11. Pleural and pedal ganglia were placed in two chambers separated by a Plexiglas wall, which was sealed with petroleum jelly. Each chamber was independently perfused. A single 1.5-hour exposure of 5-HT was used to induce long-term facilitation because the barrier separating the chambers was easily disrupted by repeated bath exchanges required for multiple 5-HT applications.
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Mutation of Glycine Receptor Subunit Creates β-Alanine Receptor Responsive to GABA

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The amino acid at position 160 of the ligand-binding subunit, α1, is an important determinant of agonist and antagonist binding to the glycine receptor. Exchange of the neighboring residues, phenylalanine at position 159 and tyrosine at position 161, increased the efficacy of amino acid agonists. Whereas wild-type α1 channels expressed in *Xenopus* oocytes required 0.7 millimolar β-alanine for a half-maximal response, the doubly mutated (F159Y,Y161F) α1 subunit had an affinity for β-alanine (which was more potent than glycine) that was 110-fold that of the wild type. Also, γ-aminobutyric acid and D-serine, amino acids that do not activate wild-type α1 receptors, efficiently gated the mutant channel. Thus, aromatic hydroxyl groups are crucial for ligand discrimination at inhibitory amino acid receptors.

Neurotransmitter receptors mediate synaptic transmission in the nervous system and constitute important target sites of many therapeutic drugs. The ligand-gated ion channel subclass of neurotransmitter receptors consists of families of oligomeric proteins, which are composed of homologous ligand-binding and structural subunits (*1*). The mechanism of agonist discrimination at these receptors is not understood. Covalent labeling and site-directed mutagenesis experiments indicate that several discontinuous domains in the NH_2-terminal extracellular region of the ligand-binding subunits contribute to the formation of the binding pocket (*2–5*).

In the central nervous system, amino acids are the major excitatory and inhibitory neurotransmitters. The glycine receptor (GlyR) is the most abundant inhibitory neurotransmitter receptor in the spinal cord (*6*), whereas subtype A of the γ-aminobutyric acid receptor ($GABA_AR$) mediates inhibition in the mammalian brain (*7*). The GlyR, isolated from spinal cord (*8*), contains five membrane-spanning subunits (*9*) of apparent molecular masses of 48 kD (α) and 58 kD (β); both subunits are homologous to $GABA_AR$ and, to a much lesser extent, nicotinic acetylcholine receptor (nAChR) proteins (*1*, *10*). Several GlyR α subunits (α1, α2, α2*, and α3) have been identified by complementary DNA (cDNA) cloning (*11–13*). On heterologous expression these ligand-binding subunits generate agonist-gated Cl^- channels whose pharmacology mimics that of the postsynaptic GlyR (*11–15*).

Studies on mutated GlyR α1 and α2 subunits have identified a conserved glycine residue (corresponding to position 160 of the α1 subunit; Fig. 1) as an important determinant of ligand binding (*12*, *16*, *17*). Interestingly, two aromatic amino acids flanking this residue are conserved at the corresponding positions of the agonist-binding subunits of the $GABA_AR$ (*18*). However, residue 159, where a phenylalanine is found in all GlyR subunits, is replaced by a tyrosine in the $GABA_AR$ polypeptides (Fig. 1). We therefore introduced by site-directed mutagenesis (*19*) a tyrosine at position 159 (F159Y) and a phenylalanine at position 161 (Y161F) of the GlyR α1 subunit and exchanged both aromatic residues in the double mutant $\alpha1^{F159Y,Y161F}$. Voltage-clamp analysis of the mutated proteins expressed in *Xenopus* oocytes (*20*) revealed an unfamiliar agonist pharmacology. First, the glycine concentrations required to gate the $\alpha1^{F159Y,Y161F}$ channels were about 1/10 [concentration for eliciting a half-maximal response (EC_{50}) of 22 μM] of those required for the wild-type α1 GlyR (EC_{50} = 260 μM) (Fig. 2 and Table 1). Second, the glycinergic agonist β-alanine (*7*) became more potent than glycine (Fig. 2). Its EC_{50} value decreased about 110-fold from 720 μM for the wild-type α1 GlyR to 6.5 μM for the double mutant (Table 1). Moreover, the Hill coefficient (h) for glycine changed from 2.4 ± 0.2 (mean ± SEM) for the α1 GlyR to 1.8 ± 0.2 for the $\alpha1^{F159Y,Y161F}$ receptor. The response to β-alanine showed an inverse behavior, with $h = 1.6 \pm 0.1$ for wild-type α1, and $h = 2.5 \pm 0.2$ for the mutant $\alpha1^{F159Y,Y161F}$. Also, the mean maximal currents (I_{max}) obtained for the double mutant with β-alanine and glycine were

