A Molecular Determinant of Nickel Inhibition in CaV3.2 T-type Calcium Channels*

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Molecular cloning studies have revealed that heterogeneity of T-type Ca2+ currents in native tissues arises from the three isoforms of Ca3 channels: Ca3.1, Ca3.2, and Ca3.3. From pharmacological analysis of the recombinant T-type channels, low concentrations (<50 μM) of nickel were found to selectively block the Ca3.2 over the other isoforms. To date, however, the structural element(s) responsible for the nickel block on the Ca3.2 T-type Ca2+ channel remain unknown. Thus, we constructed chimeric channels between the nickel-sensitive Ca3.2 and the nickel-insensitive Ca3.1 to localize the region interacting with nickel. Systematic assaying of serial chimeras suggests that the region preceding domain I S4 of Ca3.2 contributes to nickel block. Point mutations of potential nickel-interacting sites revealed that H191Q in the S3–S4 loop of domain I significantly attenuated the nickel block of Ca3.2, mimicking the nickel-insensitive blocking potency of Ca3.1. These findings indicate that His-191 in the S3–S4 loop is a critical residue conferring nickel block to Ca3.2 and reveal a novel role for the S3–S4 loop to control ion permeation through T-type Ca2+ channels.

Calcium entry through low voltage-activated T-type Ca2+ channels causes a rise in cytoplasmic Ca2+ concentrations, which subsequently triggers numerous physiological functions including neuronal excitability, cardiac pacemaker activity, hormone secretion, smooth muscle contraction, fertilization, and gene expression (1–5). Overexpression of T-type channels appears to be linked to pathophysiological conditions such as absence epilepsy, pain, cardiac arrhythmia, and hypertrophy (6–9).

Metallic divalent ions such as Cd2+, Co2+, Ni2+, Pb2+, and Zn2+ have been found to inhibit Ca2+ permeation via voltage-dependent Ca2+ channels with different potencies (10–14). It was generally accepted that Cd2+ selectively blocked all types of high voltage-activated (HVA)3 channels, whereas Ni2+ was selective for low voltage-activated T-type Ca2+ channels (10, 14). T-type Ca2+ currents endogenously expressed in sinoatrial nodal cells and dorsal root ganglion neurons were shown to be selectively blocked by low concentrations of Ni2+ (<50 μM) (2, 15).

On the contrary, it has also been reported that T-type Ca2+ currents in other neuronal cells required much higher concentrations of nickel to be blocked (14, 15). The tissue-dependent variability in nickel sensitivities strongly suggests heterogeneity of T-type channels. Indeed, recent molecular cloning and expression studies demonstrated that the T-type Ca2+ channel family consists of three members, Ca3.1 (α1C), Ca3.2 (α1H), and Ca3.3 (α1I) (16–18). Consequent pharmacological analysis of nickel block of the T-type Ca2+ channel isoforms revealed that Ca3.2 was the only nickel-sensitive isoform (IC50 for block = 5–10 μM), whereas Ca3.1 and Ca3.3 were as nickel-insensitive (IC50 for block ≥ 100 μM) as most HVA channels (11, 19).

In the present study, we investigated the structural element(s) involved in nickel block of Ca3.2 by assaying chimeric channels between the nickel-sensitive Ca3.2 and the nickel-insensitive Ca3.1. Single point mutation experiments identified that the His-191 in the extracellular loop connecting S3 and S4 of domain I is a key structural determinant critical for nickel block of the Ca3.2.

MATERIALS AND METHODS

Chemicals—Nickel (II) chloride hexahydrate (NiCl2·6H2O) was obtained from Sigma (St. Louis). Most of the other chemicals were purchased from Sigma and USB (Cleveland, OH). A nickel stock solution (100 mM) was made in deionized water and stored at room temperature. A series of nickel solutions (in μM: 1, 3, 10, 30, 100, 300, 1000, 3000) were prepared by diluting the nickel stock solution with 10 mM Ba2+ solution just before experiments, and their pH values were adjusted to 7.6.

