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Functional Comparison of the Role of γ Subunits in Recombinant Human γ-Aminobutyric Acid$_A$/Benzodiazepine Receptors

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SUMMARY

The effect of benzodiazepines on the activity of γ-aminobutyric acid (GABA)$_A$ receptors has been shown to be influenced by different α subunits and can also be affected by the presence of different γ subunits. Previous studies have shown that receptors without a γ subunit or those containing γ1 are modulated to a lesser degree by benzodiazepines. Using the *Xenopus* oocyte expression system to express different subunit combinations, a detailed analysis of the pharmacological modulation of GABA$_A$ receptors by various benzodiazepine site ligands has been carried out. We analyzed 14 compounds, varying through full agonist, partial agonist, antagonist, and inverse agonist, with receptors consisting of α2β1, α2β1γ2S, and α2β1γ1 and we demonstrate differences in their extent of potentiation by different benzodiazepine-type ligands. Most compounds showed negligible effects on α2β1 and most agonists, particularly the imidazopyridines zolpidem, alpidem, and AHR14,749, exhibited less potentiation with α2β1γ1 than with α2β1γ2S. The inverse agonists dimethoxy-4-ethyl-β-carboline-3-carboxylate and Ro15-4513 did not act as inverse agonists and produced slight potentiation of α2β1γ1 receptors. Concentration-response curves were constructed for five selected agonists to evaluate both affinity and efficacy differences between α2β1γ2 and α2β1γ1 receptors. Most compounds showed lower efficacy and up to 10-fold lower affinity with α2β1γ1. Zolpidem showed slightly higher affinity but an extremely low efficacy; FG8205 also showed a markedly lower efficacy and was the most selective compound for α2β1γ2S versus α2β1γ1 receptors. CL218,872 showed high efficacy with α2β1γ1 and affinity similar to that with α2β1γ2 (being the least selective compound), suggesting that some low efficacy partial agonists with γ2-containing receptors may be more efficacious with γ1-containing receptors. The antagonists Ro15-1788 and CGS8216, although they blocked flunitrazepam potentiation of α2β1γ2, could not block potentiation of α2β1γ1. This study demonstrates that unique pharmacological profiles can be conferred by receptors containing different γ subunits.

The GABA$_A$ receptor is a multisubunit protein that plays a major role in inhibitory synaptic function. The receptor contains a number of binding sites for allosteric modulators, including benzodiazepines, barbiturates, and steroids. The heterogeneity of GABA$_A$ receptors expressed in the brain is due to the large number of subunits that can combine to form different molecular forms of the receptor (1, 2). It is becoming clear that the multiple forms of the receptor can exhibit different pharmacological properties, depending on the type of subunits that make up the receptor. The receptor is most likely to be a pentameric structure assembled from a gene family consisting of six α, three β, and three γ subunits, the stoichiometry of which is currently unknown. Previous work has demonstrated that the type of α subunit present can influence the benzodiazepine pharmacology, with α1-containing receptors forming the type I benzodiazepine receptor subtype, α2/α3 making up the type II class (3), and α5 producing a novel type II subclass that is insensitive to the imidazopyridine zolpidem (4). α4 and α6 make up the so-called diazepam-insensitive receptors that have low affinity for most benzodiazepines (5, 6). The γ subunit is thought to confer sensitivity of the receptor to benzodiazepines; the first of these cloned, the γ2 subunit, when combined with an α subunit and a β subunit produced receptors with a high affinity benzodiazepine binding site capable of modulating the functional receptors (7). Two other γ subunits have been identified (8, 9), and initial studies have demonstrated that these can also confer sensitivity to benzodiazepine ligands (8, 10–12). Binding to immunoprecipitated receptors or transfected cells gives a measure of the affinity of the benzodiazepine site only when a high enough affinity site is present for detection of radioligand binding. Using a functional approach the affinity can be measured even when the receptor has low affinity for benzodiazepines; the extent of potentiation or efficacy of compounds can also be measured, which is equally important in assessing their *in vivo* effects. Here we have used cloned human GABA$_A$ receptor subunits expressed in *Xenopus* oocytes to investigate the influence of combining different γ subunits with the same α and β subunits, and we show that changing this

ABBREVIATIONS: GABA, γ-aminobutyric acid; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMCM, dimethoxy-4-ethyl-β-carboline-3-carboxylate.