GlyR	α1	154	M	Q	L	E	S	F	G	Y	T	M	N	D	L
$GABA_AR$	α1	155	L	K	F	G	S	Y	A	Y	T	R	A	E	V
$GABA_AR$	β1	152	L	E	I	E	S	Y	G	Y	T	T	D	D	I
nAChR	β1	144	M	K	L	G	T	W	T	Y	D	G	S	V	V

Fig. 1. Alignment of partial GlyR, $GABA_AR$, and nAChR subunit sequences. The regions homologous to amino acids 154 to 166 of the GlyR α1 subunit are shown; this segment is conserved in the mammalian GlyR α1, α2, and α3 subunits (*13*). Amino acids homologous to residues 159 to 161 of the GlyR α1 subunit are boxed. Sequences are from the following references: human GlyR α1 subunit (*11*); bovine $GABA_AR$ α and β subunits (*18*); and bovine nAChR α1 subunit (*28*).

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almost equal, but for the α1 subunit, β-alanine produced an I_{max} that was only 54 ± 8% of the glycine I_{max} [Fig. 2 and (5)].

Analysis of the singly mutated $\alpha1^{Y161F}$ and $\alpha1^{F159Y}$ subunits revealed a modest increase in agonist efficacies for $\alpha1^{Y161F}$ and a much higher increase for $\alpha1^{F159Y}$ (Table 1). The corresponding Hill coefficients decreased to h = 1.74 ± 0.19 and 2.17 ± 0.11 for glycine and increased to h = 1.85 ± 0.23 and 2.16 ± 0.28 for β-alanine, respectively. Thus, introduction of a OH^- group at position 159 generated most of the double mutant phenotype, but both substitutions contributed to increased agonist efficacy. In contrast with a recently described nAChR mutant (*21*), this increase cannot be attributed to receptor desensitization, because agonist responses declined with similar kinetics for both the α1 and the mutant channels (*22*).

To investigate the contribution of amino acid side chains to ligand discrimination, we determined the dose-response relations of D- and L-alanine, D- and L-serine, GABA, and the putative neurotransmitter taurine. All mutants responded to reduced concentrations of these amino acids, with the largest effect being observed for the $\alpha1^{F159Y,Y161F}$ double mutant and the agonists taurine, D-alanine, and β-alanine (Table 1). For example, D-serine, which produces no current at 100 mM with wild-type receptors, activated the double mutant channels with an EC_{50} of 4 mM. Moreover, GABA, which fails to evoke Cl^- currents in α1- and $\alpha1^{Y161F}$-expressing oocytes, produced a significant current in $\alpha1^{F159Y}$ and a large response in $\alpha1^{F159Y,Y161F}$-injected cells, with I_{max} values of 26 and 80%, respectively, of the maximal glycine-induced current (Fig. 3). These results demonstrate similarities between the agonist binding pockets of the inhibitory amino acid receptors because the exchange of a single OH^- group is sufficient to convert the GlyR into a GABA-responsive protein. Moreover, our findings suggest that the still controversial β-alanine and taurine receptors proposed by some investigators (*23*) may originate from minor modifications of the GlyR α subunit design.

