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# Assembly of $GABA_A$ Receptor Subunits: Analysis of Transient Single-Cell Expression Utilizing a Fluorescent Substrate/Marker Gene Technique

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**$GABA_A$ receptor channels (GABARs) composed of varying combinations of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits were transiently expressed in mammalian cell lines. The whole-cell patch-clamp recording technique was used to determine which combinations of GABAR subunits produced functional receptor channels and whether assembly of GABAR subunits into receptor channels followed a random or preferred sequence. To identify rapidly cells expressing GABARs, mammalian cell lines were cotransfected with combinations of GABAR subunit cDNAs and the *Escherichia coli* β-galactosidase gene as a transfection marker. Positively transfected cells were identified by staining with the enzyme substrate fluorescein di-β-galactopyranoside. Using this technique, we confirmed that functional $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs were assembled in transfected mouse L929 fibroblast cells, but surprisingly, functional $\alpha_1\gamma_{2S}$ and $\beta_1\gamma_{2S}$ GABARs were not expressed. It was determined that after transient transfection, levels of expressed receptors varied little among individual cells permitting comparison of absolute whole-cell GABA-evoked current values. Whole-cell currents recorded from cells coexpressing $\alpha_1\beta_1\gamma_{2S}$ subunits were three to four times larger than those recorded from cells coexpressing $\alpha_1\beta_1$ subunits, and they were always enhanced by coapplied diazepam. The increase in whole-cell current was due in part to the larger single-channel current of the $\alpha_1\beta_1\gamma_{2S}$ GABARs. GABARs comprised of $\alpha_1\beta_1\gamma_{2S}$ subunits were formed preferentially over GABARs of $\alpha_1\beta_1$ subunits alone, since only after substantially increasing the ratio of the $\beta_1$ expression vector over the $\alpha_1$ and $\gamma_{2S}$ subunit expression vectors were $\alpha_1\beta_1$ GABARs formed in the presence of the $\gamma_{2S}$ subunit. These findings suggest that assembly of GABARs from constituent subunits did not proceed randomly to form all possible combinations, but that certain subunit combinations were preferred intermediates during the assembly process.**

***[Key words: assembly, fluoresence, β-galactosidase, Hill slope, patch clamp, recombinant DNA, transient expression]***

Unlike nicotinic cholinergic receptors, the final assembled form of $GABA_A$ receptor channels (GABARs) is uncertain. The stoichiometry of subunits and which subunits are found assembled *in vivo* are not known. Initial studies of GABAR subunit expression in different heterologous expression systems suggested that subunits assembled into multiple combinations, producing ion channels with distinct electrophysiological and pharmacological properties. For example, injection of various single, double, and triple combinations of mRNAs encoding $\alpha$, $\beta$, and $\gamma$ GABAR subunits into *Xenopus* oocytes produced functional ion channels with differing GABA and diazepam sensitivities (Sigel et al., 1990). In general, it appeared that $\alpha\beta$ and $\alpha\beta\gamma$ subunit combinations produced either more ion channels or channels that conducted more current than single $\alpha$, $\beta$, and $\gamma$ subunit combinations or $\alpha\gamma$ and $\beta\gamma$ combinations. Analogous experiments using cotransfection of HEK 293 cells with various combinations of $\alpha_1$, $\beta_2$, $\gamma_{2S}$ and $\alpha_3$, $\beta_1$, $\gamma_{2S}$ subunit cDNAs produced similar results (Verdoorn et al., 1990; Knoflach et al., 1992). Based upon these recent experimental observations, it has been proposed that assembly of GABARs may proceed randomly from a given set of subunits to form a heterogeneous mixture of receptor subtypes (Burt and Kamatchi, 1991). It still remains to be determined if there is an ordered pathway(s) of assembly, where certain combinations of subunits form preferred intermediates, mature functional GABARs, or do not assemble.

To analyze GABAR assembly more thoroughly, we wished to combine whole-cell and single-channel electrophysiological and pharmacological characterization of different receptor subunit combinations in the same cell. GABARs composed of various combinations of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits were chosen for study, since previous *in situ* hybridization studies have suggested that these subunits may be found in specific neurons in the CNS (Malherbe et al., 1990; Wisden et al., 1992). Coexpression of these subunits in mammalian cell lines produced type I benzodiazepine receptors (Pritchett et al., 1989a); the benzodiazepine sensitivity of the receptor was due to the incorporation of the $\gamma$ subunit (Pritchett et al., 1989b). Theoretically, these neurons could assemble a mixture of different subunit combinations (e.g., $\alpha\beta$, $\alpha\gamma$, $\beta\gamma$, $\alpha\beta\gamma$), unless there were preferred forms of GABARs. By combining whole-cell patch-clamp recording

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with single-channel kinetic analysis of controlled subunit expression, it might be possible to characterize more completely the properties of the different potential subunit combinations and to identify them by their single-channel gating properties. Then, it might be possible to determine if a cell that expresses multiple GABAR subunits assembles all subunit combinations equally (random assembly), or if alternatively some or only one preferred combination exists (ordered assembly).

To study assembly of GABARs properly, it was necessary to control the expression of individual subunits or subunit combinations in a cell. This has routinely been performed by using a heterologous expression system, such as *Xenopus* oocytes or mammalian cell lines (Gu et al., 1990; Sigel et al., 1990; Knoflach et al., 1992). However, the choice of the system could affect the results obtained or limit the analytical techniques that could be performed. Ideally, a cell line for electrophysiological study of transiently expressed receptor ion channels would possess the following properties. First, it should grow as individual cells without long processes and not as a syncytium to permit adequate space clamp of the cells. Second, the cell line should be easily transfected with expression vector constructs or injected with mRNA and with high efficiency. Third, the cell line should be of mammalian and optimally neuronal origin to provide appropriate posttranslational regulation of receptor expression. Last, the cell line should not be engineered to translate large amounts of vector-encoded protein. Although high expression of ion channels on the cell membrane would enhance the sensitivity of whole-cell current recording and other biochemical assays, it would preclude proper single-channel analysis because the higher receptor density would make it difficult to excise membrane patches with few ion channels. Also, high subunit expression might cause the promiscuous assembly of the protein, as has been described for some seven transmembrane receptors and G-proteins (Macnulty et al., 1992). *Xenopus* oocytes are not mammalian in origin, but amphibian, and thus they may potentially modify exogenous proteins with different posttranslational machinery (e.g., glycosylation). On the other hand, oocytes can be injected with mRNA with high efficiency. Alternatively, mammalian eukaryotic cell lines, such as HEK 293 and COS 1 fibroblast cells, have been commonly used. These cells, however, grow as electrotonically coupled syncytia, and therefore it has been difficult to identify and voltage-clamp the specific transfected cell.

Mouse L929 fibroblast cells fulfill most of the criteria set forth above. They are small cells (approximately 30 $\mu$m) without processes, and are not electrotonically coupled to one another in culture. Also, although L929 cells have been immortalized, they are not genetically engineered to produce large quantities of vector encoded protein. Their greatest limitations are that they are non-neuronal in origin and transfect with a low efficiency (approximately 2–5% on average). L929 cells have been used for the stable expression of other ligand-gated and voltage-gated ion channels, and the electrophysiological and pharmacological properties of the expressed ion channels were similar to the native proteins when all of the appropriate subunits were present (Sine and Claudio, 1991; Varadi et al., 1991; Isom et al., 1992). Thus, use of mammalian neuronal cells for heterologous expression of ion channel proteins is not always necessary. However, to use L929 cells to investigate GABAR assembly after transient transfection with various subunit expression vectors, a method to locate positively transfected cells for study was needed.

