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Differences in Agonist/Antagonist Binding Affinity and Receptor Transduction Using Recombinant Human γ-Aminobutyric Acid Type A Receptors

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SUMMARY

Using human γ-aminobutyric acid type A ($GABA_A$) receptor subunit combinations, expressed in cell lines and *Xenopus laevis* oocytes, the pharmacology of a number of ligands interacting directly with the GABA recognition site has been studied in [^{3}H]muscimol binding and electrophysiologically. The binding affinity of $GABA_A$ agonist and antagonist ligands showed small but statistically significant dependence on the subunit composition of receptors that include γ2 and different α and β subunits. The potency of antagonist ligands was largely independent of receptor subunit composition, whereas the composition of receptors expressed in oocytes strongly influenced the EC_{50} value of agonists. An apparent reciprocal correlation between subunits favoring agonist binding and antagonist binding, respectively, was observed. Whereas antagonists showed comparable potencies in binding and functional studies, the potency of agonists in binding studies was generally two to three orders of magnitude higher than the agonist potencies measured electrophysiologically. 5-(4-Piperidyl)isothiazol-3-ol, which behaves as a low efficacy partial agonist at $GABA_A$ receptors in cultured cortical neurons, showed no efficacy in oocytes, but produced pure antagonist effects with a binding/functional affinity ratio between those observed for the agonists and antagonists. It is concluded that the $GABA_A$ receptor mechanisms transducing binding into physiological response, but not the binding *per se*, is dependent on the receptor subunit composition.

The $GABA_A$ receptor complex is believed to be a heteropentameric assembly of transmembrane protein subunits, which exist in several forms and/or splice variants. At present, several families of $GABA_A$ transmembrane proteins have been identified: α1–6, β1–3, γ1–3, δ, and ϵ (1–13). The composition and configuration of native $GABA_A$ receptor complex(es) are as yet largely unknown, although a large body of experiments indicates that a prerequisite for full functionality of $GABA_A$ receptors is the presence of α, β, γ, δ, or ϵ subunits (14, 15). We have shown previously that both the EC_{50} and relative efficacy of GABA agonists are highly dependent on subunit composition of recombinant $GABA_A$ receptors (16). Photoaffinity labeling and mutagenesis studies have identified amino acid residues located on both the α and β subunits, essential for the binding of the agonist radioligand [^{3}H]muscimol or receptor activation (17–19), suggesting that the GABA binding site is formed at the interface between these receptor subunits.

To determine if the large variations in EC_{50} values and relative efficacies described in our previous study (16) were reflected in the binding properties as measured in [^{3}H]muscimol binding, we now have carried out studies on the relationship between binding affinity, functional EC_{50}, and $GABA_A$ receptor subunit composition, using a series of $GABA_A$ receptor ligands so that they covered a large variation in affinity, structure, and relative efficacy. Thus, the highly flexible molecule GABA, the conformationally restricted GABA analogues muscimol and thiomuscimol, where the terminal carboxyl group of GABA has been replaced by the 3-hydroxyisoxazol and 3-hydroxyisothiazol group, respectively, were chosen as full agonists (Fig. 1). As partial receptor agonists the restricted GABA analogues THIP and P4S, which have been demonstrated previously to be partial agonists at recombinant human $GABA_A$ receptors (16, 20), and Thio-4-PIOL, a low-efficacy partial agonist at the native $GABA_A$ receptor in cultured cortical neurons (21), were chosen. Bicuculline and SR95531 (22) were selected as competitive $GABA_A$ receptor antagonists (Fig. 1). $GABA_A$ receptor

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ABBREVIATIONS: GABA, γ-aminobutyric acid; Thio-4-PIOL, 5-(4-piperidyl)isothiazol-3-ol; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol; P4S, piperidine-4-sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

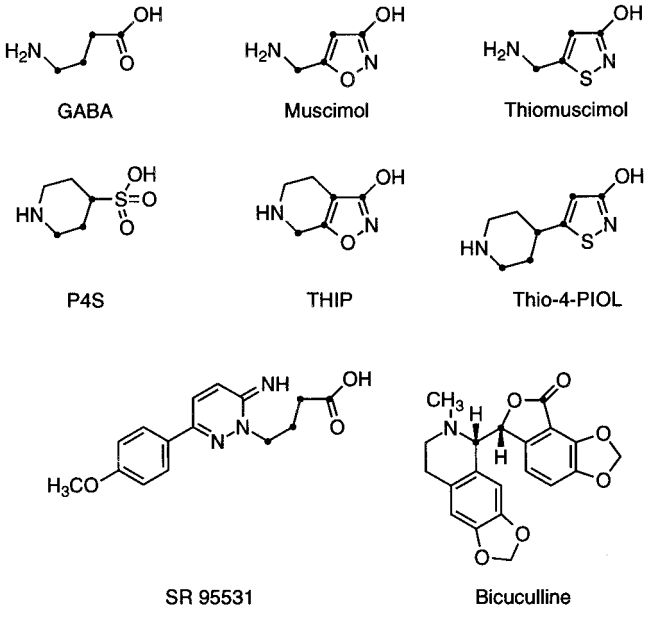

Fig. 1. Structures of $GABA_A$ receptor ligands characterized in the present study.

