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The Extracellular Disulfide Loop Motif of the Inhibitory Glycine Receptor Does Not Form the Agonist Binding Site

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SUMMARY

Inhibitory (glycine and γ-aminobutyric acid type A) and excitatory (nicotinic acetylcholine and serotonin 5-hydroxytryptamine type 3) receptors of the ligand-gated ion channel superfamily are related by both structural similarities and primary sequence identity. One invariant feature of all members of this receptor superfamily is the presence of an extracellular disulfide loop motif. This structural motif has been modeled, and Cockcroft *et al.* [*Proteins* **8**:386–397 (1990)] have suggested that it forms the agonist binding site of the ligand-gated ion channel receptors. Using site-directed mutagenesis of the inhibitory glycine receptor (GlyR), we have specifically tested this hypothesis. The lysine residue at position 143 is proposed to form the binding site for the negatively charged carboxyl group of the agonist glycine. Differing residues at this position in other ligand-gated receptors are proposed to confer agonist specificity. The aspartic acid residue at position 148 is an invariant residue in every known subunit of the ligand-gated ion channel receptor superfamily. This residue has been proposed as the binding site for the positively charged amino group of the various agonists. Mutation of the lysine at position 143 to alanine resulted in essentially unaltered GlyRs, showing only modest decreases in strychnine affinity (K_d, 8.1 ± 1.4 nM versus 13.4 ± 1.3 nM), glycine displacement of strychnine binding (K_i, 25 ± 5 μM versus 49 ± 9 μM), and glycine activation of chloride currents (EC_{50}, 27 ± 6 μM versus 114 ± 14 μM). Thus, we conclude that Lys-143 does not play a major role in either agonist or antagonist binding or agonist activation of the GlyR. Mutation of Asp-148 to either alanine or asparagine disrupted the expression and/or assembly of the receptor, and no binding sites or ion channels were expressed on the cell surface. The conservative mutation of the aspartic acid at position 148 to glutamic acid (D148E) allowed the expression of receptors, although with reduced efficiency. The D148E GlyRs showed a 1 order of magnitude decrease in strychnine affinity (K_d, 8.1 ± 1.4 nM versus 82 ± 21 nM), without any change in the glycine displacement of strychnine binding (K_i, 25 ± 5 μM versus 29 ± 8 μM) or glycine activation of chloride currents (EC_{50}, 27 ± 6 μM versus 20 ± 1 μM). Thus, Asp-148 plays an important role in receptor assembly and in antagonist binding. We conclude that neither Lys-143 nor Asp-148 plays any significant role in the agonist binding or receptor activation of the inhibitory GlyR, thus refuting Cockcroft's hypothesis. Moreover, as a consequence of the conservation of both the structure and the primary sequence of the disulfide loop motif in all ligand-gated ion channel receptors, we suggest that this domain does not play a role in agonist binding of the other members of this receptor superfamily. However, our results show that the invariant residue Asp-148 forms part of the antagonist binding site, and we suggest that this structural motif may play a role in antagonist binding to the other related receptors.

The ligand-gated ion channel receptors mediate rapid (millisecond) changes in the distribution of ions across the postsynaptic membranes of neurons. Members of this family include receptors for both excitatory neurotransmitters such as acetylcholine and serotonin and inhibitory neurotransmitters such as glycine and GABA (reviewed in Ref. 1). The ligand-gated ion channel receptors each consist of five subunits, which associate to form a pentameric channel structure. cDNAs encoding a number of subunits for each receptor have been cloned and the primary structures determined (1–5). The subunits of all four classes of receptor show significant topological and sequence similarities. Each subunit contains an amino-terminal extracellular domain of approximately 220 amino acids, which contains a disulfide-bonded loop motif, four putative α-helical transmembrane regions, and an intracellular domain between the third and fourth transmembrane regions. In addition, the subunits share varying degrees of primary sequence identity, ranging from 20% to 50%.

