Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1765–1769, March 1992
Neurobiology

Antagonism of ligand-gated ion channel receptors: Two domains of the glycine receptor α subunit form the strychnine-binding site

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Communicated by W. J. Peacock, November 21, 1991

ABSTRACT **The inhibitory glycine receptor (GlyR) is a member of the ligand-gated ion channel receptor superfamily. Glycine activation of the receptor is antagonized by the convulsant alkaloid strychnine. Using *in vitro* mutagenesis and functional analysis of the cDNA encoding the α1 subunit of the human GlyR, we have identified several amino acid residues that form the strychnine-binding site. These residues were identified by transient expression of mutated cDNAs in mammalian (293) cells and examination of resultant [^{3}H]strychnine binding, glycine displacement of [^{3}H]strychnine, and electrophysiological responses to the application of glycine and strychnine. This mutational analysis revealed that residues from two separate domains within the α1 subunit form the binding site for the antagonist strychnine. The first domain includes the amino acid residues Gly-160 and Tyr-161, and the second domain includes the residues Lys-200 and Tyr-202. These results, combined with analyses of other ligand-gated ion channel receptors, suggest a conserved tertiary structure and a common mechanism for antagonism in this receptor superfamily.**

The inhibitory action of glycine in the spinal cord and brain stem is generated through activation of the glycine receptor (GlyR), with subsequent opening of the integral chloride channel. The GlyR complex, isolated from rat spinal cord, consists of two distinct subunits with apparent molecular masses of 48 kDa (α) and 58 kDa (β), which associate to form a pentameric structure (for review, see ref. 1). Rat and human cDNAs encoding three different receptor α subunit subtypes and a β subunit have been cloned and sequenced (2–5). The predicted amino acid sequences and hydrophobicity profiles of these subunits are markedly similar to those of the γ-aminobutyric acid type A receptor ($GABA_AR$) subunits and also with the nicotinic acetylcholine receptor (nAChR) subunits, thus defining the ligand-gated ion channel receptor superfamily (2, 6, 7). The similarities among the various subunits of the GlyR, $GABA_AR$, and the nAChR include amino acid sequence identity of between 20 and 35% and predicted topological features, such as a large amino-terminal extracellular domain containing a highly conserved disulfide-bonded loop motif and four α-helical transmembrane domains, the latter forming the integral ion channel of this receptor superfamily.

Inhibitory glycinergic neurotransmission is antagonized by the convulsive alkaloid strychnine (8). Young and Snyder (9, 10) showed that [^{3}H]strychnine could bind with high affinity (K_d = 2–12 nM) (9, 11) to membranes from rat spinal cord. This binding was displaced by glycine, β-alanine, taurine, and β-aminoisobutyric acid. Although the binding sites for glycine and strychnine are mutually interactive or overlapping, they can be distinguished by their differential sensitivity to pH, ionic strength, and chemical modification of GlyRs (for review, see ref. 11). None the less, the molecular nature of strychnine antagonism of glycine-binding and action remains unclear.

UV light-induced photoaffinity-labeling of the GlyR with [^{3}H]strychnine shows specific, glycine-inhibited incorporation into the α subunit (12). Peptide mapping of the [^{3}H]strychnine photoaffinity-labeled α subunit indicates that the strychnine-binding site is located within the amino-terminal extracellular domain of the receptor. The site was initially mapped to residues 100–220 (2, 12), and recently Ruiz-Gomez *et al.* (13) have further localized the site of [^{3}H]strychnine photoaffinity-labeling to between residues 177 and 220.

Modification of tyrosine or arginine residues of the GlyR markedly decreases specific [^{3}H]strychnine binding (10, 13). Additionally, modification of lysine residues decreases the ability of glycine to displace specifically bound [^{3}H]strychnine, although lysine modification has been reported not to affect [^{3}H]strychnine binding to the receptor (14, 15). There are four tyrosine residues, located at positions 128, 161, 197, and 202, which may contribute to the formation of the strychnine-binding site (2). The presence of charged amino acids in the vicinity of Tyr-197 and Tyr-202 has led to the hypothesis that the strychnine-binding site is located between residues 190 and 202 and includes Tyr-197 and Tyr-202 (2, 13).