subunit combinations containing $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ were chosen so that the relative efficacies of the known partial receptor agonists P4S and THIP covered a wide range. $\alpha6$-Containing receptors were also included, as GABA agonists have been shown previously to possess very high potency and efficacy at $\alpha6\beta1\gamma2$ (23).

Materials and Methods

Cell lines. Cell lines stably expressing the $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, $\alpha3\beta3\gamma2$, $\alpha5\beta3\gamma2$, $\alpha6\beta3\gamma2$, $\alpha1\beta1\gamma2$, and $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtypes were used to prepare membranes for [^{3}H]muscimol binding.

cDNAs. cDNAs encoding human $\alpha1$, $\alpha2$, a3, $\alpha5$, $\alpha6$, $\beta1$, $\beta2$, $\beta3$, $\gamma1$, and $\gamma2$ subunits have been described elsewhere (24–26).

[^{3}H]Muscimol binding. For binding assays both freshly prepared or frozen membranes were used with similar results, and membranes could be stored at −80° for up to 3 months before use. Membranes were resuspended in 10 mM KH_2PO_4, 100 mM KCl, pH 7.4, at 1 mg/ml, and 100-μl aliquots were incubated with [^{3}H]muscimol (8 nM) for 1 hr at 20° in a total volume of 0.5 ml in the presence of various $GABA_A$ agonists or antagonists at concentrations ranging from 10^{-10} to 10^{-6} M. Nonspecific binding was defined with 1 mM GABA. Membranes were filtered through Whatman GF/B filters. These were washed three times with ice-cold buffer, and radioactivity was counted by liquid scintillation counting.

Oocyte expression. *Xenopus laevis* oocytes were removed from anesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment to remove follicle cells [type IA (0.5 mg/ml) for 8 min] the oocyte nuclei were then directly injected with 10–20 nl of injection buffer [88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0 (nitrocellulose-filtered)] containing different combinations of human $GABA_A$ subunit cDNAs (20 ng/μl) engineered into the expression vector pCDM8 or pcDNAAmp. After incubation for 24 hr, oocytes were placed in a 50-μl bath and perfused with modified Barth's medium consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 7.5. Cells were impaled with two 1–3-MΩ electrodes containing 2 M KCl and voltage clamped between −40 and −70 mV. The cell was continuously perfused with saline at 4–6 ml/min, and drugs were applied in the perfusate. GABA or $GABA_A$ agonists were applied until the peak of the response was observed, usually 30 sec or less. At least 3 min of wash time was allowed between each agonist application to prevent desensitization. Data from each oocyte were analyzed with respect to the maximum response, relative to either the plateau level for a full concentration-response curve for GABA or the response to 3 mM GABA (no difference). Concentration-response curves were calculated using a nonlinear squares fitting program to the equation $f(x) = B_{max}/(1 + (EC_{50}/x)^n)$ where EC_{50} is the concentration of drug eliciting a half-maximal response, x is the drug concentration, and n is the Hill coefficient. Antagonist experiments were carried out with three to five concentrations of GABA followed by three to five concentrations of GABA in the presence of the antagonist. The shift of the dose-response curve to GABA was determined in the response range, where the two obtained curves were parallel. The dose ratio, calculated as the ratio between the concentration of GABA in the presence and absence of antagonist, was transformed to a K_i value by the equation: log (dose ratio − 1)= −log (K_i) −log ([antagonist]). Values presented are mean values ± standard error of at least four individual experiments. Thio-4-PIOL, muscimol, thiomuscimol, P4S, and THIP were synthesized as described previously (26). All other compounds were obtained from Sigma Chemical (Poole, Dorset, UK) or Research Biochemicals (Natick, MA). The computer program GraFit 3.0 (Erithacus Software, Staines, UK) was used to analyze and plot data.

Results

Binding of agonists and antagonists to stable cell lines using [^{3}H]muscimol. [^{3}H]Muscimol binding was characterized with respect to K_d value and B_{max} value in Ltk cells stably expressing human $GABA_A$ receptors of various subunit compositions. As illustrated in Table 1, a high level of receptor expression was detected in all subunit compositions, resulting in a specific binding level of 75–80%. Displacement curves, as illustrated in Fig. 2, were obtained using at least eight concentrations of inhibitor and the determined IC_{50} values were converted to K_i values using the equation $K_i = IC_{50}/[(1 + [^3H]muscimol/K_d\,(muscimol)]$. Binding affinities expressed as pK_i values are summarized in Tables 2 and 3. In general, only small differences in K_i values were observed between different subunit combinations. Thus, a maximum difference of approximately 10-fold in affinity between all subtypes examined was observed. However, from these data, a pattern of agonist and antagonist preferring subtypes was evident.

