

Effect of α Subunit on Allosteric Modulation of Ion Channel Function in Stably Expressed Human Recombinant γ -Aminobutyric Acid_A Receptors Determined Using ³⁶Cl Ion Flux

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ABSTRACT

Inhibitory γ -aminobutyric acid (GABA)_A receptors are subject to modulation at a variety of allosteric sites, with pharmacology dependent on receptor subunit combination. The influence of different α subunits in combination with $\beta 3\gamma 2$ s was examined in stably expressed human recombinant GABA_A receptors by measuring ³⁶Cl influx through the ion channel pore. Muscimol and GABA exhibited similar maximal efficacy at each receptor subtype, although muscimol was more potent, with responses blocked by picrotoxin and bicuculline. Receptors containing the $\alpha 3$ subunit exhibited slightly lower potency. The comparative pharmacology of a range of benzodiazepine site ligands was examined, revealing a range of intrinsic efficacies at different receptor subtypes. Of the diazepam-sensitive GABA_A receptors ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$), $\alpha 5$ showed the most divergence, being discriminated by zolpidem in terms of very low affinity, and CL218,872 and CGS9895 with different efficacies. Benzodiazepine potentiation at $\alpha 3\beta 3\gamma 2$ s with nonselective agonist

chlordiazepoxide was greater than at $\alpha 1$, $\alpha 2$, or $\alpha 5$ ($P < 0.001$). The presence of an $\alpha 4$ subunit conferred a unique pharmacological profile. The partial agonist bretazenil was the most efficacious benzodiazepine, despite lower $\alpha 4$ affinity, and FG8205 displayed similar efficacy. Most striking were the lack of affinity/efficacy for classical benzodiazepines and the relatively high efficacy of Ro15-1788 ($53 \pm 12\%$), CGS8216 ($56 \pm 6\%$), CGS9895 ($65 \pm 6\%$), and the weak partial inverse agonist Ro15-4513 ($87 \pm 5\%$). Each receptor subtype was modulated by pentobarbital, loreclezole, and 5α -pregnan-3 α -ol-20-one, but the type of α subunit influenced the level of potentiation. The maximal pentobarbital response was significantly greater at $\alpha 4\beta 3\gamma 2$ s ($226 \pm 10\%$ increase in the EC₂₀ response to GABA) than any other modulator. The rank order of potentiation for pregnanolone was $\alpha 5 > \alpha 2 > \alpha 3 = \alpha 4 > \alpha 1$, for loreclezole $\alpha 1 = \alpha 2 = \alpha 3 > \alpha 5 > \alpha 4$, and for pentobarbital $\alpha 4 = \alpha 5 = \alpha 2 > \alpha 1 = \alpha 3$.

The inhibitory neurotransmitter GABA plays a major role throughout mammalian central nervous system via activation of GABA_A and GABA_B receptors. GABA_A receptors are multisubunit oligomers with an integral chloride channel belonging to the ligand-gated ion channel receptor family. To date, cloning studies have revealed the gene family encompasses a multitude of different subunits, falling into seven families ($\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , π , and θ) (Whiting et al., 1999). Studies on subunit assembly and stoichiometry suggest the subunits coassemble as pentamers to form a variety of receptor subtypes that are differentially expressed in mammalian brain (Wisden et al., 1992; Fritschy and Mohler, 1995; McKernan and Whiting, 1996). In vitro, an α , a β , and a γ subunit are required to form fully functional GABA_A receptors resembling those in native tissues (Pritchett et al., 1989) where 2:2:1 stoichiometry is often observed (Im et al., 1995; Chang et al., 1996), although (α)₂(β)₁(γ)₂ may also be possible (Sieghart et al., 1999). The presence of different

subunits confers different pharmacological, functional, and modulatory properties on receptor subtypes. The largest population of GABA_A receptors in rat brain has subunit composition of $\alpha 1\beta 2\gamma 2$, whereas $\alpha 2\beta 3\gamma 2$ and $\alpha 3\beta 2\gamma 3$ together constitute the next most prevalent subtypes (McKernan and Whiting, 1996). There is also a variety of other combinations that form more minor receptor populations (Sieghart et al., 1999), although the precise physiological roles of many of these are not fully defined.

GABA_A ion channel function can be modulated via a variety of allosteric sites (Sieghart, 1992) that are the target for a number of therapeutic agents, such as benzodiazepines, barbiturates, steroids, convulsants, and anesthetics (for review, see Sieghart, 1995; Rabow et al., 1995). Affinity and efficacy at the benzodiazepine site, one of the better characterized, are influenced by α and γ (McKernan et al., 1995; Buhr et al., 1996; Wingrove et al., 1997) but not β subunits (Hadingham et al., 1993). The benzodiazepine site is the

ABBREVIATIONS: GABA, γ -aminobutyric acid; DMEM, Dulbecco's modified Eagle's medium; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

target for commonly prescribed anxiolytic drugs, occurring at the interface of an $\alpha(1-6)$ and a $\gamma(1-3)$ subunit, with residues in both influencing modulation (Smith and Olsen, 1995; Wingrove et al., 1997). The presence of $\gamma 2$ confers a classical benzodiazepine pharmacology to GABA_A receptors (Pritchett et al., 1989), whereas receptors containing the less abundant $\gamma 1$ subunit (Benke et al., 1996) display atypical benzodiazepine pharmacology, including a much lower affinity for Ro15-1788 (Wafford et al., 1993). Introduction of a $\gamma 3$ subunit affects the affinity and efficacy of some but not all benzodiazepine ligands (Hadingham et al., 1995). Of the α subunits, the presence of $\alpha 4$ or $\alpha 6$ results in low affinity for classic benzodiazepines such as diazepam but high affinity for the imidazobenzodiazepine Ro15-4513 (Knoflach et al., 1996; Wafford et al., 1996).

Cloning and stable expression of selected GABA_A subunits permits detailed analysis of pharmacological profiles associated with specific receptor subtypes and this, together with the knowledge of which combinations predominate in vivo, their localization, and relative abundance (McKernan and Whiting, 1996), may help to dissect the physiological roles of these inhibitory receptors. We have examined the influence of subunit composition on activity at human recombinant GABA_A receptors stably expressed in mammalian cells, with particular emphasis on compounds that modulate ion channel function. The methodology involves measuring ³⁶Cl influx through the GABA_A receptor ion channel pore and, in 96-well format, offers the potential for multiple manipulations simultaneously within the same experiment without the need for washout and recovery associated with electrophysiological experiments where a single cell or oocyte is patch-clamped. Many previous studies used primary cultures, synaptoneurosome, or microsac preparations from native tissues to examine GABA-activated transmembrane ion flux (Kardos, 1993) but the precise array of subunits is generally unknown and the measurable response may arise from a mixed receptor population. The other primary techniques used to examine GABA_A receptor function are electrophysiological, with much of the evaluation done in transient expression systems such as *Xenopus laevis* oocytes (Rabow et al., 1995). Comparisons of GABA_A receptor function in stably transfected Ltk⁻ cells and transiently transfected oocytes reveal some differences, primarily with regard to the actions of ethanol (Harris et al., 1997). Stable expression of recombinant receptors, however, offers the advantage of a homogeneous clonal population of cells where expression can be carefully monitored, reducing the likelihood of misassembly and overcoming some of the variability seen with transient expression such as multiple transient assembly (Ebert et al., 1996), poor efficiency of transfection, and change of receptor expression with time. In this study, the pharmacological consequences of stably expressing different GABA_A receptor α subunits in combination with $\beta(3)$ and $\gamma(2s)$ in mouse Ltk⁻ cells are examined.

Materials and Methods

Tissue Culture and Cell Preparation. Human GABA_A receptor subunits were stably expressed in mouse Ltk⁻ fibroblast cells by transfection of the appropriate subunit cDNAs in vector pMSGneo using standard calcium phosphate transfection techniques (Hadingham et al., 1992). Cells were maintained in stock trays in Dulbecco's

modified Eagle's medium (DMEM) with 580 mg/l glutamine (Life Technologies Europe, Paisley, Scotland, UK), supplemented with 10% (v/v) FetalClone II serum (Hyclone, Logan, UT) and 1 mg/ml geneticin. Stocks were split 1:8 into fresh trays once a week. For seeding, cells (passage 5–25) were removed from stock plates with 0.05% trypsin/0.53 mM EDTA solution (Life Technologies, Gaithersburg, MD) and resuspended in DMEM supplemented with 10% FetalClone II serum but without geneticin. Cells with subunit composition $\alpha\beta 3\gamma 2$ were seeded into Costar 96-well plates at densities of between 3 and 8 × 10⁴ cells/ml in a volume of 200 μ l/well and grown in the presence of 10% serum for 5 to 8 days in an incubator at 37°C. Receptor expression, which is under the control of a dexamethasone-sensitive promoter, was induced 24 h before experiment in the confluent cell monolayers using serum-containing DMEM supplemented with 1 μ M dexamethasone (Sigma Chemical, Poole, Dorset, UK).