In this article, we describe a method utilizing cotransfection of the *Escherichia coli* $\beta$-galactosidase gene (*lacZ*) along with cDNAs encoding the above subunits to identify rapidly single positively transfected L929 cells by staining with the fluorescent LacZ substrate fluorescein di-$\beta$-galatcopyranoside (FDG) (Nolan et al., 1988). Using this procedure, we examined whole-cell GABA-evoked currents from single cells transiently expressing double- and triple-subunit combinations of GABAR subunit cDNAs in L929 cells. Pharmacological analysis of the expressed GABARs demonstrated that not all combinations of subunits readily assembled into functional GABARs and that there appeared to be a preferred form of the mature GABAR.

## Materials and Methods

*Sources.* FDG and Imagene were purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals, drugs, sera, media, and cell attachment factors were obtained from GIBCO–Bethesda Research Labs (BRL) (Bethesda, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), or Sigma Chemicals (St. Louis, MO). Tissue culture dishes were purchased from Corning Glass Works (Corning, NY). All cell lines were purchased from American Type Culture Collection (Rockford, MD). Restriction and DNA modification enzymes were obtained from either BRL, Boehringer Mannheim Biochemicals, or New England Biolabs, Inc. (Beverly, MA). Diazepam was kindly provided by Hoffmann–LaRoche (Nutley, NJ).

*Plasmid construction.* Full-length cDNAs encoding the bovine $\alpha_1$ and $\beta_1$, rat $\alpha_5$, and human $\gamma_{2S}$ subunits of the $GABA_A$ receptor were kindly provided by Drs. E. Barnard (MRC, London) and A. Tobin (UCLA) in the vectors described previously [$\alpha_1$, $\beta_1$, $\gamma_{2S}$, E. Barnard (Schofield et al., 1987; Zaman et al., 1992); $\alpha$5, A. Tobin (Khrestchatisky et al., 1989)]. All plasmids were cut with appropriate restriction enzymes to release the complete open reading frames and approximately 10–100 base pairs of the 5′ and 3′ untranslated regions, including the Kozak sequences (Kozak, 1981, 1984) and were subcloned individually into the *Bgl* II site of the mammalian expression vector pCMVNeo (Huggenvik et al., 1991) to form the plasmids pCMVb$\alpha_1$, pCMVb$\beta_1$, pCMVr$\alpha_5$, and pCMVh$\gamma_2$. The vector pCMV$\beta$Gal was created by subcloning a 3000 base pair *Bgl* II fragment of pSV$_2\beta$Gal (Hall et al., 1983) (from Dr. Audrey Seasholtz, University of Michigan) into pCMVNeo.

*Preparation of gridded plates.* A Mecanex BB Form 2 device (Medical Systems, Inc., Greenvale, NY) was used to imprint a 26 × 26 grid (300 $\mu$m per grid edge) on the bottom of a 35 mm tissue culture dish, according to the manufacturer's instructions. Individual grid spaces are identified by a corresponding two-letter alphabetic code (see Fig. 1). After plating at low density, cells could be accurately located on the dish relative to a particular grid while switching between the fluorescent and electrophysiology microscopes. The process of imprinting the grid removed some of the net negative charge on the dish necessary for cell adherence; thus, some cell lines tested required the plates to be coated with adhesion factors. A coating of one or two drops of fibronectin (100 $\mu$g/ml), poly-L-lysine (50000 Da, 50 $\mu$g/ml), or collagen (0.5 mg/ml) in phosphate-buffered saline was used to obtain optimal cell adherence (data not shown). Alternatively, retreatment of the gridded plates with a corona discharge (Amstein and Hartman, 1975; Ramsey et al., 1984) reimparted the lost negative charge and returned the cellular adherence, abrogating the need for attachment factors (data not shown). Corona discharge treatment of the gridded plates was performed by Corning Inc.

*Cell culture and DNA transfection.* All cell lines were grown in Dulbecco's modified Eagle's medium with 100 IU/ml of penicillin and 100 $\mu$g/ml of streptomycin, at 37°C in 5% $CO_2$, 95% air. COS 1 and CV-1 cells were supplemented with 10% fetal bovine serum; L929 and HEK 293 cells were grown with 10% horse serum. For transfections, cell lines were passaged the night before with trypsin/EDTA solution (0.5%/0.2%, respectively) and plated at approximately 70% confluency in a 60 mm dish. The next day, cells were transfected using the modified calcium phosphate precipitation method (Chen and Okayama, 1987) with various combinations of CsCl-banded pCMVb$\alpha_1$, pCMVr$\alpha_5$, pCMVb$\beta_1$, pCMVh$\gamma_2$, and pCMV$\beta$Gal. Unless otherwise stated, plasmids were mixed in a 1:1:1:1 ($\alpha$:$\beta$:$\gamma$:$\beta$Gal) or 1:1:1 (subunit 1:subunit 2:$\beta$Gal) ratio while maintaining the total amount of DNA added per 60 mm dish at 9.6 $\mu$g in 300 $\mu$l of transfection buffer. Cells were shocked with 15% glycerol, 1 × PBS 4 hr later. Twenty-four hours after the addition of

