0026-895X/01/5905-1108–1118$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
*Mol Pharmacol* 59:1108–1118, 2001

Vol. 59, No. 5
340/899063
*Printed in U.S.A.*

MOL PHARM

MOLECULAR PHARMACOLOGY

# Effect of $\alpha$ Subunit on Allosteric Modulation of Ion Channel Function in Stably Expressed Human Recombinant $\gamma$-Aminobutyric Acid$_A$ Receptors Determined Using $^{36}$Cl Ion Flux

ALISON J. SMITH, LUANDA ALDER, JONATHAN SILK, CHARLES ADKINS, ALAN E. FLETCHER, TIM SCALES, JULIE KERBY, GEORGE MARSHALL, KEITH A. WAFFORD, RUTH M. MCKERNAN, and JOHN R. ATACK

*Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, United Kingdom*

Received June 7, 2000; accepted January 26, 2001

This paper is available online at http://molpharm.aspetjournals.org

## ABSTRACT

Inhibitory $\gamma$-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 $\alpha$ subunits in combination with $\beta3\gamma2$s was examined in stably expressed human recombinant GABA$_A$ receptors by measuring $^{36}$Cl 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 $\alpha3$ 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 ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$), $\alpha5$ 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 $\alpha3\beta3\gamma2$s with nonselective agonist chlordiazepoxide was greater than at $\alpha1$, $\alpha2$, or $\alpha5$ ($P < 0.001$). The presence of an $\alpha4$ subunit conferred a unique pharmacological profile. The partial agonist bretazenil was the most efficacious benzodiazepine, despite lower $\alpha4$ 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 ± 12%), CGS8216 (56 ± 6%), CGS9895 (65 ± 6%), and the weak partial inverse agonist Ro15-4513 (87 ± 5%). Each receptor subtype was modulated by pentobarbital, loreclezole, and 5$\alpha$-pregnan-3$\alpha$-ol-20-one, but the type of $\alpha$ subunit influenced the level of potentiation. The maximal pentobarbital response was significantly greater at $\alpha4\beta3\gamma2$s (226 ± 10% increase in the EC$_{20}$ response to GABA) than any other modulator. The rank order of potentiation for pregnanolone was $\alpha5 > \alpha2 > \alpha3 = \alpha4 > \alpha1$, for loreclezole $\alpha1 = \alpha2 = \alpha3 > \alpha5 > \alpha4$, and for pentobarbital $\alpha4 = \alpha5 = \alpha2 > \alpha1 = \alpha3$.

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 ($\alpha1$–6, $\beta1$–3, $\gamma1$–3, $\delta$, $\epsilon$, $\pi$, and $\theta$) (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 $\alpha$, a $\beta$, and a $\gamma$ 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 $(\alpha)_2(\beta)_1(\gamma)_2$ 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 $\alpha1\beta2\gamma2$, whereas $\alpha2\beta3\gamma2$ and $\alpha3\beta\gamma2/\gamma3$ 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 $\alpha$ and $\gamma$ (McKernan et al., 1995; Buhr et al., 1996; Wingrove et al., 1997) but not $\beta$ subunits (Hadingham et al., 1993). The benzodiazepine site is the

**ABBREVIATIONS:** GABA, $\gamma$-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.



Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

target for commonly prescribed anxiolytic drugs, occurring at the interface of an α(1–6) and a γ(1–3) subunit, with residues in both influencing modulation (Smith and Olsen, 1995; Wingrove et al., 1997). The presence of γ2 confers a classical benzodiazepine pharmacology to GABA$_A$ receptors (Pritchett et al., 1989), whereas receptors containing the less abundant γ1 subunit (Benke et al., 1996) display atypical benzodiazepine pharmacology, including a much lower affinity for Ro15-1788 (Wafford et al., 1993). Introduction of a γ3 subunit affects the affinity and efficacy of some but not all benzodiazepine ligands (Hadingham et al., 1995). Of the α subunits, the presence of α4 or α6 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 $^{36}$Cl 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, synaptoneurosomes, 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 β(3) and γ(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 αnβ3γ2 were seeded into Costar 96-well plates at densities of between 3 and 8 × 10$^4$ cells/ml in a volume of 200 μl/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 μM 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 $^{36}$Cl and appropriate drugs for 5 s, and then washed in ice-cold stop buffer (HEPES/Krebs' buffer containing 100 μM 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 × 10$^4$cells/ml in a volume of 200 μl/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-μl 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 $^{36}$Cl for a further 7 s.

**Measurement of $^{36}$Cl 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 $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM $NaH_2PO_4$, 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 $^{36}$Cl 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 μM) and furosemide (100 μM). Modulatory compounds or assay buffer was added to the wells in a volume of 40 μl for a 30-s preincubation using a Robbins Hydra 96 dispenser. At $t$ = 30 s a subsequent 40-μl addition of a 2× ($^{36}$Cl 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 $^{36}$Cl influx was terminated by aspiration of incubation buffer and immediate washing with 5× 200 μl of ice-cold picrotoxin stop buffer using a second Dynatech plate washer. Stock $^{36}$Cl 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 μCi/ml.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

Scintillation fluid (200 $\mu$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 $\mu$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 $\mu$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,

