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Differences in Affinity and Efficacy of Benzodiazepine Receptor Ligands at Recombinant γ-Aminobutyric Acid$_A$ Receptor Subtypes

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SUMMARY

Two forms of the multisubunit γ-aminobutyric acid (GABA)$_A$ receptor were expressed in *Xenopus* oocytes by injecting mRNA encoding the bovine GABA$_A$ receptor subunit cDNAs for $\alpha1\beta1\gamma2L$ and $\alpha3\beta1\gamma2L$. The properties of these two combinations were examined by electrophysiological recording of GABA currents using the two-electrode voltage-clamp method. The actions of several benzodiazepine site ligands were compared in terms of their affinity for and efficacy at these two subunit combinations. Flunitrazepam potentiated control GABA responses to a maximum of 77% with $\alpha1\beta1\gamma2L$ and 105% with $\alpha3\beta1\gamma2L$, with EC_{50} values of 29 ± 11 nM and 23 ± 10 nM, respectively. Flunitrazepam also produced a greater shift to the left of the GABA concentration-response curve with $\alpha3\beta1\gamma2L$ than with $\alpha1\beta1\gamma2L$. Concentration-response curves for the type I benzodiazepine receptor-preferring compounds zolpidem and CL218 872 showed a selectivity for the $\alpha1\beta1\gamma2L$ receptor, with respective affinity ratios 7-fold and 17-fold higher, compared with $\alpha3\beta1\gamma2L$. The inverse agonist methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate produced a maximum inhibition of 30% with both receptor combinations and also had a higher affinity for $\alpha1\beta1\gamma2L$ than $\alpha3\beta1\gamma2L$. For the first time CL218 872 and FG8205 were shown to be partial agonists of individual receptor combinations, compared with a full agonist such as flunitrazepam. The FG8205 concentration-response curve reached a maximum approximately 60% that of a full agonist with both $\alpha1\beta1\gamma2L$ and $\alpha3\beta1\gamma2L$. CL218 872 reached a lower maximum efficacy with $\alpha3\beta1\gamma2L$ (30%) than with $\alpha1\beta1\gamma2L$ (51%), demonstrating not only that different compounds can have varying levels of partial agonist activity but also that the same compound can have differing degrees of efficacy at different receptor combinations.

The GABA$_A$ receptor is a multisubunit receptor that forms an ion channel that is selectively permeable to chloride ions. The channel is directly operated by GABA, and the proteins that make up the receptor have a number of sites to which a variety of drugs can bind, thus modulating the activity of the receptor/channel complex. A whole gene family exists for the mammalian GABA$_A$ receptor, including six α, three β, three γ, and δ subunits, which can combine together to form a heterogeneous population of receptors throughout the brain (for review, see Ref. 1). Genetic reconstitution of expressed subunits has indicated that at least an α, β, and γ subunit are required to show modulation by benzodiazepines (2); however, barbiturates and steroids potentiate single subunits or combined pairs of subunits.

Before the cloning of GABA$_A$ receptor subunits, two pharmacologically separable subtypes of GABA$_A$ receptor had been identified, based on brain regional differences in affinity for several ligands that bound to the benzodiazepine site (3–5). These two subtypes, which bind most benzodiazepines with high affinity, were classified as BZ I and BZ II, with several compounds such as zolpidem, CL218 872, and alpidem having higher affinity at BZ I. Both receptors are found in different proportions throughout the central nervous system; for example, the cerebellum is rich in BZ I-type GABA receptors, whereas GABA receptors in the spinal cord are predominantly BZ II (6). Both subtypes are found in the hippocampus and cortex in varying proportions. *In situ* hybridization experiments using different α subunits identified a correlation showing α1 mRNA in regions with high affinity for these compounds and α2 and α3 in regions with low affinity for β2 1-selective compounds (7). In transfected cells expressing α1, α2, or α3 subunits, combined with β2γ2, α1-containing receptors exhibited high binding affinity for CL218 872 and other BZ I-selective compounds, whereas α2 and α3 had lower affinity, suggesting a molecular basis for these subtypes in that the α1 subunit is present in BZ I-type receptors and BZ II-type receptors contain α2 or α3 subunits (8). With the cloning and expression of other GABA$_A$ receptor subunits, receptors with novel pharmacology have been described and are likely to exist in the brain (9–11).