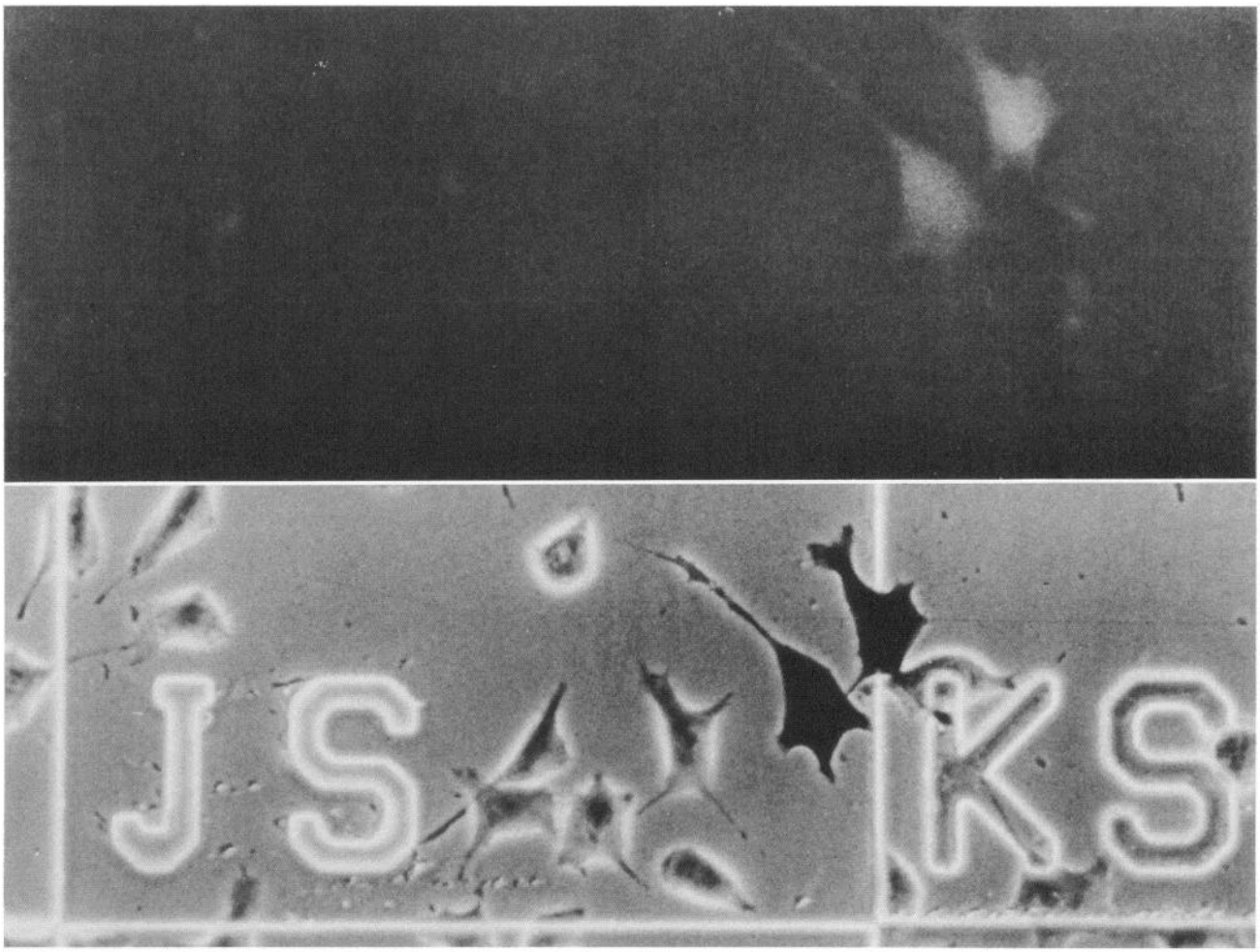

*Figure 1.* Localization of LacZ+ cells plated on gridded plates. Forty-eight hours posttransfection, *lacZ* expression by the cells was verified by both FDG staining (*top*) and X-Gal counterstaining (*bottom*). At this plating density, individual LacZ+ cells are easily discernable relative to the grid letters and borders. *Letters* are 62.5 μm in height.

precipitate, cells were passaged as above, placed into sterile 15 ml conical tubes, and treated with 350 μg/ml tissue culture grade DNase I for 10 min at 37°C. After pelleting the cells at 400 × *g*, they were plated onto either standard 35 mm or Mecanex-gridded plates. Electrophysiological analysis was performed 24 hr later.

*β-Galactosidase staining protocols.* Three methods of β-galactosidase staining were utilized. 5-Bromo-4-chloro-3-indoyl β-D-galactoside (X-Gal) staining of cells was performed as described previously (Sanes et al., 1986) to determine the transfection efficiency. FDG staining was performed as originally described (Nolan et al., 1988), with the following slight modifications made for use with adherent cells. Cells were washed twice in PBS to remove the medium and then incubated for 5 min at 37°C with 1 ml of PBS to reequilibrate the cells to this temperature. During the incubation step, 20 mM FDG solution (prepared by the manufacturer) was diluted 1:20 (25 μl) into 0.5 ml of 0.5× PBS in a microcentrifuge tube, and placed in a 37°C water bath. The PBS was aspirated from the cells, and the warmed 1 mM FDG solution (final concentration) was added to the cells. The plate was warmed in the 37°C water bath for 1 min and then placed on ice, and 2.5 ml of ice cold 1× PBS was added. After 5 min on ice, the cells were viewed with a fluorescence microscope fitted with fluorescein filters. Imagene staining for *lacZ* activity was performed according to the manufacturer's instructions.

*Electrophysiological analysis.* Prior to recording, the PBS/FDG solution or culture medium on the plate of cells was exchanged with three 2 ml washings of external recording medium containing the following (in mM): 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 10 mM glucose, 10 HEPES (pH 7.4). The intrapipette solution contained (in mM) 153 KCl, 1 $MgCl_2$, 5 EGTA, and 10 HEPES (pH 7.3). This combination of external and intrapipette solutions produced a chloride equilibrium potential ($E_{Cl}$) of approximately 0 mV and a potassium equilibrium potential ($E_K$) of −75 mV across the patch membrane. GABA and diazepam were diluted with external recording solution from a stock solution (10 mM or 1 mM, respectively) to the indicated final concentration on the day of the experiment. Drugs were applied by a pressure ejection micropipette (10–15 μm tip diameter, 0.5–1.0 psi) placed next to the cell or patch. Whole-cell current recording was performed with methods described previously for mouse spinal cord neuron recordings (Macdonald et al., 1989; Porter et al., 1990) using a List L/M EPC-7 amplifier (Darmstadt, Germany). All recordings were done at room temperature (22–24°C). GABA concentration–response curves and $EC_{50}$ values were fitted with the GraphPAD INPLOT program (GraphPAD, San Diego, CA).

*Whole-cell and single-channel data collection and analysis.* Currents were recorded simultaneously on a video cassette recorder (Sony SL-2700, modified to 0–20 kHz) via a digital audio processor (Sony PCM-501 ES, 14-bit, 44 kHz) and a chart recorder (Gould Inc., Cleveland, OH) for later computer analysis. Whole-cell and single-channel recordings were low-passed filtered (3 dB at 1 kHz, eight-pole Bessel filter, Frequency Devices) before the chart recorder. The peak whole-cell current amplitude was measured by hand from the chart output. Single-channel records were analyzed using methods described previously for mouse spinal cord neurons (Macdonald et al., 1989; Porter et al., 1990).

## Results

### *Identification of LacZ+ cells*

Mammalian cells expressing *lacZ* cDNA constructs (LacZ+ cells) have been identified by staining with the fluorescent substrate

FDG as was shown by flow cytometry (Nolan et al., 1988). We wished to determine if the method could be adapted to the staining of adherent cells on the above gridded plates, thus enabling us to use *lacZ* cotransfection with GABAR subunit cDNAs to identify cells that were expressing β-galactosidase and, presumably, the ion channel proteins. L929 cells transfected with the construct pCMVβGal were plated on gridded dishes (see Materials and Methods), stained with FDG using a modified protocol for adherent cells (Fig. 1, *top*), and subsequently fixed and counterstained with the chromogenic LacZ substrate X-Gal (Fig. 1, *bottom*). FDG accurately stained LacZ$^+$ cells as demonstrated by the X-Gal counterstain. FDG staining was more sensitive than X-Gal staining since some FDG positive cells did not stain with X-Gal (data not shown). All cell lines tested gave a sufficient fluorescent signal, with COS 1, HEK 293, and CV-1 cells fluorescing most intensely, most likely due to higher levels of *lacZ* expression (data not shown). Cell-specific staining could be reversed easily by incubating the FDG loaded cells at 37°C for 15 min to allow for diffusion of the enzymatically produced free fluorescein (Bruning et al., 1980) (data not shown). The membrane-permeant FDG analog Imagene was also used, but it produced nonspecific staining of all cells due to high background levels of endogenous β-galactosidase (data not shown). The addition of chloroquine to inhibit the endogenous β-galactosidase activity removed the nonspecific staining, but was too toxic to the cells to permit further experimentation.

*Correlation of FDG staining and GABAR expression*

Since cells take up large numbers of plasmid molecules during transfection, acute transfection of mammalian cells with several expression vectors should result in the translation of all cDNA-encoded proteins in successfully transfected individual cells (Loyter et al., 1982a,b). This hypothesis was tested by cotransfecting several cell lines with the *lacZ* cDNA and various α, β, and γ GABAR subunit cDNA expression vectors and determining the correlation between *lacZ* and GABAR channel expression (Table 1). Cotransfected L929 cells that were randomly selected and analyzed for GABAR expression by whole-cell recording produced a GABA-evoked response rate of 10%. This expression rate was similar to the 7% transfection efficiency of these cells as determined by X-Gal staining for β-galactosidase activity. In contrast, identification of individual LacZ$^+$ cells by FDG staining with subsequent analysis of GABAR expression demonstrated the utility of the cotransfected marker gene as a method to identify a population of cells expressing GABARs for study. The transfection efficiency for most preparations was between 1% and 20% as determined by X-Gal staining, but with electrophysiological analysis 75% of the FDG-stained cells (four different cell lines) were also responsive to GABA. This result confirmed the feasibility of cotransfection of individual cells with a marker gene and receptor cDNAs and decreases by almost an order of magnitude the number of recordings necessary to obtain a GABAR current. The findings were reproducible through several experimental trials (Table 1). Interestingly, there was no correlation between the intensity of FDG staining or cell size and the final whole-cell current response. Large differences in staining intensity were most likely due to the variable loading of FDG into cells and not due to changes in the levels of LacZ expression, as measured by visual inspection of the X-Gal staining (data not shown). Cell input resistances were similar among all cells during both whole-cell and single-channel patch clamp studies, suggesting that the staining protocol was not deleterious to the cell membrane (data not shown). Though some cells did not survive the FDG staining protocol due to the osmotic shocks, they were easily recognized by their morphology and not chosen for study.

