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RESEARCH PAPER

Function of hyperekplexia-causing α1R271Q/L glycine receptors is restored by shifting the affected residue out of the allosteric signalling pathway

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BACKGROUND AND PURPOSE

Glycine receptor α1 subunit R271Q and R271L (α1R271Q/L) mutations cause the neuromotor disorder, hereditary hyperekplexia. Studies suggest that the 271 residue is located within the allosteric signalling pathway linking the agonist binding site to the channel gate. The present study aimed to investigate a possible mechanism for restoring the function of the α1R271Q/L glycine receptor.

EXPERIMENTAL APPROACH

A 12-amino-acid segment incorporating the 271 residue on the glycine receptor α1271Q/L subunit was replaced by the homologous segment from the glycine receptor β subunit ($\alpha1_{Ch}$271Q/L). The function of the $\alpha1_{Ch}$271Q/L glycine receptor was examined by whole-cell patch-clamp recording and voltage-clamp fluorometry techniques.

KEY RESULTS

The function of the $\alpha1_{Ch}$271Q/L glycine receptor was restored to the level of the wild-type (WT) α1 glycine receptor. Moreover, in the $\alpha1_{Ch}$ glycine receptor, in contrast to the α1 glycine receptor, the channel function was not sensitive to various substitutions of the 271 residue, and the conformational change in the vicinity of the 271 residue was uncoupled from the channel gating.

CONCLUSIONS AND IMPLICATIONS

The 271 residue is shifted out of the allosteric signalling pathway in the $\alpha1_{Ch}$ glycine receptor. We propose that this mechanism provides a novel drug design strategy not only for glycine receptor α1R271Q/L-caused hereditary hyperekplexia, but also for any pathological condition that is caused by missense mutation- or covalent modification-induced disorders involving residues in allosteric signalling pathways. Such a strategy makes it possible to design an ideal drug, which only corrects the function of the mutant or modified protein without affecting the WT or naive protein.

LINKED ARTICLE

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Abbreviations

ECD, extracellular domain; MTSR, sulphorhodamine methanethiosulphonate; PPF, propofol; TMD, transmembrane domain; TMRM, tetramethylrhodamine methyl ester; VCF, voltage-clamp fluorometry; WT, wild-type

Introduction

Missense mutations and abnormal covalent modifications of certain residues in proteins are causes of a huge body of pathological conditions. Hereditary hyperekplexia (startle disease), which is a neuromotor disorder characterized by exaggerated startle reflexes and hypertonia in response to sudden unexpected auditory or tactile stimuli, is mainly

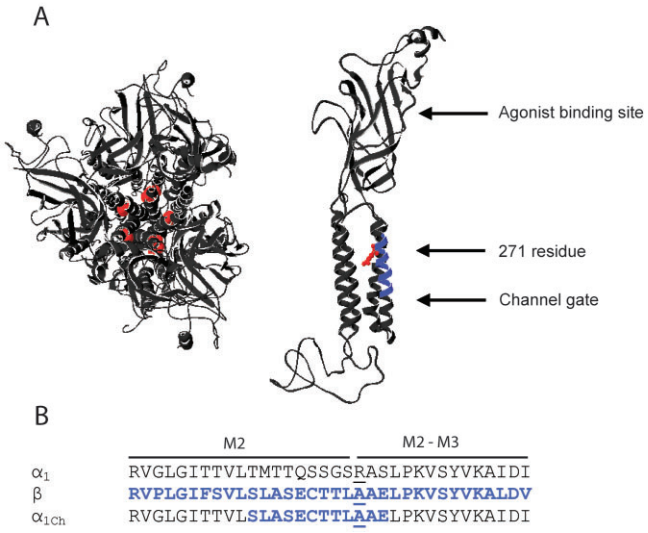

Figure 1

Location of the 271 residue on the glycine receptor α1 subunit. (A) In structural models of the pentameric glycine receptor (top view, left panel) and single α1 subunit (side-view, right panel) (Chung *et al.*, 2010), the 271 residue (red) is physically located between the agonist binding site and the channel gate. The 12-amino-acid segment incorporating the 271 residue is highlighted in blue in the model of single α1 subunit. (B) Sequences of the M2 and M2–M3 domains of the glycine receptor α1, β and $\alpha1_{Ch}$ subunits are shown. The 271 residues are underlined.

caused by hereditary mutations to the inhibitory postsynaptic neurotransmitter receptor, the glycine receptor chloride channel (Harvey *et al.*, 2008; Chung *et al.*, 2010).

The glycine receptor exists as a pentamer. Each subunit is composed of an N-terminal extracellular domain (ECD) and four transmembrane domains (TMD) M1–4. Agonist binding to the ECDs (Brejc *et al.*, 2001; Unwin, 2005; Hibbs and Gouaux, 2011), via an allosteric signalling pathway (channel-gating pathway), leads to the opening of the channel pore, which is lined by the M2 TMDs (Figure 1A) (Bouzat *et al.*, 2004; Lummis *et al.*, 2005; Unwin, 2005; Hilf and Dutzler, 2008; 2009; Bocquet *et al.*, 2009; Lee *et al.*, 2009; Hibbs and Gouaux, 2011).

The most commonly occurring hyperekplexia-causing mutations are R271Q and R271L (R271Q/L) in the glycine receptor α1 subunit (Zhou *et al.*, 2002). This residue lies at the extracellular mouth of the channel pore, physically located between the agonist-binding sites and channel gate (Unwin, 2005; Hilf and Dutzler, 2008; 2009; Hibbs and Gouaux, 2011) (Figure 1A). The R271Q/L mutations exert their pathological effects by reducing agonist glycine sensitivity (Figure 2A and B) (Lynch, 2004). Many other residue substitutions at this site, such as R271A, also reduce glycine sensitivity (Figure 2B) (Langosch *et al.*, 1994; Rajendra *et al.*, 1994; Lynch *et al.*, 1997; 2001). Furthermore, taurine, which is a low-efficacy glycine receptor agonist, completely fails to activate the α1R271Q/L/A glycine receptor channel opening (Figure 3A and B) (Rajendra *et al.*, 1995). Moreover, this residue and those in its vicinity also experience a conformational change during channel gating and more importantly this change is coupled to the channel-gating process (Pless *et al.*, 2007). Taken together, these results suggest that the 271 residue is located within the channel-gating pathway that functionally links the agonist-binding site to the channel gate in the glycine receptor (Figure 1A).