The exchange of the aromatic residues at positions 159 and 161 generates receptors that are preferentially gated by agonists with longer carbon chains, such as β-alanine, taurine, or GABA (Fig. 4). On the basis of site-directed mutagenesis of the α1 and α2 subunits, we proposed that the ligand-binding region of the GlyR contains two distinct subsites for agonists (*5*). Accordingly, subsite I of the α1 subunit mediates activation by glycine, whereas subsite II allows gating by β-alanine and taurine. Because mutations of glycine 160 selectively affect subsite I (*5*), our results are consistent with the assumption that the mutations described here render subsite I more accessible to β-alanine, taurine, GABA, and D-amino acids by eliminating steric constraints within the binding site. Interestingly, inhibition by the antagonist strychnine was not significantly affected by our mutations (*24*), but chemical modification of tyrosines (*25*) and substitution of tyrosine 161 by alanine (*16*) reduced strychnine binding to the GlyR.

A motif of aromatic-small-aromatic side chains equivalent to positions 159 to 161 of the GlyR α1 subunit is highly conserved at the homologous positions of ligand-binding $GABA_AR$ and nAChR proteins (Fig. 1). In the *Torpedo* nAChR α1 subunit, the corresponding residues tryptophan 149 and, to a much lesser extent, tyrosine 151 are covalently labeled

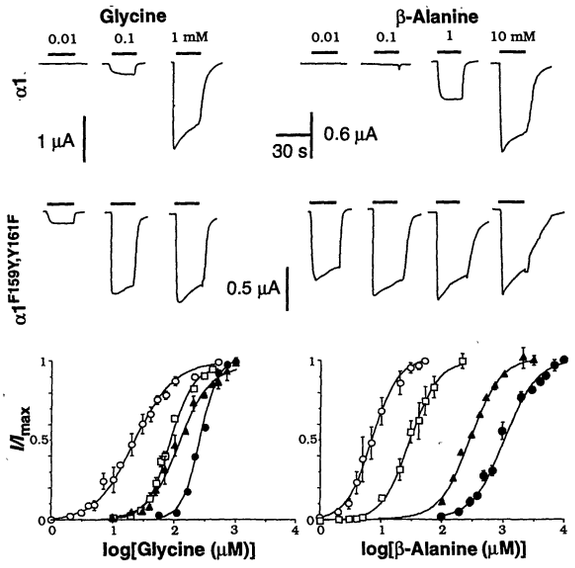

Fig. 2. Agonist dose-response relations recorded from oocytes injected with GlyR wild-type α1 and the mutant $\alpha1^{F159Y}$, $\alpha1^{Y161F}$, and $\alpha1^{F159Y,Y161F}$ cRNAs. (**Top**) Membrane currents elicited by superfusion of 0.01, 0.1, and 1 mM glycine (left) and of 0.01, 0.1, 1, and 10 mM β-alanine (right). Bars indicate duration of agonist application. (**Bottom**) Glycine (left) and β-alanine (right) dose-response curves obtained for the wild-type α1 subunit (●) and the $\alpha1^{F159Y}$ (□), $\alpha1^{Y161F}$ (▲), and $\alpha1^{F159Y,Y161F}$ (○) mutants. Current values are given as responses relative to those at saturating concentrations of agonist. The data are mean values (± SEM) of three to five experiments. Smooth curves represent a least squares fit of the Hill equation to the experimental data.

Table 1. Agonist pharmacology of wild-type and mutant GlyR α1 subunits. Dose-response curves for the different agonists were obtained from oocytes injected with the different cRNAs. Data represent the mean (± SEM) of two to seven experiments (*n*). No detectable membrane current, n.d. The amino acids glutamate, proline, arginine, aspartate, and 2-aminoisobutyric acid (50 to 100 mM each) failed to elicit current responses.

