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Stable Expression of Type I γ-Aminobutyric Acid$_A$/ Benzodiazepine Receptors in a Transfected Cell Line

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SUMMARY

Expression plasmids were constructed with cDNAs encoding the rat γ-aminobutyric acid-A (GABA$_A$) receptor α1, β2, and γ2 subunits and were cotransfected into cultured human embryonic kidney 293 cells. A single cell line (WS-1) was established after G-418 treatment and clonal selection. This cell line contained saturable, high affinity binding sites for the benzodiazepines [^{3}H] Ro 15-4513 and [^{3}H]flunitrazepam that were modulated by GABA. Competition experiments with benzodiazepine receptor ligands suggest a profile characteristic of native "type I" benzodiazepine receptors, because strong correlations were observed between the K_i values of these ligands in WS-1 cells and in both cerebellar homogenates ($r = 0.97$, $p < 0.0001$) and 293 cells transiently transfected with the corresponding cDNAs ($r = 0.96$, $p < 0.001$). Fluorescence intensity in WS-1 cells loaded with the Cl^--specific probe 6-methoxy-*N*-(3-sulfopropyl)-quinolinium was reliably increased by GABA. This effect was blocked by bicuculline and augmented by midazolam, consistent with the presence of GABA-gated, benzodiazepine receptor-modulated, Cl^- channels. Northern blot analysis revealed the presence of mRNAs encoding α1 and γ2 receptor subunits. Southern blot analysis confirmed genomic integration of transfected α1 and γ2 cDNAs. The β2 subunit was not detected in either Northern or Southern blot analysis, indicating that a functional type I GABA$_A$/benzodiazepine receptor complex can be constituted without a β subunit.

GABA$_A$ receptors are constituted as a heterogeneous group of multimeric, ligand-gated, Cl^- channels. These ligand-gated ion channels are involved in diverse (patho)physiological processes and mediate the pharmacological actions of many clinically important substances including benzodiazepines, imidazopyridines, barbiturates, anesthetic steroids, and ethanol (1). The GABA$_A$ receptor isoform commonly referred to as the type I BzR was initially described using the triazolopyridine CL 218,872 (2–4). Thus, whereas the seminal descriptions of BzR reported a single class of binding sites with no remarkable differences in the affinities of 1,4-benzodiazepines among brain regions (5–8), the apparent affinity of CL 218,872 for cerebellar BzR (designated as type I) was ~9-fold higher than that in hippocampus, with an intermediate potency being obtained in cerebral cortex (2, 9) (reviewed in Ref. 10). Type I BzR are widely distributed throughout the central nervous system but appear to be the predominant receptor isoform in cerebellum (2, 9, 11) (but see Refs. 12 and 13). These findings were among the first providing definitive evidence of GABA$_A$ receptor heterogeneity (reviewed in Refs. 14 and 15). Using similar neurochemical criteria (higher ligand affinity in cerebellum than hippocampus), subsequent studies have described other structurally unrelated type I-selective ligands such as quazepam, zolpidem, CGS 9895, and 3-carboethoxy-β-carboline (16) (reviewed in Ref. 15). The anxioselective actions of CL 218,872 (3, 10) led to the hypothesis that type I BzR were responsible for the anxiolytic and anticonvulsant actions of CL 218,872, whereas other (type II) BzR mediated the muscle-relaxant and hypnotic effects of BzR ligands. However, the findings that other type I-selective ligands (e.g., zolpidem and quazepam) did not share this pharmacological selectivity, coupled with reports of anxioselective BzR ligands (e.g., CGS 9896 and PK 8165) that do not exhibit subtype selectivity, suggest that these pharmacologically defined receptor subtypes cannot adequately explain selective drug action (reviewed in Refs. 14 and 17).

Subsequent studies have established that various combinations of different α, β, and γ subunits form the basis of GABA$_A$ receptor heterogeneity (reviewed in Refs. 18–20). Despite the potential for large numbers of isoforms based on different combinations of identified subunits (six α, three β, and three γ subunits), only three or four distinct isoforms (e.g., type I, type II, and diazepam insensitive) can be distinguished in native tissues using radioligand-binding techniques (reviewed in Refs. 17 and 20). Expression studies performed with either transiently transfected cDNAs in eukaryotic cell lines or mRNAs injected into *Xenopus* oocytes have established that an α1

ABBREVIATIONS: GABA, γ-aminobutyric acid; CL 218,872, 3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-b]pyridazine; βCCM, methyl β-carboline-3-carboxylate; DMCM, dimethoxy-4-ethyl-β-carboline-3-carboxylate; SPQ, 6-methoxy-*N*-(3-sulfopropyl)-quinolinium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; kbp, kilobase pair; CMV, cytomegalovirus; PBS, phosphate-buffered saline; SSC, standard saline citrate; BzR, benzodiazepine receptor(s).

subunit, any one of β1–3 subunits, and γ2 subunit receptor conformation (of undetermined stoichiometry) can mimic the neurochemical and functional properties of native type I BzR (21, 22). Such transient expression systems have proven useful in identifying and elucidating the role these subunits play in the formation and function of $GABA_A$ receptors.

We wished to expand the repertoire of expression systems available to study the function of these receptors by constructing a cell line capable of stably expressing type I BzR as a tool to understand the mechanisms and pharmacological actions of ligands acting through this isoform. We now report the establishment of a cell line that stably expresses a $GABA_A$/BzR isoform with pharmacologies that mimic those of both native cerebellar type I BzR and cell lines transiently expressing these subunits.

