

A Novel Domain of the Inhibitory Glycine Receptor Determining Antagonist Efficacies: Further Evidence for Partial Agonism Resulting from Self-Inhibition

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ABSTRACT

Different amino side chains in the N-terminal extracellular region of the inhibitory glycine receptor (GlyR) have been shown to be crucial for ligand recognition. Here we describe a novel domain of the GlyR α 1 subunit that constitutes an important determinant of antagonist activity. The antagonists strychnine, nipecotic acid, and isobutyric acid displayed reduced potencies at recombinant GlyRs formed from α 1 subunits, in which lysine 104, phenylalanine 108, or threonine 112 were replaced by alanine. Agonist affinities, in contrast, were slightly in-

creased at these mutant receptors. Taurine and β -aminoisobutyric acid, which are partial agonists at the wild-type GlyR, behaved as full agonists at the mutant GlyRs and failed to inhibit glycine-induced currents. This is consistent with apolar residues at positions 104, 108, and 112 of the α 1 subunit reducing the antagonistic, but not the agonistic, binding of β -amino acids. Our data support a model in which the partial agonism of β -amino acids results from their self-inhibitory activity.

The inhibitory glycine receptor (GlyR) is a member of the ligand-gated ion channel family that mediates synaptic inhibition by increasing the chloride permeability of the postsynaptic membrane. Biochemical and molecular studies indicate that the GlyR is a pentameric membrane protein composed of ligand-binding α and structural β subunits. The α subunit exists in various isoforms (α 1– α 4), which all form functional homo-oligomeric receptor channels upon heterologous expression in *Xenopus laevis* oocytes or mammalian cell lines (reviewed in Kuhse et al., 1995).

Site-directed mutagenesis indicates that different domains within the extracellular amino-terminal region of the GlyR α 1 subunit contribute to ligand binding. These include amino acid residues at positions 52 (Ryan et al., 1994; Saul et al., 1994), 159 to 161 (Vandenberg et al., 1992a; Schmieden et al., 1993), and 200 to 206 (Vandenberg et al., 1992b), respectively. Substitution of these positions has been shown to alter the apparent affinities of agonists and/or competitive antagonists. Interestingly, the homologous positions of type A γ -aminobutyric acid receptors (GABA_ARs) and nicotinic ace-

tylcholine receptors also have been found to be crucial for ligand binding (Galzi and Changeux, 1995). In addition, mutations causing hereditary hyperekplexia have been located in the short loop connecting transmembrane segments 2 and 3 and shown to drastically alter both agonist affinity and channel gating (Langosch et al., 1994; Rajendra et al., 1995; Lewis et al., 1998).

Previous comparisons of the agonist response properties of α 1 and α 2 GlyRs have identified residue 111 of the α 1 subunit as a crucial determinant of activation by the partial agonist taurine (Schmieden et al., 1992). Taurine displays a highly variable efficacy of GlyR gating in different preparations and has been proposed to act as a GlyR subtype- or cell type-specific ligand (Lewis et al., 1991). However, some of the reported differences in agonist efficacy may result from the antagonistic properties of β -amino acids (Horikoshi et al., 1988; Schmieden and Betz, 1995). Here, we mutated residues around position 111 of the GlyR α 1 subunit and found that substitution of aromatic, polar, and charged side chains at positions 104, 108, and 112 decreases antagonist but increases agonist potencies. Our data are consistent with the low efficacy of β -amino acid partial agonists resulting from self-inhibition.

Materials and Methods

In Vitro Mutagenesis and RNA Synthesis. Oligonucleotide-directed mutagenesis was performed on single-stranded cDNA of the

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ABBREVIATIONS: β -ABA, β -aminobutyric acid; GABA, γ -aminobutyric acid; β -AIBA, β -aminoisobutyric acid; GABA_AR, type A γ -aminobutyric acid receptor; GlyR, inhibitory glycine receptor; wt, wild-type; 5,7ClQA, 5,7-dichloro-4-hydroxy-quinoline-3-carboxylic acid.

human GlyR $\alpha 1$ subunit cloned into pBluescript (Grenningloh et al., 1990) using an in vitro mutagenesis kit (In Vitro Mutagenesis System II; Amersham, Amersham, UK). All mutants were identified and verified by dideoxy sequencing of the mutated regions. *EcoRV* linearized plasmid DNAs were used for synthesis of RNA (mRNA Capping Kit; Stratagene, Inc., La Jolla, CA) with T3 RNA polymerase as described (Schmieden et al., 1992).

Oocyte Expression and Electrophysiology. The methods used for analyzing the pharmacology of recombinant GlyRs have been described previously (Schmieden et al., 1989). Briefly, *Xenopus laevis* oocytes were removed from frogs anesthetized with urethane (Sigma, Munich, Germany), dissected after collagenase (Sigma) treatment, and injected with cRNAs (10–20 ng per oocyte) of the human GlyR $\alpha 1$ subunit and mutants thereof. Voltage-clamp recording of whole-cell currents was performed 24 to 48 h after injection at a holding potential of -70 mV. Experimental values are presented as the mean \pm S.E.M. of peak current responses. For the evaluation of half-maximal effective agonist concentrations (EC_{50}) and Hill coefficients (h) from dose-response curves, data from several oocytes were fitted by the logistic equation:

$$I = \frac{I_{\max}}{\left(\frac{EC_{50}}{L}\right)^h + 1} \quad (1)$$

where I corresponds to the current obtained, I_{\max} to the maximal agonist-induced current, and L to the concentration of the ligand used. Inhibition curves were obtained by coapplying glycine at a concentration corresponding to its EC_{50} value with increasing concentrations of antagonist. Wash-out times between each application were 3 to 5 min.

Data Analysis. Analysis of the pharmacological data was performed on a MacIntosh computer using a fitting program written by V. Schmieden. The algorithm used calculates the differences between the measured data (Y_x) and the values (Y) obtained by equation fitting. By reiterating the parameters EC_{50} from eq. 1, and K_a , K_b , and h , respectively, from eq. 3, an optimal fit was established by minimizing the sum of the square of residuals (SS; Bowen and Jerman, 1995) according to:

$$SS = \sum (Y - Y_x)^2 \quad (2)$$

Partial agonist dose-response curves were fractionated into their agonistic and antagonistic contributions by assuming a mole fraction factor of $F = 1$ (see below). This results in:

$$I = \frac{I_{\max}[L]^h}{\left(K_a + F \frac{K_a[L]}{K_b}\right)^h + [L]^h} \quad (3)$$

where K_a and K_b represent the binding constants for the respective agonistic and antagonistic conformers of β -amino acids. $[L]$ denotes the total concentration of β -amino acid, h is the Hill coefficient, and I_{\max} represents the maximal current evoked by the full agonist glycine.

