Amino Acid Volume and Hydropathy of a Transmembrane Site Determine Glycine and Anesthetic Sensitivity of Glycine Receptors*

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Two specific amino acid residues in transmembrane segments (TM) 2 and 3 are critical for the enhancement of glycine receptor (GlyR) function by volatile anesthetics. To determine which physicochemical characteristics of these sites determine their roles in anesthetic actions, an extensive series of single amino acid mutations at amino acid residue 288 (Ala-288) in TM3 of the α1 GlyR subunit was tested for modulation by volatile anesthetics. The mutations changed the apparent affinities of receptors for glycine; replacements with larger volumes and less hydropathy exhibited higher affinities for glycine. Potentiation by anesthetics was reduced by specific mutations at Ala-288. The molecular volume of the substituents was negatively correlated with the extent of potentiation by isoflurane, enfurane, and 1-chloro-1,2,2-trifluorocyclobutane, whereas there was no correlation between anesthetic enhancement and polarity, hydropathy, or hydrophilicity of substituents. In contrast to anesthetics, no correlation was found between the effects of the nonanesthetics 1,2-dichloro-octafluorobutane, whereas there was no correlation between anesthetic enhancement and polarity, hydropathy, or hydrophilicity of substituents. In contrast to anesthetics, no correlation was found between the effects of the nonanesthetics 1,2-dichloro-octafluorobutane, whereas there was no correlation between anesthetic enhancement and polarity, hydropathy, or hydrophilicity of substituents. In contrast to anesthetics, no correlation was found between the effects of the nonanesthetics 1,2-dichloro-octafluorobutane, whereas there was no correlation between anesthetic enhancement and polarity, hydropathy, or hydrophilicity of substituents. In contrast to anesthetics, no correlation was found between the effects of the nonanesthetics 1,2-dichloro-octafluorobutane, whereas there was no correlation between anesthetic enhancement and polarity, hydropathy, or hydrophilicity of substituents.

The mechanisms of general anesthesia still remain unclear. During the past few years, however, a consensus has emerged that general anesthetics act on the ligand-gated ion channel superfamily. Among them, the neurotransmitter receptors for the inhibitory amino acids γ-aminobutyric acid (GABA) and glycine were intensively studied as likely targets of general anesthetics in the brain and spinal cord (1, 2).

The GlyR is composed of two families of subunits, the α (α1–α4) and β subunits (3–5). In adult brains, the α1 and β subunits are expressed not only in the brainstem and spinal cord but also in higher brain regions such as the cerebral cortex, hippocampus, and cerebellum. The native GlyR is considered to assemble in a pentameric structure with a proposed subunit stoichiometry of 3α1 and 2β subunits, but when expressed in Xenopus oocytes or mammalian cells, the α1 subunit can also form highly active homomeric GlyR, which has most of the pharmacological properties of the native GlyR. Each GlyR subunit has four putative transmembrane (TM) segments with the second (TM2) proposed to form the lining of the pore. This transmembrane topology is also believed to exist in other ligand-gated ion channels such as GABA<sub>Α</sub> receptors and nicotinic acetylcholine receptors.

Recombinant GlyR function is enhanced by volatile anesthetics (isoflurane, enfurane, halothane, sevoflurane, methoxyflurane, and 1-chloro-1,2,2-trifluorocyclobutane (F3)) (6–8) as well as alcohols (6, 9) but not by the nonanesthetic 1,2-dichloro-octafluorocyclobutane (F6) (6). Recently, site-directed mutagenesis revealed that two specific amino acid residues in TM2 and TM3 (Ser-267 and Ala-288 for the α1 GlyR subunit, respectively) are critical for the enhancement of both the GlyR and GABA<sub>Α</sub> receptors by volatile anesthetics and alcohols (10). In the GABA<sub>Α</sub> receptor, corresponding or nearby amino acid residues in TM2 and TM3 of the β subunits also govern modulation by the intravenous anesthetics etomidate, pentobarbital, and propofol (11–14). However, the physicochemical characteristics that determine their critical role in anesthetic actions are not known. Although the Meyer-Overton rule described a correlation between anesthetic potency and lipid solubility (15), the physicochemical nature of the anesthetic site of action is not necessarily hydrophobic. Some investigations suggest hydrophilic, polar, or amphipathic properties of the anesthetic target site (16–19). In this investigation, we constructed an extensive series of replacements of Ala-288 in TM3 of the α1 GlyR subunit by other amino acid residues with a variety of physicochemical properties and have examined the effects of each mutation on modulation by volatile anesthetics isoflurane, enfurane, and F3, and nonanesthetics F6 and 2,3-dichloro-octafluorocyclobutane (F8).