Three distinct extracellular domains involved in ligand binding have been identified in the ligand-gated ion channel receptors, especially within the nAChR (reviewed in Ref. 6). In the

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ABBREVIATIONS: GABA, γ-aminobutyric acid; $GABA_AR$, γ-aminobutyric acid type A receptor; GlyR, glycine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; nAChR, nicotinic acetylcholine receptor.

GlyR, the antagonist strychnine interacts with the extracellular domain of the α1 subunit of the receptor at residues Gly-160, Tyr-161, Lys-200, and Tyr-202 (7, 8), whereas the agonist glycine interacts with residue Thr-204 (9).

Barnard *et al.* (10) have suggested that the highly conserved disulfide-bonded loop, located between residues Cys-138 and Cys-152 in the GlyR, may be involved in agonist recognition. Similar suggestions have been made for the nAChR (2). However, in the nAChR the disulfide loop motif is not apparently involved in agonist binding (11, 12) and no data demonstrate that the loop forms a ligand binding site.

The disulfide loop motif contains a number of highly conserved residues, and computer-aided modeling of the structure of this loop suggests that it is a rigid amphiphilic β-hairpin (13). An invariant aspartic acid residue, at position 148 in the GlyR, has been suggested to form part of an anionic site on the receptor that interacts with the positively charged amino group of the respective agonist of each receptor, i.e., glycine, GABA, acetylcholine, and serotonin. The residue at position 143 in the GlyR, predicted to be opposite the aspartic acid residue, would provide the specificity required for the distinction between the various agonists. In the α1 subunit of the GlyR position 143 is a lysine residue, which has been proposed to be the site of interaction for the carboxyl group of the agonist glycine (13).

We have used site-directed mutagenesis and functional expression studies to investigate the contributions of the lysine and aspartic acid residues in the formation of the agonist and/or antagonist binding sites of the α1 subunit of the human GlyR. We demonstrate that Lys-143 and Asp-148 are not involved in either agonist recognition or channel gating. Lys-143 is also not involved in antagonist recognition. However, we show that Asp-148 is a novel determinant of antagonist binding. Furthermore, the functional expression of GlyRs on the cell surface is particularly sensitive to mutations at position Asp-148, suggesting that this residue plays a critical role in the assembly of the receptor.

Materials and Methods

Site-directed mutagenesis. The cDNA encoding the α1 subunit of the human GlyR (14) was subcloned into the pCIS expression vector (15). Mutations in the cDNA were constructed using the oligonucleotide-directed polymerase chain reaction mutagenesis method of Ho *et al.* (16) and were confirmed by sequencing of the cDNA clones. The single-letter code for amino acids is used to describe mutations. The letter preceding the position number refers to the amino acid in the wild-type GlyR and the letter after the number refers to the amino acid replacing the wild-type amino acid. For example, D148E refers to the mutation of aspartic acid at position 148 to glutamic acid.

Expression of mutated GlyR α1 subunit cDNAs. Plasmid DNA encoding wild-type or mutated α1 subunits of the GlyR was transfected, using the method of Chen and Okayama (17), into exponentially growing 293 cells (adenovirus-transformed human embryonic kidney cells; ATCC CRL 1573) (15, 18, 19). After 24 hr, the cells were washed twice and then cultured in fresh culture medium (Eagle's minimum essential medium with Hanks' salts, supplemented with 2 mM glutamine and 10% fetal calf serum).

Electrophysiological analysis. Coverslips (1-cm diameter) on which transfected cells were grown were placed in a small (1-ml capacity) chamber continually superfused at a rate of 2 ml/min with standard bath solution (140 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4 with NaOH). In experiments measuring strychnine inhibition, strychnine (10 nM to 10 μM) was dissolved in standard bath solution and passed through the chamber for 5–10 min before application of 1 mM glycine. Glycine (2 μM to 2 mM) in standard bath solution was rapidly applied to the chamber using a microperfusion tube system (8). Glycine-activated currents were recorded from cells held at −55 mV, using the whole-cell configuration (20) of the patch-clamp technique, by using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Patch pipettes of resistance 1–4 MΩ were filled with the following solution: 145 mM CsCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.4 with CsOH. At least 80% series resistance compensation was used when currents larger than 1.5 nA were recorded, and recordings were not made from cells with maximal currents greater than 5 nA. Although this minimized series resistance errors, it resulted in I_{max} values that may not accurately reflect the level of GlyR expression. EC_{50} values for glycine-activated currents were calculated by fitting data, using a nonlinear least squares algorithm, to the Hill equation, $I/I_{max} = [G]^h/(EC_{50}^h + [G]^h)$, where I is the magnitude of the peak current elicited by glycine concentration [G], I_{max} is the magnitude of the maximum peak current elicited by saturating concentrations of glycine, and h is the Hill coefficient.