ABBREVIATIONS: GABA, γ-aminobutyric acid; MBS, modified Barth's saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BZ I, type I benzodiazepine receptor; BZ II, type II benzodiazepine receptor, DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carobxylate.

Here, using a functional approach, i.e., expressing recombinant GABA receptor subunits in *Xenopus* oocytes and using electrophysiological methods to assay the affinity and efficacy of various modulators of the receptor, we have demonstrated selectivity for $\alpha 1$ of CL218 872 and zolpidem, as well as partial agonist activity of CL218 872 and FG8205. Flunitrazepam, triazolam, and zolpidem were full agonists and the inverse agonist DMCM had a high affinity for both receptor combinations. Both full and partial agonist responses were sensitive to blockade by the benzodiazepine antagonist flumazenil (Ro15-1788).

Methods

Frogs were maintained in aquarium tanks at room temperature with a 12/12-hr light/dark cycle and were fed a regular diet of chopped steak and crickets. To obtain oocytes, frogs were anesthetized by immersion in a solution of 0.5% tricaine and a small piece of ovary was removed through an incision in the abdominal wall. Stage V and VI oocytes were isolated and the theca and epithelial cell layer were dissected away using watchmaker forceps. Follicle cells were removed by a 10-min treatment in Sigma type IA collagenase (0.5 mg/ml) dissolved in MBS [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]. Oocytes were injected with 50 nl of a solution containing mixtures of subunit cRNAs (1–2 mg/ml), using a 10-μl micropipette with an internal diameter of 20 μm. Oocytes were incubated for 2 days in MBS supplemented with 2 mM sodium pyruvate, 100 units/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml gentamycin. For recording, oocytes were placed in a 50-μl bath and perfused with MBS at 10–13 ml/min. Cells were impaled with two 1–3-MΩ electrodes containing 3 M KCl and were voltage-clamped at −70 mV. Drugs were applied in the perfusate and GABA modulators were preapplied for 30 sec before GABA was applied together with drug. GABA was applied until the peak of the response was observed, which for the majority of oocytes was 30 sec or less. Concentration-response curves were fitted to the data using a nonlinear least-squares fitting program and 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. EC_{50} values and B_{max} values are shown in Table 1, with corresponding standard errors. Zolpidem was obtained from Synthelabo and CL218 872 was obtained from Lederle. FG8205 was synthesized at Merck Sharp and Dohme, Hoddesdon, and all other compounds were obtained from Sigma Biochemicals.

Results

Concentration-response curves for GABA were obtained for different subunit-containing $GABA_A$ receptors. For the combinations $\alpha 1\beta 1\gamma 2L$ and $\alpha 3\beta 1\gamma 2L$ the GABA EC_{50} values were 41 ± 7.3 μM and 98 ± 19 μM, respectively. The benzodiazepine flunitrazepam potentiated responses to GABA for both subunit receptor combinations (Fig. 1). Concentration-response curves for flunitrazepam potentiation of GABA responses (30 μM) demonstrated a similar affinity for $\alpha 1$- and $\alpha 3$-containing receptors, with EC_{50} values of 29 ± 11 nM and 23 ± 10 nM, respectively (see Fig. 3a). These are similar to affinities measured in transfected cells and hippocampal brain slices (8, 12). A higher maximum degree of potentiation was observed with $\alpha 3$-containing receptors (100% above control responses, compared with 60% for $\alpha 1$). In keeping with this a maximum concentration of flunitrazepam (1 μM) produced a 2-fold shift to the left of the GABA concentration-response curve with $\alpha 1\beta 1\gamma 2L$ receptors but a 3-fold shift with $\alpha 3\beta 1\gamma 2L$ receptors (Fig. 1). This relative efficacy was also seen with other full