Materials and Methods

Transient and stable expression of $GABA_A$ receptor subunits. CMVα1 was prepared by digestion of the rat $GABA_A$ receptor α1 subunit cDNA entire coding sequence from pSKII (23) with *Hind*III and *Xba*I and subsequent ligation to RcCMV vector (Invitrogen, San Diego, CA). The RcCMV vector contains the gene encoding aminoglycoside phosphotransferase (*neo*$_r$), which confers resistance to the antibiotic G-418. CMVβ2 was prepared by digestion of the rat $GABA_A$ receptor β2 subunit cDNA entire coding sequence from pGEM7(−) (23) with *Bam*HI and *Xba*I and ligation to pcDNAI vector (Invitrogen). CMVγ2 (renamed from "rat GABA γ2 sense" for convenience) containing the rat γ2 $GABA_A$ receptor subunit cDNA cloned into the expression vector pCDM8 (Invitrogen) was the gift of Drs. H. Lüddens and P. Seeburg, University of Heidelberg (24).

Expression plasmids CMVα1, CMVβ2, and CMVγ2 were transfected into *Escherichia coli* strains HB101 (CMVα1) or MC1061/P3 (CMVβ2 and CMVγ2) and isolated as previously described (25). Human kidney 293 cells (ATCC 1573) were maintained in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) supplemented with 5% horse serum (stable cell line selection) or 10% fetal calf serum (transient transfections) (GIBCO), 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin in 75-cm^2 tissue culture flasks. Cultures at ~60% confluence were transfected by calcium phosphate precipitation with 5 μg of each plasmid, essentially as described (26). Five hours after transfection, cells were shocked with 15% glycerol in medium for 30 sec, rinsed twice with PBS, and refed with serum-containing medium. Transient transfection studies were carried out 48 hr later. For clonal selection, cells were maintained in the presence of 400 μg/ml G-418 (GIBCO) at 37° in 5% CO_2 for 14 days. The G-418-resistant cells were trypsinized, washed, and seeded at <1 cell/well in 96-well microtiter plates (Costar, Cambridge, MA). Twenty to 25 days after seeding, a total of 10 clones were grown successively in 24-well plates, 25-cm^2 culture flasks, and 75-cm^2 culture flasks. All binding and functional experiments were carried out after at least 10 cell passages.

Radioligand binding. For membrane preparation, cells grown in tissue culture flasks were scraped into 5 ml of PBS. The cells were then centrifuged at 1000 × *g*, the medium was removed, and the cells were dispersed in 4–5 ml of PBS/flask. This rinsing procedure was repeated once. The cells were pelleted at 1000 × *g*, resuspended in 1 ml of 50 mM Tris-citrate buffer (pH 7.8)/flask (~4 × 10^6 cells), and homogenized in a Brinkmann homogenizer (setting 6–7 for 10 sec). The membranes were then centrifuged for 20 min at 20,000 × *g*. This "washing" procedure was repeated three more times. The 10 clones were initially screened for the presence of BzR with [^{3}H]Ro 15-4513 (5 nM). Screening of individual cell lines was carried out in a total volume of 0.5 ml, consisting of 0.3 ml of cell membranes (~150–200 μg of protein), 0.05 ml of [^{3}H]Ro 15-4513 (specific activity 29 Ci/mmol), and buffer to volume. Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. For saturation studies, cell membranes were prepared as described and divided into six pools of tissue. Three experiments for each ligand were performed to determine the B_{max} and K_d. Studies were performed in a total volume of 0.5 ml, consisting of 0.3 ml of cell membranes (~150–200 μg of protein), 0.05 ml of either [^{3}H]Ro 15-4513 (final concentrations, 1–42.1 nM) or [^{3}H]flunitrazepam (final concentrations, 0.5–25.4 nM), and 0.15 ml of Tris-citrate buffer (pH 7.8). Nonspecific binding was determined in the presence of 10 μM Ro 15-1788 or 10 μM diazepam (transient transfection studies) and was typically 10–20% of total binding. For competition studies, membranes from WS-1 cells were prepared as described above. Cerebellar membranes from male adult Sprague-Dawley rats were prepared as described (27). Assays were performed in a total volume of 0.5 ml, consisting of 0.3 ml of WS-1 cell membranes (~150–200 μg of protein) or 0.1 ml of cerebellar membranes (~80–100 μg of protein), 0.05 ml of [^{3}H]Ro 15-4513 (5 nM), 0.05 ml of competitor, and Tris-citrate buffer (pH 7.8) to volume. At least four concentrations of competitor were used. Nonspecific binding was determined as described above. GABA modulation assays were performed in a total volume of 0.5 ml, consisting of 0.3 ml of cell membranes (150–200 μg of protein), either 0.05 ml of [^{3}H]Ro 15-4513 (5 nM) or 0.05 ml of [^{3}H]flunitrazepam (specific activity, 93 Ci/mmol; 5 nM), 0.05 ml of GABA (final concentrations, 0.01–1000 μM), and Tris-citrate buffer to volume. Nonspecific binding was determined as described above. All incubations (0–4°) were terminated after 60 min by rapid filtration through GF/B filters with a Brandel M48R cell harvester (Gaitherburg, MD). The radioactivity retained on the filters was measured in a Beckman LS 5801 liquid scintillation counter. Data were analyzed with GraphPad Inplot 3.15 (GraphPad, San Diego, CA).

Chloride flux. Cl^- flux in WS-1 cells was measured using the Cl^--sensitive fluorescent probe SPQ essentially as described by Engblom *et al.* (28). In brief, cells were cultured on autoclaved coverslips (9 × 35 mm) (coated with poly-L-lysine; Sigma, St. Louis, MO) in culture dishes overnight. Cells were washed twice with Hanks' balanced salt solution (GIBCO) and loaded with SPQ (10 mM) under subdued light (60 min at room temperature). The coverslips were then rinsed twice with Hanks' balanced salt solution. Before the experiment, coverslips were placed in a cuvette containing 3 ml of low-Cl^- buffer (137 mM D-glucuronic acid, 1 mM $MgSO_4$, 5 mM $KHSO_4$, 4.2 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 20 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$, pH 7.4 with NaOH) and 1 mM furosemide. A magnetic stirrer was used to ensure adequate mixing of added substances. Fluorescence was measured with a SPEX Fluorolog 2 (SPEX, Edison, NJ) using excitation and emission wavelengths of 350 nm and 445 nm, respectively.