Hereditary hyperekplexia, including those resulting from the R271Q/L glycine receptor mutations, are currently treated by using benzodiazepines, such as clonazepam, which act presumably by potentiating another inhibitory postsynaptic receptor, the type A GABA ($GABA_A$) receptor (Zhou *et al.*, 2002; Bakker *et al.*, 2009; Thomas *et al.*, 2010). However, the treatment is non-specific and symptomatic. Although there are barely any case reports, due to the limited literature, on the side effects of using clonazepam to treat hyperekplexia, drowsiness, ataxia and behaviour problems have often been listed as side effects when using clonazepam to treat other more common neurological disorders, such as epilepsy (Browne, 1976). Moreover, in contrast to the majority of hyperekplexia-causing mutations, which are recessive and do not require life-long treatment, the R271L/Q glycine receptor mutations are dominant, present life-long symptoms and require long-term treatment (Rees *et al.*, 2006; Harvey *et al.*, 2008; Chung *et al.*, 2010). This posits a high chance of potential serious side effects if the benzodiazepine clonazepam is used. To minimize the occurrence of side effects, the ideal treatment would be one that specifically corrects the structural or functional defect imposed by the disease mutation.

Here we report that the replacement of a 12-amino-acid (12-AA) segment incorporating the 271 residue on the glycine receptor α1 subunit with the homologous segment from the glycine receptor β subunit restores the function of the α1R271Q/L glycine receptor. Further experiments suggest that such a restoration is achieved by altering the local microenvironment in the vicinity of the 271 residue and in consequence shifting this residue out of the dominant channel-gating pathway.

Like residue replacement, the binding of a small molecule could also alter local conformation (Todd and Freire, 1999; Kumar *et al.*, 2000; del Sol *et al.*, 2009; Kar *et al.*, 2010), and therefore, our proposal could form the basis for a universal mutant or modified residue-specific drug design strategy: an allosteric drug (Kar *et al.*, 2010) can be designed to alter the microenvironment in the vicinity of the affected residue and thereby eliminate the residue from the dominant allosteric signalling pathway. Such a strategy may make it possible to design an 'ideal' drug that simply corrects the function of the mutant or modified protein without affecting the wild-type (WT) or naive protein.

Methods

Mutagenesis and chimera construction of the glycine receptor cDNAs

Nomenclature used in this article conforms to the *Guide to Receptors and Channels* published in the British Journal of Pharmacology (Alexander *et al.*, 2011).

The human glycine receptor α1 cDNAs were subcloned into the pcDNA3.1zeo+ (Invitrogen, Carlsbad, CA, USA) or pGEMHE (Liman *et al.*, 1992) plasmid vectors for expression

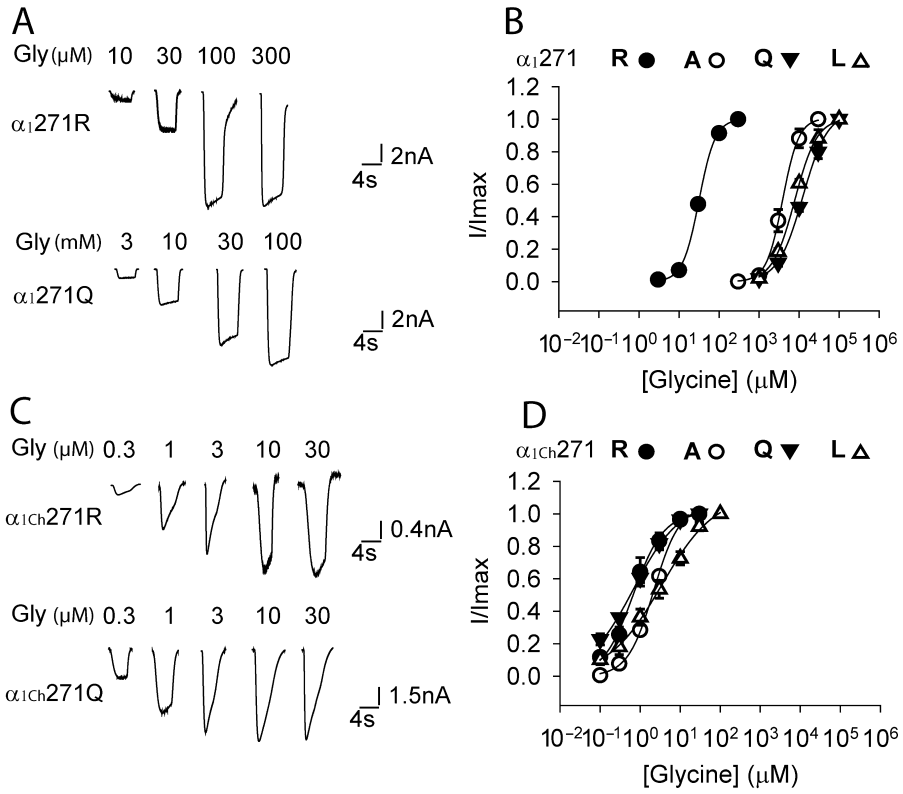

Figure 2

Effects of various substitutions of the 271 residue on the glycine (Gly) sensitivity of the α1 and $\alpha1_{Ch}$ glycine receptors. Example traces of currents induced by increasing glycine concentrations in the indicated constructs of the α1 and $\alpha1_{Ch}$ glycine receptors are shown in (A) and (C), respectively. Averaged normalized glycine dose–response curves for various substitutions of the 271 residue of the α1 and $\alpha1_{Ch}$ glycine receptors are shown in (B) and (D), respectively (n = 3 or 4).

in HEK293 cells or *Xenopus* oocytes, respectively. Site-directed mutagenesis and chimera construction were performed using the QuickChange (Stratagene, La Jolla, CA, USA) mutagenesis and multiple-template-based sequential PCR protocols, respectively.

