28.2 ± 8.8 μM, n = 13; Fig. 3, B and C), whereas point mutation of D189E enhanced its zinc sensitivity by about 3-fold (IC50 = 1.0 ± 0.1 μM, n = 6; p < 0.001; Fig. 3, B and C). These results indicated that an acidic residue just before the critical Glu190 and His191 residues is also required to endow the Ca3.2 with high-affinity zinc inhibition. Taken together, these findings indicate that Asp189, Glu190, and His191 compose a major part of the zinc-binding motif.

Crystallographic studies of the voltage-sensor paddle of voltage-gated K+ channels show that the S1–S2 loop and the S3 and S4 loops are in close proximity (25). Therefore, we hypothesized that the Asp-Gly-His motif in the IS3–IS4 loop is likely to be structurally close to the IS1–IS2 loop of Ca3.2 (Fig. 4A). To test the possibility that the region from the amino-terminal end to the IS1–IS2 loop influences zinc sensitivity, we constructed Ca3.3.1/I122N, IS3.2L+I171G+Q172H. The zinc sensitivity of this mutant was similar to that of Ca3.3.1/I171G+Q172H displaying an IC50 of 5.5 ± 0.5 μM (n = 10) (Fig. 4, B and C). This finding suggests that non-conserved residues in the NH2 terminus to IS1–IS2 loop do not contribute to the zinc metal binding site.

Zinc inhibition profiles of Ca3.3.3.2/I34L and Ca3.3.3.2/I34S showed that, despite both mutants containing the Asp-Gly-His motif, the former was still about 2-fold less sensitive to zinc than Ca3.2, whereas the latter was about 2-fold more sensitive (Fig. 2). Compared with Ca3.3.3.3.3/I34L, Ca3.3.3.3/I34S additionally contained the IS2–IS3 and IS4 regions from Ca3.3.2, implying that these regions are responsible for the difference in zinc sensitivity. Sequence comparison from IS2 to IS4 revealed that “Phe-Asp-Ala” containing one negatively charged residue was present at the outer portion of IS2 of Ca3.3.2, whereas “Phe-Asp-Asp” containing two negatively charged residues was at the corresponding portion in Ca3.3.1 (Fig. 4A). We hypothesized that the position and number of aspartate residues at this position might alter zinc sensitivity. To test this hypothesis, we first replaced Asp115 in Ca3.3.1/I115G+Q172H with a non-polar alanine residue, converting “Phe-Asp-Asp” into “Phe-Asp-Ala.” Ca3.3.1/I122A+I171G+Q172H was about 2- and 4-fold more sensitive to zinc than wild-type Ca3.3.2 and Ca3.3.1/I171G+Q172H, respectively (IC50 = 1.4 ± 0.2 μM, n = 14; p < 0.001; Fig. 4, B and C). In contrast, Ca3.3.2/A114I was 4-fold less sensitive to zinc than wild-type Ca3.3.2 (Ca3.3.2/A114D, IC50 = 12.5 ± 2.1 μM, n = 8; Fig. 4, B and C). In addition, mutation of D140A (Ca3.3.2/D140A) reduced zinc sensitivity of Ca3.3.2 by 2.6-fold (IC50 = 7.9 ± 1.3 μM, n = 8; Fig. 4, B and C), whereas mutation of D140E (Ca3.3.2/D140E) did not significantly alter zinc sensitivity (IC50 = 3.4 ± 0.7 μM, n = 10; p = 0.4601; Fig. 4B). These consistent changes in zinc sensitivity by diverse mutations of aspartate residues in this region support the hypothesis that the acidic residue(s) in the outer portion of IS2 may also play a role in zinc binding. Another implication is that the presence of only one negatively charged residue in this region is more likely to form a structural conformation favorable to zinc block.