Northern blot analysis. mRNAs were isolated from either nontransfected 293 cells or the WS-1 clone by the Micro-fast Track mRNA isolation kit (Invitrogen) and were quantified by spectrophotometry. Two micrograms of mRNA from 293 cells or the WS-1 clone were electrophoresed on an 0.8% agarose/8% formaldehyde gel, transferred to Nytran filters (Schleicher and Schull, Keene, NH), and fixed by UV cross-linking (Stratalinker; Stratagene, LaJolla, CA). The full length cDNAs of the $GABA_A$ receptor subunits were ^{32}P-labeled by random priming (Prime-It random labelling kit; Stratagene) to specific activities of 1.0 × 10^9 cpm/μg of DNA (α1), 7.9 × 10^8 cpm/μg of DNA (β2), and 1.3 × 10^9 cpm/μg of DNA (γ2). Filters were hybridized in 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 7% SDS, 0.5% bovine serum albumin, at 65° for 24 hr. After hybridization, filters were washed for 30 min at 25° in 2× SSC, 0.1% SDS, and for 30 min at 55° in 0.1× SSC, 0.1% SDS, air dried, and exposed to X-OMAT film (Kodak, Rochester, NY) with an intensifying screen for the indicated times (25). After exposure, filters were stripped by washing at 95° for 20 min in 0.1× SSC, 0.1% SDS, twice and then rehybridized as described above with a human β-actin cDNA probe (3.7 × 10^8 cpm/μg of DNA).

Southern blot analysis. Genomic DNA from 293 cells or the WS-1 clone were obtained as follows. Cells were scraped from culture flasks, washed twice with PBS, and then lysed in 6 ml of 200 mM NaCl, 20 mM EDTA, 40 mM Tris·HCl, pH 8.0, 0.5% SDS, per 75-cm^2 flask.

Proteinase K was added (0.2 mg/ml) and the lysate was incubated for 4 hr at 50°. Three milliliters of saturated NaCl were added. After 10 min on ice, the lysate was centrifuged at 5000 × *g*. The DNA was aspirated, precipitated with 2 volumes of ethanol, and dried. Ten micrograms of genomic DNA from 293 cells or the WS-1 clone were digested by the appropriate restriction enzyme (*Hind*III, α1 subunit; *Hind*III, β2 subunit; *Xba*I, γ2 subunit) in a volume of 300 μl. The digested DNAs were precipitated with ethanol, dried, redissolved in 30 μl of H_2O, and electrophoresed on an 0.8% agarose gel. The nucleic acids were transferred to GeneScreen (DuPont, Wilmington, DE) and hybridized to radiolabeled probes as described above. Full length cDNAs were prepared as probes with specific activities of 3.4×10^8 cpm/μg of DNA (α1), 1.2×10^8 cpm/μg of DNA (β2), and 1.0×10^9 cpm/μg of DNA (γ2). The filters were washed under conditions described above and were exposed to X-OMAT film with an intensifying screen for the indicated times.

Compounds. Radioligands were obtained from DuPont/NEN (Boston, MA). Ro 15-1788, Ro 19-4603, diazepam, and flunitrazepam were donated by Hoffmann-LaRoche (Nutley, NJ). Alpidem and zolpidem were donated by Laboratoires d'Etudes et de Recherches Synthelabo (Paris, France). CL 218,872 was donated by Lederle (Pearl River, NY), and quazepam was donated by Schering-Plough Corp. (Bloomfield, NJ). DMCM was purchased from Research-Biochemicals Inc. (Natick, MA). GABA and bicuculline were purchased from Sigma. SPQ was purchased from Molecular Probes (Eugene, OR). Other materials were from standard sources.

Results

Cell lines established from 293 cells transfected with the expression plasmids CMVα1, CMVβ2, and CMVγ2 (Fig. 1) were selected with [^{3}H]Ro 15-4513 as described in Materials and Methods. Of 10 cell lines originating from single cells resistant to G-418, four (clones 4, 5, 10, and 11) were found to contain significantly greater (>2.5-fold) specific [^{3}H]Ro 15-4513 binding than did untransfected 293 cells (Table 1). Clone 4 (renamed WS-1) contained ~20-fold higher specific binding (107.1 fmol/mg of protein) than did 293 cells and was selected for subsequent experiments.

Saturation analysis of [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam binding to WS-1 membranes was performed (Fig. 2 and *inset*). The B_{max} values obtained were 356 ± 44 and 181 ± 24 fmol/mg of protein and the K_d values were 5.3 ± 0.7 and 3.8 ± 0.6 nM for [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam, respectively. B_{max} values for these radioligands were significantly different ($p < 0.05$; paired *t* test). No significant changes in [^{3}H]flunitrazepam B_{max} values were observed in cells after 10 passages or 40 passages. Transient expression of α1 and γ2 subunits revealed high affinity, saturable BzR (B_{max} = 136 fmol/mg of protein; K_d = 6.3 nM). Two other independent transient transfections confirmed the presence of BzR constituted by α1 and γ2 subunits.

Competition experiments were performed with a variety of BzR ligands using [^{3}H]Ro 15-4513 as the radioligand (Table 2). Strong correlations were observed between the potencies of these ligands to inhibit [^{3}H]Ro 15-4513 binding to WS-1 cells and their potencies in cerebellum (using values obtained in this and other laboratories (16, 39–41); $r = 0.97$, $p < 0.0001$, and $r = 0.93$, $p < 0.0001$, respectively) and in 293 cells transiently transfected with the corresponding cDNAs (21, 29, 38) ($r = 0.96$, $p < 0.001$). Moreover, consistent with previous reports in both cerebellum and transiently transfected cells, a ~4-fold reduction in the potency of CL 218,872 was obtained when the incubation temperature was elevated from 0 to 37° (Table 1).