The multiple-template-based sequential PCR protocol for chimera construction was developed in our laboratory and has recently been described in detail elsewhere (Shan and Lynch, 2010). This procedure does not require the existence of restriction sites or the purification of intermediate PCR products, and needs only two or three simple PCRs followed by general subcloning steps. Most importantly, the chimera joining sites are seamless and the success rate for construction is nearly 100% (Shan and Lynch, 2010).

In the voltage-clamp fluorometry (VCF) experiments, to eliminate non-essential background cysteines, the C41A mutation was introduced into the glycine receptor α1 cDNAs in the pGEMHE vector (Shan *et al.*, 2003), and a further C267S mutation was introduced into the 12-AA region of the glycine receptor $\alpha1_{Ch}$ cDNA. This manipulation did not alter channel function.

HEK293 cell culture, expression and electrophysiological recording

The effects of various substitutions of the 271 residue on the glycine and taurine sensitivity of the α1 and $\alpha1_{Ch}$ glycine receptors were determined by experiments on HEK293 cells. Details of the HEK293 cell culture, glycine receptor expression and electrophysiological recording of the HEK293 cells are described elsewhere (Shan *et al.*, 2001b). Briefly, HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected using a calcium phosphate precipitation protocol. In addition, the pEGFP-N1 (Clontech, Mountain View, CA, USA) was co-transfected to facilitate identifying the transfected cells.

Glycine and taurine-induced currents were measured using the whole cell patch-clamp configuration. Cells were treated with external Ringer's solution and internal CsCl solution (Shan *et al.*, 2001b). Cells were voltage-clamped at –40 mV.

Xenopus *oocyte preparation, expression and VCF recording*

VCF experiments were performed on glycine receptors expressed in *Xenopus* oocytes. Details of oocyte preparation, glycine receptor expression and VCF recording are described elsewhere (Pless *et al.*, 2007). Briefly, the mMessage mMachine kit (Ambion, Austin, TX, USA) was used to generate capped mRNA. The mRNA was injected into oocytes of the female *Xenopus laevis* frog with 10 ng per oocyte. After the

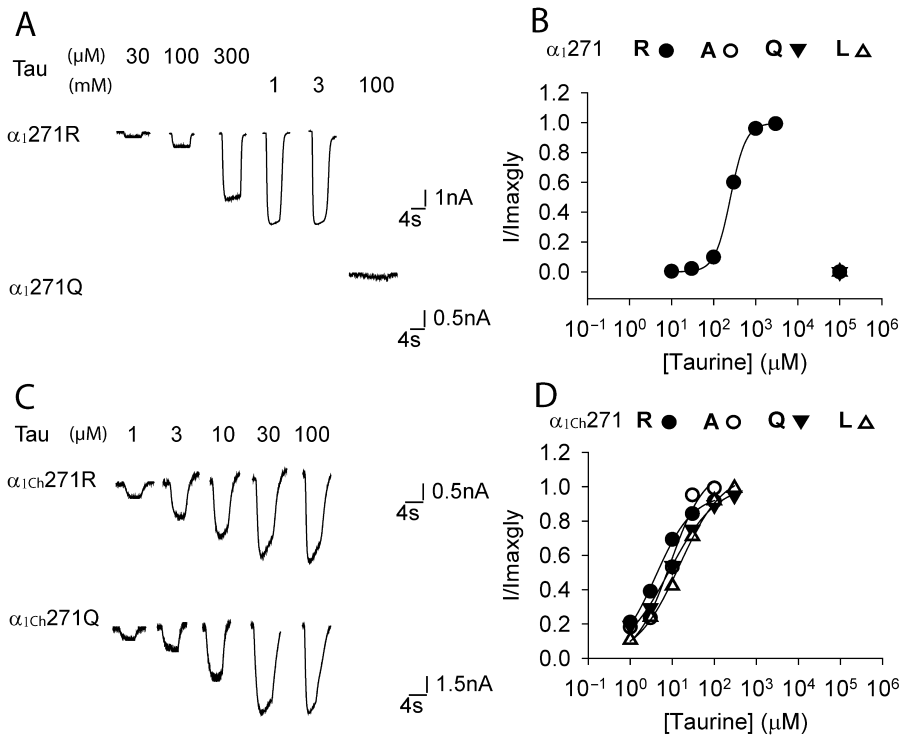

Figure 3

Effects of various substitutions of the 271 residue on the taurine sensitivity of the α1 and $\alpha1_{Ch}$ glycine receptors. Example traces of currents induced by increasing taurine concentrations in the indicated constructs of the α1 and $\alpha1_{Ch}$ glycine receptors are shown in (A) and (C), respectively. Averaged normalized taurine dose–response curves for various substitutions of the 271 residue of the α1 and $\alpha1_{Ch}$ glycine receptors are shown in (B) and (D), respectively (n = 3 or 4).

injection, the oocytes were incubated in ND96 solution (Pless *et al.*, 2007) for 3–4 days at 18°C before recording.

The sulphhydryl-reactive reagents, sulphorhodamine methanethiosulphonate (MTSR, Toronto Research Chemicals, North York, Ontario, Canada) and tetramethylrhodamine methyl ester (TMRM; Invitrogen), were used to label the 271C residues. On the day of recording, the oocytes were labelled with 10 μM MTSR for 25 s or 10 μM TMRM for 60 min, either in the absence or presence of glycine. The oocytes were then transferred to the recording chamber and perfused with ND96 solution. The current was recorded by the two-electrode voltage-clamp configuration and the recording electrode was filled with 3 M KCl. Cells were voltage-clamped at –40 mV. The fluorescence was recorded using the PhotoMax 200 photodiode detection system (Dagan Corp., Minneapolis, MN, USA).