**Table 1. Correlation of *lacZ* expression of GABA-evoked responses in various transfected cell lines**

| Cell line | % transfection efficiency | Number with GABA-evoked responses | % GABA responsive |
|---|---|---|---|
| Randomly tested cells | | | |
| L929 | 6.82 ± 0.54[a] | 2/20 ($n$ = 1) | 10.0 |
| LacZ$^+$-tested cells | | | |
| L cells[b] | 1–8 | 106/141 ($n$ = 19) | 75.2 |
| COS 1 | 5–15 | 17/18 ($n$ = 5) | 94.4 |
| CV-1 | 5–10 | 15/15 ($n$ = 5) | 100.0 |
| HEK-293 | 15–20 | 3/3 ($n$ = 1) | 100.0 |

Cells were cotransfected with *lacZ* and GABAR subunit cDNAs ($\alpha_1$ or $\alpha_5$, $\beta_1$, $\gamma_{2S}$) and analyzed 48 hr later. For the randomly tested cells, cells were plated on standard 35 mm tissue culture dishes and not stained with FDG; cells were randomly chosen for electrophysiological testing. For the LacZ$^+$-tested cells, LacZ$^+$ cells for electrophysiological testing were identified by FDG staining after plating on gridded plates. The transfection efficiency was determined visually by staining a parallel plate of cells with the chromogenic LacZ substrate X-Gal and calculating the percentage of blue, transfected cells. The values given are approximate determinations, except where noted. The number of cells with GABA-evoked responses was determined by eliciting whole-cell currents from selected cells with applications of 1–30 μM GABA and is listed as the number of GABA-responsive cells over the total number of cells tested. The numbers in parentheses represent the numbers of individual experiments performed.

[a] The transfection efficiency was determined accurately by calculating the percentage of blue, transfected cells in eight low-power microscopic fields and is given as the average ± SEM.

[b] Represents the combined total of results obtained with L929 cells and other derivative clones and was similar for each individual cell line.

*Comparison of GABAR whole-cell currents*

Development of this transfection marking technique enabled us to determine if double and triple GABAR subunit combinations were assembled at high levels in individual cells and what percentage of transfected cells produced functional ion channels, a rough estimate of assembly efficiency. We initiated our studies with double-subunit combinations of the $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits and not single subunits since it was reasoned that if no whole-cell responses were seen with certain double-subunit combinations, then assembly of GABARs from either single subunit could not have occurred. Whole-cell recordings were obtained from individual LacZ$^+$ L929 cells cotransfected with expression vectors encoding either $\alpha_1\beta_1$, $\alpha_1\gamma_{2S}$, $\beta_1\gamma_{2S}$, or $\alpha_1\beta_1\gamma_{2S}$ subunit combinations. Surprisingly, unlike previous reports (Draguhn et al., 1990; Sigel et al., 1990; Verdoorn et al., 1990), only the expression of $\alpha_1\beta_1$ ($n$ = 6) and $\alpha_1\beta_1\gamma_{2S}$ subunits ($n$ = 5), and not the other double-subunit combinations ($\alpha_1\gamma_{2S}$ and $\beta_1\gamma_{2S}$, $n$ = 6 each), produced functional GABARs in L929 cells (Fig. 2). The threshold of sensitivity for the whole-cell recordings was 2 pA. The GABAR currents elicited from $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunit combinations were GABA concentration dependent, although the pattern of whole-cell current responses differed between the two populations of responding GABARs (Fig. 3). By using a pressure ejection micropipette to administer drugs, GABA could be administered more quickly than by standard perfusion techniques and fairly accurate peak GABA-elicited responses could be measured. However, an accurate measure of the rate of desensitization was not possible. By using the LacZ marker gene,

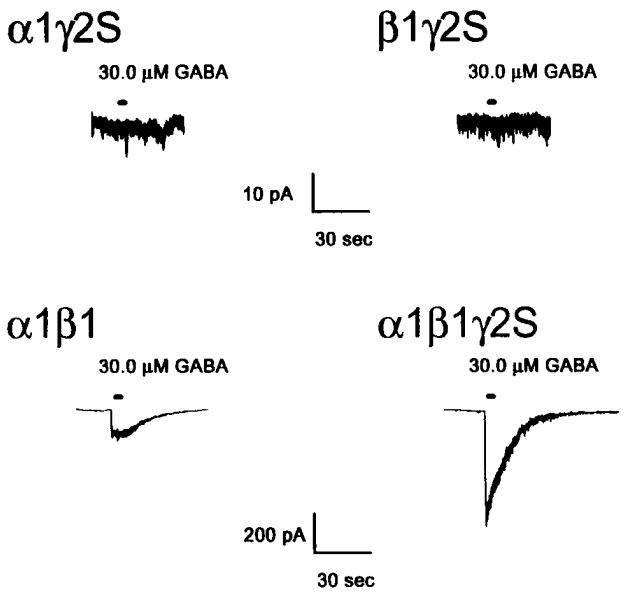


*Figure 2.* Representative whole-cell currents elicited after coexpression of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunit combinations. Whole-cell currents were recorded from individual LacZ$^+$ cells expressing either $\alpha_1\gamma_{2S}$, $\beta_1\gamma_{2S}$, $\alpha_1\beta_1$, or $\alpha_1\beta_1\gamma_{2S}$ subunit expression vector combinations. A 3 sec application of 30 μM GABA was applied to each cell to elicit a response, while maintaining the cell at a holding potential of −75 mV. *Downward deflections* indicate outward $Cl^-$ conductance. Note the differences in the scale for the different recordings.

the relative expression efficiency for GABARs composed of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunits was calculated to be 90% (data not shown). Since expression of $\alpha_1\gamma_{2S}$ and $\beta_1\gamma_{2S}$ subunit combinations did not produce functional GABARs (0% relative efficiency), this was interpreted to mean that $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ GABARs were also not assembled, or they would have been detected. Hence, single-subunit GABARs were not studied.

Peak whole-cell current amplitudes obtained from several cells expressing the four different combinations of subunits were averaged, and the resulting concentration–response curves were compared (Fig. 4). Note that the whole-cell current response curves were plotted as the average of the actual current observed (pA), not as the average of the current after normalization to the maximum current (%). This result demonstrated that levels of receptor expression in transfected L929 cells were reproducible from cell to cell and allowed for direct comparison of the amplitude of single-cell currents elicited from the various double- and triple-subunit combinations. The maximum amplitudes of $\alpha_1\beta_1\gamma_{2S}$ GABAR currents (847 ± 212 pA, $n$ = 5) were four to five times larger than $\alpha_1\beta_1$ GABAR currents (172 ± 83 pA, $n$ = 6). A comparison of whole-cell responses of cells expressing $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunits suggested that the receptors formed had different pharmacological properties (Table 2). For $\alpha_1\beta_1\gamma_{2S}$ GABARs, GABA concentration–response curves had a maximum response of 847 pA, an $EC_{50}$ of 7.4 μM, and a Hill slope of 1.7. In contrast, $\alpha_1\beta_1$ GABARs had a smaller maximum response of 172 pA, an $EC_{50}$ value of 1.0 μM, and a reduced Hill slope of approximately 1.1. The GABARs formed could be further differentiated by their sensitivity to diazepam. GABAR currents from receptors formed with $\alpha_1\beta_1$ subunits were not sensitive to diazepam, whereas GABAR currents from receptors formed with $\alpha_1\beta_1\gamma_{2S}$ subunits were enhanced 52 ± 8% ($n$ = 5) by diazepam (coapplication of 50 nM diazepam with 3 μM GABA; Fig. 5, Table 2). In all cells tested, transient expression of all three subunit cDNAs produced GABAR currents that were enhanced by diazepam, suggesting that the $\gamma_{2S}$ subunit was incorporated into the receptors. These results were similar to previous experiments from other laboratories showing diazepam enhancement of GABARs incorporating the $\gamma_{2S}$ subunit (Pritchett et al., 1989a,b).

