Molecular Basis for Zinc Potentiation at Strychnine-sensitive Glycine Receptors

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The divalent cation Zn2+ is a potent potentiator at the strychnine-sensitive glycine receptor (GlyR). This occurs at nanomolar concentrations, which are the predicted endogenous levels of extra-cellular neuronal Zn2+. Using structural modeling and functional mutagenesis, we have identified the molecular basis for the elusive Zn2+ potentiation site on GlyRs and account for the differential sensitivity of GlyR α1 and GlyR α2 to Zn2+ potentiation. In addition, juxtaposed to this Zn2+ site, which is located externally on the N-terminal domain of the α subunit, another residue was identified in the nearby Cys loop, a region that is critical for receptor gating in all Cys loop ligand-gated ion channels. This residue acted as a key control element in the allosteric transduction pathway for Zn2+ potentiation, enabling either potentiation or overt inhibition of receptor activation depending upon the moiety resident at this location. Overall, we propose that Zn2+ binds to a site on the extracellular outer face of the GlyR α subunit and exerts its positive allosteric effect via an interaction with the Cys loop to increase the efficacy of glycine receptor gating.

The glycine receptor is a major component of inhibitory neurotransmission in the spinal cord and brainstem. It forms part of the Cys loop receptor family, which includes acetylcholine, γ-aminobutyric acid type A (GABA_A)3 and serotonin type 3 receptors (2). Glycine receptors are pentameric assemblies of ligand binding N-terminal domain followed by four transmembrane (TM) segments and transduction pathways remain controversial.

However, Zn2+ binding sites do provide realistic targets for identification, as these are traditionally compact, consisting of only 3–4 residues, and their coordination chemistry is understood (9). With regard to GlyRs, Zn2+ exhibits biphasic activity, potentiating receptor activation at submicromolar Zn2+ concentrations and causing inhibition at concentrations >10 μM (8). A previous study demonstrates that the mutation D80A in the extracellular domain of the GlyR α1 subunit ablated Zn2+ potentiation, and it is proposed that this residue participates in the direct coordination of Zn2+ (10, 11). However, an additional report indicates that the Zn2+ potentiation of responses to the partial agonist taurine, which binds to the same agonist site as glycine, is unaffected by mutating Asp-80. This suggests that either multiple Zn2+ binding sites exist or that this mutation induces an indirect allosteric effect on receptor function that selectively disrupts Zn2+ potentiation of responses to glycine rather than those to taurine (12). In accord with an indirect allosteric effect, mutations of several other residues in the TM2-TM3 linker are capable of disrupting Zn2+ potentiation, though none of these residues are chemically suitable for the direct coordination of Zn2+.

The high sensitivity of the strychnine-sensitive GlyR to Zn2+ potentiation makes this receptor an ideal substrate for modulation by basal levels of Zn2+. In a physiological context, Zn2+ is released following neuronal stimulation (13, 14) and can also modulate inhibitory neurotransmitter receptors at basal concentrations (15, 16). Furthermore, Zn2+ is concentrated into synaptic boutons that also contain either glutamate, GABA, or glycine in many areas of the brain, including the cortex, hippocampus, and spinal cord (17–19). Although the predicted concentration of Zn2+ resulting from presynaptic release is probably <10 μM (20), this is more than sufficient to modulate N-methyl-D-aspartate receptors, certain GABA_A receptor subtypes, and GlyRs (8, 21). Indeed, low nanomolar basal Zn2+ concentrations are adequate to prolong the decay phase of glycineric inhibitory postsynaptic currents (22).

In this study, we accounted for the differential sensitivity to Zn2+ potentiation of GlyR α1 and GlyR α2 by identifying the location of a single conserved residue in the N-terminal domain. Subsequently, by using structural homology modeling together with the identified residue underlyng Zn2+ sensitivity, we established the molecular determinants for the elusive Zn2+ potentiation binding site. In doing so, we uncovered a prospective transduction residue for this site that is located in the Cys loop-gating domain, providing a plausible molecular pathway for Zn2+ potentiation of glycine receptor gating.

MATERIALS AND METHODS

cDNA Constructs—Human (h) wild-type cDNA constructs were used for hGlyR α1L, hGlyR α2V and hGlyR β. Site-specific mutant cDNAs were prepared using the Stratagene QuickChange mutagenesis kit. The mutated sequences were confirmed by complete sequencing of the cDNA insert using an ABI sequencer.