Data analysis

Results are expressed as mean ± SEM of three or more independent experiments. The empirical Hill equation, fitted by a non-linear least squares algorithm (SigmaPlot 9.0; Systat Software, Point Richmond, CA, USA), was used to calculate the EC_{50} values for glycine- or taurine-induced current and fluorescence changes. Statistical significance was determined using Student's *t*-test.

Results

Replacement of the 12-AA segment incorporating the 271 residue restores the function of α1R271Q/L glycine receptor

The glycine receptor and the $GABA_A$ receptor, two major chloride-permeable postsynaptic neurotransmitter receptors, share common structural and functional characteristics and possibly even the same evolutionary origin (Lynch, 2004; Miller and Smart, 2010; Thompson *et al.*, 2010). It has long been recognized that, with few exceptions, an Arg at sites corresponding to the 271 position of the glycine receptor α1 subunit is a signature of both the glycine receptor and $GABA_A$ receptor subunit members (including the glycine receptor α1 subunit) (Supporting Information Figure S1). One of the exceptions is the glycine receptor β subunit, where an Ala exists at this position (Figure 1B). The heteromeric glycine receptor that incorporates three Ala-carrying β subunits together with two α1 subunits exhibits a glycine sensitivity similar to that of the homomeric α1 glycine receptor (Shan *et al.*, 2001b; Grudzinska *et al.*, 2005). On the other hand, replacing the Arg in the α1 glycine receptor with Ala compromises channel function and mimics the phenotype of the α1R271Q/L glycine receptor (Figure 2A and B) (Lynch *et al.*, 1997).

Table 1

Properties of glycine- and taurine- induced currents of glycine receptors

	Glycine		Taurine		
Glycine receptor	EC_{50} (μM)	*n*	EC_{50} (μM)	Imax,tau/ Imax,gly (%)	*n*
α1					
271R(WT)	33 ± 2	4	261 ± 32	99 ± 1	3
271A	5700 ± 1600	4	N.D.	0	3
271Q	13200 ± 2000	4	N.D.	0	3
271 L	8000 ± 490	3	N.D.	0	3
$α1_{Ch}$					
271R	0.87 ± 0.19	4	3.7 ± 0.3	92 ± 5	4
271A(WT)	2.2 ± 0.2	4	8.7 ± 0.9	99 ± 1	4
271Q	0.65 ± 0.06	4	7.3 ± 0.7	95 ± 2	4
271 L	3.4 ± 1.1	4	16 ± 3	100 ± 0	4

N.D., not determined because taurine exhibited no agonist efficacy.

Supposing that this paradox might be due to a local effect, we replaced the 12-AA segment (262–273 residues) incorporating the 271 residue in the glycine receptor α1 subunit with the homologous segment from the glycine receptor β subunit (Figure 1B). The modified subunit was named the glycine receptor $α1_{Ch}$ subunit (Ch is short for chimera) (Figure 1B). Surprisingly, the $α1_{Ch}$ glycine receptor, which has an Ala at the 271 position, showed a glycine sensitivity 2600 times higher than the α1R271A glycine receptor and even an order of magnitude higher than the α1WT glycine receptor (Figure 2B and D, Table 1). Because the α1R271A glycine receptor mimics the phenotype of α1R271Q/L glycine receptors, we wondered whether this 12-AA segment replacement also restored the function of α1R271Q/L glycine receptors. We next introduced either Gln or Leu to the 271 position of the $α1_{Ch}$ glycine receptor. Both constructs demonstrated glycine sensitivities 20000 (Gln) and 2500 (Leu) times higher than their corresponding substitutions in the α1 glycine receptor (Figure 2B–D, Table 1). We concluded that the 12-AA segment replacement restored the function of the α1R271Q/L glycine receptors.

As noted above, the α1R271Q/L glycine receptors are completely insensitive to activation by the low-efficacy agonist, taurine (Figure 3A and B) (Rajendra *et al.*, 1995). We thus investigated whether the 12-AA replacement also restored the taurine sensitivity of α1R271Q/L glycine receptors to WT levels. As shown in Figure 3C and D and Table 1, taurine behaved as a full-agonist in activating the $α1_{Ch}$271Q/L/A glycine receptors with a sensitivity even higher than in the WT α1 glycine receptor. We therefore concluded that the 12-AA replacement also restored taurine sensitivity of the α1R271Q/L glycine receptors.

Replacement of the 12-AA segment incorporating the 271 residue diminishes the residue's contribution to channel gating

To further characterize the 271 residue in the $α1_{Ch}$ glycine receptor, we replaced the 271 Ala with Arg, which is the residue at the 271 position of the α1WT glycine receptor. Surprisingly, the $α1_{Ch}$271R glycine receptor indicated a glycine sensitivity similar to that of the $α1_{Ch}$271Q/L/A glycine receptors (Figure 2C and D, Table 1), in sharp contrast to the case of the α1 glycine receptor, where the α1271R(WT) glycine receptor showed a glycine sensitivity 180–400 times higher than the α1R271Q/L/A glycine receptors (Figure 2A and B, Table 1). Consistently, the $α1_{Ch}$271R glycine receptor also demonstrated taurine sensitivity and maximal response similar to those of the $α1_{Ch}$271Q/L/A glycine receptors (Figure 3C and D, Table 1). Such insensitivity of the $α1_{Ch}$ glycine receptor to various residue substitutions at the 271 position implies that this residue might have a diminished contribution to channel gating in the chimeric receptor.