IC_{50} values for strychnine inhibition of glycine-activated currents were calculated using the equation $I/I_0 = 1/[1 + ([S]^h/IC_{50}^h)]$, where I is the peak current elicted by 1 mM glycine in the presence of strychnine concentration [S] and I_0 is the peak current elicited by 1 mM glycine in the absence of strychnine. Data are presented as the mean ± standard error.

[^{3}H]Strychnine binding assays. Intact cells were harvested 48 hr after transfection by using phosphate-buffered saline containing 0.5 mM EDTA and were washed three times in the same buffer without EDTA. Whole cells (5×10^5 cells) expressing the recombinant GlyRs were incubated with [^{3}H]strychnine (1–100 nM, 17.0 Ci/mmol; New England Nuclear), with and without 100 mM glycine to determine nonspecific binding. After incubation to equilibrium at 4°C for 60 min, the cells were collected by centrifugation using an Eppendorf microcentrifuge and the supernatant was discarded. The amount of radioactive strychnine remaining bound to the pelleted cells was determined by liquid scintillation counting. The dissociation constants for [^{3}H]strychnine binding to the GlyRs were estimated by fitting the data to the equation $Y = B_{max}[S]/(K_d + [S])$, where Y is the amount of [^{3}H]strychnine bound, B_{max} is the maximal level of binding, [S] is the [^{3}H]strychnine concentration, and K_d is the dissociation constant.

Displacement of 20 nM [^{3}H]strychnine, bound to cells expressing the recombinant GlyRs, by 0–100 mM glycine was measured using the same procedure. IC_{50} values for glycine displacement of [^{3}H]strychnine were calculated by fitting the data to a transformed Hill equation, $Y = [A + (B - A)]/[1 + (10^c/10^{[G]})^h]$, where Y is the calculated dpm of [^{3}H]strychnine bound at glycine concentration [G], A is the nonspecific binding of [^{3}H]strychnine, B is the [^{3}H]strychnine bound in the absence of glycine, c is the IC_{50}, and h is the Hill coefficient (set at −1). IC_{50} estimations for glycine displacement of [^{3}H]strychnine were converted to K_i values using the Cheng-Prusoff equation, $IC_{50} = K_i(1 + [S]/K_d)$, where [S] is the concentration of [^{3}H]strychnine used in the assay (20 nM) and K_d is the dissociation constant for [^{3}H]strychnine bound to the GlyR. Data are presented as mean ± standard error from triplicate experiments.

Results

Properties of the expressed GlyRs. Expression of the human GlyR α1 subunit cDNA in the mammalian 293 cell line generates functional GlyRs. Glycine-activated chloride currents were observed with an EC_{50} of 27 ± 6 μM (Fig. 1; Table 1) and strychnine inhibited these currents with an IC_{50} of 28 ± 7 nM (Fig. 2; Table 2). The dissociation constant (K_d) for [^{3}H]strychnine binding to the wild-type GlyR was 8.1 ± 1.4 nM (Fig. 3; Table 3), and the inhibition constant (K_i) for glycine displacement of bound [^{3}H]strychnine was 25 ± 5 μM (Fig. 4; Table 3).