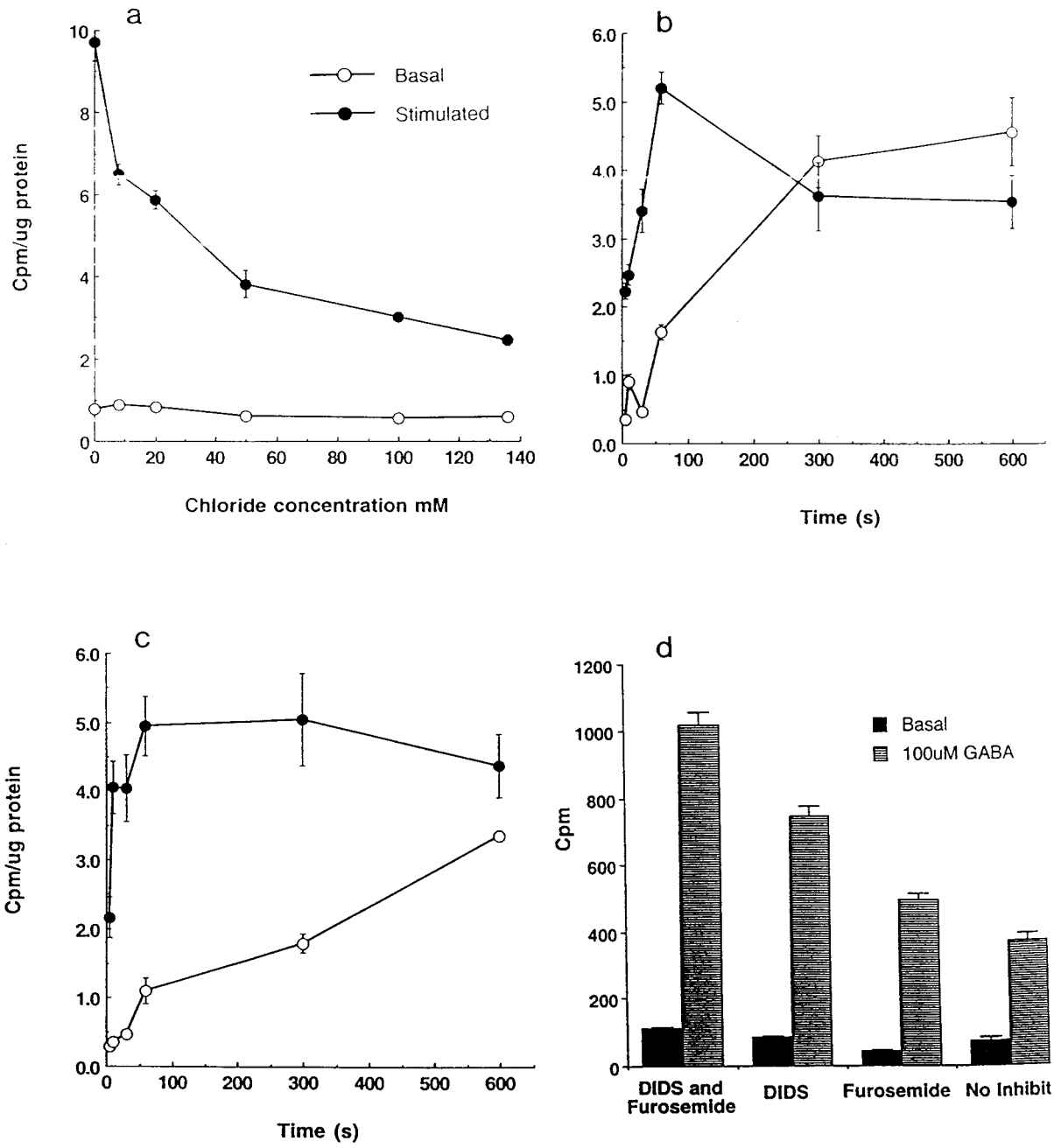


**Fig. 1.** Optimization of flux assay using $\alpha1\beta3\gamma2$ cells. a–c, performed in coverslip format with stimulation by 10 $\mu$M muscimol. a, effect of varying $[Cl^-]_{ext}$. b, time course at 4°C in the presence of 8 mM $[Cl^-]_{ext}$. c, effect of DIDS and furosemide on 4°C time course. d, effects of DIDS and furosemide on signal in final 96-well format [$3 \times 10^4$cells/ml, induced 1 day, $[Cl^-]_{ext}$ = 0, 4°C, 30-s preincubation, 10-s ($^{36}Cl$ ± GABA) addition].

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

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 [$^3$H]Ro15-1788 for receptors containing α1, 2, 3, and 5 subunits and displacement of [$^3$H]Ro15-4513 binding for α4β3γ2 receptors.

**Whole-Cell Patch-Clamp of Ltk$^-$ Cells.** Responses to GABA were also determined electrophysiologically in Ltk$^-$ cells stably expressing α1-5β3γ2 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 $CaCl_2$, 2 mM $MgCl_2$, 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 μM, with resistances around 4 MΩ. The intracellular solution contained 130 mM CsCl, 10 mM HEPES, 10 mM BAPTA.Cs, 5 mM ATP.Mg, 0.1 mM leupeptin, 1 mM $MgCl_2$, 100 μM $NaVO_3$, 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. Noncumulative 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 α1β3γ2 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 μM muscimol, whereas agonist-independent basal uptake remained constant (Fig. 1a). Time course experiments were performed in α1 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 $^{36}$Cl accumulation with increasing incubation time, such that no specific signal remained at 5 min (Fig. 1b). Inclusion of the chloride transport inhibitors DIDS (10 μM) and furosemide (100 μM) 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 × $10^4$ 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 $^{36}$Cl 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 α1β3γ2 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 $^{36}$Cl 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 α4 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 α6-containing receptors but also shows inhibitory effects at α4β3γ2 receptors with an $IC_{50}$ value of 162 μM (Wafford et al., 1996).

**$^{36}$Cl Influx: GABA Agonists and Antagonists.** The effect of expressing different α subunits in combination with β3γ2s subunits was examined on the response to GABA$_A$ receptor agonists. GABA (Fig. 2) and muscimol both elicited concentration-dependent increases in $^{36}$Cl influx, an effect not observed in noninduced controls. Both agonists elicited similar maximal increases in $^{36}$Cl influx, although muscimol exhibited higher potency than GABA at each of the subunit combinations. $EC_{50}$ values (μM) 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 α3 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 α4β3γ2 in flux.

The magnitude of maximal agonist stimulation varied across subtypes with α3β3γ2 receptors exhibiting a significantly larger GABA response than any other subtype ($P <$ 0.001; analysis of variance followed by Bonferroni post hoc

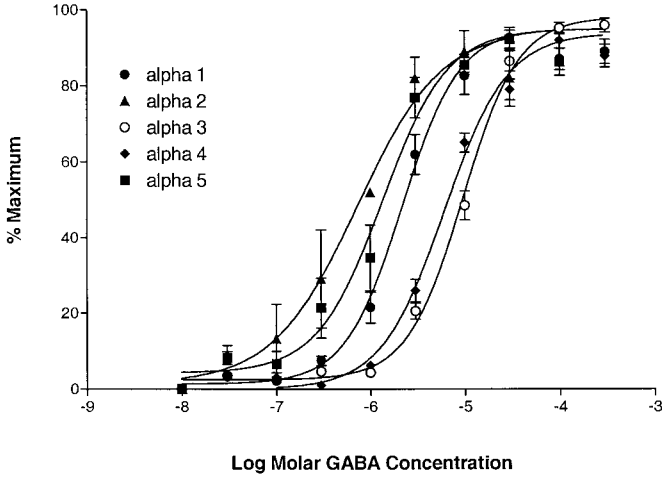


**Fig. 2.** GABA elicited concentration-dependent increases in $^{36}$Cl influx in Ltk$^-$ cells stably expressing GABA$_A$ receptors of composition α1-5β3γ2s. Each curve represents mean data from at least 12 independent experiments (mean ± standard error of mean).