Results

Characterization of Agonist Responses. We first analyzed oocytes injected with the GlyR $\alpha 1$ subunit mutants K104A, F108A, and T112A for their responses to several α -amino acids. This revealed significant changes in agonist pharmacology resulting from these substitutions.

Superfusion of glycine at a concentration of 1 mM evoked inward current responses of up to several μA from all three mutants (Fig. 1A). The onset and desensitization of agonist-induced currents were similar to those obtained for the wild-type (wt) $\alpha 1$ subunit GlyR. This indicates that the amino acid

substitutions introduced had no major effects on expression efficiency or gating properties. The glycine concentrations generating half-maximal responses (EC_{50}) calculated from dose-effect curves were 0.15 ± 0.01 and 0.11 ± 0.02 mM for the K104A and T112A mutants, respectively (Fig. 1B). These values resemble that found for the wt $\alpha 1$ subunit ($EC_{50} = 0.20 \pm 0.03$ mM). Substitution of phenylalanine at position 108 by alanine in mutant F108A generated receptor channels displaying a 3-fold higher affinity for glycine ($EC_{50} = 0.06 \pm 0.01$ mM). Hill coefficients ranged between 2.1 and 2.3 for all these homo-oligomeric GlyRs.

As reported previously (Tokutomi et al., 1989; Schmieden and Betz, 1995), α -amino acid derivatives with $C\alpha$ substitutions displayed stereospecific agonist activity. L-alanine and L-serine behaved as full agonists at all mutants tested (data not shown), with EC_{50} values between 0.73 to 1.64 and 0.97 to 5.9 mM, respectively (see Table 1). This represents a 2- to 4-fold increase in the agonistic potency of these amino acids as compared with the wt $\alpha 1$ GlyR. In contrast, a strong increase in the relative current response was obtained for the stereoisomers D-alanine and D-serine. Whereas D-serine does not gate the wt $\alpha 1$ GlyR channel (Schmieden and Betz, 1995), it elicited strong currents in oocytes expressing the mutants K104A, F108A, and T112A. Compared to saturating concentrations of glycine, D-serine exhibited maximal responses of $64 \pm 13\%$ at K104A and of 90% at F108A and T112A, respectively (data not shown), with EC_{50} values ranging between 6 and 22 mM (Table 1). Remarkably, the EC_{50} values of D-alanine were found to be 6- to 13-fold lower with the different mutants than that determined for the wt $\alpha 1$ subunit (Table 1). These data show that substitutions at positions 104, 108, or 112 alter the stereoselective binding of D-amino acids to the inhibitory GlyR.

β -Amino acids are known to behave as partial agonists at the $\alpha 1$ GlyR, with a rank order of β -alanine > taurine > β -aminobutyric acid (β -ABA) when maximal current responses were compared to that of glycine (Schmieden and Betz, 1995). Whereas application of 10 mM taurine produced relative responses of about 0.32 at the wt $\alpha 1$ GlyR, the mutants K104A, F108A, and T112A were very efficiently gated by taurine, with relative responses of 0.74, 0.90, and 0.86, respectively (Fig. 1A, Table 1). Figure 1C indicates that this increased efficacy was not the result of a change in affinity, because the EC_{50} values of taurine were very similar for the wt $\alpha 1$ subunit and the various mutants. Another important difference from oocytes injected with wt $\alpha 1$ cRNA became apparent when the mutant receptors were exposed to β -ABA. This β -amino acid analog generated maximal currents of 63 to 77% of the glycine I_{\max} at all three mutants (Table 1); this represents an increase in gating efficacy of about 10-fold. The respective dose-effect curves differed much more from the wt $\alpha 1$ GlyR than those obtained for taurine. Although concentrations of 4.3 ± 0.7 mM β -ABA were sufficient to evoke a half-maximal response at K104A, the mutants F108A and T112A exhibited EC_{50} values of 1.9 ± 0.5 and 9.7 ± 1.7 mM, respectively (Fig. 1D and Table 1). The Hill coefficient for β -ABA of about 1.3 at all mutants was significantly lower than that obtained for glycine.

The third known partial agonist of the GlyR is β -aminoisobutyric acid (β -AIBA), whose maximal responses at the wt $\alpha 1$ GlyR correspond to only about 5% of the glycine I_{\max} (Schmieden and Betz, 1995). Interestingly, even 50 mM

β -AIBA failed to evoke saturating responses at K104A-expressing oocytes. At 6 out of 10 oocytes, no current response to β -AIBA was detectable, whereas the respective glycine current was $>1 \mu\text{A}$. A least-squares fit of the resulting data indicated a relative efficacy of 0.11 and an EC_{50} value of 21 mM (Table 1). Thus, β -AIBA was significantly less potent at the mutant receptors than the related molecule β -ABA.

Antagonist Pharmacology. To investigate whether the substitutions described above also affect antagonist efficacy, we examined several antagonists for their potency to inhibit glycine responses.

Nanomolar concentrations of strychnine are known to potentially suppress glycine-induced currents at the wt $\alpha 1$ GlyR (Sontheimer et al., 1989; Grenningloh et al., 1990). Here,

strychnine was tested using glycine at a concentration corresponding to its EC_{50} value. Figure 2A shows that the mutants K104A and F108A were about 6- to 10-fold less sensitive to the alkaloid (IC_{50} values of 172 ± 9 and 293 ± 6 nM, respectively) than the $\alpha 1$ subunit receptor (IC_{50} of about 15 nM). A much stronger shift of the inhibition curve to low affinity was obtained for T112A (IC_{50} of $1.6 \pm 0.1 \mu\text{M}$; Table 2).

Piperidin-3-carboxylic acid (nipecotic acid) has been described as a competitive antagonist of the GlyR, with an IC_{50} value of 0.8 ± 0.1 mM (Schmieden and Betz, 1995). At all mutants, the potency of this heterocycle was significantly reduced (Fig. 2B), resulting in IC_{50} values of about 3 mM for K104A and F108A, and of about 7 mM for T112A (Table 2).