Construction of Chimeras between Ca3.1 and Ca3.2—The chimeric channels were constructed by modification of the cDNAs encoding the rat Ca3.1 (α1C, GenBank™ accession number AF027984) and human Ca3.2 (α1H, GenBank™ accession number AF051946) channels, for which PCR was used to add silent or non-silent restriction enzyme sites. The chimeric channels were subcloned into the pGEM-HEA vector containing 5′ and 3′ untranslated regions of a Xenopus β-globin gene for better expression in oocytes (20). The methods for construction of the chimeric channels have been reported previously (21). Details of their construction are given below, where the restriction endonuclease sites and the nucleotide positions in the respective channel cDNAs are designated in parentheses. In some cases a restriction site was introduced by PCR, and these are indicated by asterisks for silent mutations and crosses for mutations that change the protein sequence.

HHGG—A HindIII site was introduced at 3959, corresponding to the loop connecting domain II and III of the Ca3.2 by PCR. The forward primer was 5′-CCCAGGAAGCTGTAAATGCT-3′, and reverse primer was 5′-AAAAGCTTGTTGTGATGACCTT-3′. The full-length cDNA of HHGG was constructed by ligating Clal (5′-polylinker)-PvuI

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3 The abbreviation used is: HVA, high voltage-activated.
Structural Determinant of Ca_{3.2} for Nickel-sensitive Block

(3725, Ca_{3.2}), PvuI (3728, Ca_{3.2})-HindIII' (3963, Ca_{3.2}), HindIII' (4246, Ca_{3.1})-KpnI (6170, Ca_{3.1}), and KpnI (6175, Ca_{3.1})-AflIII (3'-polylinker) into the Clal- (5'-polylinker) and AflIII-digested (3'-polylinker) plasmid pGEM-HEA.

GHH—The plasmid GHH was constructed by ligating Clal (5'-polylinker)-SpeI (2304, Ca_{3.1}) and SpeI (2301, Ca_{3.1})-HindIII' (4246, Ca_{3.1}) into the Ca_{3.2-}HindIII' plasmid pGEM-HEA, which was opened by Clal (5'-polylinker) and HindIII' (3960).

HGG—The plasmid HGG was constructed by ligating Clal (5'-polylinker)-HindIII (1424, Ca_{3.2}) into the plasmid Ca_{3.1} pGEM-HEA, which was opened by Clal (5'-polylinker) and HindIII (1758, Ca_{3.1}).

GHGG—The plasmid GHGG was constructed by ligating the following fragments, Clal (5'-polylinker)-BspEI (2696, Ca_{3.1}) and BspEII (2437, Ca_{3.2})-HindIII' (3963, Ca_{3.2}) into the plasmid HHGG, which was opened by Clal (5'-polylinker) and HindIII' (3960, HHGG).

HGPGHGG—The plasmid HHGG/GSs-pore was constructed by ligating the fragments Clal (5'-polylinker)-BamHI (733, Ca_{3.2}), BamHI (1076, Ca_{3.1})-Sall (1555, Ca_{3.1}) and Sall' (1221, Ca_{3.2})-HindIII (1424, Ca_{3.2}) into the plasmid Ca_{3.1} pGEM-HEA, which was opened by Clal (5'-polylinker) and HindIII (1758, Ca_{3.1}).

GGGH/H_{N-IS4}—The plasmid GGG/H_{N-IS4} was constructed by digesting the fragments Clal (5'-polylinker) and HindIII (1424, Ca_{3.2}) into the plasmid Ca_{3.1} pGEM-HEA, which was opened by Clal (5'-polylinker) and HindIII (1758, Ca_{3.1}).

GGGH/H_{N-IS5-Ipore}—The plasmid GGG/H_{N-IS5-Ipore} was constructed by digesting the fragments Clal (5'-polylinker)-BspEI (2696, Ca_{3.1}) and BspEII (2437, Ca_{3.2})-HindIII' (3963, Ca_{3.2}) into the plasmid HHGG, which was opened by Clal (5'-polylinker) and HindIII' (3960, HHGG).