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

test). In contrast, $\alpha4\beta3\gamma2$ 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 $\alpha$ subunits in mediating different pharmacologies at the benzodiazepine site was examined. With respect to benzodiazepine binding, the nature of the $\alpha$ 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 $EC_{20}$-equivalent concentration of GABA was significantly potentiated by chlordiazepoxide (30 $\mu$M) at $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunit-containing receptors ($P < 0.001$) and typical data are illustrated in Fig. 5. Chlordiazepoxide efficacy was not significantly different between $\alpha1$, $\alpha2$, and $\alpha5$, although the potentiation observed at $\alpha3$-containing receptors was significantly larger ($P < 0.001$).

In contrast, $\alpha4\beta3\gamma2$ 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 $\mu$M). In this case, the benzodiazepine giving a maximal potentiation at $\alpha4$ (bretazenil, 30 $\mu$M) was used instead. The relative efficacies for bretazenil at each receptor subtype, with the amount of potentiation expressed as percentage of increase in the $EC_{20}$ response, were as follows: $\alpha1$ 50 ± 4 ($n = 19$), $\alpha2$ 21 ± 6 ($n = 18$), $\alpha3$ 70 ± 7 ($n = 24$), $\alpha5$ 44 ± 4 ($n = 23$), and $\alpha4$ 56 ± 6% ($n = 11$).

To enable comparison of the effect of different $\alpha$ subunits 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 $\mu$M) at $\alpha1\beta3\gamma2$ and $\alpha3\beta3\gamma2$ receptors.

**$^{36}Cl$ Influx: Effect of $\alpha$ Subunit on Modulation at Other Sites.** The influence of the $\alpha$ 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 ($EC_{20}$) 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 $EC_{20}$ GABA response.

A neuroactive steroid, the progesterone metabolite 5$\alpha$-pregnan-3$\alpha$-ol-20-one (10 $\mu$M), the nonvolatile anesthetic barbiturate pentobarbital (100 $\mu$M), and the anticonvulsant loreclezole (10 $\mu$M) potentiated the response to an $EC_{20}$ 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 $\alpha$ subunits (Fig. 6).

At $\alpha1$ subunit-containing receptors loreclezole elicited a significantly greater potentiation of $^{36}Cl$ influx (147 ± 9% increase in $EC_{20}$) than either pentobarbital (106 ± 16%) or pregnanolone (64 ± 5%) ($P < 0.01$ and $P < 0.001$, respectively). Pregnanolone and loreclezole produced a similar degree of potentiation at $\alpha2\beta3\gamma2$ ($P > 0.05$), whereas at $\alpha3\beta3\gamma2$ receptors the potentiation with loreclezole (148 ± 5%) slightly exceeded that with pregnanolone (108 ± 11%). At $\alpha5$ subunit-containing receptors, pregnanolone and pentobarbital were equally efficacious (213 ± 5 and 216 ± 7%, respectively), whereas loreclezole exhibited significantly lower efficacy (117 ± 7%, $P < 0.001$). Receptors of subunit composition $\alpha4\beta3\gamma2$ were characterized by a pentobarbital response that was significantly larger ($P < 0.001$) than any other modulator (226 ± 10%). Pregnanolone was the second most efficacious modulator at $\alpha4\beta3\gamma2$ receptors (99 ± 14%), whereas loreclezole only exhibited a modest potentiation (39 ± 4%). For reference, the potentiation observed with the most efficacious benzodiazepine at $\alpha4\beta3\gamma2$, bretazenil, was 56 ± 6% increase in $EC_{20}$.

The rank order of efficacy for pregnanolone across receptors expressing different $\alpha$ subunits was $\alpha5 > \alpha2 > \alpha3 = \alpha4 > \alpha1$. For loreclezole the rank order of potentiation was $\alpha1 = \alpha2 = \alpha3 > \alpha5 > \alpha4$ and for pentobarbital $\alpha4 = \alpha5 = \alpha2 > \alpha1 = \alpha3$. Although 100 $\mu$M 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 $\mu$M pentobarbital was also examined and produced the following potentiations (% increase in $EC_{20}$ GABA response, $n = 12$): $\alpha1\beta3\gamma2$, 26 ± 2; $\alpha2\beta3\gamma2$, 33 ± 3; $\alpha3\beta3\gamma2$, 28 ± 2; $\alpha4\beta3\gamma2$, 46 ± 3; and $\alpha5\beta3\gamma2$, 30 ± 2%.

TABLE 1

$EC_{50}$ values ($\mu$M) for GABA- and muscimol-stimulated $^{36}Cl$ influx are shown for $GABA_A$ receptors of composition $\alpha1$-5$\beta3\gamma2$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 ± 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 |
| | $\mu M$ | | |
| $\alpha1$ | 0.54 ± 0.06 (17) | 3.9 ± 0.5 (13) | 0.86 ± 0.06 (8) |
| $\alpha2$ | 0.27 ± 0.04 (14) | 1.6 ± 0.4 (12) | 1.1 ± 0.07 (8) |
| $\alpha3$ | 2.0 ± 0.23 (17) | 10 ± 0.7 (12) | 15 ± 0.7 (8) |
| $\alpha4$ | 2.8 ± 0.5 (9) | 6.4 ± 0.6 (17) | 2.6 ± 0.05 (8) |
| $\alpha5$ | 0.36 ± 0.03 (16) | 1.6 ± 0.3 (17) | 2.8 ± 0.3 (8) |

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY

## 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 $\alpha$ 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 $\alpha3\beta3\gamma2$ 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 $\mu$M, 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.