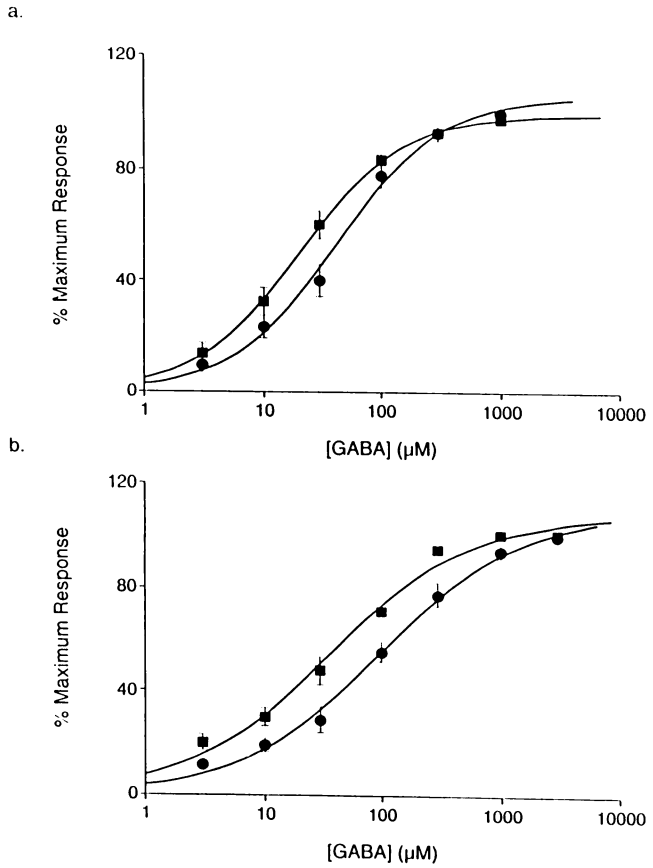

Fig. 1. GABA concentration-response curves in the absence (●) and presence (■) of a maximal potentiating concentration of flunitrazepam (1 μM). Two different combinations of GABA receptor were examined, $\alpha 1\beta 1\gamma 2$ (a) and $\alpha 3\beta 1\gamma 2$ (b). Currents were normalized to the maximum GABA response, and *points* represent means ± standard errors of four to six oocytes.

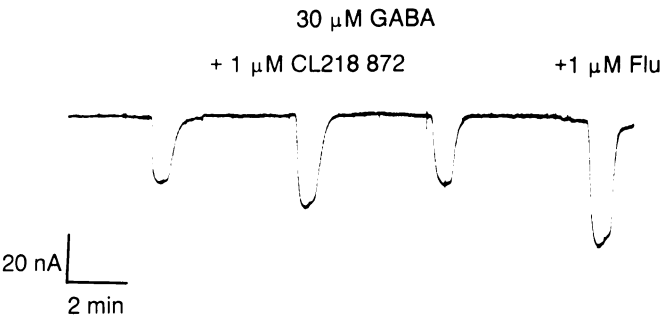

Fig. 2. Potentiation of sequential 30 μM GABA responses in an oocyte expressing $\alpha 1\beta 1\gamma 2$ $GABA_A$ receptors, by maximum concentrations of CL218 872 (1 μM) and flunitrazepam (*Flu*) (1 μM).