Point mutations were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene), and the entire region derived from PCR products was sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). The PCR-generated DNA cassette digested with Clal and HindIII into the plasmid Ca_{3.1} pGEM-HEA, which was opened by Clal (5'-polylinker) and HindIII (1755, Ca_{3.1}).

All PCRs were performed using Fru Ultra DNA polymerase (Stratagene), and the entire region derived from PCR products was sequenced to verify correct introduction of point mutated site(s) and that there were no inadvertent mutations.

Preparation of Oocytes and Expression of Chimeric Channels—Several ovary lobes were surgically removed from mature female Xenopus laevis (Xenopus Express, France) and torn into small clusters of 3–5 oocytes in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl_{2}, 1 mM MgCl_{2}, 5 mM HEPES, 2.5 mM pyruvic acid, and 50 μM/ml gentamicin, pH 7.6). To remove follicle membranes, isolated oocytes were treated with collagenase (Type IA, 2 mg/ml) and were treated for 30 min in Ca^{2+}-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl_{2}, 5 mM HEPES, pH 7.6).

All cDNAs encoding Ca_{3.1}, Ca_{3.2}, and chimeric channels were linearized by AflIII and used as templates. Capped cRNAs were synthesized in vitro using T7 RNA polymerase provided in the mMessage mMachine transcription kit in accordance to the manufacturer’s instruction (Ambion, Austin, TX). The cRNAs were injected into oocytes at concentrations of 10–50 ng/50 nl using a Drummond Nanoject pipette injector (Parkway, PA) attached to a Narishige micromanipulator (Tokyo, Japan). SOS solution was changed daily.

Electrophysiology and Data Analysis—Barium currents were measured using a two-microelectrode voltage clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) between the third and eighth day after cRNA injection. Microelectrodes (Warner Instruments) were broken to decrease the electrode resistance to 0.2–1.0 megohms and filled with 3 M KCl. The bath solution contained 10 mM Ba(OH)_{2}, 90 mM NaOH, 1 mM KOH, and 5 mM HEPES (pH 7.4 with methanesulfonic acid). The currents were acquired at 1 kHz and low pass-filtered at 1 kHz using the pClamp system (Digidata 1320A and pClamp 8, Axon Instruments). Data were analyzed using the Clampfit software (Axon Instruments) and presented graphically using the Prism software (GraphPad, San Diego, CA). Dose-response curves were fitted using the Hill equation:

\[
\text{Dose-response curve} = \frac{\text{max} \times \text{concentration}}{\text{IC}_{50} + \text{concentration}}
\]

where max is the maximum response, concentration is the concentration of nickel on wild-type Cav3.1 and Cav3.2 channels. Peak Ba^{2+} currents through the T-type Ca^{2+} channel were detected as having half-maximal inhibition, and n is the Hill coefficient. Data are presented as means ± S.E. and tested for significance using Student’s t-test.

RESULTS

Prior to testing the chimeric channels, we first confirmed the effects of nickel on wild-type Ca_{3.1} and Ca_{3.2} channels. Peak Ba^{2+} currents were elicited by test pulses to 20 mV from a holding potential of –90 mV every 15 s. Expression of the T-type Ca^{2+} channel was detected as robust inward currents from the third day after cRNA injection. Application of serial nickel solutions inhibited Ba^{2+} currents through the Ca_{3.1} or Ca_{3.2} channels in a dose-dependent manner, and the inhibited currents could be reversed by washing (Fig 1, A and B). The Ca_{3.1} currents required high concentrations of nickel to be blocked. In contrast, the Ca_{3.2} currents were highly sensitive to nickel block. On aver-
FIGURE 1. Nickel-blocking profiles of the GGGG (Ca<sub>v</sub>3.1) and HHHH (Ca<sub>v</sub>3.2) channels. Currents were evoked by a test potential to −20 mV from a holding potential of −90 mV every 15 s. A and B, time courses of nickel inhibition of Ca<sub>v</sub>3.1 (GGGG) and Ca<sub>v</sub>3.2 (HHHH) currents, respectively. Their representative currents before and after application of serial doses of nickel were shown in each inset. C and D, cumulative dose-response curves of nickel inhibition on Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2. Currents were normalized to the peak current in the absence of nickel, and the normalized percent block was plotted against nickel concentrations. The smooth curves were obtained from fitting the average data with the Hill equation. The estimated IC<sub>50</sub> values of Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 were 304.8 ± 6.2 μM (Hill coefficient, 0.92 ± 0.08) and 4.9 ± 2.0 μM (Hill coefficient, 0.8 ± 0.07), respectively. E and F, current-voltage (I-V) relationships of Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 currents before (●) and after (○) nickel treatment (300 μM for Ca<sub>v</sub>3.1; 5 μM for Ca<sub>v</sub>3.2). Peak currents measured at various test potentials were normalized to the maximum peak current, and their averaged values are plotted against potentials. All data are presented as mean ± S.E. (n = 5–6).
Structural Determinant of Cav3.2 for Nickel-sensitive Block