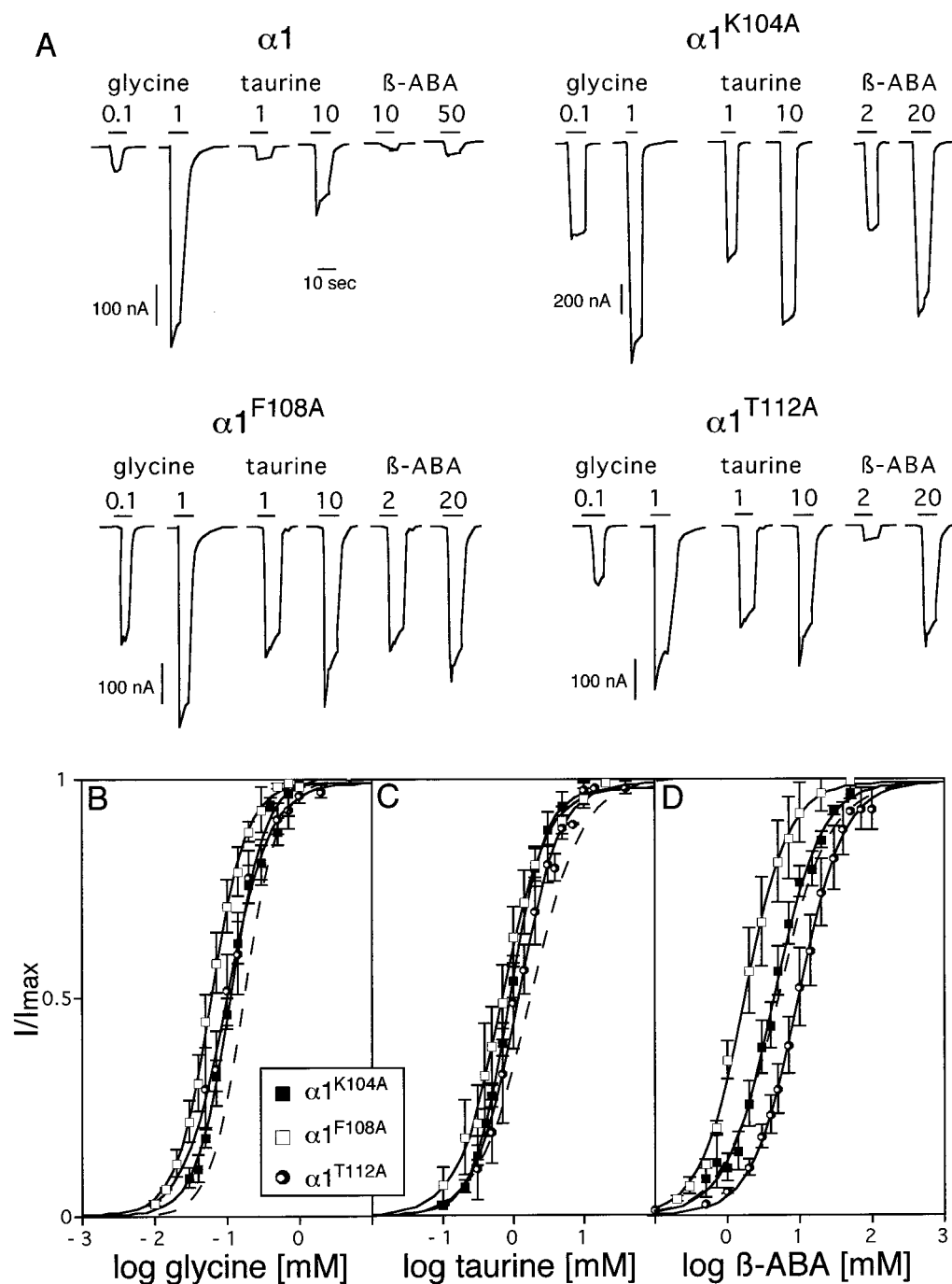


Fig. 1. Current responses elicited by GlyR agonists (A) in oocytes expressing wt GlyR and the mutants $\alpha 1$ K104A, $\alpha 1$ F108A, and $\alpha 1$ T112A, respectively. Bars above the traces indicate duration of agonist application, and numbers indicate agonist concentration in mM. Dose-response curves of glycine (B), taurine (C), and β -aminobutyric acid (β -ABA; D) are shown for the respective mutants as indicated by the inset. Fractional current values normalized to the maximal response obtained for each agonist (I/I_{max}) are given. For comparison, the dose-response curves for glycine, taurine, and β -ABA, respectively, obtained at the wt $\alpha 1$ GlyR are shown (dotted line; see Schmieden and Betz, 1995).

The piperidine derivative isonipecotic acid was also analyzed on K104A and showed a significant reduction in affinity (Table 2). Finally, inhibition of glycine currents by the α -amino acid isobutyric acid ($IC_{50} = 20.4 \pm 1.3$ mM; $n = 3$) was strongly reduced (Table 2).

A new class of GlyR antagonists is represented by the kynurenic acid analog 5,7-dichloro-4-hydroxy-quinoline-3-carboxylic acid (5,7ClQA). This compound inhibits in a mixed competitive/noncompetitive fashion, which originates from the chloride substitution at the aromatic ring system (Schmieden et al., 1996). On K104A and wt $\alpha 1$ GlyR expressing oocytes, IC_{50} values for 5,7ClQA were similar (Table 2).

To examine whether the effects described above might be potentiated upon multiple substitution, we designed a triple (K104A, F108A, T112A) $\alpha 1$ mutant. The resulting receptor was potentially gated by agonists, but showed a pharmacological profile related to that of the mutant F108A. Briefly, the EC_{50} value for glycine was 0.063 ± 0.01 mM ($n = 4$); taurine and β -ABA exhibited EC_{50} values of 0.61 ± 0.09 mM ($n = 3$) and 3.17 ± 0.78 mM ($n = 4$), respectively. Maximal responses for taurine were 100%, and for β -ABA 60% of the glycine I_{max} . Furthermore, nipecotic acid and strychnine antagonized current responses with IC_{50} values of 3.18 ± 0.9 mM ($n = 3$) and 0.32 ± 0.1 μ M ($n = 3$), respectively.