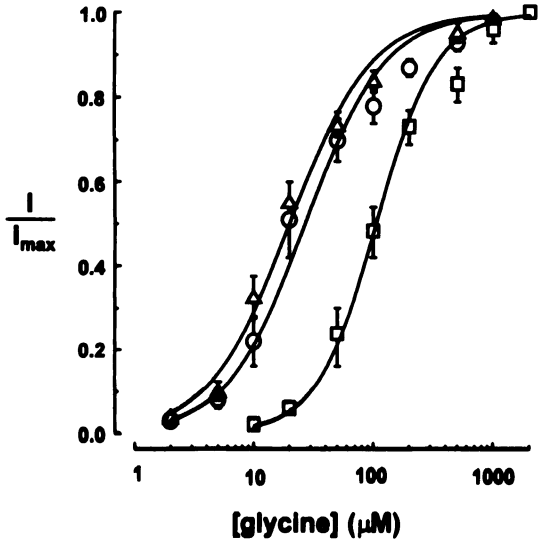

Fig. 1. Glycine activation of wild-type and mutated GlyR chloride channels. Increasing doses of glycine were applied by a perfusion tube system to cells transfected with the wild-type (○), K143A (□), or D148E (△) GlyR cDNA. The cells were voltage clamped at −55 mV as described in Materials and Methods. The data presented are mean values ± standard errors from four or five different cells.

TABLE 1

Channel activation properties of wild-type and mutated GlyRs

EC_{50} values for glycine activation of chloride channels, maximum currents (I_{max}), and Hill coefficients (h) were determined as described in Materials and Methods. The values of the parameters and their standard errors from n determinations are presented. The fold change (values in parentheses) was calculated relative to the values obtained for the wild-type GlyR.

GlyR	EC_{50}	I_{max}	h	n
	μM	nA		
Wild-type	27 ± 6 (1)	2.3 ± 1.1 (1)	1.3 ± 0.2	4
K143A	114 ± 14 (4)	0.83 ± 0.31 (0.4)	1.6 ± 0.3	5
D148E	20 ± 1 (0.7)	2.1 ± 0.6 (0.9)	1.4 ± 0.2	5

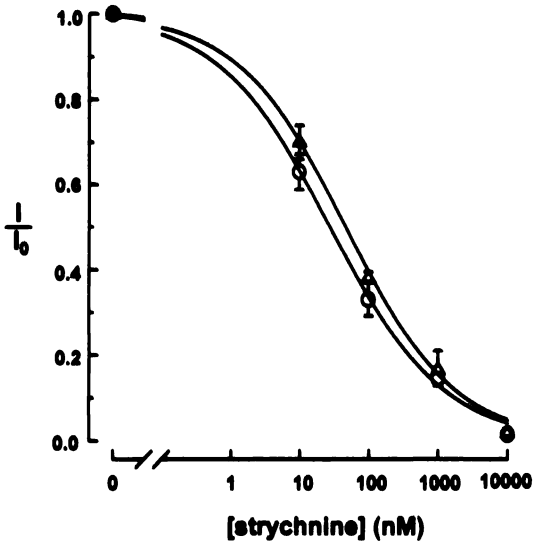

Fig. 2. Strychnine inhibition of glycine-activated wild-type and mutated GlyR chloride channels. Cells transfected with wild-type (○) or D148E (△) GlyR cDNA were preincubated with various concentrations of strychnine before the application of 1 mM glycine by a perfusion tube system. The data presented are mean values ± standard errors from three different cells.

TABLE 2

Strychnine inhibition of channel activation of wild-type and mutated GlyRs

IC_{50} values for strychnine inhibition of glycine-activated chloride channels and Hill coefficients (h) were determined as described in Materials and Methods. The values of the parameters and their standard errors from three different cells are presented. The fold change (values in parentheses) was calculated relative to the values obtained for the wild-type GlyR.