The IC₅₀ values were obtained from fitting data with the Hill equation. Time constants of inactivation (τᵢ) before and after nickel block were obtained from exponential fits to the current traces measured at a test potential of -20 mV before and after nickel treatment. The Hill equation was used to fit the data, and the Hill coefficients (n) were obtained. The Hill coefficients (n) were found to be positively shifted by nickel (Table 1).

Next, we postulated that nickel may interact with regions preceding the S4 domain, such as with residue(s) in the extracellular loops between IS1 and IS4, to IS4, such as with residue(s) in the extracellular loops between IS1 and IS4. The HGGG current was found to be blocked by low concentrations of nickel. The IC₅₀ values for blocking HGGG and HGGG were 4.7 ± 1.8 μM (n = 5) and 291.1 ± 52 μM (n = 6), respectively. Taken together, the potency of nickel block for the HGGG was very close to that of the wild-type Cav3.2, indicating that the domain I of the Cav3.2 contains the essential structural element(s) determining the nickel-sensitive block.

The IC₅₀ values for inhibiting the Cav3.1 and Cav3.2 channels were 304.8 ± 6.2 and 4.9 ± 2.0 μM, respectively (Fig. 1, C and D), being consistent with previous studies (19). Comparison of current-voltage (I-V) relationships of Cav3.1 or Cav3.2 currents before and after nickel treatment showed that nickel inhibition positively shifted the I-V relationships (Fig. 1, E and F). Consistently, the half-activation potentials were found to be positively shifted by nickel (Table 1).

Based on the different potencies of nickel block between the Cav3.1 (GGGG) and Cav3.2 (HHHH), we investigated what structural portion(s) endowed Cav3.2 with nickel sensitivity. In this regard, sensitivities of nickel block were examined for a series of chimeric channels (Fig. 2). Of the two half-chimeras, the GGHG currents were blocked by relatively high concentrations of nickel. On average, the IC₅₀ value of GGHG was 307.3 ± 8.1 μM (n = 6), similar to that for Cav3.1 (GGGG). On the contrary, the HHHG currents were sensitively blocked by low concentrations of nickel. On average, the IC₅₀ value was 7.3 ± 2.2 μM (n = 5), similar to that for the Cav3.2. These findings suggested that the structural element(s) contributing to high nickel sensitivity were located in the first half (domain I and II) but not on the second half (domain III and IV) of the Cav3.2.

Our next step was to transfer a single domain of Cav3.2 into Cav3.1. The HGGG currents were found to be blocked by low concentrations to nickel. In contrast, the GHHG currents required much higher concentrations of nickel to be blocked. The IC₅₀ values for blocking HGGG and GHGG were 4.7 ± 1.8 μM (n = 5) and 291.1 ± 52 μM (n = 6), respectively. Taken together, the potency of nickel block for the HGGG was very close to that of the wild-type Cav3.2, indicating that the domain I of the Cav3.2 contains the essential structural element(s) determining the nickel-sensitive block.