Effects of different α subunits. The standard $GABA_A$ agonists, GABA, muscimol, thiomuscimol, THIP, and P4S, had the highest affinity for the $\alpha6/\alpha5$-containing $GABA_A$ receptors and the lowest affinity for the $\alpha2/\alpha3$-containing receptors, whereas the antagonists, Thio-4-PIOL, bicuculline, and SR95531, had the highest affinity for the $\alpha2/\alpha5$-containing receptors and lowest affinity for the $\alpha6$-containing

TABLE 1

Equilibrium binding parameters for [^{3}H]muscimol binding in Ltk cells stably expressing human $GABA_A$ receptors

Values represent mean ± standard error of at least four experiments.

	K_d	B_{max}
	nM	*fmol/mg protein*
$\alpha1\beta3\gamma2$	5.0 ± 2.3	3900 ± 340
$\alpha2\beta3\gamma2$	9.1 ± 0.93	2200 ± 950
$\alpha3\beta3\gamma2$	6.1 ± 1.6	5600 ± 1200
$\alpha5\beta3\gamma2$	4.3 ± 0.8	3800 ± 700
$\alpha6\beta3\gamma2$	1.3 ± 0.40	1900 ± 340
$\alpha1\beta1\gamma2$	6.8 ± 0.69	1300 ± 850
$\alpha1\beta2\gamma2$	8.4 ± 0.84	6140 ± 850

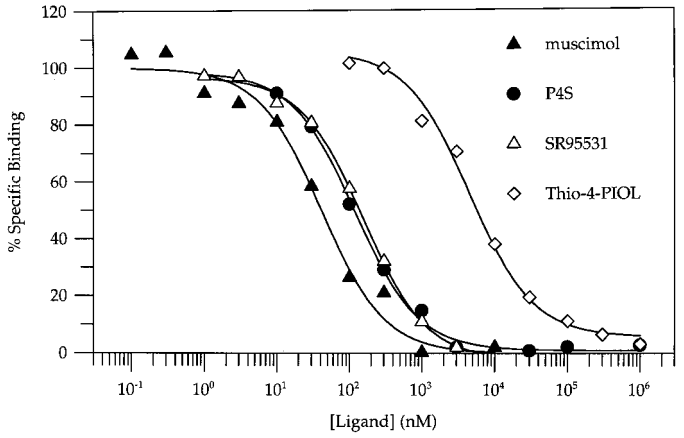

Fig. 2. Inhibition curves for muscimol, P4S, SR95531, and Thio-4-PIOL in displacement experiments using [^{3}H]muscimol in Ltk cells stably expressing human $\alpha6\beta3\gamma2$ $GABA_A$ receptors. Values are from a representative experiment, where each data point is determined in triplicate.

receptor. A rank order correlation of the affinity for all the agonists gave $\alpha6 = \alpha5 > \alpha1 > \alpha3 > \alpha2$, whereas the rank order for antagonists was $\alpha2 = \alpha5 > \alpha3 > \alpha1 > \alpha6$. By plotting the rank order of affinity versus subunit it was observed that with the exception of $\alpha5$, an inverse relationship was maintained for agonist versus antagonists (Fig. 3).

Effects of different β subunits. The compounds were studied at receptors containing a constant $\alpha1\gamma2$ with varying β subunit composition. Only minor differences in affinities of the compounds for the different β subunits were detected. Thus, a 10-fold difference in K_i value was seen for thiomuscimol between $\alpha1\beta1\gamma2$ (6.6 ± 0.04) and $\alpha1\beta3\gamma2$ (7.6 ± 0.03), whereas only minor differences were seen for the other compounds. The compounds had in general highest affinity for the $\beta3$-containing receptors, and slightly weaker affinity for $\beta1$- and $\beta2$-containing receptors. No significant difference in selectivity was observed between agonists and antagonists.

Electrophysiology. $GABA_A$ agonists were characterized with respect to their EC_{50} value and maximum response relative to the full agonist GABA (Fig. 4A). Antagonists were characterized with respect to their pK_i value, using the shift of the GABA concentration-response curve in oocytes injected with cDNA encoding for the same $GABA_A$ subunits as those expressed in the cell lines (Fig. 4B). Results for agonists and antagonists are summarized in Tables 2 and 3, respectively.

