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A Novel Hyperekplexia-causing Mutation in the Pre-transmembrane Segment 1 of the Human Glycine Receptor α_1 Subunit Reduces Membrane Expression and Impairs Gating by Agonists*[S]

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In this study, we have compared the functional consequences of three mutations (R218Q, V260M, and Q266H) in the α_1 subunit of the glycine receptor (GlyRA1) causing hyperekplexia, an inherited neurological channelopathy. In HEK-293 cells, the agonist EC_{50s} for glycine-activated Cl^- currents were increased from 26 μM in wtGlyRA1, to 5747, 135, and 129 μM in R218Q, V260M, and Q266H GlyRA1 channels, respectively. Cl^- currents elicited by β-alanine and taurine, which behave as agonists at wtGlyRA1, were decreased in V260M and Q266H mutant receptors and virtually abolished in GlyRA1 R218Q receptors. Gly-gated Cl^- currents were similarly antagonized by low concentrations of strychnine in both wild-type (wt) and R218Q GlyRA1 channels, suggesting that the Arg-218 residue plays a crucial role in GlyRA1 channel gating, with only minor effects on the agonist/antagonist binding site, a hypothesis supported by our molecular model of the GlyRA1 subunit. The R218Q mutation, but not the V260M or the Q266H mutation, caused a marked decrease of receptor subunit expression both in total cell lysates and in isolated plasma membrane proteins. This decreased expression does not seem to explain the reduced agonist sensitivity of GlyRA1 R218Q channels since no difference in the apparent sensitivity to glycine or taurine was observed when wtGlyRA1 receptors were expressed at levels comparable with those of R218Q mutant receptors. In conclusion, multiple mechanisms may explain the dramatic decrease in GlyR function caused by the R218Q mutation, possibly providing the molecular basis for its association with a more severe clinical phenotype.

Fast inhibitory neurotransmission in the adult mammalian central nervous system is primarily mediated by the amino acids γ-aminobutyric acid (GABA)[1] and Gly, which, by activating Cl^--selective ligand-gated ion channels (LGICs), hyperpolarize the membrane of the postsynaptic neuron and inhibit firing (1). Glycine receptors (GlyRs) are heteropentameric proteins made up of the assembly of two types of homologous subunits: α subunits, which carry the molecular determinants for agonist and antagonist binding, and β subunits, which determine the subsynaptic localization of GlyRs (2). In humans, only one gene encoding for β subunits has been identified (3); this gene is widely expressed in the central nervous system at all developmental stages of vertebrates. On the other hand, four genes encoding distinct α subunits (α_{1-4}) are known, each showing a specific regional distribution and ontogenetic profile (4). In particular, α_1 subunits seem to be the predominant isoform expressed postnatally in the spinal cord and brainstem; GlyRs of mature vertebrates are thought to have a $3\alpha_1 2\beta$ stoichiometry. The topological organization of the GlyR subunits is believed to be similar to that of other members of the LGIC super-family (5), with both the N and the C termini located extracellularly and four transmembrane segments; the second transmembrane segment (M_2) determines permeation properties such as anion selectivity and conductance.

GlyRs play a central role in the pathophysiology of hyperekplexia (HE), a rare inherited human neurological disease characterized by muscle stiffness, hyperreflexia, and an exaggerated startle response to sensory stimuli (6). The muscle stiffness and hyperreflexia, usually present at birth, decline during the first year of life, although an exaggerated startle response persists during the adult life. The gene associated with hyperekplexia was linked to chromosomal locus 5q33–35 in 1992 (7); within this region, mutation analysis in HE-affected families proved that the *GlyRA1* gene, encoding the α_1 subunit of GlyR, was the defective gene in HE (8). Several HE-causing mutations are known in the *GlyRA1* gene; these include both dominant and recessive inherited mutations, as well as *de novo* mutations found in sporadic cases. Further support for the hypothesis that abnormal GlyR function causes neurological disturbances derives from the observation that the phenotypes of *spasmoid* (9), *spastic* (10), and *oscillator* (11) mice, all of which carry genetically determined defects in GlyR-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplementary figure showing the alignment of the predicted primary sequence of LGIC subunits.

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; GlyR, glycine receptor; LGIC, ligand-gated ion channel; HE, hyperekplexia; TAU, taurine; β-ALA, β-alanine; STR, strychnine; wt, wild-type.

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encoding genes, bear a striking resemblance to some of the sensorimotor manifestations of human HE.

In the present study, we have characterized the functional properties of three GlyRA1 mutations associated with HE. The R218Q mutation, recently described by members of our group (12), affects a residue that is located at the boundary between the extracellular N-terminal domain and M_1 and is highly conserved among subunits forming both anion-selective (GlyR and $GABA_{A,C}R$) and cation-selective (nicotinic α_{1-7} and α_9, β_{1-4}, γ, δ, ϵ; 5HT-3_A and 5HT-3_B; some purinergic receptors) LGICs (Supplemental Fig. 1). The V260M (13) and the Q266H (14) mutations affect residues located in the M_2 domain of GlyRA1 subunits but are not believed to participate in permeation (5, 15); they are less well conserved among LGIC subunits. We have used pharmacological, biochemical, and molecular modeling techniques to investigate the molecular basis of these three human "channelopathies" and found evidence that all three residues may cause a variable degree of impairment in GlyR gating, with the most dramatic phenotype associated with the R218Q mutation.

EXPERIMENTAL PROCEDURES

Mutagenesis of hGlyRA1 cDNA—Mutations were engineered into the human GlyRA1 cDNA, cloned into the pCIS2 vector, by sequence overlap extension PCR with the *Pfu* DNA polymerase, as already described (16). DNA sequences were verified using an ABI PRISM 310 sequencing apparatus (Applied Biosystem, Foster City, CA).

