Molecular Determinants of Proton Modulation of Glycine Receptors*

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Extracellular pH regulates glycine receptors through an unknown mechanism. Here we demonstrate that acidic pH remarkably inhibited glycine-activated whole-cell currents in recombinant glycine α1 and αβ receptors transiently expressed in human embryonic kidney 293 cells. The proton effect was voltage-independent and pharmacologically competed with glycine receptor agonist glycine and antagonist strychnine. Using site-directed mutagenesis, we have identified an N-terminal domain that is essential for proton-induced inhibition of glycine current. In α1 homomers, removal of the hydroxyl group by mutation of residue Thr-112 to Ala or Phe abolished inhibition of glycine currents by acidification. In contrast, mutation of Thr-112 to another hydroxylated residue (Tyr) produced receptors that retained partial proton sensitivity. In αβ heteromers, a single mutation of the β subunit T135A, which is homologous to α1 Thr-112, reduced proton sensitivity, whereas the double mutation α1(T112A)/β(T135A) almost completely eliminated the proton sensitivity. In addition, the mutation α1 H109A greatly reduced sensitivity to protons in homomeric α1 receptors. The results demonstrate that extracellular pH can regulate the function of glycine α1 and αβ receptors. An extracellular domain consisting of Thr-112 and His-109 at the α1 subunit and Thr-135 at the β subunit plays a critical role in determining proton modulation of glycine receptor function.

Glycine is a fast inhibitory neurotransmitter in the mammalian central nervous system. It acts by binding to C1– conducting glycine receptors that belong to a superfamily of ligand-gated ion channels including nicotinic acetylcholine, γ-aminobutyric acid type A, and 5-hydroxytryptamine type 3 receptors. Glycine receptors are pentameric membrane proteins composed of α1–4 and β subunits. Only the α subunits can form functional homomeric receptors that contain major determinants of agonist and antagonist binding. Homomeric glycine receptors (primarily α2) appear to be expressed during embryonic and early postnatal development and might serve as extrasynaptic receptors throughout maturity (1, 2). Adult postsynaptic glycine receptors are composed of α1–4 and β subunits that form functional heteromeric receptors (3α:2β) and are located in spinal cord, brainstem, and many regions in brain (hippocampus, amygdala, striatum, cortex, etc.) (3, 4). Each subunit consists of a large extracellular N-terminal region, four transmembrane domains, and a large cytoplasmic domain. Transmembrane domain II forms the channel lumen (5), whereas the extracellular N-terminal region at α subunits contains the glycine and strychnine binding sites (6–11).

The brain is exposed to both transient changes in extracellular pH under physiological conditions, such as spontaneous neuronal firing and respiratory changes (12) and more sustained acidosis (up to a 1-unit drop in pH) after various pathophysiological conditions such as seizure, ischemia, and stroke (13, 14). It is well known that protons modulate neuronal excitability, and this effect is partially mediated through pH modulation of activity of ion channels. Previous studies have shown that extracellular acidification decreases the opening of voltage-gated (Na+, K+, and Ca2+) (15, 16) and ligand-gated channels (acetylcholine, N-methyl-D-aspartate, γ-aminobutyric acid, type A) (17–21). The pH modulation of glycine receptors has been only preliminarily assessed (22).

In the present study, we have characterized the sensitivity of homomeric α1 and heteromeric α1β receptors to extracellular pH and performed site-directed mutagenesis to investigate the structure-function relationship of glycine receptors in pH sensing. We have identified three residues at N terminus, Thr-112 and His-109 of the α1 subunit and Thr-135 of the β subunit, which play a critical role in determining proton modulation of glycine receptor function.

**EXPERIMENTAL PROCEDURES**

**Cloned Receptors**—Wild type human glycine α1 and β cDNA were generous gifts from H. Betz. The wild type or mutant receptor cDNA was expressed in human embryonic kidney cell lines via the mammalian expression vector pCI2. Cells were transfected using calcium phosphate precipitation technique to achieve transient expression. Briefly, human embryonic kidney cells were plated onto coverslips and transfected with wild type or mutant subunits. Typically, α1 or α1 plus β (1:10) cDNA (24) was added to cells growing exponentially on one coverslip placed in a 35-mm culture dish. After 6–8 h, cells were washed and placed in fresh culture medium. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection.