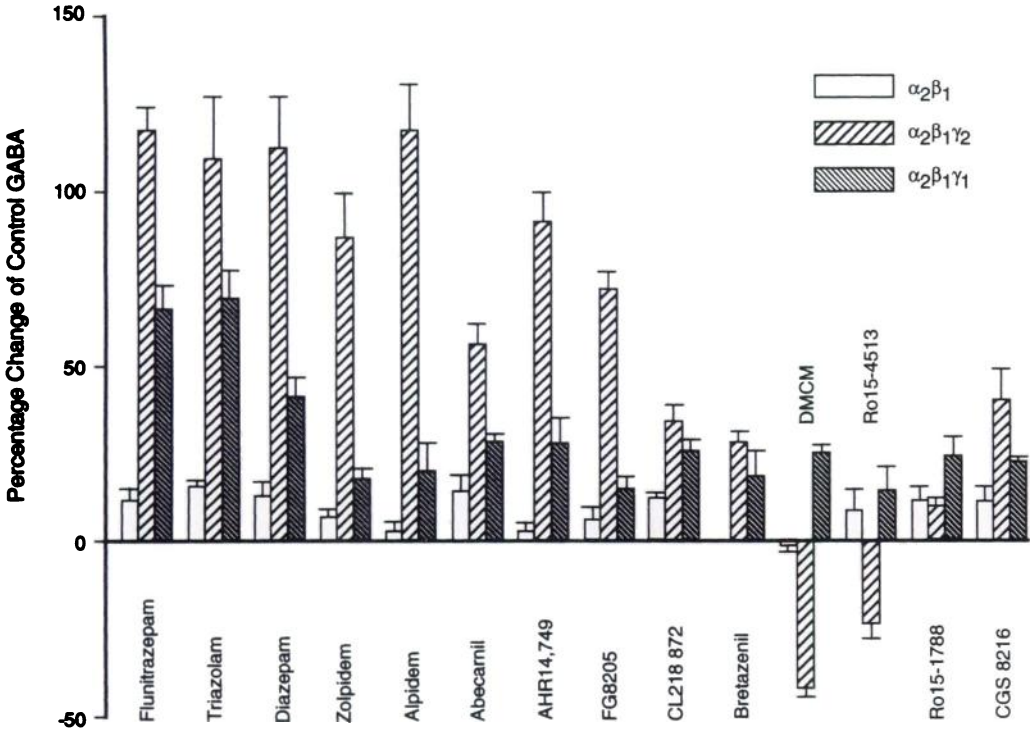

Fig. 1. Modulation of control GABA currents by benzodiazepine ligands with $\alpha2\beta1$, $\alpha2\beta1\gamma2S$, and $\alpha2\beta1\gamma1$ $GABA_A$ receptor subunit combinations. Each value is the mean ± standard error of at least four oocytes. Control GABA responses were obtained by selecting a GABA concentration approximately 20% of maximum for each individual oocyte, usually 1–10 μM. All drugs were applied at a concentration of 1 μM.

subunit can also have profound effects on the pharmacology of the benzodiazepine binding site produced by these receptors.

Materials and Methods

Human $GABA_A$ receptor cDNAs. The cloning and sequencing of human $\alpha1$, $\alpha2$, $\beta1$, and $\gamma2S$ subunit cDNAs will be reported elsewhere,[1] and these were subcloned into pCDM8 vector for expression studies. A human $\gamma1$ subunit cDNA was obtained by screening a human fetal brain λZAP cDNA library (Stratagene) under moderate stringency (13) with a ^{32}P-labeled rat $\gamma1$ probe (bases 27–1596 of the published rat $\gamma1$ nucleotide sequence) (8) obtained by PCR. A 4.1-kilobase $\gamma1$ cDNA was obtained. This cDNA lacked the first 400 bases of the coding region, which had been replaced by an irrelevant cloning artifact sequence. Anchored PCR was used to obtain the missing 5′ sequences. The antisense oligonucleotide 5′-AATGTTCATCCATGGGAAAGTTAT-3′, corresponding to the sequence 580 base pairs into the human $\gamma1$ coding region and containing an *Nco*I site, and an oligonucleotide based upon the T7 primer sequence of Bluescript SK− (Stratagene) were used in PCR, as described previously (14). A 650-base pair PCR product was digested with *Nco*I and *Kpn*I and subcloned into the truncated $\gamma1$ cDNA, which had also been cut with these enzymes, to yield a full length human $\gamma1$ cDNA. This was sequenced on both strands using Sequenase II enzyme (United States Biochemicals). The sequence of this human $\gamma1$ cDNA differed at a single residue from the published amino acid sequence (8); Leu-37 is an isoleucine in this cDNA.