Effects of different α subunits. Concentration-response curve analysis demonstrated that the potency of GABA agonists, THIP, P4S, thiomuscimol, muscimol, GABA, varied with the type of α subunit incorporated into the $GABA_A$ receptor complex. A larger variation in affinity was seen in oocytes compared with that found in binding. Thus, a maximum ratio between the highest and lowest affinity of 50 times was observed. In agreement with results from the binding experiments, the agonists showed a similar profile in α subunit selectivity, the highest potency being at $\alpha6$-containing receptors, slightly higher than that at $\alpha5/\alpha2$-containing receptors. The lowest EC_{50} was seen at $\alpha3$-containing receptors. The rank order of potency for all agonists was $\alpha6 > \alpha5 = \alpha2 > \alpha1 > \alpha3$.

SR95531, bicuculline, and Thio-4-PIOL were shown to be competitive antagonists, producing a parallel shift of the GABA concentration-response curve. Thio-4-PIOL, which has been characterized previously as a partial $GABA_A$ agonist in dissociated neurons (21), had no agonist activity on any of the combinations studied, and was therefore characterized as an antagonist. In contrast to the agonists, the antagonists showed little difference in K_i between receptors containing different subunits. The three antagonists studied covered a range of potencies from thio-4-PIOL which had an average K_i of 25 μM, bicuculline which had a K_i of 1.8 μM, and SR95531 with a K_i of 0.15 μM. Thio-4-PIOL and SR95531 did not distinguish between different subunit combinations, whereas bicuculline was unique in showing a significantly lower affinity at the $\alpha6\beta3\gamma2$ receptor (8.5 μM, $p < 0.001$) compared with other α-containing receptors.

Effect of different β subunits. Variations of the β subunit when expressed as the combination $\alpha1\beta x\gamma2$, where β is $\beta1$, $\beta2$, or $\beta3$, had no significant effect on agonist or antagonist affinity. SR95531 was the most potent antagonist, followed by bicuculline, and Thio-4-PIOL being the least potent. To investigate if the significantly lower potency of bicuculline at $\alpha6\beta3\gamma2$-containing receptors compared with other α-containing receptors was due to a unique quality of this receptor combination, SR95531, bicuculline, and Thio-4-PIOL were further characterized as functional antagonists in oocytes injected with either $\alpha6\beta x\gamma2$ or $\alpha3\beta x\gamma2$, using the three different β subunits (Fig. 5A and Table 3). None of the three compounds showed significant differences in K_i value between $\alpha3\beta1\gamma2$, $\alpha3\beta2\gamma2$, and $\alpha3\beta3\gamma2$. However, variation of the β subunit and combination with $\alpha6\gamma2$ showed that bicuculline was significantly weaker at $\alpha6\beta3\gamma2$ receptors compared with $\alpha6\beta1\gamma2$ or $\alpha6\beta2\gamma2$ receptors, whereas SR95531 and Thio-4-PIOL showed no difference in affinity as a function of the variation in subunit composition (Fig. 5B).

Correlations and comparisons between binding data and oocyte data. The functional pEC_{50} (agonists) or pK_i (antagonists) values for all compounds in all subunit combinations from electrophysiological recording in oocytes, was plotted as function of the pK_i values obtained in the binding assay (Fig. 6). A high correlation between data in the two test systems was obtained, with R for agonists being 0.80 and for antagonists 0.95. The absolute antagonist affinities were similar in both assays; however, the agonist affinities maintained their correlation but were approximately 1000-fold different in the two assay systems. The antagonists displayed less subunit-dependent variation than did the agonists in both assays. Thio-4-PIOL did not correlate well for either the antagonists or agonists, and fell between the two groups (Fig. 6). One possible reason for this discrepancy may be that although Thio-4-PIOL behaves as an antagonist, it is structurally more closely related to the agonists, and shows a relatively higher affinity in [^{3}H]muscimol binding experiments.

Discussion

Molecular pharmacological studies on the $GABA_A$ receptor complex have focused mainly on the benzodiazepine binding sites (1–11), and in a number of studies [^{3}H]TBPS, which interacts with a site near or within the chloride channel (28), has been used as a tool to study agonists and antagonists (29, 30). In continuation of previous studies (16), the present investigations were directed toward the GABA binding site of

this receptor complex, using [^{3}H]muscimol as a radioligand and a series of ligands ranging from full agonists through partial agonists to compounds showing purely competitive antagonist profiles.