The identified domain I of the Cav3.2 was further dissected to narrow down the exact region(s) contributing to the nickel block. We initially hypothesized that the pore loop and S6 of the domain I, known to be essential for ion permeation and selectivity, are involved in the nickel block. However, it is unlikely that these structural portions contribute to the high nickel sensitivity, because the Cav3.1 and Cav3.2 contain identical amino acid sequences in these regions. Our next hypothesis was that the extracellular loop connecting the S5 and the pore is involved in the nickel block, because the extracellular loop sequences are quite different between the two T-type channels. However, the extracellular loop mutant channel, HGGG/G55pore, (where the IS5-pore loop of HGGG was replaced with the corresponding one of the Cav3.3) was still sensitive to nickel (IC₅₀ = 4.7 ± 1.9 μM, n = 5). These results restricted the nickel interacting site(s) within the remaining region from the amino terminus to S4 (IS4) of domain I of the Cav3.2. To examine the relevance of this region, GGGG/Hₙ-IS₅₄ was constructed. As expected, the chimeric channel currents were blocked by low concentrations of nickel (IC₅₀ = 3.9 ± 2.1 μM, n = 9), for the GGGG/Hₙ-IS₅₄, which was slightly lower than that for the wild-type Cav3.2. These findings indicate that essential structural determinant(s) for the nickel-sensitive block reside between the amino terminus and IS4.

Next, we postulated that nickel may interact with regions preceding to IS4, such as with residue(s) in the extracellular loops between IS1 and IS2, and/or IS3 and IS4. To identify putative nickel-interacting residue(s), we aligned the amino acid sequences in the regions prior to IS4 of the three T-type channel isoforms (Fig. 3A). Ni²⁺ can interact with histidine (H) and cysteine (C) residues and the acidic amino acids, aspartic acid (D) and glutamic acid (E) (23–26). Involvement of Glu-127 and Glu-131 in the IS1-IS2 loop of the Cav3.2 seems unlikely because aspartate (D), a negatively charged amino acid similar to glutamate (E), is found in the corresponding position of the nickel-insensitive Cav3.3.

Table 1

| Construct          | IC₅₀ (μM) | Vᵢ (mV) | Slope |
|--------------------|----------|---------|-------|
| HGGG               | 304.8 ± 6.2 | 0.99 ± 0.08 | 25.9 ± 0.04 |
| GHHG               | 304.8 ± 6.2 | 0.99 ± 0.08 | 25.9 ± 0.04 |
| GGHG               | 304.8 ± 6.2 | 0.99 ± 0.08 | 25.9 ± 0.04 |
| GGGG               | 304.8 ± 6.2 | 0.99 ± 0.08 | 25.9 ± 0.04 |
| GGGG/Hₙ-IS₅₄      | 304.8 ± 6.2 | 0.99 ± 0.08 | 25.9 ± 0.04 |

*P < 0.05, **P < 0.01.
FIGURE 2. Nickel-blocking profiles of chimeras derived from Cav3.1 and Cav3.2. Left, schematic diagrams of constructed chimeras with GGGG (Cav3.1) and HHHH (Cav3.2) were linearly represented. The transmembrane segments and connected loops of the T-type channels were displayed with cylinders and lines, respectively. White cylinders and thin lines indicate the regions from the Cav3.1, whereas gray cylinders and thick lines represent the regions from Cav3.2. Right, I_{50} values of chimeric channels were shown with bar graphs. These values were obtained from dose-response curves fitted to the data with the Hill equation. GGHH and GHGG channels were blocked by high concentrations of nickel (I_{50} = 307.3 ± 8.1 and 291.1 ± 5.2 μM, respectively). Their nickel-blocking sensitivities were similar to that of Cav3.1. In contrast, HHGG and HGGG channels required lower concentrations of nickel to be blocked (I_{50} = 7.3 ± 2.2 and 4.7 ± 1.8 μM, respectively). These findings suggested that the structural portion contributing to the nickel-sensitive block was located in domain I of the Cav3.2. Further dissected chimeras (HGGG/GIS5-ipore and GGGG/HN-IS4) were also sensitively blocked by low concentration of nickel (I_{50} = 4.7 ± 1.9 and 3.9 ± 2.1 μM, respectively), which was similar to that of Cav3.2. All data are presented as mean ± S.E. (n = 5–9).