Heterologous Expression of GlyRA1 Subunits—Wild-type (wt) and mutant GlyRA1 subunits were transiently expressed via transfection of human embryonic kidney (HEK-293) cells. HEK-293 cells were grown in minimal essential medium containing 10% fetal bovine serum, nonessential amino acids (0.1 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml), in an humidified atmosphere at 37 °C with 5% CO_2 in 100-mm plastic Petri dishes. For electrophysiological experiments, the cells were seeded on glass coverslips (Carolina Biological Supply Company, Burlington, NC). One day after plating, the cells were transfected with 2 μg of the appropriate cDNAs using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's protocol, together with 2 μg of a plasmid encoding the enhanced green fluorescent protein (Clontech) used as a transfection marker. When lower amounts of GlyRA1 cDNA were used for transfection (see Fig. 5), the amount of enhanced green fluorescent protein cDNA was proportionally increased. All of the experiments were performed 1–2 days after transfection.

Electrophysiology—Currents were recorded from HEK-293 cells at 20–22 °C using a commercially available amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The whole cell configuration of the patch clamp technique was adopted using glass micropipettes of 3–5 megaohms of resistance (17). The extracellular and intracellular (pipette) solution contained (in mM): 138 NaCl, 2 $CaCl_2$, 5.4 KCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4, with NaOH; and 140 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, 0.25 mM cAMP, pH 7.3–7.4, with KOH, respectively. The pCLAMP software (v. 6.0.4, Axon Instruments) was used for data acquisition and analysis. Statistical differences were evaluated with the Student's t test ($p < 0.05$). Solution exchanges were achieved by means of a cFlow 8 flow controller attached to a cF-8VS 8-valve switching apparatus and a MPRE8 miniature flow outlet positioned within 200 μm of the cell (Cell Microcontrols, Norfolk, VA).

Cell Surface Biotinylation and Western Blotting—Plasma membrane expression of GlyRA1 subunits in HEK-293 cells was investigated by surface biotinylation of membrane proteins in intact cells 2 days after transfection using a cell membrane-impermeable reagent (sulfosuccinimidyl-6-(biotinamido) hexanoate; Pierce) according to the manufacturer's protocol (18). Following biotinylation, the cells were lysed, and biotinylated proteins were isolated by reacting cell lysates with ImmunoPure streptavidin beads (Pierce). GlyR α subunits in streptavidin precipitates and total lysates were revealed in Western blots using an anti-GlyRA monoclonal antibody (mAb4a; Alexis Biochemicals, Lausen, Switzerland; dilution 1:4000); an alkaline phosphatase-conjugated sheep anti-mouse IgG (Amersham Biosciences; dilution 1:2000) was used as secondary antibody. Reactive bands were detected by chemiluminescence (SuperSignal, Pierce), using a ChemiDoc station (Bio-Rad). To confirm that intracellular proteins were not labeled by the biotinylation reagent, blots were stripped and reprobed with an anti-α-tubulin antibody (Sigma; dilution 1:5000).

Molecular Modeling—A molecular model of a GlyRA1 subunit was built using a previously described model of the $GABA_AR$ α_1 subunit as a template (19–22). We built loops to fill in the gaps between the GlyRA1 sequence and the template sequence and refined the side chains in the model using the auto-rotomer feature in the Biopolymer Module of Insight II (v. 2000.1, Accelrys, San Diego, CA) as described previously (19, 22). The backbone atoms (C, Cα, N) of each GlyR residue were tethered to the coordinates of corresponding residues in the template with a force constant of 100 kcal/A^2, and the structure was optimized with the Discover_3 module of Insight II. Coulombic interactions were calculated with a constant dielectric of one and a cutoff at 9.5 A. The structure was relaxed by performing 5000 2-fs steps of molecular dynamics at 298 K and was then reoptimized with the Discover_3 module to a derivative of 1 kcal/A.

RESULTS

Glycine Sensitivity of Homomeric GlyRA1s Carrying HE-causing Mutations—The putative topological arrangement of a single GlyR α_1 subunit, highlighting the Arg-218, the Val-260, and the Gln-266 residues, is shown in Fig. 1*A*. To assess the functional consequences of the HE-causing GlyRA1 mutations affecting these residues, we transfected HEK-293 cells with the cDNAs encoding wtGlyRA1 or R218Q, V260M, or Q266H GlyRA1 mutants and recorded Cl^- currents carried by the expressed channels upon perfusion with the agonist Gly. In wtGlyRA1-expressing cells, perfusion with concentrations of Gly above 3 μM (0.01–30 mM) activated robust inward Cl^- currents when the cell was held at −60 mV (Fig. 1*B*); the reversal potential of these currents approximated the Cl^- equilibrium potential (data not shown), confirming anionic selectivity. The peak amplitude of these Gly-gated Cl^- currents increased with Gly concentration up to 300 μM; larger Gly concentrations (3–30 mM) failed to increase Cl− current peak size, although they accelerated the time course of Gly-gated current desensitization. Peak amplitudes of Gly-gated currents were normalized to the maximum, expressed as a function of Gly concentrations, and fitted to a standard form of the Hill equation ($y = y_{max}$ ([Gly]nH/[Gly]nH + $EC_{50}n$H). The EC_{50} for Gly was 26 ± 4 μM, with a Hill coefficient (n_H) of 2.0 ± 0.1 (Fig. 1*C*). These values concur with those reported previously (23, 24).