**Mutagenesis**—Mutations of receptor cDNA were performed using commercially available QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with commercially produced mutagenic primers (Integrated DNA Technologies). All mutants were verified by DNA sequencing (Biotechnology Core Facility, Texas Tech University, Lubbock, TX).

**Electrophysiology**—Whole-cell patch recordings were made at room temperature (22–25 °C). Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1–2.5 MΩ. The pipette solution contained 140 mM CsCl, 10 mM EGTA, 10 mM Hepes, 4 mM MgATP, pH 7.2. Coverslips containing cultured cells were placed in a small chamber (∼1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5–8 ml/min) with the following external solution containing 125 mM NaCl, 5.5 mM KCl, 0.8 mM MgCl2, 3.0 mM CaCl2, 20 mM Hepes, 10 mM Glucose.

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mm D-glucose, pH 7.3. Glycine-induced Cl− currents from the whole-cell patch clamp technique were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. Glycine-induced Cl− currents were low pass-filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis. 60–80% series resistance compensation was applied at the amplifier. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5-mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed during the recording period, the patch was aborted, and the data were not included in the analysis. Except during acquisition of current-voltage (I-V) relationships, cells were voltage-clamped at −60 mV. I-V relationships were determined by subtracting responses to continuous voltage ramp (−50 to +50 mV in 0.5 s) before EC50 glycine concentration (15 μM for α1 and 10 μM for α1β) application from the responses to the same voltage protocols during glycine application.

Heteromeric α1β glycine receptors are 17-fold less sensitive to picrotoxin than homomeric α1 receptors (25). Thus, the incorporation of β subunits with α1 subunits was confirmed by assessing picrotoxin sensitivity. If 500 μM picrotoxin caused 30% or less inhibition of the current activated by EC50 glycine, it was assumed that heteromeric α1β receptors were expressed.

Experimental Protocol—pH of external solutions was altered by the addition of NaOH or HCl and routinely checked before and during experiments. Glycine was prepared in the extracellular solution and applied from independent reservoirs by gravity flow for 10 s to cells using a T-shaped tube positioned within 100 μm of the cells. With this system, the 10–90% rise time of the junction potential at the open tip was 12–51 ms (21). Once a control glycine response was determined, the effect of pH on the response was examined. To assess the pH effect cells were first bathed in media that was set to the test pH, then glycine, 51 ms (21). Once a control glycine response was determined, the effect of pH on the response was examined. To assess the pH effect cells were first bathed in media that was set to the test pH, then glycine, was applied to the cells. Because in the whole-cell recordings, external solution of low pH elicited, as in other studies (20, 21, 23), a transient whole-cell inward current through acid-sensing ion channels, glycine application at various pH test values was made after the transient current had recovered and stabilized.

Glycine applications were separated by at least 3-min intervals to ensure both adequate washout of glycine from the bath and recovery of receptors from desensitization if present.

Chemicals—All drugs were obtained from Sigma. Glycine, strychnine, and ZnCl2, stocks were made in double distilled H2O.

Data Analysis—Glycine concentration-response profiles were fitted to the following equation: \( I/I_{\text{max}} = \frac{[\text{glycine}]^n}{EC_{50} + [\text{glycine}]^n} \), where \( I \) and \( I_{\text{max}} \) represent the normalized glycine-induced current at a given concentration and the maximum current induced by a saturating concentration of glycine, respectively, EC50 is 50% effective glycine concentration, and \( n \) is the Hill coefficient.