In conclusion, substitution of lysine 104, phenylalanine 108, or threonine 112 by alanine reduced the potency of antagonists in a rank order of strychnine > isobutyric acid > nipecotic acid, whereas the apparent affinities of agonists were inversely increased (Fig. 3). This indicates a critical role for these amino acid side chains and/or the region defined by these mutations in determining the consequences of ligand binding.

Modeling of Partial Agonist Function. We have previously shown that the β -amino acids taurine and in particular β -ABA and β -AIBA exhibit antagonistic activity when coapplied with glycine (Schmieden and Betz, 1995). To test whether a similar behavior is found for the mutants de-

scribed above, we performed inhibition experiments with taurine on oocytes expressing mutant K104A. As shown in Fig. 4A, increasing concentrations of taurine generated strong currents when applied alone. In the presence of 100 μ M glycine, both ligands acted synergistically as agonists. Similar results were found for β -ABA; again the dose-effect curve of glycine was not shifted when 2 mM β -ABA was added (Fig. 4B). These data indicate a loss of the antagonistic properties of these β -amino acids upon mutation of lysine 104. In contrast, β -AIBA still potently inhibited glycine responses (Fig. 4C). The current elicited by 0.1 mM glycine was drastically reduced when increasing concentrations of β -AIBA were coapplied. Analysis of the data indicated an IC_{50} value for β -AIBA of 2.4 mM (Table 2).

It is worth noting that the current responses elicited by the partial agonists taurine and β -ABA were strongly enhanced upon mutation, whereas those of β -AIBA were not. To unravel the basis of this observation, we plotted the determined I_{max} values against the respective EC_{50} values for each mutant (Fig. 5A). Neither taurine nor β -ABA or β -AIBA showed a good correlation between agonist efficacy and apparent affinity. For example, the relative I_{max} value of β -ABA found at the T112A mutant was much larger (75%) than that (7%) obtained with the wt $\alpha 1$ subunit. The EC_{50} value for this ligand, however, was lower at wt $\alpha 1$ than at T112A GlyRs. This analysis indicates that the different binding affinities of β -amino acids as defined by their EC_{50} values are not sufficient to explain their vastly different gating efficacies.

In a previously published model of partial agonist action, we speculated that the low current responses of β -amino acids might be due to a dual action as both activators and competitive inhibitors (Schmieden and Betz, 1995). This was proposed to reflect different molecular conformations of the ligand. The dose-response profiles of partial agonists may thus be considered as the sum of both an agonist and an antagonist concentration-effect curve. Under conditions of full binding site saturation, the relative maximal current

TABLE 1

Agonist pharmacology of the GlyR $\alpha 1$ subunit mutants K104A, F108A, and T112A

Amino acid responses were recorded from oocytes injected with the different mutant $\alpha 1$ cRNAs as described. The agonist concentrations eliciting a half-maximal response (EC_{50} ; mean \pm S.E.M.) of n experiments were calculated from least-square fits of the corresponding dose-effect curves. I_{max} values of taurine, β -AIBA, and β -ABA are given as a fraction of the maximal glycine current.

Ligand	$\alpha 1^{**}$		$\alpha 1^{K104A}$		$\alpha 1^{F108A}$		$\alpha 1^{T112A}$	
	EC_{50}	n	EC_{50}	n	EC_{50}	n	EC_{50}	n
	mM		mM		mM		mM	
Glycine	0.20 ± 0.03	(5)	0.15 ± 0.01	(19)	0.06 ± 0.01	(7)	0.11 ± 0.02	(9)
L-alanine	3.10 ± 0.80	(5)	1.14 ± 0.11	(5)	0.73 ± 0.13	(3)	1.64 ± 0.48	(5)
D-alanine	9.00 ± 1.30	(6)	1.44 ± 0.29	(4)	0.72 ± 0.08	(3)	0.99 ± 0.35	(4)
L-serine	5.10 ± 0.70	(5)	4.26 ± 0.97	(5)	0.97*		5.95 ± 1.19	(3)
D-serine	^a		22.37 ± 7.00	(4)	6.41*		22.32 ± 7.00	(4)
β -alanine	0.60 ± 0.30	(5)	0.24 ± 0.04	(4)	0.09 ± 0.02	(5)	0.23 ± 0.04	(4)
Taurine	1.70 ± 0.10	(18)	0.95 ± 0.09	(5)	0.79 ± 0.24	(4)	1.10 ± 0.24	(6)
β -ABA	5.60 ± 0.80	(8)	4.32 ± 0.66	(10)	1.88 ± 0.45	(2)	9.67 ± 1.73	(3)
β -AIBA	8.70 ± 0.8	(5)	21.22 ± 4.47	(4)	n.d.		n.d.	
	I_{max}	n	I_{max}	n	I_{max}	n	I_{max}	n
	%		%		%		%	
Taurine	31.9 ± 2.8	(18)	73.2 ± 1.7	(5)	90.7 ± 3.5	(4)	85.9 ± 5.6	(7)
β -ABA	7.1 ± 1.9	(8)	63.7 ± 2.5	(10)	66.5 ± 0.2	(2)	77.6 ± 6.8	(3)
β -AIBA	6.5 ± 2.8	(5)	10.8 ± 0.04	(4)	n.d.		n.d.	

n.d., not determined.

** Data taken from Schmieden and Betz, 1995.

* Data from a single experiment.

^a No agonistic function detectable.

(I_{rel}) obtained with a partial agonist should be proportional to the ratio of K_a/K_b , i.e., the binding constants for the activating and inhibiting conformers, as follows:

$$I_{rel} = \frac{I_{max}}{\left(\frac{K_a}{K_b}\right)^h + 1} \quad (4)$$

We therefore examined whether the concentration-effect curves of taurine, β -ABA, and β -AIBA could be described as a fraction of two components (eq. 3; see *Materials and Methods*). Figure 5B shows a least-squares fit for the partial agonists tested, in which I_{max} was normalized to the maximal glycine current. For taurine, a ratio of K_a/K_b of 0.63 ($K_a = 0.51$; $K_b = 0.8$) and a Hill coefficient of 2.6 were calculated. Whereas this K_a value is comparable to the determined EC_{50} value (Table 1), the resulting Hill coefficient was significantly higher than the slope obtained by a simple fit (eq. 1) and very similar to that found for glycine. Correspondingly, for β -ABA and β -AIBA, ratios of 0.707 ($K_a = 2.9$; $K_b = 4.1$) and 1.88 ($K_a = 17.2$; $K_b = 9.13$), respectively, were found.

