Research Paper

Mutations within the agonist-binding site convert the homomeric α₁ glycine receptor into a Zn²⁺-activated chloride channel

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Abbreviations: AChBP, acetylcholine binding-protein; AMPA, (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CNS, central nervous system; GABA, γ-aminobutyric-acid; GlyR, glycine receptor; NMDA, N-methyl-D-aspartate; Iₘₜₐₓ, maximal inducible current; nAChR, nicotinic acetylcholine receptor; TM, transmembrane segment; wt, wild-type; ZAC, Zn²⁺-activated channel

Key words: biosensor, inhibitory neurotransmission, chloride channel, glycine receptor, ligand-gated ion channel, zinc, ZAC, AChBP

The divalent cation Zn²⁺ has been shown to regulate inhibitory neurotransmission in the mammalian CNS by affecting the activation of the strychnine-sensitive glycine receptor (GlyR). In spinal neurons and cells expressing recombinant GlyRs, low micromolar (<10 μM) concentrations of Zn²⁺ enhance glycine currents, whereas higher concentrations (>10 μM) have an inhibitory effect. Mutational studies have localized the Zn²⁺ binding sites mediating allosteric potentiation and inhibition of GlyRs in distinct regions of the N-terminal extracellular domain of the GlyR α₁-subunits. Here, we examined the Zn²⁺ sensitivity of different mutations within the agonist binding site of the homomeric α₁-subunit GlyR upon heterologous expression in Xenopus oocytes. This revealed that six substitutions within the ligand-binding pocket result in a total loss of Zn²⁺ inhibition. Furthermore, substitution of the positively charged residues arginine 65 and arginine 131 by alanine (α₁R₆⁵A, α₁R₁₃¹A), or of the aromatic residue phenylalanine 207 by histidine (α₁F₂₀⁷H), converted the α₁ GlyR into a chloride channel that was activated by Zn²⁺ alone. Dose-response analysis of the α₁F₂₀⁷H GlyR disclosed an EC₅₀ value of 1.2 μM for Zn²⁺ activation; concomitantly the apparent glycine affinity was 1000-fold reduced. Thus, single point mutations within the agonist-binding site of the α₁ subunit convert the inhibitory GlyR from a glycine-gated into a selectively Zn²⁺-activated chloride channel. This might be exploited for the design of metal-specific biosensors by modeling-assisted mutagenesis.

Introduction

The divalent metal ion Zn²⁺ serves as an important structural component of many proteins and as a co-factor in several enzymatic reactions. In addition, different lines of evidence suggest that Zn²⁺ functions as a signaling molecule in the nervous system, where it is stored and released from synaptic terminals of distinct neuronal cell populations, such as hippocampal mossy fibers, spinal interneurons and neurons of the retina. Several studies have shown that Zn²⁺ regulates the function of ligand-gated ion channels, including N-methyl-D-aspartate (NMDA), (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), P2X, nicotinic acetylcholine (nACh) and γ-aminobutyric-acid type A (GABAₐ) receptors by either increasing or decreasing agonist-induced current responses. Zn²⁺ also modulates inhibitory glycine receptors (GlyR) currents in a biphasic fashion by potentiating them at low micromolar Zn²⁺ concentrations, and inhibiting them at higher concentrations (≥10 μM), via distinct Zn²⁺ binding sites. By using a knockin approach, the high-affinity potentiating site has been shown to be essential for proper glycinergic transmission in vivo.