Concentration-response curves for strychnine inhibitory effect were fitted to the following equation: \( I/I_{\text{max}} = \frac{[\text{strychnine}]^n}{[\text{strychnine}]^n + IC_{50}} \), where \( I \) is the Cl− current amplitude at the end of drug application normalized to control at a given strychnine concentration, IC50 is the half-blocking concentration, and \( n \) is the Hill coefficient. Concentration-response profiles were evaluated using approximately the EC30 glycine concentration. A minimum of three individual experiments was conducted for each paradigm. All data are presented as the means ± S.E. Student’s t test (paired or unpaired) or a one-way analysis of variance was used to determine statistical significance (\( p < 0.05 \)).

RESULTS

Extracellular Protons Inhibit Glycine-activated Currents—Based on the characterization of recombinant glycine receptors at the control condition pH 7.3, 15 and 10 μM glycine are approximately EC50 concentrations for α1 and α1β receptors, respectively (see Fig. 2, A and B). The EC50 concentrations generate a stable current, elicit minimal receptor desensitization, and allow -fold modulator-induced potentiation of the glycine-evoked response. In assessing the effect of pH on the EC50 glycine response, we varied the pH of the external medium between 8.4 and 5.4. The modulatory effect of glycine-activated current by extracellular pH in human glycine α1 and α1β receptors is illustrated in Fig. 1. The amplitude of current activated by EC50 glycine was increased when the pH was increased from 7.3 to 7.8 and 8.4 and markedly attenuated when pH was decreased from 7.3 to 6.8 and 6.4 (Figs. 1, A and B). The effect of pH on glycine current was rapid and completely reversible (Fig. 1, A and B). No adaptation to proton effect was observed during a prolonged perfusion (up to 10 min) with acidic or alkaline medium (data not shown). Fig. 1C shows the average sensitivity of glycine-activated current to protons over the range of pH 5.4–8.4. In both receptors the acidic pH had a more profound effect than alkaline pH on glycine current. For homomeric α1 receptors, the glycine current activated by 15 μM glycine was enhanced to 118 ± 4.8% at pH 7.8 and to 129 ± 10% of the control at pH 8.4 (\( n = 3–5 \)). The response to 15 μM glycine was inhibited to 43 ± 6.6% at pH 6.8, 22 ± 3.4% at pH 6.4, and 12 ± 1.8% of the control at pH 5.4 (\( n = 3–4 \)). For heteromeric α1β receptors, glycine-activated currents were also sensitive to acidic pH and hardly sensitive to alkaline pH. The current activated by 10 μM glycine was inhibited to 39.1 ± 5.4 and 1.3 ± 1.3% of the control at pH 6.4 and 5.4, respectively.
curves for glycine-activated current were determined at pH 7.3, to distinguish between these possibilities, concentration-response decreasing the efficacy of glycine at receptors or both. To dis-
cantly Competitive—Protons might inhibit glycine-activated cur-
reversal potential in glycine—ions reversed at the calculated equilibrium potential for Cl
Modulation of Glycine Receptors by Protons

Inhibition of Glycine Currents by Protons Is Pharmacologically Competitive—Protons might inhibit glycine-activated current by decreasing the affinity of the receptors for glycine or decreasing the efficacy of glycine at receptors or both. To dis-
tinguish between these possibilities, concentration-response curves for glycine-activated current were determined at pH 7.3, 8.4, and 6.4 for α1 and α1β receptors. As shown in Fig. 2, A and B, change in pH shifted the glycine EC50 values for both receptor configurations. For α1 homomers, EC50 values were 53.7, 23.0, and 19.1 μM at pH 6.4, 7.3, and 8.4, respectively. The EC50 value at pH 6.4 was increased significantly compared with pH 7.3 (p < 0.05, n = 4, paired t test, Fig. 2A). For α1β heteromers, the EC50 values were 32, 18, and 19 μM at 6.4, 7.3, and 8.4, respectively. The EC50 value at pH 6.4 was significantly different from that at pH 7.3 (p < 0.05, paired t test, n = 6, Fig. 2B). In contrast, the maximal glycine-activated current and Hill coefficient values were not significantly affected by changes in pH in either receptor (p > 0.05). The lack of shift in EC50 at pH 8.4 is comparable with minimal effect of alkaline pH on the glycine receptors (Fig. 1).