Cell Culture and Transfection—Human embryonic kidney (HEK) cells (American Type Culture Collection CRL1573) were grown and transfected as previously documented (23). Plasmids of hGlyR cDNA clones were co-transfected in a ratio of 1:1 with enhanced green fluorescent protein (24). To co-express GlyR αβ heteromers, β subunit cDNA was added in excess at a ratio of 20:1 to a subunit cDNA. HEK cells were plated onto poly-l-lysine-coated coverslips (100 μg/ml) sufficient to achieve 20% confluence and used for recording on the next day.
Solutions—The internal pipette solution contained (mM): 140 KCl, 2 MgCl$_2$, 1 CaCl$_2$, 10 HEPES, 11 EGTA, and 2 ATP, pH 7.2 (~300 mosM). The external Krebs solution consisted of (mM): 140 NaCl, 4.7 KCl, 1.2 MgCl$_2$, 25 CaCl$_2$, 10 HEPES, and 11 d-glucose, pH 7.4 (~300 mosM). For those experiments requiring control over the basal levels of Zn$^{2+}$ (see Fig. 1), tricine was used with an assumed $K_d$ for Zn$^{2+}$ complexation of $10^{-7}$ M (25). Thus, 0.26, 0.78, 2.6, 7.8, 26, 77.5, 254, and 775 $\mu$M Zn$^{2+}$ provided effective free Zn$^{2+}$ concentrations of 0.8, 2.68, 8, 26.8, 80, 268, 2680, and 8620 nM, respectively (calculated with WINMAXC software, version 2.4). The cysteine accessibility experiments required the incubation of 2-aminophenylmethanesulphonate (MTSEA), prepared in Krebs-Ringer solution immediately before application, with HEK cells for 1 min at a concentration of 3 mM (Insight Biotechnology, Ltd).

Electrophysiology—An Axopatch 200B amplifier (Axon Instruments) recorded whole-cell currents from single HEK cells using the patch clamp technique. HEK cells exhibited resting potentials between 40 and 40 mV and were voltage-clamped at a –40 mV holding potential. The cells were visualized with differential interference contrast optics using a Nikon Optiphot microscope with an epifluorescence attachment to identify green fluorescent protein-transfected cells. A Y-tube was used to rapidly apply drugs and Krebs solutions (exchange rate 50–100 ms) to the recorded cells. Patch electrodes were fabricated using a Narashige PC-10 puller with resistances, after polishing, of 4–5 megohms. All recordings were performed in constantly perfusing Krebs-Ringer solution at room temperature (20–22°C).

Data Acquisition and Analysis—Recorded currents were filtered using a high pass Bessel filter at 3 kHz (–36 db/octave), and series resistance compensation was achieved up to 70%. Data were recorded in 20-s acquisition epochs directly to a Pentium IV, 1.8 GHz computer using Clampex software, version 8.0, via a Digidata 1322A (Axon Instruments) sampling at 200-µs intervals. Zn$^{2+}$ was co-applied with the agonist to attenuate any delayed onset of Zn$^{2+}$-mediated inhibition. Strychnine and picrotoxin were pre-incubated for 15 s, sufficient to attain equilibrium, and then co-applied with the agonist. The digitized membrane current records were analyzed off-line using Axoscope, version 8.2. Biphasic (potentiation and inhibition) Zn$^{2+}$ concentration response curves were fitted according to a modified Hill equation as previously described (26). Where a single component to the concentration response relationships was evident, it was fitted with a form of the Hill equation. For the agonist and Zn$^{2+}$-potentiating concentration potentiation curves, $I = I_{\text{max}} + (I_{\text{max}} - I_{\text{min}})((1/(1 + (EC_{50}/A)^{m}))$, and for the strychnine and Zn$^{2+}$ concentration-inhibition curves, $I/\text{max} = 1 - ([1/(1 + (IC_{50}/B)^{n})]$).

The EC$_{50}$ represents either the concentration of agonist-inducing or Zn$^{2+}$-potentiating (A) 50% of the maximal current ($I_{\text{max}}$) evoked or potentiated by a saturating concentration of agonist or Zn$^{2+}$, and $n$ is the Hill coefficient. For Zn$^{2+}$ potentiation, $I_{\text{max}}$ represents the control glycine current in the absence of Zn$^{2+}$ and was set to 100%. For inhibition, the IC$_{50}$ defines the antagonist concentration (B) producing a 50% inhibition of the current, and $m$ is the Hill coefficient. When Zn$^{2+}$ induced a biphasic inhibition, the concentration response data were fitted with a two-component inhibitory curve using $I/\text{max} = 1 - (aB^{m+}(B^{m+} + IC_{50})) + (b(B^{m+})(B^{m+} + IC_{50}))$, where $a$ and $b$ represent the relative proportions of each inhibitory component. All statistical comparisons used an unpaired t test.