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis of the human α1 GlyR subunit cDNA (20) in the modified pBK-CMV vector (pBK-CMV-NB-200) (21) was performed by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Degenerate complementary oligonucleotides were used for the simultaneous creation of multiple mutants. All mutations were verified by partial sequencing of both plasmid strands.

Oocyte Expression—Preparation of Xenopus laevis oocytes and microinjection of the cDNA was performed as described previously (22, 23). Isolated oocytes were placed in modified Barth’s saline (MBS) containing (in mM): NaCl 88, KCl 1, HEPES 10, MgSO<sub>4</sub> 0.82, NaHCO<sub>3</sub> 2.4, CaCl<sub>2</sub> 0.91, and Ca(NO<sub>3</sub>)<sub>2</sub> 0.33 adjusted to pH 7.5. Wild-type and
TABLE I
The EC<sub>50</sub> and Hill coefficient values and the maximal current for the wild-type and mutant α1 GlyRs

| α1 GlyR | EC<sub>50</sub> (μM) | Hill coefficient | I<sub>max</sub> (pA) |
|---------|----------------|-----------------|------------------|
| A288Y   | 1.0            | 1.1             | 800–12,000       |
| A288K   | 1.4            |                 | 800–14,000       |
| A288E   | 8.3            | 1.5             | 1,200–18,500     |
| A288G   | 14             | 1.7             | 1,600–25,000     |
| A288Q   | 31             | 1.4             | 1,800–35,000     |
| A288M   | 37             | 2.2             | 1,600–35,000     |
| A288H   | 68             | 2.4             | 1,300–20,000     |
| A288N   | 70             | 2.0             | 2,400–28,000     |
| A288I   | 76             | 1.5             | 1,700–25,000     |
| A288L   | 154            | 1.5             | 2,200–19,500     |
| A288T   | 195            | 1.5             | 1,000–25,000     |
| A288V   | 243            | 1.7             | 1,300–22,000     |
| A288S   | 263            | 1.3             | 1,700–20,500     |
| Wild-type | 333            | 2.1             | 1,800–34,000     |
| A288C   | 874            | 2.3             | 2,300–25,000     |
| A288F   | 1015           | 2.5             | 11,000–42,500    |

The EC<sub>50</sub> and Hill coefficient values and the maximal current for the wild-type and mutant α1 GlyRs. Concentration-response curves for glycine were examined for the wild-type and mutant α1 GlyRs (Fig. 1). The α1-A288F, A288R, A288D, and A288W receptors are not included because of little or no current response to glycine. The data were normalized to the maximal current in each oocyte, and fitted according to the equation of the form: $I = I_{\text{max}}/[1 + (EC_{50}/A)^n]$, where $A$ is current, $I_{\text{max}}$ is the maximal current recorded in a given oocyte, EC<sub>50</sub> is the glycine concentration for half-maximal current response, and $n$ is the Hill coefficient. The EC<sub>50</sub> and Hill coefficient values were obtained for each receptor from 4 to 8 oocytes taken from at least two different frogs, and $I_{\text{max}}$ represents the lowest and highest maximal current recorded from all oocytes used in this investigation.

Fig. 1. Correlation of physicochemical properties of the mutated amino acids at position 288 of the α1 subunit with the EC<sub>50</sub> values for glycine. Panel A, concentration-response curves for glycine of the wild-type and mutant α1-A288Y, A288F, A288Q, A288L, and A288F receptors. Each point represents the mean ± S.E. of the normalized current from 4–8 oocytes. The theoretical curves have been drawn according to the equation $I = I_{\text{max}}/[1 + (EC_{50}/A)]$, where $A$ is current, $I_{\text{max}}$ is the maximal current recorded in a given oocyte, EC<sub>50</sub> is the glycine concentration for half-maximal current response, and $A$ is the glycine concentration, and $n$ is the Hill coefficient. Panels B–E, correlations of the volume ($\text{panel B}$), polarity ($\text{panel C}$), hydropathy ($\text{panel D}$), or hydrophilicity ($\text{panel E}$) of mutated amino acids with the log(EC<sub>50</sub>)(μM) values for glycine. The obtained log EC<sub>50</sub> value of each GlyR was correlated with the volume (40), polarity (41), hydropathy (28), and hydrophilicity (42) of the substituted amino acid residues. For the hydropathy of the amino acid residues, the slope of linear regression (dashed) was significantly different from 0 (p < 0.02) with the r<sup>2</sup> value of 0.29. No significant correlation was noted for the volume, polarity, or hydrophilicity. Panel F, correlations of the combination of volume and hydropathy of the substituted amino acid residues with the log EC<sub>50</sub> values for glycine. The log EC<sub>50</sub> value for glycine of each GlyR was plotted against the volume and hydropathy of the amino acid residues. The best fitting regression plane of the equation form: $z = 2.96 + (-0.013) \times x + 0.179 \times y$, where $x$, $z$, and $y$ represent the log EC<sub>50</sub>, the scale value of volume and hydropathy of the substituted amino acids, respectively, is shown. The filled or shaded circles represent the point above or below the regression plane, respectively. See Tables 1 and 2 for values calculated from these figures.