The GlyR is a member of the Cys-loop family of pentameric ligand-gated ion channels that is mainly expressed in spinal cord, brain stem and retina. So far, four GlyR α (α₁-α₄) subunits and a single GlyR β subunit have been identified which all comprise a large N-terminal domain, four transmembrane segments (TMs) and an extended intracellular loop between TM3 and TM4 (reviewed in ref. 12). In contrast to the α subunits, which generate functional homo-oligomeric GlyRs upon heterologous expression, the β subunit forms channels only when co-assembled with α subunits in a 2α:3β stoichiometry.13,14 Agonist binding occurs at the interface of two adjacent N-terminal domains. By homology modeling and site-directed mutagenesis, the residues involved in agonist recognition have been identified and shown to be highly conserved between α and β subunits. Furthermore, amino acid side chains essential for Zn²⁺ modulation have been identified in the α₁ subunit (reviewed in...
The $\alpha_1$1 R131A mutation converts the GlyR into a Zn2+-activated ion channel. In a previous study, we have used site-directed mutagenesis to map amino acid residues that contribute to binding of the agonist glycine and/or the antagonist strychnine at the GlyR $\alpha_1$-subunit.13 When routinely analyzing whether the mutations introduced might affect GlyR modulation by Zn2+, we found that the $\alpha_1$1 R131A substitution resulted in GlyRs that could be activated by Zn2+ alone (Fig. 1A), with a maximal activation efficiency of 42 ± 17% (Fig. 1B and Table 1) as compared to the maximal glycine-inducible current ($I_{\text{max}}$).

Furthermore, the $\alpha_1$1 R131A mutation completely abolished inhibition of glycine responses by Zn2+ at concentrations as high as 1 mM (Table 1). Dose-response analyses revealed an $EC_{50}$ value for Zn2+ activation of the $\alpha_1$1 R131A GlyR of 1.1 ± 0.6 μM (Fig. 1B and Table 1). Both the glycine and Zn2+ induced currents of the $\alpha_1$1 R131A receptor were inhibited by the chloride channel blocker picrotoxin with IC50 values of 7.5 ± 1.8 μM and 6.2 ± 0.4 μM, respectively, which were not significantly different from that determined at the wt $\alpha_1$1 GlyR (IC50 = 4.7 ± 1.2 μM). This is consistent with both Zn2+ and glycine activating chloride flux through the homomeric $\alpha_1$1 GlyR.

Zn2+ activation of the $\alpha_1$1 R131A GlyR is not mediated by the previously identified modulatory Zn2+ binding sites. Substitutions that reduce Zn2+ inhibition do not affect Zn2+ activation. Since mutating T133 located in the vicinity of R131 results in a similar loss of Zn2+ inhibition as seen with the $\alpha_1$1 R131A GlyR (Table 1; reviewed in ref. 17), we first examined whether residues thought to form the inhibitory Zn2+ binding site determine the agonist activity of Zn2+ at this mutant receptor. To this end, residues previously shown to be crucial for the inhibitory action of Zn2+ (H109, T112 and T133; reviewed in refs. 7–9) were substituted by alanines in the $\alpha_1$1 R131A subunit. Expression of the double mutants $\alpha_1$1 R131A,H109A, $\alpha_1$1 R131A,T112A and $\alpha_1$1 R131A,T133A all resulted in GlyRs that were activated by Zn2+ with maximal efficiencies of 46 ± 19%, 58 ± 14% and 39 ± 4%, respectively, i.e., values similar to that obtained with the single R131A substitution alone (Table 1). Furthermore, apparent Zn2+ affinities as indicated by the respective $EC_{50}$ values also were not significantly different from that of the $\alpha_1$1 R131A receptor (Table 1). We conclude from these results that inactivation of the inhibitory Zn2+ binding site defined by residues