Transfection of HEK-293 cells with R218Q GlyRA1 cDNA also led to the appearance of Gly-gated Cl^- currents (Fig. 1*B*), although the agonist sensitivity of these currents was dramatically decreased when compared with that of homomeric wtGlyRA1 channels. Gly concentrations larger than 0.3 mM (1–30 mM) were required to activate Cl^- currents in R218Q GlyRA1-transfected cells. Dose-response analysis revealed that the homomeric GlyRA1 channels carrying the R218Q mutation displayed a 200-fold decrease in Gly sensitivity when compared with wtGlyRA1 channels (EC_{50} of 5747 ± 270 μM), with no apparent change in the Hill coefficient n_H (2.3 ± 0.2) (Fig. 1*C*). In addition, the maximal Cl^- current density in cells transfected with the R218Q GlyRA1 cDNA (19.6 ± 8.9 pA/pF; n = 26) was markedly decreased when compared with that of wtGlyRA1-transfected cells (204.7 ± 32.9 pA/pF; n = 20; $p < 0.05$; see also Fig. 5).

The two other HE-associated GlyRA1 mutations, V260M and Q266H, caused a more modest 5-fold increase in the EC_{50} for the agonist Gly (the EC_{50} was 135 ± 3 μM for V260M and 129 ± 8 μM for Q266H mutants) (Fig. 1*C*). Also in this case, no significant differences were detected in the Hill coefficients of V260M (n_H = 1.7 ± 0.2) and Q266H (n_H = 1.6 ± 0.2) GlyRA1 receptors when compared with wtGlyRA1 receptors.

To gain further insight into the molecular defect prompted by the R218Q mutation, we measured the rates of channel activation, desensitization, and deactivation by fitting with a single exponential function the early phases of agonist (30 mM Gly)-induced current activation, the time course of agonist (30

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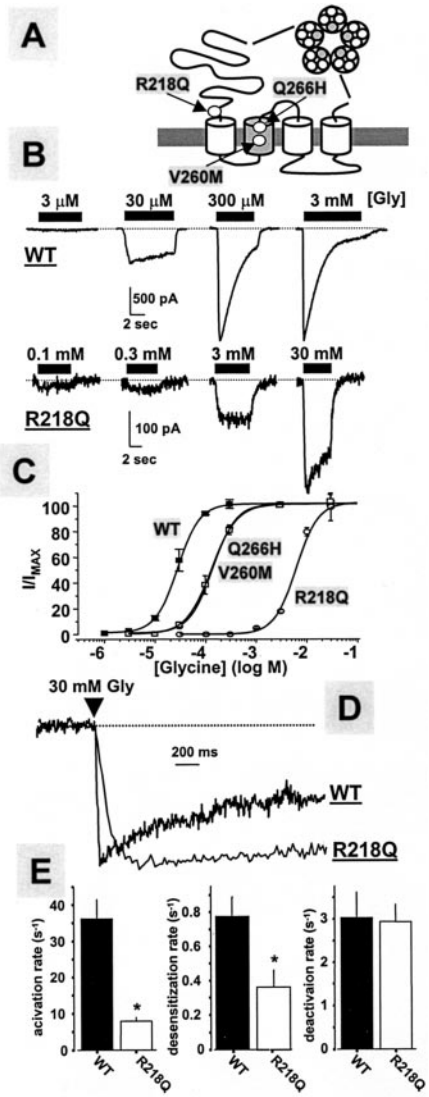

FIG. 1. **Glycine sensitivity of homomeric channels formed by GlyRA1 subunits carrying HE-causing mutations.** *A*, schematic representation of the putative topological arrangement of a single GlyRA1 subunit (1). *B*, whole cell current responses to Gly in HEK-293 cells expressing wt and R218Q GlyRA1 subunits. In this and the following figures, the holding potential was −60 mV, and the *bars* on *top* of the *current traces* show the duration of drug perfusion. *C*, Gly dose-response curves for wt (*filled squares*), V260M (*empty squares*), Q266H (*filled circles*), and R218Q (*empty circles*) GlyRA1 mutants. The *solid lines* represent the fits of the experimental data to the form of the Hill equation described under "Results." EC_{50s} and n_H values for each curve are given under "Results." Each point is the mean ± S.E. of 4–10 determinations in separate cells. *D*, superimposed traces showing current responses elicited by exposure to 30 mM Gly in wtGlyRA1 and GlyRA1 R218Q channels. Agonist-activated responses have been normalized to facilitate comparison. *E*, effect of the R218Q mutation on the rate of channel activation, desensitization, and deactivation in the presence of Gly. Each *bar* is the mean ± S.E. of 6–15 determinations in separate cells.

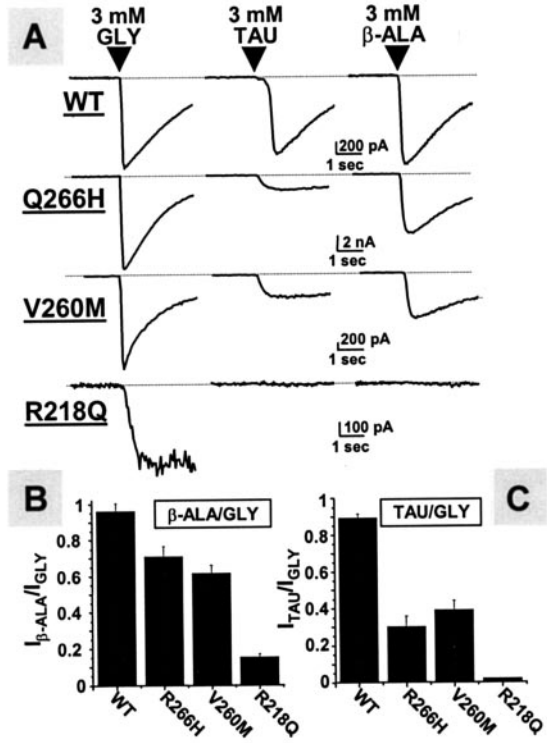

FIG. 2. **Taurine and β-alanine sensitivity of GlyRA1 mutants.** *A*, whole cell current responses to the application of Gly, TAU, and β-ALA (each at 3 mM) in the same cell expressing wt or mutant GlyRA1 subunits. *B* and *C*, $I_{\beta\text{-ALA}}/I_{Gly}$ (*B*) and I_{TAU}/I_{Gly} (*C*) ratios in wt and GlyRA1 mutants, as indicated. The ratios of the peak current amplitude elicited by 3 mM β-ALA/3 mM Gly and 3 mM TAU/3 mM Gly (for wt, V260M, and Q266H) or by 30 mM β-ALA/30 mM Gly and 30 mM TAU/30 mM Gly (for R218Q) are plotted in *B* and *C*, respectively. Each *bar* is the mean ± S.E. of 3–8 determinations in separate cells.