### *Analysis of GABAR subunit preference*

The absence of $\alpha_1\gamma_{2S}$ and $\beta_1\gamma_{2S}$ GABAR expression in L929 cells greatly simplified further study of potential GABAR heterogeneity. Whole-cell currents elicited from individual cells after transfection with $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits could thus only be the summation of single-channel currents from either $\alpha_1\beta_1\gamma_{2S}$ and

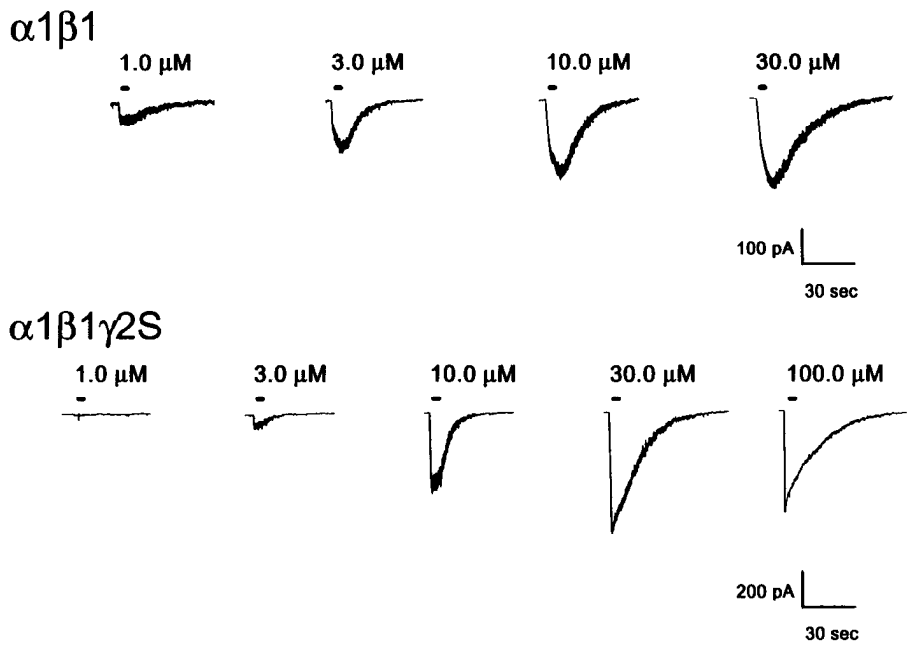


*Figure 3.* $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ whole-cell GABAR currents. Representative whole-cell currents evoked with increasing concentrations of GABA (noted above the curve), from individual L929 cells after cotransfection with either $\alpha_1\beta_1$ (*top*) or $\alpha_1\beta_1\gamma_{2S}$ (*bottom*) subunit cDNAs. Downward deflections indicate outward $Cl^-$ conductance. Cells were held at a membrane potential of −75 mV.

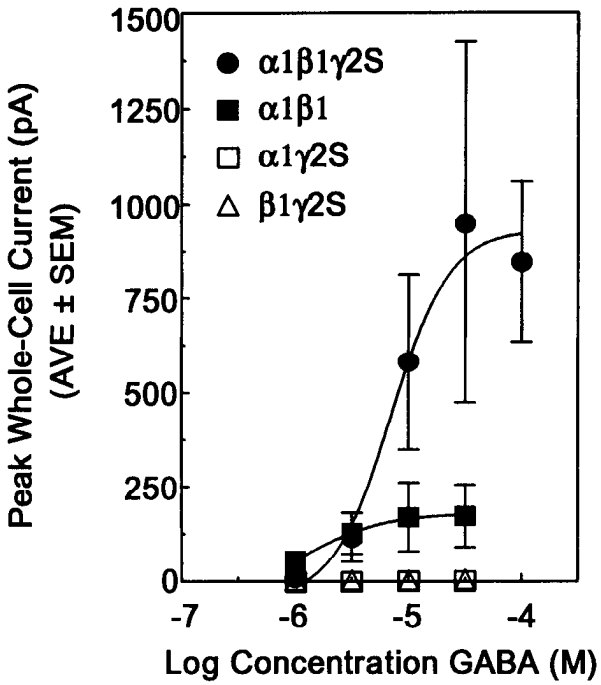


*Figure 4.* GABA concentration–response curves of double- and triple-subunit combinations. The averaged peak amplitude of whole-cell GABA currents evoked from single LacZ$^+$ L929 cells after transfection with equal amounts by weight of cDNAs encoding various receptor subunits and *lacZ*: $\alpha_1\beta_1\gamma_{2S}$ ($n = 5$), $\alpha_1\beta_1$ ($n = 6$), $\alpha_1\gamma_{2S}$ ($n = 6$), and $\beta_1\gamma_{2S}$ ($n = 6$). No curves were drawn for the latter two double-subunit combinations since no responses were recorded at any GABA concentration tested. The threshold for detection was 2 pA at a holding potential of −75 mV.

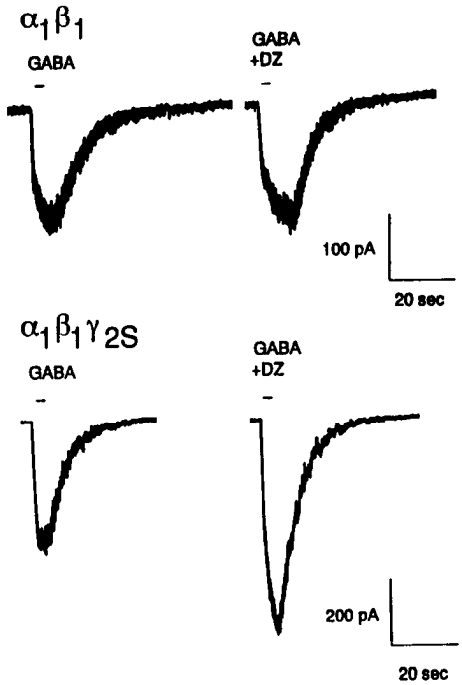


*Figure 5.* Demonstration of diazepam modulation of $\alpha_1\beta_1\gamma_{2S}$ GABARs. Typical diazepam (*DZ*) modulation of GABA-evoked currents from single cells expressing either $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ subunit combinations held at −75 mV. Drugs (3 μM GABA or 3 μM GABA + 50 nM diazepam) were applied by pressure ejection for the duration indicated by the *bar*. After the application of diazepam, reapplication of the same concentration of GABA alone elicited an identical whole-cell current as seen prior to diazepam exposure (data not shown).

$\alpha_1\beta_1$ GABARs inserted into the membrane. To test if GABARs formed after expression of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunit cDNAs preferentially formed a GABAR composed of all three subunits, L929 cells were transfected with increasing amounts of the $\beta_1$ subunit expression vector relative to the $\alpha_1$ and $\gamma_{2S}$ subunit cDNAs. GABA-evoked whole-cell currents were recorded from LacZ$^+$ cells and cells were classified as expressing either currents insensitive or sensitive to diazepam, and the percentage enhancement by diazepam was measured (Table 3). In cells transfected with a 1:1:1 ratio of subunit cDNAs ($\alpha_1$:$\beta_1$:$\gamma_{2S}$), all GABA-evoked currents were diazepam sensitive ($n = 5$). Doubling the amount of the $\beta_1$ subunit expression vector to a 1:2:1 ratio ($\alpha_1$:$\beta_1$:$\gamma_{2S}$) still produced only diazepam-sensitive currents in all cells tested ($n = 8$). In fact, no diazepam-insensitive currents were recorded until the ratio of the $\beta_1$ subunit had increased to 1:4:1 ($\alpha_1$:$\beta_1$:$\gamma_{2S}$). Even at this ratio of subunits, less than half of the GABAR currents (4 of 10) were insensitive to diazepam. This suggested that the diazepam-sensitive form of GABARs composed of $\alpha_1\beta_1\gamma_{2S}$ subunits was preferred. Interestingly, the diazepam enhancement seen for all diazepam-sensitive GABAR currents was similar, no matter what ratio of $\beta_1$ subunit was transfected into the cell (Table 3).