GlyR	IC_{50}	h	n
	nM		
Wild-type	28 ± 7 (1)	0.53 ± 0.07	3
D148E	47 ± 10 (1.7)	0.55 ± 0.06	3

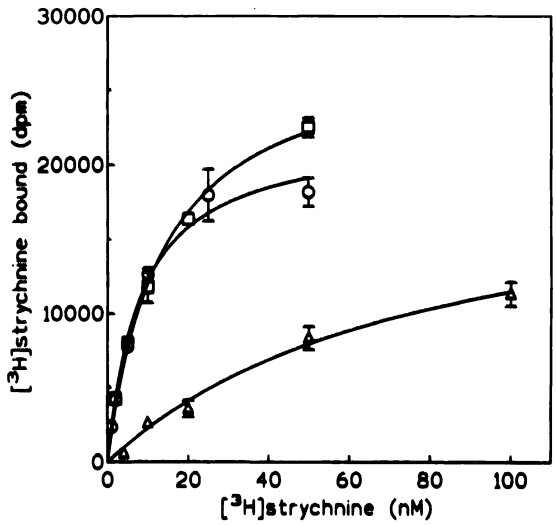

Fig. 3. Saturation isotherm of [^{3}H]strychnine binding to wild-type and mutated GlyRs. [^{3}H]Strychnine binding to cells transfected with the wild-type (○), K143A (□), or D148E (△) GlyR cDNA was determined. The data presented are from a representative experiment and show the mean values ± standard errors of triplicate measurements.

TABLE 3

Ligand-binding properties of wild-type and mutated GlyRs

K_d values for [^{3}H]strychnine binding and K_i values for glycine displacement of bound [^{3}H]strychnine (20 nM) were determined as described in Materials and Methods. The values of the parameters and their standard errors from three determinations are presented. The fold change (values in parentheses) was calculated relative to the values obtained for the wild-type GlyR.

GlyR	[^{3}H]Strychnine K_d	Glycine K_i
	nM	μM
Wild-type	8.1 ± 1.4 (1)	25 ± 5 (1)
K143A	13.4 ± 1.3 (1.7)	49 ± 9 (2)
D148E	82 ± 21 (10)	29 ± 8 (1.2)

The involvement of Lys-143 and Asp-148, located within the disulfide loop of the α1 subunit of the GlyR, in forming the strychnine and/or glycine binding sites was investigated by using site-directed mutagenesis. The two residues were initially changed to alanine (21), to minimize any secondary structure perturbations while removing the capacity of the side chains to interact with the ligands.

Role of Lys-143 in ligand binding and GlyR activation. The glycine concentration required for half-maximal activation of currents of the K143A GlyR was increased 4-fold, to 114 ± 14 μM (Fig. 1; Table 1), and the K_i for glycine displacement of [^{3}H]strychnine was increased 2-fold, to 49 ± 9 μM (Fig. 4; Table 3). Because the K143A GlyR showed a normal [^{3}H]strychnine affinity (K_d = 13.4 ± 1.3 nM) (Fig. 3; Table 3),

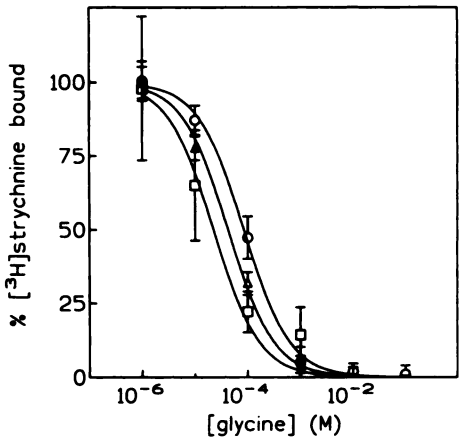

Fig. 4. Glycine displacement of bound [^{3}H]strychnine from wild-type and mutated GlyRs. [^{3}H]Strychnine (20 nM) bound to cells transfected with the wild-type (○), K143A (□), or D148E (△) GlyR cDNA was displaced by varying concentrations of glycine. The data presented are from a representative experiment and show the mean values ± standard errors of triplicate measurements.

strychnine inhibition of glycine-activated currents was not examined. Although there was a modest decrease in glycine sensitivity of the K143A GlyR, Lys-143 is unlikely to be directly involved in either glycine or strychnine recognition by the GlyR. These results may be compared with similar experiments in which a distant residue, Thr-204 of the α1 subunit, was mutated to alanine, resulting in a 2900-fold increase in the K_i for glycine displacement of [^{3}H]strychnine (9) and a 400-fold increase in the EC_{50} for glycine activation of chloride currents.[2] Thus, the small change in affinity of glycine for the K143A GlyR, compared with the wild-type GlyR, is unlikely to reflect a major alteration of the glycine binding site.