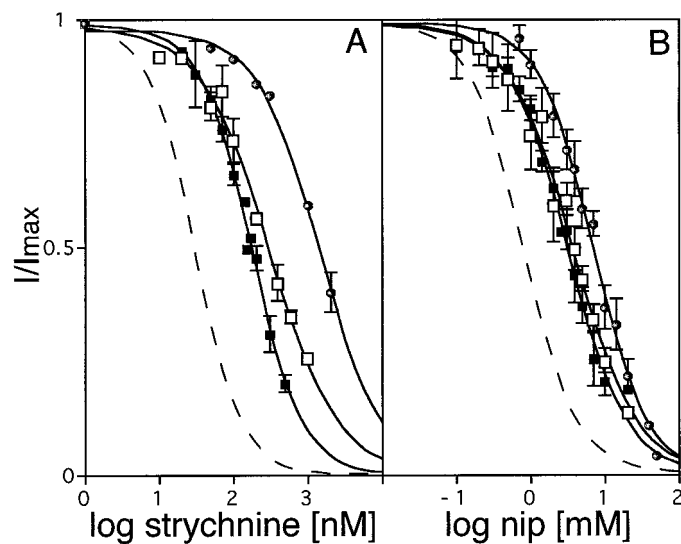


Fig. 2. Inhibition of glycine responses of the mutants $\alpha 1K104A$ (■), $\alpha 1F108A$ (□), and $\alpha 1T112A$ (●) by strychnine (A) and nipecotic acid (nip; B). Glycine at half-saturating concentration was coapplied with increasing concentrations of the indicated antagonists. Current values were normalized to the glycine response obtained in the absence of antagonist and were fitted by a single sigmoidal curve. Error bars often were smaller than the symbols used, and are not indicated. For comparison, the inhibition curves for strychnine and nipecotic acid obtained at the wt $\alpha 1$ GlyR are shown (dotted line; see Schmieden and Betz, 1995).

TABLE 2

Antagonist pharmacology

Inhibition of glycine currents by antagonists was analyzed in oocytes injected with the mutant $\alpha 1$ cRNAs. IC_{50} values were determined using a glycine concentration corresponding to the respective EC_{50} value (see Table 1). Data represent mean \pm S.E.M. of n experiments and are given for strychnine in nM, 5,7CIQA in μM , and nipecotic acid, isonipecotic acid, isobutyric acid, and β -AIBA in mM, respectively.

Ligand	$\alpha 1$		$\alpha 1K104A$		$\alpha 1F108A$		$\alpha 1T112A$	
	IC_{50}	n	IC_{50}	n	IC_{50}	n	IC_{50}	n
Strychnine	*16.0 \pm 2.0	(6)	172.0 \pm 8.8	(5)	293.1 \pm 5.7	(2)	1605 \pm 100	(2)
Nipecotic a.	#0.82 \pm 0.1	(13)	3.62 \pm 0.54	(9)	3.09 \pm 0.27	(5)	7.11 \pm 0.99	(6)
Isonipecotic a.	#0.23 \pm 0.03	(5)	0.61 \pm 0.1	(7)	n.d.		2.35 \pm 0.5	(5)
Isobutyric a.	#2.9 \pm 0.5	(3)	20.4 \pm 1.28	(4)	n.d.		n.d.	
5,7CIQA	†19.4 \pm 5.1	(5)	26.0 \pm 1.0	(2)	n.d.		n.d.	
β -AIBA	#0.8 \pm 0.1	(3)	2.37 \pm 0.45	(4)	n.d.		n.d.	

n.d., not determined.

*, #, † Data taken from Schmieden et al., 1989, Schmieden and Betz, 1995, and Schmieden et al., 1996, respectively.

Again, for both ligands the calculated Hill coefficients were 2.1 and 2.6. This result strengthens the view that the reduced agonist efficacies of these partial agonists are due to competitive self-inhibition.

This approach was also used to model the low current responses of partial agonists at the $\alpha 1$ wt GlyR and the high current responses obtained for mutants F108A and T112A. To this end, the individual dose-response relations of taurine, β -ABA, and β -AIBA were fitted by eq. 3. The parameters calculated from this analysis are summarized in Table 3. It is obvious that the calculated K_a values and the experimentally determined EC_{50} values were rather similar. A high current response is thus the consequence of a low K_b value. In other words, the mutations described minimize the antagonistic binding of partial agonists and increase the efficacy of agonistic interaction. In Fig. 5C, the maximal currents evoked by saturating concentrations of the β -amino acids were plotted versus the logarithm of the calculated K_a/K_b ratios. All determined maximal currents showed a good correlation with the affinity ratios K_a/K_b . The sigmoidal shape of the data fit is consistent with eq. 4 and exhibits a slope of -2 .

In conclusion, mutations at positions 104, 108, and 112 of the $\alpha 1$ subunit generate GlyRs displaying increased agonist and reduced antagonist affinities. Notably, the efficacies of partial agonists were significantly higher than at the wt $\alpha 1$ GlyR. This is consistent with an impairment of β -amino acid antagonism.

Discussion

In this study, we describe the pharmacology of three GlyR $\alpha 1$ subunit mutants, in which lysine 104, phenylalanine 108, and threonine 112, respectively, were replaced by alanine. Like the wt $\alpha 1$ subunit, these mutants generated fully functional receptor proteins that displayed moderate increases in agonist affinity. The largest reductions in EC_{50} values were seen for D-alanine and D-serine, whereas glycine, L-alanine, L-serine, and taurine affinities were detectably affected only upon substitution of phenylalanine 108. Notably, β -ABA and β -AIBA displayed rather low apparent affinities at mutants T112A and K104A. This might be due to the exposed methyl groups at their $C\alpha$ and $C\beta$ atoms, which may restrict interactions with the mutated binding pocket.