FIGURE 3. Nickel-blocking profiles of the HGGG/E137Q and HGGG/H191Q channels. A, the amino acid sequences between S1 and S5 of domain I of the Cav3.1, Cav3.2, and Cav3.3 channels are aligned. The putative membrane-spanning segments are marked with horizontal bars above the sequence, and conserved residues among the three isoforms are highlighted in gray. The putative nickel-interacting residues are displayed with bold letters and amino acid numbers are based on the Cav3.2 sequence. B and C, single point mutations (E137Q or H191Q) were introduced into the HGGG channel, and their expressed currents were evoked by the same protocol. Representative current traces of HGGG/E137Q (B) and HGGG/H191Q (C) before and after application of serial concentrations of nickel were superimposed. D, the dose-response curves of nickel block on HGGG/E137Q (○) and HGGG/H191Q (●). Data represent the average blocking percentages. The smooth curves represent the fits to the data with the Hill equation. The IC_{50} values of HGGG/E137Q and HGGG/H191Q were 5.1 ± 1.2 μM (Hill coefficient, 1.01 ± 0.02) and 312.5 ± 4.2 μM (Hill coefficient, 0.90 ± 0.20), respectively. All data are presented as mean ± S.E. (n = 8–10).
FIGURE 4. Nickel blocking profiles of the Cav3.2/H191Q and Cav3.1/Q172H channels. Currents were elicited by the same protocol used in Fig. 1. A and D, time courses of nickel inhibition of Cav3.2/H191Q and Cav3.1/Q172H currents, respectively. Their representative currents before and after application of various doses of nickel were shown in each inset. B and E, the dose-response curves of nickel inhibiting on Cav3.2/H191Q (○), Cav3.2/H191A (●), and Cav3.1/Q172H (△). Data represent the average inhibition percentages. The smooth curves represent the fit to the data using the Hill equation. The IC50 values of Cav3.2/H191Q, Cav3.2/H191A, and Cav3.1/Q172H were 306.6 ± 7.1 (Hill coefficient, 0.96 ± 0.05), 285.9 ± 3.1 (Hill coefficient, 0.90 ± 0.10), and 61.3 ± 3.7 μM (Hill coefficient, 0.85 ± 0.12), respectively. C and F, current-voltage (I-V) relationships of Cav3.2/H191Q and Cav3.1/Q172H before (open symbols) and after (closed symbols) nickel treatment (300 μM for Cav3.2/H191Q; 60 μM for Cav3.1/Q172H). Peak currents at various test potentials were normalized to the maximum peak current, and their averaged values are plotted against potentials. All data are presented as mean ± S.E. (n = 6–9).
(IC_{50} = 87 \text{ M} for the nickel block; 19), Glu-137 in the IS1–IS2 loop and His-191 in the IS3–IS4 loop are found only in Ca_{3.2} channels. Therefore, Glu-137 and His-191 of HGGG were individually point-mutated into glutamine (Q), which is found in the corresponding positions of Ca_{3.1}. HGGG/E137Q currents were blocked by low concentrations of nickel. The IC_{50} value for the nickel block was 5.1 ± 1.2 \text{ M} (n = 8), similar to that of HGGG, suggesting that Glu-137 in the IS1–IS2 loop is not a crucial residue determining nickel block (Fig. 3, B and D). In contrast, HGGG/H191Q currents required much higher concentrations of nickel to be blocked, showing an IC_{50} of 312.5 ± 4.2 \text{ M} (n = 10). These findings show that the single point mutation of H191Q induced a 25-fold change in nickel sensitivity and suggest that His-191 accounts for the high nickel sensitivity observed with the HGGG chimera.