Evidence for other amino acid residues being involved in the formation of the strychnine-binding site has come from a comparison of the functional properties of rat and human neonatal GlyRs. Neonatal rats are "immune" (resistant) to strychnine poisoning (16), and Becker *et al.* (17) have identified an α subunit (α2) expressed only in neonates, which has low strychnine-binding activity. The lower strychnine affinity of the neonatal rat GlyR subunit, relative to the equivalent human subunit, has been attributed to a single amino acid change in the extracellular domain from glycine in the human GlyR to glutamate in the rat GlyR (18). This glycine/glutamate residue corresponds to Gly-160 in the human α1 subunit. Thus, residues in the vicinity of Gly-160 in the α1 subunit may also play a role in formation of the strychnine-binding site.

To more fully understand the mechanism of strychnine antagonism of glycine binding and channel activation, we have mapped the strychnine-binding site on the α1 subunit of the human GlyR. The approach used has been to selectively

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Abbreviations: GlyR, glycine receptor; nAChR, nicotinic acetylcholine receptor; $GABA_AR$, γ-aminobutyric acid type A receptor; DDF, (*N,N*-dimethyl)aminobenzenediazonium fluoroborate. For mutations, the letter preceding position number refers to the amino acid in the wild type; the letter after the number refers to the amino acid replacing the wild-type amino acid.

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mutate particular codons, corresponding to the selected amino acid residues, in the cDNA encoding the α1 subunit and then compare the functional properties, in terms of ligand binding and electrophysiological responses, of wild-type and mutated GlyR α1 subunits. We demonstrate that at least two separate domains of the α1 subunit contribute to formation of the strychnine-binding site. Specifically, mutation of residues Gly-160, Tyr-161, Lys-200, and Tyr-202 in the human GlyR α1 subunit markedly reduces strychnine antagonism of glycine-gated channel activation.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The cDNA encoding the α1 subunit of the human GlyR (4) was subcloned into the pCIS expression vector (19). Mutations in the cDNA were constructed by using the oligonucleotide-directed PCR mutagenesis method of Horton *et al.* (20) and were confirmed by sequencing the cDNA clones (21). The single-letter code for amino acids is used to describe mutations.

Expression of Mutated GlyR α1 Subunit cDNAs. Plasmid DNA encoding wild-type or mutated α1 subunits of the GlyR was transfected by using the method of Chen and Okayama (22) into exponentially growing 293 cells (adenovirus-transformed human embryonic kidney cells; ATCC CRL 1573) (19, 23). After 24 hr the cells were washed twice with culture medium (Eagle's minimum essential medium in Hanks' salts supplemented with 2 mM glutamine and 10% fetal calf serum) and refed.

[^{3}H]Strychnine-Binding Assays. Intact cells were harvested 48 hr after transfection by using phosphate-buffered saline/0.5 mM EDTA and washed three times in the same buffer without EDTA. Whole cells (1×10^6 cells) were incubated with [^{3}H]strychnine (0.1–100 nM), (23.7 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) with and without 10 mM glycine, to determine nonspecific binding. After incubation to equilibrium, 4°C for 60 min, the cells were collected by filtration on Whatman GF/B filter paper, with a Brandel cell harvestor, and washed three times with ice-cold phosphate-buffered saline. The radioactive strychnine remaining bound to the cells was determined by liquid scintillation counting with biodegradable scintillant (Amersham). Displacement of 1 nM [^{3}H]strychnine by strychnine (0–100 μM) and glycine (0–10 mM) was determined by using the same procedure.

Electrophysiological Measurements. The standard bath perfusion solution contained 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. Electrophysiological recording of glycine-activated currents was done with the tight-seal patch-clamp in the whole-cell recording configuration (24), using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Transfected cells were grown on 1-cm round coverslips and then placed in a small volume chamber (0.6 ml capacity) continuously superfused with the standard bath perfusion solution, at a rate of ≈2 ml/min. Solutions containing agonists or antagonists could be rapidly passed through the chamber for bath application. Glycine (3 mM) in the standard bath perfusion solution was applied via a 0.25-mm (i.d.) plastic microperfusion tube positioned ≈0.1 mm from the cell being recorded. An electrically operated valve allowed rapid application of the glycine (<0.5 s) via the perfusion tube. Patch pipettes of resistance 200 kΩ–1 MΩ were filled with a solution usually containing 120 mM KF, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, and 10 mM Hepes. In later experiments the patch pipette was filled with a solution in which the KF and KCl were replaced by 145 mM CsCl, so that the intracellular and extracellular chloride concentrations were equal. Series resistance compensation was not used because of the relatively low amplitude of the currents (typically < 1 nA) and the low resistance of the patch pipettes.