Competition for [^{3}H]muscimol binding sites revealed that small but statistically significant differences in binding affinities for a range of compounds were observed using recombinant $GABA_A$ receptors containing different α or β subunits. The observed variation in affinity as a consequence of varying the α subunit may be interpreted as evidence that the α subunit forms part of the binding site for both agonists and antagonists. Alternatively, the binding site for both agonists and antagonists could be located on one receptor subunit with the α subunit exerting an allosteric effect on the GABA binding site, thereby modulating the affinity for the ligands. Based on binding studies it is impossible to distinguish between these possibilities. Further, it appears that the α subunit-dependent affinity of agonists and antagonists is related to each other in a reciprocal fashion (Fig. 2), suggesting that the α subunit not only serves as the co-determinant of potency, but also as an "agonist/antagonist selectivity filter." Thus, agonists generally are most potent at α6-containing receptors, and weakest at α2-containing receptors, whereas the converse was observed for antagonists. The subunit-dependent profiles of the partial $GABA_A$ agonists, THIP and P4S, which have been shown previously to express variable degrees of efficacy in oocytes containing different α subunits (16), show the characteristics of $GABA_A$ agonists in the present studies (Fig. 3). Interestingly, P4S has been shown to act as an antagonist at $\alpha4\beta1\gamma2$ $GABA_A$ receptors expressed in oocytes (23).

Thio-4-PIOL, which has been shown previously to act as a low efficacy partial $GABA_A$ agonist at native $GABA_A$ receptors in cultured cortical neurons (21), shows the characteristics of a competitive antagonist at $GABA_A$ receptors expressed in oocytes. Nevertheless, in the correlation of binding affinity with function (Fig. 6), Thio-4-PIOL shows a behavior intermediate between the agonists and the antagonists. Thus, in agreement with previous observations (16), there is no obvious correlation between agonist affinity and efficacy, and the mechanisms determining efficacy remain to be elucidated.

The nature of the β subunit also is a determinant of the $GABA_A$ receptor ligands under study. However, in contrast to the observed effects at the α subunit (Tables 2 and 3), none of the compounds, except bicuculline, showed significant subunit selectivity (Fig. 5B). Inasmuch as the $\alpha6\beta3\gamma2$ subunit combination may represent a native $GABA_A$ receptor in brain (31–33), the low affinity of bicuculline for this receptor configuration is interesting. pK_B values for bicuculline on $\alpha1\beta1\gamma2$ and $\alpha1\beta1$ have been reported as 5.9 (34), a value similar to most combinations in this study. To establish if the reduced receptor affinity at $\alpha6\beta3\gamma2$ was due to a specific quality of the $\alpha6$ or $\beta3$ subunit receptor composition, bicuculline was further characterized at $\alpha6\beta x\gamma2$ and $\alpha3\beta x\gamma2$ combinations. From these studies it was apparent that the low affinity actually was specific to the combination $\alpha6\beta3\gamma2$, rather than a property of any individual subunit, as all other combinations had a significantly higher affinity.

Although the effects of the γ subunit have not been mapped out in this study, published data suggest that muscimol,

TABLE 2

Pharmacology of $GABA_A$ agonists using a [^{3}H]muscimol binding assay (pK_i value) and oocyte electrophysiology (pEC_{50} and % of maximum response to GABA) using different recombinant receptor subunit combinations

Values represent mean ± standard error of at least four experiments; some data was taken from Ref. 16.

	GABA			Muscimol			Thiomuscimol			THIP			P4S		
	pK_i	pEC_{50}	% of max	pK_i	pEC_{50}	% of max	pK_i	pEC_{50}	% of max	pK_i	pEC_{50}	% of max	pK_i	pEC_{50}	% of max
$\alpha1\beta1\gamma2$	7.11 ± 0.079	4.60 ± 0.042	100	8.17 ± 0.16	5.15 ± 0.12	99	6.62 ± 0.044	4.61 ± 0.13	99	6.06 ± 0.027	3.45 ± 0.13	78	7.40 ± 0.031	4.28 ± 0.041	32
$\alpha1\beta2\gamma2$	7.24 ± 0.031	4.70 ± 0.041	100	8.08 ± 0.079	5.30 ± 0.13	97	7.38 ± 0.097	4.57 ± 0.19	92	5.79 ± 0.18	3.84 ± 0.028	76	7.04 ± 0.27	4.60 ± 0.043	38
$\alpha1\beta3\gamma2$	7.67 ± 0.12	5.10 ± 0.041	100	8.34 ± 0.059	5.40 ± 0.073	97	7.58 ± 0.034	4.22 ± 0.11	98	6.57 ± 0.036	3.62 ± 0.079	70	6.82 ± 0.068	4.36 ± 0.081	21
$\alpha2\beta3\gamma2$	7.64 ± 0.090	5.40 ± 0.041	100	8.05 ± 0.14	6.04 ± 0.056	96	7.07 ± 0.20	4.85 ± 0.027	99	6.43 ± 0.10	3.93 ± 0.095	98	7.01 ± 0.153	4.63 ± 0.070	96
$\alpha3\beta3\gamma2$	7.30 ± 0.10	4.55 ± 0.056	100	8.24 ± 0.14	5.05 ± 0.097	98	7.43 ± 0.12	3.83 ± 0.083	96	6.78 ± 0.052	3.63 ± 0.060	53	6.97 ± 0.187	4.19 ± 0.045	75
$\alpha5\beta3\gamma2$	8.06 ± 0.12	5.52 ± 0.11	100	8.54 ± 0.087	5.70 ± 0.048	98	7.88 ± 0.029	4.53 ± 0.11	98	7.33 ± 0.059	4.40 ± 0.11	99	7.23 ± 0.092	4.66 ± 0.12	92
$\alpha6\beta3\gamma2$	7.11 ± 0.079	5.82 ± 0.097	100	8.27 ± 0.15	5.79 ± 0.080	101	8.25 ± 0.18	5.15 ± 0.12	97	6.99 ± 0.071	4.06 ± 0.15	95	7.14 ± 0.123	4.72 ± 0.042	15