| Glycine | Zn2+ activation | Zn2+ inhibition |
|---------|-----------------|-----------------|
| $EC_{50}$ [μM] | $IC_{50}$ [μM] | IZn/Igly [%] | $IC_{50}$ [μM] |
| $\alpha_1$ wt | 190 ± 50# | NA | 35 ± 14 |
| $\alpha_1$ R131A | 110 ± 90# | 1.1 ± 0.6 | 42 ± 17 |
| $\alpha_1$ R131A,H109A | 21 ± 10* | 5.6 ± 2.9 | 46 ± 19 |
| $\alpha_1$ R131A,T112A | 15 ± 6* | 1.1 ± 1.0 | 58 ± 14 |
| $\alpha_1$ R131A,T133A | 26 ± 12* | 2.5 ± 1.3 | 39 ± 4 |
| $\alpha_1$ R131A,D80A | 72 ± 21 | 8.8 ± 2.3 | 11 ± 3* |
| $\alpha_1$ R131A,E192A | 16 ± 6* | 2.0 ± 0.9 | 50 ± 5 |
| $\alpha_1$ R131A,D194A | 21 ± 5* | 7.3 ± 2.7 | 41 ± 5 |
| $\alpha_1$ R131A,R65A | 25,000 ± 8,700* | 1.2 ± 0.5 | 38 ± 8 |
| $\alpha_1$ R131A,R188A | >100,000* | NA** | NA** |
| $\alpha_1$ R131A,R190A | >100,000** | 1.9 ± 1.1 | 20 ± 9 |
| $\alpha_1$ R131A,R202A | >100,000** | 7.0 ± 0.8* | 23 ± 3 |
| $\alpha_1$ R131A,F207A | >100,000** | 1.2 ± 0.9 | 3 ± 2* |
| $\alpha_1$ R131A,F207Y | 51 ± 9 | 0.4 ± 0.2 | 44 ± 14 |
| $\alpha_1$ R131A,F207C | >100,000** | 0.64 ± 0.33 | 11 ± 4* |

Data are means ± SD from 5–10 experiments. Zn2+ inhibition was determined at the respective $EC_{50}$ value of glycine. Values significantly different from $\alpha_1$1 GlyRs (p < 0.01; **p < 0.001) are indicated.

NI, no inhibition with 1 mM Zn2+; NA, no activation with Zn2+ (up to 1 mM). (Taken from ref. 13.) Zn2+ potentiation of currents elicited by subsaturating glycine concentrations ($EC_{50}$) was seen with all mutants tested (data not shown).
H109, T112 and T133 does not account for Zn\(^{2+}\) activation of the α\(^{1}\)R131A GlyR.

Substitution of residues contributing to Zn\(^{2+}\) potentiation. Next, we addressed the question whether mutating the residues D80, E192 and D194 known to contribute to Zn\(^{2+}\) potentiation of the wt α\(^{1}\) GlyR (reviewed in refs. 9, 15 and 16) would affect Zn\(^{2+}\) activation of the α\(^{1}\)R131A mutant receptor. Table 1 shows that the double mutants α\(^{1}\)R131A,D80A, α\(^{1}\)R131A,E192A and α\(^{1}\)R131A,D194A all were activated by Zn\(^{2+}\), with EC\(_{50}\) values and relative activation efficiencies similar to that of the α\(^{1}\)R131A GlyR. Although with one of the mutants (α\(^{1}\)R131A,D80A) a reduction (p < 0.05; n = 6) in relative Zn\(^{2+}\) activation efficacy was seen, these results are consistent with residues mediating Zn\(^{2+}\) potentiation of the wt GlyR not being essential for Zn\(^{2+}\) activation of the α\(^{1}\)R131A mutant receptor.