Early Optimization of Flux Conditions. The assay was developed initially in cells grown on 25-mm coverslips contained in six-well plates. Coverslips were immersed in HEPES/Krebs' buffer containing ³⁶Cl and appropriate drugs for 5 s, and then washed in ice-cold stop buffer (HEPES/Krebs' buffer containing 100 μ M picrotoxin, pH 7.4 at 4°C using 1 M Tris) and influx determined by digestion in NaOH and scintillation counting. Although agonist responses and benzodiazepine modulation could be measured, the signal was small and the methodology time-consuming. Assay conditions were optimized, including examining the effects of assay temperature, extracellular chloride concentration, optimizing agonist exposure times, and using chloride transport inhibitors. The assay was then transferred into 96-well format to improve throughput. Cells were initially seeded at densities of 2 × 10⁴ cells/ml in a volume of 200 μ l/well with 7 days of induction. Following cell washing, ligand, GABA, and test compounds were added simultaneously for 10 s to each well in a 50- μ l volume ($n = 6$ wells/treatment), followed by aspiration and washing with stop buffer. Effects of inducing receptor expression with dexamethasone for different periods were investigated. A progressive improvement in the signal was observed as days of induction were reduced from 7 days to 1 day, with benzodiazepine modulation by flunitrazepam being optimal at 1 day. The current protocol was therefore adopted in which cells are seeded and allowed to grow for 5 to 8 days before being induced 1 day before use. The assay procedure was also amended at this stage to include a 30-s preincubation step with test compounds to allow equilibration before addition of GABA and ³⁶Cl for a further 7 s.

Measurement of ³⁶Cl Influx. Following the 24-h induction period with dexamethasone, 96-well plates were removed from the incubator. Cells were initially washed at room temperature with buffer of the following ionic composition 162 mM NaCl, 6.4 mM KCl, 1.7 mM MgCl₂, 1.2 mM CaCl₂, 1.2 mM NaH₂PO₄, 1.2 mM HEPES, pH 7.4, using a Dynatech Ultrawash Plus 96-well plate washer. Induction medium was aspirated and, after a prewash, cells were washed twice for 2-min periods, aspirating after the final wash. Measurement of ³⁶Cl influx was performed in assay buffer where chloride ions were absent to favor uptake of radioactive chloride. Chloride salts were replaced by acetate salts and the chloride-free assay buffer, pH 7.4, at 4°C using 1 M Tris) also contained 11 mM glucose and the chloride transport inhibitors DIDS (10 μ M) and furosemide (100 μ M). Modulatory compounds or assay buffer was added to the wells in a volume of 40 μ l for a 30-s preincubation using a Robbins Hydra 96 dispenser. At $t = 30$ s a subsequent 40- μ l addition of a 2 × (³⁶Cl ligand solution ± GABA in chloride-free assay buffer) was then made to each well using a Sagian Beckman Multipette 96-well dispenser, incubating for a further 7 s. At $t = 37$ s ³⁶Cl influx was terminated by aspiration of incubation buffer and immediate washing with 5 × 200 μ l of ice-cold picrotoxin stop buffer using a second Dynatech plate washer. Stock ³⁶Cl ligand was supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK) as NaCl in aqueous solution (122 mg Cl/ml) at a concentration of 1 mCi/ml. The final concentration of radioligand in the assay was 4.44 μ Ci/ml.

Scintillation fluid (200 μ l) was finally added to each well, the plate sealed, and influx of 36 Cl was determined by scintillation counting on a Packard TopCount.

Data Handling and Analysis. 36 Cl influx was measured on each plate in replicates of six wells per treatment. Each 96-well plate included the following controls: six wells for basal or background influx in the absence of GABA mimetics (chloride-free buffer plus 36 Cl only); six wells treated with a maximal concentration of GABA (buffer plus 36 Cl solution containing GABA, 100 μ M final concentration); and for modulation of GABA-stimulated flux, six wells treated with 36 Cl plus an EC_{20} concentration of GABA. For benzodiazepine modulation, each plate also contained a full benzodiazepine agonist in the presence of an EC_{20} concentration of GABA as an internal standard, relative to which everything else was expressed, to control

for any minor variations in cell responsiveness from day to day. For $\alpha 1$, 2, 3, and 5, this was the nonselective benzodiazepine agonist chlordiazepoxide (30 μ M). Modulation at other sites on the $GABA_A$ receptor is shown as the amount of potentiation of the EC_{20} .

Agonist stimulation and modulatory responses were assessed by performing one-way analysis of variance using Prism version 2.0b (GraphPad Software, San Diego, CA) followed by post hoc testing using the Bonferroni test to determine significance. Concentration-response curves were fitted by nonlinear least-squares regression analysis using RS1 (BBN Research Systems, Cambridge, MA). Mean EC_{50} , IC_{50} , and efficacy values are presented as the arithmetic mean and standard error of the mean from a number of independent determinations. Data from experiments to determine the relative efficacy of modulatory agents were calculated using ActivityBase,