For expression studies the $\gamma1$ cDNA was subcloned as a *Xho*I (site in the polylinker of the Bluescript vector at the 5′ end of the cDNA insert)-*Spe*I (site in the 3′ untranslated region) fragment into pCDM8 vector.

Oocyte expression. *Xenopus* oocytes were removed from anesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment (type IA, 0.5 mg/ml, for 10 min) to remove follicle cells, the oocyte nuclei were directly injected with 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0; filtered through nitrocellulose) containing different combinations of human $GABA_A$ subunit cDNAs (6 ng/μl) engineered into the expression vector pCDM8. 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 were voltage clamped between −40 and −70 mV. The cell was continuously perfused with saline at 10–13 ml/min and drugs were applied in the perfusate. GABA modulators were preapplied for 30 sec before the addition of GABA. GABA was applied until the peak of the response was observed, usually 30 sec or less. At least 3 min of wash time were allowed between each GABA application, to prevent desensitization. Concentration-response curves were calculated using a nonlinear least squares fitting program with the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. Zolpidem and alpidem were obtained from Synthelabo, CL218,872 was obtained from Lederle, AHR14,749 was obtained from Wyeth, bretazenil was a gift from Roche, and CGS8216 was obtained from Ciba Geigy. FG8205 was synthesized at Merck, Sharp, and Dohme (Hoddesdon, UK). Abecarnil was a gift from Schering. All other compounds were obtained from Sigma Biochemicals or Research Biochemicals Inc.

Results

$\alpha2\beta1\gamma1$ and $\alpha2\beta1\gamma2$ subtypes. Data from *in situ* hybridization (15, 16) and subunit-specific antibody experiments[2] suggest that the $\alpha2$ and $\gamma1$ subunits coassemble *in vivo*, forming a significant population in the brain. Evidence to date suggests that the β subunit does not play a critical role in modulating the benzodiazepine binding site (3, 4, 7). As a result, these

[1] Hadingham, K. L., P. Wingrove, B. Le Bourdelles, K. J. Palmer, C. I. Ragan, and P. J. Whiting. Cloning of cDNA sequences encoding human $\alpha2$ and $\alpha3$ γ-aminobutyric $acid_A$ receptor subunits and characterization of the benzodiazepine pharmacology of recombinant $\alpha1$-, $\alpha2$-, $\alpha3$-, and $\alpha5$-containing human γ-aminobutyric $acid_A$ receptors. *Mol. Pharmacol* **43**:970–975 (1993).