Structural Homology Modeling—The mature N-terminal extracellular domain of the hGlyR $\alpha_1$ subunit was modeled on the crystal structure of the acetylcholine-binding protein (27) using SwissProt DeepView, version 3.7, in accordance with a ClustalW protein alignment. All three-dimensional images were subsequently rendered using the freeware program POV-Ray.

RESULTS

GlyR $\alpha_1$ and $\alpha_2$ Exhibit Distinct Sensitivities to Zn$^{2+}$ Potentiation—The sensitivity of the GlyR $\alpha_1$ and $\alpha_2$ subtypes to Zn$^{2+}$ potentiation was assessed from whole-cell recordings of half-maximal (EC$_{50}$) responses to glycine obtained from transfected HEK cells maintained at –40 mV
TABLE ONE

| Glycine | Taurine | Zn²⁺ (on glycine) |
|---------|---------|-------------------|
|         | Max current | EC₅₀ | n | Iₘ₅ᵃ | n | EC₅₀ | Max increaseᵇ | IC₅₀ | n |
| α₁      | 4.5 ± 0.5    | 16 ± 2  | 5 | 90 ± 10 | ~100 | 5 | 0.8 ± 0.3 | 39 ± 8 | 20 ± 5 | 4 |
| R10       | 5.9 ± 0.3    | 25 ± 4  | 4 | 130 ± 30 | ~100 | 4 | 0.8 ± 0.2 | 42 ± 6 | >3000 | 4 |
| R10, H109Fa | 5.1 ± 0.2    | 28 ± 5  | 3 | ND | ND | 1.8 ± 0.5 | 55 ± 4 | >10000 | 6 |
| R10, E192A | 3.6 ± 0.7    | 19 ± 4  | 3 | 190 ± 60 | ~100 | 3 | None | None | 710 ± 100 | 7 |
| R10, D194A | 4.6 ± 0.4    | 48 ± 4  | 3 | 310 ± 70 | ~100 | 3 | None | None | 270 ± 50 | 3 |
| R10, H215A | 3.7 ± 0.2    | 21 ± 3  | 3 | 100 ± 30 | ~100 | 3 | 22 ± 4 | 28 ± 12 | 1320 ± 210 | 4 |

Acidic non-binding residues

R10, D141A | 2.9 ± 1.1    | 46 ± 7  | 2 | 240 ± 80 | ~100 | 2 | 0.5 ± 0.2 | 41 ± 21 | >3000 | 3 |
| R10, D148A | <0.04       | ND | 9 | ND | ND | ND | ND | ND | ND |
| R10, E191A | 4.8 ± 0.6    | 22 ± 5  | 3 | 190 ± 40 | ~100 | 3 | 0.8 ± 0.4 | 80 ± 50 | >3000 | 3 |

Residues around Zn²⁺ binding site

R10, K190A | 4.5 ± 0.3    | 9 ± 2   | 3 | 80 ± 20 | ~100 | 3 | 0.9 ± 0.7 | 29 ± 6 | ND | 3 |
| R10, R196A | 5.0 ± 0.5    | 27 ± 6  | 4 | 150 ± 50 | ~100 | 4 | 0.6 ± 0.1 | 34 ± 7 | ND | 4 |
| R10, R213A | 5.3 ± 0.2    | 9 ± 7   | 3 | 60 ± 10 | ~100 | 3 | 0.5 ± 0.3 | 58 ± 30 | ND | 3 |
| R10, E217A | 4.2 ± 0.8    | 21 ± 5  | 3 | 120 ± 50 | ~100 | 3 | 0.9 ± 0.3 | 37 ± 3 | ND | 4 |

α The maximal inducible current by taurine as a percentage of the maximal glycine-evoked current in the same cell.

ᵇ The maximum percentage potentiation by Zn²⁺ of a 50% maximal (EC₅₀) agonist-induced response.

This mutation did cause some disruption to other receptor properties, such as a decrease in the rate of activation (unpublished observations). For this reason it was not used as a general background mutant, in preference to H107N, to disrupt inhibition in this study.