RESULTS
Affinities of the Mutant GlyRs for Glycine—The homomeric GlyRs composed of the α1 subunit mutated at Ala-288 displayed glycine-activated currents after expression in Xenopus oocytes, except for those substituted with proline, arginine, aspartate, or tryptophan (α1-A288P, A288R, A288D, and A288W).

Mutant α1 GlyR subunits cDNAs (0.4 ng in 30 nl) were injected into the animal poles of oocytes by the “blind” method of Colman (24) using a digital microdispenser (Drummond Scientific, Broomall, PA) loaded with a micropipette (10–15 mm tip size). The injected oocytes were singly placed in Corning cell wells (Corning Glass Works, Corning, NY) containing incubation medium (sterile MBS supplemented with 10 mg/liter streptomycin, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, and 220 mg/liter pyruvate) and incubated at 15–19 °C. On days 1–4 after injection, oocytes were used in electrophysiological recording.

Electrophysiological Recording—Oocytes were placed in a rectangular chamber (approximately 100 μl volume) and perfused (2 ml min<sup>−1</sup>) with MBS with or without drugs, via a roller pump (Cole-Parmer Instrument Co., Berkeley, CA). Analyses were carried out using StatView software (Abacus Concepts Pad Software Inc., San Diego, CA), and linear and multiple regression analyses were carried out using StatView software (Abacus Concepts Inc., Berkeley, CA).
A288W, respectively), which produced little or no current in response to glycine (10 mM). However, the α1-A288D and A288W receptors produced outward currents when tested with 100 μM of the chloride-channel blocker picrotoxin, consistent with closure of channels that are tonically open in the absence of glycine. To determine the effects of mutations at Ala-288 on the apparent affinities for glycine, dose-response relationships for glycine of the wild-type and mutant GlyRs were examined (Table I, Fig. 1A). The mutant receptors showed a wide range of apparent affinities for glycine. Substitution of Ala-288 with tyrosine (α1-A288Y) produced a receptor with the lowest EC50 value (1.0 μM) among all GlyRs, whereas replacement by phenylalanine (α1-A288F) exhibited the highest EC50 value (1015 μM). The mutant receptors with lower EC50 values (α1-A288Y, A288K, A288E, and A288G) produced small outward currents (100–800 nA) by 100 μM of picrotoxin. We analyzed the correlations between physicochemical properties (volume, polarity, hydropathy, and hydrophilicity) of the mutated amino acids and the EC50 values for glycine of the resultant mutant GlyRs (Table II, Fig. 1, B–E). For the hydropathy of the amino acid residues, the slope of linear regression was significantly different from 0 (p < 0.02) with the r2 value of 0.29. The hydropathy is a measure in which the hydrophilic and hydrophobic properties of each amino acid side chain are taken into consideration (28). No significant correlation was noted for the volume, polarity, or hydrophilicity. However, multiple regression analysis showed that the fitting of regression for the log EC50 was improved by combining the volume and hydropathy as two independents (p < 0.02, r2 = 0.37), with regression coefficient values of −0.013 and 0.179, respectively (Table II, Fig. 1F). The importance of both volume and hydropathy in determining the EC50 values is especially indicated by the results of the α1-A288Y and A288F receptors. The EC50 values for glycine were drastically different between mutations with tyrosine (Y) and phenylalanine (F) which have large molecular volume and only differ in one hydroxyl group, whereas EC50 values were not so different between mutations with serine (S) and alanine (A), which also differ in a hydroxyl group but have small molecular volume.