The effects of GABA on the binding of [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam were measured in order to determine whether a functional coupling between GABA and BzR was present (Fig. 3). GABA increased the binding of [^{3}H]flunitrazepam in a concentration-dependent manner (Δ_{max}, 36.8 ± 9.7%; EC_{50}, 0.1 μM; Fig. 3). GABA reduced the binding of the partial inverse agonist [^{3}H]Ro 15-4513 to WS-1 membranes in a concentration-dependent manner (Δ_{max}, −11%, EC_{50}, 0.2 μM; Fig. 3).

In order to determine whether the WS-1 clone contained functional GABA-gated Cl^- channels, cells were loaded with the fluorescent probe SPQ, which is quenched in the presence of Cl^- ions. In 293 cells, GABA did not significantly increase fluorescence (Fig. 4a). In contrast, GABA (100 μM) significantly increased (11.3 ± 1.4%, $p < 0.01$) fluorescence quenched by Cl^- in WS-1 cells (Fig. 4b; Table 3). This effect was abolished by the GABA antagonist bicuculline (Fig. 4c; Table 3). Midazolam (10 μM) had no effect on Cl^- flux in the absence of GABA (Table 3) but significantly increased ($p < 0.01$) fluorescence in the presence of subeffective GABA concentrations (10 μM) (Fig. 4d; Table 3). This augmentative action of midazolam was blocked by Ro 15-1788 (10 μM) (Fig. 4e). Results of multiple trials are summarized in Table 3.

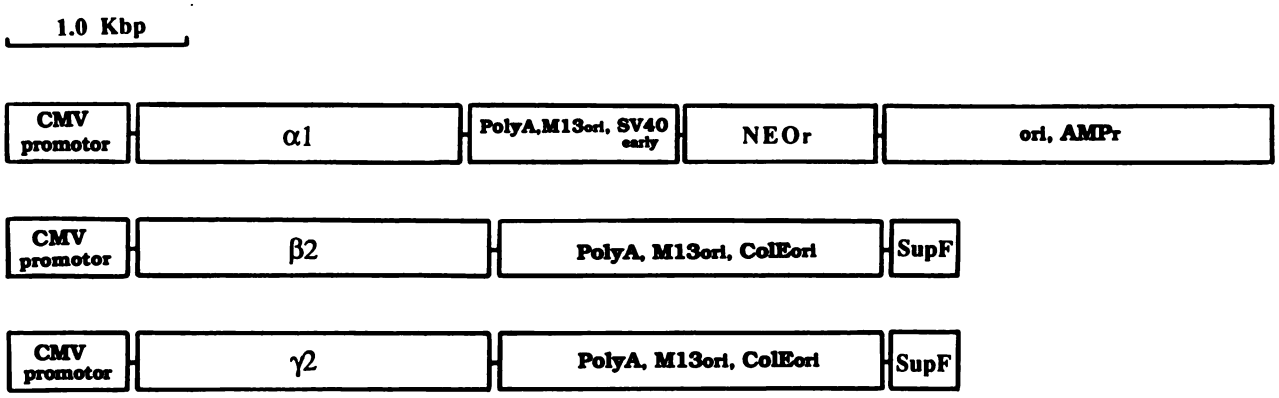

Fig. 1. $GABA_A$ receptor subunit plasmids used for transfecting 293 cells. The plasmid vectors constructed (CMVα1 and CMVβ2) and supplied (CMVγ2) (24) are shown in linear form. CMVα1 was constructed from the parent plasmid RcCMV and CMVβ2 was constructed from the parent plasmid pcDNAI, as described in Materials and Methods. The CMVα1 plasmid contained the G-418 resistance gene (*neo*$_r$) utilized for selection. Other important DNA elements are shown, i.e., CMV promoter, cDNA insert (α1, β2, and γ2), polyadenylation signal (*PolyA*), origins of replication (*M13Ori*, *ColEori*, and *ori*), antibiotic selection markers (*AMP*, and *SupF*), and promoter for *neo*, gene (*SV40 early*).

TABLE 1

Specific [^{3}H]Ro 15-4513 binding to cell lines after clonal selection

293 cells were cotransfected with CMV expression plasmids containing α1, β2, and γ2 $GABA_A$ receptor subunits. After 4 weeks of G-418 selection, resistant single cells were isolated by limiting dilution and expanded as described in Materials and Methods. Membranes from ~6 × 10^6 cells were isolated for each clonal line and were incubated in the presence of 5 nM [^{3}H]Ro 15-4513. Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. Total binding was measured in duplicate, with one nonspecific determination.

Clone	Specific binding
	fmol/mg of protein
1	8.6
2	3.6
4 (WS-1)	107.1
5	17.0
7	8.9
8	2.9
9	3.2
10	12.1
11	11.4
15	2.8
HK 293	4.1