[2] K. Quirk, N. P. Gillard, C. I. Ragan, P. J. Whiting, and R. M. McKernan. $GABA_A$ receptors in the rat brain can contain both $\gamma2$ and $\gamma3$ subunits but $\gamma1$ does not exist in combination with another γ subunit.
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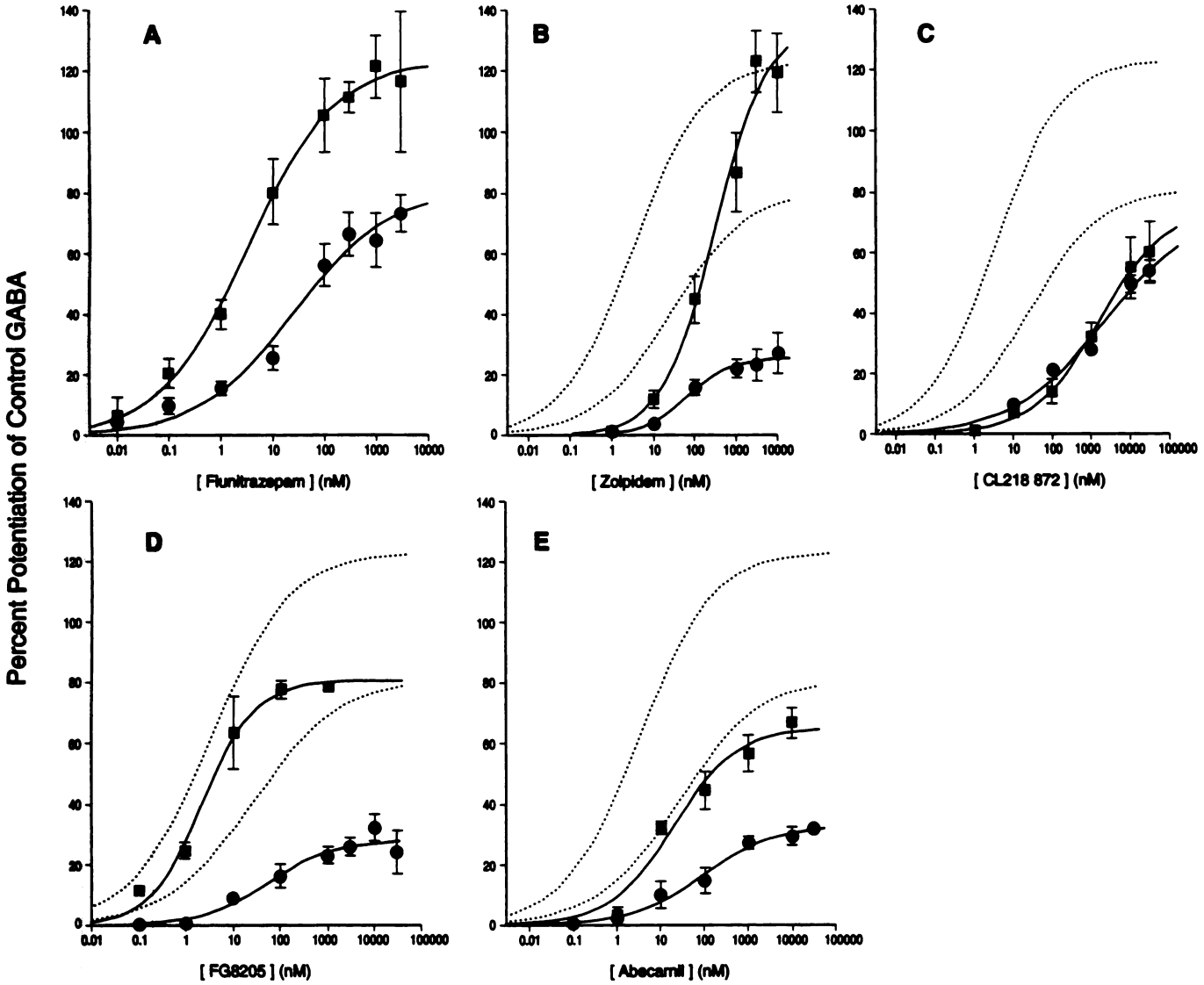

Fig. 2. Concentration-response curves for compounds acting at the benzodiazepine binding site of $\alpha2\beta1\gamma2S$ (■) and $\alpha2\beta1\gamma1$ (●), using a GABA concentration approximately 20% of maximum for each individual oocyte. Each *point* represents the mean ± standard error of at least three oocytes and represents the potentiation of a control GABA current by flunitrazepam (A), zolpidem (B), CL218,872 (C), FG8205 (D), and abecarnil (E). *Dotted lines* in B-E show the flunitrazepam curves from A, for comparison with other agonists.

different subunit combinations were chosen for the comparative study of the role of γ1 and γ2 in conferring benzodiazepine pharmacology to GABA$_A$ receptors. GABA concentration-response curves with $\alpha2\beta1$, $\alpha2\beta1\gamma1$, and $\alpha2\beta1\gamma2S$ revealed slightly differing affinities for GABA. The $\alpha2\beta1$ receptor had the highest affinity of 11.3 ± 1.9 μM (mean ± standard error of 5 cells). The $\alpha2\beta1\gamma2S$ and $\alpha2\beta1\gamma1$ receptors had GABA affinities of 30.6 ± 11 μM (n = 7) and 39.8 ± 4.1 μM (n = 5), respectively. To compensate for differences in GABA affinity, benzodiazepines were applied together with a concentration of GABA that elicited approximately 20% of the maximum response for each individual oocyte.