Dose response data for glycine and taurine activation and Zn²⁺ modulation of wild-type and mutant GlyRs

Data represent the mean ± S.E. for n number of experiments. ND, not determined.

Zn²⁺ Potentiation and Glycine Receptors

Table 1 shows the dose response data for glycine and taurine activation and Zn²⁺ modulation of wild-type and mutant GlyRs. The table includes columns for Max current, EC₅₀, n, Iₘ₅, EC₅₀, Max increase, and IC₅₀. The data represent the mean ± S.E. for n number of experiments. ND, not determined.

- **Glycine**: Max current values range from 4.5 ± 0.5 to 4.2 ± 0.8, with EC₅₀ values ranging from 16 ± 2 to 21 ± 5. The n values range from 3 to 5.
- **Taurine**: Max current values range from 5.9 ± 0.3 to 5.0 ± 0.5, with EC₅₀ values ranging from 25 ± 4 to 27 ± 6. The n values range from 3 to 4.
- **Zn²⁺ (on glycine)**: Max current values range from 0.8 ± 0.3 to 0.9 ± 0.3, with EC₅₀ values ranging from 39 ± 8 to 37 ± 3. The n values range from 4 to 3.

The table also includes acidic non-binding residues and residues around Zn²⁺ binding site. The mutations and their effects on Zn²⁺ potentiation are also presented.

To assess the significance of prospective binding site residues for Zn²⁺ potentiation, the data were compared to previous studies. The results showed that mutating α₁, Asp-194 to alanine, a residue incapable of coordinating Zn²⁺. However, because of the biphasic nature of Zn²⁺ action at the GlyR, where Zn²⁺ potentiates at nanomolar to low micromolar concentrations and inhibits at doses >10 μM, any attempt to measure a reduced sensitivity to Zn²⁺ potentiation might be occluded by the onset of Zn²⁺ mediated inhibition. To obviate this problem, all experiments to identify the Zn²⁺ potentiation binding site were performed using an H107N “background” mutation (hereafter referred to as “reduced inhibition” (R1)). This mutation dramatically attenuated the GlyR sensitivity to Zn²⁺ inhibition, increasing the Zn²⁺ IC₅₀ from 15 μM to >3 mM, without affecting other macroscopic properties of the receptor, particularly the sensitivity of the receptor to Zn²⁺ potentiation (see below paragraph and Table ONE).

To investigate the high potency Zn²⁺ potentiation phenomenon on the background of a low sensitivity Zn²⁺ inhibition component, it was necessary to fully characterize the modulatory curves over a wide concentration range from 0.01 μM to 3 mM. As tricine can only effectively buffer Zn²⁺ concentrations below 1 μM, it was not included in these comparisons. In accord with previous studies (10, 22), the apparent sensitivities to potentiating Zn²⁺ of the wild-type receptor and R10 were lower in the absence of tricine because of the competing background Zn²⁺ present in the external solution, although the relative EC₅₀ values remained indistinguishable between the two receptors, (Zn²⁺ EC₅₀ values: α₁ wild-type, 0.8 ± 0.3 μM; R1α₁, 0.8 ± 0.2 μM; n = 4; p > 0.05) (TABLE ONE).

To assess the significance of prospective binding site residues for Zn²⁺ potentiation, the consequences of their replacement were compared for two GlyR agonists, glycine and taurine. This is necessary, as previously identified residues, which have been postulated to be part of a potentiating Zn²⁺ binding site, ablated enhancement of responses to one agonist but not the other presumably due to indirect affects on downstream transduction mechanisms (12). This emphasized the potential importance of Asp-194, because the Zn²⁺ potentiation of both glycine and taurine activation was abolished by mutation to alanine.
Zn\(^{2+}\) Potentiation and Glycine Receptors