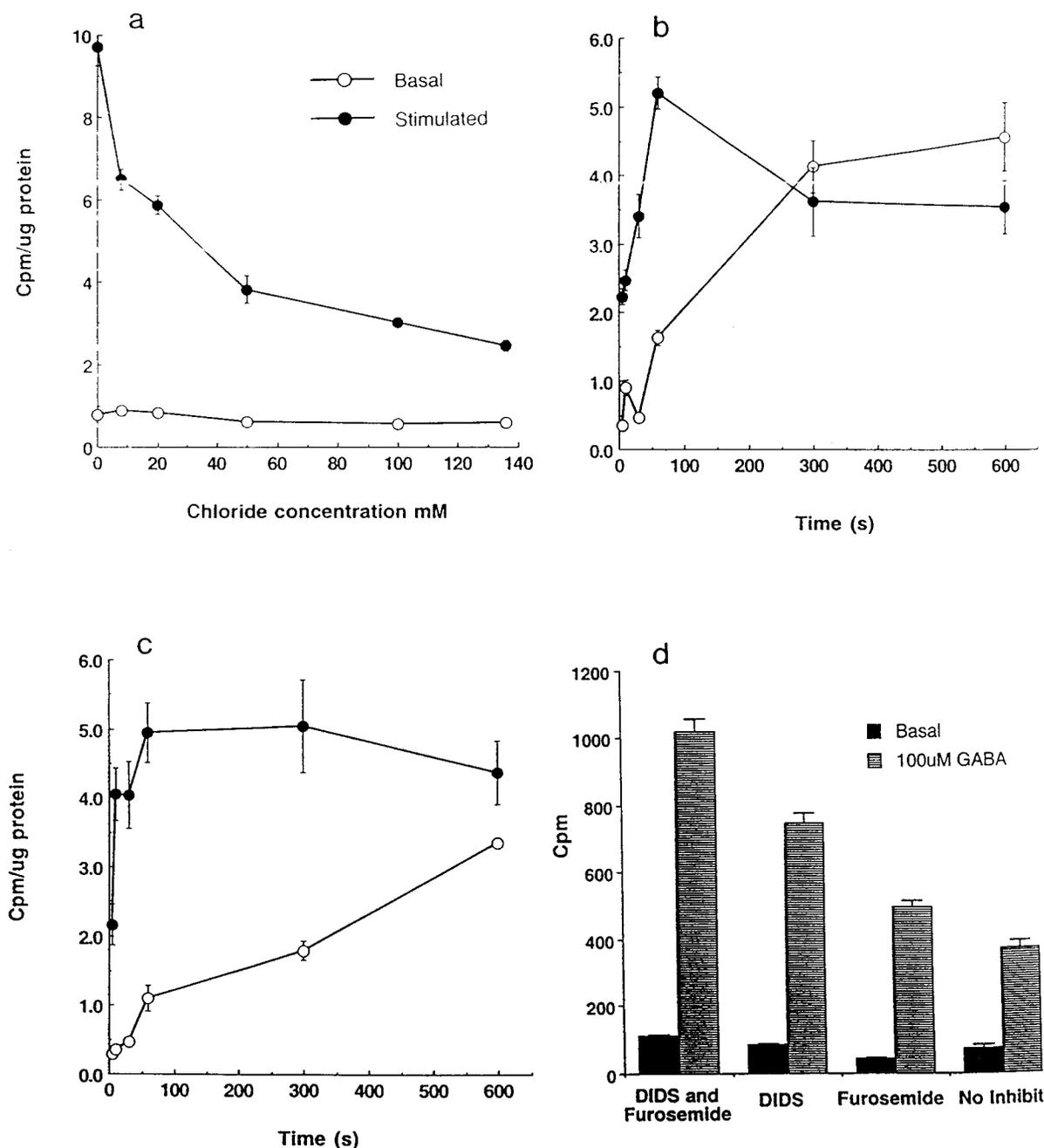


Fig. 1. Optimization of flux assay using $\alpha 1\beta 3\gamma 2$ cells. a–c, performed in coverslip format with stimulation by 10 μ M muscimol. a, effect of varying $[Cl^-]_{ext}$. b, time course at 4°C in the presence of 8 mM $[Cl^-]_{ext}$. c, effect of DIDS and furosemide on 4°C time course. d, effects of DIDS and furosemide on signal in final 96-well format [3×10^4 cells/ml, induced 1 day, $[Cl^-]_{ext} = 0$, 4°C, 30-s preincubation, 10-s (36 Cl \pm GABA) addition].

version 3.2.0 (ID Business Solutions Ltd., Guildford, Surrey, UK) and Microsoft Excel, version 5.0 (Microsoft, Redmond, WA). Benzodiazepine binding affinities quoted represent displacement of specific binding of [³H]Ro15-1788 for receptors containing α 1, 2, 3, and 5 subunits and displacement of [³H]Ro15-4513 binding for α 4 β 3 γ 2 receptors.

Whole-Cell Patch-Clamp of Ltk⁻ Cells. Responses to GABA were also determined electrophysiologically in Ltk⁻ cells stably expressing α 1-5 β 3 γ 2 GABA_A receptors after 24-h induction with dexamethasone. Glass coverslips containing a cell monolayer were placed in a chamber on the stage of a Nikon Diaphot inverted microscope and perfused continuously with artificial cerebral spinal fluid containing 149 mM NaCl, 3.25 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 11 mM D-glucose, D-(+)-sucrose, pH 7.4, and observed with phase-contrast optics. Fire-polished patch pipettes were pulled using conventional 120TF-10 electrode glass and tip diameter was approximately 1.5 to 2.5 μ m, with resistances around 4 M Ω . The intracellular solution contained 130 mM CsCl, 10 mM HEPES, 10 mM BAPTA-Cs, 5 mM ATP.Mg, 0.1 mM leupeptin, 1 mM MgCl₂, 100 μ M NaVO₃, pH adjusted to 7.3 with CsOH and 320 to 340 mOsm. Cells were voltage-clamped at -60 mV via an Axon 200B amplifier (Axon Instruments., Foster City, CA) and drugs applied via a multibarrel drug delivery system, which could pivot the barrels into place using a stepping motor, ensuring rapid application and washout of drug. GABA was applied to the cell for 5 s with a 30-s washout period between applications. Non-cumulative concentration-response curves to GABA were constructed and curves fitted using a nonlinear least-squares-fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^{n_H}]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n_H is the Hill coefficient.

Results

Assay Optimization. In initial experiments on α 1 β 3 γ 2 cells grown on 25-mm coverslips, progressively decreasing the extracellular chloride ion concentration by substituting acetate salts resulted in a progressive increase in the magnitude of stimulation with 10 μ M muscimol, whereas agonist-independent basal uptake remained constant (Fig. 1a). Time course experiments were performed in α 1 cells under different conditions to select an optimal assay duration. At room temperature in the presence of 8 mM extracellular chloride, a specific signal was obtained at early time points with 10 s being optimal, followed by a progressive decline with no specific signal remaining in excess of 60 s. The temperature was reduced to 4°C to minimize nonspecific membrane transport of chloride and this extended the period during which a specific signal could be observed. However, there was still a progressive rise in basal ³⁶Cl accumulation with increasing incubation time, such that no specific signal remained at 5 min (Fig. 1b). Inclusion of the chloride transport inhibitors DIDS (10 μ M) and furosemide (100 μ M) further extended the period of time over which a specific window could be observed, with the magnitude of agonist-stimulated accumulation being maintained at a plateau and basal accumulation increasing at a slower rate (Fig. 1c). In 96-well format, a high enough seeding density was critical for optimal responses and density is amended occasionally according to cell growth and receptor expression, currently between 3 and 8 \times 10⁴ cells/ml. The number of days of induction was important in maximizing observation of benzodiazepine modulation, with 1 day being optimal. Additionally, preincubation with benzodiazepines for 30 s before addition of GABA and ³⁶Cl was important to accurately measure compounds with slow kinetics such as abecarnil. The effect of DIDS and

furosemide on the magnitude of agonist stimulation in α 1 β 3 γ 2 cells, both individually and together, is shown in the final 96-well format (Fig. 1d) with a 30-s preincubation and a 10-s flux incubation.

Increasing the ligand concentration did not increase the overall specific signal, which was proportional to that at lower ligand concentration. Assay times (during which GABA and ³⁶Cl are present) of 10, 15, 30, and 45 s did not result in a significant increase in counts, implying most of the GABA-stimulated chloride influx occurs in the first few seconds and it is likely that desensitization ultimately limits chloride entry into the cell.

For flux assays on receptors containing the α 4 subunit, the presence of furosemide markedly inhibited the response to GABA and the chloride transport inhibitors were therefore excluded for this subtype. Furosemide has been reported to be a subtype-selective antagonist at α 6-containing receptors but also shows inhibitory effects at α 4 β 3 γ 2 receptors with an IC₅₀ value of 162 μ M (Wafford et al., 1996).

³⁶Cl Influx: GABA Agonists and Antagonists. The effect of expressing different α subunits in combination with β 3 γ 2s subunits was examined on the response to GABA_A receptor agonists. GABA (Fig. 2) and muscimol both elicited concentration-dependent increases in ³⁶Cl influx, an effect not observed in noninduced controls. Both agonists elicited similar maximal increases in ³⁶Cl influx, although muscimol exhibited higher potency than GABA at each of the subunit combinations. EC₅₀ values (μ M) for GABA and muscimol are shown in Table 1, together with a comparison with patch-clamp data. The GABA affinities of diazepam-sensitive recombinant receptors showed little discrimination between subtypes, with the exception of α 3 subunit-containing receptors, where both GABA and muscimol were approximately an order of magnitude less potent. Both agonists also displayed slightly lower potency at receptors of subunit composition α 4 β 3 γ 2 in flux.

The magnitude of maximal agonist stimulation varied across subtypes with α 3 β 3 γ 2 receptors exhibiting a significantly larger GABA response than any other subtype ($P < 0.001$; analysis of variance followed by Bonferroni post hoc

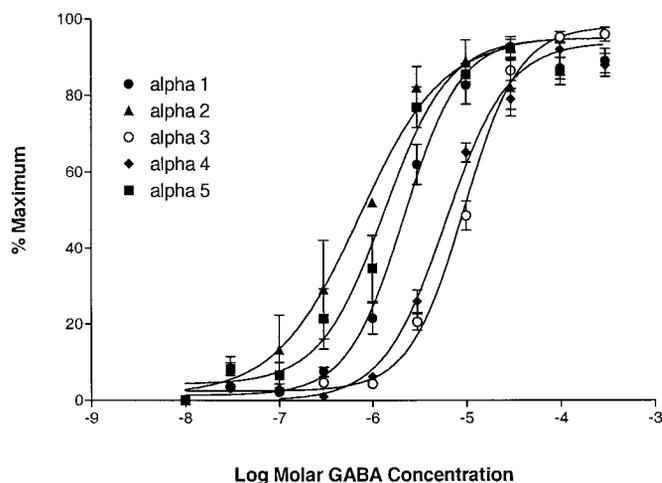


Fig. 2. GABA elicited concentration-dependent increases in ³⁶Cl influx in Ltk⁻ cells stably expressing GABA_A receptors of composition α 1-5 β 3 γ 2s. Each curve represents mean data from at least 12 independent experiments (mean \pm standard error of mean).

test). In contrast, $\alpha 4\beta 3\gamma 2$ receptors displayed the smallest maximal agonist effect, as observed in Fig. 5.