Based on the experimental results and potassium channel models (25, 26), we developed a model to illustrate how zinc coordinates to the Asp-Gly-His motif and the acidic residue at the outer portion of IS2 (Fig. 5). We chose a model of the closed channel (26) rather than the crystal structure of the open channel (25), because our previous studies demonstrated that nickel had a lower affinity for the open state (9). The model suggests zinc might inhibit channel opening by disrupting the function of the voltage-sensor paddle in repeat I, rather than a direct action on Ca2+ ion permeation as observed with Cd2+ block of HVA channels. We next sought biophysical data to support this hypothesis, reasoning that Zn2+ might alter charge movement that precedes channel opening. Due to limitations in the two microelectrode voltage clamp of oocytes, we used patch clamp recording of HEK-293 cells in the whole cell mode. On-gating currents can be measured at the reversal potential after series resistance compensation and proper cancellation of residual capacitance charge, and quantitated by integrating the area of the outward gating current (27). Expression of recombinant Ca3.3.2 in HEK-293 cells generated ~4000 pA of inward current during step depolarizations to −20 mV and ~750 pA outward current during step depolarizations to +55 mV (Fig. 6). Addition of 10 μM zinc inhibited the inward current by 91% (±1%, n = 6), and inhibited the outward gating current 23% (±1%, n = 6, p = 0.08, paired t test; Fig. 6, C and D). Time matched controls (Control 2) showed no change in gating current, ruling out effects due to rundown. These results indicate that zinc is capa-
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A

| IS1 | 122 | IS2 |
|-----|-----|-----|
| Ca\textsubscript{v}3.1 LLNCVTLMFRPCEDIAACDSQRCRILQADFDFIFAFAVEMVVKMVALG |
| Ca\textsubscript{v}3.2 MLNCVTLMFRPCEDVCGSERCNILEAFDAFIFAFAVEMVIKMVALG |

B

**Domain I**

| IC\textsubscript{50} values (µM) |
|-------------------------------|
| Ca\textsubscript{v}3.1/3,2;N-IS12L+L171G+Q172H | 5.5 ± 0.5 |
| Ca\textsubscript{v}3.1/D122A+L171G+Q172H | 1.4 ± 0.2 |
| Ca\textsubscript{v}3.2/A141D | 12.5 ± 2.1 |
| Ca\textsubscript{v}3.2/D140A | 7.9 ± 1.3 |
| Ca\textsubscript{v}3.2/D140E | 3.4 ± 0.7 |

C

Percent Inhibition

![Graph showing percent inhibition vs. [Zn\textsuperscript{2+}], µM]

- Ca\textsubscript{v}3.2
- Ca\textsubscript{v}3.1/3,2;N-IS12L+L171G+Q172H
- Ca\textsubscript{v}3.1/D122A+L171G+Q172H
- Ca\textsubscript{v}3.2/A141D
- Ca\textsubscript{v}3.2/D140A
- Ca\textsubscript{v}3.2/D140E
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FIGURE 5. A structural model for zinc binding to the identified elements on the Ca$_{3.2}$ channel. This model is modified from the structural model of a closed voltage-gated potassium channel (26). For clarity, only the voltage-sensor paddle is shown (left panel). The zinc interacting residues are shown by individual amino acids, of which oxygen, carbon, and nitrogen atoms are marked red, white, and blue, respectively. A possible location of the zinc ion is displayed with a blue sphere. Because the amino acid sequence of the IS3–IS4 linker of Ca$_{3.2}$ is profoundly different from the Shaker potassium channel, we modeled the Asp-Gly-His motif to that predicted for the albumin ATCUN motif (29).

FIGURE 6. Zinc inhibition of ionic and gating currents of Ca$_{3.2}$ expressed in HEK-293 cells. Whole cell patch recordings were made using the Hh8-5 stable cell line expressing Ca$_{3.2}$ (41). Currents were measured using 10 mM Ca$^{2+}$ in the external solution and 155 TEA-Cl in the internal solution as charge carriers. A, ionic currents recorded during a step pulse to −20 mV from a holding potential of −100 mV. Currents were measured before (control) and after application of 10 µM ZnCl$_2$. B, average results of the peak currents were measured in control and zinc. C, gating currents were measured from the same cell using a step depolarization to +55 mV. Each trace represents the average of 20 consecutive sweeps. As in A, data recorded in the presence of zinc is represented by a thick line. D, average gating currents were normalized to control. Control 2 is a time-matched control where cells were continuously perfused with the 10 mM external solution.