mM Gly)-induced current desensitization, and the current decay upon agonist (3 mM) removal in both wtGlyRA1 and R218Q channels. Comparison of these kinetic properties showed that wtGlyRA1 receptors displayed an increased activation rate and a faster desensitization rate during agonist exposure when compared with GlyRA1 R218Q channels (Fig. 1, *D* and *E*), without significant changes in the deactivation rate upon removal of the agonist (Fig. 1*E*).

Taurine and β-Alanine Sensitivity of HE-causing GlyRA1 Mutants—β-amino acids such as β-alanine (β-ALA) and taurine (TAU) behave as agonists at wtGlyR, but they appear to be of lower efficacy than Gly in some mutant receptors, acting as partial agonists (25, 26). As shown in Fig. 2*A*, the peak Cl^- currents elicited by exposure to 3 mM β-ALA or TAU in wtGlyRA1 receptors were similar in amplitude to those elicited by an identical Gly concentration (Fig. 2, *B* and *C*). By contrast, the currents elicited by both β-ALA and TAU in GlyRA1 carrying the V260M or the Q266H mutation were significantly smaller than those triggered by an identical concentration of Gly (3 mM) in the same cells (Fig. 2, *A–C*). In cells expressing GlyRA1 subunits carrying the R218Q mutation, 3 mM TAU or β-ALA application failed to elicit detectable Cl^- currents (Fig. 2*A*). In the large majority of the R218Q-transfected cells, perfusion with a higher β-ALA concentration (30 mM) also failed to elicit measurable Cl^- currents. In the only three cells in which these currents were clearly above background, the $I_{\beta\text{-ALA}}/I_{Gly}$ ratio was 0.13 ± 0.03 ($p < 0.05$ *versus* controls) (Fig. 2*B*); in R218Q-transfected cells, 30 mM TAU failed to activate measurable Cl^- currents (Fig. 2*C*).

Strychnine-induced Blockade of GlyRA1 Channels Carrying the R218Q Mutation—Strychnine (STR) is an antagonist of Gly, which is known to bind at sites that partially overlap the agonist binding site on the GlyR (1, 4, 25). The competitive nature of STR-induced antagonism is shown in Fig. 3*A*. Cl^- currents carried by channels composed of wtGlyRA1 subunits were activated by 30 μM Gly, an agonist concentration close to the EC_{50}. During agonist application, 400 nM STR caused a rapid inhibition of the Gly-gated Cl^- currents, which were almost fully abolished. The time course of STR-induced Cl^- current inhibition reflects the kinetics of antagonist binding to its site on the receptor, which depend upon antagonist concentration. Lower STR concentrations (100 nM) also inhibited Gly-

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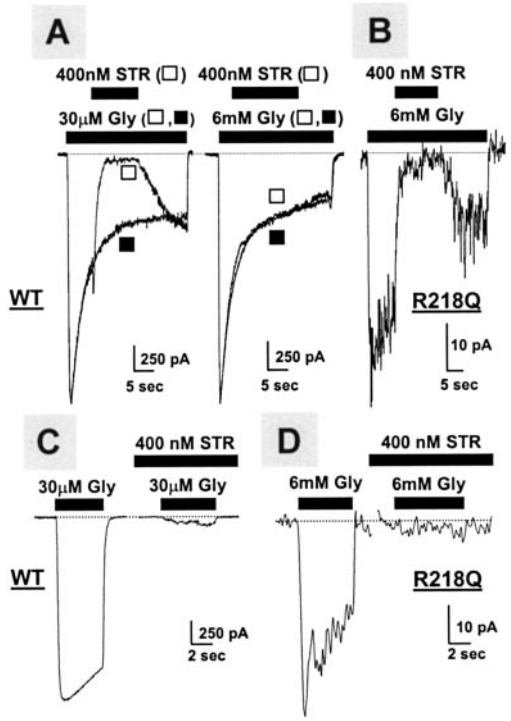

FIG. 3. **Blockade of wt and R218Q GlyRA1 channels by strychnine.** *A*, whole cell current responses to Gly (30 μM, *left panel*; 6 mM, *right panel*) application in cells expressing wtGlyRA1 subunits. In the trace labeled with an *empty square*, 400 nM STR was co-applied with Gly; in the traces labeled with a *filled square*, no STR was co-applied with Gly (control). *B*, whole cell current response to 6 mM Gly application in a cell expressing R218Q GlyRA1 subunits. 400 nM STR was co-applied with Gly. *C*, *left panel*, whole cell current response to 30 μM Gly application in a cell expressing wtGlyRA1 subunits. *Right panel*, whole cell current response to 30 μM Gly in the same cell, after preincubation for 1 min with 400 nM STR. *D*, whole cell current response to 6 mM Gly application in the same cell expressing R218Q GlyRA1 subunits in control conditions (*left panel*) or after preincubation for 1 min with 400 nM STR (*right panel*). The same experiments were performed in at least three additional cells, with comparable results.

gated Cl^- currents, although the time required to reach steady-state inhibition was much longer (data not shown). Upon removal of 400 nM STR, and in the continuous presence of the agonist, the Cl^- current amplitude recovered slowly, reflecting the kinetics of unbinding of the antagonist from its receptor. When a supramaximal Gly concentration (6 mM, 200-fold higher than the EC_{50}) was applied, 400 nM STR did not inhibit the agonist-induced current in wtGlyRA1 channels, consistent with the competitive nature of STR blockade.