### *Comparison of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABAR single-channel currents*

To investigate further the potential dissimilarities between the two receptor populations, single-channel currents were recorded from outside-out membrane patches excised from L929 cells after transfection with either $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ subunit cDNAs. Application of 3 μM GABA, at a holding potential of −75 mV, evoked a series of openings and closings in both receptor populations (Fig. 6). Openings were not seen in the absence of applied GABA with either receptor. Application of GABA to non-transfected LacZ$^-$ cells (Fig. 6, top trace) did not elicit any channel

**Table 2. Pharmacological and electrophysiological properties of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs**

| Property | $\alpha_1\beta_1$ | $\alpha_1\beta_1\gamma_{2S}$ |
|---|---|---|
| Hill slope | 1.1 ± 0.2 ($n = 6$) | 1.7 ± 0.2 ($n = 5$) |
| $EC_{50}$ GABA (μM) | 1.0 ± 0.2 ($n = 6$) | 7.1 ± 0.9 ($n = 5$) |
| % Enhancement with 50 nM diazepam[a] | 0 ($n = 6$) | 52 ± 8 ($n = 5$) |

Whole-cell GABA currents and concentration–response curves were obtained from single LacZ$^+$ transfected cells as described in the Figure 3 caption. The Hill slope and GABA $EC_{50}$ value were calculated using absolute current values. All values in the table are the average ± SEM.

[a] Diazepam (50 nM) was coapplied with GABA (3.0 μM) and the percentage enhancement was calculated based upon the response to GABA (3.0 μM) alone.

**Table 3. The effect of GABAR subunit ratios on receptor assembly**

| Ratio of $\alpha_1$:$\beta_1$:$\gamma_{2S}$ cDNA | No. of diazepam-insensitive currents | No. of diazepam-sensitive currents | % Diazepam enhancement |
|---|---|---|---|
| 1:1:1 | 0 | 5 | 55 ± 6[a] |
| 1:2:1 | 0 | 8 | 42 ± 7 |
| 1:4:1 | 4 | 6 | 51 ± 6 |

L929 cells were transfected with the indicated ratios of subunit cDNAs (see Materials and Methods). The $\alpha_1$ vector (pCMVb$\alpha_1$) was held constant at 1.2 μg per 60 mm dish, and the other vector concentrations were varied accordingly; 1.2 μg of pCMV$\beta$GAl was also added to all precipitates. Parent plasmid pCMVNeo was added to the precipitate to maintain a constant 9.6 μg of vector DNA in the 300 μl precipitate. LacZ+ cells were assayed for GABA-evoked whole-cell currents as described in the Table 1 notes using 3 μM GABA. The percentage enhancement seen upon coadministered 50 nM diazepam was measured and cells were classified as being either diazepam-insensitive or -sensitive.

[a] Results between rows are not statistically significant (two-tailed Student's $t$ test, $p > 0.05$).

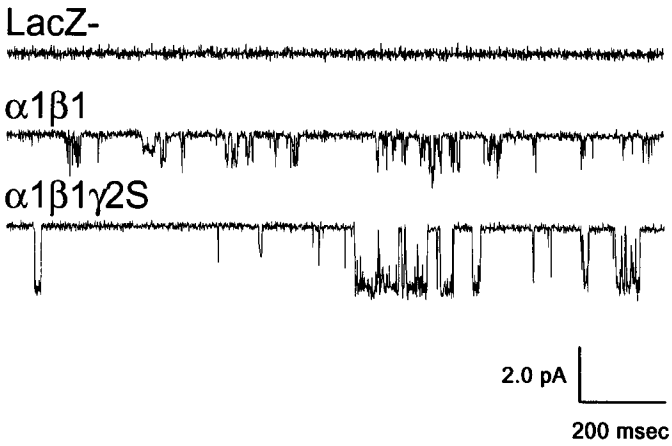


*Figure 6.* Raw data tracings of single-channel openings from $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs. GABA (3 μM) was applied to excised outside-out membrane patches from nontransfected LacZ⁻ L929 cells (*top*), $\alpha_1\beta_1$ transfected LacZ+ (*middle*), and $\alpha_1\beta_1\gamma_{2S}$ transfected LacZ+ (*bottom*) L929 cells at a holding potential of −75 mV. *Downward deflections* indicate outward $Cl^-$ conductance.

openings, thus demonstrating the lack of endogenous GABARs in this cell line. A comparison of the single-channel currents evoked from outside-out patches excised from L929 cells expressing either $\alpha_1\beta_1$ (middle trace) and $\alpha_1\beta_1\gamma_{2S}$ GABARs (bottom trace) demonstrated a difference in the amplitude and gating between the two receptor populations. GABA-evoked openings of $\alpha_1\beta_1$ GABARs exhibited mostly brief, single opening bursts with some bursts of sequential openings and bursts. In contrast, the single-channel openings evoked by GABA from $\alpha_1\beta_1\gamma_{2S}$ subunit transfected cells consisted mainly of multiple opening bursts with a longer duration. Single-channel openings of $\alpha_1\beta_1$ GABARs opened to at least two small current levels of 0.88 and 1.21 pA, whereas $\alpha_1\beta_1\gamma_{2S}$ GABARs opened to at least two larger current levels of 1.51 and 2.23 pA ($n$ = 3 patches each). No openings to the largest current level (2.23 pA) were observed from patches excised from cells expressing only $\alpha_1$ and $\beta_1$ subunits (data not shown), suggesting that this current level of the receptor was unique to GABARs possessing $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits. Conversely, it appeared that no small current openings (0.88 and 1.21 pA) were present in patches excised from cells expressing all three subunits (data not shown), implying that $\alpha_1\beta_1$ GABARs were not formed as readily in the presence of the $\gamma_{2S}$ subunit. Simultaneous opening of multiple ion channels in a given patch was a relatively rare event (data not shown).

## Discussion

*Assembly of GABAR subunits*

We examined the pharmacological and electrophysiological properties of various double- and triple-subunit combinations of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits expressed in single L929 cells and determined that $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$, but not $\alpha_1\gamma_{2S}$ or $\beta_1\gamma_{2S}$, GABARs were functionally expressed. This result was contrary to previous work by others showing that $\alpha\gamma$ GABARs were expressed at similar levels (based on averaged whole-cell GABA-evoked current amplitude) as the $\alpha\beta$ or $\alpha\beta\gamma$ GABARs and that $\beta\gamma$ and single-subunit GABARs expressed poorly (Draguhn et al., 1990; Sigel et al., 1990; Verdoorn et al., 1990). However, interpretation of GABAR expression levels purely by comparison of whole-cell current levels was difficult (see below). Some of these previous experiments were performed using the rat $\beta_2$ subunit instead of the bovine $\beta_1$ subunit used in these studies. The use of the $\beta_2$ instead of the $\beta_1$ subunit may have an effect, but no such drastic difference between $\beta$ subunit isoforms or subunit species has been shown previously (Ymer et al., 1989).