DISCUSSION

We recently identified His$^{191}$ in the IS3–IS4 extracellular linker as a critical determinant of nickel, copper, zinc, and redox sensitivity of Ca$_{3.2}$ (15–17). To localize other partners of His$^{191}$ required for interacting with high affinity zinc, we systematically transferred various portions in domain I of Ca$_{3.2}$ into Ca$_{3.1}$/Q172H, which was only slightly more sensitive to zinc than wild-type Ca$_{3.1}$. Analysis of their zinc dose-response relationships revealed that introduction of the region(s) only including the IS3–IS4 loop of Ca$_{3.2}$ into Ca$_{3.1}$/Q172H (or Ca$_{3.1}$) dramatically increased its zinc sensitivity, indicating that the IS3–IS4 loop contains additional residues involved in metal binding. Consequent point mutations uncovered the role of an "Asp-Gly-His" motif involved in zinc inhibition of Ca$_{3.2}$. Chimeras and point mutations also revealed an important role of aspartate residues in IS2 in zinc block, thereby providing the basis for a structural model where trace metals bind and block movements of the repeat I voltage-sensor paddle. This hypothesis was supported by measuring the ability of zinc to reduce on-gating charge movements.

The critical role of Gly in the middle of the Asp-Gly-His motif was shown by two experimental results: 1) single mutation of Q172H into Ca$_{3.1}$ only partially increased zinc sensitivity, but double mutations of L171G and Q172H into Ca$_{3.1}$ had a larger effect, bringing zinc potency close to that of wild-type Ca$_{3.2}$, and 2) single mutation of G190L decreased the zinc sensitivity of Ca$_{3.2}$. Glycine residues are known for providing flexibility for polypeptide(s) because glycine has hydrogen as its side chain, rather than a carbon, as is the case in all other amino acids (28). Based on its structural property, we simply interpret that the Gly in the Asp-Gly-His motif provides not only a deprotonated amide nitrogen atom, but also flexibility for its neighboring Asp$^{189}$ and His$^{191}$ residues to interact with zinc more efficiently. This may explain why introduction of histidine into the corresponding position of Ca$_{3.1}$ was not sufficient to convert zinc block to the potency observed in Ca$_{3.2}$. We hypothesize the bulky leucine residue in Ca$_{3.1}$ interferes with zinc coordination by Asp$^{170}$ and His$^{172}$.

The amino-terminal Cu(II)- and Ni(II)-binding (ATCUN) motif of serum albumin is composed of an "Asp-Glu-Ala-His" motif (29). Based on the similarity of the Ca$_{3.2}$ Asp-Gly-His motif to the ATCUN motif, we predicted that the acidic residue in the motif would also be critical for the micromolar zinc sensitivity of Ca$_{3.2}$. This prediction was tested by mutating Asp$^{189}$ in Ca$_{3.2}$ into either a neutral residue or another acidic residue, followed by examining their zinc sensitivities. Point mutation

ble of inhibiting gating charge movement, and is consistent with a model whereby zinc binding stabilizes the closed conformation of the voltage-sensor paddle.
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of D189A decreased the zinc sensitivity by about 10-fold. Notably, point mutation of D189E enhanced the zinc sensitivity by about 3-fold. These results support that, in addition to Gly$^{190}$ and His$^{191}$, the preceding Asp$^{189}$ is another requirement for the high zinc sensitivity to Ca$_{3.2}$. The structure of the ATCUN motif (Asp/Glu-Ala-His) was proposed to be a pentacoordinated structure formed by the carboxyl group of the acidic residue and four nitrogen ligands (one from the amino terminus, two from the peptide backbone, and one from the imidazole nitrogen) (29). In addition to an ATCUN motif, our data support the hypothesis that Ca$_{3.2}$ includes an additional ligand, coming from the carboxyl group of the aspartate residue in IS2, thereby providing a model of the zinc binding site in Ca$_{3.2}$.