Agnostic binding site mutants abolish Zn\(^{2+}\) activation. Since residue R131 is oriented into the ligand-binding pocket of the GlyR,\(^{13}\) we next hypothesized that Zn\(^{2+}\) may activate the α\(^{1}\)R131A GlyR via its agonist-binding site by mimicking the positively charged amino group of glycine. To test this hypothesis, we combined the α\(^{1}\)R131A mutation with substitutions that destabilize binding of the amino group of glycine (α\(^{1}\)E157D and α\(^{1}\)F207A), and thus result in GlyRs with strongly reduced agonist affinities (EC\(_{50}\) of glycine >100 mM).\(^{13}\) Indeed, the mutant GlyRs α\(^{1}\)R131A,E157D and α\(^{1}\)R131A,F207A were not or only marginally activated by Zn\(^{2+}\) as compared to the respective I\(_{\text{max}}\) values obtained with glycine (0% and 3%, respectively; Fig. 2 and Table 1). Since the additional substitutions at both the 157 and 207 positions caused a loss of Zn\(^{2+}\) activation, we decided to introduce residues that might assist Zn\(^{2+}\) binding, i.e., cysteine and tyrosine side chains.\(^{21}\) The double mutants α\(^{1}\)R131A,E157C, α\(^{1}\)R131A,F207Y and α\(^{1}\)R131A,F207C all were activated by Zn\(^{2+}\) with relative activation efficiencies of 11–44% and apparent Zn\(^{2+}\) affinities similar to that of the α\(^{1}\)R131A receptor (Fig. 2 and Table 1). In addition, we analyzed mutations which disrupt binding of the carboxyl group of glycine (substitutions at R65A and E157D)\(^{13}\) GlyRs formed from the double mutants α\(^{1}\)R131A,R65A and α\(^{1}\)R131A,E157D were activated by Zn\(^{2+}\) with similar efficiencies as the α\(^{1}\)R131A GlyR (Table 1), indicating that the loss of Zn\(^{2+}\) activation seen with mutants α\(^{1}\)R131A,E157D and α\(^{1}\)R131A,F207A is position-specific.

In conclusion, Zn\(^{2+}\) activation of α\(^{1}\)R131A receptors was not altered when residues of the previously identified inhibitory or the potentiating Zn\(^{2+}\) sites or residues interacting with the carboxyl group of glycine were mutated (Table 1). Only substitution of E157 and F207, i.e., of residues, which have been shown to complex the amino group of glycine, drastically impaired Zn\(^{2+}\) activation. Apparently, Zn\(^{2+}\) activation involves the cationic subsite of the receptor's glycine binding pocket.\(^{13}\)

| Table 2 | Differential effects on Zn\(^{2+}\) pharmacology of mutations within the agonist-binding site of the α\(^{1}\) GlyR |
|-----------------|-----------------|-----------------|-----------------|
| Glycine        | Zn\(^{2+}\) activation | Zn\(^{2+}\) inhibition |
|                | EC\(_{50}\) [μM] | IC\(_{50}\) [μM] | I\(_{Zn}/I_{gly}\) [%] | IC\(_{50}\) [μM] |
| α\(^{1}\) WT   | 190 ± 50        | NA              | -               | 35 ± 14         |
| α\(^{1}\)R65A  | >100,000**      | 5.5 ± 1.0**     | 7 ± 4**         | NI**            |
| α\(^{1}\)R131A | 110 ± 90        | 1.1 ± 0.6**     | 42 ± 17**       | NI**            |
| α\(^{1}\)E157C | >100,000**      | NA              | -               | NI**            |
| α\(^{1}\)F159Y | 26 ± 11**       | -               | 20 ± 11         | -               |
| α\(^{1}\)F159H | >100,000**      | -               | >800**          | -               |
| α\(^{1}\)Y202F | 11,000 ± 2,900**| NA              | -               | >900**          |
| α\(^{1}\)Y202H | 16,000 ± 5,000**| NA              | -               | 435 ± 210**     |
| α\(^{1}\)T204S | 4,400 ± 500     | NA              | -               | 49 ± 24         |
| α\(^{1}\)T204H | >100,000**      | NA              | -               | NI**            |
| α\(^{1}\)F207Y | 220 ± 43        | NA              | 190 ± 74*       | -               |
| α\(^{1}\)F207C | >100,000**      | NA              | -               | NI**            |
| α\(^{1}\)F207H | >100,000**      | 1.2 ± 0.9**     | 62 ± 11**       | NI**            |