However, it is also possibly because the energy barrier of the channel-gating pathway, which is reflected by the glycine EC_{50}s in this case (Colquhoun, 1998), has reached its lowest limit ('ceiling effect') in the $α1_{Ch}$271Q/L/A glycine receptors, as these constructs had very low glycine EC_{50}s, around 1 μM (Table 1). In this scenario, the energy barrier (glycine EC_{50}) would not reduce further when a more gating-favourable Arg is in place. If that is the case, we argue that the channel function would not be enhanced by a potentiator. To test this possibility, we applied the glycine receptor potentiator, propofol (PPF), to the $α1_{Ch}$271Q glycine receptor, which exhibited the lowest glycine EC_{50} among the Q/L/A substitutions (Table 1). As shown in Figure 4A, PPF enhanced the subsaturating glycine induced current by 93 ± 10 % (n = 4). Moreover, PPF left-shifted the glycine dose–response curve of the $α1_{Ch}$271Q glycine receptor (EC_{50} < 0.3 μM, n = 4 in the presence of PPF vs. EC_{50} = 0.65 ± 0.06 μM, n = 4 in the absence of PPF, Figure 4B and C). Note that it was not possible to quantify glycine concentrations less than 0.3 μM due to a variable contribution from the glycine that inevitably contaminates salt solutions (0.01–0.1 μM). These data imply that the energy barrier of the channel-gating pathway of the $α1_{Ch}$271Q glycine receptor has not reached the lowest limit,

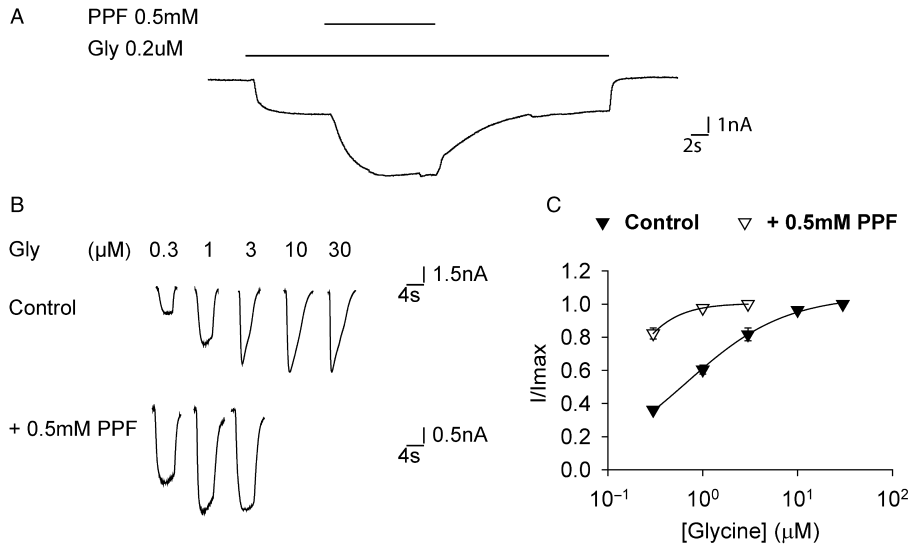

Figure 4

Propofol (PPF) potentiation of $\alpha1_{Ch}$271Q glycine receptor function. (A) Example of propofol potentiating sub-saturating glycine-induced $\alpha1_{Ch}$271Q glycine receptor currents. (B) Example traces of $\alpha1_{Ch}$271Q glycine receptor currents induced by increasing glycine concentrations in the absence and presence of propofol. (C) Averaged normalized glycine dose–response curves of the $\alpha1_{Ch}$271Q glycine receptor in the absence and presence of propofol (n = 4).

confirming that the insensitivity of the $\alpha1_{Ch}$ glycine receptor to various residue substitutions at the 271 position is due to this residue's diminished contribution to channel gating.

Replacement of the 12-AA segment incorporating the 271 residue alters its local microenvironment

We next sought to determine the underlying mechanism for the different contributions of the 271 residue to channel gating in the α1 and $\alpha1_{Ch}$ glycine receptors. To achieve this, we turned to the VCF technique. VCF detects local conformational changes in the vicinity of a residue when the residue is labelled with a fluorescent dye (Gandhi and Isacoff, 2005; Pless and Lynch, 2008). Rhodamine fluorescent dyes are usually used, because rhodamine fluorescence exhibits an increase in quantum efficiency as the hydrophobicity of its environment is increased. Thus, rhodamine fluorescence intensity reports the change of hydrophobicity of its immediate microenvironment, which is often caused by local conformational changes. The VCF experiments were carried out in *Xenopus* oocytes, as fluorescence detection is not routinely possible in glycine receptors expressed in HEK293 cells (Pless and Lynch, 2008).

To label the 271 position with a rhodamine fluorescent dye, a cysteine was introduced to this position so that the dye can be attached through a disulphide bond (Gandhi and Isacoff, 2005; Pless and Lynch, 2008). Interestingly, the α1271C and $\alpha1_{Ch}$271C glycine receptors exhibited glycine EC_{50} values of 4300 ± 200 μM (n = 4) and 2.1 ± 0.4 μM (n = 5), respectively. It is thus evident that the 271C residue behaves in the same manner as the Q/L substitutions, in both the $\alpha1_{Ch}$ and α1 glycine receptors. The result of the VCF investigation is therefore expected to reflect the behaviour of the 271Q/L substitutions.

As previously reported (Pless *et al.*, 2007), we confirmed that the rhodamine fluorescent dye MTSR, when attached to the 271C residue in the α1 glycine receptor, exhibited an increase in fluorescence intensity (reflected by the upwards step of the fluorescence trace) upon glycine application (Figure 5A). This implies that MTSR detected an increase of hydrophobicity in the vicinal microenvironment due to a local conformational change during channel gating. Moreover, as the fluorescence and current glycine dose–response relationships overlapped, we concluded that the local conformational change is coupled with a channel-gating process. This conclusion is consistent with the suggestion that the 271 residue in the α1 glycine receptor lies within the dominant channel-gating pathway, as previously proposed (Langosch *et al.*, 1994; Rajendra *et al.*, 1994; 1995; Lynch *et al.*, 1997; 2001).