Proton Inhibitory Effect Is Voltage-independent—To evaluate whether protons inhibit the glycine response by altering the Cl⁻ ion driving force, the effect of protons on the reversal potential of glycine-activated current was examined. Fig. 2, C and D, shows the current-voltage relationship for current activated by EC50 glycine at pH 7.3 and 6.4 for α1 and α1β receptors recorded from the same cell. The glycine-induced currents reversed at the calculated equilibrium potential for Cl⁻ ions (ECl⁻ = −3.14 mV). For α1 receptors the reversal potential was −4.9 ± 1.6 mV at pH 8.4, −6.9 ± 2.5 mV at pH 7.3, and −3.9 ± 1.8 mV at 6.4 (n = 4–6). These values are not significantly different (p > 0.05, one-way analysis of variance). In addition, the average percentage inhibition by protons (at pH 6.4) did not significantly differ at membrane holding potentials between −50 and +50 mV (71 ± 7.4% at −50 mV, 73 ± 11% at −30 mV, 63 ± 11% at +30 mV, 62 ± 12% at +50 mV, n = 4–6; p > 0.05, one-way analysis of variance). The proton effect on I-V relationship for α1β receptors was similar to that for α1 receptors.
on glycine-activated currents in wild type (WT), apparent affinity of strychnine, a competitive antagonist. The concentration-response curve from wild type receptors (34). Inspection of the amino acid sequence alignment for acetylcholine-binding protein (AChBP), human γ-aminobutyric acid type A subunits (hGABA\(_A\)), human (h)-p1-2, and glycine subunits (hGLY; α-3 and β) at the N terminus. A threonine and a histidine residue (except in AChBP), indicated with asterisks, are conserved in all subunits. The solid dots indicate a aspartic acid (D) or glutamic acid (E) residue on either side of threonine residue in glycine subunits.

Strychnine is a weak base with a \( pK_a \) value of 8.26 (26). Based on the Henderson-Hasselbalch equation, ~90% of the strychnine molecules are estimated to remain in the zwitterionic form within the pH range from 6.4 to 7.3. As shown in Fig. 3A, a change of pH from 7.3 to 6.4 significantly shifted strychnine inhibition of currents activated by equal potency of glycine (EC\(_{50}\)) to 111±12 nM at pH 7.3 to 102±32 nM at pH 6.4 (n = 6, p < 0.05, paired \( t \) test). The maximal inhibition of strychnine was not affected by the change in pH.

**Mutation α1 T112A Abolishes Protons Sensitivity**—It has been reported that the \( Zn^{2+} \)-induced inhibition of glycine current was greatly attenuated in acidic pH (22, 25), suggesting that \( Zn^{2+} \) may interact with protons at the same site(s). Previous studies indicate that the residues Thr-112 on the α1 subunits are critical to the inhibition by \( Zn^{2+} \) (22, 25). Furthermore, Thr-112 is also important for inhibition by the competitive antagonist strychnine (34). Inspection of the amino acid sequence alignment for ligand-gated anionic channels revealed that Thr is conserved at the equivalent position across all the subunits among γ-aminobutyric acid type A, glycine, and γ-aminobutyric acid type C receptors (residues marked with an asterisk in Fig. 4). In addition, both \( Zn^{2+} \) and protons exert an inhibitory effect on these receptors (21, 22, 28). Therefore, the Thr-112 residue is a candidate for proton action sites on glycine α1 receptors.