For explanation and details, see legend to Table 1. Values significantly different from wt α\(^{1}\) receptors (*p < 0.01; **p < 0.001) are indicated.
a glycine-gated into a selectively Zn$^{2+}$-activated chloride channel. The activation by Zn$^{2+}$ of these mutant receptors involves binding of Zn$^{2+}$ to the glycine binding site, since (i) substitutions within the previously identified potentiating and inhibitory allosteric Zn$^{2+}$ binding sites had no effect on Zn$^{2+}$ activation, and (ii) distinct substitutions of residues crucial for binding of the $\alpha$-amino group of glycine generated receptor channels primarily activated by Zn$^{2+}$. Since detailed structural information is available for the N-terminal ligand-binding domain of members of the Cys-loop receptor family, our results suggest that this class of ion channels may be engineered into biosensors that display specificity for novel ligands including divalent metal ions.

**Generation of a Zn$^{2+}$-sensitive receptor.** Here several substitutions within the glycine-binding site of the $\alpha_1$ GlyR resulted in either a loss of Zn$^{2+}$ inhibition or, more remarkably, activation of the receptor by Zn$^{2+}$ alone. By mutational analysis and molecular modeling, several residues located at the interface of adjacent $\alpha_1$ subunits have been shown to contribute to agonist binding within the $\alpha_1$ GlyR binding pocket. Here, we show that substitution of seven of these residues, two positively charged arginines located on the - side ($\alpha_1 R65$ and $\alpha_1 R131$) and five negatively charged, polar or aromatic residues on the + side, drastically reduced ($\alpha_1 F159$, $\alpha_1 T112$ and $\alpha_1 T133$) or totally abolished ($\alpha_1 E157$, $\alpha_1 F207$) Zn$^{2+}$ inhibition of the recombinant $\alpha_1$ GlyR (Table 2), whereas Zn$^{2+}$-mediated potentiation was still preserved in all mutated $\alpha_1$ GlyRs (see legend to Table 1). The respective wt side chains are thought to interact with the $\alpha$-carboxyl and $\alpha$-amino groups of glycine. When molecular modeling was used to dock Zn$^{2+}$ into the glycine binding pocket followed by several energy-minimization runs (see Materials and Methods), the lowest energy docking mode oriented Zn$^{2+}$ towards the + side of the binding pocket. Figure 4 shows an enlarged view of the wt $\alpha_1$ GlyR subunit interface with Zn$^{2+}$ computationally docked into the agonist-binding site. Accordingly, Zn$^{2+}$ may bind by interacting with the negatively charged residue $\alpha_1 E157$, the polar residue $\alpha_1 F207$ and, via cation-$\pi$-interactions, the aromatic side chains of $\alpha_1 F159$, $\alpha_1 Y202$ and $\alpha_1 F207$, all located on the + side of the agonist-binding pocket. Consistent with this binding model, the substitutions $\alpha_1 F157C$, $\alpha_1 F159H$, $\alpha_1 Y202FH$ and $\alpha_1 F207C$ drastically reduced the IC$_{50}$ value of Zn$^{2+}$. We hence conclude that the most likely explanation for the inhibitory effect of Zn$^{2+}$ on glycine currents is binding within the ligand-binding site of the GlyR. This conclusion is supported by previous studies in which Zn$^{2+}$ was shown to inhibit glycine currents of the wt $\alpha_1$ GlyR in a competitive fashion. This interpretation implies that the previously described inhibitory Zn$^{2+}$ binding site defined by the mutations $\alpha_1 H109A$, $\alpha_1 T112A$ and $\alpha_1 T133A$ (reviewed in refs. 8, 9, 17 and 22) does not constitute a separate metal ion binding pocket but likely represents a sub-domain of the subunit interface. Consistent with this assumption, two of the three substitutions interfering with Zn$^{2+}$ inhibition (i.e., $T112$, $T133$) are, as deduced from our current model of the extracellular domain of the $\alpha_1$ GlyR, located at the subunit interface and oriented towards the ligand-binding site. Accordingly, substitutions at this interface will distort the agonist binding pocket formed between two adjacent $\alpha$ subunits and thus affect both glycine affinity and Zn$^{2+}$ inhibition.