RESULTS

Expression of cloned rat or human GlyR α subunits in *Xenopus* oocytes or in the mammalian 293 cell line yields functional, homomeric, glycine-gated chloride channels, which show strychnine-dependent antagonism of glycine-activated currents, typical of GlyRs found in rat spinal cord (3, 4, 23). Thus, analysis of homomeric channels not only provides a very good approximation to the native receptor but also provides a much simpler system to study in comparison with native multimeric ligand-gated ion channel receptors. Accordingly, the wild-type human GlyR α1 subunit cDNA was transfected into 293 cells, [^{3}H]strychnine binding was measured, and electrophysiological recordings were made to establish a basis for comparisons with the mutated subunits. The wild-type α1 subunit cDNA expressed in 293 cells bound [^{3}H]Strychnine with a dissociation constant (K_d) of 28 ± 2 nM and B_{max} of 3.7×10^5 [^{3}H]strychnine molecules bound per cell (Fig. 1). This B_{max} value represents an average number of receptors on all cells. The transfection efficiency was ≈10–40%; thus, the number of [^{3}H]strychnine-binding sites on expressing cells is ≈2.5- to 10-fold higher than the value for the B_{max}. [^{3}H]Strychnine was displaced by glycine with an IC_{50} of 65 ± 11 μM (see Fig. 3*A*). Corresponding K_d values for strychnine binding to rat spinal cord membranes have been reported in the range 2–12 nM (9), and IC_{50} values for glycine displacement of [^{3}H]strychnine binding on membrane receptors are in the range of 2–40 μM (for review, see ref. 11). Homomeric human α1 GlyRs, expressed in oocytes, show strychnine inhibition (K_i = 16 ± 2 nM) of glycine-activated currents (EC_{50} = 290 ± 20 μM) (4). The higher concentration of glycine required for 50% inhibition of [^{3}H]strychnine binding we observed may reflect a lower affinity of glycine for the homomeric α1 GlyR than to rat brain-membrane GlyRs, consistent with the reported EC_{50} values (4). Whole-cell recordings of 293 cells transfected with the human α1 subunit cDNA demonstrated activation of an inward current, at a holding potential of −100 mV, in response to 3 mM glycine. Measurements of the reversal potential of the currents in equal concentrations of NaCl in the bath and CsCl in the pipette gave potentials near zero, consistent with the channels being selective to Cl^-, as expected. These currents were blocked by 1 μM strychnine (Fig. 2). Application of glycine to mock-transfected cells failed to activate inward currents.

Mutation of Gly-160. To confirm and extend the observations of Kuhse *et al.* (18) we analyzed responses of the human α1 mutation G160E (Gly-160 → Glu). High-affinity

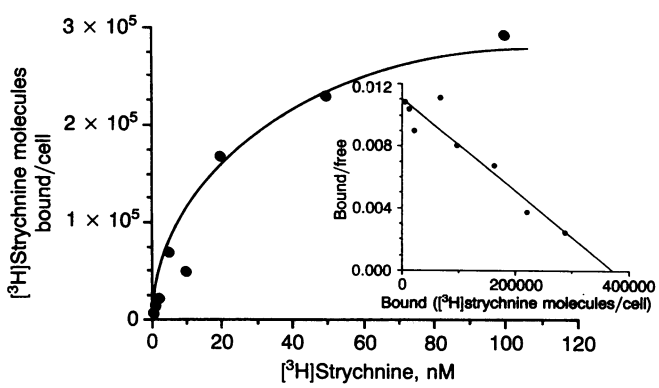

FIG. 1. Saturation isotherm of [^{3}H]strychnine binding to 293 cells transfected with the wild-type α1 subunit. (*Inset*) Corresponding Scatchard plot.

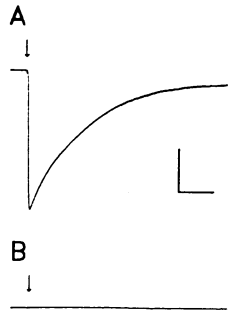

FIG. 2. Effect of strychnine on glycine-evoked currents in 293 cells expressing the wild-type α1 subunit. (*A*) Glycine (3 mM) was applied by perfusion tube to a transfected cell, voltage clamped at −100 mV (start of pulse marked by arrow), resulting in a large inward current that appeared to rapidly desensitize. (*B*) Addition of 1 μM strychnine in the recording chamber abolished the response to glycine at the same holding potential and in the same cell. (Vertical bar = 200 pA; horizontal bar = 2 s in *A* and 1.4 s in *B*.)