FIGURE 2. GlyR \(\alpha_1\) subunit residues that affect Zn\(^{2+}\) potentiation. Zn\(^{2+}\) concentration-response curves for the modulation of EC\(_{50}\) responses to glycine (A) and taurine (B) constructed for mutant homomeric GlyRs RI\(\alpha_1\), RI\(\alpha_1\), E192A, RI\(\alpha_1\), H215A, and one heteromeric GlyR, RI\(\alpha_1\), E192A. All experiments were performed on a background mutant receptor, GlyR \(\alpha_1\), H1107N, that exhibited a reduced inhibition (RI) to Zn\(^{2+}\). Note the absence of any Zn\(^{2+}\) potentiation for most of the mutant receptors, apart from RI\(\alpha_1\), and RI\(\alpha_1\), H215A. The insets show typical glycine and taurine (EC\(_{50}\)-activated) currents in the absence (continuous line) and presence of 10 \(\mu\)M Zn\(^{2+}\) (dotted line). C, color-coded amino acid motifs identified in the N-terminal domain of the GlyR \(\alpha_1\) subunit and homologous GlyRs and \(\beta\) subunits that reside in close proximity to the previously identified \(\alpha_1\), D194A. The single extracellular domain in the GlyR structural model illustrates the side chains of three putative Zn\(^{2+}\) binding residues. The \(\alpha\)-helical section at the start of the mature protein is shown in pink. The inset is a plan view of the GlyR pentamer (red circles represent the extracellular subunit N-terminal domains), and the arrow denotes the viewing angle. The protein alignments show potential Zn\(^{2+}\) binding residues in bold in each motif, whereas important residues for Zn\(^{2+}\) potentiation are on color-coded backgrounds. A divergent \(\alpha\) subunit residue in the Cys loop is also highlighted in gray.

To elucidate which residues were capable of interacting with GlyR \(\alpha_1\) and taurine-activated responses was ablated in the GlyR RI\(\alpha_1\), E192A mutant (Fig. 2A and B). The EC\(_{50}\) for Zn\(^{2+}\) potentiation of glycine responses was increased from 0.8 \pm 0.2 \(\mu\)M for RI\(\alpha_1\) to 22 \pm 4 \(\mu\)M for RI\(\alpha_1\), H215A \((n = 4; p < 0.05)\). This reduction in Zn\(^{2+}\) sensitivity was directly comparable with that observed when the GlyR was activated by taurine (EC\(_{50}\) 0.9 \pm 0.3 to 21 \pm 4 \(\mu\)M, respectively; \(n = 4; p < 0.05\)). As previously, removing or reducing Zn\(^{2+}\) potentiation increased the apparent sensitivity to Zn\(^{2+}\) inhibition for both of these mutants. Moreover, substituting other acidic candidate residues for alanines (highlighted in Fig. 2C) had no effect on the potency of Zn\(^{2+}\) potentiation. For all three RI mutants \(\alpha_1\), E192A, \(\alpha_1\), D194A, and \(\alpha_1\), H215A, the maximal responses evoked by the agonists glycine and taurine were indistinguishable from the wild-type receptor, and only RI\(\alpha_1\), D194A exerted a modest 3-fold increase in the agonist EC\(_{50}\) values (TABLE ONE), suggesting that these mutations selectively affected the Zn\(^{2+}\) potentiation binding site and did not exert a general perturbation on GlyR function. Most importantly, the GlyR homology model (Fig. 2C) predicts that Glu-192, Asp-194, and His-215 reside in close proximity to one another on the outside face of the N-terminal extracellular domain. Typically, functional groups involved in direct coordination of Zn\(^{2+}\) lie within 2–5 Å of the divalent ion (9), which is easily accommodated by the predicted distances between the three residues identified on the GlyR model (Fig. 2C). To highlight the localized specific role this domain plays in Zn\(^{2+}\) potentiation, scanning alanine mutagenesis was performed on other residues that lie immediately to either side of the putative Zn\(^{2+}\) binding site and that are also predicted to have externally oriented side chains. These mutated GlyRs (RI mutants \(\alpha_1\), K190A, \(\alpha_1\), R196A, \(\alpha_1\), R213A, and \(\alpha_1\), E217A) did not affect the glycine, taurine, or Zn\(^{2+}\) EC\(_{50}\) values, the maximal Zn\(^{2+}\) potentiation, or the maximal glycine-activated current (supplemental Fig. 1). In addition, co-expression of a GlyR RI\(\alpha_1\), E192A with the \(\beta\) subunit did not recover Zn\(^{2+}\)-mediated potentiation, suggesting the \(\beta\) subunit is unable to compensate or provide a Zn\(^{2+}\) potentiation site of its own \((n = 3)\) (Fig. 2A).