The substitutions $\alpha_1 R65A$, $\alpha_1 R131A$ and $\alpha_1 F207H$ produced an unexpected additional effect, a pronounced agonist activity of Zn$^{2+}$ in the nano- to micromolar range (Table 2). We assume that the Zn$^{2+}$...
activation seen with these mutant GlyRs is due to a conversion of the inhibitory into an activating Zn\textsuperscript{2+} site within the glycine-binding pocket. In case of residues R65 and R131, this conversion may be explained by a loss of electric repulsion mediated by the positively charged arginine side-chains, which results in a conformational rearrangement in the center of the binding pocket that enables channel activation by Zn\textsuperscript{2+} (see also Fig. 4). Consistent with this conclusion, the related human Zn\textsuperscript{2+}-activated channel (ZAC) subunit displays all structural motifs characteristic of Cys-loop receptors but carries noncharged side chains at the positions homologous to R65 and R131 of the GlyR α1 subunit.\textsuperscript{23} Notably, the α1\textsubscript{F207H} substitution located in the C-loop also was effective in conferring Zn\textsuperscript{2+}-gating with high-affinity and efficacy. This presumably reflects histidine's ability to stabilize cation interactions,\textsuperscript{21} thereby enabling Zn\textsuperscript{2+} activation of the α1\textsubscript{F207H} mutant receptor. Since agonist-induced conformational rearrangements of the C-loop seem to be sufficient to transduce agonist binding into channel opening (reviewed in refs. 24–26), we propose that complexation of Zn\textsuperscript{2+} by C-loop interactions and/or removal of positive repulsive effects in the GlyR binding pocket are important for triggering channel opening of the mutant α1 GlyRs. This implies that subtle changes within the binding pocket suffice for allowing conversion of antagonists into agonists, or for altering substrate specificity. This conclusion is supported by site-directed mutagenesis experiments on other Cys-loop receptors, in which agonist binding site substitutions homologous to those shown here to cause Zn\textsuperscript{2+} activation of the α1 GlyR were found to be important determinants of agonist selectivity (reviewed in ref. 27).

**Perspectives.** At many synapses of the mammalian CNS, Zn\textsuperscript{2+} is highly enriched in synaptic vesicles and co-released together with neurotransmitters in an activity-dependent manner.\textsuperscript{28} Recent studies have shown that Zn\textsuperscript{2+} potentiates synaptic GlyRs,\textsuperscript{29} and that this Zn\textsuperscript{2+} potentiation is required for proper glycinergic neurotransmission in vivo.\textsuperscript{10} Presently, free Zn\textsuperscript{2+} concentrations in the synaptic cleft have not been precisely determined but have been suggested to vary between 100 nM and >100 μM within specific CNS regions.\textsuperscript{1} For obtaining more precise estimates of synaptic Zn\textsuperscript{2+} concentrations, specifically targeted biological Zn\textsuperscript{2+} sensors would be of considerable value. The mutant α1 GlyRs described here could be developed into such a tool. Moreover, due to its fast kinetics the α1 GlyR-derived Zn\textsuperscript{2+}-activated channel described here may be useful for identifying and monitoring Zn\textsuperscript{2+} release in biological systems in real time.

**Materials and Methods**

**Site-directed mutagenesis.** Mutations were introduced into the human α1 subunit cDNAs subcloned in the pNKS2 vector by using the QuickChange site-directed mutagenesis Kit (Stratagene). All substitutions were confirmed by DNA sequencing. cRNAs of mutant and wild-type (wt) GlyRs were synthesized and injected as described previously.\textsuperscript{9}