In GlyRA1 channels carrying the R218Q mutation, 400 nM STR also inhibited the Cl^- currents activated by Gly, with kinetics similar to those observed in wtGlyRA1 (Fig. 3*B*). In these experiments, similarly to those described for wtGlyRA1 channels, a concentration of Gly close to the EC_{50} (6 mM) for GlyRA1 R218Q channels was used to activate Cl^- currents.

In cells expressing R218Q GlyRA1 subunits, preincubation with 400 nM STR markedly reduced the response to 6 mM Gly (always in the presence of 400 nM STR) (Fig. 3*C*); the peak amplitude of the Cl^- current elicited by 6 mM Gly in the presence of 400 nM STR was 8.0 ± 1.1% that of control ($n = 4$). This result was very similar to that obtained in cells expressing wtGlyRA1 subunits using the EC_{50} concentration of 30 μM (Fig. 3*C*), in which the peak amplitude of the Cl^- current elicited by 30 μM Gly in the presence of 400 nM STR was 5.3 ± 1.7% ($n = 5$) of those recorded in the absence of STR. Measurements of the true equilibrium dissociation constant for STR by the method of Schild (27, 28) were precluded in GlyRA1 R218Q channels due to the very low potency of Gly, requiring the use of concentrations higher than 10 mM to elicit even small currents.

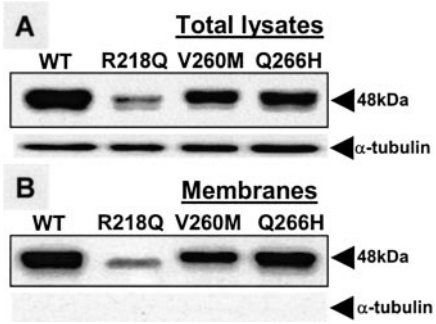

FIG. 4. **Membrane localization of GlyRA1 subunits carrying HE mutations.** Western blot experiments performed in total lysates (*A*) or streptavidin-purified biotinylated plasma membrane proteins (*B*) from HEK-293 cells transfected with wt or mutant GlyRA1 cDNAs are shown. The *higher* and *lower* blots in each panel were probed with the mAb4a monoclonal antibody recognizing GlyRA1 subunits or with anti-α-tubulin antibodies, respectively.

Membrane Localization of GlyRA1 Subunits Carrying HE Mutations—To determine whether the HE-causing mutations modified membrane insertion of GlyRA1 subunits, in addition to causing changes in the functional properties of correctly processed channels, Western blots were performed in total cell lysates and streptavidin-isolated plasma membrane proteins from HEK-293 cells transfected with the cDNAs encoding wt-GlyRA1 or mutant GlyRs. The mAb4a monoclonal antibody was used, which recognizes an extracellular epitope (amino acids 96–105) shared by all GlyRA subunits; GlyRA1 subunits were revealed by the appearance of an immunoreactive band of an approximate molecular mass of 48 kDa (29). This band was not detected in extracts from untransfected HEK-293 cells (data not shown).

The intensity of this 48-kDa band did not show significant differences between cells expressing wtGlyRA1 subunits or GlyRA1 subunits carrying the V260M or the Q266H mutation, both in total lysates (Fig. 4*A*) and in streptavidin-isolated plasma membrane proteins (Fig. 4*B*). By contrast, cells expressing GlyRA1 R218Q subunits showed a significant decrease in the intensity of this 48-kDa immunoreactive band, both in total lysates (Fig. 4*A*) and in isolated plasma membrane fractions (Fig. 4*B*) when compared with wtGlyRA1-expressing cells. In fact, densitometric analysis revealed that the intensity ratios between the 48-kDa band in total lysates and the band corresponding to α-tubulin (~55 kDa) in total lysates were 2.61 ± 0.94, 1.65 ± 0.45, 1.88 ± 0.64, and 0.46 ± 0.17 for cells transfected with wt, V260M, Q266H, and R218Q GlyRA1 cDNAs, respectively ($n = 4$; $p < 0.05$ between R218Q GlyRA1- and wtGlyRA1-transfected cells). Furthermore, the ratios between the intensity of the GlyRA1-specific band in streptavidin-isolated plasma membrane proteins and the band corresponding to α-tubulin in total lysates were 3.67 ± 1.66, 1.96 ± 0.39, 1.74 ± 0.33, and 0.25 ± 0.16 for cells transfected with wt, V260M, Q266H, and R218Q GlyRA1 cDNAs, respectively ($n = 5$; $p < 0.05$ between R218Q GlyRA1- and wtGlyRA1-transfected cells).

Decreased Subunit Expression Does Not Explain the Reduced Agonist Sensitivity of GlyRA1 R218Q Channels—In *Xenopus* oocytes, the level of heterologous GlyRs expression affects agonist sensitivity (30, 31). In fact, when GlyRA1s are expressed at high density, they display an increased affinity for glycine, as well as for other agonists such as TAU or GABA. Therefore, the possibility existed that, in the present experiments, the reduced sensitivity of mutant R218Q receptors might have