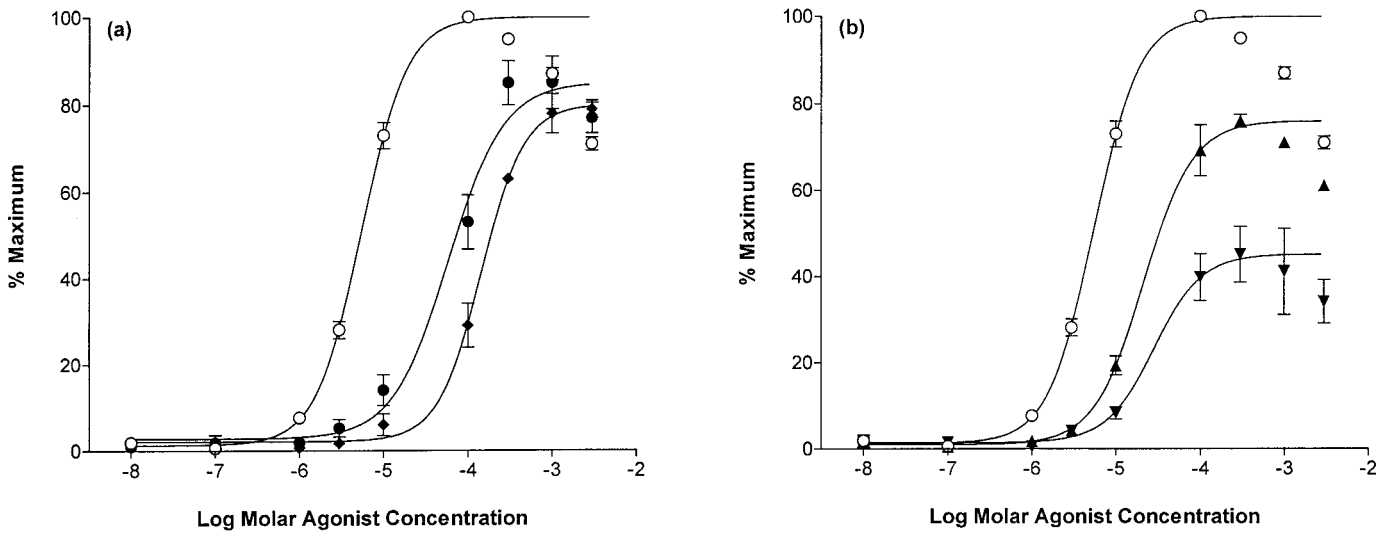


**Fig. 3.** Agonist-stimulated $^{36}Cl$ influx was blocked in a concentration-dependent manner by the competitive $GABA_A$ antagonist bicuculline (a) and the chloride channel blocker picrotoxin (b). Mean effects of 30 and 100 $\mu$M bicuculline and picrotoxin are shown on the response to GABA in $\alpha3\beta3\gamma2$-containing cells (mean of $n = 5$ independent experiments). a, ○, GABA alone; ●, +30 $\mu$M bicuculline; ◆, +100 $\mu$M bicuculline. b, ○, GABA alone; ▲, +30 $\mu$M picrotoxin; ▼, +100 $\mu$M picrotoxin.

TABLE 2

Binding affinity (nanomolar) of benzodiazepine site ligands at $\alpha1\text{-}5\beta3\gamma2s$ $GABA_A$ receptors

Results are mean ± S.E.M. ($n$ = 3–6) (or mean % inhibition of binding at 10 $\mu$M). Data for $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ represent displacement of specific [$^3$H]Ro15-1788 binding, whereas $\alpha4$ data represent displacement of specific [$^3$H]Ro15-4513 binding, measured for 1 hr at 4°C.

| Compound[a] | $K_i$ | | | | |
|---|---|---|---|---|---|
| | $\alpha1$ | $\alpha2$ | $\alpha3$ | $\alpha4$ | $\alpha5$ |
| | | | *nM* | | |
| β-CCM | 2.4 ± 0.3 | 7.4 ± 1.4 | 72 ± 14 | 1040 ± 90 | 44 ± 6 |
| CL218,872 | 66 ± 0.3 | 720 ± 110 | 850 ± 120 | (7.2%) | 460 ± 13 |
| Zopiclone | 28 ± 3 | 64 ± 5 | 29 ± 2 | (2.7%) | 46 ± 8 |
| Bretazenil | 0.22 ± 0.02 | 0.37 ± 0.07 | 0.43 ± 0.09 | 43 ± 2 | 0.86 ± 0.22 |
| Chlordiazepoxide | 890 ± 250 | 460 ± 99 | 980 ± 110 | (3.1%) | 620 ± 200 |
| Diazepam | 12 ± 1 | 7 ± 0.5 | 33 ± 3 | (8.4%) | 11 ± 1 |
| Ro15-1788 | 1.1 ± 0.04 | 1.5 ± 0.1 | 1.0 ± 0.1 | 240 ± 27 | 0.50 ± 0.08 |
| Ro15-4513 | 4.8 ± 0.1 | 7.3 ± 1.2 | 2.4 ± 0.4 | 5.1 ± 0.6 | 0.13 ± 0.01 |
| FG8205 | 0.9 ± 0.2 | 2.4 ± 0.2 | 1.8 ± 0.3 | 440 ± 55 | 5.1 ± 1.8 |
| Abecarnil | 1.3 ± 0.2 | 2.4 ± 0.3 | 4.4 ± 0.2 | 1190 ± 22 | 5.0 ± 0.3 |
| Zolpidem | 23 ± 5 | 110 ± 23 | 320 ± 4 | (7.1%) | (19.3%) |
| Triazolam | 0.41 ± 0.01 | 0.32 ± 0.04 | 1.5 ± 0.1 | (9.5%) | 0.42 ± 0.05 |
| Flunitrazepam | 3.9 ± 0.5 | 1.1 ± 0.3 | 5.9 ± 0.7 | (1.8%) | 1.7 ± 0.5 |
| CGS9895 | 0.32 ± 0.05 | 1.1 ± 0.2 | 0.28 ± 0.05 | 103 ± 8 | 0.96 ± 0.15 |
| CGS8216 | 0.17 ± 0.01 | 0.49 ± 0.09 | 0.30 ± 0.06 | 35 ± 11 | 1.3 ± 0.2 |
| DMCM | 10 ± 0.7 | 13 ± 3 | 7.5 ± 0.7 | 30 ± 8 | 2.2 ± 0.6 |

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

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

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 spontaneous 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 $EC_{50}$ 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 $^{36}Cl$ accumulation over 7 s (i.e., area under the curve rather than peak), the similarity between $EC_{50}$ 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.