**Oocyte expression.** For the isolation of stage V and VI oocytes, ovarian lobes were surgically removed from adult female Xenopus laevis frogs anaesthetized by immersion in 0.3% (w/v) tricaine methane sulfonate (Sigma) through a 1–2 cm incision in the abdomen. All protocols were approved by the local animal care and use committee (II25.3-19c20/15; RP Darmstadt, Germany). Oocytes were carefully dissected and stored in sterile Barth’s medium (composition in mM: 88 NaCl, 1 KCl, 0.4 CaCl\textsubscript{2}, 0.3 Ca(NO\textsubscript{3}), 8.2 MgSO\textsubscript{4}, 2.4 NaHCO\textsubscript{3}, 10 Tris, pH 7.2). Before injection, the follicle cell layer was removed by treatment with 1 mg/ml collagenase (type IIa, Sigma) for 1 h at room temperature to enzymatically dissociates the oocytes followed by mechanical disruption using forceps. RNA (50 nl per oocyte) was injected as detailed previously.\textsuperscript{7}

**Electrophysiological recordings and data analysis.** Two-electrode voltage clamp recording of whole-cell currents was performed in Ringer’s solution containing (in mM): 115 NaCl, 1 KCl, 1.8 CaCl\textsubscript{2} and 10 Hepes (pH 7.2), at a holding potential of -70 mV.\textsuperscript{9} Dose-response curves for the agonist glycine were generated by measuring the inward currents elicited by increasing agonist concentrations; in cases where saturation was not reached at 300 mM glycine, EC\textsubscript{50} values were stated to be >100 mM. Antagonist inhibition curves were obtained by co-applying glycine at a concentration corresponding
to the respective $EC_{50}$ value with increasing concentrations of the antagonist; for low-affinity mutants (glucose $EC_{50} > 100$ mM), Zn$^{2+}$-inhibition was measured at a glucose concentration of 300 mM. For data analysis, currents were digitally stored on a computer using pCLAMP 8 (Axon Instruments, Inc., CA). Results were analyzed using the KaleidaGraph program (Synergy Software, Reading, PA) and GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Agonist-induced peak currents ($I$) were normalized to the maximal current value ($I_{\text{max}}$) and fitted with the Hill equation as described.\textsuperscript{18} To determine half-maximal effective antagonist concentrations ($IC_{50}$) from inhibition curves, results were fitted by the following equation:

$$I = I_{\text{max}} \cdot \left( \frac{1}{\left( \frac{1}{B} \right)^{n_H} + \left( \frac{1}{C} \right)^{n_H}} \right)$$

where $I$ corresponds to the current obtained, $I_{\text{max}}$ to the maximal agonist-induced current, $B$ to the concentration of the antagonist, $C$ to the $IC_{50}$ value and $n_H$ to the Hill coefficient. Activation efficiency of the Zn$^{2+}$-responses ($I/I_{\text{max}}$; %) was calculated by determining the ratio of maximal inducible currents of saturating Zn$^{2+}$ and glucose concentrations. For mutants with a glucose $EC_{50}$ value $> 100$ mM, the current responses elicited with 300 mM glucose was used to calculate the activation efficiency of Zn$^{2+}$. Experimental values are presented as means ± S.D. of peak current responses; statistical significance was determined by Student's t-test and considered to be significant at $p < 0.01$ (n.s., not significant).

### Molecular modeling of GlyR N-terminal domains

Sequence alignment of the extracellular domain of the GlyR $\alpha_1$ subunit with the acetylcholine binding protein from Lymnea stagnalis (AChBP) was taken from Grudzinska et al.\textsuperscript{13} Molecular modeling was based on the crystal structure of AChBP,\textsuperscript{13} Brookhaven Protein Data Bank entry 119B) by using the SYBYL 6.9 program (Tripos Associates, St. Louis, MO) according to Grudzinska et al.\textsuperscript{13} Zn$^{2+}$ was introduced into the binding pocket of the wild-type receptor by positioning it with the help of DOCK in a conformation that closely matched that of bound glucose, and then subjected to MD simulation as described in ref. 13. The resulting structure was used to evaluate changes in Zn$^{2+}$ binding site interactions caused by mutation of binding site residues. Pymol\textsuperscript{20} was used to make the figure.

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