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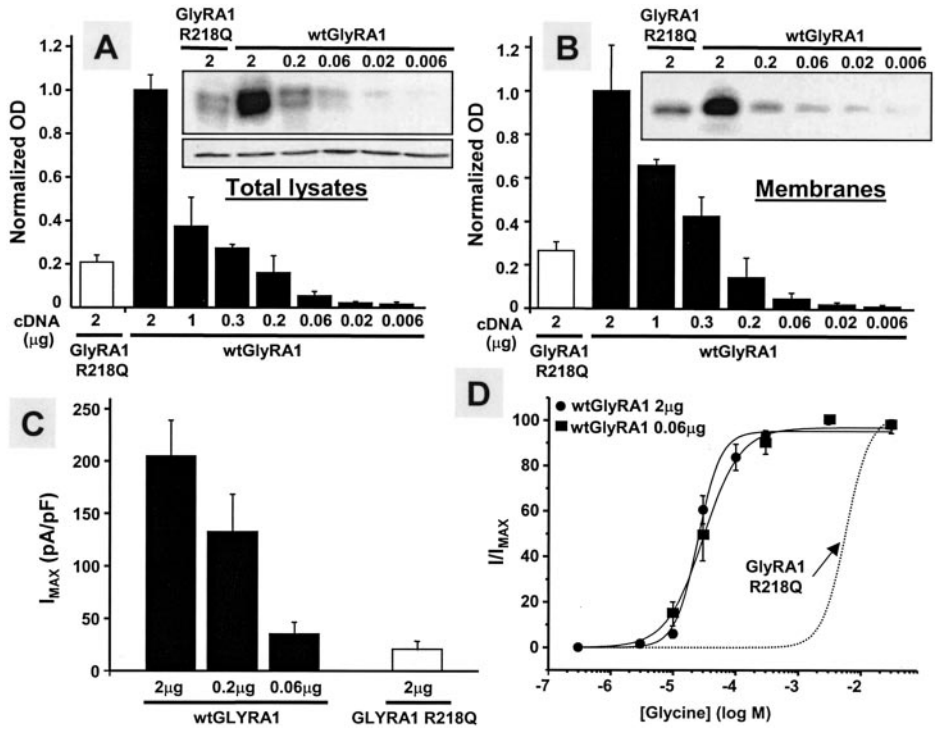

FIG. 5. **Comparison of the functional properties of wtGlyRA1 channels when expressed at levels comparable with those of GlyRA1 channels carrying the R218Q mutation.** Western blots of total cell lysates (*A*) and plasma membrane proteins (*B*) from HEK-293 cells transfected with GlyRA1 R218Q (2 μg) and decreasing concentrations (from 2 to 0.006 μg) of wtGlyRA1 cDNA are shown. Each *bar* represents the optical density readings from each band, normalized to the value of the band corresponding to 2 μg of wtGlyRA1 cDNA. Each *bar* is the mean ± S.E. of determinations made in three separate experiments. The *insets* in each panel show the results of a representative Western-blot experiment probed with the mAb4a monoclonal antibody or, for the lower blot of *A*, with the anti-α-tubulin antibody. *C*, Gly (3 mM)-activated current densities in HEK-293 cells transfected with the indicated concentrations of wtGlyRA1 cDNA (2, 0.2, and 0.06 μg), when compared with those recorded from GlyRA1 R218Q-transfected cells (2 μg). For each recorded cell, the peak value of the Gly-activated current was divided by membrane capacitance. Each *bar* is the mean ± S.E. of 16–24 cells for each group. *D*, Gly dose-response curves for wtGlyRA1 receptors expressed at high (2 μg cDNA, *filled circles*) or low (0.06 μg cDNA, *filled squares*) densities. The *solid lines* represent the fits of the experimental data to the form of the Hill equation described under "Results." EC_{50s} and n_H values were 25 ± 2 μM and 2.4 ± 0.5 for cells transfected with 2 μg wtGlyRA1 cDNA and 31 ± 3 μM and 1.5 ± 0.2 for cells transfected with 0.06 μg wtGlyRA1 cDNA. Each point is the mean ± S.E. of 4–10 determinations in separate cells.

been partially due to a decreased membrane expression of subunits. To verify this point, we decreased membrane expression of wild-type GlyRA1 to the same levels of those observed with the R218Q mutant, by progressively reducing (from 2 to 0.006 μg) the amount of wtGlyRA1 cDNA used to transfect HEK-293 cells. Western blotting experiments on total lysates and biotinylated membrane proteins from cells transfected with different cDNA concentrations demonstrated that we could achieve a protein expression level of wtGlyRA1 subunits comparable with that of GlyRA1 R218Q mutant subunits when transfecting the cells with approximately one-tenth (0.2 μg) of wtGlyRA1 cDNA (Fig. 5, *A* and *B*). Cells transfected with lower cDNA amounts (0.2–0.06 μg) and expressing smaller Gly-induced currents (<50 pA/pF; Fig. 5*C*) did not show significant differences in the apparent sensitivity to Gly when compared with cells expressing higher amounts of GlyRA1 receptors (2 μg of cDNA; >200 pA/pF of maximal Gly-gated currents) (Fig. 5*D*). These results provide convincing evidence for the hypothesis that the reduced agonist sensitivity of homomeric receptors carrying the R218Q mutation cannot be simply explained by a reduced membrane expression of mutant subunits. This view is also supported by the observation that, in HEK-293 cells expressing wtGlyRA1 subunits at a level comparable with those of GlyRA1 R218Q subunits (36.5 ± 20 pA/pF; n = 3), the I_{TAU}/I_{Gly} ratio was 0.67 ± 0.14 (at 3 mM of each agonist) and 0.78 ± 0.07 (at 30 mM of each agonist) (data not shown); these values are similar to those observed in cells expressing higher wtGlyRA1 receptor densities and clearly different from those of R218Q-expressing cells (Fig. 2).

DISCUSSION

Characterization of the functional consequences of mutations causing human diseases often reveals novel aspects of the structural and functional determinants of the affected proteins. In the present study, we have evaluated the functional consequences of three GlyRA1 mutations causing HE. The position of these three residues is illustrated in a molecular model of a single GlyRA1 subunit (Fig. 6). Two of these mutations, V260M (13) and Q266H (14), alter residues located in the pore-lining M_2 domain, whereas the R218Q substitution (12) affects a highly conserved residue located at the boundary between the N terminus and the M_1 segment (Supplemental Fig. 1).