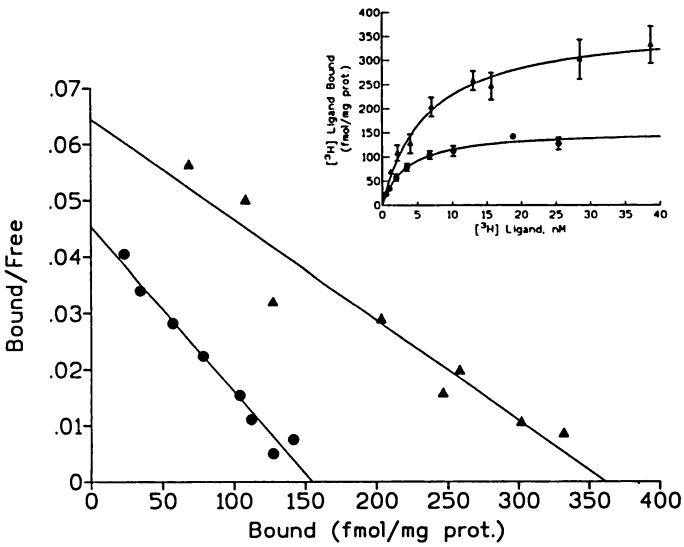

Fig. 2. [^{3}H]Benzodiazepine binding to WS-1 membranes. 293 cells were transfected with $GABA_A$ receptor subunits and selected for stable expression as described in Materials and Methods. Membranes were incubated with [^{3}H]Ro 15-4513 (1.0–42.1 nM) (▲) or [^{3}H]flunitrazepam (0.5–25.4 nM) (●), as described. Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. Data points are the mean of three independent experiments conducted in duplicate. *Inset*, saturation isotherms. *Bars*, standard error. The *curves* were fitted and the r^2 for each curve was >0.92. K_d values were 5.3 ± 0.7 nM and 3.8 ± 0.6 nM and B_{max} values were 356 ± 44 and 181 ± 24 fmol/mg of protein for [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam, respectively.

TABLE 2

Potencies of BzR ligands to inhibit [^{3}H]Ro 15-4513 binding to WS-1 and rat cerebellar membranes

K_i (mean ± standard error) values were determined using 2 × 10^6 cells (~200 μg of protein) or 1 nM [^{3}H]flunitrazepam from cerebellar membranes (~80–100 μg of protein). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation. K_d values used were 5.3 nM for [^{3}H]Ro 15-4513 (WS-1) and 3.8 nM for [^{3}H] flunitrazepam (cerebellum). Values presented are the results of at least three independent experiments. K_i data for transiently expressed $\alpha1\beta1$-$3\gamma2$ receptor subunits were taken from Pritchett *et al.* (21), Lüddens *et al.* (29), and Pritchett and Seeburg (38). K_i literature values for rat cerebellar type I receptors were taken from Gee and Yamamura (39), Sieghart and Schuster (40), Langer *et al.* (41), and Sieghart and Schlerka (16).

Compound	K_i			
	WS-1	Cerebellum	$\alpha1\beta1$-$3\gamma2$	Cerebellum (lit.)
		nM		
CL 218,872	62 ± 11	53 ± 8	108 ± 28	74.9 ± 13
CL 218,872[a]	234 ± 39	257 ± 32	405 ± 75	200 ± 15
Alpidem	47 ± 4	14 ± 4	ND[b]	26
Zolpidem	23 ± 4	21 ± 2	19 ± 3.5	23.6 ± 2.0
Quazepam	56 ± 6	36 ± 0.4	ND	17.6 ± 2.0
βCCM	1.0 ± 0.2	1.5 ± 0.2	0.8 ± 0.1	0.6 ± 0.2
DMCM	1.1 ± 0.3	2.6 ± 0.3	5.3 ± 1.5	5.1 ± 1.4
Ro 15-1788	0.6 ± 0.2	0.8 ± 0.1	0.6 ± 0.2	0.6 ± 0.2
Ro 19-4603	0.2 ± 0.0	0.5 ± 0.1	ND	ND
Flunitrazepam	2.8 ± 0.2	2.4 ± 0.2	2 ± 0.3	2.1 ± 0.3[c]
Diazepam	21 ± 2.3	8.7 ± 0.6	16.3 ± 0.5	7.6 ± 0.8

[a] Incubations performed at 37°.
[b] ND, not determined.
[c] K_d (nM) value.

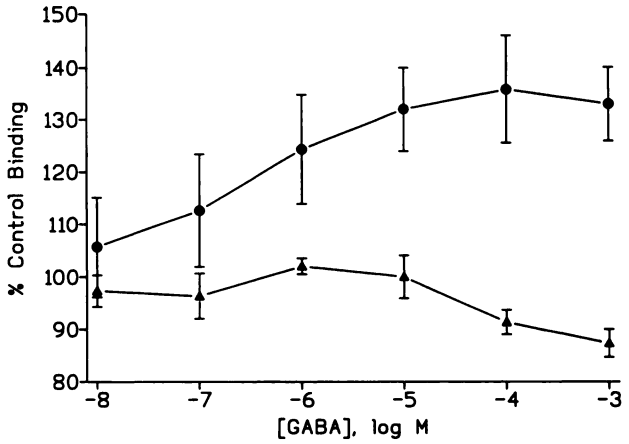

Fig. 3. Modulation of [^{3}H]flunitrazepam (●) and [^{3}H]Ro 15-4513 (▲) binding by GABA. WS-1 membranes were incubated with ~5 nM radioligand and GABA (0.01–1000 μM). Values are the mean ± standard error of three experiments.

Northern blot analysis was performed in order to determine the expression of the various transfected subunits (Fig. 5). A 1.8-kilobase transcript was detected with an α1 cDNA probe among mRNAs isolated from WS-1 cells but not in untransfected 293 cells (Fig. 5). Under identical conditions the β2 subunit mRNA was not detected (Fig. 5). A 1.6-kilobase transcript was detected among WS-1 but not 293 mRNAs probed with the γ2 subunit (Fig. 5).