Following the same protocol, the $\alpha1_{Ch}$271C glycine receptor was labelled with MTSR and subjected to VCF investigation. Surprisingly, no fluorescence change was detected upon glycine application (Figure 5A). The 271C residue was possibly not labelled by the MTSR due to structural inaccessibility. Alternatively, this residue was labelled, but during channel gating, either no conformational change occurred in the vicinity of the 271 residue, or the microenvironment hydrophobicity detected by the MTSR fluorophore was not altered even though a local conformational change took place. Nevertheless, such different behaviours of the 271 residue between the α1 and $\alpha1_{Ch}$ glycine receptors suggest that either the static microenvironment or the dynamic microenvironment change during channel gating, or both, in the vicinity of the 271 residue in the $\alpha1_{Ch}$ glycine receptor are altered by

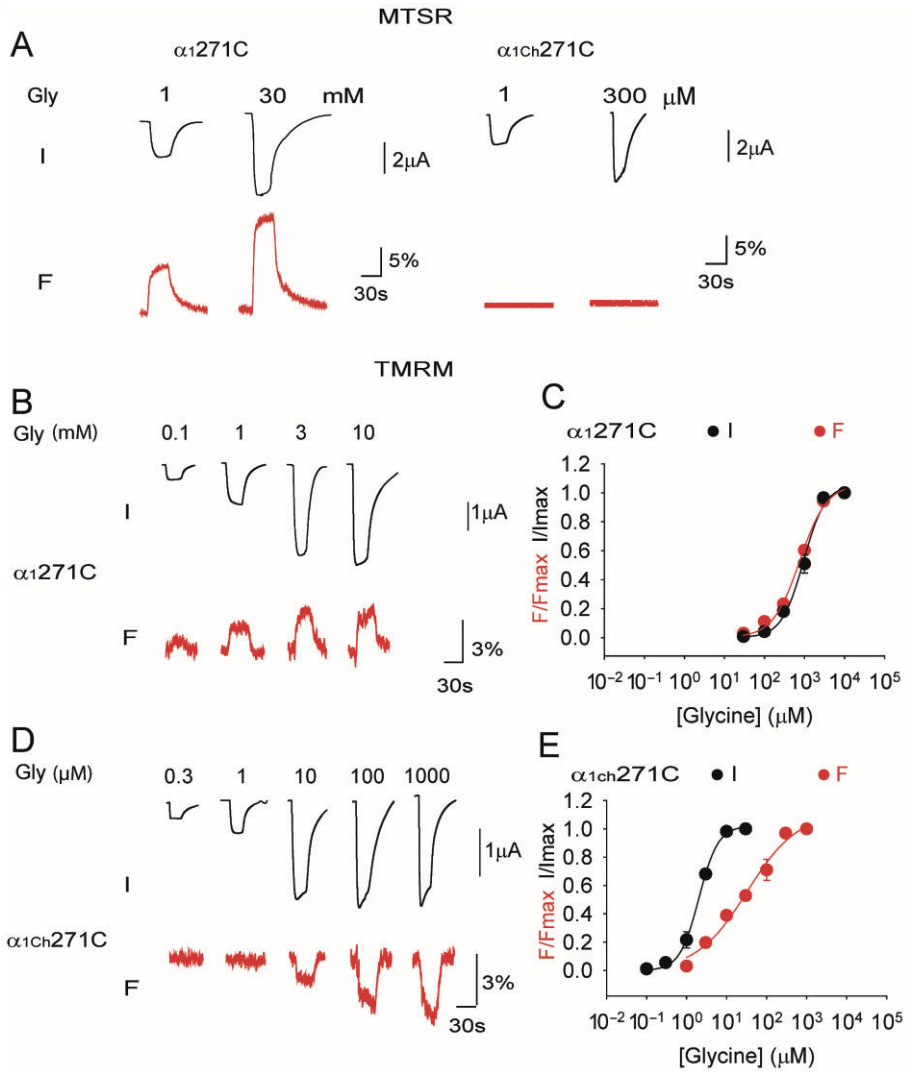

Figure 5

VCF of the α1 and $\alpha1_{Ch}$ glycine receptors. Example current (I) and fluorescence (F) traces of the α1271C and $\alpha1_{Ch}$271C glycine receptors labelled with MTSR or TMRM are shown in (A), (B) and (D). Averaged normalized glycine dose-response curves of current (I) and fluorescence (F) of the α1271C and $\alpha1_{Ch}$271C glycine receptors labelled with TMRM are shown in (C) and (E), respectively (n = 4 or 5).

the 12-AA segment replacement from those in the α1 glycine receptor.

Considering that rhodamine fluorophores are structurally different and may thus respond differently to a given conformational change when attached to the α1271C glycine receptor (Pless *et al.*, 2007), we next investigated the response of another rhodamine fluorescent dye TMRM in the α1271C and $\alpha1_{Ch}$271C glycine receptors. In the TMRM-labelled α1271C glycine receptor, the fluorescence intensity was increased upon glycine application (reflected by the upwards step of the fluorescence trace, Figure 5B). In contrast, in the TMRM-labelled $\alpha1_{Ch}$271C glycine receptor, the fluorescence intensity was decreased upon glycine application (reflected by the downwards step of the fluorescence trace, Figure 5D). Such different direction of fluorescence intensity change provides a more direct indication that either the static microenvironment or the dynamic microenvironment change, or both, during channel gating, in the vicinity of the 271 residue in the $\alpha1_{Ch}$glycine receptor, are distinct from those in the α1 glycine receptor.

More interestingly, the dose–response curve of fluorescence was right-shifted from that of the current in the $\alpha1_{Ch}$271C glycine receptor when TMRM was used (fluorescence EC_{50} = 36 ± 8 μM, n = 4 vs. current EC_{50} = 2.0 ± 0.2 μM, n = 5, P < 0.01, Figure 5E). This is in contrast with the α1271C glycine receptor, where the dose–response curves of fluorescence and current overlapped (fluorescence EC_{50} = 770 ± 150 μM, n = 5 vs. current EC_{50} = 960 ± 120 μM, n = 5, P > 0.05, Figure 5C), consistent with what was observed when MTSR was used (Pless *et al.*, 2007). These data suggest that the conformational change in the vicinity of the 271 residue in the $\alpha1_{Ch}$271C glycine receptor, unlike in the α1271C glycine receptor, is uncoupled from the channel-gating process. We hence propose that, in the $\alpha1_{Ch}$ glycine receptor, the 271

residue is not essential for channel gating and might not reside within the dominant channel-gating pathway. Such a proposal is also supported by the fact that the $\alpha1_{Ch}$ glycine receptor channel function is not sensitive to various residue substitutions at the 271 position, as described earlier.