The V260M mutant GlyRA1 receptors displayed a 5-fold increase in the EC_{50} for Gly; a similar effect was observed in Q266H GlyRA1 mutant receptors, as reported previously (23). Theoretically, these changes in agonist sensitivity could be caused either by changes in agonist binding or by gating properties (or both) (27). The decreased effectiveness of maximal concentrations of β-amino acids (TAU and β-ALA) suggests that the coupling mechanism between agonist binding and channel gating is rendered less efficient in these GlyRA1 mutants, implying that these two M_2 residues may participate in the molecular motions that occur during the gating process (24). However, these changes are relatively modest when compared with those produced by mutations in other critical gating residues, most notably R271Q, the first HE mutation to be identified (8, 26).

Our results with the R218Q substitution (12) were remark-

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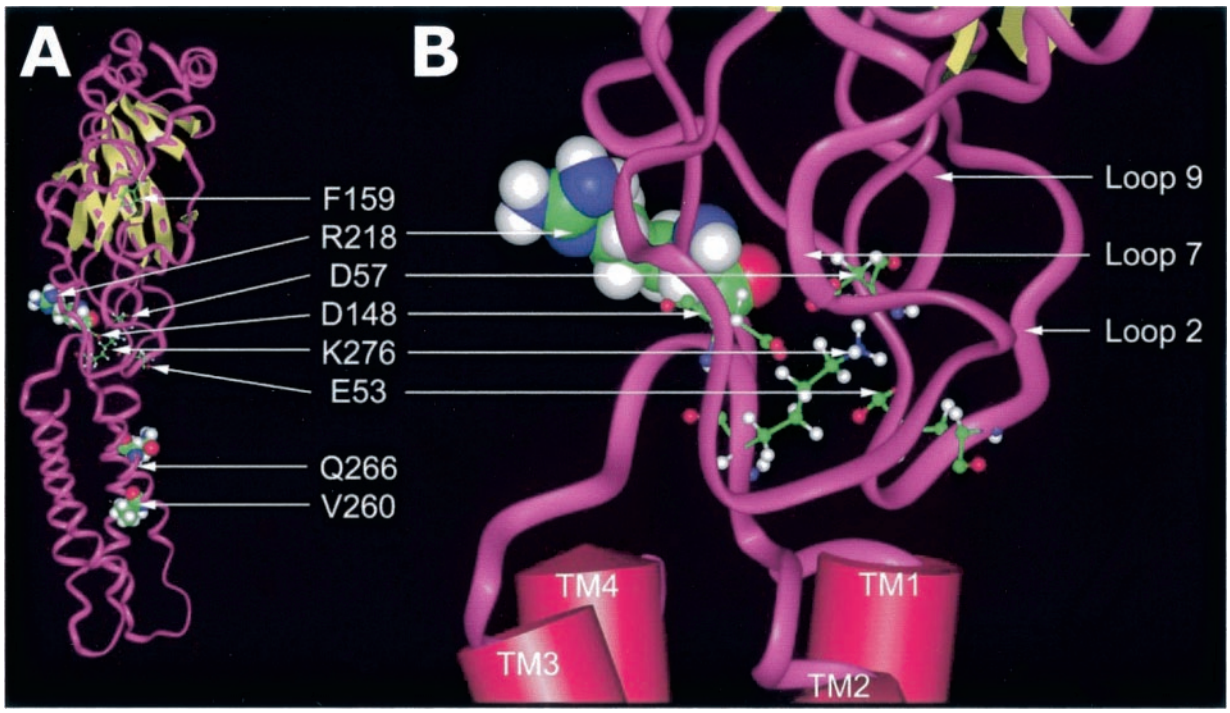

FIG. 6. **Molecular model of a GlyR α_1 subunit.** *A*, the three amino acid residues at which mutations were studied (Arg-218, Val-260, Gln-266) are rendered with space-filling surfaces; carbon, oxygen, nitrogen, and hydrogen atoms are colored *green*, *red*, *blue*, and *white*, respectively. β sheets are shown as *yellow ribbons*, and the peptide backbone is rendered as a *purple tube*. *TM1–4* refer to transmembrane segments 1–4 (19). Phe-159 is rendered in *stick format* to illustrate the location of the glycine agonist binding site. *B*, an enlarged view of the interface between the ligand binding and transmembrane domains. Amino acid residues that may interact with Arg-218 are rendered in *ball-and-stick format*, and α helices are rendered as *red cylinders*. The designation of loops is from the crystal structure of the acetylcholine binding protein (AChBP, Protein Data Bank file 1L9B) (43).

able. The R218Q mutation caused a 200-fold decrease in the apparent affinity of Gly at its receptor. In these channels, maximal concentrations of β-ALA and TAU were virtually unable to activate Cl^- currents. Once again, these drastic changes could reflect alterations in the agonist binding site, but the observation that Gly-gated currents were similarly antagonized by low concentrations of the competitive antagonist STR in both wt and R218Q GlyRA1 channels suggests that any changes at the agonist/antagonist binding site must be subtle. The efficacy of β-ALA and TAU was reduced close to zero in R218Q GlyRA1 channels, suggesting a very deleterious change in the coupling machinery by the R218Q mutation in a residue close to the M_1 segment of the GlyRA1 subunit. However, it should be emphasized that, because of technical limitations (limited solubility and osmolarity changes), agonist concentrations up to 30 mM were used in these experiments; thus, we cannot exclude the possibility that higher (>100 mM) β-ALA and TAU concentrations might have elicited partial agonist responses in R218Q GlyRA1 channels.