Benzodiazepine profile with $\alpha2\beta1$, $\alpha2\beta1\gamma2$, and $\alpha2\beta1\gamma1$. A selection of benzodiazepine receptor ligands ranging from full agonist through antagonist to inverse agonist were tested at a single concentration of 1 μM with three different subunit combinations (Fig. 1). α2 and β1 were expressed together or in combination with γ2S or γ1. With $\alpha2\beta1\gamma2S$, the nonselective full agonists flunitrazepam, triazolam, and diazepam elicited a maximum potentiation of GABA responses of approximately 125% above control. Using these criteria, alpidem was also a full agonist. From this study, all the compounds tested required the presence of a γ subunit for any significant potentiation. At this fixed concentration all nonselective full agonists produced less potentiation with γ1-containing receptors. However, most benzodiazepine type I receptor-selective compounds had a considerably lower potentiating effect on γ1-containing, compared with γ2S-containing, receptors. The inverse agonists DMCM and Ro15–4513 paradoxically potentiated $\alpha2\beta1\gamma1$, whereas they inhibited $\alpha2\beta1\gamma2$, and the antagonists Ro15–1788 and CGS8216 had a small potentiating effect on all the combinations.

Benzodiazepine concentration-response curves. From the previous series of experiments it was not possible to determine whether the small potentiations with $\alpha2\beta1\gamma1$ represented low affinity or low maximum efficacy. Therefore, five com-

TABLE 1

Benzodiazepine affinity and efficacy for $GABA_A$ receptors expressed in *Xenopus* oocytes

EC_{50} values and standard errors from the curves fitted in Fig. 2 are listed for the combinations $\alpha2\beta1\gamma2S$ and $\alpha2\beta1\gamma1$. The maximum potentiations are expressed as percentage of the control GABA response and were calculated using B_{max} values and standard errors from the curve-fitting program as described. By assuming flunitrazepam to be a full agonist and setting this value to 100% for each receptor combination, the maximum values for other drugs are expressed relative to the maximum flunitrazepam potentiation, thus giving a measure of relative efficacy.

	$\alpha2\beta1\gamma2$		$\alpha2\beta1\gamma1$	
	Maximum potentiation	Efficacy	Maximum potentiation	Efficacy
	% above control	%	% above control	%
Flunitrazepam	123 ± 3.2	100	81 ± 9.6	100
Zolpidem	132 ± 17.1	108	25 ± 3.8	31
CL218,872	74 ± 7.8	61	79 ± 2.8	97
FG8205	80 ± 3.3	65	28 ± 3.0	34
Abecarnil	64 ± 8.0	52	32 ± 2.4	39

	EC_{50}	
	$\alpha2\beta1\gamma2$	$\alpha2\beta1\gamma1$
	nM	
Flunitrazepam	3.3 ± 0.7	26.5 ± 20.3
Zolpidem	301 ± 174	64 ± 22.5
CL218,872	1660 ± 914	3292 ± 858
FG8205	2.2 ± 0.7	56.6 ± 40
Abecarnil	19.5 ± 15	93.7 ± 48

pounds, flunitrazepam, CL218,872, zolpidem, FG8205, and abecarnil, were selected for further investigation. By constructing full concentration-response curves with $\alpha2\beta1\gamma2S$ and $\alpha2\beta1\gamma1$ (Fig. 2), their maximum ability to modulate GABA responses and their affinity for these receptors were determined. Table 1 shows the affinities and efficacies for the benzodiazepine ligands investigated, from the concentration-response curves in Fig. 2. Efficacies were calculated for each receptor combination by assuming flunitrazepam to be a full agonist and normalizing the other compounds relative to this value, giving an estimate of the degree of agonist activity. The affinity values were taken from the fitted curves in each case. Most compounds had a higher affinity for $\alpha2\beta1\gamma2S$ versus $\alpha2\beta1\gamma1$; zolpidem, however, had a 4-fold selectivity for $\alpha2\beta1\gamma1$, and CL218,872 had very similar affinities for the two combinations. Furthermore, zolpidem became a low efficacy partial agonist with $\alpha2\beta1\gamma1$, and from Fig. 1 it appears that other selective compounds, such as AHR14,749 and alpidem, may also show a similar profile. Abecarnil and FG8205 were partial agonists with both combinations, showing lower efficacy with $\alpha2\beta1\gamma1$. The partial agonist CL218,872, however, became almost a full agonist, compared with flunitrazepam, on $\alpha2\beta1\gamma1$, with a very similar concentration-response curve. By comparing concentration-response curves it appeared that FG8205 showed the greatest selectivity between $\gamma2S$ and $\gamma1$, whereas CL218,872 showed the least selectivity.