Agonist-induced increases in ^{36}Cl influx were specifically blocked in a concentration-dependent manner by the competitive GABA_A receptor antagonist bicuculline and the chloride channel blocker picrotoxin (Fig. 3). In the case of picrotoxin, there was a marked and concentration-dependent suppression of the maximum response.

^{36}Cl Influx: Modulation at the Benzodiazepine Site.

Next, the role of varying α subunits in mediating different pharmacologies at the benzodiazepine site was examined. With respect to benzodiazepine binding, the nature of the α subunit can exert a marked influence on the observed pharmacology (Table 2) (representative concentration-response curves for selected compounds shown in Fig. 4). From the GABA concentration-response curve at each receptor isoform, a concentration of GABA was selected that produced an increase in ^{36}Cl influx approximately 20% of maximum and the modulatory effects of benzodiazepine site ligands were examined. The net effect of benzodiazepine agonists was to shift the GABA concentration-response curve to the left, whereas compounds exhibiting inverse agonist activity caused a rightwards shift. The nonselective benzodiazepine full agonist chlordiazepoxide was used as a standard, relative to which the effects of other benzodiazepine ligands were expressed. The response to an EC₂₀-equivalent concentration of GABA was significantly potentiated by chlordiazepoxide (30 μM) at $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunit-containing receptors ($P < 0.001$) and typical data are illustrated in Fig. 5. Chlordiazepoxide efficacy was not significantly different between $\alpha 1$, $\alpha 2$, and $\alpha 5$, although the potentiation observed at $\alpha 3$ -containing receptors was significantly larger ($P < 0.001$).

In contrast, $\alpha 4\beta 3\gamma 2$ receptors have very low binding affinity for classical benzodiazepines such as chlordiazepoxide, flunitrazepam, triazolam, and diazepam (Table 2), and chlordiazepoxide elicited no significant effect on ^{36}Cl flux at the highest concentration that could be tested (30 μM). In this case, the benzodiazepine giving a maximal potentiation at $\alpha 4$ (bretazenil, 30 μM) was used instead. The relative efficacies for bretazenil at each receptor subtype, with the amount of potentiation expressed as percentage of increase in the EC₂₀ response, were as follows: $\alpha 1$ 50 ± 4 ($n = 19$), $\alpha 2$ 21 ± 6 ($n = 18$), $\alpha 3$ 70 ± 7 ($n = 24$), $\alpha 5$ 44 ± 4 ($n = 23$), and $\alpha 4$ $56 \pm 6\%$ ($n = 11$).

To enable comparison of the effect of different α subunits

TABLE 1

EC₅₀ values (μM) for GABA- and muscimol-stimulated ^{36}Cl influx are shown for GABA_A receptors of composition $\alpha 1-5\beta 3\gamma 2$ s, stably expressed in Ltk⁻ cells

Electrophysiological responses to GABA in Ltk⁻ cells, measured using a whole-cell patch-clamp technique, are also shown for comparison. Data are presented as arithmetic mean \pm S.E.M. with the number of determinations in parentheses.

Subunit	EC ₅₀		
	^{36}Cl Flux in Ltk ⁻		Patch-Clamp in Ltk ⁻
	Muscimol	GABA	GABA
	μM		
$\alpha 1$	0.54 ± 0.06 (17)	3.9 ± 0.5 (13)	0.86 ± 0.06 (8)
$\alpha 2$	0.27 ± 0.04 (14)	1.6 ± 0.4 (12)	1.1 ± 0.07 (8)
$\alpha 3$	2.0 ± 0.23 (17)	10 ± 0.7 (12)	15 ± 0.7 (8)
$\alpha 4$	2.8 ± 0.5 (9)	6.4 ± 0.6 (17)	2.6 ± 0.05 (8)
$\alpha 5$	0.36 ± 0.03 (16)	1.6 ± 0.3 (17)	2.8 ± 0.3 (8)

on benzodiazepine efficacy and to control for differences in benzodiazepine binding affinity between subtypes, the relative efficacy of each benzodiazepine site ligand is shown in Table 3 at a single saturating concentration, ($1000 \times K_i$). The potentiation caused by benzodiazepine site agonists was blocked by Ro15-1788 (1 μM) at $\alpha 1\beta 3\gamma 2$ and $\alpha 3\beta 3\gamma 2$ receptors.

^{36}Cl Influx: Effect of α Subunit on Modulation at Other Sites. The influence of the α subunit was also examined on potentiation of the GABA response by a variety of allosteric modulators that act at other sites on the GABA_A receptor, including the barbiturate, steroid, and loreclezole sites. Again, an equieffective concentration of GABA (EC₂₀) was used at each receptor subtype to examine the modulatory effects. The amount of potentiation in each case has been expressed as percentage increase in the EC₂₀ GABA response.

A neuroactive steroid, the progesterone metabolite 5α -pregnan-3 α -ol-20-one (10 μM), the nonvolatile anesthetic barbiturate pentobarbital (100 μM), and the anticonvulsant loreclezole (10 μM) potentiated the response to an EC₂₀ concentration of GABA at each of the receptor subtypes examined ($\alpha 1$, 2, 3, 4, and 5). However, there were quantitative differences in both the relative efficacy between the three modulators at any particular receptor isoform and in the degree of efficacy for a particular modulator across different α subunits (Fig. 6).

At $\alpha 1$ subunit-containing receptors loreclezole elicited a significantly greater potentiation of ^{36}Cl influx ($147 \pm 9\%$ increase in EC₂₀) than either pentobarbital ($106 \pm 16\%$) or pregnanolone ($64 \pm 5\%$) ($P < 0.01$ and $P < 0.001$, respectively). Pregnanolone and loreclezole produced a similar degree of potentiation at $\alpha 2\beta 3\gamma 2$ ($P > 0.05$), whereas at $\alpha 3\beta 3\gamma 2$ receptors the potentiation with loreclezole ($148 \pm 5\%$) slightly exceeded that with pregnanolone ($108 \pm 11\%$). At $\alpha 5$ subunit-containing receptors, pregnanolone and pentobarbital were equally efficacious (213 ± 5 and $216 \pm 7\%$, respectively), whereas loreclezole exhibited significantly lower efficacy ($117 \pm 7\%$, $P < 0.001$). Receptors of subunit composition $\alpha 4\beta 3\gamma 2$ were characterized by a pentobarbital response that was significantly larger ($P < 0.001$) than any other modulator ($226 \pm 10\%$). Pregnanolone was the second most efficacious modulator at $\alpha 4\beta 3\gamma 2$ receptors ($99 \pm 14\%$), whereas loreclezole only exhibited a modest potentiation ($39 \pm 4\%$). For reference, the potentiation observed with the most efficacious benzodiazepine at $\alpha 4\beta 3\gamma 2$, bretazenil, was $56 \pm 6\%$ increase in EC₂₀.

The rank order of efficacy for pregnanolone across receptors expressing different α subunits was $\alpha 5 > \alpha 2 > \alpha 3 = \alpha 4 > \alpha 1$. For loreclezole the rank order of potentiation was $\alpha 1 = \alpha 2 = \alpha 3 > \alpha 5 > \alpha 4$ and for pentobarbital $\alpha 4 = \alpha 5 = \alpha 2 > \alpha 1 = \alpha 3$. Although 100 μM pentobarbital is likely to produce maximal potentiation of the receptor subtypes examined here (Thompson et al., 1996), it is likely that there will also be some direct activation at this concentration. Consequently, a lower concentration of 10 μM pentobarbital was also examined and produced the following potentiations (% increase in EC₂₀ GABA response, $n = 12$): $\alpha 1\beta 3\gamma 2$, 26 ± 2 ; $\alpha 2\beta 3\gamma 2$, 33 ± 3 ; $\alpha 3\beta 3\gamma 2$, 28 ± 2 ; $\alpha 4\beta 3\gamma 2$, 46 ± 3 ; and $\alpha 5\beta 3\gamma 2$, $30 \pm 2\%$.