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TABLE 3

Pharmacology of $GABA_A$ antagonists using a [^{3}H]muscimol binding assay (pK_i value) and oocyte electrophysiology (pK_i) using different subunit combinations

Values represent mean ± standard error of at least four experiments

	SR95531		Bicuculline		Thio-4-PIOL	
	pK_i (binding)	pK_i (oocytes)	pK_i (binding)	pK_i (oocytes)	pK_i (binding)	pK_i (oocytes)
$\alpha1\beta3\gamma2$	7.42 ± 0.038	6.65 ± 0.083	5.34 ± 0.085	5.64 ± 0.10	5.77 ± 0.040	4.59 ± 0.16
$\alpha2\beta3\gamma2$	8.05 ± 0.073	6.83 ± 0.24	5.73 ± 0.17	5.79 ± 0.097	6.20 ± 0.099	4.55 ± 0.10
$\alpha3\beta3\gamma2$	7.46 ± 0.11	6.61 ± 0.12	5.55 ± 0.053	5.71 ± 0.13	5.84 ± 0.061	4.47 ± 0.083
$\alpha5\beta3\gamma2$	7.52 ± 0.12	6.98 ± 0.22	5.35 ± 0.097	6.04 ± 0.074	6.35 ± 0.041	4.52 ± 0.066
$\alpha6\beta3\gamma2$	7.46 ± 0.14	6.87 ± 0.067	4.71 ± 0.49	5.08 ± 0.090	5.75 ± 0.041	4.72 ± 0.12
$\alpha1\beta1\gamma2$	6.50 ± 0.081	6.97 ± 0.12	5.32 ± 0.0070	5.62 ± 0.073	5.26 ± 0.11	4.63 ± 0.14
$\alpha1\beta2\gamma2$	7.22 ± 0.19	6.70 ± 0.086	4.76 ± 0.080	5.68 ± 0.042	5.44 ± 0.099	4.64 ± 0.019
$\alpha1\beta3\gamma2$	7.42 ± 0.038	6.65 ± 0.083	5.34 ± 0.085	5.64 ± 0.10	5.77 ± 0.040	4.59 ± 0.16
$\alpha3\beta1\gamma2$		6.81 ± 0.11		5.65 ± 0.11		4.46 ± 0.058
$\alpha3\beta2\gamma2$		6.64 ± 0.061		5.65 ± 0.19		4.58 ± 0.13
$\alpha3\beta3\gamma2$		6.61 ± 0.13		5.71 ± 0.13		4.46 ± 0.084
$\alpha6\beta1\gamma2$		7.01 ± 0.061		5.65 ± 0.087		4.52 ± 0.12
$\alpha6\beta2\gamma2$		6.90 ± 0.046		5.33 ± 0.081		4.93 ± 0.072
$\alpha6\beta3\gamma2$		6.86 ± 0.064		5.07 ± 0.087		4.70 ± 0.12

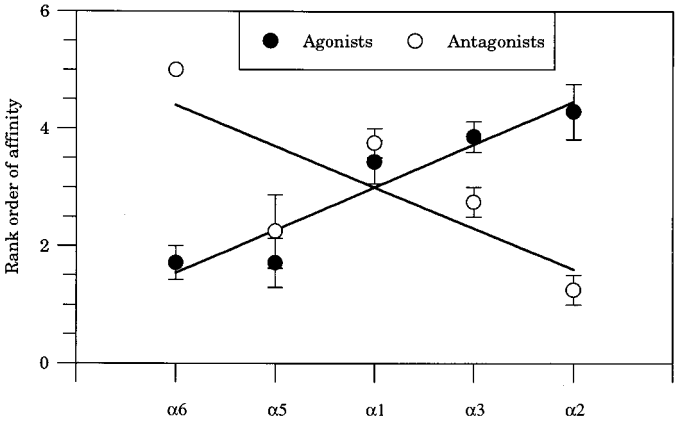

Fig. 3. Meaned rank order of affinity for agonists (muscimol, GABA, THIP, P4S, and thiomuscimol) and antagonists (Thio-4-PIOL, bicuculline, and SR95531), plotted versus the α subunit included in the receptor, when expressed as $\alpha x\beta3\gamma2$s. Linear regression shows significant subunit dependence and an inverse relationship for agonists versus antagonists.