Effects of benzodiazepine antagonists. The benzodiazepine antagonists were tested for their ability to inhibit potentiation of $\alpha2\beta1\gamma2S$ and $\alpha2\beta1\gamma1$ by flunitrazepam. When tested with $\alpha2\beta1\gamma1$, the potentiation by 300 nM flunitrazepam was not inhibited by either 300 nM Ro15–1788 or 1 μM CGS8216; however, with $\alpha2\beta1\gamma2S$ the large potentiation by 300 nM flunitrazepam was markedly inhibited by preincubation with either compound (Fig. 3). This suggests that these antagonists have a low affinity for $\alpha2\beta1\gamma1$ receptors.

Discussion

The present study provides a detailed functional comparison of the effects of introducing a γ subunit into the $GABA_A$ receptor and demonstrates how different variants of the γ subunit can influence both the affinity and efficacy of various benzodiazepine site ligands. Without a γ subunit the receptors expressed a higher affinity for GABA than when a γ subunit was included. This finding is in keeping with other studies (17–19), and it appeared that the γ1-containing receptors were slightly less sensitive to GABA. A larger difference in GABA affinity is observed when different α subunits are expressed with the same βγ subunits (17, 20, 21).

The type of α subunit can influence the affinity and efficacy of a number of benzodiazepine-type modulators, such as CL218,872, zolpidem, and alpidem, and photoaffinity labeling experiments suggest a benzodiazepine binding site on the α subunit (22, 23). However, the presence of a γ subunit appears to be necessary to observe high affinity benzodiazepine binding and functional modulation of the receptor by benzodiazepines (7, 17). As well as conferring benzodiazepine sensitivity, the three γ subunits that have been cloned also appear to confer different pharmacological properties to the populations of GABA receptors they constitute (7, 8, 12). The γ2 subunit is also alternatively spliced in the intracellular loop region, with the long form possessing eight extra amino acids. Although these forms differ in response to potentiation by ethanol (24), in our experience they do not differ with respect to their benzodiazepine pharmacology.[3] Here we have compared the pharmacological profile of γ2- and γ1-containing receptors by performing concentration-response experiments to determine the affinity and efficacy of various benzodiazepine site ligands. The initial study examining 14 different compounds at a concentration of 1 μM with three different receptors, $\alpha2\beta1$, $\alpha2\beta1\gamma2$, and $\alpha2\beta1\gamma1$, demonstrated results similar to those reported in transfected cells (10). Full agonists with $\alpha2\beta1\gamma2$ gave slightly lower potentiation at 1 μM with γ1-containing receptors (approximately 80% of control), compared with γ2-containing receptors (approximately 125% of control), and the imidazopyridines zolpidem, alpidem, and AHR14,749 all gave significantly lower potentiation (20–30% of control with γ1-containing receptors, compared with 80–130% with γ2-containing receptors). The imidazobenzodiazepine FG8205, which was a partial agonist with $\alpha2\beta1\gamma2$ and has been shown to be a partial agonist with receptors containing α1 or α3 (20), gave a markedly lower potentiation with $\alpha2\beta1\gamma1$ (28% of control with $\alpha2\beta1\gamma1$ but 80% of control with $\alpha2\beta1\gamma2S$). The difference in affinity and efficacy of FG8205 made this compound one of the most selective between γ2S- and γ1-containing receptors. The inverse agonist DMCM and partial inverse agonist Ro15–4513 lost their inverse agonist activity and potentiated slightly the receptors containing $\alpha2\beta1\gamma1$; this has also been observed in transfected cells (10). The partial agonist CL218,872 gave a similar potentiation of γ2- and γ1-containing receptors, and concentration-response curves for both combinations were very similar. In this respect, CL218,872 was the least selective compound between γ1- and γ2S-containing receptors. The percent efficacy level shown in Table 1 was calculated with reference to flunitrazepam, assuming that flunitrazepam can be classified as a full agonist with $\alpha2\beta1\gamma1$-containing receptors,

[3] K. A. Wafford, Unpublished observations.