[^{3}H]strychnine binding to 293 cells transfected with human α1 G160E cDNA was not detected. This may be due to either reduced affinity of [^{3}H]strychnine for the receptor or inefficient expression of the receptor protein. To demonstrate the presence of functional receptors we measured chloride current activation by glycine. Whole-cell recordings revealed an inward current at a holding potential of −100 mV upon application of 3 mM glycine. Measurement of the reversal potential for this and all other mutated subunits, under similar conditions as for the wild-type homomeric α1 GlyR, gave potentials near zero, showing that the channels are selective for Cl^-. This current was not blocked by 1 μM strychnine, but 100 μM strychnine inhibited current flow (Table 1), which is consistent with the inability to detect high-affinity [^{3}H]strychnine-binding sites on cells transfected with the G160E α1 GlyR. Thus, these results extend the conclusions of Kuhse *et al.* (18) and indicate that Gly-160 in the human α1 subunit may play a role in formation of the strychnine-binding site. The Gly-160 residue was also mutated to alanine so as to minimize structural and conformational changes (26) that may influence strychnine binding. This mutation did not affect the affinity of [^{3}H]strychnine or the IC_{50} of glycine to displace [^{3}H]strychnine bound to the receptor (Fig. 3*B*). This result suggests that the G160E mutation is insensitive to strychnine binding due to either the steric or charge effects of the glutamic acid substitution.

Mutation of Tyr-161. All remaining site-directed mutations involved changing the selected amino acid residue to alanine to minimize any perturbations of secondary structure (26) that may affect the correct assembly and expression of receptors on the surface of the 293 cells. High-affinity [^{3}H]strychnine binding to cells expressing mutant Y161A (Tyr → Ala) was not detected. This result suggests either that Tyr-161 plays a role in formation of the binding site for strychnine or, alternatively, that this particular tyrosine residue, which is invariantly conserved among all subunits of the ligand-gated ion channel receptor superfamily, plays a critical role in assembly of the receptor, and the Y161A mutation may have disrupted correct protein folding. This second possibility was eliminated by results from whole-cell recordings of 293 cells transfected with the Y161A subunit cDNA. Application of 3 mM glycine activated an inward chloride current, showing functional GlyRs. Strychnine at 1 μM had no effect on current flow, and 100 μM strychnine was required to completely inhibit the current (Table 1). These results are very similar to those obtained at the adjacent G160E mutation. Thus, α1 subunits of the GlyR with mutations G160E and Y161A may be activated by glycine, yet these subunits are relatively insensitive to strychnine. Strychnine antagonism of glycine is only seen at significantly higher concentrations than those required for antagonism of glycine responses on the wild-type α1 subunit of the GlyR. Thus, residues Gly-160 and Tyr-161 of the GlyR α1 subunit define a domain involved in mediating strychnine binding.

Table 1. Current responses to glycine application to 293 cells transfected with wild-type and mutated α1 subunits of the GlyR

Wild-type or mutated GlyR	Current range,* pA	Strychnine concentration required for inhibition,† μM
Wild type	505–1500	1
G160E	166–900	100
Y161A	210–2300	100
Y197A	360–1080	1
K200A	100–1500	100
Y202A	220–590	100

Transfected cells were analyzed under voltage clamp (−100 mV) in the whole-cell mode. Inward current flow from four to six transfected cells was measured in response to 3 mM glycine.

*Current flow depends upon transfection efficiency (receptor density per cell). As each recording is from a separately transfected cell, a broad range of responses is seen (25).

†Only two concentrations of strychnine, 1 μM and 100 μM, were tested.