Discussion

The function of α1R271Q/L glycine receptors is restored by shifting the affected residue out of the dominant channel-gating pathway

Here we report that replacement of a 12-AA segment incorporating the 271 residue of the glycine receptor α1 subunit with the homologous segment of the glycine receptor β subunit restores channel function of the hereditary hyperekplexia-causing α1R271Q/L glycine receptors. More interestingly, through residue substitution and VCF investigation, we concluded that this rescue effect is achieved by adjusting the local microenvironment and in consequence, diminishing the 271 residue's contribution to channel gating. It has been proposed that multiple allosteric signalling pathways exist in proteins, and which pathways dominate is determined by protein topologies, specific binding events, covalent modifications and cellular conditions (del Sol *et al.*, 2009). Residue replacement, which potentially changes the protein topology (Sinha and Nussinov, 2001), can shift the dominant signalling pathway from one pathway to another. In our experiment, the 271 residue lies within the dominant channel-gating pathway in the α1 glycine receptor. However, the 12-AA segment replacement induces a local conformational change and, in consequence, shifts the dominant channel-gating pathway to an alternative one, where the 271 residue does not reside (Figure 6A). The hypothesis that the 271 residue does not reside within the dominant channel-gating pathway is reminiscent of ivermectin-induced glycine receptor channel activation. Ivermectin is a glycine receptor agonist that binds to the glycine receptor and gates the channel opening in a manner distinct from the physiological agonist glycine (Shan *et al.*, 2001a; Pless *et al.*, 2007; Hibbs and Gouaux, 2011). For example, the α1 glycine receptor function activated by ivermectin is almost conserved when the R271Q mutation is introduced (Shan *et al.*, 2001a). Moreover, the MTSR-labelled α1271C glycine receptor does not show any fluorescence change upon ivermectin application (Pless *et al.*, 2007). Both observations imply that the 271 residue does not reside within the ivermectin-mediated channel-gating pathway.

Implications for a residue-specific drug design strategy

Many pathophysiological conditions are caused by residues being either missense mutated or abnormally covalently modified (for example, by phosphorylation). The relevant treatment strategy is usually symptomatic. For example, to treat glycine receptor mutation-caused hereditary hyperekplexia, benzodiazepines, such as clonazepam, are used (Zhou *et al.*, 2002; Thomas *et al.*, 2010). The benzodiazepines, which are $GABA_A$ receptor potentiators, can counter the overexcitation symptoms due to the compromised glycine receptor function. However, such an 'off-target' treatment strategy is the source of a wide range of side effects.

A more specific treatment strategy is to directly target the affected protein. A drug is usually designed either to enhance (in loss-of-function) or to inhibit (in gain-of-function) the function of the affected protein. However, these effects are usually global rather than mutation- or modification-specific, as the drug affects the WT or naïve protein as well as the mutant or modified protein (Wang *et al.*, 2003; Joerger and Fersht, 2007). This will lead to a lack of specificity as proteins usually have multiple subtypes (e.g. α1, α2 and α3 glycine receptors) of different genomic origins, which share a high degree of homology and, in consequence, similar structure and function relationships. Any drug acting on one subtype (e.g. the mutant protein, glycine receptor α1R271Q/L) has a very high chance of affecting other subtypes (e.g. other WT subtypes of the mutant protein such as glycine receptor α2 and α3) as well. As protein subtypes are usually distributed in various tissues and thus have different physiological or pathological roles from each other, a drug that is supposed to only act on the specific target subtype in the ideal state but affects multiple other subtypes in reality, will cause undesirable side effects. Another consideration is that abnormal residue covalent modification of a given protein under a certain pathological condition usually only occurs in a localized region of the human body. A drug that affects the naïve as well as the modified proteins may correct the modifications in the localized region, but would also interfere with processes in other regions where the target protein expresses but without any modification. This is another source of undesirable side effects.

One way to circumvent this 'global effect' is to design a mutant or modified residue-specific drug. This ideal drug should affect the mutant or modified protein but not the WT or naïve protein. Despite many attempts, this goal has been successfully achieved in only a few cases. One successful case is the mutant p53-targeting drug, PRIMA-1. PRIMA-1 affects the function of mutant p53 but not the WT p53 (Bykov *et al.*, 2002a,b), through a mechanism involving modification of thiol groups within the protein (Lambert *et al.*, 2009). However, such a mechanism apparently cannot become a universal strategy for mutant or modified protein-specific drug design.

We proposed in this article that the affected residue could be shifted out of the dominant allosteric signalling pathway by the local conformational change induced by residue substitutions. Since binding of a small molecule, like residue substitutions, can also induce conformational change and redistribute the dominant signalling pathway (Todd and Freire, 1999; Kumar *et al.*, 2000; del Sol *et al.*, 2009; Kar *et al.*, 2010), our proposal could form the basis for a universal mutant or modified residue-specific drug design strategy: an allosteric drug (Kar *et al.*, 2010) can be designed to alter the microenvironment in the vicinity of the affected residue and to activate an alternative allosteric signalling pathway that excludes the affected residue (Figure 6A). This drug action can be realized to have a neutral effect on the WT or naïve protein through activating the alternative allosteric signalling pathway with a strength equivalent to the original one (Figure 6A). However, the drug should restore the function of the mutant or modified proteins to the WT level, because the