We next sought to confirm the critical role of His-191 by mutating this residue in wild-type T-type channels. The application of nickel solutions dose-dependently inhibited Ca_{3.2}/H191Q (HHHH/H191Q), and the inhibited currents could be reversed by washing (Fig. 4A). On average, the IC_{50} for the nickel block was 306.6 ± 7.1 \text{ M} (n = 6), indicating that the H191Q mutation greatly reduced the nickel sensitivity of the channel. Another point mutation of H191Q currents in the absence and presence of 300 \text{ M} nickel showed that nickel shifted the I-V relationship to more depolarized potentials (Fig. 4C, Table 1). These results support our findings in the chimeric channels and show that His-191 in the IS3–IS4 loop confers the high nickel sensitivity to the Ca_{3.2}.

Finally, we examined whether Ca_{3.1} could be transformed into a nickel-sensitive channel by simply switching the corresponding glutamine (Q) of the Ca_{3.1} to histidine (H). Accordingly, Ca_{3.1}/Q172H (GGGG/Q172H) was constructed, and its nickel sensitivity was assayed. Ca_{3.1}/Q172H currents were inhibited by nickel solutions in a dose-dependent manner, and the inhibited currents were rapidly recovered by washing (Fig. 4D). Consistent with our hypothesis, the nickel blocking sensitivity of GGGG/Q172H was increased 5-fold (IC_{50} = 61.3 ± 3.7 \text{ M}, n = 9), although it was not as sensitive as that of the Ca_{3.2} (Fig. 4, D and E). These results clearly show that His-191 is a key residue in the nickel binding pocket (Fig. 5). Alternatively, mutation of His-191 might have led to a rearrangement of the channel that disrupted the nickel binding pocket. Data arguing against this latter hypothesis are that the biophysical (Table 1) and pharmacological properties (mibefradil dose-response studies, results not shown) were not altered by H191Q mutation.

Voltage-gated ion channels contain many conserved amino acids and are likely to be similar in structure-function. Therefore we have modeled repeat I of Ca_{3.2} using current models for the Shaker K^+ channel (27). Voltage-dependent gating is thought to begin with outward movement of S4 segments, which in turn leads to opening of the channel walls formed by S6 segments. In these models the S3–S4 linker is in close proximity to the extracellular face of S4, S5, and S6 segments; therefore it is likely that there is a nickel binding pocket on the extracellular surface of Ca_{3.2} channels. If so, then nickel should be able to bind to closed channels in the rested state and block their transition to open states. To test this prediction we expressed oocytes expressing Ca_{3.2} channels to nickel at a holding potential of −100 mV, then tested for channel availability (Fig. 6). Nickel evoked the same degree of block in the absence and presence of depolarizing test pulses, indicating that it could bind to closed channels.

**DISCUSSION**

Voltage-gated calcium channels are highly selective for Ca^{2+} ions because they bind Ca^{2+} in the pore, thereby preventing permeation by monovalent cations. This binding site also binds other divalent cations such as Cd^{2+} with higher affinity, leading to the block of Ca^{2+} permeation. This binding site was localized to the pore loops by site-directed mutagenesis (10) and in HVA channels is formed by glutamates (E) in each of the four repeats (EEEΕ locus). The mutation of these residues in Ca_{1.2} channels significantly decreased the affinity for cadmium binding (28). In low voltage-activated channels two of these glutamates were replaced by aspartates (D), creating an EEDΕ locus. Replacement of these aspartates in Ca_{3.1} channels with glutamates confers a cadmium-sensitive block to Ca_{3.1}, which requires much higher concentrations of cadmium to be blocked than HVA calcium channels (29). Our previous studies indicated that nickel blocks Ca_{3} channels in part by binding within the permeation path (19), presumably because of binding at the
molecular determinant contributing to the high affinity block of Ca₃.2 channels by nickel. This residue was localized using a series of chimeras between channels that show high affinity block (Ca₃.2) and low affinity block (Ca₃.1). Interestingly His-191 resides in the short (9-amino-acid long) loop that connects IS3 to the IS4 voltage sensor, rather than residing in the pore loops as might be expected from work on cadmium binding sites. Based on observations that nickel appears to block Ca₃ channels at two sites (11, 19), we propose that nickel binding to His-191 blocks the gating of channels to the open state by interrupting the coupling between S4 and the pore.

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