Role of Asp-148 in ligand binding and GlyR activation. Glycine activation of chloride currents and [^{3}H]strychnine binding was not detected in cells transfected with the GlyR α1 subunit cDNA containing the mutation of Asp-148 to alanine (D148A). Either this residue is critical for the recognition of both ligands or the mutation has altered the structure of the GlyR in such a way as to prevent expression of the GlyR at the cell surface. To further investigate the role of this residue in GlyR structure and function, conservative substitutions of Asp-148 by asparagine and by glutamic acid were analyzed. Transfection of the D148N α1 GlyR cDNA into 293 cells again did not yield detectable GlyRs, using either electrophysiological or ligand-binding assays. However, the D148E α1 GlyR did generate functional GlyRs on the cell surface, and these were analyzed by both electrophysiological and ligand-binding techniques. The efficiency of D148E GlyR formation was lower than that of wild-type GlyRs, with B_{max} values reduced by about 80%. The EC_{50} for glycine-activated chloride currents (20 ± 1 μM) was unchanged, compared with wild-type GlyRs (27 ± 6 μM) (Fig. 1; Table 1), whereas a minor increase in the IC_{50} for strychnine inhibition of these currents, to 47 ± 10 nM, was observed (Fig. 2; Table 2). The K_d for [^{3}H]strychnine bound to D148E GlyR increased significantly by 10-fold, to 82 ± 21 nM, compared with the wild-type GlyR (8.1 ± 1.4 nM) (Fig. 3; Table 3). No change in the K_i for glycine displacement of [^{3}H]strychnine (29 ± 8 μM) was seen (Fig. 4; Table 1). The D148E mutation therefore appears to affect antagonist binding without any appreciable effect on glycine binding and activation of the GlyR.

[2] S. Rajendra and C. R. French, unpublished observations.

Discussion

Our studies clearly show that neither the K143A nor the D148E mutation has any significant effect on agonist binding or activation of the GlyR. Thus, we suggest that the hypothesis that these two residues form the glycine binding site (13) is unlikely to be correct. Comparison of the wild-type GlyR with the K143A GlyR revealed similar strychnine sensitivities and a small reduction in glycine sensitivity. The conservative mutation D148E was also characterized by no significant changes in agonist binding, activation, or displacement properties. However, such changes may not be as pronounced with a conservative substitution as with removal of the charged group. If the Lys-143 and Asp-148 residues were responsible for binding the agonist glycine, a large decrease in glycine sensitivity would be expected for the mutated GlyR, as has been observed for other agonist binding site mutations, such as that at position Thr-204 (9). The lack of any significant change in glycine sensitivity leads us to conclude that Lys-143, Asp-148, and the disulfide loop motif do not form the agonist binding site. The specific lack of involvement of these residues in agonist activation of the GlyR suggests that the equivalent residues in other ligand-gated ion channel receptors also are not involved in agonist binding or activation. In the case of the nAChR, ligand binding and antibody studies have also provided evidence against the involvement of the disulfide loop motif in agonist binding and activation (11, 12).