Previous studies have established that negatively charged residues in transmembrane segments S1, S2, and S3 also act as parts of voltage sensing machinery by electrostatically interacting with positively charged residues in the S4 segment of voltage-gated ion channels (30). The electrostatic interaction between charged residues helps fold the channel in a proper conformation that efficiently targets the channels to the plasma membrane (31, 32). Papazian and colleagues (33, 34) also found that divalent ions such as nickel and magnesium can bind to the extracellular ion binding pocket formed by the negatively charged residues at S2 and S3 in ether-à-go-go K$^+$ channels, decelerating activation kinetics and/or inhibition of currents. Based on these previous findings, we tested whether the negatively charged residue(s) at the outer portion of IS2 were involved in zinc sensitivity. Sequence comparison showed that Ca$_{3.1}$ has Phe-Asp-Asp, whereas Ca$_{3.2}$ has Phe-Asp-Ala at this position. Ca$_{3.1}$/D122A+L171G+Q172H$^*$ constructed by replacing Phe-Asp-Asp of Ca$_{3.1}$/L171G+Q172H$^*$ with Phe-Asp-Ala, was 4.6-fold more sensitive to zinc than Ca$_{3.2}$/L171G+Q172H$^*$. Ca$_{3.2}$/A141D constructed by mutating Phe-Asp-Ala into Phe-Asp-Asp became 4.2-fold less sensitive to zinc than wild-type Ca$_{3.2}$. These data imply that the presence of one acidic residue in the middle of this triplet renders higher zinc sensitivity than that of two acidic residues. This implication is further supported by the findings that the presence or absence of a negatively charged residue in the middle of the triplet significantly affected zinc inhibition sensitivity (Ca$_{3.2}$/D140E versus Ca$_{3.2}$/D140A, IC$_{50} = 3.4 \pm 0.7 \text{ versus } 7.9 \pm 1.3 \mu M$). These results suggest that Phe-Asp-Ala at the outer portion of IS2 is more favorable than the other sequence combinations in improving zinc inhibition sensitivity, together with the major contribution of the Asp-Gly-His motif.

All of the mutant channels where the negatively charged residue at the outer portion of IS2 was neutralized showed a common positive shift of their activation and inactivation curves compared with those of wild-type Ca$_{3.3}$ (Table 1). For example, activation and inactivation curves of Ca$_{3.2}$/D140A were positively shifted by ~10 mV, and its functional expression in Xenopus oocytes was dramatically decreased as well (data not shown). The voltage dependence of activation and inactivation of all the other mutants were similar to their respective wild-type channels, ruling out any artificial shift in sensitivity due to less activation under the voltage protocols used. By analogy to findings with K$^+$ channels, the decrease in expression may suggest that Asp$^{140}$ is electrostatically interacting with positively charged residues in IS4, influencing structural conformation of the channel and trafficking of the channels to the plasma membrane (30, 35).

We identified two structural elements critical for rendering the high zinc sensitivity to Ca$_{3.2}$: the Asp-Gly-His motif in the IS3–IS4 loop and an Asp residue in IS2. Based on the chimeric approach used in this study, we cannot rule out possible involvement of conserved residues in other parts of the channel, such as S4 voltage-sensor paddle regions, as identified in Cu$^{2+}$ block of BK channels (36). In addition, this study does not localize the lower affinity zinc binding sites involved in block of Ca$_{3.1}$. Although the underlying mechanism of how the negatively charged residue in IS2 contributes to zinc sensitivity remains to be further investigated, a simple interpretation is that the acidic residue acts as another member for zinc coordination with the Asp-Gly-His motif, based on the findings that its mutation into a neutral residue decreases zinc sensitivity. The ability of zinc to decrease gating charge 25% is consistent with immobilization of one of the four voltage-sensor paddles. It is interesting to note that repeat I plays a dominant role in the opening of both HVA and LVA channels (37, 38). These studies also provide evidence for considerable structural similarity between voltage-gated K$^+$ and Ca$^{2+}$ channels, and combined with the established role of Ca$_{3.2}$ in pain and epilepsy, provide a structural model for the development of novel therapeutics (17, 24, 39, 40).

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