To investigate the functional role of Thr-112 in modulation of glycine-activated currents, site-directed mutagenesis was employed to replace the threonine residue at 112 with alanine, which has similar residue volume to threonine but lacks a hydroxyl group (-OH). The mutation T112A did not cause a significant shift in glycine EC\(_{50}\) (from 23±2.1 μM in wild type to 42±5.6 μM (n = 4) in mutant receptors at pH 7.3, p > 0.05, unpaired \( t \) test), which is consistent with previous investigations (11, 34). However, mutation T112A greatly reduced the inhibition of glycine currents by strychnine at pH 7.3 compared with wild type receptors. Fig. 3B shows that receptors expressing T112A were about 3-fold less sensitive to strychnine (IC\(_{50}\) value of 120±30 nM, n = 4) than the wild type (IC\(_{50}\) value of 44±6.1 nM, n = 6, p < 0.05, unpaired \( t \) test). The Hill coefficient and maximal inhibition were not altered by the mutation. As shown in Fig. 5A, T112A completely abolished proton modulatory effect on glycine α1. The current activated by EC\(_{50}\) glycine (25 μM) was 111±14.5% of the control at pH 5.4 and 90±3.1% of the control at pH 8.4 for mutant receptors. This compares to 12±1.8% at pH 5.4 and 129±10% at pH 8.4
in wild type receptors (Fig. 5B). As shown in Fig. 5C, a shift of pH from 7.3 to 6.4 was unable to affect EC_{50} values for glycine or maximal available glycine-induced current for T112A mutant receptors (EC_{50} = 27.6 ± 3.8 μM at pH 7.3 and 28.7 ± 5.78 μM at pH 6.4, respectively, n = 5) compared with a right shift of concentration-response curve observed in wild type receptors (Fig. 2A). These data suggest Thr-112 is sufficient to account for the effect of protons on glycine α1 receptors.

To further explore whether the volume of amino acid or hydroxyl group is responsible for action of proton, Thr-112 was mutated into tyrosine (Tyr), which has a hydroxyl group but is of larger volume. As shown in Fig. 3B, the strychnine potency was reduced in mutant T112Y beyond that observed in T112A (IC_{50} value of 333 ± 78 nM, n = 4, p < 0.05, compared with T112A or wild type, unpaired t test). In contrast, the T112Y mutation only partially attenuated the response to acidic pH compared with wild type α1 receptors; + +, p < 0.01, compared with α1 (T112A) receptors, unpaired t test.)

**Effect of Charged Residues Neighboring Thr-112 on pH Sensitivity**—In addition to the polar residue Thr-112, charged residues neighboring Thr-112 were tested for potential proton coordination. By examining residues in the vicinity of α1 Thr-112, we found that two acidic amino acid residues, Glu-110 and Asp-114, are conserved in all glycine subunits (marked with dots in Fig. 4), and these flank residue Thr-112. Both Glu-110 and Asp-114 on glycine α1 receptors contain a negatively charged carboxyl group, which may provide an electrical requirement for interaction with protons. To test this hypothesis, Glu-110 and Asp-114 were mutated individually to the neutral amino acid alanine. As shown in Fig. 6A, the mutation of E110A or D114A caused an approximate 4-fold shift of EC_{50} for sensitivity to protons in T113A, tested with glycine EC_{30} (5 μM, was similar to wild type receptors (n = 5, data not shown). Therefore, the role of the threonine residue in modulation of glycine receptors seems site-specific.