agonists such as triazolam and zolpidem. A maximum concentration of CL218 872 could not elicit a full response, compared with flunitrazepam on the same cell (Fig. 2), suggesting that CL218 872 was acting as a partial benzodiazepine agonist at both $\alpha 1$- and $\alpha 3$-containing receptors. Concentration-response curves confirmed this and showed that CL218 872 produced a lower maximum potentiation with $\alpha 3\beta 1\gamma 2L$ than with $\alpha 1\beta 1\gamma 2L$ (Fig. 3b). CL218 872 was also 14-fold selective for those receptors containing an $\alpha 1$ subunit. By defining the flunitrazepam maximum as 100% for both receptor combinations and comparing this with the effects of other drugs, it was determined

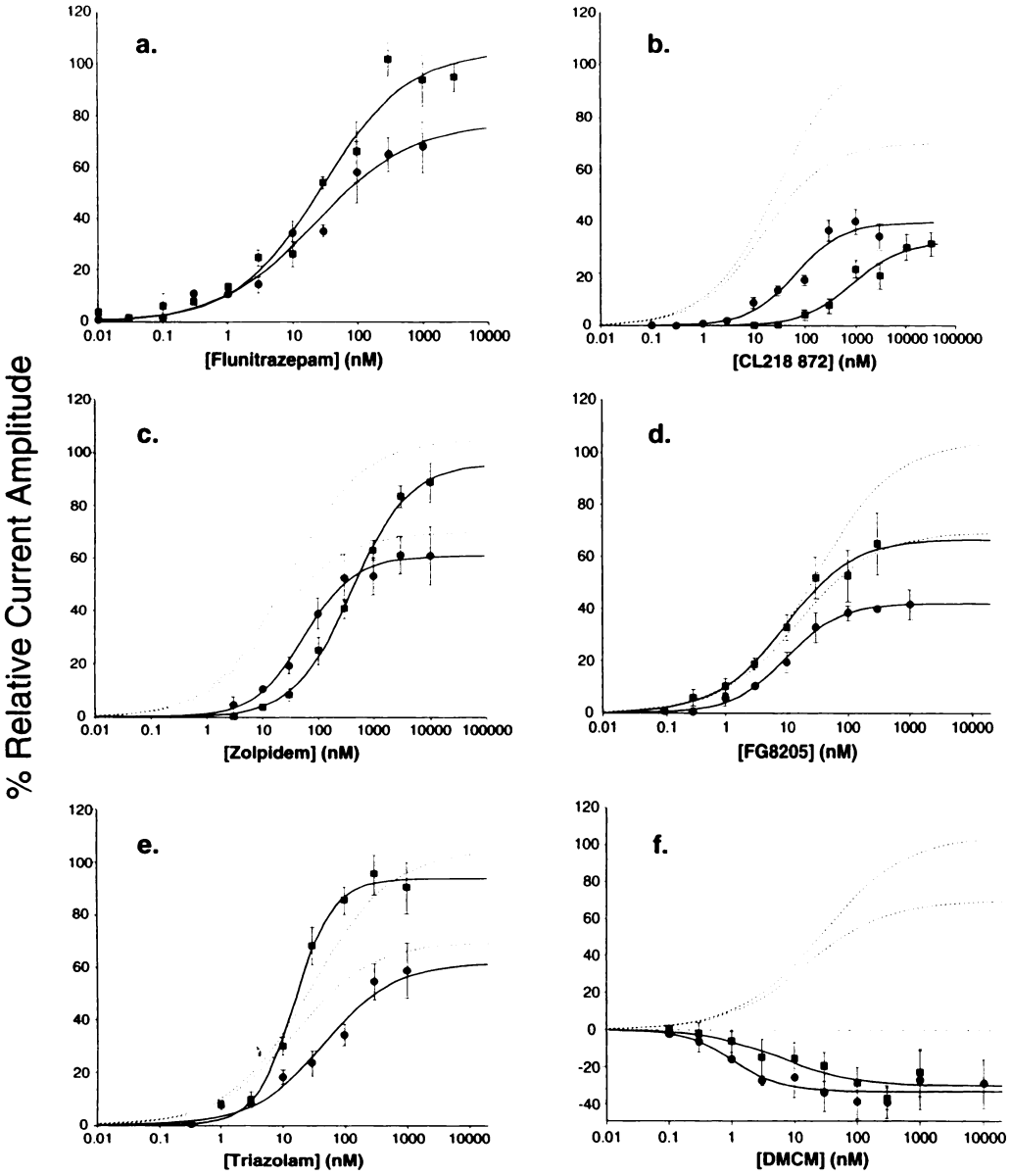

Fig. 3. Concentration-response curves for compounds acting at the benzodiazepine binding site on $\alpha1\beta1\gamma2L$ (●) and $\alpha3\beta1\gamma2L$ (■), using a single GABA concentration of 30 μM, which was below the EC_{50} for both combinations. Each *point* indicates the mean ± standard error of at least three oocytes and represents the percentage of potentiation of a control 30 μM GABA current by flunitrazepam (a), CL218872 (b), zolpidem (c), FG8205 (d), triazolam (e), and DMCM (f). *Dotted lines* in b–f, flunitrazepam curves from a, for comparison with other agonists and the inverse agonist.

that zolpidem behaved as a full agonist at both receptors, but again it had a 7-fold higher affinity for receptors containing the $\alpha1$ subunit (Fig. 3c). Triazolam was a full agonist at both $\alpha1$- and $\alpha3$-containing receptors and was 3-fold more potent at the $\alpha3\beta1\gamma2L$ receptor (Fig. 3e). FG8205 was a partial agonist with a higher level of efficacy than CL218 872, reaching a maximum of approximately 60% of full agonist activity at both $\alpha1$ and $\alpha3$. It was of high affinity but was not selective for either subunit combination (Fig. 3d). The inverse agonist DMCM inhibited the GABA currents, and concentration-response curves showed a high affinity for both subunit combinations (Fig. 3f). The EC_{50} values and maximum efficacies of all the benzodiazepine site ligands examined are summarized in Table 1. All of the benzodiazepine agonists were sensitive to blockade by the antagonist flumazenil; examples are shown of inhibition of FG8205 and flunitrazepam potentiation by 300 nM flumazenil in Fig. 4.