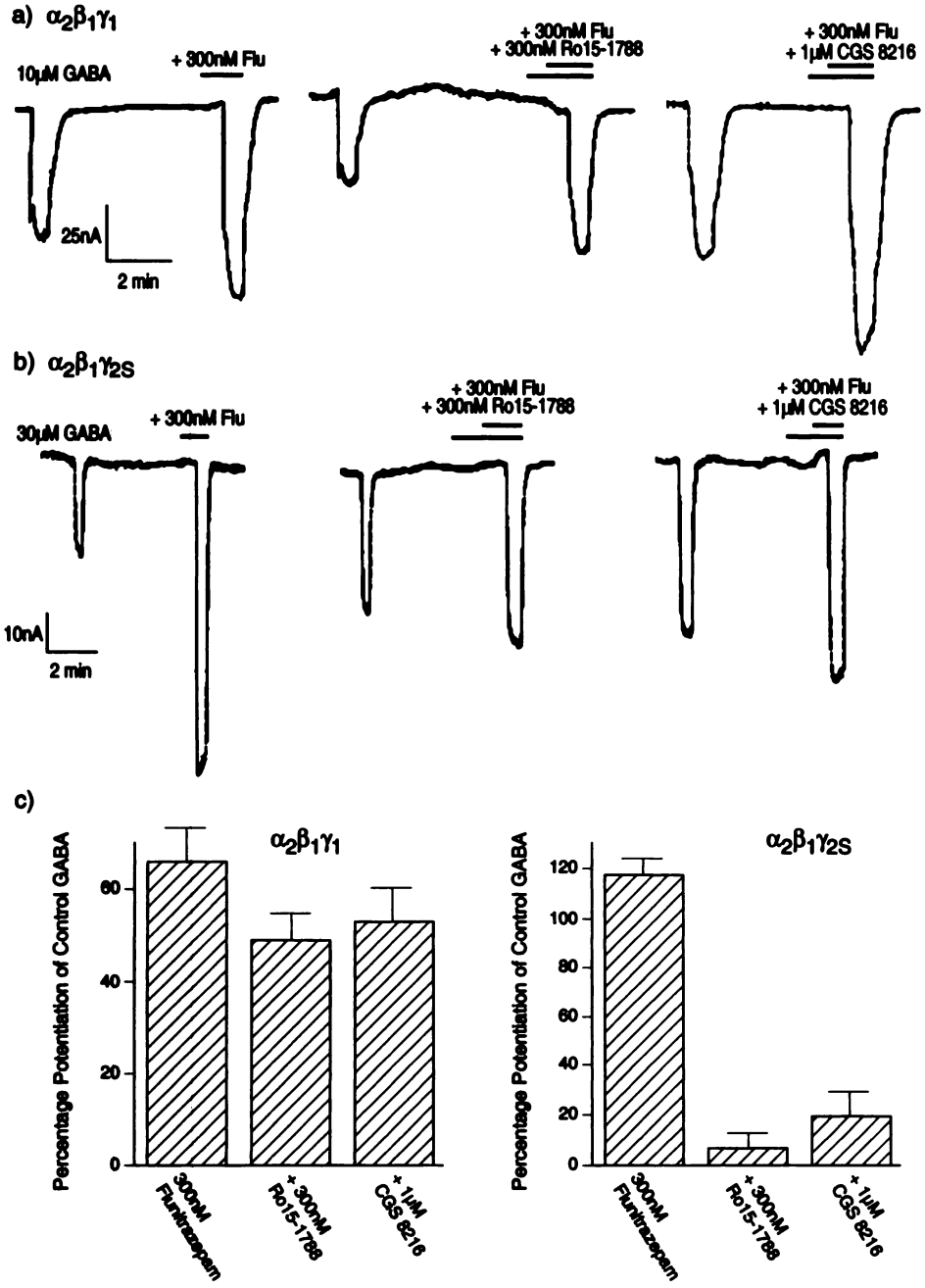

Fig. 3. Potentiation by flunitrazepam (*Flu*) (300 nM) of currents in response to 10 or 30 μM GABA, followed by antagonism using Ro15–1788 (300 nM) or CGS8216 (1 μM). a, With α2β1γ1 flunitrazepam potentation is not blocked by antagonists. b, With α2β1γ2S flunitrazepam exhibits a large potentiation that is blocked by preapplication of either Ro15–1788 or CGS8216. Drugs were applied as indicated by the *bars*. c, Mean data ($n \geq 3$) showing inhibition of flunitrazepam potentiation with α2β1γ2S but not α2β1γ1.