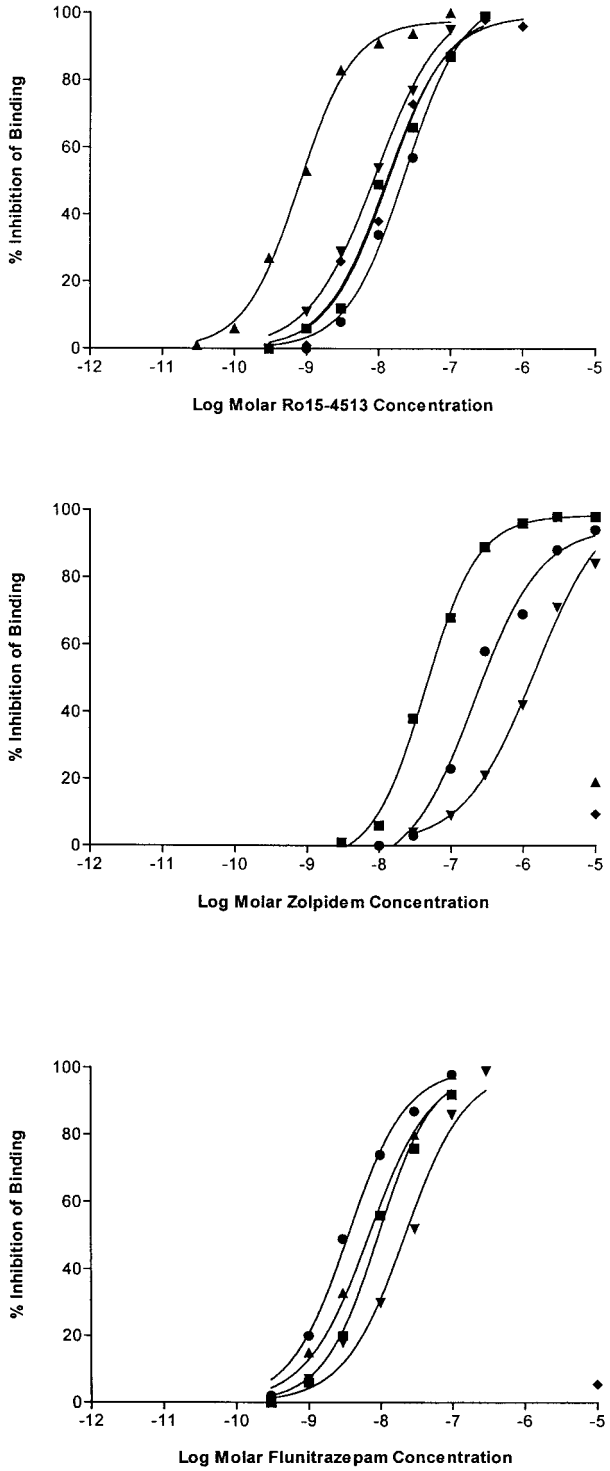


**Fig. 4.** Representative concentration-response curves showing inhibition of binding to the benzodiazepine site for selected benzodiazepine site ligands at $\alpha1$-5$\beta3\gamma2$s $GABA_A$ receptors. The radioligand for $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ is [$^3$H]Ro15-1788 and for $\alpha4$ subunit-containing receptors, [$^3$H]Ro15-4513. a, Ro15-4513; b, zolpidem; and c, flunitrazepam. ■, $\alpha1$; ●, $\alpha2$; ▼, $\alpha3$; ◆, $\alpha4$; ▲, $\alpha5$.

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 $\alpha$ 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 $EC_{20}$ 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 $\alpha1\beta3\gamma2$s 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 $\beta$-carbolines, methyl 6,7-dimethoxy-4-ethyl-$\beta$-carboline-3-carboxylate, abecarnil, and 3-carbomethoxy-$\beta$-carboline were relatively consistent in their action at $\alpha1$-, $\alpha2$-, $\alpha3$-, and $\alpha5$-containing receptors, being either agonist or inverse agonist at all. The exception was Ro15-1788, which was a pure antagonist at $\alpha5$ but exhibited a small degree of partial agonist activity at $\alpha3$. Compounds of different structural types (CL218,872, zopiclone, zolpidem, CGS9895, and CGS8216) exhibited the most subtype selectivity, mainly with regard to $\alpha5$-containing receptors. Zolpidem, a full agonist at $\alpha1$, $\alpha2$ and $\alpha3$, was without activity at

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

α5, although it should be noted that the α5 affinity is very low. The α1 binding-selective compound CL218,872 was an α5 antagonist, whereas at α1, α2, and α3 receptors it was a partial agonist. CGS9895 also had different efficacy at α5β3γ2 receptors, being a partial inverse agonist. However, α4β3γ2 receptors were the most distinct pharmacologically. The imidazobenzodiazepine Ro15-4513 has the highest affinity at α4β3γ2 receptors but many other ligands have much lower α4 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 chlodiazepoxide, flunitrazepam, and zolpidem. The com-