Interestingly, at the highest Gly concentration (30 mM), both activation and desensitization rates of wtGlyRA1 channels were faster than those observed in GlyRA1 R218Q channels, with no change in deactivation rates. These data are consistent with the hypothesis of a deleterious effect of the R218Q mutation on channel gating since at these high agonist concentrations, the activation rate approximates the channel opening rate. Given the positive coupling between channel desensitization and opening demonstrated for nicotinic acetylcholine receptors (32), it seems plausible that an enhancement in the channel opening rate also increases the proportion of receptors entering the desensitized state from the open state. An interference with the gating process has been also proposed to explain the results recently obtained upon mutation of an arginine residue (Arg-222) located immediately past the residue corresponding to Arg-218 in GlyRA1 receptors in another member of the LGIC family, the $5HT_{3A}$ receptor (33). The possible alteration of GlyRA1 gating by the R218Q mutation is also supported by our molecular model (Fig. 6*A*), in which Arg-218 is located a long distance from the agonist binding site (which is close to Phe-159) (34). In fact, the distance between the Cα atoms of Phe-159 and Arg-218 is 29 Å in our model; however, Arg-218 lies relatively close to other residues that have been implicated in the gating process, notably Arg-271 (8, 26), Lys-276, and Asp-148 (24). The function of Arg-218 cannot easily be surmised from this model, but several negatively charged residues (Glu-53, Asp-57, Asp-148) are located close to Arg-218 and might interact with it during the gating process (Fig. 6*B*), as has been proposed for other residues within the corresponding region of the closely related $GABA_A$ receptor (20). It is also striking that this residue is strictly conserved among subunits forming both cation-selective (nicotinic, serotoninergic, some purinergic) and anion-selective ($GABA_{A,C}$, Gly) receptors within the LGIC super-family, indicating that it is likely to play a vitally important structural or functional role.

In addition to those mutations that cause functional changes in channels that have been properly inserted into the plasma membrane, disease-causing human mutations may affect channel function by interfering with the complex mechanism of protein folding, maturation, assembly, membrane trafficking, and retrieval (35, 36). Our results show that the steady-state levels of R218Q GlyRA1 subunit protein were markedly decreased when compared with wtGlyRA1 subunits, whereas the levels of the V260M or Q266H subunit protein were normal, as reported previously for another HE-causing GlyRA1 mutant (P250T) located in the intracellular linker between the transmembrane regions M_1 and M_2 (29). Thus, in addition to profoundly affecting gating, the R218Q mutation reduces GlyR function by drastically decreasing the number of receptors in-

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corporated into the plasma-membrane, possibly because of a decreased synthesis and/or increased degradation of the receptor protein (37, 38). Interestingly, wtGlyRs can be internalized and cleaved proteolytically after ubiquitination at the plasma membrane (39). It will be of interest to verify whether GlyRA1 subunits carrying the R218Q mutation undergo an enhanced protein turnover as a consequence of an increased plasma membrane ubiquitination prompted by the mutation.

It should be underlined that, although in *Xenopus* oocytes expression level affects GlyR agonist sensitivity (30, 31), the reduced sensitivity of R218Q mutant receptors to Gly seems not to be a consequence of the decreased expression of mutant subunits in HEK-293 cells. In fact, agonist sensitivity of wtGlyRA1s was unchanged when these receptors were expressed at levels comparable with those of GlyRA1s carrying the R218Q mutation. Interestingly, the observation that the maximal macroscopic currents recorded from cells expressing GlyRA1 R218Q mutant subunits are considerably smaller than those recorded from cells expressing a comparable amount of wtGlyRA1 subunits seems to suggest that the R218Q mutation might affect single channel conductance or maximal opening probability, although this possibility remains to be verified.

When compared with other GlyRA1 mutations found among HE-affected patients, the R218Q mutation represents the most dramatic end of the spectrum in terms of its effects on the receptor function and expression. Interestingly, the clinical evolution of the disease in the child carrying this mutation also lies at one extreme end of an ample spectrum of phenotypic severity; in fact, in addition to the classical HE symptoms, the affected child also showed a considerable degree of neurocognitive impairment, requiring special education in school at 6 years of age (12). This result is of considerable interest in view of the fact that neurocognitive development is usually reported to be normal in HE patients (6), and no perinatal environmental factor could be identified that may explain the neurocognitive impairment of our patient (12). In a similar case of a 6-year-old child affected by a severe recessive form of HE (40), a progressive decrease of the classical HE symptoms occurred with time, but mental retardation developed during childhood. The GlyRA1 mutation described in this case was a complete deletion of exons 1–6 of the *GlyRA1* gene, which would result in a complete loss of protein. This is in close analogy with our results in the R218Q mutant, in which a 200-fold decrease in Gly potency, regardless of the molecular nature of the defect, can be considered as a functional *GlyRA1* knockout. Thus, GlyRA1 subunits, in addition to their role in brainstem reflex modulation and spinal control of muscle tone, might also participate in neurocognitive development in humans. GlyRs have been characterized in septal cholinergic neurons, which play a major role in learning and memory by means of their connections with the hippocampus and the cerebral cortex (41), and their function can be modulated by several psychotropic drugs (4). However, compensatory mechanisms for the lack of GlyRA1 subunits seem to operate more efficiently in humans than in mice. The frameshift mutation found in the *oscillator* mouse (11) produces a complete loss of GlyR α_1 polypeptide in the mouse central nervous system (42), and homozygous *oscillator* mice all die around the 10th postnatal day.

In conclusion, our analysis of the molecular defects associated with three HE-causing mutations in GlyRA1 subunits confirm a modular hypothesis for LGIC subunits, in which distinct regions participate in agonist and antagonist binding, gating, and post-translational stability (4), and reveals a potential novel role for the Arg-218 residue in GlyR gating. This mutation has a dramatic effect on several aspects of GlyR function and is associated with a more severe clinical phenotype, possibly highlighting a previously under-recognized role of GlyRA1 subunits in higher cognitive functions in humans.

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