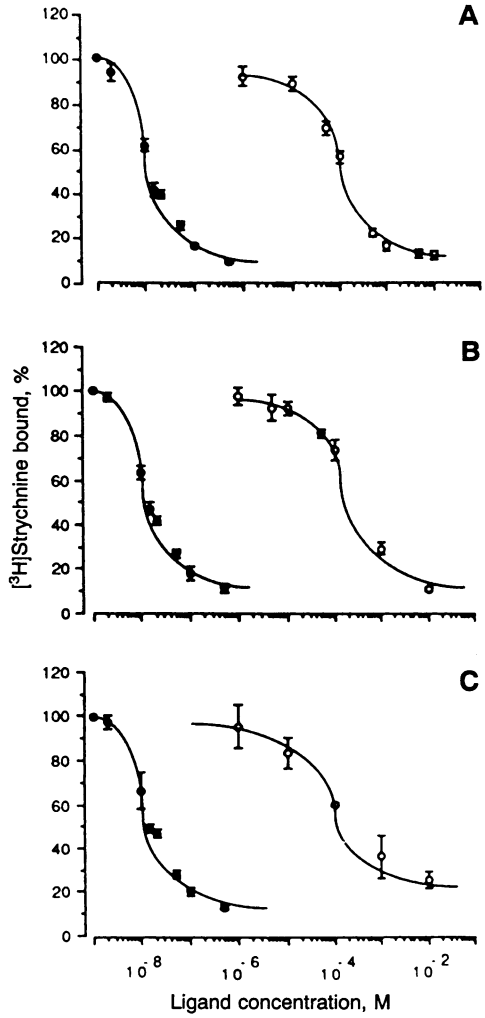

FIG. 3. Displacement of [^{3}H]strychnine from the wild-type α1 subunit of the GlyR (*A*), the G160A α1 subunit (*B*), and the Y197A α1 subunit (*C*) by strychnine (●) and glycine (○).

Mutation of Tyr-197 and Tyr-202. Both Grenningloh *et al.* (2) and Ruiz-Gomez *et al.* (13) have hypothesized that the strychnine-binding site is located between residues 190 and 202 of the GlyR and includes Tyr-197 and Tyr-202. We therefore constructed the α1 subunit mutations Y197A and Y202A (Tyr → Ala). The Y197A mutation mirrored the responses of the wild-type α1 subunit, in terms of strychnine

displacement of [^{3}H]strychnine (IC_{50} = 16 ± 4 nM), glycine displacement of [^{3}H]strychnine (IC_{50} = 87 ± 45 μM) (Fig. 3*C*), inward chloride current flow in response to glycine application, and blockade of such currents by 1 μM strychnine (Table 1). Thus, Tyr-197 is unlikely to be involved in the recognition or binding of strychnine. Expression of the Y202A mutated α1 subunit cDNA resulted in the loss of high-affinity binding sites for [^{3}H]strychnine. However, inward chloride currents were detected upon application of 3 mM glycine to the transfected cells (Table 1). The currents were not inhibited by 1 μM strychnine, and 100 μM strychnine was required to completely block the current.

Mutation of Lys-200. Lys-200 is the only charged residue located between Tyr-197 and Tyr-202. Modification of lysine residues has been reported to decrease the ability of glycine to displace specifically bound [^{3}H]strychnine but not to affect [^{3}H]strychnine binding (14). Thus, the K200A (Lys → Ala) mutation was constructed, and the mutated cDNA was expressed. Unexpectedly, the K200A α1 subunit did not form high-affinity [^{3}H]strychnine-binding sites. However, electrophysiological analysis showed that, as with the Y202A mutation, the GlyRs formed with the K200A α1 subunits passed inward chloride currents in the presence of 3 mM glycine, showing functional GlyRs. The currents were not inhibited by 1 μM strychnine but were completely blocked by 100 μM strychnine (Table 1). Thus, Lys-200 and Tyr-202 define a second domain required for formation of the strychnine-binding site on the α1 subunit of the GlyR.

DISCUSSION

Mutational analysis of the α1 subunit of the GlyR demonstrates that two distinct domains of the receptor contribute to the formation of the high-affinity strychnine-binding site. Mutation of amino acid residues Gly-160, Tyr-161, Lys-200, and Tyr-202 destroys high-affinity strychnine binding. However, the subunits, assembled as a receptor complex, remained sensitive to glycine and conducted chloride ions through the receptor channel. This suggests that each mutation either sterically inhibits strychnine binding (e.g., Gly-160) or removes the specific chemical groups that determine strychnine affinity (e.g., Tyr-161, Tyr-202, and Lys-200).