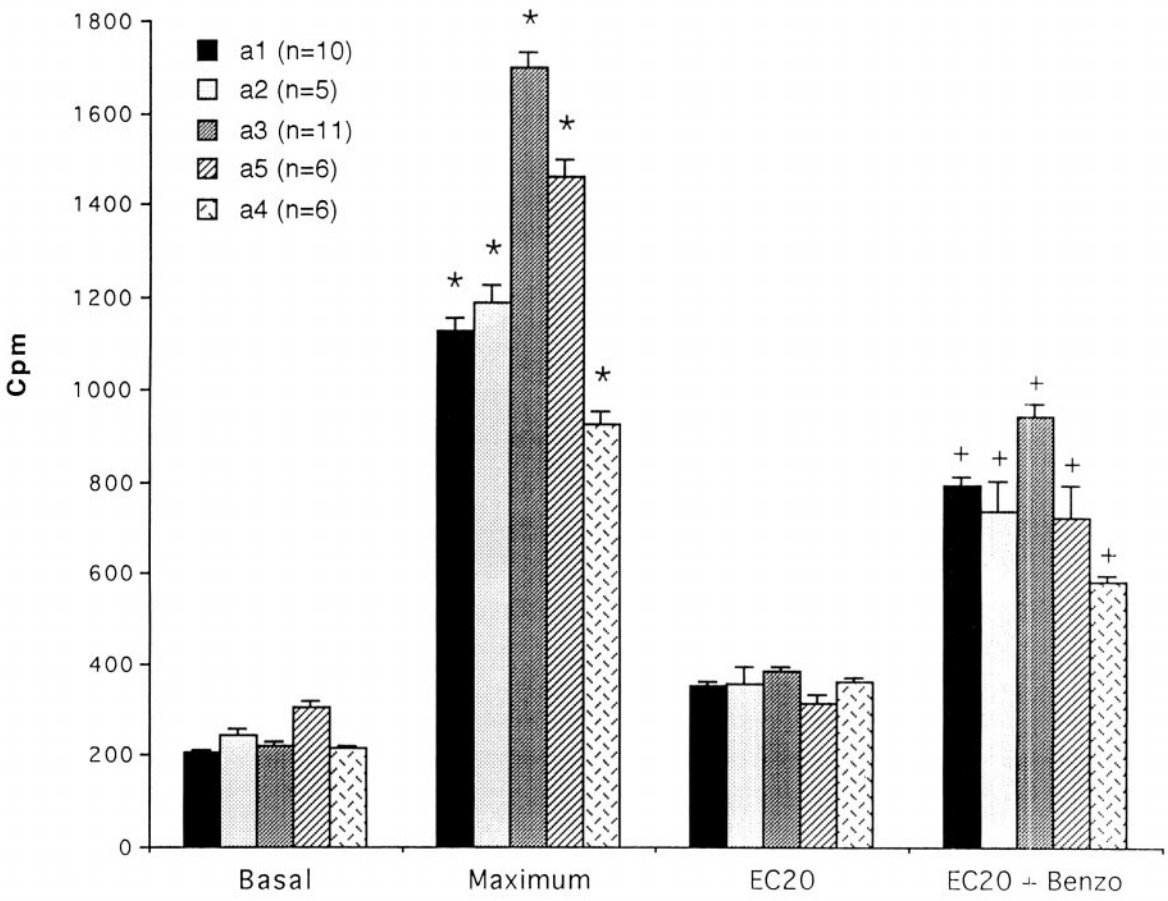


**Fig. 5.** Typical flux responses for maximal agonist and benzodiazepine modulation in cells expressing α1, α2, α3, α4, or α5 subunits with β3γ2s. Maximum agonist effects were defined with 100 μM GABA (*$P < 0.001$ with respect to basal); maximal potentiation of the GABA $EC_{20}$ response by benzodiazepine was with 30 μM chlordiazepoxide for α1, α2, α3, and α5 and 30 μM bretazenil for α4-containing receptors ($^+P < 0.001$ with respect to $EC_{20}$). Seeding densities: α1, $8 \times 10^4$ ml$^{-1}$; α2, $5 \times 10^4$ ml$^{-1}$; α3, $6 \times 10^4$ ml$^{-1}$; α5, $6 \times 10^4$ ml$^{-1}$; and α4, $6 \times 10^4$ ml$^{-1}$.

TABLE 3

Allosteric modulation of GABA-stimulated flux by benzodiazepine ligands in cells expressing different α subunits with β3γ2s, expressed as percentage of maximum benzodiazepine potentiation (mean ± S.E.M.) from at least eight independent experiments

Chlordiazepoxide is 100% by definition for α1, α2, α3, and α5 and bretazenil 100% at α4.

| Compound | % Maximum | | | | |
|---|---|---|---|---|---|
| | α1 | α2 | α3 | α4 | α5 |
| β-CCM | −16 ± 3 | −22 ± 5 | −19 ± 2 | −33 ± 8 | −27 ± 2 |
| CL218,872 | 59 ± 3 | 33 ± 4 | 21 ± 3 | N.D. | 1 ± 4 |
| Zopiclone | 82 ± 4 | 88 ± 7 | 98 ± 4 | N.D. | 80 ± 4 |
| Bretazenil | 32 ± 2 | 20 ± 5 | 33 ± 3 | | 44 ± 3 |
| Chlordiazepoxide | | | | 4 ± 9 | |
| Diazepam | 71 ± 2 | 81 ± 7 | 88 ± 3 | N.D. | 57 ± 3 |
| Ro15-1788 | 6 ± 3 | 10 ± 3 | 19 ± 3 | 53 ± 12 | −2 ± 2 |
| Ro15-4513 | −9 ± 3 | −6 ± 5 | 2 ± 2 | 87 ± 5 | −4 ± 5 |
| FG8205 | 46 ± 3 | 41 ± 3 | 57 ± 4 | 106 ± 8 | 37 ± 5 |
| Abecarnil | 101 ± 6 | 57 ± 6 | 71 ± 4 | 15 ± 7 | 43 ± 5 |
| Zolpidem | 116 ± 6 | 103 ± 8 | 105 ± 6 | −22 ± 6 | −16 ± 5 |
| Triazolam | 119 ± 8 | 95 ± 5 | 112 ± 5 | N.D. | 78 ± 5 |
| Flunitrazepam | 91 ± 1 | 95 ± 9 | 110 ± 5 | 15 ± 11 | 101 ± 8 |
| CGS9895 | 12 ± 3 | 26 ± 6 | 19 ± 1 | 65 ± 6 | −13 ± 5 |
| CGS8216 | −18 ± 2 | −9 ± 3 | −3 ± 2 | 56 ± 6 | −26 ± 5 |
| DMCM | −24 ± 5 | −28 ± 4 | −26 ± 1 | −15 ± 5 | −28 ± 5 |

N.D., not determined.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

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 ± 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 $\alpha$ or $\gamma$ subunit (both $\alpha 1\beta 2$ and $\beta 2\gamma 2$ receptors were sensitive), their presence may influence the level of potentiation When different $\alpha$ 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 $\alpha 1\beta 2\gamma 2$L receptors than $\alpha 4\beta 2\gamma 2$L.

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 $\alpha$ subunit, depending on the steroid examined and the experimental conditions (Lambert et al., 1995). Although the type of $\beta$ 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 $\alpha$ subunit altered the efficacy of pregnanolone to potentiate GABA ($\alpha 2\beta 1\gamma 2$L > $\alpha 1\beta 1\gamma 2$L ≫ $\beta 1\gamma 2$L), in agreement with the current findings, and efficacy was also influenced by the $\gamma$ subunit. In the case of the barbiturate site, the $EC_{50}$ value for pentobarbitone was not dependent on receptor subtype but maximum potentiation differed with the type of $\alpha$ and $\beta$ 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 $\alpha 4\beta 2\gamma 2$L (Whittemore et al., 1996) and $\alpha 4\beta 1\gamma 2$s 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 $\alpha 4\beta 2\gamma 2$L than at $\alpha 1\beta 2\gamma 2$L 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 $EC_{20}$ response by pentobarbital was reported to be of the same magnitude for $\alpha 4\beta 1\gamma 2$s and $\alpha 6\beta 1\gamma 2$s 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