Discussion

Previous experiments using displacement of [^{3}H]Ro15-1788 binding to transiently transfected cells showed that cells expressing $\alpha1\beta1\gamma2$ subunits exhibited higher affinity for the BZ I-selective compounds CL218 872, 2-oxoquazepam, and zolpidem than did those expressing $\alpha2\beta1\gamma2$ and $\alpha3\beta1\gamma2$ GABA receptors (8). Other studies using electrophysiological methods to describe expressed recombinant $GABA_A$ receptors have shown potentiation by benzodiazepines (13–16); however, a detailed examination of agonist and inverse agonist affinity and efficacy has not been described previously. We have used an electrophysiological approach to show that the functional modulation of receptors containing $\alpha1\beta1\gamma2L$ and $\alpha3\beta1\gamma2L$ corresponds to BZ I and BZ II GABA/benzodiazepine-type pharmacology, respectively. Due to the heterogeneous distribution of different GABA receptor subunits in the brain, other receptor combinations are also likely to be functionally equivalent to BZ I and BZ II, as well as making up other novel pharmacological subtypes.

We observed responses to GABA that were concentration dependent, with EC_{50} values of 41 μM and 98 μM for $\alpha1\beta1\gamma2$ and $\alpha3\beta1\gamma2$, respectively, and using 30 μM GABA we consist-

TABLE 1

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

EC_{50} values (means ± standard errors) from the curves fitted in Fig. 3 are given for the combinations $\alpha1\beta1\gamma2L$ and $\alpha3\beta1\gamma2L$. The maximum potentiations were calculated using B_{max} values (means ± standard errors) from the curve-fitting program 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 then expressed relative to the maximum flunitrazepam potentiation.