**Fig. 5. Proton effect on wild type and mutant glycine α1 receptors.** A, typical recording of responses of the α1(T112A) receptors to 25 μM glycine (EC_{30}) over a pH range of 5.4–8.4. Note that the mutation T112A causes a loss of sensitivity to protons. B, summarized data of proton sensitivity of wild type (WT) and mutant glycine α1 receptors. The data are normalized to the control current at pH 7.3 and represent the mean ± S.E. of at least 4 cells. The mutation T112A completely abolished proton sensitivity, whereas receptors expressing T112Y retained partial sensitivity to protons. α1(T112F) receptors lost proton sensitivity to pH 6.4 but remain sensitive to pH 5.4 (n = 5). C, proton effect on concentration-response relationship of the mutant α1(T112A) glycine receptors. The currents are normalized to the maximal response elicited by glycine at pH 7.3. Changes in pH did not affect glycine EC_{50} or Hill coefficient in α1(T112A) receptors. The EC_{50} values and Hill coefficients (in parentheses) were: 28 ± 3.8 μM (1.94 ± 0.38) at pH 7.3; 29 ± 5.8 μM (1.84 ± 0.28) at pH 6.4. Each data point represents the mean of five cells. The proton effect on concentration-response profile in α1(T112A) receptor is different from one in wild type α1 receptors (see the inset adapted from Fig. 2A). ( **, p < 0.01, compared with wild type α1 receptors; + +, p < 0.01, compared with α1 (T112A) receptors, unpaired t test.)
glycine receptors (22, 27). We, thus, examined the role of the His-109 residue in proton sensitivity in glycine α1 receptors. Fig. 6A shows that the replacement of histidine with alanine did not change the EC50 value for glycine (EC50, 24 ± 2.4 μM; Hill coefficient, 1.64 ± 0.12, n = 3, p > 0.05, unpaired t test, compared with wild type). As shown in Fig. 6B, the mutation H109A resulted in significant reduction of proton sensitivity. The mutation H109A eliminated 38–54% of the inhibition caused by protons as the percentage of current inhibition was 54 ± 4.5% at pH 5.4 and 39 ± 6.4% at pH 6.4 compared with 88 ± 1.4% inhibition at pH 5.4 and 78 ± 3.4% inhibition at pH 6.4 observed in wild type (n = 4–5, p < 0.01, unpaired t test, compared with wild type) (Fig. 6B). The potentiation of glycine currents by alkaline pH (pH 8.4) was also abolished (Fig. 6B). These data indicate that His-109 also takes part in proton modulatory effect of glycine α1 receptors, although His-109 cannot fully account for the proton effect.

β Subunits Contribute to Proton Sensitivity—Finally, we examined the role of β subunits in proton modulation of heteromeric glycine α1β receptors. If β subunits do not contribute to pH modulation, heterologous expression of mutant α1(T112A) with β subunits would produce receptors that remain insensitive to protons, as observed in α1(T112A) homomers. In contrast to complete loss of proton sensitivity observed in mutant homomeric α1(T112A) (see Fig. 5), proton sensitivity was reduced but still maintained in α1(T112A)β heterooligomers (Fig. 7A), suggesting that β subunits may participate in proton modulation in heteromeric receptors. To further confirm this, Thr-135 of the β subunit, which is the homologue of α1 Thr-112 (Fig. 4), was mutated to Ala. Compared with wild type α1β receptors, mutation β T135A caused a small (−10%) but significant reduction of proton-induced inhibition of EC50 glycine currents (n = 8, p < 0.05 at pH 5.4, unpaired t test). As expected, double mutation α1(T112A) and β(T135A) showed further reduction of the sensitivity to acidic pH (Fig. 7A). The EC50 glycine currents were 105 ± 3.3% at pH 8.4, 93 ± 0.9% at pH 6.4, and 82 ± 4.5% of the control at pH 5.4 (n = 4). On the average, acidic pH inhibited EC50 glycine currents in a rank order of α1β > α1β(T135A) > α1(T112A)β > α1(T112A)β(T135A) (Fig. 7A).

Fig. 6. Effect of mutations H109A, E110A, and D114A on proton sensitivity of glycine α1 receptors. A, glycine concentration-response curves for the wild type and mutant receptors. The currents are normalized to the maximal response of each cell. The EC50 values and Hill coefficients (in parentheses) were: 23 ± 2.1 μM (1.6 ± 0.11) for wild type (WT); 24.2 ± 2.4 μM (1.64 ± 0.12) for H109E; 95.5 ± 10.5 μM (1.27 ± 0.14) for E110A; 76.3 ± 16.1 μM (1.87 ± 0.31) for D114A. Each data point represents the mean of at least three cells. B, proton sensitivity for wild type and mutant receptors. The currents were normalized to the current activated by EC50 glycine at pH 7.3. Note that sensitivity to acidic or alkaline pH was not significantly different between α1(E110A) or α1(D114A) and wild type receptors. In contrast, the sensitivity to acidic or alkaline pH was significantly reduced in α1(H109A) receptors. Each data point represents the mean of at least four cells (**, p < 0.01, unpaired t test compared with proton sensitivity in wild type).