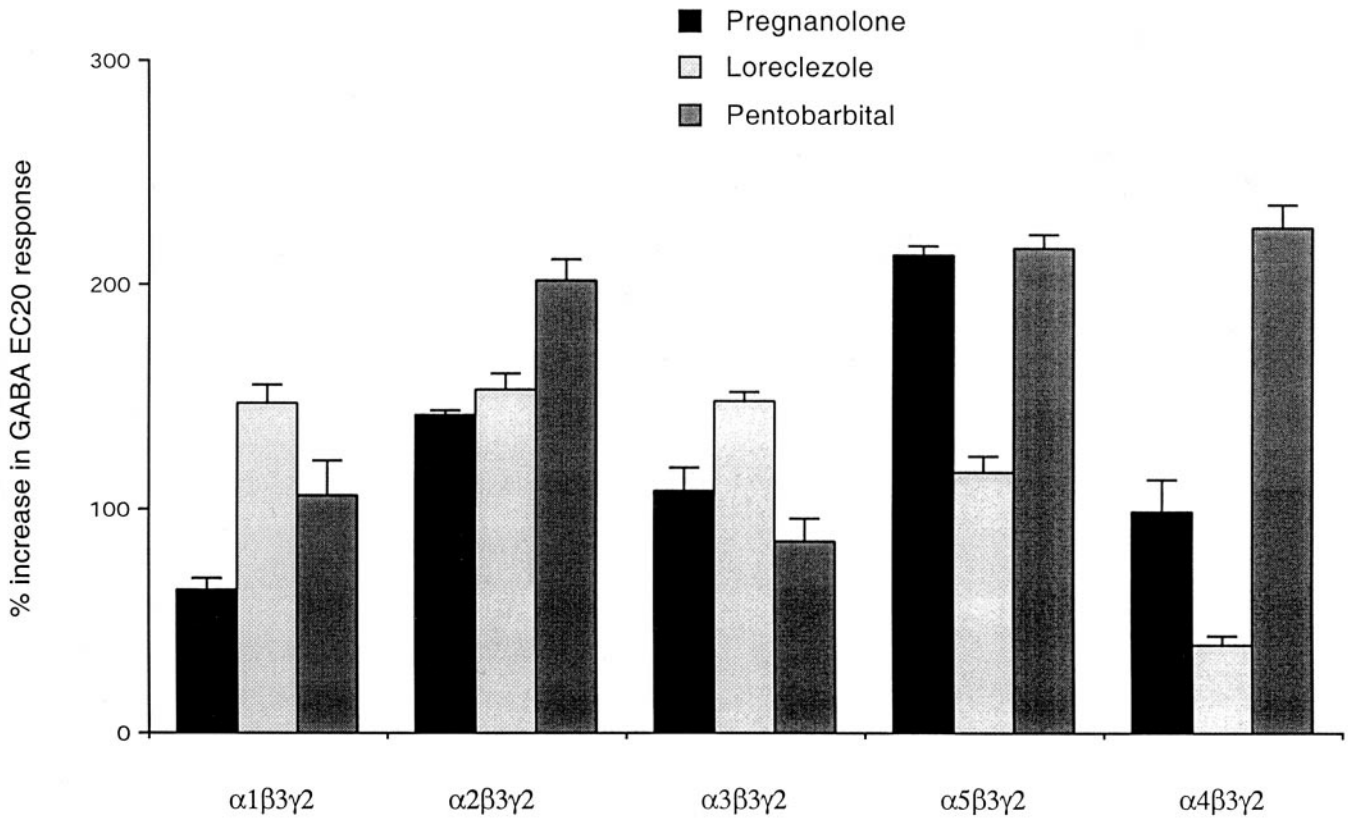


**Fig. 6.** Effect of expressing different $\alpha$ subunits with $\beta 3\gamma 2$s on modulation of GABA-stimulated flux at the steroid, barbiturate, and loreclezole sites on the $GABA_A$ receptor. Results are expressed as percentage of increase in the response to GABA at the $EC_{20}$ (mean ± S.E.M.) from at least seven independent experiments.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

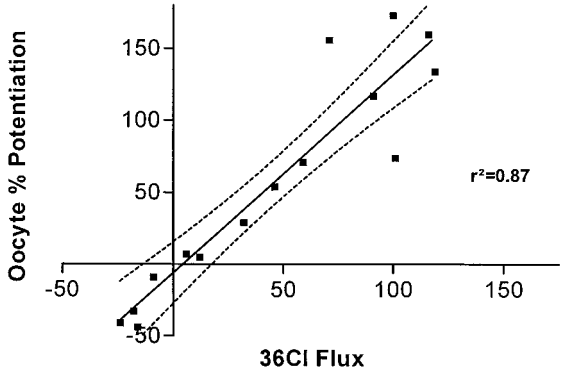


**Fig. 7.** Correlation is shown for benzodiazepine site ligands at $\alpha1\beta3\gamma2$s GABA$_A$ receptors determined using two different methodologies. $^{36}Cl$ influx was measured in Ltk$^-$ cells as described herein and electrophysiological responses were determined in *X. laevis* oocytes expressing $\alpha1\beta3\gamma2$s receptors, impaled with two 1 to 3 MΩ 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 $EC_{20}$ 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 $\alpha6\beta3\gamma2$s was only about 50% higher than $\alpha1\beta3\gamma2$s in Ltk cells, whereas in oocytes the potentiation of $\alpha6\beta3\gamma2$s was 3- to 4-fold greater than other subtypes. In addition, the potentiation of $\alpha5\beta3\gamma2$s 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 $\alpha$ and $\beta$ subunits. Direct activation typically occurs at concentrations above 100 $\mu$M except for $\alpha6$-containing receptors where direct activation is observed above 10 $\mu$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 $\mu$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 $\mu$M) was also examined, with the largest potentiation being observed at $\alpha4\beta3\gamma2$ receptors (46 ± 3%).

Although the nature of the $\alpha$ 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.

## References


Alder L, Smith AJ, Priestley T, Silk J, Adkins C, McKernan RM and Atack JR (1998) Use of a 96 well format 36Cl flux assay to measure GABA$_A$ ion channel function in stably expressed recombinant receptors: constitutive channel opening in the absence of agonist. *Br J Pharmacol* **123:**P199.

Benke D, Honer M, Michel C and Mohler H (1996) GABA$_A$ receptor subtypes differentiated by their γ-subunit variants: prevalence, pharmacology and subunit architecture. *Neuropharmacology* **35:**1413–1423.

Buhr A, Baur R, Malherbe P and Sigel E (1996) Point mutations of the $\alpha1\beta2\gamma2$ γ-aminobutyric acid$_A$ receptor affecting modulation of the channel by ligands of the benzodiazepine binding site. *Mol Pharmacol* **49:**1080–1084.

Carlson BX, Engblom AC, Kristiansen U, Schousboe A and Olsen RW (2000) A single glycine residue at the entrance to the first menbrane-spanning domain of the γ-aminobutyric acid type A receptor β2 subunit affects allosteric sensitivity to GABA and anaesthetics. *Mol Pharmacol* **57:**474–484.

Cestari IN, Min KT, Kulli JC and Yang J (2000) Identification of an amino acid defining the distinct properties of murine β1 and β3 subunit-containing GABA$_A$ receptors. *J Neurochem* **74:**827–838.

Chang Y, Wang R, Barot S and Weiss DS (1996) Stoichiometry of a recombinant GABA$_A$ receptor. *J Neurosci* **16:**5415–5424.

Cox ED, Diaz-Arauzo H, Huang Q, Reddy MS, Ma C, Harris B, McKernan RM, Skolnick P and Cook JM (1998) Synthesis and evaluation of analogues of the partial agonist 6-(propyloxy)-4-(methoxymethyl)-β-carboline-3-carboxylic acid ethyl ester (6-PBC) and the full agonist 6-(benzyloxy)-4-(methoxymethyl)-β-carboline-3-carboxylic acid ethyl ester (Zk 93423) at wild type and recombinant GABA$_A$ receptors. *J Med Chem* **41:**2537–2552.