**Volatility by Anesthetics of Multiple Mutants at Alanine-288**—To determine whether size, polarity, hydropathy, or hydrophilicity of the mutated amino acid residues dictated the ability of volatile anesthetics to potentiate GlyR function, effects of anesthetics and nonanesthetics (nonimmobilizers) on the wild-type and mutant GlyRs were examined. Each compound was tested at concentrations corresponding to 2 MAC, and was preapplied before being coapplied with glycine. Preapplication of compounds in the absence of glycine did not produce any current response in both wild-type and mutant GlyRs, except for the α1-A288W receptor, which exhibited an outward current by application of isoflurane alone as described previously (10). Because mutant receptors showed a wide range of apparent affinities for glycine, we standardized the experiment by using an EC5 concentration of glycine in each case, i.e. a concentration of glycine that gave 5% of the maximal response obtained in that mutant receptor. The wild-type GlyR was effectively potentiated by volatile anesthetic isoflurane, whereas mutant receptors at Ala-288 generally exhibited less potentiation by isoflurane. The correlations of the isoflurane potentiation with physicochemical properties of the mutated amino acid side chains or the EC50 value of each receptor were analyzed (Fig. 2). We found a significant inverse correlation between the volume of amino acid side chain and the extent of potentiation by isoflurane. The slope of linear regression for the volume of the amino acid residues was significantly different from 0 (p < 0.0001) with r2 = 0.73. On the other hand, no correlation was found between isoflurane responsiveness and the polarity, hydropathy, hydrophilicity of the amino acid residues, or log EC50 values for glycine of mutant receptors. The fitting of regression for the potentiation by isoflurane was not improved by any combination of two properties of amino acid residues.

Similarly, the extent of potentiation by enfurane and F3 was significantly correlated with the volume of amino acid side chain (p < 0.0001, r2 = 0.75 for both enfurane and F3) but not with the polarity, hydropathy, hydrophilicity, or log EC50 values for glycine (Figs. 3 and 4).

Nonanesthetics F6 and F8 had only slight effects on the wild-type α1 GlyR as well as on the mutant receptors except for the α1-A288F mutant on which F6 and F8 had strong inhibitory effects (Figs. 5 and 6). Unlike anesthetics, the effects of F6 and F8 were not correlated with any physical properties of the amino acid residues or log EC50 values for glycine of mutant receptors.

Fig. 7 shows correlations of the extent of potentiation of each mutant receptor between anesthetics/nonanesthetics. The extent of potentiation by isoflurane was highly correlated with those by enfurane and F3 (p < 0.0001, r2 = 0.93, and p < 0.0001, r2 = 0.96, respectively), whereas no significant correlation was found between F3 and F6 (p > 0.12, r2 = 0.16).

**DISCUSSION**

In this investigation, we found that a series of single amino acid replacements at Ala-288 in the TM3 of the α1 GlyR subunit reduced the extent of potentiation of the resulting GlyRs by volatile anesthetics. Furthermore, we found that the molec-
The mutations of Ala-288 significantly affected the apparent affinity for glycine. Because the agonist binding site of the GlyR subunit is proposed to reside in the aminoterminal domain (3–5), altered apparent affinities of the mutant receptors for glycine may imply that amino acid residue 288 is involved in the transduction of agonist binding into channel opening but not agonist binding per se. Similarly, mutations of a critical amino acid residue Ser-270 in TM2 of the α2 GABA<sub>A</sub> receptor subunit (which corresponds to Ser-267 in TM2 of the α1 GlyR subunit) alter channel gating (30). Thus, both critical amino acid residues in TM2 and TM3 might reside on the transduction pathway responsible for converting agonist binding energy into channel opening. Furthermore, the efficacies of mutants in increasing glycine affinities depend on the volume and hydrophathy of the substituted amino acid residues; residues with larger volume and less hydrophathy exhibit higher affinities for glycine. The volume of the amino acid residue is also linked to channel gating at position 270 in TM2 of the α2 GABA<sub>A</sub> receptor subunit; mutants with a larger volume of side chains had higher affinities for glycine (30). Because anesthetics increase the apparent affinity for glycine. In this context, physicochemical properties of side chains might reflect characteristics necessary
for anesthetics to exert their effects after binding to the GlyR, i.e. larger volume and less hydrophathy appear to be necessary for anesthetics after binding. This hypothesis is supported by the comparison of the molecular volume of isoflurane or F3 (140 or 127 Å³, respectively) with those of side chains of large amino acids such as tyrosine or tryptophan (side chain volumes 133 or 127 Å³, respectively) (31). Furthermore, the importance of a hydrophatic component of anesthetics is consistent with the reports that complete halogenation or full halogenation of end methyl groups of alkanes and ethers tends to decrease the anesthetic potencies of these agents and to enhance convulsant activity (32–35).