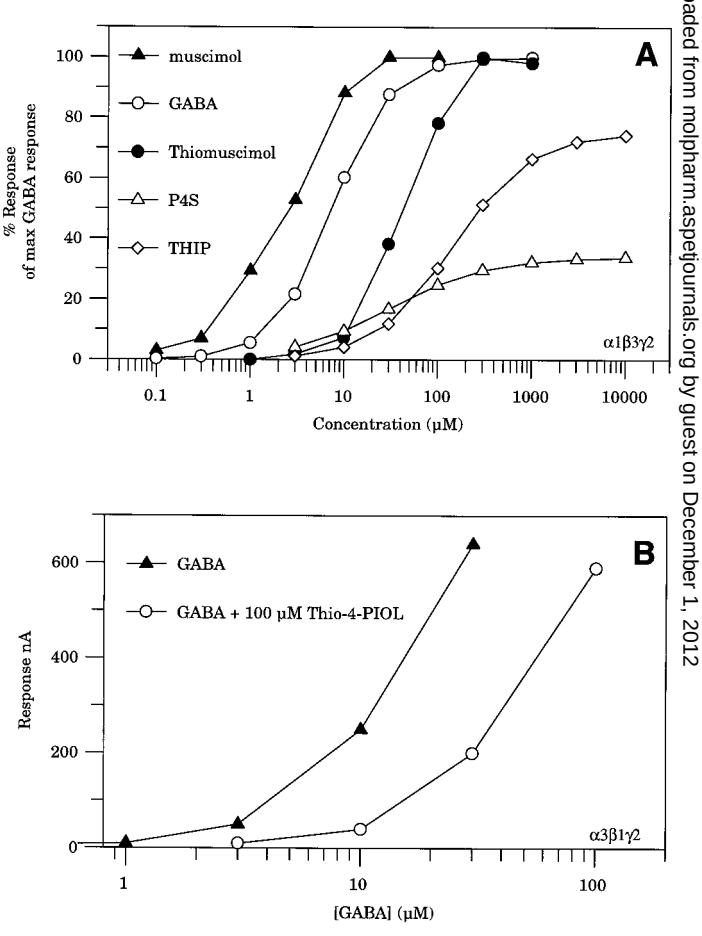

Fig. 4. A, Concentration response curves for muscimol, GABA, thiomuscimol, P4S, and THIP in oocytes injected with $\alpha1\beta3\gamma2$. B, Shift of concentration response curve to GABA using 100 μM Thio-4-PIOL. Values shown represent data obtained in single oocytes.

GABA, bicuculline, and SR95531 affinity are not significantly affected by changes in γ subunit structure (14).

The rank order of potency observed in subunit affinity using [^{3}H]muscimol binding for agonists is similar to that seen when measuring the EC_{50} in *X. laevis* oocytes; however, in the functional assay, the absolute values are 2–3 orders of magnitude lower. This suggests that the two measures are reflecting the same subunit-dependent phenomena.

Several amino acids have been implicated in the $GABA_A$ binding site. The primary interaction on the α subunit is with Phe64, which is conserved in all α subunits (17, 19). On the β subunit, two regions have been found to be critical for agonist and antagonist affinity, a YG*X*T and a TG*X*Y motif in the amino terminus of the peptide have been proposed to form part of the GABA binding site (18). These motifs are identical in all of the β subunits.

The difference of two to three orders of magnitude in binding affinities and functional potencies seen for the agonists is consistent with other studies, and is a common feature of agonist profiles at other ligand-gated ion channels (35, 36). Several hypotheses have been suggested to account for this. Relevant to this phenomenon may be the detection of a high and low affinity binding site for GABA (37). Whereas the low affinity site appears to be the functionally relevant recognition site, the role of the high affinity site remains unclear, although it may be related to the desensitized state of the receptor. Our observations of the same trend in subunit af-