Most previous work was performed using cells or oocytes that were genetically or naturally engineered to produce large amounts of nucleic acid–encoded protein as evidenced by the size of the maximal GABA-evoked currents found in these cell systems. After transfection or injection with $\alpha$, $\beta$, and $\gamma$ subunit cDNAs or mRNAs, oocytes and HEK 293 cells produced GABAR currents in the nanoamp to microamp size range, relative to transfected L929 cells, which produced picoamp-sized GABAR currents. In our laboratory, transfection of $\alpha_1\beta_1\gamma_{2S}$ subunits into COS 1 cells, which are similar to HEK 293 cells, also produced nanoamp currents on average after application of maximum GABA concentrations (data not shown). Together, these results could be seen as a continuum, from oocytes, which efficiently assemble almost all triple-, double-, and single-subunit combinations (Sigel et al., 1990), to HEK 293 cells, which effectively assemble triple- and certain double-subunit, but poorly assemble single-subunit, combinations (Verdoorn et al., 1990), to L929 cells, which assemble $\alpha_1\beta_1\gamma_{2S}$ and only the $\alpha_1\beta_1$ double-subunit combination.

Assembly of mature GABARs from various subunits may proceed by an ordered process, as has been described for nicotinic cholinergic receptors (Blount et al., 1990; Paulson et al., 1991; Saedi et al., 1991), or it may proceed in part by mass action. The differences in the types of GABARs formed in the various cells mentioned above may be due to the levels of expressed protein within the cell, which could allow for assembly by mass action of less preferred combinations. Another possible level of assembly regulation may be found with the different biochemical environments in these cell lines and oocytes, which may further influence the final oligomeric assembly rates. For example, Claudio and colleagues have shown that phosphorylation of the $\gamma$ subunit of nicotinic cholinergic receptors can increase the assembly of the mature channel (Green et al., 1991a,b; Ross et al., 1991). Thus, differences in phosphorylation states within the different cell types may alter the pattern of GABAR expression. Preliminary evidence from our laboratory suggests that phosphorylation of the $\beta_1$ subunit by cAMP-de-

pendent protein kinase may also effect the assembly of $\alpha_1\beta_1\gamma_{2S}$ GABARs (Angelotti et al., 1991).

Efficiency of GABAR expression as determined by the number of LacZ$^+$ cells that expressed functional GABARs was similar for both $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs, approximately 90%. This parameter could not be measured without an external transfection marker, and it was normally determined by comparing the transfection efficiency separately from the expressed receptor (Pritchett et al., 1988). Using a fluorescent marker gene technique, we were able to determine receptor expression in known transfected cells, and this result suggested that the assembly of these two GABARs was not a rare event.

*Properties of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs*

Based on whole-cell currents, L929 cells expressing $\alpha_1\beta_1$ GABARs had approximately one-fourth the total current of cells expressing $\alpha_1\beta_1\gamma_{2S}$ GABARs and produced receptor channels with different GABA concentration–response relationships and diazepam sensitivities. Previous whole-cell current analyses of GABARs expressed in oocytes and mammalian cells have demonstrated a noncooperative GABA activation of the ion channel after expression of $\alpha$, $\beta$, and $\gamma$ subunits (Sigel et al., 1990; Knoflach et al., 1992). Verdoorn and colleagues (Verdoorn et al., 1990), using fast drug application demonstrated that GABARs incorporating a $\gamma_2$ subunit, had GABA concentration–response curves with Hill slopes approaching 2.0. Their results suggested that the lack of cooperativity seen before was possibly due to rapid desensitization of the peak whole-cell response that was not detected when administering drug(s) by perfusion. Using a pressure-ejection technique to administer drug to $\alpha_1\beta_1\gamma_{2S}$-subunit-transfected L929 cells, we also observed a Hill slope of approximately 1.7, whereas previously $\alpha_1\beta_1\gamma_{2S}$ GABARs expressed in oocytes were shown to have a Hill slope of 1.2 (Malherbe et al., 1990). The small size of the L929 cells probably allowed for a more quick and uniform application of drug and enabled us to record the true peak whole-cell current before desensitization. This result may not be true for the $\alpha\beta$ GABAR currents, which possibly desensitize more rapidly, assuming that $\alpha_1\beta_1$ GABAR currents desensitize as quickly as $\alpha_1\beta_2$ GABAR currents (Verdoorn et al., 1990). However, the similarity in Hill slopes calculated for $\alpha_1\beta_1$ and $\alpha_1\beta_2$ GABARs (approximately 1.1 and 1.18, respectively) suggests that the true peak currents were measured.

Coexpression of the $\gamma_{2S}$ subunit along with $\alpha_1$ and $\beta_1$ subunits produced GABAR currents that were enhanced by diazepam. Currents elicited by 3 $\mu$M GABA were enhanced approximately 50% upon coapplication of 50 nM diazepam. This level of enhancement by diazepam was similar to that seen after expression of the same subunits in HEK 293 cells, suggesting that differences in the heterologous expression systems did not affect the benzodiazepine binding site (Puia et al., 1991). Single-channel openings of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs also differed, with $\alpha_1\beta_1\gamma_{2S}$ GABARs having longer open and burst durations and opening to a larger conductance level(s) than $\alpha_1\beta_1$ GABARs. These results are similar to previously published single-channel tracings obtained from mammalian cells expressing either $\alpha_1\beta_1$, $\alpha_1\beta_2$, or $\alpha_1\beta_2\gamma_{2S}$ subunit cDNAs (Moss et al., 1990; Verdoorn et al., 1990). The relative lack of the largest current level recorded from $\alpha_1\beta_1\gamma_{2S}$ GABAR patches (2.23 pA) in $\alpha_1\beta_1$ GABAR patches would suggest that the two populations of receptors can be discriminated by their single-channel current levels as well as pharmacological criteria. A more intriguing result was the apparent absence of the smaller $\alpha_1\beta_1$ GABARs in patches from cells expressing all three subunits. This result further suggested that receptor channels assembled after coexpression of all three subunits were distinct from receptor channels assembled after coexpression of only the $\alpha_1$ and $\beta_1$ subunits, and also they may be the preferred final form of the receptor. Confirmation of this hypothesis will require a more complete analysis of single-channel currents and kinetic properties of the two GABARs (Angelotti and Macdonald, 1993).

Low concentrations of GABA (1–5 $\mu$M) evoked similar peak whole-cell currents from cells expressing GABARs composed of $\alpha_1\beta_1$ subunits or $\alpha_1\beta_1\gamma_{2S}$ subunits, even though the single-channel currents producing these whole-cell currents had different amplitudes. This result demonstrated that the peak whole-cell current for a single concentration of GABA could not be used as a measure of the relative level of expression of different subunit combinations, unless the concentration was maximal for all of the compared receptors and the underlying single-channel properties were known. Further understanding of the differences between the whole-cell current behavior of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs will require a complete single-channel kinetic analysis of the open, closed, and burst properties of the two receptor populations (Angelotti and Macdonald, 1993).

*$\alpha_1\beta_1\gamma_{2S}$ GABARs were the preferred final form of the receptor channel*