The most interesting result obtained with our GlyR mutants is that the apparent changes in antagonist affinities were opposite to those found for the α -amino acid agonists. Similar observations have recently been reported for the GABA_AR (Ebert et al., 1997). Strychnine, isobutyric acid,

nipecotic acid, and isonipecotic acid all inhibited the glycine response only with high IC_{50} values. This decrease in antagonist affinity was most pronounced with mutant T112A. Interestingly, the partial agonist β -AIBA inhibited glycine responses at mutant K104A to >90%; however, its IC_{50} value was 3-fold higher than that obtained at the wt $\alpha 1$ subunit. A similarly reduced antagonistic affinity was also found for nipecotic and isonipecotic acid. From this data one may speculate that β -AIBA and the other GlyR antagonists use similar subsites within the binding pocket.

Our results imply that aromatic, polar, and charged amino acid residues in the mutated region contribute to antagonist efficacy in a rank order of threonine 112 > phenylalanine 108 > lysine 104. Agonist affinities, in contrast, increased when introducing apolar side chains at these positions. Interestingly, the pharmacological profile of the triple mutant (K104A, F108A, T112A) resembled that of the single mutant

F108A. This suggests that the domain harboring positions 104 to 112 contributes only to a limited extent to antagonist/receptor interaction. Consequently, multiple substitutions in this region may not cause further reductions in antagonist affinities. Similarly, substitution of histidine 107 and glutamate 110 had no significant effect on antagonist recognition (Schmieden et al., 1992).

An important role of aromatic residues in ligand recognition by neurotransmitter receptors has been demonstrated in various studies. For the GlyR, photoaffinity labeling by strychnine has been proposed to involve energy transfer from aromatic side chains, such as tyrosine (Graham et al., 1983). Mutational analysis identified tyrosines 161 and 202 as crucial determinants of strychnine binding (Vandenberg et al., 1992a; Rajendra et al., 1995). Furthermore, the exchange of phenylalanine 159 and tyrosine 161 has been found to increase agonist but not antagonist affinity (Schmieden et al., 1993). In addition, charged amino acid residues like lysine 200 (Rajendra et al., 1995) promote both strychnine and glycine recognition, whereas mutations of the polar residue threonine 204 selectively reduces agonist binding (Rajendra et al., 1995). These findings lead us to propose that the agonist and antagonist recognition site of the GlyR contains two domains (positions 159–161 and 200–204), where agonists bind to hydroxyl moieties, whereas antagonists interact directly with aromatic ring systems (see also Rajendra et al., 1995). The domain harboring positions 104 to 112 includes both hydroxylated and aromatic side chains; however, it appears crucial primarily for antagonist efficacy.

The question whether the amino acid residues mutated here are directly involved in ligand binding cannot be answered presently. Homologous positions of the GABA_AR have been implicated in benzodiazepine recognition (Siegel and Buhr, 1997). Threonine 142 of the γ subunit and histidine 101 of the $\alpha 1$ subunit of the GABA_AR are thought to contribute to the interface between both subunits (Galzi and Changeux, 1995). Mutation of position 100 in the GABA_AR $\alpha 6$

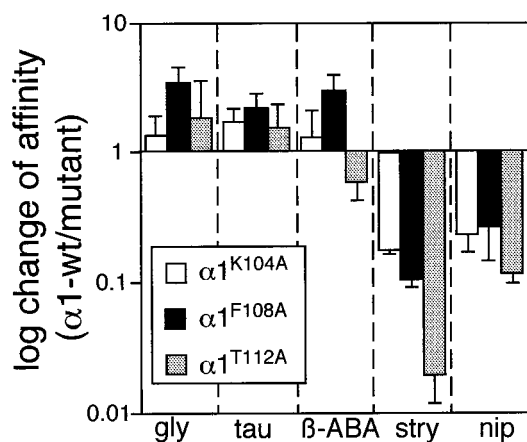


Fig. 3. Histogram of the relative affinities of agonists and antagonists at the $\alpha 1$ subunit mutants. The EC_{50} values of glycine (gly), taurine (tau), and β -ABA and the IC_{50} values of the antagonists strychnine (stry) and nipecotic acid (nip) are plotted as a fraction \pm S.D. of that found for the wt $\alpha 1$ GlyR (see Schmieden and Betz, 1995). Note that downward deflection indicates a decrease in affinity.

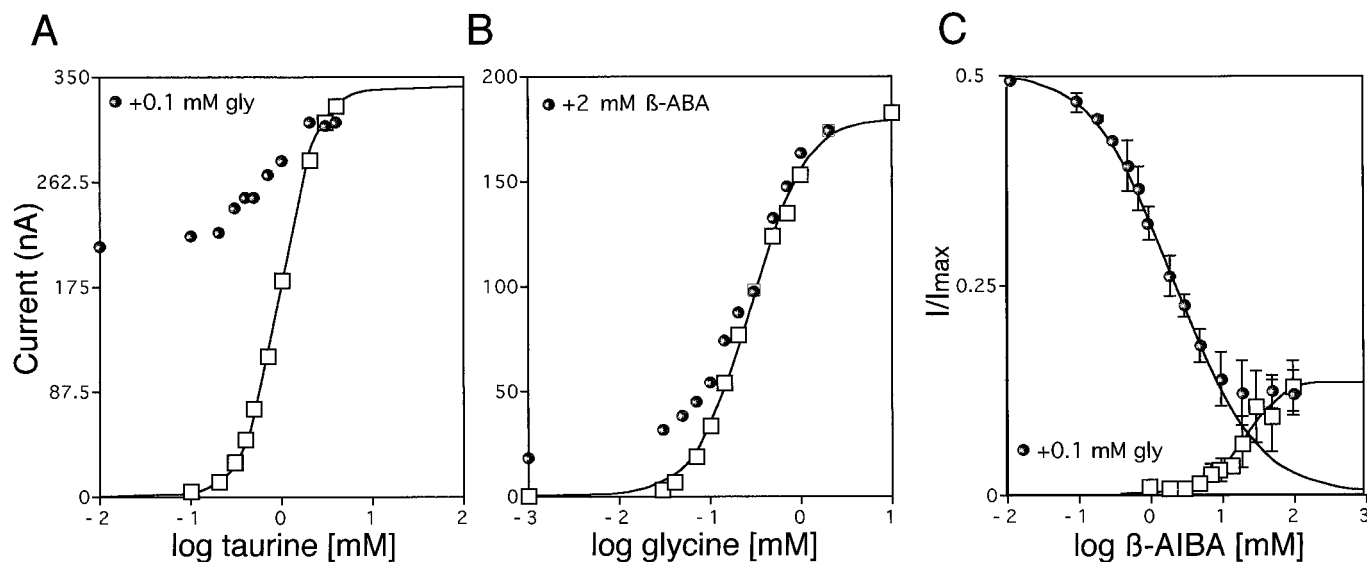


Fig. 4. Competition of glycine responses by partial agonists. A, representative dose-response curve of taurine from an individual $\alpha 1$ K104A cRNA-injected oocyte in the absence (\square) and presence (\bullet) of 0.1 mM glycine. B, representative dose-response curve of glycine from a second $\alpha 1$ K104A cRNA-injected oocyte in the absence (\square) and presence (\bullet) of 0.2 mM β -ABA. C, mean dose-effect curves of β -AIBA in the absence (\square) and presence (\bullet) of 0.1 mM glycine ($n = 4$). Note the high potency of β -AIBA to inhibit glycine-induced currents. Peak amplitudes of glycine responses were plotted as current relative to that obtained with saturating glycine concentrations (I/I_{max}).