Discussion

A functional ion flux assay using ^{36}Cl has been established in 96-well plate format to measure efficacy at human recombinant GABA_A receptors and examine the influence that the α subunit exerts on receptor pharmacology. The pharmacology of flux responses was consistent with data described previously using electrophysiological techniques. Muscimol and GABA stimulated ^{36}Cl influx in a concentration-dependent manner and were specifically blocked by picrotoxin and bicuculline. The magnitude of maximal agonist stimulation varied across subtypes, with $\alpha 3\beta 3\gamma 2$ receptors having the largest GABA response, possibly reflecting different levels of receptor expression. Picrotoxin caused a rightward shift of the curve and significant, concentration-dependent depression of the maximum, as previously reported. In contrast, bicuculline, a competitive antagonist that should not depress

the maximum, although it has some potential to act as an allosteric inhibitor of channel opening at the GABA site (Ueno et al., 1997), did produce some depression in the current study in addition to a rightward shift. However, there was no significant difference between 30 and 100 μM , in contrast to picrotoxin. The reasons for this depression are unclear, although a slow dissociation rate or incomplete equilibrium with agonist may make an antagonist seem pseudo-irreversible.

In time course experiments during assay development, stimulation with muscimol peaked and then declined to a plateau at longer assay times, possibly due to removal of accumulated cellular chloride or receptor desensitization (Fig. 1b). In the presence of chloride pump inhibitors furosemide and DIDS, this decline was attenuated (Fig. 1c) and agonist-induced accumulation of chloride was enhanced (Fig.

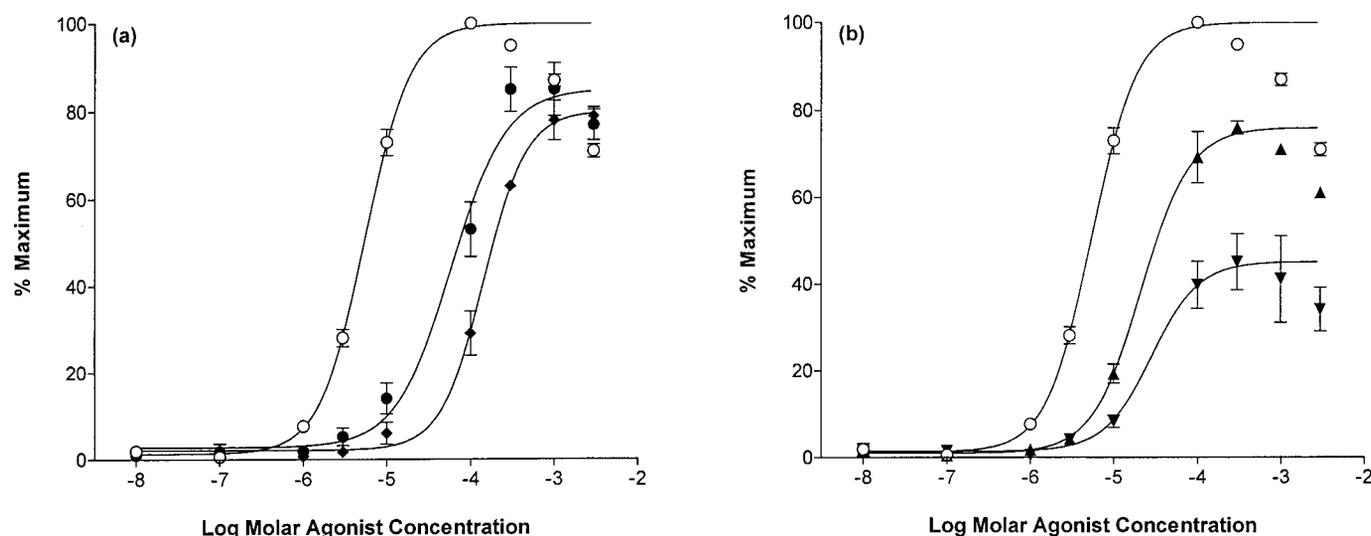


Fig. 3. Agonist-stimulated ^{36}Cl influx was blocked in a concentration-dependent manner by the competitive GABA_A antagonist bicuculline (a) and the chloride channel blocker picrotoxin (b). Mean effects of 30 and 100 μM bicuculline and picrotoxin are shown on the response to GABA in $\alpha 3\beta 3\gamma 2$ -containing cells (mean of $n = 5$ independent experiments). a, \circ , GABA alone; \bullet , +30 μM bicuculline; \blacklozenge , +100 μM bicuculline. b, \circ , GABA alone; \blacktriangle , +30 μM picrotoxin; \blacktriangledown , +100 μM picrotoxin.

TABLE 2

Binding affinity (nanomolar) of benzodiazepine site ligands at $\alpha 1-5\beta 3\gamma 2\text{s}$ GABA_A receptors

Results are mean \pm S.E.M. ($n = 3-6$) (or mean % inhibition of binding at 10 μM). Data for $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ represent displacement of specific [^3H]Ro15-1788 binding, whereas $\alpha 4$ data represent displacement of specific [^3H]Ro15-4513 binding, measured for 1 hr at 4°C.

Compound ^a	K_i				
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$
	<i>nM</i>				
β -CCM	2.4 \pm 0.3	7.4 \pm 1.4	72 \pm 14	1040 \pm 90	44 \pm 6
CL218,872	66 \pm 0.3	720 \pm 110	850 \pm 120	(7.2%)	460 \pm 13
Zopiclone	28 \pm 3	64 \pm 5	29 \pm 2	(2.7%)	46 \pm 8
Bretazenil	0.22 \pm 0.02	0.37 \pm 0.07	0.43 \pm 0.09	43 \pm 2	0.86 \pm 0.22
Chlordiazepoxide	890 \pm 250	460 \pm 99	980 \pm 110	(3.1%)	620 \pm 200
Diazepam	12 \pm 1	7 \pm 0.5	33 \pm 3	(8.4%)	11 \pm 1
Ro15-1788	1.1 \pm 0.04	1.5 \pm 0.1	1.0 \pm 0.1	240 \pm 27	0.50 \pm 0.08
Ro15-4513	4.8 \pm 0.1	7.3 \pm 1.2	2.4 \pm 0.4	5.1 \pm 0.6	0.13 \pm 0.01
FG8205	0.9 \pm 0.2	2.4 \pm 0.2	1.8 \pm 0.3	440 \pm 55	5.1 \pm 1.8
Abecarnil	1.3 \pm 0.2	2.4 \pm 0.3	4.4 \pm 0.2	1190 \pm 22	5.0 \pm 0.3
Zolpidem	23 \pm 5	110 \pm 23	320 \pm 4	(7.1%)	(19.3%)
Triazolam	0.41 \pm 0.01	0.32 \pm 0.04	1.5 \pm 0.1	(9.5%)	0.42 \pm 0.05
Flunitrazepam	3.9 \pm 0.5	1.1 \pm 0.3	5.9 \pm 0.7	(1.8%)	1.7 \pm 0.5
CGS9895	0.32 \pm 0.05	1.1 \pm 0.2	0.28 \pm 0.05	103 \pm 8	0.96 \pm 0.15
CGS8216	0.17 \pm 0.01	0.49 \pm 0.09	0.30 \pm 0.06	35 \pm 11	1.3 \pm 0.2
DMCM	10 \pm 0.7	13 \pm 3	7.5 \pm 0.7	30 \pm 8	2.2 \pm 0.6

^a Certain of these data have already been published (Cox et al., 1998).