Though the stoichiometry of the different subunits in a native or recombinant GABAR has not been determined, the subunits probably assemble into a pentameric structure analogous to another ligand-gated supergene family member, the nicotinic ACh receptor (Stroud et al., 1990). By transfecting cells with a 1:1:1 ratio of $\alpha_1$:$\beta_1$:$\gamma_{2S}$ subunit cDNAs, though this cannot be their true stoichiometry, GABARs composed of all three subunits were formed as evidenced by their sensitivity to diazepam and GABA pharmacology. It was possible that whole-cell currents observed were the combination of single-channel openings from both the $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs described above. If this did occur and the relative ratios of the $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs varied from cell to cell, then the diazepam enhancement seen due to $\alpha_1\beta_1\gamma_{2S}$ GABARs would have varied accordingly. Alternatively, as the initial single-channel records suggest (see above), the $\alpha_1\beta_1\gamma_{2S}$ form of GABARs in cells expressing $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits may be preferred over $\alpha_1\beta_1$ GABARs. If true, then the amount of diazepam enhancement seen for any given cell expressing all three subunits should be similar. By varying the ratio of different subunits inside of a cell, an attempt was made to alter the final form of the assembled GABAR current, as determined by the enhancement by diazepam. Whole-cell currents were measured after transfection of L929 cells with either a 1:1:1 or 1:2:1 ratio of $\alpha_1$:$\beta_1$:$\gamma_{2S}$ subunit expression vectors. In both cases, all cells tested ($n = 5$ and $n = 8$, respectively) produced diazepam-sensitive currents, suggesting that they were $\alpha_1\beta_1\gamma_{2S}$ GABAR currents. This subunit configuration appeared to be favored over $\alpha_1\beta_1$ GABARs, since only after increasing the $\beta_1$ subunit level fourfold within a cell, relative to the $\alpha_1$ and $\gamma_{2S}$ subunits, were any diazepam-insensitive GABAR currents recorded ($n = 4$ of 10 diazepam-insensitive currents). Further support for the hypothesis that the $\alpha_1\beta_1\gamma_{2S}$ form of the GABAR may be the preferred form of the receptor can be found in the magnitude of the diazepam enhancement measured. For all diazepam-sensitive currents, regardless of the level of transfected $\beta_1$ expression vector, the enhancement was approximately 50%.

These results further support the idea that assembly of GABARs from their subunit components did not occur randomly with $\alpha$, $\beta$, and $\gamma$ subunits freely substituting for one another, but instead that assembly proceeded by a more ordered process as seen with nicotinic ACh receptors (Gu et al., 1991; Saedi et al., 1991).

By using a marker gene to identify L929 cells that had been transfected, it was possible to ascertain the relative expression of functional GABARs in individual cells that were cotransfected with various receptor subunit cDNAs. More importantly, it was then possible to determine the true level of GABAR current produced in an individual cell, not a syncytium of cells, and to relate the differences in absolute currents to the expression of different subunits. The pharmacological differences in the whole-cell GABA concentration–response curves for $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs suggested that the receptor channels formed were unique. Also, it appeared that expression of $\alpha_1\beta_1\gamma_{2S}$ GABARs occurred preferentially over $\alpha_1\beta_1$ GABARs, even in the presence of a fourfold higher level of the $\beta_1$ subunit. These conclusions were based upon whole-cell current experiments. Single-channel currents from the two receptor populations also appeared to be different (Fig. 6), but more detailed analyses of the single-channel openings will be required to determine if coexpression of $\alpha_1$, $\beta_1$, and $\gamma_{2S}$ subunits in a cell produces a mixture of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs, or only a unique $\alpha_1\beta_1\gamma_{2S}$ GABAR. Theoretically, assuming random assembly of all subunits, expression of all three subunits in a cell could lead to the expression of 51 different GABAR configurations (Burt and Kamatchi, 1991). Results with L929 cells suggest that random assembly did not occur since not all double-subunit combinations were expressed, but it still remains to be determined if all of the remaining configurations were produced. Single-channel kinetic analysis of $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ GABARs should permit the discrimination of different GABAR configurations and possibly the resolution of a single preferred form of the GABAR, if one exists.

*Analysis of transient gene expression in single cells*

Proper determination of the relative assembly rates of various combinations of GABAR subunits would require biochemical analysis of a population of cells, after transfection with expression vectors encoding different subunits (Ross et al., 1991). However, biochemical analyses would not differentiate between functional and nonfunctional ion channels. Electrophysiological analyses would discriminate functional from nonfunctional GABARs, but must be performed on single cells. An approximation of the efficiency of GABAR assembly could be obtained from whole-cell recording techniques if the following criteria were met. First, it was known that the cell to be studied had indeed been transfected and thus could possibly express a GABAR assembled from the various subunit expression vectors. Second, the cell to be assayed was electrically isolated from all other cells to obtain a true measure of the GABA-evoked current from a single cell. Previous attempts to determine which GABAR subunits assemble to form functional ion channels were performed by randomly assaying transfected cells for GABA-evoked currents (Verdoorn et al., 1990; Knoflach et al., 1992). This technique did not permit the determination of the assembly efficiency, since nontransfected cells that were accidentally selected for study would decrease the apparent efficiency.

Recent synthesis of new fluorescent substrates for *lacZ* has made it possible to use this marker gene to stain living cells for this enzyme activity while maintaining cell viability (Nolan et al., 1988). This article extends this finding and demonstrates that cotransfection of the *lacZ* gene with GABAR subunit cDNAs into individual adherent cells can be used to identify single cells that have been successfully transfected. The stain was reversible and did not damage the cell membranes, making it possible to perform patch-clamp recordings. More importantly, all L929 cells that had been transfected with equal amounts of $\alpha$, $\beta$, $\gamma$ subunit cDNAs and were $LacZ^+$ expressed functional receptors composed of all three subunits. This result was confirmed by the demonstration that all resulting currents were sensitive to diazepam. In these transfected cells, no currents composed predominantly or exclusively by $\alpha_1\beta_1$ GABARs were observed and neither $\alpha\gamma$ nor $\beta\gamma$ GABARs were expressed when only $\alpha_1\gamma_{2S}$ or $\beta_1\gamma_{2S}$ subunit expression vectors were transfected into L929 cells. Thus, if a cell had been transfected with *lacZ*, it had been transfected by all of the plasmids. These results extend the findings of Ruddle and colleagues (Loyter et al., 1982a,b) and demonstrate at the single-cell level that each cell phagocytizes multiple plasmids during transfection. This technique thus fulfills the first criteria set forth above.

The amplitude of whole-cell GABA-evoked currents elicited from single L929 cells transfected with GABAR subunit cDNAs varied around a mean with a relatively small standard error. Studies examining the level of expression of a transiently transfected cDNA have not been performed at the level of the single cell. Since transfection occurs by an uncharacterized process of DNA-particle phagocytosis (Loyter et al., 1982a,b), it would be expected that the amount of plasmid DNA that entered the cell and was eventually expressed would vary widely among individual cells within a population, even though the overall level of expression within the population, after normalization for transfection efficiency, would vary much less. The latter observation makes it possible to study the expression of a transfected cDNA among large populations of cells. The present experiments demonstrate that the former hypothesis was not true and that the levels of transiently expressed cDNAs varied little among individual cells. This result was more surprising in that the parameter being measured (whole-cell GABA-evoked currents) depended upon the coexpression of three different cDNAs. This result was significant for the study of whole-cell currents elicited from single transfected cells, since it permitted the comparison of absolute whole-cell current values, and not the normalized current value, from two different populations of individual cells, as performed previously (Levitan et al., 1988; Malherbe et al., 1990).

With the advent of molecular cloning, it is now possible to study receptor protein(s) after expression in a heterologous cell system and, more importantly, to modify protein function by mutating specific amino acid residues (Verdoorn et al., 1991) or by changing its biochemical or cellular environment (Hoger et al., 1991; Karschin et al., 1991). These experiments are difficult to perform, though, since some cell lines that do not transfect at a high efficiency or grow as a syncitium may be desirable for their biochemical or physiological traits. The development of the reversible staining technique and several L929 derivative cell lines that express various G-protein–linked receptors (muscarinic, histaminergic, adrenergic) (Liao et al., 1990; Machida et al., 1990; Gantz et al., 1991), ion channels ($Ca^{2+}$) (Varadi et al., 1991), growth factor receptors (epidermal growth factor) (Baccarini et al., 1991), and altered levels of protein kinases (Uhler and Abul-Chebl, 1992) will make it possible to study the effect of various second messengers on the function and ex-

pression of ligand-gated and voltage-gated ion channels. We believe that this staining method also should be applicable to other experimental systems where reversible identification of individual viable and transfected adherent cells would be necessary to study single-cell events, such as single cell voltammetry (Leszczyszyn et al., 1991) and fluorescent microscopic analysis of ion fluxes (Rich et al., 1990).

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