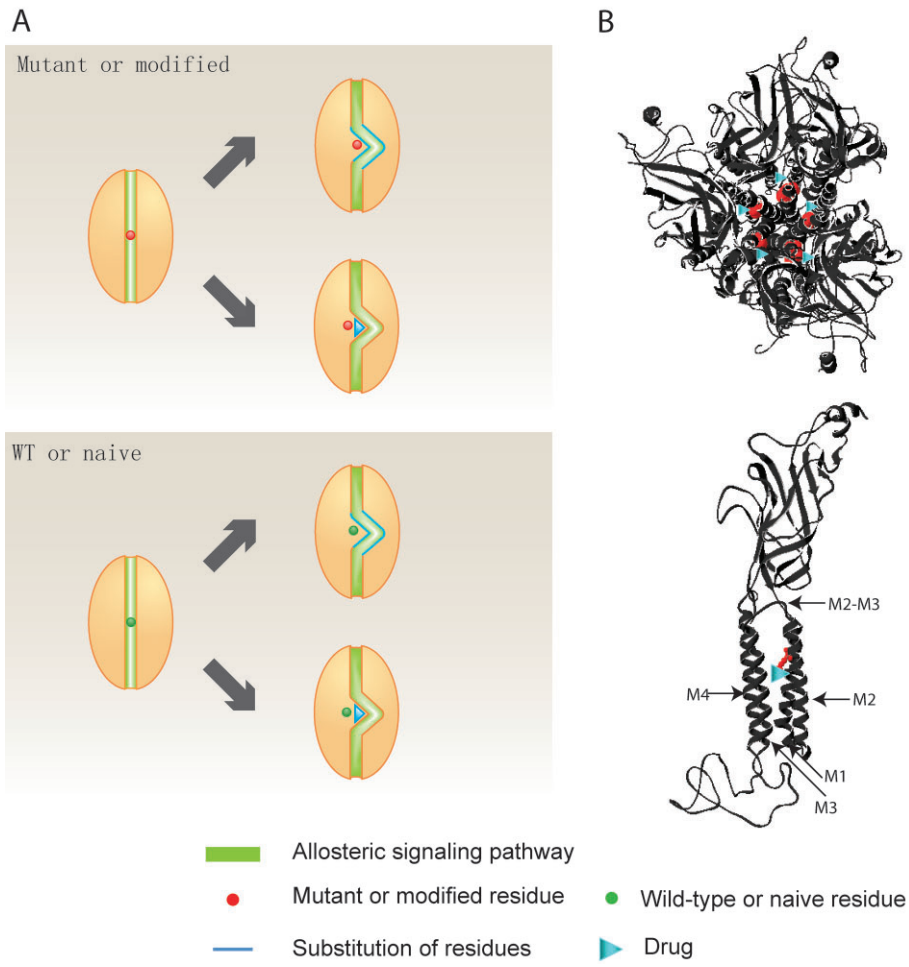

Figure 6

Model of the residue-specific drug design strategy. (A) In a protein with a certain residue, either mutant or modified (red circle), the protein function is compromised because the affected residue blocks the dominant allosteric signalling pathway (green strip). The protein function can be restored by activating an alternative allosteric signalling pathway that does not include the affected residue. This restoration can be achieved through adjusting the local microenvironment, either internally, by substituting the amino acids in the vicinity of the affected residue (blue line), or externally, by applying a drug (blue triangle) that has an equivalent effect as the vicinal amino acid substitution. If the newly activated alternative allosteric signalling pathway has equivalent strength as the original one in the WT or naïve protein, neither the vicinal amino acid substitution nor external drug application apparently affects the WT or naïve protein function. (B) When designing a drug (blue triangle) that specifically corrects the glycine receptor 271Q/L mutations (red residue), a possible docking site for this drug is the cavity formed by the extracellular halves of the M1, M2 and M3 segments and the M2–M3 domain, which is the binding site of many clinically related drugs and substances. The location of the 271 residue and the potential drug molecule are indicated in the structural models of the pentameric glycine receptor (top view, top panel) and single α_1 subunit (side view, bottom panel) (Chung *et al.*, 2010).

affected residue is no longer within the dominant allosteric signalling pathway and hence does not affect the protein function (Figure 6A). This missense mutation- or covalent modification-specific drug design strategy would help tackle one of the most serious problems existing among the drugs clinically used today: lack of specificity.

Possible drug design strategy for the glycine receptor R271Q/L hereditary hyperekplexia

The 12-AA segment that restores the function of the α1R271Q/L glycine receptor is located along the extracellular half of the M2 segment and the M2-M3 domain (Figures 1A and 6B). Both domains, together with the extracellular halves of the M1 and M3 segments, form a cavity, which contains the binding site of many clinically related drugs or substances including alcohol (Mihic *et al.*, 1997), neurosteroids (Hosie *et al.*, 2006), general anaesthetics (Nury *et al.*, 2011) and ivermectin (Collins and Millar, 2010; Lynagh and Lynch, 2010; Hibbs and Gouaux, 2011), and therefore can be used as the potential docking site for drugs that specifically correct the glycine receptor R271Q/L mutations. Interestingly, the general anaesthetic, PPF, which binds into this cavity and potentiates the glycine receptor function, restores the WT phenotype of the hyperekplexic glycine receptor R271Q transgenic mice (O'Shea *et al.*, 2004), although a wide range of side effects would be expected, since PPF also potentiates

the $GABA_A$ receptor and inhibits the nAChR (Franks, 2008). Nevertheless, PPF could possibly serve as the seeding backbone for designing a drug specifically correcting the glycine receptor R271Q/L mutations. The final ideal glycine receptor R271Q/L mutation corrector, by exploiting the novel drug design strategy proposed in the article, could be achieved to affect the function of the α1R271Q/L glycine receptor but not any other protein including the α1WT, α2 and α3 glycine receptors and closely related $GABA_A$ receptor and nAChR.

It should be noted however that this mutation corrector is only effective in treating hereditary hyperekplexia caused by α1R271Q/L mutations, but not by any mutation arising from other sites of the glycine receptor α1 subunit, from the glycine receptor β subunit or from the SLC6A5 glycine transporter. Considering that the absolute number of patients diagnosed with hyperekplexia caused by α1R271Q/L mutations is low, it might not be commercially feasible to develop a specific α1R271Q/L mutation corrector. Instead, the target-specific drug design strategy we propose here provides a general principle for developing drugs that correct mutations or abnormal residue-modifications in proteins.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Alignment of the protein sequences covering the M2 segments and M2–M3 domains of human glycine and GABAA receptor subunits. The residues corresponding to the 271 position of the glycine receptor α1 subunit are highlighted in **bold**.

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