Agonist	EC_{50} (μM)			
	α1	$\alpha1^{F159Y}$	$\alpha1^{Y161F}$	$\alpha1^{F159Y,Y161F}$
Glycine	260 ± 22 (13)*	87 ± 5 (4)	120 ± 19 (5)	22 ± 4 (7)
β-Alanine	850 ± 200 (4)	29 ± 4 (4)	283 ± 77 (3)	7 ± 1.6 (5)
Taurine	1,920 ± 316 (7)	135 ± 15 (2)	876 ± 161 (3)	45 ± 15 (2)
L-Alanine	3,100 ± 775 (6)	990 ± 111 (2)	1,250 ± 50 (2)	290 ± 110 (2)
L-Serine	6,070 ± 520 (3)	815 ± 85 (2)	1,250 ± 50 (2)	270 ± 50 (2)
D-Alanine	12,700 ± 2,500 (3)	910 ± 292 (2)	2,330 ± 676 (3)	127 ± 27 (3)
D-Serine	>50,000 (4)	6,300 ± 200 (2)	>50,000 (2)	4,000 ± 1,000 (2)
GABA	n.d. (5)	7,030 ± 559 (3)	n.d. (3)	3,600 ± 700 (2)

*Value includes data from (*5*).

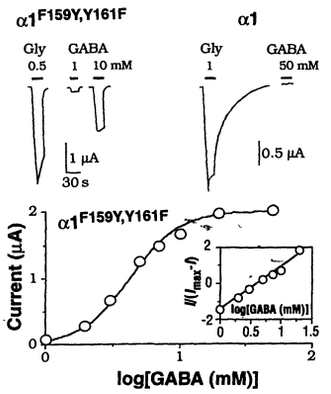

Fig. 3. Responses to the indicated concentrations of glycine and GABA of the $\alpha1^{F159Y,Y161F}$ and the wild-type α1 subunits expressed in oocytes are shown as current traces above the GABA dose-response curve for the $\alpha1^{F159Y,Y161F}$ subunit. (**Inset**) A Hill plot yields a Hill coefficient of 2.4 for this experiment (mean 2.13 ± 0.26).

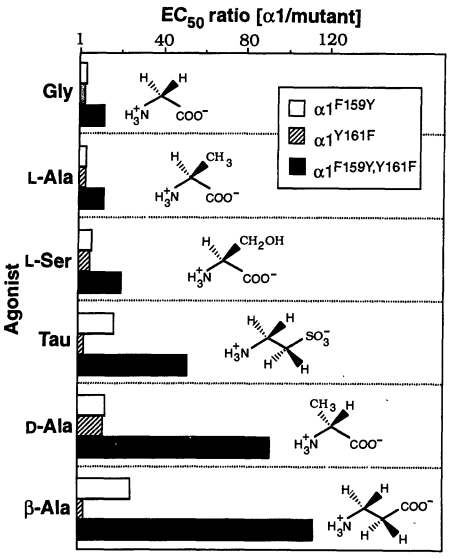

Fig. 4. Chemical structure and relative EC_{50} values of different amino acids. Data represent ratios of α1 wild-type to $\alpha1^{F159Y}$, $\alpha1^{Y161F}$, and $\alpha1^{F159Y,Y161F}$ mutant EC_{50} values. Large ratios indicate a strong increase in agonist efficacy for the mutant.

by a photoreactive acetylcholine analog (*26*). Moreover, photoaffinity labeling of tryptophan 149 increases with receptor desensitization (*27*). These observations support the notion (*2*, *26*) that these aromatic residues are directly oriented toward the bound ligand in this family of ligand-gated ion channel proteins. That aromatic side chains are important for the binding of inhibitory amino acid transmitters may be relevant for the future design of drugs acting at their receptor recognition sites.

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20. Oocytes were prepared and injected with cRNA as described (*14*). Voltage-clamp recordings of agonist responses were obtained at a holding potential of −70 mV. For the determination of EC_{50} values, a computer-controlled superfusion system was used for sequentially applying different agonist concentrations and a reference solution of glycine to elicit a maximal response. Application was for 16 s for each, followed by washout periods of 4 min. Dose-response curves were constructed by normalizing current values to the I_{max} obtained with the respective agonist.
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