A role for tyrosine residues in forming the strychnine-binding site has been suggested. The mechanism of photoaffinity-labeling seems to involve energy transfer from aromatic amino acids, such as tyrosine (12). In addition, Ruiz-Gomez *et al.* (13) have suggested that the nitrogen at position 19 and the carbonyl group at position 10 of strychnine are important binding-site determinants and that these functional groups may interact with tyrosine or arginine residues. [^{3}H]Strychnine does not photoaffinity-label Tyr-161 (13) and, from our results, Tyr-197 is not involved in strychnine binding; therefore, we conclude that Tyr-202 is the residue that is the [^{3}H]strychnine photoaffinity-labeling site of the GlyR α1 subunit. This conclusion is validated by the mutational analysis, which shows that conversion of Tyr-202 to an alanine abolishes high-affinity strychnine binding while not altering formation of functional glycine-gated receptor ion channels. Identification of Tyr-202 as part of the strychnine-binding site may provide the basis for determining the relative orientation of the strychnine molecule when bound to the GlyR. Such modeling could be useful for predicting additional interactions between the GlyR α subunit and strychnine.

Identification of Tyr-161 as forming part of the strychnine-binding site is unexpected. This residue forms part of the disulfide-bonded loop motif and is conserved among all subunits of all members of the ligand-gated ion channel receptor superfamily (2, 6, 7). For such an invariant residue, a basic structural or functional role, common to all receptor subunits, would be expected; yet mutation of this residue to alanine does not prevent ligand (glycine) activation of the receptor, a central feature of this receptor superfamily. Examination of the effects of similar mutations in other ligand-gated ion channel receptors, should, therefore, be of interest.

Glycine-induced currents seen in transfected cells expressing the GlyR mutants G160E, Y161A, K200A, and Y202A are blocked by high concentrations of strychnine (100 μM). This indicates that strychnine can still interact with these receptors, albeit with lower affinity than for the wild-type homomeric α1 receptors. From these observations, the interactions between strychnine and the GlyR would appear to involve a number of specific molecular contacts, the alter-

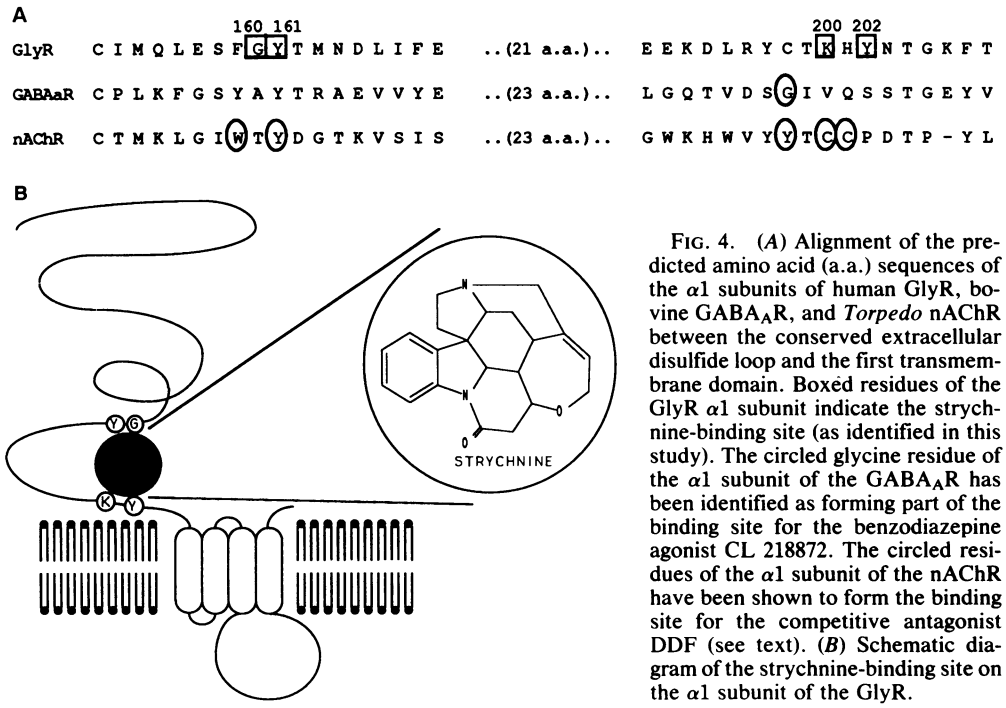

FIG. 4. (*A*) Alignment of the predicted amino acid (a.a.) sequences of the α1 subunits of human GlyR, bovine $GABA_AR$, and *Torpedo* nAChR between the conserved extracellular disulfide loop and the first transmembrane domain. Boxed residues of the GlyR α1 subunit indicate the strychnine-binding site (as identified in this study). The circled glycine residue of the α1 subunit of the $GABA_AR$ has been identified as forming part of the binding site for the benzodiazepine agonist CL 218872. The circled residues of the α1 subunit of the nAChR have been shown to form the binding site for the competitive antagonist DDF (see text). (*B*) Schematic diagram of the strychnine-binding site on the α1 subunit of the GlyR.

ation of any one of these leading to decreased affinity and specificity.