Asymmetry of Function at the Putative Zn\(^{2+}\) Potentiation Binding Site—Demonstrating that this discrete domain is accessible to water is a vital requirement for any dynamic Zn\(^{2+}\) binding site and would strengthen the conclusion that the identified residues may act as direct coordinators of Zn\(^{2+}\). We determined this by individual cysteine substitutions of GlyR \(\alpha_1\), Gly-192, Asp-194, and His-215, which were then exposed to the cysteine-modifying reagent MTSEA. If MTSEA covalently binds to the potentiation site, it will replace a Zn\(^{2+}\)-coordinating Cys moiety with a positively charged amine group, which should attenuate the Zn\(^{2+}\) potentiation of glycine-activated currents. Pre-application of 3 mM MTSEA for 1 min to the control GlyR RI\(\alpha_1\), did not affect Zn\(^{2+}\) potentiation (Fig. 3A) or the glycine EC\(_{50}\) and glycine maximal responses (data not shown). Upon individual replacement of Gly-192, Asp-194, and His-215 with cysteine, GlyRs were generated that retained high sensitivities to Zn\(^{2+}\) potentiation, with EC\(_{50}\) values for Zn\(^{2+}\) within 10-fold of the wild-type receptor \((n = 4)\) (Fig. 3, B–D). This was not surprising, as cysteine is quite capable of coordinating Zn\(^{2+}\). However, following exposure to MTSEA, RI\(\alpha_1\), D194C and RI\(\alpha_1\), H215C were rendered unresponsive to Zn\(^{2+}\) potentiation, suggesting that the side chains of both of these residues are surface-exposed and important for Zn\(^{2+}\) potentiation. The sensitivity of RI\(\alpha_1\), E192C, however, was largely unaffected...
(n = 4) (Fig. 3, B–D). The binding of MTSEA alone was insufficient to induce potentiation of glycine-activated responses at Rlα1,D194C or Rlα1,H215C (Fig. 3, B and C). Surprisingly, MTSEA did increase glycine potency in the absence of Zn^{2+} for Rlα1,E192C, potentiating the glycine response by 61 ± 16% (n = 4, p < 0.05) (Fig. 3E). Thus, Glu-192 appears to be accessible to MTSEA, although this covalent modification did not affect the ability of Zn^{2+} to bind to the receptor. If the carboxyl side chain of Glu-192 was not directly coordinating Zn^{2+}, then perhaps substitution of this residue with alanine would perturb the β-strand backbone, which would either indirectly disrupt the Zn^{2+} potentiation site or disrupt a possible contribution of the polar peptide backbone at this locus for Zn^{2+} coordination. To determine the relevance of the backbone at Glu-192, this residue was mutated to proline (Rlα1,E192P), an amino acid associated with placing conformational restraints upon peptide backbones (31). Even though proline cannot coordinate Zn^{2+}, potentiation in this mutated receptor was retained, although at a 5-fold reduced sensitivity (0.8 ± 0.2 μM for Rlα1, and 4.2 ± 0.8 μM for Rlα1,E192P (n = 4, p < 0.05) (Fig. 3F). This suggested that, to retain Zn^{2+}-mediated potentiation, this region must retain a specific conformation of the backbone at Glu-192, which can be accommodated by the introduction of a proline but not by insertion of an alanine. Of course, without precise structural data, it is not possible to infer any specific details about the structural organization at this locus other than that the backbone is particularly sensitive to perturbation in a fashion that affects Zn^{2+} potentiation. As a control for this strategy, the mutant GlyR Rlα1,D194P ablated Zn^{2+}-mediated potentiation, an outcome that is expected if, indeed, the side chain moiety is contributing to Zn^{2+} coordination at this particular position (data not shown).

Although the current data most strongly support a role for GlyR α1 Asp-194 and His-215 in direct Zn^{2+} coordination, the GlyR Rlα1,H215A substitution attenuated the sensitivity to Zn^{2+}, whereas GlyR Rlα1,D194A entirely removed Zn^{2+} potentiation (Fig. 2, A and B). To examine the actual extent by which Zn^{2+}-mediated potentiation was affected by perturbation of Asp-194, it was necessary to further disrupt the competing inhibitory Zn^{2+} site such that inhibition does not occlude any remaining sensitivity to Zn^{2+} potentiation. A number of GlyRs were generated to ablate Zn^{2+}-mediated inhibition based on previously identified targets (10, 26, 29). Of these, the one that most effectively attenuated Zn^{2+}-mediated inhibition while still retaining mostly "normal" receptor function in terms of agonist specificity, sensitivity,
and maximal activation was the Rlα1,H109F receptor (TABLE ONE). On this new mutant background, we introduced the mutation D194K to prevent any Zn$^{2+}$ binding by replacing aspartate with a positively charged amine group. The resultant GlyR Rlα1,H109FD194K was unaffected by 0.1–100 μM Zn$^{2+}$ (n = 4), a concentration range over which the wild-type GlyR α1 underwent its full Zn$^{2+}$ modulatory profile. This demonstrated that the potentiating Zn$^{2+}$ site was effectively ablated up to 100 μM Zn$^{2+}$, a concentration that is 125-fold greater than the Zn$^{2+}$ EC$_{50}$ value of 0.8 ± 0.3 μM on the wild-type GlyR α1 (Fig. 4).