and this makes CL218,872 almost a full agonist with this combination. This finding suggests that compounds that are partial agonists with some combinations may be full agonists with others. Concentration-response curves for flunitrazepam with both combinations revealed a slightly lower maximum efficacy with γ1-containing receptors and an 8-fold lower affinity. Other full agonists examined in the initial screen, such as triazolam and diazepam, had efficacy similar to that of flunitrazepam at 1 μM with α2β1γ2 and a similarly lower level with α2β1γ1. Full concentration-response curves for zolpidem revealed an extremely low efficacy with γ1-containing receptors but a slightly higher affinity, although this EC_{50} estimate should be regarded with caution because the maximum level of potentiation was only 20% of control. This general lack of intrinsic activity of zolpidem with γ1-containing receptors may also be true for other imidazopyridines and imidazobenzodiazepines, as observed in the initial screen (alpidem, AHR14,749, and FG8205). Abecarnil appeared to be a partial agonist with α2β1γ2 and had lower affinity (5-fold) and efficacy with α2β1γ1. With both subunit combinations, Hill coefficients for all the benzodiazepine concentration-response curves were <1, with no significant differences for individual compounds.

Previously published data using [^{3}H]flunitrazepam to label α1β1γ1 receptors expressed in transiently transfected cells demonstrated a 10-fold lower affinity for flunitrazepam, a lower affinity for other agonists, and negligible effects of DMCM and Ro15–1788 (8). This would agree with our findings with regard to DMCM losing its inverse agonist activity with α2β1γ1.

The benzodiazepine antagonists Ro15–1788 and CGS8216 exhibited a low level of potentiation at 1 μM with γ1- and γ2-

containing receptors and inhibited potentiation by flunitrazepam of $\alpha 2\beta 1\gamma 2$ receptors (Fig. 3a). However, neither compound was able to antagonize the potentiation produced by flunitrazepam with $\alpha 2\beta 1\gamma 1$ (Fig. 3b). This observation that benzodiazepine potentiation of $\alpha 2\beta 1\gamma 1$ cannot be inhibited functionally by Ro15–1788 is in agreement with the low affinity of Ro15–1788 at $\gamma 1$-containing receptors (8).

We have demonstrated here that, compared with the $\gamma 2$ subunit, the $\gamma 1$ subunit can confer a different pharmacological profile with regard to benzodiazepine site ligands and that the γ subunit plays a role as important as that of the α subunit in determining benzodiazepine sensitivity. Recent evidence also suggests that the $\gamma 3$ subunit can confer yet another different pharmacological profile to the $GABA_A$ receptor (11, 12). Whether these receptors occur *in vivo* and their relative importance remain to be determined. Results obtained using subunit-specific antibodies with immunoprecipitated receptors strongly suggest that $\alpha 2$ and $\gamma 1$ coassemble *in vivo*.[2] It is also interesting to note that the genes encoding the $\alpha 2$ and $\gamma 1$ subunits occur together on human chromosome 4 (25), and it is suggested that these are expressed together. *In situ* hybridization studies suggest that $\gamma 1$ has a unique distribution in the brain, being localized to specific regions such as the amygdala, hypothalamus, and septum (26) and particularly areas that do not express large amounts of $\gamma 2$ (27). The $\alpha 2$ and $\gamma 1$ subunits are also abundant in Bergmann glia (15, 16), as well as cultured astrocytes (28), and may form a glial form of the $GABA_A$ receptor. Also, a pharmacological profile very similar to that which is conferred by $\gamma 1$ has been described in cultured astrocytes (28, 29). Although the functional role of $\gamma 1$-containing receptors is currently unknown, their unique pharmacology may allow the development of drugs targeted at this particular subclass of receptor.

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