The D148A and D148N GlyRs did not form strychnine or glycine binding sites at the cell surface. This is most likely to be due to the lack of correct assembly of the receptor, suggesting a structural role for the loop motif. The more conservative D148E mutation did generate functional GlyRs, although with reduced efficiency, and produced no change in glycine binding or activation, compared with the wild-type GlyR. However, the D148E GlyR showed a significant (10-fold) reduction in affinity for [^{3}H]strychnine but only a 2-fold change in the IC_{50} for strychnine inhibition of glycine-activated currents. The relatively small difference between the IC_{50} values for inhibition of glycine-activated currents, compared with the 10-fold difference in the K_d values for strychnine binding, may reflect the different techniques used to determine these values. The K_d value is an estimate of strychnine binding to a desensitized receptor at equilibrium, whereas the IC_{50} value is a measurement of a dynamic process, the inhibition of glycine-activated currents by strychnine. Thus, although the two measurements are related, they reflect different properties of the system and can therefore differ. A possible explanation for this contrast is that the D148E mutation may have affected the desensitization properties of the receptor. This appears unlikely, because cells expressing the D148E GlyR showed currents that desensitized in a manner similar to that observed for the wild-type GlyR (data not shown). Our results suggest that the D148E GlyR was not structurally altered and that the decrease in strychnine affinity is a reflection of the change in the amino acid residue. Thus, residue Asp-148 plays an important role in antagonist recognition. If nonconservative mutations could be expressed, an even greater decrease in strychnine affinity would be predicted.

Akagi *et al.* (22) have investigated the role of the two GlyR cysteine residues, required for the formation of the disulfide loop, by mutating each of them to alanine. However, those authors were able to detect only very small (<0.5% of control) glycine-activated currents and concluded that adequate expression of the altered subunits was not achieved. Similar studies of the corresponding cysteine residues of the nAChR α subunit have also been reported (23, 24). Again, in those studies the level of receptor expression was significantly reduced as a result of the mutations. Thus, it appears that the functional expression of the ligand-gated ion channels is particularly sensitive to mutations in the extracellular disulfide loop. It is interesting to note that Cockcroft *et al.* (13) simulated the structural effects of an alanine replacement at each residue in the disulfide loop and predicted that the integrity of the structural motif would not be compromised. Assuming that this is the case, the D148A and D148N mutations must have affected the way in which the loop associates with other regions of the receptor and, in so doing, prevented correct assembly.

Asp-148 provides a novel domain that contributes to the formation of the strychnine binding site. Additional residues identified as being involved in strychnine recognition are from two distinct domains of the extracellular portion of the GlyR and include Gly-160, Tyr-161, Lys-200, and Tyr-202 (7, 8). It is possible that the carboxyl group of the aspartic acid residue at position 148 may provide a negatively charged group to interact with the positively charged alkaloid strychnine. The D148E mutation retains the carboxyl group and thus still allows alkaloid binding, although with some decrease in affinity. Unfortunately, the lack of expression of the D148A and D148N GlyRs prevents more detailed investigation.

Molecular pharmacological studies of the various ligand-gated ion channel receptors have resulted in the identification of three major domains of ligand binding. Each of the sites identified in these domains has been mapped within a single subunit. However, for the nAChR, ligand binding is also mediated by the interaction of sites located on adjacent subunits (25, 26). In the nAChR, domain 1 includes residues Trp-86 and Tyr-93, domain 2 includes residues Trp-149 and Tyr-151, and domain 3 includes residues Tyr-190, Cys-192, Cys-193, and Tyr-198 (6, 27–30). GlyR antagonist binding site residues have previously been reported to be Gly-160 and Tyr-161 (7, 8), corresponding to domain 2 of the nAChR, and Lys-200 and Tyr-202 (8), corresponding to domain 3 of the nAChR. Additionally, a GlyR agonist binding site residue, Thr-204, is also located in domain 3 (9), whereas taurine-responsive residues have been mapped to Ile-111 and Ala-212 (31), being in close proximity to domains 1 and 3. In the $GABA_AR$, benzodiazepine binding sites have also been mapped to domains 1 and 3, being at His-101 (32) and Gly-201 (33). An additional ligand binding site is Phe-64 in the $GABA_AR$ (34). Because our results demonstrate that Asp-148 has a significant role in antagonist binding, we suggest that this residue defines a fourth domain of ligand interaction in this superfamily of receptors. As such, we predict that the disulfide loop motif may play a role in antagonist binding in other ligand-gated ion channel receptors.

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