Drug	$\alpha1\beta1\gamma2$		$\alpha3\beta1\gamma2$	
	EC_{50}	Efficacy	EC_{50}	Efficacy
	nM	%	nM	%
Flunitrazepam	29 ± 11.2	100 ± 8.9	24 ± 10.4	100 ± 7.4
Triazolam	46 ± 10.2	81 ± 3.4	16 ± 1.6	90 ± 2.6
FG8205	10 ± 1.1	54 ± 1.4	10 ± 3.1	64 ± 5.1
CL 218 872	68 ± 28.9	51 ± 5.3	885 ± 456.8	30 ± 4.0
Zolpidem	57 ± 7.5	80 ± 2.2	410 ± 55.4	90 ± 3.2
DMCM	1 ± 0.4	−33 ± 2.9	6 ± 4.7	−30 ± 4.5

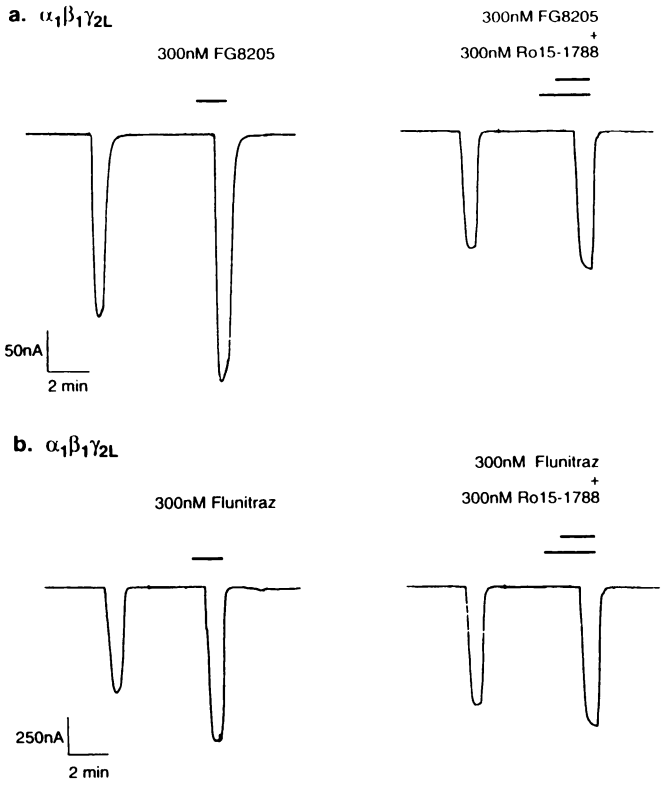

Fig. 4. Inhibition of individual current responses to 30 μM GABA together with flunitrazepam (300 nM) (a) and the partial agonist FG8205 (300 nM) (b) by the antagonist flumazenil (Ro15-1788) at 300 nM. Data are from separate oocytes expressing the combination $\alpha1\beta1\gamma2$, and drugs were applied for the times indicated by the *bars above each trace*.

ently observed a high degree of potentiation by benzodiazepine agonists with these combinations.

The selectivity of CL218 872 and zolpidem was examined with expressed $\alpha1\beta1\gamma2$ and $\alpha3\beta1\gamma2$ receptors in *Xenopus* oocytes. In brain membranes CL218 872 has a 5–10-fold selectivity and zolpidem a 4–11-fold selectivity for cerebellum (predominantly BZ I) over hippocampus (predominantly BZ II) (17, 18). The selectivity is larger with cells transfected with $\alpha1\beta\gamma2$ and $\alpha3\beta\gamma2$, with a 20-fold selectivity of zolpidem and an 11-fold selectivity of CL218 872, although this selectivity is reduced to 4-fold when binding is carried out at 37° (8, 11). This temperature dependence is also observed with brain membranes (19), suggesting a lower degree of selectivity under more physiological conditions. We found that the selectivity for these receptors in oocytes was 7-fold for zolpidem and 17-fold for CL218 872 at room temperature (22°), indicating that, functionally, a high degree of selectivity is maintained for these receptors under physiological conditions. Mutagenesis studies have identified critical residues in the amino acid sequence of the $\alpha1$ subunit that influence affinity of benzodiazepine site ligands. Single amino acid changes dramatically change the affinity for benzodiazepines (20) or, in other cases, only BZ I-selective compounds (21).