Southern genomic blot analysis was performed in order to characterize the integration of the transfected subunits into the genome. A unique 11-kbp band was identified in *Hind*III-digested genomic DNAs from WS-1 cells but not from 293 cells probed with the α1 subunit (Fig. 6). Other bands, of 9.8 kbp, 5.8 kbp, 2.5 kbp, 1.5 kbp, and 1.0 kbp, were detected in both cell lines. No unique band was detected in *Hind*III-digested DNAs from WS-1 cells probed with the β2 subunit. Three bands, of 13.2 kbp, 7.8 kbp, and 3.8 kbp, were observed hybridizing to the β2 cDNA probe. Restriction digestions performed with four other enzymes (*Pst*I, *Bam*HI, *Eco*RI, and *Xba*I) and hybridization to the β2 subunit revealed different restriction patterns but no bands unique to the WS-1 clone (data not shown). Unique 1.9- and 0.6-kbp bands hybridized to the γ2 subunit cDNA were detected in *Xba*I-digested DNAs from WS-1 but not 293 cells (Fig. 6). Three other bands, of 11.2 kbp, 8.2 kbp, and 7.3 kbp, were detected in both cell lines. Taken together, these data indicate integration of the $GABA_A$ receptor subunits α1 and γ2 into the WS-1 clone but not the β2 subunit.

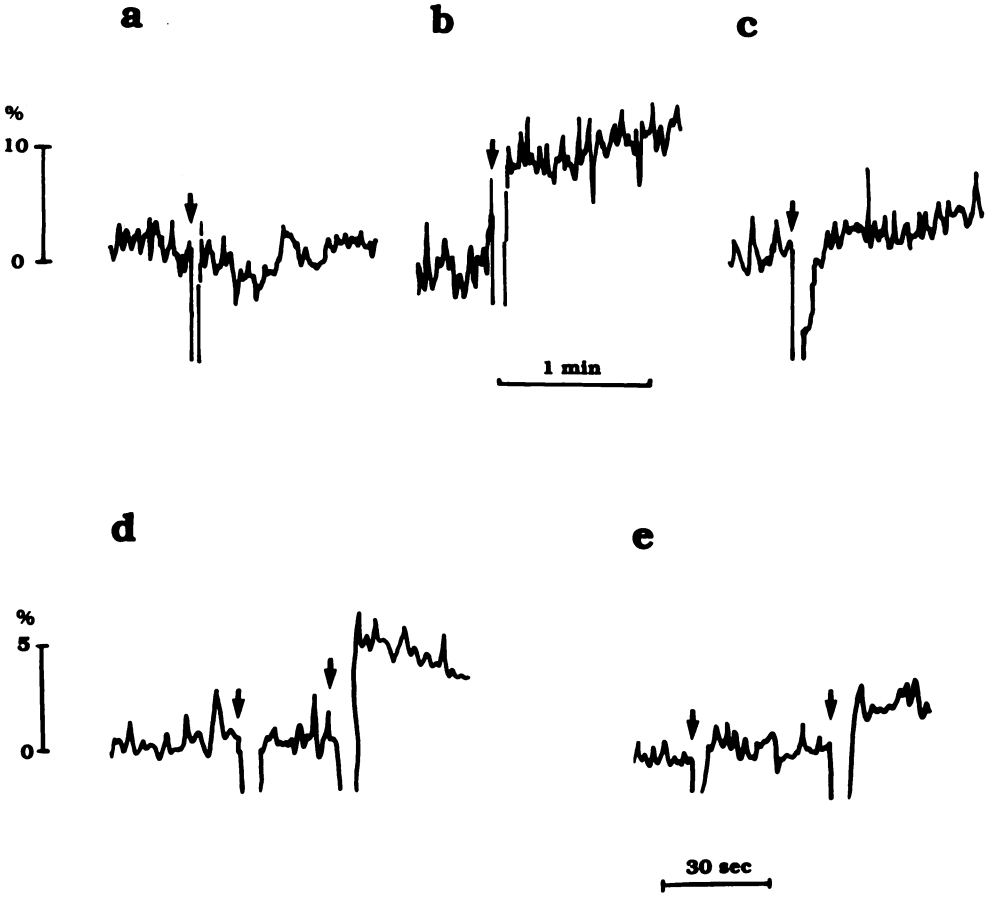

Fig. 4. Effect of GABA and BzR ligand treatment on 293 or WS-1 cells loaded with the dye SPQ. Cells were loaded with SPQ and fluorescence measurements were made as described in Materials and Methods. GABA (100 μM) was added to 293 cells (a) or WS-1 cells (b) as indicated by the *arrow*. c, Pretreatment with bicuculline (100 μM) blocked the increase in fluorescence intensity observed after addition of GABA. d, Treatment of WS-1 cells with GABA (10 μM) (*first arrow*) was followed by midazolam (10 μM) (*second arrow*). e, Treatment of WS-1 cells with GABA (10 μM) (*first arrow*) was followed by midazolam (10 μM) and Ro 15-1788 (10 μM) (*second arrow*). The scale is given as percentage increase in SPQ fluorescence from basal levels (6,000–12,000 counts/sec). *Tracings* shown are representative results from three or four experiments.

TABLE 3

SPQ fluorescence changes produced in 293 and WS-1 cells by various agents

Cells growing on lysine-coated slides were loaded with the dye SPQ as described in Materials and Methods. After furosemide treatment, fluorescence intensity was measured and agents were added 30–45 sec later. The antagonists bicuculline and Ro 15-1788 were added simultaneously with the agonists. In the GABA plus midazolam study, midazolam was added 15–30 sec after GABA. Analysis of variance revealed significant treatment effects (F = 13.0, degrees of freedom = 45, $p < 0.000001$).

Cell line	Treatment	n	Change in SPQ fluorescence
			%
293	GABA, 100 μM	4	1.3 ± 0.64
WS-1	GABA, 100 μM	9	11.3 ± 1.4[a]
WS-1	GABA, 100 μM, + bicuculline, 10 μM	4	1.6 ± 0.82
WS-1	Midazolam, 10 μM	4	1.8 ± 1.0
WS-1	GABA, 10 μM	14	3.8 ± 0.72
WS-1	GABA, 10 μM, + midazolam, 10 μM	7	8.9 ± 0.87[b]
WS-1	GABA, 10 μM, + midazolam, 10 μM/Ro 15-1788; 10 μM	4	3.3 ± 1.33

[a] Significantly different from the same treatment in 293 cells (Newman-Keuls, $p < 0.01$).