GlyR α1 Thr-151 Is a Critical Control Element for Zn$^{2+}$ Potentiation—Besides the classical Zn$^{2+}$ binding moieties of the potentiating site, we also identified a nearby polar residue at position 151 located in the Cys loop called L7 (27). Alanine substitution of threonine 151 generated an Rlα1,T151A GlyR that was insensitive to the potentiating effect of Zn$^{2+}$ (Fig. 5, A and D). Although the lack of potentiation was clear, there was also an unusual additional effect revealed in the form of a novel biphasic sensitivity to Zn$^{2+}$ inhibition, with high (IC$_{50}$ = 1.6 ± 0.6 μM, n = 5) and low potency components (IC$_{50}$ = 1040 ± 290 μM, n = 5). This biphasic inhibitory profile was also apparent when taurine was the agonist (IC$_{50}$ = 3.2 ± 0.8 μM and 2840 ± 820 μM, n = 5) (Fig. 5B). Intriguingly, the IC$_{50}$ value for the high sensitivity inhibitory component is directly comparable with the original Zn$^{2+}$ EC$_{50}$ value of 0.8 ± 0.2 μM for potentiation at Rlα1. Conceivably, the α1 T151A substitution may have converted the Zn$^{2+}$ potentiation site to a high sensitivity Zn$^{2+}$ inhibitory site. In support of this hypothesis, incorporating the mutation E192A (producing GlyR Rlα1,T151A,E192A), designed to disrupt the Zn$^{2+}$ potentiation site, mostly removed the high potency inhibitory component (Fig. 5A and TABLE TWO). Furthermore, the lower potency component was attributed to the previously identified Zn$^{2+}$ inhibitory site (26), because restoration of this site by reinserting His-107 (producing GlyR Rlα1,T151A) increased the relative sensitivity to inhibition (Fig. 5G and B). The effects of similar experiments, introducing D194A or H215A onto the Rlα1,T151A-mutated GlyR, were not possible, as these receptors (Rlα1,T151AD194A, n = 24; and Rlα1,T151AH215A, n = 16) were effectively non-functional with very low maximal currents (TABLE TWO).

To further characterize the role of Thr-151, this residue was mutated to two variants selected from the Cys loop receptor family, including an arginine from the GlyR β subunit, which previously appeared incapable of supporting Zn$^{2+}$ potentiation, and an Asn from the serotonin type 3A receptor, which displays a comparable sensitivity profile to the GlyR α subunit with regard to Zn$^{2+}$ potentiation (32, 33). These mutated GlyRs, Rlα1,T151R and Rlα1,T151N, failed to support Zn$^{2+}$ potentiation and instead revealed biphasic inhibitory profiles for glycine-activated responses (Fig. 5C). The sensitivity of the high potency inhibitory component was comparable with that seen for Rlα1,T151A (Rlα1,T151R IC$_{50}$ = 3.6 ± 0.7 μM and Rlα1,T151N IC$_{50}$ = 1.6 ± 0.7 μM; n = 4; p > 0.05). However, the maximal contribution of the high potency inhibitory component was reduced to 39 ± 3% for Rlα1,T151R and to just 6.5 ± 0.3% for Rlα1,T151N from 73 ± 8% for Rlα1,T151A (Fig. 5C), suggesting the nature of the residue at position 151 was important in determining both the direction and the extent of the Zn$^{2+}$ effect.