DISCUSSION

Protons could potentially modulate glycine receptors through several sites of action. Protons could change the charge(s) of the glycine molecule itself, thus altering its interaction with the agonist binding site. This alternative is doubtful, as it is estimated from the Henderson-Hasselbach equation that the charge(s) of the glycine molecule itself, thus altering its interaction with the agonist binding site. Protons could also change the electrostatic forces at the channel pore. Inhibition by protons was not impaired by these mutations, suggesting that Glu-110 and Asp-114 residues are unlikely involved in the inhibitory effect of protons on glycine receptors (Fig. 6B).

The titratable residue histidine has been reported to be involved in determining proton sensitivity in a number of ion channels (29–32). His-109 is highly conserved across all subunits of the ligand-gated anionic channels (Fig. 4) and has been identified as a Zn2+ binding site for Zn2+ -induced inhibition of glycine receptors (22, 27). We, thus, examined the role of the
The mechanism by which Thr-112 influences pH sensitivity is complex. Mutation of Thr-112 to Ala converts the partial agonists taurine and β-aminoisonobutyric acid into full agonists and dramatically reduces sensitivity to inhibition by strychnine and Zn\(^{2+}\) (11, 34), indicating that Thr-112 plays a fundamental role in actions of several modulators. T112A does not, however, affect glycine sensitivity (11, 34). Consistent with these studies, we found that mutation of Thr-112 to Ala, Tyr, or Phe decreased sensitivity (i.e. increased IC\(_{50}\)) to inhibition by strychnine or Zn\(^{2+}\) (data not shown) but had no effect on apparent glycine affinity, indicating that residue Thr-112 might participate in an antagonistic effect but not glycine affinity. With regard to the role of Thr-112 in proton-mediated inhibition, the presence of a hydroxyl group at this position appears to be critical. Receptors in which the Thr-112 residue was mutated to another hydroxylated residue (Tyr) retained sensitivity to protons, whereas the presence at the 112 position of Phe, which is similar in molecular volume to Tyr but does not have an hydroxyl group, conferred resistance to protons. Receptors with the T112Y mutation did not, however, retain sensitivity to inhibition by strychnine or Zn\(^{2+}\). Our results, coupled with those of others (11, 34) define a central role for Thr-112 in mediating inhibitory actions of protons, strychnine, and Zn\(^{2+}\), although the molecular determinants at this site for the three modulators are somewhat distinct.

Titrable histidine residues are major determinants of pH modulation in many ion channels (29–32). His-109, which is highly conserved among several ligand-gated ion channels, is likely one of the binding sites for protons in glycine α1 receptors. His-109 has been identified as the site of Zn\(^{2+}\) coordination. Harvey et al. (22) show that Zn\(^{2+}\) inhibition of glycine α1 receptors was greatly attenuated by acidification, suggesting that protons may compete with the Zn\(^{2+}\) action. In good agreement with previous investigations (22, 27), we found that mutation of His-109 into a non-charged residue abolished Zn\(^{2+}\) inhibition (data not shown). Meanwhile, mutation H109A also led to partial loss of proton sensitivity, suggesting His-109 is involved in proton-induced inhibition. It seems that both Zn\(^{2+}\) and H\(^+\) modulate glycine receptors through His-109. In addition, partial loss of proton sensitivity in mutation H109A indicates that protons might bind to other ionizable amino acid residues in addition to His-109. We have excluded two negatively charged residues, Glu-110 and Asp-114, in a neighboring position to Thr-112, from potential binding sites for protons because mutation of either of the residues failed to affect proton sensitivity.