[b] Significantly different from 10 μM GABA treatment (Newman-Keuls, $p < 0.01$).

Discussion

The construction, establishment, and characterization of a stable cell line expressing a type I $GABA_A$/BzR is described. After >1 year and >70 cell passages, no observable changes in benzodiazepine binding sites have been observed. Whereas the K_d values of both [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam were similar to those found in native and transiently transfected BzR (29), the B_{max} values of [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam in the WS-1 cell line (356 ± 44 and 181 ± 24 fmol/mg of protein respectively; ~20,000 and ~10,000/cell, respectively) were ~20–40% of those observed in rodent cerebellar membranes (30, 31). The density of [^{3}H]Ro 15-4513 binding sites was nearly 2-fold higher than that of [^{3}H]flunitrazepam sites in WS-1 cells (Fig. 2). This finding is consistent with reports that the B_{max} of Ro 15-4513 is significantly greater (~50%) than that obtained with flunitrazepam (30).[1] The number of [^{3}H]Ro 15-4513 binding sites can be differentially modulated in cerebellum cortex, hippocampus, and neurons in primary culture, but not striatum, by chronic ethanol treatment (31–33), suggesting that this larger B_{max} may not be a ligand-specific artifact. The larger number of [^{3}H]Ro 15-4513 sites present in native receptors and a stably transfected cell line (Fig. 2) may represent a unique benzodiazepine binding site, other than the "diazepam-insensitive" BzR that appears largely confined to cerebellar granule cells in the mammalian central nervous system (29) and that can accommodate [^{3}H]Ro 15-4513 but not other BzR ligands. Nonetheless, competition experiments using a variety of BzR ligands with [^{3}H]Ro 15-4513 in native type I BzR and the WS-1 cell line resulted in Hill coefficients near unity.[1]

Competition studies with several different structural classes of BzR ligands demonstrated that BzR expressed in the WS-1 cell line mimic those in both native type I $GABA_A$/BzR and

[1] G. Wong and P. Skolnick, unpublished observations.

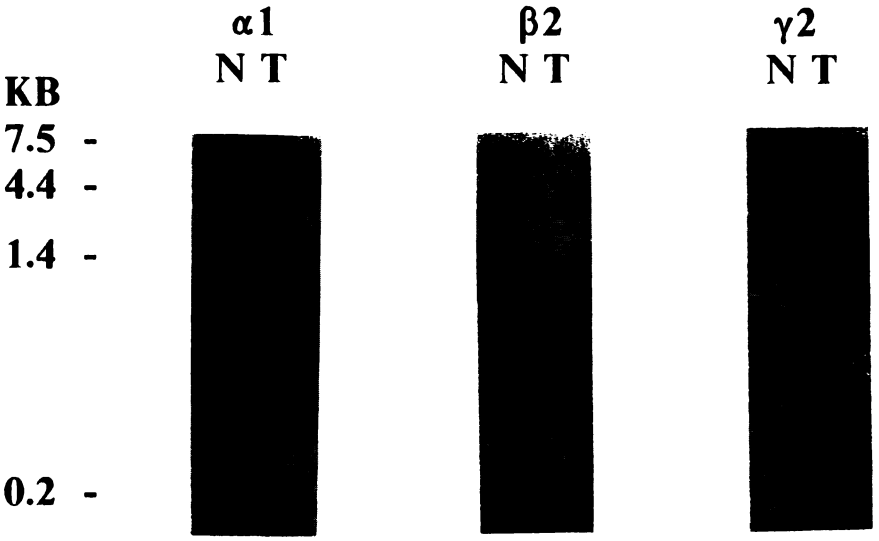

Fig. 5. Northern blots of 293 and WS-1 cell lines with $GABA_A$ receptor subunit probes. Two micrograms of mRNA from nontransfected 293 cells (*N*) or transfected WS-1 cells (*T*) were electrophoresed, transferred to Nytran, and blotted as described in Materials and Methods. Complete cDNA coding sequences of the indicated subunits were ^{32}P-labeled and used as probes. Autoradiographs were exposed for 72 hr. The filters were subsequently stripped, reprobed with the labeled human β-actin cDNA, and exposed to X-OMAT film with an intensifying screen for 24 hr.