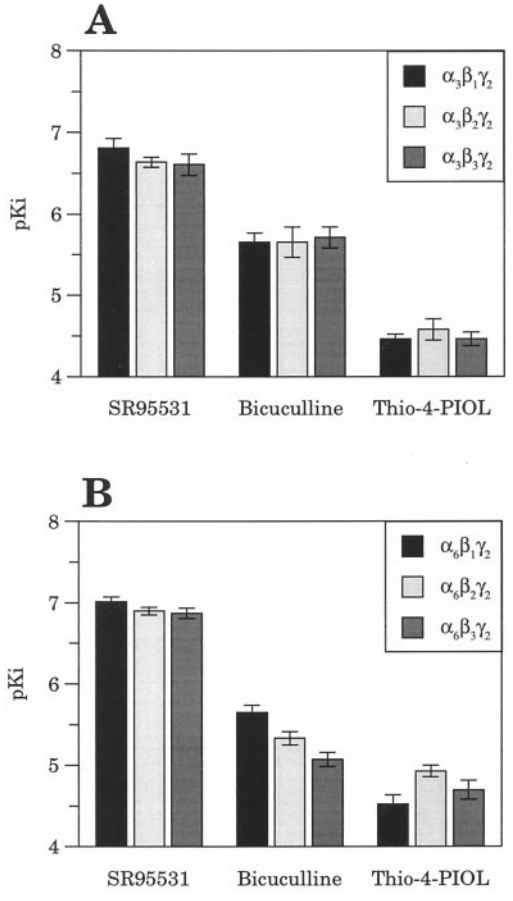

Fig. 5. Effect of β subunit on the pK_i value of thio-4-PIOL, bicuculline, and SR95531 against GABA responses in oocytes. A, $\alpha3\beta3\gamma2$; B, $\alpha6\beta3\gamma2$. Values represent mean ± standard error of at least four experiments.

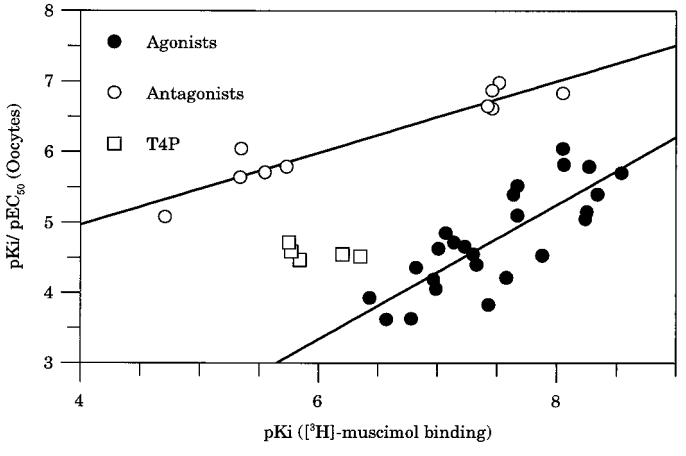

Fig. 6. Correlation between data obtained in [^{3}H]muscimol binding and electrophysiological experiments for all compounds tested in the study. Data are presented in Tables 2 and 3.

finity in both assays might suggest that the high and low affinity measures reflect the same site of interaction, rather than two separate binding sites. The physical conditions imposed on the tissue for radioligand binding (i.e., disruption of the membranes and changed ionic conditions that are optimized for detection of high levels of specific binding) may affect the binding site, although an association of this with an increase in receptor affinity is not obvious. An alternative explanation is that [^{3}H]muscimol binding measures the true affinity of the receptor for the agonist, whereas the functional measure reflects activation of the entire receptor/ion channel complex. If this is the case then the difference should be observed only for agonists and not for antagonists. The present data seem to support this hypothesis, in that the competitive antagonists bicuculline and SR95531 have similar affinities in both assays. Similarly, the wide variation in subunit-dependent functional receptor affinity for agonists is not reflected in the [^{3}H]muscimol binding assay, where much smaller differences in K_i values are observed. Thus, other factors related to the transduction process might account for the larger apparent differences between subunits. This is supported by observed differences in channel kinetics between $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ (38), where the presence of $\alpha3$ slowed activation, desensitization, and deactivation. Further evidence for this comes from studies on GABA receptors, as well as other ligand-gated ion channels, where mutations in the ion channel region that affect channel gating can result in up to 1000-fold increases in receptor affinity (39–41).

In determining K_i values for the antagonists using oocytes, shifts of the GABA concentration-response curves were used. This means that the subtype selectivity for the agonist GABA has already been taken into account. Thus, the shift in the dose-response curve is purely a consequence of the antagonist affinity for the receptor complex. Using this method, the data demonstrate that, unlike agonists, a minor difference in antagonist K_i is observed when the subunit composition is varied. Similarly, the affinities correspond well with those found using [^{3}H]muscimol binding to the same receptor combinations.

In conclusion, data presented here provide evidence for the role of the α subunit in the $GABA_A$ receptor as a co-determinant of ligand binding affinity and a determinant of receptor selectivity for agonists or antagonists. Furthermore, the data clearly demonstrate that whereas the $GABA_A$ receptor mechanisms transducing binding into a physiological response is highly dependent on the receptor subunit composition, the binding step of receptor ligand interaction may play only a minor role in the subunit dependency of GABA ligand potency.

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