In this context, it is of interest to compare the structurally related compounds F3 and F6. Both F3 (c(CClF_CC_F2CH2CH2)) and F6 (c(CClFCCIFCF2CF2)) are polyhalogenated cyclobutane derivatives soluble in lipids and should be potent anesthetics according to the Meyer-Overton hypothesis. However, only F3 produces anesthesia in vivo (25). The wild-type α1 GlyR was potentiated by F3 but not by F6, indicating that the GlyR is a rational target for anesthetic actions. Because of the position of halogenation, F3 is considered to possess less hydrophathy than F6 (36, 37). Thus, the distinct action on the GlyR between F3 and F6 may be at least partly explained by their differences in hydrophathy.

Some of the mutations, especially the α1-A288F, converted the effects of anesthetics from enhancement of agonist responses to inhibition of responses. This observation is consistent with the effects of mutations of the α1 GlyR and the ρ1 GABA receptor on alcohol actions; some mutants at Ser-267 of the α1 GlyR, such as 1-A288F mutant was strongly inhibited. A possible explanation is that F6 and F8, only the α1-A288F mutants at Ser-267 of the α1 GlyR and the ρ1 GABA receptor (p1-I307S/W328A) changed the alcohol effects from inhibition to potentiation (21, 38). To explain the change in anesthetic effects from enhancement to inhibition, we hypothesize that the effects of mutations would be to change the relative affinity for anesthetics to bind to and stabilize the closed states of the channel relative to the open states in a manner dependent on the volume of amino acid residues at Ala-288 (38). As for nonanesthetics F6 and F8, only the α1-A288F mutant was strongly inhibited. A possible explanation is that F6 and F8 preferentially stabilize the closed states of the α1-A288F channel but do not bind to other α1 receptors (F6 and F8 are not antagonists of anesthetic actions on GlyRs3). In any case, these hypotheses await further evaluation with single channel recording techniques.

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3 J. R. Trudell, personal communication.

4 T. Yamakura, S. J. Mihic, and R. A. Harris, unpublished data.
The two amino acid residues in TM2 and TM3 are critical for enhancement not only by volatile anesthetics but also by alcohols (10). The effects of ethanol on the $\alpha_1$ GlyR also depend upon the molecular volume of the substituents at position 267 in TM2; replacement of the Ser-267 residue with large residues resulted in inhibition (38). With regard to volatile anesthetics, others found a negative association between the molecular volume of the critical amino acid Ser-270 in TM2 of the $\alpha_2$ GABA$\_A$ receptor subunit and the degree of enhancement of GABA responses by isoflurane (30). Similar observations of the critical amino acid residues in TM2 and TM3 in terms of contribution of the molecular volume of amino acid residues to modulation of the GlyR and GABA$\_A$ receptors by alcohols and anesthetics, as well as the involvement in channel gating, are consistent with the proposal that these small amino acid residues at the critical positions in TM2 and TM3 might contribute together to make a space for a binding cavity of volatile anesthetics and alcohols on both the GlyR and GABA$\_A$ receptors. However, it is important to note the limitation of these types of studies. Even for effects of mutation on agonist binding to receptors, it is not easy to differentiate between effects on binding and effects on the transductional process (39), and the same is true in anesthetic binding. Thus, at this time we cannot rule out the possibility that anesthetics bind elsewhere on the GlyR subunit and that the molecular volume at Ala-288 plays a critical role in transductional modifications by anesthetics.

In summary, we studied mutations at a site (Ala-288) in TM3 near the extracellular surface of homomeric $\alpha_1$ GlyR and found marked effects on the ability of glycine to activate this receptor and on the actions of volatile anesthetics. In particular, large amino acids with low hydropathy mimicked the action of volatile anesthetics (increased apparent affinity of the receptor for glycine). Large amino acids also reduced or prevented the actions of volatile anesthetics, but this was independent of amino acid hydropathy. We propose that anesthetics enhance GlyR function because they bind in the vicinity of position 288 and provide appropriate changes in molecular volume and hydropathy. A corollary to this proposal is that large amino acids at position 288 prevent access of anesthetics to this site.
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