1d), which may imply that the chloride pumps in these cells act to remove accumulated chloride ions. Theoretically, agonist-independent basal accumulation is likely to be the net result of a variety of different processes, including passive diffusion, chloride pump activity, or entry via any spontane-

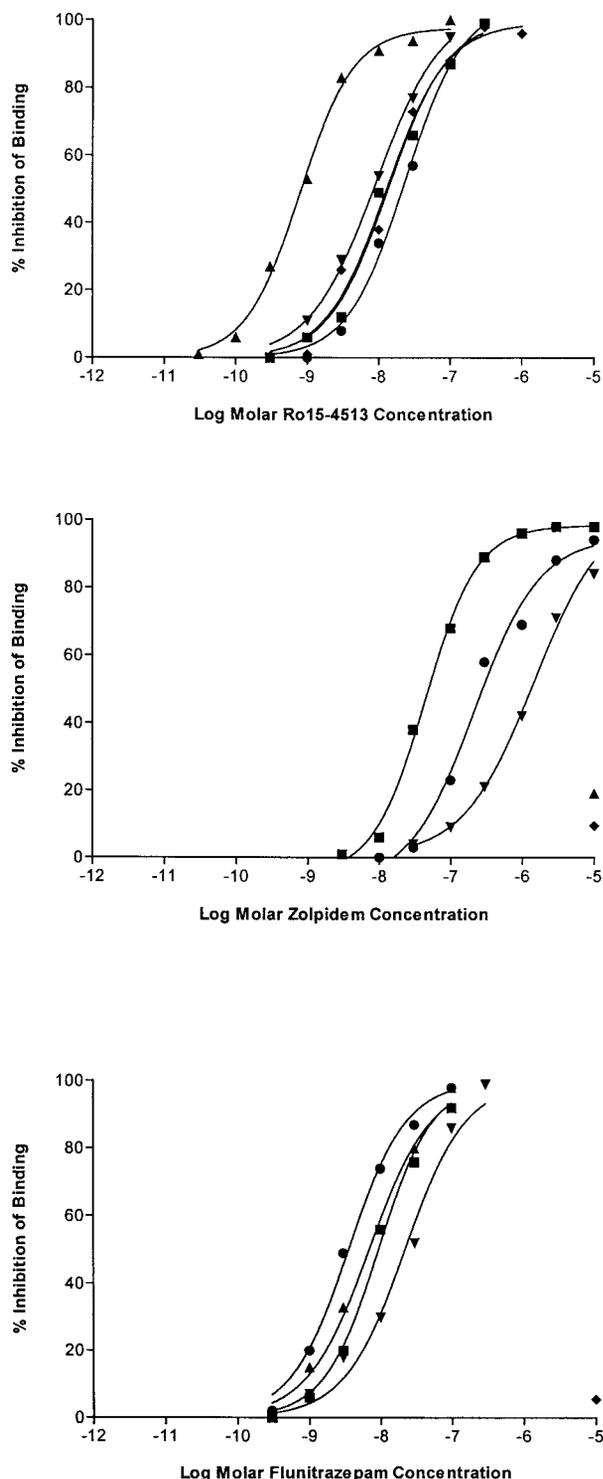


Fig. 4. Representative concentration-response curves showing inhibition of binding to the benzodiazepine site for selected benzodiazepine site ligands at $\alpha 1$ - $5\beta 3\gamma 2s$ GABA_A receptors. The radioligand for $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ is [³H]Ro15-1788 and for $\alpha 4$ subunit-containing receptors, [³H]Ro15-4513. a, Ro15-4513; b, zolpidem; and c, flunitrazepam. ■, $\alpha 1$; ●, $\alpha 2$; ▼, $\alpha 3$; ◆, $\alpha 4$; ▲, $\alpha 5$.

ous opening of the GABA_A receptor ion channel. The importance of the 30-s modulator preincubation highlights kinetic considerations. Optimal flux responses are a fine balance between on-rate, which controls initiation, and possible desensitization, which controls termination. GABA_A receptors desensitize rapidly with agonist exposure and clearly an important consideration with the flux assay is to what extent receptors are in a desensitized state during ion flux measurements. However, when GABA EC₅₀ values were determined electrophysiologically in the same Ltk⁻ cells by patch-clamp measurement (Table 1), flux data seemed to be in reasonably good agreement. Electrophysiology is the most direct and widely used method of measuring receptor function and the temporal resolution clearly offers more refinement in terms of examining rate of onset and measuring peak responses. Although one cannot determine the true peak of flux by backward extrapolation and the overall flux measurement is potentially more sensitive to desensitization measuring total net ³⁶Cl accumulation over 7 s (i.e., area under the curve rather than peak), the similarity between EC₅₀ values determined by the two methods suggests that desensitization is not a major limiting issue with the current protocol. The current methodology offers a noninvasive means of quantitatively examining large homogeneous populations of GABA_A ion channels and constitutes a slightly different measure to electrophysiology, measuring an integrated response over time in the whole cell.

Evidence suggests desensitization and current activation/deactivation kinetics, which contribute to the time course of native GABA_A receptor-mediated inhibitory postsynaptic currents, and synaptic function vary with GABA_A receptor subunit composition (Tia et al., 1996). The α subunit isoform is at least partially responsible for determining the gating kinetics of recombinant GABA_A receptors (Lavoie et al., 1997). Clearly, if comparisons of efficacy are to be made between different receptor isoforms, it is important to compare the actions of modulators at the same effective GABA concentrations at each receptor subtype. This could also apply to electrophysiology. Accordingly, GABA concentration-response curves were constructed regularly to determine accurate EC₂₀ values before modulation studies. Flux efficacies generally compare well to those reported in a variety of electrophysiological assays and, as additional evidence to support this, a correlation is shown between efficacies determined using electrophysiology and flux for $\alpha 1\beta 3\gamma 2s$ receptors (Fig. 7). The fact that there is a good correlation between data from two different expression systems and methodologies (*X. laevis* oocytes and Ltk⁻ cells, respectively) lends further weight to the validity of the flux assay.

Benzodiazepine ligands displayed a wide range of intrinsic efficacies at different receptor subtypes. Broadly, the 1,4-benzodiazepines and β -carbolines, methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate, abecarnil, and 3-carbomethoxy- β -carboline were relatively consistent in their action at $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -, and $\alpha 5$ -containing receptors, being either agonist or inverse agonist at all. The exception was Ro15-1788, which was a pure antagonist at $\alpha 5$ but exhibited a small degree of partial agonist activity at $\alpha 3$. Compounds of different structural types (CL218,872, zopiclone, zolpidem, CGS9895, and CGS8216) exhibited the most subtype selectivity, mainly with regard to $\alpha 5$ -containing receptors. Zolpidem, a full agonist at $\alpha 1$, $\alpha 2$ and $\alpha 3$, was without activity at

$\alpha 5$, although it should be noted that the $\alpha 5$ affinity is very low. The $\alpha 1$ binding-selective compound CL218,872 was an $\alpha 5$ antagonist, whereas at $\alpha 1$, $\alpha 2$, and $\alpha 3$ receptors it was a partial agonist. CGS9895 also had different efficacy at $\alpha 5\beta 3\gamma 2$ receptors, being a partial inverse agonist. However, $\alpha 4\beta 3\gamma 2$ receptors were the most distinct pharmacologically.

The imidazobenzodiazepine Ro15-4513 has the highest affinity at $\alpha 4\beta 3\gamma 2$ receptors but many other ligands have much lower $\alpha 4$ affinity and the concentrations tested in flux were increased accordingly, although the highest concentration that could be tested (30 μM) is unlikely to have been maximal for chlordiazepoxide, flunitrazepam, and zolpidem. The com-