subunit (corresponding to H101 of the $\alpha 1$ GABA_AR subunit) caused a complete loss of diazepam binding, whereas the affinity for GABA was not altered (Korpi et al., 1993). Furthermore, tyrosine 93 in the α -subunit of the nicotinic acetylcholine receptor, involved in acetylcholine binding, is equivalent to histidine 101 of the GABA_AR $\alpha 1$ subunit. Although benzodiazepines are allosteric modulators rather than agonists or antagonists of the GABA_ARs, it is interesting to speculate that this binding domain of GABA_ARs may be equivalent to one for GlyR competitive antagonists. Notably, quinolinic acid compounds behave differently from the other GlyR antagonists. The IC₅₀ value of 5,7ClQA was similar for both mutant and wt GlyRs. 5,7ClQA has been described previously as a mixed competitive/noncompetitive antagonist (Schmieden et al., 1996). Neither the competitive binding nor the allosteric interaction of 5,7ClQA were found

to be affected by the mutations analyzed in this study (V.S., unpublished data). Consequently, a separate subsite for quinolines may exist.

The maximal current responses elicited by taurine and β -ABA were significantly higher with the mutant receptors (70–90% of the maximal glycine response) than with the wt $\alpha 1$ GlyR (30 and 7%, respectively). In contrast, β -AIBA appeared similarly effective at both the mutants and the wt $\alpha 1$ subunit. To examine whether this change in ligand efficacy reflected differences in agonist affinity, we compared the EC₅₀ values obtained for taurine, β -ABA, and β -AIBA to the respective maximal current responses and found no correlation. This may reflect a nonequivalence of measured EC₅₀ value and true binding affinities. For recombinant GlyRs, glycine affinities determined by displacement of bound [³H]strychnine and EC₅₀ values calculated from dose-effect

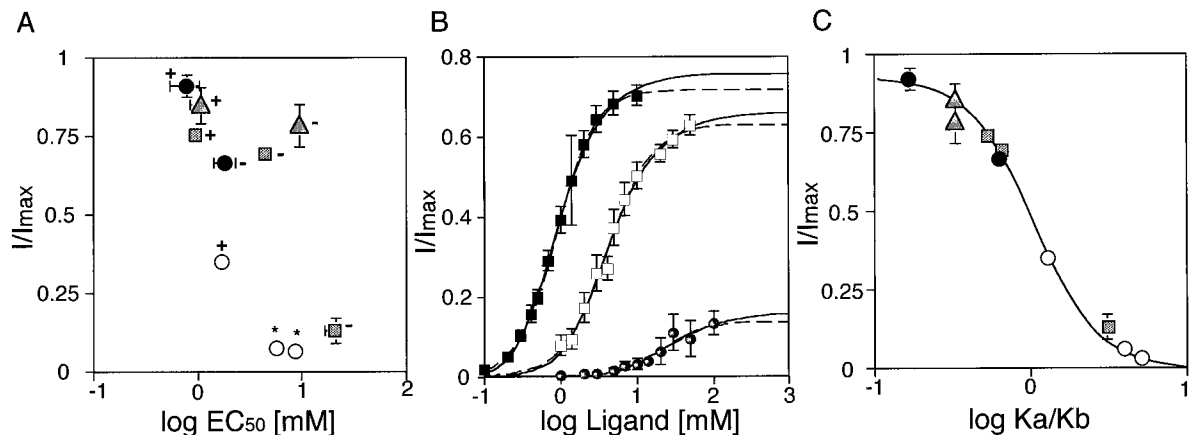


Fig. 5. A, correlation of the maximal currents (I_{\max}) induced by the partial agonists taurine (+), β -ABA (–), and β -AIBA (*), and their respective EC₅₀ values determined in oocytes expressing wt $\alpha 1$ subunit GlyR (○) and the mutants $\alpha 1$ K104A (■), $\alpha 1$ F108A (●), and $\alpha 1$ T112A (▲), respectively. I_{\max} values were plotted as current relative to that induced by a saturating glycine concentration. Bars represent the mean \pm S.E.M. of three to six experiments. B, dose response curves of taurine (■), β -ABA (□), and β -AIBA (●) at mutant K104A. For comparison, data points were fitted by eq. 1 (broken line) and eq. 3 (solid line) as described in the text. EC₅₀ and slope (h) values calculated for ligand-induced currents using eq. 1 were for taurine 0.8 mM ($h = 1.7$), β -ABA 4.14 mM ($h = 1.4$), and β -AIBA 22 mM ($h = 1.5$). K_a and K_b values ($K_a:K_b$) calculated according to eq. 3 were for taurine 0.5:0.8 mM, for β -ABA 2.9:4.1 mM, and for β -AIBA 17:9.6 mM. The Hill coefficients were about 2.6. Note that I_{\max} values for all curves were set to 1. C, the maximal induced current of the β -amino acids shown in (A) are plotted against the ratio of the binding equivalents K_a and K_b . Both parameters are the results of the least square fitting procedure of eq. 3 as shown in Table 3. The slope of the sigmoidal curve resulting was -2 .