Despite identifying two domains involved in forming the strychnine-binding site on the α subunit of the GlyR, we have not identified residues involved in glycine recognition. All mutants described could mediate glycine-activated inward currents in cells expressing the subunits. Chemical modification of lysine residues on the GlyR does not alter [^{3}H]strychnine binding but does destroy the capacity of glycine to displace [^{3}H]strychnine (10, 15). Lys-200 is unlikely to be involved in glycine binding, as the K200A mutant was still responsive to glycine. It may, therefore, be concluded either that other lysine residues are involved in glycine recognition, such as lysine residues at positions 190, 193, or 206, or that chemical modification of lysine residues affects a number of residues—thus altering conformation of the glycine-binding site(s) and preventing glycine displacement of strychnine.

Location of the strychnine-binding site at residues 160, 161, 200, and 202 within the extracellular domain of the GlyR closely corresponds to the location of residues in the α subunit of the nAChR, which form the binding site for the competitive antagonist (*N,N*-dimethyl)aminobenzenediazonium fluoroborate (DDF; 27–29). DDF has been covalently incorporated into the nAChR through UV light-induced photoaffinity-labeling, and the residues labeled include Tyr-93, Trp-149, Tyr-151, Tyr-190, Cys-192, and Cys-193. Alignment of the amino acid sequences of the GlyR α subunit and the nAChR α subunit (Fig. 4*A*) shows that the strychnine-binding sites on the GlyR correspond to the residues labeled by DDF on the nAChR. Thus, the binding sites for antagonists on the GlyR and the nAChR would appear to be formed by residues in similar positions within the extracellular domain (Fig. 4). This hypothesis suggests that the amino acid residues in this region form similar tertiary structures and a common mechanism for antagonism of these receptors exists. This finding further reinforces the notion that these receptors form a superfamily of ligand-gated ion channel receptors with related tertiary structures and functional properties (Fig. 4). An extension of this idea of similar structural and functional units within this family of receptors would predict that residues in corresponding positions on the $GABA_A$ subunits may play a role in formation of binding sites for competitive GABA antagonists.

Analysis of the α1 and α3 subunits of the $GABA_AR$ has identified an amino acid residue that determines the affinity of the benzodiazepine-like agonist CL 218872 (30). $GABA_ARs$ containing the α1 subunit (type I benzodiazepine receptors) have a dissociation constant of 108 nM for CL 218872, whereas $GABA_ARs$ containing the α3 subunit (type II benzodiazepine receptors) have a 10-fold lower affinity. Replacement of Glu-225 in the α3 subunit with a glycine residue, which is the corresponding residue in the α1 subunit, and expression of this mutated cDNA in combination with the β2 and γ2 subunits of the $GABA_AR$, generates $GABA_ARs$ with type I benzodiazepine receptor pharmacology. Alignment of the amino acid sequences of the α1 subunit of the $GABA_AR$ and the α1 subunit of the nAChR (Fig. 4*A*) shows that Gly-201 (Glu-225 in the α3 subunit) corresponds to Tyr-200 in the α1 subunit of the nAChR. The coincident location of the Gly-201 residue to one domain of the strychnine-binding site further suggests that residues in this region of all members of the ligand-gated ion channel receptor superfamily form a pocket that is accessible to various ligands (both antagonists and allosteric modulators) and plays an important role in the transduction of ligand binding into channel opening (Fig. 4*B*). This observation opens the experimental and therapeutic possibility that allosteric modulators of the GlyR, and other ligand-gated ion channel receptors, may be developed.

We acknowledge the technical assistance of Vicki Falls, Marjorie Liu, and Cheryl Handford and advice of Tiina Iismaa. This work was supported by grants from the Cystic Fibrosis Foundation (U.S.A.) and the Australian National Health and Medical Research Council.

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