We demonstrate that heteromeric glycine α1β receptors are sensitive to pH change in a manner similar to that observed in homomeric α1 receptors. Unlike in homomeric α1 receptors, mutation of α1(T112A) in α1β receptors did not completely abolish pH sensitivity. Effects of protons were only dramatically reduced when the equivalent β subunit T residue was also mutated. This indicates the β subunit contributes to pH sensitivity in α1β receptors. Although it is usually believed that the β subunits alone neither form a functional channel nor form a ligand binding pocket (25), incorporation of β subunits changes the pharmacological and functional properties of glycine receptors (for review, see Ref. 4). α1β glycine receptors have a stoichiometry of three α1 and two β subunits (5). The presence of only two Ala residues in α1β receptors (α1β(T135A)) elicited only a small reduction of proton sensitivity, whereas the presence of three (α1(T112A)β) or five (α1(T112A)ββ(T135A) Ala residues had a significantly larger impact on proton inhibition. This indicates that multiple residues in both α and β subunits are required for proton modulation of heteromeric α1β receptors.

**Fig. 7.** Effect of mutation on proton sensitivity in heteromeric glycine α1β receptors. A, the currents activated by EC\(_{50}\), glycine (10 μM for α1β, 30 μM for α1(T112A)β, 35 μM for α1β(T135A), 25 μM for α1(T112A)ββ(T135A)) are normalized to the control current at pH 7.3. Data plotted are the means ± S.E. of at least three cells. For a better comparison, the response of wild type (WT) α1β receptors to protons taken from Fig. 1C is shown in a dashed line. (**, p < 0.01, compared with wild type α1β receptors, unpaired t test.) B, concentration-response relationship for wild type (dashed line, from Fig. 2B) and mutant α1β receptors. The EC\(_{50}\) values and Hill coefficients (in parentheses) were: α1(T112A)β, 46 ± 8.2 μM (1.45 ± 0.22); α1β(T135A), 57 ± 7.4 μM (1.43 ± 0.20); α1(T112A)ββ(T135A), 48 ± 8.0 μM (1.11 ± 0.09). Data plotted are means ± S.E. of at least three cells.

\[^{3}H\]strychnine binding is decreased in acidic pH. We, thus, studied the pH sensitivity of glycine receptors expressing N-terminal mutations that have been reported to be important for receptor binding and modulation (22, 25, 27, 34).

Complete loss of proton sensitivity in homomeric α1 receptors with mutation of T112A indicates that Thr-112 is a key residue in determining pH sensitivity. Because threonine is not titratable within the pH range we tested here (pK\(_{a}\) = 15), Thr-112 is likely not to be a binding site per se for protons. It is worth noting that the residues involved in proton sensitivity may not be necessarily ionizable. Non-ionizable residues have been reported to play a major role in proton sensitivity of N-methyl-β-aspartate receptors (alanine residues) (35), Kir6.2 channels (threonine) (36), and Kir2.3 channels (threonine) (37). The role of the threonine residue in the modulation of protons is site-specific because mutation of another highly conserved threonine residue at position 113 did not affect receptor sensitivity to protons.
Given the widespread distribution of glycine receptors in spinal cord, brainstem, and other regions of the brain (38), modulation of glycine receptors by protons is of significant physiological importance. Although we only studied α1 and αβ glycine receptor subtypes, the pH modulatory effect may be universal for other glycine subtypes because the critical residues identified here (Thr-112 and His-109) are conserved across the different α subunits. Indeed, we have observed that pH modulates recombinant glycine α2 receptors in a way similar to α1 receptors.1 The postsynaptic glycine receptors (predominantly αβ) might be saturated in the synaptic cleft at the moment of release of glycine (39). Considering that protons may modify the kinetics of glycine receptor subtypes because the critical residues across the different α subunits are conserved (Thr-112 and His-109) are conserved across the different α subunits, the pH modulatory effect may be similar to wild type glycine receptors.1

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Note Added in Proof—While this paper was under review, Li et al. (Li, Y.-F., Wu, L.-J., Li, Y., Xu, L., and Xu, T.-L. (2003) J. Physiol. (Lond.) 552, 73–87) reported similar proton effects on native glycine receptors.1 The postsynaptic glycine receptors (presumably homomeric α receptors) seem to be most effectively activated by sub-saturating concentrations of glycine spillover from the adjacent synaptic cleft (40). Proton-mediated modulation of postsynaptic glycine receptors would result in alternation of tonic inhibition of neurons (4).

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