TABLE 3
Parameters of fitting procedures

The data of the concentration-effect relations of the partial agonists taurine, β -ABA, and β -AIBA were fitted by eq. 3 (see text) using a least squares procedure. The equivalents of agonistic and antagonistic binding are described by K_a and K_b , respectively (mean \pm S.E.M.); n is the Hill coefficient. The numbers of experiments are given in brackets. The quality of fits was determined by the sum of squares (SS1). In addition, fits were designed by the logistic function 1 (see *Materials and Methods*); their quality is represented by SS2. All values of SS1 and SS2 are multiplied by hundred. Values for the wt $\alpha 1$ subunit GlyR were calculated from the data given in Schmieden and Betz, 1996.

Parameter	$\alpha 1$	$\alpha 1$ K104A	$\alpha 1$ F108A	$\alpha 1$ T112A
Taurine	(5)	(5)	(4)	(6)
K_a [mM]	1.79 ± 0.5	0.68 ± 0.09	0.61 ± 0.11	0.8 ± 0.3
K_b [mM]	1.38 ± 2.5	1.32 ± 0.3	2.63 ± 0.70	10.4 ± 5.9
n	2.5 ± 0.6	2.4 ± 0.3	2.1 ± 0.14	2.5 ± 0.2
SS1	0.11 ± 0.04	0.14 ± 0.02	0.3 ± 0.06	0.34 ± 0.15
SS2	0.11 ± 0.05	0.14 ± 0.05	0.4 ± 0.09	0.28 ± 0.10
β -ABA	(5)	(10)	(2)	(3)
K_a [mM]	7.5 ± 2.3	2.72 ± 0.44	1.56 ± 0.84	9.1 ± 2.4
K_b [mM]	2.07 ± 0.4	4.49 ± 0.59	2.44 ± 1.46	17.7 ± 1.8
n	2.6 ± 0.5	2.7 ± 0.28	2.2 ± 0.7	2.0 ± 0.1
SS1	0.005 ± 0.004	0.12 ± 0.02	0.16 ± 0.02	0.3 ± 0.1
SS2	0.005 ± 0.004	0.12 ± 0.03	0.18 ± 0.04	0.23 ± 0.01
β -AIBA	(5)	(3)		
K_a [mM]	15.8 ± 7.9	26.3 ± 20.7	n.d.	n.d.
K_b [mM]	4.6 ± 1.6	10.9 ± 4.5	n.d.	n.d.
n	2.4 ± 0.4	2.9 ± 0.6	n.d.	n.d.
SS1	0.005 ± 0.002	0.04 ± 0.03	n.d.	n.d.
SS2	0.011 ± 0.004	0.02 ± 0.02	n.d.	n.d.

n.d., not determined.

curves have been found to be similar (Rajendra et al., 1995). We therefore conclude that the low efficacy of β -amino acids must be due to other properties of these ligands.

In an previous report we speculated that the low gating efficacy of partial agonists originates from their simultaneous agonistic and antagonistic binding within the pharmacophore of the GlyR (Schmieden and Betz, 1995). This assumption is based on the fact that β -amino acids can exist in different conformations. The *trans* configuration is structurally related to nipecotic acid, which is a full antagonist, whereas the *cis* configuration has been proposed to represent the agonistic conformation (Schmieden and Betz, 1995). Accepting these predictions and assuming that the binding site of the GlyR can be occupied by both the *trans*- and *cis*-conformers, a simple competitive interaction may occur between the latter. Here, we examined this proposal by fitting the current responses of partial agonists with the logistic function 3, which considers the respective affinities of both conformers. Based on visual inspection of the resulting fits it can be concluded that this procedure results in a good description of the observed current responses. Accordingly, the efficacy (I_{rel}) of the respective ligand is proportional to its K_a/K_b ratio. It is worth noting that the K_b , but not the K_a , value depends critically on the mole fractions of agonistic and antagonistic conformers (F) within the agonist solution. Here, we assumed the mole fraction ratio to be 1, because this value is close to the mole ratios obtained for β -alanine and taurine in NMR studies (Ham, 1974). As shown in Fig. 5, this resulted in excellent fits of the predicted and our experimentally determined relative current values.

According to the proposal made above, the high current responses of taurine and β -ABA found at our mutants reflect an increase in agonistic binding (low K_a value) with a simultaneous loss of GlyR antagonism (high K_b value). Indeed, our competition experiments clearly show that both ligands failed to inhibit glycine-evoked currents. In contrast, β -AIBA was still capable of antagonizing glycine responses at mutant K104A. Although the determined EC_{50} and IC_{50} values for this ligand changed (both about 2.5-fold), the calculated K_a/K_b ratios and the evoked current responses appeared similar for both the mutants and the wt $\alpha 1$ GlyR.

Our model also gives a reasonable explanation for the differences in Hill coefficients reported between full and partial agonists. When using a uniform cooperativity for all agonists of about $h = 2.5$, our model calculations generated dose-response curves for taurine and β -ABA with a slope < 2 . Similar results were observed in experiments with adrenergic $\alpha 2$ autoreceptors (Feuerstein et al., 1994) and the $\alpha 1$ GlyR (Schmieden and Betz, 1995), in which partial agonist function was mimicked by applying mixtures of a full agonist and an antagonist at various concentration ratios.

Allosterical two-state receptor models (Monod et al., 1965; Leff, 1995) have also been used to explain partial agonist activity. In these models, ligands are thought to bind to active (R) or inactive (R') receptor conformations with corresponding K_a and K_b values. The affinities of a particular ligand for R' and/or R then define its full agonistic, partial agonistic, or antagonistic activities, respectively. Consequently, receptors in which the R:R' ratios were changed ("L-phenotype" according to Galzi et al., 1996) may yield different gating efficiencies. Using this approach, the low

glycine responses of GlyR $\alpha 1$ mutants causing hyperekplexia (Rajendra et al., 1994; Langosch et al., 1994) have been explained theoretically (Galzi et al., 1996). However, these models consistently correlated low current responses with low affinities for agonists. Assuming that our mutations solely changed the allosteric R/R' ratio of the resulting GlyRs by favoring a high open probability as required for increased current responses, the resulting EC_{50} values should be significantly lower than for the wt GlyR. This prediction contradicts our findings. We therefore conclude that the amino acid exchanges at positions 104, 108, and 112 had no major effect on allosteric transitions. Rather, these amino acids appear crucial for the recognition of antagonists and the *trans*-conformation of β -amino acids. All presently available data are compatible with the interpretation that the altered gating efficiencies of partial agonists seen upon substitution of residues 104, 108, and 112 reflect a loss of self-inhibition resulting from an increased K_a/K_b ratio.

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