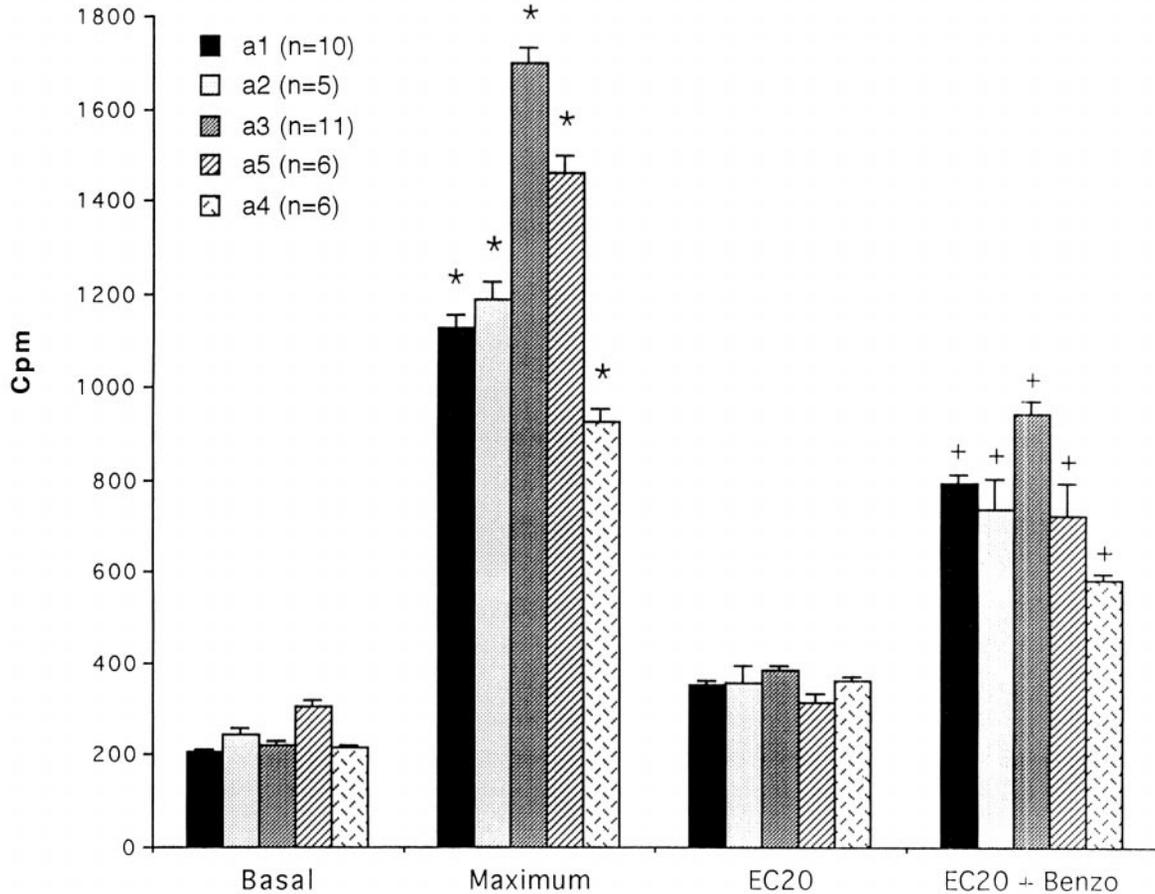


Fig. 5. Typical flux responses for maximal agonist and benzodiazepine modulation in cells expressing $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 5$ subunits with $\beta 3\gamma 2$ s. Maximum agonist effects were defined with 100 μM GABA (* $P < 0.001$ with respect to basal); maximal potentiation of the GABA EC₂₀ response by benzodiazepine was with 30 μM chlordiazepoxide for $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ and 30 μM bretazenil for $\alpha 4$ -containing receptors (+ $P < 0.001$ with respect to EC₂₀). Seeding densities: $\alpha 1$, $8 \times 10^4 \text{ ml}^{-1}$; $\alpha 2$, $5 \times 10^4 \text{ ml}^{-1}$; $\alpha 3$, $6 \times 10^4 \text{ ml}^{-1}$; $\alpha 5$, $6 \times 10^4 \text{ ml}^{-1}$; and $\alpha 4$, $6 \times 10^4 \text{ ml}^{-1}$.

TABLE 3

Allosteric modulation of GABA-stimulated flux by benzodiazepine ligands in cells expressing different α subunits with $\beta 3\gamma 2$ s, expressed as percentage of maximum benzodiazepine potentiation (mean \pm S.E.M.) from at least eight independent experiments
Chlordiazepoxide is 100% by definition for $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ and bretazenil 100% at $\alpha 4$.

Compound	% Maximum				
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$
β -CCM	-16 \pm 3	-22 \pm 5	-19 \pm 2	-33 \pm 8	-27 \pm 2
CL218,872	59 \pm 3	33 \pm 4	21 \pm 3	N.D.	1 \pm 4
Zopiclone	82 \pm 4	88 \pm 7	98 \pm 4	N.D.	80 \pm 4
Bretazenil	32 \pm 2	20 \pm 5	33 \pm 3		44 \pm 3
Chlordiazepoxide				4 \pm 9	
Diazepam	71 \pm 2	81 \pm 7	88 \pm 3	N.D.	57 \pm 3
Ro15-1788	6 \pm 3	10 \pm 3	19 \pm 3	53 \pm 12	-2 \pm 2
Ro15-4513	-9 \pm 3	-6 \pm 5	2 \pm 2	87 \pm 5	-4 \pm 5
FG8205	46 \pm 3	41 \pm 3	57 \pm 4	106 \pm 8	37 \pm 5
Abecarnil	101 \pm 6	57 \pm 6	71 \pm 4	15 \pm 7	43 \pm 5
Zolpidem	116 \pm 6	103 \pm 8	105 \pm 6	-22 \pm 6	-16 \pm 5
Triazolam	119 \pm 8	95 \pm 5	112 \pm 5	N.D.	78 \pm 5
Flunitrazepam	91 \pm 1	95 \pm 9	110 \pm 5	15 \pm 11	101 \pm 8
CGS9895	12 \pm 3	26 \pm 6	19 \pm 1	65 \pm 6	-13 \pm 5
CGS8216	-18 \pm 2	-9 \pm 3	-3 \pm 2	56 \pm 6	-26 \pm 5
DMCM	-24 \pm 5	-28 \pm 4	-26 \pm 1	-15 \pm 5	-28 \pm 5

N.D., not determined.

pound with the highest $\alpha 4$ efficacy was FG8205, which was equivalent to bretazenil. The partial inverse agonist Ro15-4513 and the antagonist/partial agonist Ro15-1788 both exhibited high $\alpha 4$ efficacy (87 ± 5 and $53 \pm 12\%$ of bretazenil, respectively). Other compounds of note were CGS9895 and the partial inverse agonist CGS8216, which both caused significant $\alpha 4$ potentiation.

All subtypes were sensitive to potentiation by the anticonvulsant loreclezole, in keeping with the presence of a $\beta 2$ or $\beta 3$ subunit (Wafford et al., 1994). Although sensitivity to loreclezole does not require an α or γ subunit (both $\alpha 1\beta 2$ and $\beta 2\gamma 2$ receptors were sensitive), their presence may influence the level of potentiation. When different α subunits were expressed in oocytes with $\beta 2\gamma 2$, the rank order of efficacy was $\alpha 1 = \alpha 2 = \alpha 3$, $\alpha 5$ was slightly less efficacious, $\alpha 6$ produced significantly less potentiation, and $\alpha 4$ was not determined. This is the same as the rank order observed here with $\beta 3$ subunit-containing receptors in Ltk⁻ cells ($\alpha 1 = \alpha 2 = \alpha 3 > \alpha 5 > \alpha 4$). Whittemore et al. (1996), describing the pharmacology of the human $\alpha 4$ subunit, also found greater potentiation with loreclezole at $\alpha 1\beta 2\gamma 2L$ receptors than $\alpha 4\beta 2\gamma 2L$.

In contrast to the benzodiazepine site, barbiturate and neurosteroid modulatory sites of GABA_A receptors are less well defined. In particular, studies concerning which subunits influence steroid sensitivity have yielded contradictory results, particularly with regard to the α subunit, depending on the steroid examined and the experimental conditions (Lambert et al., 1995). Although the type of β subunit seems to have little impact, direct and modulatory effects of steroids are preserved even at homomeric $\beta 1$ receptors. In a recent

report (Maitra and Reynolds, 1999), changing or removing the α subunit altered the efficacy of pregnanolone to potentiate GABA ($\alpha 2\beta 1\gamma 2L > \alpha 1\beta 1\gamma 2L \gg \beta 1\gamma 2L$), in agreement with the current findings, and efficacy was also influenced by the γ subunit. In the case of the barbiturate site, the EC₅₀ value for pentobarbitone was not dependent on receptor subtype but maximum potentiation differed with the type of α and β subunits present (Thompson et al., 1996). Progress is currently being made in defining residues which influence anesthetic action using site-directed mutagenesis (Carlson et al., 2000; Cestari et al., 2000). The larger potentiation with pentobarbital at $\alpha 4$ compared with $\alpha 1$, observed here, has been reported for $\alpha 4\beta 2\gamma 2L$ (Whittemore et al., 1996) and $\alpha 4\beta 1\gamma 2s$ receptors (Wafford et al., 1996) expressed in oocytes. In the former study, the authors also examined pregnanolone and loreclezole. Pregnanolone produced a smaller potentiation of $\alpha 4$ than pentobarbital but had higher efficacy than loreclezole, as observed here. The efficacy of pregnanolone was greater at $\alpha 4\beta 2\gamma 2L$ than at $\alpha 1\beta 2\gamma 2L$ receptors, in agreement with the present observations, although in the current study the efficacy for pregnanolone at $\alpha 2$ and $\alpha 5$ exceeded that at $\alpha 4$.