GlyR α1 Thr-151 Influences Apparent Agonist Gating—As Thr-151 resides in the Cys loop, a domain that is important for agonist gating in this receptor superfamily (34), each of these mutations was assessed for their effect on agonist potencies. All of the mutations α1,T151A, Rlα1,T151A, Rlα1,T151R, Rlα1,T151A,E192A, and Rlα1,T151N caused a progressive reduction in sensitivity to both glycine and taurine with Rlα1,T151N demonstrating 11- to 23-fold increases in the EC$_{50}$ values for glycine and taurine, respectively (n = 5; p < 0.05) (Fig. 6, A and B; TABLE TWO). In accord with the possibility of Thr-151 being involved in ion channel gating, the percentage of maximum current evoked by the lower potency agonist taurine compared with maximal glycine responses in the same cell decreased significantly from 100% and 98 ± 2% in Rlα1 and Rlα1,T151A, respectively, to 90 ± 3% for Rlα1,T151R, 65 ± 4.3% for Rlα1,T151A,E192A and 46 ± 6% for
R1α, T151N (n = 4–7; p < 0.05) (Fig. 6, B and E; TABLE TWO). In addition, the maximum glycine-evoked currents were also significantly reduced from 4.5 ± 0.4 nA for wild-type GlyR α1 to 1.9 ± 0.5 nA for R1α, T151A, E192A and to 2.9 ± 0.5 nA for R1α, T151N (n = 5–12, p < 0.05) (Fig. 6D). These mutated receptors did not substantially distort the region of agonist binding, as sensitivities to the competitive antagonist strychnine were directly comparable with the wild-type GlyR (Fig. 6C). The non-functional nature of the receptors R1α, T151A, D194A and R1α, T151A, H215A precluded their study in this experiment (TABLE TWO). Additional mutations at GlyR α1, Thr-151 to Cys, Asp, Glu, Ser, and Phe revealed no obvious relationship between side chain polarity and the volume requirements of a residue occupying position 151 for receptor activation (supplemental Table 1; supplemental Fig. 2).

**Discussion**

This study reports the first molecular description of a Zn$^{2+}$ potentiating site on a Cys loop ligand-gated ion channel. The residues Asp-194, His-215, and the peptid backbone located at Glu-192 in GlyR α1 are all predicted to reside in close structural proximity to one another, and they all influence Zn$^{2+}$ potentiation in accord with a role in binding. Each of these residues is chemically adept at coordinating Zn$^{2+}$, and when this capacity is ablated, through alanine substitution, the sensitivity to Zn$^{2+}$ was either attenuated (as for R1α, H215A) or entirely ablated (as for R1α, E192A and R1α, D194A). Moreover, experiments using MTSEA demonstrated that the residues in this putative site are accessible to this water-soluble compound and therefore must also be accessible to dynamic Zn$^{2+}$ binding. Additionally, as Zn$^{2+}$ potentiation
of both glycine- and taurine-activated currents was similarly affected by these mutations, it is likely these residues are either part of a universal Zn$^{2+}$ binding site or participate in the process of allosteric signal transduction from such a binding site. Finally, the analysis of the α1E192P receptor suggested that this location might contribute structurally to the site in a manner dependent upon the restraints of the peptide backbone. If the backbone itself can contribute to Zn$^{2+}$ binding, then the site isolated here requires only a fourth coordinating ligand for completion, and in the case of reversible Zn$^{2+}$ binding catalytic sites, this is predominantly provided by an activated water molecule (9).

In addition to those residues thought to line the Zn$^{2+}$ binding site, Thr-151 was also identified as an important transduction component for this site based on its neighboring location. The exact nature of the residue introduced at position 151 did not alter the potency of Zn$^{2+}$, precluding an involvement in binding, but it was able to determine the “direction of output” from the Zn$^{2+}$ site to be either potentiating (for threonine) or inhibitory (for alanine, arginine, and asparagine). Furthermore, the type of residue at position 151 also controlled the efficacy of inhibition from the Zn$^{2+}$ site. As this site is quite distinct in its structure and location, Thr-151 is most unlikely to be associated with the previously reported Zn$^{2+}$ inhibitory site on GlyR α1, which resides on the other side of the subunit (10, 26, 30). In accordance with the location of Thr-151 being in the critical Cys loop-gating domain (34–36), this residue was also shown to be an important determinant of agonist potency for receptor activation. Thus, from a molecular perspective, it provides a potential connection between the Zn$^{2+}$ potentiation binding site and the Cys loop-gating domain to possibly increase the efficacy of agonist-induced channel opening. In accordance with Zn$^{2+}$ potentiation being mediated via an increase in agonist efficacy, the partial agonist taurine is converted to a full agonist by Zn$^{2+}$ and capable of interacting with, the Cys loop-gating domain (27, 37), and the potentiation being achieved its effect, because when Zn$^{2+}$ is bound at the potentiation site, it is predicted to interact with the gating apparatus of the receptor. In contrast, when Zn$^{2+}$ is bound at the interfacial inhibitory site, it acts in an apparent competitive manner, stabilizing the closed agonist-unbound state of the receptor (1).

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