DMCM has been shown to have a slightly higher binding affinity for $\alpha1$, compared with $\alpha2$ or $\alpha3$ (8); our results also suggest some selectivity for $\alpha1$- versus $\alpha3$-containing receptors. Unlike some previous reports (13, 15), we did not observe any agonist-like effect at concentrations up to 10 μM.

In addition to affinity, the level of efficacy was measured with the different subunit-containing receptors. In general, full agonists were more efficacious with $\alpha3$ than $\alpha1$. This is in agreement with concentration-response curves for flunitrazepam with the same rat brain subunit combinations in transiently transfected 293 cells (16). It was considered possible that the higher degree of potentiation may be due to the lower GABA affinity of $\alpha3\beta1\gamma2L$, which would place 30 μM GABA, the concentration at which benzodiazepines were tested, at a lower position on the GABA concentration-response curve than with $\alpha1\beta1\gamma2L$. However, when the shifts of the GABA concentration-response curve produced by flunitrazepam were compared (see Fig. 1), a greater shift to the left was observed for $\alpha3\beta1\gamma2$ than $\alpha1\beta1\gamma2$, which confirms the greater degree of potentiation observed with this subunit combination.

Different levels of maximum agonist efficacy of benzodiazepine site ligands have previously been described for native $GABA_A$ receptors, for instance on frog dorsal horn neurons (22) and cultured chick spinal cord neurons (23); however one possible explanation for this result could be differential affinity for and efficacy with the mixture of individual receptor subclasses that may be present on these neurons. By using a single $\alpha\beta\gamma$ combination we have demonstrated for the first time that the partial agonist effect is due to a lower maximal efficacy of an individual receptor subtype. We have also demonstrated that the agonists CL218 872 and FG8205 act as partial agonists in oocytes expressing a single population of receptors with different relative levels of efficacy. When compared with a maximum potentiation by flunitrazepam on the same cell, the maximum potentiation by these agonists reached a lower level. FG8205, however, had a higher level of efficacy than CL218 872. It was also found that the maximum potentiation by a compound varied with different subunit combinations, because CL218 872 had 51% the efficacy of flunitrazepam with $\alpha1\beta1\gamma2L$, compared with only 30% with $\alpha3\beta1\gamma2L$. Thus, these results suggest not only that affinity can change with different subunit combinations but also that the intrinsic activity of a compound can vary.

The affinity and maximum efficacies of the drugs measured in oocytes expressing $\alpha1\beta1\gamma2L$ were similar to those measured using binding techniques (8, 11, 24) and whole-cell patch-clamping of L cells stably expressing this subunit combination (25), suggesting that a different surrounding membrane environment and possible variations in the post-translational modifications provided by different cell types are not critical factors

for the interactions of these drugs at their binding sites. Flunitrazepam and DMCM also have affinities similar to those measured in hippocampal slices using electrophysiological and binding techniques (12). EC_{50} values are similar to the affinities described for immunoprecipitated $GABA_A$ receptors from rat brain containing $\alpha 1$ and $\alpha 3$ subunits (26), suggesting that the compounds investigated here act in a similar fashion on native $GABA_A$ receptors containing these subunits.

The *Xenopus* oocyte can be used as a device for expressing different combinations of GABA receptor subunits and measuring both affinity and efficacy of benzodiazepine receptor ligands. This is particularly useful because the present results indicate that compounds active at the benzodiazepine binding site have differing degrees of efficacy at, as well as affinity for, different subunit combinations. These factors will be important in the development and evaluation of new compounds that are putative modulators of the $GABA_A$ receptor at the benzodiazepine site.

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