Potentiation of the EC₂₀ response by pentobarbital was reported to be of the same magnitude for $\alpha 4\beta 1\gamma 2s$ and $\alpha 6\beta 1\gamma 2s$ expressed in oocytes (Wafford et al., 1996) and, in the same system, potentiation of $\alpha 6$ by pentobarbital was significantly greater than that for $\alpha 1$, 2, 3, or 5 (Thompson et al., 1996). Therefore, from oocyte work, it would be predicted that the magnitude of maximal pentobarbital potentiation at $\alpha 4$ should greatly exceed $\alpha 1$, 2, 3, or 5. Interestingly, in a

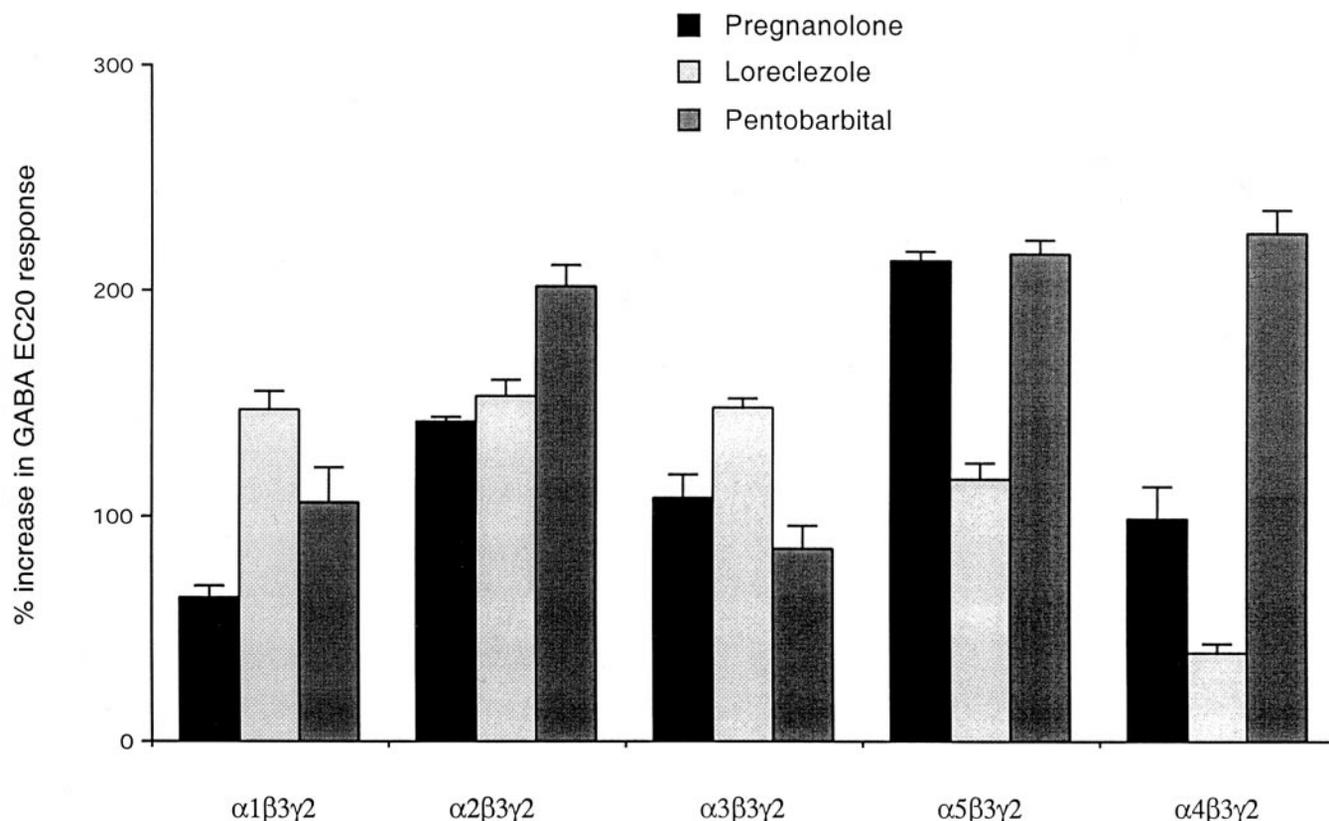


Fig. 6. Effect of expressing different α subunits with $\beta 3\gamma 2s$ on modulation of GABA-stimulated flux at the steroid, barbiturate, and loreclezole sites on the GABA_A receptor. Results are expressed as percentage of increase in the response to GABA at the EC₂₀ (mean \pm S.E.M.) from at least seven independent experiments.

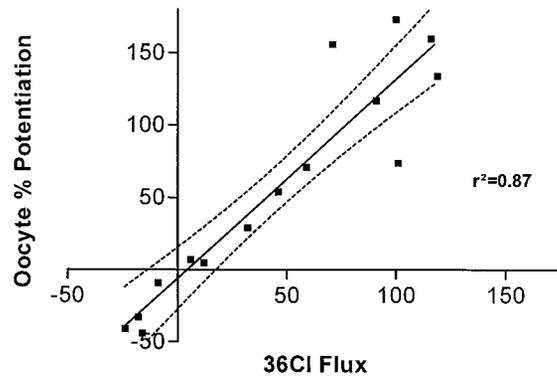


Fig. 7. Correlation is shown for benzodiazepine site ligands at $\alpha 1\beta 3\gamma 2s$ GABA_A receptors determined using two different methodologies. ³⁶Cl influx was measured in Ltk⁻ cells as described herein and electrophysiological responses were determined in *X. laevis* oocytes expressing $\alpha 1\beta 3\gamma 2s$ receptors, impaled with two 1 to 3 M Ω electrodes containing 2 M KCl and voltage-clamped at -70 mV (further details in Wafford et al., 1993). In each case, effects of benzodiazepines were examined on control GABA EC₂₀ responses with a preapplication time of 30 s. Dotted lines represent 95% confidence intervals of the linear regression.

study where expression in Ltk cells was compared with oocytes (Harris et al., 1997), some interesting differences were noted between the two expression systems. Pentobarbital potentiation of $\alpha 6\beta 3\gamma 2s$ was only about 50% higher than $\alpha 1\beta 3\gamma 2s$ in Ltk cells, whereas in oocytes the potentiation of $\alpha 6\beta 3\gamma 2s$ was 3- to 4-fold greater than other subtypes. In addition, the potentiation of $\alpha 5\beta 3\gamma 2s$ by pentobarbital was much greater in cells than oocytes, consistent with the present observations.

It is well documented that pentobarbital can directly activate GABA_A receptors in the absence of agonist and the magnitude of activation varies with receptor subtype, depending on both α and β subunits. Direct activation typically occurs at concentrations above 100 μ M except for $\alpha 6$ -containing receptors where direct activation is observed above 10 μ M (Thompson et al., 1996). Although the focus of the current study was to compare maximal potentiation of different GABA_A receptor subtypes via a variety of modulatory sites, some direct activation of receptors with 100 μ M pentobarbital is likely. Although pentobarbital was tested in the absence of GABA, it was not possible to resolve this component of the response due to some degree of spontaneous opening of the ion channel, which has previously been reported using this assay and confirmed electrophysiologically (Alder et al., 1998). Therefore a submaximal concentration of pentobarbital (10 μ M) was also examined, with the largest potentiation being observed at $\alpha 4\beta 3\gamma 2$ receptors (46 \pm 3%).

Although the nature of the α subunit is a critical determinant of binding and efficacy at the benzodiazepine modulatory site on the GABA_A receptor, producing distinct pharmacological profiles, its influence on other modulatory sites examined here seems to be more subtle. Although all receptor subtypes were sensitive to modulation by pentobarbital, loreclezole, and pregnanolone, there were differences in the level of potentiation at different subunit combinations. This 96-well format flux assay should aid further investigation of recombinant GABA_A receptors and the structural features that influence and modulate ion channel function.

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