Ebert V, Scholze P and Sieghart W (1996) Extensive heterogeneity of recombinant γ-aminobutyric acid$_A$ receptors expressed in $\alpha4\beta3\gamma2$-transfected human embryonic kidney 293 cells. *Neuropharmacology* **35:**1323–1330.

Fritschy J-M and Mohler H (1995) GABA$_A$ receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359:**154–194.

Hadingham KL, Harkness PC, McKernan RM, Quirk K, Le Bourdelles B, Horne AL, Kemp JA, Barnard EA, Ragan CI and Whiting PJ (1992) Stable expression of mammalian type A g-aminobutyric acid receptors in mouse cells: demonstration of functional assembly of benzodiazepine-responsive sites. *Proc Natl Acad Sci USA* **89:**6378–6382.

Hadingham KL, Wafford KA, Thompson SA, Palmer KJ and Whiting PJ (1995) Expression and pharmacology of human GABA$_A$ receptors containing γ3 subunits. *Eur J Pharmacol* **291:**301–309.

Hadingham KL, Wingrove PB, Wafford KA, Bain C, Kemp JA, Palmer KJ, Wilson AW, Wilcox AS, Sikela JM, Ragan CI and Whiting PJ (1993) Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol Pharmacol* **44:**1211–1218.

Harris RA, Mihic SJ, Brozowski S, Hadingham K and Whiting PJ (1997) Ethanol, flunitrazepam and pentobarbital modulation of GABA$_A$ receptors expressed in mammalian cells and *Xenopus* oocytes. *Alcoholism Clin Exp Res* **21:**444–451.

Im WB, Pregenzer JF, Binder JA, Dillon GH and Alberts GL (1995) Chloride channel expression with the tandem construct of α6-β2 GABA-A receptor subunit requires a monomeric subunit of α6 or γ2. *J Biol Chem* **270:**26063–26066.

Kardos J (1993) The GABA$_A$ receptor channel mediated chloride ion translocation through the plasma membrane: new insights from $^{36}Cl^-$ ion flux measurements. *Synapse* **13:**74–93.

Knoflach F, Benke D, Wang Y, Scheurer L, Luddens H, Hamilton BJ, Carter DB, Mohler H and Benson JA (1996) Pharmacological modulation of the diazepam-insensitive recombinant γ-aminobutyric acid$_A$ receptors $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$. *Mol Pharmacol* **50:**1253–1261.

Lambert JL, Belleli D, Hill-Venning C and Peters JA (1995) Neurosteroids and GABA$_A$ receptor function. *Trends Pharmacol Sci* **16:**295–303.

Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB and Twyman RE (1997) Activation and deactivation rates of recombinant GABA$_A$ receptor channels are dependent on α-subunit isoform. *Biophys J* **73:**2518–2526.

McKernan RM, Wafford KA, Quirk K, Hadingham KL, Harley EA, Ragan CI and Whiting PJ (1995) The pharmacology of the benzodiazepine site of the GABA$_A$ receptor is dependent on the type of γ-subunit present. *J Recept Res* **15:**173–184.

McKernan RM and Whiting PJ (1996) Which GABA$_A$ receptor subtypes really occur in the brain? *Trends Neurosci* **19:**139–143.

Maitra R and Reynolds JN (1999) Subunit dependent modulation of GABA$_A$ receptor function by neuroactive steroids. *Brain Res* **819:**75–82.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR and Seeburg PH (1989) Importance of a novel GABA$_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond)* **338:**582–585.

Rabow LE, Russek SJ and Farb DH (1995) From ion currents to genomic analysis: recent advances in GABA$_A$ receptor research. *Synapse* **21:**189–274.

Sieghart W (1992) GABA$_A$ receptors: ligand-gated $Cl^-$ ion channels modulated by multiple drug-binding sites. *Trends Pharmacol Sci* **13:**446–450.

Sieghart W (1995) Structure and pharmacology of γ-aminobutyric acid$_A$ receptor subtypes. *Pharmacol Rev* **47:**181–234.

Sieghart W, Fuchs K, Tretter V, Ebert V, Jechlinger M, Hoger H and Adamiker D (1999) Structure and subunit composition of GABA$_A$ receptors. *Neurochem Int* **34:**379–385.

Smith GB and Olsen RW (1995) Functional domains of GABA$_A$ receptors. *Trends Pharmacol Sci* **16:**162–168.

Thompson SA, Whiting PJ and Wafford KA (1996) Barbiturate interactions at the human GABA$_A$ receptor: dependence on receptor subunit combination. *Br J Pharmacol* **117:**521–527.

Tia S, Wang JF, Kotchabhakdi N and Vicini S (1996) Distinct deactivation and desensitization kinetics of recombinant GABA$_A$ receptors. *Neuropharmacology* **35:**1375–1382.

Ueno S, Bracamontes J, Zorumski C, Weiss DS and Steinbach JH (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABA$_A$ receptor. *J Neurosci* **17:**625–634.


MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

Wafford KA, Bain CJ, Quirk K, McKernan RM, Wingrove PB, Whiting PJ and Kemp JA (1994) A novel allosteric modulatory site on the $GABA_A$ receptor $\beta$ subunit. *Neuron* **12:**775–782.
Wafford KA, Bain CJ, Whiting PJ and Kemp JA (1993) Functional comparison of the role of $\gamma$ subunits in recombinant human $\gamma$-aminobutyric $acid_A$/benzodiazepine receptors. *Mol Pharmacol* **44:**437–442.
Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS and Whiting PJ (1996) Functional characterization of human $\gamma$-aminobutyric $acid_A$ receptors containing the $\alpha$4 subunit. *Mol Pharmacol* **50:**670–678.
Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Bourdelles BL, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, et al. (1999) Molecular and functional diversity of the expanding $GABA_A$ receptor gene family. *Ann NY Acad Sci* **868:**645–653.
Whittemore ER, Yang W, Drewe JA and Woodward RM (1996) Pharmacology of the human $\gamma$-aminobutyric $acid_A$ receptor $\alpha$4 subunit expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **50:**1364–1375.
Wingrove PB, Thompson SA, Wafford KA and Whiting PJ (1997) Identification of key amino acids in the gamma subunit of the $GABA_A$ receptor which contribute to the benzodiazepine binding site. *Mol Pharmacol* **52:**874–881.
Wisden W, Laurie DJ, Monyer H and Seeburg PH (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* **12:**1040–1062.

**Send reprint requests to:** Dr. Alison Smith, Department of Biochemistry, Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Rd., Harlow, Essex, CM20 2QR, UK. E-mail: alison_smith@merck.com

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012