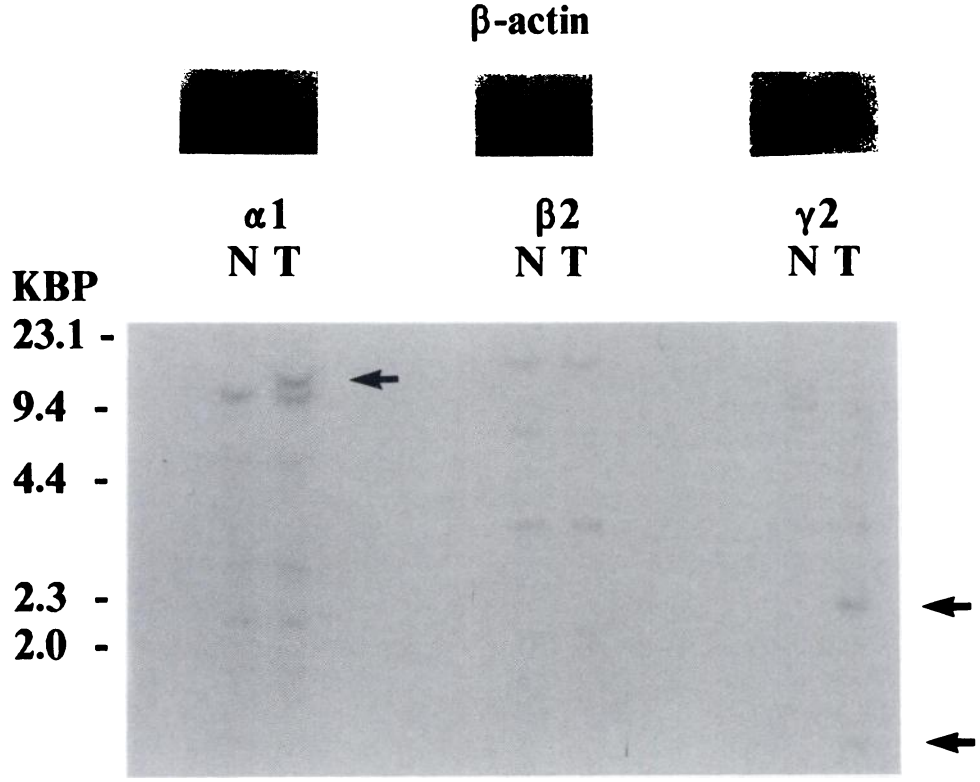

Fig. 6. Genomic Southern blot of 293 and WS-1 cells. Genomic DNA from 293 (*N*) and WS-1 (*T*) cell lines were obtained as described in Materials and Methods. Ten micrograms of genomic DNA were digested with the appropriate restriction enzymes, *Hind*III (α1), *Hind*III (β2), or *Xba*I (γ2), and loaded into each lane. The DNAs were electrophoresed, transferred to Nytran filters, and blotted with the labeled subunit cDNAs. The probes used are indicated (α1, β2, and γ2). Band sizes were estimated by coelectrophoresis with *Hind*III-digested λ DNA fragments. *Arrows*, bands unique to WS-1.

cells transiently transfected with α1β1–3γ2 cDNAs (Table 2). Thus, strong correlations were obtained between the potencies of these ligands in the WS-1 cell line and in both cerebellar homogenates and transient transfection studies. Furthermore, as previously reported in these latter systems, a significant reduction in the apparent affinity of CL 218,872 was observed when incubation temperatures were elevated to 37°. This is characteristic of type I receptors but not type II receptors, where elevated temperatures do not affect the apparent affinity of CL 218,872 (21).

Several other lines of evidence indicate a functional coupling between the benzodiazepine and GABA recognition sites in this stably transfected cell line. Thus, GABA increased [^{3}H]flunitrazepam and decreased [^{3}H]Ro 15-4513 binding, respectively, in a concentration-dependent fashion (Fig. 3). Nonetheless, the magnitude of these shifts was somewhat lower and the potency of GABA somewhat higher than those observed in native type I receptors (34). This bidirectional modulation also demonstrates the ability of BzR in this cell line to discriminate agonist/inverse agonist ligands, which may prove useful in drug discovery studies.

The presence of GABA-gated BzR-modulated ion channels in this stably transfected cell line was demonstrated using a fluorescent probe (SPQ) sensitive to changes in Cl^- concentration. A similar technique was recently used (28) to measure GABA-mediated Cl^- efflux in cultured cerebellar granule neu-

rons. Thus, no GABA-induced changes in fluorescence intensity were observed in SPQ-loaded 293 cells (Fig. 4a). In contrast, GABA (100 μM) produced a reliable increase in fluorescence intensity in WS-1 cells (Fig. 4b) that could be blocked by the GABA antagonist bicuculline (Fig. 4c; Table 3). Moreover, the absolute magnitude of these changes in response to GABA (11.3 ± 1.4%) were comparable to those observed in cultured cerebellar granule cells (~7.5%) (28). A concentration of midazolam (10 μM) that did not significantly affect Cl^- flux ($p < 0.01$) augmented the effects of a suboptimum concentration of GABA (10 μM) (Table 2). This augmentation was antagonized by the BzR antagonist Ro 15-1788 (10 μM), providing additional evidence that the WS-1 cell line contains a functional $GABA_A$/BzR complex. Sigel *et al.* (22) were unable to detect GABA-gated Cl^- currents in *Xenopus* oocytes injected with $\alpha\gamma$ mRNAs, whereas Verdoorn *et al.* (35) demonstrated GABA-gated Cl^- channels in 293 cells transiently transfected with $\alpha1\gamma2$ cDNAs with conductance properties different from those in cells transfected with α, β, and γ subunits. The effects of BzR ligands were not examined in this latter study. Electrophysiological studies (36) reported while this manuscript was in preparation have demonstrated GABA-gated currents that are augmented by benzodiazepines in *Xenopus* oocytes transfected with $\alpha3\gamma2$ cDNAs, which is consistent with the present report indicating that $\alpha1\gamma2$ expression is sufficient to produce BzR-mediated, GABA-gated, Cl^- currents. The data obtained in transient transfection studies demonstrating that high affinity BzR can be constituted by $\alpha1$ and $\gamma2$ subunits provide additional evidence that this subunit combination mimics the native receptor. Yet another report demonstrated that DMCM modestly antagonized GABA responses in cells transfected with cDNAs encoding $\alpha1\gamma2$ (37). More detailed electrophysiological studies using patch-clamp techniques will provide additional insights into the functional properties of the WS-1 cell line. These studies are currently underway.

β Subunit expression or integration was not detected in either Northern or Southern blots, respectively, which lends additional support to the hypothesis that $GABA_A$ receptors can be reconstituted without a β subunit. The possibility that an $\alpha\gamma$ subunit composition is sufficient for full benzodiazepine recognition and GABA sensitivity but the β subunit contributes to efficacy is consistent with the present results and those reported elsewhere (35–37) (but see Ref. 23). It should be possible then to construct other type I conformations with the addition of different β subunits. The properties predicted include selectivity for type I ligands but varying responses to GABA.

A cell line expressing type I $GABA_A$ receptors has been constructed and characterized; it mimics the biochemical and pharmacological properties of those observed in cerebellar tissues. Studies using this cell line have provided new insights into the interdependent relationships between subunit composition, ligand binding, and functional properties of $GABA_A$ receptors.

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